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Combination of procedure for ultra rapid extraction (PURE) and loop-mediated isothermal amplification (LAMP) for rapid detection of *Mycoplasma bovis* in milk

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ABSTRACT. Polymerase chain reaction (PCR) is typically used for the early detection of mycoplasma in bovine milk; it requires 3 days to obtain results because of the necessary enrichment process. A more rapid, simple, and accurate detection method is required to directly detect the *Mycoplasma bovis* (*M. bovis*) gene in milk. In this study, we assess the utility of combining the following two methods to achieve this goal: the loop-mediated isothermal amplification (LAMP), which is more sensitive than PCR, and the procedure for ultra rapid extraction (PURE), which adsorbs and filters components that inhibit DNA amplification/detection. LAMP was examined using DNA extracts obtained by four methods. This showed that PURE had the highest sensitivity and specificity and that the combination of PURE and LAMP was able to detect *M. bovis* in milk. We then showed that the detection limit of *M. bovis* was 10^2 colony-forming units per milliliter of milk using the PURE–LAMP. Finally, the respective sensitivities of the PURE–LAMP and PCR were 57% and 86% for bulk tank milk, 89% and 74% for mature milk, 85% and 92% for colostrum/transitional milk, and 97% and 95% for mastitis milk. The specificity was 100% for all milk samples in both LAMP and PCR. We conclude that PCR was suitable for detecting mycoplasma in bulk tank milk and that the PURE–LAMP could detect mycoplasma within 2 hr and was also effective for mature and mastitis milk.

KEY WORDS: loop-mediated isothermal amplification (LAMP), milk infection, *Mycoplasma bovis*, procedure for ultra rapid extraction (PURE), rapid detection

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Bovine mycoplasma mastitis is a contagious disease that affects dairy cows. It can devastate the economy of dairy farms and the health of their livestock because of its severe symptoms, strong infectivity, undetectability by common mastitis tests, and inability to cure with typical antibiotics [2, 4, 7]. In some regions in Japan, screening tests for mycoplasma are regularly conducted in bulk tank milk to prevent the spread of infection by early detection and early response. Conventionally, culture tests were performed for the diagnosis of bovine mycoplasma mastitis, but these require 5–10 days in mycoplasma culture media to produce results, by which time any infection may have spread. Recently, polymerase chain reaction (PCR) was therefore introduced to enable earlier detection [6], and although this also requires enrichment in culture, results can be obtained in as short as 3 days, thereby helping to prevent the spread of mycoplasma mastitis. However, *Mycoplasma bovis* (*M. bovis*) mastitis, the most infectious and prevalent form of mycoplasma in cattle [4], can spread before even these results are known, leaving farmers understandably stressed while waiting for the results. Thus, efforts must be made to further shorten the inspection time.

The loop-mediated isothermal amplification (LAMP) is a gene amplification technique that was developed in Japan [14, 15]. In this method, DNA is incubated at a constant temperature for approximately 60 min with four different primers to identify six distinct regions on the target gene and a polymerase with high-strand displacement activity. This is a simple amplification method

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that allows inspection and detection in a single step and short time. Considering that the amplification efficiency is estimated to be 10–100 times greater than that of PCR [1, 20], it may be possible to use it to detect mycoplasma DNA directly from milk samples without the need for enrichment culture. The LAMP determines positivity based on an increase in turbidity, yet milk is highly turbid at sample collection because it contains fat and casein. This necessitates pretreatment to remove the turbidity.

The procedure for ultra rapid extraction (PURE) is a rapid and simple technique of DNA extraction and purification [12] in which components that inhibit DNA amplification and detection are adsorbed on porous material and filtered. It has been confirmed that this method is suitable for use with blood, throat swab, and sputum [8, 10, 12, 13] samples, indicating that it should be suitable for use with milk. We hypothesized that combining DNA extraction by the PURE and gene amplification by the LAMP could further shorten the time to obtain results.

In this study, we aimed to clarify the effectiveness of direct rapid detection of *M. bovis* in milk by the PURE–LAMP by examining the DNA extraction method (study 1), confirming the detection limit (study 2) and comparing the sensitivity/specificity of the PURE–LAMP with that of PCR using an enriched solution (study 3).

MATERIALS AND METHODS

Study 1: Effectiveness of PURE in DNA extraction from milk

We examined 16 samples of milk (*M. bovis* positive, 8 [mastitis milk, 4. mature milk, 4]; *M. bovis* negative, 8 [mycoplasma negative, 4; other mycoplasmas positive, 4]). For DNA extraction, we examined the PURE (PURE DNA extraction kit, Eiken Chemical, Tokyo, Japan: sample volume, 300 *µl*; reagents, 2 kinds; sample concentration in solution; 25%, incubation, 90°C for 5 min, with filtration). This was compared with commercially available DNA simple extraction kits A (Cica Genius® DNA Extraction Reagent, Kanto Chemical, Tokyo, Japan: sample volume, 10 *µl*; reagents, 2 kinds; sample concentration in solution; 9%, incubation, 72°C for 6 min and 94°C for 3 min), B (SR DNA extraction kit, Eiken Chemical: sample volume, 50 *µl*; reagents, 3 kinds; sample concentration in solution; 45%, incubation, 95°C for 5 min, spin-down centrifuge), and C (Mighty Prep reagent for DNA, Takara Bio, Kusatsu, Japan: sample volume, 20 *µl*; reagent, 1 kind; sample concentration in solution; 17%, incubation, 95°C for 10 min, high-speed centrifuge). DNA extracts obtained by the PURE, A, B, or C methods were then mixed with primers [5] and DNA amplification regents (DNA amplification reagent D, Eiken Chemical). Using a real-time turbidimeter (Loopamp EXIA, Eiken Chemical), we attempted to detect *M. bovis* using the LAMP at 63°C over 60 min. A turbidity of 0.1 or more was considered positive [5].

Study 2: Detection limit of M. bovis in milk by PURE–LAMP

Nine positive milk samples containing *M. bovis* (Sample No. 1–5: somatic cell count (SCC) <200/*µl*, 6–7: SCC 200–1,000/*µl*, 8–9: SCC >1,000/*µl*) within 24 hr of collection were diluted with *M. bovis* negative milk of similar SCC levels to achieve × 10, × 10^2, × 10^3, × 10^4, and ×10^5 dilutions, and the PURE–LAMP was performed according to the approach described in study 1. Furthermore, 10 *µl* of each diluted solution was plated on Hayflick agar plates and incubated in 5% CO_2 at 37°C for 3–10 days to produce typical mycoplasma colonies. The mycoplasma counts in the milk were calculated based on the number of colony-forming units (CFUs), and the detection limit of *M. bovis* in milk was clarified by the PURE–LAMP.

Study 3: Sensitivity and specificity of M. bovis between PURE–LAMP and enriched-broth PCR

We examined 12 samples of bulk tank milk (*M. bovis* positive, 7; *M. bovis* negative, 5), 73 of mature milk (positive, 38; negative, 35), 74 of colostrum or transitional milk (second milking after parturition) (positive, 13; negative, 61), and 122 of mastitis (modified California mastitis test positive) milk (positive, 58; negative, 64) from eight farms in the Tokachi, Hokkaido, Japan. The PURE–LAMP was performed according to the approach describe in study 1, using a directly obtained 300 *µl* sample of milk (direct-milk PURE–LAMP). In addition, 100 *µl* of milk was inoculated in 3 m*l* of Hayflick broth and incubated at 37°C for 3 days. *M. bovis* DNA in the enriched broth was analyzed using a DNA extraction kit (Cica Genius® DNA Extraction Reagent, Kanto Chemical) and a PCR kit (Cica Genius® *M. bovis* Detection Plus Kit, Kanto Chemical) for enriched-broth PCR [6]. The sensitivity and specificity of the direct-milk PURE–LAMP and enriched-broth PCR were clarified, and the effectiveness of LAMP was evaluated using Kappa coefficient as follows: <0.4, poor; 0.41–0.6, moderate; 0.61–0.8, good; and >0.8, excellent [19].

The determination of *M. bovis* positive / negative in milk sample was performed by the following method. One hundred microliter of milk was inoculated in 3 m*l* of Hayflick broth and incubated at 37°C for 5–7 days. Ten microliter of the incubated broth sample was applied to Hayflick agar plates and incubated in 5% CO_2 at 37°C for 3–10 days. If the mycoplasma colony did not grow, it was determined to be negative. If the mycoplasma colony grew, the colony was separated and cultured, and the strain was identified by species-specific PCR [9], SDS-PAGE [11], and 16S rRNA sequencing [17].

We confirmed beforehand that the PURE-LAMP did not show false positive results for *M. arginini, M. canadense*, *M. adleri*, *M. bovigenitalium*, and *M. californicum* (cause of mycoplasma mastitis other than *M. bovis*) using infected milk and American Type Culture Collection strains.

RESULTS

Study 1: Effectiveness of PURE in DNA extraction from milk (Table 1)

DNA extraction methods A, B, and C produced turbid samples, whereas the PURE produced a transparent sample that was pale

yellow in color (Fig. 1). The baseline determinations by real-time turbidimeter were also unstable for extraction methods A, B, and C. Of note, extraction method B could not distinguish the difference from increased turbidity due to DNA amplification. Some samples obtained by extraction methods A and C could discern an increase in turbidity due to DNA amplification reaction despite the unstable baseline result. However, the PURE produced a stable baseline that was clearly distinguishable from the DNA amplification reactions. *M. bovis*-negative samples were negative using LAMP reaction for all DNA extraction methods, and no false positives were observed. The false negative rate for *M. bovis*-positive samples was 0% (0/8 samples) for the PURE, compared to 50% (4/8 samples), 100% (8/8 samples), and 25% (2/8 samples) for extraction methods A, B, and C, respectively. The time required for the PURE–LAMP test was within 2 hr.

Study 2: Detection limit of M. bovis in milk by PURE–LAMP (Table 2)

The PURE–LAMP was able to detect *M. bovis* in milk about >10^2 CFU/m*l* for most milk samples. In sample 3, although an increase in turbidity was observed, the turbidity after 60 min was 0.07, which was determined to be a false negative. For other samples, the corresponding times to positive results (turbidity of >0.1) were between 43.3 and 56.1 min. The false negative milk was retested and found positive in 57.2 min. However, *M. bovis* could not be detected in milk for levels below 10^2 CFU/m*l* for the milk samples of No.1–9.

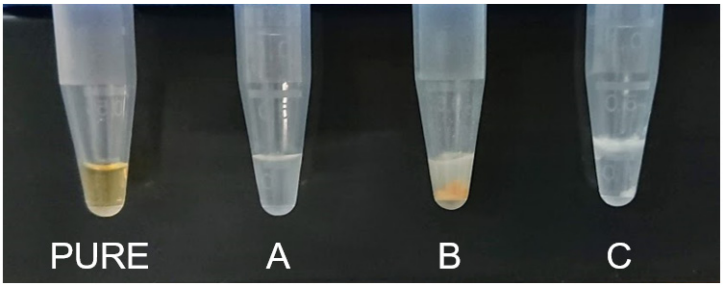

Fig. 1. DNA extract prepared using procedure for ultra rapid extraction (PURE), method A, B, and C.

Table 1. Results of loop mediated isothermal amplification (LAMP) of *Mycoplasma bovis* (*M. bovis*) positive and negative milk using procedure for ultra-rapid extraction (PURE), method A, B and C as DNA extraction method

DNA extraction method*	Milk sample			
	M. bovis + (n=8)		*M. bovis* – (n=8)	
	LAMP test +	LAMP test –	LAMP test +	LAMP test –
PURE	8	0	0	8
A	4	4	0	8
B	0	8	0	8
C	6	2	0	8

+: positive, -: negative. *PURE: sample volume, 300 *µl*; reagents, 2 kinds; sample concentration in solution; 25%, incubation, 90°C for 5 min, with filtration. A: sample volume, 10 *µl*; reagents, 2 kinds; sample concentration in solution; 9%, incubation, 72°C for 6 min and 94°C 3 min. B: sample volume, 50 *µl*; reagents, 3 kinds; sample concentration in solution; 45%, incubation, 95°C for 5 min, spin-down centrifuge. C: sample volume, 20 *µl*; reagent, 1 kind; sample concentration in solution; 17%, incubation, 95°C for 10 min, high-speed centrifuge

Table 2. Detection limit of *Mycoplasma bovis* (*M. bovis*) in milk by procedure for ultra rapid extraction and loop-mediated isothermal amplification (PURE-LAMP)

M. bovis (CFU/m*l*)	LAMP test								
	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6	Sample 7	Sample 8	Sample 9
	SCC <200 cells/*µl*					SCC 200–1,000 cells/*µl*		SCC >1,000 cells/*µl*	
1 (1–9)	–	–	–	–	–	–	–	–	–
10 (10–99)	–	–	–	–	–	–	–	–	–
10^2 (100–999)	+	+	–[a)]	+	+	+	+	+	+
10^3 (1,000–9,999)	+	+	+	+	ND	+	+	+	+

+: positive, –: negative. a) False negative (turbidity increased to 0.1 after 60 min). ND: no data (because the number of mycoplasmas in the sample was <1,000 CFU/m*l*). CFU: colony forming unit, SCC: Somatic cell count.

Study 3: Sensitivity and specificity analysis between PURE–LAMP and enriched-broth PCR (Table3)

In the bulk milk, seven samples were positive and five samples were negative for *M. bovis*. By the direct-milk PURE–LAMP, four samples were positive and eight samples were negative, with a sensitivity of 57%, a specificity of 100% and kappa coefficient of 0.526 (moderate). By the enriched-broth PCR, six samples each were positive and negative, giving a sensitivity of 86%, a specificity of 100% and kappa coefficient of 0.833 (excellent). The three samples that were false negative by the direct-milk PURE-LAMP and the one sample that was false negative by the enriched-broth PCR were different samples.

In the mature milk, 38 samples were positive and 35 samples were negative for *M. bovis*. By the direct-milk PURE–LAMP, 34 samples were positive and 39 samples were negative, with a sensitivity of 89%, a specificity of 100% and kappa coefficient of 0.891 (excellent). By the enriched-broth PCR, 28 samples were positive and 45 samples were negative, with a sensitivity of 74%, a specificity of 100% and kappa coefficient of 0.729 (good). One of the four samples that were false negative by the direct-milk PURE-LAMP and seven of the ten samples that were false negative by the enriched-broth PCR were different samples.

In the colostrum or transitional milk, 13 samples were positive and 61 samples were negative for *M. bovis*. By the direct-milk PURE–LAMP, 11 samples were positive and 63 samples were negative, with a sensitivity of 85%, a specificity of 100% and kappa coefficient of 0.901 (excellent). By the enriched-broth PCR, 12 samples were positive and 62 samples were negative, with a sensitivity of 92%, a specificity of 100% and kappa coefficient of 0.952 (excellent).

In the mastitis milk, 58 samples were positive and 64 samples were negative for *M. bovis*. By the direct-milk PURE–LAMP, 56 samples were positive and 66 samples were negative, with a sensitivity of 97%, a specificity of 100% and kappa coefficient of 0.967 (excellent). By the enriched-broth PCR, 55 samples were positive and 67 samples were negative, with a sensitivity of 95%, a specificity of 100% and kappa coefficient of 0.951 (excellent). The two samples that were false negative by the direct-milk PURE-LAMP and the three samples that were false negative by the enriched-broth PCR were different samples.

DISCUSSION

Various methods exist for extracting DNA from samples for genetic examination. In this study, we examined simple DNA extraction methods with three or fewer regents and one or fewer other equipment items, together with incubation times and temperatures of 10 min or less and 2 levels or less, respectively. In the PURE, we used 300 *µl* of milk. Given that the amount of milk was highest for this extraction method, the amount of *M. bovis* DNA was also highest, potentially improving the chance of obtaining an accurate result. As the amount of milk in a sample increased, however, the amount of fat and casein also increased, making the DNA extract more turbid. Using the PURE, substances other than DNA were adsorbed and filtered to produce a transparent extract from the milk sample. In method A, the amount of milk sample was small, but the DNA extract remained turbid. Centrifugation effectively removed turbidity from milk, leaving a casein layer at the bottom, a fat layer at the top, and a whey layer in the middle [16]. It was only necessary to sample the whey layer to obtain DNA extract without turbidity, but we could not avoid some mixing with the other layers. The DNA extracts obtained by methods B and C were centrifuged, but the turbidity could not be removed completely. Among the DNA extraction methods that we examined, the greatest accuracy for *M. bovis* detection was achieved when using the PURE.

Currently, the most widely used method for detecting mycoplasma in milk is to incubate 100 *µl* in a liquid medium specific for mycoplasma (e.g., Hayflick broth) for 3 days, to extract DNA from the enriched medium, and then to subject that material to PCR [6]. Theoretically, this method can detect one live mycoplasma in 100 *µl* of milk, so the detection limit is considered to be 10 CFU/m*l*. In this study, the detection limit of *M. bovis* in milk was predicted to be 10^2 CFU/m*l* when using the PURE–LAMP, which is about 10 times less sensitive than the PCR after enrichment. However, the examination time can be reduced from 3 days to 1.5–2 hr by using directly obtained milk in the PURE–LAMP with no enrichment. The milk of cows with mastitis contains 10^5–10^9 CFU/m*l* of mycoplasma, and infected cows excrete a large amount of mycoplasma before symptoms develop [18]. Therefore, even at the

Table 3. Comparison of positive number, negative number, sensitivity and specificity of *Mycoplasma bovis* (*M. bovis*) in bulk tank milk, mature milk, colostrum/transitional milk, and mastitis milk by direct-milk rocedure for ultra rapid extraction and loop-mediated isothermal amplification (PURE-LAMP) and enriched-broth polymerase chain reaction (PCR)

	M. bovis	Direct-milk PURE-LAMP					Enriched-broth PCR				
		Number of positive	Number of negative	Sensitivity (%)	Specificity (%)	Kappa coefficient	Number of positive	Number of negative	Sensitivity (%)	Specificity (%)	Kappa coefficient
Bulk tank milk	Positive (n=7)	4	3	57	-	0.526	6	1	86	-	0.833
	Negative (n=5)	0	5	-	100		0	5	-	100	
Mature milk	Positive (n=38)	34	4	89	-	0.891	28	10	74	-	0.729
	Negative (n=35)	0	35	-	100		0	35	-	100	
Colostrum/ transitional milk	Positive (n=13)	11	2	85	-	0.901	12	1	92	-	0.952
	Negative (n=61)	0	61	-	100		0	61	-	100	
Mastitis milk	Positive (n=58)	56	2	97	-	0.967	55	3	95	-	0.951
	Negative (n=64)	0	64	-	100		0	64	-	100	

Kappa cefficient: <0.4, poor; 0.41–0.6, moderate; 0.61–0.8, good; >0.8, excellent.

detection limit of 10^2 CFU/ m*l*, it is considered that *M. bovis* is unlikely to be missed. If *M. bovis* in milk has already died, it may not be detected by enrichment and PCR, but can be detected by the PURE-LAMP. We consider that the PURE–LAMP was effective for detecting mycoplasma infection in milk. In this study, "false negative samples" were found that could not be determined positive within 60 min, although turbidity increased. It is recommended that such samples be retested.

When testing mycoplasma in milk on dairy farms, bulk tank milk is typically used for screening; mature milk is used to identify the positive cow on the farm; and colostrum, transitional, and mastitis milk are used to detect newly infected cows. We showed that the specificity of the direct-milk PURE–LAMP was 100% for all milk samples, with no negative samples incorrectly categorized as positive. In mature and mastitis milk samples, the direct-milk PURE–LAMP detected *M. bovis* with higher sensitivity than the enriched-broth PCR and achieved an excellent grade. However, it was lower sensitivity than the enriched-broth PCR for the bulk tank and colostrum/transitional milk samples.

The screening test for bulk tank milk is effective for detecting mycoplasma-positive farms. However, if the number of positive cows or if the bacterial discharge is small, there is a risk of a false negative result [4]. In this study, three bulk tank milk samples were *M. bovis* positive by the enriched-broth PCR but negative by the direct-milk PURE–LAMP. According to interview survey, at the farms where these samples were collected, the milk of all cows was examined at a later date. As a result, it was reported that 1 of 294 cows, 1 of 170 cows, and 1 of 168 cows were positive for *M. bovis* at three farms. The three cows had discharged mycoplasma in their milk but were reported to be free of any clinical signs. It was considered that the amount of mycoplasma discharged was relatively small. Given that the amount of mycoplasma in the bulk tank milk was small, it was considered to have been above the detection limit in the enriched-broth PCR but below the detection limit for the direct-milk PURE–LAMP. However, one bulk tank milk sample that was negative by the enriched-broth PCR was positive by the direct-milk PURE–LAMP. Screening of bulk tank milk is imperfect, but on balance, the enriched-broth PCR appears to be more suitable than the direct-milk PURE–LAMP for detecting mycoplasma in this setting.

Detecting *M. bovis* from colostrum or transitional milk was also less sensitive with the direct-milk PURE–LAMP than with the enriched-broth PCR, but the difference was marginal. Only one sample was *M. bovis* positive by the enriched-broth PCR but negative by the direct-milk PURE-LAMP. Colostrum contains many components, such as fat and protein, which lower the accuracy of DNA extraction compared with mature milk [3]. The transitional milk used in this study was obtained from the second sample after parturition and, as such, was the second most concentrated milk after colostrum. Not only was the amount of mycoplasma in these colostrum and transitional milk below the detection limit, but the high concentration of milk components may have been caused the slightly lower sensitivity of the direct-milk PURE–LAMP. In Japan, milk obtained within 5 days after parturition cannot be shipped for sale. Hence, farmers often wish to bring colostrum or second milk for mycoplasma examination to know the results within this period and ensure smooth shipments and prevent exposure to other milking cows. Existing methods require at least 3 days to obtain results from such tests, but our proposed direct-milk PURE–LAMP offers the potential to obtain valid results within 2 hr. Even using transitional milk milked on the fifth day after parturition, accurate results could be obtained within the restricted shipping period. The effectiveness of using the direct-milk PURE–LAMP with transitional milk that is closer to mature milk (milked 3–5 days after parturition) is certainly worth considering in future research.

In samples of both mature and mastitis milk, the sensitivity of the direct-milk PURE–LAMP was non-inferior to that of the enriched-broth PCR. The direct-milk PURE–LAMP was available for these milks. Although the presence of false negative samples suggested that bacterial counts may be low even in mastitis milk, we considered that PURE–LAMP was able to reliably detect infected cows with high concentrations of *M. bovis* at high risk of spreading other cows. However, there was a discrepancy in false negatives detected by the two methods. Samples that were positive by the enriched-broth PCR but that were negative by the direct-milk PURE–LAMP was one in mature milk and two in mastitis milk. We considered that these samples contained only about 10 CFU/m*l* of *M. bovis* and were below the PURE-LAMP detection limit. Seven samples in mature milk and three in mastitis milk were positive by the direct-milk PURE–LAMP and negative by the enriched-broth PCR, respectively. It was considered that these samples did not sufficiently grow *M. bovis* in the enrichment-broth, though *M. bovis* concentration was at least 100 CFU/m*l*. But the cause is unknown. Of these, one sample of mature milk and three samples of mastitis were mixed infections with another mycoplasma. The growth of *M. bovis* may have been inhibited because another mycoplasma grew preferentially. This should be considered in future studies.

In conclusion, the PURE–LAMP could detect *M. bovis* directly in milk within 2 hr and was both effective and non-inferior to the enriched-broth PCR, especially for cases of mature and mastitis milk.

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REFERENCES

1. Bai, Z., Shi, L., Hu, C., Chen, X., Qi, J., Ba, X., Peng, Q., Chen, Y., Chen, H. and Guo, A. 2011. Development of a loop-mediated isothermal amplification assay for sensitive and rapid detection of *Mycoplasma bovis*. *Afr. J. Biotechnol.* **10**: 12333–12338.
2. Fox, L. K., Kirk, J. H. and Britten, A. 2005. Mycoplasma mastitis: a review of transmission and control. *J. Vet. Med. B Infect. Dis. Vet. Public Health* **52**: 153–160. [Medline] [CrossRef]
3. Gamberale, F., Pietrella, G., Sala, M., Scaramella, P., Puccica, S., Antognetti, V., Arrigoni, N., Ricchi, M. and Cersini, A. 2019. Management of Mycobacterium avium subsp. paratuberculosis in dairy farms: Selection and evaluation of different DNA extraction methods from bovine and

buffaloes milk and colostrum for the establishment of a safe colostrum farm bank. *MicrobiologyOpen* **8**: e875. [Medline] [CrossRef]
4. González, R. N. and Wilson, D. J. 2003. Mycoplasmal mastitis in dairy herds. *Vet. Clin. North Am. Food Anim. Pract.* **19**: 199–221. [Medline] [CrossRef]
5. Higa, Y., Uemura, R., Yamazaki, W., Goto, S., Goto, Y. and Sueyoshi, M. 2016. An improved loop-mediated isothermal amplification assay for the detection of *Mycoplasma bovis*. *J. Vet. Med. Sci.* **78**: 1343–1346. [Medline] [CrossRef]
6. Higuchi, H., Iwano, H., Kawai, K., Ohta, T., Obayashi, T., Hirose, K., Ito, N., Yokota, H., Tamura, Y. and Nagahata, H. 2011. A simplified PCR assay for fast and easy mycoplasma mastitis screening in dairy cattle. *J. Vet. Sci.* **12**: 191–193. [Medline] [CrossRef]
7. Jasper, D. E. 1982. The role of Mycoplasma in bovine mastitis. *J. Am. Vet. Med. Assoc.* **181**: 158–162. [Medline]
8. Kawano, S., Maeda, T., Suzuki, T., Abe, T., Mikita, K., Hamakawa, Y., Ono, T., Sonehara, W., Miyahira, Y. and Kawana, A. 2015. Loop-mediated isothermal amplification with the Procedure for Ultra Rapid Extraction kit for the diagnosis of pneumocystis pneumonia. *J. Infect. Chemother.* **21**: 224–226. [Medline] [CrossRef]
9. Kobayashi, H., Hirose, K., Worarach, A., Paugtes, P., Ito, N., Morozumi, T. and Yamamoto, K. 1998. In vitro amplification of the 16S rRNA genes from *Mycoplasma bovirhinis, Mycoplasma alkalescens* and *Mycoplasma bovigenitalium* by PCR. *J. Vet. Med. Sci.* **60**: 1299–1303. [Medline] [CrossRef]
10. Kobayashi, M., Aoki, S., Koike, K., Moriya, A., Katou, M. and Saito, T. 2015. Evaluation of the PURE-LAMP method for diagnosing *Mycobacterium tuberculosis* complex. *J. Jpn. Soc. Clin. Microbiol.* **25**: 106–110.
11. McNulty, M. S., Gowans, E. J., Houston, M. J. and Fraser, G. 1975. Neuraminidase content of strains of Newcastle disease virus which differ in virulence. *J. Gen. Virol.* **27**: 399–402. [Medline] [CrossRef]
12. Mitarai, S., Okumura, M., Toyota, E., Yoshiyama, T., Aono, A., Sejimo, A., Azuma, Y., Sugahara, K., Nagasawa, T., Nagayama, N., Yamane, A., Yano, R., Kokuto, H., Morimoto, K., Ueyama, M., Kubota, M., Yi, R., Ogata, H., Kudoh, S. and Mori, T. 2011. Evaluation of a simple loop-mediated isothermal amplification test kit for the diagnosis of tuberculosis. *Int. J. Tuberc. Lung Dis.* **15**: 1211–1217, i. [Medline] [CrossRef]
13. Miyazaki, H., Shibano, M., Nakano, T., Hayakawa, S., Okamura, T. and Saitoh, T. 2018. Comparison of QIAGEN method and PURE method, which is a DNA extraction method by the LAMP method using loopamp mycoplasma pneumoniae detection reagent kit. *Jpn. J. Med. Technol* **67**: 456–461.
14. Nagamine, K., Hase, T. and Notomi, T. 2002. Accelerated reaction by loop-mediated isothermal amplification using loop primers. *Mol. Cell. Probes* **16**: 223–229. [Medline] [CrossRef]
15. Notomi, T., Okayama, H., Masubuchi, H., Yonekawa, T., Watanabe, K., Amino, N. and Hase, T. 2000. Loop-mediated isothermal amplification of DNA. *Nucleic Acids Res.* **28**: E63. [Medline] [CrossRef]
16. Oyeniyi, O. O. and Hunter, A. G. 1978. Colostral constituents including immunoglobulins in the first three milkings postpartum. *J. Dairy Sci.* **61**: 44–48. [Medline] [CrossRef]
17. Pettersson, B., Uhlén, M. and Johansson, K. E. 1996. Phylogeny of some mycoplasmas from ruminants based on 16S rRNA sequences and definition of a new cluster within the hominis group. *Int. J. Syst. Bacteriol.* **46**: 1093–1098. [Medline] [CrossRef]
18. Pfützner, H. and Sachse, K. 1996. *Mycoplasma bovis* as an agent of mastitis, pneumonia, arthritis and genital disorders in cattle. *Rev. - Off. Int. Epizoot.* **15**: 1477–1494. [Medline] [CrossRef]
19. Spekhorst, L. M., Visschedijk, M. C., Alberts, R., Festen, E. A., van der Wouden, E. J., Dijkstra, G., Weersma R. K. and Dutch Initiative on Crohn and Colitis. 2014. Performance of the Montreal classification for inflammatory bowel diseases. *World J. Gastroenterol.* **20**: 15374–15381. [Medline] [CrossRef]
20. Wang, J., Zhang, Y., Cui, Y., Yan, Y., Wang, X., Wang, R., Jian, F., Zhang, L. and Ning, C. 2017. A rapid, simple and sensitive loop-mediated isothermal amplification method to detect *Anaplasma bovis* in sheep and goats samples. *Parasitol. Int.* **16**: 30568–305672. [Medline]