Conventional multiplex polymerase chain reaction (PCR) versus real-time PCR for species-specific meat authentication

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Abstract: Simple and reliable conventional multiplex-polymerase chain reaction (multiplex-PCR) as well as real-time PCR approaches for the partial-length *cytochrome b* or *cyt b* gene of mitochondrial DNA (mtDNA) were compared to identify seven animal species for the authenticity of low non Halal (pig, dog, cat and donkey) and high value Halal (cattle, sheep and goat) meats. Either analysis indicated the successful detection of as little as 0.05 pg (5%) adulteration in cattle meat. As compared to real-time PCR, the approach of conventional multiplex-PCR can also be applied to detect authentication with equal efficiency to fresh, cooked or putrefied mixed samples of cattle meat.

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Key words: Mitochondrial DNA, *cyt b* gene, meat species identification, multiplex polymerase chain reaction (PCR).

1. Introduction

Identification of the species of origin in meat samples is relevant to consumers for the possible economic loss from fraudulent adulterations, medical requirements of individuals that might have specific allergies and for religious reasons (Asensio *et al*., 2008a). For some consumer groups, such as vegetarians, the contamination of food with meat residue is strictly prohibited. Another example of meat identification is the Halal food for the Muslim consumers, who are prohibited from consuming pork (Unajak *et al*., 2011). The extensive development of nucleic acid-based technologies over the past decades reflects their importance in food analysis. Various polymerase chain reactions (PCR)-based approaches were attempted for meat authentication. In recent years, PCR coupled with molecular techniques, such as hybridization, nucleotide-sequencing, single-strand conformation polymorphism (SSCP) or forensically informative nucleotide sequencing (FINS), were used for differentiation of buffalo meat from cattle meat (Murugaiah *et al*., 2009). The advantages of DNA-based analysis include the ubiquity, abundance and stability of DNA in all cell type (Jain *et al*., 2007).

PCR analysis of species-specific mitochondrial DNA (mtDNA) is the most common method currently used for meat species identification. The method is more sensitive in the identification of closely-related species as compared to nuclear DNA because each cell has only one set of genomic DNA in the nucleus, but bearing 10^4 copies of mtDNA (Parodi *et al*., 2002; Rastogi *et al*., 2007) with high mutation rate due to the poor corrective replication of polymerase and lack of proof-reading system in mitochondria. Following this approach, species-specific DNA in picograms (pg) can be detected in both processed and unprocessed meat samples. Genes to be targeted for amplification can be *12S*, *16S* and *18S rRNA*, *actin*, *cytochrome b*, *cytochrome oxidase*-II, *NADH dehydrogenase* 5/6 and *mtD*-loop (Kesmen *et al*., 2009; Singh and Neelam, 2011; Unajak *et al*., 2011). Very old samples of more than 100 million years can also be identified (Girish and Nagappa, 2009). Using conventional multiplex PCR, many targets can simultaneously be amplified, which helps in detection of many species in a short period of time (Bai *et al*., 2009; Ghovvati *et al*., 2009; Girish and Nagappa, 2009).

Heat-stable proteins have been reported to be useful targets for both the detection of animal remains and species identification in foods of animal origin, such as meat (Chen and Hsieh, 2000). However, methods based on DNA

amplification are still preferred, as they are less affected by industrial processing (Pascoal *et al*., 2005). Generally, mitochondrial DNA (mtDNA)-based PCR methods were proved to be good option for the analysis of putrefied samples or those submitted to heat treatment in which DNA has been partly degraded (Rodríguez *et al*., 2004). With the emergence of real-time PCR technology, PCR methods for mitochondrial encoded targets (Lahiff *et al*., 2002) have been reported for the detection of bovine material in feedstuffs (Fajardo *et al*., 2008; Mafra *et al*., 2008).

In the present work, conventional multiplex-PCR was proposed to identify seven meat species for the authenticity of low non Halal (pig, dog, cat and donkey) and high value Halal (cattle, sheep and goat) animal meats species. The study also aimed to establish the detection limit (sensitivity) and specificity of the PCR methods developed. Application of these species-specific PCR assays for detection of contaminating material from these species in industrial processed meat samples was also evaluated. In addition, real-time PCR was used for the detection of meat species to compare sensitivity to the conventional multiplex-PCR.

2.Materials and Methods

Meat samples

Thirty five meat samples, five from each of the seven species were collected. Cattle (*Bos taurus*), sheep (*Ovis aries*) and goat (*Capra hircus*) were collected from slaughterhouse, while pig (*Sus scrofa*) was obtained from meat markets in Egypt. Meat samples of cat (*Felis catus*) and dog (*Canis familiaris*) were collected from Veterinary Hospital, Faculty of Veterinary Medicine, Mansoura University, Egypt and donkey (*Equus asinus*) meat from Mansoura Zoo, Egypt. Samples from each species were collected under aseptic conditions in sterile plastic bags, then, stored at -20°C until processed.

DNA extraction

DNA was extracted from skeletal muscular fresh tissues of the seven different animal species using the commercial AxyPrep Multisource Genomic DNA Miniprep kit (cat. no. AP-MN-GDNA-50, Axygen Bioscience, CA, USA) following the manufacturer's manual.

Species-specific primers and PCR amplification

The primer sequences were derived from the *cyt b* gene sequences of various species. The primers were synthesized from Metabion, Germany to amplify partial-length *cyt b* gene. Primer sequences designed by Matsunaga *et al*. (1999) were used as one universal forward primer (SIM) and five different reverse primers (R) for amplifying species-specific mtDNA segments of the gene from goat, cattle, sheep, pig and donkey. Primers designed by Abdulmawjood *et al*. (2003) were used as two forward (F) and two reverse (R) primers for amplifying the gene from cat and dog (Table 1).

Table 1. Primer sequences utilized for PCR to amplify partial-length *cyt b* gene from DNAs of different animal species.

Name	Primer*	Sequences (5' – 3')
SIM	F	GAC CTC CCA GCT CCA TCA AAC ATC TCA TCT TGA TGA AA
Sheep	R	CTA TGA ATG CTG TGG CTA TTG TCG CA
Goat	R	CTC GAC AAA TGT GAG TTA CAG AGG GA
Cattle	R	CTA GAA AAG TGT AAG ACC CGT AAT ATA AG
Pig	R	GCT GAT AGT AGA TTT GTG ATG ACC GTA
Donkey	R	CTC AGA TTC ACT CGA CGA GGG TAG TA
Dog	F	GGA GTA TGC TTG ATT CTA CAG
	R	AGA AGT GGA ATG AAT GCC
Cat	F	CTC ATT CAT CGA TCT ACC CA
	R	GTG AGT GTT AAA ACT AGT ACT AGA AGA

*See Matsunaga *et al*. (1999) and Abdulmawjood *et al*. (2003).

Original conventional PCRs for different species were performed in reaction volumes of 50 µl using 1 ng of genomic DNA of each species (gathered from five replicates), 25 pmoles of each primer, 1x Taq DNA polymerase buffer, 2 mM $MgCl_2$, 0.2 mM dNTPs and 0.2 Taq DNA polymerase (Finnzymes, Thermo Scientific, Finland). PCR (Mastercycler Gradient, Eppendorf, Hamburg, Germany) was carried out by initial denaturation at 94°C for 4 min, followed by 35 cycles each at 94°C for 60 s, annealing temperature at 48 to 58°C for 60 s, polymerization temperature at 72°C for 60 s and final extension at 72°C for 10 min, then, the samples were held at 4°C. The amplified mtDNA fragments were separated on 2% agarose gel, stained with ethidium bromide, visualized on a UV Transilluminator and photographed by Gel Documentation system (Alpha Imager M1220, Documentation and Analysis System, Canada).

Real time-PCR was carried out using the Agilent Mx3000P qPCR Systems (Agilent technologies, Palo Alto, CA, USA) with different primers. The reaction components were 12.5 µl

Maxima™ SYBR Green/ROX qPCR Master Mix (Thermo Fisher Scientific, Fermentas, Lithuania), 25 pmoles of each forward or reverse primer, and water (nuclease-free water) up to 22.5 µl. Then, 2.5 µl of diluted cDNA template (1/10) was added. Amplification was carried out in triplicates along with a no-template negative control (nuclease-free water). To avoid false positives due to DNA contamination, PCR reaction was carried out for all RNA samples (data not shown). The thermal cycling conditions were similar to those for conventional PCR. Data were collected and amplification plots of ΔRn versus cycle number were generated for analysis.

Table 2. Binary mixtures (in ng) of different DNAs for conventional multiplex PCR.

Cattle	Goat	Sheep	Pig	Donkey	Cat	Dog
0	1	1	1	1	1	1
0.75	0.25	0.25	0.25	0.25	0.25	0.25
0.9	0.1	0.1	0.1	0.1	0.1	0.1
0.95	0.5	0.5	0.5	0.5	0.5	0.5
1	0	0	0	0	0	0

Preparation of heat-treated and putrefied meats

Twenty milligrams of meat were wrapped from each species (cattle, goat, sheep, pig, donkey, cat or dog) in aluminum foils and heated in autoclave at 120°C for 30 min. Other meat samples were allowed to putrefy in a natural condition at room temperature by leaving 10 g from samples of each species (cattle, goat, sheep, pig, donkey, cat or dog) in seven different small Petri dishes for seven days. Then 20 mg meat samples were taken from each species for each treatment. DNA extractions and PCRs were done as previously indicated for fresh samples.

Preparation of meat binary mixtures

Binary mixtures were prepared for multiplex PCR by adding DNA from each species (goat, sheep, pig, donkey, cat or dog) to DNA of beef (cattle). The earlier DNAs were added in the percentages of 100 (1 ng), 25 (0.25 + 0.75 ng cattle DNA), 10 (0.1 + 0.9 ng cattle DNA), 5 (0.05 + 0.95 ng cattle DNA) or 0 (1 ng cattle DNA) (Table 2).

3. Results and Discussion

Original amplification of partial-length cyt b gene from different species

Original PCR was planned to amplify partial-length *cyt b* gene differing in amplicon sizes for the identification of different meat species samples. The results indicated successful amplification of the target *cyt b* gene sequences with the expected amplicon sizes (157, 274, 331, 398, 439, 672 and 808 bp for goat, cattle, sheep, pig, donkey, cat and dog, respectively (Figure 1). PCRs to detect cross species amplification were negative, that is, goat-specific primers gave amplicons only in goat mtDNA and not in any mtDNAs of the other species (data not shown).

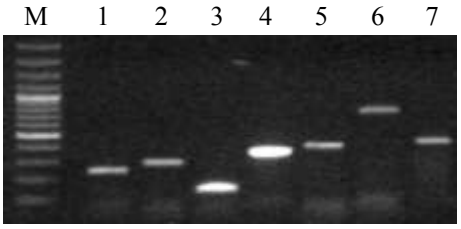

Figure 1. Electrophoretic analysis of partial-length *cyt b* amplicons of DNAs of different meat species samples. Lanes 1 to 7 represent cattle, sheep, goat, pig, donkey, dog and cat DNA samples. Lane M represents molecular size marker (100-bp DNA ladder, New England Biolabs, Ipswich, MA, USA, cat. no. N3231S).

PCR profiles of binary mixtures of different foreign meat samples with cattle

The PCR sensitivity test of binary meat mixtures shown in Figure 2 indicated that contaminants with as low as 5% DNA (5 pg) of different foreign meat species (goat, sheep, pig, donkey, cat or dog) mixed with 95% DNA from cattle (0.95 ng) were successfully detected (Figure 2a to f, respectively) with the expected amplicon sizes. As for the different reactions with 100% (1 ng) cattle (Figure 2a to f, lane 1), the results indicated no cross species amplification has been found for any species-specific pair of primers.

PCR profiles of heat-treated and putrefied meat samples

Results of PCR with the meat samples subjected to heat treatment to simulate cooking (120°C for 30 min) as well as those subjected to putrefaction before DNA was extracted (from 20 mg muscle samples) and tested via conventional PCR to amplify partial-length *cyt b* gene are shown in Figure 3. Amplicons resulting from either treatment were quite similar to those of the fresh meat samples. In other words, heat or purification did not affect efficiency of amplification of partial-length *cyt b* gene of different meat species.

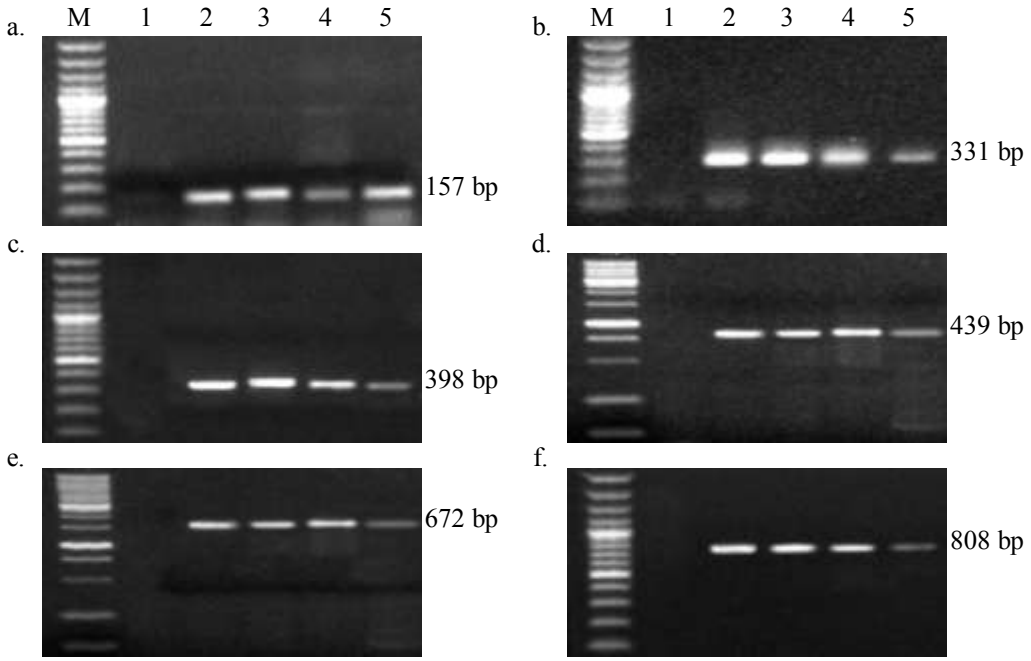

Figure 2. Electrophoretic analysis of partial-length *cyt b* amplicons (Lanes 1 to 5) from binary DNA mixtures (0, 1, 0.25, 0.1 and 5 ng, respectively) of different meat species samples mixed with cattle (1, 0, 0.75, 0.9 and 0.95 ng, respectively). A to f represent mixtures involving goat, sheep, pig, donkey, cat or dog DNAs, respectively.

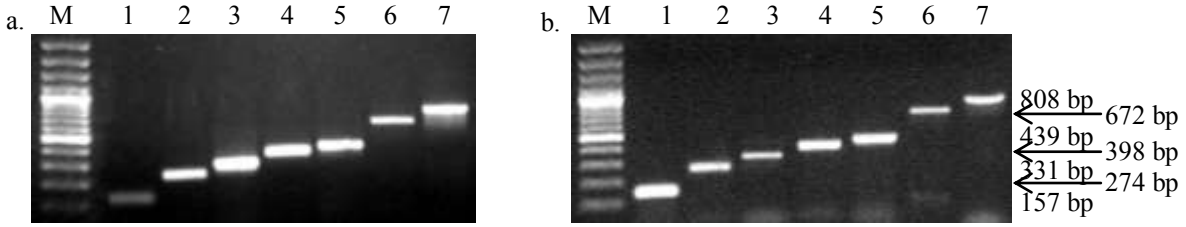

Figure 3. Electrophoretic analysis of partial-length *cyt b* amplicons from DNAs of heat-treated (a) and putrefied (b) meat samples of the seven animals species. Lanes 1 to 7 represent DNAs of goat, cattle, sheep, pig, donkey, cat and dog.

Conventional multiplex PCR

Multiplex PCR of individual animal species was conducted using the seven species-specific primer mixtures. All the primers were mixed in the following proportions (5:1:1:1:1:1:1:1:1:1) for SIM-F : goat-R : cattle-R : sheep-R : pig-R : donkey-R : cat-F : cat-R : dog-F : dog-R, respectively. Two microlitres of this mixture was incorporated in the PCR to give 10 pmoles of each primer except for SIM-F that was represented by 50 pmoles. This multiplex PCR (1 ng/reaction) was tested on DNA samples from each species as well as DNA mixture of the equally mixed seven species. The electrophoretic banding pattern is shown in Figure 4. The results indicated the presence of intense target amplicon for each single species with the absence of any cross reaction (Figure 4, lanes 1 to 7) regardless of primer multiplexing. Additionally, multiplex PCR with the seven DNA samples was successful in detecting the target seven amplicons in the reaction, however, with lower band intensities (Figure 4, lane 8) probably due to multiplexing of DNAs and primer pairs of different animal species.

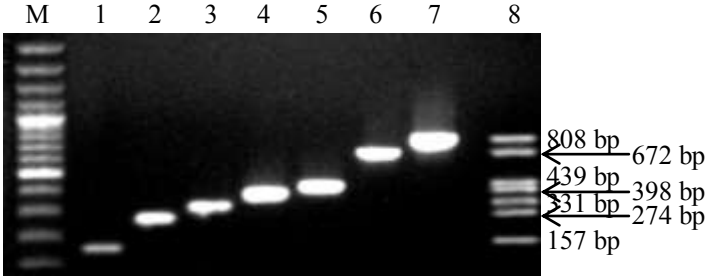

Figure 4. Electrophoretic analysis of conventional multiplex PCR products from DNAs of meat samples from the seven animals species individually (1 to 7) and mixed. Lanes 1 to 7 represents goat, cattle, sheep, pig, donkey, cat and dog DNA samples. Lane 8 represents DNA mixture of the seven animal species samples.

Real-time PCR

In the present study, real-time PCR was supplied with dye detection software (Agilent, USA) to determine the SYBR Green I dye spectrum using a multicomponent algorithm. The Rn value expresses the magnitude of the normalized fluorescence signal generated by the dye for each cycle during PCR amplification. The point at which the amplification plot crosses the threshold is defined as C_t (threshold cycle). The specificity of real-time PCR was evaluated by the amplification of *cyt b* partial-length gene from the mixed mitochondrial DNAs (1 ng/reaction) of different animal species with the seven sets of primers. The results indicated the presence of the target species-specific amplification curve with different C_t value specific for each animal DNA (Figure 5 and Table 3).

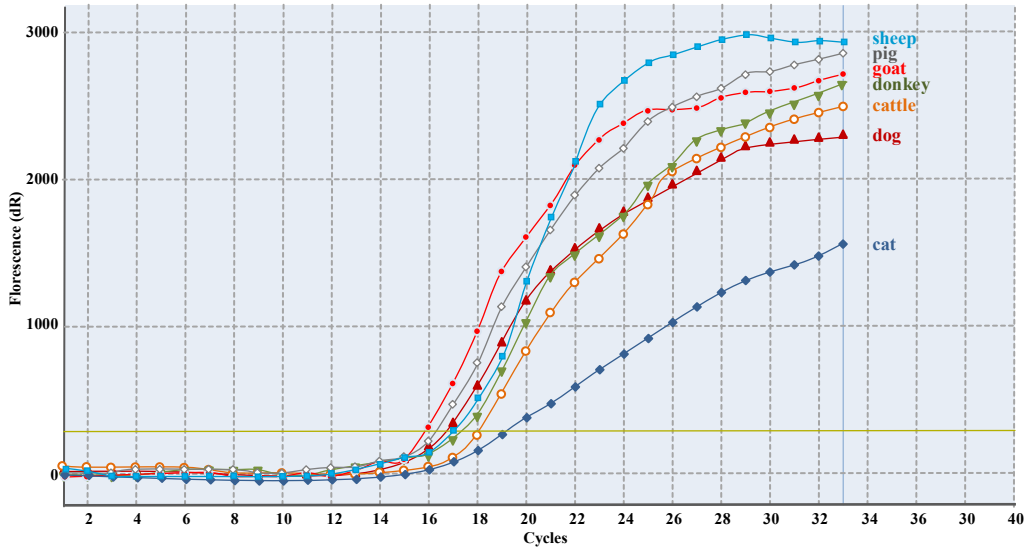

Figure 5. Real-time PCR amplification curves of *cyt b* gene fragments of mtDNAs of the seven animal species.

Table 3. Real-time PCR results for the partial-length *cyt b* gene amplification with DNAs of different meat species.

Animal species	Fragment size (bp)	Threshold (C_T) value	Melting temperature (T_m) (°C)
Goat	157	15.79	78.45 - 81.75
Cattle	274	17.55	82.25 - 84.95
Sheep	331	17.09	80.75 - 83.20
Pig	398	15.94	80.35 - 83.65
Donkey	439	17.28	84.15 - 86.45
Cat	672	19.54	81.75 - 84.55
Dog	808	16.48	81.25 - 84.55

Generally speaking, PCR has proved to be reliable, sensitive and fast technique in detecting species-specific mtDNA fragments (Fajardo *et al.*, 2007; Kesmen *et al.*, 2007; Martín *et al.*, 2007). We successfully developed a species-specific multiplex-PCR assay to examine seven kinds of animal species in which four of them are commonly used in meat adulteration. In the present work, the universal specific primers in combination with species-specific primers for *cyt b* gene vastly increase the specific sensitivity. The results for mtDNA templates coming from fresh, cooked or putrefied meat were optimistic. The present method has similar sensitivity with that of previous methods (Bai *et al.*, 2009; Mane *et al.*, 2009) in which no cross-reactivity was shown with mtDNAs of other animal species.

Food composition and authenticity assessment is becoming a very important issue by avoiding unfair competition among producers, allowing consumers to have accurate information about the acquired products. Following the European Union labeling regulations, meat products should be accurately labeled regarding their species content (European Commission, 2001). Quality evaluation in these products encompasses many issues, such as the fraudulent substitution of higher commercial value meats by lower value meats (Fajardo *et al.*, 2008), the presence of undeclared species (Aida *et al.*, 2005) and the use of vegetable proteins, since they have a considerably lower price than muscle proteins (Belloque *et al.*, 2002). Furthermore, the presence of undeclared ingredients may be troublesome for health reasons, such as in the case of bovine spongiform encephalopathy due to the addition of infected neurological tissue and because of allergic reactions in sensitised individuals (Asensio *et al.*,

2008b). The most important problem to be considered in meat species adulteration is related to religious practices as in some religions, such as Islam and Judaism pork meat consumption which is forbidden. Although, the species of origin in raw meats can be identified by using most of protein-based methods, some authors showed that they are significantly less sensitive in the evaluation of thermally processed foods because of specific epitopes alterations (Rodríguez *et al*., 2004).

Mitochondrial DNA molecules coupled with polymerase chain reaction (PCR) represents a fast, sensitive and highly specific alternative to protein-based methods (Mafra *et al*., 2008). Conventional PCR techniques are generally able to produce qualitative results of the identified species, while real-time PCR has demonstrated to be a useful tool for the determination of minute amounts of different species, even in complex foodstuffs (Fajardo *et al*., 2008; Mafra *et al*., 2008). Real-time PCR is probably the most used quantitative mtDNA-based method, however, the high cost of the equipment and reagents is still a drawback for the application of this technique in most laboratories. Alternatively, other approaches based on conventional multiplex PCR for quantitative analysis have been tested (Mafra *et al*., 2007) and recommended (the present work). We claimed that the latter process is efficient enough to detect as little as 5% (5 pg DNA contaminants) of other animal species. When committing adulteration of meat, it is not logical to mix low quality meats at percentages lower than 5%.

In conclusion, this study suggests an accurate analytical technique for detecting meat adulteration by conventional multiplex PCR analysis of the *cyt b* gene of animal mtDNA. This technique was used to detect and trace meat adulteration and to differentiate species present in meat mixture. The test could also be used and applied by researchers and quality control laboratories for verification and control of industrial meat products, such as Halal authentication and raw material origin certification

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