Species determination and authentication of meat samples by mitochondrial 12S rRNA gene sequence analysis and conformation-sensitive gel electrophoresis

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Partial fragment of 450 bp of mitochondrial (mt) 12S rRNA gene was PCR-amplified and sequenced from cooked and uncooked meat samples of various known and unknown origin, viscera, and biological fluids like blood and semen of suspected origin, using universal primers. Homology search for obtained sequences was carried out using BLAST at NCBI. The technique was found to identify the meat tissue, semen, blood and viscera up to species level, even when the tissues were preserved at ambient temperature for prolonged periods of time. Further, to detect the adulteration in meat conformation-sensitive gel electrophoresis (CSGE) was used, which successfully detected adulteration in meat tissue even when the level of adulteration was as low as 10%. Overall, the simplicity of CSGE patterns could make this technique suitable for meat authentication in routine food forensic analysis.

IDENTIFICATION of meat offered for sale and in eating joints is necessary for consumer preference and regulatory surveillance. In India, it becomes especially important due to socio-religious issues associated with the preference. The conventionally available methods include various forms of electrophoresis[1] and use of immune sera in agar gel diffusion[2]. However, these methods depend on protein expression pattern and suffer from the disadvantage that after cooking the proteins get heat denatured. This results in the alteration of electrophoresis pattern. Moreover, immune sera often show cross species reactivity. Hence there is need for a method that would give unambiguous conclusion and also could work with cooked samples. Methods based on PCR amplification of specific genes could be used for this purpose, because even after subjecting to heat during cooking, the DNA would still be amenable to PCR amplification. Moreover, even if there is partial degradation after prolonged storage, smaller fragments could still be amplified. Mitochondrial 12S rRNA gene sequence analysis is extensively used in molecular taxonomy and phylogeny[3]. In our laboratory, we have shown utility of these for species identification and detection of cross-contamination in cell lines[4–7] and also for identification of a disputed skin sample received in a forensic laboratory[8]. In a recent publication[9], the same technique has been used for species identification of meat; however the samples used were processed in the laboratory to simulate the cooking procedure. In this communication, we successfully employ the technique for the identification of species origin of various cooked and uncooked meat samples, viscera, and biological fluids like blood and semen. The specimens used here were actually collected from households and restaurants. We also successfully demonstrate utility of conformation-sensitive gel electrophoressis (CSGE)[10] for the detection in adulteration in meat.

Blood, semen, viscera, and meat tissues of unknown origin were obtained from Regional Forensic Science Laboratory, Pune. Meat samples of buffalo, cow, goat, pig, sheep and chicken of known origin were collected from the local slaughterhouse and from restaurants cooked in a conventional way. DNA extraction from the uncooked meat tissue was carried out directly, whereas cooked meat was washed thoroughly with sterile distilled water to remove traces of spices and oil. DNA extraction was carried out as described earlier[8]. A 450 bp mt 12S rRNA gene fragment was amplified from this DNA using 'Universal Primers'[11]. Primer sequence and PCR conditions were as described earlier[8]. The PCR product was then directly used for sequencing by Big Dye Terminator v3.1 cycle sequencing kit (Applied Biosystems, Foster City, USA) using ABI 310 Automated DNA sequencer. We were able to get a clean and unambiguous sequence even with the PCR product from the cooked meat. Homology search for these sequence was done using BLAST[12] at the National Center for Biotechnology Information (NCBI), USA. All the meat samples showed a high degree of homology to corresponding sequences in GenBank, irrespective of whether cooked or uncooked meat was used for extracting the DNA (Table 1). This indicated that mt 12S rRNA gene could be amplified from cooked meat and the sequence of the PCR product could be used for species identification.

The samples that arrive in the forensic laboratories are not always fresh. In order for the method to have wider application, it should also be able to give conclusive results on those samples that are stored under non-sterile conditions for varying periods of time. For testing this, we kept cooked as well as uncooked meat at various periods of time at ambient temperature (25–40°C) and then performed DNA extraction and PCR. It was observed that even after storing at ambient temperature for 10 days under non-sterile conditions, PCR amplifiable DNA could be obtained even though there is some amount of degradation of DNA (as seen by streaking in the agarose gel, Figures 1 and 2).Thereby indicating that the method works well on the samples that might be at ambient temperature for prolonged periods of time.

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Table 1. Homology for sequences of PCR products of known tissue samples

Sample	Closest match (first five hits in BLAST analysis)	Accession number	Score	*E*-value
Goat meat	*Capra hircus*	AJ490504	733	0.0
	C. hircus	AF533441	726	0.0
	C. hircus	M55541	726	0.0
	Capra falconeri	AY141133	702	0.0
	Capra cylindricornis	AF363778	686	0.0
Pig meat	*Sus scrofa* breed Chinese	AF304200	805	0.0
	S. scrofa breed Large White	AF486874	805	0.0
	S. scrofa breed Chinese Wannanhua	AF486873	805	0.0
	S. scrofa breed Chinese Jiangquahai	AF486872	805	0.0
	S. scrofa breed Chinese Yushanhei	AF486871	805	0.0
Chicken meat	*Gallus gallus*	AP 003318	745	0.0
	G. gallus	AP003580	745	0.0
	Gallus varius	AY235571	745	0.0
	G. gallus	X52392	737	0.0
	G. varius	AP003317	737	0.0
Cow meat	*Bos indicus*	AJ 490501	406	e-110
	B. taurus	AJ630114	398	e-108
	B. taurus	V00654	398	e-108
	Bison tarus	AB074968	398	e-108
	B. tarus	AB074967	398	e-108
Sheep meat	*Ovis aries*	AJ490503	765	0.0
	O. aries	AF091699	751	0.0
	O. aries	AF010406	741	0.0
	Ovis ammon	AY141135	678	0.0
	O. aries	AJ000850	672	0.0
Buffalo meat	*B. indicus*	AJ 490501	400	e-111
	B. taurus	AJ630114	400	e-109
	B. taurus	V00654	400	e-109
	B. taurus	AB074968	345	e-109
	B. taurus	AB074967	323	e-109

Closest match refers to the closest match in BLAST search. Accession number is the number for the GenBank sequence. Score and *E*-value are statistical parameters. *E*-value decreases exponentially with the score that is assigned to a match between two sequences. This means that lower the *E*-value, or closer it is to zero, more 'significant' is the score (match). Detailed explanation can be found at the NCBI website (www.ncbi.nlm.nih.gov).

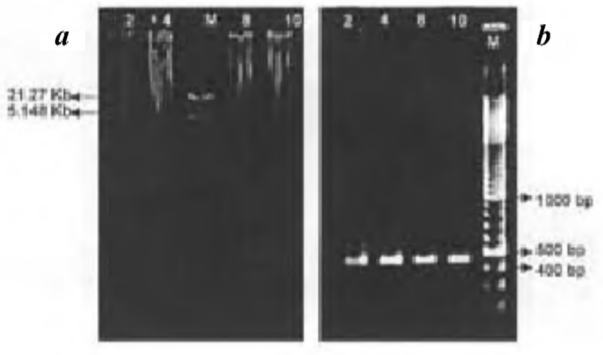

Figure 1. DNA extraction and mt 12S rRNA gene PCR from uncooked meat samples. (*a*) Genomic DNA and (*b*) PCR products after 2, 4, 8 and 10 days respectively. M, Molecular weight marker; Lambda *Hin*dIII digest (Roche Molecular Biology) for genomic DNA and 100 bp step DNA ladder (Gibco BRL) for PCR product.

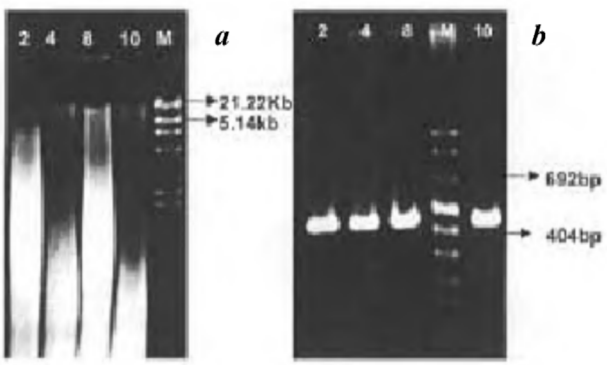

Figure 2. DNA extraction and mt 12S rRNA gene PCR from cooked meat samples. (*a*) Genomic DNA and (*b*) PCR products after 2, 4, 8 and 10 days respectively. M, Molecular weight marker; Lambda *Hin*dIII digest (Roche Molecular Biology) for genomic DNA and Marker VIII (Roche Molecular Biology) for PCR product.

Table 2. Homology for sequences of PCR products from unknown tissue samples

Sample	Closest match	Accession number	Score	*E*-value
Unknown A (meat)	*Capra hircus*	AF533441	795	0.0
	C. hircus	M55541	795	0.0
	C. hircus	AJ490504	777	0.0
	C. falconeri	AY141133	763	0.0
	C. cylindricornis	AF363778	765	0.0
Unknown B (meat)	*B. indicus*	AJ490501	365	5e-98
	B. taurus	AJ630114	357	1e-95
	B. taurus	V00654	357	1e-95
	B. taurus	AB074968	357	1e-95
	B. taurus	AB074967	357	1e-95
Unknown C (meat)	*G. gallus*	AP003318	753	0.0
	G. gallus	AP003580	753	0.0
	G. gallus	AY235571	753	0.0
	G. gallus	X52392	745	0.0
	G. gallus	AP003317	745	0.0
Unknown D (meat)	*O. aries*	AF091699	787	0.0
	O. aries	AF010406	777	0.0
	O. aries	AJ490503	767	0.0
	O. aries	AJ000850	710	0.0
	Hemitragus jemlahicus	AF401511	700	0.0
Unknown E (blood)	*Panthera onca*	AF416456	759	0.0
	P. onca	AY012151	706	0.0
	P. leo	Y08505	698	0.0
	P. pardus	D28895	674	0.0
	P. tigris	Y08504	668	0.0
Unknown F (viscera)	*P. onca*	AF416456	739	0.0
	P. onca	AY012151	702	0.0
	P. leo	Y08505	698	0.0
	P. tigris	Y08504	668	0.0
	P. pardus	D28895	668	0.0
Unknown G (viscera)	*P. onca*	AF416456	660	0.0
	P. onca	AY012151	617	e-174
	P. leo	Y08505	609	e-171
	P. pardus	D28895	595	e-167
	P. tigris	Y08504	589	e-165
Unknown H (semen)	*Homo sapiens* haplotype As9Y	AY 195792	593	e-166
	H. sapiens haplotype As10F	AY 195791	593	e-166
	H. sapiens haplotype As8D	AY 195790	593	e-166
	H. sapiens haplotype A3L1B1	AY 195789	593	e-166
	H. sapiens haplotype A5L2A1	AY 195788	593	e-166

Closest match refers to the closest match in BLAST search. Accession number is the number for the GenBank sequence. Score and *E*-value are statistical parameters. *E*-values decreases exponentially with the score that is assigned to a match between two sequences. This means that lower the *E*-value, or closer it is to zero, more 'significant' is the score (match). Detailed explanation can be found at the NCBI website (www.ncbi.nlm.nih.gov).

In order to confirm the validity of the method, we obtained actual cooked meat preparations from households, butcheries, restaurants and from the Regional Forensic Science Laboratory and labelled them as A–H. These were then subjected to DNA extraction, PCR and sequencing as described above. Homology values for the sequence of these PCR products are shown in Table 2, indicating that sample A was from goat, B from cow, C from chicken, D from sheep, E–G from leopard and H from human. The analysis has been repeated several times and consistent results could be obtained each time (data not shown). Thus, PCR amplification and sequencing of mt 12S rRNA gene could be used for unambiguous species identification of meat by the regulatory authorities. The technique has the advantage that only a small amount of material is required. The method could be used even for cooked meat and meat stored at ambient temperature for prolonged periods of time.

Surveillance to detect adventitious or fraudulent contamination in meat products serves to reassure all parties within the food supply chain. This is particularly true of ethnic groups whose religious beliefs forbid the consumption of particular species and who require assurance that they are purchasing food that conform to their beliefs. The ability to intercept adulterated meat before it enters do-

mestic commerce is a high priority issue. CSGE involves heteroduplex analysis of PCR products in a novel, mildly denaturating, polyacrylamide gel matrix using a different cross-linker, bis-acrolyl-piperazine, instead of the conventional bis-acrylamide. The technique was originally introduced for the detection of mutation in breast cancers[10]. We have employed this technique for detection of adulteration in meat. In order to ascertain the reliability of the technique, we first mixed amplified PCR products of mt 12S rRNA gene of buffalo, cow, goat and pig in various proportions. The PCR product, were prepared and subjected to CSGE as described earlier[10]. The formation of heteroduplexes could be seen even when PCR products are mixed in the ratio 1 : 9 (data not shown). CSGE analysis of PCR products from pure meat tissue does not result in heteroduplex band formation (Figure 3). Identical results were obtained when the tissues of different meat samples were mixed in different proportions prior to DNA extraction and PCR (Figure 4). Thus adulteration of up to 10% could be detected using this technique. The actual levels of adulteration being much higher than this, the technique thus becomes extremely useful for practical applications.

Thus the study indicates that DNA being a robust molecule, does not get damaged during extended periods of meat storage or by cooking. DNA can be amplified by PCR and amplified products can be directly used for sequencing and analysis for species identification. Similarly, the purity of meat samples can be easily detected by heteroduplex analysis done on the PCR product obtained from meat samples. This method can detect as low as 10% adulteration. Further studies are being carried out to simplify the process so that it can be applied at local forensics laboratories.

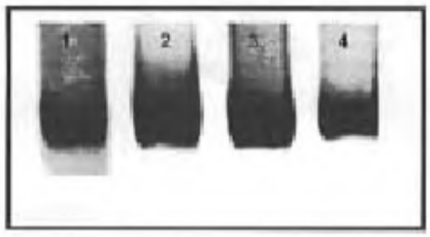

Figure 3. Conformation-sensitive gel electrophoresis of PCR product of 12S rRNA gene from pure meat samples. Lane 1, Buffalo; lane (2), Cow; lane 3 Goat; and lane 4, Pig. No heteroduplex was formed.

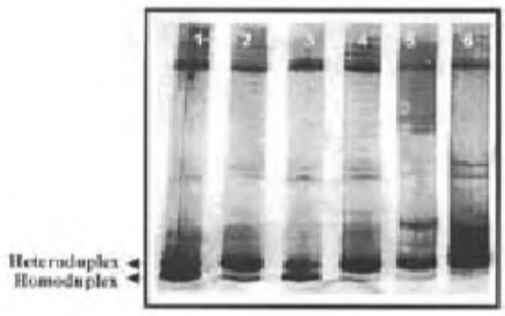

Figure 4. mt 12S rRNA gene PCR product obtained from the mixture of two different meat samples subjected to CSGE analysis by gel electrophoresis as described in text. Different lanes indicate the mixture of meat samples of different origin [ratio of 1 : 9] to carry out PCR amplifications. Lane 1, Buffalo + cow; lane 2, Buffalo + goat; Lane 3, Cow + goat; lane 4, Pig + buffalo; lane 5, Pig + cow and lane 6, Pig + goat.

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