

Detection of Caprine-Specific Nucleic Acid Sequences in Goat Milk Using Polymerase Chain Reaction

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Abstract: This study was carried out to evaluate, PCR-based method for detection of DNA in goat milk. It utilized primers targeting the mitochondrial cytochrome b (*mtcyt-b*) gene which was used as a target DNA for PCR amplification. For the specific identification of goat *mtcyt-b* gene, pair of primers (GSL1, GSR2) were used which produced a 428 base pair (bp) PCR product from milk samples as well as from peripheral blood. Amplification products were visualized on ethidium bromide-stained agarose gels. Amplification products were not detected when the PCR was applied to DNA from animal species including cattle, sheep, swine, camel, deer, horse, donkey and human which indicates that the 2 pairs of primers are specific for goat. In conclusion, DNA can be extracted from goat milk and would be advantageous in the variety of application such as species identification in milk and milk.

Key words: Goat milk, DNA, rapid detection, PCR assay, cattle

INTRODUCTION

Collection of milk is a non invasive procedure. The technical convenience of milk as a source of DNA can be expected to increase the field of application of the marker-based methods for genetic analysis of goat genome. Recently, species identification of dairy products has received great attention. It has a remarkable importance for several reasons related to governmental regulation and public health. Mixture in dairy products and species substitution should be observed to be a cause of human adverse reactions (Bottero *et al.*, 2003). The source of DNA in milk could be from somatic cells (Fahr *et al.*, 1999). Recently DNA was detected from human milk (Abdalla *et al.*, 2009).

Goat milk is similar to cow milk with around 87% water, 67% energy, 3.3% protein, 4.0% fat and 4.6% carbohydrates (Belanger, 2001). Goat milk differs from cow and human milk in several ways among them higher digestibility and lower lactose (Larson, 1978). One difference is that goat milk has smaller fat globules present due to the lack of the enzyme that aggregates the globules in the milk (Belanger, 2001).

The objective of the present study was to evaluate milk as a source of goat DNA and as a substrate for PCR amplification using mitochondrial *cytochrome-b* gene as a target DNA for PCR amplification.

MATERIALS AND METHODS

Study design: This was prospective study in which samples are collected throughout the study.

Study area: This study was conducted at the Molecular Biology Laboratory, Faculty of Veterinary Medicine, University of Khartoum and the National Ribat University, Khartoum, Sudan. During the period from February to July 2010.

Study population: Twenty five Nubian Goats, maintained at the dairy farm of the Faculty of Veterinary medicine, University of Khartoum, Shmbat were included. All goats were in good health particularly with no clinical mastitis.

Data collections

Milk samples collection from goats: Before collection, the teats were cleaned with alcohol to avoid samples

contamination from skin. Milk (2.5 mL) was collected in sterile 2.5 mL centrifuge tube by hand milking, samples were stored at 4°C until testing for extraction of total genomic and mitochondrial cytochrome-b (mtcyt-b) DNA. The extracted DNA was used as a target DNA for PCR amplification.

Blood samples collection: Blood samples were collected for preparation of positive control, blood samples were collected in clean sterile vacutainers, containing Ethylene Diamine Tetra Acetic Acid (EDTA) from goats (positive controls) from the animals attended at the veterinary teaching hospital.

The blood samples then centrifuged in bench centrifuge (Hettich Zentrifugen, D-785320, Germany) in order to separate the buffy coat which is rich in white blood cells and used for extraction of total genomic and mitochondrial cytochrome-b (mtcyt-b) DNA. The extracted DNA was used as a target DNA for PCR amplification.

DNA extraction from milk samples: Extraction of DNA from goat milk and peripheral blood was made possible using a commercially available QIAamp blood kit (QIAGEN Inc. Chatsworth, Canada) according to the manufacturer's instructions. In details, 200 µL of milk samples, 20 µL of proteinase K enzyme stock solution and 200 µL of Lysing buffer (LA buffer) were pipetted into 1.5 mL eppendorf tube and the mixture was vortexed on the vortexing machine (Janke and Kunkel, GmbH, CoKG, Germany) and incubated at 60°C for 10 min. About 200 µL of absolute ethanol were added to the sample and mixed by vortexing. The mixture then was transferred to the QIAamp spin column and was placed in a clean 2 mL collection tube and centrifuged in microcentrifuge (Hettich Zentrifugen, 12-24, Tuttlingen, Germany) at 8000 rpm for 1 min. The QIAspin column was washed firstly with 500 µL of washing buffers 1 (AW1) at the same earlier mentioned centrifugation speed and rewashed using washing buffer 2 (AW2) at speed 1200 rpm centrifugation speed for 3 min. The QIAamp spin column was then placed in a clean 1.5 mL eppendorf tube and the DNA was eluted with 200 µL of double distilled water preheated at 70°C. Maximum DNA yield will be obtained by spinning at 1200 rpm for 1 min after remaining for 1 min in the room temperature. The DNA concentration was determined by spectrophotometer at 260 wave length. The 5 µL of the suspended nucleic acid will be used in the PCR amplification.

Selection of goat primers for PCR amplification: For PCR amplification a pair of goat-specific primers (GSL1 and GSR2) was designed from the caprine *mtcyt-b* gene

sequences. GSL1 included bases 284-303 of the positive sense strand (5)-TCA TAC ATA TCG GAC GAC GT. whereas GSR2 included bases 693-712 of the complementary strand (5)-CAA GAA TTA GTA GCA TGG CG. Using of this pair of primers (GSL1 and GSR2) in PCR assay resulted in amplification of a 428 bp PCR product from caprine mtcyt-b DNA.

The primers were synthesized on a DNA synthesizer (Milligen/Biosearch, 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 (PCR): A stock buffered solution containing 250 µL 10X PCR buffer, 100 µL of MgCl₂, 12.5 µL of each dATP, dTTP, dGTP and dCTP was prepared in 1.5 mL eppendorf tube and double distilled water was added to bring the volume of the stock buffer solution to 1.5 mL. The primers were used at a concentration of 20 µmole L⁻¹ which appears to 2 µL. Next, 5.0 µL of the target DNA was added to 42 µL of the stock solution in 0.5 mL PCR tubes and mixed by vortexing. This is followed by 1.0 µL of Taq DNA polymerase (Perkin Elmer) which was used at a concentration of 2.5 units. All PCR amplification reactions were carried out in a final volume of 50 µL. The thermal cycling profiles were as follows: 2 min incubation at 95°C followed by 40 cycles at 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 will be performed on a Techne PHC-2 thermal cycler (Techne, Princeton, NJ, USA).

Visualization of PCR products: All PCR amplification product samples were visualized using agarose gel electrophoresis. The 10X Tris Borate EDTA (TBE) buffer was diluted to 1X solution which was used to prepare 1.0% agarose gels and as running buffer in electrophoresis after it was stained with ethidium bromide as 0.5 µg mL⁻¹. About 15 µL from each PCR reaction containing amplified product was loaded onto gels of 1.0% agarose (FMC Bioproduct, Rockland ME) and was electrophoresed. The results were visualized under UV light transilluminator. The results were then photographed by gel documentation system.

RESULTS AND DISCUSSION

DNA was successfully extracted from goat milk samples used in this study. The DNA yield varies with the different stages of lactation as compared with the yield from peripheral blood leukocytes. The PCR-based assay described in this study afforded sensitive and specific identification of goat mtcyt-b DNA. Using the pair of primers (GSL1 and GSR2). The sensitivity studies indicate

that the 428 bp PCR products were detected from not <1.0 pg of goat *mtcyt-b* gene (Fig. 1). The specificity studies for goat primers indicate that the described PCR assay failed to amplify the specific PCR product from DNA extracted from other animal species including cattle, sheep, swine, camel, deer, horse, donkey and human (Fig. 2). Using primers GSL1 and GSR2, amplification of

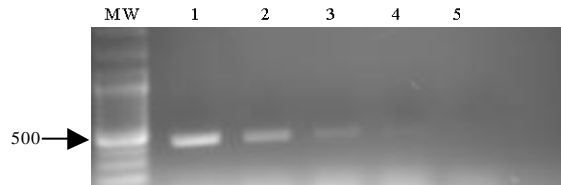


Fig. 1: The sensitivity was tested for the same reaction using 10 fold serial dilution for the initial concentration $1 \text{ ng } \mu\text{L}^{-1}$. The results of PCR amplification using the pair of primers (GSL1 and GSR2) indicate that this reaction can amplify as far as 1 pg of goat *mtcyt-b* gene. The size of each product was 428 bp. Sensitivity of the PCR assay for the detection of the goat specific *mtcyt-b* DNA. Lane MW: Molecular Weight marker. Lanes 1-5: 428 bp PCR products amplified from goat milk DNA at concentrations of 1 ng, 100 pg, 10 pg, 1 pg, 100 fg, respectively (Sensitivity test)

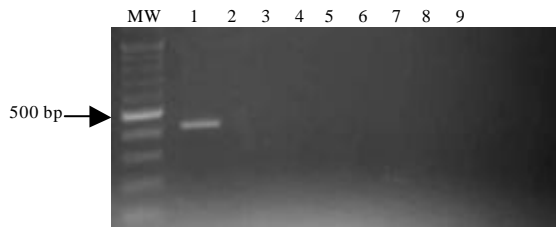


Fig. 2: The study of the goat specific *mtcyt-b* gene PCR amplification, the pair of primers (GSL1 and GSR2) was used. Target DNA produced 428 bp PCR products. The other target DNAs included cattle, sheep, swine, camel, deer, horse, donkey and human, respectively, gave negative results to amplification with goat specific *mtcyt-b* gene. The laboratory specificity in this case was measured as follow: Specificity of the PCR assay for the detection of the goat specific *mtcyt-b* DNA. Lane MW: Molecular Weight marker (100 bp DNA ladder). Lane 1: goat milk DNA (positive control). Lane 2-9: *mtcyt-b* DNA of cattle, sheep, swine, camel, deer, horse, donkey and human, respectively (Specificity test)

the goat-specific 428 bp PCR product was produced from different milk samples collected from goats included in this study (Fig. 3-5).

In goat, peripheral blood leukocytes are the usual source of DNA for genotyping. However, obvious

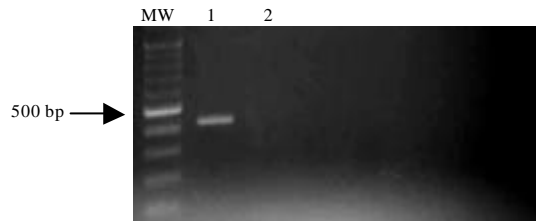


Fig. 3: Detection of the specific 428 bp PCR product from goat blood (positive control). Lane MW: Molecular Weight marker. Lane 1: goat DNA, extracted from goat blood (positive control). Lane 2: Nucleic acid free sample (negative control)

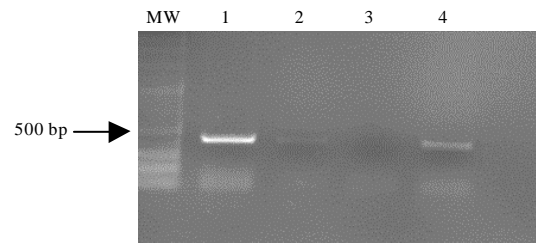


Fig. 4: Amplification of the PCR assay for the detection of the goat-specific 428 bp PCR product in milk samples. Lane MW: Molecular Weight marker. Lane 1: Goat DNA (positive control). Lane 2 and 4: 2 different goat DNA extracted from milk samples; Lane 5: Cattle DNA (negative control)

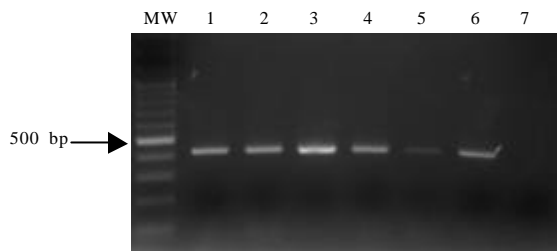


Fig. 5: Amplification of the PCR assay for the detection of the goat-specific 428 bp PCR product in milk samples. Lane MW: Molecular Weight marker; Lane 1: Goat DNA (positive control); Lane 2-6: 5 different goat DNA extracted from milk samples; Lane 7: Sheep DNA (negative control) study of the goat *mtcyt-b* DNA PCR amplification, the pair of primers (GSL1 and GSR2) was used. Target DNA produced 428 pb PCR products

technical difficulties exist in the collection of blood samples from large numbers of individuals among widely separated herds, limiting the application of marker-based methods for genetic analysis and for genetic improvement of economic traits in dairy goat. The scientific data presented in this study indicate that DNA extracted from goat milk could serve as substrates for PCR amplification of the full length of the caprine mitochondrial *cytochrome-b* gene or a fragment of the gene. Since, collection of milk is a non invasive procedure, it can often substitute for blood as a source of DNA. The technical convenience of milk as a source of DNA can be expected to increase the field of application of the Marker-based methods for genetic analysis of goat genome.

Recently, species identification of dairy products has received great attention. It has a remarkable importance for several reasons related to governmental regulation and public health. Protection against species substitution or admixture in dairy products is of significant importance (Bottero *et al.*, 2003). Milk is known to be frequent cause of food allergies. It was found that most milk proteins even at low concentration are potential allergens (Sampson, 2003). Also, cow's milk was reported as the main dairy product responsible for human adverse reaction (Rance *et al.*, 2005). Thus, the counterfeiting of goat's milk with cow's milk may be considered as a health risk making species identification an important issue in current food safety requirement. The common fraudulent practice found in the dairy production line is the use of a cheaper type of milk in substitution of more expensive ones. Currently, different methods are used for species identification in milk and milk products including immunological (Addeo *et al.*, 1995) electrophoretic (Cartoni *et al.*, 1998) and chromatographic techniques (Pellegrino *et al.*, 1991). Among these methods capillary electrophoresis, two dimensional electrophoresis, iso-electric focusing of milk caseins which is the European Community reference method for cow's milk detection (ECR, 1996) also ELISA are reported (Molina *et al.*, 1999). However these methods can't always distinguish milk from closely related species and not suitable for heat treated milk. The PCR amplification technology, described in this study, provides a simple, rapid, reliable and sensitive method for species identification and differentiation. The time required for the PCR amplification was approximately 3 h this means that confirmatory diagnosis could be obtained within the same working day.

Lipkin *et al.* (1993) reported that milk is less reliable source of DNA than is blood because it requires large size of sample and high concentration of somatic cells (Lipkin *et al.*, 1993). But in the study researchers

used only 200 μ L of milk sample for extraction of DNA and the PCR products were the same in length and approximate quantity for milk for DNA extracted from milk and for DNA extracted from blood, this difference may reflect the detection procedures described above.

The intensity of DNA signal in gel electrophoresis, show that there is a decline in the DNA content in the late lactation periods. This may indicate the milk DNA content is high in the first and second lactation periods and then declines. For more significant results DNA quantification by spectrophotometer is required which provide information about DNA concentration through lactation periods.

In this study, PCR-based assay use DNA targets in the mitochondrial genome. These non-nuclear targets possess several advantages over nuclear genes (Unsold *et al.*, 1995). They are generally more abundant in any given sample than single-copy nuclear genes and because mitochondrial DNA has a relatively high mutation rate compared with the nuclear DNA, they contain a greater accumulation of point mutations that can be used to better define species differences. Moreover, mitochondrial DNA tends to be inherited through the maternal germ line and the resulting lack of heterozygosity in the alleles under study simplifies analysis (Kocher *et al.*, 1989). The mtcyt-b DNA was selected in this research as the target sequence for species identification.

CONCLUSION

PCR-based assay described in this study would be advantageous in the variety of conditions including comparative genomics, species identification in milk and milk products, experimental physiology and can be recommended in the quality control departments in order to support policies and regulation of import export of milk and milk products.

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