

Real-time loop-mediated isothermal amplification (LAMP) of *mgc2* gene of *Mycoplasma gallisepticum*

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Abstract

Introduction: *Mycoplasma gallisepticum* is considered the most pathogenic and economically significant avian *Mycoplasma* spp. for the worldwide poultry industry. The aim of this study was to develop a novel and sensitive real-time loop-mediated isothermal amplification (LAMP) assay based on the amplification of its *mgc2* gene sequence for its rapid molecular detection in poultry. **Material and Methods:** Blood samples from 300 broiler and layer chickens were screened using a rapid serum agglutination (RSA) test. A real-time LAMP reaction was conducted with seropositive swab samples at 60°C for 90 min in an ESEQuant tube scanner using 6-carboxyfluorescein as the reporting dye. **Results:** The sensitivity of the developed assay was 10 fg/μL of DNA. The assay was found 100% specific, showing no cross-reactivity with other avian *Mycoplasma* species. The proportion found of the positive samples by the real-time LAMP was 58%. In comparison, the RSA was found to detect 52% of positive cases. **Conclusion:** The *mgc2* real-time LAMP emerged as a more sensitive and accurate method for molecular detection of *M. gallisepticum* than RSA. Robustness and precision give it applicability as a potential field diagnostic tool for *M. gallisepticum* control. The study will be beneficial in reducing economic losses that *M. gallisepticum* inflicts on the poultry industry. This is the first reported development of a real-time LAMP assay based on the amplification of the *mgc2* gene sequence using an ESEQuant tube scanner for galline *M. gallisepticum* detection.

Keywords: poultry, *Mycoplasma gallisepticum*, real-time LAMP, *mgc2* gene.

Introduction

Mycoplasma gallisepticum is still considered the most pathogenic and economically significant *Mycoplasma* spp. for the worldwide poultry industry (4). *M. gallisepticum* infection is commonly known as chronic respiratory disease (CRD) in avian species (11). Timely monitoring and rapid diagnosis are the most crucial steps in prevention and control of *M. gallisepticum* infection. Culture isolation, serological assays, and molecular detection methods (PCR, real-time PCR, and nested PCR) have been applied for the diagnosis of avian mycoplasmas for decades (3, 18). However, these methods are expensive and require an extended period of time to detect the infection (2). Real-time PCR is rapid but sophisticated equipment and a well-established laboratory are necessary for this technique (5). The loop-mediated isothermal amplification

(LAMP) assay has emerged as an affordable and rapid molecular diagnostic technique (6, 12). This isothermal technology does not require specialised and expensive instrumentation for amplification or for post amplification procedures; thus is able to obviate the limitations associated with PCR (1, 9). A LAMP reaction takes place under isothermal conditions of 60–65°C with high strand displacement activity performed by *Bst* or *Bsm* DNA polymerase (19). It uses four or six primers (two inner, two outer, or two loop primers) recognising six or eight specific sequences on the target DNA. Inner primers are FIP (forward inner primer) and BIP (backward inner primer), outer primers are F3 (forward outer primer) and B3 (backward outer primer), and loop primers consist of FL (forward loop) and BL (backward loop) primers (6, 17).

Here, we have presented a novel and sensitive real-time LAMP assay using an ESEQuant tube

scanner (Qiagen, Germany) for rapid molecular detection of *M. gallisepticum*. The scanner is a rapid fluorescence measurement system for real-time detection of pathogens. The device has an eight-tube holder and an LCD panel to show the positive and negative results. To the best of our knowledge, this is the first report on development of a real-time LAMP assay based on the amplification of *mgc2* gene sequence for the detection of *M. gallisepticum* in chickens.

Material and Methods

Rapid serum agglutination test. Commercially available stained *M. gallisepticum* antigen (SPAFAS *M. gallisepticum* Plate Antigen, Charles River Laboratories, USA) was used for initial screening of serum samples. *M. gallisepticum* reagent serum (Charles River Laboratories) served as the control positive serum. A rapid serum agglutination (RSA) test was performed (14) to screen 300 sera samples which were collected from commercial broiler and layer flocks.

Field samples and reference strains. Seropositive tracheal and nasal swab samples (n = 156) were collected from chickens of commercial broiler and layer flocks. DNA of reference species of avian *Mycoplasma* (*M. synoviae*, *M. gallisepticum*, and *M. imitans*) on Indicating FTA Classic cards (Whatman, USA) procured from the Poultry Diagnostic and Research Center (PDRC), University of Georgia, USA, was used to evaluate the specificity of the assay (Table 1).

Table 1. Reference strains of *Mycoplasma* spp. used in the study

Name	Strain	Origin
<i>Mycoplasma gallisepticum</i>	A5969	PDRC
<i>Mycoplasma synoviae</i>	F10-2AS	PDRC
<i>Mycoplasma imitans</i>	4229	PDRC

DNA extraction. The swab samples were processed directly for genomic DNA extraction through a phenol-chloroform extraction method as previously described (16). DNA of reference strains was purified from Indicating FTA Classic cards as previously reported (10).

Primer design for real-time LAMP. A set of four LAMP primers was designed targeting the *mgc2* gene sequence of *M. gallisepticum* strain K4669ATK98 (GenBank accession number: AY556303). The primers consisting of two outer (F3 and B3) and two inner (FIP and BIP) primers were generated online by Primer Explorer V 5 software (<https://primerexplorer.jp/lampv5/>) (Fujitsu, Japan). The names and sequences of designed primers are detailed in Table 2. Their location within

the *mgc2* gene sequence of *M. gallisepticum* is shown in Fig. 1. The theoretical specificity of all primers was analysed using the NCBI BLAST search tool (<http://www.ncbi.nlm.nih.gov/BLAST/>). Primer purity was also kept under consideration and in this regard additional purification of the FIP and BIP primers was carried out using a reverse phase cartridge (RPC), (Gene Link, USA).

Reaction conditions for real-time LAMP. The real-time LAMP reaction was carried out in an ESEQuant tube scanner (V 2.7.5) (Qiagen, Germany) with 6-carboxyfluorescein (FAM) as the reporting dye, as previously described for *M. mycoides* (8). The reaction with a total volume of 25 μ L per tube was conducted using an experimental DNA amplification kit with *Bsm* DNA polymerase (Cat. no. V0524, Thermo Scientific, USA). The reaction mixture contained Isothermal MasterMix, 40 pmol each of FIP and BIP primers, 5 pmol each of F3 and B3 primers, and 1 μ L of template DNA. The reaction mixture was incubated from 55°C to 65°C for different duration times from 30 to 90 min in the ESEQuant tube scanner and fluorescence signals were recorded using FAM (13). The tubes were heated at 80°C for 5 min to terminate the reaction.

Confirmation of LAMP amplified products. The amplified products of LAMP were analysed by gel electrophoresis on a 2% agarose gel, stained with 0.5 μ g/mL of ethidium bromide. The GeneRuler 50-bp DNA ladder (Cat no. SM0373, Fermentas, USA) was incorporated as standard. The reaction solutions of 5 μ L along with 6x loading dye were subjected to electrophoresis at 90 V for 1 h. After electrophoresis, the gel was visualised using a gel documentation system (DigiGenius, Syngene, UK) under UV light (7).

Results

Rapid serum agglutination test. The overall prevalence of *M. gallisepticum* in serologically tested broiler and layer chickens was 52% (156/300), as detailed in Table 3.

Evaluation of LAMP amplified products. Overall 58% (90/156) field samples (tracheal and nasal swabs) were detected as positive by the real-time LAMP assay (Table 3). Amplification curves were obtained through the ESEQuant tube scanner in the case of positive samples. No amplification was seen with *M. synoviae* or *M. imitans* (negative controls), nor with the no template control (NTC) (Fig. 2).

Real-time LAMP optimisation. To determine the optimum reaction conditions of real-time LAMP, its reaction mixtures were incubated from 55°C to 65°C for 30, 45, 60, and 90 min. The reaction temperature of 60°C was considered the optimal temperature, and the optimal duration time of the LAMP reaction was found to be 90 min. After 90 min, no effect on the final amplification was observed.

Real-time LAMP sensitivity. To evaluate the sensitivity of the developed real-time LAMP assay, genomic DNA of *M. gallisepticum* was tested in 10-fold serial dilutions from 1 pg/ μ L to 1 fg/ μ L. The lowest detection limit of the LAMP assay was recorded as 10 fg/ μ L of DNA (Fig. 3).

Real-time LAMP specificity. In order to evaluate the specificity of the LAMP assay, it was performed with genomic DNA templates of two other species of *Mycoplasma* (*M. synoviae* and *M. imitans*). At optimum

conditions, no amplification was observed in the cases of both other species of *Mycoplasma* and NTC, showing the 100% specificity of the developed assay (Fig. 2).

Confirmation of LAMP amplified products.

The amplified products of the successful LAMP assay were detected by demonstration of a specific ladder-like pattern on electrophoresed gel. Negative controls did not show any amplified product on the gel (Fig. 4).

Table 2. Designed real-time LAMP primers targeting *mge2* gene sequence of *M. gallisepticum*

Primer Name	Sequence (5–3')	Length
F3	AAGCGATTGAGCCAAC TG	18
FIP (F1c-F2)	GATCCCTATCTGAGGGTTATTAGCT-CTGAAGAAGTTAATACTCAAGAACC	50
B3	TAAACCCTGGTCGCATTC	18
BIP (B1c-B2)	ACCTCAGATTAATCCGCAATTTGGT-TTGGTTAGGTGGCATTGG	43

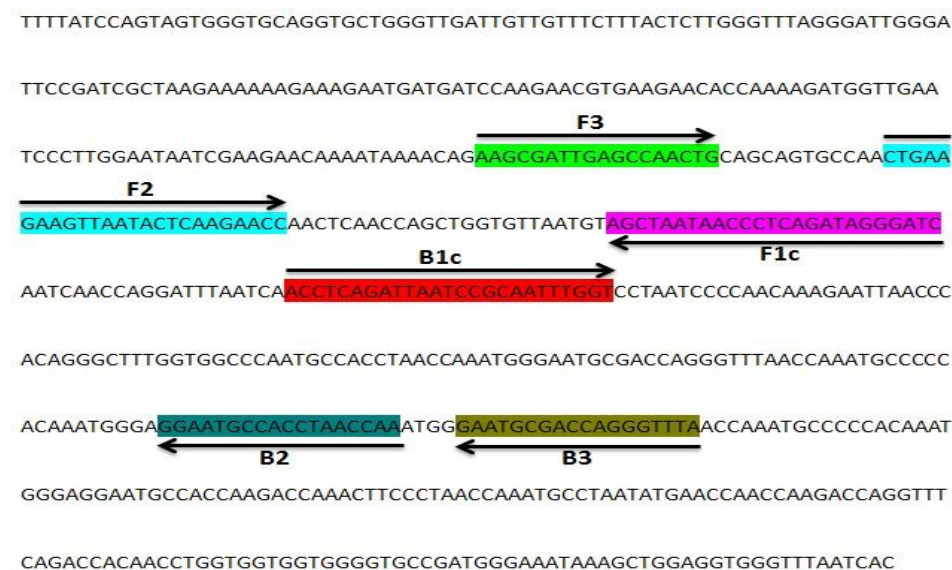


Fig. 1. LAMP primer location sites in *mge2* nucleotide sequence of *M. gallisepticum*. FIP consisted of F1c (complementary sequence of F1) and F2 sequence. BIP comprised B1c (complementary sequence of B1) and B2 sequence. Forward and backward outer primers are F3 and B3

Table 3. Distribution of samples for *M. gallisepticum* screened with RSA and confirmed through real-time LAMP assay from broiler and layer flocks

Flock	Number of samples	RSA positive samples	Real-time LAMP positive samples (out of RSA positive samples)
A	40	23	10
B	36	19	13
C	37	21	11
D	33	15	10
E	39	30	16
F	41	11	05
G	35	18	18
H	39	19	07
Total number	300	156	90
%	-	52	58

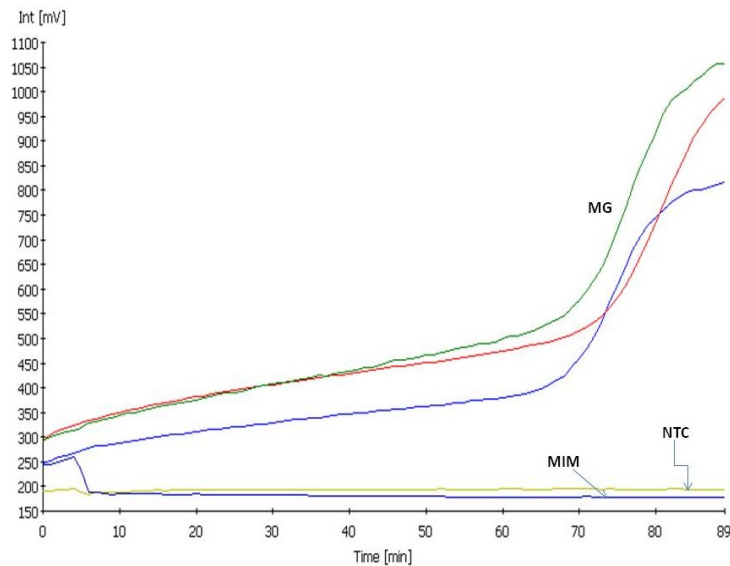


Fig. 2. Amplification curves of *M. gallisepticum*-positive samples detected through *mgc2*-gene-based real-time LAMP. *M. synoviae*, *M. imitans*, and NTC were not amplified

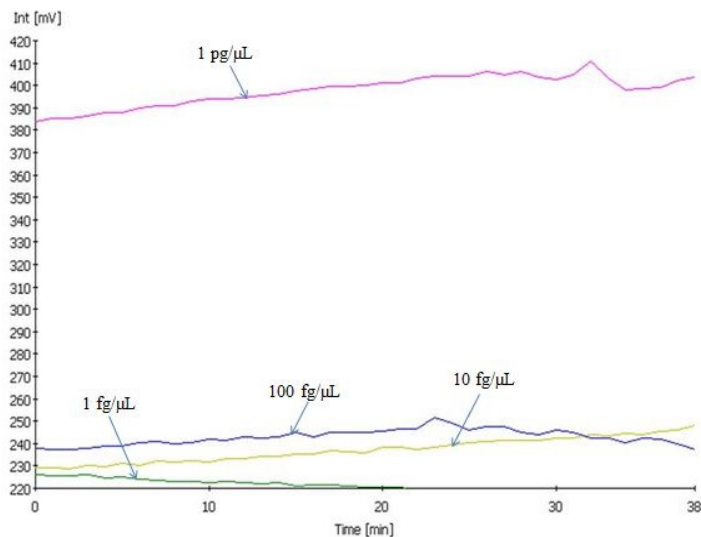


Fig. 3. Sensitivity of real-time LAMP by serial 10-fold dilutions of *M. gallisepticum* DNA

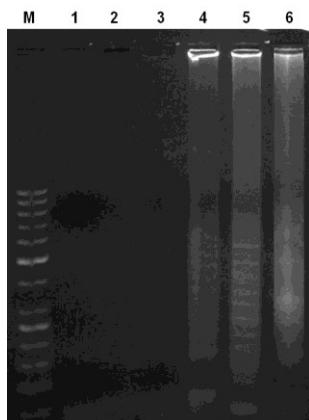


Fig. 4. Positive samples of *M. gallisepticum* on agarose gel after gel electrophoresis. Lane M – Gene Ruler 50-bp DNA ladder; Lanes 4, 5, and 6 – *M. gallisepticum* positive samples; Lane 1 – MS; Lane 2 – MIM; Lane 3 – negative reagent sample

Comparison of real-time LAMP and RSA tests.

The proportion of the positive samples detected by real-time LAMP was 58%. In comparison, the performed RSA was found to detect 52% of the positive cases. Therefore, the LAMP assay has been proved the more sensitive and confirmatory test for molecular detection of *M. gallisepticum* in poultry.

Discussion

Rapid diagnosis of *Mycoplasma gallisepticum* is required for its effective control. Sensitive and reliable molecular diagnostic methods based on real-time PCR are available for the detection of the bacteria in surveillance and control programmes, and preferred over serological assays, but are expensive and not applicable to field conditions. The high specificity, rapidity, and efficiency of the LAMP assay persuaded

us to establish real-time LAMP-based detection of *M. gallisepticum*. Although LAMP technology has been developed for the diagnosis of a range of pathogens, its application in the detection of avian mycoplasmas is very limited. Recently, Zhang *et al.* (19) reported a conventional LAMP assay for *M. gallisepticum* targeting a conserved region of the *pdhA* gene. But so far there has been no report on real-time LAMP based detection of the bacterium in poultry. In the present study, we developed a real-time LAMP assay based on the amplification of the *mgc2* gene sequence for the detection of *M. gallisepticum*, using an ESEQuant tube scanner with FAM dye. We designed the primers to target the *mgc2* gene of *M. gallisepticum*, which is a very apt gene to amplify the genome of *M. gallisepticum*, whereas in a previously reported conventional LAMP (19), primers were designed from a conserved gene region (*pdhA*) of the bacterium. In the current study, the primers were synthesised over a 200 nmol scale and subjected to additional purification through a reverse phase cartridge (RPC) (15). The RPC purification method provides 85%–95% purity of the oligonucleotides (Gene Link, USA) and thus makes the assay more accurate. To confirm the specificity of the newly designed primers, DNA templates of other non-target *Mycoplasma* spp. were also used, including the genetically closely related *Mycoplasma imitans*. The LAMP technique successfully distinguished between *M. gallisepticum* and other species of *Mycoplasma*, as the assay revealed its inability to produce amplified products from *Mycoplasma* spp. other than *M. gallisepticum*, thus confirming that the primers designed in this study are highly specific for *M. gallisepticum*.

Mycoplasmas are fastidious organisms and take a long period of incubation (7–21 days) to culture. So, LAMP-based detection following prior cultivation of the organism is not applicable for on-site field and point-of-care testing. Therefore, we amplified the target DNA directly from swab samples, whereas in a previously reported conventional LAMP assay (19), it was amplified using culture isolates. The designed primer set in this study successfully amplified the target sequence of *M. gallisepticum* from swab samples, thus making the assay less laborious and less tedious. In the present study, the use of an ESEQuant tube scanner proved fit for the purpose of rapid molecular diagnosis of infection at any location (point-of-care or field site) because this small and easy-to-use device is portable enough to carry into remote field areas. While the sophisticated thermal cycler requires a standard laboratory set up, the real-time LAMP using the tube scanner gave us the positive and negative results on the spot *via* the fluorescence signals in the form of amplification curves. We additionally confirmed the amplified products through gel electrophoresis. The use of lyophilised MasterMix and primer mix made the assay more suitable for field conditions, because these

ready mixes saved time and avoided errors. Moreover, the lyophilised MasterMix is thermostable for at least one year and so imposes no need for storage in the refrigerator. The percentage of the positive samples detected through real-time LAMP was 58%, and this percentage was higher than that of RSA (53%). RSA is also associated with false positive results, so the real-time LAMP served as a confirmatory test for the detection of *M. gallisepticum*. In this way, the newly developed real-time LAMP assay significantly improved the efficiency of *M. gallisepticum* detection in poultry.

In conclusion, the present LAMP assay is more sensitive, 100% specific, rapid, and culture-free technology for the molecular detection of *M. gallisepticum* in poultry. Moreover, the LAMP assay using an ESEQuant tube scanner has proved its potential for the real-time molecular detection of *M. gallisepticum*. This promising technique is applicable in *M. gallisepticum* control and eradication programmes. This is the first report on real-time LAMP based detection of *M. gallisepticum*. It recommends itself highly for precise and robust point-of-care testing for *M. gallisepticum* under field conditions. The study will be beneficial in reducing the economic losses caused by *M. gallisepticum* in the poultry industry. This novel and exciting molecular technique introduces simplicity lacked by previously reported molecular methods for the detection of *M. gallisepticum* in chickens.

Conflict of Interests Statement: The authors declare that there is no conflict of interests regarding the publication of this article.

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