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Evaluation of methods of DNA extraction from *Staphylococcus aureus* in milk for use in real-time PCR

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ABSTRACT. The aim of this study was to evaluate the repeatability and performance of 4 methods of extracting DNA from *Staphylococcus aureus* (SAU) and the gene encoding bovine mitochondrial cytochrome B (BMCB) in milk samples from cows with subclinical mastitis for use in amplification by real-time polymerase chain reaction. Two milk samples were obtained from cows naturally infected with *S. aureus* and subjected to the following extraction methods: Qiagen DNA extraction kit; Axyprep DNA extraction kit; in silica column boil and in silica column method. After extraction in duplicate, eluates were subjected to purification and precipitation to determine purity (A_{260}/A_{280} ratio) and concentration (μg/μL) by spectrophotometry and amplification by real-time polymerase chain reaction of target genes (*SAU* and *BMCB*). There was no effect of

the DNA extraction method on DNA concentration and threshold cycle for *BMCB* and *SAU*. The purity ratio (A_{260}/A_{280}) was higher when using Qiagen DNA extraction (1.76 ± 0.136) compared to the other methods tested. Our results indicate that the DNA extraction kit from Qiagen produces samples of the highest purity ratio compared to other methods.

Key words: DNA; Extraction; Mastitis; *Staphylococcus aureus;* Real-Time Polymerase Chain Reaction

INTRODUCTION

Bovine mastitis is considered an important illness in dairy cattle worldwide (Ribeiro et al., 2003), as it results in economic losses to the farmer (Bes et al., 2000) and dairies (Auldist and Hubble, 1998). Most disease cases have a bacterial etiology; therefore, it is necessary to identify the causative agent to adopt control and treatment measures (Oliver et al., 2004).

Isolation of the causative pathogen from intramammary infection in milk samples is considered the definitive diagnosis of bovine mastitis (Oliver et al., 2004). The conventional method for bacterial identification is based on the microbiology culture by phenotypic, biochemical, and enzymatic characteristics (Hogan et al., 1999). However, the microbiology culture of milking samples presents some limitations such as high analysis time an insufficient sensitivity to detect mammary quarters with intermittent elimination of *Staphylococcus aureus* in cases of subclinical mastitis (Phuektes et al., 2001; Taponen et al., 2009).

Because of the limitations of microbiology culture for mastitis diagnosis, qualitative protocols to detect pathogens based on polymerase chain reaction (PCR) have been described for microbial identification (Jayarao et al., 1991, Meiri-Bendek et al., 2002). Real-Time Polymerase Chain Reaction (qPCR) allows amplification and detection of the target gene to occur simultaneously in a closed system, from a coupled thermocycler to a monitoring system for fluorescence emission allowing for quantification of the number of DNA copies (Higuchi et al., 1993; Molina and Tobo, 2004). PCR is a rapid technique that allows the amplification of specific genome regions from minimal amounts of DNA, even if the DNA is degraded (Barea et al., 2004). The use of qPCR for detection of bovine mastitis-causing microorganisms shows diagnostic sensitivity and specificity similar to conventional qualitative methods of microbiological diagnosis (Taponen et al., 2009).

The qPCR method serves not only as a diagnostic means for pathogen detection (Koskinen et al., 2009; Taponen et al., 2009), but can also simultaneously quantify the target. However, qPCR still has had limited application for quantification of mastitis pathogen (Koskinen et al., 2009; Taponen et al., 2009), as the repeatability of results of quantification of the amplified target depends on the repeatability, quality, and yield, which is affected by the method used to extract genomic DNA (Mackay, 2004).

There is a strong demand for qPCR detection and enumeration of causative agents of bovine mastitis in milk samples. However, no previous study has determined which extraction methods produce the highest quality DNA with repeatability, absence of genomic DNA degradation, and genomic yield. Thus, the aim of this study was to evaluate genomic yield (DNA concentration), purity (A_{260}/A_{280} ratio), and repeatability of 4 methods used to extract DNA from *S. aureus* in milk samples from cows with subclinical mastitis.

MATERIAL AND METHODS

Two composite milk samples were collected aseptically from dairy cows that had been positively diagnosed with *S. aureus* mastitis identified by microbiological culture, according to the method described by the National Mastitis Council (Hogan et al., 1999). The electronic somatic cell count in milk samples was performed by flow cytometry using the Somacount 300 (Bentley Instruments; Dublin, Ireland).

Four methods for extraction of bacterial genomic DNA were analyzed. Extractions were performed in duplicate accordingly using the method described below.

Qiagen kit

DNA extraction using a kit from Qiagen DNA isolation kit (Hilden, Germany) was performed according to manufacturer guidelines. In a microcentrifuge tube, 20 µL Qiagen protease (or proteinase K) was pipetted into the bottom of the tube. Next, 200 µL milk sample and 200 µL lysis buffer (AL buffer) were added to the microcentrifuge tube. After homogenization by vortexing for 15 s, the solution was incubated at 95°C for 10 min. The solution was centrifuged to remove adhered contents inside the cap. Next, 200 µL ethanol (96-100%) was added and the sample was vortexed for 15 s. The solution was centrifuged to remove adhered contents inside the cap. The solution was carefully transferred to a QIAamp mini spin column (in a 2-mL collection tube), followed by centrifugation at 6000 *g* for 1 min. The mini spin column contents were transferred into a clean 2-mL collection tube (supplied with the kit). After centrifugation, the collection tube with the filtrate was discarded. Next, 500 µL wash buffer 1 (AW1 buffer) was added without wetting the tube edge. The solution was centrifuged at 6000 *g* for 1 min, after which the spin mini column was transferred to a new collection tube. The collection tube used was discarded. Again, 500 µL wash buffer 2 (AW2 buffer) was added. The solution was then centrifuged at 20,000 *g* for 3 min. After centrifugation, the solution was centrifuged for 1 min under the same conditions. The mini spin column was transferred to a new 1.5-mL collection tube for recovery of extracted DNA, the used collection tube was discarded, and 100 µL elution buffer (AE buffer) or distilled water was added. The solution was incubated at room temperature (15°-25°C) for 5 min and then centrifuged at 6000 *g* for 1 min. After centrifugation, the solution was centrifuged for 1 min under the same conditions. The extracted DNA was stored at -20°C.

AxyPrep

The protocol of the DNA extraction kit AxyPrep (Bacterial Genomic DNA Miniprep Kit protocol, Axygen Biosciences, Union City, CA, USA) was used after collecting 1.0 x 10^9 bacteria in a 2-mL microcentrifuge tube (supplied). The material collected was centrifuged at high speed (20,000 *g*) for 10 min. The supernatant was discarded and the bacterial pellet was resuspended in 150 µL bacterial preparation buffer (S buffer) containing RNAse. Next, 20 µL lysozyme was added followed by stirring. The sample was incubated at room temperature for 15 min, and then 30 µL 0.25 M EDTA, pH 8.0, was added and the sample was incubated on ice for 5 min. After adding 450 µL lysis buffer (GA buffer), the sample was homogenized by vortexing for 15 sec, heated in a water bath at 65°C for 10 min, and then 400 µL protein removal buffer (GB buffer) was added, followed by 1 mL partition buffer (DV buffer) (pre-chilled to

4°C). Vigorous mixing was followed by centrifugation at 12,000 *g* for 2 min. The upper layer of the solution (blue phase) was discarded without disturbing the interface. Next, 1 mL partition buffer (DV buffer) (pre-chilled to 4°C) was added for the remainder of the interface and lower phase and mixed vigorously to achieve homogeneity. The sample was centrifuged at 12,000 *g* for 2 min. The upper phase of the solution (blue color) was completely removed, with only the lower phase (clear coloration) of the solution remaining in the tube. The lower phase was transferred to a silica filter by placing it in a 2-mL microcentrifuge tube followed by centrifugation at 12,000 *g* for 1 min. The filtrate was discarded and 400 µL digestion buffer (BV buffer) was added to the filtrate and homogenized by vortexing. The base of a suction vacuum pump was attached and the mixture transferred to an AxyPrep column. The vacuum source was turned on and adjusted to 25-30 inches Hg. A total of 500 µL wash buffer 1 (W1 buffer) were added to the AxyPrep column and filtered through a vacuum system. Next, 700 µL wash buffer 2 (W2 buffer) was added along the wall of the AxyPrep column after buffer AW1 and again filtered using the vacuum system. The column was washed again with 700 µL wash buffer 2 (W2 buffer) and the AxyPrep column was placed inside a 2-mL microcentrifuge tube and centrifuged at 12,000 *g* for 1 min. The AxyPrep column was transferred to a clean 1.5-mL microcentrifuge tube (supplied), and 100-200 µL water or eluant was added to the center of the membrane to elute the DNA. The sample was incubated for 1 min at room temperature and then centrifuged at 12,000 *g* for 1 min. The DNA samples were stored at -20°C.

Silica column method

Reagents and buffers used for the silica column method (adapted method from Cremonesi et al., 2006 using the silica column) and boil on silica column method (adapted Barea et al., 2004), were prepared based on the protocol described by Cremonesi et al. (2006). First, 0.5 M EDTA, pH 8.0, 100 mM Tris-HCl, pH 6.8, and 1 M Tris-HCl, pH 8.0, solutions were prepared. Next, lysis buffer (AL buffer) was prepared by adding 2 g Triton, 5 mL 100 mM Tris-HCl, pH 6.8, 2 mL 0.5 M EDTA, 17.724 g guanidine thiocyanate (Sigma-Aldrich, St. Louis, MO, USA), 0.5 g DL.T, and 50 mL QSP MilliQ water. The washing buffers 1 and 2 (AW1 and AW2 buffers) were prepared by adding 0.146 g NaCl, 6.25 mL absolute ethanol, 6.25 mL isopropanol, 8.862 g guanidine thiocyanate (Sigma-Aldrich), 250 µL 1 M Tris-HCl, pH 8, and 35 mL QSP MilliQ water. However, guanidine hydrochloride (Sigma-Aldrich) was not added to wash buffer 2 (AW2 buffer); instead, 25 mL QSP MilliQ water was used. The elution buffer (AE buffer) was prepared by adding 150 µL 1 M Tris-HCl, pH 8.0, 30 µL 0.5 M EDTA, and 15 mL QSP MiliQ water.

Silica column boil

For the silica column boil method (adapted method from Nogueira et al., 2004 using the silica column), 200 µL sample and 200 µL AW1 buffer were added to a microcentrifuge tube. After boiling at 95°C for 10 min, the solution was transferred to a microcentrifuge silica tube and centrifuged at 6000 *g* for 1 min. The filtrate resulting from the centrifugation was discarded. Next, 100 µL AW2 buffer was added to the same microcentrifuge silica tube, which was again centrifuged at 20,000 *g* for 1 min. The filtrate was again discarded. Another 100 µL AW2 buffer was added and the tube was centrifuged at 20,000 *g* for 1 min. The filtrate was again discarded. The bottom of the microcentrifuge tube was exchanged for final storage and

100 µL AE buffer was added, then centrifuged at 6000 *g* for 1 min. The centrifugation procedure was repeated and the filtrate reserved. DNA samples were stored at -20°C.

Silica column

For the silica column method, 180 µL milk sample and 20 µL proteinase K were mixed with 200 µL AL buffer, and the samples were heated at 70°C for 10 min to inactivate residual proteolytic activity. Next, 200 µL ethanol were added, followed by 15 s of agitation. The resulting solution was transferred to a silica microcentrifuge tube and centrifuged at 6000 *g* for 1 min. The column was removed from the tube and placed into a clean tube. Addition of 500 µL AW1 buffer to column was followed by centrifugation at 6000 *g* for 1 min. The wash procedure was repeated with the addition of 500 µL AW2 buffer, followed by centrifugation at 20,000 *g* for 3 min. Extracted DNA from the column was eluted by adding 100 µL AE buffer. First, 50 µL were added to the solution, and after 1 min of rest the tube was centrifuged at 6000 *g* for 1 min. The samples were transferred to a recovery tube, and then 50 µL AE buffer was again added to the solution. After a 5-min incubation, the tube was centrifuged at 6000 *g* for 1 min. DNA samples were stored at -20°C.

Analysis

After performing DNA extraction, samples were subjected to precipitation and purification procedures to determine DNA concentration using a spectrophotometer (Sambrook, 2000). To evaluate concentration and purity of extracted DNA, 7 µL extracted sample was diluted in 63 µL 1X Tris-HCl pH 8.0 EDTA buffer in spectrophotometer (GeneQuant® Pro; Amersham Biosciences; Florida, USA), to determinate the A_{260}/A_{280} ratio and concentration of genomic material in units of µg/µL.

After extraction, samples were amplified by qPCR using the StepOne method (Applied Biosystems, Foster City, CA, USA) according to the manufacturer protocol. The primer for the positive control was based on the bovine mitochondrial cytochrome B (BMCB) gene, with sense 5'-GCAATACACTACACATCCGACACAA-3' and antisense 5'-GCGTGTATGTATCGGATGATTCAG-3', the primers for amplification reactions of *S. aureus* were, respectively, senso 5'-CCTGAAGCAAGTGCATTTACGA-3' e antisenso 5'-CTTTAGCCAAGCCTTGACGAACT-3', designed using the PrimerExpress software (Applied Biosystems). For the amplification reaction, the reaction mixture was composed of 10 µL SYBR Green PCR Master Mix, 3.6 µL sense and antisense primers, and 1.8 µL autoclaved MilliQ water. Next, 1 µL of each extracted DNA sample was added to 19 µL reaction mixture into each well of an qPCR plate. The main primers used were BMCB (gene encoding bovine mitochondrial cytochrome B) and SAU (*S. aureus*). The thermocycler program was run for 50 cycles at 95°C for 15 s and 60°C for 1 min, with an initial cycle of 95°C for 10 min. After amplification, the results were expressed in threshold cycles (Ct).

Results were subjected to descriptive statistics and analysis of variance using the Minitab software, version 16.1.1 (State College, PA, USA).

RESULTS

Treatment means were compared using the Tukey test with a significance level of 5%.

In this study, there was no effect of extraction method on Ct values and DNA concentration; however, there was an effect of the extraction method on the A_{260}/A_{280} ratio (Table 1). The Qiagen method showed a higher purity ratio (A_{260}/A_{280} ratio) of 1.76 ± 0.136 µg/µL than the other extraction methods. The Axyprep, boiling, and silica column methods showed A_{260}/A_{280} ratios of 1.36 ± 0.064, 1.33 ± 0.057, and 1.47 ± 0.087 µg/µL, respectively.

Table 1. Effect of DNA extraction method on DNA concentration (µg/µL), A_{260}/A_{280} ratio, and threshold cycle.

	Axyprep®	Qiagen®	Boil	Silica Column	SE	P value
Concentration (µg/mL)	449.25	414.1[a]	330.75	626.50	177.1	0.184
A_{260}/A_{280} ratio	1.36[b]	1.76[a]	1.33[b]	1.47[b]	0.182	0.021
Threshold cycle¹	36.363	38.689	39.391	36.172	7.949	0.934
Threshold cycle²	19.385	19.305	23.225	24.130	4.044	0.249

Means followed by the same letter in the same row do not differ at the 5% level according to the Tukey test. Threshold cycle¹ represents Ct for SAU (*Staphylococcus aureus*) and Threshold cycle² represents Ct for BMCB (gene encoding bovine mitochondrial cytochrome B).

Using the coefficient variation of purity (A_{260}/A_{280} ratio), all methods were shown to have lower values, or values close to 5%, indicating good repeatability during amplification.

DISCUSSION

In a study by Lusk et al. (2013), the commercially available Qiagen extraction method showed the highest performance for threshold cycle (Ct ≤ 31) among all tested methods for DNA extraction from milk samples. In the present study, Ct values were not affected by the extraction method used. The results of the present study are similar to those of Simonato et al. (2007), who showed higher amplification and purity of samples extracted using the Qiagen method. However, compared with other DNA extraction methods, such as Chelex and boiling, the cost of the Qiagen extraction method was higher (Barea et al., 2004; Simonato et al., 2007). The Axyprep method showed questionable repeatability; it showed variable results in eliminating the "supernatant" considering that the handler directly interferes with repeatability. The supernatant was derived from reagents of the extraction kit, with the milk fat forming a fat layer in the tube, preventing direct contact of the pipette tip with the liquid fraction of the sample without physically contacting the supernatant. The other extraction methods studied did not generate a fat layer (supernatant).

Replacement of the silica cap with a silica column for immediate use in the column silica method provides reliability, ease of use, and shorter execution time, compared to the original protocol using the silica cap, as described by Cremonesi et al. (2006).

Although the results were limited because only 2 samples were analyzed, the coefficients of variation in relation to the concentration methods were less than 5%. One possible source of variation, as described by Phillips et al. (2012), may be pipetting errors during transfer of DNA into the wells of the qPCR plate; if a pipetted volume is reduced, less DNA is extracted from the sample. Another source of variation is the fat concentration present in milk samples, which directly interferes in the extraction of DNA from milk.

qPCR can be used to detect mastitis-causing agents, but the DNA extraction technique requires improvement. The qPCR technique is limited to detecting agents with purity values (A_{260}/A_{280} ratio) of 1.6-1.9, the possibility of repetition (coefficient of variation <5%),

and quality and yield of genetic material, and the possibility of amplification by qPCR from a sample that has had a successful DNA extraction. The Qiagen DNA Isolation Kit method showed the highest purity (1.76), as well as high repeatability (coefficient of variation <5%). Thus, the DNA extraction kit from Qiagen showed the highest purity ratio compared with the other methods tested.

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