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Development of two novel SYBR green-based quantitative PCR assays for detection and quantification of *Mycoplasma gallisepticum* and *Mycoplasma synoviae*

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Abstract: *Mycoplasma gallisepticum* (MG) and *Mycoplasma synoviae* (MS) are considered the most pathogenic and economically devastating avian mycoplasmas. A definite MG and MS diagnosis depends on three main approaches, including isolation and identification, demonstration of specific antibodies, and detection of genetic materials. A fast and precise diagnosis technique is an indispensable prerequisite to prevent and control avian pathogens more effectively. This study aimed to develop and validate convenient SYBR green-based quantitative PCR (qPCR) assays using genome specific primers to rapidly detect and quantify MG and MS. Two sets of primers were designed based on the highly conserved regions of *mgc2* and *vlhA* genes in MG and MS, respectively. The efficiencies of both reactions were calculated around 96% with a detection limit of as few as 10 copy numbers of DNA/µL in a sample. The standard curves showed linearity over a dynamic detection range of 6 log units from 10^1 to 10^6 DNA copy numbers/µL without any significant variations in inter- and intra-assays. The melting temperatures of the amplicons for MG and MS were determined to be 79.90 °C and 80.79 °C, respectively. The results showed that only MG and MS were detected as a single melt peak without cross reaction with other non-targeted pathogenic agents. The developed assays were further applied to the 60 swab specimens from commercial poultry with unknown MG and MS statuses and compared with the conventional PCR assays. The current assays detected 16 and 11 samples as MG and MS, respectively. However, five MG and three MS positive samples were tested positive in gel-based assays. The protocols developed here have the potential for use as a diagnostic research tool to detect and quantify MG and MS in clinical samples. Furthermore, the proposed qPCR techniques can provide an alternative approach for diagnosis and surveillance in epidemiological studies.

Key words: *Mycoplasma gallisepticum*, *Mycoplasma synoviae*, qPCR assay, SYBR green

1. Introduction

Mycoplasma gallisepticum (MG) and *Mycoplasma synoviae* (MS) are two common primary avian mycoplasma pathogens that exclusively infect poultry around the world. Chickens and turkeys infected by MG and MS manifest a wide range of respiratory symptoms, including rales, coughing, nasal discharge, conjunctivitis, and infraorbital sinusitis. Furthermore, these two mycoplasmas result in great economic losses due to carcass condemnation, reduced egg production, feed efficiency, and egg shell quality, and costs of control. The rate of MG and MS populations in respiratory tracts can be alleviated by antimicrobial therapy. However, antibiotics cannot be effective in eliminating these mycoplasmas from infected flocks. Thus, the method of choice to control and prevent MG and MS is achieved using mycoplasma-free replacement stock and stringent biosecurity measures. Besides, vaccination against MG and MS offers an alternative control strategy by initially providing protection against clinical signs, drops in egg production, and egg transmission [1,2].

A positive MG and MS diagnosis may be made using three approaches: (i) isolation and identification, (ii) serology, and (iii) molecular assays. Although the culture method is still considered the gold standard for direct MG and MS detection, it is often costly, laborious, and time-consuming, requiring two weeks or more for results turnaround. Serologic procedures, such as rapid plate agglutination test, ELISA, and hemagglutination inhibition, are usually used for flock monitoring. Because of low sensitivity and specificity of these tests, MG and MS positive reactors should be confirmed by isolation and identification and/or PCR [1,2]. Several PCR-based techniques have been developed for rapid, sensitive, and highly specific detection of MG and MS DNAs. Compared to conventional PCR, real-time PCR has many benefits, including rapidity, high sensitivity and specificity, fast turnaround time, and quantitative measurement and also does not need to follow postamplification steps [3].

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Several intercalating SYBR green- and probe-based quantitative PCR (qPCR) assays have been developed to detect MG and MS in the format of monoplex or duplex [4–8]. SYBR green-based qPCR assays are simpler and less costly than their counterparts, as they do not require quite expensive dye-labelled oligonucleotide probes. Nonspecific amplification and primer-dimers are considered the only drawbacks of the real-time PCR approach based on SYBR green dye. However, melt curve analysis confirms the specificity of post-PCR amplification. Furthermore, this assay is still faster than conventional PCR and is found to be economical for large-scale routine testing of clinical samples. All previous SYBR green-based qPCRs utilized previously published primers for conventional PCR and had limitations in differentiating and quantifying mycoplasmas [5,6]. It is necessary to develop rapid and efficient methods to detect MG and MS, and reliable diagnostic assays are essential prerequisites to control and prevent these avian pathogens effectively. Thus, this study established a real-time PCR system using SYBR green and melt curve analysis to detect and quantify avian mycoplasmas in clinical samples.

2. Materials and methods

2.1. Bacterial strains

MG strain S6 and MS strain MS-H were obtained from the depository of the Mycoplasma Reference Laboratory, the Razi Vaccine and Serum Research Institute, Karaj, Iran. The strains were propagated and cultured in Frey Mycoplasma Broth Base (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% heat inactivated horse serum. Other bacteria, such as *Acholeplasma laidlawii* (AL), avian pathogenic *Escherichia coli* (EC), *Avibacterium paragallinarum* (AP), *Pasteurella multocida* (PM), *Ornithobacterium rhinotracheale* (ORT), and *Salmonella* Enteritidis (SE) were isolated in our laboratory.

2.2. DNA extraction

Genomic DNA was extracted using the method described by Markham et al. (1993) with some modifications. Briefly, after collecting cells from broth culture or tracheal swabs by centrifugation at 13000 RPM for 30 min, they were washed with PBS buffer once. The pellet cells were resuspended in 50 mM Tris-HCl-50 mM EDTA-100 mM NaCl and then lysed by 10% SDS. Afterward, 100 µg/mL proteinase K was added to the lysate, and the mixture was incubated at 56 °C for 4 h. Moreover, 100 µg/mL RNase A was added to the mixture and incubated at 37 °C for 30 min. The lysate was extracted according to the following order with tris-saturated phenol, phenol-chloroform (1:1), and chloroform-isoamyl alcohol (24:1). Sodium acetate (3 M, pH = 5, 0.1 volume) was added to the solution, and the DNA was precipitated with absolute ethanol. After washing the pellets with 70% ethanol and centrifugation, the DNAs were eluted in 50 µL nuclease-free water. The purity of the extracted DNAs was assessed by calculating the ratio of the readings at 260 and 280 nm using NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA).

2.3. Primer design

Two sets of qPCR primer pairs specific to the conserved regions of *mgc2* and *vlhA* genes in MG and MS were designed using the software Primer 3 version 4 [9]. The description of primer pairs used in the study is included in Table 1. The specificity of the primers was verified in silico using the NCBI Primer-BLAST module to ensure that the primers had no relevant cross reactivity with other genes of either MG and MS or any organisms. The primers specific to *mgc2* and *vlhA* genes amplified 98 and 133-211 bp products, respectively. The primers used for plasmid construction were previously described and amplified the complete *mgc2* and partial *vlhA* genes [10,11]. Additionally, two previously established primer pairs were used in conventional PCR to detect MG and MS in clinical samples [12,13]. All the primers were synthesized by Macrogen (Seoul, South Korea).

2.4. Preparation of standard plasmid DNA templates

After amplifying complete *mgc2* and partial *vlhA* genes using conventional PCR, the amplicons were inserted into the pJET1.2/blunt cloning vector (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's guideline. Afterward, the ligated vectors were transformed into chemically *Escherichia coli* competent cells (TOP10) by heat shock at 42 °C for 1 min. Plasmids (pMG and pMS) were extracted using the High Pure Plasmid Isolation Kit (Roche, Mannheim, Germany) according to the kit's instruction. The recombinant pMG and pMS plasmids were commercially Sanger sequenced (Macrogen, Seoul, South Korea) to confirm successful ligation of the desired sequence. The concentration of the extracted plasmid DNAs was determined using NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). Then, the recombinant plasmids were subjected to calculate copy numbers, as described previously [14]. Ten-fold serial dilutions of the standard plasmids pMG and pMS with copy numbers ranging from 10^1 copies/µL to 10^6 copies/µL were used to construct a standard curve. All the samples, either unknown samples or dilutions, were assayed in triplicates.

2.5. Optimization of SYBR green real-time PCR assays

Initially, eight different concentrations of the primers (0.1–0.8 µM) were used to optimize the real-time PCR assays. The assays were performed on an ABI StepOne real-time PCR system (Applied Biosystems, Foster, CA, USA) in a reaction volume of 20 µL, containing the RealQ Plus 2x Master Mix Green with High Rox (Ampliqon, Odense, Denmark), 0.5 µM of each primer, and 2 µL of

Table 1. Primer sequences used in this study.

Primer names	Target gene	Primer sequences (5'-3')	Size of amplified product (bp)	References
MB-qMG-F	*mgc2*	TCCAAGAAAGATTACCTCCGAACCC	98	This study
MB-qMG-R		TCCCAATCCCTAAACCCAAGAGTAAA		
MB-qMS-F	*vlhA*	GCCATTGCTCCTGCTGTTAT	133-211	This study
MB-qMS-R		GGTCAACTGTACCACCTCCT		
MB-*mgc2*-F	*mgc2*	TGATTGCTATGGTGGGCTTG	1384	[10]
MB-*mgc2*-R		ACCGATTAAGGCAAGAGGAGTT		
MB-Link	*vlhA*	TACTATTAGCAGCTAGTGC	380	[11]
MB-MSCons		AGTAACCGATCCGCTTAAT		
MB-MG-10F	16S rRNA	AACACCAGAGGCGAAGGCGAGG	531	[12]
MB-MG-11R		ACGGATTTGCAACTGTTTGTATTGG		
MB-MSLF	16S rRNA	GAGAAGCAAAATAGTGATATC	214	[13]
MB-MSLR		TCGTCTCCGAAGTTAACAA		

the extracted DNA. The thermal cycling profile was a single cycle of TEMPase hot start enzyme activation at 95 °C for 15 min followed by 40 PCR cycles of 15 s at 95 °C for denaturation and 45 s at 60 °C for both annealing and extension. Following amplification, the melting curves were generated by monitoring the fluorescence signal from 60 °C to 95 °C according to standard machine settings (raising 0.3 °C each step). Each sample was tested and repeated independently three times.

2.6. Sensitivity, specificity, and precision of the assays

Ten-fold serial dilutions of the standard plasmids pMG and pMS with copy numbers ranging from 10^1 copies/µL to 10^6 copies/µL were used to determine the sensitivity of the assays using conventional PCR. Afterward, the amplicons were analyzed by electrophoresis in 2% agarose gel to confirm the sensitivity of the proposed assays. The serial dilutions were subjected to qPCR to determine detection limit. Specificity was assessed using DNAs from other avian bacterial pathogens, namely AL, EC, AP, PM, ORT, and SE. The standard DNAs with three different 10-fold concentrations (10^4–10^6 copies/µL) were tested repeatedly to evaluate the precision of the assays. The intra-assay variations were measured using three separate dilution samples and performed on the same day in triplicates. The assays were performed independently at one-week intervals to check the inter-assay precisions. Then, the coefficient of variation (CV) was individually calculated between the quantification cycle (Cq) of runs for each assay.

2.7. SYBR-green qPCR and conventional PCR assays of field samples

A total of 60 samples suffering from multi-causal respiratory disease were collected from commercial poultry. The samples were taken from trachea and/or choanal cleft using cotton swabs. After sampling, each swab was placed into 1 mL transport medium (brain heart infusion) and immediately transported on ice to the laboratory. The tubes were vortexed and kept at –70 °C until needed for DNA extraction according to the method described above.

3. Results

3.1. Preparation of plasmid DNA standards

The complete *mgc2* (1384 bp) and partial *vlhA* (380 bp) genes of MG and MS were amplified using the primer pairs and cloned into the pJET1.2/blunt vector. The recombinant plasmids were then sequenced to verify correct inserts. The plasmid concentration of pMG and pMS was measured as 542 ng/µL and 574 ng/µL, containing 1.135×10^{11} and 1.582×10^{11} copies/µL, respectively.

3.2. Generation of standard curves

Series of dilutions ranging from 10^1 copies/µL to 10^6 copies/µL were used to generate standard curves for MG and MS (Figure 1 and Figure 2). Linear regression was plotted between Cq values (y axis) and plasmid DNA copy numbers over six-log serial dilutions (x axis). The coefficient of determination (R^2) of the MG and MS qPCR assays was 0.999 and 0.998, with the slope values of –3.416 and –3.431, respectively. These values reflect the optimum PCR efficiency and linearity of the standard curve.

3.3. Assessment of sensitivity, specificity, and precision

The sensitivity of the assays was ascertained using the 10-fold serial dilution of plasmid DNA ranging from 10^1 copies/µL to 10^6 copies/µL. The amplicons were visualized by 2% agarose gel electrophoresis (Figure 3 and Figure 4).

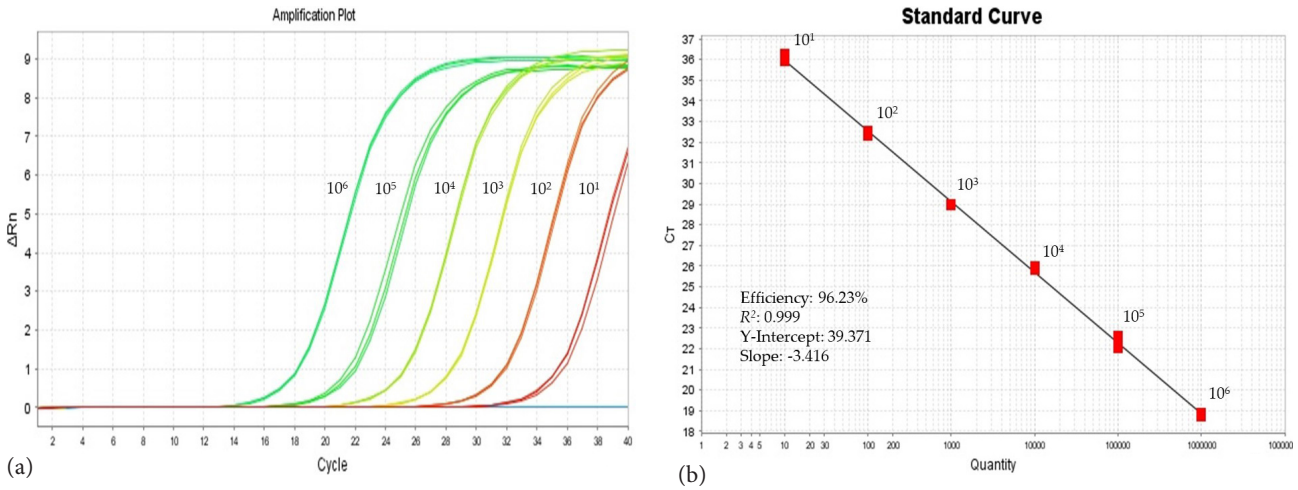

Figure 1. SYBR Green-based qPCR amplification plot and standard curve for MG. (a) Ten-fold dilutions of standard plasmid DNA ranging within 10^1–10^6copies/µL were amplified by SYBR Green based qPCR on the ABI StepOne Real-Time PCR System. (b) The standard curve was generated by plotting the mean Cq values versus the 10-fold serial dilutions of standard plasmid DNA over a range of concentrations from 10^1 to 10^6copies/µL in triplicate.

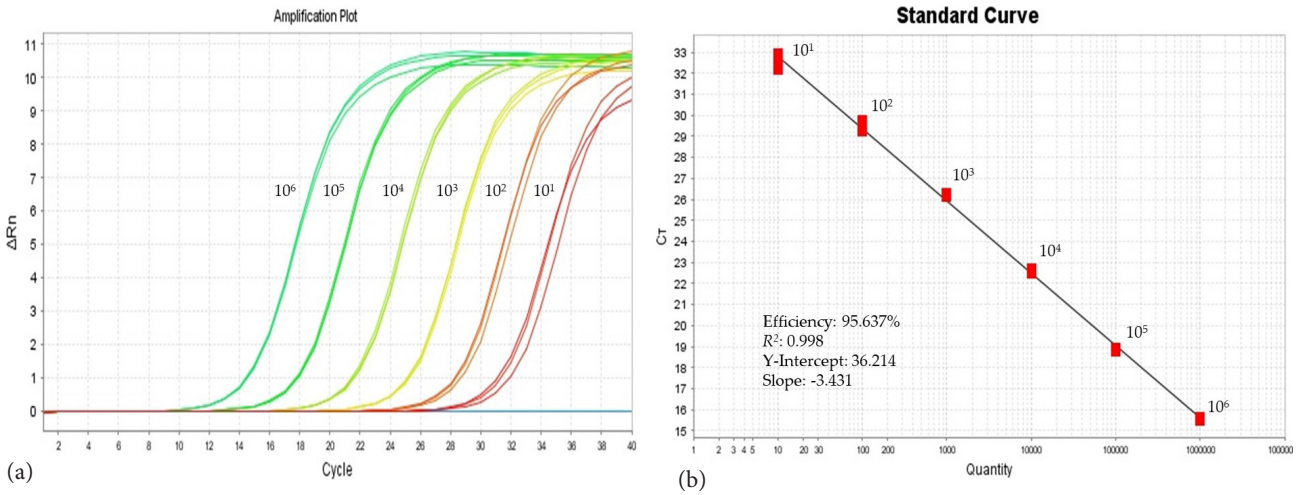

Figure 2. SYBR Green-based qPCR amplification plot and standard curve for MS. (a) Ten-fold dilutions of standard plasmid DNA ranging from 10^1 to 10^6copies/µL were amplified by SYBR Green based qPCR on the ABI StepOne Real-Time PCR System. (b) The standard curve was generated by plotting the mean Cq values versus the 10-fold serial dilutions of standard plasmid DNA over a range of concentrations from 10^1 to 10^6copies/µL in triplicate.

Both assays were able to detect as few as 10 copy numbers of DNA/µL in the sample. The Cq value higher than 35 was considered as cut-off value for negative samples. Six other poultry pathogens were chosen to check the specificity of the assays. No positive signals of these pathogens and negative controls were observed in the assays. Moreover, the florescence melting curve showed a single specific amplification by generating a melt peak at 79.90 °C and 80.79 °C for MG and MS, respectively, suggesting no evidence of primer dimers (Figure 5 and Figure 6). The mean and CV values were calculated according to the Cq values obtained from inter- and intra-assays (Table 2). The CV values related to inter-assay variability (repeatability) for MG and MS were in the range between 0.23 and 1.65% and 0.4-0.5%, respectively. Moreover, the CV values of intra-assay variability (reproducibility) ranged between 0.82 and 1.23% for MG and 0.31 and 0.66% for MS.

3.4. Evaluation of the assays on clinical samples

Sixty trachea and/or choanal cleft swabs were examined using the currently developed real-time PCR and previously described conventional PCR assays to evaluate the practical use of the SYBR green-based real time PCR. In total, 16 and 11 samples were detected as positive for MG and MS by qPCR assays, respectively, compared with

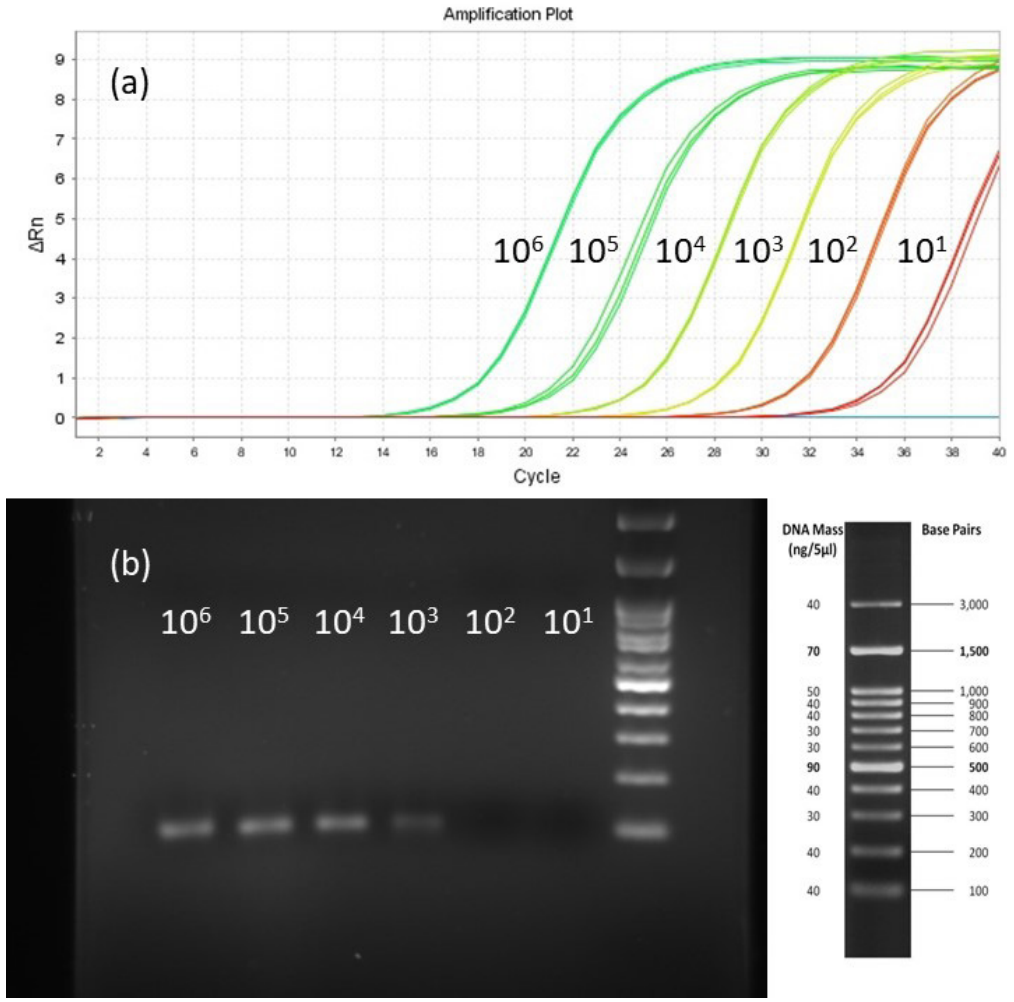

Figure 3. Sensitivity of the SYBR Green qPCR for MG. (a) Amplification of the 10-fold dilutions of standard plasmid ranging from 10^1 to 10^6copies/µL. (b) Conventional end-point PCR products were tested by 2% gel electrophoresis.

five MG and three MS positive samples by gel-based PCR assays.

4. Discussion

Culture-based and several serological assays are applied for diagnosing MG and MS infection. However, these approaches are tedious and not always sensitive and specific. Besides, DNA-based detection methods are increasingly used as dependable diagnostic tools for MG and MS detection. Compared with conventional PCR, qPCR provides accuracy in determining the exact starting template copy number and sensitivity to have a broad dynamic range. Although, several SYBR green- and probe-based qPCR methods have been utilized for MG and MS detection, they have some limitations in quantification and sensitivity and may detect other mycoplasmas. Moreover, the high expansion rate of the poultry industry requires a rapid and inexpensive high-throughput screening approach. Thus, qPCR can be a helpful tool for avian mycoplasma diagnosis [7]. The SYBR green qPCR technique described here was developed based on the *mgc2* and *vlhA* genes of MG and MS, respectively.

Several SYBR green-based protocols were applied to detect part of the 16S rRNA and lipoprotein genes in MG and MS. All these assays provided distinct advantages of yielding rapid results and preventing post amplification analysis over conventional PCR [5,6,15,16]. However, these primers were not specific enough to distinguish MG and detect different mycoplasmas [5,6]. In silico examination of both primer sets showed no cross reactivity with gene sequences of other organisms available in the database. Moreover, no amplification signals in the reactions with the above-mentioned bacteria were observed. The single melting

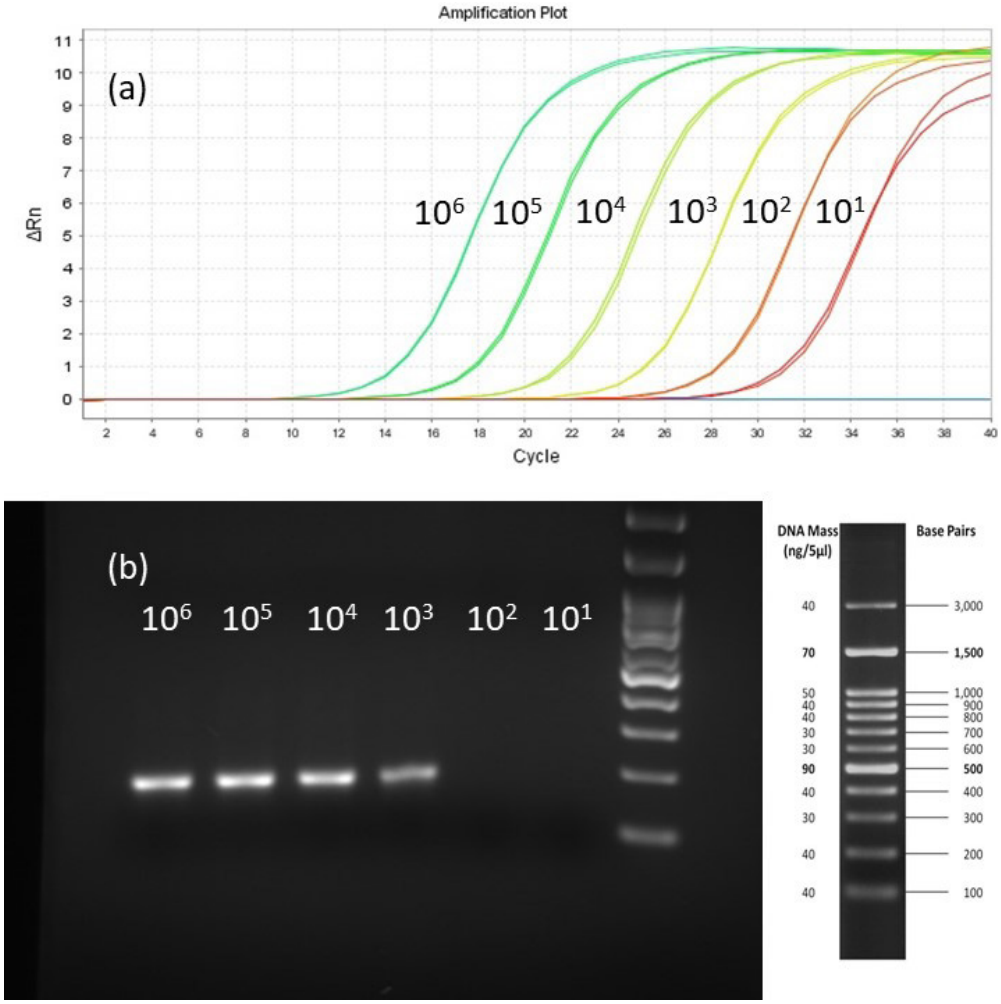

Figure 4. Sensitivity of the SYBR Green qPCR for MS. (a) Amplification of the 10-fold dilutions of standard plasmid ranging from 10^1 to 10^6copies/µL. (b) Conventional end-point PCR products were tested by 2% gel electrophoresis.

curve peak during dissociation analysis also indicated amplification specificity in both MG and MS SYBR green-based qPCR assays.

Sensitivity is a key determinant of a screening test to detect pathogens as a diagnostic tool [17]. The newly developed assays were capable of detecting only 10 copy numbers of DNA/µL of MG and MS in the samples, which are more sensitive than previously developed probe-based qPCR protocols [4,8,18]. The MS detection sensitivity using the presented qPCR method was found to be 10 copies/µL, showing that the method is as sensitive as the previously reported TaqMan qPCR assay [7]. The suggested qPCR approach for MG detection had a lower sensitivity (10 copies/µ) compared to the probe-based qPCR (one copy per reaction) proposed by Raviv and Kleven [7]. Although, the sensitivity of the SYBR green qPCR assay was 10-fold lower than that of previously developed probe-based qPCR, low-cost fluorochrome and simpler approach are the main advantages of SYBR green over other qPCR detection formats.

Compared to the conventional end-point PCR approach, the two newly developed assays proved to be highly sensitive and specific for MG and MS detection and quantification without the need for post-PCR procedures and expensive fluorescent probes. Due to their rapidity and reproducibility, these methods can be easily implemented for high throughput screening of clinical poultry samples and control of avian mycoplasmas. An excellent coefficient of variation, PCR efficiencies, and relatively small intra- and inter-assay variability confirmed the validity of the methods. The developed assays could be used as alternative economic approaches to improve MG and MS surveillance worldwide and allow developing better preventive measures.

Table 2. Precision of the current SYBR green qPCR assays of MG and MS.

Assay	MG						MS					
	Inter-assay variance			Intra-assay variance			Inter-assay variance			Intra-assay variance		
	Cqs of Standard DNA			Cqs of Standard DNA			Cqs of Standard DNA			Cqs of Standard DNA		
	10^4	10^5	10^6	10^4	10^5	10^6	10^4	10^5	10^6	10^4	10^5	10^6
1	24.20	21.82	18.10	24.43	21.91	17.27	23.01	19.25	15.36	22.63	18.81	15.57
2	24.29	21.83	17.03	24.64	21.99	17.39	22.68	18.81	15.57	22.54	18.94	15.71
3	24.30	22.09	17.34	24.47	22.12	17.44	22.63	18.89	15.59	22.68	18.93	15.51
Mean ± SD	24.26 ± 0.05	21.91 ± 0.15	17.32 ± 0.28	24.50 ± 0.12	22.00 ± 0.10	17.36 ± 0.08	22.77 ± 0.20	18.98 ± 0.12	15.50 ± 0.45	22.61 ± 0.07	18.89 ± 0.07	15.59 ± 0.10
CV	0.23	0.7	1.65	0.50	0.40	0.50	0.91	1.23	0.82	0.31	0.38	0.66

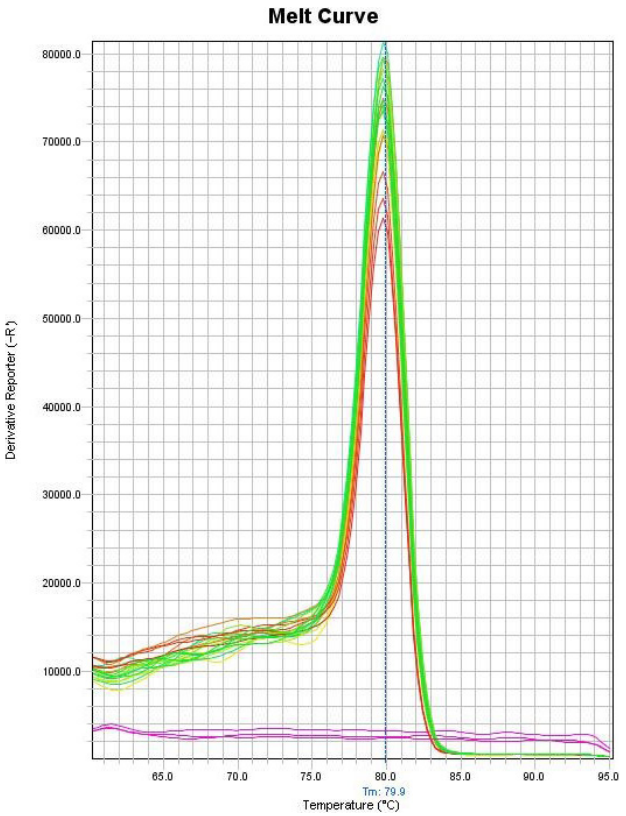

Figure 5. Specificity of the SYBR Green qPCR for MG with melting curve analysis constructed with 0.3 °C increment.

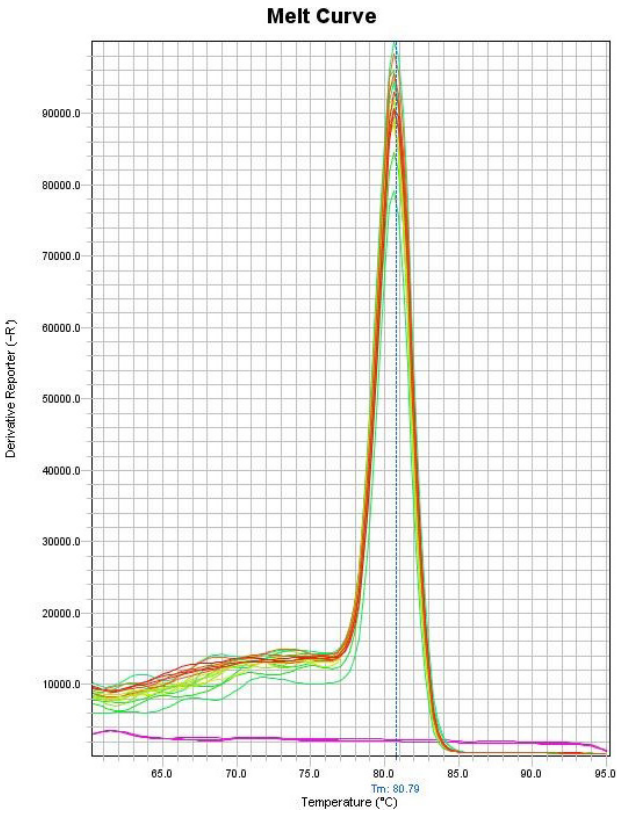

Figure 6. Specificity of the SYBR Green qPCR for MS with melting curve analysis constructed with 0.3 °C increment.

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