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Original Research Article

Screening of commercial meat products from supermarket chains for feline derivatives using SP-PCR-RLFP and lab-on-a-chip

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ABSTRACT

Determination of feline meat in food products is an important issue for social, health, economic and religious concern. Hence this paper documented the application of species specific polymerase chain reaction-restriction fragment length polymorphism (SP-PCR-RFLP) assay targeting a short-fragments (69 bp) of mitochondrial cytochrome *b* (*cytb*) gene to screen feline meat in commercial meat products using lab-on-a-chip. The SP-PCR assay proved its specificity theoretically and experimentally while testing with different common animal, aquatic and plant species of DNA. The feline specific (69 bp, 43- and 26-bp) characteristic molecular DNA pattern was observed by SP-PCR and RFLP analysis. For assay performance, it was tested in three different types of commercial dummy meat products such as frankfurters, nuggets and meatballs and digested with *Alu*I-restriction enzyme. The highest sensitivity of the assay using lab-on-a-chip was as low as 0.1 pg or 0.01 % (w/w) in commercial dummy meat products. We have also applied this assay to screen three important commercial meat products of six different brand from six supermarket chains located at three different states of Malaysia. Thus total 378 samples were tested to validate the specificity, sensitivity, stability of the assay and utilization of it for commercial meat product screening.

1. Introduction

Currently, food safety is an important issue due to increasing consumer's awareness relate to the foods either from the restaurant or from different supermarket chains. Numerous features such as lifestyles (organic food and vegetarianism), health-hazardous problems (microbial risk and allergens), diet (nutritional balance and calories), economic situation (fair trade) and religious taboo (cat, pork, dog is banned according to religious dietary rules in Islam) are the major factors to verify the ingredients in various commercial food products. Furthermore, reports of fake labelling and stating false ingredients on food products have made a negative impact on customers' confidence. To gain economic benefits use of lower-priced meats in the higher-priced meat products has become a common exercise in the meat industry (Hsieh et al., 1995). Therefore, usage of proper meats is a prerequisite to maintain fair trade and consumer's trust for commercial meat products in the market chain.

The estimated value of the Halal market is $2.3 trillion and expected to reach 10 trillion US Dollars by 2030 (Amin et al., 2016). The price of halal foods is higher compared to conventional foods due to the requirement of particular processing and supply chain. For more profit mislabeling of 'Halal' symbols on food products has been reported (Ali et al., 2015d) Furthermore, the meat of certain sources are a prospective carrier of hepatitis (chimpanzee, gorilla and orangutan species) (Sa-Nguanmoo et al., 2008), influenza (pig and other species) (Mubareka et al., 2009), avian influenza-H5N1 virus (avian species) (Beigel et al., 2005), anthrax (horse, cattle and other species), plague (cat and dog species), severe acute respiratory syndrome (SARS) (cat and other species) (Ali et al., 2015) and human immunodeficiency virus (HIV) (chimps species) (Girish et al., 2004). Therefore, it becomes important to screen the commercial meat products from different supermarket chains to maintain sound health, fair trade, consumer trust and religious faith.

Frankfurters, nuggets and meatballs are very popular meat products

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almost every part of the world including Malaysia, India, China, Indonesia, Vietnam, Europe and the USA (Rahman et al., 2014). Recently, reports have been published about fraudulent use of horse meat in commercial beef meatball (Rahman et al., 2016), frozen meatballs (Ali et al., 2015a) and frankfurter sausages (Amin et al., 2016). According to the religious faith of Islam consumption of feline meat is forbidden. However, it has been consumed as exotic meals in South Korea, Vietnam, Thailand, Cambodia, and China. No legal market has been allotted to sell feline meats and roaming of feline species across the household without owner tag, make it as a source of meat without any offered prices in some countries. Thus feline meat is a potential adulterant in the food chain for gaining of deceitful economical profits.

It is a challenging job to check the substituted materials from the real products as it is seemed very similar to the original ingredients (Ghovvati et al., 2009). For meat species detection several methods based on DNA, protein and lipid have been described (Ali et al., 2015c; Fajardo et al., 2008; Rahman et al., 2015). Protein and lipid-based platform can be used for the species detection, but these methods are not economic and require well-trained experts for complicated data analysis (Pischetsrieder and Baeuerlein, 2009). Furthermore, protein and lipid-based biomarkers are not indubitable as they could be denatured or degraded or ramified due to the commercial meat products preparation steps (heat, pressure or chemical treatments) (Fajardo et al., 2010). In contrast, DNA based short-length biomarker ($\leq$150 bp) are more stable and highly sensitive for meat product analysis (Rojas et al., 2010). Thus it has been chosen over the protein and lipid-based biomarkers.

Several DNA based assay such as species-specific polymerase chain reaction (SP-PCR) (Karabasanavar et al., 2014; Rahman et al., 2014), multiplex PCR (Ali et al., 2015d; Hou et al., 2015), real-time PCR (Kesmen et al., 2013), Restriction Fragment Length Polymorphism (RFLP) (Chen et al., 2014; Rashid et al., 2015), PCR product sequencing (Girish et al., 2004), Randomly Amplified Polymorphic DNA (RAPD) (Arslan et al., 2006) and DNA barcoding (Di Pinto et al., 2013; Lamendin et al., 2015) are described for determining the animal species. Among these SP-PCR is simple, cost-effective and able to amplify specific DNA target from food matrix. Multiplex PCR is less time consuming and can detect more than one species in a single response, but performing multiplex-PCR for screening commercial products with a large number of the sample will be expensive. Sequencing is a most recent technique to authentic specific DNA target, however, it needs sophisticated instruments and not available in common laboratories. Reverse transcription-polymerase chain reaction (RT-PCR) provide exact quantitative conclusions through real-time ascertainment of the accumulating PCR product utilizing fluorescence resonance energy transfer (FRET) process. But it also entail with expensive instruments, chemicals and mostly useful for highly sensitive sample analysis. On the contrary, SP-PCR assay with a post-PCR restriction digestion (RFLP) analysis is more convenient for commercial product analysis. It not only amplifies specific PCR amplicon but also provides unambiguous DNA fingerprinting pattern from a complex pool of DNAs or closely related species detection. Thus PCR-RFLP analysis was utilized for determining the closely related species such as yak and cattle (Chen et al., 2009), wild pig and swine (Fajardo et al., 2008). Traditional agarose gel-based detection has the limitation to resolute restriction digested PCR products of smaller size (< 50 bp). The micro-fluidic innovation coupled with a lab-on-a-chip can effectively separate the specific short-length DNA target (< 50 bp). Therefore, we have applied a short-amplicon based SP-PCR-RFLP assay for the commercial meat product screening for feline meat from different supermarket chains across Malaysia.

2. Materials and methods

2.1. Samples collection

Fresh meat samples of goat (*Capra hircus)*, beef *(Bos taurus)*, sheep (*Ovis aries*), lamb (*Ovis aries*), buffalo (*Bubalus bubalis)*, pork *(Sus scrofa),* chicken *(Gallus gallus)*, duck *(Anas platyrhynchos*), pigeon (*Columba livia)*, turkey *(Meleagris gallopavo)*, shrimp (*Litopenaeus vannamei*), tilapia (*Oreochomis aureus)*, carp (*Cyprinus carpio)*, cod (*Gadus morhua*), potato (*Solanum tuberosum)*, cuttle (*Sepia officinalis)*, wheat *(Triticum aestivum*), cucumber (*Cucumis sativus),* tomato (*Solanum lycopersicum)* and onion (*Allium cepa*) were procured in triplicates from various supermarkets and wet markets in Malaysia. Cat (*Felis catus*) and dog (*Canis familiaris*) meat samples were obtained in triplicates from euthanized animals according to animal welfare protocol by authorized personnel from Kuala Lumpur City Hall, Health Ministry, Malaysia. Monkey (*Macaca fascicularis* sp.) sample was collected from Wildlife Malaysia, Cheras, Kuala Lumpur; rat (*Rattus norvegicus)* and turtle (*Cuora amboinensis)* was bought from Chinese wet market at Selangor, Malaysia. For commercial sample analysis commonly available meat product's samples of total six different brands of chicken and beef frankfurters, nuggets and meatballs were collected from six different superstore/supermarket chains located across three different states of Malaysia. The samples were collected in triplicate and carried by using the ice-cold box (4 °C) and refrigerated at −20 °C for further use and extraction of DNA.

2.2. Preparation of dummy meat products

To identify meat adulteration, dummy meat products of beef and chicken frankfurters, nuggets and meatballs were prepared (Table 1) by adding 1, 0.5, 0.1 and 0.01 % (w/w) of cat meat into beef and chicken meats according to Rahman et al. (2016). The raw frankfurters and nuggets were autoclaved for 2.5 h under 45-psi pressure at 120 °C and the meatballs were boiled for 90 min at 100 °C (Rahman et al., 2016) to check its consistency under meat processing steps. All the prepared dummy meat products were refrigerated at −20 °C for further use and DNA extraction.

2.3. DNA extraction

Meat and fish samples were sliced into small pieces using sterilized surgical scalpels. Total DNA was extracted from 25 mg of raw meat samples from each specimen using commercial genomic DNA extraction kit (Yeastern Biotech Co. Ltd, Taipei, Taiwan). For the extraction of DNA from plants, commercial and lab-made beef and chicken frankfurters, nuggets and meatballs; 1 g of each sample was used by applying cetyl trimethylammonium bromide (CTAB) method according to Amin et al. (2016). To measure the purity and concentration of extracted DNAs UV–vis NanoPhotometer® (Nano Life Quest Sdn. Bhd; Selangor, Malaysia) were used.

2.4. Feline-specific primer

A pair of feline specific primers based on mitochondrial cytochrome *b* gene (GenBank: AB194817.1) was developed using an online bioinformatics tool Primer3plus (www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi), which possessed a 22 bp *Alu*I cut-site as an internal oligo (Ali et al., 2015a; Amin et al., 2016). In the in-silico analysis, primer specificity was confirmed by extracting the nucleic acid sequences of more species by using the BLAST (Basic Local Alignment Tool) of NCBI database (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Retrieved sequences of 14 most important animal species such as goat, beef, sheep, lamb, buffalo, chicken, duck, pigeon, turkey, pork, dog, Malaysia box turtle, monkey and rat; 5 fish samples of Atlantic shrimp, tilapia, cuttle, carp and cod and 5 plant sources of wheat, tomato, cucumber, potato and onion were aligned by ClustalW (Clustal Omega) sequence alignment tool (https://www.ebi.ac.uk/ Tools/msa/clustalo/) to verify the degree of interspecies polymorphism of primer binding site. The total number of mismatch for both primers binding sites were calculated by using MEGA5 software. Another set of primer designed by

Table 1
Formulation of lab-made dummy beef and chicken frankfurters, nuggets and meatballs (≥100 g).

Ingredients	Frankfurters			Nuggets			Meatballs		
	Beef	Chicken	Feline	Beef	Chicken	Feline	Beef	Chicken	Feline
Ground beef meat	80 g[a]	–	–	83g	–	–	85 g[a]	–	–
Ground chicken meat	–	85 g[a]	–	–	83 g	–	–	85 g[a]	–
Ground feline meat	–	–	85 g[a]	–	–	83 g	–	–	80 g[a]
Fresh breadcrumbs	6 g	6 g	6 g	–	–	–	7.5 g	7.5 g	7.5 g
Dry breadcrumbs	8 g	–	–	–	–	–	–	–	5 g
Onion paste	2 g	2 g	2 g	1 g	1 g	1 g	1 g	1 g	1 g
Eggs	0.3 g	0.3 g	0.3 g	2.5 g	2.5 g	2.5 g	0.5 g	0.5 g	0.5 g
Garlic paste	0.5 g	0.5 g	0.5 g	1.5 g	1.5 g	1.5 g	1.3 g	1.3 g	1.3 g
Ginger paste	–	1.8 g	1.8 g	–	–	–	1.2 g	–	1.2 g
Soybean oil	–	–	–	1.2 g	1.2 g	1.2 g			
Tomato paste	–	–	–	–	–	–	0.4 g	0.4 g	0.4 g
Textured soya protein	–	–	–	1.8 g	1.8 g	1.8 g	–	–	–
Butter	–	1.5 g	1.5 g	–	–	–	–	2.5 g	2.0 g
Red pepper powder	–	0.25 g	–	0.5 g	0.5 g	0.5 g	–	–	–
Chilled water	–	–	–	2 g	2 g	2 g	–	–	–
Finely chopped carrot	–	–	–	2.5 g	2.5 g	2.5 g	–	–	–
Sodium nitrite	–	–	–	0.005 g	0.005 g	0.005 g	–	–	–
Tetra-sodium pyrophosphate	–	–	–	0.2 g	0.2 g	0.2 g	–	–	–
Sodium chloride	–	–	–	0.5 g	0.5 g	0.5 g	–	–	–
Black pepper powder	0.25 g	–	–	0.5 g	0.5 g	0.5 g	–	0.15 g	0.15 g
White pepper powder	–	–	0.25 g	0.5 g	0.5 g	0.5 g	0.3 g	–	–
Wheat flour	–	–	–	1.5 g	1.5 g	1.5 g	–	–	–
Tapioca starch	2.5 g	2.5 g	2.5 g	–	–	–	1.5 g	1.5 g	1.0 g
Chesses	–	–	–	–	–	–	–	0.2 g	0.2 g
Salt	AA	AA	AA	AA	AA	AA	AA	AA	AA
Sugar	–	–	–	AA	AA	AA	–	–	–
Others additives	AA	AA	AA	AA	AA	AA	AA	AA	AA

AA, adjustable amounts.
[a] 1%, 0.5 %, 0.1 % and 0.01 % of feline meat were mixed with a well-adjusted amount of chicken and beef meat to make 100 g specimen of each frankfurters, nuggets and meatball products.

Rojas et al. (2010) targeting a conserved region of eukaryotic 18S rRNA gene (141-bp) was employed as an internal control for experimental analysis. The consensus feline specific 69 bp cytb gene sequences were used to determine the pairwise genetic distance and dendrogram was constructed with molecular evolution and phylogenetic analysis software, MEGA version 5. 3D plot was created based on pairwise distances of 69 bp feline cytb gene-specific site and the number of oligonucleotide mismatches by XLSTAT software (Addinsoft, 2013) to define the potentiality of the primers for feline species detection.

All the primers for eukaryotic (Forward: 5′ GGT AGT GACGAA AAATAA CAATAC AGGAC 3′, Reverse: 5′ ATACGC TATTGG AGC TGG AATTACC 3′) and feline specific (Forward 5′ ACTATT ATTTAC AGTCAT AGCCAC AGC 3′, Reverse: 5′ CAGAAG GACATT TGGCCT CA 3′) were bought from First BASE Laboratories Sdn Bhd, Selangor, Malaysia.

2.5. PCR amplification

A 69 bp of feline specific target and 141 bp of eukaryotic control were selectively amplified in a 25 μL of total reaction mixtures containing 20 ng of each DNA template, 5 μL of 5x colourless GoTaq Flexi Buffer, 0.1 μL of GoTaq Flexi DNA polymerase, 0.5 μL of 0.2 mM each of dNTP, 0.5 μL of each primer, 1.5 μL of 25 mM $MgCl_2$ (Promega, Madison, USA). PCR parameters 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, elongation at 72 °C for 30 s and final elongation at 72 °C for 5 min were used for the successful amplification of feline specific DNA target. All the PCR assay were done by using Veriti 96-Well Gradient Thermal cycler machine (Applied Biosystems; California, USA). Finally, amplified PCR products were analyzed by lab-on-a-chip based microfluidic automated electrophoretic platform (Experion DNA 1000 Analysis Kit, Bio-Rad Laboratories, Inc., USA) following the manufacturer's direction.

2.6. Enzymatic digestion and RFLP analysis

We successfully digested the PCR products of the dummy and commercial meat products of frankfurters, nuggets and meatballs by restriction endonuclease in 30 μl reaction volume composed of 1 μl of *Alu*I restriction enzyme (New England Biolab, CA, USA), 10 μl of PCR product, 2 μl of 10× digestion buffer and appropriate volume of nuclease free water. Restriction digestion was performed by using water shaking bath at 37 °C for 30 min. To inactivate the restriction digestion enzyme reheating of the reaction mixture was done for 25 min at 65 °C. For the final RFLP analysis and separation of specific DNA fragments, 1 μl of digested PCR-products were loaded in the lab-on-a-chip-well of 1 K DNA kit and run by using Experion Automated Electrophoresis Station (Bio-Rad Laboratories, USA).

2.7. Commercial meat products analysis

Extracted DNAs of commercial chicken and beef frankfurters, nuggets and meatballs were exploited for the detection of cat meat using the SP-PCR (feline-specific) primer. We have considered the affirmative detection of deliberately cat meat spiked dummy commercial frankfurters, nuggets and meatballs as a positive control. The presence of 141-bp of endogenous control was analyzed to confirm the quality of DNA of the samples collected from the six different supermarket chains located across three different states of Malaysia.

3. Results and discussions

3.1. Feline species specificity analysis

For the in-silico analysis, nucleotide sequences of mitochondrial cytb gene of all species were taken from the NCBI database and aligned with the feline cytb specific SP-PCR primer binding sites. It

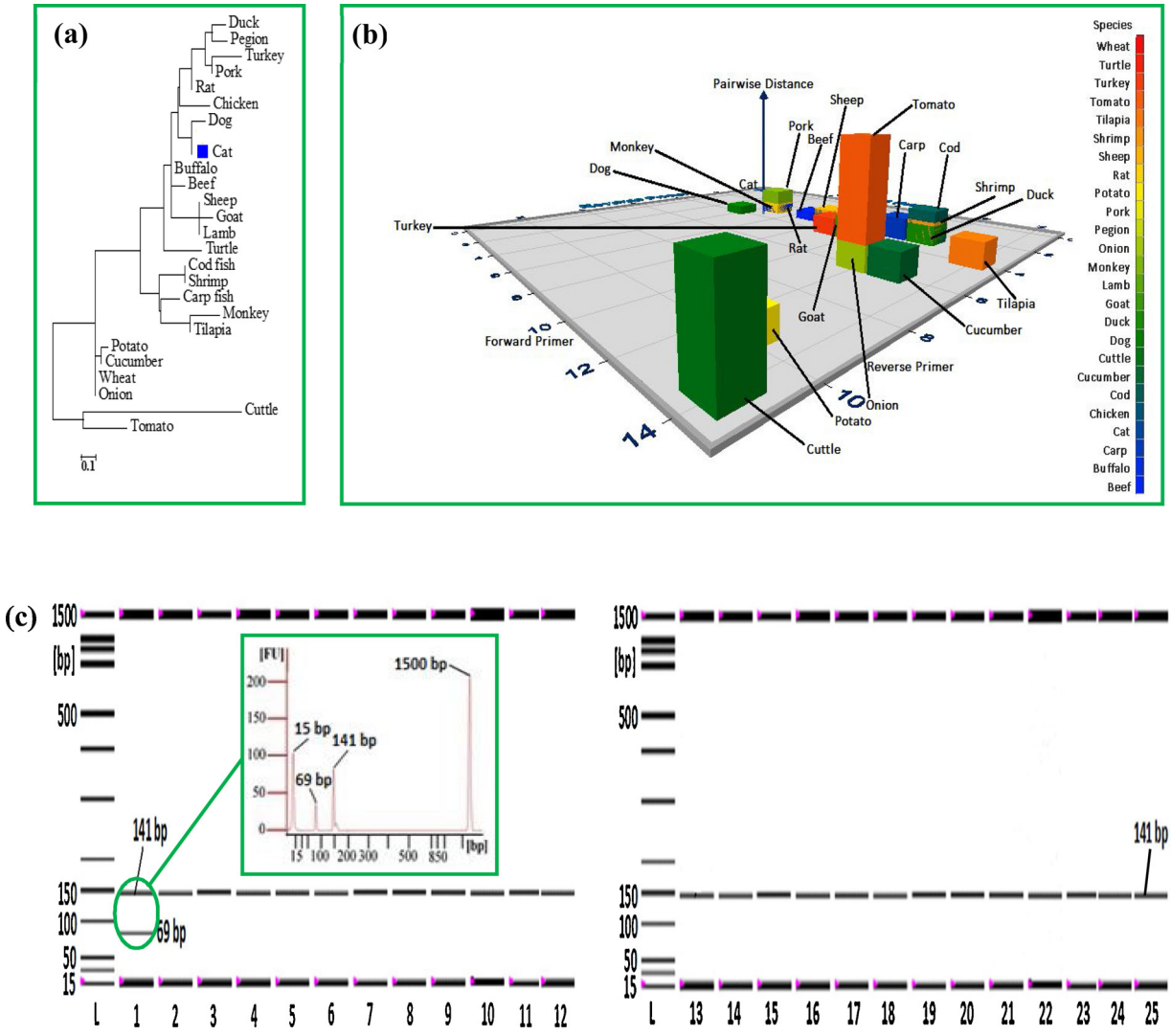

Fig. 1. In silico (a, b) and experimental analysis (c) of short-amplicon based feline specific primers. Phylogenetic tree (a) and mismatch calculation of twenty-four (24) species DNA sequences of *cytb* gene with feline specific primer pairs shown on 3D (b). Test of cross amplification of feline specific primers against 10 ng DNA of twenty-four (24) different non target animal, fish and plant species (c); lane L: DNA ladder; lane 1: feline-specific target (69 bp) and lanes 2-25: endogenous control (141 bp) for beef, buffalo, chicken, dog, goat, duck, sheep, lamb, pork, pigeon, rat, turkey, turtle, monkey, cod, carp, tilapia, shrimp, cuttle, potato, onion, wheat, cucumber, tomato, respectively. Electropherograms of 69 bp target and 141 bp of endogenous control is presented by respective labels.

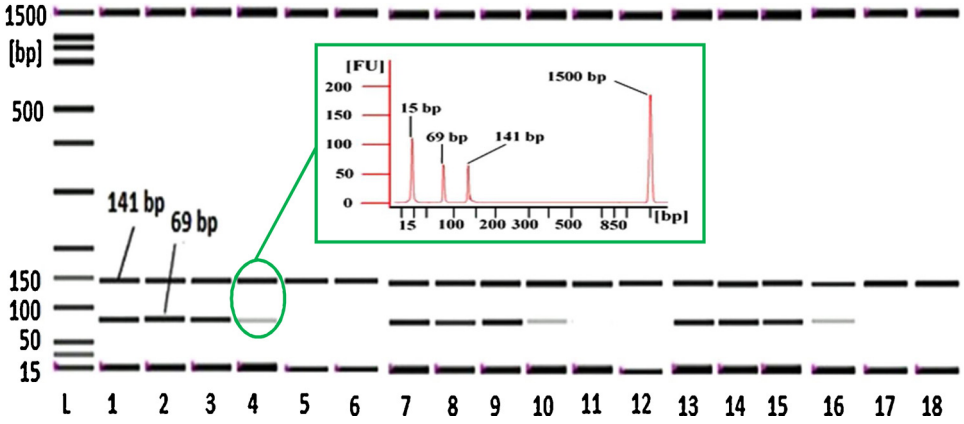

Fig. 2. Specificity and sensitivity analysis in lab made dummy meat products. LOD of 1, 0.5, 0.1 and 0.01 % feline meat spiked dummy beef frankfurters (lanes 1-4), chicken nuggets (lanes 7-10) under extensive autoclaving treatment (120 °C for 2.5 h at 45 psi) and beef meatballs (lanes 13-16) under boiling treatment (100 °C for 90 min) respectively and lane L is DNA ladder. No amplification from dummy beef and chicken frankfurters, nuggets and meatballs without spiking any feline meats (lanes 5-6, 11-12 & 17-18) respectively. Electropherograms are also shown by respective labels.

demonstrated the feline specificity by complete matching with feline cytb gene and three to nineteen (14–86%) nucleotide mismatching with the other non-target species (Fig. 1b) providing the theoretical supports to our findings. Moreover, phylogenetic tree analysis (Fig. 1a) and the number of mismatches (Fig. 1b & 3a) reflected the divergence between the target and rest of the species DNA. The maximum and minimum genetic distance were observed between cat with cuttlefish (1.608) and cat with beef and buffalo (0.160), which reduced the risks of cross

positive amplification during the experimental study. In this experiment, the feline specificity was extended to check with the total 14 non targets of animal, 5 fish and 5 common plant species which are frequently used for making different type of food products. In Fig. 1c (lane 1) and Fig. 2 (lane 1, 7 & 13), clearly showed that the primers are cat or feline specific, as the target amplicon (69 bp) was amplified only from the samples of feline meat or feline meat spiked product. Moreover, a pair of eukaryotic primer amplified the 141-bp target of the 18S rRNA gene from all the tested samples (Fig. 1c, lanes 1–25), which confirmed the existence of adequate DNA template.

Previously, six different PCR assays using *cytb* gene (331 bp) (Irine et al., 2013); *cytb* gene (180 bp) (Tobe and Linacre, 2008); *cytb* gene (672 bp) (Abdel-Rahman et al., 2009), (Abdulmawjood et al., 2003); 12S rRNA (108 bp) (Martin et al., 2007) and ND4 gene (274 bp) (Ilhak and Arslan, 2007) have been reported for cat meat detection. However, the majority of these assays have been regarded with longer amplicons size (≥180 bp) which would possibly be fragmented underneath different meat products processing treatments and lead PCR amplification failure. The recent studies esteemed the short-amplicon based PCR assays due to its higher stability over the longer-ones (≥150 bp) for species authentication under the complex background of food products (Rahman et al., 2016). Therefore, we have used this short amplicon-based (69-bp) SP-PCR assay with mitochondrial-*cytb*-gene target for *F. catus* detection in commercial meat products.

3.2. Feline meat spiked dummy meat products analysis

Lower priced meat are deliberately mixed in commercial meat products (Amin et al., 2016) and the food forgeries were reported in a number of meat products such as frankfurters, dried meats, burgers, ground meat, nuggets, meatballs and sausage (Kane and Hellberg, 2016). Recently, the 'Food Safety Authority of Ireland' discovered 37 % horsemeat in beef burgers and ground beef products and 85 % pork meat in salami (Kane and Hellberg, 2016). The latest scandal of cat meat in curry and selling of cat meat as rabbit meat (Amin et al., 2016) headed us to screen the commercial meat products adulteration with cat meat.

We observed the feline specific DNA from all lab-made (Table 1) feline meat spiked dummy beef and chicken frankfurters, nuggets and meatballs (Fig. 2 & Table 2). The results proved its sensitivity and efficiency to identify feline derivatives from any food processing products. Recently, Rahman et al. (2015) and Rashid et al. (2015) was identified 0.01 % (w/w) and 0.1 % (w/w) dog and monkey meat in dummy burgers and meatballs respectively, using a lab-on-a-chip-based detection method. Thus the LOD of 0.01 % in three different meat products (Fig. 2, lanes 4, 10 & 16) was an acceptable conclusion, as we used the short-length (69 bp) target in a highly sensitive microfluidic lab-on-a-chip based automated electrophoretic platform.

3.3. Lab-on-a-chip based SP-PCR-RFLP analysis

Lab-on-a-chip-based capillary electrophoretic assay made SP-PCR-RFLP patterns more specific, reliable and highly sensitive to distinguish and differentiate digested banding profiles in commercial meat products. It also proved its dominance over other capillary electrophoresis based assay such as DNA sequencing and probe hybridization methods for commercial sample analysis. Therefore, the amplified feline-specific PCR products (69 bp) was digested using *Alu*I restriction enzyme since the theoretical study (Fig. 3a) revealed the availability of restriction sites yielding 43- and 26-bp length of two distinct fragments. Moreover, internal control of 141 bp of 18S rRNA gene produced two fragments (14- and 127 bp) with one *Alu*I restriction cut-site during the in-silico analysis. In Fig. 3b, *Alu*I digested feline meat contaminated (beef and chicken) frankfurters (lanes 2 & 4), (beef and chicken) nuggets (lanes 6 & 8) and (beef and chicken) meatballs (lanes 10 & 12) were separated by the Bio-Rad Experion Automated Electrophoresis Station. The 43-, 26- and 127-bp fragments were clearly visible both in the gel image (Fig. 3b) and the electropherograms (Fig. 3b) reflecting the target amplicon. However, 14-bp of eukaryotic control (141 bp) was not visible (Fig. 3b, lanes 2, 4, 6, 8, 10 & 12) because it integrated with the lower marker (15 bp) of the DNA ladder (1500 bp). The molecular sizing statistics of the amplified products and restriction digestion of feline frankfurters, nuggets and meatballs using lab-on-chip are presented in Table 3. Among the previously developed feline specific assays, Abdulmawjood et al. (2003) described PCR-RFLP method with a longer fragment of 981 bp without any heat treating effect. Besides, this method was not verified in commercial meat products under the extensive autoclaving condition and might not be appropriate for processed meat products sample analysis due to larger-sized DNA target (981 bp). Therefore, the importance of this developed short-amplicon based SP-PCR-RLFP assay for commercial sample analysis using lab-on-a-chip is easily intelligible.

3.4. Screening of commercial meat products from supermarket chains

Frankfurters, nuggets and meatballs are the most common and popular meat products which are consumed all over the world including Malaysia, China, Indonesia and Europe (Rohman et al., 2011). Low-cost meat is usually added to higher valued meat products to reduce the production cost and financial benefit. Recently, horse meat was found in meatballs (Ali et al., 2015a) and chicken nuggets in Europe (Rahman et al., 2016). Therefore, for the screening of commercial beef and chicken frankfurters, nuggets and meatballs, 0.01 % (w/w) cat meat-spiked beef and chicken frankfurters (Fig. 4b, lanes 1 & 2), nuggets (Fig. 4b, lanes 3 & 4) and meatballs (Fig. 4b, lanes 5 & 6) were used as a positive control in the presence of 141 bp of eukaryotic 18S rRNA gene. In Fig. 4b (lanes 1–6), all the positive control (0.01 %)

Table 2
Analysis result of feline meat spiked dummy commercial meat products.

Item	No	Feline meat spiked dummy commercial products	Existence of target	No of Total sample	Positive detection	Detection Probability (%)
Frankfurter	1	Raw beef frankfurters	+	9	9/9	100%
	2	Autoclaved beef frankfurters	+	9	9/9	100%
	3	Raw chicken frankfurters	+	9	9/9	100%
	4	Autoclaved chicken frankfurters	+	9	9/9	100%
Nuggets	1	Raw beef nuggets	+	9	9/9	100%
	2	Autoclaved beef nuggets	+	9	9/9	100%
	3	Raw chicken nuggets	+	9	9/9	100%
	4	Autoclaved chicken nuggets	+	9	9/9	100%
Meatballs	1	Raw beef meatballs	+	9	9/9	100%
	2	Boiled beef meatballs	+	9	9/9	100%
	3	Raw chicken meatballs	+	9	9/9	100%
	4	Boiled chicken meatballs	+	9	9/9	100%
	Total feline meat spiked sample		+	108	108/108	100%

"+" denotes presence of target feline DNA.

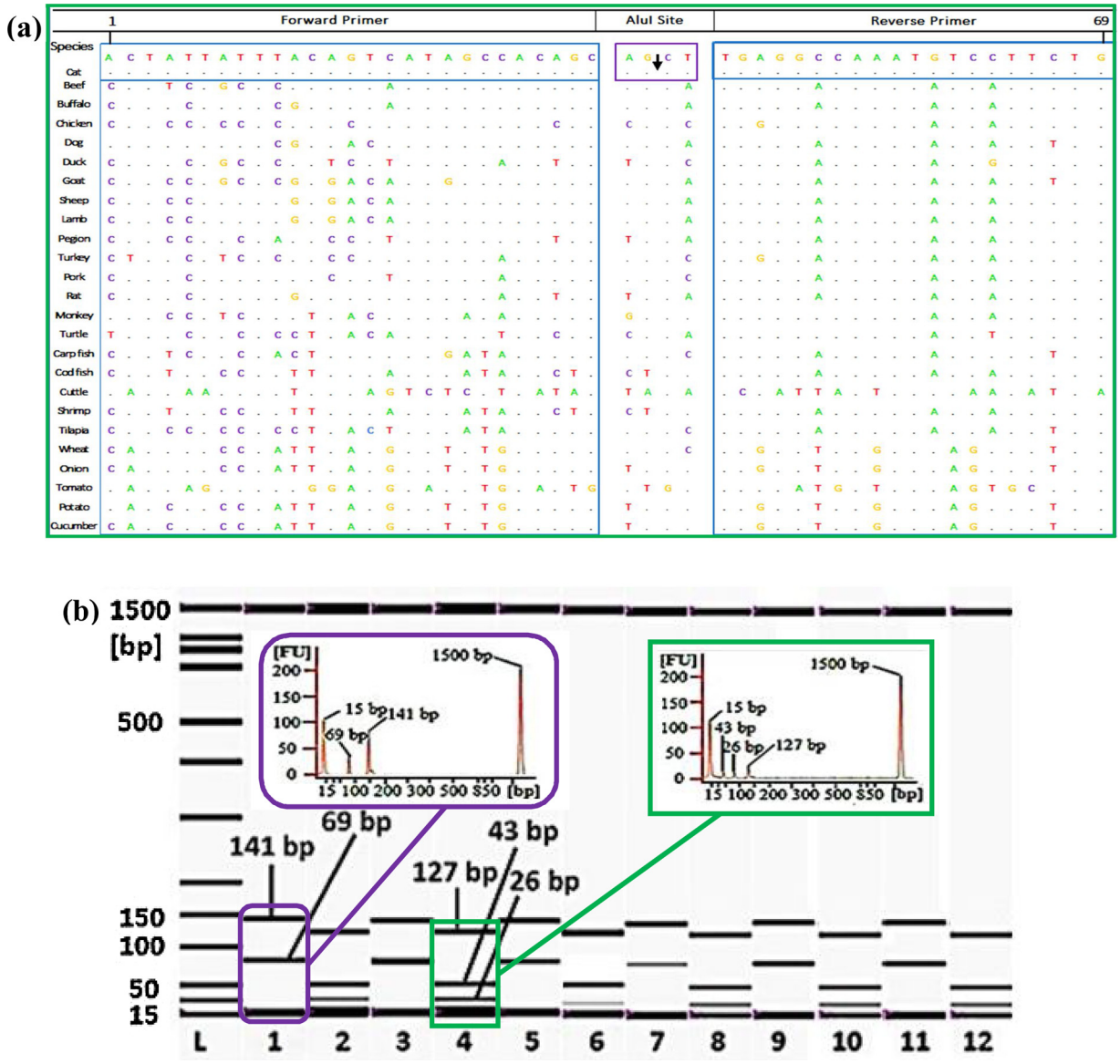

Fig. 3. (a) In-silico analysis with restriction cut site and (b) amplification of feline specific DNA target (69 bp) and internal control (141 bp) using SP-PCR-RFLP assay from deliberately cat meat spiked commercial meat products with the endogenous control. Clear 69 bp feline specific and 141 bp sized endogenous control before *Alu*I digestion (lanes 1, 3, 5, 7, 9 & 11) and with 127 bp, 43bp and 26 bp size (lanes 2, 4, 6, 8, 10, and 12) using lab on a chip. In the gel view, SP-PCR products and endogenous control from (beef and chicken) frankfurters (lanes 1 & 3), (beef and chicken) nuggets (lanes 5 & 7) and (beef and chicken) meatballs (lanes 9 & 11) before *Alu*I digestion; and (beef and chicken) frankfurters (lanes 2 & 4), (beef and chicken) nuggets (lanes 6 & 8) and (beef and chicken) meatballs (lanes 10 & 12) after *Alu*I digestion. Electropherograms are demonstrated by respective labels.

Table 3
Molecular sizing of feline specific PCR amplicon and Lab-on-a-chip based restriction patterns from raw and treated feline meat spiked dummy commercial chicken and beef frankfurter, nuggets and meatballs samples.

PS (bp)	Frankfurters				Nuggets				Meatball			
	Frankfurters -Beef		Frankfurters-Chicken		Nuggets-Beef		Nuggets-Chicken		Meatball-Beef		Meatball-Chicken	
	Raw	Autoclaved	Raw	Autoclaved	Raw	Autoclaved	Raw	Autoclaved	Raw	Boiled	Raw	Boiled
141	144 ± 1	147 ± 0.8	140 ± 0.2	145 ± 1.5	143 ± 0.5	149 ± 1.7	141 ± 1.5	151 ± 1	147 ± 0.3	142 ± 0.1	149 ± 1	140 ± 1.2
127	128 ± 0.6	130 ± 1.8	133 ± 0.5	127 ± 0.9	131 ± 0.7	129 ± 0.3	132 ± 0.2	133 ± 0.1	128 ± 0.8	127 ± 0.2	131 ± 0.9	130 ± 1.8
14	–	–	–	–	–	–	–	–	–	–	–	–
69	72 ± 0.2	69 ± 0.8	72 ± 0.4	65 ± 0.7	70 ± 0.9	66 ± 0.1	76 ± 0.8	67 ± 0.3	71 ± 0.9	68 ± 0.5	72 ± 0.2	69 ± 0.4
43	44 ± 0.2	49 ± 0.1	42 ± 0.0	45 ± 0.7	43 ± 0.5	46 ± 0.4	41 ± 0.1	42 ± 0.9	43 ± 0.0	40 ± 0..3	44 ± 0.2	45 ± 0.00
26	24 ± 0.7	23 ± 0.3	20 ± 0.1	24 ± 0.0	23 ± 0.4	22 ± 0.1	21 ± 0.7	24 ± 0.6	20 ± 0.7	21 ± 0.8	23 ± 0.2	24 ± 0.5

PS-product size; bp-base pair.

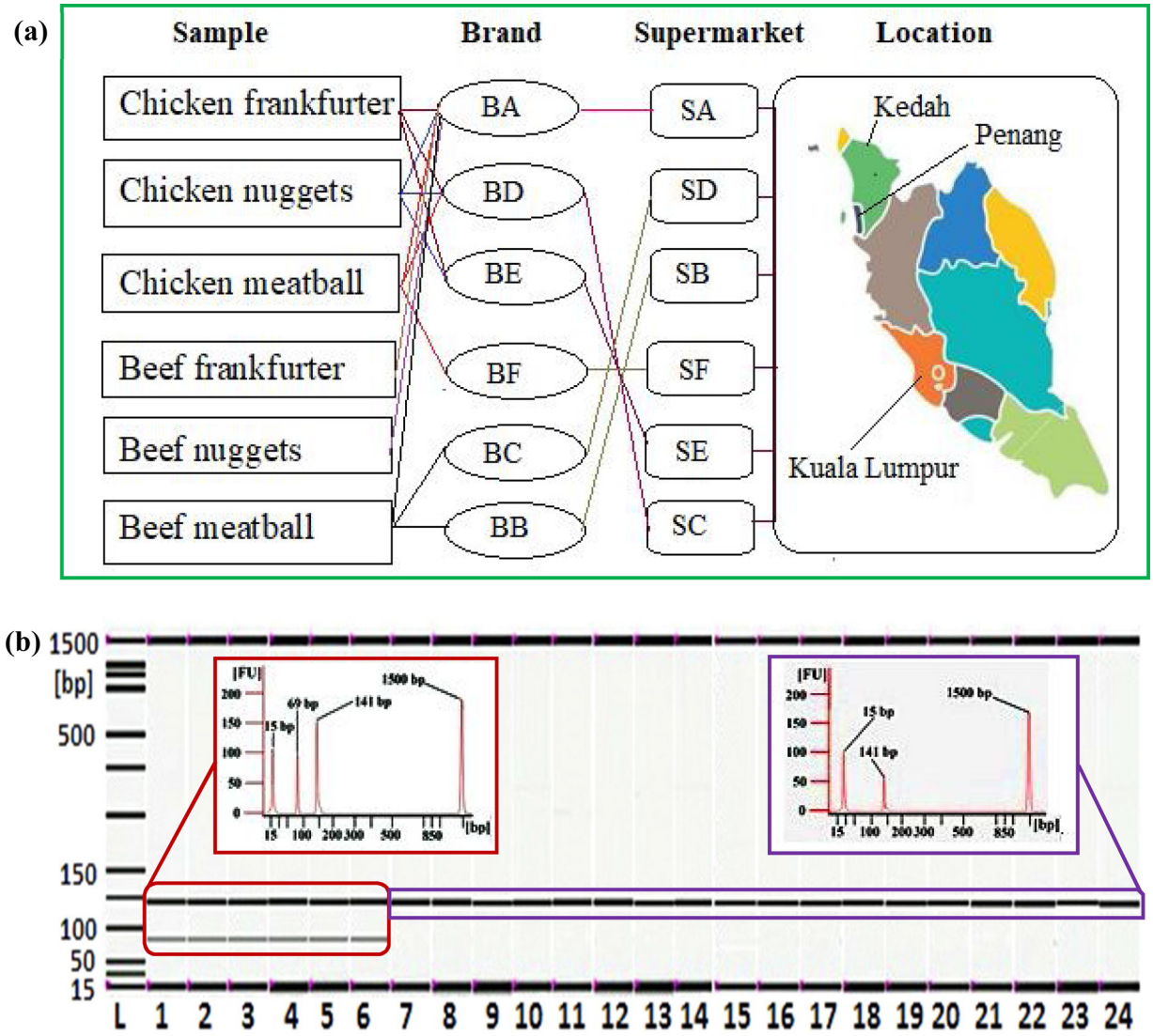

Fig. 4. Geographical location of different supermarket chain and brands of collected commercial meat products (frankfurters, nuggets and meatballs) (a) and screening of commercial meat products (frankfurters, nuggets and meatballs) using 69 bp feline specific PCR assay with endogenous control (141 bp) (b). No PCR product was detected from of different brands of commercial meat samples (lanes 7-24) from different supermarket chains (lanes 7-24). Lane L: DNA ladder; lanes 1-6: 0.01 % cat meat spiked chicken and beef frankfurters, nuggets and meatballs respectively and lanes 7-24 different brands of commercial chicken and beef frankfurter, nuggets and meatball collected from different supermarket chain across Malaysia. Lanes 7-9: commercial chicken frankfurter; lane 10-12 chicken nuggets; lanes 13-15 chicken meatball of three different brands. Lanes 16-18: beef frankfurter, Lane: 19-21 beef nuggets; lane 22-24 beef meatball from three different supermarket chains. Electropherograms are presented with labels in insets on the right side.

was amplified with feline specific SP-PCR products from each of the spiked commercial samples of frankfurters, nuggets and meatballs. On the other hand, there was no feline specific SP-PCR amplicon from pure commercial products of beef and chicken frankfurters, nuggets and meatballs (Fig. 4b, lanes 7–24). Note that, homogeneous amplification of eukaryotic primers (141 bp) form all the commercial products (Fig. 4b, lanes 1–24) reflected the presence of ample DNA template and the assay performance was validated with the absence of cat meat derivatives in commercial meat products (Fig. 4b lanes 7–24 & Table 4). Thus, total 378 samples of two types (chicken and beef) with three commercial meat products (frankfurter, nuggets and meatball) of total six (6) different brands purchased from total six (6) supermarket located at Kedah, Penang and Kuala Lumpur of Malaysia (Fig. 4) were negative for feline meat detection using feline SP-PCR based on lab-on-a-chip (Table 4). The result is acceptable as 'the Government of Malaysia' is devoted to set up Malaysia as a 'Halal-hub' and strongly observing for the assessment of 'Halal Status' of foods in different food products.

4. Conclusion

Note that, a highly sensitive SP-PCR-RFLP assay using electrophoresis based lab-on-a-chip is documented for the repetitive analysis of cat meat detection in popular commercial meat products. A set of feline specific primers were analyzed using cat and other twenty-four (24) species of animal, plant and fish DNA. Thus the feline specific target was amplified only from the feline DNA template, which ratified the specificity and self-standing capacity of the applied biomarkers. The LOD of 0.01 % (w/w) feline meats was found in all commercial dummy meat products (frankfurters, nuggets and meatballs) using SP-PCR assay. The successful digestion of the SP-PCR products using *Alu*I restriction enzyme yield feline specific restriction patterns (43- and 26-bp) from all the deliberately cat meat spiked dummy commercial products. Thus the endpoint detection and separation of restriction digested SP-PCR-RFLP products using lab-on-a-chip confirmed the feline species authenticity and declined any dubious results. Henceforward, the amazing stability and established sensitivity of this assay initiates

Table 4
Analysis results of screening commercial meat products of different brands and supermarket chains across Malaysia.

Item	No	Commercial meat product	Existence of target	No of Total sample	Positive detection	Detection Probability (%)
Meat Products	1	Beef frankfurters (B = 1, S = 1, L = 3, r = 3)	—	27	0/27	100%
	2	Chicken frankfurters (B = 3, S = 3, L = 3, r = 3)	—	81	0/81	100%
	3	Beef nuggets (B = 1, S = 1, L = 3, r = 3)	—	27	0/27	100%
	4	Chicken nuggets (B = 3, S = 3, L = 3, r = 3)	—	81	0/81	100%
	5	Beef meatballs (B = 3, S = 3, L = 3, r = 3)	—	81	0/81	100%
	6	Chicken meatballs (B = 3, S = 3, L = 3, r = 3)	—	81	0/81	100%
Brands	1	BA (P = 6, S = 1, L = 3; r = 3)	—	162	0/162	100%
	2	BB(P = 1, S = 1, L = 3; r = 3)	—	27	0/27	100%
	3	BC (P = 1, S = 1, L = 3; r = 3)	—	27	0/27	100%
	4	BD (P = 3, S = 1, L = 3; r = 3)	—	81	0/81	100%
	5	BE (P = 2, S = 1, L = 3; r = 3)	—	54	0/54	100%
	6	BF (P = 1, S = 1, L = 3; r = 3)	—	27	0/27	100%
Supermarkets	1	SA (P = 6, S = 1, L = 3; r = 3)	—	162	0/162	100%
	2	SB(P = 1, S = 1, L = 3; r = 3)	—	27	0/27	100%
	3	SC (P = 3, S = 1, L = 3; r = 3)	—	81	0/81	100%
	4	SD (P = 1, S = 1, L = 3; r = 3)	—	27	0/27	100%
	5	SE (P = 2, S = 1, L = 3; r = 3)	—	54	0/54	100%
	6	SF (P = 1, S = 1, L = 3; r = 3)	—	27	0/27	100%
		Total commercial product's sample	—	378	0/378	100%

"B" brand, "S" Supermarket, "L" Location, "r" for replicate and "−" denotes absence of target DNA.

its application for the screening of larger quantity of samples of three major commercial meat products (frankfurters, nuggets and meatballs) of total six brands from different supermarket chains across Malaysia (three-states) for feline species detection. The screening of total of 378 samples of commercial meat products from six different supermarket chain of Malaysia, were negative for feline species detection. Thus the assay showed it acceptability to the regulatory bodies and quality control laboratories for commercial meat products screening. It also showed the reflection of the reliability of the halal status of Malaysian commercial meat products.

Authors statement

All the authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Compliance with ethics requirements

All experiments were conducted with the Ethical clearance ref. no: NANOCAT/25/04/3013/MMR (R), University of Malaya.

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Declaration of Competing Interest

The authors do not have any conflict of interest to publish this manuscript.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jfca.2020.103565.

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