



Analytical Methods

Direct-multiplex PCR assay for meat species identification in food products



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ABSTRACT

This is the first time that direct PCR – DNA amplification without prior DNA extraction – was successfully developed and fully validated for rapid and economical simultaneous identification of six commonly consumed meat species. To achieve this, six species-specific primers were selected from previous reports and newly designed from the mitochondrial cytochrome *b* (*cyt b*), cytochrome oxidase I (COI), and 12s rRNA gene. The assay generated PCR products of 100, 119, 133, 155, 253, and 311 bp for pork, lamb/mutton, chicken, ostrich meat, horsemeat and beef, respectively. Validation showed that the assay is robust, rapid, economical, reproducible, specific, and sensitive down to 12,500 mitochondrial copy (equating to seven fg). It could be used with a variety of raw meats and products, including highly degraded and processed food samples. This proposed method will be greatly beneficial to the consumers, food industry, and law enforcement.

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1. Introduction

Unlisted, mislabeled, or fraudulent ingredients in food products is a serious problem and impacts humans in several ways. Several manufacturers have exploited consumers by adding or substituting ingredients in food products for cheaper ones to earn more profits (Moore, Spink, & Lipp, 2012). Certain ingredients in high demand could boost the sales of food products, and this serves as an incentive for mislabeling, as evidenced in the case of crocodile meat (Unajak et al., 2011). These occurrences negatively impact the health and safety of the consumers and the economy, as well as breach religious laws. Both national and international regulations are therefore issued to protect consumer rights; thus, a reliable technique for meat identification is needed by regulatory agencies.

There are a multitude of options available for species identification in raw meats and food products: proteins, lipid, volatile organic compounds, and DNA analyses (Ali et al., 2012). Among these, DNA-based techniques are reliable, robust, and rapid. High variations in nucleotide compositions between individuals and species enable differentiation and DNA withstands environmental degradation better than the other compounds (Che Man, Aida, Raha, & Son, 2007; Che Man, Mustafa, Khairil Mokhtar, Nordin, & Sazili, 2012; Rojas et al., 2010). Both nuclear and mitochondrial

DNA have been used, for example, the nuclear DNA's porcine leptin gene, bovine beta-actin gene, turkey prolactin receptor (Köppel, Ruf, & Rentsch, 2010; Köppel, Ruf, Zimmerli, & Breitenmoser, 2008; Köppel, Zimmerli, & Breitenmoser, 2009), and the mitochondrial D-loop region, cytochrome *b*, cytochrome oxidase subunit I (COI), 16s rRNA, 12s rRNA, and NADH dehydrogenase subunit 5/6 genes (Ali, Hashim, Mustafa, & Che Man, 2011; Branicki, Kupiec, & Pawlowski, 2003; Che Man et al., 2007, 2012; Dalmasso et al., 2004; Fajardo, Gonzalez, Rojas, Garcia, & Martin, 2010; Unajak et al., 2011). These markers are used with several techniques, such as species-specific amplification (Linacre, 2006, 2009), real-time PCR (Köppel et al., 2008, 2009, 2010; Rojas et al., 2010), PCR-RFLP (Ali et al., 2011; Murugaiah et al., 2009), and sequencing (Ali et al., 2012). However, these methods are complicated and required purification of DNA from food products. DNA degradation from acidity regulation and heating process, as well as trace contamination, can also prevent successful detection (Köppel et al., 2008, 2009).

Direct PCR, the amplification of DNA directly from samples with no prior DNA extraction, was first proposed in the 1990s (Mercier, Gaucher, Feugeas, & Mazurier, 1990; Tjhi et al., 1994). With improved polymerase enzymes and PCR buffer in recent years (Hedman, Nordgaard, Rasmusson, Ansell, & Radstrom, 2009; Hedman et al., 2010, 2011; Kermekchiev, Kirilova, Vail, & Barnes, 2009; Zhang, Kermekchiev, & Barnes, 2010), direct PCR has been touted as a sensitive and robust technique, as well as a time-saver. Direct PCR possesses several advantages. First, it is rapid as the extraction and quantification step are bypassed. Second, it is more

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sensitive than conventional PCR (Linacre, Pekarek, Swaran, & Tobe, 2010; Swaran & Welch, 2012), as the process of DNA extraction has very low efficiency (16–30%) (Colussi, Viegas, Beltramo, & Lojo, 2009; Kishore, Reef Hardy, Anderson, Sanchez, & Buoncristiani, 2006). Lastly, it improves DNA analysis results from several types of biological sample, including trace, degraded, aged, and difficult samples (Kitpipit, Chotigeat, Linacre, & Thanakiatkrai, 2014; Linacre et al., 2010; Park, Kim, Yang, & Lee, 2008; Swaran & Welch, 2012). Diluting the samples in phosphate-buffered saline (PBS) increases the amplification success rate for various sample types (Kitpipit, Thanakiatkrai, & Chotigeat, 2013b; Kitpipit et al., 2014). This dilution protocol has been used in a recently published direct-triplex PCR assay for pork, lamb, and chicken and it has been shown to overcome inhibitors found in food products (Kitpipit, Sittichan, & Thanakiatkrai, 2013a); however, a larger multiplex that includes other commonly consumed meat, including another *haram* meat (horsemeat), will shorten and simplify the source identification process, as well as brings additional benefit the halal food industry.

Thus, the aim of the study was to develop a direct-hexaplex PCR assay for simultaneous identification of six commonly consumed meats (pork, lamb/mutton, chicken, ostrich meat, horsemeat, and beef). Using the dilution protocol for sample preparation, the assay was then fully validated for its specificity, reproducibility, sensitivity, and robustness.

2. Materials and methods

2.1. Meat samples

Raw meats of six species (pork – *Sus scrofa*, lamb/mutton – *Ovis aries*, chicken – *Gallus gallus*, ostrich meat – *Struthio camelus*, horsemeat – *Equus ferus caballus*, and beef – *Bos indicus*) commonly consumed worldwide were collected from morphological-verified samples and used. One hundred and fifteen food products, including raw frozen meats, cold cuts, instant frozen foods, street foods, and halal-certified foods were collected from local retailers and markets in five provinces (Songkhla, Pattalung, Krabi, Yala, and Pattani) of Southern Thailand. All samples were cut into small pieces using a sterile scalpel, kept in a sterile plastic bag, and stored at –20 °C to prevent DNA degradation.

2.2. Primer design

Cytochrome oxidase I subunit (COI) gene sequences of six target species and other nine possible meat species were downloaded from GenBank and aligned. Species-specific primers previously reported were checked for their specificity and new primers were also designed. The GenBank accession numbers for these sequences were AF034253.1, AF492350.1, AF492351.1, CK203026.1, CK203028.1, EF588275.2, EU755253.1, GU068049.1, HQ857210.1, HQ857212.1, JQ996232.1, KC461238.1, NC_001700.1, NC_002785.1, NC_005044.2, NC_005129.2, NC_006295.1, NC_009684.1, NC_014703.1, NC_015647.1, NC_015651.1, NC_016178.1, NC_018596.1, NC_020615.1, NC_020622.1, NC_020683.1, NC_011218.2, NC_012095.1, U20753.1, and Y12025.1.

Candidate primers were checked for their physical parameters – melting temperature, self-complementarity, and secondary structures – using Oligo Calc (<http://www.basic.northwestern.edu/bio-tools/OligoCalc.html>). Primers cross-reactivity was also inspected using AutoDimer (<http://www.cstl.nist.gov/strbase/AutoDimer-Homepage/AutoDimerProgramHomepage.htm>). Details of primers used in this study are shown in Table 1.

2.3. Direct multiplex amplification

All samples were prepared and amplified by the dilution protocol of direct PCR technique (Kitpipit et al., 2014): approximately 1 mm² punch of sample (from the inside of the specimen) was added to 20 µL of phosphate-buffered saline (PBS) and heated at 98 °C for 2 min. The mixed meat sample was prepared by pooling 1.5 µL of each meat pre-PCR dilution sample.

The PCRs were performed in a total volume of 20 µL, containing 10 µL Phire[®] Animal Tissue PCR Buffer (Thermo Scientific, Thailand), 1.0 unit Phire[®] Hot Start II DNA Polymerase (Thermo Scientific, Thailand), optimized concentrations of six primer pairs (Table 1), 1.5 µL supernatant of pre-PCR dilution samples (in PBS), and sterile water. Amplifications were performed using a T100[™] Bio-Rad thermal cycler with the following PCR condition: initial denaturation at 98 °C for 5 min; 35 cycles of denaturation at 98 °C for 5 s, annealing at 66 °C for 5 s, and extension at 72 °C for 25 s; and a final extension at 72 °C for 1 min. The reactions were stored at 4 °C until further analysis. A negative control was included with all sets of reactions to monitor for contamination.

2.4. DNA separation and detection

The PCR products were separated on a 2% low melting point gel electrophoresis. PCR products were stained using ethidium bromide and electrophoresis was run at 100 V for 30 min. The gels were visualized and photographed in a Bio-rad GelDoc 1000 gel documentation system (Bio-rad, USA).

2.5. Direct multiplex assay validation

The direct multiplex assay developed in this study was validated for its specificity, reproducibility, sensitivity, and robustness (food product testing). For the specificity test, the assay was cross-tested with all six target species and 13 possible domestic and wildlife meat species consumed in Asia: crocodile (*Crocodylus siamensis*), duck (*Anas platyrhynchos*), deer (*Cervidae* spp.), rabbit (*Oryctolagus cuniculus*), goose (*Anser domesticus*), buffalo (*Bubalus bubalis*), cat (*Felis catus*), dog (*Canis lupus familiaris*), muntjac (*Muntiacus muntjak*), elephant (*Elephas maximus*), tiger (*Panthera tigris*), bear (*Ursus malayanus*) and human (*Homo sapiens*). For the reproducibility test, 19 voucher target meat samples from different sources (five pork samples, two lamb/mutton samples, five chicken samples, two ostrich meat samples, two horsemeat samples and three beef samples) were tested with the assay. For the sensitivity test, quantified mitochondrial copies of the six target species were prepared following the method of Tobe and Linacre (2008). Five concentrations (100,000, 50,000, 25,000, 12,500, and 6250 mitochondrial copies/µL) were prepared by serial dilution and amplified by the assay to determine the minimum mitochondrial copy that can be detected. For robustness and real-world performance testing, the developed assay was used to test 115 food products, including 20 raw frozen meats, 17 cold cuts, 13 instant frozen foods, 27 street foods, and 38 halal-certified foods.

3. Results

3.1. The direct multiplex assay development

3.1.1. Singleplex amplification

The direct singleplex amplifications of all six species-specific primers used in this study were first performed using voucher meat samples. This was done to test the primers' efficiency and specificity and to evaluate the possibility of applying direct PCR for meat authentication. The result showed that direct PCR was

Table 1

Details of primer sequence, melting temperature, PCR product size, and reference are shown.

Meat species	Primer name	Sequences (5'–3')	Gene	Final conc. (μM)	Product size	References
Pork	Sus-F1	5'-GAA AAA TCA TCG TTG TAC TTC AAC TAC A-3'	<i>cyt b</i>	2.0	100 bp	Lopez-Andreo, Lugo, Garrido-Pertierra, Prieto, and Puyet (2005)
	Sus-R1	5'-GGT CAA TGA ATG CGT TGT TGA T-3'		2.0		
Lamb	Ovi-F2	5'-GAA AAA CCA TCG TTG TCA TTC AAC T-3'	<i>t-Glu</i>	1.8	119 bp	Lopez-Andreo et al. (2005)
	Ovi-R2	5'-AAA TAT TTG ATG GAG CTG GGA GA-3'		1.8		
Chicken	Gal-F3	5'-AGC AAT TCC CTA CAT TGG ACA CA-3'	<i>cyt b</i>	0.8	133 bp	Zhang, Fowler, Scott, Lawson, and Slater (2007)
	Gal-R3	5'-GAT GAT AGT AAT ACC TGC GAT TGC A-3'		0.8		
Ostrich	Str-F4	5'-CCC TTT AAA GAC ATC TGG TAT TGT GAG-3'	12s rRNA	1.2	155 bp	Rojas et al. (2011)
	Str-R4	5'-TAA ATT GTA GGC TCT CTG GGG TTC-3'		1.2		
Horse	Equ-F5	5'-CGT TTG ATC TGT CCT TAT TAC GGC A-3'	COI	2.0	253 bp	This study
	Uni-R	5'-CCG AAT GGT TCT TTT TTY CCY GAG TAG TA-3'		2.0		
Cow	Bos-F6	5'-CAT CAA CTT CAT TAC AAC AAT TAT CAA CAT AAA G-3'	COI	1.0	311 bp	This study
	Uni-R	5'-CCG AAT GGT TCT TTT TTY CCY GAG TAG TA-3'		1.0		

successful in identifying meat species from raw meat samples. In these experiments, each species-specific primer pair produced PCR products only from its target species and generated the expected PCR products of 100, 119, 133, 155, 253, and 311 bp for pork (*Sus scrofa*), lamb/mutton (*Ovis aries*), chicken (*Gallus gallus*), ostrich meat (*Struthio camelus*), horsemeat (*Equus ferus caballus*) and beef (*Bos indicus*), respectively. No PCR product was observed from the negative controls. Direct amplification from a large sample size (>1 mm²) could occasionally cause amplification failure; further dilution (1:10 to 1:100) of pre-PCR solution using sterile water resolved this problem (data not shown).

3.1.2. Multiplex amplification

The direct multiplex assay for simultaneous detection of the six meat species were successfully developed. Fig. 1 shows six different PCR fragments with sizes corresponding to the expected sizes of the six target meat species. No PCR fragment was observed from the negative control.

3.2. Direct multiplex assay validation

3.2.1. Specificity test

The developed direct multiplex assay was used to analyze six target species (pork, lamb/mutton, chicken, ostrich meat, horsemeat and beef) and 13 possible domestic and wildlife meat species (crocodile, duck, deer, rabbit, goose, buffalo, cat, dog, muntjac, elephant, tiger, bear and human) in order to examine the assay specificity. The result showed that the assay was highly specific to the targets: the expected PCR fragments were generated only from the target species as predicted, i.e. no cross-reaction from non-target species was found (data not shown).

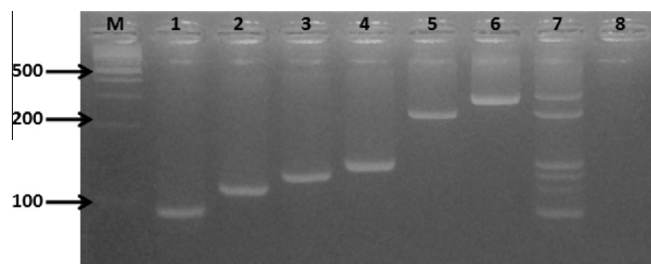


Fig. 1. Multiplex direct amplification of six target species separated on a 2% agarose gel. Lane M: 100 bp ladder, 1: pork (100 bp), 2: lamb/mutton (119 bp), 3: chicken (133 bp), 4: ostrich meat (155 bp), 5: horsemeat (253 bp), 6: beef (311 bp), 7: six target species mixed (pooled from pre-PCR dilution samples of six meats), and 8: negative control. Expected PCR products were detected in all lanes.

3.2.2. Reproducibility test

The assay reproducibility was tested using 19 voucher meat samples (of the six targets) obtained from different sources. The expected PCR products were generated from all target meat samples, as shown in Fig. 2. This resulted in 100% accuracy in meat identification.

3.2.3. Sensitivity test

The direct multiplex assay was used to amplify five mtDNA concentrations to determine the assay sensitivity – the minimum mtDNA amount of each target meat species that could still be detected by the assay. The detectable mtDNA amounts for each target species were different (Table 2). PCR products of pork, lamb/mutton, chicken, and beef were detected from as low as 12,500 mtDNA copies. PCR products of ostrich meat and horsemeat were detected from 25,000 mtDNA copies and 50,000 mtDNA copies, respectively.

3.2.4. Application to commercial food products

The real-world use of the developed assay with commercial meats and food products was demonstrated. Table 3 shows the results from all food products. The result showed that the direct multiplex assay was robust and could be successfully applied; only three of 115 samples (2.6%) did not amplify. Approximately 25% of all samples (29 of 115) had meat species that were not listed as an ingredient. The falsification was found in all food product types – raw frozen meat, cold cut, instant frozen food, street food, and halal food. The highest number of falsification (64.7%) was found in cold cuts while raw frozen meats had the lowest percentage (5.0%). No pork was detected in halal-certified food.

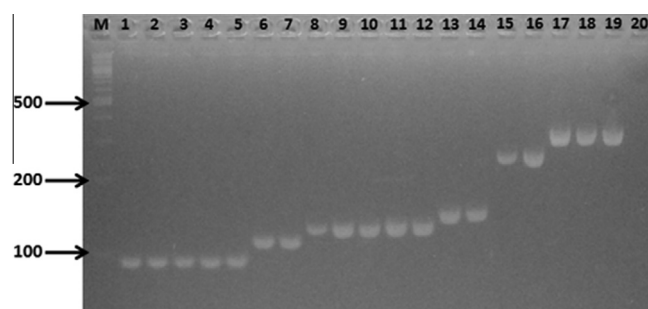


Fig. 2. Reproducibility test of the multiplex direct assay with 19 voucher meat samples. Lane M: 100 bp ladder, 1–5: pork (100 bp), 6,7: lamb/mutton (119 bp), 8–12: chicken (133 bp), 13,14: ostrich meat (155 bp), 15,16: horsemeat (253 bp), 17–19: beef (311 bp), 20: negative control. Expected PCR products were detected in all lanes.

Table 2

Sensitivity test of the multiplex direct assay. +++ stands for high intensity, ++ for medium intensity, + for low intensity, and – denotes band absence).

Meat species detection	mtDNA amount (copies)				
	100,000	50,000	25,000	12,500	6250
Pork	+++	++	+	+	–
Lamb/Mutton	+++	++	++	+	–
Chicken	+++	++	++	+	–
Ostrich meat	++	++	+	–	–
Horsemeat	++	+	–	–	–
Beef	+++	++	++	+	–

4. Discussion

The direct-multiplex PCR assay developed in this study could be completed in only 90 min, does not require expensive equipment (e.g. no real-time PCR instrument and no capillary electrophoresis), and saves DNA extraction time and cost. This is the first time that a direct-hexaplex PCR assay has been evaluated, successfully developed, and fully validated for simultaneous meat species identification from raw meats and food products.

We employed the Phire® Hot Start II DNA Polymerase and its proprietary buffer. Modified and novel polymerases have been shown to perform better than the traditional *Taq* polymerase (Hedman et al., 2009, 2010, 2011; Kermekchiev et al., 2009; Zhang et al., 2010). One mechanism of how this is achieved is the stronger polymerase-DNA bond, i.e. via the SSo-binding domain (Wang et al., 2004). Certain compounds, such as BSA and Tween-20, have also been shown to increase inhibitor tolerance when they are added to the PCR buffer (Kreader, 1996). Since the buffer is proprietary, its composition is unknown to the users. Additionally, the inhibitors would also have been diluted using the dilution protocol (Kitpipit et al., 2014). As the direct PCR procedure requires no extensive and lengthy pre-extraction and extraction step, this novel assay is therefore cost-effective and rapid. It addresses the drawbacks of conventional PCR-based methods previously reported.

The assay required a very small amount of meat sample (approximately 0.3–1.0 mm²) to achieve a successful amplification.

This illustrates that these trace amounts contain sufficient DNA for amplification. This sensitivity could be achieved as the direct PCR procedure requires no DNA extraction step, resulting in no DNA loss (Colussi et al., 2009; Linacre et al., 2010; Swaran & Welch, 2012). The optimal meat sample size found in this study corresponds to our previous experience with wildlife muscle samples, in which approximately one mm² yields the highest amplification success rate and PCR product using direct PCR (Kitpipit et al., 2014). Requiring small amount of samples is very beneficial for meat authentication as contamination or fraudulent ingredients could be in trace amounts. The direct amplification technique is therefore proved to provide a high chance of detecting falsifications, even in trace amounts.

Primers used for the assay produced small amplicons in the range of 100 to 311 bp, increasing the chance of detection from degraded/processed raw meats and food products. Because even traces of pork are *haram* (forbidden) for Muslims, the amplicon size for pork was deliberately chosen to be the smallest at 100 bp in order to maximize the chance of detecting pork. Previous reports have shown that amplicon size of less than 150 bp has a higher chance of survival in degraded samples (Ali et al., 2011, 2012; Hughes-Stamm, Ashton, & van Daal, 2011; Köppel et al., 2009; Senge, Madea, Junge, Rothschild, & Schneider, 2011). Thirty-eight halal food products were tested with the developed assay and none showed contamination from pork. The primers were designed from the *cyt b*, *COI*, and 12s rRNA genes in the mtDNA. This brings three benefits. Firstly, these genes contain high DNA variations among different species and low DNA variation among individuals of the same species, providing high confidence in meat species discrimination (Linacre, 2006; Tobe, Kitchener, & Linacre, 2010). In other words, the short amplicon length does not compromise specificity. Secondly, the approximately 3500 mitochondrial copies in a single skeletal muscle cell make the assay very robust and sensitive (Miller, Rosenfeldt, Zhang, Linnane, & Nagley, 2003). Lastly, the sequence data from *cyt b* and *COI* genes are available on DNA databases for many species, which enables additional comparisons using sequencing if needed. The species-specific primer technique employed here is better than barcoding because it is much faster

Table 3

Robustness test with commercial food products and falsification survey. The numbers of samples that contains the target species out of the total number of samples tested are shown, e.g. 11/16 under the chicken column in the cold cut with pork category means chicken were detected in 11 samples that should have had only pork. Fraud ratio for each category is calculated by dividing the number of samples with falsification by the total number of samples in that category. The bolded rows show different categories of samples and the total number of samples in that category.

Food products	No.	Multiplex 6 species					Fraud ratio (%)
		Pork	Mutton	Chicken	Ostrich	Horse	
Raw frozen meat	20						5.0
Pork	5	5/5	–	–	–	–	
Mutton	2	–	2/2	–	–	–	
Chicken	5	–	–	5/5	–	–	
Ostrich meat	3	1/3	–	–	2/3	–	
Horsemeat	2	–	–	–	–	2/2	
Beef	3	–	–	–	–	–	3/3
Cold cut	17						64.7
with pork	16	15/16	–	11/16	–	–	
with beef	1*	–	–	–	–	–	
Instant frozen food	13						7.7
with pork	5	4/5	–	1/5	–	–	
with chicken	8*	–	–	7/8	–	–	
Street food	27						33.3
with pork	15	15/15	–	9/15	–	–	
with chicken	12	–	–	12/12	–	–	
Halal food	38						18.4
with chicken	30*	–	–	29/30	–	–	
with beef	8	–	2/8	5/8	–	–	1/8

A minus (–) denotes no PCR product detected. An asterisk (*) denotes one sample did not yield any product for all six target species.

and cheaper. The results are easier to interpret, but it cannot be used for species not included in the multiplex. The six species selected are a combination of commonly eaten meats and two *haram* meats (horsemeat and pork), which will aid food control agencies around the world, particularly for halal foods. Comparing to our previously published triplex assay (Kitpipit et al., 2013a), the hexaplex in this study adds three more targets (including one more *haram* meat). The numbers of samples for specificity, reproducibility, and commercial products have all been increased, which results in additional confidence in the assay's results.

The proposed assay has two limitations. First, the assay cannot provide quantitative data, unlike real-time PCR (Köppel et al., 2010). Nonetheless, accurate quantification for real-time PCR could only be achieved when an appropriate reference material is used, as the matrix in food could interfere with the amplification process. In other words, serially diluted DNA from raw meats could not be used as a reference for DNA extracted from food samples, as there might be ingredients in the recipe that are co-extracted with the DNA and interferes with the quantification process (Köppel et al., 2008, 2009). The second limitation is the unequal amplification efficiency and sensitivity for the six species, but this same problem is encountered in all types of multiplex PCR assays (Ali et al., 2012).

The assay is fully validated for its accuracy, specificity, sensitivity, and applicability in analysis of food samples. The assay was found to provide 100% accuracy in the identification of the six meat species obtained from different sources. It is specific only to target meat species, indicating no chance of error or misidentification found. The assay is also extremely sensitive, being able to detect samples as little as 12,500 mtDNA copies or seven femtogram of mtDNA (equating to less than five muscle cells) (Tobe & Linacre, 2008), which makes it viable for thermally processed food samples. This sensitivity is comparable to published conventional and real-time singleplex and multiplex PCR assays, e.g. 300 cells (calculated from 1 ng of DNA from a 3 billion bp genome of cow) (Murugaiah et al., 2009), ten cells (calculated from 0.32 ng of DNA) (Köppel et al., 2008), and a single cell (calculated from 0.0035 ng of DNA) (Zhang, Fowler, Scott, Lawson, & Slater, 2007). Comparisons with some studies are difficult as they reported assay sensitivity in terms of percentages of a mixed sample. In addition, the optimized multiplex assay is illustrated to be robust enough to detect various meat fraudulent acts in real-world food samples. All these points reveal confidence in the use of the developed direct multiplex assay for the six-target meat identification from a variety of food samples.

5. Conclusion

This is the first time a direct-hexaplex PCR assay has been applied to meat species identification in raw meats and food products. The assay has been successfully developed and proven to be efficient in simultaneously identifying six commonly consumed meat species, which are pork, lamb/mutton, chicken, ostrich meat, horsemeat and beef. The assay has also been fully validated to meet the recommended standards in order to be used in real-world law enforcement. It is accurate, specific, sensitive, and applicable to detect adulterations and misbranding from a variety of meats and food product, including highly degraded or processed food samples.

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