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A lab-on-a-chip-based multiplex platform to detect potential fraud of introducing pig, dog, cat, rat and monkey meat into the food chain

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Food forgery has posed considerable risk to public health, religious rituals, personal budget and wildlife. Pig, dog, cat, rat and monkey meat are restricted in most religions, but their sporadic adulteration are rampant. Market controllers need a low-cost but reliable technique to track and trace suspected species in the food chain. Considering the need, here we documented a lab-on-a-chip-based multiplex polymerase chain reaction (PCR) assay for the authentication of five non-halal meat species in foods. Using species-specific primers, 172, 163, 141, 129 and 108-bp sites of mitochondrial ND5, ATPase 6 and cytochrome b genes were amplified to detect cat, dog, pig, monkey and rat species under complex matrices. Species-specificity was authenticated against 20 different species with the potential to be used in food. The targets were stable under extreme sterilisation (121°C at 45 psi for 2.5 h) which severely degrades DNA. The assay was optimised under the backgrounds of various commercial meat products and validated for the analysis of meatballs, burgers and frankfurters, which are popular fast food items across the globe. The assay was tested to detect 0.1% suspected meats under commercial backgrounds of marketed foods. Instead of simplex PCR which detects only one species at a time, such a multiplex platform can reduce cost by at least fivefolds by detecting five different species in a single assay platform.

Keywords: lab-on-a-chip-based multiplex PCR; haram meat-species; extreme sterilisation; commercial food matrices

Introduction

The world is getting increasingly busier, and a growing proportion of the population faces time-constraints in making their own meals. Consequently, they are forced to accept whatever they can have from a nearby restaurant or grocery store (Ali et al. 2013; Rahman et al. 2014). Thus, the demands and prospects of restaurant businesses and ready-made foods, such as burgers, pizzas, hot dogs, sandwiches, soups, cookies, candies and creams, are growing fast (Van Der Spiegel et al. 2012). However, labelling does not necessarily always guarantee total control “from farm-to-fork”. One of the obvious regions might be the massive and ongoing innovations in processing and packaging technologies (McMillin 2008; Cammà et al. 2012). Furthermore, the recent scandals of horse meat in Europe and pig and rat meats in China (Ali et al. 2014; Premanandh 2013) and the repeated addition of prohibited food items such as pork, dog and cat, meats with various dishes have alarmed consumers into demanding testing for the presence of prohibited ingredients in marketed foods (Mohamad et al. 2013; Karabasanavar et al. 2014; Kitpipit et al. 2014; Rashid et al. 2015). As a result, the European Commission has emphasised food safety as a key priority in food policy and health (Regulation EC No. 178/2002). Some other countries, such as Malaysia, Indonesia, China, Thailand, Singapore and Brazil, have established trustworthy certification bodies to control and protect public health, religious credence, fair-trade business and wildlife linked to food falsification and/or mislabelling, especially of animal origin (Rohman et al. 2011; Rahman et al. 2015).

The enforcement of labelling regulations requires sensitive, reliable and easily performable scientific methods to verify trace ingredients in processed and unprocessed foods (Ghovvati et al. 2009; Fajardo et al. 2010; Doosti et al. 2011; Herrero et al. 2011; Cawthorn et al. 2013; Kesmen et al. 2013; Hou et al. 2014; Karabasanavar et al. 2014). Although lipid- and protein-based methods can be applied for the biomarker-based molecular authentication schemes (Buckley et al. 2010; Sentandreu & Sentandreu 2011; Von Bargen et al. 2013), these techniques are laborious, cumbersome and need expensive instruments and skilled manpower for operation and complicated statistical analysis to draw a conclusion (Che Man et al. 2005; Nurjuliana et al. 2011; Van Der Spiegel et al. 2012). Moreover, protein-and lipid-based methods are less reliable because both the amount and type of fats and fatty acids could be modified during the processing treatments (Rohman et al. 2011). In contrast, Codon

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degeneracy, superior stability, and universal traceability of DNA in all cells as well as lower-cost instrumentation have made DNA based methods feasible for practical analysis (Kitpipit et al. 2014; Hou et al. 2014; Safdar & Junejo 2015) and hence a myriad of DNA-based assays including species-specific polymerase chain reaction (PCR; Karabasanavar et al. 2014; Rahman et al. 2014), PCR-RFLP (Dooley et al. 2005; Chen et al. 2014; Rashid et al. 2015), PCR product sequencing (Ali et al. 2013), real-time PCR (Kesmen et al. 2013) and DNA barcoding (Di Pinto et al. 2013; Lamendin et al. 2015) have been documented for meat species authentication. Species-specific PCR seems to be the best because it is often conclusive and low-cost in comparison to other methods such as single nucleotide polymorphism (SNP) analysis, PCR-RFLP, PCR-RAPD and DNA barcoding (Ballin 2010; Bottero & Dalmasso 2011; Ali et al. 2014; Karabasanavar et al. 2014). Multiplex PCR assays with species-specific primers are very promising because they offer multiple target detection in a single assay platform, reducing both cost and time (Matsunaga et al. 1999; Zha et al. 2010, 2011; Safdar et al. 2014). Although several multiplex PCR assays have been documented for the identification of various animal species (Matsunaga et al. 1999; Dalmasso et al. 2004; Di Pinto et al. 2005; Zhang 2013; Kitpipit et al. 2014), none of them have aimed at the authentication of five prohibited species in religious food, such as halal food. Here we report the development and detailed verification of a multiplex PCR assay for the detection of pig, dog, cat, rat and monkey DNA under various commercial food matrices such as beef and chicken meatballs, burgers and frankfurters, which are popularly consumed across the world.

Methods and materials

Meat samples collection

Dog (Canis lupus familiaris), cat (Felis catus) and rat (Rattus rattus) meats were collected in triplicates from Dewan Bandaraya Kuala Lumpur, Air Panas, Kuala Lumpur, Malaysia. Monkey (Macaca fascicularis) meats from three different monkeys were obtained from Wildlife Malaysia, Cheras, Kuala Lumpur. Pork was purchased in triplicates from three different vendors from Chinese wet market in Seri Kembangan, Selangor, Malaysia. The most commonly used commercial meat (beef (Bos taurus), chicken (Gallus gallus), goat (Capra hircus), lamb (Ovis aries), buffalo (Bubalus bubalis), venison (Odocoileus virginianus), duck (Anas platyrhynchos), pigeon (Columba livia) and quail (Coturnix coturnix)), expensive fish species (salmon (Salmo salar), cod (Gadus morhua), tuna (Thunnus orientalis), carp (Cyprinus carpio), rohu (Labeo rohita) and tilapia (Oreochromis niloticus)) and five different halal-branded (each of beef and chicken) meatballs, seven burgers, and eight frankfurter items were purchased in triplicates on three different days from the various wet and supermarkets across Malaysia. All meat samples and products were transported under ice-chilled condition (4°C) and stored frozen at −20°C until use to prevent natural and enzymatic decompositions of meats and DNAs.

Preparation of dummy meat products

The most popularly consumed ready-to-eat meat products – namely, meatball, burger and frankfurter of both beef and chicken – were prepared to validate the reliability of the developed assay. To simulate commercial meat products, meatballs, burgers and frankfurters were prepared following Ali, Hashim, Mustafa, et al. (2012), Ali et al. (2013) and Rahman et al. (2014), respectively (Table 1). Prepared ready-to-eat meat products were subjected to autoclave at 121°C under 45 psi pressure for 2.5 h to simulate extensive cooking and boiling effects (Rahman et al. 2014, 2015). All prepared samples were stored at −20°C prior to DNA extraction.

Table 1. Formulation of ready-to-eat model meat products (g).

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Meatball (≥35 g/piece)</th>
<th>Burger (≥85 g/piece)</th>
<th>Frankfurter (≥70 g/piece)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Minced meat</td>
<td>Beef</td>
<td>Chicken</td>
<td>Beef</td>
</tr>
<tr>
<td>Soy protein</td>
<td>3</td>
<td>3</td>
<td>60</td>
</tr>
<tr>
<td>Starch/ breadcrumb</td>
<td>5</td>
<td>5</td>
<td>10.8</td>
</tr>
<tr>
<td>Chopped onion</td>
<td>1</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>Chopped ginger</td>
<td>0.1</td>
<td>0.1</td>
<td>0.2</td>
</tr>
<tr>
<td>Cumin powder</td>
<td>0.75</td>
<td>0.75</td>
<td>1</td>
</tr>
<tr>
<td>Garlic powder</td>
<td>0.5</td>
<td>0.5</td>
<td>0.75</td>
</tr>
<tr>
<td>Black pepper</td>
<td>0.14</td>
<td>0.14</td>
<td>0.25</td>
</tr>
<tr>
<td>Tomato paste</td>
<td>1.5</td>
<td>1.5</td>
<td>2.5</td>
</tr>
<tr>
<td>Butter</td>
<td>1.5</td>
<td>1.5</td>
<td>2.5</td>
</tr>
<tr>
<td>Egg</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Salt</td>
<td>SA</td>
<td>SA</td>
<td>SA</td>
</tr>
<tr>
<td>Others</td>
<td>SA</td>
<td>SA</td>
<td>SA</td>
</tr>
</tbody>
</table>

Notes: *1%, 0.5%, and 0.1% of all target meats (pig, dog, cat, monkey and rat) were added both individually and collectively with a balanced amount of minced chicken and beef to make ≥35 g, 85 g, and 70 g specimen of each meatball, burger, and frankfurter, respectively. *Amounts are in approximate values and some items were taken in teaspoon measurements. *Enhancers and flavouring agents. *SA, suitable amounts.
**DNA extraction from raw meats and meat products**

Total DNA of all meat and fish samples were extracted using Yeastern Genomic DNA Mini Kit (Yeastern Biotech Co., Ltd, Taipei, Taiwan) from 20 mg of muscle tissues following manufacturer’s instruction. DNA from model and commercial meat products was extracted from 100 mg specimen. First, the sample was grinded with a micropestle in a 1.5-ml micro-centrifuge tube to make a pulp followed by the addition of 20 µl of Proteinase K. The mixture incubated at 60°C for 30 min to lyse the sample. After adding 400 µl of lysis buffer, the sample mixture was incubated again at 60°C for 20 min to ensure the clarity of sample lysate. Subsequent steps followed the instructions given by the kit manufacturer (Yeastern Biotech Co., Ltd, Taipei, Taiwan). DNA from plant species (wheat (Triticum aestivum), tomato (Solanum lycopersicum), garlic (Allium sativum) and pepper (Capsicum annuum)) was extracted using the CTAB method according to Rahman et al. (2014). Concentration and purity of the extracted DNA were determined by UV-Vis Spectrophotometer (Libra S80, Biochrom Ltd, Cambridge, UK).

**Biomarker selection**

Species-specific primers were designed by targeting mitochondrial genes because they are well protected by mitochondrial membrane, maternally inherited and present in multiple copies per cell (Xin et al. 2006). Among the mitochondrial genes, NADH dehydrogenase subunit 5 (ND5) and ATPase subunit 6 offer appropriate target length, sufficient degree of intra-species conserved regions and interspecies polymorphism, and available sequence database for most animals and plants (Da Fonseca et al. 2008; Kitpipit et al. 2014). On the other hand, moderate evolutionary rate and clear patterns have made cytochrome b (cytb) gene a suitable candidate to study phylogenetic evolution at the intra- and inter-species levels and target for specific primers and probes (Brown et al. 1979; Xin et al. 2006). These features grew our interest to design species-specific primers targeting ND5 gene for pig and monkey, ATPase 6 for dog and rat, and cytochrome b for cat species. The whole genome sequences of pig (AF034253.1), monkey (FJ906803.1), dog (NC_002008.4), rat (NC_012374.1) and cat (NC_001700.1) were retrieved from the NCBI database (http://www.ncbi.nlm.nih.gov/) and were aligned using the ClustalW sequence alignment tool (Thompson et al. 1994) to select the inter-species hyper-variable and intra-species conserved regions. The regions of the noted gene sites so found were used to design species-specific primers for pig, dog, rat, monkey and cat (Table 2). The further checking of mismatches to all other species either at the 3’ position or, where possible, for both forward and reverse primers was performed by MEGA5 software (Tamura et al. 2011). The designed primers were also screened for unique specificity to eliminate cross-species binding with other animal or plant species using the online BLAST local alignment tool in the NCBI database (http://blast.ncbi.nlm.nih.gov/Blast.cgi). All designed primers were purchased from IDT, USA.

**Simplex PCR optimisation**

In a preliminary phase of this investigation, species-specificity of the designed primers was assessed separately using DNA extracted from cat, dog, pig, monkey and rat muscle tissues. PCR amplification was accomplished in a 25 µl total volume containing 0.5 U GoTaq Flexi DNA Polymerase (Promega, Madison, USA), 5 µl of 5X GoTaq Flexi Buffer, 200 µM each of dNTP, 1.5 mM MgCl₂, 0.2 µM primers and 0.5 µl (20 ng/µl) of total DNA in ABI 96-well verity thermal cycler. Concentration and purity of the extracted DNA were assessed using Yeastern Genomic DNA Mini Kit (Yeastern Biotech Co., Ltd, Taipei, Taiwan). DNA from plant species were purchased from IDT, USA.

**Table 2. Species-specific oligonucleotide primers for five target meat species.**

<table>
<thead>
<tr>
<th>Species</th>
<th>Genes</th>
<th>Primers</th>
<th>Primer sequence (5’–3’)</th>
<th>Amplicon size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cat</td>
<td>Cytochrome b</td>
<td>Fwd</td>
<td>GGAATAATGTTTTCGACCACCTAAGC</td>
<td>172</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Rev</td>
<td>TGCCCTGAGATGGTAATTAGGAT</td>
<td></td>
</tr>
<tr>
<td>Dog</td>
<td>ATPase 6</td>
<td>Fwd</td>
<td>TGGCTCTAGCCGTTCGATTA</td>
<td>163</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Rev</td>
<td>TGGCCTAGCCGTTCGATTA</td>
<td></td>
</tr>
<tr>
<td>Pig</td>
<td>ND5</td>
<td>Fwd</td>
<td>CCATCCCAATTTAATCCACCAACTC</td>
<td>141</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Rev</td>
<td>TGATATTTCCTGACCTGTTG</td>
<td></td>
</tr>
<tr>
<td>Monkey</td>
<td>ND5</td>
<td>Fwd</td>
<td>TGGACCTCCAACATATTTTACTAGC</td>
<td>129</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Rev</td>
<td>CCCTATGCAAGGATGTACG</td>
<td></td>
</tr>
<tr>
<td>Rat</td>
<td>ATPase 6</td>
<td>Fwd</td>
<td>ATCATCAGACGCCTTATTAGC</td>
<td>108</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Rev</td>
<td>AGGTTCGTCCTTGTGTA</td>
<td></td>
</tr>
</tbody>
</table>
stained with Florosafe DNA stain (1st Base Laboratories, Selangor, Malaysia) using a gel image documentation system (AlphaImager HP, California, USA; data not shown) and second on gel-image and electrochromatograms of Bio-Rad Experion Automated Electrophoresis Station (Bio-Rad Laboratories, Inc., USA; Figure 1).

**Multiplex PCR optimisation**

The tetraplex PCR was carried out for cat, dog, monkey and rat and finally multiplex PCR to detect cat, dog, pork, monkey and rat in ABI thermal cycler (Applied Biosystems, Foster city, CA) in a 25 µl total volume containing 1 U GoTaq Flexi DNA Polymerase (Promega, Madison, USA), 5 µl of 5X GoTaq Flexi Buffer, 200 µM each of dNTP, 1.5 mM MgCl₂, 0.2–0.4 µM primers and 2.5 µl (0.5 µl of 20 ng/µl for each species) of total DNA with the cycling parameters of initial denaturation at 94°C for 3 min followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 59.5°C for 30 s and elongation at 72°C for 30 s and final elongation at 72°C for 5 min. Negative template control of PCR reaction (PCR reaction mixture without template DNA) was included to ensure the purity of the PCR reaction mixture from contaminating DNA. The amplified PCR products were analysed by Bio-Rad Experion Automated Electrophoresis Station (Bio-Rad Laboratories, Inc.) using Experion DNA 1 K Analysis Kit (Bio-Rad Laboratories, Inc.). Typically, 1 µl of diluted (50 ng/µl) PCR products was applied with 5 µl of specialised buffer into each of the 11 sample wells and 9 µl of gel-stain into gel-stain and priming wells of the Experion DNA Chip. One microlitre of DNA 1000 ladder containing 1500, 1000, 850, 700, 500, 400, 300, 200, 150, 100, 50, 25 and 15-bp marker DNAs was applied into the ladder well. The samples were vortex-mixed for 1 min at 2000 rpm and were immediately run on the bioanalyser. The separation was achieved within 30 min by the application of high voltage in the sieving polymer and specialised buffer in the microfluidic channels through independent electrodes for each well and the automated electrophoretic patterns and the results were displayed as gel image and electropherograms (Figures 1–4).

**Results and discussion**

**Comparative perspectives of biomarker-based species detection schemes**

The authentication of meat species is of increasing concern and vital to ensure quality food in compliance with health, religion and fair prices (Rohman et al. 2011; Ali, Hashim, Mustafa, et al. 2012; Ali, Kashif, Uddin, et al. 2012). Current analytical methods for species identification are based on detecting lipid, protein or DNA biomarkers with identifying fingerprints (Di Pinto et al. 2013; Von Bargen et al. 2013). The principle of lipid-based methods includes the positional analysis of fatty acid in triacylglycerol (TAG) and 2-
monoacylglycerol (2-MAG; Szabó et al. 2007), as well as the analysis of volatile organic compounds (VOCs; Acevedo et al. 2011). However, these methods have important limitations; for example, the content and varieties of fatty acids in TAGs and MAGs can be extensively modified during the cooking process (Marikkar et al. 2011) and it is not sensitive enough to analyse the food samples without extracting and pre-concentrating the VOCs they contain (Ali, Kashif, Uddin, et al. 2012). In the case of protein-based methods, quantification of histidine dipeptides (Aristoy & Toldrá 2004), analysis of sarcoplasmic muscle proteins such as parvalbumins (Addis et al. 2010; Von Bargen et al. 2013), species-specific osteocalcin (Balizs et al. 2011), analysis of single collagen peptide (Buckley et al. 2010), as well as detection of species-specific proteins by enzyme-linked immunosorbent assay (ELISA; Hsieh et al. 2002; Asensio et al. 2008) are in wide practices. However, these methods not only need complicated machineries and extensive analytical skills, but also

Figure 2. (colour online) The gel image and the electropherograms of multiplex PCR (M-PCR) of beef (a) and chicken (b) meatballs with sensitivity. In the gel images; L, Ladder; Lanes 1–5, species-specific PCR with multiplex optimisation to detect cat (Felis catus), dog (Canis lupus familiaris), pig (Sus scrofa), monkey (Macaca fascicularis) and rat (Rattus rattus) DNA, respectively, spiked in beef and chicken meatballs; Lane 6, multiplex PCR to detect five target species in beef and chicken meatball in raw state and Lane 7, in heat-treated (121°C for 2.5 h) states; Lanes 8 and 9, M-PCR of beef and chicken meatball spiked with 0.5 and 0.1% target meat species, respectively; Lane 10 of figure (a), M-PCR of heat-treated (121°C for 2.5 h) target meat species and Lane 10 of figure (b) and 11 of figure (a), positive control (M-PCR of beef and chicken meatball in raw state); Lane 11 of figure (b), negative control. Electropherograms of Lanes 7 and 9 show the detection of five meat species in heat-treated state and from 0.1% spiked target meat species, respectively.
provide conflicting outcomes (Ali, Kashif, Uddin, et al. 2012). Furthermore, sensitivity is greatly compromised in proteomic approaches as given by Von Bargen et al. (2013), who detected 0.55% horse or pork in a beef matrix and 0.13% pork contamination in beef, which is much lower than PCR-based techniques (0.00001 ng
DNA in pure and 0.1% (w/w) of meat in complex matrices; Rashid et al. 2015). Another drawback of the “shotgun proteomics” is that after trypsin digestion of the whole protein mixture, samples become much more complex to analyse compared to individual protein spots, thus making it necessary to use powerful mass...
spectrometers together with high-throughput bioinformatics resources which are capable of analysing the enormous amount of the generated MS/MS spectra (Sentandreu & Sentandreu 2011). Moreover, cross-reactivity between closely related species may occur as has happened in case of chicken and turkey (Sentandreu & Sentandreu 2011). Furthermore, lipidomic and proteomic approaches require expensive instrumentations like GC-MS, HPLC, e-nose, MALDI-TOF and Q-TOF mass spectrometry for which skilled manpower is a must to deal with the experiments and resulting analysis (Ali, Kashif, Uddin, et al. 2012, Ali et al. 2014). On the other hand, DNA and ubiquitous and robust biomarkers and could be easily amplified from a single copy to millions to ensure easy analysis using simple instrumentations and comparatively fewer skill sets (Ali et al. 2011).

**DNA extraction and PCR optimisation**

To prevent DNA loss at aqueous and organic phase separations (Karabasanavar et al. 2011), a spin column-based extraction technique was used. This measure effectively increased the yield of good quality DNA. Because DNA in heat-treated meat products is highly susceptible to degradation, 100 mg commercial samples were used for DNA extraction. The quantity and quality of total extracted DNA was estimated based on absorbance at 260 nm and 280 nm in a UV-Vis Spectrophotometer (Biochrom Ltd, Cambridge, UK). The $A_{260}/A_{280}$ ratios of extracted DNA were between 1.7 and 2, which indicated a high quality of DNA in all specimens (Parchami Nejad et al. 2014). Because higher annealing temperature increases primer specificity and reduces non-specific PCR amplification (Wu et al. 2009; Ali, Hashim, Mustafa, et al. 2012; Rahman et al. 2015), a repeated run of gradient PCR (data not shown) was performed to optimise the higher end of annealing temperatures for all simplex and multiplex PCR assays.

After optimising the individual simplex PCR assays, multiplex PCR systems were adjusted by sequential addition of cat, dog, pig, monkey and rat species as shown in lane 6 of Figure 1. This eliminated the probability of potential primer dimer or multimer formation (Matsunaga et al. 1999; Zhang 2013). As the agarose gel eventually fails (data not shown) to separate the products of multiplex PCR systems with shorter-length differences in amplified products, (Bottero & Dalmasso 2011), a micro-fluidic-based capillary electrophoresis system incorporated in a bioanalyser was used to ensure accuracy, precision, resolution (~10 bp) with minimum consumption of samples and time (Chen et al. 2014; Rahman et al. 2015). Bio-Rad Experion Automated Electrophoresis Station provided automated banding patterns along with the electropherograms for all of the five targets amplified in this multiplex system (Figures 1–4).

**Species-specificity of designed primers**

The specificity, precision and melting temperature ($T_m$) of the primer play crucial role in the development of a multiplex PCR because its success depends on the ability of the primers to be selectively annealed with their respective targets under a single set of PCR conditions, including reaction volume, cycling and annealing (Ali et al. 2014). Because the presence of a single mismatch in the primer binding site may reduce the efficiency of the PCR assay or may lead to failure of PCR amplification (Wu et al. 2009), the estimation of oligonucleotide mismatch is a key factor in designing species-specific primers (Rahman et al. 2015). The primers designed here contained 5–13 bp (23–45%) mismatches with other relevant species and very closely spaced $T_m$ (61–62°C), ensuring the primers’ annealing only with the DNA template of target species and not with any non-target species (Matsunaga et al. 1999). Pairwise distance analysis of primer sequences (data not shown) against 15 animal and 5 plant species computed using the maximum composite likelihood method (Tamura et al. 2011) revealed a minimum distance between monkey and pigeon and maximum distance between rat and tuna, reflecting very high genetic distance and very little or no probability of cross-amplification of non-target species (Ali et al. 2013).

In the PCR run, simplex PCRs were performed on DNA extracted from muscle tissues of each target species to verify the specificity of the designed primers. The species-specific primers amplified 172, 163, 141, 129 and 108 bp fragments from cat, dog, pig, monkey and rat DNA template, respectively. The specificity of the each pair of the designed primers was checked one-by-one and also against the 15 meat-providing and expensive terrestrial (beef, chicken, goat, lamb, buffalo, venison, duck, pigeon and quail) and aquatic species (salmon, cod, tuna, carp, rohu and tilapia) and 5 plant materials (wheat, tomato, garlic, onion and pepper) which are commonly used in the preparation of meat products. In each individual assay, each pair of primers was first tested against the other four species of the multiplex system as non-target DNA and then with the most commonly used commercially important meat, fish, and plant species and no cross-amplification was detected (data not given).

All assays were carried out in triplicate on three different days by three independent analysts to avoid any operator bias or prejudice. The results indicated no cross-amplification even on repetition in blind experiments. On the other hand, primer specificity of the
other species was also examined through multiplex PCR optimised with DNA isolated from other non-target species (data not shown). Moreover, successful amplification of DNA extracted from prolonged and severely heat-treated (121°C for 2.5 h) pure meat samples through both simplex (Figure 1; lanes 7–11) and multiplex (Figure 2a; lane 10) PCR assays were attained.

Specificity and sensitivity under complex food matrices
Adulteration often happens in commercial minced meat products such as meatball, burger and frankfurter (Tanabe et al. 2007; Ali et al. 2013; Rahman et al. 2014, 2015). Therefore, the performance of the developed multiplex PCR assay was tested under a complex background of beef and chicken meatballs, burgers and frankfurters adulterated with 1%, 0.5% and 0.1% of target meats, both individually and collectively. The prepared meat products were autoclaved at 121°C at 45 psi for 2.5 h to reflect the effects of heat treatment of processed foods.

In the case of individual target meat adulteration, only specific target was amplified (Figure 2–4, lanes 1–5), suggesting the stringent specificity of the designed primers. The DNAs of target meat species from all dummy meat products containing as low as 0.1% adulteration were successfully amplified by use of the developed multiplex PCR system (Figures 2–4; lanes 6–9). The substitution of lower-valued meats with higher-priced ones has long been done to gain economic profits. However, the mixing of less than 1% low-priced meats does not make any significant profit while weighing a great risk (Ali, Asing, Hamid, et al. 2015). Thus, the detection of 1% adulteration was considered sufficient to prove the sensitivity and reliability of multiplex PCR assays (Hou et al. 2014; Ali, Razzak, Hamid, et al. 2015). Consequently, we spiked 1% minced meat with dummy meat products (Figure 2–4, lanes 1–5). However, some researchers have carried out multiplex PCR to detect 0.1% adulteration (Safdar et al. 2014; Safdar & Junejo 2015). Therefore, we also tested the sensitivity of the developed assay with 0.5% and 0.1% adulteration in various meat products (Figure 2–4, lanes 8–9).

An extensive literature search demonstrated the previously developed multiplex PCR assays targeted relatively longer amplicons (≥200 bp) and in most of the cases, the assays were not tested under food-processing conditions and complex matrices of commercial meat products. Recent studies showed that ≤150 bp amplicons have a higher chance of survival in degraded samples and thus offer better sensitivity and method validity (Rodriguez et al. 2004; Cammà et al. 2012; Rahman et al. 2015). Lin and Hwang (2008) reported that highly degraded DNA could not be detected if ≥300 bp amplicons are used.

Furthermore, earlier multiplex PCR assays included only pig and horse meats among the haram (not allowed) meat species (Matsunaga et al. 1999; Di Pinto et al. 2005; Kitppit et al. 2014; Safdar et al. 2014), which have limited scope in halal authentication. Additionally, the validity of previously developed multiplex PCR assays was determined by analysing one dummy meat product (Hou et al. 2014; Parchami Nejad et al. 2014; Safdar et al. 2014; Safdar & Junejo 2015). We tested here five different beef and chicken meatballs, eight burgers and seven frankfurters spiked with target meats to validate the assay.

Analysis of commercial meat products
The robustness, validity and reliability of the developed multiplex assay was determined by screening commercially available popular meat products such as beef and chicken meatballs, burgers and frankfurters, which are widely consumed across Malaysia, Indonesia, China and most parts of the world (Rohman et al. 2011; Ali, Hashim, Mustafa, et al. 2012; Ali et al. 2013; Ali, Razzak, Hamid, et al. 2015; Rahman et al. 2014; Rahmania et al. 2015). To fulfil the selected goal, five different halal branded beef and chicken meatballs, eight burgers and seven frankfurters of different manufacturers were collected and tested from three different Malaysian outlets on three different days. The statistical data of the tested samples are given in Table 3. While in all model meatballs with deliberate adulteration the target species were positively detected in blind experiments, all commercial samples were found with negative targets. All of these experiments were carried out in triplicate, by three different analysts on three different days to avoid any prejudice.

The finding of this paper of non-fraudulent labelling of pig, dog, rat, cat and monkey in commercial products was against the conventional wisdom because fraudulent labeling is quite common around the globe (Fajardo et al. 2010; Doosti et al. 2014). However, in Malaysian perspectives it was not a surprise because the Malaysian government is highly committed to develop “Halal Hub” and has been strictly monitoring local markets from time to time. Thus, the study finding was logical and suggested the halal sanctity of Malaysian meatballs, burgers and frankfurters.

Conclusion
Adulterated meat in the food supply chain impacts on public sentiments, and jeopardises health and religious faiths. Further, rat, cat, dog and monkey are potential carriers of plague, ringworm, hydrophobia (rabies), and herpes virus (Herpevirus simiae), simian virus 40 (SV40) and other zoonotic diseases and hence are not safe and hygienic for public consumption. Religion has always been a key determinant in meat consumption, such as pork consumption which is prohibited in Islam.
and Judaism. Some incidents such as food allergy due to pork consumption and porcine DNA in meat pies and pasties supplied to the prisons of the UK have greatly promoted the authentication of porcine derivatives in various meals and menus. Additionally, Islamic law has prohibited Muslims from eating animals having canine teeth or fangs such as dog, cat, monkey and rat. Furthermore, the availability of stray dogs and cats in certain countries has made meat from these species a potential adulterant in costly meats such as beef, lamb and chicken. On the other hand, monkey (macaque) is a widespread species and often can be hunted free of charge. Thus, the chances of its falsification are very high. Carefully designed species-specific PCR under optimised conditions is conclusive to detect and identify species, eliminating the need for restriction digestion and/or sequencing of PCR products. Instead of single species, the detection of five different species in a single assay platform clearly cut the analysis cost by at least five times, offering easy analysis of food components in the industry and retail outlets. The multiplex PCR systems developed here conveniently detected five meat species forbidden in Islamic foods from spiked meat products with as low as 0.1% adulteration, clearly showing its appeal in the halal food industry and halal regulatory bodies. Shorter amplicon lengths (108–172 bp) and successful amplification of target species under raw and extremely heat-treated meat species suggested that the method would be used in the screening of target species under compromised states and complex matrices. In addition to pentaplexing, the assay could be used in simplex, duplex, triplex, and tetraplex PCR systems based on the requirements and needs.

### Acknowledgements

The authors acknowledge wildlife Malaysia and Dewan Bandaraya Kuala Lumpur (DBKL) for providing monkey, dog and cat meat samples.

### Ethical compliance

The ethical permission was obtained from the University of Malaya Ethical Committee (ref. no.: NANOCAT/23/07/2013/MEA(R)) and all the animals and meats used in this study were handled following the institutional and national guideline for animal care.

### Disclosure statement

No potential conflict of interest was reported by the authors.

### Table 3. Wide screening of model and commercial ready to eat meat products sold in markets using developed multiplex PCR.

<table>
<thead>
<tr>
<th>Meat products</th>
<th>Pig</th>
<th>Dog</th>
<th>Cat</th>
<th>Monkey</th>
<th>Rat</th>
<th>PCR accuracy (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Different meat products spiked with</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pig meat</td>
<td>54/54</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>100</td>
</tr>
<tr>
<td>Dog meat</td>
<td>–</td>
<td>54/54</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>100</td>
</tr>
<tr>
<td>Cat meat</td>
<td>–</td>
<td>–</td>
<td>54/54</td>
<td>–</td>
<td>–</td>
<td>100</td>
</tr>
<tr>
<td>Monkey meat</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>54/54</td>
<td>–</td>
<td>100</td>
</tr>
<tr>
<td>Rat meat</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>54/54</td>
<td>100</td>
</tr>
<tr>
<td>Beef meatballs spiked with all target meat species (raw)</td>
<td>9/9</td>
<td>9/9</td>
<td>9/9</td>
<td>9/9</td>
<td>9/9</td>
<td>100</td>
</tr>
<tr>
<td>Beef meatballs spiked with all target meat species (heat-treated)</td>
<td>9/9</td>
<td>9/9</td>
<td>9/9</td>
<td>9/9</td>
<td>9/9</td>
<td>100</td>
</tr>
<tr>
<td>Chicken meatballs spiked with all target meat species (raw)</td>
<td>9/9</td>
<td>9/9</td>
<td>9/9</td>
<td>9/9</td>
<td>9/9</td>
<td>100</td>
</tr>
<tr>
<td>Chicken meatballs spiked with all target meat species (heat-treated)</td>
<td>9/9</td>
<td>9/9</td>
<td>9/9</td>
<td>9/9</td>
<td>9/9</td>
<td>100</td>
</tr>
<tr>
<td>Chicken burgers spiked with all target meat species (raw)</td>
<td>9/9</td>
<td>9/9</td>
<td>9/9</td>
<td>9/9</td>
<td>9/9</td>
<td>100</td>
</tr>
<tr>
<td>Chicken burgers spiked with all target meat species (heat-treated)</td>
<td>9/9</td>
<td>9/9</td>
<td>9/9</td>
<td>9/9</td>
<td>9/9</td>
<td>100</td>
</tr>
<tr>
<td>Beef frankfurter spiked with all target meat species (raw)</td>
<td>9/9</td>
<td>9/9</td>
<td>9/9</td>
<td>9/9</td>
<td>9/9</td>
<td>100</td>
</tr>
<tr>
<td>Beef frankfurter spiked with all target meat species (heat-treated)</td>
<td>9/9</td>
<td>9/9</td>
<td>9/9</td>
<td>9/9</td>
<td>9/9</td>
<td>100</td>
</tr>
<tr>
<td>Chicken frankfurter spiked with all target meat species (raw)</td>
<td>9/9</td>
<td>9/9</td>
<td>9/9</td>
<td>9/9</td>
<td>9/9</td>
<td>100</td>
</tr>
<tr>
<td>Chicken frankfurter spiked with all target meat species (heat-treated)</td>
<td>9/9</td>
<td>9/9</td>
<td>9/9</td>
<td>9/9</td>
<td>9/9</td>
<td>100</td>
</tr>
<tr>
<td>Commercial meat products*</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Beef meatball</td>
<td>0/9</td>
<td>0/9</td>
<td>0/9</td>
<td>0/9</td>
<td>0/9</td>
<td>100</td>
</tr>
<tr>
<td>Chicken meatball</td>
<td>0/9</td>
<td>0/9</td>
<td>0/9</td>
<td>0/9</td>
<td>0/9</td>
<td>100</td>
</tr>
<tr>
<td>Beef burger</td>
<td>0/9</td>
<td>0/9</td>
<td>0/9</td>
<td>0/9</td>
<td>0/9</td>
<td>100</td>
</tr>
<tr>
<td>Chicken burger</td>
<td>0/9</td>
<td>0/9</td>
<td>0/9</td>
<td>0/9</td>
<td>0/9</td>
<td>100</td>
</tr>
<tr>
<td>Beef frankfurter</td>
<td>0/9</td>
<td>0/9</td>
<td>0/9</td>
<td>0/9</td>
<td>0/9</td>
<td>100</td>
</tr>
<tr>
<td>Chicken frankfurter</td>
<td>0/9</td>
<td>0/9</td>
<td>0/9</td>
<td>0/9</td>
<td>0/9</td>
<td>100</td>
</tr>
</tbody>
</table>

Notes: *Five different brands of each beef and chicken meatballs, 8 burgers and 7 frankfurter items were analysed to validate the developed multiplex PCR assay. The numerator and denominator of each fraction denote the number of positive detection and total number of samples analysed using the multiplex PCR assay.
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References


