Development of Molecular Methods for Rapid Differentiation of *Mycoplasma gallisepticum* Vaccine Strains from Field Isolates

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ABSTRACT *Mycoplasma gallisepticum* is among the most economically significant mycoplasmas causing production losses in poultry. Seven melt-curve and agarose gel-based mismatch amplification mutation assays (MAMAs) and one PCR are provided in the present study to distinguish the *M. gallisepticum* vaccine strains and field isolates based on mutations in the *crmA*, *gapA*, *lpd*, *plpA*, *potC*, *glpK*, and *hlp2* genes. A total of 239 samples (*M. gallisepticum* vaccine and type strains, pure cultures, and clinical samples) originating from 16 countries and from at least eight avian species were submitted to the presented assays for validation or in blind tests. A comparison of the data from 126 samples (including sequences available at GenBank) examined by the developed assays and a recently developed multilocus sequence typing assay showed congruent typing results. The sensitivity of the melt-MAMA assays varied between 10¹ and 10⁴ *M. gallisepticum* template copies/reaction, while that of the agarose-MAMAs ranged from 10³ to 10⁵ template copies/reaction, and no cross-reactions occurred with other *Mycoplasma* species colonizing birds. The presented assays are also suitable for discriminating multiple strains in a single sample. The developed assays enable the differentiation of live vaccine strains by targeting two or three markers/vaccine strain; however, considering the high variability of the species, the combined use of all assays is recommended. The suggested combination provides a reliable tool for routine diagnostics due to the sensitivity and specificity of the assays, and they can be performed directly on clinical samples and in laboratories with basic PCR equipment.

KEYWORDS DIVA, *Mycoplasma gallisepticum*, molecular methods, poultry, vaccine, wild-type strain

Infection with *Mycoplasma gallisepticum* has a wide variety of clinical manifestations, but the most important disease presentation is chronic respiratory disease in chickens and infectious sinusitis in turkeys, resulting in reduced meat and egg production. Therefore, *M. gallisepticum* is among the economically most significant mycoplasmas of poultry worldwide (1, 2). Like other pathogenic avian mycoplasmas, *M. gallisepticum* can be disseminated horizontally, but the major route of transmission is from infected breeder birds to progeny, and this is the prime consideration for international trade (2).

Control programs for *M. gallisepticum* are based on maintaining commercial breeder stocks free of infection. In other cases, targeted antibiotic medication and vaccination are being evaluated as feasible options (2, 3). The commercially available agents for *M.*

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gallisepticum vaccination are bacterins, live vaccines, and an *M. gallisepticum* antigen expressing recombinant fowl pox vaccine (4). Currently, the worldwide used live *M. gallisepticum* vaccine strains are the 6/85 (Nobilis MG6/85; MSD Animal Health), ts-11 (VaxSafe MG; Bioproperties Pty Ltd.), and the F strain (Cevac MG-F; Ceva, Inc.). Strain 6/85 was developed by serial passages of a field isolate originating from the United States (5, 6). The temperature-sensitive vaccine strain ts-11 was selected from an Australian field isolate (strain 80083) after chemical mutagenesis (7). The F strain was probably first isolated in 1956 in the United States (8), and it is a field strain with moderate virulence (9). Since live vaccines are used in many parts of the world, differentiation of *M. gallisepticum* vaccine strains from wild virulent isolates has become essential in the control programs.

The discrimination of vaccine strains from field isolates is a complicated challenge, as the genetics behind the attenuation of the vaccine strains are not well understood. Moreover, reversion of the virulence of *M. gallisepticum* vaccine strains (10) or mixed infection with the vaccine and related strain types (11–13) can occur.

Several attempts were made in the past to discriminate *M. gallisepticum* vaccine strains and field isolates, including DNA fingerprinting methods, like amplified fragment length polymorphism (AFLP) (14) and random amplified polymorphic DNA (RAPD) analyses (15). However, these methods have low reproducibility, and they require the isolation of the tested organisms. Sequence-based methods with higher reproducibility and reliability, lower labor intensity, and applicability on clinical samples have been designed as well, such as gene-targeted sequencing (16), the TaqMan assay (17), and high-resolution melt-curve analysis (18–20). These techniques are usually tested on a very limited number of samples from a limited geographical region, need special equipment, or are highly expensive. Recently, PCR tests have been developed for the differentiation of ts-11 from field isolates (21), but these are not suitable in situations when multiple strains are present in a sample. For the genotyping of *M. gallisepticum* isolates, a core-genome multilocus sequence typing (MLST) system with improved discriminatory power has been established (22), but this method needs the previous isolation and whole-genome sequence of the bacteria. Ultimately, a six-gene-based MLST assay has also been released, which proved to be suitable for discrimination of the ts-11, 6/85, and F vaccine strains (23).

The current study describes the development and characterization of rapid and cost-effective PCR-based assays for the simultaneous discrimination of 6/85, ts-11, and F vaccine strains from field isolates. To better evaluate the system, the results were compared with the data from MLST analysis (23) and PCR assays described by Ricketts et al. (21), and a total of 239 *M. gallisepticum* strains and clinical samples originating from 16 countries were examined.

MATERIALS AND METHODS

***M. gallisepticum* strains and samples.** For the validation of the developed assays, vaccine strains 6/85 (Nobilis MG6/85; MSD Animal Health), ts-11 (VaxSafe MG; Bioproperties Pty Ltd.), and F (Cevac MG-F; Ceva, Inc.) were obtained from their commercial distributors. The *M. gallisepticum* type strain (ATCC 19610) was used as a wild-type control in the assays. Fourteen *M. gallisepticum* field isolates were recovered from clinical submissions between 2010 and 2017 originating from Europe (Hungary, *n* = 7; Romania, *n* = 3; Ukraine, *n* = 2; Czech Republic, *n* = 1; and Spain *n* = 1) (see Data Set S1 in the supplemental material). The field isolates originated from tracheal swabs or lung samples from unvaccinated turkeys and chickens. Ethics approval and specific permission were not required for the study, as all samples were collected by the authors during routine diagnostic examinations or necropsies with the consent of the owners. Isolation of the strains was performed by washing the tracheal swabs or the lung samples in 2 ml of Frey's broth medium (pH 7.8) (24) and incubating at 37°C under a 5% CO₂ atmosphere. Filter cloning was used to gain pure cultures from the isolates.

The DNA was extracted from the strains using the QIAamp DNA minikit (Qiagen, Inc., Hilden, Germany) according to the manufacturer's instructions. All isolates were identified by quantitative PCR (qPCR) targeting the *mgc2* gene of *M. gallisepticum* (25). In order to exclude the presence of other contaminant mycoplasmas in the cultures, the DNA of the isolates was submitted to a universal *Mycoplasma* PCR system (26), followed by sequencing on an ABI Prism 3100 automated DNA sequencer (Applied Biosystems, Foster City, CA), sequence analysis, and a BLAST search.

A further 185 *M. gallisepticum* strains (cultures or DNA; Italy, *n* = 75; Spain, *n* = 42; United Kingdom, *n* = 22; Israel, *n* = 20; the United States, *n* = 7; Australia, *n* = 6; The Netherlands, *n* = 4; Germany, *n* = 3;

Portugal, $n = 2$; Austria, $n = 1$; France, $n = 1$; Jordan, $n = 1$; and Slovenia, $n = 1$) and 36 DNA samples from clinical samples (Spain, $n = 17$; Israel, $n = 8$; Italy, $n = 6$; Iraq, $n = 3$; Albania, $n = 1$; and Jordan, $n = 1$) were provided for a blind test from the sample collections (Data Set S1). The presence of *M. gallisepticum* in the samples was checked with the PCR system described by Raviv and Kleven (25). Nuclease-free water was used as a negative control in all PCR assays.

Whole-genome sequencing, sequence analysis, and target selection. *M. gallisepticum* genomic DNAs of vaccine strains 6/85 and ts-11 were extracted from 5 ml of logarithmic-phase broth cultures using a QIAamp DNA minikit (Qiagen, Inc.). Next-generation sequencing was performed on an Ion Torrent platform (New England BioLabs, Hitchin, United Kingdom), as previously described (27, 28). Reads were mapped to *M. gallisepticum* strain R_{low} (GenBank accession number [AE015450.2](#)) as a reference genome and annotated using the Geneious software version 10.2.3 (29). The average numbers of reads and read lengths were 215,429 reads and 167.7 bp, respectively. The mean coverages were 45.7× and 31.3× for the whole genome of the 6/85 and ts-11 strains, respectively.

Candidate genes were selected according to previous publications (30–35). The candidate genes were retrieved from the genomes of the *M. gallisepticum* ts-11, 6/85, and F vaccine strains (GenBank accession number [NC_017503.1](#)) and published *M. gallisepticum* genomes (strain S6, GenBank accession number [NC_023030.2](#); strain R_{low}, GenBank accession number [AE015450.2](#); strain R_{high}, GenBank accession number [NC_017502.1](#); house finch isolates, GenBank accession numbers [NC_018412.1](#), [NC_018409.1](#), [NC_018406.1](#), [NC_018407.1](#), [NC_018408.1](#), [NC_018410.1](#), [NC_018411.1](#), and [NC_018413.1](#); and ts-11 reisolates, GenBank accession numbers [MAFU000000000](#), [MAFV000000000](#), [MAFW000000000](#), [MADW000000000](#), [MATM000000000](#), [MATN000000000](#), [MAGQ000000000](#), and [MAGR000000000](#)) and aligned by Geneious (29) (Data Set S1). The validity of single nucleotide polymorphisms (SNPs) was confirmed by manual examination of the assembled sequences. Numbering of nucleotide positions was according to the individual genes of *M. gallisepticum* strain R_{low} (GenBank accession number [AE015450.2](#)). SNPs and mutations present in one of the *M. gallisepticum* vaccine strains (6/85, ts-11, or F) but absent in other publicly available *M. gallisepticum* strains were selected for primer design.

Assay design. A mismatch amplification mutation assay (MAMA) is a PCR-based technique used for SNP discrimination in many bacteria (36). In brief, the technique is based on SNP-specific primers at the 3' end, with one being marked with an additional 15- to 20-bp-long GC clamp at the 5' end. A single destabilizing mismatch at the 3' end of each allele-specific primer enhances the discriminative capacity of the assay. The GC clamp increases the melting temperature and the size of the amplicon. The temperature shift can be easily detected in the presence of intercalating fluorescent dye on a real-time PCR platform (melt-MAMA), and the difference in the sizes of the amplicons can be observed in agarose gel electrophoresis (agarose-MAMA), which enable the differentiation of the SNP-specific genotypes.

In the present study, MAMAs and a PCR (amplifying products with different lengths) were designed and tested for the detection of *M. gallisepticum* vaccine-specific alterations. Melt-MAMA tests and the melt analysis of a PCR assay were optimized on an Applied Biosystems StepOnePlus real-time PCR system with the StepOne software version 2.3 (Thermo Fisher Scientific, Waltham, MA, USA). Primer melting temperature (T_m) and general suitability were calculated using NetPrimer (Premier Biosoft International, Palo Alto, CA). The primer sequences and thermocycler parameters for the assays can be found in Table 1.

A PCR mixture of melt-MAMAs and the PCR-6/85-crmA (analyzed by melting) consisted of 2 μ l of 5× colorless GoTaq Flexi buffer (Promega, Inc., Madison, WI), 1 μ l MgCl₂ (25 mM), 0.3 μ l dinucleoside triphosphate (dNTP; 10 mM; Qiagen, Inc., Valencia, CA), 0.5 μ l EvaGreen (20×; Biotium, Inc., Hayward, CA), primers (10 pmol/ μ l, according to Table 1), 0.08 μ l GoTaq G2 Flexi DNA polymerase (5 U/ μ l; Promega, Inc.), nuclease-free water, and 1 μ l DNA template at a final volume of 10 μ l. The thermocycling parameters were 95°C for 10 min, followed by 30 or 40 cycles (according to Table 1) of 95°C for 15 s and 60°C for 1 min. PCR products were subjected to melt analysis using a dissociation protocol comprising the steps 95°C for 15 s, followed by 0.3°C incremental temperature ramping from 60°C to 95°C. EvaGreen fluorescence intensity was measured at 525 nm at each ramp interval and plotted against temperature. All specimens were tested in duplicate.

Agarose-MAMAs and the PCR-6/85-crmA (analyzed by gel electrophoresis) were performed in a C1000 Touch thermal cycler (Bio-Rad Laboratories, Inc., Berkeley, CA, USA) in 25 μ l total volume containing 1 μ l target DNA diluted in 5 μ l of 5× Green GoTaq Flexi buffer (Promega, Inc.), 2.5 μ l MgCl₂ (25 mM; Promega, Inc.), 0.5 μ l dNTP (10 mM, Qiagen, Inc.), primers (10 pmol/ μ l) according to Table 1, 0.25 μ l GoTaq G2 Flexi DNA polymerase (5 U/ μ l; Promega, Inc.), and nuclease-free water under the following PCR conditions: 95°C for 5 min, followed by 40 cycles at 95°C for 30 s, 60°C for 30 s, and 72°C for 30 s. The final elongation step was performed at 72°C for 5 min. Electrophoresis was carried out on a 3% agarose gel (MetaPhor agarose; Lonza Group Ltd., Basel, Switzerland), and a 20-bp DNA ladder (O'RangeRuler, 20 bp; Thermo Fisher Scientific, Inc.) was used as a molecular weight marker.

Validation of the assays. Initially, the targeted mutations were selected according to *in silico* analysis of available *M. gallisepticum* whole-genome sequences (19 isolates from GenBank and the 3 vaccine strains). For further evaluation, tests were challenged with the DNA of the live vaccine strains 6/85, ts-11, and F and the type strain, and with the DNA of *M. gallisepticum* field isolates originating from unvaccinated flocks ($n = 14$, Data Set S1).

In order to test the sensitivity of the assays, 10-fold dilutions of each genotype were used in the range of 10⁶ to 10⁰ copies/ μ l. Template copy number was determined by a qPCR system targeting the *mgc2* gene, which is present in a single copy in the *M. gallisepticum* genome (25). The 10-fold dilution series of a synthetic sequence (500 ng, gBlock; Integrated DNA Technologies, Inc., Coralville, IA) was used as a control for the template copy number determinations, and contains a 94-bp-long fragment of the *mgc2* gene (between nucleotides 220622 and 220716, according to nucleotide numbering of the *M. gallisepticum* strain R_{low}).

TABLE 1 Primer sequences of assays for the differentiation of 6/85, ts-11, and F vaccine strains from field isolates designed in this study

Vaccine strain	Gene	Mutation (amino acid change) ^a	Assay name	Primer name	Primer sequence (5'→3')	Primer vol (μl) ^b		No. of cycles	
						Melt-MAMA	Agarose-MAMA	Melt-MAMA	Agarose-MAMA
6/85	<i>lpd</i>	G1372T (A→S)	MAMA-6/85-lpd	lpd-1372-6/85	ggggcgggcccggggggtttttgtttraagtggttataaatcga	0.15	1	40	40
				lpd-1372-wt	gtttttgtttraagtggttataaatagc	0.6	4	40	40
				lpd-1372-con	gaacaagcaattcacccacacc	0.15	1	40	40
	<i>gapA</i>	A1306G (R→G)	MAMA-6/85-gapA	gapA-1315-6/85	ggggcgggcccggggggttttttagaaactaaatttgaaatcg	0.15	1	40	40
				gapA-1315-wt	gggttttttagaaactaaatttgaaagca	0.15	1	40	40
				gapA-1315-con	ataaaataccgtatggataaccaacag	0.15	1	40	40
	<i>crmA</i>	48-nt del; 16-aa del	PCR-6/85-crmA	PCR-crmA-F	tgcctgctgctaaacctggtgc	0.15	1	40	40
				PCR-crmA-R	ggagcgggtggttttgagca	0.15	1	40	40
ts-11	<i>plpA</i>	C953G (T→S)	MAMA-ts11-plpA	plpA-971-ts11	ggggcgggcccggggccttctagatgaggtgtgattgtgc	0.15	1	30	40
				plpA-971-wt	gcttctagatgaggtgtgattgagg	0.15	4	30	40
				plpA-971-con	ggattattacctgaacttgcacag	0.15	1	30	40
	<i>glpK</i>	G67A (D→N)	MAMA-ts11-glpK	glpK-67-ts11	ggggcgggcccggggacatcttgcgttcaatcggttgta	0.15	1	40	40
				glpK-67-wt	acatcttgcgttcaatcggttctg	0.15	1	40	40
				glpK-67-con	ggaagatattgcgtaaattcgttttg	0.15	1	40	40
	<i>potC</i>	C526G (Q→E)	MAMA-ts11-potC	potC-526-ts11	ggggcgggcccggggatgaaccctaaatctaatcttagctttag	0.15	1	30	40
				potC-526-wt	atgaaccctaaatctaatcttagcttaac	0.6	4	30	40
				potC-526-con	gcgggtgttaataagatagatgaatct	0.15	1	30	40
F	<i>hlp2</i>	G5542C (E→Q)	MAMA-F-hlp2	hlp2-5542-F	ggggcgggcccggggccttctagtggtggttttttaattcttg	0.15	1	40	40
				hlp2-5542-wt	gtcttagtggtggttttttaattcttctc	0.15	1	40	40
				hlp2-5542-con	gaagtgcataaagaaataaactgatctg	0.15	1	40	40
	<i>crmA</i>	C2116G (Q→E)	MAMA-F-crmA	crmA-2116-F	ggggcgggcccggggacacccattcggaaacactctcg	0.15	4	40	40
				crmA-2116-wt	acaaccattcggaaacactcac	0.15	1	40	40
				crmA-2116-con	ctaattcttaattgatgagaactgatcac	0.15	1	40	40

^aNucleotide numbering is according to *M. gallisepticum* R_{low} (GenBank accession no. [AE015450.2](#)). nt, nucleotides; aa, amino acids; del, deletion.
^bPrimer (10 pmol/μl) volume in 10 μl (melt-MAMA) and in a 25-μl reaction mixture (agarose-MAMA). Lowercase sequence bases at the 5' end of the vaccine-specific primers indicate the additional GC clamp.

ticum strain NCTC 10115 at GenBank). The lowest template copy numbers yielding a melting temperature (T_m) specific to the genotypes were considered as the detection limit of the assays.

The specificity of the assays was tested by involving the following avian *Mycoplasma* species in the analysis: *M. anatis* (ATCC 25524), *M. anseris* (ATCC 49234), *Mycoplasma* sp. strain 1220 ("*M. anseris*alpin-gitis" ATCC BAA-2147), *M. cloacale* (ATCC 35276), *M. columbinasale* (ATCC 33549), *M. columbinum* (ATCC 29257), *M. columborale* (ATCC 29258), *M. gallinaceum* (ATCC 33550), *M. gallinarum* (ATCC 19708), *M. gallopavonis* (ATCC 33551), *M. iners* (ATCC 19705), *M. iowae* (ATCC 33552), *M. meleagridis* (NCTC 10153), and *M. synoviae* (ATCC 25204) type strains. All *M. gallisepticum* vaccine strains (6/85, ts-11, and F) were also included as a control in all vaccine strain-specific assays.

In order to assess the capability of the assays to identify a mixed population of wild-type and vaccine strains in a single specimen, different template copy number combinations of the *M. gallisepticum* type strain and the vaccine strains (6/85, ts-11, or F) were tested in separate PCRs. The mixtures contained the type strain and one of the vaccine strains in the following combinations: a constant template copy number (10^6 copies/ μ l) of one strain was paired with a member of a series of 10-fold DNA dilutions (10^6 to 10^3 copies/ μ l) of the other strain, and vice versa.

M. gallisepticum vaccine strains and the type strain were used in the stability testing of the mutations targeted by the designed assays. Each strain was passaged 10 times in Frey's medium and submitted for the assays after DNA extraction. Genotype calls of the 10th clones and the parent strains were compared.

Blind tests, multilocus sequence typing, and strain-specific PCR. A blind test of the developed assays was performed on the DNA of 185 *M. gallisepticum* isolates and 36 clinical samples originating from flocks of unknown vaccination status.

Genetic diversity and relatedness to vaccine strains of the 14 *M. gallisepticum* strains used for the validation tests and 89 *M. gallisepticum* strains and clinical samples examined in the blind tests were previously determined by MLST analysis using six housekeeping genes (*atpG*, *dnaA*, *fusA*, *rpoB*, *ruvB*, and *uvrA*) (23). The MLST profiles of the live vaccine strains, the type strain, and the 19 publicly available *M. gallisepticum* genomes have also been defined (23). Based on the genetic relatedness to vaccine strains, four MLST profiles are presented in the current study, those for 6/85, ts-11, F, and the wild type (no relatedness to any of the live vaccine strains detected). The genotype calls of the presented assays were compared with the genotype assignment of the MLST (Data Set S1).

Samples which appeared to be ts-11 reisolates by the developed MAMA tests and/or those originating from Australia were further tested according to Ricketts et al. (21). In brief, the presence of three additional genes (*vlhA3.04a*, *vlhA3.05*, and *mg03659*) was investigated for the discrimination of field isolates from ts-11 vaccine strains.

Statistical analyses. The adjusted Rand coefficient was used to determine the congruency of the assays in the comparisons. Values were calculated with the help of the online tool Comparing Partitions (<http://www.comparingpartitions.info/?link=Tool>). Samples which showed false-negative results in any of the compared assays were excluded from the analyses.

Data availability. The nucleotide sequences of the *M. gallisepticum* amplicons included in the MLST were submitted to the National Center for Biotechnology Information (NCBI) under GenBank accession numbers [MH544230](#) and [MH544241](#) and [MK288880](#) to [MK289516](#).

RESULTS

Sequence analysis and target selection. For the differentiation of *M. gallisepticum* vaccine strains 6/85, ts-11, and F, a total of 8, 15, and 9 nonsynonymous mutations were targeted using the MAMA and PCR assays, respectively (data not shown). The number of assays was narrowed to two MAMAs and one PCR (examining the presence of a deletion) to strain 6/85, three MAMAs to strain ts-11, and two MAMAs to strain F. The targeted mutations are located in virulence-associated genes (*crmA*, *gapA*, *hlp2*, *lpd*, *plpA*, and *glpK*) or in the gene coding for an ABC transporter protein (*potC*). Selection of the assays was performed according to preliminary examinations using the following criteria: (i) the peaks of the melting curves of the vaccine and wild-type strains were distinguishable; (ii) the peak of the negative control did not overlap the peaks of the vaccine or wild-type strains; and (iii) the mutation was specific to the vaccine or vaccine reisolates when all available samples with known vaccination status were tested. Amplicons containing the targeted mutations of the vaccine and the wild-type strain are presented in Text S1.

Validation of the assays. The results of the validation tests of all selected assays are shown in Table 2. The melting temperatures and sizes of the amplicons are listed in Table 2 and shown in Fig. 1.

The detection limit of the melt-MAMA assays varied between 10^1 and 10^4 template copies/reaction, while that of the agarose-MAMAs varied between 10^3 and 10^5 template copies/reaction depending on the assay and the genotype. Negative controls or templates of other avian *Mycoplasma* species were either not amplified or generated nonspecific products with melt profiles differing from the profiles of the expected two

TABLE 2 Validation results of assays designed in the present study based on the analyses of 18 *M. gallisepticum* strains^a

Vaccine	Assay name	Genotype ^b	T_m (°C) ^c	Amplicon length (bp)	Negative-control conditions ^d	Sensitivity (template copies/reaction) ^e		Template copies/reaction for mixed samples (v:wt) ^f									
						Melt-MAMA		Agarose-MAMA		10 ⁶ :10 ³	10 ⁶ :10 ⁴	10 ⁶ :10 ⁵	10 ⁶ :10 ⁶	10 ⁵ :10 ⁶	10 ⁴ :10 ⁶	10 ³ :10 ⁶	wt
						v	wt	v	wt								
6/85	MAMA-6/85-lpd	6/85	79.8 ± 0.1	102	T_m 72.4 ± 0.1°C C_T 29 ± 0.2	10 ³	10 ³	10 ⁴	10 ⁴	6/85	6/85	6/85	bm	wt	wt	wt	wt
	MAMA-6/85-gapA	wt	76.5 ± 0.3	88	ND/ T_m 71.8 ± 0.3°C	10 ²	10 ³	10 ³	10 ⁴	6/85	6/85	6/85	bm	wt	wt	wt	wt
		6/85	80.3 ± 0.1	99	C_T 33 ± 2.9	10 ⁴	10 ⁴	10 ⁴	10 ⁵	6/85	6/85	6/85	6/85	bm	wt	wt	wt
	PCR-6/85-crma	6/85	82.2 ± 0.1	90	T_m 77.2 ± 0.1°C C_T 24.1 ± 0.4	10 ⁴	10 ⁴	10 ⁴	10 ⁵	6/85	6/85	6/85	6/85	bm	wt	wt	wt
ts-11	MAMA-ts11-plpA	ts-11	82.2 ± 0.0	82	ND	10 ³	10 ³	10 ⁴	10 ⁴	ts-11	ts-11	ts-11	bm	wt	wt	wt	wt
		wt	77.5 ± 0.1	68	ND/ T_m 61.3 ± 0.0°C	10 ¹	10 ¹	10 ³	10 ³	ts-11	ts-11	ts-11	bm	wt	wt	wt	wt
	MAMA-ts11-glpK	ts-11	79.3 ± 0.1	94	C_T 32.5 ± 0.7	10 ⁴	10 ³	10 ⁴	10 ⁴	ts-11	ts-11	ts-11	bm	wt	wt	wt	wt
		wt	76.3 ± 0.1	80	T_m 72.8 ± 0.3°C C_T 30.1 ± 0.2	10 ⁴	10 ³	10 ⁴	10 ⁴	ts-11	ts-11	ts-11	bm	wt	wt	wt	wt
F	MAMA-F-hlp2	ts-11	77.6 ± 0.1	106	ND/ T_m 70.7 ± 0.1°C	10 ³	10 ²	10 ³	10 ³	F	F	F	bm	wt	wt	wt	wt
		wt	74.0 ± 0.1	88	C_T 37.1 ± 0.2	10 ⁴	10 ³	10 ⁴	10 ⁴	F	F	F	bm	wt	wt	wt	wt
	MAMA-F-crma	ts-11	81.0 ± 0.0	89	T_m 80.4, 72.6°C	10 ⁴	10 ³	10 ⁴	10 ⁴	F	F	F	bm	wt	wt	wt	wt
		wt	75.5 ± 0.2	75	C_T 31.8 ± 0.9	10 ⁴	10 ³	10 ⁴	10 ⁴	F	F	F	bm	wt	wt	wt	wt

^aTested with the following avian *Mycoplasma* species (type strain): *M. anatis* (ATCC 25524), *M. anseris* (ATCC 49234), *Mycoplasma* sp. 1220 ("M. anseris/paratuberculosis" ATCC BAA-2147), *M. cloacale* (ATCC 35276), *M. columbinasale* (ATCC 33549), *M. columbinum* (ATCC 29257), *M. columborale* (ATCC 29258), *M. gallinaceum* (ATCC 33550), *M. gallinarum* (ATCC 19708), *M. gallopavonis* (ATCC 33551), *M. iners* (ATCC 19705), *M. iowae* (ATCC 33552), *M. meleagridis* (NCTC 10153), and *M. synoviae* (ATCC 25204). No cross-reactions were detected.

^bwt, wild type.

^cND, not detected. C_T , cycle threshold.

^d T_m , melting temperature.

^eTested with both genotypes. v, vaccine.

^fbm, bimodal peak indicating the presence of both genotypes in the sample.

FIG 1 Detection of A1306G substitution in *gapA* gene. (A to C) y axis, derivative reporter, the negative first derivative of the normalized fluorescence generated by the reporter during PCR amplification. x axis, temperature melt curve. (A) Melting curves of the melt-MAMA showing melting temperatures of the 6/85 vaccine strain (red line; T_m , $80.3 \pm 0.1^\circ\text{C}$) and the *M. gallisepticum* reference strain (ATCC 19610, blue line; T_m , $76.0 \pm 0.6^\circ\text{C}$). Negative controls (gray lines) may show nonspecific amplicons above a C_T of 33 ± 2.9 (T_m , $71.8 \pm 0.3^\circ\text{C}$) or no amplicons. (B) Samples containing smaller amounts of wild-type *M. gallisepticum* DNA can form a bimodal melting peak by the melt-MAMA; next to the wild-type-specific melting peak (sample IZSVE/2015/2062-4f, with approximately 10^3 template copies/ μL ; $76.0 \pm 0.6^\circ\text{C}$), the peak of negative sample (T_m , $71.8 \pm 0.3^\circ\text{C}$) also appears (green line). (C) Samples containing mixed DNA of the 6/85 vaccine (10^5 template copies/ μL) and wild-type strain (10^6 template copies/ μL) can form a bimodal melting peak by the melt-MAMA; next to the wild-type-specific melting peak ($76.0 \pm 0.6^\circ\text{C}$), the peak of 6/85 (T_m , $80.3 \pm 0.1^\circ\text{C}$) also appears (green line). (D) PCR product sizes of MAMA-6/85-gapA in agarose gel. Electrophoresis was performed in 3% agarose gel (MetaPhor Agarose, Lonza Group Ltd., Basel, Switzerland), and a 20-bp DNA ladder (O'RangeRuler 20 bp, Thermo Fisher Scientific, Inc.) was used as a molecular weight marker (m). Lane 1, nonspecific amplicons in the negative control below 60 bp, lane 2, 99-bp fragments specific for the 6/85 vaccine strain; lane 3, wild-type strains yielded 85-bp fragments; lane 4, sample containing smaller amount of wild-type *M. gallisepticum* DNA (sample IZSVE/2015/2062-4f, with approximately 10^3 template copies/ μL); lane 5, sample containing mixed DNA of the 6/85 vaccine (10^5 template copies/ μL) and wild-type strain (10^6 template copies/ μL).

allelic states. The nonspecific melting temperatures or band sizes should be omitted from further analyses. Assays differentiating one of the vaccine strains (6/85, ts-11, or F) resulted in a wild-type-specific amplicon when tested on the other two vaccines, discriminating *M. gallisepticum* vaccine strains from each other (Table 2).

Considering the sensitivity of the assays in general, the tests showed a sensitivity similar to those of the wild-type and vaccine type *M. gallisepticum* DNA. Two assays specific for the ts-11 vaccine strain (MAMA-ts11-glpK and MAMA-ts11-potC) and one assay specific for the F vaccine (MAMA-F-crmA) showed higher sensitivity to the

wild-type DNA, and one assay specific for the 6/85 vaccine strain (MAMA-6/85-crmA) showed higher sensitivity to the vaccine type DNA when mixtures of the wild-type and vaccine type DNA were tested (Table 2). Bimodal melting peaks at the specific melting temperatures or two amplicons with the specific band sizes indicated the presence of both *M. gallisepticum* variants (Fig. 1).

In vitro stability tests were based on the comparison of genotype calls of the 10th clones of the three vaccine and type strains and of the parent strains. Identical genotype calls were detected between clones and parent strains in all assays; however, it should be noted that the test may not reflect completely the genetic stability of the strains under field conditions.

Blind tests. The quantity of *M. gallisepticum* DNA in the samples submitted for blind tests varied largely and showed a wide range of cycle threshold (C_T) values in the *mgc2* gene-based qPCR (25) (Data Set S1). In samples with higher C_T values (usually C_T values above 20 in the *mgc2* gene-based qPCR), the nonspecific PCR product of the negative control was often visible beside the genotype-specific amplicon in the developed assays, detected by real-time PCR as a bimodal peak or by agarose gel electrophoresis as multiple bands (Fig. 1B and D). The nonspecific melting temperatures or band sizes were omitted from the analyses.

In 11 cases, results were evaluable only in one or none of the differentiating assays (C_T values above 28 in the *mgc2* gene-based qPCR [25]); thus, these samples were omitted from further analysis. A further 9 DNA samples (C_T values above 20 in the *mgc2* gene-based qPCR [25]) showed false-negative results in at least one of the following assays: MAMA-6/85-lpd ($n = 2$), MAMA-ts11-plpA ($n = 6$), MAMA-ts11-glpK ($n = 1$), MAMA-ts11-potC ($n = 5$), and MAMA-F-crmA ($n = 3$) (Data Set S1). The validity tests showed that PCR-6/85-crmA has the lowest sensitivity, and accordingly, the highest number of false-negative results was detected with this assay ($n = 50$, C_T values above 15 in the *mgc2* gene-based qPCR [25]).

The MAMA-6/85-lpd, MAMA-6/85-gapA, and PCR-6/85-crmA assays designed for the differentiation of the 6/85 vaccine strain showed high congruency (range of adjusted Rand coefficients, 0.876 to 0.938). The results of two samples from Italy, namely IZSVE/2013/4693-4f and IZSVE/2014/6259-35f, showed discrepancies when tested with the developed assays. Sample IZSVE/2013/4693-4f was characterized as wild-type *M. gallisepticum* by the MAMA-6/85-gapA assay and as the 6/85 vaccine strain with the other two methods, while sample IZSVE/2014/6259-35f was discriminated as the 6/85 vaccine with only the MAMA-6/85-lpd assay (Data Set S1).

The MAMA-ts11-plpA, MAMA-ts11-glpK, and MAMA-ts11-potC assays also showed high congruency (range of adjusted Rand coefficients, 0.761 to 0.887). Excluding the differences caused by the distinct sensitivity of the assays, contradictory results were found in two cases. Sample 99179 from Australia was characterized as a wild strain by MAMA-ts11-plpA and as a vaccine strain by the remaining two assays. It is also notable that sample IZSVE/2013/4693-4f from Italy showed the mutations specific for the ts-11 vaccine with the MAMA-ts11-glpK assay but was characterized as a field strain with the rest of the assays.

In the case of assays differentiating strain F from *M. gallisepticum* field isolates, only one strain (MYCAV391) of the 221 tested *M. gallisepticum* samples was characterized as a vaccine type. Two tests, MAMA-F-hlp2 and MAMA-F-crmA, showed maximum congruency (adjusted Rand coefficient, 1.000) (Data Set S1).

Multilocus sequence typing and strain-specific PCR. A total of 126 samples (including *M. gallisepticum* vaccine strains, the type strain, 19 *M. gallisepticum* publicly available whole-genome sequences, 14 strains used for validation, and 89 samples used for a blind test) were analyzed by MLST. The eight developed typing methods showed high congruency with the MLST (range of adjusted Rand coefficients, 0.896 to 1.000), taking into consideration the sensitivity of the assays.

Out of the three samples which showed incongruent results with the vaccine-differentiating assays, IZSVE/2014/6259-35f showed the 6/85 vaccine type with only

MAMA-6/85-lpd, and it was characterized as a wild-type strain by MLST. IZSVE/2013/4693-4f showed the mutations specific for ts-11 vaccine with the MAMA-ts11-glpK assay and for the 6/85 vaccine with MAMA-6/85-lpd and PCR-6/85-crmA, while based on MLST analysis, it proved to be a field isolate closely related to strain 6/85 (7/2,636 nucleotide differences from 6/85 on 1 of 6 examined genes). Sample 99179 showed the vaccine type by the MAMA-ts11-glpK and MAMA-ts11-potC assays, while based on MLST analysis, it proved to be a field isolate closely related to strain ts-11 (10/2,636 nucleotide differences from ts-11 on 3 of 6 examined genes).

The ts-11 vaccine-specific genotype was determined for strain K6216D based on *in silico* analysis of the targeted mutations in the strain's whole-genome sequence (GenBank accession no. [MATM00000000](#)). However, MLST analysis defined a unique sequence type (ST50) for this strain, differing in only one nucleotide from the ts-11 MLST profile (23). Similarly, strain IZSVE/2013/4957-D5d (MLST ST48), which originated from a chicken sample from Italy in 2013, also differed only in one nucleotide from the ts-11 MLST profile (23). This strain showed the ts-11 genotype by the MAMA-ts11-glpK and MAMA-ts11-potC assays but proved to be false negative by the MAMA-ts11-plpA assay. In the case of vaccine 6/85, the vaccine-specific genotype was determined for strain IZSVE/2014/1779-12f in the blind test of the developed assays. This strain belonged to the MLST (ST13) most similar to the 6/85 MLST profile, showing only two nucleotide differences on one allele.

According to the method of Ricketts et al. (21), out of the 12 examined *M. gallisepticum* samples, all six Australian samples and one from Italy (IZSVE/2013/3185-5f) were characterized as ts-11 isolates, the results of which reveal poor agreement with the MLST and MAMA-ts11-plpA, MAMA-ts11-glpK, and MAMA-ts11-potC assays (range of adjusted Rand coefficients, 0.198 to 0.327) (Data Set S1).

DISCUSSION

M. gallisepticum infections have great impact on the poultry industry, and vaccination is a cost-effective option to reduce economic losses. The use of *M. gallisepticum* live vaccines led to the need for a reliable technique which can differentiate vaccine strains from wild-type isolates. This is crucial in epidemiological investigations, vaccination, animal trading, and eradication programs.

DNA fingerprinting methods have limitations, such as low reproducibility, a lengthy procedure, and the lack of comparable data between laboratories (14, 15). Other sequence-based methods can only differentiate *M. gallisepticum* vaccine strains from strains of limited genetic variability, or they are time- and resource-intensive processes or require the isolation of pure cultures (16–20, 22).

This study revealed mutations in *M. gallisepticum* vaccine strains that are absent in R_{low} and other publicly available *M. gallisepticum* field isolates. Targeted mutations are located in genes whose significance in virulence has already been investigated. Cytadhesins, encoded by the *gapA* and *crmA* genes, play a major role in *M. gallisepticum* host colonization and virulence (32). The *hlp2* gene, similar to *hlp3*, encodes a cytodherence-associated protein (high-molecular-weight 2-like protein), while *plpA* encodes pneumoniae-like protein A, which is capable of binding fibronectin (35). The dihydro-lipoamide dehydrogenase (encoded by *lpd*), a component of the pyruvate dehydrogenase complex, is also identified as a virulence-associated determinant, as it is required for *in vivo* growth and survival in the host (33). The glycerol kinase gene (*glpK*) has a role in H_2O_2 production, thereby affecting host cell cytotoxicity (30, 37). PotC is the permease component of the ABC-type spermidine/putrescine transport system; however, direct evidence of its role in virulence is lacking. The plasticity of the ABC transporter component genes is likely important for survival in the host environment (30). As numerous factors have a role in virulence and its alteration, several mutations were targeted by the assays designed in the present study.

Real-time and conventional PCR assays were developed for the detection of these vaccine-specific candidate mutations, and the assays were tested on 258 highly diverse *M. gallisepticum* strains and clinical samples (including vaccine strains, the type strain,

and whole-genome sequences). The diversity and genetic relatedness of 126 *M. gallisepticum* samples were previously investigated using an MLST assay, determining strains with identical genotypes as 6/85, ts-11, or F vaccine strains (23). Considering the different sensitivities of the assays, congruent results were observed among the assays developed in this study for the differentiation of vaccine strains 6/85, ts-11, and F from field isolates and the MLST results as well. However, evaluation of additional F strain reisolates should further increase the reliability of the presented assays. Dissimilar genotype calls of the eight assays and comparison of the results with MLST indicate that MAMA-6/85-gapA is the most reliable assay to distinguish strain 6/85, while MAMA-ts11-plpA proved to be the most reliable assay for the discrimination of the vaccine ts-11 (Data Set S1).

In the case of vaccine type ts-11, samples harboring at least one SNP specific to strain ts-11 and/or originating from the same country (Australia) as the parent strain of the ts-11 vaccine were checked with PCR systems specific to ts-11 sequences described by Ricketts et al. (21). The disagreement was remarkable between the results from assays developed in the current study and those from the PCR systems of Ricketts et al. (21), as all five Australian wild-type samples showed the ts-11-specific regions, while 5 of 6 samples containing ts-11-specific SNP lacked the ts-11-specific sequences. The interpretation of negative results is difficult because besides the presence of the specific regions in the samples, the quality of the DNA and the sensitivity of the PCR systems also influence the results. Although the detection limit is not published for the PCR systems of Ricketts et al. (21), according to our results, the detection limit of these assays was similar to that of the currently developed assays for the detection of the vaccine strain (10^3 template copies/reaction). As with the PCRs of Ricketts et al. (21), the developed assays were unable to discriminate ts-11 strains with reverted virulence, as all nonvirulent and virulent ts-11 reisolates contained the targeted mutations according to the sequences available at GenBank.

It is noteworthy that in the case of a ts-11 reisolat with reverted virulence (strain K6216D, isolated from a progeny flock of a ts-11-vaccinated broiler flock which was not distinguishable from the ts-11 vaccine strain by previous DNA sequence and RAPD analyses [10]), a unique MLST was determined before (23). Likewise, the MLST system could distinguish other closely related (differing at 1 to 10 positions in the examined 2,636-bp-long concatenated sequences) strains from vaccines ts-11 and 6/85, which showed the vaccine type (MLST difference, 1 to 2 positions) or incongruent results (MLST difference, 7 to 10 positions) with the assays developed in this study. Among the currently available molecular tools, the combined use of the presented assays provides a feasible option for the rapid differentiation of vaccine strains from field isolates with high approximation.

The developed assays aim to support routine diagnostics by determining the successful vaccination of the animals or confirming the *M. gallisepticum*-free status of a flock. Based on the diagnostic application of previously established MAMAs for the discrimination of live *Mycoplasma* vaccine strains from wild strains (38), submitting the DNA pool of samples from a small group of animals (at least 4 pools from 20 birds/house) to test the presence of the vaccine/pathogen is the most appropriate method to reflect the status of a flock. In order to achieve the most definite results of the discrimination of *M. gallisepticum* vaccine and wild-type strains, the combined use of all presented PCR tests is recommended. Nonspecific melting temperatures or band sizes should be omitted from the analyses. During the interpretation of the results, congruent data indicate the presence of the vaccine strains.

The developed method is highly specific; thus, it is applicable directly on clinical samples, avoiding technical problems associated with isolation, which is particularly important in the case of mycoplasmas. However, due to the moderate sensitivity of certain assays, clinical specimens with lower DNA loads may show false-negative results, and in these cases, strain isolation or enrichment may be required. The presented assays are suitable for the detection of mixed infections and show a sensitivity similar to those of the wild-type and vaccine type strains. Further advantages of the assays are that they were all

designed with the same thermal profile, allowing their simultaneous application, and they can be performed on basic real-time PCR platforms (without high-resolution melt function) and on conventional PCR equipment coupled with agarose gel electrophoresis. The strain-specific methods for the 6/85, ts-11, and F vaccines reported here represent convenient, rapid, and cost-efficient tools for control programs against *M. gallisepticum* infections.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/JCM.01084-18>.

SUPPLEMENTAL FILE 1, PDF file, 0.1 MB.

SUPPLEMENTAL FILE 2, XLSX file, 0.1 MB.

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