Original Article

Rapid detection of *Mycoplasma bovis* by real-time Loop-mediated isothermal amplification

Yan Pan[1§] Ying Ming Wei[2§] Garmendia AE[3] Hao Peng[1] Shi Wen Feng[1] Li Tao[1] Jun Li[1*]

Abstract

Mycoplasma bovis (*M. bovis*) is one of the most significant pathogens in cattle. The aim of this study was to develop a rapid real-time LAMP assay, specific for *M. bovis* with designed primers targeting the OPPD/F gene. The assay was tested for 1 h at 63°C. The results could be visualized by the addition of calcein dye. Real-time LAMP was specific exclusively for *M. bovis* without cross-reactivity with other bacteria. The sensitivity of the real-time LAMP was determined to be 2.29×10^{-3}ng/µL of DNA and was 100 times higher than conventional PCR. Lamp assay was conducted on 65 clinical samples to test its usefulness and reliability and compared to the PCR assay results. A concordance of 100% was observed between the two assays. This real-time LAMP assay can be applied by diagnostic laboratories and for field detection of *M. bovis* infection.

Keywords: Mycoplasma bovis, LAMP, OPPD/F gene

[1]*Guangxi Key Laboratory of Veterinary Biotechnology, Guangxi Veterinary Research Institute, Nanning, Guangxi Province 530001, China*
[2]*College of Animal Sciences and Veterinary Medicine, Guangxi University, Nanning, Guangxi Province 530004, China*
[3]*Department of Pathobiology, University of Connecticut, Storrs, Connecticut 06268, USA*
[§]*Contributed equally*
***Correspondence**: jlee9981@163.com* (J. Li)
Received March 13, 2020.
Accepted July 20, 2020.

Introduction

Mycoplasma bovis (*M. bovis*) is one of the most significant pathogens causing respiratory disease, mastitis, arthritis, keratoconjunctivitis and otitis (Nicholas *et al*., 2003; Maunsell *et al*., 2011; Castillo-Alcala *et al*., 2012; Dyer *et al*., 2013). *M. bovis* was first isolated from the milk of a mastitic cow in the United States in 1961 (Hale *et al*., 1962) and now it is recognized all over the world and causes great economic loss (Caswell and Archambault, 2007). The first case isolated from the lungs of cattle with pneumonia was in China in 2008 (Xin *et al*., 2008). Since 2008, *M. bovis* has had a higher prevalence in China (Xin *et al*., 2008; Shi *et al*., 2008; Ran *et al*., 2010; Fu *et al*., 2011). Currently, *M. bovis* has become a serious threat to beef and dairy cattle and this has resulted in huge economic losses due to the non-provision of effective vaccine in China (Mustafa *et al*., 2013). The total of 455 serum samples of *M. bovis* infection was 7.69% (35/455) in dairy cows of subtropical southern China (Fu *et al*., 2011). Because of the lack of an effective vaccine and effective antimicrobial drugs, "early diagnosis, early treatment" is a rule to prevent and control the disease. However, to the best of our knowledge, *M. bovis* diagnostic methods including culture (Sachse *et al*., 1993), PCR assays (Ayling *et al*., 1997) and ELISA assays (Heller *et al*., 1993; Register *et al*., 2013; Fu *et al*., 2014), have not absolutely fulfilled the need for a rapid diagnostic test to detect infection. Culturing for the detection of *M. bovis* in clinical samples is often time-consuming and sensitivity is low (Maunsell *et al*., 2011). PCR and ELISA assays require special technical kills and specialized equipment (Maunsell *et al*., 2011). So the development of tools for the rapid identification of cattle infected with *M. bovis* is very critical.

Loop-mediated isothermal amplification (LAMP) assay, is a novel detection technology based on nucleic acid amplification, for highly specific and rapid detection of mycoplasma organisms that has recently been developed. Thus LAMP has been widely applied for the diagnosis of mycoplasma such as *Mycoplasma synoviae* (Kursa *et al*., 2015), *Mycoplasma hyopneumoniae* (Liu *et al*., 2015), *Mycoplasma pneumonia* (Ishiguro *et al*., 2015), etc. Previous research has developed LAMP assays to detect *M. bovis*in clinical (nasal) samples and in milk (Higa *et al*., 2016; Appelt *et al*., 2018). Appelt *et al*., (2018) developed and compared the LAMP and qPCR assays for *M. bovis* detection in bovine milk. Overall, the qPCR assay was more robust than LAMP. Higa *et al*., (2016) improved the LAMP assay permitting sensitive and rapid *M. bovis* detection. However, the improved LAMP assay showed a slightly lower specificity (90.9%) meaning that some LAMP results were false-positives or, alternatively, that the false-negative frequency of culture results was elevated. The objective of our research was to provide more rapid, sensitive, specific and lower cost molecular detection methods for *M. bovis*. For this purpose, we designed species-specific primers and established a rapid real-time LAMP method, specific for *M. bovis* from clinical samples.

Materials and Methods

Mycoplasma Strains and DNA Extraction: *M. bovis, Mycoplasma ovipneumoniae, Acholeplasma modicum, Mycoplasma mycoides SC, Escherichia coli, Arcanobacterium pyogenes, Salmonella* subsp. enterica serovar Enteritidis, *Klebsiella pneumoniae* were utilized in the study (Table 1). DNA was extracted with a Bacteria Genomic DNA Kit (CWBIO, Beijng, China) and stored at -80°C until use.

Primer Design: LAMP primers targeting the conserved regions of the OPPD/F gene of *M. bovis* HB0801 strain were designed (Accession: CP002058.1). Four complementary primers designed with PrimerExplore V4 software (NetLaboratory, Tokyo, Japan) are listed in Table 2. All the primers were purified by PAGE (BGI, Beijing, China).

Table 1 Bacterial strains used in the real-time LAMP assay

Species	Strain (origin)
M. bovis	PG45 ATCC 2553 (reference strain)
M. ovipneumoniae	Y98 ATCC 29419 (reference strain)
M. Mycoides SC	PG1 (reference strain)
A. modicum	PG49 ATCC 29102 (reference strain)
E. coli	ATCC 25922 (reference strain)
A. pyogenes	ATCC 19411 (reference strain)
Salmonella subsp. enterica serovar Enteritidi	ATCC 13076 (reference strain)
K. pneumoniae	ATCC 700603 (reference strain)

Table 2 Sequences of LAMP primers used in the study

Primer	Sequence (5′-3′)	Length (nt)
F3	AGGAGGAGAAAAAAGCCTAT	20
B3	GCTAAAACTGAAGCTTGCA	19
FIP	CGCCACTTAATGTATGTGGATATCTGGCCTTTTGGAAAGATTTGG	45
BIP	ACAATGGTTGTTGCTTTAAAACCACAGTTGGATCTAAAGCAGTAG	45

F3, forward outer primer; B3, backward outer primer; FIP, forward inner primer; BIP, backward inner primer.

Real-time LAMP Assay: The assay was carried out in 25 µl total reaction volume using the commercially available Loopamp DNA amplification kit (Eiken China Co., Ltd., Shanghai, China) containing 1 µl of Bst DNA polymerase, 12.5 µl of reaction mix, 5 pmol each of outer primers F3 and B3, 40 pmol each of inner primers FIP and BIP, 1µl and 2 µl of extracted DNA. To visualize the results, 1µl of the fluorescent detection reagent calcein was added (Eiken China Co., Ltd., Shanghai, China) to the mix. For real-time monitoring,

the LAMP reaction was incubated at 63°C for 1 hour in an LA-320C Loopamp Realtime Turbidimeter (Eiken Chemical Company Limited, Japan). The turbidity at OD 400 nm was plotted against time to positivity while the threshold value was set at 0.2, and the time of positivity (Tp) value was less than 45 mins. The LAMP reaction was also observed with the naked eye under UV light.

Testing Specificity and Sensitivity of the Real-time LAMP Assay: To explore the specificity of the real-time LAMP assay, other species with high homology to M. bovis, including *M. ovipneumoniae, M. mycoides* SC , *A. modicum* were tested under identical conditions. In addition, *E. coli, A. pyogenes, Salmonella subsp. enterica serovar Enteritidis, K. pneumoniae* were tested.

To analyze the sensitivity of real-time LAMP assay, 10-fold serial dilutions (2.29×10^{2} ng/μL to 2.29×10^{-5}ng/μL) of DNA extracted from *M. bovis* were tested. The sensitivity was also confirmed by conventional PCR using a specific primer set of OPPD/F preciously reported by Tao Li, which produced a 448 bp specific amplicon (Tao *et al*., 2015).

Application of the Assay on Clinical Samples: The efficacy of the developed LAMP assay to detect *M. bovis* was assessed by comparison with conventional PCR using 65 nasal swab samples collected from cattle with clinical signs (respiratory symptom) in Guangxi, China between 2012 and 2016.

Results

Development of Real-time LAMP Assay for the Detection of M. bovis: A LA-320C Loopamp Realtime Turbidimeter to amplify DNA isothermally equipped with temperature settings was utilized for the detection of *M. bovis* OPPD/F during LAMP amplification. The *M. bovis* LAMP, only amplified OPPD/F from *M. bovis* but not from any of the other Mycoplasma species (such as *Mycoplasma ovipneumoniae, Acholeplasma modicum, Mycoplasma mycoides*) or the other bacteria tested. A positive reaction was observed when the turbidity reached 0.1 in 32 mins. This confirmed a very high assay specificity of the designed primers and a rapid real-time LAMP procedure (Fig. 1).

Sensitivity and Standard Curve Analysis: Ten-fold serial dilutions of DNA extracted from *M. bovis* were used to assess the sensitivity of the newly developed real-time LAMP assay compared with the conventional PCR method. The turbidity rate showed that the real-time LAMP assay could detect at least approximately 0.0229 pg/μL of DNA (Fig. 2A). Therefore, the LAMP assay was 100-fold more sensitive than the conventional PCR as the latter detected approximately 2.29 pg/μL of DNA (Fig. 2B, 2C).

The results also show a good correlation between the corresponding concentration of DNA ($R^2 > 0.99$, $P < 0.05$) and the threshold time (T_t), indicating that the developed LAMP assay could be used to reliably quantify DNA extracted from pathogens utilizing standard curves (Fig. 2D).

Application of real-time LAMP Assays on Clinical Samples: Sixty-five clinical samples were tested for *M. bovis* both by real-time LAMP and conventional PCR assays. The real-time LAMP assay was able to detect *M. bovis* in 12 of 65 positive and 53 of 65 negative samples. The concordance, between the two assays was 100%. There were no false positives observed (Table 3). Based on this data, the positive predictive value of the real-time LAMP was 100%. Additionally, the real-time LAMP assay was more rapid and had a lower cost, in comparison to the conventional PCR.

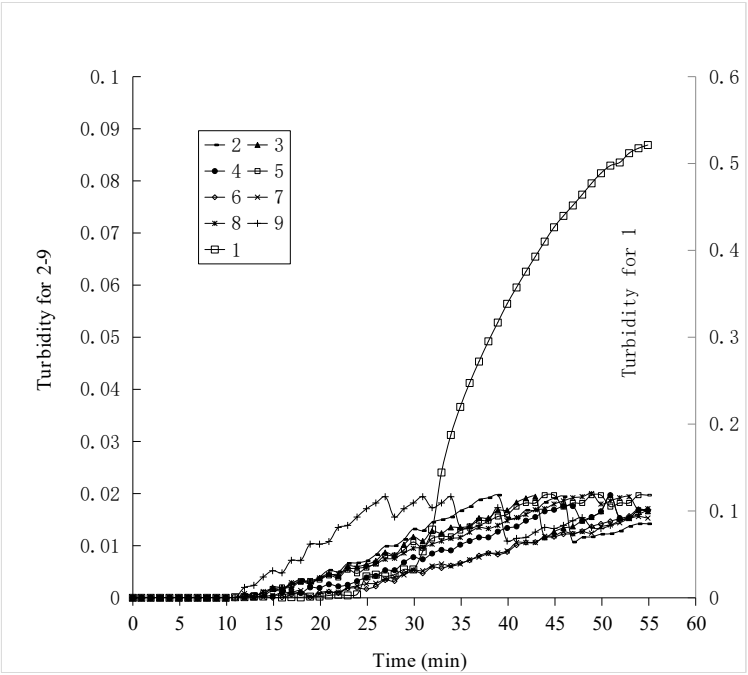
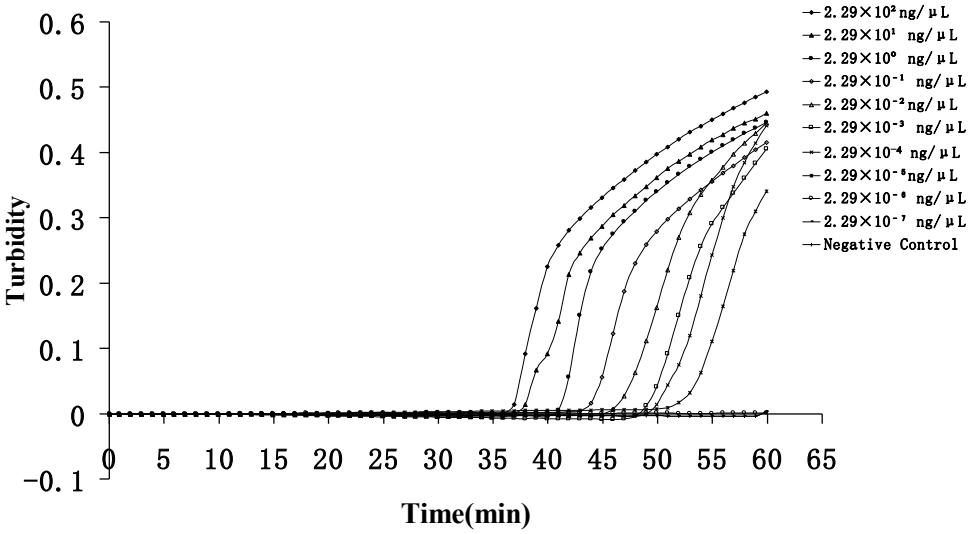
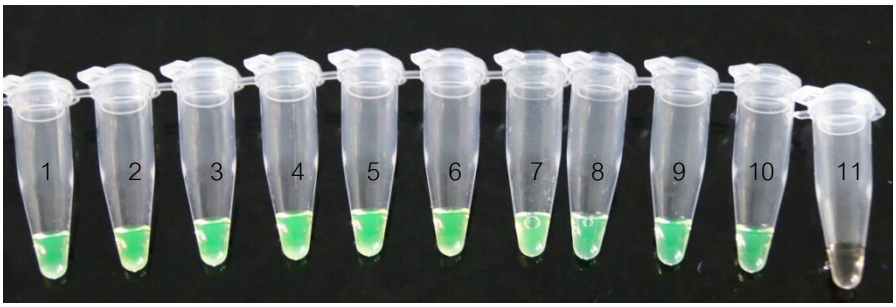


Figure 1 Specificity of the real-time fluorescence LAMP assay for the detection of M bovis OPPD/F. 1, *M. bovis*; 2, *M. ovipneumoniae*; 3, *A. modicum*; 4, *M. mycoides SC*; 5, *E. coli*; 6, *A. pyogenes* ; 7, *Salmonella* subsp. enterica serovar Enteritidi; 8, *K. pneumoniae*; 9, negative control.

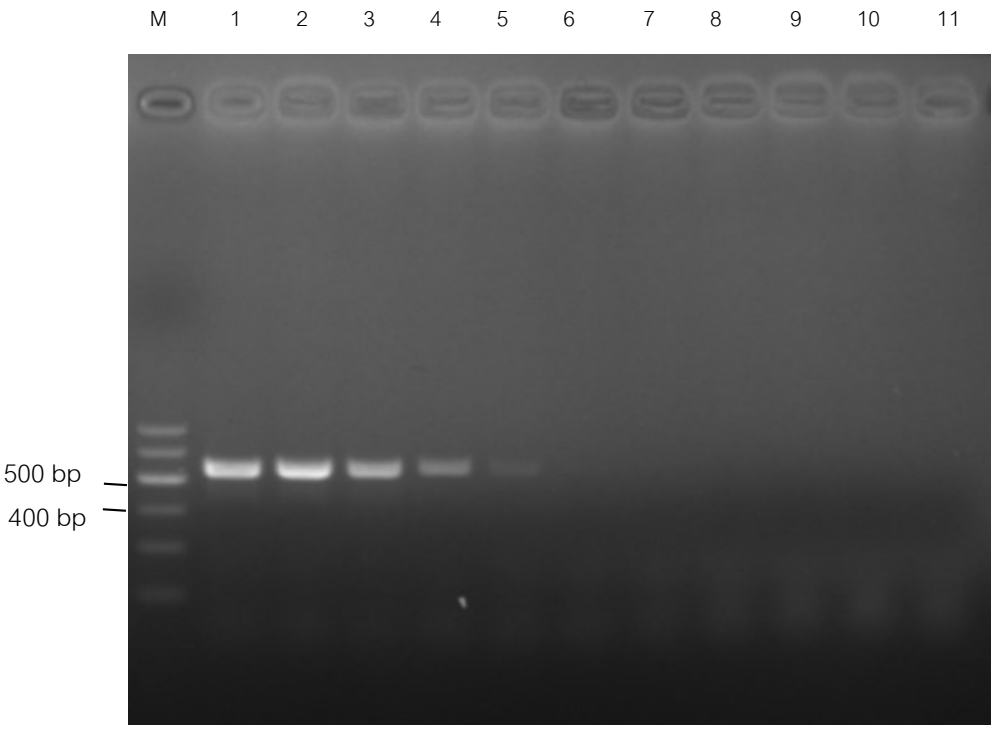
A


Turbidity
Time(min)
0.6
0.5
0.4
0.3
0.2
0.1
0
-0.1
0 5 10 15 20 25 30 35 40 45 50 55 60 65
2.29×10² ng/μL
2.29×10¹ ng/μL
2.29×10⁰ ng/μL
2.29×10⁻¹ ng/μL
2.29×10⁻² ng/μL
2.29×10⁻³ ng/μL
2.29×10⁻⁴ ng/μL
2.29×10⁻⁵ ng/μL
2.29×10⁻⁶ ng/μL
2.29×10⁻⁷ ng/μL
Negative Control

B


1
2
3
4
5
6
7
8
9
10
11

C


M 1 2 3 4 5 6 7 8 9 10 11
500 bp
400 bp

D

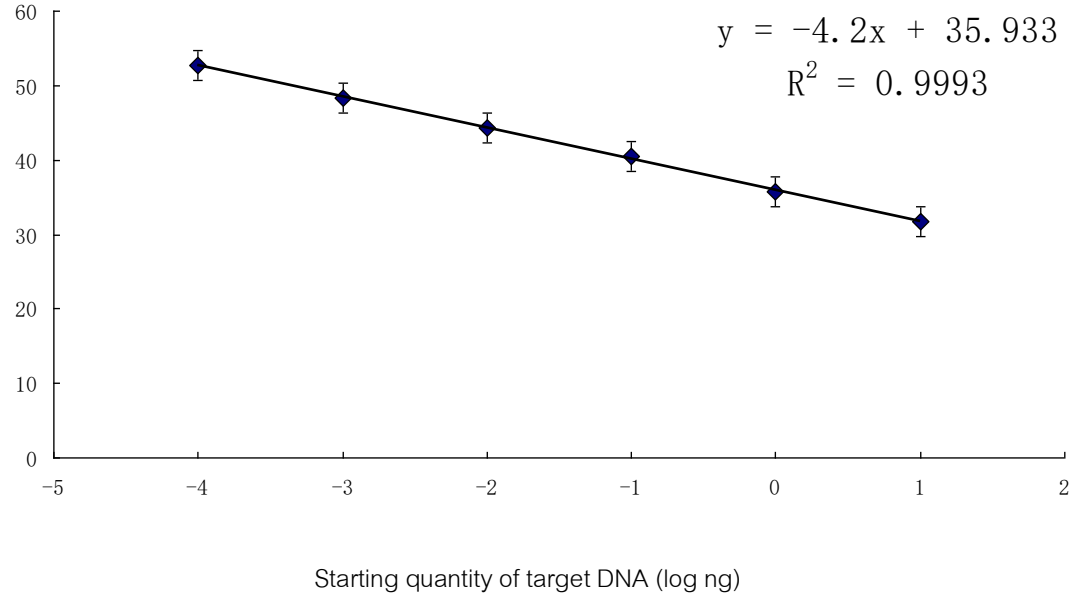


Figure 2 The sensitivity of real-time LAMP assay and standard curve. (A) The turbidity units versus time amplification curves plotted automatically using a LA-320C Loopamp Realtime Turbidimeter. (B) Visible colour change in *M. bovies* samples, greenish fluorescence under UV light. (C) Agarose gel (1%) showing conventional PCR amplification products. In each case, reactions were performed using 10-fold serial dilutions of total DNA. (D) Standard curve for real-time LAMP assay. The threshold time (T_t) and the amount of initial template plasmid DNA were plotted using a LA-320C Loopamp Realtime Turbidimeter. Error bars represent standard deviations from triplicate reactions. 1, 2.29×10^{2} ng/ul; 2, 22.9×10^{1}ng/ul; 3, 2.29×10^{0} ng/ul; 4, 2.29×10^{-1} ng/ul; 5, 2.29×10^{-2} ng/ul; 6, 2.29×10^{-3} ng/ul; 7, 2.29×10^{-4} ng/ul; 8, 2.29×10^{-5} ng/ul; 9, 2.29×10^{-6} ng/ul; 10, 2.29×10^{-7} ng/ul; 11, negative control.

Table 3 Real-time LAMP assay results from *M. bovis* clinical samples

Real-time LAMP	PCR	
	Positive	**Negative**
Positive	TP 12	FP 0
Negative	FN 0	TN 53

Diagnostic
Sensitivity = TP/ (TP+FN) ×100% =100%
Specificity = TN/ (TN+FP) ×100% =100%
Positive predictive value (PPV) = TP/ (TP+FP) ×100% =100%
Negative predictive value (NPV) =TN/ (TN+FN) ×100% =100%

Discussion

M. bovis is a significant pathogen in cattle. *M. bovis* infections have been reported worldwide, with prevalences of 60 to 100% in France, 70% in USA, 25 to 50% in UK, and 13 to 23% in Ireland (Arcangioli *et al*., 2008; Nicholas and Ayling, 2003; Byrne *et al*., 2001), causing great economic losses in cattle. Since 2008, *M. bovis* has been reported in Hubei, Jiangxi, Anhui, Guizhou, Chongqing, Shandong, Hunan, Guangxi, etc., provinces in China (Xin *et al*., 2008; Shi *et al*., 2008; Ran *et al*., 2010; Fu *et al*., 2011). Thus, *M. bovis* has rapidly become a serious threat to dairy cattle and beef in China. *M. bovis* played a primary role in the etiopathogenesis of bovine respiratory disease complex (BRDC) caused by mixed bacterial infections, such as *M. bovis* and *Pasteurella multocida*; *M. bovis* and *Haemophilus somnus*; or all 3 organisms *M. bovis*, *P. multocida*, and *H. somnus* (Buchvarova and Vesselinova, 1989). Co-infection of *M. bovis* as an initiating agent and viruses such as bovine respiratory syncytial virus, parainfluenza-3 virus and bovine viral diarrhoea virus infections do occur in BRDC (Arcangioli *et al*., 2008).Thus, rapidly and accurately diagnosing *M. bovis* infection is very important to control the disease.

The culture of *M. bovis* is the "gold standard" in diagnosis. However, isolation of *M. bovis* is very difficult, takes time and has low sensitivity. PCR methods have a higher sensitivity and specificity. However, the latter method requires expensive equipment such as PCR thermocycler, gel electrophoresis systems, etc., making it very difficult to carry out diagnosis in the field (Nicholas and Ayling, 2003).

The *M. bovis* OPPD/F gene is an excellent target for specific amplification assays as it has a low homology compared to the *M. agalactiae* P80 gene or the *M. mycoides subsp. mycoides Small Colony Type* P72 gene (Li *et al*., 2011). In our research, two sets of *M. bovis*-specific primers were designed with inner and outer primer pairs targeting the conserved regions of the OPPD/F gene, which amplified only *M. bovis* DNA. No amplifications were detected when DNAs from seven other bacteria including *M. ovipneumoniae, A. modicum, M. mycoides, E. coli, S. suis 2, Salmonella, Bordetella* and negative control were tested. These results confirmed

that the developed LAMP assay was highly specific for the diagnosis of *M. bovis*. Furthermore, during the application of real-time LAMP assays on clinical samples, the concordance with a conventional PCR was 100%. There were no false-positives or false-negatives observed. The real-time LAMP assay showed sensitivity, specificity and positive predictive value and negative predictive value were 100%. Appelt *et al*., (2018) and Higa *et al*., (2018) developed a LAMP assay for detection of *M. bovis*. showing a sensitivity and specificity of 87.5% and 82.4%, 97.2% and 90.9%, respectively. Our real-time LAMP assay was more sensitive and specific than previously reported LAMP assays.

Due to its powerful amplification efficiency, in a 45 mins reaction at 63°C, the real-time LAMP assay was 100- fold more sensitive than the conventional PCR assay. Hence, the real-time LAMP method is more suitable than PCR for detection of *M. bovis*.

In summary, we developed a real-time LAMP assay with increased sensitivity and specificity compared to previous methods. This research has proved that the real-time LAMP assay has great potential for field use in the detection of *M. bovis*.

Conflict of Interest: All authors declare no competing interests.

Authors contribution: Jun Li and Yan Pan conceived and designed the study. Yan Pan performed the experiments. Ying Ming Wei, Garmendia AE, Hao Peng, Li Tao and Shi Wen Feng analyzed and interpreted the data. Yan Pan wrote the manuscript. All authors have read and approved the final manuscript.

Acknowledgements

This study was supported by the Guangxi key technologies research and development program (No. AA17204057), Guangxi Science and technology planning project (No. AB19245010), the National modern agriculture industry technology system (No.nycytxgxcxtd-09-05) and the Guangxi basic scientific research project (No. 16-3).

References

Appelt S, Aly SS, Tonooka K, Glenn K, Xue Z, Lehenbauer TW and Marco ML 2019. Development and comparison of loop-mediated isothermal amplification and quantitative polymerase chain reaction assays for the detection of Mycoplasma bovis in milk. J Dairy Sci. 102(3): 1985-1996.

Arcangioli MA, Duet A, Meyer G, Dernburg A, Bézille P, Poumarat F and Le Grand D 2008. The role of *Mycoplasma bovis* in bovine respiratory disease outbreaks in veal calf feedlots. Vet J. 177(1): 89-93.

Ayling RD, Nicholas RAJ and Johansson KE 1997. Application of the polymerase chain reaction for the routine identification of *Mycoplasma bovis*. Vet Rec. 141(12): 307–308.

Byrne WJ, McCormack R, Brice N, Egan J, Markey B and Ball HJ 2001. Isolation of *Mycoplasma bovis* from bovine clinical samples in the Republic of Ireland. Vet Rec. 148(11): 331-333.

Buchvarova Y and Vesselinova A 1989. On the aetiopathogenesis of *Mycoplasma pneumonia* in calf. Arch Exp Veterinarmed. 43(5): 685-689.

Caswell JL and Archambault M 2007. *Mycoplasma bovis* pneumonia in cattle. Anim Health Res Rev. 8(2): 161–186.

Castillo-Alcala F, Bateman KG, Cai HY, Schott CR, Parker L, Clark ME, McRaild P, McDowall RM, Foster RA, Archambault M and Caswell JL 2012. Prevalence and genotype of *Mycoplasma bovis* in beef cattle after arrival at a feedlot. Am J Vet Res. 73(12): 1932-1943.

Dyer N, Register KB, Miskimins D and Newell T 2013. Necrotic pharyngitis associated with *Mycoplasma bovis* infections in American bison (Bison bison). J Vet Diagn Invest. 25(2): 301–303.

Fu JH, Liu QY, Xu MJ, Shi DS, He XH, Pan Y, Guo RB, Gao Q, Yi SX, Si HS and Zhu XQ 2011. Seroprevalence of *Mycoplasma bovis* infection in dairy cows in Subtropical southern China. Afr J Biotechnol. 10(54): 11313-11316.

Fu P, Sun Z, Zhang Y, Yu Z, Zhang H, Su D, Jiang F and Wu W 2014. Development of *Mycoplasma bovis* infection based on a monoclonal antibody of P48 protein. BMC Vet Res. 10: 42.

Heller M, Berthold E, Pfützner H, Leirer R and Sachse K 1993. Antigen capture ELISA using a monoclonal antibody for the detection of *Mycoplasma bovis* in milk. Vet Microbiol. 37(1-2): 127-133.

Hale HH, Helmboldt CF, Plastridge WN and Stula EF 1962. Bovine mastitis caused by a *Mycoplasma* species. Cornell Vet. 52: 582–591.

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(8): 1343-1346.

Ishiguro N, Koseki N, Kaiho M, Kikuta H, Togashi T, Watanabe T, Ariga T and Hokkaido Pediatric Respiratory Infection Study Group 2015. Sensitivity and specificity of a loop-mediated isothermal amplification assay for the detection of *Mycoplasma Pneumonia* from nasopharyngeal swab samples compared with those of Real-time PCR. Clin Lab. 61(5-6): 603-606.

Kursa O, Woźniakowski G, Tomczyk G, Sawicka A and Minta Z 2015. Rapid detection of *Mycoplasma synoviae* by loop-mediated isothermal amplification. Arch Microbiol. 197 (2): 319-325.

Liu MJ, Du GM, Bai FF, , Wu YZ, Xiong QY, Feng ZX, Li B and Shao GQ 2015. A rapid and sensitive loop-mediated isothermal amplification procedure (LAMP) for *Mycoplasma hyopneumoniae* detection based on the p36 gene. Genet Mol Res. 14(2): 4677-4686.

Li DW, Huang CP, Zhang YM, Xie JH, Ran ZG, Xiong ZL and Fan XW 2011. Establishment and application of the triplex PCR for detecting *M.Bovis, M.mycoides subsp.mycoides Small Colony Type* and *M.agalactiae*. Acta Vet et Zoo Sinica. 42(2): 306-310.

Mustafa R, Qi JJ, Ba XL, Chen YY, Hu CM, Liu XL, Tu LL, Peng QJ, Chen HC and Guo AZ 2013. In vitro quinolones susceptibility analysis of Chinese *Mycoplasma bovis* isolates and their phylogenetic

scenarios based upon QRDRs of DNA topoisomerases revealing a unique transition in ParC. Pak Vet J 33(3): 364–369.

Maunsell FP, Woolums AR, Francoz D, Rosenbusch RF, Step DL, Wilson DJ and Janzen ED 2011. *Mycoplasma bovis* infections in cattle. J Vet Intern Med. 25(4): 772–783.

Nicholas RA and Ayling RD 2003. *Mycoplasma bovis*: disease, diagnosis, and control. Res Vet Sci. 74(2): 105-112.

Register KB, SaccoRE, and OlsenSC 2013. Evaluation of enzyme-linked immunosorbent assays for detection of *Mycoplasma bovis*-specific antibody in bison sera. Clin Vaccine Immunol 20(9): 1405-1409.

Ran ZG, Xie JH, Luo L, Yang ZL, Ling HQ, Su CZ, Xiong ZL, Fan WX and Mi ZY 2010. Etiological diagnosis of *Mycoplasma bovis* pneumonia and arthritis in some provinces in China. Chinese J Pre Vet Med. 32 (1): 40~43.

Shi L, Gong R, Yin ZY, Zhou Y, Pei J, Hu ZB, Wang LX, Hu CM, Liu T, Chen YY, Liao JH, Zhao JL, Chen HC and Guo AZ 2008. Diagnosis of cattle infectious *Mycoplasma bovis* Pneumonia. J Huazhong Agric Univ. 27 (5): 629~633.

Sachse K, Pfützner H, Hotzel H, Demuth B, Heller M and Berthold E 1993. Comparison of various diagnostic methods for the detection of *Mycoplasma bovis*. Rev Sci Tech. 12(2): 571–580.

Tao L, Li J, Pang GL, Zhou QA, Pan Y, Qin RF, Li CT, Chen ZX, Xu LG and Yang W 2015. Isolation and identification of *Mycoplasma bovis* from Nanning, Guangxi. Heilongjiang Ani Sci & Vet Med. 483(8): 169-171.

Xin JQ, Li Y, Guo D, Song NH, Hu SP, Chen CP, Pei JP and Cao PL 2008. First isolation of *Mycoplasma bovis* from calf lung with pneumoniae in China. Chinese J Pre Vet Med. 30(9): 661–664.