Food Additives & Contaminants: Part A

Lab-on-a-chip-based PCR-RFLP assay for the confirmed detection of short-length feline DNA in food

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Lab-on-a-chip-based PCR-RFLP assay for the confirmed detection of short-length feline DNA in food

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Abstract

Wider availability but lack of legal market trades has given feline meat a high potential for use as an adulterant in common meat and meat products. However, mixing of feline meat or its derivatives in food is a sensitive issue, since it is a taboo in most countries and prohibited in certain religions such as Islam and Judaism. Cat meat also has potential for contamination with of SARS, anthrax and hepatitis and its consumption might lead to an allergic reaction.

Here we developed a very short-amplicon-length (69 bp) PCR assay, authenticated the amplified PCR-products by \textit{AluI}-restriction digestion followed by its separation and detection on a lab-on-a-chip-based automated electrophoretic system and proved its superiority over the existing long-amplicon-based assays. Although it has been assumed that longer DNA targets are susceptible to breakdown under compromised states, scientific evidence for this hypothesis has been rarely documented. We produce here strong evidence that shorter targets are more stable than the longer ones. We confirmed feline specificity by cross-challenging the primers against 10 different species of terrestrial, aquatic and plant origins in presence of
a 141bp site of 18S rRNA gene as an universal eukaryotic control. RFLP analysis separated 43 and 26-bp fragments of AluI-digest both in the gel-image and electropherograms, confirming the original products. The tested detection limit was 0.01% (w/w) feline meat in binary and ternary admixed as well as meatball matrices. Shorter-target, better stability and higher sensitivity convinced anyone to think that such an assay would be valid for feline identification even in degraded specimens.

**Keywords:** Short-amplicon-length PCR assay; Lab-on-a-chip-based automated electrophoretic system; Restriction digestion; Binary, ternary and meatball matrices; Taboo and religious prohibition.

**Introduction**

Food component authentication is an ever increasing demand and a key point in policy making and market regulation. It is an undeniable need to safeguard public health, consumers’ lifestyles religious faith, fair-trade and of course sustainability in wildlife and natural habitats (Cawthorn et al. 2013; Fajardo, González, Rojas, García, & Martín, 2010; Ghovvati et al. 2009; Hou et al. 2014; Karabasanayar et al. 2014; Kesmen, Celebi, Gülüce, & Yetim, 2013). Food falsification is a serious issue since consumption of certain meats such as pork and its derivatives are strictly prohibited in several religions such as Islam and Judaism (Ali et al. 2015). Furthermore, the recent scandals of horse meat in Europe and pig and rat meat in China (Ali, Razzak, & Hamid, 2014, Premanandh, 2013) have put consumers on red alert in determining the presence of prohibited ingredients in marketed foods.

Cat is a domesticated animal and its population is huge across the globe. In addition to religious restriction, cat meat consumption is a taboo in most countries. It is also a potential carrier of hepatitis, SARS, anthrax and some other deadly diseases (Anitei 2006). Consequently, there is no legal market for the sale of feline meats in any part of the world. However, cat was served as Indian curry in UK restaurant in 2013 (Chatterji 2013). Thus the chances of cat meat mixing in halal, kosher and other common meat and meat products are very high or at least could not be ruled out.

On the other hand, food component authentication using physical attributes is impossible under current perspectives due to massive and ongoing innovations in processing and packaging technologies (Ali et al. 2011; Bottero & Dalmasso, 2011; McMillin 2008). In meat speciation, DNA biomarkers, especially the short-length ones which are extremely
stable even under harsh processing conditions such as heat, pressure and chemical additives where protein-based markers are denatured and lipid based biomarkers are ramified, are particularly interesting. Consequently, DNA-based techniques have been the methods of choice over the recent years (Fajardo et al. 2010). Mitochondrial DNAs (mtDNA) or genes are especially suitable for meat identification applications since they are present in multiple copies in each cell with adequate polymorphisms or species-specific fingerprints (Murugaiyah et al. 2009). Among the DNA-based methods, polymerase chain reaction (PCR)-based detection schemes are highly appreciated since they are simple, cost-effective and robust and can amplify marker DNA targets even from a single or few copies to easily detectable quantities (Ong et al. 2007, Verkaar et al. 2002), species-specific PCR (Che Man et al. 2007, Haunshi et al. 2009, Karabasanavar et al. 2014, Mane et al. 2012), multiplex PCR (Ali et al. 2015, Dooley et al. 2004), randomly amplified polymorphic DNA (RAPD) (Arslan et al. 2006), PCR restriction fragment length polymorphism (RFLP) (Ali et al. 2012b; Rashid et al. 2015), and real-time PCR (Ali et al. 2012a, Kesmen et al. 2013) are some of the significant reports for the identification of meat species. The species-specific PCR-RFLP assays are particularly advantageous since they not only amplify specific targets but also confirm the authentic PCR products through the analysis of restriction-patterns (Ali et al. 2014). Further, they have special interest in meat speciation because they exploit the sequence variations that exist within a defined region of target DNA, allowing differentiation of even closely related species by digestion of selected DNA fragments with appropriate restriction enzymes (Fajardo et al. 2008). PCR-RFLP assays have been documented to distinguish between the closest species such as cattle-buffalo and sheep-goat (Girish et al. 2004), swine and wild boar (Fajardo et al. 2008), various fish species (Wolf et al. 2000) and cattle and yak (Chen et al. 2009). However, no PCR-RFLP assay has been reported for feline meat authentication. Here we successfully amplified a very short (69bp) target of cytb gene using feline specific primers, digested it with Alul restriction-enzyme and obtained some distinctive restriction fingerprints for the identification of feline species.

Materials and Methods

Collection of Samples

Raw meat samples of beef (Bos taurus), chicken (Gallus gallus), sheep (Ovis aries), goat (Capra hircus), pig (Sus scrofa), rat (Rattus rattus), duck (Anas platyrhynchos), carp (Cyprinus carpio) and tilapia (Oreochromis aureus) were purchased in triplicates from the
various super markets in Kuala Lumpur, Petaling Jaya and Selangor in Malaysia. Plant samples (wheat (*Triticum aestivum*), tomato (*Solanum lycopersicum*), garlic (*Allium sativum*), onion (*Allium cepa*), cucumber (*Cucumis sativus*), potato (*Solanum tuberosum*), and pepper (*Capsicum annuum*) were collected from the groceries across Kuala Lumpur. Cat (*Felis catus*) meat samples from three different animals were collected from Jabatan Kesihatan Dewan Bandaraya Kuala Lumpur (DBKL) located at Air Panas in Kuala Lumpur and Faculty of Veterinary Sciences in University of Putra Malaysia in Selangor. The species authenticity of the animal and plant species were verified by a taxonomy expert and transported to the laboratory under ice-chilled environment (4 °C) and stored at (-20 °C) until further used.

**Preparation of Admixtures and Meatballs**

In order to identify cat meat adulteration in processed food products, two sets of admixed (i) binary (cat-goat) and (ii) ternary (cat-chicken-beef) were prepared by spiking 10, 1, 0.1, and 0.01 % cat meat in goat, chicken and beef in a 100-g specimen (Ali et al. 2011a). Thus, prepared samples were mixed vigorously with bench top blender to make a homogeneous admixture. All admixtures were prepared on three separate days by three independent analysts and were autoclaved at 120°C under 0.4 MPa pressure for 2.5 h before extracting DNA.

On the other hand, to mimic commercial meatballs, model raw, autoclaved, and ready to eat meatballs were prepared using cat, chicken, and beef meat according to Ali et al. (2012a). For pure meatballs, to a 100 g specimen of minced meat of cat, chicken, and beef were added to 7.5g fresh breadcrumbs, 1.25-g minced garlic, 0.32 tomato paste, 0.2-g salt and 0.03-g tapioca starch and mixed well. To simulate cat meat adulteration, chicken and beef meatballs were prepared by spiking 10, 1, 0.1, and 0.01 % of cat meat into chicken and beef meats, mixed and minced well. Then the raw meatballs were boiled in 100°C for 90 min and autoclaved at 120°C at 0.4 MPa for 2.5 h (Rahman et al. 2014). All the prepared meatballs were kept at -20°C and were blended well into a homogenous mixture prior to DNA extraction.

**Extraction of DNA**

The samples were cut into small pieces and the DNA was extracted from 25 mg of raw meat samples using DNA Mini Kit for Animal Tissues (Yeastern Biotech Co. Ltd, Taipei) following manufacturer’s instructions. A total of 1 g sample was used to extract DNA for
admixed, meatball and plant samples using CTAB method according to Rahman et al. (2014). The concentration and purity of all the extracted DNAs were checked by UV-vis NanoPhotometer (IMPLEN, Nano Life Quest Sdn. Bhd; Selangor, Malaysia).

**Designing of Primer**

A publicly available primer designing software primer3Plus (www.bioinformatics.nl/cgi-bin/primer3plus.cgi) was used for designing feline specific primer pairs. Firstly, the feline specificity of the primers was theoretically determined using NCBI blast analysis (www.ncbi.nlm.nih.gov). Secondly, cytb gene sequences of animals, fish and plant species (cat: AB194812.1, beef: EU807948.1, chicken: EU839454.1, duck: HQ122601.1, sheep: EU365990.1, buffalo: D32193, pig: GU135837.1, turkey: HQ122602.1, goat: EU130780.1, rat: HM222710.1, pigeon: KC811464.1, carp: AB158807.1, cattle: AB240155.1, tilapia: AF015020, shrimp: EU069446.1, cucumber: AF288044.2, Onion: GU253304.1, wheat: X02352.1, tomato: XM004251454.1 and potato: X58437.1) were retrieved from publicly available NCBI database and aligned by ClustalW multiple-sequence alignment tool (Thompson et al. 1994) to identify the degree of interspecies polymorphism. The amplicon contained one \textit{Alu}I sites within it, offering opportunities to separate similar-sized cross-amplified PCR products (if any) by way of RFLP analysis. However, a 141-bp fragment of eukaryotic 18S rRNA gene was used as a positive control (Rojas et al., 2010) to evaluate the quality of DNA in all samples. However, all the designed primers were purchased from the 1st Base Laboratories (1st Base Laboratories, Sdn. Bhd., Selangor, and Malaysia).

**PCR Amplification**

We successfully amplified 69 bp feline and 141-bp eukaryotic targets using a PCR assay containing 25 µl of reaction volume having 5 µl of 5x colourless GoTaq Flexi Buffer (Promega, Corporation, Madison, USA), 1.5 µl of 25 mM MgCl$_2$ (Promega), 0.5 µl of 0.2 mM each of dNTPs mix (sodium salts of dATP, dCTP, dGTP and dTTP 10 mM in water) (Promega, Madison, USA), 100 nM each primers (IDT, Inc.), 50 units/ml of Taq polymerase (Promega), 20 ng of total DNA template. All the PCR reaction was performed in Veriti 96-Well gradient Thermal cycler system (Veriti Thermal Cycler, Applied Biosystems; Foster City, California, USA) with an initial denaturation at 95°C for 3 min followed by 35 cycles of denaturation at 95°C for 20 s, annealing at 58°C for 20 s and extension at 72°C for 30 s and
the final extension was completed by 72°C for 5 min and hold at a 4°C for 2 min. A negative control was developed by adding nuclease free water in place of template DNA for experimental validation of all PCR reaction. The PCR products were kept at -20°C for further analysis. Thus amplified PCR products were identified on a microfluidic-based lab-on-a-chip incorporated in Experion DNA 1K analysis kit following manufacturer’s guidelines (Bio-Rad Laboratories, USA).

**RFLP Analysis**

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 digest of each sample in Experion lab-on-a-chip well using 1K DNA analysis kit (Bio-Rad Laboratories, USA).

**Comparison of Target DNA Stability**

To study the comparative stability of the newly designed 69 bp amplicon and one of the previously documented 108 bp targets (the shortest of the previously documented targets 672 (Abdel-Rahman et al. 2009), 331 (Irine et al. 2013), 274 (Ilhak and Arslan 2007), 180 (Tobe and Linacre 2008) and 108 bp (Martin et al. 2007)). PCR was performed with 50 ng feline DNA template after autoclaving feline meats at 135 °C for 30, 60, 90, 120 and 150 min under 0.3 MPa and microwave cooking at 600 W and 700 W for 30 min.

**Results and Discussions**

**Feline Species Specificity**

Species-specific PCR (Ali et al. 2013, Mane, Mendiratta and Tiwari 2012) is a simple, low-cost, sensitive and reliable authentication technic capable of detecting even a minute level of
defilement in raw, processed and commercial food products (Mafra et al. 2007). However, certain pros and cons must be ensured to harness a reliable outcome. The presence of a single mismatch in the primer binding region might cause PCR failure under complex matrices (Wu et al. 2009). Hence, the estimation of mismatch nucleotides in primer annealing site is one of the important step in designing a species-specific primer set. Designing primers with perfect matching with the specific target and multiple mismatches with non-target species definitely increase the selectivity of detection, limiting the chances of non-target amplification during a PCR experiment. Multiple alignment analysis using in-silico tool of the selected 69 bp site of the cytb gene *Felis catus* (AB194812.1) with 17 other non-target species reflected perfect matching only with feline cytb gene and counted 3-19 nt (14-86%) mismatching with other non-target species (Figure 1b). Pairwise distance (Table 1), phylogenetic tree (Figure 1a), 3D plot (Figure 1c) and number of mismatches in restriction and primer-binding sites (Figure 1b) reflected clear dissimilarity of the cat primers with all other tested species (Figure 1a) (Tamura et al. 2004). The highest pairwise distance (1.29) was observed between cat and cuttle fish and the lowest (0.21) was found in beef and buffalo (Tamura et al. 2004) demonstrating enough genetic distance and unlikelihood of cross-species amplification in a real PCR experiment. Finally, a practical PCR run amplified only the 69 bp product from *F catus* species (Figure 2a) and no product from 7 commercially important meats (beef, chicken, sheep, goat, rat, pig and duck) species, 1 plant (cucumber), and 2 fish (carp and tilapia) species, supporting the theoretical conclusion that the newly developed assay was highly specific for the feline species. However, an universal eukaryotic primer pair amplified 141-bp site of 18S rRNA gene from all species, revealing the presence of good quality DNA in all tubes and eliminating the probability of any false negative detection (Figure 2a) (Rojas et al., 2010).

Previously developed feline-specific PCR assays were based on mitochondrial whole genome (672 bp (Abdulmawjood et al. 2003); 672 bp (Abdel-Rahman, El-Saadani, Ashry and Haggag 2009); ND4 gene (274 bp) (Ilhak and Arslan 2007); 12S rRNA (108 bp) (Martin, Garcia, Fajardo, Rojas, Hernandez, Gonzalez and Martin 2007); cytb gene (180 bp) (Tobe and Linacre 2008) and cytb gene (331 bp) (Irine, Nuraini and Sumantri 2013). These assays involved considerably longer amplicons which are assumed to be fragmented under commercial food processing treatments, causing PCR failure or truncated PCR products. We used here short-length DNA amplicon (69 bp) and found that it was stable under several food processing conditions which breakdown DNA (Ali et al., 2013; Rahman et al., 2014). However, little scientific efforts have been made to validate or prove this hypothesis.
Consequently, we subjected here two different PCR targets (69 bp which was developed in this study and 108 bp that was the shortest in length among the published reports) under harsh autoclaving (133°C for 30, 60, 90, 120 and 150 min under 0.3 MPa) and microwaving treatments (600 and 700W for 30 min). We found here that 69 bp targets survived and hence were amplified under all treatment conditions (Figure 4). But 108 bp targets failed to withstand 150 min of autoclaving at 133°C and 30 min of microwaving stress at 700W (lanes 10 and 14 in Figure 2 (b)). These clearly revealed that the assay we developed here was more stable and robust than those of the previously reported assays.

**Sensitivity in Admixed and Meatballs**

Two sets of admixtures were prepared according to Ali et al. (2012b) to mimic commonly used meat adulteration practices in industry. Set 1 was cat-goat binary and set 2 was cat-beef-chicken admixtures containing different percentages (10, 1, 0.1 and 0.01 %) of spiked cat meat. Therefore, we subjected both sets (1 and 2) of admixtures under extensive autoclaving (120°C under 0.4MPa at 2.5 h) to check the constancy of the target DNA under extensive heat and pressure conditions. We found 69bp product from all cat-goat and cat-beef-chicken admixed containing as low as 0.01 % (w/w) of spiked feline meats (Figure 3a) as well as endogenous eukaryotic target (141bp) from all admixed and pure meat products (lanes 1 & 6 in Figure 3(a) and lanes (5 & 10 in Figure 3(b)). These reflected good quality DNA in all meat products and confirmed no false negative detection (Figure 3a). Martin and his coworkers (2007) detected as low as 0.1% (0.125 ng) of 108 bp amplicon of the 12S rRNA gene in oat and cat meat binary mixtures under normal autoclaving condition (133°C for 30 min under 0.3 MPa). Our newly developed assay was more stable and sensitive in terms of autoclaving treatments (133°C for 30 min under 0.3 MPa vs. 120°C for 150 min under 0.4 MPa), amount of template DNA (125 ng vs. 25 ng) and admixed complex (binary vs. ternary) compare to the previous studies. The lowest detection limit 0.01% of cat meat (Abdulmawjood, Schönenbrücher and Bülte 2003) was obtained in a PCR-RFLP assay that targeted a 672-bp site of mt-cytb gene in beef-goat-lamb meat mixtures. However, the authors did not check their target stability in any food processing treatments and such a long-length target is unlikely to be stable under harsh condition (Figure 3 (b)) (Ali et al., 2012a; Rojas et al., 2010). Additionally, capillary electrophoresis (CE) based end-point detection
technique of the existing method provided a higher sensitivity of 0.01% (w/w) feline meats or 0.001ng feline DNA in binary and ternary admixtures.

More importantly, we tested the developed assay in commercial meatballs matrices since no report has been published for feline meat detection in commercial products. To fill-up this research gap, different amount (10%, 1%, 0.1% and 0.01%) of cat meat was spiked in deboned beef and chicken following Rahman et al. (2014). To simulate the normal cooking and extensive autoclaving, thus prepared meatballs were boiled at 100 °C for 90 min and autoclaved at 120 °C for 2.5 h under 0.4 MPa, respectively. In Figure 3a; (lanes 1-4) and (lanes 6-10) clearly showed that PCR products was obtained from autoclaving cat meat adulterated beef (Figure 3a) and boiled chicken meatballs (Figure 3b). Feline PCR product was amplified from all contaminated meatballs, showing the tested LOD of the assay was 0.01% (w/w) feline meat. Previously, Ali et al. (2013) and Rahman et al. (2015) detected 0.1% (w/w) and 0.01% (w/w) canine DNA in frankfurters and burgers formulations, respectively. However, we do not think the need of adulteration detection below 0.01% since adulteration at this level is not profitable and unlikely to be done.

RFLP Analysis

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 the recognition sites, creating 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).
Here we digested the 69-bp feline-specific PCR products by AluI since in-silico analysis showed (data not presented) available restriction sites for these enzymes with suitable fragment-lengths. The PCR products of meatballs were digested with restriction enzyme AluI and generated two fragments (43 bp and 26 bp) with one restriction site after digestion. Additionally, in Figure 4, demonstrates 2 fragments of length 43 and 26 bp which were resulted following AluI digestion of raw, boiled and autoclaved beef and chicken meatballs PCR product. The molecular size statistics of the 69-bp feline-specific site from raw, boiled and autoclaved, cat meat contaminated ready to eat model meatballs are shown in Table 2. Previously, a 672-bp longer DNA sized PCR-RFLP assay was documented by Abdulmawjood et al. (2003) and was not tested these assay in commercial meat products or food processing treatments. Henceforth, such a longer-size amplicon had the countless probability of breaking down during the treatments and causing PCR failure while testing in commercial foods matrices. Therefore, the advantage of our PCR-RFLP technique is easily understandable in terms of sensitivity, product size, and stability.

**Analysis of Commercial Meatballs**

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, Hashim, Mustafa, Che Man, Dhahi, Kashif, Uddin and Abd Hamid 2012a). Among these items, meatball is very popular all of the world (Ali et al. 2012b). The ‘Administration of Czech State Veterinary’ discovered horsemeat in frozen meatballs marketed as beef and pork meatballs in Sweden (Pollak 2013), while turkey and chicken were detected in 100% beef meatballs in Turkey (Ulca et al. 2013). Therefore, we screened here a four different commercial branded halal logo containing beef (A-D) and chicken (E-H) meatball samples purchased from different outlets across Malaysia and digested the amplified PCR products with AluI restriction enzyme. The model meatballs was prepared following Ali et al. (2012b) described earlier and were tested (Table 3). However, no commercial meatball was found to be positive for feline meat, reflecting the absence of cat-meat adulteration in meatball formulations in Malaysia. Amplification of endogenous eukaryotic control, reflected good quality DNA in all commercial products (Figure 3b). 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.
**Assay Superiority**

We believe the superiority of a food forensic assay largely depends on its stability, sensitivity, robustness and precision under various food processing conditions (Ali et al. 2012b). It has been assumed for long time that short-length PCR targets better performs over the longer ones (Rojas et al. 2010, Ali et al. 2013, and Rahman et al. 2014). However, little scientific efforts have been made available to validate or prove this hypothesis. Consequently, we subjected here two different PCR targets (69 bp which was developed in this study and 108 bp that was shortest in length among the published reports) under harsh autoclaving (135°C for 30, 60, 90, 120 and 150 min under 0.3 MPa) and microwaving treatments (600 and 700W for 30 min). We found here that 69 bp targets survived and hence were amplified under all treatment conditions (Figure 2b). However, 108 bp targets failed to withstand 150 min of autoclaving at 135°C and 30 min of microwaving stress at 700W (lanes 10 and 14 in Figure 2b). These clearly revealed that the assay we developed here is more stable and robust than those of the previously reported assays.

**Conclusion**

The chances of bringing feline meat into the food chain is very high because of its wide availability, but lack of any trade opportunities in legal meat markets. In addition to incite religious outcry, this might bring new diseases hosted by the species into the human race. Compared to longer DNA targets, shorter targets offer several advantages such as better stability, sensitivity and of course reliability. However, the development of shorter-targets is often challenging since within a short sequence region adequate fingerprints for target-species have to be accommodated. Thus there is a need of product authentication by a trustworthy method. PCR-RFLP assay coupled with an automated electrophoresis technique is a highly encouraging technique to identify and authenticate short-length PCR targets. We successfully amplified and detected here a 69 bp-site of feline cytb gene and found distinctive restriction patterns for AluI under pure and complex matrices. The PCR product was amplified in presence of a 141bp universal site of eukaryotic 18SrRNA gene which eliminated the risks of any false negative detection. The assay was optimized and adapted for the screening of commercial meatballs for potential feline meat adulteration. The tested limit of detection was 0.01% feline DNA under binary and ternary admixtures and 0.01% (w/w) feline meats in commercial food products of meatballs. We further proved here that this newly developed assay was more stable and hence more reliable than existing assays for
detection of feline species. The assay should find application for the identification of feline species in food forensics or any archaeological investigations.

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Compliance with Ethics Requirements

Ethical clearance of ref. No: NANOCAT 26/09/3013/ MAA (R) was obtained from the Institutional Animal Care and Use Committee, University of Malaya (UM IACUC) and all experiments were conducted following the national and institutional guideline while handling animal meats used in this study.

Conflict of Interest

All the authors declare that they have significantly contributed to this research and they do not have any conflict of interest to publish this article in journal.

References


Figure 1. In-silico analysis of the feline specific primer pairs. Phylogenetic tree (a), built from the 69-bp regions of cytb/cob-gene sequences of cat and other 19 non target animal and plant species using neighbor-joining method; mismatch comparison of the studied species in the primer annealing regions and AluI-cut sites (b); and 3D plot showing primer mismatch and pairwise distance (c).
Figure 2. Primer specificity (a) and target stability (b) analysis. In (a) shown are lane L: DNA ladder; lanes 1-11: PCR products from cat, beef, chicken, sheep, goat, pig, duck, rat, carp, tilapia and cucumber, respectively. The inset is the electropherograms of target (69 bp) and endogenous control (141 bp). In (b), comparative stability analysis of a shorter (69 bp) (developed in this study) (lanes 1-5 and 11 & 12) and longer (108 bp) (the shortest of the previously documented assays) (lanes 6-10 and 13 & 14) feline DNA targets after autoclaving (lanes 1-10) at 133°C for 30 (lanes 1 & 6), 60 (lanes 2 & 7), 90 (lanes 3 & 8), 120 (lanes 4 & 9) and 150 (lanes 5 & 10) min under 43.51 psi and microwave cooking (lanes 11-14) at 600W (lanes 11 & 13) and 700W (lanes 12 & 14) for 30 min. Please note that while the 69 bp was amplified under all treatments (lanes 1-5 and 11 & 12), the 108 bp target was not detected after 150 min of autoclaving (lane 10) and microwaving at 700W for 30 min (lane 14).
Figure 3. Specificity and sensitivity test under autoclaved (a) cat-goat binary and cat-beef-chicken ternary admixtures and (b) model meatballs (boiled and autoclaved). In (a), 10, 1, 0.1, and 0.01 % feline meat admixed into goat meats (lanes 2-5) and beef-chicken binary admixtures (lanes 7-10) under autoclaving condition (120°C for 2.5 h at 310 kPa). Lanes 1 and 6 are pure beef and chicken meats, respectively. In (b), 10, 1, 0.1, and 0.01 % feline meat mixed with beef meatball under boiling treatment (100°C for 90 min) (lanes 1-4) and chicken meatball under extensive autoclaving treatment (120°C for 2.5 h at 310 kPa) (lanes 6–9). Lanes 5 and 10 are pure beef and chicken meatballs, respectively and lane L is DNA ladder. The 69-bp and 141-bp PCR products represent feline target and eukaryotic endogenous control, respectively.
Figure 4. RFLP analysis of feline PCR product before (*lanes* 1, 3, 5, 7, 9, 11) and after (*lanes* 2, 4, 6, 8, 10, 12) *Alu*I digestion. In the gel view, PCR products from raw (1,2,7,8), boiled (3,4,9,10) and autoclaved (5,6, 11,12) beef (1-6) and chicken (7-12) meatballs and *lane L*: DNA ladder. The inset is the electropherograms as shown by respective labels.
Table 1. Pairwise distances between 69 bp cytb feline-specific site and 19 other species.

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Table 2. Molecular sizing statistics analysis of the lab-on-a chip based feline specific PCR-RFLP assay before and after digested with *Alu*I restriction endonucleases.

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<th>Autoclaved beef meatball with <em>Alu</em>I digestion</th>
<th>Boiled chicken meatball with <em>Alu</em>I digestion</th>
<th>Boiled chicken meatball with <em>Alu</em>I digestion</th>
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Table 3. Analysis of feline meat in commercial meatball products.

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<td>B</td>
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<td>D</td>
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<td>100%</td>
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Lab-on-a-chip-based PCR-RFLP assay for the confirmed detection of short-length feline DNA in food

Md. Eaqub Ali a,b*, Md. Al Amin a, Sharifah Bee Abd Hamid a, Shuhaimi Mustafa c, M.A. Motalib Hossain a

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Graphical Abstract

RFLP analysis of feline PCR product before (lanes 1, 3, 5, 7, 9, 11) and after (lanes 2, 4, 6, 8, 10, 12) AluI digestion. In the gel view, PCR products from raw (1,2,7,8), boiled (3,4,9,10) and autoclaved (5,6,11,12) beef (1-6) and chicken (7-12) meatballs and lane L: DNA ladder. The inset is the electropherograms as shown by respective labels.