BMC Veterinary Research

RESEARCH

Open Access

Development of polymerase chain reaction-lateral flow dipstick assay for detection of *Mycoplasma bovis* in cattle

Shengnan Song[1†], Jia Guo[1†], Yang Zhao[1†], Feng Shi[2], Yong Wang[1], Qian Zhang[3*], Zhen Wang[1*] and Chuangfu Chen[1*]

Abstract

Mycoplasma bovis (*M. bovis*) is capable of causing a range of diseases in cattle, encompassing calf pneumonia, arthritis, conjunctivitis, meningitis, and mastitis. It is widely recognized as one of the predominant pathogens posing a significant threat to the global cattle industry. Therefore, accurate and sensitive methods are urgently needed to detect *M. bovis*. This study aims to detect *M. bovis* by combining colloidal gold with biotin-labeled oligonucleotides to improve detection sensitivity and form a chromogenic detection probe based on signal amplification technology. Here, we developed a sensitive and specific polymerase chain reaction-lateral flow dipstick assay (PCR-LFD) strip for efficient nucleic acid detection of *M. bovis*. A pair of specific primers with 5′ ends labeled with biotin and digoxigenin probes was designed for PCR experiments. Colloidal gold particles-labeled anti-digoxigenin IgG coated gold-labeled test strip was prepared, streptavidin was used as the detection probe, and nitrocellulose membrane coated goat anti-mouse IgG was used as the control line. Our results showed that the detection limit of the PCR-LFD was 89 fg/μL for the *M. bovis* DNA. The results from the test strip were highly consistent with those from real-time qPCR. This assay were highly specific for *M. bovis*, as there were no cross-reactions with other microorganisms tested and the detection sensitivity of the test was also relatively high (97.67%). The novel strips present a promising tool for the cost-effective and sensitive diagnosis of *M. bovis*.

Keywords *Mycoplasma bovis*, Biotin-labeled oligonucleotides, Colloidal gold-based, Polymerase chain reaction-lateral flow dipstick assay, Nucleic acid, Visual detection

Introduction

Mycoplasma bovis is one of the primary bacterial pathogens contributing to the multifactorial bovine respiratory disease complex, which is associated with pneumonia in cattle [1, 2]. This disease is highly contagious and characterized by coughing, nasal discharge, and acute respiratory distress accompanied by extensive lesions in the lung (pneumonia) [3, 4]. The economic losses caused by *M. bovis* are estimated to be approximately 150 million euros across Europe as well as over $100 million per year in the USA [5]. *M. bovis* is recognized as a prominent emerging pathogens in the cattle population of industrialized nations, posing a significant threat to livestock

†Shengnan Song, Jia Guo and Yang Zhao Equally contributed to this work as a first author.

*Correspondence:
Qian Zhang
845614587@qq.com
Zhen Wang
530469380@qq.com
Chuangfu Chen
chenchuangfu_ccf@163.com
[1]State International Joint Research Center for Animal Health Breeding, College of Animal Science and Technology, Shihezi University, Shihezi, China
[2]College of Life Science, Shihezi University, Shihezi, China
[3]State Key Laboratory for Sheep Genetic Improvement and Healthy Production, Xinjiang Academy of Agricultural and Reclamation Science, Shihezi 832003, China

© The Author(s) 2024. **Open Access** This article is licensed under a Creative Commons Attribution-NonCommercial-NoDerivatives 4.0 International License, which permits any non-commercial use, sharing, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons licence, and indicate if you modified the licensed material. You do not have permission under this licence to share adapted material derived from this article or parts of it. The images or other third party material in this article are included in the article's Creative Commons licence, unless indicated otherwise in a credit line to the material. If material is not included in the article's Creative Commons licence and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this licence, visit http://creativecommons.org/licenses/by-nc-nd/4.0/.

production and causing substantial economic and production losses in both beef and dairy industries [3, 6].

Given the absence of efficacious vaccines against *M. bovis* [6, 7], cattle farmers worldwide continue to encounter substantial obstacles in managing this formidable disease. Consequently, the implementation of effective husbandry practices, early diagnosis, and efficient antimicrobial therapies are imperative for disease control. Although culturing of *M. bovis* represents the gold standard method, the presence of vira co-infections and easily cultivable, which are frequently encountered in bovine respiratory diseases (BRD) associated with *M. bovis*, can potentially complicate diagnostic procedures [5]. The serological diagnosis based on the detection of specific antibodies to *M. bovis* is a suitable and practical approach for assessing prevalence and conducting epidemiological studies in herds [8], including indirect haemagglutination, growth inhibition, immunohistochemistry, complement binding, agar diffusion, indirect ELISA (I-ELISA) and PCR [9–11]. Despite numerous approaches employed for the determination of *M. bovis*, certain limitations persist in terms of ease of use, sensitivity, specificity, cost-effectiveness, and reliance on specialized equipment or expertise [12]. Recently, nucleic acid amplification combined with lateral flow dipstick (LFD) technique has become apromising molecular technology for low-resource, rapid diagnostics for point-of-care diagnosis in the detection of *M. bovis* species compared to the aforementioned methods [7, 9].

However, the concentration of specific nucleic acids and proteins in biological samples is typically low, while also being susceptible to interference from numerous substances, thereby potentially resulting in erroneous test outcomes [11, 13, 14]. The utilization of biotin-avidin amplified cascade gold nanoparticle labeling technology for highly sensitive detection represents a pivotal aspect in the realm of analytical chemistry research. The particles in colloidal gold state can form a firm electrostatic bond with the positive charge groups of protein molecules and other biological macromolecules without affecting the biochemical properties of the protein. Additionally, the intense color of colloidal gold provides good contrast for visual detection. Therefore colloidal gold can be used as an ideal immunomarker.

In this study, the signal amplification technology that colloidal gold can be combined with antibody or biotin-labeled oligonucleotides is used to improve the detection sensitivity to form a colorimetric detection probe, which integrates the specificity and sensitivity of PCR. A biotin and digoxigenin probes-based lateral flow strip (used colloidal gold as the labeled materials) were developed and analyzed for their sensitivity and specificity for detection of *M. bovis* in milk samples from cattle. The established of *M. bovis* nucleic acid detection test strip method can serve as an alternative method for clinical investigation, epidemic surveillance and disease diagnosis of *M. bovis*.

Materials and methods

Safety

The care and use of the animals were conducted in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. Procedures involving animals were approved by the Shihezi University Institutional Animal Care and Use Committee.

Reagents and materials

The biosafety trials involved in this study were conducted at Xinjiang Center for Disease Control and Prevention, using the following strains: *M. bovis* (strain PG45), *M. bovis* Xinjiang isolates, *Mycoplasma ovine pneumonia*, *Escherichia coli*, *Pasteurella multocida*, *Brucella abortus*, *Bovineviral diarrhea virus*, and *Mycobacterium bovis*. The hydrogen tetrachloroaurate hydrate, trisodium citrate, potassium carbonate, and bovine serum albumin derived from bovine serum were obtained from Sinopharm (Shanghai, China). The mouse anti-digoxigenin immunoglobulin G (IgG) and goat anti-mouse IgG were procured from Luoyang Bai Aotong Experimental Materials Center. The Nitrocellulose (NC) membrane, glass cellulose membrane absorbent pads, sample pads, conjugate pads, and polyvinyl chloride (PVC) sheets were provided by Bioadvantage Co., Ltd.

DNA extraction and generation of standard DNA

The *M. bovis* and bacterial genomic DNA were extracted with the Bacteria DNA Kit (Takara, Beijing, China), following the manufacturer's instructions. 2 mL of each sample was centrifuged at 16,000 *g* for 20 min at 4 °C, phosphate-buffered saline (PBS, pH 7.4) was used to wash the pellet twice after removing the supernatant with impurities and excess liquid. DNA was extracted from the washed pellet and stored at −80 °C. In order to generate a *M. bovis*-standard DNA, a PCR product with 448 bp covering the region of interest gene, was amplified from the *M. bovis* DNA using *oppD* as primers [15] (*oppD*-F: CGTTATGCAAGATTAAATACTTACGAC, *oppD*-R: TGAAACTTTCTCAGCATTAGCC) and cloned into the pMD19-T (Takara, Shanghai, China) for standards. *Escherichia coli DH5α* cells were transformed with the generating plasmid. Aliquots of the standard DNA were prepared in 10-fold serial dilutions from 1.0×10^7 to 1.0×10^0 copies/mL in nuclease-free water and stored at −80 °C.

PCR reaction and PCR-LFD assay

The 5' ends of the upstream and downstream primers of *M. bovis* were designed to label biotin probes and

digoxigenin probes, respectively. The reaction conditions were pre-denaturation at 94 °C for 5 min; denaturation at 94 °C for 30 s, annealing at 53 °C for 30 s, and extension at 72 °C for 45 s, a total of 30 cycles; and a final extension at 72 °C for 10 min. The above procedure is performed in Applied Biosystems MiniAmp Plus Thermal cycler (Thermo Fisher Scientific, USA). Using the established PCR reaction system and reaction conditions, the templates of 8 test strains were amplified respectively.

The test strip included a sample pad, a conjugate pad, an absorbent pad, an NC membrane. The conjugate pad contained the colloidal gold particles-labeled anti-digoxigenin IgG, which produced an easily visible red color. Streptavidin and goat anti-mouse IgG were applied as the test (T) and control (C) lines, respectively. Carefully dispense the PCR product onto the nucleic acid test strip and allow it to incubate for approximately 5 min, observing for the appearance of a discernible red band (Fig. 1).

Specificity, sensitivity, repeatability, and concordance rate of the PCR-LFD strip

For the sensitivity test of the strip, *M. bovis* standard-DNA was diluted with 0.01 mol/L PBS (pH 7.2) to concentrations of 10^{-1} (8.9 ng/μL), 10^{-2} (890 pg/μL), 10^{-3} (89 pg/μL), 10^{-4} (8.9 pg/μL), 10^{-5} (890 fg/μL), 10^{-6} (89 fg/μL), and 10^{-7} (8.9 fg/μL). The test should be conducted in accordance with the reaction system, where the samples are applied onto sample pads of the same batch of test strips. Subsequently, the results are observed and a sensitivity test is performed.

The genome DNA of *Mycobacterium bovis*, *Pasteurella multocida*, *Escherichia coli*, *Mycoplasma filamentosa*, *Bovine Viral Diarrhea Virus*, *Brucella abortus*, *Mycoplasma Sheepneumoniae*, *M. bovis* PG45 and *M. bovis* Xinjiang isolates were used as templates for PCR amplification. Simultaneously, agarose gel electrophoresis was performed to detect the amplified products and a specificity test was conducted by applying the product onto the sample pad of the test strip.

For the repeatability analysis, three randomly selected PCR products were diluted with 0.01 mol/L PBS (pH 7.2) to concentrations of 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, and 10^{-7}.

Comparison of PCR-LFD strips to real-time qPCR

A total of 197 bovine milk samples collected from seven dairy cow farms in Xinjiang Province, China, were screened for *M. bovis* using the developed strips in parallel to real-time qPCR, which were used according to the manufacturer's instructions, to compare their specificity, sensitivity, and accuracy.

Results

Evaluation of PCR-LFD nfo primer

The analytical specificity of the PCR methods was evaluated using DNAs of a panel of target and non-target *M. bovis* (strain PG45), *M. bovis* Xinjiang isolates,

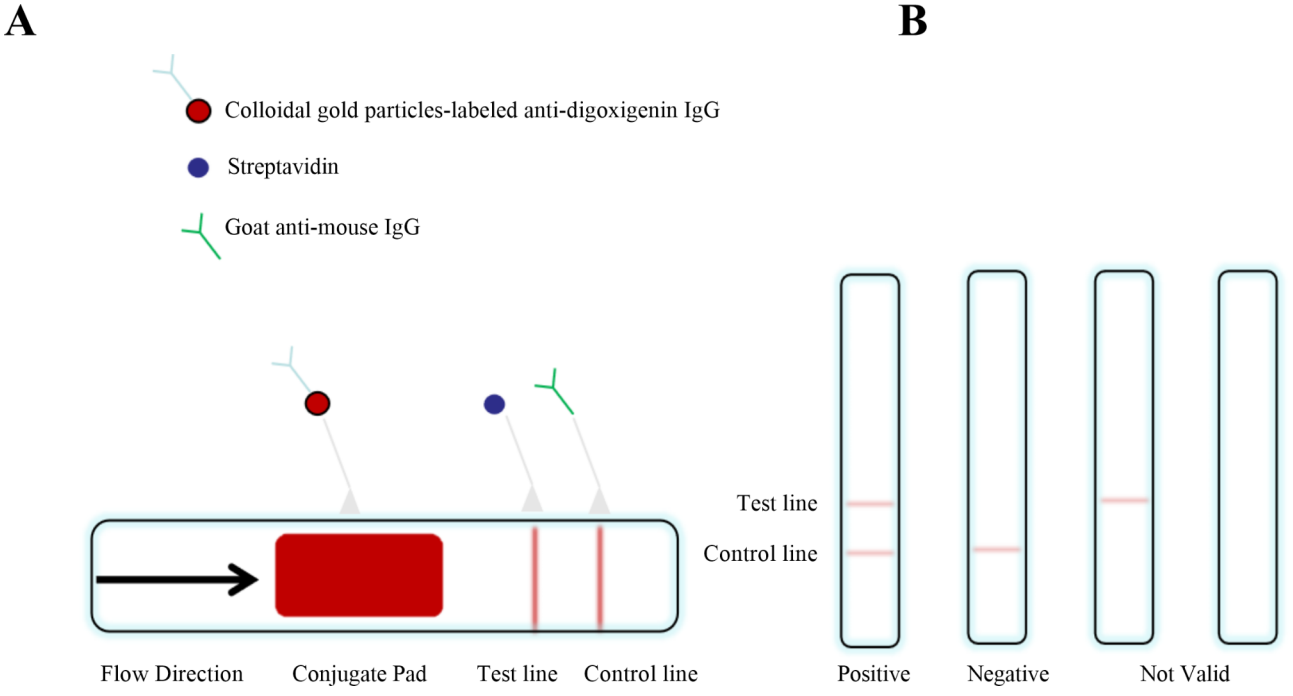

Fig. 1 Schematic representation of the PCR-LFD mechanism. (**A**) Primer labeled with streptavidin and digoxigenin at 5′ end were used for subsequent LFD assay steps. (**B**) Evaluation of results using PCR-LFD

Mycoplasma ovine pneumonia, *Escherichia coli*, *Pasteurella multocida*, *Brucella abortus*, *Bovineviral diarrhea virus*, and *Mycobacterium bovis*. All real-time qPCR tests of DNA samples from which *M. bovis* was expected to be absent showed negative results with Ct values greater than or equal to 37 (Fig. 2A). Furthermore, results from end-point PCR were in agreement with those of real-time qPCRs. The PCR analysis fully confirmed that primer set *oppD* probe yielded specific amplification efficiency for the detection assay, and produced the expected size of the product of 448 bp (Fig. 2B).

Cross-reactivity of the PCR-LFD strips

The cross-reactivity of the test strips was assessed using bovine-susceptible pathogens, including *M. bovis* (strain PG45), *M. bovis* Xinjiang isolates, *Mycoplasma ovine pneumonia*, *Escherichia coli*, *Pasteurella multocida*, *Brucella abortus*, *Bovineviral diarrhea virus*, and *Mycobacterium bovis*. While the sample positive for *M. bovis* yielded positive results on the test strip, all other samples exhibited negative results (Fig. 2C). These findings convincingly demonstrate the suitability of the test strip for detecting *M. bovis*.

Detection limit of the PCR-LFD strips

To assess the detection limit of the PCR-LFD strip, various concentration gradients were generated by diluting the standard *M. bovis* DNA with buffer solution. Notably, when the standard *M. bovis* DNA was diluted to a concentration of 10^{-7} (8.9 fg/μL), the PCR-LFD strip yielded a negative result (Fig. 3A). The positive result of the PCR-LFD strip when the standard *M. bovis* DNA was diluted to 10^{-6} indicates that the detection limit of the PCR-LFD strip is 89 fg/μL (Fig. 3A). While the detection limit results of PCR was 890 fg/μL (Fig. 3B), and the detection limit of the PCR-LFD strips was in agreement with real-time qPCRs (Fig. 3C).

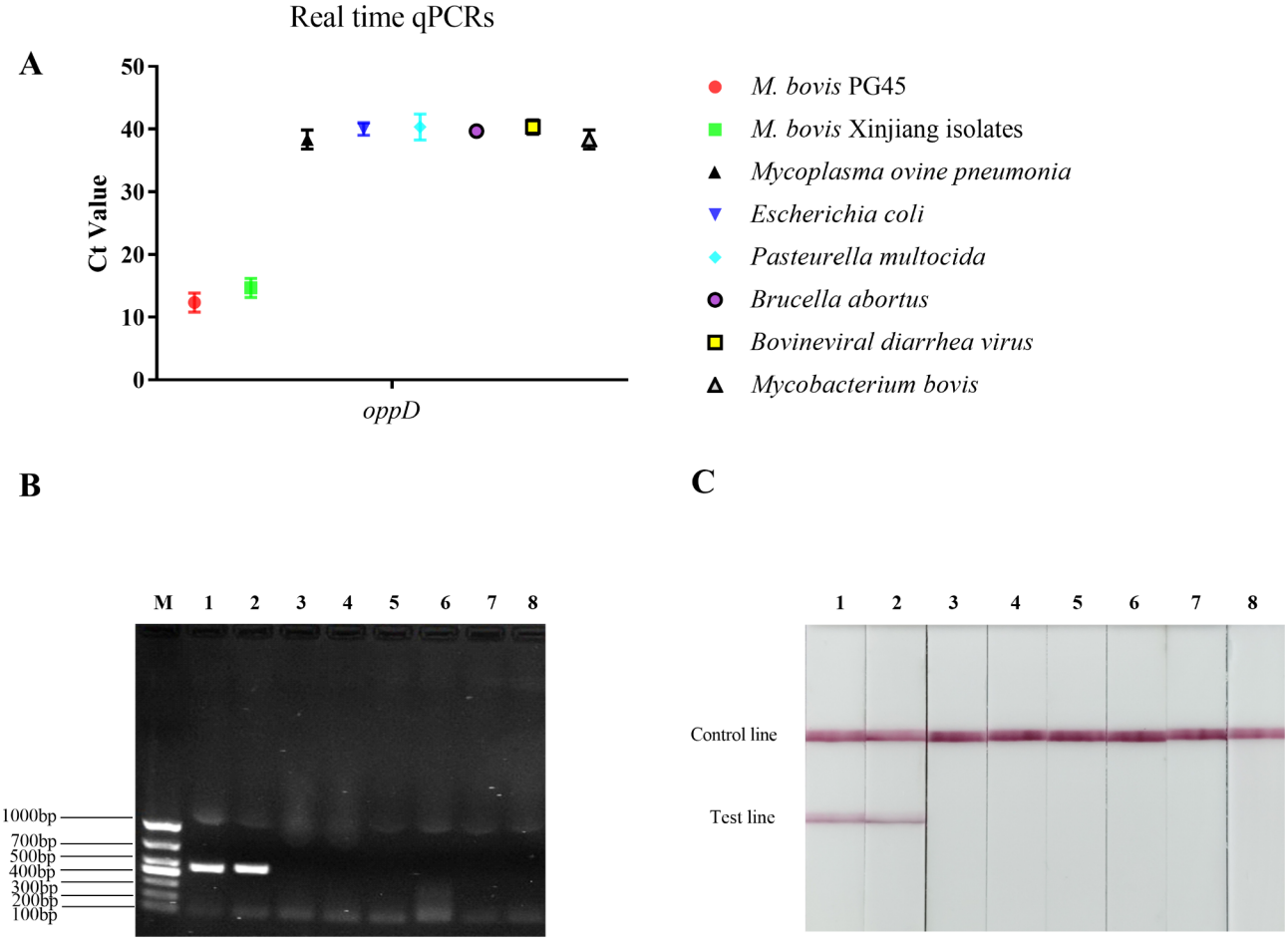

Fig. 2 The Primer and PCR-LFD strips specificity verification. (**A**) Determination of the analytical specificity of *oppD* by real-time qPCR. (**B**) Analytical specificity of the PCR assay. (**C**) Cross-reactivity of the PCR-LFD strips. Only the *M. bovis* was amplified and there were no cross reactions with other pathogens tested. Line 1, *M. bovis*; line 2, *M. bovis* Xinjiang isolate; line 3, *Mycoplasma ovine pneumonia*; line 4, *Escherichia coli*; line 5, *Pasteurella multocida*; line 6, *Brucella abortus*; line 7, *Bovineviral diarrhea virus*; line 8, *Mycobacterium bovis*

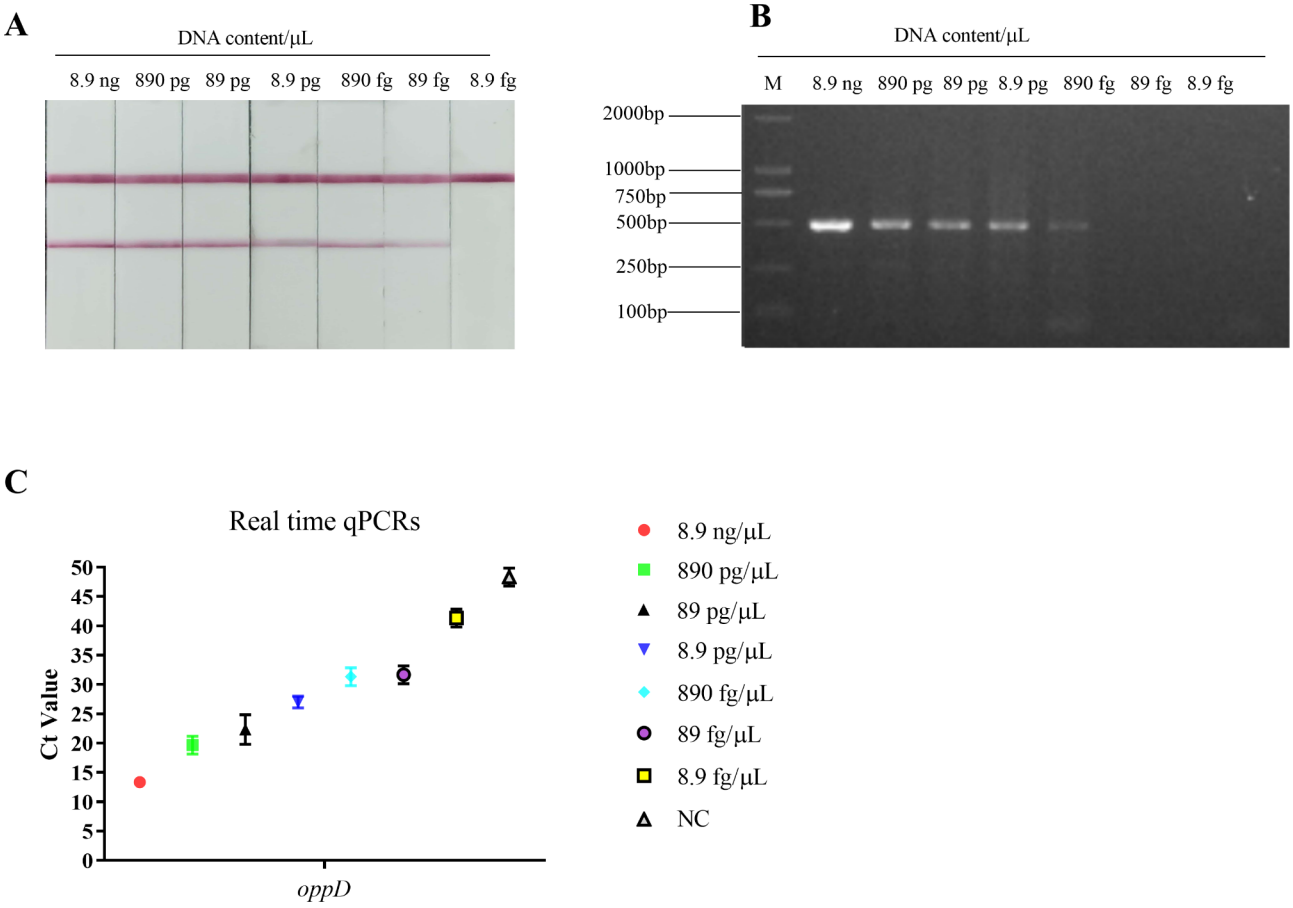

Fig. 3 Limit of detection of PCR-LFD strips in detecting *M. bovis* DNA. (**A**) Results by PCR-LFD (with a detection limit of 89 fg/μL). (**B**) Results by PCR (with a detection limit of 890 fg/μL). (**C**) Results by real-time qPCR (with a detection limit of 89 fg/μL)

Table 1 Camparision of polymerase chain reaction-lateral flow dipstick assay and real-time qPCR assay on clinical samples

PCR-LFD	Real-time qPCR						
	Positive	Negative	Total	Sensitivity (%)	Specificity (%)	Accuracy (%)	Positive rate (%)
Positive	42	0	42	97.67			
Negative	1	154	155		100		
Total	43	154	197			99.49	21.82 (43/197)
Positive rate (%)	21.32 (42/197)						

Both positive = 42, both negative = 154, number of samples = 197. The following equations were used: sensitivity (%) = (both positive/positive in real-time qPCR) × 100%; specificity (%) = (both negative/negative in real-time qPCR) × 100%; accuracy (%) = (both positive + both negative)/total) × 100%

Comparison of PCR-LFD strip results to real-time qPCR

To verify the accuracy of the PCR-LFD and the real-time qPCR, the test strip was used for the detection of *M. bovis* DNA in 197 clinical samples, including 42 positive samples and 155 negative samples. The results of the comparison of the strip results with those of real-time qPCR were shown in Table 1. The PCR-LFD showed 100% and 97.67% specificity and sensitivity, respectively, compared to real-time qPCR. The lateral flow strips had higher specificity and sensitivity. The results obtained with the lateral flow strips exhibited 99.49% concordance with those of real-time qPCR.

Discussion

Mycoplasma mastitis in dairy cattle is commonly caused by *M. bovis* [16]. Among the primary ways it is transmitted is through ingestion of milk from cows with mastitis, and it is capable of surviving in the milk of asymptomatic and clinically healthy cows [8]. Milk samples from individual cows and bulk tanks have both been used to detect *M. bovis* in surveillance and eradication efforts [17]. In this study, we present a PCR-LFD strip that can detect the presence of *M. bovis* DNA in milk in a sensitive and specific manner, which was independent of professional personnel and equipment and can be used for the diagnosis of *M. bovis* and in molecular diagnosis.

To the best of our knowledge, this is the first instance in which biotin-avidin amplified cascade colloidal gold has been chosen as a labeling material to establish a method for detecting *M. bovis* DNA in bovine milk, and to describe the results achieved with conjugated proteins and lateral flow test strips using colloidal gold labeling. The biotin-avidin amplified cascade colloidal gold label proved to be effective for the studied lateral flow strips.

Colloidal gold technology is a visual immunochromatographic detection method that is simple to operate, has low production costs, and is widely used in the field of detection and diagnosis. However, this technology has low detection sensitivity. Streptavidin combined with colloidal gold, with the multi-stage amplification effect of the biotin-avidin system, greatly enhances the sensitivity of colloidal gold immunoassay technology. Finally, the red line on a white nitrocellulose background exhibits excellent contrast ratio, as confirmed by visual inspection. Leveraging these characteristics and properties, biotin-avidin amplified cascade gold nanoparticle labeling can be effectively utilized as labels in lateral flow strips.

DNA detection methods for *M. bovis* are highly dependent on the quality of the targeted *Mycoplasma* genes used in the test. Thus, to develop reliable molecular methods, it is essential to prepare primers with high specificity. We selected the *oppD* gene region of *M. bovis*, which encodes ATP-binding proteins of the ABC-transporter family, was also used as a specific target region in a PCR test and was capable of direct detection of the organism in milk [18]. As a result of DNA amplification, the target band size of *M. bovis* PG45 and *M. bovis* Xinjiang isolates was 448 bp in size. In contrast, the empty control and other pathogenic strains failed to detect the amplified band. This shows that the primer has strong specificity, and the reaction system and reaction conditions are suitable.

We further examined the accuracy of the result, including its specificity, sensitivity, and consistency in comparison with the real-time qPCR results. As demonstrated by the results of this study, the detection limit of the PCR-LFD strip is 89 fg/μL. The specificity of the test strip was analyzed by testing several common cattle pathogens, including *M. bovis*, *Pasteurella multocida*, *Bovineviral diarrhea virus*, *Brucella abortus*, *Mycoplasma mycoides*, *Escherichia coli*, *Mycoplasma ovipneumoniae* and *M. bovis* Xinjiang isolate. There was no cross-reaction between the PCR-LFD strip and DNA of those pathogens. In order to assess its practicality, the test strip was utilized for the detection of 197 clinical samples. Out of these, the results obtained from the test strip were in agreement with the real-time qPCR results 99.49% of the time.

To the best of our knowledge, this study represents the pioneering utilization of biotin-avidin amplified cascade colloidal gold as a labeling material for establishing a method to detect *M. bovis* DNA, and we present here the outcomes achieved through conjugated biotin and lateral flow test strips employing colloidal gold labeling. The strip results demonstrate a relative increase in specificity, with no observed cross-reactivity to other bovine pathogens; furthermore, the detection sensitivity has also been enhanced. This technique exhibits suitability for implementation in regions characterized by a high incidence of *M. bovis* due to its straightforward operation, visually interpretable outcomes, and cost-effectiveness.

Conclusions

The present study describes the development of a novel PCR-LFD strip for rapid and sensitive detection of *M. bovis*. Its sensitivity and specificity were shown to be comparable to a previously published real-time qPCR. This cost-effective and practical tool holds great promise for diagnosis of *M. bovis* in clinical samples.

Acknowledgements
We thank our laboratory members who helped us to improve the research and the manuscript, with their skillful technical assistance, invaluable comments, and suggestions.

Author contributions
C.F., Z.W., and Q.Z. conceived and designed the experiments. S.N. and J.G. participated in the experiments and wrote the manuscript. Y.Z., F.S., Y.W., and Q.Z. performed the experiments. S.N. and J.G. collect the experimental materials. All authors read and approved the final manuscript.

Funding
This work was supported by grants from the Transformation and Application Demonstration of Rapid Screening Technology Achievements for Important Animal Diseases in Intensive Breeding (grant no. 21322912D), Young Innovative Talent Program (grant no. CXPY202323) and Tianchi talent Project.

Data availability
Datasets generated during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

Received: 26 October 2023 / Accepted: 16 August 2024
Published online: 27 August 2024

References

1. Pardon B, Callens J, Maris J, Allais L, Van Praet W, Deprez P, Ribbens S. Pathogen-specific risk factors in acute outbreaks of respiratory disease in calves. J Dairy Sci. 2020;103:2556–66.
2. Oliveira TES, Pelaquim IF, Flores EF, Massi RP, Valdiviezo MJJ, Pretto-Giordano LG, Alfieri AA, Saut JPE, Headley SA. *Mycoplasma bovis* and viral agents

associated with the development of bovine respiratory disease in adult dairy cows. Transbound Emerg Dis. 2020;67(Suppl 2):82–93.
3. Dudek K, Nicholas RAJ, Szacawa E, Bednarek D. *Mycoplasma bovis* infections-occurrence. Diagnosis Control Pathogens (Basel Switzerland). 2020;9:640.
4. Fox LK, Kirk JH, Britten A. *Mycoplasma mastitis*: a review of transmission and control. J Vet Med B Infect Dis Vet Public Health. 2005;52:153–60.
5. Nicholas RA, Ayling RD. *Mycoplasma bovis*: disease, diagnosis, and control. Res Vet Sci. 2003;74:105–12.
6. Nicholas RA. Bovine mycoplasmosis: silent and deadly. Vet Rec. 2011;168:459–62.
7. Zhao G, Hou P, Huan Y, He C, Wang H, He H. Development of a recombinase polymerase amplification combined with a lateral flow dipstick assay for rapid detection of the *Mycoplasma bovis*. BMC Vet Res. 2018;14:412.
8. Maunsell FP, Woolums AR, Francoz D, Rosenbush RF, Step DL, Wilson DJ, Janzen ED. *Mycoplasma bovis* infections in cattle. J Vet Intern Med. 2011;25:772–83.
9. Li R, Wang J, Sun X, Liu L, Wang J, Yuan W. Direct and rapid detection of *Mycoplasma bovis* in bovine milk samples by recombinase polymerase amplification assays. Front Cell Infect Microbiol. 2021;11:639083.
10. Nielsen PK, Petersen MB, Nielsen LR, Halasa T, Toft N. Latent class analysis of bulk tank milk PCR and ELISA testing for herd level diagnosis of *Mycoplasma bovis*. Prev Vet Med. 2015;121:338–42.
11. Parker AM, House JK, Hazelton MS, Bosward KL, Morton JM, Sheehy PA. Bulk tank milk antibody ELISA as a biosecurity tool for detecting dairy herds with past exposure to *Mycoplasma bovis*. J Dairy Sci. 2017;100:8296–309.
12. Calcutt MJ, Lysnyansky I, Sachse K, Fox LK, Nicholas RAJ, Ayling RD. Gap analysis of *Mycoplasma bovis* disease, diagnosis and control: an aid to identify future development requirements. Transbound Emerg Dis. 2018;65(Suppl):91–109.
13. Voltarelli DC, de Alcântara BK, Lunardi M, Alfieri AF, de Arruda Leme R, Alfieri AA. A nested-PCR strategy for molecular diagnosis of mollicutes in uncultured biological samples from cows with vulvovaginitis. Anim Reprod Sci. 2018;188:137–43.
14. Hotzel H, Sachse K, Pfützner H. Rapid detection of *Mycoplasma bovis* in milk samples and nasal swabs using the polymerase chain reaction. J Appl Bacteriol. 1996;80:505–10.
15. Li D, Huang C, Zhang Y, Xie J, Ran Z, Xiong Z, Fan W. Establishment and application of the triplex PCR for detecting M. Bovis, M. mycoides subsp. mycoides small colony type and M. agalactiae. Acta Vet et zootechnica Sinica. 2011;42:306–10. (in Chinese).
16. Gioia G, Werner B, Nydam DV, Moroni P. Validation of a *Mycoplasma* molecular diagnostic test and distribution of *Mycoplasma* species in bovine milk among New York State dairy farms. J Dairy Sci. 2016;99:4668–77.
17. Pinho L, Thompson G, Machado M, Carvalheira J. Management practices associated with the bulk tank milk prevalence of *Mycoplasma* spp. indairy herds in Northwestern Portugal. Prev Vet Med. 2013;108:21–7.
18. Sachse K, Salam HS, Diller R, Schubert E, Hoffmann B, Hotzel H. Use of a novel real-time PCR technique to monitor and quantitate *Mycoplasma bovis* infection in cattle herds with mastitis and respiratory disease. Vet J. 2010;186:299–303.

Publisher's note

Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.