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A Simple and Rapid Assay for Specific Identification of Bovine Derived Products in Biocomplex Materials

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Abstract: A simple and rapid method for specific identification of beef or bovine-derived products in processed food and in animal feed concentrates was developed and evaluated using Polymerase Chain Reaction (PCR). The mitochondrial cytochrome-b (mtcyt-b) gene was used as a target DNA for PCR amplification. Three primers derived from a highly conserved region of bovine mtcyt-b gene were used. The outer pair of primers (RSL1 and CSR2) produced a 365 base pair (bp) PCR amplicon from bovine DNA, while the internal semi-nested pair of primers (CSL1 and CSR2) were used to amplify a 284 bp PCR amplicon, internal to the annealing sites of primers (RSL1 and CSR2). Both amplicons were identified easily after visualization on agarose gel stained with ethidium bromide. The specificity studies indicated that the primary or the semi-nested PCR products were not amplified from DNA extracted from different ruminant species including, sheep, goat and ghazals; or from non-ruminant animals including camels, horses and pigs. Also was found very sensitive because could detect 0.001% (W/V) of bovine mtcyt-b gene. The semi-nested amplification was necessary to increase the sensitivity of the PCR assay and to confirm the identity of the primary PCR amplicons. The described PCR assay detected the primary and the semi-nested PCR amplicons from different animal feed concentrates containing bovine-derived product including, canned food, poultry and dairy feed concentrates. The described PCR assay should facilitate rapid detection of beef and bovine-derived products in processed food and in animal feed concentrates.

Key words: Mitochondrial cytochrome DNA, bovine, PCR, molecular diagnostic

INTRODUCTION

It is well documented that beef and bovine-derived products are not generally recognized by regulatory agencies to be safe for use in ruminant feed (Aradaib *et al.*, 1998a). This will either ban or severely restricts feeding of bovine proteins back to ruminants. Therefore, the development of a molecular diagnostic technique for rapid detection and identification of beef or bovine-derived product will be advantageous in quality control in food industry (Allmann *et al.*, 1993). Recently, we described a PCR assay for specific identification of pork in processed human food and in commercial animal feed concentrate (Khairalla *et al.*, 2006). Because of the stability of the DNA, diagnostic assays, which make use of DNA in the product, can be used to authenticate the animal species utilized in the processed human food or in

animal feed concentrates. Nucleic acid hybridization, sequencing of oligonucleotides and DNA amplification technology have been successfully applied for species identification in human food (Allmann *et al.*, 1993; Meyer *et al.*, 1995, Aradaib *et al.*, 1998b; 2001; Aradaib, 2004). In the present investigation, we described a simpler, reliable, rapid, sensitive and specific method that could be used for specific identification of bovine-derived products in biocomplex materials including, processed food and in animal feed concentrates.

MATERIALS AND METHODS

This study was conducted at the molecular biology laboratory, Faculty of Veterinary Medicine, University of Khartoum, Sudan, during the period from June 2005 to March 2006.

DNA extraction from samples: Extraction of DNA from bovine blood, meat and offals was made possible using a commercially available QIAamp tissue kit (QIAGEN Inc. Chatsworth, CA) according to the manufacturer's instructions. Briefly, 200 mg of minced meat were treated by freezing and thawing to rupture the cell wall of the myocytes. Two hundred microliter of the supernatant, 20 μ L of proteinase K stock solution and 200 μ L of lysing buffer were pipetted into 1.5 mL eppendorf tube and the mixture was incubated at 37°C for one hour and then at 70°C for 30 min 200 μ L of absolute ethanol was then added to the sample and mixed by vortexing. The mixture was then transferred to the QIAamp spin column and placed in a clean 2 mL-collection tube and centrifuged at 8000 RPM for 1 min at room temperature. The QIAspin column was washed twice using 500 μ L of washing buffers and spinning for 1 min. The QIAamp spin column was placed in a clean 1.5 mL eppendorf tube and the DNA was eluted with 200 μ L of double distilled water preheated at 70°C at room temperature. A maximum DNA yield was obtained by spinning at 12,000 RPM for 1 min at room temperature. The DNA concentration was determined by spectrophotometer at 260 nm wave length. Five microliter of the suspended nucleic acid was used in the PCR amplification.

Selection of the primers for PCR amplification: For the first amplification step, a pair of primers was selected from the published sequences of the bovine mtcyt-b gene and used in the PCR assay. Primers (RSL1 and CSR2) were selected for the synthesis of the primary bovine-specific PCR product. Primer RSL1 included bases 12-40 of the positive sense strand (5)-CCC AGC CCC CTC AAA CAT CTC A. CSR2 included bases 357-376 of the complementary strand (5)-GGCTATTACTGTGAGCAGA. Using primers RSL1 and CSR2, the primary PCR amplification will produce a 365 bp PCR amplicon from bovine DNA.

For identification of bovine species, a pair of semi-nested primers (CSL1 and CSR2) was designed from the same sequence of bovine mtcyt-b gene sequences cited above. CSL1 included bases 93-111 of the positive sense strand (5)-GAA TTT CGG TTC CCT CCT G. CSR2 included bases 357-376 of the complementary strand (5)-GGC TAT TAC TGT GAG CAG A. Using primers CSL1 and CSR2, the PCR assay will result in a 284 bp amplicon.

The primers were synthesized on a DNA synthesizer (Milligen/Bioscience, a division of Millipore/Burlington, MA) and purified using oligo-pak oligonucleotide purification columns (Glen Research Corporation, Sterling, VA.) as per manufacturer's instructions.

Polymerase chain reaction: A stock buffered solution containing 250 μ L 10X PCR buffer, 100 μ L of $MgCl_2$, 12.5 μ L of each dATP, dTTP, dGTP and dCTP was prepared in 1.5 mL eppendorf tube. The primers were used at a concentration of 20 pg μ L⁻¹ and double distilled water was added to bring the volume of the stock buffer solution to 1.5 mL. Two microliter of the primers, 5.0 μ L of the target DNA and 42 μ L of the stock solution were added onto 0.5 mL PCR tubes and mixed by vortexing. One microliter of Taq DNA polymerase (Perkin Elmer) at a concentration of 5.0 U μ L⁻¹ were used. All PCR amplification reactions were carried out in a final volume of 50 μ L. The thermal cycling profiles were as follows: a 2 min initial incubation at 95°C, followed by 40 cycles of 94°C for 1 min, 57°C for 30 sec and 72°C for 45 sec and a final incubation at 72°C for 10 min. Thermal profiles were performed on a Techne PHC-2 thermal cycler (Techne, Princeton, NJ.). Following amplification, 15 μ L from each PCR containing amplicons were loaded onto gels of 1.0% SeaKem agarose (FMC Bioproduct, Rockland ME) and electrophoresed. The gels were stained with ethidium bromide and the bovine-specific PCR amplicons were easily identified following visualization under UV light.

Semi-nested polymerase chain reaction: For the semi-nested PCR amplification, 2.0 μ L of the primary amplicons produced by CSL1 and CSR2 were transferred to 0.5 mL PCR tube containing (2 μ L of semi-nested primers and; 45 μ L of stock PCR buffer and 1 μ L Taq DNA polymerase was used at a concentration of 5.0 U μ L⁻¹. The semi-nested pair of primers (CSL1 and CSR2) was expected to amplify a 284 bp PCR amplicons, internal to the annealing sites of primers RSL1 and CSR2. All PCR amplifications were carried out in a final volume of 50 μ L. The thermal cycling profiles were as follows: a 2 min incubation at 95°C, followed by 30 cycles of 94°C for 1 min, 57°C for 30 sec and 72°C for 45 sec and a final incubation at 72°C for 10 min. Thermal profiles were performed on a Techne PHC-2 thermal cycler (Techne, Princeton, NJ.). Following amplification, 15 μ L from each PCR containing amplicon were loaded onto gels of 1% SeaKem agarose (FMC Bioproduct, Rockland ME) and electrophoresed. The gels were stained with ethidium bromide and the bovine-specific PCR amplicons were easily identified following visualization under UV light.

RESULTS

The described PCR-based assay afforded sensitive and specific identification of bovine mtcyt-b DNA used in this study. Using the outer pair of primers (RSL1 and CSR2), the PCR-based assay detected the primary bovine-

specific 365 bp PCR amplicon from not less than 1.0 pg DNA from bovine species (Fig. 1). The sensitivity studies indicated that the semi-nested pair of primers (CSL1 and CSR2) increased the sensitivity of the assay and the semi-

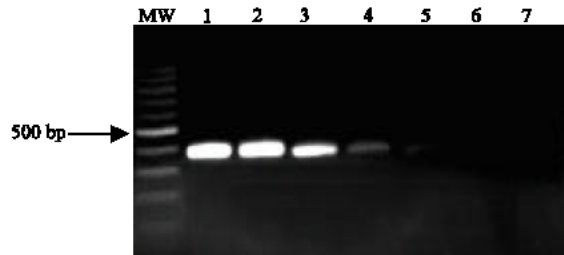


Fig. 1: Sensitivity of the PCR assay for the detection of the bovine-specific 365 bp PCR amplicons, using primers RSL1 and CSR2. Visualization of the 365 bp PCR amplicons on ethidium bromide-stained agarose gel from 100 fg of bovine DNA. Lane MW: molecular weight marker (100 bp ladder); lanes 1-7: bovine DNA at concentrations of 1 ng, 100 pg, 10 pg, 1 pg, 100 fg, 10 fg and 1 fg, respectively

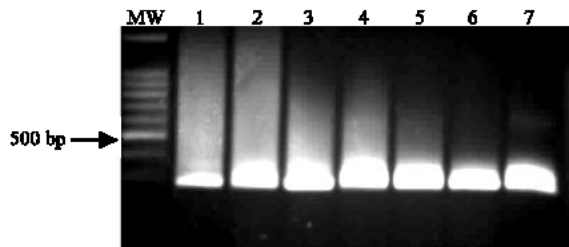


Fig. 2: Semi-nested amplification of the 284 bp PCR amplicons from the primary PCR product. The nested PCR amplification detected as little as 0.001 fg of swine DNA. Lane MW: molecular weight marker (100 bp ladder); lanes 1-7: bovine DNA at concentrations of 1 ng, 100 pg, 10 pg, 1 pg, 100 fg, 10 fg and 1 fg, respectively

nested 284 bp specific PCR amplicons were amplified from as little as 0.001 fg of bovine DNA (equivalent to a single copy of mtcyt-b genome) as shown in Fig. 2.

The specificity studies indicated that the described PCR assay failed to amplify the primary or the semi-nested bovine-specific PCR amplicons from DNA extracted from other animal species including, sheep, goat, deer, horse, camel and pig (Fig. 3). Using primers RSL1 and CSR2, amplification of the bovine-specific 365 bp PCR product was produced from different biocomplex materials containing bovine DNA (Fig. 4).

Application of this PCR assay to DNA extracted from animal feed concentrates containing bovine-derived product resulted in detection of the primary and the semi-nested PCR amplicons from different commercial animal feed concentrates including, canned dog food, dairy feed concentrates and poultry feed (Fig. 5).

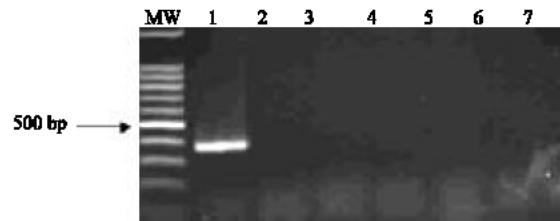


Fig. 3: Specificity of the polymerase chain reaction for specific identification of bovine DNA using primers (CSL1 and GSR2). Amplification was not detected from other ruminant mtcyt-b DNA. Lane MW: molecular weight marker; Lane 1: 1.0 ng of bovine mtcyt-b DNA (positive control); Lane 2-4: 1.0 ng of ruminant mtcyt-b DNA extracted from other ruminant species including sheep, goat and deer, respectively. Lane 5-7: 1.0 ng of non ruminant mtcyt-b DNA extracted from other ruminant species including horse, camel and pig, respectively

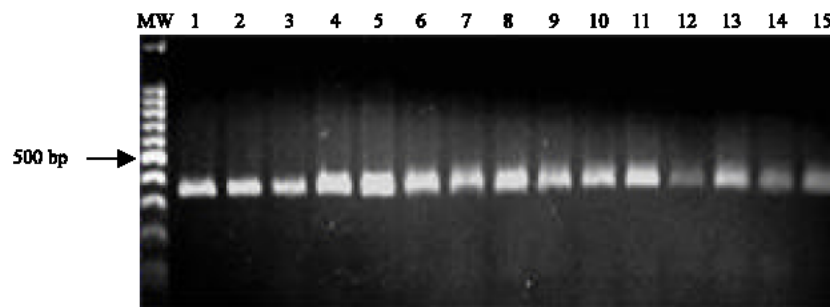


Fig. 4: Amplification of the PCR assay for the detection of the bovine-specific 365 bp PCR amplicons, using primers RSL1 and CSR2. Lane MW: Molecular weight marker; Lane 1: 1.0 ng of bovine mtcyt-b DNA (positive control); Lane 2-15: 14 different bovine DNA samples

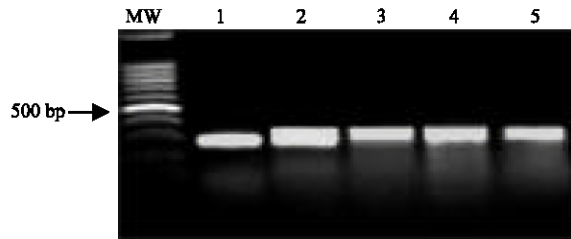


Fig. 5: Visualization of the semi-nested PCR amplicons from bovine DNA extracted from different preparations of animal feed concentrates. Lane 1: Bovine DNA (positive control), Lane 2 and 3: candies containing bovine gelatin, Lane 4: Dog Food; Lane 5: Commercial cattle feed concentrates

DISCUSSION

Epidemiological studies have implicated animal feed containing ruminant-derived protein, such as meat and bone meals, may contain the etiologic agent that causes Transmissible Spongiform Encephalopathy (TSE), specifically Bovine Spongiform Encephalopathy (BSE), commonly known as mad cow disease (Aradaib *et al.*, 1998a; Aradaib, 2004). The development of a molecular diagnostic technique for identification of biomaterials from complex sources would be advantageous in a variety of circumstances including comparative genomics and investigative forensics.

In a previous report, differentiation of sheep and goat meat was shown by comparison of their nucleotide sequences through direct sequencing techniques and digestion of PCR product with restriction endonuclease enzymes. Analysis of the PCR fragment with endonuclease enzymes to detect Restriction Fragment Length Polymorphisms (RFLPs) was also described. This analysis is useful to differentiate species instead of sequencing the common fragment (Rodriguez *et al.*, 2004). Application of PCR-RFLP technique was described for differentiation of meat species particularly food animals (Allmann *et al.*, 1993; Mayers *et al.*, 2004; Lanzilao *et al.*, 2005). In a previous study, pork was differentiated from sheep and goat meat by comparison of their nucleotide sequences through direct sequencing techniques and digestion of PCR product with restriction endonuclease enzyme (Mayer *et al.*, 2004). However, this method is tedious, laborious, time consuming and expensive. The semi-nested PCR amplification technology, described in this study, provides a simple, rapid and sensitive method for detection and identification of bovine-derived product in processed food and in animal feed concentrates DNA extraction was a simple procedure that takes only half an

hour using QIAamp extraction kit. The time required for the primary and the nested amplifications was approximately 4 h. The electrophoresis, staining of the agarose gel with ethidium bromide and visualization of the specific PCR products usually takes 1h. Confirmatory results of submitted samples could be obtained within the same working day. The semi-nested amplification increased the sensitivity of the PCR assay particularly, when the concentration of the bovine DNA in the sample is less than 100 fg, which is beyond the detection limit of the agarose gel electrophoresis. The described PCR assay detected as little as 0.001 fg of bovine DNA which is equivalent to a single copy of bovine mtcyt-b gene. To our knowledge, this semi-nested PCR assay provides the most sensitive method for detecting beef or bovine-derived contaminants in a variety of complex biomaterials compared to previous methods described by Calvo *et al.* (2002) Myer *et al.* (2004) and Walker *et al.* (2004).

The specificity studies indicated that the primary 386 bp PCR amplicon was not amplified even from a relatively high concentration of 1.0 ng of DNA extracted from other ruminant species including, sheep, goat and deer; and from other animal species including, camel, horse and pig under the same stringency condition described in this study. Using the pair of semi-nested primers, the nested amplification confirmed the identity of the primary amplified product and thereby avoiding the hybridization confirmation procedure, which is cumbersome and usually takes overnight.

It is worth mentioning that the semi-nested PCR, described in this study, could be used as a valuable tool in forensic veterinary medicine to authenticate the presence of bovine-derived product in processed food or in animal feed concentrates. In a previous report, it has been shown that heat treatment or cooking of meat of various animal species resulted in decreased sensitivity of the PCR assay (Mayer *et al.*, 2004; Khairalla *et al.*, 2006). Nevertheless, in the present study, treatment of beef with high temperature did not affect the detection limit of the semi-nested PCR assay. This could be attributed to the different methods of DNA extraction, which subsequently resulted in different DNA yield from heat treated samples. Thus, application of this PCR could be useful for detection of heat-treated products including microwaved cooked beef and smoked luncheon. In previous studies, different genes were targeted for PCR amplifications including growth hormone gene; mtcyt-b gene and interspersed repetitive elements (Walker *et al.*, 2004; Aradaib, 2004). In the present study, selection of the primers was based on the observation that the mtcyt-b gene has high copy numbers (hundred to thousand copy

numbers per cell). In addition, the occurrence of conserved regions within the mtcy-b gene provides high sensitivity for the nested PCR (Aradaib *et al.*, 1998a, Aradaib *et al.*, 2001). More over, the described semi-nested PCR assay does not require sophisticated laboratory equipments such like DNA sequencer and hybridization facilities. Further more, analysis of the PCR fragments with endonuclease enzymes to detect. Restriction Fragment Length Polymorphisms (RFLPs) was not employed in this study. This analysis is also useful to differentiate animal species. However, PCR-RFLPs is time consuming and expensive. The nested PCR, described in this study, could be used as a valuable tool in forensic veterinary medicine to authenticate the presence of bovine in complex biomaterials utilized in processed food or in animal feed concentrates.

It is worth mentioning that complex biomaterials containing bovine tissues are not accepted as a source of protein in animal feed concentrates. This concern would require strict screening for bovine product in animal feed concentrates, which could be provided by the above described PCR-based assay. This semi-nested PCR assay should provide verification of the bovine protein status of specified processed food or animal feeds concentrate.

CONCLUSIONS

The described semi-nested PCR assays should be used as simple and rapid methods for detection and specific identification of bovine-derived products in processed food and animal feed concentrates. In addition, application of this assay would be advantageous in a variety of circumstances including, comparative genomics and investigative forensics and can be recommended in quality control departments.

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