DEVELOPMENT AND VALIDATION OF SHORT AMPLICON-LENGTH PCR ASSAYS FOR THE DETECTION OF FELINE SPECIES IN PROCESSED FOODS

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INSTITUTE OF GRADUATE STUDIES
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DESSERTATION SUBMITTED IN FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF PHILOSHOPY

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ABSTRACT

Food falsification is a common concern, but has been mutually practiced in the meat industry, especially for processing food products, for realizing an extra profit. The everyday happenings such horse, porcine, rat and dog meats forgeries in various foods have made consumers increasingly worried to safeguard their religious faith, health, money and wildlife in natural habitats. The consumers of the Halal food market have reached to 1.8 billion and turnover has crossed US Dollar 700 billion in 2012 and it has been projected to reach at US$ 1.6 trillion by the 2030. Since the market is quite large and opportunities in halal food business are huge, it has been targeted for adulteration for a long time.

Consumption or mixing of feline ingredients in halal and kosher foods is forbidden and various diseases such as SARS, anthrax and hepatitis could be transmitted through feline meats. However, since feline species are abundant across the world without market price and their meats are consumed in exotic foods, the chances of their adulteration in common meats are very high. For meat specification, DNA-based techniques are preferred over protein and lipid-based molecular identification schemes since DNA biomarkers, especially the short-length one, is extremely stable even under harsh processing condition (heat, pressure and additives chemicals) and compromised states (natural decomposition) where most protein-based markers are denatured or degraded.

Although several PCR assays have been proposed for feline species detection, those assays are based on longer length target amplicon which are assumed to break down under food processing treatments. Thus, a reliable detection of feline ingredients is crucial for the safety of consumer health, religious faith and fair-trade economy. In this study, a 69-bp target of feline mitochondrial cytochrome b gene was selectively amplified using

a pair of primers of the said species. The assay was specific for feline species under raw, processed, admixed and commercial food matrices. The specificity of the developed assay was checked against commercially important 14 terrestrial 5 aquatic and 5 plants species. The target DNA stability under various food processing conditions such boiling, autoclaving and microwaving that degrade DNA and exceptional constancy were found in all treatments. The lower limit of detection of the assay was reflected by its ability to detect 0.1 pg of feline DNA from raw meats, 0.01% (w/w) in different admixes and 0.1% (w/w) of feline meats in burger as well as meatball formulations, respectively.

The PCR product was further authenticated by restriction digestion followed by RFLP analysis in microfluidic-based lab-on a chip system. Theoretical analysis revealed two RFLP fragments of length 43 and 26-bp which will be separated using a highly sensitive microfluidic-based lab-on a chip system with a resolution of ≤10-bp. Very short amplicon-length, extreme stability and high sensitivity suggested that this assay could be used by the regulatory bodies for the routine assessments of feline species in food forensics or archaeological investigations. Therefore, a short amplicon-length polymerase chain reaction (PCR) was developed and validated it by restriction fragment length polymorphism (RFLP) analysis for the authentication of feline meat in processed foods.

ABSTRAK

Pemalsuan makanan merupakan kebimbangan umum, tetapi telah saling diamalkan di dalam industri daging, terutama untuk hasil makanan, bertujuan mencapai keuntungan lebih. Penipuan yang berlaku setiap hari melibatkan daging kuda, babi, tikus dan anjing di dalam pelbagai makanan telah meningkatkan kebimbangan pengguna di dalam menjaga larangan agama, kesihatan, kewangan dan hidupan liar di habitat asalnya. Pengguna pasaran makanan Halal sudah mencecah 1.8 bilion dan perolehan telah melampaui 700 bilion dolar AS pada 2012 dan disasarkan akan mencecah AS$1.6 trilion menjelang 2030. Memandangkan pasaran yang meluas dan peluang perniagaan makanan halal yang besar, ia telah menjadi sasaran pengadukan makanan sejak sekian lama.

Pengambilan atau pencampuran bahan berasaskan famili kucing ke dalam makanan halal dan kosher adalah dilarang dan pelbagai penyakit seperti SARS, antraks dan hepatitis boleh berjangkit melalui daging tersebut. Namun memandangkan spesies famili kucing banyak terdapat di seluruh dunia tanpa harga pasaran dan dagingnya dimakan di dalam makanan eksotik, peluang untuk pengadukan daging ini ke dalam daging biasa sangat tinggi. Untuk spesifikasi daging, teknik-teknik berasaskan DNA lebih cenderung digunakan berbanding skema pengesanan protein dan molekul berasaskan lipid memandangkan penanda bio DNA, terutamanya amplikon yang bersaiz kecil teramat stabil walau pun mengalami pemprosesan yang tinggi (tahap kepanasan, tekanan dan bahan kimia tambahan) dan keadaan terkompromi (penguraian semulajadi) yang mana kebanyakan penanda berasaskan protein akan ternyahasli atau ternyahgred.

Walaupun beberapa penilaian PCR telah dicadangkan untuk pengesanan spesies famili kucing, penilaian-penilaian tersebut berasaskan saiz amplikon sasaran yang lebih besar yang dijangka akan terurai apabila melalui pemprosesan makanan. Oleh yang demikian, pengesanan campuran spesies famili kucing yang berkesan sangat penting

dalam menjamin keselamatan dan kesihatan pengguna, agama dan ekonomi perdagangan adil. Dalam kajian ini, 69-bp gen sitokrom b mitokondria dari spesies famili kucing telah diperincikan secara terpilih menggunakan sepasang primer daripada spesies tersebut. Penilaian adalah spesifik kepada spesies famili kucing daripada matriks makanan mentah, terproses, campuran dan komersial. Kekhususan penilaian yang dibangunkan telah disemak dengan spesies yang penting secara komersial iaitu 14 spesies darat, 5 spesies akuatik dan 5 spesies tumbuhan. Kestabilan DNA sasaran dalam pelbagai keadaan pemprosesan makanan seperti pendidihan, pengautoklafan dan penggunaan gelombang mikro yang mendegradasi DNA dan ketetapan luar biasa dilihat dalam semua rawatan. Pengesanan had bawah untuk penilaian dilihat melalui kebolehan mengesan 0.1 pg DNA spesies famili kucing dalam daging mentah, 0.01% (w/w) dalam pelbagai campuran berbeza dan 0.1% (w/w), masing-masing dalam burger dan formulasi bebola daging.

Produk PCR seterusnya telah disahkan melalui pembatasan pencernaan diikuti analisis RFLP dalam sistem cip makmal berasaskan mikro berbendalir. Analisis teori mendedahkan dua cebisan RFLP berukuran 43 dan 26-bp yang akan dipisahkan menggunakan sistem cip makmal berasaskan mikro berbendalir dengan peleraian $\leq$10-bp. Amplikon bersaiz sangat kecil, kestabilan ekstrem dan sensitiviti tinggi mencadangkan bahawa penilaian ini boleh digunapakai oleh badan kawal selia untuk menjalankan ujian berkala terhadap spesies famili kucing di dalam forensik makanan dan penyiasatan arkeologi. Dengan itu, satu reaksi rantai polimerase (PCR) amplikon bersaiz kecil telah dihasilkan dan disahkan melalui analisis pembatasan panjang cebisan polimorfisme (RFLP) untuk pengesahan kewujudan daging famili kucing di dalam makanan terproses.

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LIST OF SYMBOLS AND ABBREVIATIONS

$	: dollar
%	: percent
′	: prime
˚C	: degree celsius
µg	: microgram
µl	: microliter
µM	: micromolar
pg	: picogram
≥	: greater than or equal to
3D	: three dimensional
A	: adenine
A_{260}/A_{280}	: ratio of UV at absorbance 260 nm and 280 nm
ABI	: applied biosystem
AIDS	: acquired immune deficiency syndrome
ATP 6	: ATPase subunit 6
BLAST	: basic local alignment search tool
bp	: base pairs
C	: cytosine
COI	: cytochrome c oxidase subunit I
Cq	: quantification cycle
CE	: capillary electrophoresis
Ct	: threshold cycle
CTAB	: Cetyl trimethyl ammonium bromide
cyt b	: cytochrome b
DBKL	: dewan bandaraya Kuala Lumpur
dH_2O	: distilled water
D-loop	: displacement loop
DNA	: deoxyribonucleic acid
dNTP	: deoxyribonucleoside triphosphate
ds-DNA	: double stranded- deoxyribonucleic acid
EC	: European Commission
ELISA	: enzyme-linked immunosorbant assay

E-nose	: electronic nose
FTIR	: fourier transformed infrared
g	: gram
G	: guanine
GC-MS	: gas chromatography-mass spectrometry
GHR	: growth hormone receptor
h	: hour
HIV	: human immunodeficiency virus infection
HPLC	: high performance liquid chromatography
IDT	: integrated DNA technology
LC	: liquid chromatography
LOD	: limit of detection
MEGA5	: molecular evolutionary genetics analysis version 5
mg	: milligram
$MgCl_2$	: magnesium chloride
min	: minute
ml	: mililitre
mM	: milimolar
mt	: mitochondrial
mtDNA	: mitochondrial deoxyribonucleic acid
NCBI	: national center of biological information
ND2	: NADH dehydrogenase subunit 2
ND5	: NADH dehydrogenase subunit 5
ND6	: NADH dehydrogenase subunit 6
ng	: nanogram
NI	: not indicated
nt	: nucleotide
NM	: not mentioned
NR	: not reported
PAGE	: polyacrylamide gel electrophoresis
PCR	: polymerase chain reaction
PCR-RFLP	: polymerase chain reaction- restriction fragment length polymorphism
psi	: pounds per square inch
rpm	: rotations per minute

rRNA	: ribosomal ribonucleic acid
s	: second
SARS	: severe acute respiratory syndrome
T	: thymine
Taq	: *Thermus aquaticus*
Tm	: melting temperature
UV	: ultraviolet
w/w	: weight/weight

CHAPTER ONE

INTRODUCTION

CHAPTER 1: INTRODUCTION

1.1 Background of the Study

Now a days consumers are over anxious about the ingredients of food which they are purchasing and consuming from the food court, road side restaurant and groceries. A list of factors including lifestyles (e.g. vegetarianism and organic food), diet (e.g. calories and nutritional value), health hazardous issues (e.g. lack of allergens), and economic (e.g. additional earnings) are potential reasons for differential values of various foods and falsification of ingredients (Fajardo, González, Rojas, García, & Martín, 2010). More importantly, consumers are not ready to accept minute level of tarnishing in their foods regarding the spiritual devotion issues (e.g. pork is banned in some religious laws) (Arun, Ciftcioglu, & Altunatmaz, 2014). Additionally, unlisted, misdescription, fake labeling, or fraudulent ingredients in food products have been a serious issue having adverse effects on humans in a several ways. The substituted materials appear very similar to the original materials and thus it is really challenging to identify the false ingredients form the original one (Ghovvati, Nassiri, Mirhoseini, Moussavi, & Javadmanesh, 2009). Moreover, mixing of low-cost products as a substitute of original elements in high valued products is quite a common practice in meat industry to cut down production cost and gain extra profit (Hsieh and others, 1995). Thus the proper descriptions of food products has become a mandatory to support a sustainable fair economy and prevent food forgery and restore consumers trust (LMG, 1992; LMV, 1995 and TSV, 1995). It has been confirmed by different food regulatory agencies and food control authorities that higher valued meats have been replaced with lower valued ones in many countries (Liu, 2006).

"Halal" logo on food products indicates that the products are prepared according to the food consumption guidelines of Islamic law (S'hariah) and there is no prohibition to consume those foods in the view of religious concerns (Ali et al., 2013). Global halal food markets are rapidly expanding because of its special health and religion compliant attributes (Ali et al., 2014). The current consumers of this food market has been reached to 1.8 billion (Anonymous, 2014) and turnover of the overall halal business has crossed 2.3 trillion US Dollars in 2012 (Salama, 2013) and predicted to reach $10 trillion by 2030 (Salama, 2015). Due to specialized processing and supply chain requirements, the price of halal foods are higher than ordinary ones and hence mislabeling of 'Halal' logos have been frequently happening (Ali et al., 2015). A number of halal and non-halal consuming countries such as Malaysia, Indonesia, Turkey, China, Thailand, Australia, India, Brazil, New Zealand, and Singapore are strictly monitoring to control the purity of halal foods in order to capture the global Halal food markets (Jeddah, 2011). As a business policy, the government of Malaysia has strong commitment to create more than 10 integrated 'Halal hubs' to export the halal foods to another Muslims countries (Chen, 2008). Australia has selected Malaysia to produce halal food products for their market, while Malaysian government agreed to set up halal hub with China and Middle East for processing and packaging of Muslims' food in 2002 (www.foodproductiondaily.com, 2002). One of the major meat producer, New Zealand, is keen to pursue the concept of hub for halal meat products from Malaysia sated by Jim Sutton, minister of 'Trade Negotiations and Agriculture', New Zealand (The Star, 2003). With all these investments on halal food products will flourish Malaysia's economic growth and decrease the dependability on imported meat products. Thus it is important to establish an innovative, easily hand able, and inexpensive species authentication techniques for meat industry, marketers and food regulatory organization. In addition, detection of meat contaminant in food and food

products beneficial to the consumers, patients and followers of faiths (Islam, Judaism, Hindus, Vegetarians).

Minced meats are being frequently used in modern foods such as meatballs, burgers and frankfurters (Ali, Hashim, Mustafa, & Man, 2012a) and its detection is extremely difficult under the backgrounds of processed foods. For the detection of meat species in the raw, processed and unprocessed foods, a number of analytical methods such as DNA barcoding (Haye, Segovia, Vera, Gallardo, & Gallardo-Escárate, 2012), microarray chips (Ali, Hashim, Mustafa, Che Man, & Islam, 2012d), PCR (Colgan et al., 2001), PCR-RFLP (Ali et al., 2012a), real-time PCRs with SYBR green (Soares, Amaral, Oliveira, & Mafra, 2013), Eva green (Santos et al., 2012), molecular beacon (Yusop, Mustafa, Man, Omar, & Mokhtar, 2012) and TaqMan probe (Kesmen, Celebi, Güllüce, & Yetim, 2013; Ulca, Balta, Cagin, & Senyuva, 2013), protein based methods includes electrophoretic (Montowska & Pospiech, 2007), chromatographic (Chou et al., 2007) and spectroscopic (Ellis, Broadhurst, Clarke, & Goodacre, 2005), FTIR (Rohman, Sismindari, Erwanto, & Che Man, 2011), ELISA (Asensio, González, García, & Martín, 2008), nucleic acid based biosensor (Ahmed, Hasan, Hossain, Saito, & Tamiya, 2010) and nanoparticle sensors (Ali et al., 2011b) have been documented. Among all the methods, DNA based molecular techniques have got preference since DNA is more stable under compromised states and it provides universal information from all tissues and cells (Aida et al., 2005) to enable authentication of minute amount of defile ingredients in processed and unprocessed foods, especially of animal origins.

Cytochrome b (*cytb*) gene which is located in mitochondrial region of all species having adequate inter-species polymorphisms (highly variable among other species) (Asma, 2013) and intra-species conserved region (Hsieh, Chiang, Tsai, Lai, Huang, Linacre & Lee, 2001; Linacre & Tobe, 2011; Matsuda et al., 2005; Parson et al., 2000) and hence it has been extensively used for species identifications. A carefully chosen

sequence of *cytb* gene would generate distinctive restriction patterns following digest with one or more restriction enzymes, allowing additional confirmation by RFLP analysis. Species-specific PCR assay (Ali et al., 2013; Mane, Mendiratta, & Tiwari, 2012) have been widely used to detect a single amount of target DNA in raw, processed and unprocessed products because of its rapidity, simplicity, sensitivity and specificity (Mafra, Ferreira, & Oliveira, 2007). Therefore, in this study a short-amplicon-length feline-specific PCR assay was developed and validated by RFLP analysis using a lab-on-a-chip based automated electrophoretic system.

1.2 Study Rationale

Feline meats have been consumed in many parts of the world such as Cambodia, China, Thailand, and Vietnam for many years (Podberscek, 2009). Moreover, cat population is huge in many countries and could be easily obtained without any offered prices and having a significant chance to mix them in halal foods. Surely, cat meat consumption is forbidden in Islam and Judaism and it is a potential carrier of hepatitis, SARS, anthrax and some other deadly diseases (Anitei, 2006). It has been well believed by researchers that the most incurable and transferrable disease, HIV (Human Immunodeficiency Virus), AIDS (Acquired Immune Deficiency Syndrome), has been transmitted to human race from African chimpanzee (Fajardo et al., 2010). On the other hand, some meat scandal issue (i.e.: horse meat in Europe (Castle, 2013; Premanandh, 2013), pig and rat meat in China (Ali, Razzak, & Hamid, 2014) and cat meat in China and UK (Phillips, 2013 and Chatterji, 2013)) have contributed to the concern of Muslim consumers in defining the presence of forbidden biomaterials in the marketed foods (van der Spiegel et al., 2012). Since feline ingredients in foods is a sensitive issue, hence a

method must be developed for its reliable detection under complex and processed food matrices.

1.3 Problem Statements

Morphological, lipid and protein based species identification schemes is not reliable for the analysis of processed foods. DNA based methods for species authentication is reliable but most of the documented methods for feline species detection involve very high amplicon-lengths which often break down under compromised conditions. Thus the longer fragment might not be suitable for target species-detection in processed foods. To the best my knowledge, this a first time report for a very short amplicon-length (69-bp) PCR assay for the detection of feline meat in processed foods.

1.4 Research Objectives

The aim of this research is to develop a molecular techniques based on short amplicon-length polymerase chain reaction (PCR) and validate its reliability by restriction fragment length polymorphism (RFLP) analysis to identify the identity of feline meats in processed foods. Therefore, the objectives of this study were:

I. To develop short-length PCR-biomarkers for feline species detection.
II. To characterize the developed biomarkers under complex matrices and food processing conditions.
III. To apply the biomarker for the detection of the feline materials in processed and commercial food products.
IV. To authenticate the amplified target product by RFLP analysis.

1.5 Scopes of Work

Short-length DNA markers are of enormous significance in biosensors (Jung, Mun, Li, & Park, 2009), biochip (Iwobi, Huber, Hauner, Miller, & Busch, 2011) and forensic applications (Aboud, Gassmann, & McCord, 2010). Short DNA markers offers better target recovery from decayed samples, amplify efficiently, dispersed with higher resolution and demonstrates extraordinary stability in harsh environments (Aboud et al., 2010). Currently, a number of PCR based assays have been developed with long DNA fragments which are not reliable for the verification of highly degraded mixed biological and commercial specimens. Consequently, a short-amplicon-length PCR assay has a countless demand in forensic investigations (Hird et al., 2006). However, developing a short-length PCR assay needs extra care since within a short-sequence region, there should be adequate species-specific fingerprints. PCR technique combined with restriction fragment length polymorphism (RFLP) scheme can authenticate real PCR products if the targets contain suitable restriction site within it (Aida, Che Man, Wong, Raha, & Son, 2005; Murugaiah et al., 2009). Therefore, in this study, a PCR and PCR-RFLP assay with short-amplicon-length was proposed containing DNA markers which are present in multiple copies per cell (Murugaiah et al., 2009) and have proper restriction sites inside it. Further, the assay will be optimized and validated under complex matrices of commercial meat products to make them trustworthy for the screening of commercial foods.

1.6 Outline of the Present Work

This dissertation comprises of six chapters. The contents of the individual chapters are outlined as follows:

Chapter 1: This chapter has briefly described the background information regarding the potential reasons and negative impact of adulteration, currently available meat authentication techniques, significance of using mt-cytb gene as a target, research gap and study objectives.

Chapter 2: A review of literature on existing methods to identify animal species in foods and feeds, importance of mitochondrial gene, short length biomarker and prospect of processed foods addressed in this chapter. The reasons to select short length DNA biomarker as a target of my method is outlined.

Chapter 3: In this chapter, the materials, procedures, equipment, instruments, bioinformatics tools used for designing biomarker and in-silico analysis of restriction sites have been presented. Additionally, the details of specificity, sensitivity and target stability under various matrices have also been stated.

Chapter 4: Out comes all the analytical and experimental of short length biomarker and their application in raw, treatment, admixtures, processing and commercial meat products are illustrated in this chapter.

Chapter 5: This chapter has discussed and compared all of the experimental analysis with the previously published literatures.

Chapter 6: This is the last chapter and wraps up the dissertation with some concluding remarks and recommendations for future works.

CHAPTER TWO

LITERATURE REVIEW

CHAPTER 2: LITERATURE REVIEW

2.1 Prevalence of Meat Species Adulteration

Adulteration, i.e. substitution of more expensive materials with inexpensive materials is a mutual global practice which is forbidden by the federal and state regulations (Liu, 2006). Selling of fake meats in the name of pure meats as well as fraudulent labeling of meat ingredients have been rampant in many countries such as China, The United Kingdom, Turkey, Australia, Canada, the United States and across the Europe (Ayaz, Ayaz, & Erol, 2006; Odumeru, Boulter, Knight, Lu, & McKellar, 2003). Approximately, 19.4 % of meat products in the United States (Hsieh et al., 1995), 22 % in Turkey (Ayaz et al., 2006) 15 % in Switzerland, and 8 % in the United Kingdom were found to be mislabeled (Ballin, Vogensen, & Karlsson, 2009). In turkey undeclared species was found in fermented sausages (39.2%), cooked salami (35.7%), frankfurters (27.2%), meatballs (6.2%), and raw meats (22.2%) (Ayaz et al., 2006) and no beef was found as 5% beef in sausage sample (Ali et al., 2014). Another survey was conducted on the restaurant industries and it was confirmed that only 9 out of 37 samples contained accurate ingredients of Mediterranean fish species (Asensio, 2008a). Additionally, Zha et al. (2010) demonstrated that fraud labeling in deer products especially heart, blood, and antler has been taken place frequently. More recently, in China, rat and pork meats were sold as lamb and mutton and for involvement in this event 904 criminals were arrested by Chinese police. They were involved in an illegal marketing of pork, rat, fox, mink, and other meats after treating with additives like gelatin and sold them as a lamb (Ali et al., 2014). Another thunder bold was fallen down on the Shaanxi state in China, where more than 20 tons of fake beef which was processed with chemically treated pork were seized by police (Jeanette, 2013). On the other hand, porcine DNA in Cadbury chocolates (Rahman et al., 2014), even though it has been resolved by the religious department of

the Malaysian authority after its initial detection, and bread made up with pork, lard and casings of pig intestine in sausages sample (Che Man, Aida, Raha, & Son, 2007) are an alarming issues in Malaysia. These incidents and happenings have put us on red alert that nothing could be granted for authenticity without proper testing and verification.

2.2 Potentiality of Feline Meat Adulteration

For ethical and sympathetic concerns, certain section of population does not like to consume the meat of pigeon and pet animals (Haunshi et al., 2009) Islam and Judaism do not permit a trace amount of pork meat according to the food consume guidelines in their diet. Vegetarians and semi-vegetarians are another group of the culture who are completely negative to eat any types of meats in their dishes. A large number of consumers choose to take more chicken in the place of beef and pork while the communities of Hindus and Buddhist religion do not prefer to consume beef meat as a religious' faith (Bonne & Verbeke, 2008). Similarly, feline meats or materials are not permitted to consume in certain food consumptions guidelines such as Islam and Judaism and undeclared feline meat as adulterant in another meat products might cause food allergy. It has also been reported that cat meat was sold as a rabbit meats after repackaging by slaughterers in eastern China (Phillips, 2013) and was served as Indian curry in the UK restaurant in 2013 (Chatterji, 2013) (Figure 2.1). An illegal slaughter house was dig up by the Chinese police since the mixing of thousands of domestic cat was unearthed as a horrifying episode to the consumers (Phillips, 2013). A latest report indicated that, a large number of dead body of stray and domesticated cat were sold as profitable and illegal trading, whereas in Guangdong and Guangxi southern provinces of China it was sold at 10 yuan (£1) for each animal to be used as contaminant in the regular food chain system (Phillips, 2013).

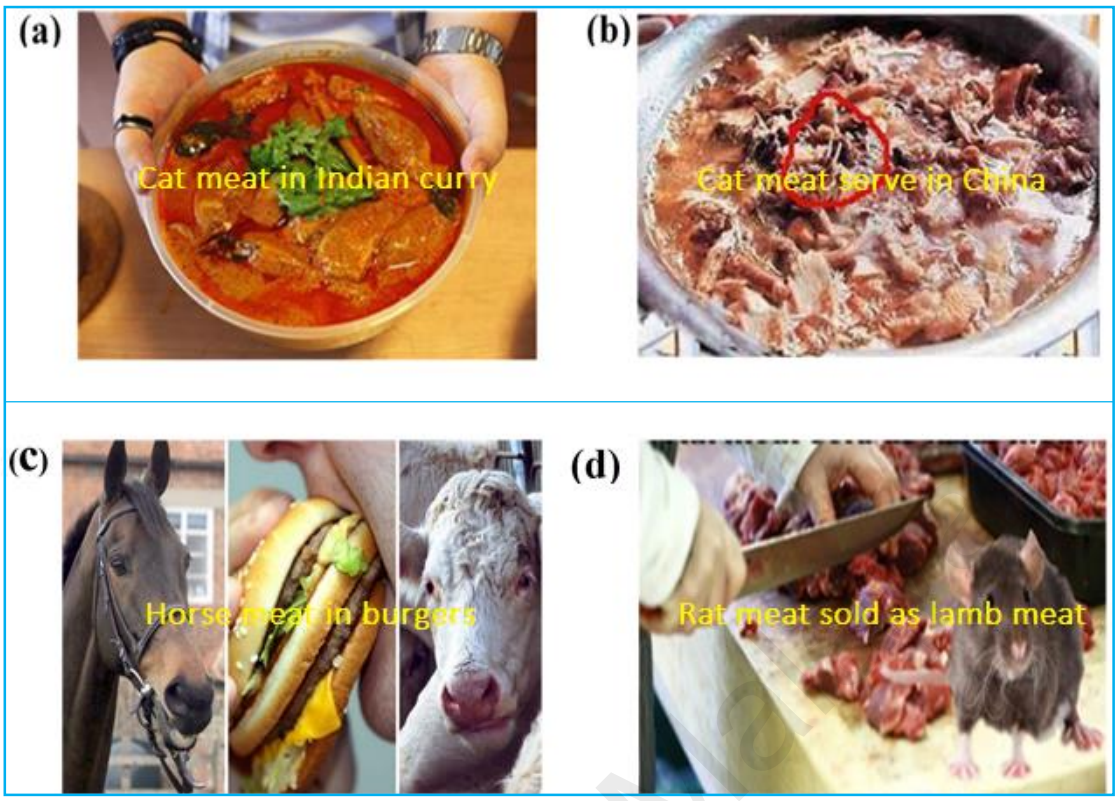


Figure 2.1 Recent meat scandal in food chain. In (a), cat meat served as Indian curry in UK restaurant in 2013. In (b), cat meat in a boiling pot to serve in China. In (c), horse meat found in burgers in Europe. In (d), rat meat sold as lamb meat in China. (Source: some public safety blog and http://www.guardian.co.uk/world/2013/may/03/china-fake-meat-rat-mutton).

2.3 History of Feline Meat Consumption

From the ancient time to still now, regardless of the aim of domestication feline meat has been used human consumption (Podberscek, 2009). In Cyprus, domestic cat was consumed in the early period of 8,500 BCE (Vigne, Guilaine, Debue, Haye, & Gérard, 2004). Later, it was spread out to the United Kingdom in the 17th century (Thomas, 1991), in China in the 14th century (Podberscek, 2009), and in France and Germany in the 18th century (Ferrieres, 2006). Nowadays, though cat meat was out lawed in certain countries

and huge protection has been given by animal welfare group, consumption of feline meat has been reported in Cambodia, China, Thailand, Vietnam, South Korea, some parts of Europe, Russia, Africa and Latin America (Podberscek, 2009).

Historically, cat meat has been consumed as part of traditional cultures, health benefits, and religious belief and in some countries its consumption has been reflected as a symbol of national pride (Podberscek, 2009). For instances, cat meat is taken as an aphrodisiac and used for a treatment of rheumatism and arthritis (Podberscek, 2009). It has been estimated that about 100,000 cats are killed in South Korea, whereas 4 million cats are eaten every year in China (Bartlett & Clifton, 2003). Additionally, a liquid or 'juice' was prepared from cats for consumption as a 'tonic' for health benefits in South Korea and China (Podberscek, 2009), thus it is most popular for giving warming effect in the winter seasons (Podberscek, 2009). Since there is no census data for cat population in many parts of the world and there is no open market for selling cat meats, thus it could be considered as a highly potential adulterant in halal foods and meat products.

2.4 Risk of Cat Meat Consumption

Feline meat are most potential carriers of hepatitis, SARS (Severe Acute Respiratory Syndrome), anthrax and some other deadly zoonoses (Anitei, 2006). Research shows that sourcing, farming, transporting, slaughtering and consumption of cats can assist in the transmission of cholera, trichinellosis and rabies (Podberscek, 2009). In addition, researchers have found that the animal originated 'zoonotic' threats like severe acute respiratory syndrome (SARS) initiated from the live animal markets of China via human to human transition (Anitei, 2006).

2.5 Biomarkers in Species Detection

Nucleic acids (i.e. DNA and RNA) are considered as the blue print of life and proteins are as the building blocks of all living organisms. When nucleic acids (DNA and RNA) and proteins work for biological indicators of the physiological state of all organisms, they are termed as biomarkers (Vu, 2011). Biomarkers or genetic marker is commonly used as a recognition elements for the precise detection of the sources of biological materials (DNA, RNA, proteins). Thus biomarker based assays are the most important advanced techniques to detect crime in forensic sciences (Andreasson, Nilsson, Budowle, Lundberg, & Allen, 2006), control food contamination and animal consumption (Maria. et al., 2011) and prevent the illegal trade of plant, animal and others endangered species (Kyle and Wilson, 2007).

2.5.1 Mitochondrial DNA

Mitochondrial DNA has played a significant role both in the field of human and animal forensics with numerous scientific applications (Matsuda et al., 2005; Nelson & Melton, 2007; Rastogi et al., 2007). It has been also popularly used in population genetics and phylogenetic analysis (Bataille, Crainic, Leterreux, Durigon, & de Mazancourt, 1999; Nakaki et al., 2007; Pereira, Meirinhos, Amorim, & Pereira, 2006). A number of reasons and benefits behind the using of mtDNA for the development of biomarkers over the nuclear DNA are: (1) mitochondrial DNA found in all biological materials; (2) Mitochondrial DNA is more abundant (100 – 1000x) than genomic DNA (Bellis, Ashton, Freney, Blair, & Griffiths, 2003; Prado, Calo-Mata, Villa, Cepeda, & Barros-Velazquez, 2007); (3) provide more information due to the codon degeneracy; (4) protected by its own mitochondrion organelle and thus preserved it from all degraded condition; (5) mitochondrial DNA is highly variable (Prado et al., 2007) and mutation fixation is much faster than nuclear DNA (Murugaiah et al., 2009).

2.5.2 Cytochrome B Gene (*Cytb* Gene)

A number of mitochondrial genes or regions namely cytochrome b, cytochrome oxidase (i.e. CO1, CO2 and CO3), ATP6, 12S rRNA, 16S rRNA, NADH dehydrogenase subunit (i.e., ND1, ND2, ND3, ND4, ND4L, and ND5) and D-loop (Figure 2.2) can be used for the design of biomarker for species identifications in food and food mixtures (Vu, 2011). Among all the mitochondrial genes, cytochrome b is an ideal candidate which is commonly used in species detection as it is species specific (Hsieh et al., 2001; Linacre & Tobe, 2011; Matsuda et al., 2005; Parsons et al., 2000), and therefore, inter and intra species relationships are additional advantageous to create molecular phylogeny (Birstein, Doukakis, Sorkin, & DeSalle, 1998) and evolutionary study (Kvist, 2000). Mitochondrial encoded cytochrome b gene is commonly used in phylogenetic work since it contains both conserved and variable regions which are adequate to resolve divergence at population level. However, cytochrome b gene was found to more informative than others gene (Aida et al., 2005). It can be easily distinguish with relatively short-amplicon-length fragment which survive under different compromised state (Tobe & Linacre, 2008).

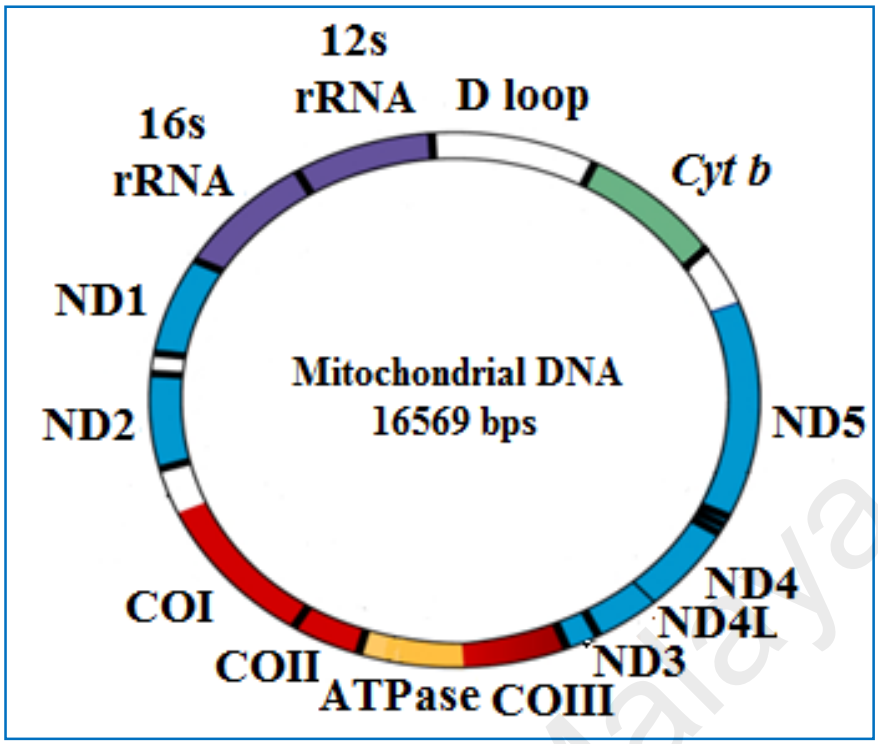


Figure 2.2 Mammalian mitochondrial genome. The gene order is the same in all mammalian species.

2.6 Current Methods in Species Authentication

Up to date, many methods have been developed to detect animal-derived materials in food and feeds. These range from conventional methods applying protein, lipid and DNA-based recognitions (i.e. species specific PCR primers, PCR-RFLP and real time PCR) to nanotechnology based assays. An overview of the current detection methods is briefly presented below:

2.6.1 Protein Based Methods for Species Authentication

Several protein based methods have been used for species identification in meat products. They are predominantly divided on the immunochemical and electrophoretic analysis of protein biomarkers.

Isoelectric focusing (IEF), gel electrophoresis can be used to separate protein profile of minced meat mixtures on polyacrylamide gel based on P^H gradient. The separated proteins are stained with coomassie blue or silver staining reagents. IEF has successfully been applied to discriminate meat and fish species in a raw state but it is not applicable in heat or processed background since the heat soluble proteins are denatured and degraded easily and quickly at extreme temperature and pressure. Furthermore, isoelectric focusing technique cannot distinguish closely related species and often provides identical protein profiles and require huge amount of high quality of proteins. On the contrary, sodium dodecyl sulfate - capillary gel electrophoresis (SDS-CGE) is an alternative form of protein analytical process as it can isolate hundreds of components simultaneously and highly sensitive and easily automated (Vallejo-Cordoba & Cota-Rivas, 1997).

Liquid chromatography (LC) and high performance liquid chromatography (HPLC) protein based methods developed to focus on protein profiles and capable to detect animal specific histidine dipeptides, carnosine, anserine, balenine, respectively in ruminant feed (Aristoy & Toldra, 2004; Schönherr, 2002). Both of these methods are sensitive and can identify as low as 1% (w/w) and ± 0.5% in pure binary mixtures of chicken and turkey (Ashoor & Osman, 1987), and known animal mixtures (Aristoy & Toldra, 2004; Schönherr, 2002) separately. However, these methods cannot specify the exact source of animal derived proteins in a complex matrices (Aristoy & Toldra, 2004). Although, mass spectrometry and gel digestion are of electrophoretic techniques combined with novel proteomic tools which were successfully used to apply species-specific sarcomeric proteins (myosin light chain) identifications in processed mixtures of different species (Martinez & Jakobsen Friis, 2004; Pischetsrieder & Baeuerlein, 2009) these techniques are laborious, costly, requires skilled personnel for handling and not reliable for complex mixtures (Pischetsrieder & Baeuerlein, 2009).

Another protein based technology is suitable for testing species determination in food and feed components through immunological approaches including enzyme linked immune-sorbent assay (ELISA) technique (Figure 2.3). In this methods, antibodies rose against a target antigen (a substance that produce an immune response) are immobilized to a solid surface followed by the detection of antigen-antibody interactions by the virtue of a labeled enzyme that converts a suitable substrate into a color product or releases an ion which reacts with another reactant to generate a detectable change in color (Bonwick & Smith, 2004). However, this approaches can be applied on site and result can be get within 15 min (Muldoon, Onisk, Brown, & Stave, 2004) but it is time consuming, need specially trained experts and sometimes give false result with blood contaminated meat from other species.

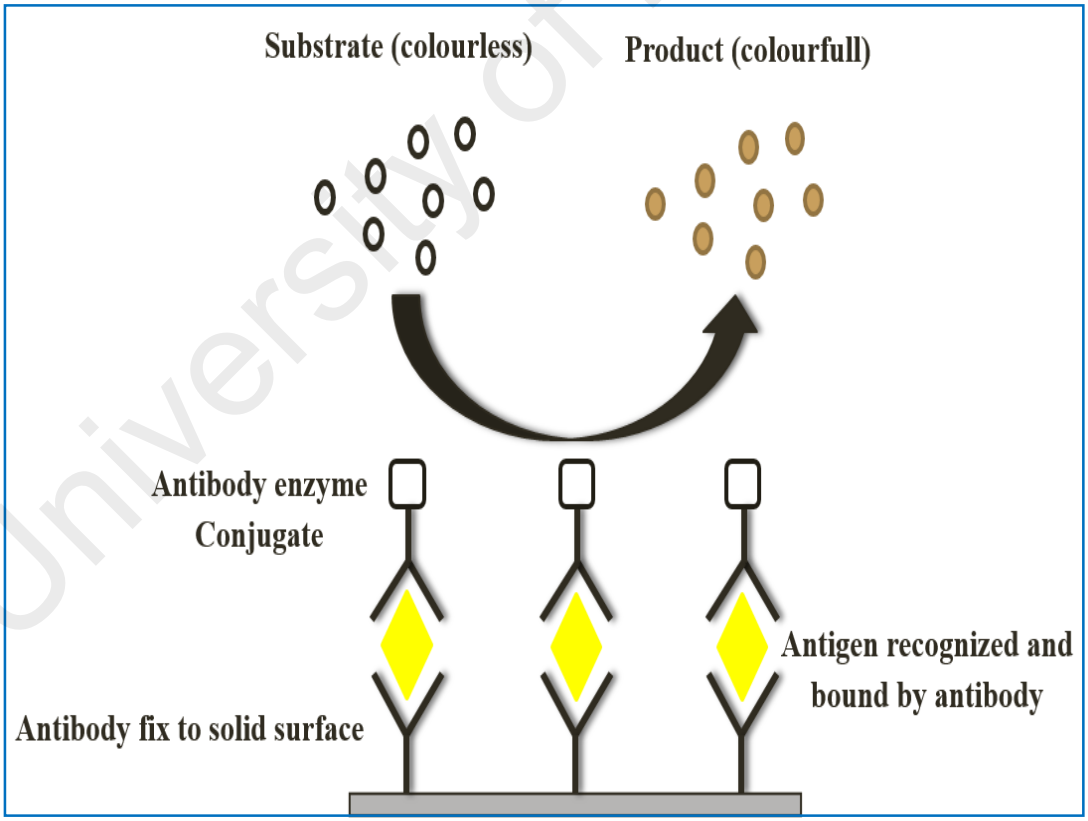


Figure 2.3 Overall process of enzyme linked immune-sorbent assay (ELISA) technique.

2.6.2 Lipid Based Methods for Species Authentication

Lipid based analytical tools have limited applications in food and foodstuff for species determinations and are less reliable. Fats and fatty acids components used in the preparation of processed food products (Marikkar, Ng, & Man, 2011; M. Nurjuliana, Che Man, Mat Hashim, & Mohamed, 2011b; Rohman et al., 2011) can be determined by Fourier transform infrared (FTIR) spectroscopy. Moreover, gas chromatography-mass spectrometry (GC-MS) is a powerful and sensitive technique that performs an important role to identify the lard content which is used to replace vegetables oil in processed foods. This techniques demands well trained experts to operate the instrument, more expensive and time consuming; though it can produce precise and reliable results (M. Nurjuliana et al., 2011b; M Nurjuliana, Man, & Hashim, 2011a). Electric nose which is also known as e-nose is an up to date non-destructive, fast and easily operative analytical tool applied in food industry to monitor quality control, processing, cleanliness, legitimacy and shelf-life (Peris & Escuder-Gilabert, 2009). Conversely, e-nose finding has to be validated by a more reliable methods such as GC-MS.

2.6.3 DNA Based Methods for Species Authentication

DNA based methods have been widely used and are becoming more popular for the identification, quantification, detection and monitoring of adulterated species in food and feeds due to its inexpensiveness, rapidity and accuracy (Ali et al., 2012b). The advantages of DNA analysis are: (1) DNA-based approach are more appropriate for analysis of heat-treated products as DNA is more stable to extreme temperature and pressure (Lockley & Bardsley, 2000); (2) the presence and characteristics of DNA are independent of the cell types, (i.e. Identical genetic information is contained in different samples such as blood, muscle or bone); (3) DNA consists numerous information due to

the genetic code degeneracy. Table 2.1, represents a summary of the key characteristics of the different DNA-based analytical approaches.

Table 2.1 Key characteristics of the different DNA-based analytical approaches

Features	**PCR**	**PCR-RFLPs**	**Real Time PCR**
Quantity of information	Adequate	Adequate	High
Prerequisite of prior information	Yes	Yes	Yes
Applicable to detection of admixtures	Yes	Yes	Yes
Inter-laboratory reproducibility	High	Poor	High
Sensitivity	Moderate	Moderate	High
Throughout capacity	Moderate	Moderate	High
Compulsion of end point detection	Yes	Yes	No
Cost of equipments and reagents	Cheap	Cheap	Expensive
Ease of use	Simple	Simple	Difficult, need trained experts

2.6.3.1 Polymerase Chain Reaction (PCR)

The polymerase chain reaction (PCR) methods used to amplify a segment of specific DNA fragment present in a complex mixtures of a other DNA molecules thus produce more than thousands of identical copies, and the technique was invented by Kary Mullis in 1983. There are three fundamental steps involved in a PCR cycling reaction known as (1) denaturation, (2) annealing and (3) elongation and every steps are completed on the basis different temperatures. Denaturation is the first step of PCR reaction starts at 94°C that breakdowns the double stranded DNA in to two single stranded DNA. During annealing, the temperature is increased from 52°C to 65°C, enables to bind each primer

specifically to the 3´ end of the target sequence on the appropriate strands of DNA. At the final or extension stage, the temperature is raised to 72 ˚C to enable the synthesis of nascent single stranded DNA from the template using the free nucleotides and the enzymatic activity of Taq DNA polymerase prior to the start of next round denaturation (Figure 2.4). Therefore, the amplified PCR products is run on agarose gel or polyacrylamide by ethidium bromide or other non-carcinogenic DNA staining with an appropriate molecular size marker after completion of all the desired cycles of amplifications. The methods, polymerase chain reaction established itself to be more accurate, highly sensitive, robust (Ali, Hashim, Mustafa, & Che Man, 2011a; Che Man, Mustafa, Khairil Mokhtar, Nordin, & Sazili, 2012; Yusop et al., 2012), easy to handle without any expensive equipment and chemicals (N.S. Karabasanavar, Singh, Kumar, & Shebannavar, 2014) and is currently the most widely used for the detection of different animal species in raw, processed and commercial food products. The brief history of species-specific PCR assays in meat species verification is documented in Table 2.2.

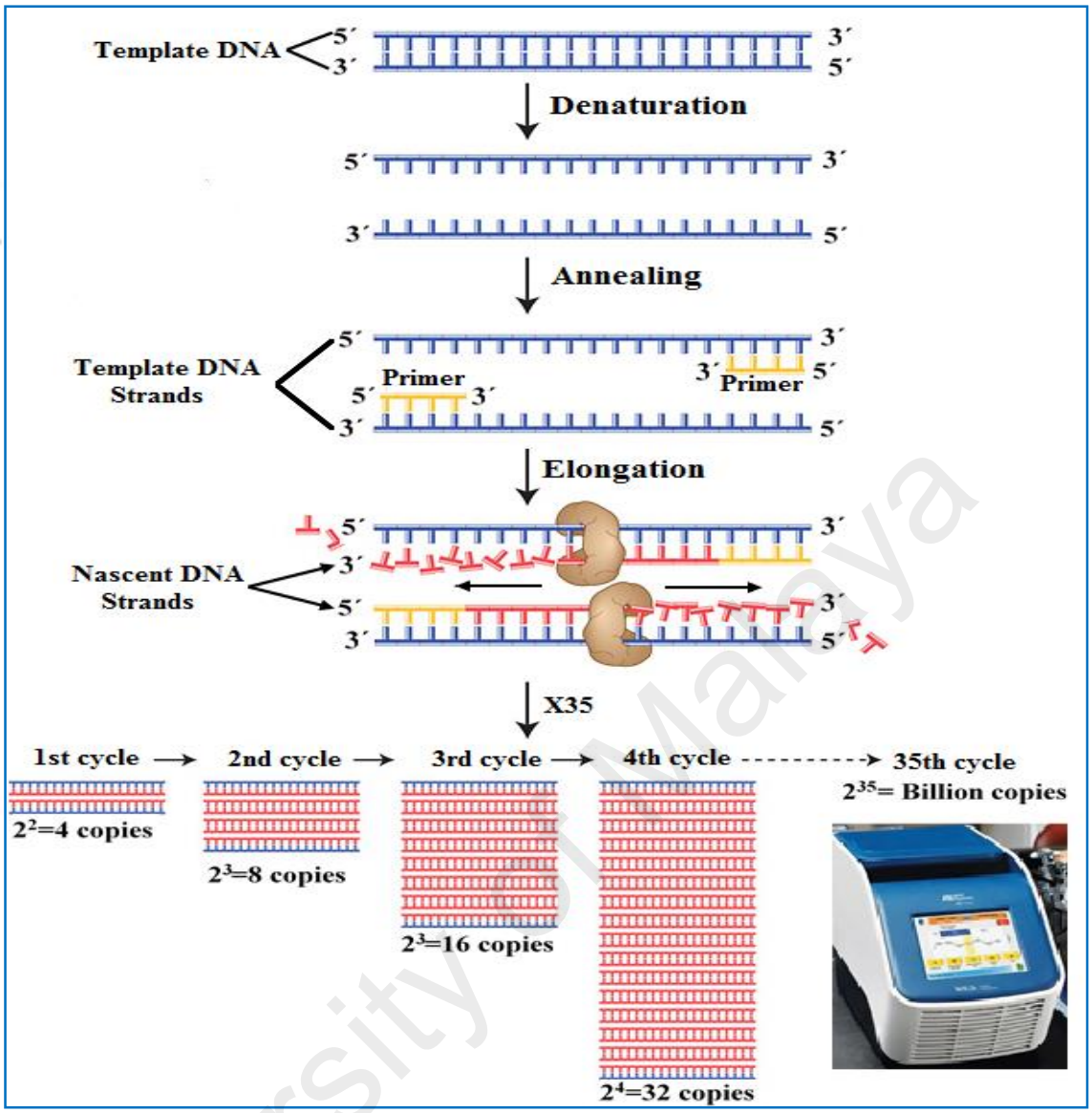


Figure 2.4 Basic principles of PCR protocol

Table 2.2 Identification of meat species using species-specific PCR assay

Detected species	**Target gene(s)**	**Amplicon-length (bp)**	**Detection limit (ng)**	**References**
Dog and cat	*Cytb*	808 and 672	0.01%	Abdulmawjood et al. (2003)
Buffalo, cattle, pig and sheep	SSR gene and *Cytb*	603, 100, 374 and 359	Not Mentioned	Ahmed et al. (2010)
Pig	*Cytb*	360	Not Mentioned	Aida et al. (2005)
Dog (*Canis familiaris*)	*Cytb*	100	0.01%	Ali et al. (2013)
Dog (*Canis familiaris*)	*Cytb*	100	0.01%	Rahman et al. (2014)
Beef and bovine	Satellite DNA	84 and	0.01%	Calvo et al. (2002)
Pork	12S rRNA	387	0.01%	Che Man et al (2007)
Pork	leptin gene	152	Not Mentioned	Farouk et al. (2006)
Chicken, duck, pigeon and pig	D-loop and *Cytb*	256 , 292, 401 and 835	Not Mentioned	Haunshi et al. (2009)
Chicken	Actin gene	391	Not Mentioned	Hopwood et al. (1999)
Bovine, ovine and caprine	12S rRNA	84, 121 and 122	0.1%	Martin et al. (2007)

Table 2.2 Continued

Detected species	**Target gene(s)**	**Amplicon-length (bp)**	**Detection limit (ng)**	**References**
Goat (*Capra hircus*)	D-loop	436	0.1 pg	Karabasanavar et al. (2011a)
Pork, horse and donkey	ND5, ATPase 6 & ATPase 8 (horse) and ND2	227, 153 and 145	0.01 ng	Kesmen et al. (2007)
Mutton (*Ovis aries*)	D-loop	404 and 329	0.1%	Karabasanavar et al. (2011b)
Goat (*Capra hircus*)	D-loop	294	Not Mentioned	Kumar et al. (2011)
Pork	AMEL	562 and 741	0.1%	Langen et al. (2010)
Horse, dog, cat, bovine, sheep, porcine, and goat	Mt-DNA	439, 322, 274, 271, 225, 212, and 157	0.5% and 0.1%	Ilhak et al. (2006)
Chicken	D-loop	442	1%	Mane et al. (2009)
Beef	D-loop	513	Not Mentioned	Mane et al. (2012)
Four duck species	12S rRNA	64 and 97	0.1%	Martin et al. (2007)
Cat, dog, and rat	12S rRNA	108, 101, and 96	0.1%	Martin et al. (2012)
Rabbit (*Oryctolagus cuniculus*)	12S rRNA	720	0.1%	Martin et al. (2009)

Table 2.2 Continued

Detected species	Target gene(s)	Amplicon-length (bp)	Detection limit (ng)	References
Cattle, pigs, sheep and chickens	COXI, 12S rRNA and 16S rRNA	90, 85, 67 and 66 bp	0.1, 0.08 and 0.09%	Natonek et al. (2013)
Goose (*Anser anser*), mule duck (*Anas platyrhynchos*), chicken (*Gallus gallus*), turkey, (*Meleagris* gallopavo), and swine (*Sus scrofa domesticus*)	12S rRNA	392, 394, 400,402 and 404	0.1%	Rodriguez et al. (2003)
Yak and cattle	12S rRNA	290 and 159	0.1%	Yin et al. (2009)
Five deer species	D-loop	140- 303	Not Mentioned	Parkanyi et al. (2013)
Cow, pig, chicken and ruminant	NM	98,134, 169 and 100	0.05%-0.0005%	Walker et al. (2003)
Chicken (*Gallus gallus*)	5-aminolevulinate (ALA) synthase gene	288	0.1%	Karabasanavar et al. (2013)
Pork (*Sus scrofa domesticus*)	D-loop	772	0.1%	Karabasanavar et al. (2014)
Goats, chickens, cattle, sheep, pigs, horses, and rats	*Cytb*	157, 227, 274, 331, 398, 439 and 603	1%	Nuraini et al. (2012)
Pork	D-loop	531	0.05%	Montiel Sosa et al. (2000)

2.6.3.2 PCR- Restriction Fragment Length Polymorphisms (PCR-RFLP)

A thoroughly applied technique PCR-Restriction Fragment Length Polymorphisms (PCR-RFLP) has been used to species detection in meat and meat products (Girish et al., 2005; Maede, 2006). It involves in the generation of species-specific band pattern after the digestion of amplified PCR products with a single or set of restriction endonucleases to find out genetic distinction between species (Doosti et al., 2011; Murugaiah et al., 2009; Sait et al., 2011) and therefore, the digestive fragment could be visualized using labelled probe on a solid support (i.e. Southern blotting) or by treating the electrophoretic gel with ethidium bromide or silver staining (Figure 2.5). Although this technique is time consuming, require additional restriction enzyme and interspecies mutation could be occurred at restriction site which may lead to dubious results (Filipe Pereira et al., 2008), it is inherently more accurate than the species-specific PCR and can distinguish closely related species by the characteristic restriction fingerprints (Ali et al., 2011a; Doosti et al., 2011; Sait et al., 2011). Nevertheless, an automated lab-on-a-chip electrophoretic technology, a recent advance to separate the complex restriction DNA fragments (>10 bp) in length, therefore subjected to need a Bioanalyzer adopted with microfluidic methods for product separation. The Agilent 2100 Bioanalyzer is the first commercially accessible system with chip based nucleic acid separation tools. Additionally, microfluidic separation device entail an extra cost but provide automation, fast, high resolution, and reproducible separation of both smaller and larger length oligo fragments (Ali et al., 2012b). In Table 2.3; summarized the PCR-RFLP assay commonly used for species authentication in meat industry.

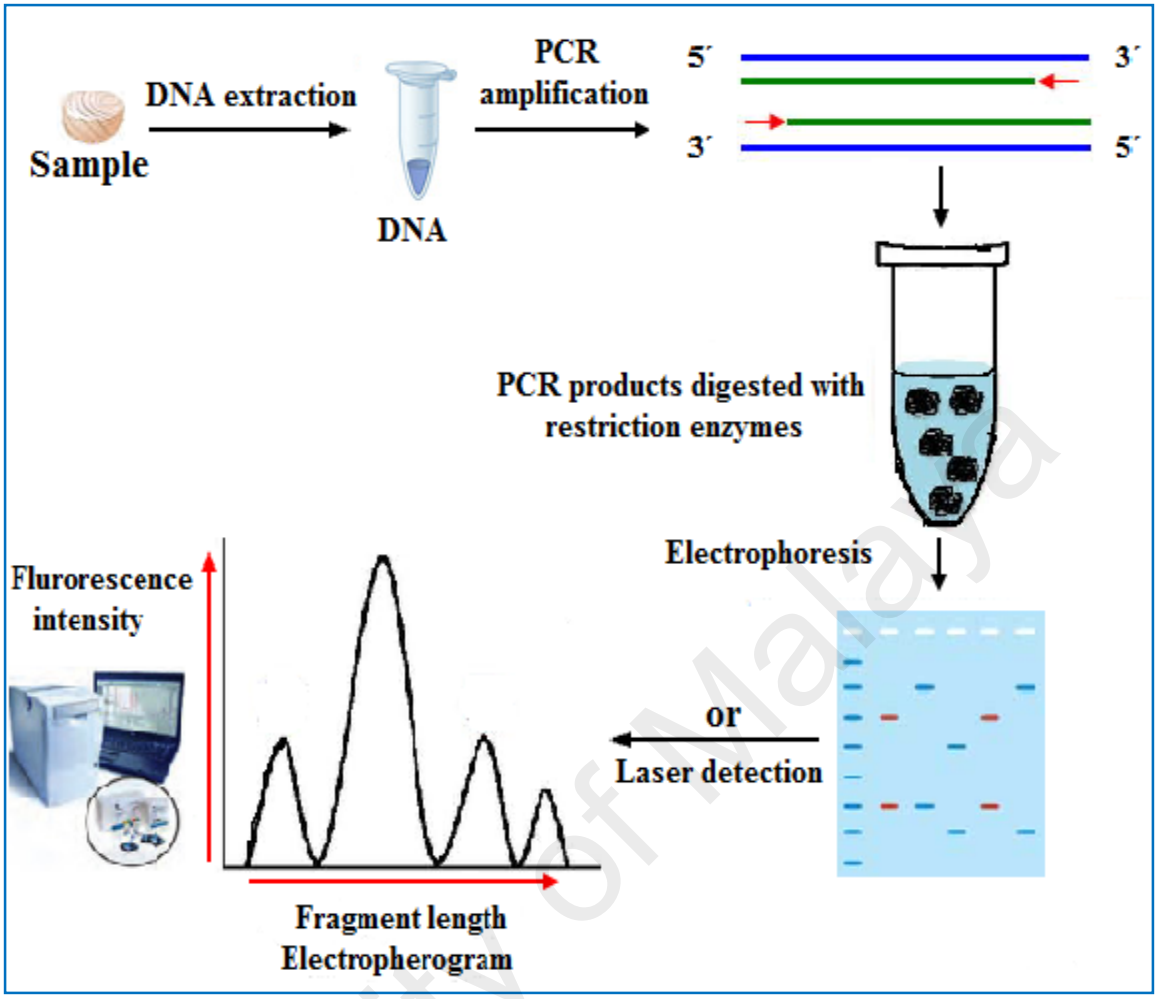


Figure 2.5 Various steps of PCR-RFLP assay

Table 2.3 Identification of meat species using PCR-RFLP assay

Detected species	**Target gene(s)**	**Restriction enzymes**	**References**
Porcine, bovine, ovine, avian, cervine and equine	*Cytb*	*Alu*I and *Hin*fI	Maede et al. (2006)
Beef, cara beef, chevon, mutton and pork	*Cytb*	*Alu*I and *Taq*I	Kumar et al. (2011)
Cows (*Bos taurus*)	*Cytb*	*Rsa*I and *Mva*I	Prado et al. (2007)
Poultry meat	*Cytb* and 12S rRNA	*Aci*I, *Alu*I, *Ava*II, *Dde*I, *Hae*III, *Hin*fI, *Hha*I, *Mbo*I, *Mse*I and *Taq*I	Stamoulis et al. (2010)
Beef, sheep, pork, chicken, donkey, and horse	*Cytb*	*Alu*I	Doosti et al. (2011)
Cow, chicken, turkey, sheep, pig, buffalo, camel and donkey	COI	*Hind*II, *Ava*II, *Rsa*I, *Taq*I, *Hpa*II, *Tru*1I and *Xba*I	Haider et al. (2011)
Cattle, buffalo, sheep and goat	12S rRNA	*Alu*I, *Hha*I, *Apo*I and *Bsp*TI	Girish et al. (2004)
Five animal species	*Cytb*	*Alu*I	Minarovic et al. (2010)
Pig, bovine, and chicken	*Cytb*	*Bse*DI	Erwanto et al. (2012)
Dog	*Cytb*	*Alu*I	Rahman et al. (2015)

Table 2.3 Continued

Detected species	**Target gene(s)**	**Restriction enzymes**	**References**
Pork	*Cytb*	*Alu*I	Ali et al. (2011c)
Commercial beef	12S rRNA	*Alu*I and *Bfa*I	Chen et al. (2010)
Beef *(Bos taurus*), pork (*Sus scrofa*), buffalo (*Bubalus bubali*), quail (*Coturnix coturnix*), chicken (*Gallus gallus*), goat (*Capra hircus*), rabbit (*Oryctolagus cuniculus)*	*Cytb*	*Alu*I, *Bsa*JI, *Rsa*I, *Mse*I, and *Bst*UI	Murugaiah et al. (2009)
Chicken	Actin gene	*Bg*I and *Hinf*III	Hopwood et al. (1999)
Cattle, buffalo, goat and sheep	12S rRNA	*Alu*I, *Hha*I, *Bsp*TI and *Apo*I	Mahajan et al. (2011)
Beef	D-loop	*Bam*HI	Mane et al. (2012)

2.6.3.3 Real Time PCR

A revolution has been made through the invention of real-time PCR because of its specificity, sensitivity, rapidity, and automation in the era of PCR technology, in the field of molecular biology, and molecular diagnostics without end point analysis (Ali et al., 2012b). In real time PCR, two types of dye chemistries are adapted, (1) non-specific detection using DNA binding dyes that introduces with double-stranded DNA in a blind way, and (2) specific detection with target DNA specific probes containing oligonucleotides which are labeled with a fluorescent reporter dye (R) on the 5´ end and a quencher (Q) on the 3´ end. The first type of fluorescence chemistries of real time PCR using SYBR green I (Farrokhi and Jafari Joozani, 2011) and Eva Green (Ihrig et al., 2006) that amplified both specific and nonspecific double stranded (ds-DNA) and sometimes provide false detection. The second category of fluorescent dye chemistry such as TaqMan probe (Fajardo et al., 2010; Koppel et al., 2011; Rojas et al., 2010) and Molecular Beacon probe (Yusop et al., 2011), allows additional target screening by the means of probe hybridization and are more specific in species determination in terms of species-specific PCR approach (Ali et al., 2012b).

In real-time PCR technology, the most important parameter is the threshold cycle (Ct) (Herrero et al., 2011) or quantification cycle (Cq) (Ali et al., 2012a & 2012b), that is defined as the cycle at which fluorescence is first observed at a statistically significant level which is above the baseline fluorescence or background signal (Figure 2.4) (Heid et al., 1996), thus the amplification detection is measured using the value of Ct. The threshold cycle (Ct) is inversely proportional to DNA amount in which higher quantity of template DNA will result in a lower Ct value. The real-time PCR can detect very short DNA fragments without end point analysis that reduces the risk of contamination (Von Wurmb- Schwark et al., 2002).

The assay works very well for the detection of single copy of quantities of a gene (Alonso et al., 2013); though this technique is restricted by comparatively extra budget derived from exclusive instruments and chemicals (Lopez-Andreo and others, 2005; Gizzi and others, 2003). An overall documentary in the field of Real Time PCR systems for different meat species identification are presented in Table 2.4.

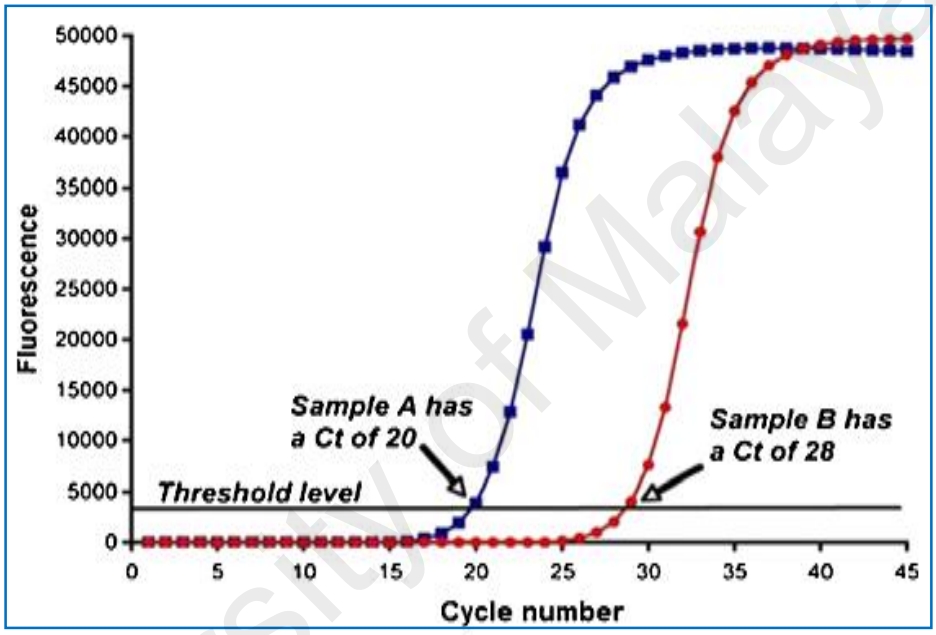


Figure 2.6 Threshold cycle (Ct) or quantification cycle (Cq) and calculation of target DNA copy number. The quantity of DNA doubles at each cycle of the exponential phase and can be calculated using the relative Ct values. Permission taken from Ali et al. (2014).

Table 2.4 Real-Time PCR in species detection. Permission taken from Ali et al. (2014)

Detected species	Target region (genes)	Limit of detection (ng)	References
TaqMan Chemistry			
Bovine (*Bos taurus*) and buffalo (*Bubalus bubalis*)	*Cytb*, 16S rRNA	Not Indicated	Drummond et al. (2013)
Seagull (*Larus michahellis*)	ND2	0.1	Kesmen et al. (2013)
Pork (*Sus scrofa*)	Not Mentioned	0.1%	Ulca et al. (2013)
Pork (*Sus scrofa*)	*Cytb*	0.001	Ali et al. (2012a)
Pork (*Sus scrofa*), cattle (*Bos taurus*)	Repetitive elements	0.001	Cai et al. (2012)
Sheep, pork, beef, chicken, turkey	16S rRNA and *Cytb*	0.00002-0.0008	Camma et al. (2012)
4 tuna species (*Thunnus obesus, Thunnus orientalis, Thunnus maccoyii*, *Thunnus albacares*)	*Cytb*, 16S rRNA, D-loop region	0.08	Chuang et al. (2012)
Pork (*Sus scrofa*)	*Cytb*	Not Indicated	Demirhan et al.(2012)
Beef (*Bos taurus*), pork (*Sus scrofa*)	*Cytb*, t-Glu gene	0.001-0.3	Lopez-Andreo et al. (2012)
Fish species	12S rRNA	0.0002	Benedetto et al. (2011)

Table 2.4 Continued

Detected Species	Target region (genes)	Limit of detection (ng)	References
Beef, Pork and Goat	D-loop region	0.1	Pegels et al. (2011)
Ostrich (*Struthio camelus*)	12S rRNA	Not Indicated	Rojas et al. (2011)
Atlantic Salmon (*Salmo salar*)	Internal transcribed spacer (ITS) 1	0.01	Herrero et al. (2011)
Chicken, turkey, duck and goose	D-loop region and 12S rRNA	Not Indicated	Pegels et al. (2012)
Donkey (*Equus asinus*), pork (*Sus scrofa*) and horse (*Equus caballus*)	ND2, ND5 & ATPase6 and 8	0.0001	Kesmen et al. (2009)
Cattle, pork, chicken, lamb, goat, turkey	Cyclic guanosine monophosphate, Phosphordiesterase, ryanodine receptor, interleukin-2 precursor and myostatin	Not Indicated	Laub et.al. (2007a)
Cattle, pork	$tRNA^{LYS}$ and ATPase 8	Not Indicated	Fumiere et al. (2006)
Cattle, pork, chicken, lamb, goat, duck, turkey	Cyclic guanosine monophosphate, Phosphordiesterase, ryanodine receptor, interleukin-2 precursor, myostatin	Not Indicated	Laube.et al. (2007b)

Table 2.4 Continued

Detected Species	Target region (genes)	Limit of detection (ng)	References
Duck, goose, chicken, turkey and pork	12S rRNA and *Cytb*	Not Indicated	Köppel et al. (2013)
Beef, pork, horse and sheep	Prolactin receptor gene, growth hormone receptor(GHR), Beta-actin-gene	0.32	Köppel et al. (2011)
Beef, pork, turkey, chicken, horse, sheep, goat	Beta-actin-gen, Prolactin receptor, Target-Function Globotriaosylceramide (TF-GB3), *Cytb*	0.32	Köppel et al. (2009)
Horse, donkey	*Cytb*	0.001	Chisholm et al. (2005)
Pork	12S rRNA	0.05	Rodriguez et al. (2005)
Beef, pork, lamb, chicken, turkey	*Cytb*	0.01- 0.1	Dooley et al. (2004)
Mallard and Muscovy duck	*Cytb*	Not Indicated	Hird et al. (2005)
Cattle, pork, lamb, chicken, turkey, and ostrich	*Cytb*, *t*-glu, ND5, nuclear 18S rRNA gene	0.000006-0.0008	Lopez-Andreo et al. (2005)

Table 2.4 Continued

Detected Species	Target region (genes)	Limit of detection (ng)	References
Haddock	Transferrin	Not Indicated	Hird et al. (2004)
Cattle	Growth hormone	0.02	Brodmann and Moor (2003)
Cattle, pork	Phosphodiesterase, ryanodine gene	Not Indicated	Laube et al. (2003)
Molecular Beacon Chemistry			
Pork	*Cytb*	0.0001	Hazim et al. (2012)
SYBR Green Chemistry			
Tuna species (*Thunnus obesus*)	ATPase 6, 16S rRNA	0.08	Chuang et al. (2012)
Pork	*Cytb*	0.01	Soares et al. (2013)
Bovine (*Bos taurus*) and buffalo (*Bubalus bubalis*)	*Cytb*, 16S rRNA	Not Indicated	Drummond et al. (2013)
Red deer (*Cervus elaphus*), fallow deer (*Dama dama*), and roe deer (*Capreolus capreolus*)	12S rRNA	0.000004	Fajardo et al. (2008)

Table 2.4 Continued

Detected species	Target gene(s)	Limit of detection (ng)	References
Ruminant (Bos taurus), Poultry (*Gallus gallus*)	16S rRNA-tRNA, 12S rRNA	0.0000245	Sakalar and Abasıyanık (2012)
Pork, cattle, horse, wallaroo	3′ end of ND6 and the 5′end of *Cytb* gene	0.00004-0.0004	Lopez -Andreo et al. (2006)
Ostrich (*Struthio camelus*)	12S rRNA	0.0000245 and 0.00023	Rojas et al. (2011)
Pork	12S rRNA	0.002	Martin et al. (2009)
A number of mammalian and avian species	Short interspersed nuclear element (SINE), long interspersed nuclear element (LINE)	0.0001-0.1	Walker et al. (2004)
Ruminant, cattle, pork, chicken	Bov-tA2 SINE, 1.711B bovine repeat, PRE-1 SINE, CR1 SINE	0.00001-0.005	Walker et al. (2003)
Eva Green Chemistry			
Hare (*Lepus* species)	*Cytb*	0.001	Santos et al. (2012)

Table 2.4 Continued

Detected species	Target gene(s)	Limit of detection (ng)	References
Cattle	16S rRNA	Not Indicated	Sawyer et al. (2003)
Beef and Soybean	ATPase 8, Lectin	0.0027 and 0.0009	Safdar and Abasıyanık (2013)

2.7 Recent Meat Scandals in Processed Foods

Addition of minced meat in commercial food products is a routine ill-practice of adulteration which are being frequently used in modern foods (Ali et al., 2012a) and the contamination rate of processed food is higher than the fresh food (Barai and others, 1992; Patterson, 1985; Hsieh and others, 1996). The Food Safety Authority of Ireland (FSAI) analyzed 27 beef burger products from 'June 2013 to March 2014' and found horse DNA in 10 samples of 27 products (37%) and 85% was found to positive for pig DNA (Reilly, 2013) and, offending burgers were supplied from Tesco stores in Ireland and the UK. More importantly, to survive in competitive markets and realize extra profit, lower valued meats such as pig and horse meats were replaced in frozen meatballs in Sweden (Pollak, 2013) while chicken and turkey were substituted in pure beef meatballs in Turkey (Ulca et al., 2013). However, the recent meat scandals in processed food such as horse meat found in beef burger in Europe (Premanandh, 2013) and human meat in McDonald's burgers in the USA (Olumide, 2014), have raised consumers doubts on labeled foods as well as their ingredients which they are consumed, hence they are now increasingly worried to safeguard their religious faith, health, money and wildlife. A list of meat scandal in commercial products such as burgers and meatballs are briefly summarized in Table 2.5.

Table 2.5 Recent meat scandal in burgers and meatballs

Target species	Adulterated products	Place of adulteration	Year of adulteration	References
Horse	Burgers	Busiest supermarkets in some countries	2013	Carty (2013) & Reilly (2013)
Pork	,,	Tesco stores	2013	Reilly (2013)
Horse	,,	Europe	2013	Embiricos (2013)
Horse	,,	Europe	2013	Janice & Jaco (2013)
Pork	,,	Not Reported	2012	Reilly (2013)
Horse	,,	Silver crest	2013	Reilly (2013)
Beef and pork	Meatballs	Sweden	2013	Pollak (2013)
Turkey and chicken	,,	Turkey	2013	Ulca et al. (2013)

2.8 Prospect of Processed Food Analysis

Misdescription of food components in processed meat products has been occurred both intentionally and unintentionally due to improper processing or handling, to survive in competitive market and realize more economic gain. It violets not only the food labelling acts but also put consumers at the risk of health and ethical compromise. A survey was made by Hsieh and others (1995) in Florida retail markets in the USA revealed adulteration rates for processed meats and raw meats are 23% and 16%, respectively. A recent test on the British food industry for horse meat adulteration in beef pasta revealed 29 samples out of 2,501 contained more than 1 % horse meat merged with beef (Castle, 2013), while turkey and chicken was found in 100% beef meatballs (Ulca et al., 2013) and pork were being served in Halal chicken sausages in west London in 2013 (Webb S., 2013). Therefore, it is more challenging to isolate substituted ingredients in processed products after grinding, cooking, smashing, salting, or mixing. Furthermore, the origin of species is easy to be buried in the meat mixture owing to the change of meat texture, color, appearance, or even flavor. Therefore, it is subjected to develop an easy, fast and reliable PCR assays for the analysis of feline materials in processed foods and meat products under complex matrices.

Table 2.6 Percentages (%) of fraud labeling from different meat products

Products analyzed	Investigated country	Substituted species	Percentage (%) of mislabeling	References
Canned products	Not Reported	Tuna	Not Mentioned	Teletchea et al. (2005)
Meat and bone meal in compound feeds	,,	Beef, sheep, pig, chicken	,,	Krcmar et al. (2003)
Baby food	,,	Ruminant, avian, fish, pig	,,	Dalmasso et al. (2004)
Canned products	,,	Sardine	,,	Jerome et al (2003)
Goat cheese	,,	Beef	,,	Maudet et al. (2001)
Dried, salted and unfrozen meat	,,	Whale	,,	Baker et al (2003)
Ground beef	,,	Cow	,,	Marfa et al. (2008)
Bovine meat products	,,	Pork	,,	,,
Cooked meat products	,,	Pork and horse	,,	,,
Hamburgers	Brazil	Undeclared soy protein	30.8	Macedo-Silva et al. (2001)
Sausages	Mexico	Undeclared animal species	29	Flores-Munguia et al. (2000)
Beef pasta	United Kingdom	Horse	1	Castle (2013)

Table 2.6 Continued

Products analyzed	Investigated country	Substituted species	Percentage (%) of mislabeling	References
Meat products	United Kingdom	Thawed meat declared as fresh	8	Anon (1996)
Halal chicken sausages	United Kingdom	Pork	Not Mentioned	,,
Chicken nuggets	Greece	Horse	,,	Embiricos (2013)
Hamburgers	Mexico	Undeclared animal species	39	Flores-Munguia et al. (2000)
Meat products	United States of America	Undeclared animal species	22.9	Hsieh et al. (1995)
Meat products	Turkey	Undeclared animal species	22	Ayaz et al. (2006)
Meat products	Switzerland	Thawed meat declared as fresh	15	Anon (2001))

CHAPTER THREE

MATERIALS AND METHODS

CHAPTER 3: MATERIALS AND METHODS

3.1 Sample Collection

Fresh meat samples of beef *(Bos taurus and Bos indicus)*, water buffalo (*Bubalus bubalis)*, chicken *(Gallus gallus)*, domestic duck *(Anas platyrhynchos*), turkey *(Meleagris gallopavo)*, sheep (*Ovis aries*), goat (*Capra hircus)*, Malaysian box turtle (*Cuora amboinensis*), monkey (*Macaca fascicularis sp*.), rat (*Rattus norvegicus)*, lamb (*Ovis aries*), pegion (*Columba livia)*, pig *(Sus scrofa)*, cuttle (*Sepia officinalis)*, carp (*Cyprinus carpio)*, tilapia (*Oreochomis aureus)*, shrimp (*Litopenaeus vannamei*), Atlantic cod (*Gadus morhua*), onion (*Allium cepa*), tomato (*Solanum lycopersicum)*, cucumber (*Cucumis sativus)*, potato (*Solanum tuberosum)* and wheat *(Triticum aestivum*) were collected in triplicates on three different days from various wet markets and super markets across Malaysia (Table 3.1). Dog (*Canis familiaris*) and cat (*Felis catus*) meat samples from three different animals were collected from Jabatan Kesihatan, Dewan Bandaraya Kuala Lumpur (DBKL) after euthanized according to animal usage guidelines by authorized personnels, Air Panas Kuala Lumpur and Faculty of Veterinary Sciences in University of Putra Malaysia in Selangor, Malaysia. For commercial products screening raw and processed samples of different halal branded commercial products like burgers and meatballs were procured from different selling-spots supermarket at Petaling Jaya and Kuala Lumpur in Malaysia in triplicates on three different days. All the collected samples were then transported to the laboratory under ice-chilled atmosphere (4°C) and kept at (-20°C) up to further use.

Table 3.1 Sources of samples used in this study

Species	Scientific name	Samples analyzed	Sources of collection
Cat	*Felis catus*	18	University Putra Malaysia, "Faculty of Veterinary Science" and "Jabatan Kesihatan Bandaraya Kuala Lumpur" (DBKL)
Dog	*Canis familiaris*	12	,,
Beef	*Bos taurus*	16	Kuala Lumpur, super markets (Aeon Big, Aeon)
Water buffalo	*Bubalus bubalis*	14	,,
Chicken	*Gallus gallus*	13	,,
Lamb	*Ovis aries*	8	,,
Goat	*Capra hircus*	15	,,
Sheep	*Ovis aries*	16	,,
Rat	*Rattus norvegicus*	17	Pasar Borong, Pudu Raya
Pig	*Sus scrofa*	13	,,
Malaysian box turtle	*Cuora amboinensis*	10	,,
Turkey	*Meleagris gallopavo*	8	,,
Domestic duck	*Anas platyrhynchos*	11	Selangor, local markets (Tesco)
Pegion	*Columba livia*	8	,,
Monkey	*Macaca fascicularis sp.*	6	Wildlife and National Parks (DWNP) Peninsular Malaysia, Kuala Lumpur; Malaysia

Table 3.1 Continued

Species	Scientific name	Samples analyzed	Sources of collection
Cuttle	*Sepia officinalis*	9	Fisheries farm (Kuala Lumpur and Selangor)
Tilapia	*Oreochomis aureus*	5	,,
Carp	*Cyprinus carpio*	4	,,
Shrimp	*Litopenaeus vannamei*	7	,,
Atlantic Cod	*Gadus morhua*	2	,,
Onion	*Allium cepa*	8	Pasar malam, local markets (Panthai dalam)
Tomato	*Solanum lycopersicum*	11	,,
Wheat	*Triticum aestivum*	16	,,
Cucumber	*Cucumis sativus*	5	,,
Potato	*Solanum tuberosum*	6	,,
Commercial (burgers and) meatballs)	-	10	KFC, MacDonald, Aeon, Tesco

3.2 DNA Extraction from Raw, Admixed, Heat Treated and Commercial Products

Total genomic DNA was extracted from 35 mg raw samples of cat, dog, beef, water buffalo, chicken, domestic duck, turkey, sheep, goat, rat, lamb, pegion, pig, cuttle, carp, tilapia, shrimp, cod, turtle, monkey and heat treated (autoclaving, boiling, and microwaving) samples of each species using Yeastern Genomic DNA Mini Kit (Yeastern Biotech Co., Ltd., Taipei, Taiwan) following the protocol given by the manufacturers.

Isolation of DNA from 35 mg of plant sources (onion, tomato, cucumber, potato and wheat) and 1g of 2 set of ternary model mixed ((i) cat: beef: chicken and (ii) feline: bovine: wheat flour), lab made (Tables 3.4 & 3.5) and commercial burgers (beef and chicken burger), and meatballs (beef and chicken meatballs) was performed using CTAB method (Cetyl Trim methyl Ammonium Bromide) according to Ma et al. (2000).

3.3 Biomarker Development

3.3.1 Salient Features of Primer Designing

There are some important key parameters that were considered to design a pair of in-silico primers are described below:

3.3.1.1 Length

Length of primers is the fundamental criteria for the specificity and annealing to the accurate targets. Cross positive amplification and lower specificity may possibly come to pass because of too short primer's' length, while too longer primer is the complex features to reduce the template-binding efficacy at standard annealing temperature (Judelson, 2000). The standard length of primers is 18-28 nucleotides base pairs and could be longer in terms of compulsion.

3.3.1.2 Melting Temperature (*Tm*) and Mismatching

Since annealing in a PCR takes place for both forward and reverse primers simultaneously, primer pairs should have comparable melting temperatures (*Tm*). A miniature difference of *Tm* of primer pairs may enhances the inefficiency of multiplex PCR platforms and lead to mis-hybridization and increase at an incorrect region of DNA to amplify. Specificity to target and non-specificity to non-target species depend on

mismatching between primers and DNA template. Each 1% mismatching of the bases in a double-stranded (ds) DNA reduces melting temperature (*Tm*) by 1 to 1.5˚C (Cheng et al., 2014; Ishii & Fukui, 2001; Köppel et al., 2013; Matsunaga et al., 1999; Sambrook et al., 1989; Zhang et al., 2014). The percentage of mismatch of newly designed primers for multiplex PCR should not be less than 15% between a species-specific primer and the other species sequences.

Therefore, to calculate melting temperature of primer only nucleotides homologous to the template are considered. The approximate melting temperature (*Tm*) of primers containing less than 25 nucleotides and calculated with the equation: *Tm* = 4 (G + C) + 2 (A + T), where G, C, A and T are number of nucleotides in the primer. If the primer have more than 25 nucleotides an online based *Tm* calculator from Promega, (http://www6.appliedbiosystems.com/support/techtools/calc/) is account for interactions of adjacent bases, effect of salt concentration thus the *Tm*'s of forward and reverse primers must be similar (2 to 5˚C differences accepted).

3.3.1.3 Secondary Structure

A minimum of intra-molecular or inter-molecular homology is important for designing primer pairs may causes for secondary structure formation (hairpins or primer dimerization (Figure 3.1)) which may lead to poor or no amplification. In a standard primer designing intra-primer homologies of 3 bp of 3´ end or more should be escaped due to "primer-dimer" formation (Figure 3.1b). Hence the internal inter-molecular interactions should also be minimized.

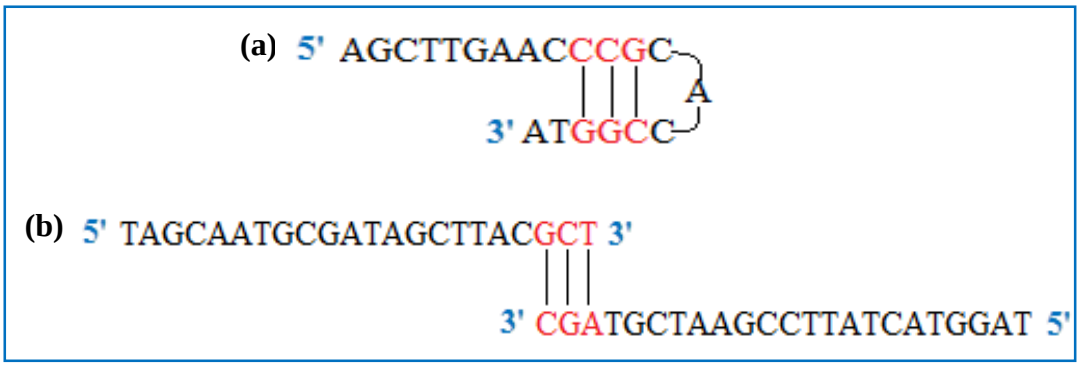


Figure 3.1 Formation of secondary structure (a) hairpin; (b) primer-dimer.

3.3.1.4 GC-Clamp

Ideally a primer has a near random mix of nucleotides and the presence of G or C within the last 4 bases from the 3' end of primers is appropriate to increase yield and to inhibit mis-priming. Nonetheless, primers with long polyG or polyC stretches essential to avoid despite of possibility to encourage non-specific annealing. Although having one or two G and/or C at 3' is acceptable but additional of Gs or Cs might badly effect the total specificity of the primers.

3.4 Feline-Specific Primer Design

Feline-specific primers (Forward-5′ ACTATTATTTACAGTCATAGCCACAGC-3′ and Reverse-5′CAGAAGGACATTTGGCCTCA-3′) were developed targeting a 69-bp site of mitochondrial gene using Primer3plus software (www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi). The selected primers were screened for primer specificity in an in-silico analysis using online Basic Local Alignment Tool (BLAST) against non-redundant nucleic acid sequences in NCBI data base (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The expected target sequence was multiple aligned with 6 common halal animal meat species like beef *(Bos taurus and Bos indicus)*,

water buffalo (*Bubalus bubalis)*, lamb (*Ovis aries*), chicken *(Gallus gallus)*, goat (*Capra hircus)*, and sheep (*Ovis aries*), 3 avian species namely domestic duck *(Anas platyrhynchos*), pegion (*Columba livia)* and turkey *(Meleagris gallopavo)*, 5 fish samples including carp (*Cyprinus carpio)*, cuttle (*Sepia officinalis)*, tilapia (*Oreochomis aureus)*, cod (*Gadus morhua*), and shrimp (*Litopenaeus vannamei*) and 5 plant origin known as onion (*Allium cepa*), cucumber (*Cucumis sativus)*, potato (*Solanum tuberosum)*, tomato (*Solanum lycopersicum)* and wheat *(Triticum aestivum*) and 5 non halal meat species such as dog *(Canis familiaris)*, turtle (*Cuora amboinensis*), monkey (*Macaca fascicularis sp.*) rat (*Rattus norvegicus)* and pork *(Sus scrofa)* several common meat, fish and plant species by ClustalW sequence alignment program (http://www.genome.jp/tools/clustalw/) to identify the variability of the primer binding regions and total mismatch between target and non-target species. The successfully designated primers were synthesized and supplied by the 1st BASE Laboratories, Pte. Ltd. in Selangor, Malaysia. In Table 3.2, represents an overview for the development of feline-specific primer pairs for the amplification of a conserved region of mitochondrial cytochrome b target region gene (*mt-cytb*).

Table 3.2 Sequences of oligonucleotide used in this study

Name	**Sequence (5´- 3´)**	**Length**	**Tm (°C)**	**GC (%)**
Forward	ACTAT TATTT ACAGT CATAG CCACA GC	27 bp	58.5	37.0
Reverse	CAGAA GGACA TTTGG CCTCA	20 bp	61.2	50.0
Product amplicon (69 bp)	ACTATTATTTACAGTCATAGCCACAGC TTTTA TGGGA TACGT CCTAC CA TGAGGCCAAATGTCCTTCTG			

The relevant sequences in the amplicon are shown on different colors

3.5 Pairwise Distance and Phylogenetic Tree Construction

Feline specificity was confirmed through theoretical analysis using bioinformatics software prior to any experimental trial in the lab. Therefore, the retrieved cytb gene sequences of animal and cob gene sequences of plant were aligned along with the primer sequences using molecular evolutionary and phylogenetic analysis software, MEGA version 5 (Tamura et al., 2011) and ClustalW alignment tool for mismatch calculation. Moreover, the experimental analysis was done in an actual PCR reaction against fourteen land animal, five fish, and five plant species. Finally, the purified PCR product was sequenced (MyTACG Bioscience Enterprise, Selangor, Malaysia) and the sequences were matched through BLAST (Basic Local Alignment Search Toll) analysis in NCBI data base. All the theoretical data revealed that developed primers were highly specific to feline cytb gene.

3.6 PCR Assay Optimization and Gel Electrophoresis

The PCR reaction was carried out using 5µl of 5x Green GoTaq Flexi Buffer (Promega, Corporation, Madison, USA), 1.5 µl of 25mM of $MgCl_2$ (Promega), 0.5µl of 0.2 mM of 150 each dNTPs mix (sodium salts of dATP, dCTP, dGTP and dTTP 10 mM each in water (40 mM Promega, Madison, USA), 100 nM each primers (IDT, Inc.), 50 units/ml of Taq polymerase (Promega), 20 ng of total DNA in 25µl of reaction volumes. All the PCR reaction was performed on the Veriti 96-Well gradient Thermal cycler system (Veriti® Thermal Cycler, Applied Biosystems; Foster City, California, USA) under the following conditions: an initial denaturation at 95˚C for 3 min followed by 35 cycles of denaturation at 95˚C for 20s, annealing at 58˚C for 20s and extension at 72˚C for 30s and the final extension was completed by 72˚C for 5 min and hold at a 4˚C for 2

min. Finally, negative control was developed by adding nuclease free water in the replacement of template DNA for experimental validation of all PCR reaction and the amplified PCR products were kept at -20˚C for further analysis.

For PCR products analysis 2% agarose gel was prepared in a horizontal agarose gel electrophoresis chamber (SUB13, Hoefer, Inc., California, and USA) with FloroSafe DNA Stain (1st Base Laboratories, Selangor, Malaysia). After that, the electrophoresis experiment was conducted at 70 min for 120V along with 50 bp DNA molecular weight marker (Fermentas, USA), passing an electrical current though the gel towards anode from cathode to separate the amplified products on the basis of molecular size. The banding pattern of PCR products was shown under UV transilluminator-gel documentation system (Alpha Imager HP; Alpha InfoTech Corp., San Leandro, CA, USA). All the chemicals and reagents for PCR products amplification and gel electrophoresis visualization were received from 1st BASE Laboratories Pte. Ltd (Selangor, Malaysia).

3.7 Stability of Target DNA

3.7.1 Raw State

The sensitivity of the target DNA was determined by 10 fold serially diluted DNA extracted from raw and pure state (10, 1, 0.1, 0.01, 0.001 and 0.0001 ng/µl). Therefore, the sensitivity of the autoclaved and boiled feline DNA was also confirmed against 10 fold (10 ng/µl - 0.0001 ng/µl) serial dilution methods. The limit of detection was 0.0001 ng/µl feline DNA from raw, autoclaved and boiling treatment.

3.7.2 Compromised States

Compromised states mean degradation of DNA either by natural decomposition or forced physical treatments. Different types of processing and cooking treatments were applied to the collected meat samples to verify the stability of the target amplicon. Therefore, the targeted meat samples were cut into smaller pieces for various processing treatments. Approximately 3-5 g of cat meats were boiled at 80˚C, 100˚C and 110˚C for 30, 90 and 30 min, respectively (Ali et al., 2013 & Karabasanavar et al., 2014) to simulate traditional cooking system. The autoclave treatment was performed at 120˚C for 50 min at 14.5-psi, 110˚C for 2 h at 14.5-psi, and 133˚C for 20 min under 43.51-psi according to the European legislation (Comission, 2002). In addition, to verify target DNA stability under extreme processing treatments, extensive autoclaving was done at 120˚C for 2.5 h under 45-psi (Ali et al., 2013). Finally, another heat treatment was carried out on raw meat using microwave oven on three different stages namely low (300W), medium (500W) and high (700W) for 10 to 30 min, respectively. All the heat treated samples were kept at – 20˚C up to further use.

3.8 Specificity and Sensitivity of Target DNA

3.8.1 Ternary Mixed Background

In order to evaluate the sensitivity and specificity of the ternary meat mixed, fresh cat meat was spiked with raw beef and chicken, respectively, at four adulteration levels: 10%, 1%, 0.1%, and 0.01% (w/w); and also mixed with wheat flour at another four different levels: 10%, 5%, 0.2% and 0.01% (w/w). Briefly, to obtain 10%, 1%, 0.1%, and 0.01% (w/w) cat meat spiked ternary admixed was prepared by adjusting cat, beef and chicken in the ratio of 10:45:45, 1:49.5:49.5, 0.1:49.95:49.95 and 0.01:49.995:49.995,

respectively. Additionally, 10%, 5%, 0.2% and 0.01% (w/w) ternary mixtures was constructed by mixing feline, bovine, and wheat flour in the percentages of 10:45:45, 5:47.5:47.5, 0.2:49.9:49.9 and 0.01:49.995:49.995, respectively. Each of admixed samples was prepared in a 100g specimen and thoroughly mixed and homogenized in a separate container and blender. Therefore, homogenous semi solid slurry was formed by adding 100 ml distilled water and grinding in a food processing blender. Henceforth, in order to examine the effect of standard cooking and autoclaving condition each parts of admixed mixtures were treated with different heating conditions namely boiling (100˚C for 90 min), extensive autoclaving (120˚C for 2.5 h under 45-psi) and oven heating (180˚C for 30 min). All admixtures were prepared on three different days by three independent analysts, thus the DNA extraction was performed using CTAB methods, and the isolated samples were preserved at -20˚C for further experimental analysis.

3.8.2 Burger Formulations

Burgers were prepared according to the Ali et al. (2012a) with an appropriate amount of deboned chicken, beef and cat minced meats and others burger ingredients as given in Table 3.3. For adulteration analysis, 100g of beef and chicken meat were mixed with specified amount in to 10%, 5%, 1% and 0.1% (w/w) of cat meat to formulate cat meat contaminated burgers in the amounts of 10:45:45, 5:47.5:47.5, 1:49.5:49.5 and 0.1:49.95:49.95, respectively. Finally, all the ingredients were mixed properly and divided into four different parts and each part was given into a burger shape (Ali et al., 2012a). Therefore, the raw burger meats were cooked in an electrical oven for 15 min under 220˚C and the prepared burger were autoclaved at 120˚C for 2.5 h in 45-psi pressure and oven heated at 180˚C for 30 min separately on three different days and kept at–20˚C.

Table 3.3 Ingredients used in the preparation beef, chicken and cat burgers (100g)

Components	**Beef burger**	**Chicken burger**	**Cat burger**
Sliced beef meat	100 g	-	-
Sliced chicken meat	-	100 g	-
Sliced cat meat	-	-	100 g
Fresh breadcrumbs	15 g	-	7.5 g
Dry breadcrumbs	-	7 g	-
Brown onion paste	0.5 g	0.5 g	0.5 g
Eggs	0.4 g	-	0.4 g
Sliced cheese	0.9 g	0.9 g	0.9 g
Olive oil	-	0.3 g	0.3 g
Tomato paste	0.35 g	0.35 g	0.35 g
Red pepper powder	0.2 g	-	0.2 g
Lettuce leaves extracts	-	0.05 g	0.05 g

3.8.3 Meatball Matrices

Pure meatballs were prepared according to the Rohman et al. (2011) by mixing a proper amount of ground beef, chicken and cat meat with tapioca starch, cooking salt, garlic and other spices as described in Table 3.4. Thus, 100g of beef and chicken meat were mixed with an appropriate amount (10%, 5%, 1%, 0.1% and 0.01%) of cat meat to formulate cat meat contaminated meatballs in the proportions of 10:45:45, 5:47.5:47.5, 1:49.5:49.5, 0.1:49.95:49.95 and 0.01:49.995:49.995, respectively. Finally, all the ingredients were blended properly and divided into four parts and mechanically each part was given into a ball shape (Ali, Hashim, Mustafa, & Man, 2012a). To mimic standard boiling and extensive autoclaving effect thus prepared raw meatballs were subjected to

boiled at 100˚C for 90 min and autoclaved at 120˚C under 45-psi pressure for 2.5 h. All the meatball samples were prepared separately on three different days by three independent analysts and extracted DNA was kept under -20˚C for commercial experimental analysis.

Table 3.4 A list of components commonly used to prepare beef, chicken and cat meatballs (100g)

Components	Beef meatballs	Chicken meatballs	Cat meatballs
Ground beef meat	100 g[a]	-	-
Ground chicken meat	-	100 g[a]	-
Ground cat meat	-	-	100 g
Fresh breadcrumbs	7.5 g	7.5 g	7.5 g
Minced garlic	1.25 g	1.25 g	1.25 g
Freshly ginger paste	1.1 g	-	1.1 g
Eggs	0.4 g	-	0.4 g
Butter	-	2.5 g	2.5 g
Olive oil	-	0.3 g	0.3 g
Tomato paste	0.35 g	0.35 g	0.35 g
Black pepper powder	-	0.15 g	0.15 g
Chesses	-	0.015	0.015
Salt	0.02 g	0.02 g	0.02 g

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

3.9 Restriction Fragment Length Polymorphisms (RFLP) Analysis

3.9.1 Restriction Enzyme Digestion

For RFLP analysis, PCR products were digested with *Alu*I restriction enzyme in a 30 µl reaction mixture containing 10 µl of PCR product, 1 µl of restriction enzyme (New England Biolab), 17 µl of nuclease free water, and 2 µl of 10× digestion buffer supplied with the enzyme (New England Biolab, CA, USA). Initially, the reaction mixtures were gently mixed and spin downed followed by incubation at 37°C in a water shaking bath for 30 min to digest the targets properly. After that the enzyme was deactivated by heating the mixture at 65°C for 25 min. Finally, RFLP analysis was completed by running 1µl of the restriction digested products of each sample in Experion lab-on-a-chip well using 1K DNA analysis kit (Bio-Rad Laboratories, USA).

3.9.2 Products Authentication by RFLP Analysis

For PCR-RFLP study, a conserved region is amplified and the amplicons are digested using an appropriate restriction enzymes resulting in a specific band pattern able to differentiate the target species. A well-known primer designing software primer3Plus (www.bioinformatices.nl/cgibin/primer3plus.cgi) used to apply feline species primer pairs using a 22-bp-*Alu*I cut site of *F. catus* of cytochrome b gene (GenBank no. AB194812.1 in the NCBI database) as an internal oligo. Multiple alignment tool ClustalW (http://www.ebi.ac.uk/Tools/ClustalW/) was applied to check the high degree of interspecies polymorphism. The amplicon contained one *Alu*I sites within it thus offering boundless opportunities to separate similar-sized cross-amplified PCR products by way of RFLP analysis. However, a 141-bp fragment of eukaryotic 18S rRNA gene was used as a positive control developed by Rojas et al. (2010) to evaluate the quality and the presence of DNA in all samples. The nucleotide sequences of the all primers for RFLP analysis in this study are presented in Table 3.5. All the designed primers and reagents

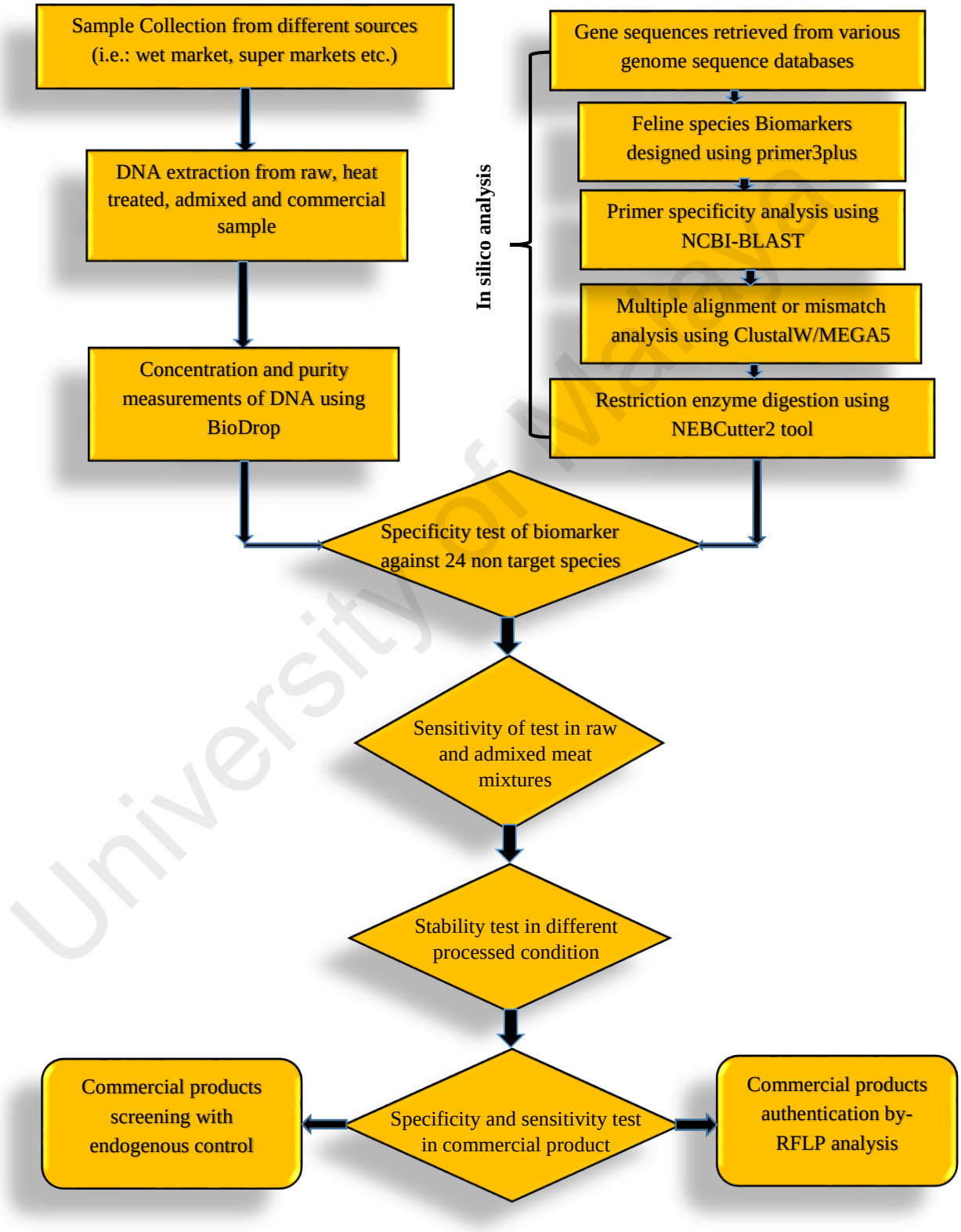
and chemicals for target digestion were synthesized and purchased from the 1st Base Laboratories (1st Base Laboratories, Sdn. Bhd., Selangor, and Malaysia).

Table 3.5 Primer pairs used in this experiment for product authentication by RFLP analysis

Primer identity	**Sequence (5´ - 3´)**	**Length (bp)**
Feline forward primer	ACT ATT ATT TAC AGT CAT AGC CAC AGC	27
Feline reverse primer	CAG AAG GAC ATT TGG CCT CA	20
Eukaryotic forward primer	GGT AGT GAC GAA AAA TAA CAA TAC AGG AC	29
Eukaryotic reverse primer	ATA CGC TAT TGG AGC TGG AAT TAC C	25

3.10 Summary of Methodology

The summary of the methodology followed for this work is schematically given in the following diagram:



CHAPTER FOUR

RESULTS

CHAPTER 4: RESULTS

4.1 DNA Extraction and Quality Analysis

In this research, two different DNA extraction methods (i.e.: commercial extraction kit and CTAB - Cetyl Tri methyl Ammonium Bromide) were used to isolate the DNA from raw, heat treated, diluted samples were extracted using commercial DNA Mini Kit for Animal Tissues following the manufacturer instructions. Furthermore, a traditional DNA extraction CTAB method was applied to extract the DNA from admixed and food processed samples according to the Ma et al. (2000). The concentration and purity of all extracted DNA were checked with Biodrop UV/VIS spectroscopy (BioChrom, Libra S70; Cambridge, UK) taking the absorbance at 260-280 nm in the ratio of A260/280. The extracted DNA was kept at -20°C for future experimental analysis. In Table 4.1, DNA concentration and purity of all extracted samples were summarized.

Table 4.1 DNA concentration and its purity obtained in this experiment

Samples	**Concentration (ng/µl)**	**Purity (A_{260}/A_{280} ratio)**
Cat	718.43	1.71
Dog	351.23	1.89
Beef	127.3	1.7
Buffalo	119.16	1.72
Chicken	259.46	1.71
Lamb	93.28	1.696
Goat	68.1	1.70
Sheep	110.08	1.74
Rat	101.06	1.85

Table 4.1 Continued

Samples	**Concentration (ng/µl)**	**Purity (A_{260}/A_{280} ratio)**
Pig	100.6	1.77
Turtle	330.91	1.73
Turkey	193.25	1.705
Duck	91.05	1.82
Pegion	248.3	1.715
Monkey	276.4	1.739
Cuttle	158.2	1.812
Tilapia	62.15	1.99
Carp	89.86	1.86
Shrimp	177.05	2.02
Cod fish	117.7	1.92
Onion	316.2	1.73
Tomato	154.9	1.75
Wheat	210.01	2.03
Cucumber	59.98	1.98
Potato	101.4	1.88
10-fold diluted DNA	**Concentration (ng/µl)**	**Purity (A_{260}/A_{280} ratio)**
10 ng	146.29	1.99
1 ng	234.02	2.02
0.1 ng	128.82	1.87
0.01 ng	114.03	1.92
0.001 ng	156.19	1.72
0.0001 ng	131.9	1.97
Heat treatments		
Autoclaving	**Concentration (ng/µl)**	**Purity (A_{260}/A_{280} ratio)**
110˚C for 2 h	129.25	1.83
120˚C for 50 min	137.26	1.72

Table 4.1 Continued

Autoclaving	**Concentration (ng/µl)**	**Purity (A_{260}/A_{280} ratio)**
133˚C for 20 min	114.09	1.79
120˚C for 2.5 h	227.3	1.705
Boiling		
100˚C for 90 min	512.7	1.91
Microwaving		
300W for 10 min	485.5	1.85
500W for 20 min	561.03	1.71
700W for 30 min	494.21	1.96
Ternary complex matrices		
Admixed matrices	**Concentration (ng/µl)**	**Purity (A_{260}/A_{280} ratio)**
Raw admixed	554.09	1.74
Boiled admixed	469.66	1.77
Oven heated	531.32	1.75
Autoclaved admixed	691.86	1.98
Processed foods	**Concentration (ng/µl)**	**Purity (A_{260}/A_{280} ratio)**
Beef burgers	339.33	1.78
Chicken burgers	200.2	2.02
Beef meatballs	238.40	1.82
Chicken meatballs	369.79	1.89

4.2 Feline Specificity Test

4.2.1 In-silico Analysis of Cross Specificity

To determine the feline specificity of the developed primers cytb gene sequence of cat (AB194812.1) and others species (cytb and cob gene), were retrieved from publicly available '**National Center for Biotechnology Information' (NCBI)** GenBank database (dog: JF489119.1, beef: EU807948.1, buffalo: D32193, chicken: EU839454.1, lamb: EU365990.1, goat: EU130780.1, sheep: EU365990.1, rat: HM222710.1, pig: GU135837.1, turtle: KF059113.1, turkey: HQ122602.1, duck: HQ122601.1, pegion: KC811464.1, monkey: FJ906803.1, cuttle: AB240155.1, tilapia: AF015020.1, carp: AB158807.1, shrimp: EU069446.1, cod fish: AM489716.1, onion : GU253304.1, tomato: XM004251454.1, wheat: X02352.1, cucumber: AF288044.2, potato: AF095281.1). The feline specific primer pairs were developed using primer designing software primer3Plus and multiple alignment was done using ClustalW (Table 4.2). Additionally, the local nucleotide alignment program 'BLAST' was applied to screen cross reactivity and species specificity of designed primers.

The maximum composite likelihood method (Tamura et al., 2011) was used to construct the phylogenetic tree (Figure 4.1) and determine the pairwise distance (Table 4.3) in the primer binding regions of the tested species (Rahman et al., 2014). The highest and lowest pairwise distance in the primer binding region was analyzed by the maximum composite likelihood method (Tamura et al., 2011). Therefore, similar results were found in the both primers when the dendogram was constructed among the other species used neighbor-joining method (Figure 4.1) (Saitou & Nei; 1987). All the obtained data confirm the high degree of discernment among *F. catus* and other animal, fish and plant species (Table 4.3).

Table 4.2 Mismatch comparison of the feline- specific forward and reverse primers against commercially important species

Forward Primer	A C T A T T A T T T A C A G T C A T A G C C A C A G C	Species	T G A G G C C A A A T G T C C T T C T G	Reverse Primer
0	. . . . .	Cat	. . . . .	0
7	C . . T C . G C . C . . . . . A	Beef	. . . . . A . . . . . A . . A	3
5	C . . . C . . . . C G . . . . A	Buffalo	. . . . . A . . . . . A . . A	3
8	C . . C C . C C . C . . . C . . . . . C . .	Chicken	. . G . . . . A . . A	3
4	. . . . . C G . . A C	Dog	. . . . . A . . . . . A . . A . . T . .	4
12	C . . . C . G C . C . . T C . T . . . . . A . . T . .	Duck	. . . . . A . . . . . A . . G	3
10	C . . C C . G C . C G . G A C A . . G	Goat	. . . . . A . . . . . A . . A . . T . .	4
8	C . . C C . . . . . G . G A C A	Sheep	. . . . . A . . . . . A . . A	3
8	C . . C C . . . . . G . G A C A	Lamb	. . . . . A . . . . . A . . A	3
9	C . . C C . . C . A . . C C . T . . . . . T . .	Pegion	. . . . . A . . . . . A . . A	3
9	C T . . C . T C . C . . C C . . . . . A	Turkey	. . G . . A . . . . . A . . A	4
5	C . . . C . . . . . C . . T . . . . . A	Pork	. . . . . A . . . . . A . . A	3
5	C . . . C . . . . . G . . . . . A . . T . .	Rat	. . . . . A . . . . . A . . A	3
9	. . . C C . T C . . . T . A C . . . . A . A	Monkey	. . . . . A . . A	3
11	T . . . C . . C . C C T . A C A . . . . . T . . C . .	Turtle	. . . . . A . . T	3
11	C . . T C . . C . A C T . . . . . G A T A	Carp fish	. . . . . A . . . . . A . . . . . T . .	3
12	C . . T . . C C . . T T . . . A . . . A T A . . C T .	Cod fish	. . . . . A . . . . . A . . A	3
14	. A . . A A . . . . T . . . A G T C T C . T . A T A .	Cuttle	. C . A T T A . T . . . . A A . A T . A	11
12	C . . T . . C C . . T T . . . A . . . A T A . . C T .	Shrimp	. . . . . A . . . . . A . . A	3
14	C . . C C . C C . C C T . A C T . . . A T A	Tilapia	. . . . . A . . . . . A . . A . . T . .	4
12	C A . . . . C C . A T T . A . G . . T . T G	Wheat	. . G . . T . . G . . . A G . . . T . .	6
12	C A . . . . C C . A T T . A . G . . T . T G	Onion	. . G . . T . . G . . . A G . . . T . .	6
13	. A . . A G . . . . . G G A . G . A . . T G . A . T G	Tomato	. . . . A T G . T . . . A G T G C . . .	10
12	. A . C . . C C . A T T . A . G . . T . T G	Potato	. . G . . T . . G . . . A G . . . T . .	6
13	C A . C . . C C . A T T . A . G . . T . T G	Cucumber	. . G . . T . . G . . . A G . . . T . .	6

A = Adenine, T = Thymine, G = Guanine, C = Cytosine

Table 4.3 Pairwise distances of 69 bp cytb site with common meat, fish, and plant species used in this study

Cat	0.00																							
Beef	0.26	0.26																						
Buffalo	0.15	0.15	0.09																					
Chicken	0.35	0.35	0.27	0.28																				
Dog	0.09	0.09	0.39	0.26	0.42																			
Duck	0.50	0.50	0.36	0.34	0.34	0.58																		
Goat	0.53	0.53	0.24	0.30	0.40	0.39	0.51																	
Sheep	0.39	0.39	0.24	0.19	0.33	0.26	0.51	0.09																
Lamb	0.39	0.39	0.24	0.19	0.33	0.26	0.51	0.09	0.00															
Pegion	0.42	0.42	0.33	0.28	0.20	0.50	0.20	0.40	0.27	0.27														
Turkey	0.42	0.42	0.43	0.35	0.34	0.50	0.26	0.60	0.52	0.52	0.34													
Pork	0.27	0.27	0.27	0.15	0.43	0.40	0.20	0.47	0.33	0.33	0.21	0.20												
Rat	0.21	0.21	0.27	0.15	0.27	0.33	0.20	0.55	0.40	0.40	0.27	0.27	0.15											
Monkey	0.46	0.46	0.56	0.57	0.48	0.33	0.70	0.47	0.40	0.40	0.70	0.48	0.56	0.48										
Turtle	0.47	0.47	0.45	0.31	0.49	0.33	0.57	0.45	0.32	0.32	0.57	0.71	0.48	0.39	0.43									
Carp fish	0.46	0.46	0.38	0.39	0.56	0.62	0.64	0.60	0.60	0.60	0.65	0.56	0.38	0.32	0.38	0.64								
Cod	0.73	0.73	0.48	0.58	0.57	0.96	0.81	0.94	0.84	0.84	0.94	0.94	0.70	0.57	0.66	0.65	0.33							
Cuttle	1.33	1.33	1.62	1.32	2.47	1.32	2.22	1.86	1.74	1.74	2.27	3.52	1.58	1.22	2.42	1.42	2.47	2.66						
Shrimp	0.73	0.73	0.48	0.58	0.57	0.96	0.81	0.94	0.84	0.84	0.94	0.94	0.70	0.57	0.66	0.65	0.33	0.00	2.66					
Tilapia	0.75	0.75	0.54	0.56	0.47	0.57	0.58	0.46	0.39	0.39	0.58	0.64	0.46	0.54	0.20	0.50	0.31	0.40	2.85	0.40				
Wheat	0.60	0.60	0.67	0.56	0.74	0.61	0.84	0.77	0.77	0.77	1.11	0.68	0.67	0.67	0.77	0.68	0.57	0.56	1.47	0.56	0.48			
Onion	0.60	0.60	0.67	0.56	0.74	0.61	0.84	0.77	0.77	0.77	1.11	0.68	0.67	0.67	0.77	0.68	0.57	0.56	1.47	0.56	0.48	0.00		
Tomato	1.29	1.29	1.60	1.30	3.52	1.33	2.22	1.62	1.37	1.37	3.39	1.58	1.35	1.48	1.62	1.50	1.58	1.58	1.30	1.58	1.65	0.97	0.97	
Potato	0.60	0.60	0.77	0.80	0.74	0.61	1.26	0.77	0.77	0.77	1.11	0.98	0.96	0.96	0.54	0.84	0.66	0.65	1.47	0.65	0.48	0.10	0.10	0.97
Cucumber	0.73	0.73	0.65	0.67	0.61	0.74	1.03	0.65	0.65	0.65	0.90	0.81	0.80	0.80	0.65	0.81	0.55	0.55	1.64	0.55	0.39	0.05	0.05	1.19	0.05

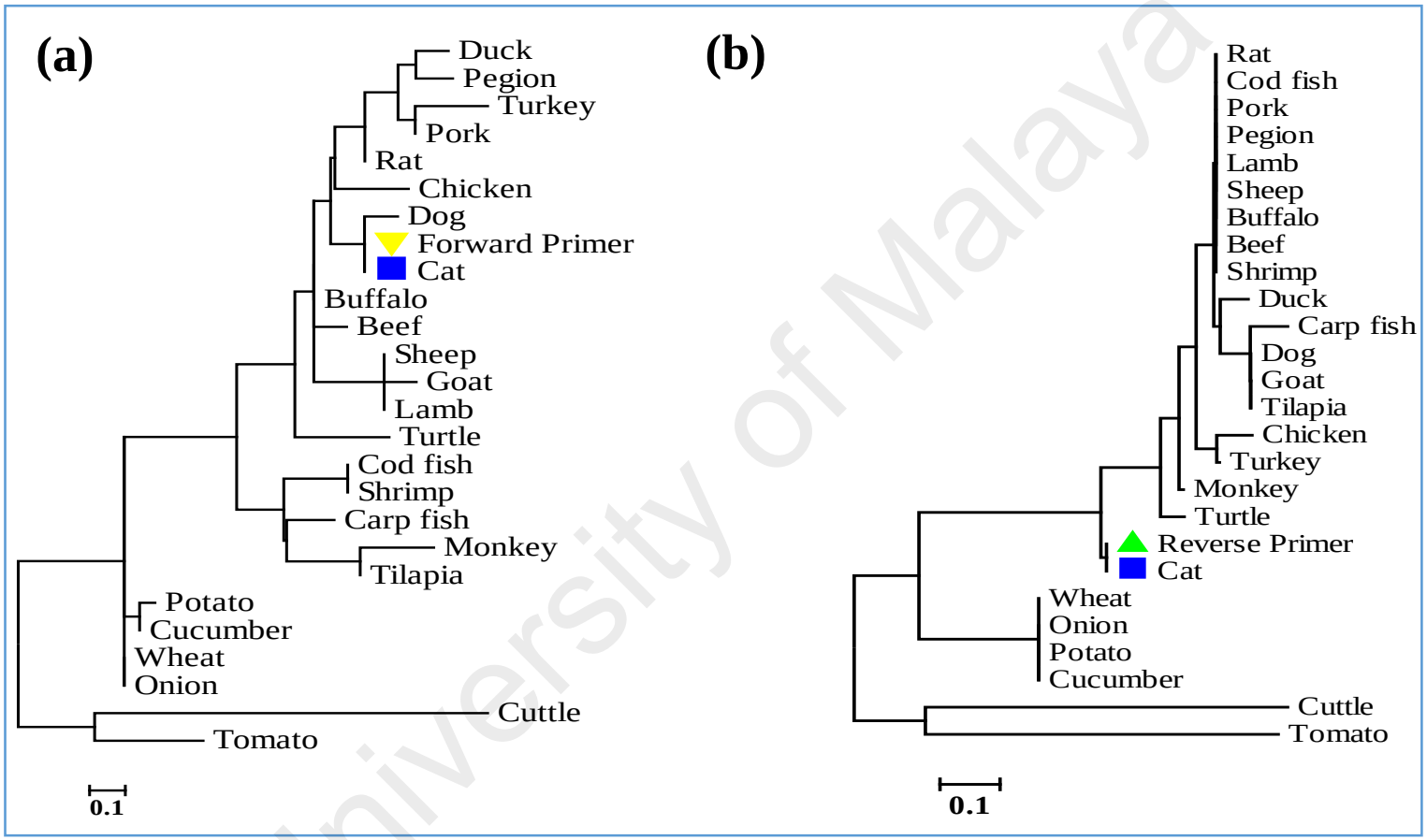


Figure 4.1 Phylogenetic tree constructed using neighbor-joining method with cytb gene sequences of land animals, fish and cob gene sequence of plant species, showing evolutionary distance between cat and other species in forward primer in (a), and reverse primer in (b).

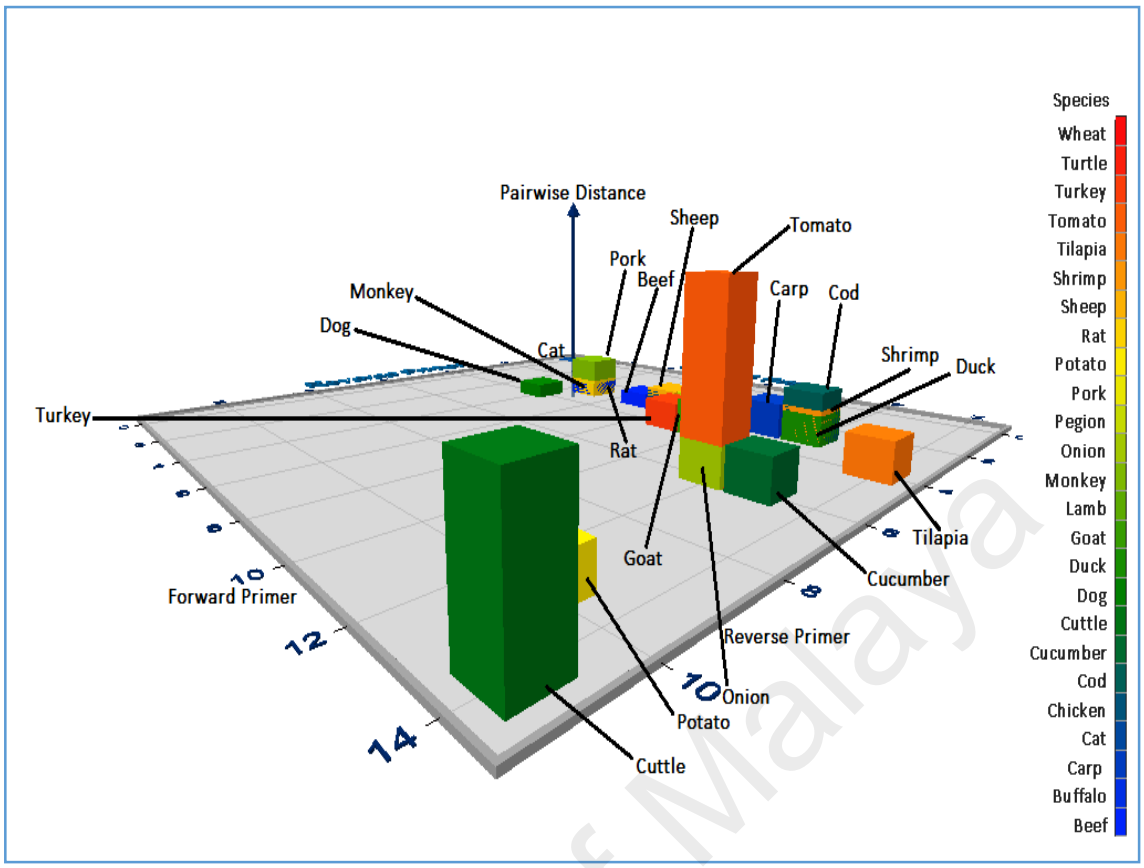


Figure 4.2. 3D plot showing mismatch and pairwise distance between cat and others 24 none target potential land animal, aquatic and plant species for theoretical analysis of primer pairs. Here, X and Y axes represent the number of forward and reverse primer mismatches and Z axis represents pair wise distance between cat and other potential terrestrial and plant species.

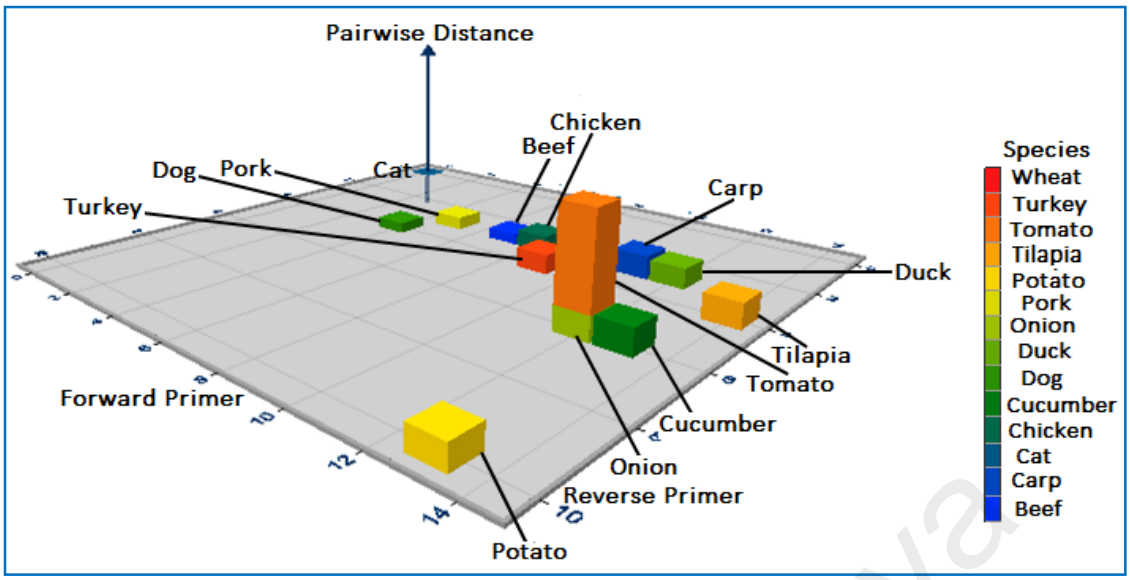


Figure 4.3 3D plot showing mismatch and pairwise distance between cat and others potential land animal, aquatic and plant species for burger formulations. Here, X and Y axes represent the number of forward and reverse primer mismatches and Z axis represents pair wise distance between cat and other potential terrestrial and plant species.

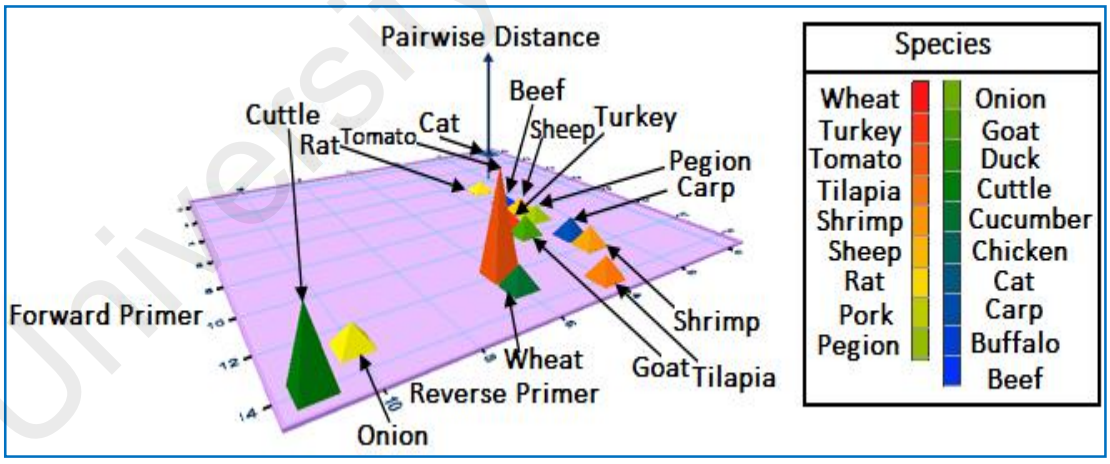


Figure 4.4 3D plot showing mismatch and pairwise distance between cat and others potential land animal, aquatic and plant species for meatball formulations. Here, X and Y axes represent the number of forward and reverse primer mismatches and Z axis represents pair wise distance between cat and other potential terrestrial and plant species.

4.2.2 In-silico Analysis of Feline Specificity in Commercial Products

The consensus gene sequences including primer binding sites were aligned with a total of 7 animal (cow, dog, chicken duck, turkey, lamb, pig), 2 fish (carp and tilapia), and 5 plant species (cucumber, onion, potato, tomato and wheat) potentially found in burger formulations. On the contrary, the 69 bp of feline species target site was aligned with 5 common halal animal meat species like beef, water buffalo, chicken, goat and sheep, 3 avian species namely domestic duck, pegion and turkey, 4 fish samples including carp, cuttle, tilapia and shrimp and 3 plant origin known as onion, tomato and wheat and 2 non halal meat species such as rat and pork for meatball formulations. Besides, ClustalW multiple sequence alignment program used to identify the variability of the primer binding region and total mismatch between target and non-target species. Pair wise distance for 69 bp feline-specific sites computed by the maximum composite likelihood method (Tamura et al., 2004) and phylogenetic tree was also build using MEGA5 software with the said species which were found as a potential ingredients for burger and meatball formulations. Therefore, pairwise distance and number of primer mismatches with the said species for burger (Figure 4.3) and meatball formulations (Figure 4.4) was shown on a 3D plot using XLSTAT software.

4.3 PCR Optimization

A 69-bp fragment (15-83 bp) was successfully amplified (Figure 4.5) from 1140-bp cytb gene of feline mitochondria (Accession number: AB194812.1), such product was never obtained from other 24 species of land and aquatic origins used in this study. The PCR reaction was conducted on a gradient thermal cycler with 25µl reaction volume by optimizing proper amount of chemicals and reagents described in previous chapter.

Conversely, 58˚C (Figure 4.5) was found to more appropriate annealing temperature for the amplification of desired products.

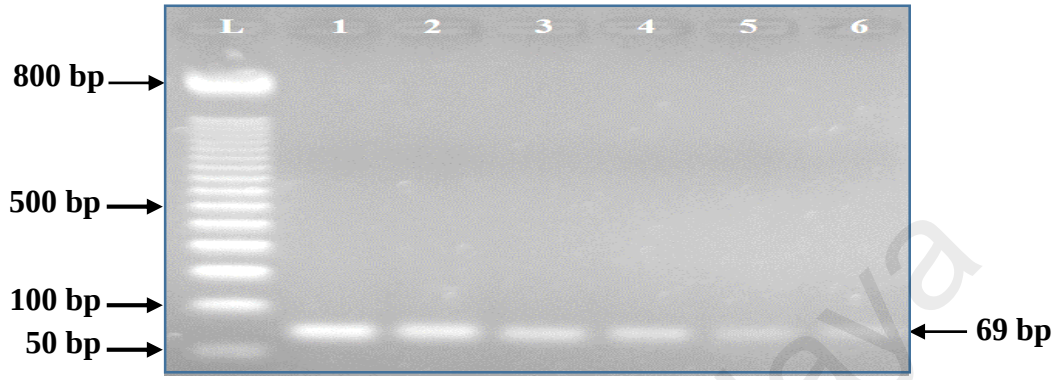


Figure 4.5 Optimization of newly developed primer pairs on gradient temperatures. Lanes 1 to 6 represents target DNA amplified at different annealing temperatures (57˚C to 62˚C). Lane L: 50 bp DNA ladder, Lane 1: 57˚C, Lane 2: 58˚C, Lane 3:59˚C, Lane 4: 60˚C, Lane 5: 61˚C, Lane 6: 62˚C, respectively.

4.4 Feline Specificity Test with Extracted DNA

To verify the feline specificity of the designed primers species specific PCR assay was performed along with twenty four non target species. The species-specific primers (69 bp) successfully amplified only from cat DNA template and no cross-species amplification was identified (Figure 4.6) with others fourteen animals, five fish and five plant species confirming the in-silico or theoretical analysis.

Figure 4.6 Cross-amplification of feline-specific target (69 bp) (Lane 1) against 24 different species (Lanes 2 to 25) separated on a 2% agarose gel using newly developed primer pairs. Lane L: 50 bp ladder, 1: cat, 2: beef, 3: buffalo, 4: chicken, 5: dog, 6: goat, 7: duck, 8: lamb, 9: sheep, 10: pegion, 11: pork, 12: monkey, 13: turtle, 14: rat, 15: turkey, 16: carp, 17: cod, 18: cuttle, 19: shrimp, 20: tilapia, 21: tomato, 22: onion, 23: potato, 24: cucumber, 25: wheat, and Lane Ntc: negative template control. Please note that, desired PCR product was amplified from Lane 1.

4.5 DNA Stability Test under Compromised Conditions

The significance of employing heat to meat sample was to check the result of various thermal process on target DNA degradation (Arslan, Ilhak, & Calicioglu, 2006; Ilhak & Arslan, 2007). Three different heat treatment schemes, namely, autoclaving, microwave cooking and boiling were performed in this research. In this study autoclaving was performed in four different stages at 110˚C for 2 h under 14.5-psi; 120˚C for 50 min under 14.5-psi; 133˚C for 20 min under 43.51-psi and 120˚C for 2.5 h under 45.5-psi (Figure 4.7). Microwave cooking (Figure 4.9), was conducted in three different phases namely low (300W for 10 min), medium (500W for 20 min) and high (700W for 30 min); whereas boiling. (Figure 4.8) was completed under 100˚C for 90 min, 110˚C for 30 min and 80˚C for 30 min. DNA extracted from all the heat-treated samples was successfully

amplified by PCR shown in Figure 4.7 (autoclaving), Figure 4.8 (boiling) and Figure 4.9 (microwaving).

Figure 4.7 Stability of the feline target after autoclaving at 110˚C for 2 h under 14.5-psi (Lane 1); 120˚C for 50 min under 14.5-psi (Lane 2); 133˚C for 20 min 43.51-psi (Lane 3) and 120˚C for 2.5 h under 45-psi (Lane 4). Lanes L & Ntc: 50 bp DNA ladder and Negative control, respectively.

Figure 4.8: Stability of the feline target after boiling at 100˚C for 90 min (Lane 1), 110˚C for 30 min (Lane 2), boiling in water bath at 80˚C for 30 min (Lane 3). Lanes L & Ntc: 50 bp DNA ladder and negative template control, respectively.

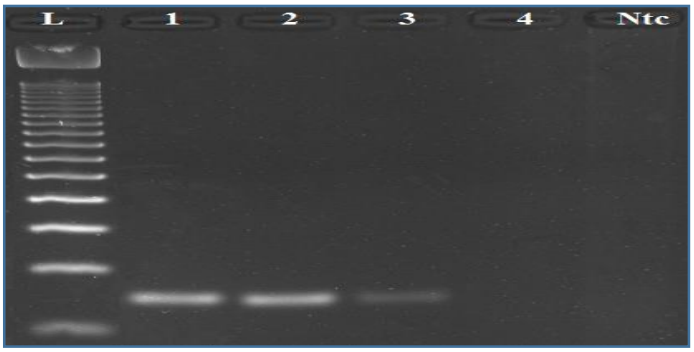


Figure 4.9 Stability of the feline target after microwave heating at 300W for 10 min (Lane 1), 500W for 20 min (Lane 2) and 700W for 30 min (Lane 3). Clear 69 bp PCR product was obtained from all lanes (1 to 3), except the lane (4). Feline meat cooked at above 700W and 30 min (not amplified) in lane 4. Lanes L & Ntc: 50 bp DNA ladder & negative control, respectively.

4.6 Sensitivity of Target DNA

4.6.1 Raw State

The sensitivity of raw and pure sample of extracted target DNA was checked by 10-fold (10, 1, 0.1, 0.01, 0.001, and 0.0001 ng) serial dilution method by three independent analysts on three different days. Sensitivity for the heat treated samples were studied in a similar way and, we clearly detected 69 bp PCR products from 0.0001ng feline DNA template both in raw and heat treated (autoclaved and boiled) and therefore defined the limit of detection (LOD) for this assay under raw and treating conditions (Figures 4.10, 4.11 and 4.12).

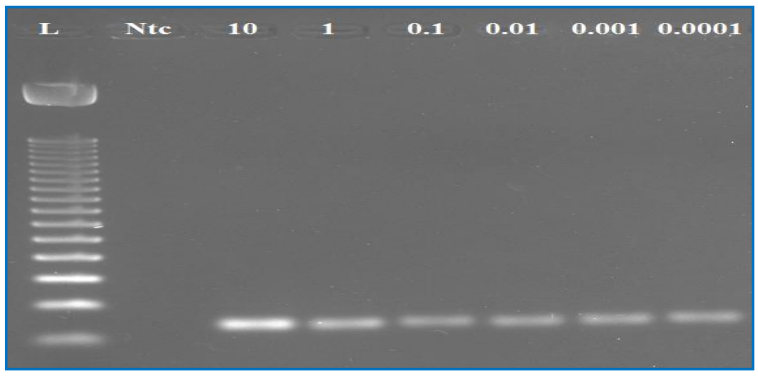


Figure 4.10 Sensitivity under 10-fold serially dilution target DNA from 10 to 0.0001 ng respectively in raw or pure state. Lane L & Ntc: 50 bp ladder & Negative template control. Lane 1: 10 ng, Lane 2: 1 ng, Lane 3: 0.1 ng, Lane 4: 0.01 ng, Lane 5: 0.001 ng and lane 6: 0.0001 ng, respectively.

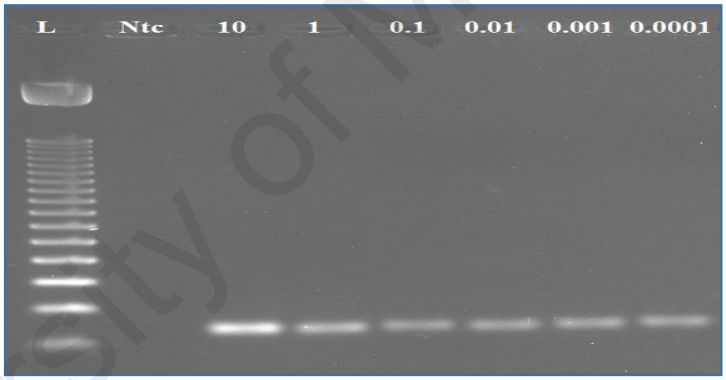


Figure 4.11 Sensitivity under 10-fold serially dilution target DNA from 10 to 0.0001 ng respectively in autoclaving treatment. Lane L & Ntc: 50 bp ladder & Negative template control. Lane 1: 10 ng, Lane 2: 1 ng, Lane 3: 0.1 ng, Lane 4: 0.01 ng, Lane 5: 0.001 ng and lane 6: 0.0001 ng, respectively.

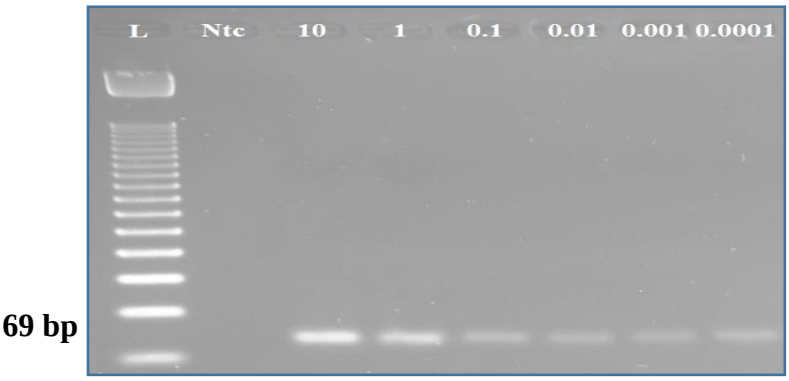


Figure 4.12 Sensitivity under 10-fold serially dilution target DNA from 10 to 0.0001 ng respectively in boiling treatment. Lane L & Ntc: 50 bp ladder & Negative template control. Lane 1: 10 ng, Lane 2: 1 ng, Lane 3: 0.1 ng, Lane 4: 0.01 ng, Lane 5: 0.001 ng and lane 6: 0.0001 ng, respectively.

1.6.2 Sensitivity Test in Ternary Mixtures

4.6.2.1 Cat-Beef-Chicken Admixes

To check the specificity and sensitivity in ternary mixtures, cat meat was properly mixed with beef and chicken meat according to base adulterated meat mixture (BAM) (Ali et al., 2012a) and demonstrates that *F. catus* specific PCR assay developed in this study was highly sensitive and specific since it unambiguously identified as low as 0.01% (w/w) feline meat in raw, autoclaved and oven heated cat-beef-chicken ternary admixes (Figure 4.13).

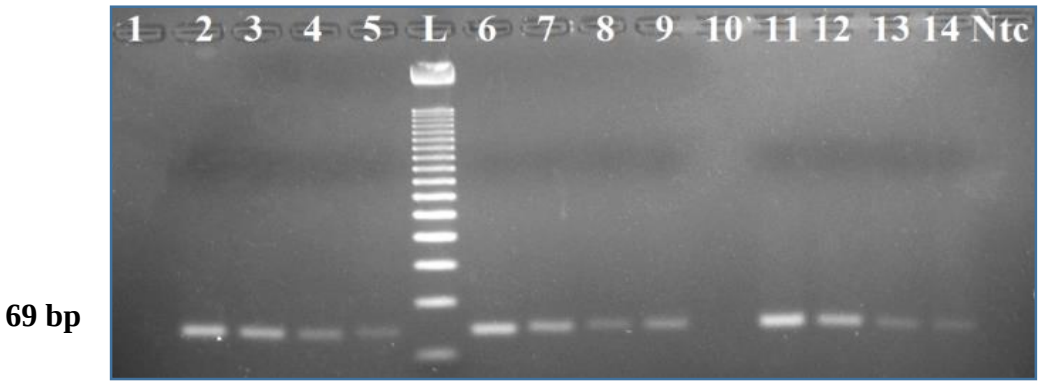


Figure 4.13 Sensitivity in cat: beef: chicken ternary admixture under raw (Lanes 2 to 5), autoclaved (Lanes 6 to 9) and oven heated (Lanes 11 to 14) states. However, Lanes 2 to 5: Raw admixtures (10, 1, 0.1 and 0.01%); Lanes 6 to 9: admixtures (10, 1, 0.1 and 0.01%) autoclaved at (120˚C for 2.5 h in 45-psi); Lanes 11 to 14: admixtures (10, 1, 0.1 and 0.01%) oven heated at 180˚C for 30 min. Lane 1: 100% beef and Lane 10: 100% chicken under raw states. Lanes L & Ntc: 50 bp ladder and negative template control, respectively.

4.6.2.2 Feline-Bovine-Wheat flour Admixes

On the other hand, to identify the specificity and sensitivity of cat-beef-wheat flour ternary mixtures that was composed of bovine and wheat flour contaminated with 10%, 5%, 0.2% and 0.01% (w/w) of cat meat and validated that the newly developed short-amplicon-length PCR assay was highly sensitive and more appropriate to track out the trace amount of DNA in complex matrices and thus able to detect feline DNA as low as 0.01% (w/w) in raw, autoclaved and boiled feline-bovine-wheat flour ternary background (Figure 4.14).

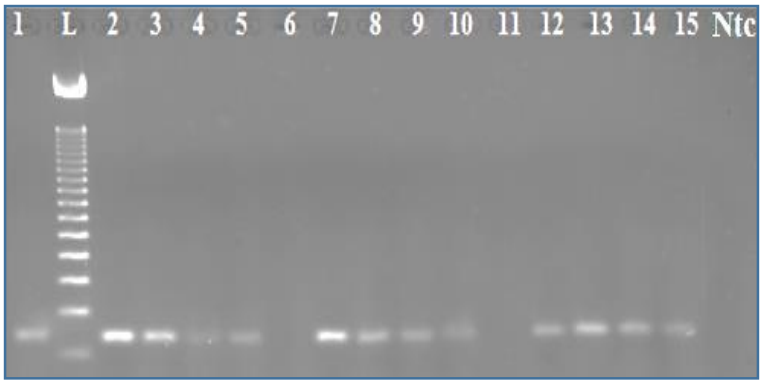


Figure 4.14 Feline adulteration detection in feline, bovine and wheat flour ternary mixtures. Lanes 1, 6 and 11: 100% raw cat meat, bovine and wheat flour, respectively. However, 10%, 5%, 0.2% and 0.01% feline meat containing admixtures before any treatments (Lanes 2 to 5), after extensive autoclaving (120˚C for 2.5 h under 45-psi pressure) (Lanes 7 to 10) and after boiling (100˚C for 90 min) (Lanes 12 to 15). Lanes L & Ntc: 50 bp ladder & negative control template, respectively.

4.6.2.3 Sensitivity in Burgers

Finally, the developed assay was tested in commercial burger matrices since no report has been published for feline meat detection in commercial products. To fill-up this research gap, different amount (10%, 5%, 1%, and 0.1 %) of cat meat was spiked in deboned beef and chicken and others burger components by following Ali et al. (2012) represents in chapter 3 (Table 3.3). The minced meats were treated in an oven under 220˚C for 15 min and standard autoclaving condition (121˚C for 2.5h at 45-psi). However, using the processed meats burgers were made and DNA was extracted from cat contaminated beef and chicken burgers. In Figures 4.15 and 4.16, (lanes 5 to 8), clearly showed that PCR products was obtained from cat meat adulterated beef (Figure 4.15) and chicken burgers (Figure 4 16). No PCR products was obtaining from commercial feline meat disinfected beef (Figure 4.15) and chicken burgers (Figure 4.16), lanes 1 to 4.

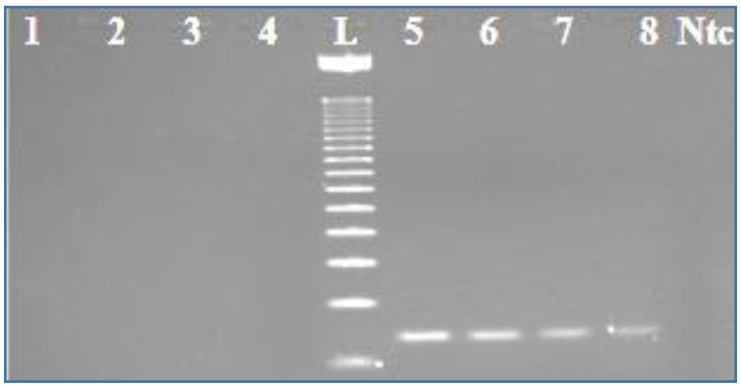


Figure 4.15 Feline meat detection in beef burger. Clear PCR products obtained from 10 %, 5%, 1%, and 0.1% feline meat adulterated model beef burgers (Lanes 5 to 8) and no PCR products from commercial beef burgers (Lanes 1 to 4). Lanes L & Ntc: 50 bp ladder & negative template control, respectively.

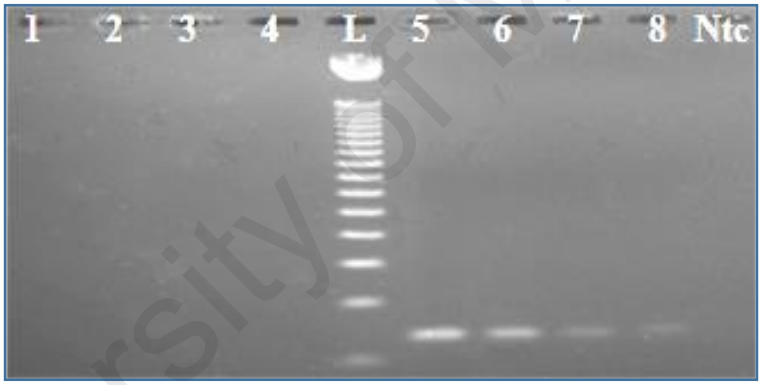


Figure 4.16 Feline meat detection in chicken burger. Clear PCR products obtained from 10 %, 5%, 1%, and 0.1% feline meat adulterated model beef burgers (Lanes 5 to 8) and no PCR products from commercial beef burgers (Lanes 1 to 4). Lanes L & Ntc: 50 bp ladder & negative template control, respectively.

Table 4.4 Screening of commercial beef and chicken burgers

Days	Pure beef burger	Pure chicken burger	Pure cat burger	Cat meat spiked beef burgers	Cat meat spiked chicken burgers	Autoclaved burgers	Oven heated burgers	Commercial beef burgers				Commercial chicken burgers				Detection probaility (%)
								A	B	C	D	E	F	G	H	
Day-1	3/3	3/3	3/3	3/3	3/3	3/3	3/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	100
Day-2	3/3	3/3	3/3	3/3	3/3	3/3	3/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	100
Day-3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	100
Day-4	3/3	3/3	3/3	3/3	3/3	3/3	3/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	100
Day-5	3/3	3/3	3/3	3/3	3/3	3/3	3/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	100

Numerator denotes feline positive samples and denominator reflects total number of analyzed samples

4.6.2.4 Sensitivity in Meatball Matrices

On the contrary, the newly designed feline specific primer pairs assay was further tested in complex background of meatballs under pure and different amount (10%, 5%, 1%, 0.1% and 0.01%) of feline meat contaminated beef and chicken meatballs (Chapter 3, Table 3.4). To simulate the normal cooking and extensive autoclaving prepared meatballs were boiled at 100˚C for 90 min and autoclaved was done at 120˚C for 2.5 h under 45-psi, respectively. Clear 69 bp feline PCR product was amplified from 10% to 0.1% (w/w) cat meat positive beef (Figure 4.17) and chicken meatballs (Figure 4.18). However, no positive amplification was found during the commercial meatballs screening with 69 bp PCR assay (Figure 4.18, lanes 1 to 6).

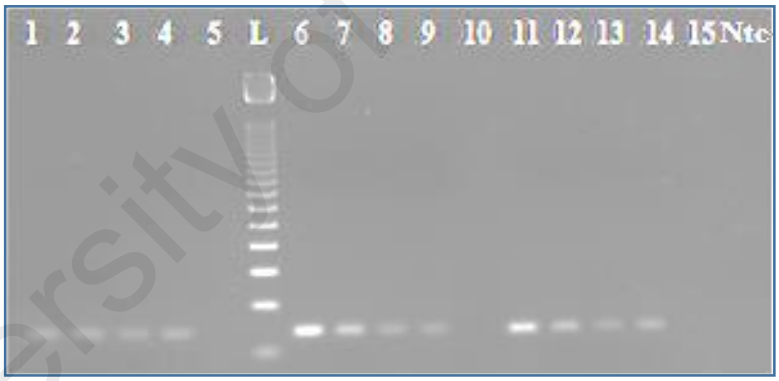


Figure 4.17 Analysis of beef meatballs for feline adulteration. Lanes 1 to 5 (raw); Lanes 6 to 10 (boiled) and Lanes 11 to 15 (autoclaved) beef meatballs spiked with 10%, 5%, 1%, 0.1% and 0.01% feline meats, respectively. Lane L & Ntc: 50 bp ladder & negative template control, respectively.

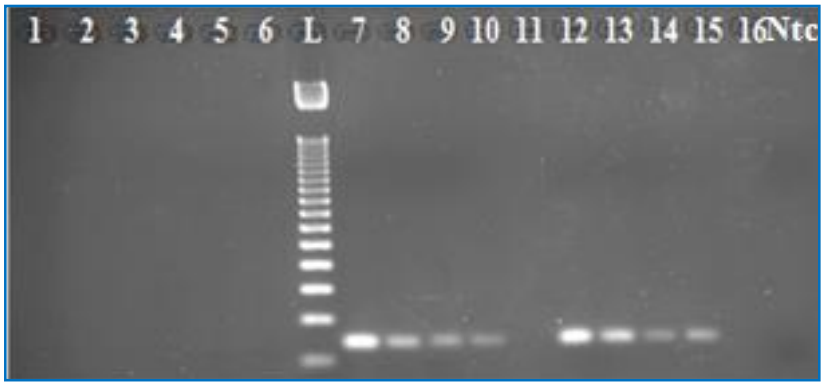


Figure 4.18 Analysis of chicken meatballs for feline adulteration under boiled (Lanes 7 to 10) and autoclaved (Lanes 12 to 15) treatment. No PCR products was amplified from commercial beef meatballs (Lanes 1 to 3) and chicken meatballs (Lanes 4 to 6). However, Lanes 7 to 10 (boiled) and Lanes 12 to 15 (autoclaved) chicken meatballs spiked with 10%, 5%, 1%, 0.1% and 0.01% cat meat, respectively. Lanes L & Ntc: 50 bp ladder & negative template control, respectively.

Table 4.5 Screening of commercial beef and chicken meatballs

Meatball samples	Day 1	Day 2	Day 3	Feline meat detection up to 0.1% (w/w)	Detection probaility (%)
Ready to eat pure beef meatball	3	3	3	0/9	100
Ready to eat pure chicken meatball	3	3	3	0/9	100
Ready to eat pure cat meatball	3	3	3	9/9	100
Ready-to-eat cat meat spiked beef meatballs	9	9	9	27/27	100
Ready-to-eat cat meat spiked chicken meatballs	9	9	9	27/27	100
Commercial beef meatballs					
A	9	9	9	0/9	100
B	9	9	9	0/9	100
C	9	9	9	0/9	100
Commercial chicken meatballs					
A´	9	9	9	0/9	100
B´	9	9	9	0/9	100
C´	9	9	9	0/9	100

Numerator reflects the total number of positive detection and denominator demonstrates total number of samples analyzed

4.7 Restriction Fragment Length Polymorphism (RFLP) Analysis

4.7.1 In-silico PCR-RFLP Digestion

To detect the any kind of non-specific PCR amplification restriction digestion analysis was performed using 69 bp fragment of the feline-species *cytochrome b* specific region. Therefore, the 69 bp feline-specific PCR products was digested by *Alu*I since in-silico analysis (Figure 4.19) using the web tool NEB cutter2 (Vincze et al., 2003), showed available restriction sites for these enzymes with suitable fragment-lengths. Restriction digestion with *Alu*I **(5´... AG ↓ CT... 3´)** enzyme using 69 bp region of this feline specific had one sites (Figure 4.19a). Additionally, it produces two fragments of 43 bp and 26 bp size (Figure 4.19b) and the mismatch analysis (Table 4.6) of other species with feline specific primers at the positions of *Alu*I-cut sites which are typical for doubtless feline species detection in raw, admixed and commercial cat meat adulterated products.

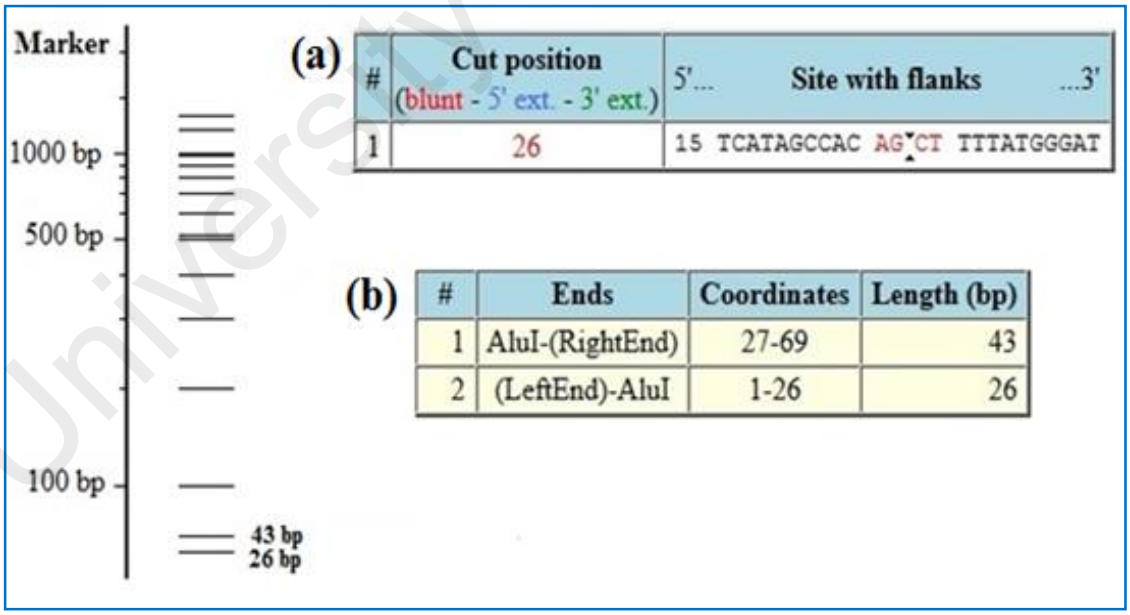


Figure 4.19 In-silico digestion of feline species primer pairs with *Alu*I restriction enzyme using an online version NEB cutter2 tool. In (a), feline species had one site after restriction digestion. In (b), two fragments 43 bp and 26 bp were produced, respectively.

Table 4.6 Mismatch bases of studied species with feline specific primers and the positions of *Alu*I restriction sites of 69 bp-amplicon-length using multiple alignment MEGA5 version software

4.7.2 Products Authentication by RFLP Analysis

An online based bioinformatics program (NEBcutter2) was applied in 69 bp target of feline cytb gene using *Alu*I restriction enzyme and found to digest with two fragments (26, and 43 bp) and one cut site. Therefore, *Alu*I digested PCR products of raw, autoclaved, oven heated and boiled burgers and meatballs were separated using lab-on-a-chip microfluidic separation technique. Both of the digested fragments (26 and 43 bp) were clearly shown in the gel-image (Figure 4.20, lanes 2, 4, 6, 8, 10 & 12) and electropherograms (Figure 4.21). In addition, the positive control (141 bp) was amplified from all the lanes (1 to 6), after screening with commercial products shown both in gel-image (Figure 4.22, lanes 1 to 6) and electropherograms (Figure 4.23). The cat meat spiked model burgers and meatballs was further amplified with endogenous control (141 bp) and digested by *Alu*I restriction enzyme which was shown in gel-image (Figure 4.22)

and electropherograms (Figure 4.23). In Figure 4.22 (lanes 7 to 12), demonstrates 3 fragments of length 127, 43, and 26 bp which were resulted following *Alu*I digestion of raw, oven heated and autoclaved burgers (lanes 7 to 9) and raw, autoclaved and boiled meatballs PCR product (lanes 10 to 12) with 141 bp. The molecular size statistics of the 69-bp feline-specific site from raw, autoclaved, oven heated and boiled cat meat spiked ready to eat model burgers and meatballs are shown in Table 4.7. Thus the analysis of commercial products screening are also presented in Table 4.8.

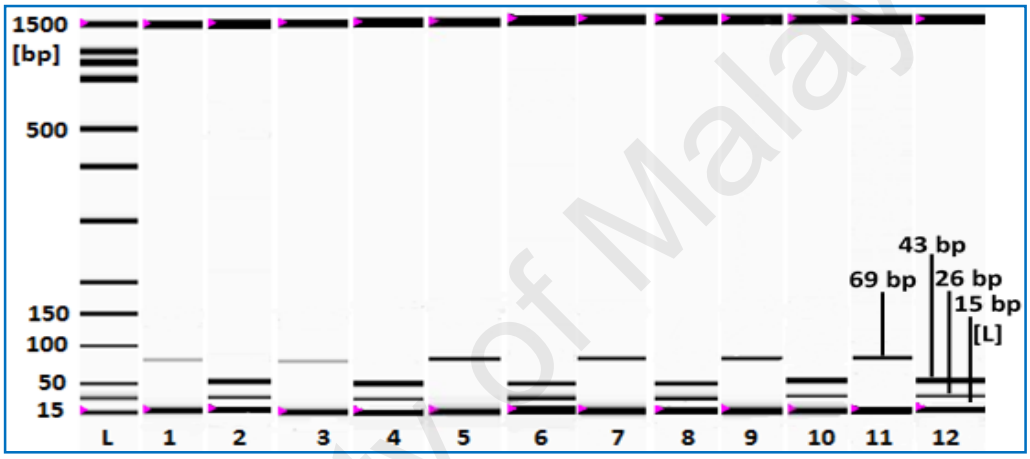


Figure 4.20 Products digested by RFLP analysis, showing 69-bp PCR and *Alu*I restriction digestion products obtained from ready to eat model burgers and meatballs. In gel image, Lane L: DNA ladder; (lanes 1, 3, 5, 7, 9, 11) before restriction digestion and (lanes 2, 4, 6, 8, 10, 12) after *Alu*I restriction digestion of PCR product obtained from raw (lanes 1 & 2), autoclaved (lanes 3 & 4), oven heated (lanes 5 & 6) in burgers (lanes 1 to 6) and raw (lanes 7 & 8), autoclaved (lanes 9 & 10), boiled (lanes 11 & 12) in meatballs (lanes 7 to 12), respectively. Corresponding, electropherograms are as shown by labels in Figure 4.21.

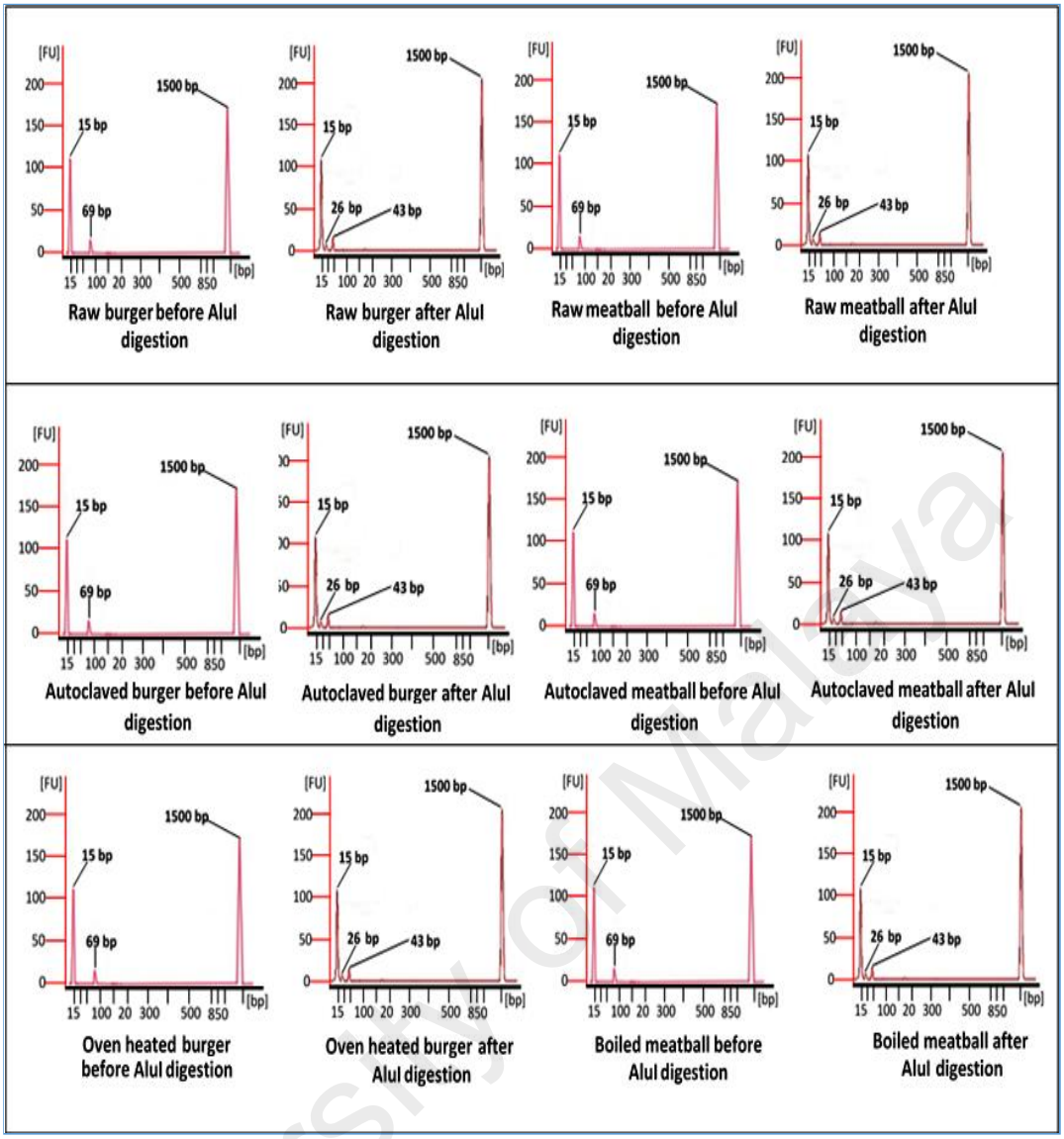


Figure 4.21 Products digested by RFLP analysis, showing 69-bp PCR and *Alu*I restriction digestion obtained from ready to eat model burgers and meatballs are shown in electropherograms are demonstrated with labels in insets in above.

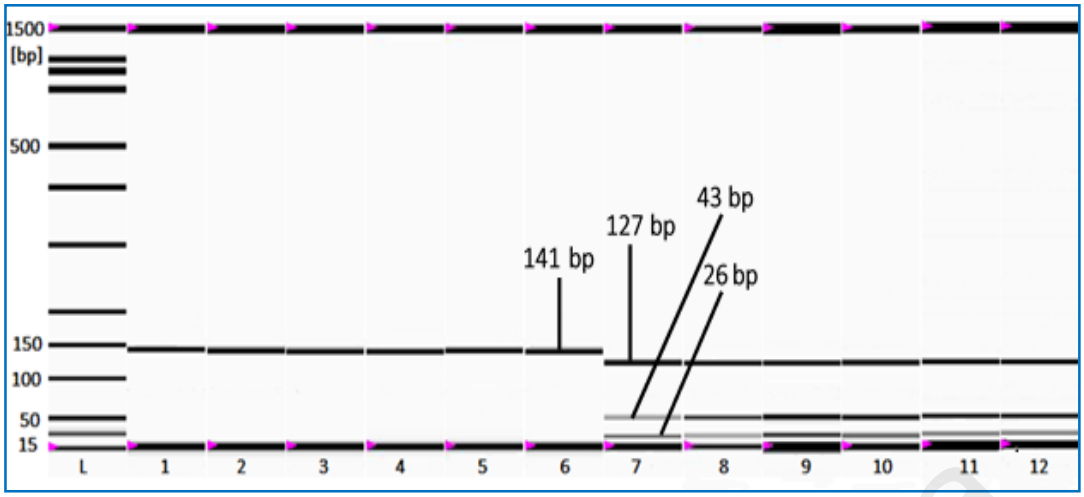


Figure 4.22 Screening of commercial products using PCR and RFLP analysis. In gel image, no false detection was observed in commercial burgers (lanes 1 to 3) and meatballs (lanes 4 to 6). Restriction pattern analysis of feline-target (69 bp) and endogenous control (141 bp) after *Alu*I (lanes 7 to 12) in adulterated model burgers (lanes 7 to 9) and meatballs (lanes 10 to 12). Briefly, Lane L: DNA ladder, lanes (7 to 9) cat meat spiked raw (lane 7), autoclaved (lane 8), oven heated burgers (lane 9) and lanes (10 to 12) cat meat adulterated raw (lane 10), autoclaved (lane 11) and boiled meatballs (lane 12). Corresponding, electropherograms are as shown by labels in Figure 4.23.

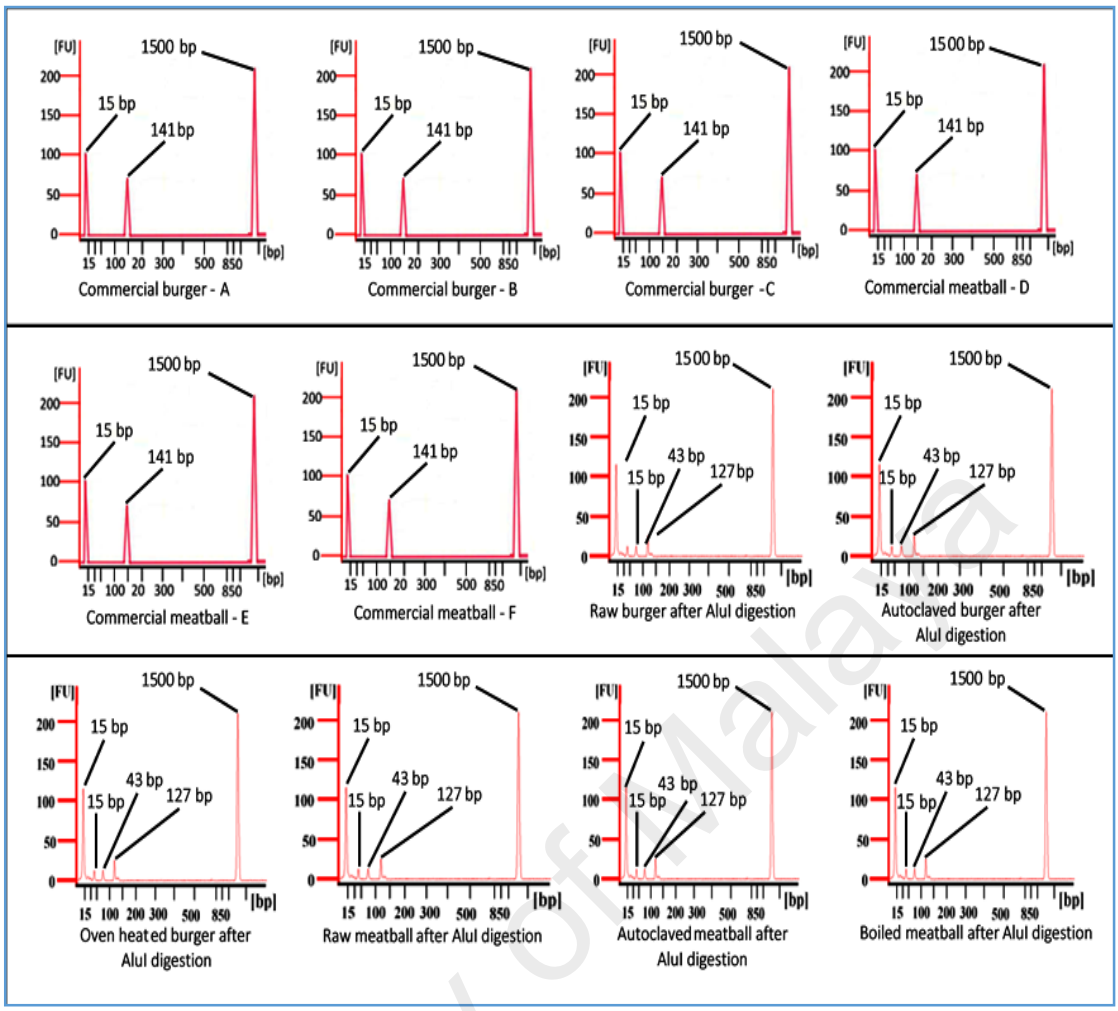


Figure 4.23 Screening of commercial products using PCR and RFLP analysis are shown in electropherograms are demonstrated by respective labels.

Table 4.7 Statistics analysis of molecular size of lab-on-a chip based feline specific PCR-RFLP assay before and after digested with restriction enzyme

Day	Size (bp)	Autoclaved burger with *Alu*I digestion		Oven heated burger with *Alu*I digestion		Autoclaved meatball with *Alu*I digestion		Boiled meatball with *Alu*I digestion		Detection probaility (%)
		Before	After	Before	After	Before	After	Before	After	
1	69	69±1.2	-	70±0.8	-	68±0.7	-	70±1.0	-	100
	43	-	42±0.9	-	47±0.9	-	47±0.0	-	45±0.6	100
	26	-	24±1.2	-	23±0.1	-	22±1.2	-	20±0.4	100
2	69	69±0.5	-	67±0.4	-	66±1.2	-	69±0.0	-	100
	43	-	43±0.8	-	44±1.2	-	46±0.6	-	44±0.5	100
	26	-	26±0.6	-	23±0.5	-	22±0.4	-	21±0.2	100
3	69	64±0.9		69±0.0	-		-	67±1.2	-	100
	43	-	40±1.2	-	43±0.6	-	44±1.0	-	46±1.2	100
	26	-	22±0.8	-	25±0.8	-	24±0.5	-	25±0.9	100

Table 4.8 Analysis of feline meat in commercial burger and meatball products

Burgers	No. of sample	*Alu*I Digested	Meatballs	No. of sample	*Alu*I Digested	Detection probability (%)
A	3	0/9	D	3	0/9	100%
B	3	0/9	E	3	0/9	100%
C	3	0/9	F	3	0/9	100%
Cat meat positive burgers	3	9/9	Cat meat positive meatballs	3	9/9	100%

The numerator and denominator of each fraction denote the number of positive detection and total number of samples analyzed

CHAPTER FIVE

DISCUSSION

CHAPTER 5: DISCUSSION

5.1 DNA Extraction

Before primer pairs was tested a selection of twenty four species including animal, aquatic and plant species was collected from various super markets and wet markets in Selangor, Petaling Jaya and Kuala Lumpur in Malaysia as stated in Chapter 3. The identity of the collected animals, fish and plant species was verified by the veterinarian, fishery and taxonomist experts from the Department of Animal Science and Crop Science under the faculty of Agriculture in the University Putra Malaysia.

Total genomic DNA was extracted from raw, heat treated (autoclaved, boiled and microwaved) meat samples using commercial DNA extraction kit and the amount of yields varies depend on the quantity of starting material, sample materials and extraction protocols (van Woerkom, 2008). Commercial Mini Kit (Yeastern Biotech Co. Ltd., Taiwan) provided higher yields of DNA from raw (59-718 ng/µl) and treated meat samples (114-561 ng/µl) than the conventional liquid-liquid extraction techniques which reduced high yield of DNA during the aqueous and organic phases of this technique (Karabasanavar et al., 2011). In addition, commercial DNA extraction kit was safe for handling to extract DNA with minimal damage while sample was preparing (Karabasanavar et al., 2011). The concentration and quality of the extraction DNA required to be checked and optimized for the test. To check the presence and amount of targets in the eluate, concentration of the extracted DNA was estimated by taking an absorbance readings at 260nm and the purity of DNA was checked by taking ratio of absorbance readings at 260 nm and 280 nm in triplicates using Bio drop UV/VIS spectroscopy (BioChrom, Libra S70; Cambridge, UK). The concentration of DNA was used 10 ng/µl initially, thus a dilution series calculation was made on these samples in which the DNA concentration of pure samples was diluted with distilled water or nuclease

free water. The A_{260}: A_{280} value of all extracted DNA was 1.7 to 2.0 which reflected good quality of yields (Adams, 2013) indicated no contamination was happening during the extraction period. However, a higher sample size (1g) was used to extract DNA from admixed meat (ternary complex) and commercial meat products (burgers and meatballs) using a modified CTAB method, popularly known to give higher yield of DNA. The yield of DNA from two set of admixed ((i) cat: beef: chicken and (ii) feline: bovine: wheat flour) samples was 554-714 ng/µl, whereas from burgers (beef and chicken burgers) was 200-340 ng/µl), and meatballs (beef and chicken meatballs) was 150-373 ng/µl), respectively. The lower yield of DNA from commercial meat products such as burgers and meatballs might be due to the presence of some PCR inhibitors compounds such as plant, spices, salts and oil that were used to formulate beef and chicken burgers and meatballs (Chapter 3; Tables 3.3 and 3.4). Moreover, heat treatments also increases the number of cells in per unit of weight of tissue though the dehydration process (Karabasanavar et al., 2011b).

5.2 Biomarker Development

Since the short-length DNA biomarkers are extraordinarily stable under processing conditions (Arslan et al., 2006) as it can detect the target DNA in raw, dry or processed condition of complex background of commercial products. In this study, intra-species conserved and interspecies hyper variable regions of mitochondrial *cytb* gene was used to design a set of feline species primers to amplify a short-length amplicons (69 bp). Based on literature study, short-length biomarker was developed by a group of researchers (Calvo et al., (2002) (84 bp), Ali et al., (2011) (109 bp), Natonek et al., (2013) (66 bp), Ali et al., (2013) (100 bp), and Rahman et al., (2014) (100 bp)) and successfully amplified their targets in the complex matrices of different compromised states. On the other hand

longer-size biomarker (≥150 bp), are not favourable in perspective of degradation. Rodriguez and his co-workers (2003) developed a 411 bp of swine-specific primers and detected in standard autoclaving condition (121°C for 20 min) but in an extensive autoclaving system (120°C for 2.5 h under 45-psi) no band was shown from the same target tested by Ali et al. (2011). Therefore, the short length amplicon 69 bp has great advantage while degraded sample material has to be analyzed.

The maximum composite likelihood method (Tamura et al., 2011) was used to analyze the phylogenetic tree (Figure 4.1) and pairwise distance (Table 4.3) in the primer binding regions of the tested species (Rahman et al., 2014). The highest pairwise distance (1.33) was observed between cat and cuttle fish in and the lowest (0.09) found between cat and dog (Table 4.3) (Tamura et al., 2011) demonstrating enough genetic distance and unlikelihood of cross-species amplification in a real PCR experiment. Similar results were found when dendogram was created among other species (Figure 4.1) (Saitou & Nei, 1987). However, 3D (Figure 4.2) plot was constructed with the number of mismatches and pairwise distance (Table 4.3) data to find the discriminating properties and molecular orbit of the primers using XLSTAT version 2014.1.01 (Addinsoft, 2013). All the obtained data represented high degree of discernment among *F. catus* and other terrestrial, fish and plant species. Additionally, the maximum (19 nt) and the minimum (3 nt) mismatches in the primer binding regions (Table 4.2, and Figure 4.2) pointed that there is no-probability of cross positive-reaction even among the closely matched species while handling the PCR in real experiment. It has been quoted that a single mismatch in the primer binding region might be sufficient to cause amplification failure in PCR reaction (Ali et al., 2012; Smith &Vigilant et al., 2002; Wu et al., 2009). All the obtained data in in-silico analysis supporting the theoretical conclusion that the newly developed assay was highly specific for the feline species. In a real PCR experiment amplified 69-bp target only from *F. catus* DNA template (Figure 4.6), confirming the in-silico or theoretical results.

5.3 Feline Specificity

Species-specific PCR assays (Ali et al., 2013; Mane, Mendiratta, & Tiwari, 2012) have been widely used to detect even a minute amount of defilement in raw, processed and commercial food products because of its rapidity, simplicity, sensitivity and specificity (Mafra, Ferreira, & Oliveira, 2007). Multiplex PCR (Dalmasso et al., 2004; Köppel, Ruf, & Rentsch, 2011) is a most recent development in the field of PCR technology. It is theoretically interesting and can distinguish many species in a single assay platform but the optimization of multiplex PCR reactions in a single tube is a challenging job (Ali, Razzak, & Hamid, 2014).

In this study, a 69-bp fragment was successfully amplified only from feline species (Figure 4.6, Lane 1) when the primers were cross-challenged against 24 other species of terrestrial (beef, buffalo, chicken, duck, goat, pig, sheep, lamb, turkey, pigeon, turtle, monkey, dog and rat) and aquatic (cod, carp, tilapia, shrimp and tilapia), and plant (onion, wheat, cucumber, potato and tomato) origins. An optimum primer concentration of 20 picomoles and preheating temperature of 95˚C for 3 min, denaturation at 95˚C for 20s containing 35 cycles, annealing at 58˚C for 20s, and 30s of extension at 72˚C and 5 min of final extension at 72˚C were found suitable for the amplification of desired products. No-cross species positives detection was found in repeated PCR run along with 24 non-target species. A higher annealing temperature increases primer specificity and reduces non-specific PCR amplification (Ali, Hashim, Mustafa, & Man, 2012a; Wu, Hong, & Liu, 2009). The optimized annealing temperature of 58˚C (Figure 4.5) was favourable for the detection of 69 bp feline target in all experimental analysis. Previously, six feline species PCR assay have been developed based on different mitochondrial genes (Abdulmawjood et al., (2003), (672 bp), Abdel Rahman et al., (2009), (672 bp); Ilhak et al., (2007), (274 bp); Martine et al., (2007), (108 bp); Tobe et al., (2008), (180 bp) and

Irine et al., (2013), (331 bp)). However, it has been an established fact that longer-amplicons are thermodynamically less stable than the shorter-ones under environmental decomposition or degradation by food processing treatment (Ali, Hashim, Mustafa, & Che Man, 2011a; Rahman et al., 2014; Rojas et al., 2010). Therefore, a very short-length amplicon (69 bp) PCR assay based on mt-cytb gene was developed and optimized for *F. catus* and demonstrated its stability under various food processing conditions for the analysis of feline meat.

5.4 DNA Stability Test

Compromised condition refers to the degradation of DNA either by natural decomposition or mechanical or forceful breakdown of specimens while processing or cooking foods (Arslan, Ilhak, & Calicioglu, 2006; Ilhak & Arslan, 2007). Three different heat treatment schemes, namely, autoclaving, microwave cooking and boiling that are commonly used in meat processing were performed in this research. Autoclaving is frequently used in steaming and canning process (Figure 4.7) is a way to destroy any potential microorganism's presents in any substrate at very high temperature (above 300˚C) under pressurized conditions (Todar, 2008). Microwave cooking (Figure 4.9), on the other hand, is a recent practice to cook or warm up food within a short-time, whereas boiling (Figure 4.8) is a traditional way of cooking in daily a life. A 69 bp of PCR products was successfully amplified from all the heat treated DNA extracted samples observed in Figure 4.7 (autoclaving), Figure 4.8 (boiling), and Figure 4.9 (microwaving). According to the European legislation (Commission, 2002), cat meat was autoclaved under three different sterilization stages (110˚C for 2 h at 14.5-psi; 120˚C for 50 min at 14.5-psi and 133˚C for 20 min at 43.51-psi) and obtained PCR products without any adverse effect. In an extreme autoclaving system (120˚C for 2.5 h at 45-psi) (Ali et al., 2013), thus the target

was successfully amplified (Figure 4.7) which proved its highly thermal stability because of short length-amplicon (Rahman et al., 2014). Additionally, Haunshi et al. (2009); Karabasanavar et al. (2011) and Mane et al. (2012) studied the effect of autoclaving on DNA by treating various type on domestic meat at 121˚C for 15-30 min and they found their sample remained thermally stable, and were not degraded under this condition. However, different autoclaving treatment was applied on target DNA and the products was amplified from all stages (Figure 4.7, lanes 1-4), compared to previous studies.

In a standard domestic practice, microwave cooking is done at three different phases known as low (300W), medium (500W) and extreme microwaving (700W) for 10-30 min and boiling was done under 100˚C for 90 min, 110˚C for 30 min; and 80˚C for 30 min in water bath. None of these treatments affected the PCR amplification (Figures.4.8 and 4.9). Meat cooked above 700W for 30 min was observed to be dehydrated, burnt in to ashes and thus was not suitable for consumption and not amplified (Figure 4.9, lane 4). Arslan et al. (2006) pan fried beef at 190˚C for 80 min and no PCR product was observed under this condition while cooking was done in non-aqueous environments. However, meats from different species were processed separately, and blender and others processing equipment were handled and cleaned carefully to avoid cross contamination for all processing conditions.

Nevertheless, two PCR assays (Abdulmawjood et al., (2003), and Martine et al., (2007)) were tested under compromised condition (boiling and autoclaving) among the developed feline meat detection techniques but none of them verified their products under extreme autoclaving (120˚C for 2.5 h under 45-psi) and microwaving (700W for 30 in) systems. In this research, the target stability was verified under extensive autoclaving and microwaving treatments and found its proven stability under extreme conditions. Previously, Ali et al. (2011 & 2013) and Rahman et al. (2014), studied the effects of

extensive autoclaving treatments on various samples and found short-length targets are more stable under compromised conditions (natural and forceful degradation while cooking). Thus, it was not unpredicted that a 69 bp feline target would be stable under extreme processing treatments.

5.5 Sensitivity Test

5.5.1 Raw and Ternary Background

The sensitivity of the newly developed simplex PCR assay was checked by template DNA dilution methods (Matsunaga et al., 1999) as shown in Figures 4.10 (raw), 4.11 (autoclaving) and 4.12 (boiling). Moreover, 69 bp PCR product was clearly amplified from 0.0001 ng feline DNA template and reflecting its LOD under raw (Figure 4.10), autoclaving (Figure 4.11) and boiling (Figure 4.12) by 10 fold serially diluted methods (Matsunaga et al., 1999). Additionally, lane 6 from Figures (4.10, 4.11 and 4.12) were appearing thin due to the low concentration of diluted DNA template. Previously, Che Man et al. (2012) and Karabasanavar et al. (2014) verified their assay sensitivity for pork DNA by serial dilution method and identified as low as 0.001ng DNA/µl. Recently, Zhang et al. (2013) found higher sensitivity up to 0.001 ng in the detection of chicken, beef, pork and mutton by semi-nested multiplex PCR. Besides, Hou et al. (2014) obtained the LOD (0.05 ng) for the detection of chicken, duck and goose. The variation of the sensitivity in a standard PCR is a common phenomenon and it differ from species to species

On the other hand, to check the specificity and sensitivity of a desired product in a complex matrices, cat meat was properly mixed with beef and chicken meat to construct a two set of ternary meat mixtures according to base adulterated meat mixture (BAM)

(Ali et al., 2012a) and demonstrates that *F. catus* specific PCR assay developed in this study was highly sensitive and specific since it unambiguously identified as low as 0.01% (w/w) feline meat in raw, autoclaved and oven heated cat-beef-chicken (Figure 4.13) and feline-bovine-wheat flour in raw, autoclaved and boiled ternary admixed (Figure 4.14). However, the sensitivity of the previously documented assays have not been systematically studied.

Previously reported works were carried out on cattle, chicken, goat, sheep, deer, lamb, ostrich, pork, dog and horse meat with amplicon-sizes between 100 and 450 bp (Kitpipit et al., 2014; Matsunaga et al., 1999; Zha et al., 2011; Zhang, 2013). Additionally, Ali et al. (2013) and Rahman et al. (2014) identified up to 0.01% (w/w) swine and canine DNA under mixed background. Earlier feline species PCR assays ((Martine at al., (2007), Ilhak and Arslan et al., (2007), Abdulmawjood et al., (2003)) detected 0.1% (w/w) and 0.01% (w/w) cat meat spiked binary (oat: cat) and ternary (cat: goat: lamb) mixtures under standard autoclaving condition (133°C for 30 min) with 108, 274, 672 bp target amplicon)), have limited scope in case of extensive autoclaving and microwaving treatments. Since, they did not check its stability under extensive autoclaving (120°C for 2.5 h under 45-psi) and extreme microwaving (700W for 30 min) which are known to massively degrade target DNA (Ali et al., 2011, Rojas et al., 2010 and Rahman et al., 2014).

More importantly, stability of such a long-length target (108-672 bp) under compromised states is seriously questioned (Ali et al., 2013). Thus the merit of this newly developed assay over the others is evident since it is well defined, has used the shortest target (69 bp) and produced high sensitivity (0.0001 ng DNA under pure, autoclaving and boiling states and 0.01% (w/w) in two set of ternary admixed).

5.5.2 Commercial Complex Background

Extensive literature search demonstrates the primers of the previously developed feline specific PCR assays targeted relatively longer amplicons (≥108 bp) and since no report has been published for feline meat detection in commercial products. Therefore, to fill-up this research gap, different amount (10%, 5%, 1%, and 0.1 %) of cat meat was spiked in deboned beef and chicken burgers by following Ali et al. (2012). The minced meats were treated in an oven under 220˚C for 15 min and standard autoclaving condition (121˚C for 2.5 at 45-psi). In Figures 4.17 and 4.18, (lanes 5-8), clearly showed that PCR products was obtained from cat meat adulterated beef (Figure 4.15) and chicken burgers (Figure 4.16). Feline PCR product was amplified from all contaminated burgers and thus the tested LOD of the assay was 0.1% (w/w).

However, the assay have been also tested in a complex background of meatballs under pure and different amount (10%, 5%, 1%, 0.1% and 0.01%) of feline meat contaminated beef and chicken meatballs. To simulate the normal cooking and extensive autoclaving prepared meatballs were boiled at 100˚C for 90 min and autoclaved was done at 120˚C for 2.5 h under 45-psi, respectively. Clear 69 bp feline PCR product was amplified from 10% to 0.1% (w/w) cat meat positive in raw, boiled and autoclaved beef (Figure 4.17) and boiled and autoclaved chicken meatballs (Figure 4.18). In Figure 4.17 (lanes 5, 10 & 15) and Figure 4.18 (lanes 11 & 16), no product was amplified with 0.01% (w/w) of cat meat contaminated beef and chicken meatballs due to some PCR inhibitor compounds (Chapter 3; Tables 3.3 & 3.4) were commonly used to prepare beef and meatballs (Ali et al., 2013).

Previously, Ali et al. (2013), Rahman et al. (2014) detected 0.1% (w/w) and 0.2% (w/w) canine DNA in frankfurters and meatballs under normal and extensive autoclaving systems. Very recently, Ali et al. (2015) detected 1% (w/w) five potential haram meat

species in meatball under raw and extensive autoclaving state. However, the chances of adulteration below 0.1% is minimal since adulteration at this level is not profitable weighing the risk of reputation damage.

Addition of minced meat in commercial food products is a routine practice (Tanabe et al., 2007) and meat replacement under mixed background and processed conditions are frequently taking place everywhere (Ali et al., 2012b). Meanwhile, inaccurate labelling description on products is a routine repeated exercise in most countries of the world (Doosti, Dehkordi, & Rahimi, 2011; Fajardo, González, Rojas, García, & Martín, 2010). Recently, the 'Administration of Czech State Veterinary Unit' discovered horsemeat in frozen meatballs marketed as beef and pork meatballs in Sweden (Pollak, 2013), while turkey and chicken were found in 100% beef meatballs in Turkey (Ulca, Balta, Cagin, & Senyuva, 2013) and horse meat was found in European burger (Premanandh 2013). Therefore, four commercial halal branded beef and chicken burgers and three of beef and chicken meatballs were collected in triplicates on three different days from, KFC, MacDonald, Aeon, Tesco and other outlets across Malaysia. In a blind experiment, four commercial brands of beef (A - D) and chicken (E - H) burgers were screened against 0.1% cat meat spiked burgers on five different days (Table 4.4). In addition, 0.1 % cat meat contaminated ready to eat meatballs was used as a positive control and screened thee halal logo containing commercial beef (A - C) and chicken (A´-C´) meatballs on three separate days (Table 4.5) to avoid any biasness and to bring variation in sampling sources thus proven itself as a highly specific with feline cytb gene. PCR products of feline specific mt-cytb gene successfully amplified from all cat meat spiked burgers and meatballs, whereas no product was amplified from commercial burgers (Figures 4.15 and 4.16; lanes 1 to 4) and meatballs in Figure 4.18 (lanes 1 to 6); ensuring no cat-meat are being contaminated in burger and meatball formulations in Malaysia.

5.6 PCR – RFLP Analysis

5.6.1 Restriction Enzyme Digestion

Although, species-specific PCR assays are often conclusive, authentication of amplified PCR products definitely increases the assay reliability. PCR-RFLP has been extensively used to distinguish two or more closest species using simple instrumentation (Ong, Cheah, Robin, Wolmon Gunsalam, Mat Isaa, Chai, Yuli, Mohamad Ghazali and Radu, 2007, Verkaar, Nijman, Boutaga and Lenstra, 2002). PCR-RFLP technique was successfully used for the differentiation of species due to its inexpensive and exactitude features, and it allows the validation of the authentic PCR products though the analysis of the restriction-digested PCR products (Ali et al., 2012a). It comprises of the generation of species-specific band profiles through restriction-digestion with one or more restriction endonucleases (Pereira et al., 2008). These restriction enzymes cleave DNA molecule at recognition sites, originating a set of fragments with different lengths that could be separated according to their molecular size by electrophoresis (Pereira, Carneiro and Amorim, 2008). Thus PCR-RFLP has been proven to be a practical, highly repeatable and reliable technique for meat species identification in food and meat industry (Haider et al., 2012).

Therefore, 69 bp feline-specific PCR products was digested by *Alu*I since in-silico analysis showed available restriction sites for these enzymes with suitable fragment-lengths (New England Biolabs; seehttp://nc2.neb.com/NEBcutter2/). Two sites for *Alu*I were found within the amplified sequence (69 bp) (Figure 20). More importantly, lanes (2, 4, 6, 8, 10 & 12) in Figure 4.20 demonstrated two fragments of length 43 and 26 bp, which resulted following *Alu*I digestion of the PCR amplification of commercial products. On the other hand, an endogenous control (141 bp) produced two *Alu*I (127 and 14 bp) fragments in Figure 4.22 (lanes 7 to 12) from cat meat adulterated raw, autoclaved,

oven heated and boiled burgers and meatballs and shown both in gel image (Figure 22) and electropherograms (Figure 23). Nonetheless, a 14 bp fragment of digested eukaryotic primers (141 bp) which was below the resolution capacity of the instrument (15 bp) could not be detected.

5.6.2 Products Authentication by RFLP Technique

In food industry, replacement of costly meats by cheaper products is quite a common practice to secure high profit, and is very frequently performed in processed meat products such as burgers, meatballs, sausages, nugget, frankfurters and others (Ali et al., 2012a). Among these items, burgers and meatballs are very common fast foods in all mainland of the world (Ali et al., 2012a). Therefore, commercial burger and meatball samples digested with *Alu*I restriction enzyme after preparing model burgers and meatballs according to Ali et al. (2012b) and Rohman et al. (2011). Total three different Halal branded commercial burgers (A-C) and meatballs (D-F) were purchased from different outlets in Malaysia and were tested (Table 4.9) and no commercial burger and meatball were found to be positive for feline meat reflecting that no cat meat is present in burgers and meatballs in Malaysia. Amplification of endogenous eukaryotic control 141-bp, reflected good quality and presence of DNA in all tubes (Figure 4.21) and eliminating the chances of any false-negative detection. The findings are acceptable in Malaysian perspectives since the country is committed to develop 'Halal-hub' industry and has been strictly monitoring the 'Halal' status of foods.

Abdulmawjood et al. (2003) developed PCR-RFLP assay with longer amplicon length (672-bp) to detect feline species in raw or pure state among the six documented assays. However, he did not verify his assay in the complex background of any commercial food products and such a longer-size had the chance of fragmentation during the food processing scheme. Therefore, the assay was evaluated to detect the feline

biomaterials in the pure and contaminated state of commercial burgers and meatballs. Thus, the advantage of the newly developed RFLP technique is easily understandable and distinguishable in terms of specificity, amplicon-size, and stability.

CHAPTER SIX

CONCLUSION AND RECOMMENDATIONS FOR FUTURE WORK

CHAPTER 6: CONCLUSION

6.1. Conclusion

In this research, two types of DNA based specification methods was successfully developed for the detection of shorter feline-mitochondrial DNA in processed mixed meats and commercial food products. The first one was the species-specific PCR assay which itself is conclusive and feline material identification under raw, pure, admixed and commercial matrices. The primers were designed targeting a 69-bp sites of cytochrome b gene which is present in multiple copies in each cells. The feline specificity was ensured by alignment analysis, mismatch comparison, phylogenetic tree and 3D plot. The primers set were challenged against 24-potential species and accurate target was amplified only from the feline targets, confirming the specificity and self-standing ability of the designed primers. Since the breakdown of target is quite common under food processing conditions, therefore the feline meats were treated under boiling (100˚C for 90 min, 110˚C for 30 min, boiling in water bath at 80˚C for 30 min), autoclaving (110˚C for 2 h under 14.5-psi; 120˚C for 50 min under 14.5-psi; 133˚C for 20 min 43.51-psi and 120 for 2.5 h under 45-psi) and microwaving (300W for 10 min, 500W for 20 min and 700W for 30 min) conditions. Extraordinary stability were revealed under all treatment conditions, reflecting the reliability of the targets under any compromised states such as natural decomposition or force full degradation of DNA by physical or chemical shocks of food processing. Therefore, the specificity of the biomarkers was further checked under ternary admixed and matrices of commercial foods such as burgers and meatballs and satisfactory results were obtained since feline targets were amplified from all backgrounds.

Secondly, shorter targets often compromise specificity since number of species-specific fingerprints is reduced within a shorter-sequence regime and verified the authentic PCR targets by RFLP analysis. The PCR product was amplified in the presence of 141bp universal site of eukaryotic 18SrRNA gene and digested with *Alu*I restriction enzymes since in-silico analysis by NEB cutter demonstrated one restriction site with fragments lengths of 43 and 26 bp within the 69 bp targets. The digests were separated in a microfluidic-based lab-on-a-chip automated electrophoresis system incorporated in Experion Bioanalyzer. The fragments were separated with good resolution and it was reflected both in gel-image and electropherograms. The tested limit of detection was 0.1 pg feline DNA under raw states, 0.01% (w/w) feline meats in ternary admixtures and 0.1% (w/w) feline meats in commercial food products such as burgers and meatballs.

To the best of my knowledge, it is the first systematic study for feline meat adulteration detection under complex matrices and compromised states as well as commercial meat based products. No study has developed a PCR assay with as low as 69 bp target with enough fingerprints for feline species. The extraordinary stability and well-established sensitivity of the study reflects its application in food authentication or archeological studies of feline species.

6.2. Recommendations for Future Work

- Recently, real-time PCR has got popularity over other PCR-based methods because of its automation, rapidity and sensitivity and ability to quantify potential targets. Thus there is a clear scope to develop various real-time PCR assays such as SYBR green, Eva Green, Molecular Beacon and TaqMan probe real-time PCRs.

- Most of the PCR assays have not been validated under various food matrices. It is also difficult to extract DNA from various matrices such as fat, dairy products, chocolates etc. Therefore, appropriate DNA extraction protocol should be developed, optimized and adapted for various food matrices.

- The PCR targets we developed here should be validated by comparing it with other exiting targets.

- Multiplex PCR assays are highly promising since they allow the detection of multiple species in a single assay platform, reducing cost and time. Therefore, the opportunity to develop such assays for multiple haram species could be explored

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APPENDIX

LIST OF PUBLICATIONS AND PAPERS PRESENTED

a) Research Articles:

Ali, M.E., **Amin, M.A**., Hamid, S.B.A., **Hossain, M.M., Mustafa, S., (2015).** Lab-on-a-chip-based PCR-RFLP assay for confirmed detection of short length feline DNA in foods. **Food Additives & Contaminant: Part A, (PUBLISHED), ISI/Scopus cited publication**.

Ali, M.E., **Amin, M.A**., Hamid, S.B.A., Rahman, M.M., Razzak, M.A., Rashid, N.R.A., Asing (2015). Short Amplicon-Length PCR Assay Targeting Mitochondrial Cytochrome b Gene for the Detection of Feline Meats in Burger Formulation. **Food Analytical Methods, (PUBLISHED), ISI/Scopus cited publication**.

Amin, M.A., Ali, M.E., Hamid, S.B.A., Rahman, M.M., Razzak, M.A., Asing, Rashid, N.R.A (2015). A suitable method for the detection of potential fraud of bringing feline meats in food chain. **International Journal of Food Properties, (UNDER REVIEW), ISI/Scopus cited Publication**.

b) Conference Proceedings:

Amin, M.A., Ali, M.E., Hamid, S.B.A., Razzak, M.A. (2014). Primer design targeting mitochondrial ND5 gene for cat meat detection in halal foods: INNOVAFOOD-2014, 27−29th August 2014, PARKROYAL Penang Resort; Penang, Malaysia. **(ISI/Scopus cited publications)**.

Hamid, S.B.A., **Amin, M.A**., and Ali, M.E.: Zeolite Supported Ionic Liquid catalyst for the Synthesis of Nanocellulose from Palm Tree biomass: BOND21 Joint International Conference on Nanoscience, Engineering, and Management, 19-21th August 2013, Bay view Beach Resort, Penang; Malaysia. Advanced Materials Research Vol. 925 (2014) pp 52-56, **(ISI/Scopus cited publications).**