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

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Running Head: *Mycoplasma gallisepticum* DIVA

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Abstract

Mycoplasma gallisepticum is among the economically most significant mycoplasmas causing production losses in poultry.

Seven melt-curve and agarose gel based mismatch amplification mutation assays (MAMA) and one polymerase chain reaction (PCR) are provided in the present study to distinguish the *M. gallisepticum* vaccine strains and field isolates based on mutations in *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. Comparison of the data of 126 samples (including sequences available at GenBank) examined by the developed assays and a recently developed multi-locus sequence typing assay showed congruent typing results. The sensitivity of melt-MAMA assays varied between 10^1-10^4 *M. gallisepticum* template copy number/reaction, while that of the agarose-MAMAs ranged between 10^3 and 10^5 template copy number/reaction and no cross-reactions occurred with other *Mycoplasma* species colonizing birds. The presented assays are also suitable to discriminate 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 that they can be performed directly on clinical samples and in laboratories with basic PCR equipment.

Keywords: *Mycoplasma gallisepticum*, vaccine ts-11, vaccine 6/85, vaccine F, MAMA

Introduction

Infection with *M. 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. gallisepticum* vaccination are bacterins, live vaccines and a *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 USA (5, 6). The temperature-sensitive vaccine strain ts-11 was selected from an Australian field isolate (strain 80083) after chemical mutagenesis (7). F strain was probably first isolated in 1956 in the USA (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) (15). However, these methods have low reproducibility and they require the isolation of the tested organisms. Sequence-based methods for the higher reproducibility and reliability, lower labour intensity and for the applicability on clinical samples have been designed also, such as gene-targeted sequencing (16), 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 highly expensive. Recently, polymerase chain reaction (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 multi-locus sequence typing (MLST) system with improved discriminatory power have 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 been released also, which proved to be suitable for the 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 of MLST analysis (23) and PCR assays described by Ricketts et al. (2016), 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. *M. gallisepticum* type strain (ATCC 19610) was used as 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) (Dataset S1). The field isolates originated from tracheal swabs or lung samples of unvaccinated turkeys and chickens. Ethical 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 with 5% CO_2 atmosphere. Filter cloning was used to gain pure cultures from the isolates.

The DNA was extracted from the strains using the QIAamp DNA Mini kit (Qiagen, Inc., Hilden, Germany) according to the manufacturer's instructions. All isolates were identified by 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 BLAST search.

A further 185 *M. gallisepticum* strains (cultures or DNA; Italy, n=75; Spain, n=42; United Kingdom, n=22; Israel, n=20; USA, 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 of clinical samples (Spain, n=17; Israel, n=8; Italy, n=6; Iraq, n= 3; Albania, n=1 and Jordan, n=1) were provided for blind test from sample collections (Dataset 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 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 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 reference genome and annotated by Geneious software version 10.2.3 (29). The average numbers of reads and read lengths were 215,429 reads and 167.7 bp. The mean coverage was 45.7 and 31.3 for the whole genome of 6/85 and ts-11 strains, respectively.

Candidate genes were selected according to previous publications (30–35). Candidate genes were retrieved from the genomes of *M. gallisepticum* ts-11, 6/85 and F vaccine strains (GenBank Acc. N.: NC_017503.1) and published *M. gallisepticum* genomes (strain S6, GenBank Acc. N.: NC_023030.2; strain R_{low} GenBank Acc. N.: AE015450.2; strain R_{high} GenBank Acc. N.: NC_017502.1, house finch isolates, GenBank Acc. N.: NC_018412.1, NC_018409.1, NC_018406.1, NC_018407.1, NC_018408.1, NC_018410.1, NC_018411.1, NC_018413.1 and ts-11 re-isolates, GenBank Acc. N: MAFU00000000, MAFV00000000, MAFW00000000, MADW00000000, MATM00000000, MATN00000000, MAGQ00000000, MAGR00000000) and aligned by Geneious (29) (Dataset S1). The validity of single nucleotide polymorphisms (SNP) 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 Acc. N.: AE015450.2). SNPs and mutations present in

146 one of the *M. gallisepticum* vaccine strains (6/85, ts-11, F) but absent in other publicly
147 available *M. gallisepticum* strains were selected for primer design.
148
149 *Assay design*
150 MAMA (Mismatch Amplification Mutation Assay) is a PCR-based technique used for SNP
151 discrimination in many bacteria (36). In brief, the technique is based on SNP specific primers
152 at the 3′ end, one being marked with an additional 15–20 bp long GC-clamp at the 5’ end. A
153 single destabilizing mismatch at the 3′ end of each allele-specific primer enhances the
154 discriminative capacity of the assay. The GC-clamp increases the melting temperature and the
155 size of the amplicon as well. The temperature shift can be easily detected in the presence of
156 intercalating fluorescent dye on a real-time PCR platform (Melt-MAMA) and the difference
157 in the sizes of the amplicons can be observed in agarose gel electrophoresis (Agarose-
158 MAMA), which enable the differentiation of the SNP-specific genotypes.
159 In the present study, MAMAs and a PCR (amplifying products with different length) were
160 designed and tested for the detection of *M. gallisepticum* vaccine-specific alterations. Melt-
161 MAMA tests and the melt analysis of a PCR assay were optimized on Applied Biosystems
162 Step-One Plus real-time PCR system with StepOne Software version 2.3 (Thermo Fisher
163 Scientific, Waltham, MA, USA). Primer melting temperature (Tm) and general suitability
164 were calculated using NetPrimer (Premier Biosoft International, Palo Alto, CA). The primer
165 sequences and thermocycler parameters for the assays can be found in Table 1.
166 PCR mixture of Melt-MAMAs and the PCR-6/85-crmA (analysed by melting) consisted of 2
167 µl 5X Color-less GoTaq Flexi Buffer (Promega Inc., Madison, WI), 1 µl $MgCl_2$ (25 mM), 0.3
168 µl dNTP (10 mM, Qiagen Inc., Valencia, CA), 0.5 µl EvaGreen (20X, Biotium Inc., Hayward,
169 CA), primers (10 pmol/ µl, according to Table 1), 0.08 µl GoTaq G2 Flexi DNA polymerase
170 (5 U/µl; Promega Inc.), nuclease-free water and 1 µl DNA template with a final volume of 10

µl. Thermocycling parameters were 95 °C for 10 min, followed by 30 or 40 (according to Table 1) cycles 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 (analysed by gel-electrophoresis) were performed in C1000™ Touch Thermal Cycler (Bio-Rad Laboratories, Inc., Berkeley, CA, USA) in 25 µl total volume containing 1 µl target DNA diluted in 5 µl 5X Green GoTaq Flexi Buffer (Promega Inc.), 2.5 µl $MgCl_2$ (25 mM, Promeg Inc.), 0.5 µl dNTP (10 mM, Qiagen Inc.), primers (10 pmol/µl) according to Table 1, 0.25 µl GoTaq G2 Flexi DNA polymerase (5 U/µl; 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 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 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, the type strain and with the DNA of *M. gallisepticum* field isolates originating from unvaccinated flocks (n=14, Dataset S1).

In order to test the sensitivity of the assays, tenfold dilutions of each genotype were used in the range of 10^6–10^0 copy number/µl. Template copy number was determined by a qPCR system targeting the *mgc2* gene, which is present as a single copy in the *M. gallisepticum* genome (25). The tenfold dilution series of a synthetic sequence (500ng; gBlock, Integrated DNA Technologies Inc., Coralville, IA) was used as control for the template copy number determinations, which contain a 94 bp long fragment of the *mgc2* gene (between nucleotides 220622-220716, according to nucleotide numbering of the *M. gallisepticum* strain NCTC 10115 at GenBank). The lowest template copy numbers yielding melting temperature (Tm) specific to the genotypes were considered 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), *M.* sp. 1220 ("*M. anserisalpingitis*", 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, F) were also included as control in all vaccine strain-specific assays.

In order to assess the capability of the assays to identify 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: constant template copy numbers (10^6 copies/µl) of one strain was paired with a member of a series of 10-fold DNA dilutions (10^6-10^3 copies/µl) 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, multi-locus sequence typing and strain-specific PCR

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). MLST profiles of the live vaccine strains, the type strain and the 19 publicly available *M. gallisepticum* genomes has been defined also (23). Based on the genetic relatedness to vaccine strains four MLST profiles are presented in the current study: 6/85, ts-11, F and wild type (no relatedness to any of the live vaccine strains detected). Genotype calls of the presented assays were compared with genotype assignment of the MLST (Dataset S1).

Samples which appeared to be ts-11 re-isolates by the developed MAMA tests and/or originating from Australia were further tested according to Ricketts et al. (21). In brief, the presence of three additional genes (*vlhA*3.04a, *vlhA*3.05 and *mg03659*) was investigated for the discrimination of field isolates from ts-11 vaccine strains.

Accession numbers. Nucleotide sequences of *M. gallisepticum* amplicons included in the MLST were submitted to National Center for Biotechnology Information (NCBI) under GenBank Accession Numbers MH544230-MH544241 and MK288880- MK289516.

Statistical analyses

Adjusted Rand co-efficient 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.

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 non-synonymous mutations were targeted with 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 an ABC transporter protein (*potC*). Selection of the assays was performed according to preliminary examinations using the following criteria: 1) peaks of the melting curves of the vaccine and wild type strains were distinguishable; 2) the peak of the negative control did not overlap the peaks of the vaccine or the wild type strains; 3) the mutation was specific to the vaccine or vaccine re-isolates 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. Melting temperatures and sizes of amplicons are listed in Table 2 and shown in Figure 1.

Detection limit of melt-MAMA assays varied between 10^1-10^4 template copy number/reaction, while agarose-MAMAs changed between 10^3 and 10^5 template copy number/reaction depending on the assay and the genotype. Negative controls or templates of other avian *Mycoplasma* species were either not amplified or generated non-specific products with melt-profiles differing from the profiles of the expected two allelic states. The non-specific melting temperatures or band sizes should be omitted from further analyses. Assays differentiating one of the vaccine strains (6/85 or ts-11 or F) resulted in 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 similar sensitivity to the wild type and vaccine type *M. gallisepticum* DNA. Two assays specific for the ts-11 vaccine strain (MAMA-ts11-glpK and –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 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 (Figure 1).

In vitro stability tests were based on the comparison of genotype calls of the 10^{th} clones of the three vaccine and the 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 wide range of cycle treshold (Ct) values in the *mgc2* gene based qPCR (25)

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(Dataset S1). In samples with higher Ct values (usually Ct values above 20 in the *mgc2* gene based qPCR) the non-specific 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 (Figure 1B and D). The non-specific 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 (Ct values above 28 in the *mgc2* gene based qPCR (25)), thus these samples were omitted from further analysis. Further 9 DNA samples (Ct 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) (Dataset 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 in the case of this assay (n=50, Ct values above 15 in the *mgc2* gene based qPCR (25)).

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

MAMA-ts11-plpA, MAMA-ts11-glpK and MAMA-ts11-potC assays also showed high congruency (range of adjusted Rand co-efficients: 0.761-0.887). Excluding the differences caused by the distinct sensitivity of the assays, contradictory results were found in two cases. Sample 99179 from Australia was characterised as wild strain by MAMA-ts11-plpA and

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vaccine strain by the remaining two assays. It is also notable, that sample IZSVE/2013/4693-4f from Italy showed the mutations specific for ts-11 vaccine with assay MAMA-ts11-glpK, but was characterised as 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 were characterised as vaccine type. The two tests (MAMA-F-hlp2 and MAMA-F-crmA) showed maximum congruency (adjusted Rand co-efficient: 1.000) (Dataset 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 blind test) were analysed by MLST. The eight developed typing methods showed high congruency with the MLST (range of adjusted Rand co-efficient: 0.896-1.000), 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 characterised as wild type strain by MLST. IZSVE/2013/4693-4f showed the mutations specific for ts-11 vaccine with assay MAMA-ts11-glpK and for 6/85 vaccine with MAMA-6/85-lpd and PCR-6/85-crmA, while based on MLST analysis it proved to be a closely related field isolate to strain 6/85 (7/2636 nucleotide differences from 6/85 on 1 of 6 examined genes). Sample 99179 showed the vaccine type by MAMA-ts11-glpK and MAMA-ts11-potC assays, while based on MLST analysis it proved to be a closely related field isolate to strain ts-11 (10/2636 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 Acc. N.:

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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 sequence type (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. (2016) out of the 12 examined *M. gallisepticum* samples, all six Australian samples and one from Italy (IZSVE/2013/3185-5f) were characterised as ts-11 isolates, which results reveal poor agreement with the MLST and assays MAMA-ts11-plpA, MAMA-ts11-glpK and MAMA-ts11-potC (range of adjusted Rand co-efficients: 0.198-0.327) (Dataset 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, 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

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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 *gapA* and *crmA* genes play a major role in *M. gallisepticum* host colonization and virulence (32). Gene *hlp2*, similar to *hlp3*, encodes cytadherence-associated protein (high molecular weight 2-like protein), while *plpA* encodes Pneumoniae-like protein A which is capable of binding fibronectin (35). The dihydrolipoamide dehydrogenase (encoded by *lpd*), a component of the pyruvate dehydrogenase complex is also identified as 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. 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 also). The diversity and genetic relatedness of 126 *M. gallisepticum* samples were previously investigated by 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 re-isolates should further increase the reliability of the presented assays. Dissimilar genotype calls of the eight assays and comparison of the results with MLST sequence types 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 (Dataset S1).

In the case of vaccine type ts-11, samples harbouring at least one SNP specific to strain ts-11 and/or originating from the same country (Australia) as the parent strain of 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 of assays developed in the current study and 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 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 copy number/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 non-virulent and virulent ts-11 re-isolates contained the targeted mutations according to the sequences available at GenBank.

It is noteworthy, that in the case of a ts-11 re-isolate with reverted virulence (strain K6216D, isolated from a progeny flock of a ts-11 vaccinated broiler flock which was not distinguishable from ts-11 vaccine strain by previous DNA sequence and RAPD analyses (10)) unique MLST ST was determined before (23). Likewise, the MLST system could distinguish other, closely related (differing at 1-10 positions in the examined 2636bp long

concatenated sequences) strains from vaccines ts-11 and 6/85, which showed the vaccine type (MLST ST difference: 1-2 positions) or incongruent results (MLST ST difference: 7-10 positions) with the assays developed in this study. Among the currently available molecular tools, the combined use of the presented assays provides 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 *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. Non-specific 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 load may show false negative results, and in these cases strain isolation or enrichment may be required indeed. The presented assays are suitable for the detection of mixed infections and show similar sensitivity to 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 6/85, ts-11 and F

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vaccines reported here represent convenient, rapid and cost-efficient tools for control programs against *M. gallisepticum* infections.

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568 the temperature sensitive (ts+) MS-H vaccine strain and wild-type *Mycoplasma*
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570

571 **Figure legends**

572 **Figure 1:** Detection of A1306G substitution in *gapA* gene. y-axis: Derivative reporter, the
573 negative first-derivative of the normalized fluorescence generated by the reporter during PCR
574 amplification. x-axis: Temperature melt curve. A) Melting curves of the melt-MAMA
575 showing melting temperatures of 6/85 vaccine strain (red line; Tm 80.3±0.1 ºC) and *M.*
576 *gallisepticum* reference strain (ATCC 19610; blue line; Tm 76.0±0.6 ºC). Negative controls
577 (grey lines) may show non-specific amplicons above Ct 33±2.9 (Tm 71.8±0.3 ºC) or no
578 amplicons. B) Samples containing lower amounts of wild-type *M. gallisepticum* DNA can
579 form a bimodal melting peak by the melt-MAMA: next to the wild type specific melting peak
580 (sample IZSVE/2015/2062-4f with approximately 10^3 template copy number/µl; 76.0±0.6 ºC),
581 the peak of negative sample (Tm 71.8±0.3 ºC) also appears (green line). C) Samples
582 containing mixed DNA of 6/85 vaccine (10^5 template copy number/µl) and wild type strain
583 (10^6 template copy number/µl) can form bimodal melting peak by the melt-MAMA: next to
584 the wild type specific melting peak (76.0±0.6 ºC), the peak of 6/85 (Tm 80.3±0.1 ºC) also
585 appears (green line). D) PCR product sizes of MAMA-6/85-gapA in agarose gel.
586 Electrophoresis was performed in 3% agarose gel (MetaPhor Agarose, Lonza Group Ltd.,
587 Basel, Switzerland) and a 20-bp DNA ladder (O'RangeRuler 20 bp, Thermo Fisher Scientific
588 Inc.) was used as molecular weight marker (m). Line 1: non-specific amplicons in the

negative control below 60 bp, Line 2: 99 bp fragments specific for 6/85 vaccine strain, Line 3: wild-type strains yielded 85 bp fragments, Line 4: sample containing lower amount of wild-type *M. gallisepticum* DNA (sample IZSVE/2015/2062-4f with approximately 10^3 template copy number/µl), Line 5: sample containing mixed DNA of 6/85 vaccine (10^5 template copy number/µl) and wild type strain (10^6 template copy number/µl).

Tables

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	Gene	Mutation[a]	Assay name	Primer name	Primer sequence (5'→3')	Primer volume (µl)[b]		Cycle number	
						melt-MAMA	agarose MAMA	melt-MAMA	agarose MAMA
6/85	*lpd*	G1372T (A→S)	MAMA-6/85-lpd	lpd-1372-6/85	ggggcggggcggggGTTTTTTGTTRAAGTGGTTATAAATCGA	0.15	1	40	40
				lpd-1372-wt	GTTTTTTGTTRAAGTGGTTATAAATAGC	0.6	4		
				lpd-1372-con	GAACAAGCAATTCACCCACACC	0.15	1		
	gapA	A1306G (R→G)	MAMA-6/85-gapA	gapA-1315-6/85	ggggcggggcggggGGTGTTTTTAGAACTAAATTTGAAATCG	0.15	1	40	40
				gapA-1315- wt	GGTGTTTTYAGAACTAAATTTGAAAGCA	0.15	1		
				gapA-1315-con	ATAAAATACCGTATGGATAACCAACAG	0.15	1		
	crmA	48 nt del 16 aa del	PCR-6/85-crmA	PCR-crmA-F	TGCTGCTGCTAAACCTGGTGC	0.15	1	40	40
				PCR-crmA-R	GGAGCGGTTGGTTTTGGAGCA	0.15	1		
ts-11	*plpA*	C953G (T→S)	MAMA-ts11-plpA	plpA-971-ts11	ggggcggggcggggGCTTCTAGATGAGGTGTGATTGTGC	0.15	1	30	40
				plpA-971- wt	GCTTCTAGATGAGGTGTGATTGAGG	0.15	4		
				plpA-971-con	GGATTATTACCTGAACTTGCCACAG	0.15	1		
	glpK	G67A (D→N)	MAMA-ts11-glpK	glpK-67-ts11	ggggcggggcggggACATCTTGTCGTTCAATCGTTTGTA	0.15	1	40	40
				glpK-67- wt	ACATCTTGTCGTTCAATCGTTTCTG	0.15	1		
				glpK-67-con	GGAAAGTATTGCGTAAATTCGTTTTG	0.15	1		
	potC	C526G (Q→E)	MAMA-ts11-potC	potC-526-ts11	ggggcggggcggggATGAACCCAAATCTAATCTTAGCTTTAG	0.15	1	30	40
				potC-526- wt	ATGAACCCAAATCTAATCTTAGCTTAAC	0.6	4		
				potC-526-con	GCGGGTGTTAAATAAGATAGAGTAATCT	0.15	1		
F	*hlp2*	G5542C (E→Q)	MAMA-F-hlp2	hlp2-5542-F	ggggcggggcggggGTCTTAGTGTGGTTTTTTTAATCTTGTG	0.15	1	40	40
				hlp2-5542- wt	GTCTTAGTGTGGTTTTTTTAATCTTCTC	0.15	1		
				hlp2-5542-con	GAAGTGCAAAAGAAATTAACTGATCTG	0.15	1		
	crmA	C2116G (Q→E)	MAMA-F-crmA	crmA-2116-F	ggggcggggcggggACAACCATTCGGAACAACTCTCG	0.15	4	40	40
				crmA-2116- wt	ACAACCATTCGGAACAACTCACC	0.15	1		
				crmA-2116-con	CTAATATTCTTAATTGATGAGAACTGATCAC	0.15	1		

[a] according to *M. gallisepticum* R_{low} (GenBank Acc. N.: AE015450.2) nucleotide numbering, amino acid changes are indicated in parenthesis

[b] Primer (10 pmol/µl) volume in 10 µl (melt-MAMA) and in 25 µl reaction mixture (agarose-MAMA)

Table 2: Results of the validation of assays designed in the present study based on the analyses of 18 *M. gallisepticum* strains

Vaccine	Assay name	Genotype	Tm (°C)	Amplicon length (bp)	NTC	Sensitivity (template copy number/reaction)[a]				Cross reaction[b]	Mixed samples (vaccine:wild type) [template copy number/reaction]						
						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
						v	wt	v	wt								
6/85	MAMA-6/85-lpd	6/85	79.8±0.1	102	Tm 72.4±0.1 °C	10^3	10^3	10^4	10^4	-	6/85	6/85	6/85	bm	wt	wt	wt
		wt	76.5±0.3	88	Ct 29±0.2												
	MAMA-6/85-gapA	6/85	80.3±0.1	99	-/Tm 71.8±0.3 °C	10^2	10^3	10^3	10^4	-	6/85	6/85	6/85	bm	wt	wt	wt
		wt	76.0±0.6	85	Ct 33±2.9												
	PCR-6/85-crmA	6/85	82.2±0.1	90	Tm 77.2±0.1 °C	10^4	10^4	10^4	10^5	-	6/85	6/85	6/85	6/85	bm	wt	wt
		wt	84.8±0.4	123-138	Ct 24.1±0.4												
ts-11	MAMA-ts11-plpA	ts-11	82.2±0.0	82	-	10^3	10^3	10^4	10^4	-	ts-11	ts-11	bm	bm	wt	wt	wt
		wt	77.5±0.1	68													
	MAMA-ts11-glpK	ts-11	79.3±0.1	94	-/Tm 61.3±0.0 °C	10^1	10^1	10^3	10^3	-	ts-11	ts-11	ts-11	bm	wt	wt	wt
		wt	76.3±0.1	80	Ct 32.5±0.7												
	MAMA-ts11-potC	ts-11	77.6±0.1	106	Tm 72.8±0.3°C	10^4	10^3	10^4	10^4	-	ts-11	ts-11	bm	bm	wt	wt	wt
		wt	74.4±0.1	92	Ct 30.1±0.2												
F	MAMA-F-hlp2	F	78.0±0.1	102	-/ Tm 70.7±0.1 °C	10^3	10^2	10^3	10^3	-	F	F	F	bm	wt	wt	wt
		wt	74.0±0.1	88	Ct 37.1±0.2												
	MAMA-F-crmA	F	81.0±0.0	89	Tm 80.4, 72.6 °C	10^4	10^3	10^4	10^4	-	F	F	bm	wt	wt	wt	wt
		wt	75.5±0.2	75	Ct 31.8±0.9												

Abbreviations: bm= bimodal peak indicating the presence of both genotypes in the sample; Ct= cyclic threshold; NTC= negative control; Tm= melting temperature; v= vaccine; wt= wild type; "-"=non-detected.

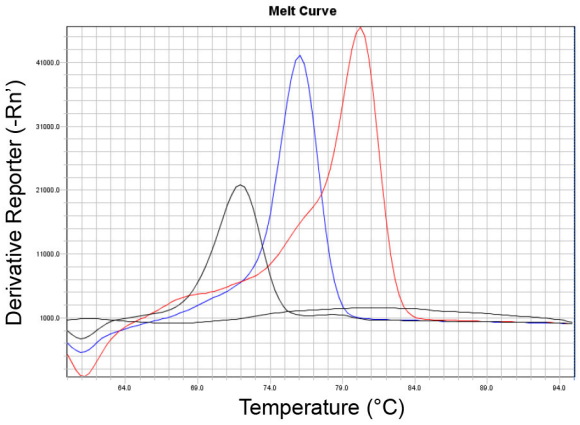
[a] tested with both genotypes

[b] tested with the following avian mycoplasma species: *M. anatis* (ATCC 25524), *M. anseris* (ATCC 49234), *M.* sp. 1220 *("M. anserisalpingitis",* ATCC BAA-2147), *M. cloacale* (ATCC 35276), *M. columbinasale* (ATCC 33549), *M. columbinum* (ATCC 29257), *M. columborale* (ATCC

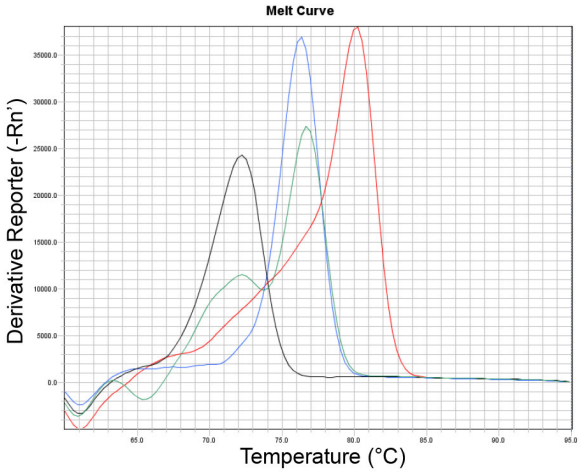
29258), *M. gallinaceum* (ATCC 33550), *M. gallinarum* (ATCC 19708), *M. gallopavonis* (ATCC 33551), *M. iners* (ATCC 19705), *M. iowae* (ATCC 33552), *M. meleagridis* (NCTC 10153) and *M. synoviae* (ATCC 25204) type strain

607

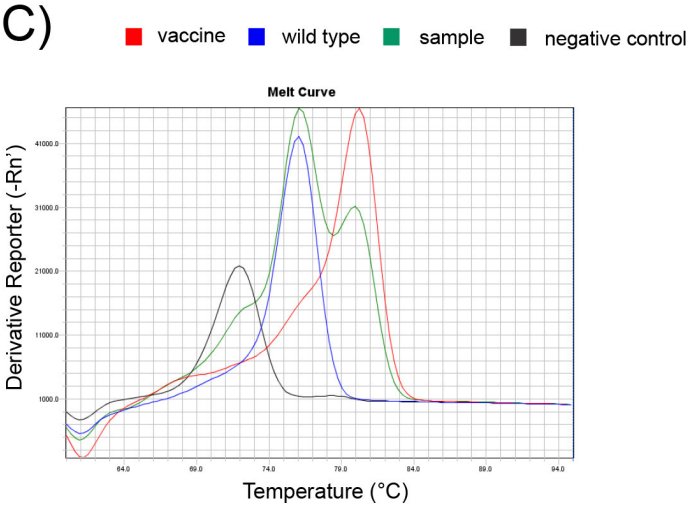
A)



B)



C)



D)