Food Chemistry 327 (2020) 126945

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

High-performance detection of *Mycobacterium bovis* in milk using digital LAMP

Yi Tao[a,b], Juanli Yun[a], Jian Wang[a], Peng Xu[a], Caiming Li[a,b], Hongtao Liu[a,b], Ying Lan[a,*], Jianzhang Pan[d,*], Wenbin Du[a,b,c,*]

[a] State Key Laboratory of Microbial Resources, Institute of Microbiology, Chinese Academy of Sciences, Beijing 100101, China
[b] College of Life Sciences, University of the Chinese Academy of Sciences, Beijing 10049, China
[c] Savaid Medical School, University of the Chinese Academy of Sciences, Beijing 10049, China
[d] Department of Chemistry, Zhejiang University, Hangzhou 310058, China

ARTICLE INFO

Keywords:
Zoonotic tuberculosis
Mycobacterium bovis
Digital loop-mediated isothermal amplification (dLAMP)
Molecular detection
Milk

ABSTRACT

This paper described a high-performance molecular test for the detection of *Mycobacterium bovis* (*M. bovis*) based on digital loop-mediated isothermal amplification (dLAMP). *M. bovis* is a persistent pathogen that causes zoonotic tuberculosis and can infect both animals and human beings. The detection of *M. bovis* in milk samples is critical for effective control and prevention of zoonotic diseases but there lacks effective and sensitive methods. Here, we developed a convenient and low-cost system for *M. bovis* detection in milk, which incorporated automated DNA extraction and dLAMP by interfacial emulsification technique. Versus real-time PCR, dLAMP provides higher accuracy and sensitivity for direct *M. bovis* detection in milk, offering a limit of detection of 14 CFU/mL within 2 h. The dLAMP system can become a powerful platform for the detection of pathogens in complex samples and provide more reliable guidance for food safety testing, epidemiological research and clinical diagnosis.

1. Introduction

Zoonotic tuberculosis (TB) is an infectious disease caused by *Mycobacterium bovis* (*M. bovis*)—a pathogen that belongs to the *Mycobacterium tuberculosis* complex (MTBC) (Barbier et al., 2016). Zoonotic TB can cause serious public health threats, including human infection. The World Health Organization (WHO) reported that there were 147,000 new cases globally and 12,500 people died due to zoonotic TB in 2016 (WHO, FAO, OIE, 2017).

The prerequisite for developing a test for *M. bovis* is to target a carrier source that can transmit the pathogen to humans. The prevalence of *M. bovis* is mainly through animal products such as meat and milk (Silva et al., 2018). Animal excretions such as sputum, feces, urine, and aerosols also act as media and reservoirs for the spreading of *M. bovis* between animals and humans (Barbier et al., 2016; Ereqat et al., 2013). As a major source of protein and fat, milk is consumed by more than six billion people throughout the world. However, milk is also a latent medium for zoonotic TB because *M. bovis* can infect the mammary glands of the animal causing mastitis and cutaneous infections (Hussien & Mahrous, 2016). Poor pasteurization of milk may lead to human infection by *M. bovis* (Zumárraga et al., 2012). Therefore, the detection of *M. bovis* in milk is indispensable for both husbandry and food safety.

The control of zoonotic diseases requires effective tests for the causative pathogens, especially for slow-growing pathogens such as mycobacteria. (Fusco & Quero, 2014; Mortari & Lorenzelli, 2014). For *M. bovis* detection in milk, there are three major approaches: microbial culture, immunological methods, and molecular tests (Zhang et al., 2016). Molecular tests based on nucleic acid amplification offer direct evidence for the presence of the pathogen in the sample—it is non-invasive, sensitive, specific, stable, and fast (Fusco and Quero, 2014). However, compared with the detection of other pathogens (Lee, Kim, Lee, Kim, & Seo, 2016; Nicolaou, Xu, & Goodacre, 2012), there only a few reports on the detection of *M. bovis* in milk using quantitative polymerase chain reaction (qPCR) (Barbier et al., 2016; Ereqat et al., 2013) or loop-mediated isothermal amplification (LAMP) (Zhang et al., 2011) and the current molecular tests of *M. bovis* are not well established or applied for the following reasons: (*i*) *M. bovis* has an unusual lipid-rich cell wall that is different from common Gram-positive or Gram-negative bacteria, (Lambrecht, Carriere, & Collins, 1988; Stewart, Robertson, & Young, 2003) which makes it very difficult to chemically disrupt *M. bovis* cells to extract its DNA; (*ii*) manual DNA extraction

* Corresponding authors at: State Key Laboratory of Microbial Resources, Institute of Microbiology, Chinese Academy of Sciences, Beijing 100101, China.
E-mail addresses: lanying@im.ac.cn (Y. Lan), kelvonpan@zju.edu.cn (J. Pan), wenbin@im.ac.cn (W. Du).

https://doi.org/10.1016/j.foodchem.2020.126945
Received 3 February 2020; Received in revised form 21 April 2020; Accepted 28 April 2020
Available online 06 May 2020
0308-8146/

methods are inconvenient with poor efficiency (Romero & Lopez-Goni, 1999); (*iii*) milk inhibitors such as calcium ions and proteinases hamper molecular amplification (Mortari & Lorenzelli, 2014; Rossen, Nørskov, Holmstrøm, & Rasmussen, 1992; Wilson, 1997); (*iv*) the low number of *M. bovis* in milk samples may be hindered by the presence of high background nucleic acids from heterogeneous resident microflora and somatic cells, that is, competitive binding of the amplification enzyme (Fusco and Quero, 2014).

Recently, digital nucleic acids amplification (dNAA) (Ding & Mu, 2016) methods such as digital PCR (dPCR) and digital LAMP (dLAMP) have been developed for quantitative and high sensitive molecular testing. dLAMP has been applied in the detection of various pathogens (Yuan et al., 2018) with high accuracy, sensitivity, specificity, as well as better tolerance to inhibitors versus dPCR (Hu, Xu, Luo, He, & Du, 2016). Recently, we developed interfacial emulsification, a chip-free monodisperse droplet generation technique, and applied it to establish a simple and affordable droplet dLAMP system (Hu et al., 2016; Xu, Zheng, Tao, & Du, 2016). To apply dLAMP technique to rapid detection of *M. bovis* in milk, here, we developed a complete pipeline by combining centrifugation enrichment, magnetic bead-based automated DNA extraction, and dLAMP based on interfacial emulsification. Our results show that the dLAMP could detect *M. bovis*-spiked milk samples within 2 h, and the 14 CFU/mL detection limit is at least 10-fold more sensitive and more tolerant to inhibitors than qPCR in milk. Furthermore, the combination of automated DNA extraction and chip-free dLAMP significantly improves the detection efficiency and standardization, which lays a foundation for future large-scale *M. bovis* assessment in animal husbandry and food industries.

2. Materials and methods

2.1. Bacteria culture and genomic DNA (gDNA) extraction

All of the strains used in this study were kindly provided by Professor Fuping Zhang from the CAS Key Laboratory of Pathogenic Microbiology& Immunology at the Institute of Microbiology, Chinese Academy of Sciences. *Mycobacterium bovis* bacille Calmette-Guérin (BCG) strain Pasteur 1173P2 , *Mycolicibacterium mucogenicum* ATCC 49650, *Mycobacterium gordonae* DSM 44160, *Mycobacterium paraense* IEC26, *Mycobacterium kansasii* ATCC 12478, and *Mycobacterium lentiflavum* ATCC 51985 were cultivated in 7H9 liquid medium at 37 ˚C for 2 to 4 weeks. *Escherichia coli* O157:H7 ATCC 43895, *Listeria monocytogenes* S3, *Staphylococcus aureus* RN4220, and *Salmonella enteria* ATCC 39184 were cultivated in Luria-Bertani (LB) liquid medium at 37 ˚C for overnight. The total DNA of each bacterial strain was extracted from 1 mL liquid culture using Minibest genomic DNA extraction kit ver3.0 (TaKaRa Bio, Dalian, China) according to the manufacturer's instruction.

2.2. Primer sequences

The *M. bovis* mpb70 gene sequence was obtained from the GenBank (EU683971). All the LAMP primers were designed by the Primer Explorer version 4. (https://primerexplorer.jp) and were synthesized by BioSune Biotechnology (Shanghai, China). (The primers are shown in Table S1).

2.3. Preparation of a plasmid carrying the target sequences

The target mpb70 gene was amplified from *M. bovis* BCG gDNA using Mpb70- F_3 and Mpb70- B_3 by PCR. The product was purified by EasyPure Quick Gel Extraction kit (Transgen Biotech, Beijing, China) and ligated into the pEASY-T1 vector using pEASY-T1 Simple Cloning Kit (Transgen) following the manufacturer's protocol. The ligation product was transferred into the Trans1-T1 Phage Resistant Chemically Competent Cell (Transgen). Plasmids were extracted from PCR-verified positive colonies, and the concentration was determined by a Qubit 3.0 Fluorometer (Thermo Scientific, Waltham, WA). The 10-fold serial diluted plasmid samples were used to make the standard curve.

2.4. Preparation of milk samples and DNA auto-extraction

The protocol used for the preparation of spiked milk samples was as previously described by Paul, Van Hekken, and Brewster (2013). Pasteurized whole cow milk was obtained from local markets. Briefly, 800 μL of 0.5 M EDTA was mixed with 10 mL milk and kept at 40 ˚C for 5 min. Then, suspended *M. bovis* BCG cells (1.4×10^7 CFU/mL) were ten-fold serial diluted by physiological saline; 100 μL diluted BCG was then added to 10 mL treated milk. The bacterial concentration was determined using a conventional plate counting method. For automated DNA extraction, the milk sample needs to be centrifuged immediately (15 min, $4696 \times g$) (Brewster & Paul, 2016). Next, the milk fat and supernatant liquid were removed by pipetting. The precipitated pellet was re-suspended by 800 μL 4% NaOH and inactivated for 10 min. Then, 800 μL of the mixture was added to the deep-well plate of Ex-DNA *M. tuberculosis* nucleic acid extraction kit (Tianlong, Xi'an, China). Afterward, the deep-well plate was placed in an automated nucleic acid extractor (Tianlong, Xi'an, China) for automated DNA extraction within 30 min. Extracted DNA samples were used for dLAMP and qPCR.

2.5. LAMP reaction preparation

Each LAMP reaction was in a 25 μL system containing 2.5 μL of 10 × ThermoPol buffer (NEB, Ipswich, MA), 1.6 μM each of FIP and BIP primers, 0.2 μM each of F_3 and B_3, 0.8 μM each of LF and LB, 8 U of the Bst 2.0 WarmStart DNA polymerase (NEB), 1.4 mM of each dNTPs (NEB), 0.6 M betaine (Sigma-Aldrich, St. Louis, MO), 6 mM $MgSO_4$ (Sigma-Aldrich), 25 μM of calcein (Sigma-Aldrich), 0.5 mM $MnCl_2$, 2 mg/mL BSA (NEB), and 1 μL of the template or ddH_2O (negative control). The reaction tubes were incubated at 66 ˚C for 1 h, and the results were displayed by direct visualization of the color change of solutions. Positive reactions are light green, and negative reactions are orange. Every experiment was conducted in triplicate and included a non-template control.

2.6. dLAMP

A 96-well plate could perform dLAMP experiments for 12 samples, and each reaction was divided into 18,400 1 nL droplets. The dLAMP experiments used 25 μL of pre-mixed LAMP mix loaded into a capillary connected to Teflon tubing to generated droplet arrays in 8 microwells of a flat-bottomed 96-well plate as previously described (Xu et al., 2016). Briefly, the reaction mix was injected through the outlet of the capillary at a flow rate of 50 nL/s while the capillary was vibrating on the surface of oil contained in a flat-bottomed 96-well plate at a frequency of 50 Hz. Monodisperse 1 nL droplets were obtained in the microwell. Each well receives 2300 droplets that self-assemble into a monolayer droplet array when an injection volume of 2.3 μL was given. The 96-well plate was incubated at 66 ˚C on a PCR machine (Mastercycler gradient, Eppendorf, Germany) for 1 h following fluorescence imaging of the droplet arrays with an inverted fluorescence microscope (Eclipse Ti, Nikon, Tokyo, Japan). We analyzed the images and counted positive droplets with ImageJ software (NIH, Bethesda, MD, USA), and then calculated the template concentrations according to the Poisson distribution (Dong, Chen, Liu, & Du, 2016).

2.7. qPCR

The qPCR system contained 10 μL 2 × SsoFast EvaGreen Supermix (Bio-Rad, Hercules, CA); 0.5 μL (10 μM each) mpb70-F_3 and mpb70-B_3; 1 μL template, and ddH_2O to a total volume of 20 μL. Non-template controls were set using ddH_2O as the template. The optimal cycling

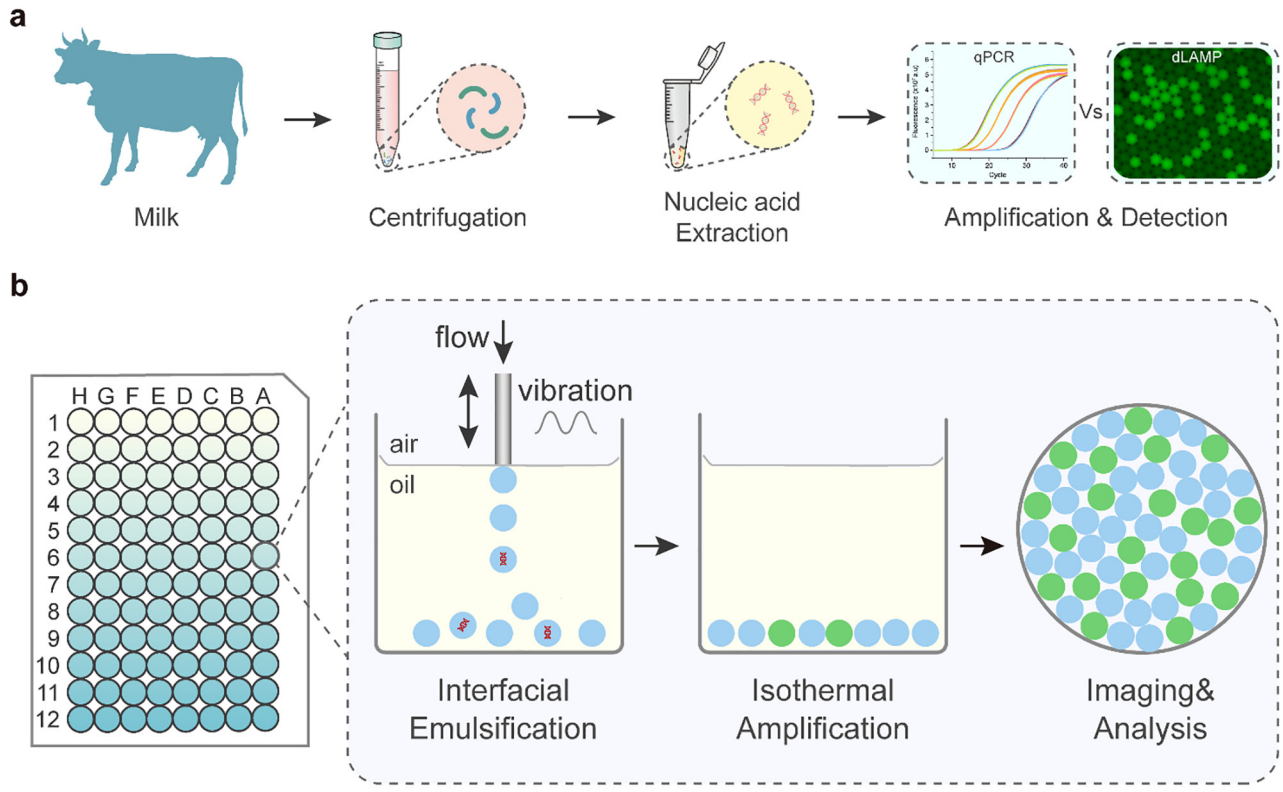

Fig. 1. (a) Schematic of the "sample-to-answer" pipeline for *M. bovis* detection in milk using digital Loop-mediated isothermal amplification (dLAMP). (b) Brief procedures of dLAMP include the generation of droplet arrays in a microwell plate by interfacial emulsification, isothermal incubation, fluorescence imaging and analysis.

program was: 98 °C for 2 min, 40 cycles of 98 °C for 5 s, 60 °C for 20 s, and storage at 4 °C. Every experiment included a non-template control and three replicates.

3. Results and discussion

3.1. Pipeline design for dLAMP detection of M. bovis in whole milk

To enable quantification of *M. bovis* in milk using dLAMP and explore its potential for epidemiological research and food safety testing, we coupled the automated DNA extraction with our self-designed automated dLAMP system for *M. bovis* testing in whole milk. As shown in Fig. 1a, the method consisted of three steps: (1) Centrifugation to enrich the *M. bovis* cells from a large volume of whole milk (e.g., 10 mL); (2) Automated DNA extraction using a commercial workstation that can process 32 samples at one time; (3) Nucleic acid amplification and absolute measurement of *M. bovis* specifically detect the conservation Mpb70 gene of *M. bovis*. Briefly, for each sample, around 18,400 droplets were produced in 8 microwells (2300 drops per well) of a flat-bottomed 96-well plate via a custom-made apparatus based on interfacial emulsification (Fig. 1b). The well plate was then incubated and imaged via an inverted fluorescent microscope.

The integrated dLAMP detection pipeline provides a convenient method for *M. bovis* detection and quantification in milk samples, which serve as the routine medium for screening in the husbandry and food industries. It has the following advantages: (1) The centrifugation step and automated DNA extraction enable efficient bacteria enrichment and DNA extraction from a large volume of whole milk. (2) The system can handle DNA extraction of 32 samples simultaneously and analyze 12 samples at one time in a single 96-well plate within 2 h. (3) The dLAMP procedure provides absolute quantification of *M. bovis* in milk, which is comparable with commercial qPCR systems with high sensitivity and specificity without relying on standard curves. (4) Compared with qPCR, dLAMP provides better tolerance to hard-to-remove inhibitory substances (Gansen, Herrick, Dimov, Lee, & Chiu, 2012; Hu et al., 2016; Nixon et al., 2014; Zhu et al., 2012). Overall, dLAMP significantly reduced the cost of the platform, consumables, shortened the 'sample-to-answer' time, and enabled more reliable detection of *M. bovis* in milk (Table 1).

3.2. The specificity of the self-designed M. bovis detection primers using dLAMP and qPCR

To verify the specificity of LAMP and PCR primers for *M. bovis* detection, we performed reactions with reference strains of untargeted bacteria that are often present in milk (Quigley et al., 2013), including *M. mucogenicum, M. gordonae, M. paraense, M. kansasii, M. lentiflavum, E. coli* O157:H7*, L. monocutogenes, S. aureus, S. enterica,* and *M. bovis*. Both qPCR and dLAMP amplification showed negative results for the reference strains, and exclusive positive results only for *M. bovis* (Fig. 2), indicating favorable specificity of the primers.

Table 1
A comparison of dLAMP with qPCR for detection of *M. bovis*.

	dLAMP	qPCR
Limit of detection (copies/μL)	3.88	31.61
Dynamic Range (Log10)	5	9
Inhibition by spiked milk (IC_{50})	2.85%	0.16%
Amplification time (h)	1	1.5 ~ 2
Quantitativeness	Absolute	Relative (rely on a standard curve)
Temperature control	Isothermal	Thermal cycling
Specificity	Extremely high	High

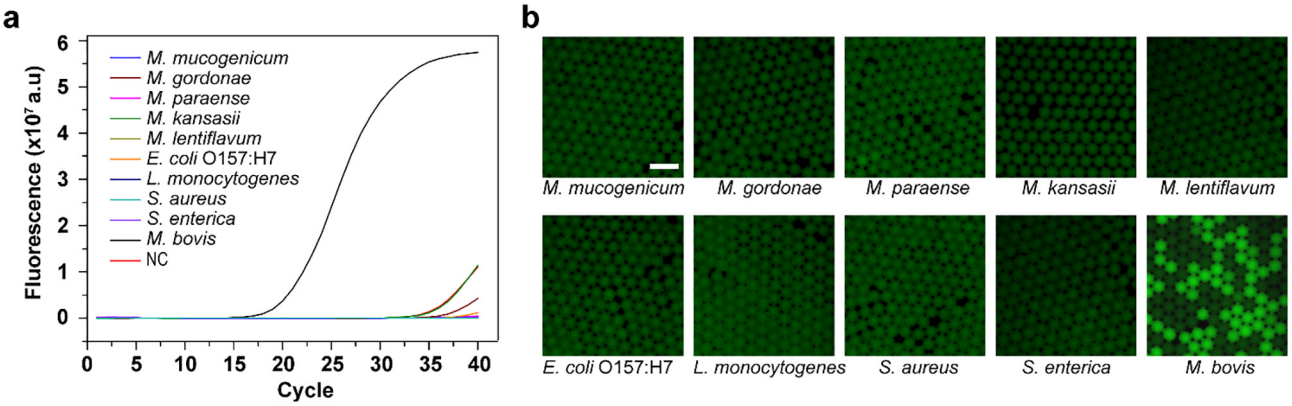

Fig. 2. The specificity of primers for *M. bovis* detection using qPCR and dLAMP. (a) Amplification curves of qPCR using Mpb70-F_3 and Mpb70-B_3 primers with 10 common zoonotic pathogens including *Mycolicibacterium mucogenicum* (*M. mucogenicum*), *Mycobacterium gordonae* (*M. gordonae*), *Mycobacterium paraense* (*M. paraense*), *Mycobacterium kansasii* (*M. kansasii*), *Mycobacterium lentiflavum* (*M. lentiflavum*), *Escherichia coli* O157:H7 (*E. coli* O157:H7), *Listeria monocytogenes* (*L. monocytogenes*), *Staphylococcus aureus* (*S. aureus*), *Salmonella enteria* (*S. enteria*), and *M. bovis*. (b) Fluorescence images of droplet arrays showing the specificity of dLAMP for *M. bovis* comparing with other 9 common pathogens mentioned above. Scale bar represents 200 μm.

3.3. Sensitivity comparison between dLAMP and qPCR

To compare the detection sensitivities between dLAMP and qPCR, we gradient diluted pEASY-T1-mpb70 plasmid and added them to dLAMP and qPCR systems, respectively. The qPCR detection resulted in good linearity between input templates and measured concentration (R^2 = 0.9977; Fig. 3); a wide range of templates can be measured (Fig. 3a). The results indicate that dLAMP has lower accuracy at higher template copy numbers (> 10^2 fg/μL, Fig. 3b) but higher sensitivity than qPCR at lower template copies of 0.13 fg. The limit of detection for dLAMP is 3.88 copies/μL, while that of qPCR is 31.61 copies/μL. These results are consistent with the previous study, which also found that qPCR allows detection over a wide dynamic range (Huggett et al., 2013). However, for *M. bovis* detection in milk, dLAMP offers better sensitivity than qPCR which is the crucial for avoiding false negative results. Moreover, the dynamic range of dLAMP can be readily extended by adjusting the droplet volumes or serial dilution when needed (Xu et al., 2016).

3.4. Evaluation of the tolerance of dLAMP and qPCR to inhibitors from milk

To evaluate the robustness of dLAMP and qPCR in tolerating inhibitors from milk, we spiked whole cow milk in reactions using 516 copies/μL pEASY-T1-mpb70 plasmids as the templates, and evaluated the amplification efficiencies of reactions with different milk concentrations in parallel. The inhibition effect of whole milk on dLAMP and qPCR is shown in Fig. 4. Adding milk to the dLAMP reaction did not inhibit the reaction at low concentrations of milk (< 1%, Fig. 4a). The interference increased gradually with increasing milk concentrations (2%–8%), and the dLAMP reaction was totally inhibited when the milk concentration reached 14%. The qPCR is more vulnerable as severe inhibition effect was observed when only trace amounts (0.03%) of milk were added (Fig. 4b). The inhibition curves in Fig. 4c illustrated a clear overview of the inhibitory effect of milk on qPCR and dLAMP. The half-maximal inhibitory concentration (IC_{50}) of spiked milk on dLAMP and qPCR is 2.85% and 0.16%, respectively. This result further proves that the quantification of qPCR depends on both the purity of the DNA templates and the standard curve (Ricchi et al., 2017). The robustness of dLAMP seen here has also been reported where dLAMP could resist the interference of high concentration humic acid and sodium dodecyl sulfate (SDS) (Hu et al., 2016).

3.5. Quantification of M. bovis in whole milk

To further evaluate the feasibility of dLAMP in the quantification of *M. bovis* in whole milk samples, we prepared BCG-spiked milk samples (Fig. 1a) and we set BCG-spiked saline samples as a control group to evaluate the interference of milk on amplification. Briefly, a series of ten-fold dilution of BCG cells predetermined by plate counting were spiked in milk and saline solutions and centrifuged for automated DNA

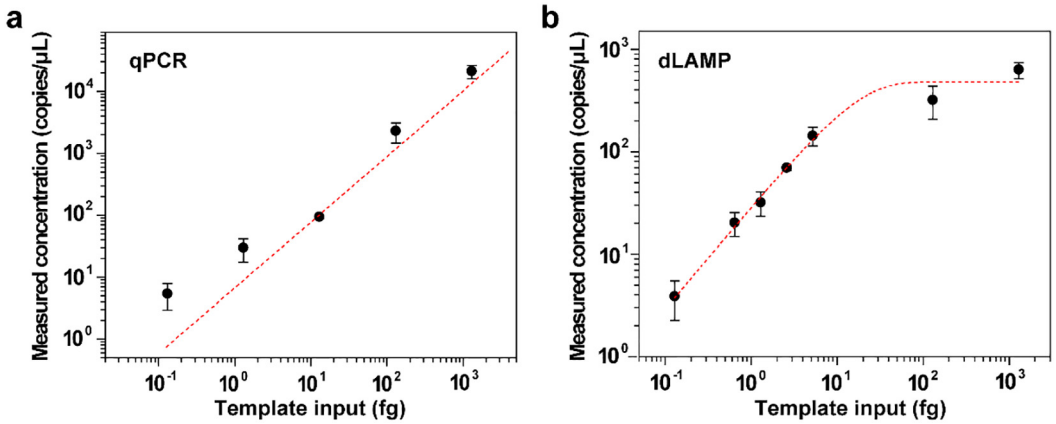

Fig. 3. Linear correlations (red dashed lines) between the input and the measured concentration of pEASY-T1-mpb70 plasmid templates based on (a) qPCR and (b) dLAMP, respectively. All data are presented as mean ± SD (standard deviation, n = 3). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

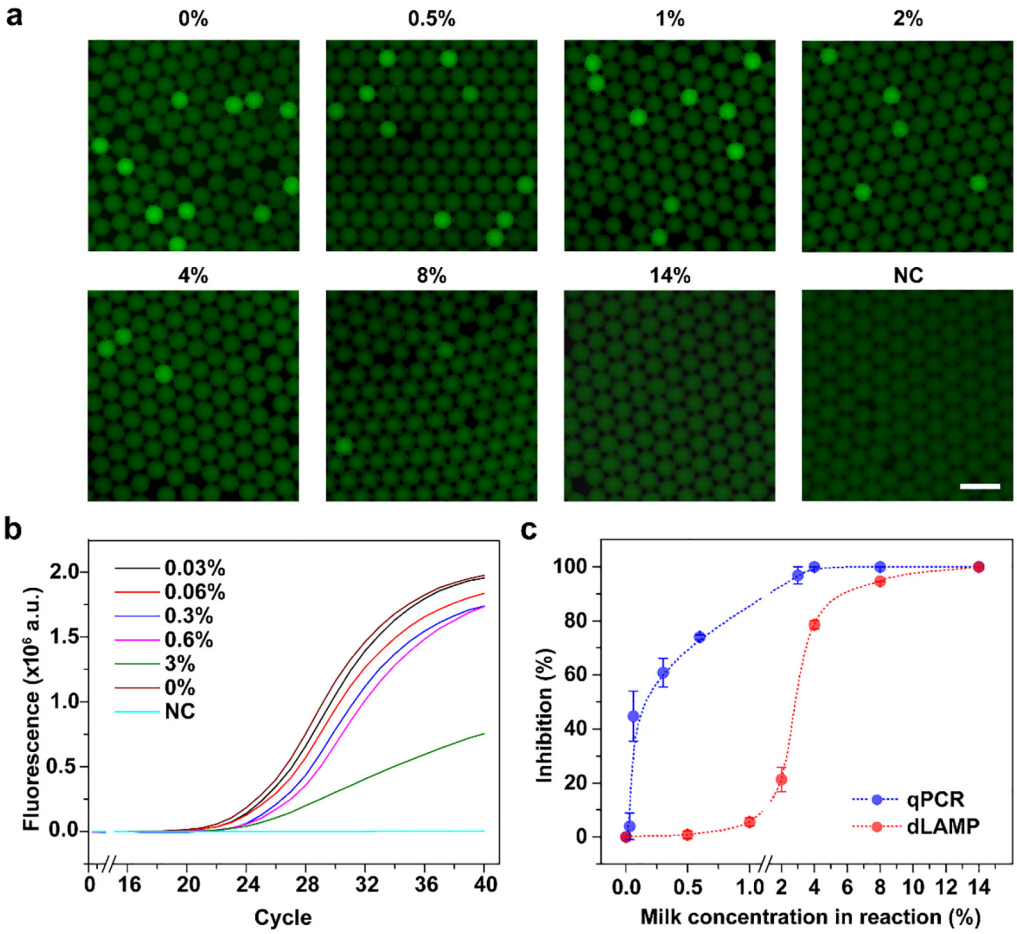

Fig. 4. Interference of spiked-in milk on dLAMP and qPCR. (a) Fluorescence images of dLAMP with increasing ratio of milk in reaction. Scale bar = 200 μm. (b) qPCR amplification curves with increasing ratio of milk. (c) Concentration-dependent inhibitory effect of milk on dLAMP (plotted with Modified Bezier fit) and qPCR (plotted with B-Spline fit); the percent inhibition is calculated considering 0% inhibition for DNA quantification with no inhibitor. All data are presented as mean ± SD (n = 3).

extraction. dLAMP has very close results between milk samples and saline controls (Figs. 5a and S4), showing linear correlations to the decreasing concentration of BCG in both milk (R^2 = 0.9893) and saline (R^2 = 0.9836). This suggests that dLAMP has strong anti-inhibition performance and good stability for quantitative detection. Compared with dLAMP, qPCR exhibited poor robustness in quantification with less consistent results and the linear regression of milk (R^2 = 0.9420) and saline (R^2 = 0.9479) are poor than dLAMP (Fig. S4) . It is worth noting that a significant high sensitivity of dLAMP was obtained (14 CFU/mL BCG; Fig. 5b), which is 10-fold higher than that of qPCR (140 CFU/mL). The measured Cq value (Cq > 35) with 14 CFU/mL BCG is higher than the negative control (Cq = 34.87), which indicates that the qPCR result for 14 CFU/mL is not reliable.

We also found that the measured concentrations of BCG by dLAMP and qPCR are higher than that of traditional plate counting. This discrepancy might be due to the decrease in the ability of Mycobacterium species to form colonies on solid culture media since this group of microorganisms is prone to clump formation (Lambrecht et al., 1988) and their growth rates vary with the depth of dormant state (Stewart et al., 2003). Moreover, This underestimation effect on the quantification of microorganisms via the culture method was also previously observed in the quantification of mycobacteria (Ricchi et al., 2017).

4. Conclusion

In summary, we developed a convenient and automated system that incorporates centrifugal enrichment, automated DNA extraction and interfacial emulsification-based dLAMP for absolute quantification of *M. bovis* in whole milk. This integrated method greatly simplifies the operation and rapid quantification of the ultra-low concentration of *M. bovis* in milk and the results demonstrate that the dLAMP is critical to both the detection limits and tolerance in hard-to-remove contaminants in milk. The use of automated DNA extraction greatly improves the detection efficiency, which lays the foundation for large-scale *M. bovis* testing. The automated dLAMP method can analyze *M. bovis* -spiked milk samples with a detection limit of 14 CFU/mL within 2 h. Compared with qPCR, dLAMP is more sensitive, offering better resistance to inhibitors and is independent of the standard curve (Table 1). We believe that the dLAMP system described in this study can also provide a powerful platform for quantitative and timely molecular testing of various pathogens in environmental or physiological samples and offers more reliable guidance for clinical diagnosis, epidemiological research, and food safety testing.

CRediT authorship contribution statement

Yi Tao: Investigation, Validation, Formal analysis, Visualization, Writing - review & editing. **Juanli Yun:** Writing - review & editing. **Jian Wang:** Supervision. **Peng Xu:** Methodology. **Caiming Li:** Visualization. **Hongtao Liu:** Resources. **Ying Lan:** Project administration, Funding acquisition, Writing - review & editing. **Jianzhang Pan:** Project administration, Writing - review & editing. **Wenbin Du:** Supervision, Project administration, Funding acquisition, Visualization, Writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

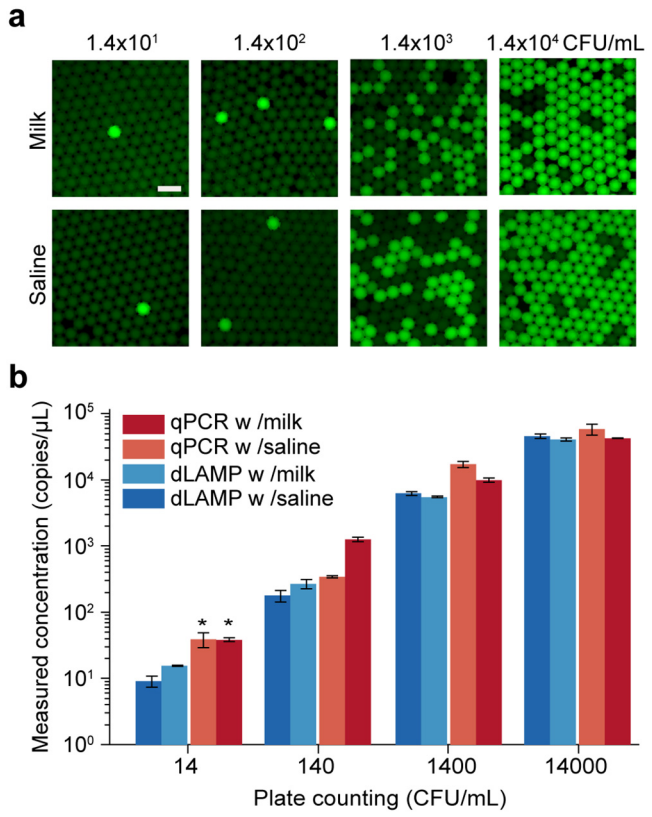

Fig. 5. Comparison of dLAMP results with qPCR in measuring *M. bovis* BCG cells in milk and saline. (a) Fluorescence images of dLAMP with increased concentrations of BCG in milk and saline. Scale bar = 200 μm. (b) Parallel test of dLAMP and qPCR with different concentrations of BCG in milk and saline; * indicates that the qPCR result was not reliable as the Cq is higher than negative control. All data are presented as mean ± SD (n = 3).

Acknowledgements

We thank Menjun Shi and Dr. Rui Wang from Beijing Prosperous Biopharm co., Ltd for technical supports. This work was supported by the National Key Research and Development Program of China (2016YFE0205800, 2018YFC0310703), National Natural Science Foundation of China (21822408, 21974122, 31970091, 91951103), Key Research Program of the Chinese Academy of Sciences (KFZD-SW-219-4), and Key Program of Frontier Sciences of the Chinese Academy of Sciences (QYZDB-SSW-SMC008).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2020.126945.

References

Barbier, E., Boschiroli, M. L., Gueneau, E., Rochelet, M., Payne, A., de Cruz, K., ... Hartmann, A. (2016). First molecular detection of Mycobacterium bovis in environmental samples from a French region with endemic bovine tuberculosis. *Journal of Applied Microbiology, 120*(5), 1193–1207.

Brewster, J. D., & Paul, M. (2016). Short communication: Improved method for centrifugal recovery of bacteria from raw milk applied to sensitive real-time quantitative PCR detection of Salmonella spp. *Journal of Dairy Science, 99*(5), 3375–3379.

Ding, X., & Mu, Y. (2016). Digital nucleic acid detection based on microfluidic lab-on-a-chip devices. In M. Stoytcheva, & R. Zlatev (Eds.). *Lab-on-a-chip fabrication and application* (pp. 125–155). London: InTech Inc.

Dong, L., Chen, D., Liu, S., & Du, W. (2016). Automated chemotactic sorting and single-cell cultivation of microbes using droplet microfluidics. *Scientific Reports, 6*(1), 24192.

Ereqat, S., Nasereddin, A., Levine, H., Azmi, K., Al-Jawabreh, A., Greenblatt, C. L., ... Bar-Gal, G. K. (2013). First-time detection of Mycobacterium bovis in livestock tissues and milk in the West Bank, Palestinian Territories. *PLoS Neglected Tropical Diseases, 7*(9), e2417.

Fusco, V., & Quero, G. M. (2014). Culture-dependent and culture-independent nucleic-acid-based methods used in the microbial safety assessment of milk and dairy products. *Comprehensive Reviews in Food Science and Food Safety, 13*(4), 493–537.

Gansen, A., Herrick, A. M., Dimov, I. K., Lee, L. P., & Chiu, D. T. (2012). Digital LAMP in a sample self-digitization (SD) chip. *Lab on a Chip, 12*(12), 2247–2254.

Hu, Y., Xu, P., Luo, J., He, H., & Du, W. (2016). Absolute quantification of H5-subtype avian influenza viruses using droplet digital loop-mediated isothermal amplification. *Analytical Chemistry, 89*(1), 745–750.

Huggett, J. F., Foy, C. A., Benes, V., Emslie, K., Garson, J. A., Haynes, R., ... Bustin, S. A. (2013). The digital MIQE guidelines: Minimum information for publication of quantitative digital PCR experiments. *Clinical Chemistry, 59*(6), 892–902.

Hussien, Heba, & Mahrous, Eman (2016). Isolation and molecular characterization of Mycobacterium tuberculosis complex isolated from raw milk in some dairy farms in Egypt. *International Journal of Basic and Applied Sciences, 2*(5), 105–109.

Lambrecht, R. S., Carriere, J. F., & Collins, M. T. (1988). A model for analyzing growth kinetics of a slowly growing Mycobacterium sp. *Applied and Environmental Microbiology, 54*(4), 910–916.

Lee, D., Kim, Y. T., Lee, J. W., Kim, D. H., & Seo, T. S. (2016). An integrated direct loop-mediated isothermal amplification microdevice incorporated with an immunochromatographic strip for bacteria detection in human whole blood and milk without a sample preparation step. *Biosensors and Bioelectronics, 79*, 273–279.

Mortari, A., & Lorenzelli, L. (2014). Recent sensing technologies for pathogen detection in milk: A review. *Biosensors and Bioelectronics, 60*, 8–21.

Nicolaou, N., Xu, Y., & Goodacre, R. (2012). Detection and quantification of bacterial spoilage in milk and pork meat using MALDI-TOF-MS and multivariate analysis. *Analytical Chemistry, 84*(14), 5951–5958.

Nixon, G., Garson, J. A., Grant, P., Nastouli, E., Foy, C. A., & Huggett, J. F. (2014). Comparative study of sensitivity, linearity, and resistance to inhibition of digital and nondigital polymerase chain reaction and loop mediated isothermal amplification assays for quantification of human cytomegalovirus. *Analytical Chemistry, 86*(9), 4387–4394.

Paul, M., Van Hekken, D. L., & Brewster, J. D. (2013). Detection and quantitation of Escherichia coli O157 in raw milk by direct qPCR. *International Dairy Journal, 32*(2), 53–60.

Quigley, L., O'Sullivan, O., Stanton, C., Beresford, T. P., Ross, R. P., Fitzgerald, G. F., & Cotter, P. D. (2013). The complex microbiota of raw milk. *Fems Microbiology Reviews, 37*(5), 664–698.

Ricchi, M., Bertasio, C., Boniotti, M. B., Vicari, N., Russo, S., Tilola, M., ... Bertasi, B. (2017). Comparison among the quantification of bacterial pathogens by qPCR, dPCR, and cultural methods. *Frontiers in Microbiology, 8*, 1174.

Romero, C., & Lopez-Goni, I. (1999). Improved method for purification of bacterial DNA from bovine milk for detection of Brucella spp. by PCR. *Applied and Environmental Microbiology, 65*(8), 3735–3737.

Rossen, L., Nørskov, P., Holmstrøm, K., & Rasmussen, O. F. (1992). Inhibition of PCR by components of food samples, microbial diagnostic assays and DNA-extraction solutions. *International Journal of Food Microbiology, 17*(1), 37.

Silva, M. R., Rocha, A. D. S., Araujo, F. R., Fonseca-Junior, A. A., de Alencar, A. P., Suffys, P. N., ... Crosland Guimaraes, M. D. (2018). Risk factors for human Mycobacterium bovis infections in an urban area of Brazil. *Memorias Do Instituto Oswaldo Cruz, 113*(8), e170445.

Stewart, G. R., Robertson, B. D., & Young, D. B. (2003). Tuberculosis: A problem with persistence. *Nature Reviews Microbiology, 1*(2), 97–105.

World Health Organization (WHO), Food and Agriculture Organization of the United Nations (FAO), & World Organisation for Animal Health (OIE). (2017). Roadmap for Zoonotic Tuberculosis. Switzerland: WHO Press.

Wilson, I. G. (1997). Inhibition and facilitation of nucleic acid amplification. *Applied and Environmental Microbiology, 63*(10), 3741–3751.

Xu, P., Zheng, X., Tao, Y., & Du, W. B. (2016). Cross-interface emulsification for generating size-tunable droplets. *Analytical Chemistry, 88*(6), 3171–3177.

Yuan, H., Chao, Y., Li, S., Tang, M. Y. H., Huang, Y., Che, Y., ... Shum, H. C. (2018). Picoinjection-enabled multitarget loop-mediated isothermal amplification for detection of foodborne pathogens. *Analytical Chemistry, 90*(22), 13173–13177.

Zhang, H., Wang, Z., Cao, X., Wang, Z., Sheng, J., Wang, Y., ... Chen, C. (2016). Loop-mediated isothermal amplification assay targeting the mpb70 gene for rapid differential detection of Mycobacterium bovis. *Archives of Microbiology, 198*(9), 905–911.

Zhang, J., Zhang, G., Yang, L., Huang, R., Zhang, Y., Jia, K., ... Li, S. (2011). Development of a loop-mediated isothermal amplification assay for the detection of Mycobacterium bovis. *The Veterinary Journal, 187*(3), 393–396.

Zhu, Q., Gao, Y., Yu, B., Ren, H., Qiu, L., Han, S., ... Mu, Y. (2012). Self-priming compartmentalization digital LAMP for point-of-care. *Lab on a Chip, 12*(22), 4755–4763.

Zumárraga, M. J., Soutullo, A., García, M. I., Marini, R., Abdala, A., Tarabla, H., ... Cataldi, A. A. (2012). Detection of Mycobacterium bovis–infected dairy herds using PCR in bulk tank milk samples. *Foodborne Pathogens and Disease, 9*(2), 132–137.

Food Chemistry **2020**, doi:10.1016/j.foodchem.2020.126945 | **Supplementary Material**

High-Performance Detection of *Mycobacterium bovis* in Milk Using Digital LAMP

Yi Tao[a,b], Juanli Yun[a], Jian Wang[a], Peng Xu[a], Caiming Li[a,b], Hongtao Liu[a,b], Ying Lan[a]*, Jianzhang Pan[d]*, Wenbin Du[a,b,c]*

[a]State Key Laboratory of Microbial Resources, Institute of Microbiology, Chinese Academy of Sciences, Beijing 100101, China.

[b] College of Life Sciences, University of the Chinese Academy of Sciences, Beijing, 10049, China

[c] Savaid Medical School, University of the Chinese Academy of Sciences, Beijing, 10049, China

[d] Department of Chemistry, Zhejiang University, Hangzhou 310058, China

* Corresponding authors: wenbin@im.ac.cn (W. D.), lanying@im.ac.cn (Y. L.), kelvonpan@zju.edu.cn (J. P.).

Optimization of incubation time of digital LAMP (dLAMP)

To optimize the dLAMP assay for better amplification efficiency and fluorescent imaging performance (Schuler et al., 2016), we evaluated incubation times ranging from 30 to 70 min (**Figure S1**). Positive droplets were detectable via fluorescence with 20 min of incubation (**Figure S2**). It is obvious that the number of positive droplets increased with longer incubation times (**Figure S1a**). The differentiation of the positive to negative ones was determined by the fluorescence intensity at a threshold that was determined by the lowest point of multiple peak fits of the histogram of fluorescence for all droplets. The peak of positive droplets was well separated from the negative droplet with over 2-fold intensity differences. The reason for the varied threshold with increased incubation times is that the positive droplets need more time to generate fluorescence signal above the threshold as the low concentration of template in one droplet (Schuler et al., 2016) and the stochastic nature of amplification initiation (Rolando, Jue, Schoepp & Ismagilov, 2018).

The dLAMP amplification efficiency was assessed with a measurement of 280 copies/μL mpb70-T1 plasmid. **Figure S1b** shows that the amplification efficiency had a linear increase with longer incubation times (R^2 =0.9979), which indicated that the dLAMP reaction took longer to initiate but proceeded more

rapidly and the amplification efficiency was highest at 60 min—the measured plasmid concentration was closest to 280 copies per microliter (275.90 copies/μL). It was reported that nonspecific amplification will appear after ~60 min, and this was attributed to primer dimers and other nonspecific products (Rolando et al., 2018). However, positive droplets were not observed in the negative control (no template) for incubation for 60 min, which indicated that there will be no nonspecific amplification within 60 min (**Figure S1a**). Therefore, we choose 60 min as the optimal amplification time for the following sample measurements.

Optimization of amplification temperature of dLAMP

The loop-mediated Isothermal Amplification method is a novel method for amplifying gene under isothermal condition. The amplification of target DNA can be conducted by incubating the reaction at a constant temperature (60~65°C) for a fixed period of time (1 hour for the standard). Mostly, the reaction condition is dependent upon the characteristics of the primers used. Therefore, it is necessary to examine the optimum condition beforehand. Through the guidance of the kit instructions (*NEB Bst2.0*), we chose 52 to 68 degrees as the temperature for optimizing. We establish the amplification efficiency of dLAMP, that is, the fraction of template copies loaded (250 copies/μL) that are detected. As seen from **Figure S3a**, increasing positive droplets is acquired after incubation from 52~66°C. The increasing temperature increased amplification efficiency to an optimum before activity decreased and the amplification efficiency reaches the highest with the incubation temperature of 66°C in **Figure S3b**. Therefore, 66°C was used for all subsequent dLAMP experiment.

Supplementary References:

Rolando, J. C., Jue, E., Schoepp, N. G., & Ismagilov, R. F. (2018). Real-Time, Digital LAMP with Commercial Microfluidic Chips Reveals the Interplay of Efficiency, Speed, and Background Amplification as a Function of Reaction Temperature and Time. *Analytical Chemistry*, *91*(1), 1034-1042.

Schuler, F., Siber, C., Hin, S., Wadle, S., Paust, N., Zengerle, R., & von Stetten, F. (2016). Digital droplet LAMP as a microfluidic app on standard laboratory devices. *Analytical Methods*, *8*(13), 2750-2755.

Supplementary Figures and Tables:

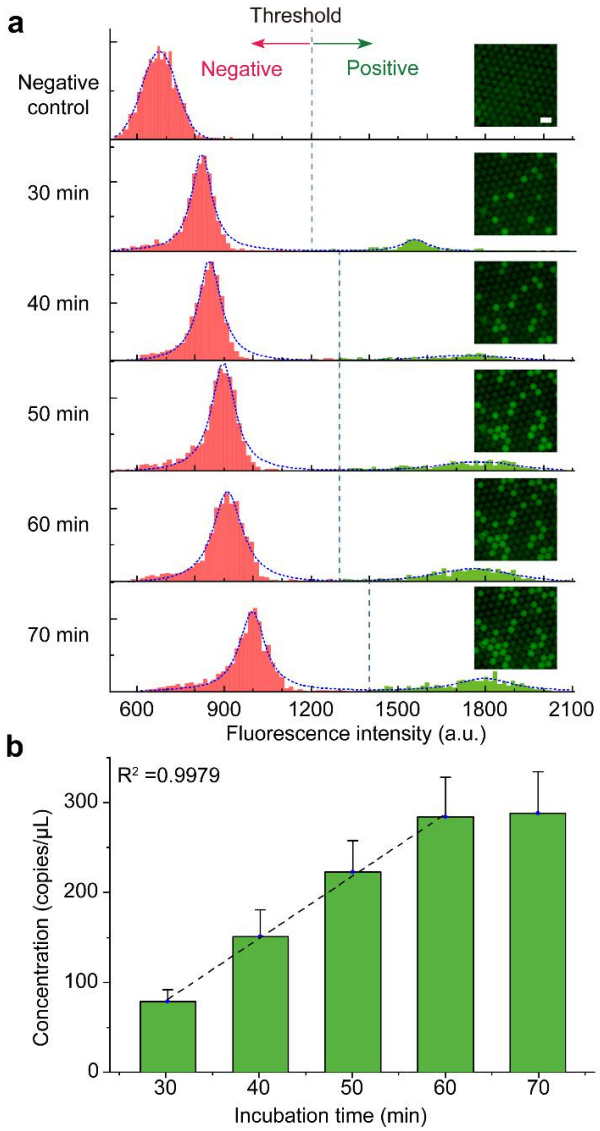

Figure S1. Reaction time optimization of dLAMP. (a) Histogram of the droplet fluorescence intensity and corresponding fluorescence images under reaction times from 30 min to 70 min; blue short dotted line indicates Lorentzian multiple peak fits; scale bar represents 200 μm. (b) dLAMP efficiency evaluation under different reaction times using a standard 280 copies/μL mpb70-T1 plasmid sample. The dashed line indicates a linear fit curve of the measured concentration of the input templates under reaction times from 30 to 70 min. All data are presented as mean ± SD (standard deviation, n=3).

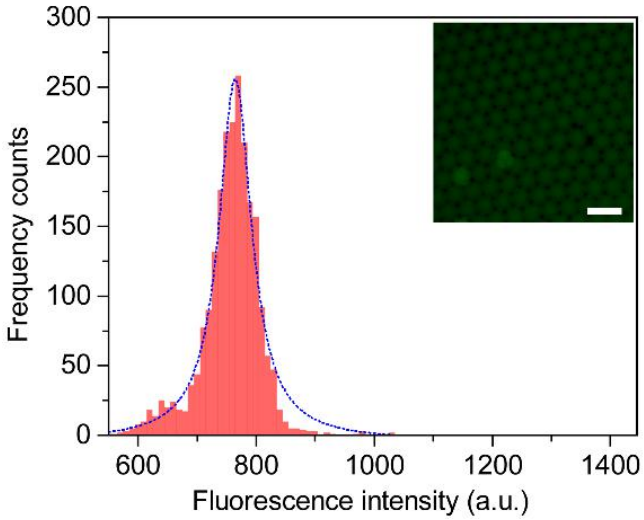

Figure S2. Histogram of the droplet fluorescence intensity and corresponding fluorescence images under the incubation of 20 min, blue short dot line indicated multiple peak fit by Lorentz, scale bar represents 200 μm.

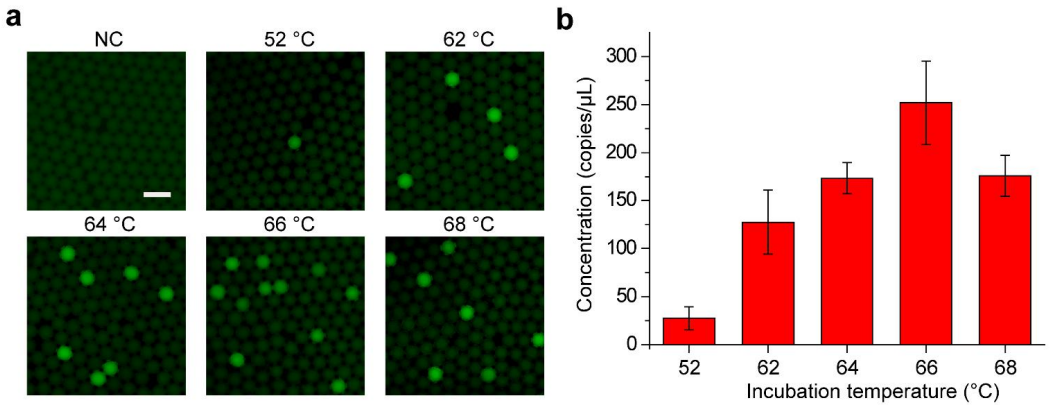

Figure S3. Influence of incubation temperature on dLAMP efficiency. (a) fluorescence imaging of droplet arrays by dLAMP after incubation ranging from 52 to 68 °C, scale bar 200 μm. (b) Evaluation of dLAMP efficiency in detection of 250 copies/μL mpb70-T1 plasmid with different incubation temperatures. All data are presented as mean value ± SD (standard deviation, n=3).

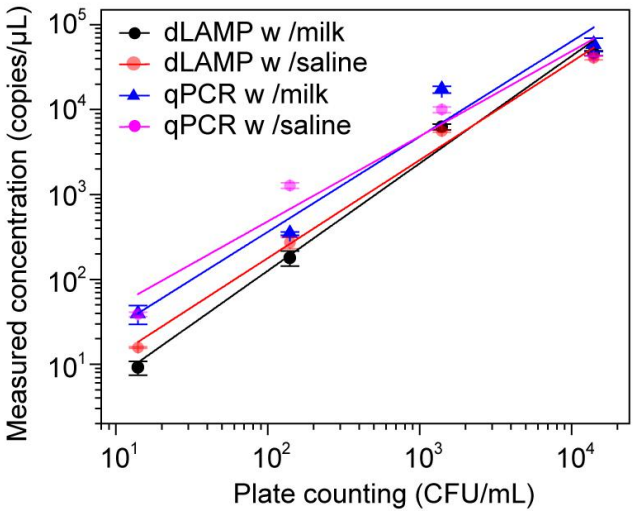

Figure S4. dLAMP and qPCR results for BCG in milk and saline sample (black and red dot represent dLAMP result for BCG spiked in milk and saline; blue triangle and magenta dot represent qPCR result for BCG spiked in milk and saline, respectively; the solid line represents linear regressions to the data points (the R^2 -values successively is 0.9893, 0.9836, 0.9420 and 0.9479); All data are presented as mean value ± SD (standard deviation, n=3).

Table S1. Primers for detection of M. bovis using dLAMP, qPCR and PCR.

Assay type	Primer name	Sequence (5'→3')
LAMP and dLAMP	Mpb70-F_3	CGAGCTCAAGACCAATTCGT
	Mpb70-B_3	ACACCCCACCACAGACG
	Mpb70-FIP	ACGTTGGCCGGGCTGGTTT-CACTGCTGACCAGCATCCT
	Mpb70-BIP	TCGGCACCCGTCAGACCCT-CGTTACCGACCTTGAGGC
	Mpb70-LF	CCGGCCACTACGTGGTAGGT
	Mpb70-LB	ACGGTGACCGGTCAGGGTA
PCR and qPCR	Mpb70-F_3	CGAGCTCAAGACCAATTCGT
	Mpb70-B_3	ACACCCCACCACAGACG