NOTE *Bacteriology*

Improved rapid and efficient method for *Staphylococcus aureus* DNA extraction from milk for identification of mastitis pathogens

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(Received 26 March 2014/Accepted 16 March 2015/Published online in J-STAGE 3 April 2015)

ABSTRACT. A rapid and efficient DNA extraction method was developed for detecting mastitis pathogens in milk. The first critical step involved cell wall disruption by bead-beating, as physical disruption using beads was more effective for DNA extraction from Gram-positive bacteria, such as *Staphylococcus aureus*, than enzymatic disruption using proteinase K. The second critical step involves the use of acetic acid and ammonium sulfate in the purification process, as these reagents effectively and efficiently remove the lipids and proteins in milk. Using these methods, DNA suitable for loop-mediated isothermal amplification was obtained within 30 min. Also, the rapid and sensitive detection of *S. aureus* in milk was possible at levels as low as 200 cfu/m*l*.
KEY WORDS: cow milk, DNA chip, DNA extraction, loop-mediated isothermal amplification (LAMP), mastitis, pathogen

doi: 10.1292/jvms.14-0159; *J. Vet. Med. Sci.* 77(8): 1007–1009, 2015

Bovine mastitis commonly affects dairy cows during the perinatal period, causing extensive economic damage to dairy farmers [1–4, 10, 12]. Mastitis results from an inflammation of a mammary gland due to infection through the teat and subsequent colonization of pathogens. *Staphylococcus aureus*, *Streptococcus uberis*, *Escherichia coli*, *Klebsiella pneumoniae*, *Mycoplasma bovis* and yeasts are well-known mastitis pathogens. Effective mastitis prevention depends on accurate identification of the pathogens responsible for infection [3, 15]. These pathogens are typically detected by cultivation on an identification medium and by biochemical analysis [8]. However, effective prevention of mastitis using traditional detection methods is difficult, as pathogen identification is time consuming and sensitivity is low [12, 14]. We previously developed a molecular assay using a DNA chip to detect mastitis pathogens [6, 13]. To ensure that a molecular assay is sufficiently sensitive, DNA extraction from the pathogens needs to be both efficient and have good reproducibility. Current techniques used to extract DNA from pathogens in milk require numerous specimens, involve a cumbersome concentration process and employ a lengthy enzymatic cell lysis step [5]. In addition, no extraction method for mastitis pathogens has been developed to date. In this study, we report the development of a rapid and highly efficient DNA extraction method for mastitis pathogens, including Gram-positive bacteria, such as *Staphylococcus aureus*, which have a thick outer cell wall.

Eighty-one milk samples from cows with clinical mastitis were obtained from dairy farmers in Hokkaido, Japan. The milk was collected aseptically in a sterile tube after cleaning the teat with cotton wool containing alcohol immediately after milking. The collected milk was stored at −80°C until DNA extraction. Mastitis pathogens in milk were detected by the cultivation method [7], which involves spreading 20 μl of milk on 5% sheep blood agar (Becton, Dickinson and Co., Franklin Lakes, NJ, U.S.A.) and then incubating the plates at 37°C for 24 hr. Pathogens were identified using a BBL Crystal Gram-Positive Identification System (Becton, Dickinson and Co.), and 14 *S. aureus* strains were selected for further analysis. A summary of the DNA extraction process for mastitis pathogens is shown in Fig. 1. In order to extract DNA for comparison with our method, we used a FAST ID Genomic DNA Extraction Kit (Genetic ID NA, Inc., Fairfield, IA, U.S.A.) that employs proteinase K for cell lysis. The loop-mediated isothermal amplification (LAMP) method was then used to evaluate DNA extraction performance [11]. The LAMP reaction was conducted in 25 μl of reaction mixture containing 1 μl of template DNA at 61°C for 60 min using a turbidity meter (LA-200, Teramecs, Kyoto, Japan). The following six primers were used for the LAMP amplification of *S. aureus* DNA: FIP: ACTGTTGGATCTTCAGAACCACT-CTCAGCAAATGCATCACAA, BIP: GAACCTGCGA-CATTAATTAAAGCG-TCTGAATGTCATTGGTTGAC, F3: ACTTTAGTTGTAGTTTCAAGTCT, B3: GATGCTTT-GTTTCAGGTGTA, LoopF: TCTATTTACGCCGTTATCT-GT and LoopB: GTGATACGGTTAAATTAATGTAC.

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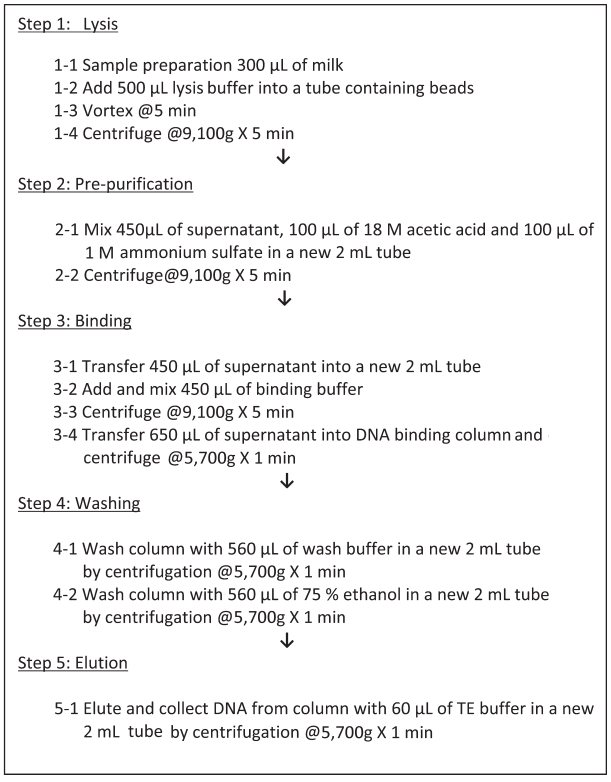

Fig. 1. Flowchart of the improved DNA extraction method.

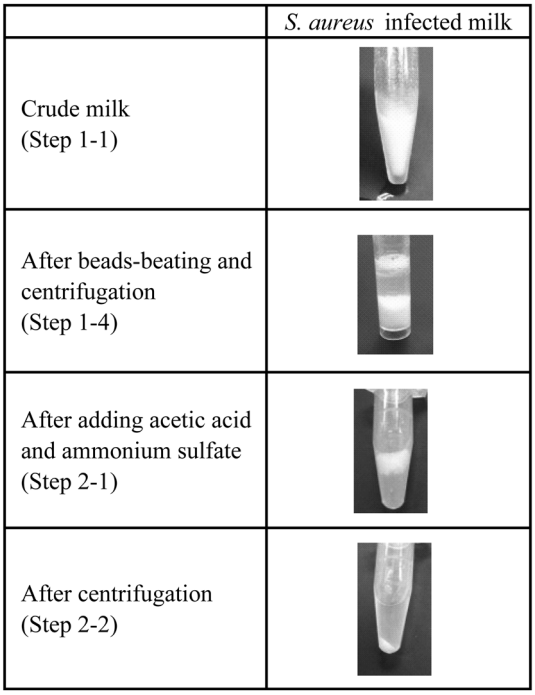

Fig. 2. Effectiveness of bead-beating and incorporation of acetic acid and ammonium sulfate addition to the improved DNA extraction protocol.

First, we evaluated the sensitivity of the LAMP reaction for detecting *S. aureus*. The threshold time (turbidity: >0.1) depended on the amount of DNA. We calculated the copy number of *S. aureus* genome based on a genome size of 2.8 Mbp and the amount of DNA by measuring the optical density at 260 nm. Experiments using DNA copy numbers of 10^4, 10^3, 10^2 and 10^1 had threshold times of 21 min, 24 min, 30 min and 36 min, respectively; tenfold differences in the DNA concentration corresponded to a threshold time of 3 to 6 min, and sensitivity was less than 10 copies of DNA. We then evaluated the distribution of pathogenic bacteria in milk using the LAMP reaction. The milk separated into two layers after being allowed to stand in a test tube for a while. The upper fraction contained large amounts of lipid, and the lower fraction contained a watery substance. The amount of pathogenic bacteria in the upper layer, lower layer and thoroughly mixed sample was measured using *S. aureus* infected milk (7,950 cfu/m*l*). The turbidity of the LAMP reaction mixture began to increase at 41 min, 46 min and 44 min, respectively, suggesting that a large number of pathogens were present in the upper lipid-rich layer. Traditional extraction methods often remove lipids from milk before DNA extraction, as lipids can inhibit enzymatic amplification in PCR and LAMP [5]. However, we considered that extraction efficiency could be improved by performing cell lysis before removing the lipids. The bead-beating method for DNA extraction from pathogens in milk was originally developed for extracting DNA from spores that have a hard covering [9]. Since this process was also effective for lipid dispersion in milk, we incorporated a bead-beating step into our DNA extraction method to disrupt the bacterial cells in the milk samples (Fig. 1).

Since extraneous protein is also known to inhibit PCR and LAMP reactions, effective removal of protein and lipids is considered to be important for improving the sensitivity of assays that detect pathogen DNA in milk. We therefore used acetic acid and ammonium sulfate to purify DNA extracted from milk. *Staphylococcus aureus* infected milk was used to evaluate the new DNA extraction method (Fig. 2). Bead-beating and centrifugation steps effectively separated lipid and protein components as a clear supernatant was obtained in all of the samples. However, the addition of acetic acid and ammonium sulfate to purify the DNA resulted in the formation of precipitates in the samples. Centrifugation alone was insufficient for removing lipid and protein from the samples. We then compared the DNA extraction efficacy between the Fast ID kit and our new method. The turbidity threshold times for the Fast ID kit and the new method using *S. aureus* infected milk (1,280 cfu/m*l*) were 43 min and 32 min, respectively, confirming that the new method for extracting DNA from the Gram-positive *S. aureus* in milk was more effective than previous methods. The effect of adding acetic acid and ammonium sulfate was then evaluated using *S. aureus* infected milk with different bacterial titers (Fig. 3). Using the modified Fast ID kit, in which the proteinase K lysis step was substituted with a bead-beating step, one-third of the *S. aureus* infected milk samples tested positive for *S.*

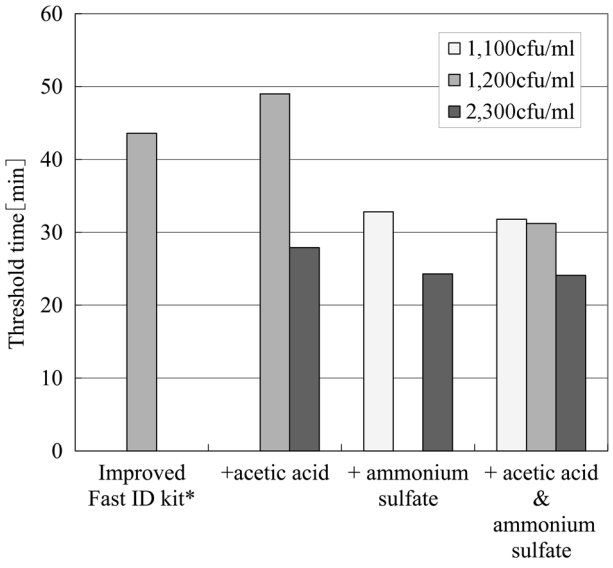

Fig. 3. Effect of acetic acid and ammonium sulfate addition on sensitivity of the LAMP method for detecting *S. aureus* in milk. *Substitution of bead-beating step for proteinase K treatment with the Fast ID Genomic DNA Extraction kit.

aureus DNA in milk. Two-thirds of the samples tested positive when either acetic acid or ammonium sulfate was used to purify the DNA, and all of the samples tested positive when both reagents were used to purify the DNA, indicating that using both reagents is optimal. The new method is thus considered to be very robust and well suited for extracting the DNA of pathogenic bacteria from milk.

Finally, we examined the effectiveness of the new DNA extraction method on fourteen *S. aureus* infected milk samples (2×10^2 to $>5 \times 10^4$ cfu/m*l*). All culture-positive samples tested positive using the LAMP method, and the lowest detected concentration of *S. aureus* was 200 cfu/m*l*. The new DNA extraction method described here was both quick and effective, requiring less than 30 min to perform, which meant that sensitive and robust milk sample analysis could be completed in less than 90 min.

ACKNOWLEDGMENT. This work was supported by a grant from the Ministry of Agriculture, Forestry and Fisheries of Japan.

REFERENCES

1. Bartlett, P. C., Miller, G. Y., Anderson, C. R. and Kirk, J. H. 1990. Milk production and somatic cell count in Michigan dairy herds. *J. Dairy Sci.* **73**: 2794–2800. [Medline] [CrossRef]
2. Beck, H. S., Wise, W. S. and Dodd, F. H. 1992. Cost benefit analysis of bovine mastitis in the UK. *J. Dairy Res.* **59**: 449–460. [Medline] [CrossRef]
3. Bramley, A. J., Cullor, J. S., Erskine, R. J., Fox, L. K., Harmon, R. J., Hogan, J. S., Nickerson, S. C., Oliver, S. P., Smith, K. L. and Sordillo, L. M. 1996. Microorganisms that cause bovine mastitis. pp. 11–14. *In*: Current Concepts of Bovine Mastitis, 4th ed. National Mastitis Council, Madison.
4. Cha, E., Bar, D., Hertl, J. A., Tauer, L. W., Bennett, G., González, R. N., Schukken, Y. H., Welcome, F. L. and Gröhn, Y. T. 2011. The cost and management of different types of clinical mastitis in dairy cows estimated by dynamic programming. *J. Dairy Sci.* **94**: 4476–4487. [Medline] [CrossRef]
5. Cremonesi, P., Castiglioni, B., Malferrari, G., Biunno, I., Vimercati, C., Moroni, P., Morandi, S. and Luzzana, M. 2006. Technical note: Improved method for rapid DNA extraction of mastitis pathogens directly from milk. *J. Dairy Sci.* **89**: 163–169. [Medline] [CrossRef]
6. Goto, K., Horiuchi, H., Shinohara, H., Motegi, K., Hashimoto, K., Hongo, S., Gemma, N., Hayashimoto, N., Itoh, T. and Takakura, A. 2007. Specific and quantitative detection of PCR products from *Clostridium piliforme, Helicobacter bilis, H. hepaticus*, and mouse hepatitis virus infected mouse samples using a newly developed electrochemical DNA chip. *J. Microbiol. Methods* **69**: 93–99. [Medline] [CrossRef]
7. Hogan, J. S., Gonzalez, R. N., Harmon, R. J., Nickerson, S. C., Oliver, S. P., Pankey, J. W. and Smith, K. L. 1999. Laboratory Handbook on Bovine Mastitis, Revised ed. National Mastitis Council, Madison.
8. Kerro Dego, O., van Dijk, J. E. and Nederbragt, H. 2002. Factors involved in the early pathogenesis of bovine *Staphylococcus aureus* mastitis with emphasis on bacterial adhesion and invasion. A review. *Vet. Q.* **24**: 181–198. [Medline] [CrossRef]
9. Kurosaki, Y., Horiuchi, H., Fujinami, Y., Hashimoto, K. and Yasuda, J. 2010. Development and application of rapid and simple method for extracting nucleic acids from microbes. *Jpn. J. Forensic Sci. Tech.* **15**: 135–142. [CrossRef]
10. Losinger, W. C. 2005. Economic impacts of reduced milk production associated with an increase in bulk-tank somatic cell count on US dairies. *J. Am. Vet. Med. Assoc.* **226**: 1652–1658. [Medline] [CrossRef]
11. Mori, Y., Nagamine, K., Tomita, N. and Notomi, T. 2001. Detection of loop-mediated isothermal amplification reaction by turbidity derived from magnesium pyrophosphate formation. *Biochem. Biophys. Res. Commun.* **289**: 150–154. [Medline] [CrossRef]
12. Morin, D. E., Petersen, G. C., Whitmore, H. L., Hungerford, L. L. and Hinton, R. A. 1993. Economic analysis of a mastitis monitoring and control program in four dairy herds. *J. Am. Vet. Med. Assoc.* **202**: 540–548. [Medline]
13. Okada, J., Horiuchi, H., Hashimoto, K., Hirosawa, D., Kurosaki, Y., Kawamoto, K., Yasuda, J., Makino, S., Gemma, N. and Nikaido, M. 2012. Mobile automatic detection system for bacillus anthracis using electrochemical DNA chip. *J. Biosens. Bioelectron.* **3**: 126 [CrossRef].
14. Schutz, M. M. 1994. Genetic evaluation of somatic cell scores for United States dairy cattle. *J. Dairy Sci.* **77**: 2113–2129. [Medline] [CrossRef]
15. Volling, O. and Krömker, V. 2008. [Udder health management practices in dairy enterprises to reduce the incidence of bovine mastitis]. *Dtsch. Tierarztl. Wochenschr.* **115**: 410–420. [Medline]