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Novel extraction method of genomic DNA suitable for long-fragment amplification from small amounts of milk

Y. F. Liu,* J. L. Gao,* Y. F. Yang,* T. Ku,* and L. S. Zan†[1]
*College of Food Engineering and Nutritional Science, Shaanxi Normal University, Xi'an, 710062, Shaanxi, China
†College of Animal Science and Technology, Northwest A&F University, Yangling, 712100, Shaanxi, China

ABSTRACT

Isolation of genomic DNA is a prerequisite for assessment of milk quality. As a source of genomic DNA, milk somatic cells from milking ruminants are practical, animal friendly, and cost-effective sources. Extracting DNA from milk can avoid the stress response caused by blood and tissue sampling of cows. In this study, we optimized a novel DNA extraction method for amplifying long (>1,000 bp) DNA fragments and used it to evaluate the isolation of DNA from small amounts of milk. The techniques used for the separation of milk somatic cell were explored and combined with a sodium dodecyl sulfate (SDS)-phenol method for optimizing DNA extraction from milk. Spectrophotometry was used to determine the concentration and purity of the extracted DNA. Gel electrophoresis and DNA amplification technologies were used for to determine DNA size and quality. The DNA of 112 cows was obtained from milk (samples of 13 ± 1 mL) and the corresponding optical density ratios at 260:280 nm were between 1.65 and 1.75. Concentrations were between 12 and 45 μg/μL and DNA size and quality were acceptable. The specific PCR amplification of 1,019- and 729-bp bovine DNA fragments was successfully carried out. This novel method can be used as a practical, fast, and economical mean for long genomic DNA extraction from a small amount of milk.
Key words: milk, DNA extraction, PCR, DNA detection

INTRODUCTION

Molecular techniques are powerful tools in quality inspection of dairy products and in research of molecular nutrition. The foundation of effective molecular biology studies, including the identification of nutritional function of food, food traceability, genetic variation among animals, marker-assisted selection of breeding, paternity testing, and hybridization of DNA by Southern blot, relies on high-quality genomic DNA (Murphy et al., 2002). Currently, the source for DNA from cows mainly comprises venous blood and organ tissues. Blood or tissue sampling from living cows requires a professional operator and sampling can affect milk production due to a stress response in the sampled animals (Buitkamp and Gotz, 2004). Therefore, it is necessary to find a more suitable material for DNA isolation and extraction from cows.

Milk somatic cells consist of polymorphonuclear neutrophilic leukocytes, macrophages, lymphocytes, and a small amount of mammary epithelial cells (Gonzalez et al., 2013). Under normal physiological conditions, 1 mL of Holstein milk contains from 2×10^4 to 2×10^5 somatic cells (VanBaale et al., 2000). The number of somatic cells in milk is affected by age, parity, stage of lactation, season, stress of body, individual characteristics, and milking operation (d'Angelo et al., 2007). Because of the unique defense system of cows, white blood cells that eliminate infection and repair damaged tissue will accumulate when the ductal system of cows is infected and damaged by diverse species of bacteria. The number of somatic cells in the secretion of mammary tissue will increase substantially from 3×10^5 to 1×10^7. The number of white cells in the blood varies, generally in the range of 7×10^6 to 1×10^7. The minimum number (7×10^6) of somatic cells in the blood is about 350 times that in the same volume of milk (Murphy et al., 2002), so DNA extraction from milk is relatively difficult (Colla et al., 2011). The average number (3×10^5) of somatic cells could reach 3×10^5 in 1 mL of milk, which is the minimum cell concentration suitable for DNA extraction. Thus, DNA extraction from milk is feasible.

Few studies have explored methods for genomic DNA extraction from milk (Maudet and Taberlet, 2001). However, in many sampling methods, the milk volume used is >50 mL. The DNA fragments obtained by traditional extraction methods can only be used to amplify products <500 bp (Phipps et al., 2003), which limits large-scale identification of genotypes and molecular analyses. Therefore, the objective of this study was

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[1]Corresponding author: zanlinsen@163.com

to develop a simpler genomic DNA extraction method suitable for large-scale genotyping operations.

MATERIALS AND METHODS

Samples

Milk samples were collected from 112 healthy adult Chinese Holstein cows. Using 15-mL centrifuge tubes, milk samples of 13 ± 1 mL were collected from each cow, and 2 drops of 5 g/mL potassium dichromate was added to each tube as a preservative. The samples were transported on ice packs (to maintain an optimum low temperature) to the laboratory and preserved at −20°C.

DNA Extraction

To separate and enrich somatic cells in milk, the cryopreserved milk samples were thawed at 4°C before being centrifuged at 2,415 × *g* and 4°C for 30 min. The top layer (cream) and middle layer (milk protein) were removed by scraping and decanting, respectively, leaving the somatic cell-containing pellet behind (Amills et al., 1997). The sediment was suspended in 600 μL of PBS (pH 7.4, 4.8 μg of NaCl, 0.12 μg of KCl, 0.864 μg of Na_2HPO_4, 0.144 μg of KH_2PO_4, 600 μL of double-distilled H_2O) before being centrifuged in a 1.5-mL centrifuge tube at 4°C for 10 min at 9,660 × *g*. After discarding the supernatant, 60 μL of emulsifiers (1.2 μL of 90% Triton-X100, 7.5 μL of 95% ethanol, and 51.3 μL of 0.009 m*M* NaCl) and 540 μL of PBS were added to the tube. After resuspension using an oscillator, the mixture was placed in a water bath at 40°C for 10 min to separate lipids from the surface of somatic cells. The mixture was recentrifuged at 4°C for 10 min at 9,660 × *g* and the supernatant was discarded. Finally, the somatic cells were resuspended in 500 μL of PBS. Then, a refrigerated centrifuge was used at 9,660 × *g* for 10 min at 4°C to concentrate precipitation of somatic cells before discarding the supernatant.

To digest somatic cells, 350 μL of DNA extraction buffer containing 50 μL of SDS (0.2 g/mL) and 10 μL of proteinase K (10 mg/mL) was added to the somatic cell concentrate. The mixture was incubated at 56°C overnight before an equal volume of 2,4,6-Tris (dimethylaminomethyl)phenol was added to the digested cell concentrate followed by repeated and gentle inverting for 10 min. The mixture was centrifuged at 4°C and 9,660 × *g* for 10 min to obtain supernatant. The supernatant was transferred to a 1.5-mL centrifuge tube with an equal volume of phenol:chloroform:isoamyl alcohol mixture (volume ratio of 25:24:1) before inverting repeatedly for 10 min to dissolve precipitation; then, the mixture was centrifuged at 4°C for 10 min at 9,660 × *g*. The supernatant was transferred to a 1.5-mL centrifuge tube with an equal volume of chloroform:isoamyl alcohol mixture (volume ratio of 24:1) before inverting repeatedly for 10 min to dissolve the precipitate. Finally, the mixture was centrifuged at 4°C for 10 min at 9,660 × *g* to collect the supernatant.

Finally, we transferred the supernatant to a 1.5-mL centrifugal tube and added 0.8 mL of ice-cold anhydrous ethanol (−20°C) to make the suspended DNA precipitate. After gentle shaking and allowing the mixture to sit for 30 min at −20°C, it was centrifuged at 4°C for 10 min at 9,660 × *g* and the top ethanol layer was removed. Next, the sediment was washed with ice-cold 70% ethanol at −20°C and centrifuged for 10 min at 9,660 × *g* at 4°C. Finally, the top ethanol layer were carefully removed, 25 μL of Tris-EDTA (1 m*M* Tris Cl and 0.5 m*M* EDTA, pH = 8.0) was added to dissolve DNA, and the mixture was stored at 4°C.

Evaluation of DNA Quality

Genomic DNA concentration and optical density ratio at 160:280 nm ($\mathbf{OD_{260/280}}$) were determined by UV spectrophotometer, and DNA size and quality were detected with 1% agarose gel electrophoresis including ethidium bromide (0.5 μg/mL; López-Calleja et al., 2004; Feligini et al., 2005) at 100 V for 30 min (Extramiana et al., 2002). After electrophoresis, the gel was observed under UV light imaging analyzer.

PCR Detection of Genomic DNA and Sequencing

According to differences in amplification primers and the size of the target sequence (based on the UV spectrophotometer results), the DNA samples were divided into 2 groups. One group of DNA samples, which had lighter bands, amplified the long (>1,000 bp) fragment of the bovine-specific functional genes. The other group, which had darker bands, amplified the short (~500 bp) target fragment.

Bovine-specific functional genes were randomly selected and their gene sequences were located in GenBank. The bovine B2 microglobulin (***B2M***) gene (GenBank accession no. NC_007308) was randomly chosen as representing bovine-specific functional genes. Two pairs of primers were designed: 5′-CAT CTG TCT TTC CCT GCC GC-3′ and 5′-CTA CAG CCT TCC TCA TCT CCC CT-3′ (primer 1; amplifying a 1,019-bp genomic sequence) and 5′-GGC TTT CCC AGC ATC ACT AAC-3′ and 5′-TCA CAG CAC CAC CAA ACT TAT CT-3′ (primer 2, amplifying 729-bp genomic sequence). The PCR was performed in 10-μL reaction mixtures containing 1 μL of genomic DNA from milk,