PCR AMPLIFICATION OF SPECIES-SPECIFIC REPEAT FOR MEAT DNA IDENTIFICATION VIA GENETIC MARKERS IN CATTLE & SHEEP

M. M. Ahmed

Abstract: The designed and evaluated four assays based upon PCR amplification of species-specific repeat (SSR) for detection, identification and authentication of cattle and sheep on the DNA level. SSR primers were applied in the polymerase chain reaction (PCR), the products has been used for the specific identification of cattle and sheep meat. PCR amplification size of the gene encoding SSR region in cattle and sheep meat was 603 bp and 374 bp respectively. The results showed that SSR analysis produced a pattern that allowed a direct identification of cattle and sheep meat in raw and heat-treated samples. Also the results showed that SSR analysis provided with a rapid and effective method to detect the meat species and it could be easily identified and authenticated. In addition, the SSR PCR methods, as species-specific, are highly sensitive and will improve the detection limits for DNA sequences derived from these species. Also indirect application is the detection of disease cow (cattle) lunacy by PCR methods for authentication of cow (cattle) meat from origin country.

Key words: Identification, authentication, meat, species-specific, cattle, cow, sheep

Introduction and literature review

During the early development of DNA sequence analysis, genomic DNA was used as a species-specific probe and was hybridized to DNA extracted from meat samples (Bauer et al. 1987; Chikuni, et al.

1 Original paper – originalni naučni rad
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1990; Ebbehoj & Thomsen 1991). The subsequent development of probes derived from species-specific satellite repetitive DNA sequences has greatly improved the specificity of the assay, now making it possible to detect admixtures that contribute as little as 5%, or less, to a product. Two satellite hybridization procedures (Buntjer et al. 1995; Buntjer et al. 1999; Hunt et al. 1997; Janssen et al. 1998 and Lenstra and Buntjer, 1999), which differ in technical implementation rather than in principle or performance.

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 (Brown, 2001). As a result, the need for sensitive detection of ruminant species remains in animal feed is a paramount agricultural issue.

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 bovine, pork, and chicken 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 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 and 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 and Montiel-Sosa et al. 2000) and animal feedstuffs (Bellagamba et al. 2001; Tartaglia et al. 1998 and Kremar and Rencova, 2001). The advantage of mitochondrial-based DNA analyses derives from the fact that there are many mitochondria per cell and many mitochondrial DNA molecules within each mitochondrion, making mitochondrial DNA a naturally amplified source of genetic variation. Recently, PCR-based methods using multicycopy nuclear DNA sequences such as satellite DNA (Guoli, et al. 1999 and Calvo, et al. 2002) and repetitive elements (Calvo et al. 2001 and Tajima et al. 2002) have been introduced. Like mitochondrial-based systems, nuclear PCR-based assays take advantage of multiple target amplification sites in the genome of interest. However, many of these systems require additional procedural steps and at least 1–250 pg of starting DNA template for species detection (Calvo et al. 2001; Calvo et al. 2002 and Tajima et al. 2002). It is recently reported that, the development of PCR assays for the detection of ruminant-, pig- and chicken-derived materials based on sequences of short and long interspersed repetitive elements. Although these assays exceed the detection limits of previously reported assays (Matsumaga, et al. 1999; Lahiff et al. 2001 and Tartaglia et al. 1998) there are several limitations to their methods. Primarily, the detection of PCR products is exclusively gel based. In addition, the size of the PCR amplifications for the assays (179–201 bp) reported by Tajima et al. (2002) might limit their utility for testing trace forensic materials that contain degraded DNA.

Hunt et al. (1997) found that the routinely by the Dutch Inspection for Health Protection. The procedure is slightly more time
consuming than the preceding one, but does not require specialized chemicals or equipment for the AP labeling and subsequent chemiluminescence’s detection steps. Samples are spotted onto a single membrane, which facilitates the testing of many samples with one probe. By using longer probes, the hybridization conditions are less critical. Probes have been validated for the most common meat species. Specific satellite or short interspersed nuclear element (SINE) probes for other species (Hunt, et al. 1997) have not yet been tested in meat samples.

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 discrimination between related species, with farm animals, and poultry...e.g., is possible (Lenstra et al. 2001).

Consumers nowadays very seldom can identify the species in the products 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 Yman 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.

Walker et al., (2003) had designed and evaluated four assays based upon PCR amplification of short interspersed elements (SINES) for species specific detection and quantization of bovine, porcine, chicken, and ruminant DNA. The need for these types of approaches has increased drastically in response to the bovine spongiform encephalopathy epidemic.

In order to overcome these and other shortcomings associated with previously reported methods for nuclear-based species-specific DNA detection, authentication and identification, the designed and evaluated a series of assays based upon PCR amplification of Cattle and Sheep meat based on species-specific repeat.
The present study was designed to investigate PCR methods for identification and authentication of cattle and sheep meat based on species-specific repeat.

**Materials and methods**

**Meat samples**

Meat samples of two species, cattle and sheep from muscle Purchase were collected in New Borg El-Arab City, Alexandria, Egypt.

**DNA Extraction**

DNA was extracted from muscle sample following the method described by Bardakci 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 NaCl, 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), the mixture was incubated at 50 °C for 30 min. DNA was purified by successive extraction with phenol, phenol: chloroform: isoamyl alcohol (25:24:1) and chloroform: isoamyl alcohol (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 mill Q water.

**PCR Primers**

<table>
<thead>
<tr>
<th>Species-specific repeat/ Replikacija specifična za vrstu</th>
<th>Sequence 5’- 3’/ Sekvenca 5’- 3’</th>
<th>Annealing Tm/Sec Vreme kaljenja/sec.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cattle/Goveda</td>
<td>AAG CTT GTG ACA GAT AGA ACG AT</td>
<td>57°C/ 1 min</td>
</tr>
<tr>
<td></td>
<td>CAA GCT GTC TAG AAT TCA GGG A</td>
<td></td>
</tr>
<tr>
<td>Sheep/Ovce</td>
<td>GTT AGG TGT AAT TAG CCT CGC GAG AA</td>
<td>57°C/ 1 min</td>
</tr>
<tr>
<td></td>
<td>AAG CAT GAC ATT GCT GCT AAG TTC</td>
<td></td>
</tr>
</tbody>
</table>
Amplification of species-specific repeat.

PCR was performed following the procedure of Lenstra et al., 2001 with some modifications. 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 of genomic 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 30 cycles of 94°C for 1 min for DNA denaturation, annealing as in table 1, extension at 72°C for 2 min and final extension at 72°C for 10 minute. The samples were cooled at 4°C.

Agarose Gel Electrophoresis

The amplified DNA fragments were separated on 3% agarose gel and stained with ethidium bromide. 100 bp DNA marker (bp 2642, 1500,.............500, 400, 300, 200, 100) was used in this study. The amplified pattern was visualized on an UV transilluminator and photographed by Gel Documentation system (Alpha ImagerTM1220, Documentation & Analysis system, Canada).

Results of investigations and discussion

PCR amplification of the gene encoding species-specific repeat in cattle and sheep using the primers yielded 603-bp amplification with Cattle and 374-bp with sheep are shown in Figure 1. 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 cattle and sheep as different meat animal species are shown in Figure 1. The results showed that SSR analysis provided with a product that allowed a direct identification of cattle and sheep meat in raw meat samples from different animal’s species. Also the results showed that SSR analysis produced a clear, rapid and effective method to authenticate the meat species, which is in agreement with the results of Lenstra et al. (2001).
Figure 1 PCR products generated by primers specific for the species

Lane M: DNA marker, Lane C: cattle, Lane S: sheep

Slika 1. Proizvodi lančane reakcije polimeraze (PCR) stvoren od strane prajmera specifičnih za vrstu odnosno oligonukleotida, staza M: DNK marker, staza C: goveda, staza S: ovce

Application of SSR analysis for species-specific gene using specific primers showed specific detection in each. 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 (Buntjer et al. 1995; Buntjer et al. 1999; Hunt et al. 1997; Janssen et al. 1998; Lenstra and Buntjer, 1999; Nowak, 1991; Brown, 2001 and Lenstra et al. 2001). However, in this study we provide a simple, rapid, and universal method for identifying animal’s meat. 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.

Inoue et al. 1990; Espinoza et al. 1996; Guoli et al. 1999; Lenstra et al. 2001; Calvo et al. 2001; Calvo et al. 2002; Tajima et al. 2002 and Walker et al. 2003 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. The use of species-specific analysis provides a simpler, quicker, and cheaper alternative to sequencing for direct identification of animal’s species.

Conclusion

In conclusion it can be emphasized that the primers amplified successfully the genomic DNA of the genotypes under study (cattle and sheep). This band on the level of DNA detection analysis provided a rapid and effective method to detect the genetic detection of different animal's meat, also use of species-specific analysis provides a simpler, quicker, and cheaper alternative to sequencing for direct identification of meat animal’s species. Also indirect application is the detection of disease cow (cattle) lunacy by PCR methods for authentication of cow (cattle) meat from origin country.

PCR AMPLIFIKACIJA PONAVLJANJA SPECIFIČNIH ZA VRSTU U IDENTIFIKACIJI DNK U MESU KORIŠĆENJEM GENETSKIH MARKERA KOD GOVEDA I OVACA

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Rezime

Urađene su i ocenjene četiri analize koje su zasnovane na PCR amplifikaciji ponavljanja specifičnih za vrste (SSR) radi detekcije, identifikacije i potvrđivanja na DNK nivou goveda i ovaca. SSR prajmeri (primarna genetička informacija) su primjenjeni u lančanoj reakciji polimeraze (PCR), proizvodi su korišćeni za specifičnu identifikaciju mesa goveda i ovaca. PCR amplifikacija veličine gena koji kodira SSR region u mesu goveda i ovaca je bila 603 bp odnosno 374 bp respektivno. Rezultati su pokazali sa je SSR analiza proizvela obrasce koji su omogućavali direktnu identifikaciju mesa goveda odnosno ovaca u sirovim i uzorcima koji su tretirani odnosno zagrevani. Takođe, rezultati su pokazali da je SSR analiza obezbedila brzu i efikasnu metodu za
otkrivanje vrste mesa, koje se lako identifikuje i potvrđuje. Dalje, SSR PCR metode, specifične za vrstu, su veoma osetljive i poboljšaće granice za otkrivanje DNK sekvenci koje potiču od ovih vrsta. Indirektna primena može biti u otkrivanju bolesti ljudskih krava (goveda) korišćenjem PCR metode za potvrđivanje mesa krave (goveda) iz zemlje porekla.

Konačno može se naglasiti da su prajmeri (primarne genetičke informacije) amplifikovali uspešno genomski DNK genotipa koji su ispitivani (goveda i ovce). To je omogućilo na nivou analize DNK detekcije brzu i efikasnu metodu za otkrivanje mesa od različitih životinja, takođe korišćenje analiye specifične za vrstu omogućilo je jednostavniju, bržu i jefitniju alternative sekveniranju za direktnu identifikaciju mesa specifične vrste životinja. Takođe, indirektna primena je u otkrivanju bolesti ljudskih krava (goveda) PCR metodom za potvrđivanje mesa krave (goveda) iz zemlje porekla.

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