A higher sensitivity and efficiency of common primer multiplex PCR assay in identification of meat origin using NADH dehydrogenase subunit 4 gene

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Abstract A Common Primer Multiplex PCR (CP-M-PCR) was developed to detect meat origin of four groups of animal (pig, ruminant, avian and rabbit). This method demonstrated higher sensitivity and efficiency than the conventional multiplex PCR. In this approach, a common forward primer was designed in the 5' end of a homologous region of mitochondrial NADH dehydrogenase subunit 4 (Nad 4) gene sequences of all the animal groups. Specific adapter reverse primers were designed by adding an adapter sequence at the 5' end. The same adapter sequence was used as the common adapter reverse primer. The primers generated specific fragments of 267, 370, 504, and 548 bp lengths for pig, ruminant, avian and rabbit meats, respectively. The use of adapter sequence at the 5' end of the common adapter reverse primers increased the efficiency of the amplification and the application of a common forward primer solved the complexity in multiplex PCR system. Bands of specific amplification can be detected in the PCR assays containing as low as 10^{-6} μ M of adapter reverse primer. This result indicated that the sensitivity was

tremendously increased as compared to the conventional multiplex PCR (10^{-3} μ M). CP-M-PCR detection limit of the DNA samples was 0.1 ng for the four groups of meats. CP-M-PCR has greatly improved the sensitivity and efficiency of the PCR system for a more reliable and accurate outcome than conventional multiplex PCR system.

Keywords Multiplex · Nad 4 · Meat · Common primer

Introduction

Global demand for meat based products to feed human population has been increasing. Global meat production for 2014 is forecast up marginally at 58.6 million tons (Record Global Meat Trade 2014). Due to their high demand, incidences of fraudulent labelling and meat adulteration with cheaper meat or non-meat materials may increase (Economic and Social Research Council 2012). These may raise a number of concerns, involving economic (e.g. EU horse meat scandal), quality (e.g. nutritional value), safety (e.g. absence of allergens) and socio-religious (e.g. absence of pork and beef) issues (Ballin 2010). Consequently some relevant authorities are prompted to upgrade the policy or acts pertaining to meat based food products. For example, European food policy has been amended and risk analysis as well as traceability has been introduced in the food safety legislation (Council Regulation (EC) No 178/2002, 2002). In Malaysia, in ensuring a high level of consumer protection, the Trade Descriptions Act 1972 has been repealed and replaced by the Trade Descriptions Act 2011 (<https://www.jetro.go.jp/world/asia/my/ip/pdf/tradedescription2011en.pdf>). The Act promotes good trade practices by prohibiting false trade descriptions and false or misleading statements, conduct and practices in relation to goods or services. However, mandatory labeling for meat origin does not provide a sufficient guarantee to protect

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consumer rights and prevent food adulteration in meat-based products.

To alleviate this problem, knowledge and skills in food science are important to provide proof of origin about the real species of a product. There are many techniques based on the analysis of the biomolecular components of cells developed for testing the species origin in industrial meat products. DNA, found in almost tissue types can be used as the target of detection since it is fairly stable under extreme processing food conditions such as thermal denaturation (Mohamad et al. 2013). Over the last decade, the use of real-time PCR in food fraudulent investigation has been increasingly advocated for species assignment in foods and feedstuffs. Real-time PCR analysis provides a distinct advantage over other PCR methods (Dalmasso et al. 2011), but it requires expensive investment in equipments. In addition, it is costly for routine species identification studies due to expensive reagents (dye labelled probe) and specialized PCR tubes. Therefore, the need for alternative PCR approaches has prompted numerous studies. On the other hand, Multiplex PCR is highly repeatable, time saving and affordable than the other mentioned methods (Dalmasso et al. 2004). It can simultaneously amplify primer mixtures in one step PCR reaction, thereby overcoming the weakness of single PCR detection, which only amplifies a pair of primers. However, multiplex PCR has several disadvantages. The low amplification efficiency, variable efficiency on different templates and poor universality, highlight the need for an advanced multiplex approach.

The aim of the present study was to develop a detection model through common primer multiplex PCR (CP-M-PCR) system to simultaneously identify some of the common meats that are widely used in meat-based product industry. DNA from raw meat samples was used as the optimization templates. The application of common primer (only one forward primer for all specific reverse primers) in multiplex PCR reduced the cost and increased the sensitivity of the analysis at a very low concentration of DNA and primer, thus reducing the limitations that generally occur in the conventional multiplex PCR. The design of a CP-M-PCR primer set has been focused on the sequence of NADH dehydrogenase subunit 4 (Nad 4) of mitochondrial gene.

Materials and methods

Samples

Samples of raw meat from different types of animal; pig, rabbit, cattle, buffalo, deer, goat, sheep, chicken, duck, turkey and quail were obtained from local markets. As there are many varieties available in the market, cattle, buffalo, deer, goat and sheep were grouped into ruminant, whereas chicken, duck, turkey and quail were grouped into avian (Table 1). All

Table 1 Samples included in the assay

Type of meat		
Pig		
Buffalo	}	Ruminant
Cattle		
Sheep		
Deer		
Goat		
Chicken	}	Avian
Quail		
Duck		
Turkey		
Rabbit		

samples were stored at -20°C until use to prevent enzymatic degradation of DNA prior to DNA extraction.

DNA extraction

The extraction of DNA from all samples was performed by using QIAamp Tissue Mini kit (QIAGEN, Germany) according to the manufacturer's instructions with minor modifications. The later consisted of an increase of the sample amount at 100 mg and a decrease of the final elution volume, at 50 μl . Each samples were incubated with 200 μl of lysis buffer ATL with 20 μl proteinase K (20 mg/ml) at 56°C overnight and then for 1 h at 70°C with 200 μl Buffer AL. The mixture was centrifuged at $4,000 \times g$ for 2 min, followed by addition of 200 μl ethanol to the transferred supernatant. The resulting mixture was applied to the QIAamp DNA spin column. The DNA bound to the column was washed in two centrifugation steps using two different wash buffers (AW1 and AW2) to improve the purity of the eluted DNA. Finally, the purified DNA was eluted from the column in 50 μl of Elution Buffer.

The DNA concentrations were determined using fluorescent dye (Quant-iTTM PicoGreen[®] dsDNA Assay Kit, Life

Fig. 1 Sequence alignment of the *nad 4* from GenBank. Ostrich (Y12025.1); chicken (AY235570.1); turkey (NC_010195.2); pig (NC_000845.1); rabbit (NC_001913.1); sheep (AF010406.1); cattle (V00654.1); red deer (NC_007704.2); deer (JN632699.1); and goat (NC_005044.2). The dotted line (•••) represents an aligned residue; the box indicates the position of primer used for PCR amplification. The alignments were performed using Bioedit 7.1

Fig. 2 The development of CP-M-PCR. **a** Multiplex PCR primer design, **b** testing and optimization

Technologies™) and measured using a microplate reader (Infinite® M200, NanoQuant, Tecan).

Mitochondrial NADH dehydrogenase subunit 4 (Nad 4) gene sequences from various animal species available in the National Center for Biotechnology Information database (www.ncbi.nlm.nih.gov) were aligned using Bioedit 7.1 software (Fig. 1). After sequence comparison and analysis, common and specific sequence fragments were identified and primers were designed for the selective amplification of 100 to 600 bp in length.

For the multiplex PCR described here, the primers were manually designed because there is no available primer design software with the capability for multiplex primer comparisons which allows user to manipulate certain parameters such as the specificity of 3'-end of the reverse primers for the different animal origins, the desired product sizes with ideal melting temperature and the acceptable secondary structure. For these purposes, guidelines as recommended by Abd-Elsalam (2003) were followed. OligoAnalyzer 3.1 software (Integrated DNA Technologies) was used to check the quality of each primer used in the CP-M-PCR. The design ensured the complementary regions between primers to be minimized to prevent the formation of secondary structures (primer-dimers, hairpins and hetero-dimers). Figure 2a illustrates the process of CP-M-PCR primer design.

In this study, common forward primer (N4CF) was designed based on the well-conserved region of Nad 4 gene of mitochondrial DNA (Fig. 1). Forward primer shared by all the species may reduce possible primer interactions. The pig-specific reverse primer (N4Pig-R) was designed in a well-conserved region for pig; the ruminant-specific reverse primer (N4Rum-R) was designed in a well-conserved region for buffalo, cattle, goat, sheep and deer; the avian-specific reverse primer (N4Avi-R) was designed for the specific detection of

Table 2 Primer sequences used in this study

Type	Primer name	Sequence (5' to 3')	Product size (bp)	T _m (°C)
Common forward primer	N4CF	agctcaatctgcctccgccaacagacctaataac		65.2 ^a
Common adapter reverse primer	N4CaR	ccttccttccttcctcc		52.1
Specific reverse primer (R)	N4Pig-R	gttgctatgagtggtaggagtgtttgc	249	59.4
	N4Rum-R	gatgttagatcatgaaaaggttgatattac	352	52.8
	N4Avi-R	aggagggtttctcgtgtgttgagtt	486	60.0
	N4Rab-R	ggctaattgatagtagaagaagtggggc	530	58.6
	N4Pig-Ra	ccttccttccttccttcggttgctatgagtggtaggagtgtttgc	267	67.9 ^a
Specific adapter reverse primers (Ra)	N4Rum-Ra	ccttccttccttccttcgatgttagatcatgaaaaggttgatattac	370	63.8 ^a
	N4Avi-Ra	ccttccttccttccttcaggagggtgtctcgtgtgttgagtt	504	68.0 ^a
	N4Rab-Ra	ccttccttccttccttcaggctaattgatagtagaagaagtggggc	548	68.1 ^a

^a Melting temperature as calculated by OligoAnalyzer 3.1. Actual annealing temperature is determined by the number of mismatch in the primer-template binding

Table 3 CP-M-PCR cycling parameters

		Temperature (°C)	Time
1 st round	10 X {	94	1 min 30 s
		94	30 s
		64	30 s
		72	30 s
2 nd round	25 X {	94	30 s
		50	30 s
		72	10 min

chicken, duck, quail and turkey; and the rabbit-specific reverse primer (N4Rab-R) was designed for the specific detection of rabbit. Specific reverse primers were linked to adapter sequence (5'-CCTTCCTTCCTTCCTCC-3') at the 5'-end and these primers were named as specific adapter reverse primers. To increase the universality of CP-M-PCR, we only selected primers that have comparable melting temperature (T_m) so that all primers anneal at similar temperatures during the PCR temperature cycling. The adapters and primers are listed in Table 2. The primers were synthesized by Integrated DNA Technologies, Pte. Ltd. (Singapore).

Primer specificity test

To ensure the specificity of the respective specific reverse primers to amplify only the targeted species, each of the specific primers was first challenged in simplex PCR against all DNAs of the 11 animal raw meat samples (Table 1) under identical amplification conditions (Zha et al. 2011). PCR amplification was performed in a final volume of 20 μ l containing 1 $\times$ i-PCR Red mix (iDNA Biotechnology), 0.25 μ M of primers and 50 ng of DNA template. Amplification was performed in a thermal cycler (Mastercycler Gradient Eppendorf, Germany) with an initial denaturation step at

Fig. 3 Schematic representation of CP-M-PCR

Fig. 4 Specificity of simplex PCR of **a** N4Pig-R, **b** N4Rum-R, **c** N4Avi-R and **d** N4Rab-R. Lane 1, pig; lane 2, buffalo; lane 3, cattle; lane 4, goat; lane 5, deer; lane 6, sheep; lane 7, chicken; lane 8, duck; lane 9, quail; lane 10, turkey; lane 11, rabbit; lane 12, control reagent; M, Kplus DNA ladder (GeneDirex®)

95 °C for 1 min 30 s, followed by 35 amplification cycles at 95 °C for 30 s, 64 °C for 30 s and 72 °C for 30 s, and lastly a

Fig. 5 Comparison of PCR products amplified with different multiplex systems **a** conventional multiplex PCR **b** common primer multiplex PCR. Lane 1–4, simplex PCR; lane 5–10, duplex PCR; lane 11–14, triplex PCR; lane 15, tetraplex PCR; lane 1, pig; lane 2, ruminant; lane 3, avian; lane 4, rabbit; lane 5, ruminant, avian; lane 6, avian, rabbit; lane 7, pig, ruminant; lane 8, pig, avian; lane 9, pig, rabbit; lane 10, avian, rabbit; lane 11, pig, ruminant, avian; lane 12, pig, ruminant, rabbit; lane 13, pig, avian, rabbit; lane 14, ruminant, avian, rabbit; lane 15, pig, ruminant, avian, rabbit; lane 16, control reagent; M, Kplus DNA ladder (GeneDirex®)

final extension at 72 °C for 10 min. Each test was repeated three times. PCR amplified product was analyzed by electrophoresis on 1.5 % (w/v) agarose gel (Invitrogen, USA) containing RedSafe™ nucleic acid staining solution at 0.4 ng/ml (Chembio) run in 1× lithium borate buffer (LB®, Faster Better Media LLC) for 20 min at 180 V.

The PCR products were purified using PCR DNA Fragments Extraction kit (Geneaid, Taiwan) and sent for sequencing (1st Base Laboratories Sdn. Bhd., Malaysia) using N4CF primer to confirm the spp. identity. Sequence analysis was performed by a BLAST search in the GenBank database (<http://www.ncbi.nlm.nih.gov>).

Optimization of multiplex PCR

For the simultaneous detection of all species, one step multiplex PCR was performed with two, three and four different groups of DNA templates, respectively. Figure 2b illustrates the process of CP-M-PCR development. The optimization of multiplex PCR parameters such as the cycling temperatures and the balance between the magnesium chloride and deoxynucleotide concentrations followed the general principles as described by Henegariu et al. (1997).

Fig. 6 Determination of CP-M-PCR sensitivity. PCR products amplified using **a** conventional multiplex PCR system and **b** CP-M-PCR system with a serial dilution of primer concentration. Lane 1, 2.5×10^{-1} μ M; lane

2, 2.5×10^{-2} μ M; lane 3, 2.5×10^{-3} μ M; lane 4, 2.5×10^{-4} μ M; lane 5, 2.5×10^{-5} μ M; lane 6, 2.5×10^{-6} μ M; M, Kplus DNA ladder (GeneDirex®)

Amplification was accomplished in a final volume of 20 μ l containing $1 \times$ i-PCR-Red mix (iDNA Biotechnology), 0.5 mM of common forward primer (N4CF), 0.25 mM of each pig, ruminant, avian and rabbit adapter reverse primers, respectively, 0.25 mM of common adapter reverse primer (N4AR) and 50 ng of each pig, sheep, chicken and rabbit DNA, respectively. Thermal cycling was programmed as shown in Table 3 with CP-M-PCR principles illustrated in Fig. 3. To ensure the reproducibility, the PCR was repeated three times.

CP-M-PCR sensitivity

Ten-fold serial dilutions of each specific adapter reverse primer (2.5×10^{-1} , 2.5×10^{-2} , 2.5×10^{-3} , 2.5×10^{-4} , 2.5×10^{-5} and 2.5×10^{-6} μ M) were used to determine and compare the primer sensitivity of CP-M-PCR against conventional multiplex PCR system. To evaluate the CP-M-PCR detection limit, DNA extracted from pig, sheep, chicken and rabbit were diluted to 10, 1, 0.1, 0.01, and 0.001 ng, respectively.

Results and discussion

Simplex PCR specificity

Mitochondrial DNA gene degrades slower than nuclear DNA (Arif and Khan 2009), making it suitable as target for meat species identification in degraded samples due to food processing (Woolfe and Primrose 2004). There have been numerous published studies describing species identification in meat and meat-based products using *12S rRNA*, *16S rRNA*, *cytochrome b*, *nad 1*, *nad 2* and *nad 5* as the target gene (Pegels et al. 2014; Mane et al. 2013; Ali et al. 2012; Sahilah et al. 2011; Di Pinto et al. 2005). In this study, the interspecies variability of Nad 4 gene sequence has been exploited to identify specific groups of animal. Nad 4 gene has been shown to contain the highest number of polymorphism as shown in bovine (Kim et al. 2010) and therefore a potential target for species identification in meat and meat-based products in future investigations.

A set of primers was designed to amplify target sequences of the four groups of animal origin (pig, ruminant, avian and

Fig. 7 Determination of detection limit. Serial dilutions of a mixed DNA template were used in the multiplex reaction. Lane 1, 10 ng; lane 2, 1 ng; lane 3, 0.1 ng; lane 4, 0.01 ng; lane 5, 0.001 ng; lane 6, control reagent; M, Kplus DNA ladder (GeneDirex®). The lower graphs are the quantity images of the intensity of the bands analyzed by UVigeltec version 12.1

rabbit) at a similar efficiency using two types of primer mixture (common primer and specific adapter reverse primers). A common forward primer (N4CF) was designed longer to be shared by all groups in this study. Ratio of mismatching between N4CF and the specific reverse primer was approximately 17 %. It was indicated that the mismatches more than 15 % decrease T_m more than 15 °C, and that make reverse primers anneal only to the specific sequence in multiplex PCR (Matsunaga et al. 1999). By using single forward primer, amplification efficiency was less affected in multiple PCR assay. Each specific reverse primer was critically designed to mismatch with different groups of animal at 3' end or next nucleotides because polymerase extends the 3' end of the primer. The primer mismatched at 3'- end was fatal for PCR amplification (Matsunaga et al. 1999).

In this study, 18 bp adapter was added to the 5' end of the specific reverse primers. It was shown that the adapters improved the specificity and reduced differences in melting temperatures among the specific adapter reverse primers. It is important that the sequence of the adapters is infrequent in the genome and the formation of secondary structure such as hairpins and dimers should be at minimum and unstable. The presence of G or C bases within the last five bases from the 3' end of primers (GC clamp) may promote specific binding at the 3' end due to the stronger bonding of G and C bases. However, more than three G's or C's should be avoided in the last 5 bases at the 3' end of the primer (Nybo 2013). Therefore, in this study 18 bases of adapter sequence 5'-CCTTCCTTCC TTCCTTCC-3' was used as common adapter reverse primer (N4AR) and in designing specific adapter reverse primers.

The specificity outcome of the N4Pig-R, N4Rum-R, N4Avi-R and N4Rab-R primers are indicated in Fig. 4. The primers generated specific fragments of 249 bp pig, 352 bp for ruminants, 486 bp for avians and 530 bp for rabbit. These showed that the primers with adapter sequence amplified only fragments from the target animal group indicating a high level of primer specificity to the target sequence. The GenBank BLAST search indicated that all these PCR products belonged to Nad 4 gene with highest match to animal of the same respective group (data not shown).

Establishment of common primer multiplex PCR

Multiplex PCR is the most efficient technique to amplify more than one desired loci in single reaction. However, multiplex PCR is a complex process and usually results in low and different efficiency on different targets. In this study, common primers and adapter primers were used in a multiplex system, a system called common primer multiplex PCR (CP-M-PCR) to overcome the disadvantages of conventional multiplex PCR. In conventional multiplex system, the design of primers is very important because the specificity and melting temperature of the primers are

critical. However, for multiplex system using adapter primers, the differences in annealing temperatures between primers is not the main concern (Wen and Zhang 2012). To assess the effectiveness of this assay for rapid detection, the annealing temperatures and PCR cycling parameters (i.e. total number of cycles and primer annealing time) were optimized (Schoske et al. 2003). CP-M-PCR amplification with N4CF, N4AR and specific adapter reverse primers was performed in two rounds of cycling conditions. In the first round of amplification, specific adapter reverse primers and N4CF annealed to the specific sequences of DNA samples. In the next round, N4AR and N4CF were allowed to amplify the fragments from the templates synthesized in the first round PCR. In this study, validation of CP-M-PCR was performed with the presence of adapter primers and compared to the results of conventional multiplex products. The amplifications products from conventional multiplex and CP-M-PCR reactions are shown in Fig. 5a and b, respectively. It was observed that the bands amplified by CP-M-PCR system were more intense than those amplified by conventional multiplex system and thus demonstrated that the amplification efficiency was increased by using the adapter primers. By using only one pair of common primers in the second round amplification cycle, disproportionate amplification can be avoided. As a result, this approach has overcome the low amplification in conventional multiplex PCR which did not result in equal signals although equal amount mixture of the primers was used (Matsunaga et al. 1999).

PCR sensitivity

In order to verify the efficiency of PCR amplification, the sensitivity of primers was investigated. In this study, the sensitivity of primers without common adapter sequence was 2.5×10^{-3} μM and there was no amplification of the specific DNA fragment when the concentration of specific reverse primers decreased (Fig. 6a). With the presence of common adapter sequence in the CP-M-PCR reaction system, the bands of specific DNA fragment were visible until 2.5×10^{-6} of reverse primer dilution (Fig. 6b, lane 6). Therefore, N4AR greatly improved the sensitivity of PCR amplification. When the N4CR was added into the reaction system, the band of specific fragment appeared until the concentration of specific primers diluted to 2.5×10^{-6} μM . This showed a greater improvement of the primer specificity by more than 1,000 times than that of conventional multiplex PCR (2.5×10^{-3} μM). However, unspecific bands appeared when primer concentration was 2.5×10^{-4} μM or less. Therefore, it is recommended to use primer at appropriate amount in order to avoid non-specific amplification. Bai et al. (2009) used a similar approach to detect chicken, cattle, pig and horse meats by targeting mitochondrial

cytochrome b gene. The study reported that the primer sensitivity was increased by 100 times in the CP-M-PCR system.

The detection limit of DNA templates was evaluated with 10-fold serial dilutions of DNA of four animal groups starting with 10 ng to 0.001 ng per reaction. The limit of detection was taken as being the lowest amount of the added DNA sample that could be amplified. Figure 7 showed the results of PCR amplification from mixed DNA templates of 10, 1, 0.1, 0.01 and 0.001 ng each. Lanes 1–3 show four bands corresponding to the four group of animal, indicating the CP-M-PCR detection limit which was 0.1 ng for the tested meat species. The present findings were consistent with Bai et al. (2009)'s study that obtained the sensitivity of 0.1 ng DNA which is sufficient to detect presence of food fraud in commercial meat products. It is encouraging to compare this result with that reported by Matsunaga et al. (1999) using conventional multiplex PCR system. In the study, a series of similar experiment was performed using a common forward primer targeting *cytochrome b* and result showed that the detection limits was 0.25 ng for the tested meat species (cattle, pig, chicken, sheep, goat and horse). This result indicated that the common adapter sequence significantly improved the sensitivity of the CP-M-PCR system.

Conclusion

PCR remains as sensitive and rapid detection technique but the CP-M-PCR approach is a further revolution of the conventional multiplex PCR system. We used raw meat samples as sources of DNA to optimize the CP-M-PCR model by taking advantage of the polymorphic Nad 4 gene sequence which serves as the detection target. As far as the animal types included in this study are concerned, we present here a multiplex detection set consisting the common primers and species specific adapter reverse primers. The developed model was shown to be fast, cost-effective and very sensitive with the ability of detecting very low amounts of animal DNA. This approach has the potential to simultaneously identify various meat origins in industrially processed meat-products such as sausages, burgers, meatballs, salami and pastrami as long as successful DNA extraction is obtained perhaps at even a lower amount. In the future, CP-M-PCR system may offer the possibility of multiplex amplification of more than four target animal groups in a single reaction, thus allowing a wider application in testing the origins of meat-based products in simpler and cheaper manners.

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Author contributions Umami KH performed the experiment, interpreted the results and drafted the manuscript. Desa MNM planned, supervised the study and revised the manuscript. Amin I and Shuhaimi M participated in the planning and design of the study.

References

- Abd-Elsalam KA (2003) Bioinformatic tools and guideline for PCR primer design. *Afr J Biotechnol* 2(5):91–95
- Ali ME, Hashim U, Mustafa S, Che Man YB, Dhahi TS, Kashif M, Md Uddin K, Abd Hamid SB (2012) Analysis of pork adulteration in commercial meatballs targeting porcine-specific mitochondrial cytochrome b gene by TaqMan probe real-time polymerase chain reaction. *Meat Sci* 91(4):454–459
- Arif IA, Khan HA (2009) Molecular markers for biodiversity analysis of wildlife animals: a brief review. *Anim Biodivers Conserv* 32(1):9–17
- Bai W, Xu W, Huang K, Yuan Y, Cao S, Luo Y (2009) A novel common primer multiplex PCR (CP-M-PCR) method for the simultaneous detection of meat species. *Food Control* 20(4):366–370
- Ballin NZ (2010) Authentication of meat and meat products. *Meat Sci* 86(3):577–587
- Dalmasso A, Fontanella E, Piatti P, Civera T, Rosati S, Bottero MT (2004) A multiplex PCR assay for the identification of animal species in feedstuffs. *Mol Cell Probes* 18(2):81–87
- Dalmasso A, Civera T, La Neve F, Bottero MT (2011) Simultaneous detection of cow and buffalo milk in mozzarella cheese by real-time PCR assay. *Food Chem* 124(1):362–366
- Di Pinto A, Forte VT, Conversano MC, Tantillo GM (2005) Duplex polymerase chain reaction for detection of pork meat in horse meat fresh sausages from Italian retail sources. *Food Control* 16(5):391–394
- Economic & Social Research Council (2012) Global food systems and UK food imports: resilience, safety and security, Discussions from the ESRC Public Policy Seminar 30 March 2012, 1–19. <http://www.foodsecurity.ac.uk/assets/pdfs/gfs-and-uk-food-imports.pdf>. Accessed 8 Mar 2014
- Henegariu O, Heerema NA, Dlouhy SR, Vance GH, Vogt PH (1997) Multiplex PCR: critical parameters and step-by-step protocol. *Biotechniques* 23:504–511
- Kim E, Cheong HS, Bae JS, Chun J, Park TJ, Lee K, Yun Y, Shin HD (2010) Identification of genetic polymorphisms in bovine mitochondrial deoxyribonucleic acid. *J Anim Sci* 88(8):2551–2555
- Mane BG, Mendiratta SK, Tiwari AK (2013) Pork specific polymerase chain reaction assay for authentication of meat and meat products. *J Meat Sci Technol* 1(1):21–27
- Matsunaga T, Chikuni K, Tanabe R, Muroya S, Shibata K, Yamada J, Shinmura Y (1999) A quick and simple method for the identification of meat species and meat products by PCR assay. *Meat Sci* 51(2):143–148
- Mohamad NA, El Sheikh AF, Mustafa S, Mokhtar NFK (2013) Comparison of gene nature used in real-time PCR for porcine identification and quantification: a review. *Food Res Int* 50(1):330–338
- Nybo K (2013) Primer design. *Bio Tech* 54(5):249–250
- Pegels N, González I, García T, Martín R (2014) Avian-specific real-time PCR assay for authenticity control in farm animal feeds and pet foods. *Food Chem* 142:39–47
- Record Global Meat Trade (2014) Livestocks and poultry: world markets and trade. November 2013. United States Department of Agriculture. http://apps.fas.usda.gov/psdonline/circulars/livestock_poultry.pdf. Accessed 8 Mar 2014

- Sahilah AM, Norhayati Y, Norrakiah AS, Aminah A, Wan Aida WM (2011) Halal authentication of raw meats using PCR amplification of mitochondrial DNA. *Int Food Res J* 18(4):1489–1491
- Schoske R, Vallone PM, Ruitberg CM, Butler JM (2003) Multiplex PCR design strategy used for the simultaneous amplification of 10 Y chromosome short tandem repeat (STR) loci. *Anal Bioanal Chem* 375(3):333–343
- Wen D, Zhang C (2012) Universal Multiplex PCR: a novel method of simultaneous amplification of multiple DNA fragments. *Plant Methods* 8(1):1, 2–9
- Woolfe M, Primrose S (2004) Food forensics: using DNA technology to combat misdescription and fraud. *Trends Biotechnol* 22(5):222–226
- Zha DM, Xing XM, Yang FH (2011) Rapid identification of deer products by multiplex PCR assay. *Food Chem* 129(4):1904–1908