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Sensitive and rapid detection of *Mycoplasma capricolum* subsp. *capripneumoniae* by Loop-mediated isothermal amplification

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A set of four specific primers was designed by targeting the *H2* gene sequences of *Mycoplasma capricolum* subsp. *capripneumoniae* (MCCP). Using *Bst* DNA polymerase, the products were amplified for 60 min at 65°C in a simple water bath. Compared with a polymerase chain reaction (PCR) test that targets the *H2* gene sequences of MCCP, the sensitivity of the loop-mediated isothermal amplification (LAMP) assay was higher (approximately 0.75 fg DNA per reaction). The LAMP products could be visualized by agar gel electrophoresis. There were no cross reactions with other strains in the *Mycoplasma mycoides* cluster, which indicates the high specificity of the LAMP procedure. The LAMP assay was able to detect MCCP in tissue.

Key words: *Mycoplasma capricolum* subsp. *Capripneumoniae*, loop-mediated isothermal amplification, rapid detection.

INTRODUCTION

Contagious caprine pleuropneumonia (CCPP) is a severe infectious disease of goats caused by *Mycoplasma capricolum* subsp. *capripneumoniae* (MCCP), which occurs in many countries of Africa and Asia (Woubit et al., 2004). It is a disease of major economic relevance characterized by high morbidity and mortality; the mortality rate often approaches 100% in susceptible flocks (Rurangirwa et al., 1987). A mycoplasma strain, designated F-38, first isolated in Kenya (MacOwan and Minette, 1976), is a member of the *Mycoplasma mycoides* cluster which includes *M. mycoides* subsp. *mycoides* SC (MmmSC), *M. mycoides* subsp. *mycoides* LC (MmmLC), *M. mycoides* subsp. *capri* (mmc), *M. capricolum* subsp. *capricolum* (Mcca) and *Mycoplasma* species bovine group7 (bg7).

CCPP is classified as a list B disease by the Office International Des Epizooties or World Organization for Animal Health (OIE). It is an infectious disease that

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Abbreviations: CCPP, Contagious caprine pleuropneumonia; **MCCP,** *Mycoplasma capricolum* subsp. *capripneumoniae*; **LAMP,** loop-mediated isothermal amplification; **PCR,** polymerase chain reaction.

affects only goats, and was first described in the late 19th century (Hutcheon, 1889; McMartin et al., 1980). Before the isolation and identification of *Mycoplasma* strain F38 by MacOwan (1976) and the subsequent demonstration of its causal relationship with CCPP (MacOwan and Minette, 1976), *M. mycoides* subsp. *capri* was considered to be the aetiological agent of CCPP (Edward, 1953; JonAs and Barber, 1969). So far, *M. capricolum* subsp. *capripneumoniae* is the only mycoplasma that fulfills the Koch postulates for CCPP, and it is believed to be the sole cause of CCPP (MacOwan, 1984). *Mycoplasma* strain F38 has been reclassified recently, and now all F38-like mycoplasmas are known as *Mycoplasma capricolum* subsp. *Capripneumoniae* (Leach et al., 1993).

CCPP has been reported to affect only goats (Thiaucourt and Bolske, 1996) and it does not cause disease in sheep, either spontaneously or experimentally (McMartin et al., 1980). However, there are some reports that describe the isolation of *M. capricolum* subsp. *capripneumoniae* from healthy sheep in Kenya that had been in contact with goat herds affected by CCPP (Litamoi et al., 1990), and from sick sheep in Uganda that had been mixed with goats suffering from the disease (Bolske et al., 1995). The isolation of *M. capricolum* subsp. *capripneumoniae* from cattle with mastitis has also been reported (Kumar and Garg, 1991), and these reports contradict the perceived host specificity of *M. capricolum* subsp. *capripneumoniae*.

Recently, a novel nucleic acid amplification method, termed loop-mediated isothermal amplification (LAMP), has been reported (MacOwan and Minette, 1976). The LAMP assay is rapid and its amplification efficiency is equivalent to that of polymerase chain reaction (PCR)-based methods (Cai et al., 2010; Gadkar and Rillig, 2008). More importantly, the approach is less costly, and all reactions can be developed in an isothermal environment. Reports of the detection of MCCP using LAMP assays have shown that the approach is easier and faster to perform than conventional PCR assays, as well as being more specific (Endo et al., 2004). In this study, a method based on the LAMP assay for the detection of CCPP was developed, and the sensitivity and specificity of the assay were evaluated. The assay was compared with a PCR test targeting the *H2* gene sequences of MCCP.

MATERIALS AND METHODS

Strains and cultivation

The origin of the 21 stains used in this study, type strains of the *M. mycoides* cluster and field isolates from China, are listed in Table 1. The mycoplasmas were cultivated in modified KM_2 (Hanks solution with 1.7% lactalbumin hydrolysate, 1% MEM, 20% de-complemented horse serum, 5% fresh yeast extract, 1% thallium acetate, 0.4% sodium pyruvate) in a high security laboratory. The DNA of *Pasteurella multocida* and *Mannheimia haemolytica* was maintained at the State Key Laboratory of Veterinary Etiological Biology.

Clinical samples

Twenty-eight (28) samples from 14 goats infected artificially with *M. capricolum* subsp. *capripneumoniae* were used in the study. The clinical samples were collected when the goats showed primary clinical signs: Cough, anorexia, laboured breathing with painful grunting, and a rise in temperature up to 41°C. Given that the gross pathological lesions were localized exclusively in the lung, the livers were collected for use in the experiment (Table 1). Sixty-one (61) clinical samples collected from western China in 2009-2011 were used for the epidemiological survey. These samples were kept at -80°C until analysis.

Sample preparation

Culture samples (1 ml) were centrifuged at 1,2000 RCF for 20 min at 4°C. The cell debris was pelleted; the pellets were washed in phosphate buffered saline (PBS) and re-suspended in 50 µl ddH_2O. After vortexing, the samples were lysed by boiling for 10 min, centrifuged and diluted 1:50. For the clinical samples, DNA extraction was performed using a kit (DNA extraction kit, Invitrogen, Carlsbad, USA) according to the protocol of the manufacturer. The extracted DNA was used for PCR both undiluted and at 1:50 dilution.

PCR conditions (He et al., 2011)

Based on the conserved sequence of the H2 gene (GenBank access number:AF162991.1) of MCCP, suitable primers were designed using the primer5.0 software: mccp f 5′ AAA AGT CCC TGA AAC ATT AC 3′ (319-338 bp) and mccp R5′ GGT GTA CCC ACT GCT AAA GA 3′ (1032-1013 bp). These primers were synthesized by TaKaRa, Dalian, China. The 50 µl reaction mixture contained 3 µl $MgCl_2$ (1.5 mM), 0.5 µl dNTP (150 µM for dCTP and dGTP, 300 µM for dATP and dTTP), 5 µl 10×Taq Buffer[b], 1 µl each primer, 0.5 µl Taq polymerase (10×Taq dNTP Buffer and Taq polymerase (1 unit, TaKaRa, Dalian, China)), 5 µl DNA sample and 34 µl ddH_2O. The PCR conditions consisted of an initial denaturation step of 5 min at 94°C, followed by 35 cycles of 45 s at 94°C, 30 s at 50°C and 45 s at 72°C, and a final extension step of 10 min at 72°C. Samples of the PCR amplification products (5 µl) were subjected to electrophoresis in a 1% agarose gel in tris/borate buffer according to standard protocols. The DNA was visualized by UV-fluorescence after staining with ethidium bromide. Each PCR test was repeated three times.

LAMP primers

The MCCP LAMP primer set was designed using the primerExplorer program http//primerexplorer.jp/e/index.html to amplify the H2 gene (GenBank access number: AF162991.1). The primers are shown in Table 2.

LAMP method

The LAMP reactions were carried out with a 25 µL reaction mixture containing 2 µL of extracted DNA, 40 pmol (each) of primers FIP and BIP, 5 pmol (each) of primers F3 and B3, 2.8 mM of each dNTP, 4 U of the large fragment of *Bst* DNA polymerase (*Bst* DNA polymerase (Biolabs@inc., New England, USA)), with the corresponding polymerase buffer. The reaction temperature and time were 65°C and 60 min. The reaction was terminated by heating at 80°C for 3 min. Positive and negative controls were

Table 1. Isolation of *Mycoplasma capricolum subsp.capripneumoniae* and LAMP and PCR-detection from artificial infected animals with MCCP.

Goat number	Macroscopic findings	Sample type	Microbiology results	LAMP	PCR
				1:50 diluted	1:50 diluted
1	Serious hepatized	Lung	MCCP	+	+
		Liquor pleurae	MCCP	+	+
2	Slight hepatized	Lung	MCCP	+	+
		Liquor pleurae	MCCP	+	+
3	Slight hepatized	Lung	MCCP	+	+
		Liquor pleurae	MCCP	+	+
4	Serious hepatized	Lung	MCCP	+	+
5	Serious hepatized	Lung	MCCP	+	+
		Liquor pleurae	MCCP	+	+
6	Slight hepatized	Lung	MCCP	+	+
		Liquor pleurae	MCCP	+	+
7	Slight hepatized	Lung	MCCP	+	+
		Liquor pleurae	MCCP	+	+
8	Slight hepatized	Lung	MCCP	+	+
		Liquor pleurae	MCCP	+	+
9	Serious hepatized	Lung	MCCP	+	+
		Liquor pleurae	MCCP	+	+
10	Serious hepatized	Lung	-	+	+
		Liquor pleurae	-	+	-
11	Serious hepatized	Lung	MCCP	+	+
		Liquor pleurae	MCCP	+	+
12	Serious hepatized	Lung	MCCP	+	+
		Liquor pleurae	MCCP	+	+
		Liquor pleurae	MCCP	+	+
13	Serious hepatized	Lung	MCCP	+	+
		Liquor pleurae	MCCP	+	+
14	Serious hepatized	Lung	MCCP	+	+
	Hepatized	Liquor pleurae	MCCP	+	+

Table 2. Primer sequences used in this study.

Name	Sequence(5`-3`)
FIP	TGCTGGTGAATATTTTGTAGCAGGTTTTTAAGCCCAAAGTTAATATACCTTGA
BIP	CAACACCAGATTCAAAGAAAGGTTTTTTTGAGTTGAAAGCTTTTTAGATTGT
F3	ACAACCTAAAGAGATTATTCACTC
B3	AACTTGACTTCCAACAACAA

included in each run, and all precautions to prevent cross contamination were observed. The LAMP products (3 µL) were detected in 2% agarose.

Specificity of LAMP

To determine the specificity of the LAMP method, it was carried out at 65°C for 60 min with the DNA of various mycoplasmas in the *M. mycoides* cluster; the templates from the eight type strains of the *M. mycoides* cluster and *Pasteurella multocida* are listed in Table 3. Each DNA sample from the strains tested was examined in triplicate. The products were separated by 2% agarose gel electrophoresis, and the target bands were visualized by staining with ethidium bromide.

Sensitivity of the LAMP method

The sensitivity of the assay was assessed by testing tenfold serial dilutions of 1 µg ml^{-1} DNA of *M. capricolum* subsp. *capripneumoniae* F1601. Reaction mix without the DNA template was included as a negative reaction control. The LAMP amplification products were analyzed visually by 2% agarose gel electrophoresis. To compare

Table 3. Collection of strains used to test the specificity of the *Mycoplasma capricolum subsp.capripneumoniae* LAMP.

Species	N	Description	Origin	PCR
M. capricolum subsp. capripeumoniae	1	M1601	M1601, China	+
M. ovipneumoniae	1	Y98	Y98, ntcc	-
M. mycoides subsp. Capri	1	PG3	PG3, China	-
M. mycoides subsp. mycoides Large colony	1	Y-goat	Cirad	-
M. capricolum subsp. capricolum	1	C.Kid	Cirad	-
M. agalactiae	1	GS. 12	China	-
M. arginini	1	PG 1	China	-
M. bovis	1	M.B1	China	-
Pasteurella multocida	1	*Pasteurella multocida*	China	-

Table 4. Isolation of *Mycoplasma capricolum subsp.capripneumoniae* and LAMP and PCR-detection from clinical samples obtained from animals suspected for being infected with MCCP.

Sample type	Number of positive results (%)	
	LAMP	PCR
Lung tissue	33 (42)	26 (42)
Liquor pleurae	14 (19)	10 (19)
Total	47	36

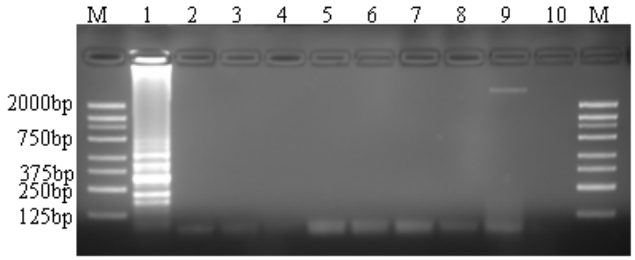

Figure 1. Gel dielectrophoresis of LAMP products showing a specific for *M. capricolum subsp. Capripeumoniae* (MCCP). Lane: M, Molecular weight; lane 1, M1601; lane 2, *M. mycoides susp. Capri* PG3; lane 3, *M. ovipneumoniae* strain Y98; lane 4, *Pasteurella multocida*; lane 5, *M. capricolum subsp. Capicolum* C.kid; lane 6, *M. agalactiae* GS.12; lane 7, *M. arginine* PG 1; lane 8, *M. bovis* strains M1; lane 9, *M. mycoides susp. mycoides large colony* Y-goat; lane 10, ddH2O.

the detection sensitivities of LAMP and PCR, PCR using *H2* gene primers that amplify a 680-bp product was carried out in a total reaction volume of 25 µL containing 2 µL of the MCCP DNA, 2 µL (10 pmol ml^{-1}) of a pair of appropriate primers, 12.5 µL Premix Ex Taq (TaKaRa, Shiga, Japan) and ddH_2O. The PCR conditions consisted of an initial denaturation step of 5 min at 94°C, followed by 35 cycles of 45 s at 94°C, 30 s at 50°C and 45 s at 72°C, and a final extension step of 10 min at 72°C. Samples of the PCR amplification products (5 µl) were analyzed by 1% agarose gel electrophoresis. Tests of the sensitivity of the LAMP and PCR assays were conducted in triplicate, and the detection limits were defined as the last positive dilutions, with a sample being considered positive if all three samples tested positive.

Results

Specificity of the LAMP assay

The specificity of LAMP was tested using DNA extracted from the eight type strains of the *M. mycoides* cluster and *P. multocida*. After incubation at 65°C for 60 min, MCCP was positively detected, whereas no other mycoplasma isolate was detected or amplified by LAMP. The other type strains of the *M. mycoides* cluster and *P. multocida*, as listed in Table 3, were negative (Figure 1).

Sensitivity of the LAMP assay

To assess the sensitivity of the LAMP assay for the detection of MCCP, the reaction was tested using 1 µL tenfold serial dilutions of MCCP DNA and compared with the PCR assay. The LAMP reaction was able to detect up to 0.75 fg DNA per reaction (Figure 2); however, the PCR could only detect MCCP up to 750 pg per reaction (Figure 3). The results indicate that LAMP has a higher sensitivity than the standard PCR method.

Clinical samples

Twenty-eight (28) clinical samples originating from 14 animals showing serious clinical signs were included in the analysis: 28 samples were positive by LAMP assay, using

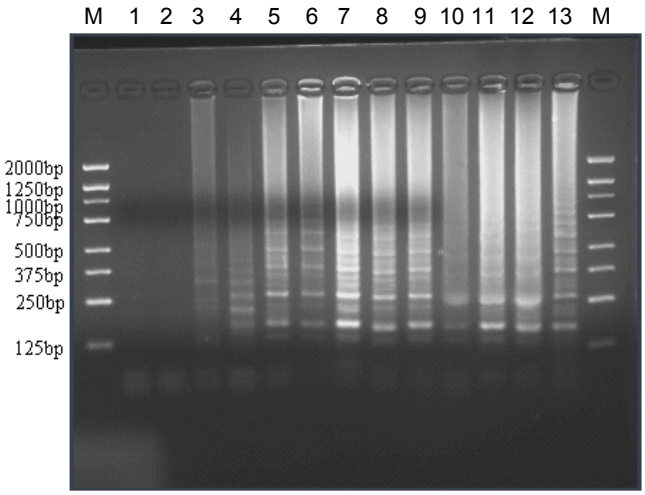

Figure 2. Sensitivity of LAMP. Lane: 13-3, 7.5 µg, 750 ng, 75 ng, 7.5 ng, 750 pg, 7.5 pg, 0.075 pg, 0.0075 pg, 0.00075 pg. The last concentration y detection was 0.00075 pg.

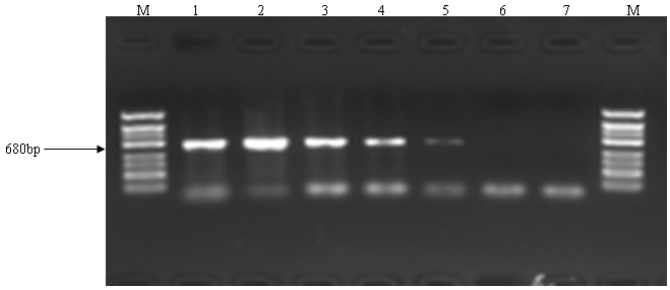

Figure 3. Sensitivity of PCR. Lanes: 1-5, represent 7.5 µg, 750 ng, 75 ng, 7.5 ng, 750 pg. The last concentration by detection was 750 pg.

1:50 diluted templates (Figure 4). To evaluate the LAMP test further, comparison with the PCR test was performed using samples from an epidemiological survey conducted in western China. The results are shown in Table 4. Overall, from 61 clinical samples, the LAMP assay gave a total of 11 more positive results than the PCR test.

DISCUSSION

The LAMP primers used in this study were based on the H2 gene sequence, which confers some advantages for molecular identification. The H2 gene is a putative membrane protein gene. It is reported that the partial sequence of the *H2* gene can be used as an epidemiological marker for the *M. mycoides* cluster because it is a conserved sequence. Such studies demonstrate the use of the *H2* gene for the molecular identification of closely related genomic species (Thiaucourt and Bolske,

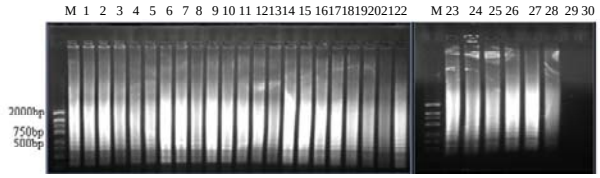

Figure 4. LAMP from artificial infected animals with MCCP and the samples was diluted as 1:50. Lane: M, Molecular weight marker; lanes 1-28, clinical samples; lane 29, negative control; lane 30, normal lung tissue of goat.

1996).

LAMP method compared with conventional PCR reported in this article has the advantages of simple operation, rapid reaction and ease of detection. The LAMP assay is a simple detection tool in which the reaction is performed in a single tube by mixing the thermopol buffer, primers and *Bst* DNA polymerase, followed by incubation of the mixture at 65°C for 60 min. The LAMP reaction is performed under isothermal conditions and it does not require expensive equipment: the only equipment needed for the LAMP reaction is a regular laboratory water bath or a heating block that can provide a constant temperature of 65°C. Moreover, the amplification efficiency is extremely high, there is no time required for thermal cycling, and inhibition reactions at later stages are less likely to occur than in standard PCR. In addition, LAMP amplifies DNA to higher concentrations than PCR, allowing convenient visualization of the products after the addition of SYBR Green I without gel electrophoresis. Hence, the LAMP assay could be developed into a field test.

In this study, the LAMP method for the detection of MCCP was found to be highly sensitive, because it could detect MCCP at 0.75 fg DNA per reaction, whereas by PCR, the detection of MCCP was possible only up to750 pg DNA per reaction. This indicates that the sensitivity of LAMP is higher than that of the standard PCR. The increased sensitivity may make LAMP a better choice than PCR for the detection of MCCP from cases of in apparent infection.

In conclusion, the LAMP method described in this study represents a new, sensitive, specific, and rapid protocol for the detection of MCCP. It may be applied in epidemiological surveys of contagious caprine pleuropneumonia.

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REFERENCES

Bolske G, Johansson KE, Heinonen R (1995). contagious caprine pleuropneumonia in uganda and isolation of mycoplasma-capricolum subspecies capripneumoniae from goats and sheep. Vet. Record 137:594-594.

Cai SH, Lu YS, Wu ZH (2010). Loop-mediated isothermal amplification method for rapid detection of Vibrio alginolyticus, the causative agent of vibriosis in mariculture fish. Letters in Appl. Microbiol. 50:480-485.

Edward DGf (1953). Organisms of the pleuropneumonia group causing disease in goats. Veterinary Record 65:873-874.

Endo S, Komori T, Ricci G (2004). Detection of gp43 of Paracoccidioides brasiliensis by the loop-mediated isothermal amplification (LAMP) method. Fems Microbiology Letters 234:93-97.

Gadkar V, Rillig MC (2008). Evaluation of loop-mediated isothermal amplification (LAMP) to rapidly detect arbuscularmycorrhizal fungi. Soil Biol. Biochem. 40:540-543.

He Y, Zhang N, Zhao P (2011). Improvement of specific polymerase chain reaction(PCR) for the identification of *Mycoplasma capricolum* subsp. *capripneumoniae* .Afri. J. Biotechnol. 10:17291-17296.

Hutcheon D (1889). Contagious pleuro-pneumonia in goats at Cape Colony, South Africa. Vet. J. 29:399-404.

JonAs AM, Barber TL (1969). Mycoplasma mycoides var. capri isolated from a goat in Connecticut. J. Infect. Dis. 119:126-131.

Kumar A, Garg DN (1991). Isolation of mycoplasma F-38 from the milk of mastitic cows. Vet. Record 128:429.

Leach RH, Erno H, Macowan KJ (1993). Proposal for designation of F38-type caprine mycoplasmas as Mycoplasma capricolum ssp. capripneumoniae, new subspecies and consequent obligatory relegation of strains currently classified as Mycoplasma capricolum (Tully, Barile, Edward, Theodore, and Erno 1974) to an additional new subspecies, Mycoplasma capricolum ssp. capricolum, new subspecies. Intl. J. Syst. Bacteriol. 43:603-605.

Litamoi JK, Wanyangu SW, Simam PK (1990). Isolation of mycoplasma biotype f38 from sheep in Kenya. Trop. Anim. Health Productn. 22:260-262.

MacOwan KJ (1984). Role of mycoplasma strain F38 in contagious caprine pleuropneumonia. Israel J. M. Sci. 20:979-981.

MacOwan KJ, Minette JE (1976). A mycoplasma from acute contagious caprine pleuropneumonia in Kenya. Trop. Ani. Health Product. 8:91-95.

McMartin DA, MacOwan KJ, Swift LL (1980). A century of classical contagious caprine pleuropneumonia: from original description to aetiology. Brit. Vet. J. 136:507-515.

Rurangirwa FR, McGuire TC, Magnuson NS (1987). Composition of a polysaccharide from mycoplasma (F-38) recognised by antibodies from goats with contagious pleuropneumonia. Res. Vet. Sci. 42:175-178.

Thiaucourt F, Bolske G (1996). Contagious caprine pleuropneumonia and other pulmonary mycoplasmoses of sheep and goats. Revue Scientifique Et Technique-Office International Des Epizooties 15:1397-1414.

Woubit S, Lorenzon S, Peyraud A (2004). A specific PCR for the identification of Mycoplasma capricolum subsp capripneumoniae, the causative agent of contagious caprine pleuropneumonia (CCPP). Vet. Microbiol. 104:125-132.