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Application of Species-Specific Polymerase Chain Reaction and Cytocrome *b* Gene for Different Meat Species Authentication

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Abstract: DNA was extracted from buffalo's, cattle's, pig's and sheep's muscles and Polymerase Chain Reaction (PCR) technique was used for detection, identification and authentication in raw meat samples by using Species-Specific Repeat (SSR). The PCR amplification size of the gene encoding SSR region in buffalo's, cattle's, pig's and sheep's meat were 603, 603, ≤100 and 374 bp, respectively. For discrimination between buffalo's and cattle's meat, amplified cytocrome *b* gene (359 bp) was digested by *Taq*I restriction enzyme. Two fragments 191 and 168 bp were generated in buffalo, whereas digested not with cattle (359 bp). The results showed that SSR and cytocrome *b* gene PCR-RFLP analysis provide a rapid and effective methods to detect the meat species and it could be easily identified and authenticated. In addition, the SSR PCR and cytocrome *b* gene PCR-RFLP methods are highly sensitive and will improve the detection limits for DNA sequences derived from these species. This finding could be easily used for authentication and detection of imported mad cow meat (lunacy) from origin countries.

Key words: Buffalo, cattle, pig, sheep, meat, species-specific, authentication, RFLP, PCR, cytocrome *b* gene

INTRODUCTION

Consumers are concerned by a variety of issues, such as food authenticity and adulteration. The identity of the ingredients in processed or composite mixtures is not always readily apparent and verification that the components are authentic and from sources acceptable to the consumer's maybe required (Lockley and Bardsley, 2000; Aida *et al*., 2005).

Consumers nowadays very seldom can identify the species in the products which they purchase: Carcasses or whole fish are rarely in display while cuts ether fresh or frozen, more or less processed (souse-vide, marinated, dried, smoked, salted, etc.) and prepared ready-to-eat products are increasingly available. This opens the possibility of fraudulent adulteration and substitution of the expected species with others of less value (Malmheden and Emanuelsson, 1998). To safeguard consumer rights, the legislation of each country should therefore impose a labeling of food products declaring the species used in their manufacture and food laboratories need to have available techniques to ascertain the species used in the manufacture of those products.

The risk associated with infectious transmissible spongiform encephalopathy in humans has discouraged many individuals around the globe from consuming beef. Hindu populations also choose not to eat beef, while Jewish and Muslim populations choose to avoid consumption of pork even in minute quantities, due to their religious beliefs. Many consumers prefer to include more chicken in their diet instead of beef or pork. In addition to infectious disease and religious concerns, many individuals are altering their eating behavior to include more chicken simply to reduce dietary fat intake in accordance with health trends. Any conceivable ambiguity in the labeling practices of commercial suppliers or grocery stores is unacceptable to these populations. The need for sensitive detection and quantization of buffalo, bovine, pork and sheep species in food and mixed food products is critical in response to this consumer demand. The quantitative detection of meat species in mixed samples has been approached using a variety

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of different systems. Early approaches to identify species-specific components within mixed samples involved the use of high-performance liquid chromatography (Espinoza *et al*., 1996; Inoue *et al*., 1990). These methods have proven useful for the identification of many animal species, but the detection limits using these approaches are restrictive. The detection of nuclear DNA sequences has also been useful in this regard, but is limited as a result of their generally low copy number (Meyer *et al*., 1994). Meat species identification using enzyme-linked immunosorbent assays (Chen and Hsieh, 2000) and protein profiles (Skarpeid *et al*., 1998) have also been used, but Polymerase Chain Reaction (PCR)-based assays are currently the method of choice for species identification (Calvo *et al*., 2001) PCR analysis of species-specific mitochondrial DNA sequences is the most common method currently used for identification of meat species in food (Herman, 2001; Lahiff *et al*., 2001; Partis *et al*., 2000; Montiel-Sosa *et al*., 2000) and animal feedstuffs (Bellagamba *et al*., 2001; Tartaglia *et al*., 1998; Krcmar and Rencova, 2001).

Bovine Spongiform Encephalopathy (BSE), commonly referred to as mad cow disease has a human form termed vCJD that is a variant of Creutzfeldt-Jakob disease, a fatal neurodegenerative disease that has caused many deaths in the United Kingdom (Brown, 2001). In response to the BSE epidemic in Europe, the United States Food and Drug Administration (FDA) imposed strict guidelines in 1997, prohibiting the use of ruminant-derived protein in the manufacture of animal feed intended for cows or other ruminants. Ruminants are defined as a suborder of the Artiodactyls, an order of mammals and represent the cud-chewing, families *Bovidae* (antelope, cattle, goats, sheep) and *Cervidae* (deer) as reported by Nowak (1991). It is widely believed that the practice of utilizing ruminant carcasses in animal feed for livestock is responsible for the spread of BSE to epidemic proportions. As a result, the need for sensitive detection of ruminant species remains in animal feed is a paramount agricultural issue. The conventional methodology used for the determination of species origin in meat and meat products have been predominantly based on the immunochemical and electrophoretic proteins analysis. More modern techniques now allow the identification of species-specific markers SSR, mt-DNA RFLP and RAPD-PCR techniques, which has two major advantages over protein analysis: samples heated to as high as 120°C can still be analyzed and discriminated between related species (Lenstra *et al*., 2001; Ahmed, 2005; Ahmed and Aliaa El-mezawy, 2005; Abdel-Rahman, 2006).

Mitochondrial DNA accumulates about 10 times as many mutations per unit as nuclear DNA and has thousands of copies per cell. Thus, amplification of a mitochondrial DNA segment is a relatively sensitive procedure and the identification of the species can be based on mutations in the amplification products. A simple and convenient way of testing for a mutation is RFLP (Restriction Fragment Length Polymorphism) analysis, which uses an enzyme with a recognition sequence created or abolished by the mutation. Species identification using PCR-RFLP of a mitochondrial cytochrome *b* segment has been well documented by Lahiff *et al*. (2001). The technique is equally applicable to the identification of species origin in cheese products (Branciari *et al*., 2000), as it is in meat products.

An alternative DNA detection system is based on the Polymerase Chain Reaction (PCR) and the amplification of a segment of the mitochondrial cytochrome *b* gene (Murray *et al*., 1995; Ahmed, 2005; Ahmed and Aliaa El-mezawy, 2005; Abdel-Rahman and Ahmed, 2007). Subsequent cleavage by a restriction enzyme gives rise to a species-specific pattern.

The present studies were application of species-specific polymerase chain reaction and cytocrome *b* gene for identification and authentication for different kind of buffalo's, cattle's, pig's and sheep's meat.

MATERIALS AND METHODS

This study was carried out at Nucleic Acid Research Department Genetic Engineering and Biotechnology Research Institute (GEBRI), Mubarak City for Scientific Research and Technology Applications, New Borg El-Arab City, Alexandria, Egypt from July-2006-to January-2007.

Meat samples: Meat samples of buffalo, cattle, pig and sheep from muscles were purchased and collected from Alexandria City, Egypt

DNA extraction: DNA was extracted from each muscle sample following the method described by Baradakci and Skibinski (1994) with some modifications. Approximately 0.5 g of the tissue was cut into small pieces and suspended in 1000 μL STE (0.1 M Na Cl, 0.05 M Tris and 0.01 M EDTA, pH 8). After adding 30 μL SDS (10%) and 30 μL proteinase K (10 mg mL^{-1}), the mixture was incubated at 50°C for 30 min. DNA was purified by successive extraction with phenol, phenol-chloroform-isoamylalcohol (25:24:1) and chloroform-isoamylalcohol (24:1), respectively. DNA was precipitated with ice-cold absolute ethanol and washed with 70% ethanol. The pellet was dried and resuspended in 200 μL dd H_2O.

Table 1: Primer sequences of species-specific repeat and cytochrome *b* gene, length PCR product and their annealing temperatures

Species	Primer sequence 5' → 3'	Length PCR product	Ann. temp. (°C)
Buffalo and cattle	AAG CTT GTG ACA GAT AGA ACG AT CAA GCT GTC TAG AAT TCA GGG A	603 bp	60
Pig	GGA GCG TGG CCC AAT GCA ATT GAA TCC ACT GCA TTC AAT C	≤100 bp	57
Sheep	GTT AGG TGT AAT TAG CCT CGC GAG AA AAG CAT GAC ATT GCT GCT AAG TTC	374 bp	62
Buffalo's and cattle's cytochrome *b*	CCA TCC AAC ATC TCA GCA TGA TGA AA GCC CCT CAG AAT GAT ATT TGT CCT CA	359 bp	57

PCR primers: SSR gene (603, ≤100 and 374 bp) and the segment of mt-DNA (359 bp) were amplified with the use of primers sequences as shown in Table 1 (Lenstra *et al*., 2001; Ahmed, 2005; Ahmed and Aliaa El-mezawy, 2005; Abdel-Rahman, 2006; Abdel-Rahman and Ahmed, 2007).

Amplification of species-specific repeat and mt-DNA cytochrome *b* gene: With some modifications, PCR was performed following the procedure of (Lenstra *et al*., 2001; Ahmed, 2005; Ahmed and Aliaa El-mezawy, 2005; Abdel-Rahman 2006; Abdel-Rahman and Ahmed, 2007). The reactions were carried out in (25 μL) consisted of 1.0 U of Taq DNA polymerase (Finnzymes), 25 pmol dNTPs, 25 pmol of random primer, 2.5 μL of 10X Taq DNA polymerase buffer and 50 ng DNA. The final reaction mixture was placed in a DNA thermal cycler (Eppendorf AG 22331, Gradient, Hambourg, Germany). The PCR program included an initial denaturation step at 94°C for 5 min followed by 35 cycles of 94°C for 30 sec for DNA denaturation, annealing temperatures as can be seen in Table 1, extension at 72°C for 30 sec and final extension at 72°C for 10 min. The samples were held at 4°C. Restriction enzyme *Taq*I was used to yield specific restriction profiles that allowed a direct identification of buffalo's and cattle's raw meat. PCR products resulted from cytochrome *b* gene amplification was digested with 10 U restriction endonuclease/12 μL of DNA products for 1 h incubation at 65°C.

Agarose gel electrophoresis: The amplified and digested DNA fragments were separated on 3% agarose gel and stained with ethidium bromide. ΦX174 DNA marker (1353, 1078, 872, …, 72 bp) and 100 bp DNA marker (2642, 1500, …, 500, 400, 300, 200, 100 bp) were used in this study. The amplified pattern was visualized on an UV Transilluminator and photographed by Gel Documentation system (Alpha Imager TM1220, Documentation and Analysis system, Canada).

RESULTS

PCR amplification of the gene encoding species-specific repeat in buffalo, cattle, pig and sheep yielded; 603, 603, ≤100 and 374, respectively. A comparison of the patterns with other farm animal species showed differences in the pattern size and allowed an identification of farm animal's meat. The results obtained after PCR amplification of the gene encoding species-specific repeat produced from buffalo, cattle, pig and sheep as different meat animal species are shown in Fig. 1. The results showed that SSR analysis provided and allowed a direct identification of cattle's, pig's and sheep's meat in raw meat samples from different animal's species, while the same SSR result (603 bp) in both buffalo and cattle was obtained as shown in Fig. 1. Obviously, the results showed that SSR technique provided a clear, rapid and effective method to discriminate the meat species of cattle, pig and sheep which is in agreement with the results of Lenstra *et al*. (2001).

For discrimination of buffalo and cattle species-specific meat, PCR-RFLP technique was used to amplify mt-DNA cytochrome *b* gene in both buffalo and cattle. As expected, the amplified fragment length of these two species was 359 bp. The digestion of cytochrome *b* gene (359 bp) using *Taq*I restriction enzyme yielded two fragments 191 and 169 bp only with buffalo, whereas with cattle the amplified cytochrome *b* gene (359 bp) was not digested (Fig. 2) allowing an identification of buffalo's and cattle's meat in addition to pig and sheep.

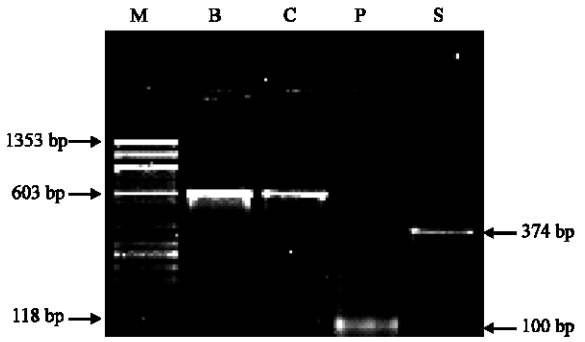

Fig. 1: PCR products generated by primers species-specific oligonucleotide. Where, lane M: DNA marker, lane B: buffalo, lane C: cattle, lane P: pig and lane S: sheep, respectively

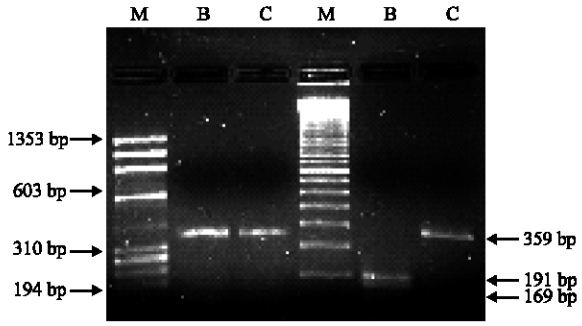

Fig. 2: PCR amplification products generated by cytochrome *b* primers. Lane M: DNA marker, lane B: buffalo and lane C: cattle. RFLP patterns of cytochrome *b* gene digested with restriction enzyme *TaqI*. Lane M: DNA marker, lane B: buffalo and lane C: cattle

DISCUSSION

PCR technology allows an amplification of specific regions of DNA, facilitating the detection of genetic differences between species or populations. With this PCR approach, it is important to establish genes showing variations for the species under study (Nowak, 1991; Bauer *et al*., 1995; Hunt *et al*., 1997; Janssen *et al*., 1998; Bauer *et al*., 1999; Lenstra and Buntjer, 1999; Brown, 2001; Lenstra, 2001; Ahmed, 2005; Ahmed and El-Mezawy, 2005; Abdel-Rahman, 2006; Abdel-Rahman and Ahmed, 2007). Application of SSR analysis for species-specific gene using specific primers and RFLP for PCR products of mt-DNA cytochrome *b* gene showed specific detection of buffalo's, cattle's, pig's and sheep's meat. However, in this study we provide a simple, rapid and universal method to specify kind of some farm animal's meat using two different techniques; species-specific PCR (buffalo, cattle, pig and sheep) and PCR-RFLP (buffalo and cattle) as shown in Fig. 1 and 2. Further studies must be done to design animal's species-specific primer and/or probe for the direct diagnosis and further analysis of the other mt-DNA regions to provide more sequence data for the differentiation of the animals.

Espinoza *et al*. (1996), Inoue *et al*. (1990), Calvo *et al*. (2001) and Lenstra *et al*. (2001) showed species specific variations, which might be useful for identifying the various species. However, the high cost of this technique and the need of individual sequences for detailed comparison make it inappropriate for the analysis of large numbers of samples from animal and poultry species. However, the use of species-specific analysis provides a simpler, quicker and cheaper alternative to sequencing for direct identification of animal species.

CONCLUSIONS

In conclusion, it can be emphasized that the primers amplified successfully the genomic DNA of the genotypes under study (buffalo, cattle, pig and sheep). This technique on the level of DNA detection analysis provided a rapid and effective method to detect the different animal's meat, also used for species-specific analysis and RFLP for PCR products of mt-DNA cytochrome *b* gene to provide us with a simpler, quicker and cheaper alternative for sequencing to direct identification of meat animal's species. Also, indirect application for detection of mad cow disease (lunacy) using PCR methods for authentication of cattle meat from origin countries.

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