



Development of qualitative and quantitative PCR analysis for meat adulteration from RNA samples



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ABSTRACT

Total RNA samples were used to establish qualitative and quantitative PCR-based methods for assessing meat adulteration. The primers were designed based on the mRNA sequences of troponin I (TnI), mitochondrial ribosomal protein (MRP) and tropomodulin genes to distinguish chicken, pork, goat, beef and ostrich. There was no cross reaction between the primers, and the detection limit of the cDNA template was 0.01 and 20 ng in simplex PCR and multiplex PCR, respectively. In the low temperature storage test, the detection limits of cDNA template with 10 and 1 ng were determined at 4 °C and –80 °C. In quantitative assay, the precision of real-time PCR analysis expressed as a coefficient of variation (CV) ranged from 0.25% to 5.24% and the trueness, expressed as an error, ranged from 0.28% to 6.98% for adulteration. Thus, herein, we provided alternative tools for the assessment of meat adulteration using mRNA-based PCR methods.

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1. Introduction

Meat adulteration frequently occurs in meat and meat products through the addition of minced meat. Preventing the adulteration of meat products using less desirable meat species is important to the consumers for economic, health, and religious reasons. Therefore, it is necessary to establish a detection system for meat adulteration and ensure food safety. Many methods have been developed for meat species identification. These techniques can be divided into two categories: protein and DNA-based methods. Protein-based methods include isoelectric focusing, electrophoresis analysis, SDS–PAGE, near-infrared spectroscopy, liquid chromatography and enzyme-linked immunosorbent assay (Chen, Hsieh, & Bridgman, 2002; Etienne et al., 2000; Gayo, Hale, & Blanchard, 2006; Hirao, Yonemura, & Miyazaki, 2004; Leitner, Castro-Rubio, Marina, & Lindner, 2006). However, most proteins are denatured after heat treatment, resulting in changes in the

antigenicity and electrophoretic mobility of molecules during food processing. DNA-based methods include polymerase chain reaction (PCR), real-time PCR, PCR-restriction fragment length polymorphism (PCR-RFLP) analysis, random amplification of polymorphic DNA (RAPD) and amplified fragment length polymorphism (AFLP) (Alves, Castellanos, Ovilo, Silio, & Rodriguez, 2002; Brodmann & Moor, 2003; Girish et al., 2005; Koh, Lim, Chua, Chew, & Phang, 1998; Martin et al., 2007). Compared with protein-based techniques, DNA-based techniques are more reliable because DNA is more stable in the food products.

PCR technology is used to multiply specific DNA sequences using specific primer pairs. PCR has been widely applied for food safety control due to the rapidity, specificity and sensitivity of this technique. One main aspect for the successful detection of a species using PCR is to identify adequate genetic markers to establish the assay. Many nuclear- and mitochondrial-specific genes or sequences, such as satellite DNA, troponin I, and mitochondrial gene including 12S rRNA and cytochrome b (Cyt b), have been broadly used as targets for the identification of meats (Alves et al., 2002; Arslan, Ilhak, & Calicioglu, 2006; Chen & Hsieh, 2002; Martin et al., 2007; Pascoal, Barros-Velazquez, Cepeda, Gallardo, & Calo-Mata, 2008; Verkaar, Nijman, Boutaga, & Lenstra, 2002). Satellite DNA comprises centromeric, highly tandem repeats in the genome of mammalian species (Erukashvily

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& Ponomartsev, 2013). Through concerted evolution, these sequences have become species-specific for use in identification (Verkaar et al., 2002). Troponin I (TnI), a myofibrillar protein and component of troponin, is involved in the regulation of muscle contractions. At least three isoforms of TnI (cardiac, fast and slow skeletal forms) are present in muscle tissues, and these proteins act as adequate targets for porcine meat identification (Chen & Hsieh, 2002; Perry, 1999). Mitochondrial genes are also good targets for species identification. Many approaches typically use sequences from a single gene, such as 12S rRNA and Cyt b, for species analysis (Safdar, Junejo, Arman, & Abasiyanik, 2014).

DNA-based PCR amplified sequence analyses can be designed using either mitochondrial or genomic DNA, wherever both single copy and repetitive sequences are used. However, there are some problems with DNA-based PCR methods, such as a low limit of detection (LOD), DNA degeneration, and difficulties in melting curve interpretation and probe design (Ballin, Vogensen, & Karlsson, 2009). Herein, we describe PCR-based methods for the identification and quantitation of meat species using total RNA. The development of RT-PCR assays requires knowledge of the intron–exon boundaries in gene sequences (Arya et al., 2005). This technique facilitates the design of primers for amplification across introns to prevent DNA contamination. We established easy, fast and reliable tools using mRNA-based simplex PCR, multiplex PCR and quantitative real-time PCR for the analysis of the sequences from TnI, MRP and tropomodulin (Tmod) genes. Messenger RNA-based RT-PCR methods utilize specific genes as markers for the detection of meat adulteration. These methods also facilitate the identification of the compositions of meat mixture through specific gene amplification using quantitative real-time PCR. We used the TnI gene as a target for the specific identification of chicken, pork and goat, and the MRP gene was used to identify beef. Moreover, we reported the first sequence of the Tmod gene to identify ostrich in adulterated meat samples. Here, we described the methods for the simultaneous and quantitative identification of contaminating chicken, pork, goat, beef and ostrich meats, with high sensitivity and specificity.

2. Materials and methods

2.1. Meat samples

Fresh meat (chicken, pork, goat, beef and ostrich) were obtained from the local supermarket. For low temperature storage assays, 1 g of each meat sample was weighed and stored at 4 °C and –80 °C until further use. Total RNA was extracted from these samples at 1, 2, 4, 6, 8, and 10 days post-storage at 4 °C, and every week for 13 weeks post-storage at –80 °C. Total RNA from all meat samples was used to determine the detection limits at each storage time and condition. For the detection of meat adulteration, mixtures containing different levels (10–60%) of meat were prepared and stored at –20 °C until further use in experiments (Supplemental Table 1). The abbreviations listed in Supplemental Table 1 are mix sample (MS), ostrich (O), goat (G), chicken (C), pork (P), beef (B), and all meat samples (A), respectively.

2.2. RNA extraction and reverse transcription

Total RNA was prepared from the meat samples using TriSolution Reagent (GeneMark, Taiwan). The RNA concentrations were measured as the absorbance at 260 nm using a spectrophotometer (U-2001, Hitachi, Japan), and the RNA integrity was assessed through agarose electrophoresis. Total RNA was purified from each meat species (pork, chicken, goat, beef and ostrich). The purity of the RNA extract was measured, showing an

A260/A280 ratio ranging from 1.79 to 2.11 and an A260/A230 ratio ranging from 1.95 to 2.76. The concentration of total RNA ranged from 462 to 1422 ng/μL. One microgram of total RNA was reverse-transcribed with oligodeoxyribonucleotide thymidine (oligo-dT). The RNA/oligo-dT mixture was incubated at 70 °C for 60 min followed by a 5-min incubation on an ice bath and the addition of 5 μL of buffer (50 mM Tris–HCl, pH 8.3, 6 mM MgCl₂, and 75 mM KCl), containing 0.5 mM of each deoxyribonucleoside triphosphate (dNTP), 1 U of recombinant RNasin Ribonuclease Inhibitor (Invitrogen, USA) and 1 μL of 200 units of Moloney murine leukemia virus reverse transcriptase (MMLV-RT) (Invitrogen, USA). The reaction was incubated at 42 °C for 60 min and successively at 95 °C for 10 min to inactivate the reverse transcriptase.

2.3. Primer design and PCR specificity and sensitivity analysis

In the present study, the primers used for PCR and real-time PCR were designed using primer express software v2.0 (Applied Biosystems, USA) and Primer Design Assistant (National Health Research Institute, Taiwan) based on the gene sequences for TnI (pork: NM_213912, chicken: NM_205417, and goat: AY033589), MRP (beef: NM_001045926) and tropomodulin (ostrich: AB254879) (Chen, Lin, Cho, Lo, & Hsiung, 2003) retrieved from the National Center for Biotechnology Information (NCBI) GenBank. The primer and amplicon sequences are shown in Supplemental Tables 2 and 3.

For the primer sensitivity test, a series of primer concentrations (1, 0.5, 0.25, 0.1 and 0.05 μM) were examined in PCR reactions containing 100 ng cDNA template. For the cDNA template sensitivity test, ten-fold serial dilutions (100, 10, 1, 0.1, and 0.01 ng) were prepared for the cDNA template generated from each species. To assess meat adulteration, 20, 2, 0.2 and 0.02 ng of cDNA were used as templates per PCR reaction.

2.4. Simplex and multiplex PCR

Simplex PCR amplification was performed in a final volume of 25 μL containing 16 mM (NH₄)₂SO₄, 67 mM Tris–HCl (pH 8.8), 0.01% Tween-20, 2.0 mM MgCl₂, 200 μM of dNTP mix, 0.3 μM of primers, 1 U *Taq* DNA polymerase (Invitrogen, USA), and 100 ng of cDNA template. Amplification was performed using the following cycling conditions: initial heat denaturation at 95 °C for 5 min, followed by 35 cycles at 95 °C for 30 s, 62 °C for 30 s, and 72 °C for 30 s, with a final extension at 72 °C for 5 min.

To detect each of the mixed species in a single reaction, one-step multiplex PCR was developed using the primer sets P-f/P-r, GT3-f/GT3-r, OS2-f/OS2-r, B6-f/B6-r and CK2-f/CK2-r (Supplemental Table 2). In the reaction, the primer concentration ranged from 0.1 to 0.2 μM, and 200 ng of cDNA template was used. Thermal cycling was programmed following the same procedure as used for simplex PCR. The amplification products were resolved through electrophoresis on 2% agarose gels (Invitrogen, USA) in TBE Buffer for 30 min at 110 V and subsequently stained with ethidium bromide (0.5 μg/mL).

2.5. Sequencing

All PCR fragments in meat adulteration experiments were purified using the Gel-M™ Gel Extraction System (Viogene, Taiwan), and sequencing was conducted at the Genomics BioSci & Tech Company using a 3730xl DNA Analyzer (Applied Biosystems). The nucleotide sequences were assessed for sequence similarity through a BLASTn search using the National Centre for Biotechnology Information database, followed by alignment and 100% matching with the chicken, pork, goat, beef and ostrich sequences available in the GenBank database.

2.6. Quantitative real-time PCR assay for meat adulteration

The quantitative real-time PCR reaction was performed in a final volume of 25 μ L containing Smart Quant Green Master Mix (AMRESCO®, Ohio, USA), 20 ng cDNA, and 1 μ M of each primer. The amplification reactions were performed using the ABI Prism™ 7900 Sequence Detection System (Applied Biosystems, USA) and a thermal cycling protocol at 50 °C for 2 min and 95 °C for 10 min, followed by 45 cycles of 95 °C for 15 s and 60 °C for 1 min. Each experiment was repeated at least three times to verify the results. The data obtained from the real-time PCR assay, including the mean, standard deviation, error and CV%, were calculated using SDS v2.2 software.

3. Results and discussion

3.1. Specific primer design and cDNA template preparation

To establish the detection system, the primer sets were designed for PCR and quantitative real-time PCR from the DNA sequences for troponin I (TnI; for pork NM_213912, chicken NM_205417 and goat AY033589), mitochondrial ribosomal protein (MRP; for beef NM_001045926) and Tropomodulin (Tmod; for ostrich AB254879) using Primer Design Assistant (Chen et al., 2003) and Primer Express Software (Applied Biosystems, Life Technologies) (Supplemental Tables 2 and 3). The high purity and high yield of total RNA suggest the adequacy of the RNA extraction protocol from meat based products (Fleige & Pfaffl, 2006). Reverse transcription was performed using total RNA to prepare the cDNA templates as described in the Materials and Methods.

3.2. Sensitivity and specificity of simplex PCR for each meat

The sensitivity of simplex PCR was evaluated after serially diluting the target DNA and spectrophotometrically measuring the DNA concentration (Hou et al., 2014). The experiment revealed a detection limit of 0.01 ng of cDNA template from different meat species (Fig. 1). Studies have suggested that the detection sensitivity was also 0.01 ng using DNA from other species (bovine, ovine, donkey,

pork, and horse) (Kesmen, Yetiman, Sahin, & Yetim, 2012). The results of the present study indicated that the detection limit for the PCR reactions was 0.01 ng, suggesting that the mRNA-based PCR assay was highly sensitive.

Species-specific PCR assays using specifically designed primers have gained popularity for the detection of meat adulteration (Karabasanavar, Singh, Kumar, & Shebannavar, 2014; Rodriguez et al., 2004). The use of specifically designed oligonucleotides with restrictive PCR conditions has made it possible to verify food authenticity through the direct and specific identification of defined DNA fragments (Rojas et al., 2010). Thus, PCR reactions were performed using the species-specific primer sets from Supplemental Table 2. In the reactions, each cDNA template was amplified to produce single DNA fragments of 500, 418, 573, 654 and 112 bp, respectively (Supplemental Table 2 and Supplemental Fig. 1). All DNA fragments were sequenced and matched with the pork, chicken, goat, beef and ostrich sequences available in the GenBank database. The limit of the diluted primer concentrations for each reaction was at 0.05 μ M, and no PCR product was generated using primer concentrations below 0.05 μ M (data not shown). The primer concentration used for the PCR conditions in the present study was better than a common DNA-based PCR reaction using 0.1–0.5 μ M of primer (Hyndman & Mitsuhashi, 2003), suggesting that this concentration of specific primer was sufficient for detection in simplex PCR.

3.3. Simplex PCR for the detection of meat adulteration

Species-specific PCR is a unique technique used to identify the specific meat species in a mixture of meat samples (Ballin et al., 2009). Two types of PCR techniques are generally used, targeting either nuclear or mitochondrial DNA. However, here, we used mRNA as a specific PCR template. To identify single species in adulterated meat, we used an equal mixture of five meat species in adulterated meat (Supplemental Table 1, MSA) and the primers OS2-f/OS2-r, CK2-f/CK2-r, P-f/P-r, GT3-f/GT3-r and COW6-f/COW6-r specific for tropomodulin and TnI genes and mitochondrial ribosomal protein (Supplemental Table 2). Each primer showed no cross reactivity with other species, and only species-specific bands were evident (Fig. 2A). The results showed

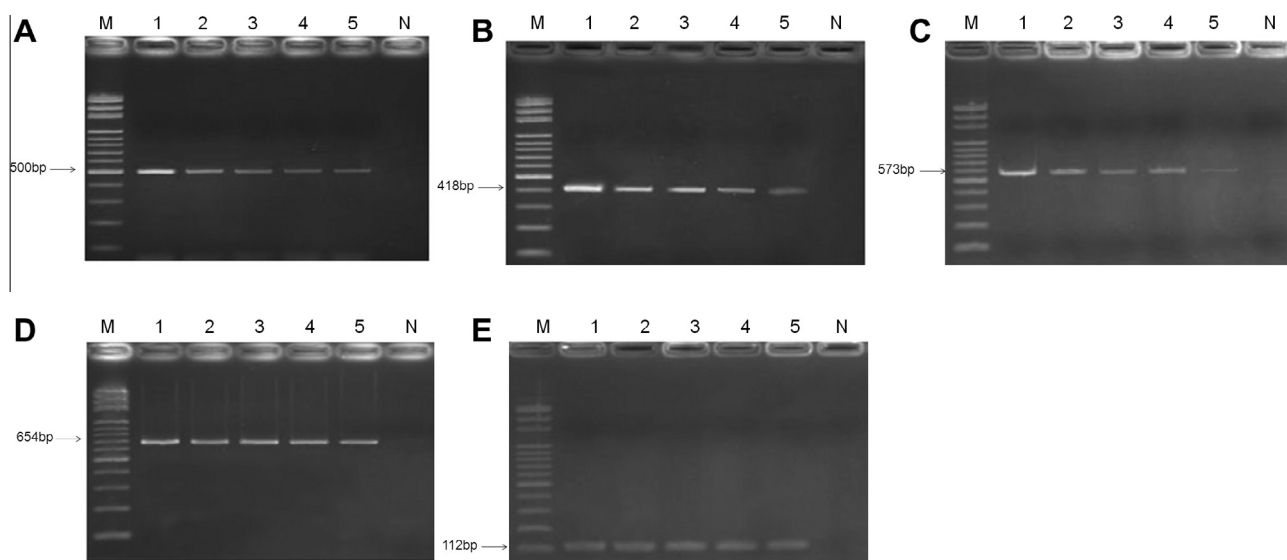


Fig. 1. The specificity of the PCR products amplified using a cDNA template generated from RNA samples of pork (A), chicken (B), goat (C), beef (D) and ostrich (E). Lane M shows the DNA marker. Lanes 1–5 show the PCR products amplified using 100, 10, 1, 0.1 and 0.01 ng cDNA with species-specific primers, respectively. Lane N shows the negative control without template in the PCR reaction.

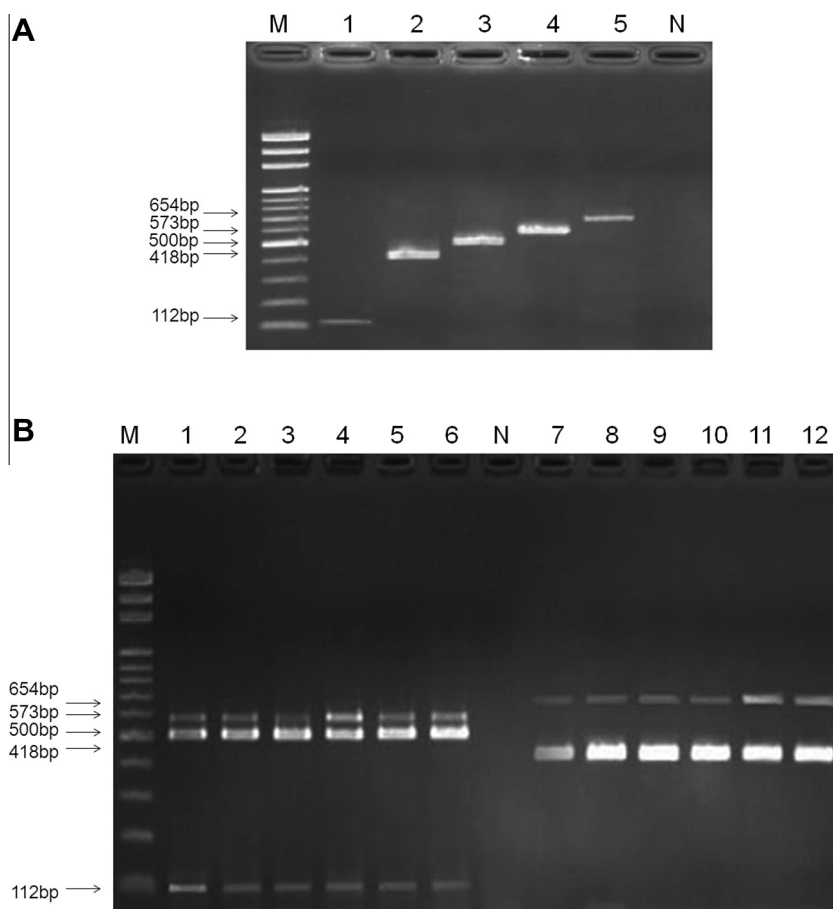


Fig. 2. The specificity of primers testing and multiplex PCR analysis. (A) In the primer testing, lane M shows the DNA marker. Lanes 1–5 show the PCR products amplified from mixed samples using specific primers for ostrich, chicken, pork, goat and beef, respectively. Lane N shows the negative control without primer in the PCR reaction. (B) In multiplex PCR, a mixture of cDNA templates (i.e., MSO, MSC, MSP, MSG, MSB, and MSA) were used as described in Material and methods. Lanes 1–6 were detected using a combination of GT3-f/GT3-r, P-f/P-r and OS2-f/OS2-r primers, and lanes 7–12 were detected using B6-f/B6-r and CK2-f/CK2-r. Lane M shows the molecular weight marker. Lane N shows the negative control without primer in the PCR reaction.

that these primers could be used in simplex PCR to simultaneously differentiate chicken, pork, goat, beef and ostrich ingredients in food.

3.4. Multiplex PCR for meat adulteration

Multiplex PCR facilitates the simultaneous detection of multiple target genes and has the advantage of reduced cost and testing time (Sint, Raso, & Traugott, 2012). The impact factors of multiplex PCR sensitivity are derived from species types, target genes and amplicon lengths (Ali, Razzak, & Hamid, 2014). For the simultaneous detection of meat adulteration, a one-step multiplex PCR technique was developed using the primers previously used for simplex PCR. A 20% ratio of each meat mixture (MSA) was used as a target (Supplemental Table 1). First, all primers were used in the PCR reaction, but no amplicons were produced (data not shown). Therefore, we optimized the primer sets for PCR. One primer set, P-f/P-r, GT3-f/GT3-r and OS2-f/OS2-r, was successful for the detection of pork, goat and ostrich (RT-PCR products: 500, 573 and 112 bp, respectively) in the mixture without cross reactivity with other species (Fig. 2B, lane 1–6). The other primer set, B6-f/B6-r and CK2-f/CK2-r, was specific for the detection of beef and chicken, generating DNA fragments of 654 and 418 bp, respectively (Fig. 2B, lane 7–12). Therefore, multiplex PCR is more functional and practical compared with simplex and real-time PCR techniques for the determination of adulteration in meat (Ali et al., 2014; Hou et al., 2014).

3.5. Effects of storage time on meat detection at low temperatures

Low temperature storage is a typical preservation method used to maintain meat freshness, as microbiological, chemical and biochemical changes are reduced under decreasing temperatures (Fernandez et al., 2007; Huang et al., 2012; Huston, Sink, Miller, & Shigley, 1965). Storage testing was examined in meat detection at 4 °C for 1, 2, 4, 6, 8 and 10 days and –80 °C for 1–13 weeks of storage. Total RNA was purified, and the RNA quality for most samples remained sufficient for reverse transcription from each storage meat at 10 days and 13 weeks after storage at 4 °C and –80 °C, respectively (data not shown). All meats maintained good quality based on physical appearance until the 4th day after storage at 4 °C. The meat started to brown and corruption was detected on the 5th day after storage at 4 °C. For PCR, 10 ng cDNA template was the detection limit for meat species stored at 4 °C for 10 days, except for goat, which showed a detection limit of 100 ng cDNA, reflecting a decline in meat quality (Fig. 3 and data not shown).

The detection limits for meat species stored at –80 °C for 13 weeks were 1 ng cDNA (Fig. 4). During storage at –80 °C, the meat started to brown from the 8th to 13th week, but without corruption. Notably, the ostrich DNA PCR fragment was clearly observed from 1 to 9 weeks of storage using 1 ng of cDNA template, but the band intensity was weak from 10 to 13 weeks (Fig. 4E, lane 1–9). These results showed that 100 ng of cDNA template was required for ostrich detection (data not shown). Altogether, these results suggest that the detection limit was

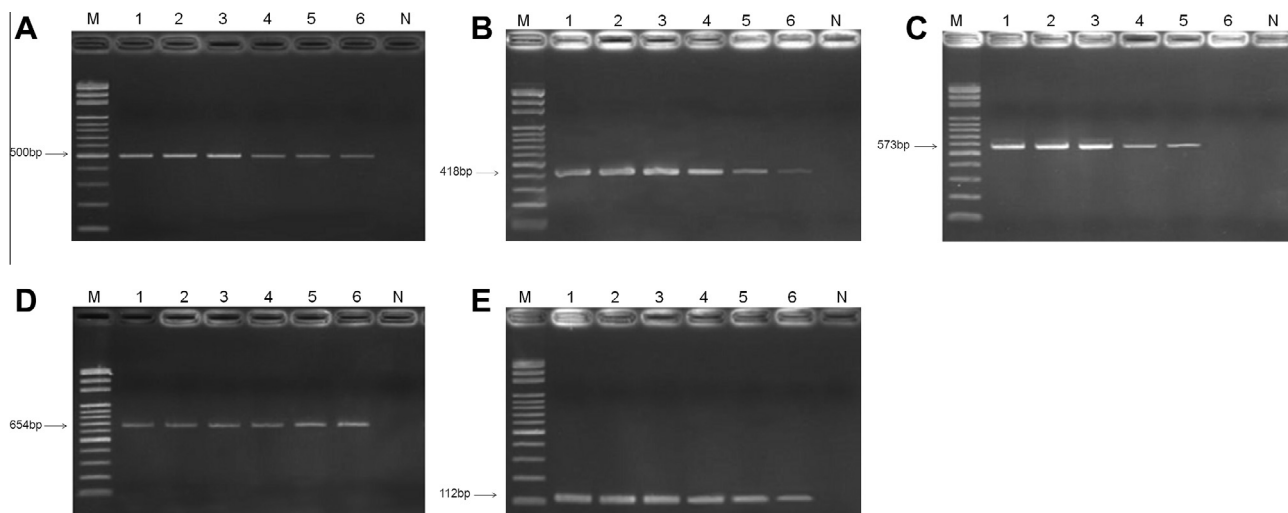


Fig. 3. Low temperature storage test. Agarose gel electrophoresis of the PCR products amplified using 10 ng cDNA from pork (A), chicken (B), goat (C), beef (D) and ostrich (E) stored at 4 °C for 1, 2, 4, 6, 8 and 10 days. Lane M shows the molecular weight marker. Lane N shows the negative control without primer in the PCR reaction.

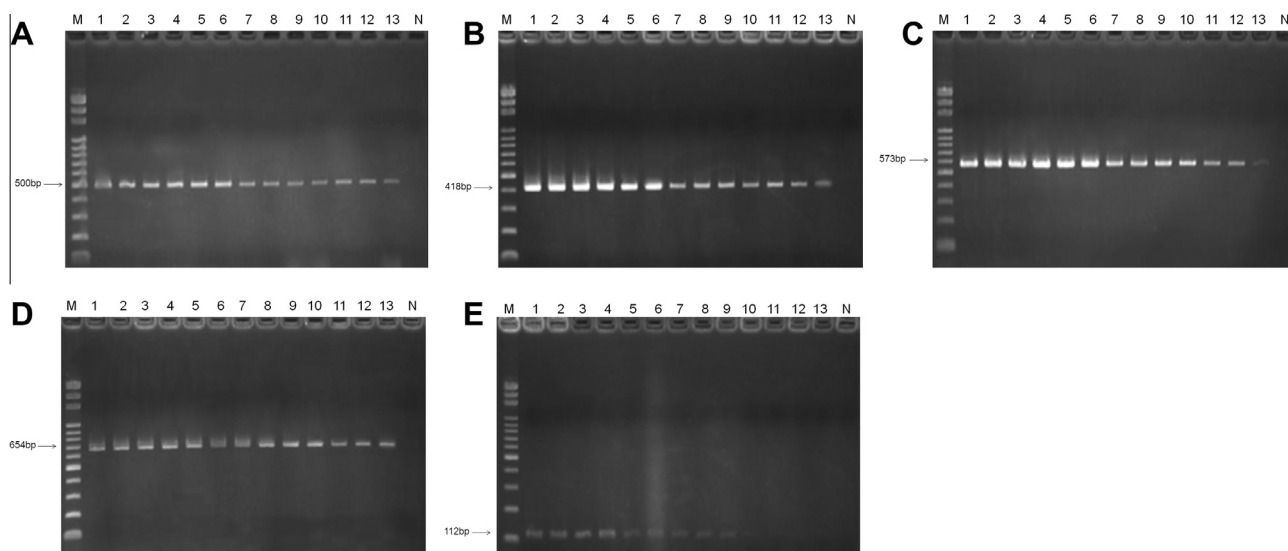


Fig. 4. Low temperature storage and freezing test. Agarose gel electrophoresis of the PCR products amplified using 1 ng cDNA from pork (A), chicken (B), goat (C), beef (D) and ostrich (E) stored at –80 °C for 1–13 weeks, corresponding to lanes 1–13, respectively. Lane M shows the molecular weight marker. Lane N shows the negative control without primer in the PCR reaction.

10–100 ng cDNA template for meat stored at 4 °C for 10 days and 1–100 ng cDNA template for meat stored at –80 °C for 13 weeks. There are few studies concerning the changes in adulterated meat characteristics during storage. In the present study, the adulterated meats were specifically and efficiently verified at different storage times under refrigeration.

3.6. Application of quantitative real-time PCR for meat adulteration

Conventional PCR techniques are typically used to obtain qualitative results for the identified species, although real-time PCR has been demonstrated as a useful tool for the determination of different species, even in adulterated foodstuffs (Fajardo, González, Rojas, García, & Martín, 2010; Fajardo et al., 2008). Because this method is easily and reliably applied to survey food products, real-time PCR is now generally utilized worldwide. In the present study, the specificity of real-time PCR was first evaluated using SYBR Green I dye and the TnI, MRP and Tmod gene sequences from pork, chicken, ostrich, goat and beef (Supplemental Table 2). The

linearity and standard curve for real-time PCR were determined using cDNA from each target species. The cDNA templates were diluted to 2000, 200, 20, 2 and 0.2 ng. The amplification curves were stably obtained when the cDNA template was lowered to 0.2 ng. The calculated R^2 values of the standard curves were 0.992, 0.992, 0.994, 0.995 and 0.990 for pork, chicken, ostrich, goat and beef cDNA, respectively, at a range of 0.2–2000 ng (data not shown). Additionally, the slopes for each species were –3.14, –3.33, –3.14, –3.07 and –3.37, consistent with the theoretical value (–3.32), achieved with a PCR efficiency of 100% (data not shown). The Ct values, presented as the means and standard deviation, obtained using the cDNA templates for the five meat species for real-time PCR are shown in Supplemental Table 4.

To assess the accuracy of this system for meat adulteration using real-time PCR, we used the Ct values to calculate the true and experiment values at different ratios of meat cDNA templates from pork (P), chicken (C), ostrich (O), goat (G) and beef (B), mixed together to generate six combinations (Table 1). The abbreviations of the six mixtures were MSC, MSO, MSG, MSB, MSP and MSA for

Table 1
Accuracy statistics for quantitative real-time PCR method.

	Species	n	True value (ng)	Experiment value (ng)	SD ^a	CV (%) ^b	Error ^c
MSC	Chicken	3	1200	1204.95	7.43	0.62	0.41
	Ostrich	3	200	224.03	0.95	0.42	12.01
	Goat	3	200	203.48	6.24	3.07	1.74
	Pork	3	200	201.98	9.74	4.82	0.99
	Beef	3	200	206.37	4.74	2.30	3.18
MSO	Chicken	3	200	201.48	5.36	2.66	0.74
	Ostrich	3	1200	1211.33	9.07	0.75	0.94
	Goat	3	200	198.44	9.68	4.88	0.78
	Pork	3	200	201.6	8.1	4.02	0.80
	Beef	3	200	204.09	0.97	0.48	2.05
MSG	Chicken	3	200	202.56	9.36	4.62	1.28
	Ostrich	3	200	213.96	3.98	1.86	6.98
	Goat	3	1200	1196.64	13.94	1.16	0.28
	Pork	3	200	201.26	10.54	5.24	0.63
	Beef	3	200	206.17	8.99	4.36	3.08
MSB	Chicken	3	200	202.96	12.78	6.30	1.48
	Ostrich	3	200	206.17	7.18	3.48	3.08
	Goat	3	200	203.39	5.16	2.54	1.69
	Pork	3	200	203.91	5.83	2.86	1.95
	Beef	3	1200	1210.47	3.08	0.25	0.87
MSP	Chicken	3	200	197.47	4.98	2.52	1.26
	Ostrich	3	200	198.34	5.63	2.84	0.83
	Goat	3	200	198.3	4.66	2.35	0.85
	Pork	3	1200	1208.29	37.32	3.09	0.69
	Beef	3	200	195.33	3.8	1.95	2.33
MSA	Chicken	3	400	407.55	1.97	0.48	1.89
	Ostrich	3	400	402.75	1.53	0.38	0.69
	Goat	3	400	414.51	10.34	2.56	1.13
	pork	3	400	412.43	8.95	2.17	3.11
	beef	3	400	404.11	3.62	0.90	1.03

^a SD is the standard deviation.

^b Coefficients of value are calculated by dividing standard deviation by mean value and are given in%.

^c $(|EXP. value - True value| / True value) * 100$.

real-time PCR using SYBR Green I. The values for the coefficient of variation (CV) for the six mixtures ranged from 0.42% to 4.82% for MSC, 0.7% to 2.05% for MSO, 0.28% to 2.98% for MSG, 0.87% to 3.08% for MSB, 0.69 to 2.33% for MSP and 0.69% to 3.11% for MSA, respectively. The error rate was 0.41–12.01% for MSC, 0.74–2.05% for MSO, 0.28–6.98% for MSG, 0.87–3.08% for MSB, 0.69–2.33% for MSP and 0.69–3.11% for MSA. Altogether, these results showed that the CV and error rate were within the acceptable range under 10% and 20% and demonstrated that this method is reliable for the analysis of meat adulteration (Karlen, McNair, Perseguers, Mazza, & Mermoud, 2007).

4. Conclusion

In this study, we developed simplex, multiplex, and quantitative real-time PCR techniques for the specific and quantitative detection of pork, chicken, beef, goat and ostrich species in raw and meat mixtures using RNA. We also established a PCR method to survey the various meats under low temperature storage using RNA. Species-specific primer sets were designed based on the DNA sequences of TnI, MRP and tropomodulin genes. The detection limit was 0.01 ng for simplex PCR, 20 ng multiplex PCR, 1–100 ng for the storage test and 0.2 ng for quantitative real-time PCR. Thus, in the present study, we successfully manipulated the RNA samples for the qualitative and quantitative analysis of individual and adulterated meats using mRNA-based PCR methods.

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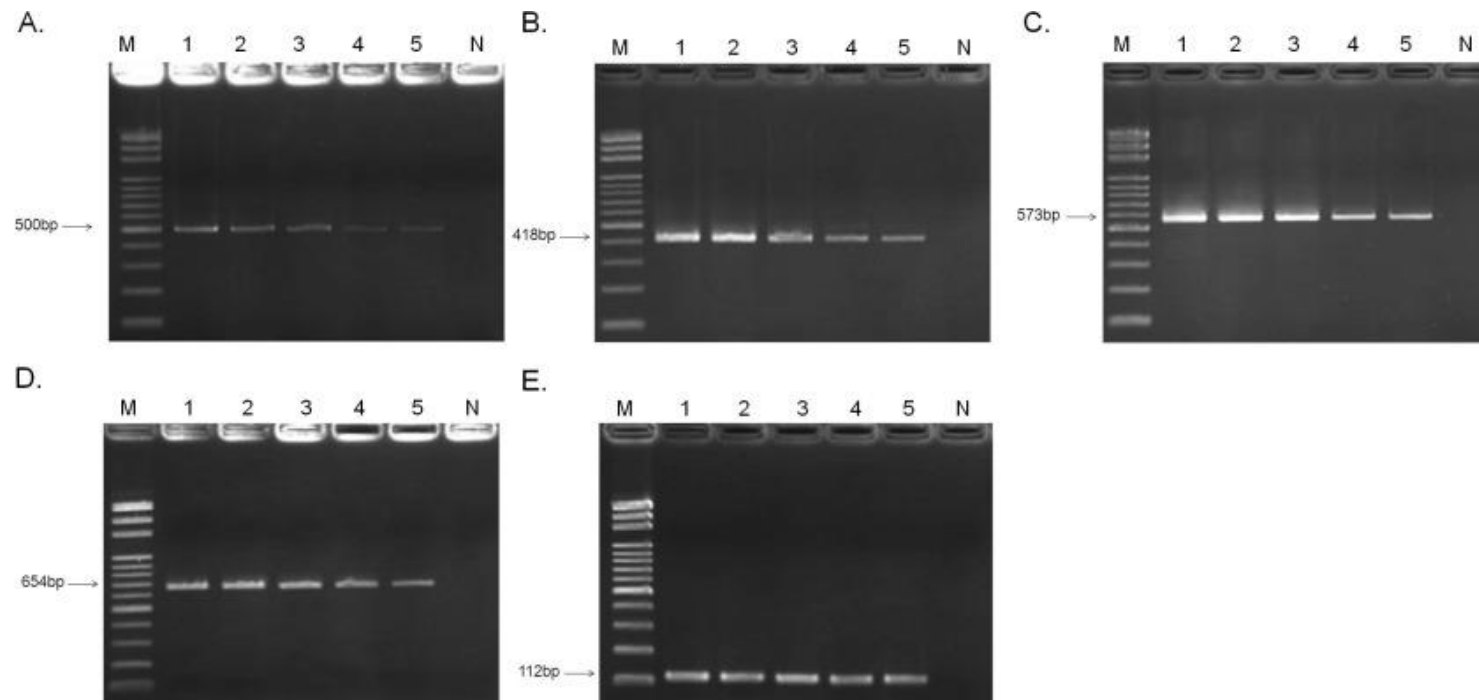
Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.foodchem.2015.06.094>.

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Supplementary Fig. S1. The sensitivity of the primers used in PCR reactions with cDNA templates obtained from RNA samples of pork (A), chicken (B), goat (C), beef (D) and ostrich (E). Lane M shows the DNA marker. Lanes 1–5 show the PCR products amplified using...

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Development of qualitative and quantitative PCR analysis for meat adulteration from RNA samples

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Supplemental Table 1. The ratio of meat adulteration.

Sample	Adulteration ratio (%)				
	B	P	C	G	O
MSO	10	10	10	10	60
MSG	10	10	10	60	10
MSC	10	10	60	10	10
MSP	10	60	10	10	10
MSB	60	10	10	10	10
MSA	20	20	20	20	20

MS: mix sample; O: ostrich; G: goat; C: chicken; P: pork; B: beef; A: all meat samples.

Supplemental Table 2. Species specific primers for PCR.

Primer	Meat species	Sequence	Gene	Amplicon (bp)
CK2-f	Chicken	5' GGC GCCAGCACCTGAAGAGTG 3'	Troponin I	418
CK2-r	(<i>Gallus gallus</i>)	5' TTCTCCTTCTCCGTGTCCTCC 3'		
P-f	Pork	5' GAGGAAGAAGCGTGCAGGGAG 3'	Troponin I	500
P-r	(<i>Sus scrofa</i>)	5' AGGCTGAGAAGGCTCGCTACC 3'		
GT3-f	Goat	5' GGGTGAAGCCATAAAGGGAGG 3'	Troponin I	573
GT3-r	(<i>Capra hircus</i>)	5' CACGCCCCGAGTGGACAAGGTG 3'		
B6-f	Beef	5' CTCACACCTCTGCTGACCCAG 3'	Mitochondrial ribosomal protein	654
B6-r	(<i>Bos Taurus</i>)	5' CACGCCAAGGACTCCCAGGTG 3'		
OS2-f	Ostrich	5' GATCCTGATAATGCGCTGCTG 3'	Tropomodulin	112
OS2-r	(<i>Struthio camelus</i>)	5' CTGCTTTTCTAGGTGGGCCA 3'		

Supplemental Table 3. Primer sequences for quantitative real-time PCR.

Primer	Meat species	Sequence	Gene	Amplicon (bp)
QCK-f	Chicken	5'GGACCTGAGCCAGAAGCTGTT3'	Troponin I	86
QCK-r	(<i>Gallus gallus</i>)	5'GCATGGCATCAGCAGACATG3'		
QP-f	Pork	5'CAACACCAGGGAGATCAAGGA3'	Troponin I	70
QP-r	(<i>Sus scrofa</i>)	5'GGGAGGGCGCTTGAAC TTC3'		
QG-f	Goat	5'CCCCATCCTGTAATTGGATACC3'	Troponin I	77
QG-r	(<i>Capra hircus</i>)	5'TGT AACTGGGCTGGGATGTG3'		
QB3-f	Beef	5' TCCGGCAACACTTTGTCCAA3'	Mitochondrial ribosomal protein	84
QB3-r	(<i>Bos Taurus</i>)	5' GCAGTTCAAACCTGCCGTTGT3'		
OS2-f	Ostrich	5'GATCCTGATAATGCGCTGCTG3'	Tropomodulin	68
QO-r	(<i>Struthio camelus</i>)	5'GCCAGTTGGTGGCTTTTGGGT3'		

Supplemental Table 4. The Ct values mean and standard deviation with five species meat of cDNA templates by real-time PCR.

Chicken					
Template conc. (ng)	2000	200	20	2	0.2
Signal rate ^a	3/3	3/3	3/3	3/3	3/3
Mean of Ct value ^b	17.22	19.69	23.06	26.39	30.52
SD Ct value ^c	0.09	0.05	0.09	0.02	0.03
Ostrich					
Template conc. (ng)	2000	200	20	2	0.2
Signal rate ^a	3/3	3/3	3/3	3/3	3/3
Mean of Ct value ^b	26.87	30.27	33.36	36.41	39.48
SD Ct value ^c	0.09	0.17	0.28	0.19	0.38
Goat					
Template conc. (ng)	2000	200	20	2	0.2
Signal rate ^a	3/3	3/3	3/3	3/3	3/3
Mean of Ct value ^b	27.23	29.81	32.98	36.25	39.36
SD Ct value ^c	0.16	0.05	0.14	0.13	0.32
Pork					
Template conc. (ng)	2000	200	20	2	0.2
Signal rate ^a	3/3	3/3	3/3	3/3	3/3
Mean of Ct value ^b	22.05	25.04	27.99	31.02	34.73
SD Ct value ^c	0.22	0.17	0.15	0.30	0.30
Beef					
Template conc. (ng)	2000	200	20	2	0.2
Signal rate ^a	3/3	3/3	3/3	3/3	3/3
Mean of Ct value ^b	29.42	32.89	36.20	39.75	42.82
SD Ct value ^c	0.13	0.17	0.37	0.26	0.52

a. Signal rate means positive signal/total reaction.

b. Ct refers to threshold cycle.

c. SD is the standard deviation.