Differentiation of *Mycoplasma gallisepticum* strains using PCR and high-resolution melting curve analysis

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Mycoplasma gallisepticum (MG) is an economically important pathogen of poultry worldwide, causing chronic respiratory disease in chickens and turkeys. Differentiation of MG strains is critical, especially in countries where poultry flocks are vaccinated with live vaccines. In this study, oligonucleotide primers were designed based on a region preceding the trinucleotide repeat of a member of the *v/hA* gene family, and amplicons of 145–352 bp were generated from cultures of 10 different MG strains, including the ts-11, F and 6/85 vaccine strains. High-resolution melting (HRM) curve analysis of the resultant amplicons could differentiate all MG strains. Analysis of the nucleotide sequences of the amplicons from each strain revealed that each melting curve profile related to a unique DNA sequence. The HRM curve profiles (for ts-11) remained consistent after at least five passages under laboratory conditions. PCR-HRM curve analysis of 33 DNA extracts derived from respiratory swabs, or mycoplasma cultures grown from respiratory swabs, of ts-11-vaccinated commercial or specific pathogen-free chickens identified all these specimens, according to their sequences, as ts-11. The potential of the PCR-HRM curve analysis was also shown in the genotyping of 30 additional MG isolates from Europe, the USA and Israel. The results presented in this study indicate that PCR followed by HRM curve analysis provides a rapid and robust technique for genotyping of MG isolates/strains using both MG cultures and clinical swabs.

INTRODUCTION

Mycoplasma gallisepticum (MG) is an important pathogen of poultry worldwide, causing chronic respiratory disease in chickens and turkeys, reduction in egg production and considerable economic losses to the poultry industry (Ley, 2008). Diagnosis of MG infection can be made by various methods, but the gold standard test for confirmation of diagnosis is isolation and identification of the organism (Ley, 2008). Such examinations typically require 2–3 weeks to complete. PCR and, recently, real-time PCR have been used for rapid detection and/or identification of MG from cultures or directly from clinical samples (Callison *et al.*, 2006; Feberwee *et al.*, 2005b; Grodio *et al.*, 2008; Hess *et al.*, 2007; Nascimento *et al.*, 1991). Since live vaccines are used in many countries, differentiation of MG strains has become increasingly important. The live vaccine strains F and 6/85 (originated from the USA), and

ts-11 (originated from Australia), are used for control of MG infection in several countries (Ley, 2008). PCR followed by RFLP (Kiss *et al.*, 1997; Lysnyansky *et al.*, 2005) and amplified fragment length polymorphism (AFLP) (Cherry *et al.*, 2006; Hong *et al.*, 2005) have been used for identification of MG strains. However, these techniques may suffer from low reproducibility, are labour intensive and time consuming, and often require extensive interpretation, leading to a relatively expensive diagnostic test. Recently, real-time PCR has been used for the differentiation of one of five vaccine strains from a challenge strain, although this was restricted to comparison of paired strains in each test only (Raviv *et al.*, 2008). Differentiation of MG strains based on multiple surface protein genes has also been reported (Ferguson *et al.*, 2005). However, this technique requires nucleotide sequencing of amplified genes and subsequent analysis and interpretation of the results. Recent studies in our laboratory have found that a combination of PCR and high-resolution melting (HRM) curve analysis provides a rapid and cost-effective alternative to the direct analysis of nucleotide sequence variation, particularly when large numbers of samples are to be analysed (Hewson *et al.*, 2009; Jeffery *et al.*, 2007; Steer *et al.*, 2009).

Abbreviations: AFLP, amplified fragment length polymorphism; GCP, genotype confidence percentage; HRM curve, high-resolution melting curve; SPF, specific pathogen-free.

The GenBank/EMBL/DDBJ accession numbers for the sequences of *Mycoplasma gallisepticum* isolates and reference strains examined in this study are FJ654137, FJ654139–FJ654146 and FJ844437.

The *vlhA* intergenic region in the MG genome contains conserved and variable regions (Papazisi *et al.*, 2003). Preliminary examination of the *vlhA* intergenic region through sequences previously available in GenBank suggested the potential of this region for detection and differentiation of MG strains.

The aim of the present study was to investigate the potential of HRM curve analysis of PCR amplicons generated from the *vlhA* intergenic region to establish a single closed-tube test method for the differentiation of MG strains. Both conventional and normalized dissociation plots were generated and genotyping applied to differentiate the MG isolates/strains, and the results were compared with DNA sequencing of PCR amplicons.

METHODS

MG strains. Twelve MG strains, including three vaccine strains (ts-11, 6/85 and F), the reference strain S6 and eight MG strains (from Australia or the USA) available in our laboratory, were used initially in this study (Table 1). All cultures were grown from a single colony in modified mycoplasma broth (Morrow *et al.*, 1998). All mycoplasma cultures were grown in mycoplasma broth containing 10% swine serum, 0.01% (w/v) NAD and 0.002% (w/v) herring sperm DNA (Sigma). The media base consisted of (per litre): 7.5 g trypticase peptone, 2.5 g phyton peptone, 0.5 g thiotone peptone (all from BBL), 5 g yeast extract (Difco), 5 g sodium chloride, 0.4 g potassium chloride, 0.35 g magnesium sulphate heptahydrate, 0.05 g disodium hydrogen orthophosphate, 0.1 g potassium dihydrogen phosphate, 1 g glucose, 1.5 ml of 1.6% phenol red, and 10 ml yeast autohydrolysate solution prepared from bakers' yeast. Mycoplasma agar was prepared as for mycoplasma broth, with the omission of glucose and phenol red, and solidified with 1.0% (w/v) Special Noble agar (Difco).

Also, three groups of MG strains from the USA, Europe and Israel were used to evaluate the newly developed PCR-HRM technique for its potential to differentiate MG strains from wider geographical locations (Table 4). These specimens were compared with each other and with MG strains analysed earlier in this study (Table 1).

DNA extraction. Total genomic DNA was extracted from mycoplasma cultures and from swabs taken from birds using a DNA extraction kit (Qiagen) according to the manufacturer's instructions. Genomic DNA was extracted from a commercial batch of Vaxsafe MG (Bioproperties Australia) and from the ts-11 master seed, which was previously passaged at least five times *in vitro*.

A group of 15 specific pathogen-free (SPF) chickens and two separate commercial layer chicken flocks were vaccinated with the ts-11 vaccine strain according to the manufacturer's instructions at 28 days of age within a positive pressure isolator and under field conditions, respectively. DNA was extracted from 15 tracheal swabs taken from SPF chickens 2 weeks post-vaccination and from 18 cultures grown from swabs taken from commercial layer chickens 7–12 weeks post-vaccination. Briefly, 0.5 ml mycoplasma culture was pelleted by centrifugation at 20 000 g for 5 min. The cells were washed twice in PBS and resuspended in 500 µl RLT lysis buffer (Qiagen), and incubated for 2 h at room temperature or overnight at 4 °C. Swabs taken from birds were also placed in 0.5 ml lysis buffer (Qiagen) and incubated at 4 °C overnight. Then, 15 µl Qiaex II matrix (Qiagen) and 300 µl 70% ethanol were added and mixed, and the lysate was loaded into a Multi-Spin MSK-100 column (Axygen), centrifuged for 30 s at 10 000 g, and the flow-through was discarded. The column was washed with 600 µl RW1 buffer (Qiagen) and twice with 500 µl RPE buffer (Qiagen), and subjected to centrifugation at 18 000 g for 90 s. The DNA was eluted from the matrix using 50 µl distilled water and used in PCR immediately or stored at –20 °C for future use.

PCR. The region preceding the trinucleotide repeat of a member of the *vlhA* gene family was chosen for amplification. This was based on the observation of poor conservation of nucleotides within this region among *vlhA* family members (Papazisi *et al.*, 2003). A pair of oligonucleotide primers, ts-11-F (5'-GTTTGGAGTTGGTGTATAGT-TAG-3') and ts-11-R (5'-TCTTCTTCGAAAACAAAGG-3'), flanking the target region was designed with the PCR amplicon expected to yield a product of 226 bp. Amplification of DNA was performed in a 25 µl reaction volume on an iCycler thermal cycler (Bio-Rad). The reaction mixture contained 3 µl extracted genomic DNA, 25 µM of each primer, 1.5 mM MgCl₂, 1250 µM of each dNTP, 5 µM SYTO 9 green fluorescent nucleic acid stain (Invitrogen), 1× Green GoTaq Flexi Reaction Buffer (Promega) and 1 U GoTaq DNA polymerase (Promega). PCR conditions were one cycle of 94 °C for 60 s, 35 cycles of 94 °C for 10 s, 50 °C for 10 s and 72 °C for 10 s, and a final cycle of 72 °C for 1 min. In each set of reactions, MG ts-11 genomic DNA and distilled H₂O were included as positive and negative controls,

Table 1. MG strains/isolates used in this study and their origin

Isolate or strain	Origin	GenBank accession no.	Reference or source
ts-11	Australia	FJ654144	Whithear <i>et al.</i> (1990)
F	USA	FJ654142	Carpenter <i>et al.</i> (1979)
S6	USA	FJ654143	Adler <i>et al.</i> (1957)
6/85	USA	FJ654146	Evans & Hafez (1992)
Ap3AS	Australia	FJ844437	Soeripto <i>et al.</i> (1989)
K1659	USA	FJ654145	Kleven <i>et al.</i> (1988b)
K1453	USA	FJ654137	Kleven <i>et al.</i> (1988b)
86134	Australia	FJ654139	This study
87006	Australia	FJ654140	This study
87081	Australia	FJ654141	This study
94001	Australia†	NA*	This study
93148	Australia†	NA	This study

*NA, Not applicable; nucleotide sequence was identical to ts-11.

†Flock vaccinated with ts-11.

Table 2. Mean $\pm$ SD of the melting points and GCP for each strain following PCR and HRM curve analysis

Genotype (number of times tested)	Number of isolates/batches tested	Peak 1 ($^{\circ}$ C)	Peak 2 ($^{\circ}$ C)	Peak 3 ($^{\circ}$ C)	GCP $\pm$ SD
ts-11 (125)	3	76.5 $\pm$ 0.3			96.1 $\pm$ 3.6
K1659 (30)	1	75.2 $\pm$ 0.1	76.9 $\pm$ 0.1		97.9 $\pm$ 2.5
K1453 (28)	1	77.7 $\pm$ 0.1			98.1 $\pm$ 1.5
87006 (21)	1	74.9 $\pm$ 0.1	79.1 $\pm$ 0.1		98.9 $\pm$ 0.3
87081 (18)	1	74.8 $\pm$ 0.9	76.4 $\pm$ 0.7	77.6 $\pm$ 0.4	95.0 $\pm$ 2.1
F (58)	1	78.2 $\pm$ 0.1			98.4 $\pm$ 1.6
S6 (28)	1	78.2 $\pm$ 0.1			99.2 $\pm$ 0.7
6/85 (38)	1	74.8 $\pm$ 0.1			96.9 $\pm$ 2.2
86134 (42)	1	75.3 $\pm$ 0.8	77.3 $\pm$ 0.3		98.4 $\pm$ 1.4
Ap3AS (21)	1	75.7 $\pm$ 0.1	77.1 $\pm$ 0.4		98.2 $\pm$ 1.2

respectively. The PCR products, together with a standard molecular mass marker, were analysed by electrophoresis in 1.7% agarose gels stained with GelRed (Biotium) and visualized by UV transillumination.

DNA from *Mycoplasma synoviae* (strain WVU-1853), a field isolate of *Mycoplasma meleagridis*, *Mycoplasma anatis*, *Mycoplasma gallinaceum* and *Mycoplasma gallinarum*, when tested by PCR (as described above), did not produce any detectable band on an agarose gel.

HRM curve acquisition and analysis. HRM curve analysis was performed in a Rotor-Gene 6000 thermal cycler (Corbett Life Science). In order to determine the optimal melting condition for differentiation of MG strains, the PCR products were subjected to three different rampings of 0.1, 0.2 and 0.3 $^{\circ}$ C s^{-1} between 72 and 82 $^{\circ}$ C. All specimens were tested in triplicate and their melting profiles analysed using Rotor-Gene 1.7.27 software and the HRM algorithm provided. Normalization regions of 73.5–74.0 and 79.5–80.0 were used for analysis. Each isolate was set as a ‘genotype’, and the average HRM genotype confidence percentage (GCP) (the value attributed to each isolate/strain compared with the genotype, with a value of 100% indicating an exact match) for the replicates was predicted by the software. The GCPs for MG ts-11 known isolates were averaged and the SDS calculated and used to establish the GCP range for the ts-11 vaccine strain cut off point. The cut off point was applied in HRM analysis to evaluate the differentiation power of the test to discriminate the ts-11 strain.

Sequencing and nucleotide sequence analysis of PCR amplicons. PCR amplicons were gel-purified using the QIAquick Gel Purification kit (Qiagen) following the manufacturer’s instructions. Purified amplicons were subjected to automated sequencing (BigDye Terminator v3.1, Applied Biosystems) in both directions, using the same primers as used for PCR. The sequences were analysed using CLUSTAL W (Thompson *et al.*, 1994) and DNADIST (Felsenstein, 1989) in BioManager (Australian National Genomic Information Service, Sydney Bioinformatics) and BioEdit Sequence Alignment Editor (version 6.0.9.0). GenBank accession numbers were assigned to the nucleotide sequences of the MG isolates and reference strains (Table 1).

RESULTS

PCR amplicons of different sizes generated from MG strains using oligonucleotide primers ts-11-F and ts-11-R

Amplified PCR products from different MG strains were analysed by gel electrophoresis (Fig. 1). MG strains ts-11,

S6, F and K1453 generated one major band, while 6/85, Ap3AS, K1659, 86134, 87006 and 87081 produced a major band with one or two minor bands visible on the agarose gel. The major amplicon band ranged in size from 145 (87006) to 352 bp (S6).

Conventional and normalized HRM curve analysis differentiates MG strains

The PCR amplicons from 10 different MG strains (Table 1) were subjected to HRM curve analysis (Fig. 2). Visual examination of the conventional melt curves at different ramp temperatures revealed that 0.3 $^{\circ}$ C s^{-1} resulted in most strains showing distinct conventional melt curves for MG strains. In the conventional melt curve, 10 distinct curve profiles were detected (Fig. 2a). MG vaccine strains ts-11 and 6/85 each generated a single peak at 76.5 and 74.8 $^{\circ}$ C, respectively (Table 2). The MG strains 86134 and Ap3AS generated only two major peaks at 75.3 and 77.3 $^{\circ}$ C, and 75.7 and 77.1 $^{\circ}$ C, respectively. The MG strains S6 and K1453 generated a major peak at 78.2 and 77.7 $^{\circ}$ C, respectively, and a shoulder peak at a lower temperature than their major peak. MG strains 87006 and K1659 generated a major peak at 74.9 and 75.2 $^{\circ}$ C, respectively, and a minor peak at a higher temperature

Fig. 1. Agarose gel electrophoresis of PCR products of the *vIhA* gene promoter region from different MG isolates/strains. MW, molecular weight marker (PCR Marker, Sigma).

Fig. 2. (a) Conventional melt curve analysis of PCR products, in duplicate, of the *v/hA* gene promoter region from different MG isolates/strains. (b) Normalized HRM curve analysis of PCR amplicons of the *v/hA* gene promoter region from different MG isolates/strains.

than their major peak. MG strain 87081 was the only strain that generated one major peak at 74.8 °C and two minor peaks at higher temperatures than the major peak.

All MG strains also generated 10 distinct normalized HRM curves (Fig. 2b). Replicates of each MG strain were genotyped with defined genotypes in a normalized HRM graph with a GCP of 95 or greater (Table 2).

HRM curve analysis for PCR amplicons using templates from DNA extractions and/or PCRs run on different days showed slight shifts in melting temperature; however, the conventional melt curve shapes and normalized HRM graphs were unchanged. The mean and SD of the melting points for the different peaks, and the GCP of SDs resulting from several runs of PCR and HRM curve analysis, are shown in Table 2.

Consistent HRM curve profile of MG after passage *in vitro* and *in vivo*

In order to evaluate the consistency of the newly developed PCR-HRM curve analysis technique following MG strain passage *in vitro* and *in vivo*, the MG ts-11 vaccine strain was used as a model. Highly similar conventional and normalized HRM curve profiles were generated from Vaxsafe MG (Bioproperties Australia) and the master seed

(results not shown). To assess the *in vivo* sensitivity and specificity of the developed PCR test, DNA from swabs taken from SPF and commercial layer chickens was subjected to PCR and HRM curve analysis. Both conventional and normalized curves were highly similar to those of ts-11 (Fig. 3), with a mean of 94.5 ± 2.0 and 87.0 ± 14.3 GCP for the 15 SPF and 18 commercial layer chicken specimens, respectively.

Fig. 3. (a) Conventional melt curve analysis of PCR products of the *vlhA* gene promoter region from strain ts-11 and MG isolates from ts-11-vaccinated birds. (b) Normalized HRM curve analysis of PCR amplicons of the *vlhA* gene promoter region from strain ts-11 and MG isolates from ts-11-vaccinated birds. Isolates 93148a, -b and -c and 94001a, -b and -c were field specimens collected 7–12 weeks post-vaccination with ts-11.

Detection of minor variations in *v/hA* nucleotide sequence by the newly developed PCR-HRM curve analysis technique

To confirm that the classification of MG strains by HRM curve analysis was related to variations in the nucleotide sequences of the amplicons, nucleotide sequences of amplicons from each distinct curve profile as well as from one MG isolate, one SPF chicken and one commercial layer chicken specimen were determined and compared. Some of the MG strains generated more than one DNA band on agarose gels (Fig. 1). In order to compare the nucleotide sequences of the amplicons from different MG strains, the major DNA band of each strain was gel-purified, sequenced and used for calculation of sequence identities. Only major DNA bands were examined, since the contribution of the other (minor) bands towards the overall sequence identity was difficult to determine, especially as the intensity of staining varied between the bands, most likely due to different numbers of amplicons per band.

The PCR amplicon for the S6 reference strain was 352 bp in length; in comparison, the ts-11 amplicon was 226 bp in

length with a 126 bp deletion at the 5' end (Fig. 4). Other strains with a distinct HRM curve profile contained deletions of from 33 to 207 nucleotides in their sequences, with a number of nucleotide substitutions throughout each of these sequences (Fig. 4). Comparison of the nucleotide sequences further revealed that PCR amplicons generated from isolates from the SPF and commercial layer chickens vaccinated with the ts-11 strain were identical to the MG ts-11 vaccine strain sequence (data not shown). The extent of sequence variability in PCR amplicons for the representative of each distinct group was determined and compared with the mean GCP of HRM analysis (Table 3).

The highest sequence identity (100 %) was observed between amplicon sequences from the 6/85, Ap3AS and 86134 strains, while the lowest (36.1 %) belonged to those of the K1659 and 87081 strains. The GCPs for the HRM curves related to 6/85/Ap3AS, 6/85/86134 and 86134/Ap3AS were 0.0, 0.0 and 13.2, respectively. The GCP for the HRM curves of K1659 and 87081 was 0.4. MG strains F and 87081, which also had high sequence identity (99.1 %) generated, however, two different conventional melt curves

Fig. 4. Comparison using CLUSTAL W of the nucleotide sequences of *v/hA* gene promoter region amplicons of MG isolates/strains. Identical nucleotides and deletions are shown by '.' and '-', respectively.

Table 3. Percentage sequence identity of MG isolates/strains compared with GCP of HRM analysis

Strain	Sequence identity (%)									
	S6	F	K1453	87081	ts-11	6/85	K1659	86134	87006	Ap3AS
S6	–	98.7	95.0	98.4	81.0	56.4	44.9	56.4	47.6	56.4
F	66.1	–	93.7	99.1	79.2	55.7	47.6	55.7	46.9	55.7
K1453	36.2	22.1	–	92.8	88.9	56.4	44.9	56.4	47.6	56.4
87081	1.5	0.1	0.8	–	79.2	56.4	36.1	56.4	42.8	56.4
ts-11	0.0	0.0	0.0	0.4	–	56.4	44.9	56.4	47.6	56.4
6/85	0.0	0.0	0.0	0.0	0.00	–	66.7	100.0	68.3	100.0
K1659	0.0	0.0	0.0	0.4	0.08	1.9	–	68.7	77.9	68.7
86134	4.8	0.4	5.6	64.2	0.57	0.0	0.0	–	68.3	100.0
87006	0.0	0.0	0.0	0.3	0.00	3.9	28.3	0.0	–	66.9
Ap3AS	0.1	0.0	0.0	39.2	0.69	0.0	2.3	13.2	3.2	–
	GCP									

with three peaks and one peak, respectively, and this difference was reflected in their normalized melt curves and GCP (0.1). Since the MG F strain produced one amplicon band while MG 87081 generated two, the difference in the shape of the normalized and conventional melt curves or the number of peaks in conventional melt curves was likely to be due to the contribution of additional DNA amplicons.

The highest GCP (66.1) was found between the S6 and F strains, while the lowest (0.0) was between ts-11 and a number of other strains, including F, S6 and 6/85. The sequence identity for the S6 and F strains was 98.7 %, while those for ts-11 and strains F, S6 and 6/85 were 79.2, 81.0 and 56.4 %, respectively.

A poor correlation (0.2) was observed when the GCP and sequence identity of all MG strains were compared. The correlation between sequence identity and GCP of MG strains (ts-11, S6, F and K1453) (that generated a single amplicon) was found to be 0.78. Thus, the poor correlation between sequence identities and GCPs when all strains were used in the comparison was due to the contribution of extra amplicons generated by MG strains 6/85, Ap3AS, K1659, 86134, 87006 and 87081.

A mathematical calculation for non-subjective grouping of MG strains using GCPs

Using GCPs for the ts-11 vaccine strain, a cut-off value was generated as a mathematical model to assess the relationship of the field isolates without visual interpretation by the operator (non-objective). The mean of the 125 genotype confidence values for the ts-11 strain was 93.7, with an SD of 10.7. A value of 3 SD (32.1) was then calculated and subtracted from the average GCP to determine a cut off point. Thus, the GCP range for the ts-11 vaccine strain and related field samples was determined to be 61.6–100. The cut off point of 61.6 was then applied for genotyping MG strains and isolates. All amplicons generated from ts-11-vaccinated SPF and commercial layer chickens had GPCs between 63.1 and 100, and were therefore genotyped

automatically as ts-11. All the other MG strains had GCPs between 0.0 and 19.5, and were therefore automatically identified as ‘variation’ (Fig. 5). Thus, the gap between the highest non-ts-11 and lowest ts-11 specimens was approximately 43 GCP.

Assessment of the potential of the PCR-HRM technique for differentiation of MG isolates from diverse geographical locations

The PCR amplicons from each of the three groups of MG isolates from the USA, Europe and Israel (Table 4) were subjected to HRM curve analysis, and isolates with conventional/normalized curves similar to each other or to those characterized earlier in this study (Table 1) were selected for further examination by nucleotide sequencing. The shape of the curves and number, relative height and temperature of the peaks, and subsequently the GCPs, were considered in this initial screening.

Visual examination of the conventional and normalized melt curves of the MG isolates from the USA (Fig. 6a) revealed that MG isolate K6216D had a single peak at

Fig. 5. Comparison of the distribution of GCPs from ts-11 and non-ts-11 strains/isolates by dot plot.

76.8 °C highly similar to ts-11, and it was therefore genotyped as ts-11 with 95 GCP. The nucleotide sequence of the amplicon (226 bp) was also identical to that of ts-11. MG isolates K5917 and K6096 had two similar conventional melt curve peaks at 76.5 and 79.7 °C, and similar normalization curves at a GCP of 99. The sequence of the PCR amplicons (323 bp) was also identical. MG isolates K5037A and K5792D also generated similar normalized and conventional melt curves, with one peak at 78.3 °C and a shoulder peak at a lower temperature. These two isolates produced single major DNA bands (319 bp) with identical nucleotide sequences. MG isolates K4181C and K4094 generated slightly different patterns to those of K5917 and K6096, with two peaks at 76.6 and 79.5 °C, and 76.6 and 79.8 °C, respectively. Nucleotide sequence analysis of these two isolates revealed 99.7% identity to each other and 93.7–100% identity to K5917/K6096. The rest of the

isolates from the USA (K4158C, K4355, K4705, K503 and K5104) had curves that were distinct from the other USA and Australian MG strains/isolates used in this study.

Visual examination of conventional and normalized melt curves of MG isolates from Europe revealed that MG isolate B40/95 and the F strain generated amplicons with similar melt curves, with one peak at 78.4 °C and a shoulder peak at a lower temperature. Nucleotide sequences of amplicons revealed 99.7% sequence identity (one nucleotide substitution in 319 bp). This similarity was also reflected in similar normalized melt curves with a GCP of 88. All other MG isolates from Europe did not have similarity to each other (Fig. 6b) or to any other reference strain/isolate characterized earlier in this study.

Examination of the Israeli MG isolates revealed that the isolates MSA-9 and MKT-6 generated highly similar conventional melt curves, with two peaks at 74.6 and 77.8 °C (Fig. 6e). The normalized melt curves for the amplicons of these isolates were also highly similar and had a GCP of 92 (Fig. 6f). The nucleotide sequence of the respective amplicons (355 bp) was found to be identical. Similarly, MG isolates BCV-6 and VR-5 generated amplicons with similar conventional melt curves, with one peak at 78.0 °C and a shoulder peak at a lower temperature. Nucleotide sequences (319 bp) of amplicons were identical to each other and to MG strain K1453 (examined earlier in this study). This similarity was also reflected in similar normalized melt curves with a GCP of 90.

Table 4. MG isolates obtained from the USA, Europe and Israel

Isolate	Host	Origin	Reference/GenBank accession no.
K415830	Turkey	USA	NRA*
K4181C	Chicken	USA	Ferguson <i>et al.</i> (2005)
K4355	Chicken	USA	Ferguson <i>et al.</i> (2005)
K4705	Chicken	USA	Ferguson <i>et al.</i> (2005)
K5037A	Turkey	USA	Raviv <i>et al.</i> (2007)/GU133050
K503	Chicken	USA	Ferguson <i>et al.</i> (2005)
K6216D	Chicken	USA	NRA/GU133047
K5792D	Chicken	USA	Raviv <i>et al.</i> (2007)/GU133051
K5917	Chicken	USA	NRA/GU133048
K6096	Chicken	USA	NRA/GU133049
K5104	Turkey	USA	Ferguson <i>et al.</i> (2005)
K4049	Housefinch	USA	NRA
B18/86	Turkey	England	NRA
B5/94	Pheasant	England	NRA
B40/95	Turkey	England	NRA/GU166688
B41/02	Chicken	England	NRA
B134/03	Pheasant	England	NRA
B7/04/50	Not known	Germany	NRA
B19/04/5	Turkey	Germany	NRA
B90/05	Turkey	England	NRA
B114/06	Partridge	Scotland	NRA
B40/07/6	Chicken	England	NRA
MYZ-8	Chicken	Israel	Lysnyansky <i>et al.</i> (2008)
MSA-9	Chicken	Israel	Lysnyansky <i>et al.</i> (2008)/ GU133053
RV-6	Chicken	Israel	NRA
VR-5	Chicken	Israel	NRA/GU133052
BCV-6	Chicken	Israel	NRA
DSD-14	Turkey	Israel	NRA
BNC-10	Turkey	Israel	NRA
MKT-6	Turkey	Israel	NRA/GU133054

*NRA, No reference available.

DISCUSSION

This study describes a rapid and reliable technique for the differentiation of MG isolates/strains. Increasing use of the MG live vaccines in poultry has led to a need for a reliable technique that can differentiate MG vaccine strains from field isolates. This is primarily for epidemiological investigation, but may also be required by registration authorities when a new MG vaccine is introduced to a country.

The use of PCR alone (Evans & Leigh, 2008; Feberwee *et al.*, 2005b) or combined with sequencing (Ferguson *et al.*, 2005; Raviv *et al.*, 2007), RFLP (Khan & Yamamoto, 1989; Kleven *et al.*, 1988a; Lysnyansky *et al.*, 2005) or AFLP (Feberwee *et al.*, 2005a; Hong *et al.*, 2005), and of random amplified polymorphic DNA (RAPD) analysis (Feberwee *et al.*, 2005a; Ferguson *et al.*, 2005; Geary *et al.*, 1994) and PFGE (Marois *et al.*, 2001; Mettifogo *et al.*, 2006), has been described for the differentiation of MG isolates/strains. Although these techniques may currently be used in some laboratories, they have limitations such as low reproducibility, lengthy procedure and the need for extensive interpretation, particularly when a large number of specimens are to be tested. In this study, initial PCR demonstrated a discriminatory power to differentiate the ts-11 vaccine strain and MG field isolates based solely on the amplicon size. The ts-11 vaccine strain produced a

Fig. 6. (a, c and e) Conventional melt curve and (b, d and f) normalized HRM curve analysis of PCR products of the *vhlA* gene promoter region from ts-11 and MG isolates from the USA (a, b), Europe (c, d) and Israel (e, f).

single DNA band (226 bp), while some isolates produced two (Ap3AS, K1659) or three bands (86134, 87006 and 87081). It has been shown that MG has a large number of *vhlA* gene copies distributed in five distinct loci throughout the genome (Baseggio *et al.*, 1996; Papazisi *et al.*, 2003). Thus, the generation of more than one amplicon is expected, since a relatively high sequence identity exists between promoter regions of the *vhlA* gene family members. Irrespective of the basis for amplification of more than one amplicon, variation in the number of the amplicons may indeed have contributed to the power of the HRM technique in differentiating different MG strains. Variation in the size and number of amplicons could have sufficed for differentiation of a number of MG strains by examination of the PCR products on an agarose gel. However, some strains were found to generate the same number of bands with similar sizes. For example, strains

K1453 and F both generated a single band of similar size (Fig. 1). Thus, discrimination of these strains using agarose gel electrophoresis may not be possible. In contrast, these strains were readily distinguishable from each other in conventional and normalized HRM curve analysis.

The melting profile of a PCR product is dependent upon length, sequence, GC content and heterozygosity (Reed *et al.*, 2007). There was no correlation between the number of *vhlA* bands and the number of peaks, although the number of peaks could be influenced by the number of bands and distribution of the nucleotides in the amplicon. The first shoulder peak of S6, F and K1453 is likely to be due to an AT-rich region which melts at lower temperature, and the second peak is likely to be due to the melting of a GC-rich region at a higher temperature. For specimens such as 87006 with three PCR bands, the major band

produces one peak and the two extra bands perhaps contribute to the shape of the curve.

In this study, ts-11 was used as a model to demonstrate the consistency of HRM curve profiles after passage *in vitro* and *in vivo*. All ts-11 vaccine reisolates generated one peak at 76.5 ± 0.3 in conventional melt curves and genotyped as ts-11 with a GCP of 63.1 or higher. In contrast, the highest GCP between ts-11 and the other strains used in this study was 19.5 %. This illustrates the discriminatory power of HRM curve analysis for the differentiation of ts-11 from other MG strains.

The PCR-HRM curve analysis could also differentiate MG strains that generated amplicons with high sequence identities. For example, MG strains 6/85, 86134 and Ap3AS had a 100 % sequence identity but had distinct conventional and normalized melt curves. Likewise, strains 87081 and K1453 generated distinct melt curve profiles, although they had only a relatively small difference (7.2 %) in their similar size (319 bp) amplicons.

The capacity of the PCR-HRM technique in differentiating MG strains/isolates was further evaluated by testing an additional 30 MG isolates from different geographical locations, including Europe, the USA and Israel. Interestingly, a field isolate from the USA (K6216D) was found to generate similar conventional and normalized melt curves to those of ts-11, with 95 GCP. This isolate had been isolated from the progeny of ts-11-vaccinated broiler breeders and found to be indistinguishable from ts-11 by RAPD (N. Ferguson, personal communication). The USA MG isolates K5917 and K6096 were found to be identical by HRM curve analysis. These two isolates had similar *mgc2* sequences and were found to be indistinguishable in the host laboratory (N. Ferguson, personal communication). The MG isolates K5037A and K5792D were also found to have similar HRM curves; however, they are reportedly different in their intergenic spacer region (IGSR) sequences (Raviv *et al.*, 2007). The cause of this discrepancy is unknown and may require further examination by nucleotide sequencing of other genes. The two Israeli MG strains VR5 and BCV-6 that were found to be indistinguishable by HRM curve analysis had been isolated from broiler breeders located at different farms, but epidemiologically appeared to be linked (I. Lysnyansky, personal communication). No apparent epidemiological links existed between the other two Israeli strains (MSA-9 and MKT-6) that were also indistinguishable by HRM curve analysis. These isolates are currently under further examination in the host laboratory.

The newly developed PCR-HRM technique provides a rapid and reliable means for detection and differentiation of MG strains from both culture and clinical swabs.

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