Poultry Science®

Development and application of a cycleave dual-probe fluorescence quantitative PCR method for simultaneous detection of Mycoplasma gallisepticum ts-11 vaccine strain and non-ts-11 strains

Xue Wang ,*,† Nana Sun,* Meng Wang,* Hui Wang,* Yuhan Liu,* Hao Shi,* Hao Zhu,* Peidong Li,* Fuyou Zhang,* Tianyao Yang,‡ Zhaoyang Li,‡ and Chunguo Liu *,1

*Group Biological Products R & D Center, Shandong Sinder Technology Co., Ltd. Qingdao, Shandong 266100, China; †College of Veterinary Medicine, Agricultural University of Hebei, Baoding, Hebei 071000, China; and ‡Shandong Sinder Technology Co., Ltd. Qingdao, Shandong 266100, China

ABSTRACT An attenuated vaccine against the *Mycoplasma gallisepticum* ts-11 strain has become an effective prevention and control method against MG infection. However, the ts-11 strain is usually difficult to distinguish from the non-ts-11 strain (including field isolates and other vaccine strains (F and 6/85)). Therefore, it is critical to establish a rapid and effective method to distinguish ts-11 strains from non-ts-11 strains. The gene sequences of the ts-11 strain (CP044225.1) and the non-ts-11 strain (including the wild-type (CP006916.3), 6/85 (CP044224.1), and F strains (NC_017503.1) were used to construct a conserved region containing a single point mutation in the potC gene in the ts-11 strain, after which a primer −probe combination method was designed. The primer −probe method was able to accurately and efficiently identify the ts-11 and non-ts-11 strains with minimum detection limits of 2.43 copies/μL and 1.65 copies/μL, respectively. Moreover, it could simultaneously distinguish the ts-11 strain from a non-ts-11 strain, and amplifications of avian influenza virus, infectious bronchitis virus, Newcastle disease virus, fowl adenovirus, infectious laryngotracheitis virus, infectious bursal disease virus, chicken anemia virus, Marek's disease virus, *Mycoplasma synoviae*, and *Ornithobacter rhinotracheale* were negative. The detection of clinical samples revealed that the established dual-probe fluorescence quantitative PCR method could be used to screen for mixed and single infections of the ts-11 strain and non-ts-11 strains effectively, with lower variation coefficients for intra- and interbatch repetition. The established cycleave dual-probe fluorescence quantitative PCR method showed good specificity, sensitivity, and repeatability and provides powerful technical support for the rapid and efficient differential diagnosis of the MG ts-11 strain from non-ts-11 strains.

Key words: *Mycoplasma gallisepticum* (MG), cycleave fluorescence quantitative PCR, differential diagnosis, ts-11 strain

2024 Poultry Science 103:103907
https://doi.org/10.1016/j.psj.2024.103907

INTRODUCTION

Approximately 23 kinds of mycoplasmas have been reported to exist in birds. Among them, the most important and common mycoplasmas in chicken include *Mycoplasma gallisepticum* **(MG)** and *Mycoplasma synoviae* **(MS)** (Dhondt, et al., 1998; Ross, et al., 2014). MG is the main pathogen of chronic respiratory disease **(CRD)** in chickens and other birds (Wang, et al., 2022). Due to the significant losses in performance and production caused by MG infection in the poultry industry, MG is considered the most important pathogenic mycoplasma affecting the economic value of poultry (Mugunthan, et al., 2023). MG infection has been reported globally and listed in the World Organization for Animal Health **(WOAH)**.

MG can cause infectious sinusitis in turkeys and was the causative agent of an epidemic of conjunctivitis in finches in North America. MG has also been reported to cause disease in some birds, including American goldfinches, purple finches, and sparrows, as well as some ornamental birds, including pheasants, partridges, and chukar partridges (Mugunthan, et al., 2023). In chickens, MG not only causes CRD but also promote the synergistic infection with *E. coli* and low-pathogenicity avian influenza virus, etc. making the respiratory disease

Received February 29, 2024.
Accepted May 23, 2024.

[1]Corresponding author: liuchunguo@sinder.cn

more severe and chronic (Naylor, et al., 1992). Due to the high pathogenicity of MG in birds and the substantial economic losses that can be incurred in the poultry industry, it is crucial to improve the diagnosis and prevention of MG.

The transmission of MG can occur horizontally and vertically. Horizontal transmission occurs mainly through droplets, saliva, aerosols carrying MG, and contaminated feed and drinking water, while vertical transmission occasionally occurs through embryo. It is difficult to completely eliminate MG infection in chickens, which leads to the long-term existence and widespread transmission of the infection in chickens, making the prevention and control of MG infection difficult (Wu et al., 2019).

Vaccine immunization is the critical strategy for preventing MG infection. At present, the 6/85, ts-11, and F strains are the major live vaccine strains used worldwide. The F strain is highly virulent and pathogenic to broilers and turkeys. Strains 6/85 and ts-11 are very safe and have a low potential risk of transmission to nearby unvaccinated chickens (Noormohammadi and Whithear, 2019; Vance, et al., 2008). The ts-11 vaccine was developed by the Bioproperties Pty., Ltd., Australia (Whithear, et al., 1990). The optimum growth temperature for the ts-11 strain is 33°C, and related studies have shown that vaccine proliferation decreases at 39.5°C, indicating the strain is sensitive to the temperature. The ts-11 strain is highly attenuated compared to its parent strain (80083), which is possibly avirulent to chickens. Moreover, the lateral transmission of the ts-11 strain among birds is also low (Alqhtani, et al., 2022; Whithear, et al., 1990). The previous studies showed that the ts-11 vaccine has a protective effect against respiratory diseases and egg drop caused by MG virulent strains (Armour and Ferguson-Noel, 2015). In contrast, the F strain is capable of transmitting and causing respiratory symptoms in broilers (Vance, et al., 2008; Rodriguez and Kleven, 1980). With the widespread use of the ts-11 vaccine, distinguishing vaccine strains from nonvaccine strains has become an urgent need in the breeding industry. Conventional isolation and culture of pathogens cannot be used to distinguish between vaccines and nonvaccine strains effectively. Currently, there is a lack of direct, fast, sensitive and quantitative methods for evaluating the colonization of ts-11 strains after vaccine immunization in China (Sulyok, et al., 2019). Therefore, establishing an effective detection method to distinguish the MG ts-11 strain from non-ts-11 strains is key for the control of MG infection.

Cycleave fluorescent PCR has been applied in studies of animal husbandry, veterinary, and medical diseases such as brucellosis and lung cancer (Fujita, et al., 2015; Nan, et al., 2016). The cycleave fluorescent PCR method is different from traditional fluorescent PCR, which combined with cycling probe technology improving the specificity. The Cycleave PCR assay can detect a single nucleotide polymorphism in real time. This assay utilizes a chimeric RNA-DNA probe and RNase H to detect the target nucleotide. The chimeric probe contains an RNA base which can be cut by RNase H when the probe forms a hybrid with a complementary DNA sequence. This cleavage results in the emission of strong fluorescence. If the RNA portion of the probe or one RNA base does not match the template, RNase H cannot cleave the probe's RNA portion. In the present study, a cycleave dual-probe fluorescence quantitative PCR (cycleave qPCR) method was developed to differentiate the MG ts-11 strain and non-ts-11 strains. Using a hybrid cycling probe composed of RNA and DNA combined with RNase H, primers for the mutation site of the ts-11 strain were designed to identify the ts-11 strain and non-ts-11 strains more efficiently and accurately. The primer−probe combination combined with the cycleave PCR detection method can identify the target strain within 1 hour, which allows rapid detection of MG strains and the rapid differential diagnosis of the ts-11 vaccine strain and non-ts-11 strains.

MATERIAL AND METHODS

Organisms

The common pathogens that infect chickens, infectious bronchitis virus (**IBV**), and Newcastle disease virus (**NDV**), avian influenza virus **(AIV)**, fowl adenovirus **(FAdV)**, infectious laryngotracheitis virus **(ILTV)**, infectious bursal disease virus **(IBDV)**, chicken anemia virus **(CAV)**, Marek's disease virus **(MDV)**, MS and *Ornithobacter rhinotracheale* **(ORT)**, used in the present study were isolated and preserved by the Group Testing Center of Shandong Sinder Technology Co., Ltd. All the viruses and MS and ORT were identified using targeted-specific PCR methods for each organism.

Primer and Probe Design

Previous research showed that the potC gene could be as the target gene for differentiating the the ts-11 vaccine strain from the field strains (Sulyok, et al., 2019). The potC gene sequences of the ts-11 strain (CP044225.1) and representative non-ts-11 strains (wild-type (CP006916.3), 6/85 (CP044224.1), and F (NC017503.1) strains) were downloaded from GenBank. Primers were designed using SnapGene software (version 3.1.4), and the conserved region containing a single point mutation in the potC gene of the ts-11 strain was targeted. GenScript real-time PCR (TaqMan) online primer design software was used to design the TaqMan probes (https://www.genscript.com/tools/real-time-pcr-taqman-primer-design-tool). The primers and probes used were synthesized by TaKaRa Bio, Inc. (TaKaRa, Dalian, China), and the sequences are shown in Table 1.

Table 1. Sequences of the primers and probes.

Primer and probe	Sequences (5′→3′)	Product length (bp)
Forward primer	5′-GTGCGGGTGTTAAATAAGAT-3′	230bp
Reverse primer	5′-TTGCGGTGTGAGTGTC-3′	
ts-11 probe	HEX-CArGAAGATTTAG-BHQ1	
non-ts-11 probe	FAM-TTrGTGAAGCTA-BHQ1	

Nucleic Acid Extraction and Preparation of Recombinant Plasmids

The nucleic acids from AIV, IBV, NDV, FAdV, ILTV, IBDV, CAV, MDV, MS, ORT and all swab samples were extracted with a VNP-32P Automatic Nucleic Acid Extraction Instrument and a DNA/RNA Extraction Kit (Vazyme, Nanjing, China) according to the manufacturer's instructions and operation manual. The recombinant plasmids pts-11 and pnon-ts-11 were used to clone the potC gene of the ts-11 strain or non-ts-11 strains, respectively, by TaKaRa Bio, Inc. (TaKaRa, Dalian, China).

Cycleave qPCR Reaction Procedure

The cycleave qPCR system was 25 μL and consisted of the following components: 12.5 μL of Cycleave PCR Mix (2×Conc.) (TaKaRa, Dalian, China), 0.5 μL each of forward primer (10 μM) and reverse primer (10 μM), 1 μL each of the ts-11 probe (5 μM) and non-ts-11 probe (5 μM), 2 μL of template, and 7.5 μL of sterile water (Supplementary Table 1). The optimal reaction conditions were as follows: predenaturation at 95°C for 30 s; 40 cycles of denaturation at 95°C for 10 s, annealing at 60°C for 30 s, and extension at 72°C for 20 s. Fluorescence signal detection was performed during extension, and the results were determined according to the amplification curve and Ct value. qPCR was performed using a Roche LightCycler 480 (Roche Diagnostics, Mannheim, Germany). The fluorescence signal at 200 RFU was set as the threshold for the cycleave qPCR assay, and a cycle threshold (Ct) $\leq$ 36 cycles was defined as positive for the samples.

Establishment of the Standard Curve

The concentrations of the recombinant plasmids of pts-11 and pnon-ts-11 were determined by a microspectrophotometer (Allsheng, Hangzhou, China), and the respective copy numbers were calculated. Then, the plasmids were subjected to 10-fold serial dilutions from 10^8 to 10^3 in sterile water, which served as the template for cycleave qPCR. Each dilution was repeated 3 times to establish a standard curve. The reaction system and procedure were performed as mentioned above, and the standard curve parameters, including efficiency, slope and R^2, were calculated with the software.

Specificity, Sensitivity, and Repeatability Analyses

For the specificity test, the extracted genomes of AIV, IBV, NDV, FAdV, ILTV, IBDV, CAV, MDV, MS, and ORT were used as templates for cycleave qPCR amplification. In addition, 10^4-fold dilutions of pts-11 and pnon-ts-11 served as positive controls, and sterile water served as a negative control.

To detect the sensitivity of cycleave qPCR, the plasmids were continuously diluted with sterile water (10^2 to 10^{11} times), which served as the template for cycleave qPCR and conventional PCR , and sterile water was used as the negative control template.

To determine the repeatability and stability of cycleave qPCR, the pts-11 and pnon-ts-11 plasmids with 10-fold serial dilutions ranging from 10^4 to 10^6 were selected as the templates for cycleave qPCR; three replicates were used for each sample in the intra- and interbatch repeated tests, and negative controls were used for each test. The interval period was 1 week for the interbatch repeated tests. The coefficient of variation **(CV)** was calculated according to the formula CV = (SD [Ct value]/overall average [Ct value]) ×100.

Detection of Clinical Samples

A total of 300 chicken choanal cleft swabs were collected from 10 large-scale poultry farms in the Shandong, Heilongjiang, and Guangdong provinces of China in 2022 after 35 d of immunization with the MG ts-11 vaccine by eye drop. The sample collection from chickens were approved by the Experimental Animal Management and Ethics Committee of Shandong Sinder Technology Co., Ltd (QXRZ: 2021-007). The established cycleave qPCR method was used to distinguish the ts-11 strain from the non-ts-11 strain, and the results were compared with those of a commercial universal real-time fluorescence PCR kit (Yingsaite, Qingdao, China) (MG universal testing kit). In addition, clinical samples were collected from two large farms with similar feeding and management conditions and different MG vaccine immunization procedures to evaluate the immune efficacy.

RESULTS

Establishment of the Standard Curve

Standard curves were created for the ts-11 vaccine strain and non-ts-11 strain for the analytical characterization of the cycleave qPCR. The standard curves for

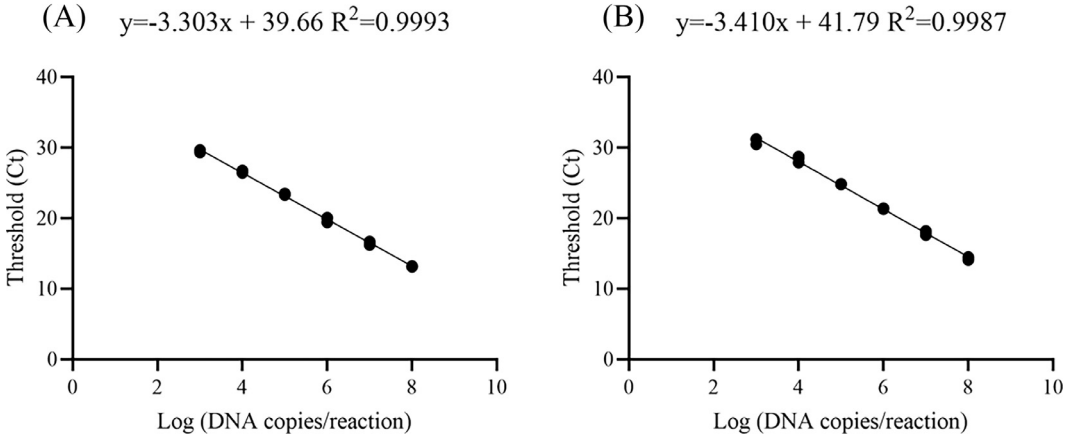

Figure 1. Standard curve for the cycleave dual-probe fluorescence quantitative PCR. The standard curves of developed cycleave qPCR were generated using ten-fold serial dilutions of plasmids of ts-11 vaccine strain (A) from 2.43×10^3 to 1.0×10^8 copies/μL and non-ts-11 strain (B) from 1.65×10^3 to 1.65×10^8 copies/μL.

the ts-11 vaccine strain and non-ts-11 strain are shown in Figure 1. The slopes were -3.303 and -3.410, the R^2 values were 0.9993 and 0.9987, and the efficiencies were 100.8% and 98.4%, respectively. As shown in Table 2, the characteristics of the assay, including R^2 values, efficiencies, slopes, and Y intercepts have been summarized.

Specificity, Sensitivity, and Repeatability of the qPCR

Common chicken-originating pathogens, including AIV, IBV, NDV, FAdV, ILTV, IBDV, CAV, MDV, MS, and ORT, were selected for testing the specificity of the developed cycleave qPCR method. The results showed that none of these pathogens were positive according to the cycleave qPCR method, which was the same as the negative control (Figure 2).

The sensitivity of the cycleave qPCR assay was assessed based on the number of nucleotide copies. The limit of detection was determined by serial 10-fold dilution of the plasmids. The sensitivities of the cycleave qPCR results were 2.43 copies/μL for the ts-11 vaccine strain (Figure 3A) and 1.65 copies/μL for the non-ts-11 strain (Figure 3B). The sensitivities of the conventional PCR method were 2.43×10^3 copies/μL for the ts-11 vaccine strain (Figure 4A) and 1.65×10^3 copies/μL for the non-ts-11 strain (Figure 4B). Therefore, the sensitivity of the established cycleave qPCR method was 1000 times greater than that of the conventional PCR method.

To evaluate the repeatability and stability of the cycleave qPCR, intra- and interbatch assays were conducted, with all the cycleave qPCRs performed in triplicate, and the mean values of Ct and CV were calculated.

Table 2. Results of the developed cycleave qPCR standard curves.

Target	Efficiency	R^2 value	Slope	Y intercept
ts-11	100.8%	0.9993	-3.303	39.66
non-ts-11	98.4%	0.9987	-3.410	41.79

The results showed that the CVs of intrabatch repeatability were 0.32 to 0.82% for the ts-11 strain and 0.91 to 1.13% for the non-ts-11 strain. Additionally, the CVs of interbatch repeatability were 0.40 to 0.51% for the ts-11 strain and 0.63 to 0.95% for the non-ts-11 strain (Table 3).

Detection in Clinical Samples

A total of 300 chicken choanal cleft swabs were collected from the chickens after 35 d of immunization with the ts-11 strain. The positive rate of the ts-11 strain was 70% (210/300), and the positive rate of the non-ts-11 strain was 20% (60/300). The other 30 tests were negative, and the concordance rate with commercial real-time fluorescence PCR (Yingsaite, Qingdao, China) results was 100%.

Company A was immunized twice with the ts-11 vaccine at 25 and 45 d, while company B was only immunized once with the ts-11 vaccine at 21 d. The choanal cleft swabs were collected at different days postimmunization, and the immune efficacy were evaluated via the established cycleave qPCR method. The results showed that the effect of twice vaccinations (Figure 5A) was better than that of once vaccination (Figure 5B). This result provides scientific data support for the establishment of immunization procedures on poultry farms.

DISCUSSION

The vaccine for MG ts-11 was first listed in Australia in 1988, was promoted more than 20 y ago and has been used in more than 60 countries worldwide (Ley, et al., 1997; Muneta, et al., 2008; Noormohammadi and Whithear, 2019). This vaccine can persistently colonize the upper respiratory tract; prevent invasion by nonvaccine strains; protect joints and oviducts; and improve egg quality (Vance, et al., 2009; Jacob, et al., 2015). However, common methods cannot accurately distinguish the ts-11 vaccine strain from non-ts-11 strains, making it difficult to understand the epidemiology of

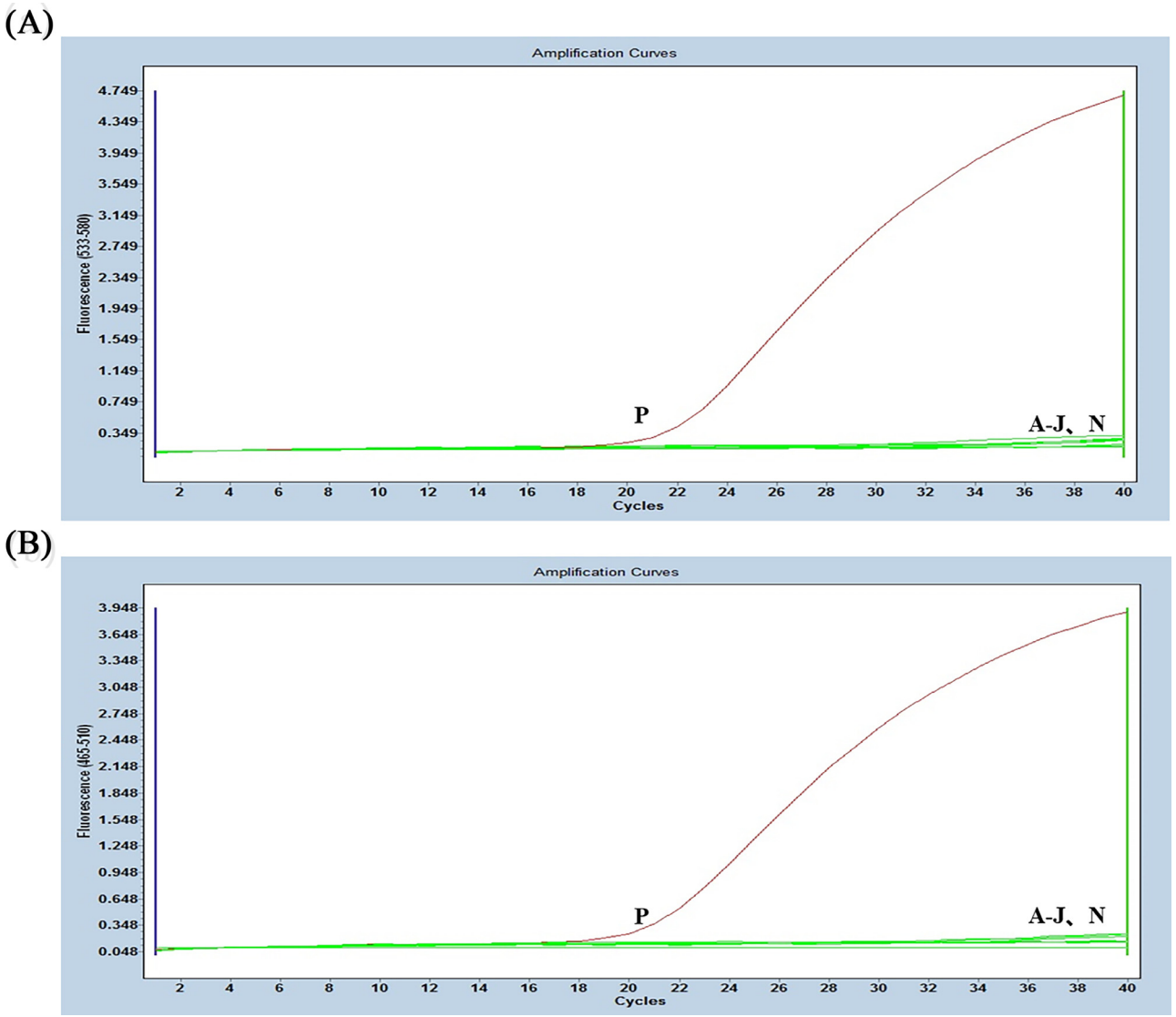

Figure 2. Specificity for the cycleave dual-probe fluorescence quantitative PCR. The ts-11 vaccine strain (A) and non-ts-11 strain (B). P: positive control; A: AIV; B: IBV; C: NDV; D: FAdV; E: ILTV; F: IBDV; G: CAV; H: MDV; I: MS; J: ORT; N: negative control.

MG infection in chickens, especially during outbreaks of MG infection in live MG ts-11 vaccine-immunized chicken flocks. The lack of accurate and rapid detection methods has dramatically limited the ability to distinguish between vaccine strains and nonvaccine strains, evaluate clinical immune efficacy, monitor chicken health, and prevent and control MG infection.

At present, the diagnosis of MG relies mainly on isolation, antibody detection, and molecular biology detection (Feberwee, et al., 2022). Isolation and culture are the gold standards for direct detection of this organism, but pathogenic MG is a slow-growing and relatively fastidious organism that may take up to 3 weeks to detect. The PCR method has the advantages of high specificity and sensitivity, is rapid, simple and relatively inexpensive, and can be used to directly detect organisms without the need for culture (Leurs, et al., 2022).

To distinguish the ts-11 and non-ts-11 strains, scientists have performed many studies. In order to differentiate a series of known MG strains, Raviv et al developed 5 qPCR methods, which could differentiate one of the five commercial and laboratory vaccine strains (F, ts-11, 6/85, K5831, K5054) from the challenge strain (Rlow). However, the applicability of this assay is limited and cannot be used in more scenarios (Raviv et al., 2008). The DNA fingerprinting analysis method requires pure cultures, shows low repeatability, requires lengthy procedures, and lacks comparable data between laboratories (Hong, et al., 2005; Naziri, et al., 2016; Mateen, et al., 2021). Melt-curve and agarose gel-based mismatch amplification mutation assays (MAMAs) were developed to distinguish MG vaccine strains and field isolates. However, due to moderate assay sensitivity, clinical specimens with low DNA loads may yield false-negative results. (Sulyok, et al., 2019).

Therefore, a simple and rapid detection method with strong sensitivity and specificity needs to be developed. Especially for single-base mutations, conventional PCR cannot distinguish strains or solve the current problem of identification. In this method, the probe is a cycling

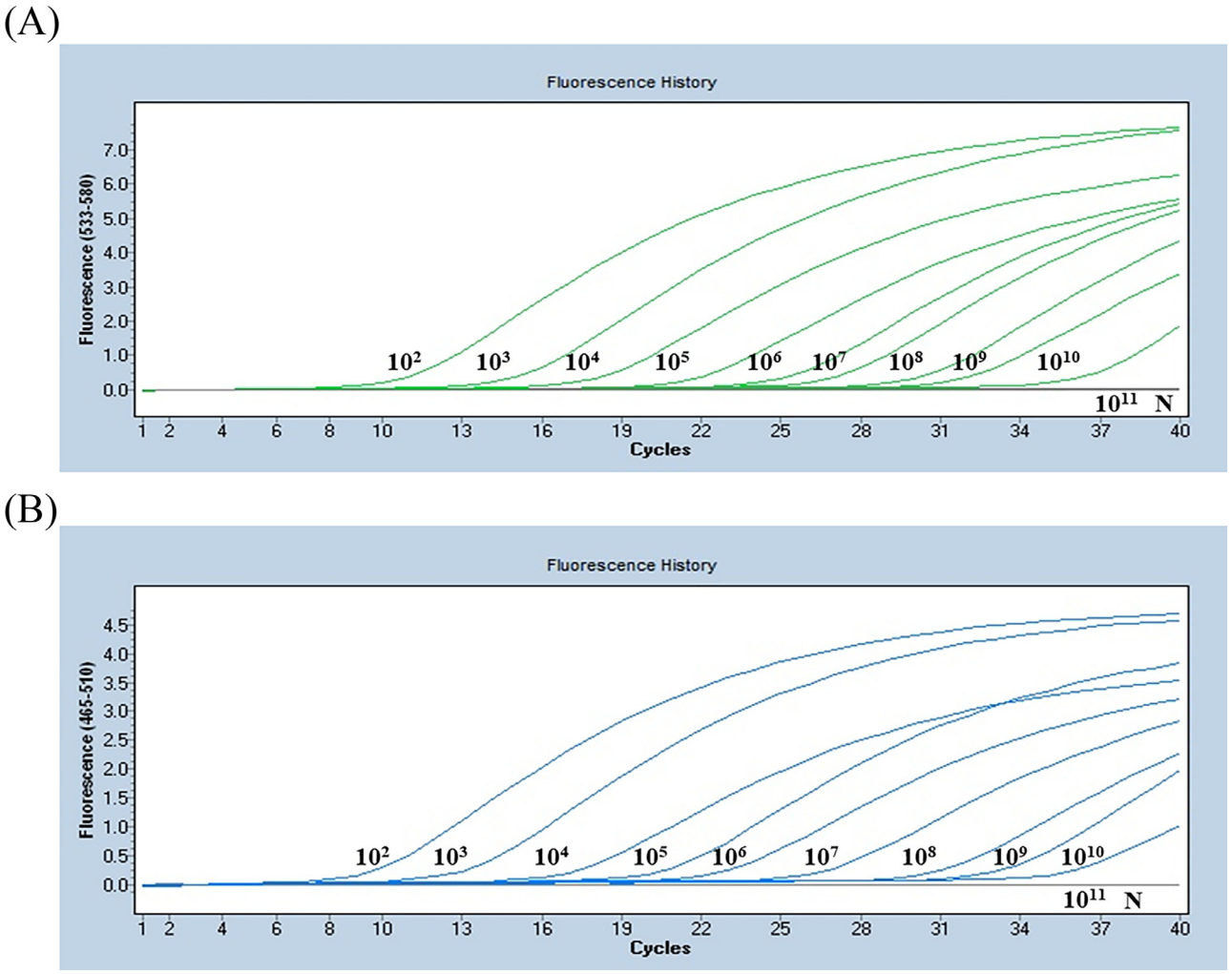

Figure 3. Sensitivity for the cycleave dual-probe fluorescence quantitative PCR. The amplification graphs of developed cycleave qPCR were generated using ten-fold serial dilutions of plasmids of ts-11 vaccine strain (A) and non-ts-11 strain (B) from 10^2 to 10^{11}. N: negative control.

probe, which is a hybrid probe composed of RNA and DNA. When used in combination with RNase H, this approach is highly sensitive and specific. When the probe hybridized with the complementary sequence of the amplified product, RNase H degraded the probe in the RNA part relieving the quenching inhibition and promoting fluorescent substance emitting fluorescence (Yamada, et al., 2021). The volume of expanded and increased production can be monitored in real time by measuring the fluorescence intensity. However, the matched probe or a base with the template would lead to the unsuccessful slicing of the probe by RNase H. Thus, this is a highly specific detection method that can recognize the differences even if there is only one base change. Compared with traditional fluorescent PCR, the probe generally consists of approximately twelve bases, with a close proximity between the fluorescent reporting group and the quenching group, good quenching effect, low fluorescence background, and a high signal-to-noise ratio.

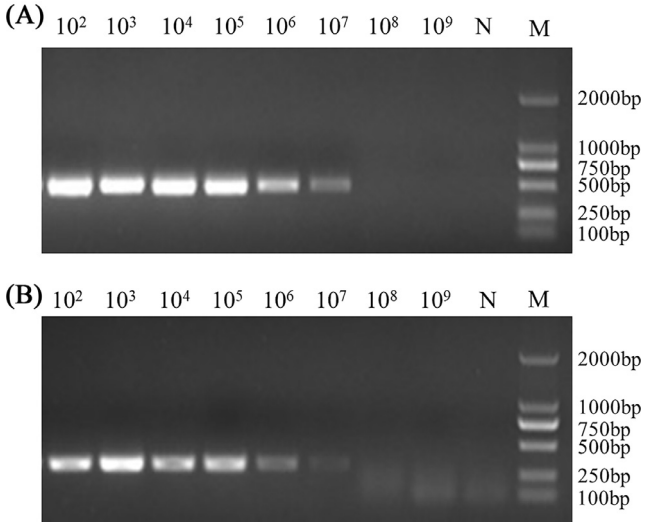

Figure 4. Electropherogram of conventional PCR detection of ts-11 vaccine strain and non-ts-11 strains. The value of each lane represents the dilution fold of plasmid of ts-11 vaccine strain (A) and non-ts-11 strain (B). N: negative control. M: DNA marker.

The cycleave qPCR method established in the present study can be used to specifically differentiate ts-11 and non-ts-11 strains within 1 h and has no cross reactivity with common poultry diseases such as AIV, IBV, or NDV. Cycleave qPCR has high sensitivity, with limit of detection of 2.43 copies/μL for the ts-11 strain and 1.65 copies/μL for the non-ts-11 strain. The intra- and

Table 3. Reproducibility of the cycleave dual-probe fluorescence quantitative PCR.

Strains	Concentration (copy/μL)	Intra-batch Ct values (means ± SD)	Intra-batch CV	Inter-batch Ct values (means ± SD)	Inter-batch CV
ts-11	2.43×10^6	21.67 ± 0.15	0.69%	21.54 ± 0.11	0.51%
	2.43×10^5	24.87 ± 0.08	0.32%	24.70 ± 0.10	0.40%
	2.43×10^4	27.96 ± 0.23	0.82%	28.04 ± 0.14	0.50%
non-ts-11	1.65×10^6	22.08 ± 0.25	1.13%	22.30 ± 0.14	0.63%
	1.65×10^5	25.58 ± 0.26	1.02%	25.63 ± 0.23	0.90%
	1.65×10^4	28.55 ± 0.26	0.91%	28.39 ± 0.27	0.95%

interbatch CVs were not more than 1.13%. For clinical sample detection, cycleave qPCR has 100% consistency with the commercial universal real-time fluorescence PCR kit and can be used to evaluate the immune efficacy of the MG ts-11 vaccine in chickens effectively. It has to clarify that this method can only distinguish ts-11 vaccine strain from non-ts-11 vaccine strain (including field strains and other vaccine strains (F and 6/85)). If the discrimination of vaccine strains (including ts-11, F and 6/85) and wild strains is needed, the combined use of other methods is recommended.

In summary, a cycleave dual-probe fluorescence quantitative PCR method was established in the present study. This method is simple, highly sensitive and specific, repeatable, and highly efficient. In particular, this method can detect both the MG-ts-11 strain and non-ts-11 strains simultaneously. Currently, the ts-11 live vaccine is the main MG vaccine type, is widely used in China, and plays an important role in the clinical practice of MG prevention and control. The establishment of cycleave qPCR will greatly promote the application of the MG ts-11 live vaccine and the prevention and control of MG infection, as has been demonstrated in clinical practice. In China, the MG eradication plan from commercial flocks has been put on the agenda at the national level, and the cycleave qPCR method developed in this study will play a critical role in the epidemiological surveillance of MG infection.

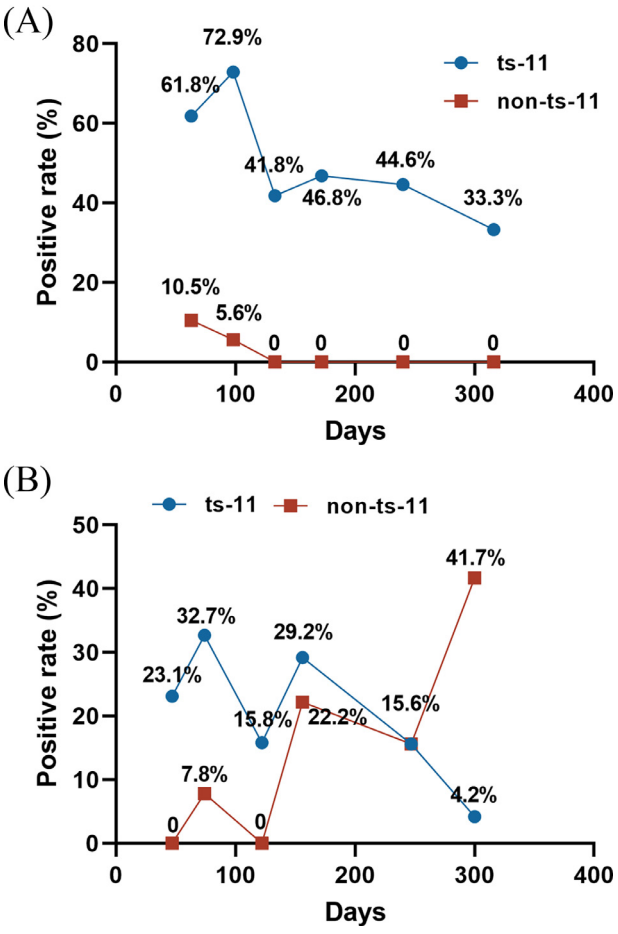

Figure 5. The positive rates of vaccine strains and wild-type strains at different days post immunization. The chickens in A company vaccinated twice MG ts-11 vaccine at 25 and 45 d, respectively (A). The chickens in B company vaccinated once MG ts-11 vaccine at 21 d (B).

ACKNOWLEDGMENTS

The study work was supported by the Crosswise project of Shandong Sinder Technology Co., Ltd. (JC20230313001). The authors thank the anonymous reviewers for their constructive comments.

Data Availability: The data that support the findings of this study are available from the corresponding author upon reasonable request.

DISCLOSURES

The authors declare no conflicts of interest.

SUPPLEMENTARY MATERIALS

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.psj.2024.103907.

REFERENCES

Alqhtani, A. H., S. A. Fatemi, K. E. C. Elliott, S. L. Branton, J. D. Evans, S. A. Leigh, P. D. Gerard, and E. D. Peebles. 2022. Effects of the in ovo vaccination of the ts-11 strain of mycoplasma gallisepticum in layer embryos and posthatch chicks. Animals (Basel) 12:1120.

Armour, N. K., and N. Ferguson-Noel. 2015. Evaluation of the egg transmission and pathogenicity of Mycoplasma gallisepticum isolates genotyped as ts-11. Avian Pathol 44:296–304.

Dhondt, A. A., D. L. Tessaglia, and R. L. Slothower. 1998. Epidemic mycoplasmal conjunctivitis in house finches from eastern North America. J. Wildl. Dis 34:265–280.

Feberwee, A., S. de Wit, and R. Dijkman. 2022. Clinical expression, epidemiology, and monitoring of Mycoplasma gallisepticum and Mycoplasma synoviae: an update. Avian Pathol 51:2–18.

Fujita, S., K. Masago, J. Takeshita, C. Okuda, K. Otsuka, A. Hata, R. Kaji, N. Katakami, and Y. Hirata. 2015. Validation of an ion torrent sequencing platform for the detection of gene mutations in biopsy specimens from patients with non-small-cell lung cancer. PLoS One 10:e0130219.

Hong, Y., M. García, S. Levisohn, P. Savelkoul, V. Leiting, I. Lysnyansky, D. H. Ley, and S. H. Kleven. 2005. Differentiation of Mycoplasma gallisepticum strains using amplified fragment length polymorphism and other DNA-based typing methods. Avian Dis 49:43–49.

Jacob, R., S. L. Branton, J. D. Evans, S. A. Leigh, and E. D. Peebles. 2015. Effects of different vaccine combinations against Mycoplasma gallisepticum on the internal egg and eggshell characteristics of commercial layer chickens 1,2,3. Poult Sci 94:912–917.

Leurs, K., E. Goossens, H. Christensen, J. G. Mainil, D. Vancraeynest, R. Ducatelle, and F. Van Immerseel. 2022. Development of a duplex qPCR for the differentiation of a live attenuated Escherichia coli aroA mutant vaccine strain from field isolates in chickens. PLoS One 17:e0278949.

Ley, D. H., J. M. McLaren, A. M. Miles, H. J. Barnes, S. H. Miller, and G. Franz. 1997. Transmissibility of live Mycoplasma gallisepticum vaccine strains ts-11 and 6/85 from vaccinated layer pullets to sentinel poultry. Avian Dis 41:187–194.

Mateen, R. M., M. F. Sabar, S. Hussain, R. Parveen, and M. Hussain. 2021. Familial DNA analysis and criminal investigation: Usage, downsides and privacy concerns. Forensic Sci. Int 318:110576.

Mugunthan, S. P., G. Kannan, H. M. Chandra, and B. Paital. 2023. Infection, transmission, pathogenesis and vaccine development against Mycoplasma gallisepticum. Vaccines (Basel) 11:469.

Muneta, Y., I. S. Panicker, A. Kanci, D. Craick, A. H. Noormohammadi, A. Bean, G. F. Browning, and P. F. Markham. 2008. Development and immunogenicity of recombinant Mycoplasma gallisepticum vaccine strain ts-11 expressing chicken IFN-gamma. Vaccine 26:5449–5454.

Nan, W., Y. Zhang, P. Tan, Z. Xu, Y. Chen, K. Mao, and Y. Chen. 2016. A rapid cycleave PCR method for distinguishing the vaccine strain Brucella abortus A19 in China. J Vet. Diagn. Invest 28:214–218.

Naylor, C. J., A. R. Al-Ankari, A. I. Al-Afaleq, J. M. Bradbury, and R. C. Jones. 1992. Exacerbation of Mycoplasma gallisepticum infection in turkeys by rhinotracheitis virus. Avian Pathol 21:295–305.

Naziri, Z., A. Derakhshandeh, R. Firouzi, M. Motamedifar, and A. Shojaee Tabrizi. 2016. DNA fingerprinting approaches to trace Escherichia coli sharing between dogs and owners. J. Appl. Microbiol 120:460–468.

Noormohammadi, A. H., and K. G. Whithear. 2019. Comparison of the short-term and long-term efficacies of the Mycoplasma gallisepticum vaccines ts-11 and 6/85. Avian Pathol 48:238–244.

Raviv, Z., S. A. Callison, N. Ferguson-Noel, and S. H. Kleven. 2008. Strain differentiating real-time PCR for Mycoplasma gallisepticum live vaccine evaluation studies. Vet. Microbiol 129:179–187.

Rodriguez, R., and S. H. Kleven. 1980. Pathogenicity of two strains of Mycoplasma gallisepticum in broilers. Avian Dis 24:800–807.

Ross, K. A., L. M. Huntimer, J. E. Vela Ramirez, J. R. Adams, S. L. Carpenter, M. L. Kohut, T. Bronich, R. Webby, K. L. Legge, S. K. Mallapragadal, M. J. Wannemuehler, and B. Narasimhan. 2014. Vaccine technologies against avian influenza: current approaches and new directions. J. Biomed. Nanotechnol 10:2261–2294.

Sulyok, K. M., Z. Kreizinger, K. Bekő, B. Forró, S. Marton, K. Bányai, S. Catania, C. Ellis, J. Bradbury, O. M. Olaogun, B. Kovács Á, T. Cserép, and M. Gyuranecz. 2019. Development of molecular methods for rapid differentiation of Mycoplasma gallisepticum vaccine strains from field isolates. J. Clin. Microbiol 57 e01084-18.

Vance, A. M., S. L. Branton, S. D. Collier, P. D. Gerard, and E. D. Peebles. 2009. Effects of time-specific F-strain Mycoplasma gallisepticum inoculation overlays on prelay ts-11-strain M. gallisepticum vaccination on digestive and reproductive organ characteristics of commercial egg-laying hens. Poult. Sci 88:980–983.

Vance, A. M., S. L. Branton, S. D. Collier, P. D. Gerard, and E. D. J. P. S. Peebles. 2008. Effects of time-specific F-strain Mycoplasma gallisepticum Inoculation overlays on prelay ts11-strain Mycoplasma gallisepticum inoculation on performance characteristics of commercial laying hens. Poult Sci 87:655–660.

Wang, S., A. Huang, Y. Gu, J. Li, L. Huang, X. Wang, Y. Tao, Z. Liu, C. Wu, Z. Yuan, and H. Hao. 2022. Rational use of danofloxacin for treatment of mycoplasma gallisepticum in chickens based on the clinical breakpoint and lung microbiota shift. Antibiotics (Basel) 11:403.

Whithear, K. G., K. E. H. Soeripto, and E. Ghiocas. 1990. Safety of temperature sensitive mutant Mycoplasma gallisepticum vaccine. Aust Vet J 67:159–165.

Wu, Z., L. Ding, J. Bao, and Y. Liu. 2019. Co-infection of Mycoplasma gallisepticum and Escherichia coli triggers inflammatory injury involving the IL-17 signaling pathway. Front Microbiol 10:2615.

Yamada, A., M. Sugimura, and T. Kuramoto. 2021. Genetic polymorphism of bovine beta-casein gene in Japanese dairy farm herds. Anim Sci J 92:e13644.