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

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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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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,

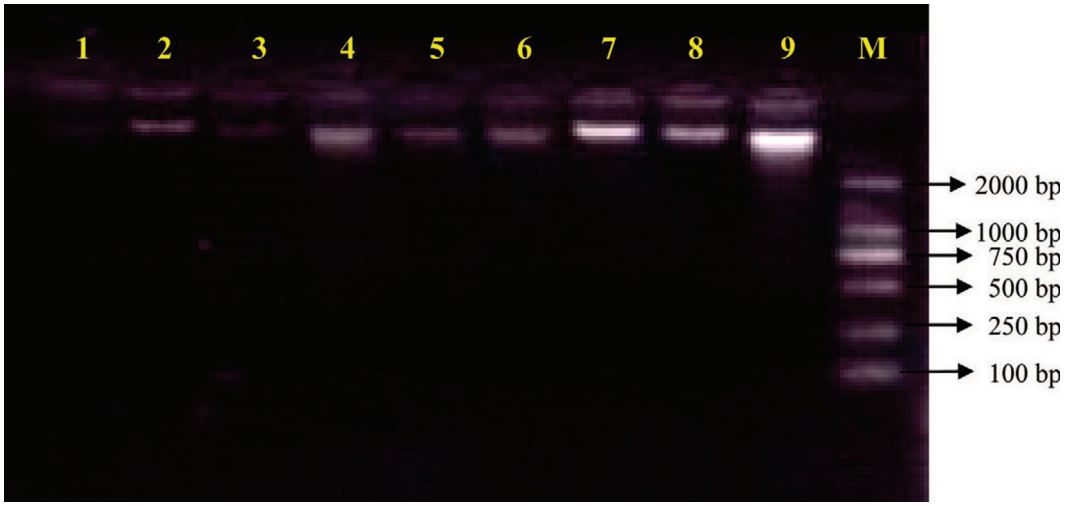

Figure 1. Representative results from agarose gel electrophoresis analysis of genomic DNA from 9 random samples in 112 milk samples. Genomic DNA from 9 random milk samples of 9 individual cows are shown in lanes 1 to 9. In each lane, 3 μL of DNA extract isolated from 12 to 14 mL of milk was analyzed. M = molecular weight marker (2,000-bp DNA ladder). Color version available in the online PDF.

10× PCR buffer, deoxynucleotide triphosphate mixture (2.5 m*M*), each primer (20 μ*M*), Taq DNA polymerase (5 U/μL), and 1 drop of liquid paraffin oil (GStorm Gradient PCR, Labtech International Ltd., Uckfield, UK). The PCR protocol of primer 1 (primer 2) was 95°C for 5 min followed by 32 cycles (30 cycles) of 94°C for 30 s, 65°C (60°C) annealing for 35s (30 s), and 72°C for 30 s, and a final extension at 72°C for 10 min. The products were purified by using a Wizard Prep PCR purification kit (Shanghai Bioasia Biotechnology, Shanghai, China) and sequenced (Beijing Aolaibo Biotechnology, Beijing, China; Applied Biosystems 3730xl DNA sequencer, Foster City, CA; Borodina et al., 2003). Genetic polymorphisms in *B2M* were identified by sequencing and comparing PCR products from 112 cows using SeqMan (DNASTAR, Inc., Madison, WI; Cler et al., 2006).

RESULTS

DNA Concentration, Purity, and Quality

We demonstrated that the somatic cell enrichment, cracking the cells by the SDS-phenol method, and nucleic acid precipitation can be used to improve processing of raw milk. Concentrations of the obtained genomic DNA were 12 to 45 μg/μL and the corresponding $OD_{260/280}$ values were 1.65 to 1.75. The results of agarose gel electrophoresis analysis are shown in Figure 1. The obtained bands were clear and consistent, with good integrity and no smearing or diffusion. The DNA extracted using the method of the present study could be used for PCR amplification. In extractive genomic DNA samples of milk, some bands were unclear, which may be related to individual differences in cows. The number of somatic cells in milk may vary with different individual animals.

PCR Amplification

The DNA samples that showed lighter bands were amplified as DNA templates; the results of PCR amplification are shown in Figure 2. The PCR amplification products of primer 1 showed bands of 1,019 bp, whose DNA sequence was consistent with the sequence of bovine *B2M* gene in GenBank. The bands were clear and single, and other bands and dimer fragments were not found. Thus, the amplification results for template DNA were excellent and the sequence results matched the nucleotide sequence of the target gene.

Similarly, in the other group, a short (~500 bp) fragment of bovine genomic sequence from the DNA samples with darker bands were amplified, and the results of PCR amplification are shown in Figure 3. The DNA sequence of primer 2 was consistent with the target sequence of bovine *B2M* gene in GenBank and the bands were clear and single. The size of the amplification product was about 729 bp, which was in line with the size of the target sequence. This finding demonstrated that even unclear DNA template can be amplified and that small quantities of DNA are suitable for PCR.

Moreover, 9 sample sequencing results amplified by the forward and reverse primers of primer pair 1 (B2M1F and B2M1R) were analyzed by DNAStar software tools (Seqman) and the results indicated no base

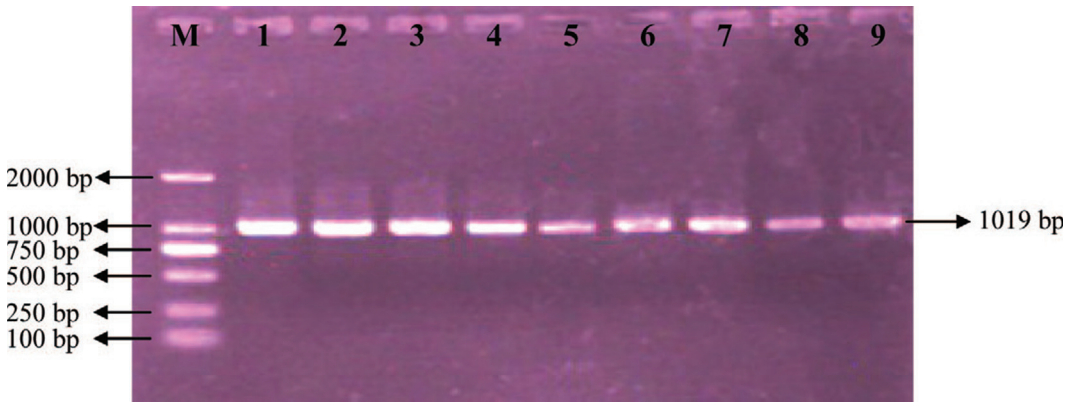

Figure 2. Representative results from agarose gel electrophoresis analysis of amplification products from 9 random genomic DNA samples with primer 1 of the bovine B2 microglobulin (*B2M*) gene. Nine random genomic DNA samples (size: 1,019 bp) with lighter bands from 9 milk samples are shown in lanes 1 to 9. M = molecular weight marker (2,000-bp DNA ladder). Color version available in the online PDF.

mutation of the entire amplification sequence. Genotype is the same indicating that the gene sequences of the segment were very conservative. The group amplified by the forward and reverse primers of primer pair 2 (B2M2F and B2M2R) showed that amplified sequences had 6 mutations, which were located at the sites of the segment sequence at 36, 168, 186, 362, 592, and 597 bp. Six sites could theoretically form 729 separate genotypes, indicating that conservation of this segment sequence was not strong and that primer 2 was not a suitable primer pair to use.

DISCUSSION

Compared with blood sampling, collection of milk samples is much easier and less stressful for dairy cows because it does not require capture, handling, or venipuncture. Classical phenol-chloroform and Chelex resin protocols have been used for extraction of genomic DNA from bovine and goat milk (Lipkin et al., 1993; Psifidi et al., 2010). However, neither of these methods is suitable for large-scale genotyping projects because consistent, quantifiable amounts of high-quality DNA cannot be obtained from milk somatic cells. Currently, new extraction techniques have been adopted with some modifications for milk somatic cells. In another study, a solid-phase absorption commercial kit (Wizard DNA clean-up kit; Promega, Madison, WI) was tested in ruminant milk, and the results were found to be reliable (Díaz et al., 2007). In that case, small volumes of milk were used and overnight incubation of samples with proteinase K was required. In another study (Psifidi et al., 2010), 6 different DNA extraction protocols [including NucleoSpin Blood Kit (Macherey-Nagel GmbH & Co. KG, Düren, Germany), NucleoSpin Tissue Kit (Macherey-Nagel GmbH & Co. KG), modified Blood

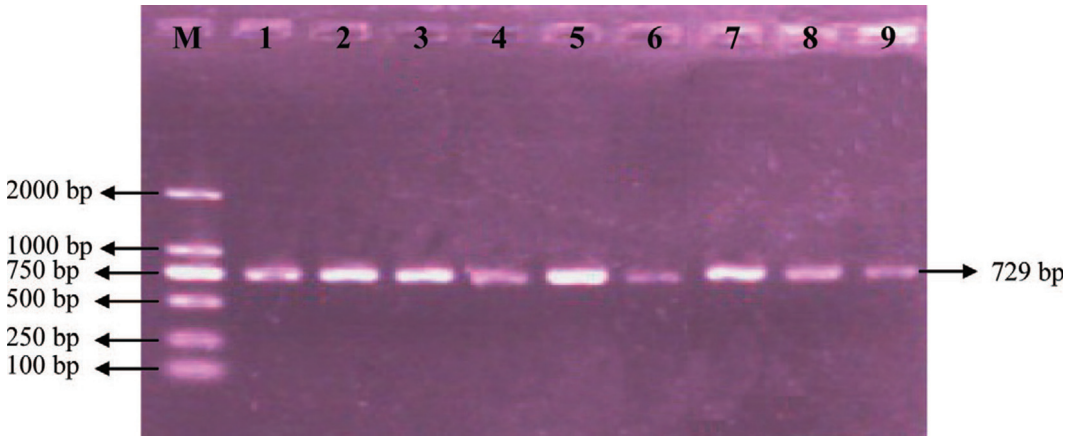

Figure 3. Representative results from agarose gel electrophoresis analysis of amplification products from 9 random genomic DNA samples (size: 729 bp) with primer 2 of the bovine B2 microglobulin (*B2M*) gene. Nine random genomic DNA sequences with darker bands from 9 milk samples are shown in lanes 1 to 9. M = molecular weight marker (2,000-bp DNA ladder). Color version available in the online PDF.

Kit protocol, modified Tissue Kit protocol, phenol-chloroform protocol, and in-house protocol] were evaluated using large-volume (50 mL) ovine milk samples. The results ($OD_{260/280}$) of the 4 commercial kits ranged from 1.71 to 1.80, which was close to the values obtained in this study; the results for the other 2 methods ranged from 1.43 to 1.55, which was lower than the values we observed. Because of the increasing usefulness of milk-pooling experiments, there is renewed interest in DNA extraction methods for milk somatic cells (Bagnato et al., 2008). Application projects using bulk milk include population screening for disease susceptibility, monitoring and eradicating genetic diseases, and detecting milk of different species in dairy products. In addition, bulk milk samples can be used for clinical diagnosis of infectious pathogens present in milk somatic cells, such as retroviruses. Using milk as a source from which to extract DNA avoids the difficulties of blood and tissue sampling and the potential stress response in cows.

The sample size of raw milk could be reduced to 13 ± 1 mL, and DNA quality and the method of DNA extraction were improved in our study. In most related studies, the samples are collected in 50-mL centrifuge tubes; however, we demonstrated that effective DNA extraction could be achieved using 15-mL centrifuge tubes to avoid the problems above (i.e., avoid wasting vessels, time, and cost). Given equal human and material resources for collecting milk samples, many more small (13 ± 1 mL) milk samples compared with large (50 mL) milk samples were collected. Therefore, our method is more convenient and faster. More importantly, it can increase the number of samples collected.

The extracted DNA could be used as a template for PCR amplification of 1,000-bp-long fragments. The DNA extracted from milk using traditional technologies only can be used as a template for amplification of mitochondrial genes (Lindquist et al., 1994), which can help determine whether a milk sample is adulterated. However, the genomic DNA obtained from the present research could serve as a bovine mitochondrial gene amplification template for the identification of adulteration in milk, and can be used for gene sequence analysis and molecular genotyping.

We have established a novel extraction method for genomic DNA that is suitable for long-fragment amplification from small amounts of milk. Milk somatic cell separation techniques were combined with SDS phenol method for DNA extraction. Then, spectrophotometry was used to determine DNA concentration and purity, and gel electrophoresis and DNA amplification technologies were used to determine DNA size and quality. This novel method could be used as a practical, fast, and economical method for long genomic DNA extraction from a small amount of milk.

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