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Determination of Genetic Distinction among Ruminant Species and Individuals Using STRs and mtDNA

B. Cinar Kul and O. Ertugrul
Department of Genetics, Faculty of Veterinary Medicine,
Ankara University, 06110 Ankara, Turkey

Abstract: In this study, researchers demonstrated powerful use of current molecular techniques for a veterinary forensic case of cattle thievery. This study aimed the identification of meat samples whether they belong to the same individuals to unveil the truth behind a case of thievery. The swab samples were collected from the crime scene and a catering factory from which the raw meet was allegedly stolen. To determine if the meat samples belonged to the same ruminant species, a PCR analysis was conducted based on amplification of the species-specific 12S-rRNA region in mtDNA. To determine if the meat samples belong to the same individiual, DNA profiling of the the autosomal micro-satellite loci common to all ruminants was employed. Using a commercial DNA isolation kit with a slight modification of test procedure, DNA was isolated from cotton swabs which were kept in improper condition for a period of longer than a year. No DNA band was obtained in agarose gel electrophoresis and no meaningful concentration value was obtained in spectrofotometric methods. However, isolated DNA was sufficient enough for PCR analysis and successful PCR results were obtained to conclude the case. As a result, the swab sample collected from the crime scene and suspected meat samples were bovine origin but their micro-satellites results exhibited that they belong to different individual from each other. Thus, the results of this study prevented the time loss due to false interpretation of the evidence and even a false accusation.

Key words: Animal forensic genetics, species-spesific PCR, mtDNA, micro-satellite, 12S-rRNA, Turkey

INTRODUCTION

Identification of biological materials collected in crime scenes and individual distinctions are vitally important to reveal the truth behind a crime. Biological materials to be used for forensic purposes are not often recovered in ample amounts and usually degraded as they are contaminated by environmental contaminants (Patrions and Ansorge, 2005). A method of individual typing based on a Variable Number of Tandem Repeat (VNTR) locus analysis developed by Jeffreys has been used since, 1985 instead of traditional typing methods based on morphology and protein analysis (Jeffreys *et al*., 1985).

As a consequence of outstanding developments in molecular technology, DNA profile analysis providing individual distinctions has become the most frequently employed technique in forensic cases (Fung and Hu, 2008). Likewise, DNA profile analysis along with other techniques is also used in veterinary forensic cases including illegal hunting, illegal food additives, abuse of animals, animal bites or attacks, theft of animals or biological materials (Taylor *et al*., 1993; Bellis *et al*., 2003; Hsieh *et al*., 2003; Eichmann *et al*., 2004; Martin *et al*., 2007). A number of methods are used in veterinary forensic cases. For instance, morphologic, biochemical and immunologic methods can be employed in identification animal species or individuals.

However if there is any interference using these methods such as degradation of samples or tearing apart to small pieces, molecular tests that analyze DNA polymorphisms give more accurate results as DNA can be isolated easily from blood, hair, semen and sloughed off epithelial cells recovered from various accessories in the surrounding.

Except monozygotic twins, every individual has a different set of DNA that positively influences DNA analyses for discrimination of individuals (Fung and Hu, 2008). For this purpose, Short Tandem Repeats (STRs) or micro-satellites in nuclear DNA (nDNA) and hypervariable Displacement loop (D-loop) region, 12S-rRNA, 16S-rRNA and Cytochrome b gene in mitochondrial DNA (mtDNA) are frequently analyzed (Jobin *et al*., 2008; Lee *et al*., 2009). In this study,

Corresponding Author: B. Cinar Kul, Department of Genetics, Faculty of Veterinary Medicine, Ankara University, 06110 Ankara, Turkey

researchers aimed to make species and individual identification of raw meat samples that had not been differentiated by morphological and immunological analyses.

MATERIALS AND METHODS

A swab sample was collected from blood stain of the slaughtered animal in place where the animal was slaughtered and two additional swab samples were also collected from blood stains and the raw meat in the holding room of the catering factory where the raw meat was transported to after allegedly stolen. Morphological identification of species of origin of the raw meat in the holding room was not possible. Each swab sample was enclosed in separate paper envelopes and transported to a molecular genetics laboratory.

After refusal of samples by three laboratories to conduct test for molecular identification, swab samples were submitted to the Molecular Genetics Laboratory of Faculty of Veterinary Medicine, Ankara University, a year after sample collection with an official request for analysis whether two swab samples collected from the catering factory and the swab sample collected from remaining parts of the slaughtered animal belonged to the same individual and whether the samples were of bovine origin.

DNA isolation: Each swab was fractionated with a disposable blade and then DNA isolation on these swab pieces was conducted using a QIAamp DNA blood mini kit (Qiagen, Hilden, Germany) according to the manufacturers's instructions. However, some procedures were modified due to storage of swabs longer than a year under improper circumstances. Volume of the chemical agents used were adjusted to a volume 8-folds higher than recommended volumes. Samples were treated with Proteinase K at 56°C for 3 h instead of half an hour. Elution was carried out with 30 μL of water in order to obtain higher amount of DNA. Isolated DNAs were run on a 0.6% agarose gel but no DNA bands were visualized and quality and quantity of DNA could not be measured using a NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). Elutions were stored at -20°C until assayed although, they were thought having inadequate templates for PCR reactions.

PCR amplification for species identification: The 12S-rRNA region of mitochondrial DNA differs among specie. Based on these sequence differences, the species origin of an unknown sample of DNA can be defined. In the present study, 12S-rRNA region species-specific primers designed by Martin *et al.* (2007) were used for species typing (Table 1). Species-spesific forward and reverse primers amplify the 12S-rRNA region of only one species. The cattle, sheep and goat species-specific forward and reverse primers were shown in Table 1. Three PCR reaction were conducted, each employing different set of species-specific primers. Each PCR reaction included known DNA samples of sheep, cattle and goat as positive controls and water as negative control in addition to isolated DNA samples from swabs. PCRs were performed in a 15 μL volume containing 1×PCR buffer (10 mM Tris-HCl, pH 8.8; 50 mM KCl; Fermentas, Thermo Fisher Scientific Inc.), 1.5 mM $MgCl_2$, 200 μM each dNTP, 5 picomole each primer and 1 U Taq DNA polymerase (Fermentas, Thermo Fisher Scientific Inc.) under the following conditions; denaturation at 94°C for 4 min followed by 30 cycles of denaturation at 94°C for 30 sec, annealing at 60°C for 30 sec and extension at 72°C for 30 sec; a final incubation was carried out at 72°C for 5 min in a Mastercycler EP gradient (Eppendorf, Hamburg, Germany) thermalcycler. The amount of template, DNAs which could not be calculated in ng were used one-tenth of the total PCR volume. PCR products (3 μL) were visualized in an Ethidium Bromide (EtBr) stained 2% agarose gel in 1×TBE buffer. The bands on gel were used as a measure of PCR success.

PCR amplifications for induvidual identification: Microsatellite loci BM1824, ETH10, ILSTS011 inRA23, SPS115, TGLA122 and TGLA227 were often used in parentage testing due to high heterozygosity values and allele numbers and mendelian inheritance. As the combined power of discrimination value and power of exclusion value of the STR/micro-satellite loci differ among populations, these values can not be calculated for the selected loci mentioned above. However, (Butler, 2005; Olowofeso *et al.*, 2005) researchers estimated the combined power of discrimination value as 0,999 based on heterozygosity values (except ILSTS011) of the Turkish Holstein population for these loci previously reported by Ozkan *et al.* (2009). The use of these seven loci as marker in identity testing in different

Table1: DNA sequences, the specificity and amplicon size of the used primers for species identification

Primers	Sequence (5'→3')	Amplicon size (bp)	Specificity
1.F:12SpVACADIR	TTAGTTGAATTAGGCCATGAAGCA	84	Cattle
1.R:12SpVACAINV	GTTTAAATAGGGTTAAGATGCACTCAATC		
2.F:12SpOVJDIR	CTAAGAATAGAGTGCTTAGTTGAACCAGG	121	Sheep
2.R:12SpOVJINV	GTCTCCTCTCGTGTGGTTCAGATA		
3.F:12SpCABRADIR	AAACGTGTTAAAGCACTACATC	122	Goat
3.R:12SpCABRAINV	GTCTTAGCTATAGTGTATCAGCTGCA		

cattle breed populations has been recommended because of their high power of exclusion value for parentage verification (Armstrong *et al*., 2006; Karthickeyan *et al*., 2008; Sharma *et al*., 2008; Van de Goor *et al*., 2011) (Eq. 1):

$$CPD=[1-(1-H_1)(1-H_2)(1-H_3)..... (1-H_n)]\times 100$$

PCR Reactions were performed in 15 μL volumes containing 1.5 μL template DNA (one-tenth of total PCR volume), 1.5 mM $MgCl_2$, 5 pmol each WellRED dye-labelled forward primer (Sigma-aldrich Inc., Canada), 5 pmol each reverse primer, 200 μM each dNTP, 1×PCR buffer (10 mM Tris-HCl, pH 8.8; 50 mM KCl; Fermentas, Thermo Fisher Scientific Inc.) and 0.5 U Taq DNA polymerase (Fermentas, Thermo Fisher Scientific Inc.). The samples were subjected to an initial denaturation step at 94°C for 4 min followed by 25 cycles of denaturation at 94°C for 30 sec, primer annealing at 58°C for 30 sec and primer extension at 72°C for 30 sec and a final extension step at 72°C for 15 min in a Mastercycler EP gradient (Eppendorf, Hamburg, Germany) thermalcycler. Fragment analysis of amplicons was conducted by capillary gel electrophoresis on a CEQ 8000 Sequence Analyzer (Beckman Coulter, Fullerton, CA, USA) with a CEQ DNA size standard-400 bp kit.

RESULTS AND DISCUSSION

Species identification: In PCR analyses, employing the cattle specific primers and specific amplifications were observed not only for control cattle DNA but also for DNA isolated from the suspected 3 test samples. However, no amplifications were observed in PCR analyses using either sheep or goat specific primers while sheep and goat control DNA samples were amplified (Fig. 1-3). Thus, it was concluded that all three samples were of bovine origin.

Individual identification: All samples were genotyped based on 7 micro-satellite loci common to all ruminants. All microsatellite loci were successfully amplified in three suspected samples. The obtained allelic profiles are shown in Table 2. As also understood from Table 2, each samples belonged to different individuals. A variety of methods are used for species and individual identification of animal materials including protein and fat electrophoresis, High Performance Liquid Chromatography (HPLC) or mass spectrometry

Table 2: Allelic profiles obtained by typing of autosomic STRs

Autosomic STRs	Sample 1*	Sample 2**	Sample 3**
BM1824	179-183	179-189	183-189
ILSTS011	265-265	265-269	267-267
INRA23	199-215	207-215	211-215
SPS115	246-262	250-256	250-262
TGLA227	97-97	89-95	82-99
ETH10	220-224	220-224	218-220
TGLA122	162-178	152-154	144-144

*From crime scene; **From holding room in the catering

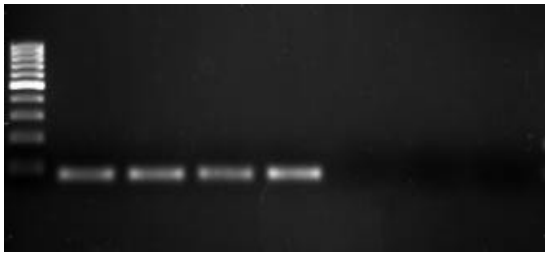

Fig. 1: The agarose gel picture of PCR assay conducted with cow specific primers. The well had 100 bp DNA marker, the 2-7 well had sample 1-3, control cow DNA, control sheep DNA and control goat DNA, respectively. The 8th well served as negative control

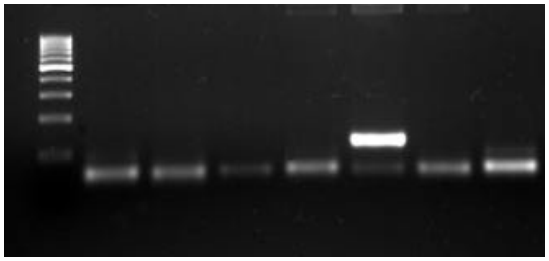

Fig. 2: The agarose gel picture of PCR assay conducted with sheep specific primers. The well had 100 bp DNA marker, the 2-7 well had sample 1-3, control cow DNA, control sheep DNA and control goat DNA, respectively. The 8th well served as negative control

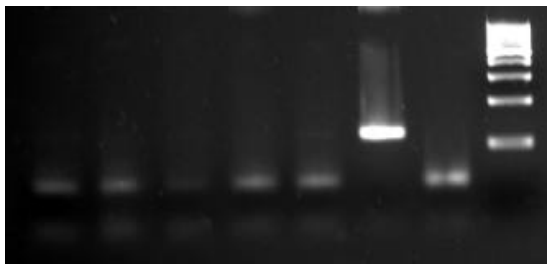

Fig. 3: The agarose gel picture of PCR assay conducted with goat specific primers. The well had 100 bp DNA marker, the 2-7 well had sample 1-3, control cow DNA, control sheep DNA and control goat DNA, respectively. The 7th well served as negative control (water)

(Taylor *et al*., 1993; Andrasko and Rosen, 1994; Espinoza *et al*., 1999; Czesny *et al*., 2000). In addition, sequencing and enzyme restriction profiles of species specific mtDNA variations and molecular techniques based on amplification using species-specific primers are also used for forensic purposes (Wolf *et al*., 1999; Tajima *et al*., 2002; Bellis *et al*., 2003; Martin *et al*., 2007; Nakaki *et al*., 2007). In the presented case, techniques based on protein analysis such as electrophoresis, chromatography and immunologic methods could not be employed due to insufficient amount of blood sample on cotton swab collected in the crime scene. Consequently, DNA based techniques were used for species and individual identification of the samples.

For species identification of the samples, researchers employed the method based on amplification of the 12S-rRNA region on mtDNA with species-specific primers (Martin *et al*., 2007). In species identification, mtDNA were preferred to genomic DNA because mtDNA has some advantages such as it has more target genome according to the mitochondria number and its uniqueness to each species.

Results of the present study supported the findings published by Martin *et al*. (2007) who reported that species-specific PCR could be used for identification of foodstuffs. The present study also shows valuable use of PCR technique for the identification of forensic evidence, especially when the amount of DNA is low. For individual identification, researchers used the method based on amplification of micro-satellite loci (STRs) which have high amplification rates in cattle, sheep and goat and often suggested for parentage cases and diversity studies by International Society for Animal Genetics (ISAG) and Food and Agriculture Organization of the United Nations (FAO) as STRs have high heterozygosity values and higher number of alleles. As these 7 loci have a high power of exclusion for different cattle breeds, amplification of these loci has been suggested for parentage and individual identification (Armstrong *et al*., 2006; Karthickeyan *et al*., 2008; Sharma *et al*., 2008; Van de Goor *et al*., 2011). Absence of 3 or higher number of alleles in all amplified suspected DNA samples excluded the possible cross contamination of samples of different origin thus, indicating each sample originated from seperate individuals.

In addition, using a commercial kit with slightly modified protocol researchers successfully isolated enough DNA from the swabs kept for a period longer a year in unsuitable conditions. The isolated DNA was not sufficient enough for agarose gel and spectrophotometric analysis but sufficient enough for PCR analysis at the same time. Thus, researchers did not have to order expensive DNA isolation kits developed for specifically forensic purposes.

CONCLUSION

As a result, the analyses for species and individual identifications indicated that all samples were of bovine origin and each sample evidently belonged to a different individual. Thus, the analysis suggested that the raw meat in the catering factory had different origins and unrelated to stolen animal. Importantly, use of molecular techniques in veterinary forensic cases provides powerful results even if the biologic material left in the crime scene is not in ample amount and stored improper conditions for a long time.

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