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Using a novel gene site to develop a duplex real-time TaqMan MGB probe PCR method for the SNP detection and differentiation between the MS-H live vaccine strain and wild-type *Mycoplasma synoviae* strains

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ABSTRACT

Mycoplasma synoviae (MS) is a globally prevalent avian pathogen responsible for airsacculitis and synovitis. The temperature-sensitive (ts)+ vaccine strain MS-H, a live attenuated variant, is the most effective and widely used vaccine for controlling infections in the poultry industry. Consequently, accurate detection is essential for a strategy known as differentiating infected from vaccinated animals (DIVA). In this study, we developed a duplex real-time TaqMan minor groove binder (MGB) probe PCR (The DRTM-probe PCR) method to differentiate the MS-H live vaccine strain from wild-type strains by targeting a single nucleotide polymorphism (SNP) in the *ktrB* gene. This gene overcomes the restoration of the genotype of wild-type 86079/7NS in specific regions. With a detection limit of 6.25 copies/μL, the DRTM-probes PCR method demonstrates a good specificity in distinguishing in one hour. For simulated clinical samples, the method achieved over 95 % sequence identity with reference fragments, confirming its accuracy. The established DRTM-probe PCR method offers a specific, rapid, and reliable approach for SNP detection with significant application potential.

Introduction

Mycoplasma synoviae (MS) and *Mycoplasma gallisepticum* (MG) are thought to be the most important avian pathogenic *Mycoplasma* species in poultry (Feberwee et al., 2022; Chaidez-Ibarra et al., 2022). Over the past few years, the incidence of MG has relatively decreased due to early prevention efforts and the widespread adoption of vaccines. Conversely, the rate of MS infection is now higher than that of MG, as reported in relevant studies (Chaidez-Ibarra et al., 2022), and has potentially become the leading pathogen cause of financial loss within the poultry industry (Landman, 2014). MS is a major avian pathogen that usually causes arthritis, synovitis, respiratory diseases, and reduced egg production and hatchability in poultry (Landman and Feberwee, 2012; Lockaby et al., 1998). The transmission can occur horizontally through direct interaction or vertically through fertilized eggs (Kleven, 1998). Although MS does not directly cause mortality, its impact on avian health is significant.

The poultry industry is still on a growing trend in the coming decades. The main strategies for MS monitoring and control programs have been present for a long time, including population cleansing, antibiotic treatment, and vaccination. However, population cleansing requires substantial human and financial investment, while prolonged antibiotics use can lead to significant resistance. Consequently, vaccination remains the most effective strategy for sustainable prevention and management. The temperature-sensitive (ts)+ vaccine strain MS-H, a live attenuated variant derived from the Australian strain 86079/7NS, was introduced in Europe in 2012. This vaccine has been widely adopted in many countries. MS-H vaccination can reduce or prevent clinical symptoms but does not provide protection against infections caused by wild-type strains (Feberwee et al., 2009; Feberwee et al., 2017). Chickens will experience prolonged bacterial colonization in their upper respiratory tract after immunization using the MS-H strain. As a result, certain existing diagnostic methods, such as serological, may not meet the requirements for the differentiation of infected from vaccinated animals (DIVA) (Shahid et al., 2014; Kreizinger et al., 2015; Dijkman et al., 2017; Sulyok et al., 2019). Molecular biology techniques can overcome certain constraints associated with serological diagnostics. Given this, reliable molecular tests with high discriminatory ability will become

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increasingly essential for effective DIVA implementation.

Real-time fluorescence quantitative PCR (qPCR) is a highly accurate method for quantitative information (Chaidez-Ibarra et al., 2022). Single nucleotide polymorphism (SNP) site-based qPCR detection has been widely applied in bacterial studies and continues to be refined for improved speed and convenience (Shen, et al., 2020; Wen et al., 2021). Dijkman (Dijkman et al., 2017) and Liu (Liu et al., 2024) have developed a qPCR method targeting a SNP site in the *obg* gene. The *obg* and *oppF* genes are currently the most frequently used targets for MS-H detection and other related specificity experiments. However, they both have the problems that could potentially enable the restoration of the mutations in the genotype to their original form (Klose, et al., 2022). Therefore, it is necessary to find a target site that is specific and stably present in the strain to guarantee the accuracy of the detection results.

In this study, we developed a duplex real-time TaqMan minor groove binder (MGB) probe PCR (DRTM-probe PCR) method targeting a novel SNP site in the *ktrB* gene. The new site could overcome the issue of restoring the genotype in those mutation regions. The established method was validated through assessments of specificity, sensitivity, interference, and simulated clinical samples evaluations. The method can accurately and rapidly differentiate between wild-type and vaccine strains of MS, thereby effectively distinguishing between immunized and non-immunized flocks. In addition, for suspected infected flocks, the established method can also detect the diseased population and provide a prerequisite for population purification, which may reduce economic losses and accelerate the prevention and control of MS (Liu et al., 2024).

Materials and methods

Ethics statement

All animal experiments were approved by Chinese Academy of Agricultural Sciences (CAAS), and were performed in CAAS with the approval of the Committee on the Ethics of Animal Experiments of CAAS (SV-20240705-Y01).

Experimental strains

MS-H (Vaxsafe MS; Bioproperties Pty., Ltd., Australia) and MG-F (Lohmann Animal Health, Winslow, ME, USA) live vaccine strains used in this study were purchased commercially. The standard strains MS WVU_{1853} and MG Rlow were purchased from the China Veterinary Culture Collection Center (CVCC). The MS (NT1, CZ1) (Zhao et al., 2024) and MG 013 (Hu et al., 2022) were different clinical isolates. The 16 other avian pathogens, including *Clostridium perfringens* (CT-1, CY-1, LH-4), *Staphylococcus aureus* SA01, *Salmonella pullorum* SP001, *Proteus mirabilis* PM01, *Enterococcus malodoratus* EM-2, *Gallibacterium anatis* GA-1, *Riemerella anatipestifer* SY-C, *Pasteurella multocida* PM-1, *Salmonella typhimurium* St-1, *Salmonella enteritidis* Se-1, and four different serotypes of *Escherichia coli* (*E. coli*) (O1, O2, O78, O157) (Zhang et al., 2024; Wang et al., 2023; Xin et al., 2022; Wu et al., 2019; Wang et al., 2020) were obtained from the laboratory of Shanghai Veterinary Research Institute, Chinese Academy of Agricultural Sciences, where they had been previously stored.

Genomic nucleic acid extraction

For bacterial cultures, centrifugation was performed, the bacterial precipitate was resuspended in phosphate-buffered saline (PBS) and lysed with boiling water for 10 min, and the supernatant was collected after low-speed centrifugation at 1,700 g as crude genomic DNA. For tracheal mucus and lung tissue samples, genomic DNA was extracted by Hi-Swab DNA Kit (Tiangen, China) and TIANamp Genomic DNA Kit (Tiangen, China), respectively. All extracted genomic DNA samples were stored at −20°C until use.

Genome comparison and design of primers and probes

To find all mutation SNP sites, we compared the whole genome of MS-H (GenBank accession CP021129) with that of 86079/7NS (GenBank accession CP029258) using MAUVE. Then, all SNP mutation sites obtained from the comparison of these two strains were further analyzed for conservation and specificity using NCBI-BLAST (http://blast.ncbi.nlm.nih.gov). Genetic stability was assessed by examining the consistency of SNP mutations across MS-H, its reisolates (TS4, AB1, AS2), its ancestral strains (86079/7NS), and other wild-type strains (NCTC 10124, ATCC 25204, 53, HN01, SD2, ZX313, WF18, 5-9, FJ-01, A4, G3, BS4S2, 18DW, 51SH). The SNP sites with the best specificity and sensitivity were selected for subsequent experiments (Fig. 1).

The screened sites were validated through extensive experiments, and the probe targeting SNP site 822 in the *ktrB* gene showed sufficient sensitivity and specificity, which was ultimately selected as the target gene. Primers for the DRTM-probe PCR were designed based on the gene sequences of MS-H and MS WVU_{1853} (Table 1). All primers and probes were synthesized by Shanghai Rui Mian Biotechnology Co., Ltd. The solutions were prepared at a concentration of 10 μM and stored at −20°C until use.

Construction and purification of standard plasmids

The *ktrB* gene from both MS-H and wild-type strains were amplified via PCR. The upstream and downstream primer sequences were as follows: 5′-gagtaccaattgcaaaaactaaaaag-3′ and 5′-gataattctacttatcatcggtggaat-3′. The amplified *ktrB* gene was subsequently cloned into the pMD18-T cloning vector (Takara, China) and transformed into *E. coli* DH5α competence cells (Tiangen, China). Recombinant plasmids were then extracted using the TIANprep Mini Plasmid kit (Tiangen, China) following the manufacturer's instructions.

Establishment and optimization of the basic reaction system

The qPCR reaction system was prepared according to the manufacturer's instructions (Vazyme, China). The crude genomic DNA from MS-H and MS WVU_{1853} were used as template. The DRTM-probe PCR was conducted using an Applied Biosystems 7500 PCR detection system (Thermo Fisher Scientific, USA). The reaction conditions were as follows: contamination digestion at 37°C for 2 min, pre-denaturation at 95°C for 3 min, followed by 45 cycles of denaturation at 95°C for 10 s, and annealing / extension at 60°C for 35 s. Signal acquisition was carried out using the FAM and CY5 channels. To optimize the reaction system, the annealing temperature was evaluated at 58°C, 60°C, and 62°C. Additionally, we employed a three-factor, five-level orthogonal experimental design to optimize the concentrations of primers and probes (Chen et al., 2023).

Sensitivity and reproducibility experiments of the method

To determine the detection limit of the optimized DRTM-probe PCR method, the positive plasmids were serially diluted 10-fold in ddH_2O. The MS-H plasmid was diluted to concentrations ranging from 1.25×10^7 copies/μL to $6.25\times10^{\circ}$ copies/μL, while the wild-type MS plasmid ranged from 1.44×10^7 copies/μL to 1.44×10^1 copies/μL. The results were used to generate a standard curve for detection limit assessment. Standard plasmids served as templates in the optimized reaction system to evaluate both intra- and inter-assay reproducibility. The coefficient of variation (CV) was used to evaluate reproducibility, with each experiment being replicated in triplicate.

Specificity experiments of the method

The specificity of the DRTM-probe PCR method was assessed using genomic DNA from 23 avian pathogens. These pathogens included MS-

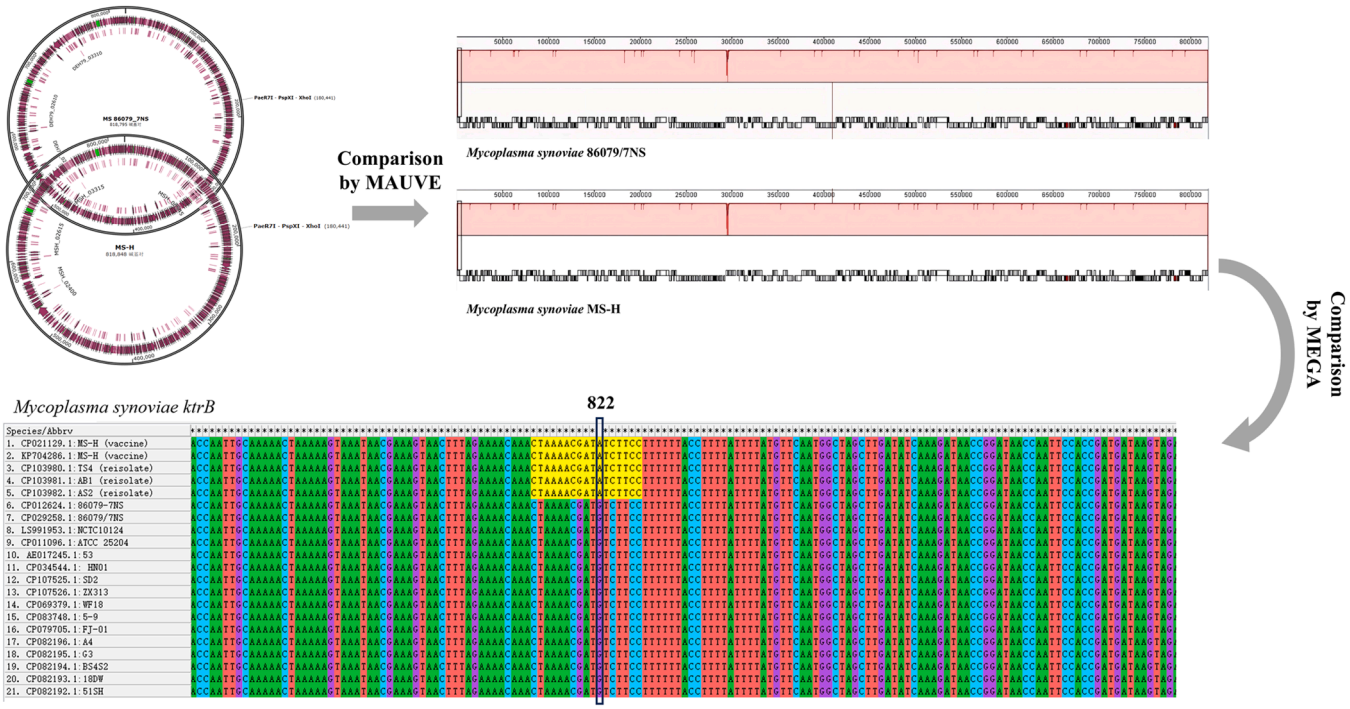

Fig. 1. Designed workflows with the target screening and probe design.

Table 1
Sequences of primers used for DRTM-probe PCR.

Primer	Sequences (5′—3′)	Base length (bp)
DRT-primer-F	ccaattgcaaaaactaaaaag	21
DRT-primer-R	cggtggaattggttatcc	18
DRT-probes-MS	CY5-taaaacgatgtcttcct-MGB	17
DRT-probes-MS-H	FAM-ctaaaacgatatcttcc-MGB	17

H, MS WVU_{1853}, MS (NT1, CZ1), MG-F, MG Rlow, MG 013, and 16 other avian pathogens, such as *Clostridium perfringens* (CT-1, CY-1, LH-4), *Staphylococcus aureus* SA01, *Salmonella pullorum* SP001, *Proteus mirabilis* PM01, *Enterococcus malodoratus* EM-2, *Gallibacterium anatis* GA-1, *Riemerella anatipestifer* SY-C, *Pasteurella multocida* PM-1, *Salmonella typhimurium* St-1, *Salmonella enteritidis* Se-1, and four different serotypes of *Escherichia coli* (O1, O2, O78, O157).

Interference experiments of the method

To evaluate the interference resistance of the MS-H vaccine strain, 0.1 μg of its genome was serially diluted in ddH_2O at ratios of 1:10, 1:50, 1:100, 1:500, 1:1000, and 1:5000. Subsequently, 1 μL of ddH_2O or 1 μL of the MS WVU_{1853} genome (100 ng/μL) was added to each dilution to establish the control and interference groups, respectively. The wild-type strain was subjected to the same interference resistance test, with 1 μL of the MS-H genome (100 ng/μL) serving as the interfering strain. All samples were then analyzed using the optimized method.

Limit of detection in simulated clinical samples

To determine the limit of detection (LOD) of the DRTM-probe PCR in simulated clinical samples, tests were conducted using genomic DNA extracted from pharyngeal swabs and lung tissue samples intentionally contaminated with MS-H or wild-type MS (MS NT1). Tracheal mucus was collected from 33 specified pathogen-free (SPF) White Leghorns using a single swab per bird, which was subsequently rinsed with 300 μL of PBS. Lung tissue samples from 11 SPF White Leghorns were taken and fully homogenized using a tissue grinder (Jingxin, China). The obtained tracheal mucus and lung tissue suspensions were spiked with serially diluted concentrations of MS-H bacteria ranging from 5.75×10^5 CFU/mL to $2.88\times10^{\circ}$ CFU/mL and MS NT1 bacteria from 1.0×10^7 CFU/mL to 1.0×10^1 CFU/mL. DNA extraction was performed using the Hi-Swab DNA Kit (Tiangen, Beijing, China) or TIANamp Genomic DNA Kit (Tiangen, Beijing, China), respectively. To verify the experimental results, the qPCR products were sequenced by Tsingke Biotechnology Co., Ltd. .

Statistical analysis

Data are presented as the mean ± standard deviation (SD) for reproducibility test, and the coefficient of variation in this experiment was calculated using the formula: (standard deviation/mean) × 100 %.

Results

Screening of SNP sites

The MAUVE comparison identified all SNP sites present in the genome. Based on this information, specificity and conservation analyses were conducted. Target SNP sites were chosen based on their genetic stability, as evidenced by the uniformity of SNP mutations across MS-H, its reisolates, ancestral strains, and other wild-type strains. Ultimately, a total of 21 SNP sites were selected for detection (Table 2). The probe targeting SNP site 822 in the *ktrB* gene showed sufficient sensitivity and specificity, resulting in its selection ($ktrB_{822}$) for further experiments.

Optimization of basic reaction system

Optimization of the annealing temperature demonstrated that at 60°C, non-specific signals were minimized, and the Ct value remained relatively low while maintaining the detection accuracy. Then, we established reaction systems with different concentrations by orthogonal to optimize primer and probe concentrations. Through a

Table 2
Genes containing MS-specific and conserved SNP sites.

Locus-tag	Product	Mutation (86079/7NS/ MS-H)	Position in MS-H
MSH_00080	tRNA (guanosine(37)-N1)-methyltransferase TrmD	G/A	14181
MSH_00325	DNA topoisomerase IV	G/A	61685
MSH_00330	Excinuclease ABC subunit B	G/A	62874
MSH_00350	tRNA-Trp	G/A	67028
MSH_01000	Hypothetical protein	G/A	201094
MSH_01150	Type I glyceraldehyde-3-phosphate dehydrogenase	G/A	242451
MSH_01205	Hemagglutinin	G/A	255672
MSH_01215	Hypothetical protein	TT/—	258578
MSH_01595	Ribonuclease R	G/A	352952
MSH_01710	Aspartate–ammonia ligase	G/A	389629
MSH_01910	Alanine–tRNA ligase	G/A	433343
MSH_01940	Alpha/beta hydrolase	G/A	438657
MSH_02150	Hemolysin C	G/A	481287
MSH_02235	Histidine–tRNA ligase	T/C	498421
MSH_02330	Hypothetical protein	G/A	522899
MSH_02455	Hypothetical protein	G/A	563391
MSH_02470	DNA-directed RNA polymerase subunit beta'	C/A	567729
MSH_02540	Hypothetical protein	G/A	584838
MSH_02655	Potassium transporter KtrB	G/A	615741
MSH_02705	DNA polymerase III subunit alpha	G/A	628272
MSH_03220	Fatty acid-binding protein DegV	G/A	737180

combination of low non-specific signals and relatively high fluorescence intensity, the optimal conditions were determined as follows: 0.8 μL of forward primer (10 μM) and reverse primer (10 μM), 0.4 μL each of the MS-H probe (10 μM) and wild-type probe (10 μM).

Establishment of the standard curve

The plasmid samples were subjected to 10-fold serial dilution and subsequently analyzed using the optimized method. Standard curves were generated for both MS-H and wild-type plasmid (Fig. 2). The standard curve equations were $y = -3.3455x + 36.821$ ($R^2 = 0.9992$) for the MS-H plasmid and $y = -3.3214x + 38.39$ ($R^2 = 0.9953$) for the wild-type MS plasmid.

Specificity experiments results

BLAST analysis confirmed that the selected primers and probes have no cross-reactivity with non-MS avian species. The presence of a CY5 signal with a characteristic S-curve indicates a wild-type MS strain. Whereas a FAM signal with an S-curve identifies the MS-H strain. No characteristic curve was observed when testing non-MS strains (Fig. 3). These results showed that the established DRTM-probe PCR method has good specificity.

Sensitivity and reproducibility experiments results

The constructed standard plasmids of the vaccine and wild-type strains were used as templates for sensitivity testing. The detection limits for MS-H and wild-type MS were 6.25 copies/μL and 14.4 copies/μL, respectively. In the reproducibility test, the results of the plasmid detection indicated that the intra-assay CVs for MS-H ranged from 0.65 % to 1.39 %, while the inter-assay CVs ranged from 0.54 % to 1.97 %. For the wild-type MS plasmid, the intra-assay CVs ranged from 0.09 % to 0.78 % and the inter-assay CVs ranged from 2.65 % to 3.61 %, with a total CV of less than 4 % (Table 3). All of these results demonstrated a strong linear relationship, confirming that the method is highly reproducible, efficient in amplification, and sensitive.

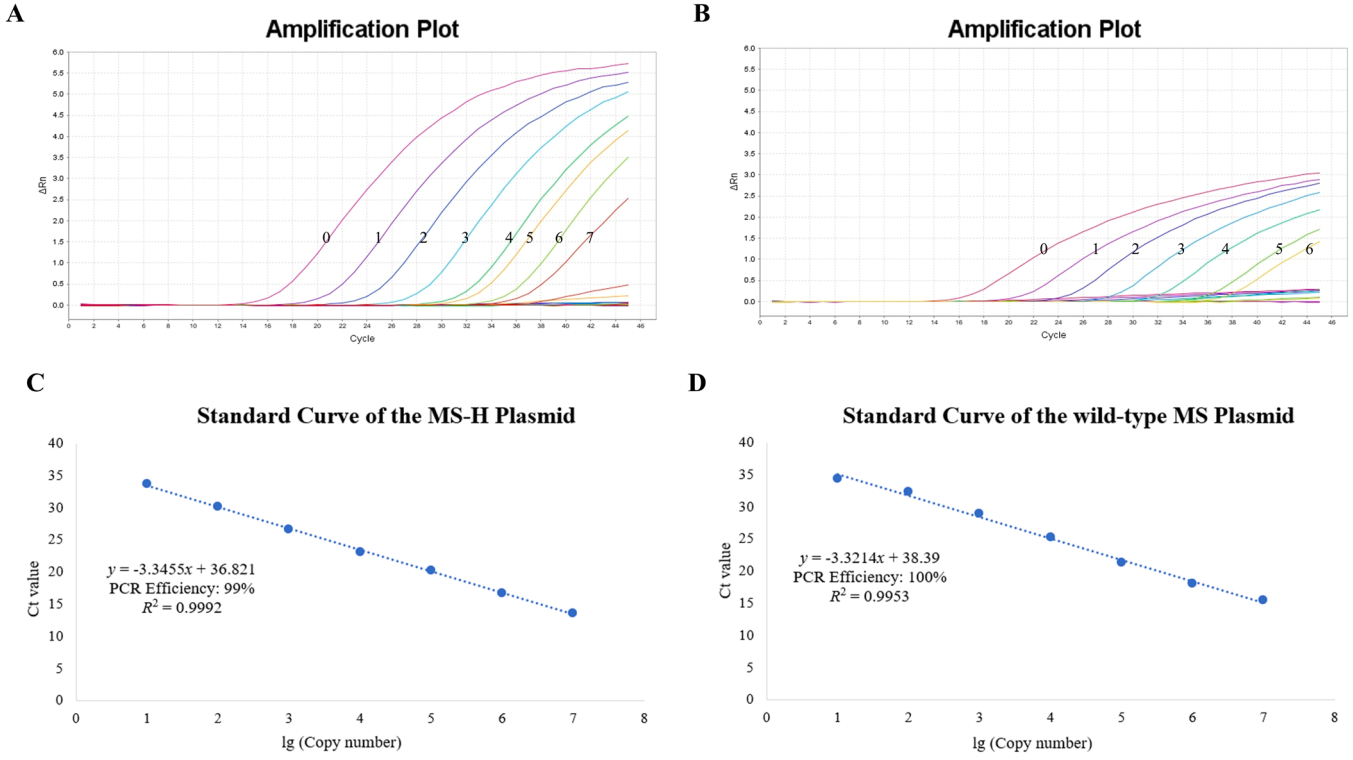

Fig. 2. Sensitivity testing of plasmid and establishment of standard curves. The amplification plot and standard curve of the developed method were generated using ten-fold serial dilutions of plasmids of MS-H vaccine strain (A, C) and wild-type MS (B, D). A (0-7): Amplification curves of MS-H plasmid with 1.25×10^7, 1.25×10^6, 1.25×10^5, 1.25×10^4, 1.25×10^3, 1.25×10^2, 1.25×10^1, 6.25×10^0 copies/μL in sequence. B (0-6): Amplification curves of wild-type MS plasmid with 1.44×10^7, 1.44×10^6, 1.44×10^5, 1.44×10^4, 1.44×10^3, 1.44×10^2, 1.44×10^1 copies/μL in sequence.

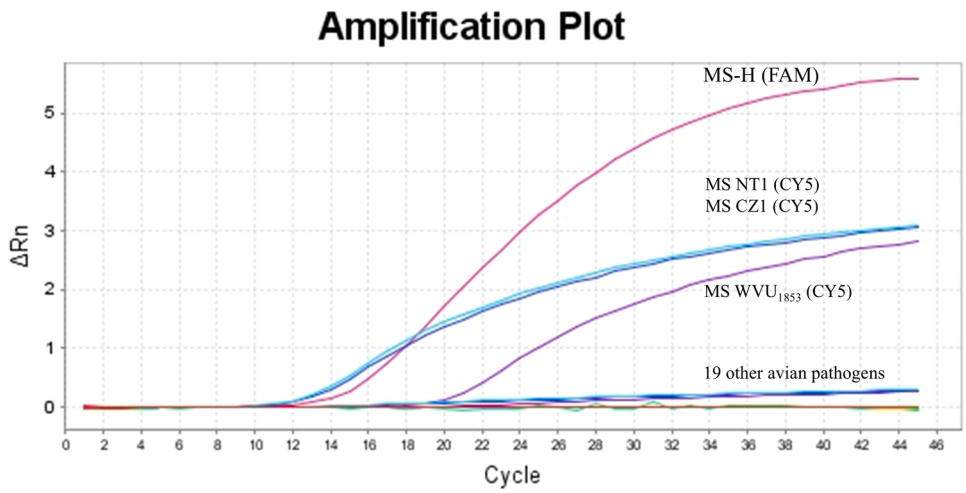

Fig. 3. Specificity test results with MS-H vaccine strain, wild-type MS strain and other 19 avian pathogens. There were 19 other avian pathogens, including MG-F, MG Rlow, MG 013, *Clostridium perfringens* (CT-1, CY-1, LH-4), *Staphylococcus aureus* (SA01), *Salmonella pullorum* SP001, *Proteus mirabilis* PM01, *Enterococcus malodoratus* EM-2, *Gallibacterium anatis* GA-1, *Riemerella anatipestifer* SY-C, *Pasteurella multocida* PM-1, *Salmonella typhimurium* St-1, *Salmonella enteritidis* Se-1, and four different serotypes of *Escherichia coli* (O1, O2, O78, O157).

Table 3
Reproducibility test results of the DRTM-probe PCR method.

Plasmid	Concentration (copies/μL)	Intra-assay		Inter-assay	
		Average Ct values ± SD	CV (%)	Average Ct values ± SD	CV (%)
MS-H	10^6	14.85±0.21	1.39	15.13±0.30	1.97
	10^5	18.67±0.12	0.65	18.71±0.20	1.09
	10^4	24.11±0.19	0.79	24.09±0.13	0.54
MS	10^6	19.06±0.06	0.33	18.56±0.67	3.61
	10^5	22.43±0.18	0.78	22.05±0.58	2.65
	10^4	26.20±0.02	0.09	25.69±0.69	2.68

Interference experiments results

The results of the interference experiments demonstrate that the detection system is capable of accurately identifying the target strain even in the presence of a strong interfering strain. No significant differences were observed between the interference group and the control group. These findings indicate that the method exhibits strong anti-interference capability and holds significant potential for precise differentiation and quantitative detection in clinically mixed samples (Table 4).

Table 4
Interference experiment results of the DRTM-probe PCR method.

Genomic DNA	Dilutions	Detecting Ct values	
		Control groups	Interference group
MS-H[1]	1:10	11.72	11.56
	1:50	13.54	12.53
	1:100	15.07	12.07
	1:500	17.74	12.65
	1:1000	18.35	11.78
	1:5000	20.79	12.27
MS WVU^2_{1853}	1:10	10.59	10.30
	1:50	13.18	12.74
	1:100	13.97	13.51
	1:500	16.46	15.84
	1:1000	17.31	17.86
	1:5000	20.28	20.81

Note:.
[1] About 0.1 μg of $MSWVU_{1853}$ genomic DNA was added to the detection system of MS-H genome dilutions as interference, and the same volume of ddH_2O was added as control. [2] About 0.1 μg of MS-H genomic DNA was added to the detection system of MS WVU_{1853} genome dilutions as interference, and the same volume of ddH_2O was added as control.

Simulated clinical samples test

The simulated clinical samples test was conducted using samples contaminated with MS-H and MS NT1 strains. The detection limit for the contaminated MS-H and wild-type strain were 5.75 CFU/mL and 1.0×10^2 CFU/mL, respectively (Fig. 4). The standard curve equations were $y = -2.7261x + 33.425$ ($R^2 = 0.9906$) for the MS-H strain and $y = -3.5115x + 40.644$ ($R^2 = 0.9985$) for the wild-type strain. These results all showed good amplification efficiency.

The results of the simulated clinical samples from tracheal mucus and lung tissues are presented in Table 5. The results demonstrate that our method effectively distinguishes between the MS-H and wild-type MS strains. Sequencing analysis confirmed that the SNP sites were accurately identified, with sequence homology exceeding 95 %, thereby validating the accuracy of this method.

Discussion

The live attenuated vaccine strain MS-H is commonly used to control MS infections in many countries. The *vlha* gene sequence is commonly used to differentiate MS-H vaccine strains from clinical isolates (Bayatzadeh et al., 2014). However, the *vlha* sequences identical to those of the MS-H strain have been identified in several field isolates from Europe (Dijkman et al., 2014). Therefore, this gene is not capable of accurately identifying vaccine strains. Consequently, for infection control and decontamination purposes, SNP-based qPCR detection has been widely used for pathogen detection in recent years (Shen, et al., 2020; Wen et al., 2021), and the *obg* and *oppF* genes are the commonly used. Dijkman (Dijkman et al., 2017) and Liu (Liu et al., 2024) had developed a qPCR method based on the *obg* gene. However, these two common detection sites can restore the genotype of wild-type 86079/7NS in those regions (Klose, et al., 2022). The possibility of restoration represents a possible inaccuracy of the test results. Therefore, the selection of gene targets is pivotal for real-time PCR development.

The aim of this study was to find new detection targets to avoid the possibility of mutations restoration and to establish accurate detection methods. To achieve this, we screened for new detection targets to reduce the likelihood of misdiagnosis. Whole genome alignment of MS-H and 86079/7NS was conducted using MAUVE. The final 21 candidate

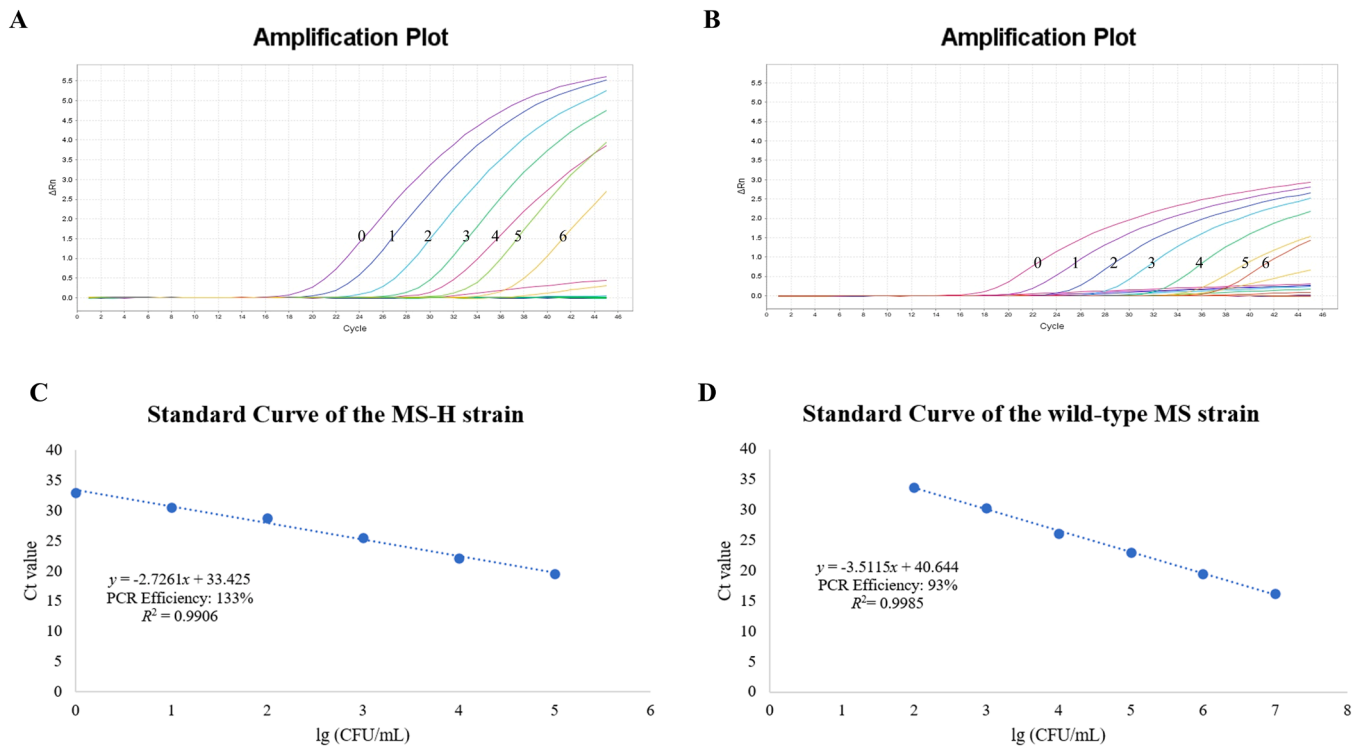

Fig. 4. Sensitivity testing of strains and establishment of standard curves. The amplification plot and standard curve of the developed method were generated using ten-fold serial dilutions of strains of (A, C) MS-H and (B, D) MS NT1. A (0-6): Amplification curves of MS-H strain with 5.75×10^{5}, 5.75×10^{4}, 5.75×10^{3}, 5.75×10^{2}, 5.75×10^{1}, $5.75\times10^{\circ}$, $2.88\times10^{\circ}$ CFU/mL in sequence. B (0-6): Amplification curves of wild-type MS strain with 1.0×10^{7}, 1.0×10^{6}, 1.0×10^{5}, 1.0×10^{4}, 1.0×10^{3}, 1.0×10^{2}, 1.0×10^{1} CFU/mL in sequence.

Table 5
Results of contamination analysis in clinical samples from tracheal mucus and lung tissues.

Type	No.	Average ± standard deviation	Results	Homology comparison (%)	Type	No.	Average ± standard deviation	Results	Homology comparison (%)
Tracheal mucus	1	25.46±0.14	+	97.83 %	Tracheal mucus	23	29.56±0.42	+	96.38 %
	2	21.83±0.12	+	97.10 %		24	22.83±0.51	+	95.65 %
	3	29.02±0.49	+	96.38 %		25	23.69±0.30	+	97.83 %
	4	32.74±0.23	+	97.83 %		26	30.74±0.09	+	99.28 %
	5	22.39±0.14	+	98.55 %		27	19.53±0.10	+	96.38 %
	6	22.16±0.27	+	95.65 %		28	21.94±0.03	+	97.10 %
	7	21.24±0.15	+	97.83 %		29	20.47±0.01	+	95.65 %
	8	28.15±0.15	+	98.55 %		30	36.03±0.21	+	97.10 %
	9	23.08±0.10	+	96.38 %		31	Undetermined	-	
	10	26.92±0.12	+	97.83 %		32	Undetermined	-	
	11	30.19±0.07	+	95.65 %		33	Undetermined	-	
	12	20.04±0.09	+	99.28 %	Lung tissues	1	15.83±0.22	+	97.10 %
	13	31.14±0.13	+	97.83 %		2	18.75±0.03	+	97.10 %
	14	26.75±0.02	+	96.38 %		3	17.87±0.22	+	96.38 %
	15	25.01±0.07	+	97.83 %		4	21.34±0.27	+	96.38 %
	16	24.16±0.12	+	96.38 %		5	22.53±0.24	+	97.83 %
	17	23.28±0.11	+	97.83 %		6	20.66±0.17	+	96.38 %
	18	23.16±0.36	+	97.10 %		7	31.71±0.48	+	97.83 %
	19	25.90±0.12	+	97.83 %		8	29.98±0.21	+	99.28 %
	20	33.23±0.28	+	99.28 %		9	Undetermined	-	
	21	23.42±0.06	+	98.55 %		10	Undetermined	-	
	22	26.50±0.04	+	97.83 %		11	Undetermined	-	

detection targets were selected by conservativeness and specificity analyses, and were using in subsequent assays.

Based on the targets screened above, we developed a duplex real-time TaqMan MGB probe PCR method utilizing a hydrolysis TaqMan oligonucleotide probe, specifically MGB probe. This probe loaded a reporter dye at the 5′-end and attached a NFQ quenching dyes at the 3′-end. The inclusion of the NFQ in the MGB probe significantly reduces background fluorescence (Kutyavin et al., 2000). Through this, the highly stable interaction between the MGB probe and the target increases the melting temperature (Tm) of the probe and its specificity (Sylvain et al., 2004; Kutyavin et al., 2000). This method is applicable in both single and multiplex formats for SNP detection across many species (Kutyavin, 2010; Tomás et al., 2012), including *Helicobacter pylori* (Zhao et al., 2022), *Mucormycosis* (Bergallo et al., 2022), and avian influenza viruses (Zhang et al., 2017). In this study, a pair of primers and two specific MGB probes were designed for the SNP mutation site of the *ktrB* gene, one probe was specific for attenuated vaccine strain MS-H and the other probe was for wild-type MS, which could simultaneously identify

MS-H and wild-type MS in one reaction. Orthogonal experiments were conducted to determine the optimal primer and probe concentrations. The optimized reaction system not only can accurately distinguish vaccine strain MS-H from wild-type MS, but also can be used for clinical detection of MS infection.

The specificity test revealed no non-specific reactions with other avian pathogens, demonstrating high specificity. In conjunction with the results from the interference test, it was further confirmed that the method could accurately identify the target strains even in the presence of a large number of clinically interfering strains. In the sensitivity test, the detection limits for MS-H and wild-type MS were 6.25 copies/μL and 14.4 copies/μL, respectively. Additionally, the detection limits for the simulated clinical samples contaminated with MS-H strain and wild-type MS were 5.75 CFU/mL and 1.0×10^2 CFU/mL, respectively.

The sensitivity of the Taqman MGB method established using this *ktrB* gene exceeds that of existing methods. Dijkman (Dijkman et al., 2017) developed a qPCR method based on the *obg* gene, which demonstrated the detection limits of $10^{2\text{-}3}$ CFU/g tracheal mucus for the MS field strain and 10^2 CFU/g tracheal mucus for the MS-H live vaccine strain, using a Ct cut-off value of 40. In contrast, our method achieved a minimum detection limit of 5.75 CFU/mL, with an optimized lower Ct detection threshold of 35. Kreizinger (Kreizinger et al., 2015) utilized melt-MAMAs and agarose-MAMAs to identify MS-H and wild-type MS, achieving sensitivities of 10^3 and 10^4 copies, respectively. Liao (Liao et al., 2024) developed a rapid quantitative method to differentiate the MS1 vaccine strain from wild-type MS, with the minimum detection limits of all qPCRs ranging from 10^1 to 10^2 copies/μL. Our method achieved a detection limit of 6.25 copies/μL, significantly surpassing these existing methods. Furthermore, comparisons between Taqman qPCR method and the melt-based MAMA method have shown that the Taqman qPCR method is more sensitive (Liao et al., 2024). We speculate that, by optimizing the DNA extraction process, even greater sensitivity can be achieved.

To evaluate the precision and sensitivity of the diagnostic method, validation studies were conducted using pharyngeal swabs and tissue samples intentionally contaminated with MS-H and wild-type MS, respectively. The results demonstrate that the method, which requires minimal sample processing and completes the detection reaction within approximately one hour, performed satisfactorily. The qPCR products were confirmed by sequencing, showing high credibility, with match rates exceeding 95 %.

Conclusion

In this study, we identified 21 SNP sites that can differentiate the MS-H vaccine strain from the wild-type MS strains, and for the first time, a duplex real-time TaqMan MGB probe PCR assay targeting $ktrB_{822}$ was developed. The established qPCR method showed high sensitivity, strong specificity, good reproducibility and excellent anti-interference capability. It can accurately differentiate between MS-H vaccinated flocks and those infected with the wild-type MS strain. Also, it facilitates the rapid diagnosis of farms clinically suspected of MS infection. Most importantly, it can circumvent the limitations associated with reversion mutations in commonly used genes, which may enhance diagnostic accuracy and provide an efficient approach for clinical testing.

Declaration of competing interest

All authors declare that they have no conflict of interest.

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