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PROJECT LEADER:

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Evaluation of High Resolution Melt Curve Analysis for detection of multiple strains in a single specimen

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Plain English Compendium Summary

Sub-Project Title:	Evaluation of High Resolution Melt Curve Analysis for detection of multiple strains in a single specimen
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Sub-Project Overview	Under a previous CRC and a number of other projects in our laboratories, High Resolution Melt (HRM) curve analysis tools have been developed for a number of poultry pathogens. Based on the general belief that only one strain can infect a particular bird at a single time, HRM curve analysis has primarily been applied for identification of a single strain/species in a given specimen in our laboratory. Exceptions to this belief have been noted in our laboratory and overseas. Examples of these exceptions are concurrent colonisation of the respiratory system with two *Mycoplasma gallisepticum* (MG) strains. In this study the capacity of PCR-HRM to detect mixed MG strains in one specimen is evaluated.
Background	The HRM curve analysis has been used to differentiate viral and bacterial genotypes based on diversity of nucleotide sequences. Recently, a PCR-HRM curve analysis for strain identification of MG has been developed in our laboratory. Since co-infection of respiratory system with two MG strains may occur in field conditions, identification of each strain/isolate would be important particularly when the flock is vaccinated with a live MG vaccine. However, the capability of PCR-HRM curve analysis for differentiation of two MG strains in one specimen has not been investigated.
Research	The strain differentiation power of HRM curve analysis was evaluated by targeting five different MG genes. Two of these genes were used for further assessment in the mix-infection study. Mixtures of two MG strains with different DNA concentrations were tested and the potential of PCR-HRM of the two genes to detect mixed infection was evaluated.
Implications	PCR-HRM curve analysis provides a useful and cost-effective alternative to mycoplasma culture using direct analysis of genetic variation, particularly when mixed strains are present in one specimen.
Publication	First manuscript entitled “Comparison of multiple genes for their capacity in high resolution melt curve analysis to differentiate *Mycoplasma gallisepticum* strains” was recently submitted to the journal of Veterinary Microbiology

Executive Summary

High Resolution Melt (HRM) curve analysis tools have been developed for a number of poultry pathogens. HRM curve analysis has primarily been applied for identification of a single strain/species in a given specimen. In this study the capacity of PCR-HRM to detect mixed MG strains in one specimen was evaluated. Results showed that PCR-HRM curve analysis provides a useful and cost-effective alternative to mycoplasma culture using direct analysis of genetic variation, particularly when mixed strains are present in one specimen.

This project was conducted in 2 phases.

In the first phase, to evaluate the existing genotyping methods for differentiation of MG strains, high resolution melt (HRM) curve analysis was applied to 5 different PCR methods targeting *vlhA*, *pvpA*, *IGSR*, *gapA* or *mgc2* genes, The *vlhA* and *pvpA* genes showed the highest capacity for strain identification and were therefore used in the second phase of the study.

In the second phase, the capability of *vlhA* and *pvpA* PCR-HRM curve analysis techniques were assessed for identification of the two MG strains in one specimen.

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Phase 1, comparison of multiple genes for their capacity in high resolution melt curve analysis to differentiate *Mycoplasma gallisepticum* strains

Abstract

Mycoplasma gallisepticum (MG) is an important avian pathogen causing significant economic losses in the global poultry industry. In an attempt to compare and evaluate against existing genotyping methods for differentiation of MG strains/isolates, high resolution melt (HRM) curve analysis was applied to 5 different PCR methods targeting *vlhA*, *pvpA*, *IGSR*, *gapA* or *mgc2* genes. To assess the power of PCR-HRM curve analysis to discriminate between examined genes, MG strains ts-11, F, 6/85 and S6, and 8 field isolates were tested. All MG strains/isolates were differentiated using PCR-HRM curve analysis and genotype confidence percentage (GCP) values of *vlhA* and *pvpA* genes, while only 0, 4 and 3 out of 12 MG strains/isolates were differentiated using *gapA*, *IGSR* and *mgc2* genes respectively. The HRM curve analysis was found to be highly correlated with the genetic diversity of the targeted genes confirmed by sequence analysis of amplicons generated from MG strains. Results from this study provides a direct comparison between genes previously used in sequencing-based genotyping methods for MG strain identification and highlights the usefulness of *vlhA* and *pvpA* HRM curve analyses as rapid and reliable tools specially for diagnosis and differentiation of MG strains used here.

Key words: high resolution melting curve analysis, *Mycoplasma gallisepticum*, MG, PCR, strain differentiation, genotyping

Introduction

Mycoplasma gallisepticum (MG) is the most important pathogenic mycoplasma of poultry and can cause chronic respiratory disease, egg reduction and condemnations at processing, resulting a considerable economic loss to the poultry industry (Ley, 2008). Since the introduction of attenuated MG vaccine strains such as F (Adler et al., 1960), 6/85 (Evans and Hafez, 1992) and ts-11 (Whithear et al., 1990), vaccination has been often used to control MG infection in poultry flocks and therefore, differentiation of MG strains has become increasingly important.

The PCR-based techniques have been used as valuable tools for detection and differentiation of MG strains/isolates. These techniques include PCR followed by restriction fragment length polymorphism (RFLP) (Kiss et al., 1997; Lysnyansky et al., 2005), amplified fragment length polymorphism (ALFP) (Cherry et al., 2006; Hong et al., 2005), pulse-field gel electrophoresis and random amplified polymorphic DNA (Marois et al., 2001). The genes for the cell membrane surface proteins such as adhesion proteins, particularly putative cytadhesin proteins, have been targeted for MG strain detection and differentiation using PCR and nucleotide sequencing. The interstrain genetic differences in 3'-end of *pvpA* gene have been extensively investigated for MG strain differentiation (Ferguson et al., 2005; Jiang et al., 2009; Liu et al., 2001; Sprygin et al., 2010). The *gapA* gene which encodes a cytadhesin protein (Goh et al., 1998) also has been used for MG strain differentiation using PCR followed by RFLP and/or sequencing (Biro et al., 2006; Evans and Leigh, 2008; Ferguson et al., 2005). Furthermore, *mgc2* (encodes a cytadherence-related surface protein) has been shown to be useful in PCR-RFLP for differentiation of MG field isolates and vaccine strains ts-11 and 6/85 (Lysnyansky et al., 2005). A DNA segment between 16S and 23S rRNA intergenic space region (*IGSR*) has also been reported to be variable between MG isolates/strains (Raviv et al., 2007) and used in PCR followed by sequencing for differentiation of MG strains.

We have recently developed a *vlhA* gene-based PCR-HRM curve analysis technique that differentiates MG strains/isolates from Australia, Europe and the USA (Ghorashi et al., 2010). The aim of this study was to apply HRM curve analysis technique to other PCR methods previously reported for differentiation of MG strains and compare their differentiation power against that of *vlhA* PCR-HRM curve analysis.

Materials and Methods

MG strains

MG vaccine strains ts-11 (Bioproperties, Australia), F (Schering-Plough Animal Health Corporation, USA) and 6/85 (Intervet, USA) were harvested from commercial vials of the vaccines or from cultures grown in Mycoplasma broth (MB) as described before (Morrow et al., 1998). The S6 strain and 8 Australian MG field isolates were also harvested from cultures in mycoplasma broth (Whithear, 1993) (Table 1).

DNA extraction

Total genomic DNA was extracted from mycoplasma cultures or from vaccines using a DNA extraction kit (QIAGEN) according to the manufacturer's instructions. Briefly, 0.5 ml of mycoplasma culture was pelleted by centrifugation at 20000 ***g*** for 5 min. The cells were washed twice in phosphate buffered saline (PBS) and resuspended in 500 µl RLT lysis buffer (QIAGEN) and incubated for two hours at room temperature or overnight at 4 ºC. Then 15 µl of Qiaex II matrix (QIAGEN) and 300 µl 70% ethanol were added and mixed, and the lysate was loaded into a multispin MSK-100 column (Axygen Inc., Hayward, CA, USA), centrifuged for 30 s at 10000 ***g*** and the flow-through discarded. The column was washed with 600 µl RW1 buffer (QIAGEN) and twice with 500 µl RPE buffer (QIAGEN) and subjected to centrifugation at 18000 ***g*** for 90 s. The DNA was eluted from the matrix using 50 µl distilled water. The concentration of extracted DNA was adjusted to 1 ng/µl and used in PCR immediately or stored at -20 ºC for future use.

PCR amplification of targeted genes

The primer sequences for the target genes, *vlhA*, *pvpA*, *gapA*, *mgc2* and *IGSR,* and expected amplicon sizes are shown in Table 2. PCR amplifications were performed in 25 µl reaction volume on a I-Cycler thermal cycler (Bio-Rad). The reaction mixture contained 1 µl extracted genomic DNA, 25 µM of each primer, 1.5 mM $MgCL_2$, 1250 µM of each dNTP, 5 µM SYTO® 9 green fluorescent nucleic acid stain (Invitrogen), 1× GoTaq® Green Flexi Reaction Buffer (Promega) and 1 U of Go Taq DNA polymerase (Promega). PCR condition for *vlhA* gene was one cycle of 94 ºC for 60 s, 40 cycles of 94 ºC for 10 s, 50 ºC for 10 s and 72 ºC for 10 s, and a final cycle of 72 ºC for one min. The optimal PCR conditions for amplification of *pvpA*, *mgc2*, *gapA* and *IGSR* genes were determined to be one cycle of 94 ºC for 2 min s, 40 cycles of 94 ºC for 30 s, 55 ºC for 30 s and 72 ºC for 30 s, and a final cycle of 72 ºC for 10 min.

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 ClustalW (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).

High-resolution melting curve acquisition and analysis

HRM curve analysis was performed in a Rotor-Gene™ 6000 thermal cycler (Corbett Life Science Pty Ltd). The PCR products were subjected to two different rampings of 0.1 °C and 0.3 °C/s between 70 °C and 90 °C. All specimens were tested in triplicate and their melting

profiles analysed using Roter Gene 1.7.27 software and the HRM algorithm provided. Different normalisation regions of 74.0-75.0 and 79.5-80.5, 79.0-80.0 and 88.0-89.0, 74.0-75.0 and 81.7-82.7, 74.0-75.0 and 79.0-80.0, and 80.0-81.0 and 88.0-89.0 were used for analysis of amplicons of *vlhA*, *pvpA*, *IGSR*, *gapA* and *mgc2*, respectively.

In order to evaluate the discrimination power of HRM curve analysis for each gene and compare it to those of other genes, differentiation of the ts-11 strain from other strains was used as a model. For each target gene, ts-11 strain was set as a 'genotype' and the average HRM genotype confidence percentage (GCP) (the value attributed to each strain being compared to the genotype with a value of 100% indicating an exact match) for the replicates were predicted by the software.

The GCPs for ts-11 strain were averaged and the standard deviation (SD) calculated and used to establish the GCP range for ts-11 strain cut off point for that particular gene. The values above/below cut off points were then used to evaluate the differentiation power of the test.

Results

PCR amplicons of expected sizes generated from MG strains using specific oligonucleotide primers for targeted genes

Amplified PCR products of different genes (*vlhA*, *pvpA*, *IGSR*, *gapA* and *mgc2*) from 4 different MG strains (ts-11, F, 6/85 and S6) were analysed by gel electrophoresis (Fig. 1). MG strains generated different amplicon sizes when *vlhA* and *pvpA* specific primers were used. The 6/85 strain also produced a minor band using *vlhA* primers and F, 6/85 and S6 also produced one or two minor bands when *pvpA* primers were used. All MG strains generated two or more minor bands when *IGSR* and/or *mgc2* primers were used. The major amplicons produced by *IGSR* and *mgc2* primers were similar, or exhibited slight difference in their size for each gene, while amplification products generated by *gapA* primers were all similar in size. The

sequence of primers and major amplicon sizes of MG strains for each gene are shown in Table 2.

Discrimination power of HRM curve analysis was different for different targeted genes

PCR amplicons of 5 different genes (*vlhA*, *pvpA*, *IGSR*, *gapA* and *mgc2*) generated from 4 different MG strains (ts-11, F, 6/85 and S6) and 8 field isolates were subjected to HRM curve analysis (Fig. 2). Visual examination of the conventional melt curves for all PCRs at 2 different ramp temperatures revealed that 0.3 °C/s resulted in distinct conventional melt curves for most strains.

When *vlhA* primers were used, the ts-11 strain generated one peak at 76.6 ± 0.6 °C which was distinct from those of other MG strains tested. The 6/85 strain produced two peaks at 76.0 ± 0.1 and 77.9 ± 0.1 ºC while F and S6 each produced one peak at 78.0 ± 0.3 and 78.3 ± 0.3 ºC respectively, both with a shoulder peak at lower temperature (Fig. 2a). The 4 MG strains generated distinct normalized curves and were visually differentiable (Fig. 2b).

When *pvpA* primers were used, all MG strains produced melt curves with two major peaks with the second peak was higher than the first peak. The mean melt curve temperatures of the first and second peaks for ts-11, 6/85, F and S6 were 81.9 ± 0.3 and 87.6 ± 0.2, 82.4 ± 0.1 and 85.7 ± 0.1,, 82.3 ± 0.2 and 87.5 ± 0.1, 82.5 ± 0.1 and 87.8 ± 0.2 ºC, respectively (Fig. 2c). The 4 MG strains generated distinct normalized curves (Fig. 2d).

When *IGSR* primers were used all MG strains generated two peaks with the first peak was higher than the second peak. The mean melt curve temperatures of the first and second peaks for ts-11, 6/85, F and S6 were 77.1 ± 0.3 and 79.2 ± 0.3, 77.4 ± 0.3 and 79.6 ± 0.3, 77.5 ± 0.3 and 79.6 ± 0.3, 77.6 ± 0.3 and 79.6 ± 0.2 ºC, respectively. The ts-11 melt curves had peaks slightly at lower temperature and therefore, could be differentiated from other MG strains. However, the melt curves for the two peaks of 6/85, F and S6 were nearly

overlapping and therefore these strains could not be differentiated from each other using either conventional or HRM melt curve analysis (Fig. 2e and f).

When *gapA* primers were used, all MG strains produced one peak between 77.8-77.9 ºC. The mean melt curve temperature for ts-11, 6/85, F and S6 were 77.8 ± 0.1, 77.8 ± 0.1, 77.9 ± 0.1 and 77.9 ± 0.1 ºC, respectively. The HRM and conventional melt curves of ts-11, 6/85, F and S6 were visually indistinguishable from each other. Note, the melt curves of all strains were overlapping (Fig. 2g and h).

When *mgc2* primers were used, the ts-11, 6/85, F and S6 each produced two melt curves at 83.0 ± 0.2 and 86.5 ± 0.2, 83.2 ± 0.1 and 86.3 ± 0.4, 83.0 ± 0.3 and 86.7 ± 0.1, 83.5 ± 0.2, and 85.9 ± 0.2 ºC, respectively. In all strains, the first peak was higher than the second one. The ts-11, 6/85, F and S6 could be differentiated based on conventional and normalized melt curves (Fig. 2i and j).

Nucleotide sequence variations in targeted genes were highly correlated with HRM curve analysis results

To assess if differences of HRM curves and GCP values for ts-11 and 6/85, F and S6 strains were reflected in the nucleotide sequences of the amplicons, nucleotide sequences of amplicons for each target gene from different MG strains were determined, aligned and compared with the consensus sequence. The divergence of nucleotide sequences from consensus sequence varied significantly between MG strains with the sequence divergence was higher in *vlhA* and *pvpA* genes and lower in *IGSR* and *gapA* genes (Fig. 3 and Table 3). Similar results were obtained when the nucleotide divergence of 6/85, F and S6 were compared directly with that of ts-11 strain (Fig. 4 and Table 4).

The mean GCP values obtained from repeated runs of PCR-HRM for target genes were also analysed to compare the discrimination power of HRM for each target gene. The ts-11 strain was set as genotype and GCP for all MG strains in replicates for targeted genes were determined. The distribution of mean GCP values obtained for tested MG strains and for

each gene is shown in Table 5 and Fig. 5. The smaller difference in GCP values between MG strains is a reflection of higher similarity of conventional and normalized HRM curves between them. Therefore, larger differences in GCP values for ts-11 and tested MG strains show higher discrimination power in that gene. The discrimination power of PCR-HRM curve analysis for *vlhA* and *pvpA* genes were significantly higher than IGSR, *mgc2* and *gapA* (Fig. 5).

Using GCP values obtained for the ts-11 strain from PCR-HRM curve analysis of *vlhA* and *pvpA* genes, cut-off values were calculated and used to assess and compare the discrimination power of PCR-HRM between these two PCR methods. The mean of 36 genotype confidence values for the ts-11 strain in *vlhA* and *pvpA* genes were 92.1±12 and 98.4±14, respectively. A value of 3 SD was subtracted from the average ts-11 strain GCPs to calculate the cut off point of ts-11 strain. Therefore, the GCP range for ts-11 strain using *vlhA* and *pvpA* genes were determined to be 55.2-100 and 56.4-100, respectively. Then calculated cut off point was applied for genotyping MG strains/isolates using respective genes. In *vlhA* PCR-HRM, ts-11 parent strain 80083 and ts-11 reisolates 69364 and 92032 (isolated from ts-11 vaccinated flocks) had means of GCP between 82.2 and 93.3, and were therefore genotyped automatically as ts-11 strain. All other MG strains had GCPs between 0.0 and 2.1, and were therefore automatically identified as 'variation'.

In *pvpA* PCR-HRM, ts-11 parent strain 80083 and ts-11 reisolates 69364, 92032 had GCPs between 58.4 and 95.1 and therefore, identified as 'ts-11' strain. All other MG strains generated GCP values between 0.0 and 41.4 and were therefore automatically identified as 'variation'. The *pvpA* primers failed to amplify field isolate 86041 in all PCR-HRM runs.

In *gapA* PCR-HRM, all field isolates generated GCP values between 85.4 and 99.9 and therefore, all genotyped as 'ts-11' strain. The *gapA* PCR-HRM could not differentiate field isolates from ts-11 strain.

In *IGSR* PCR-HRM field isolate 86064 could be differentiated from ts-11 while the rest of field isolates had GCP values between 60.2 and 98.7 and were genotyped as ts-11 strain.

In *mgc2* PCR-HRM all field isolates except for the field isolate 86041 had GCPs between 62.2 and 98.9 and were indistinguishable from ts-11 strain.

Discussion

Despite recent advances in high-throughput sequencing technology and reduction in associated cost, sequencing is still uncommon for routine examination of clinical specimens. The main problems associated with nucleotide sequencing as a routine procedure are the time required to obtain the sequences as well as the expertise needed to interpret the results. These are particularly problematic when large numbers of specimens are examined. Furthermore, nucleotide sequences may occasionally be inconclusive due to insufficient quality of the graphs and thus necessitating further submission of specimens for sequencing. Several studies have investigated the use of interstrain sequence variations in various MG genes for differentiation of MG strains (Evans and Leigh, 2008; Ferguson et al., 2005; Hong et al., 2005; Mettifogo et al., 2006; Pillai et al., 2003; Sprygin et al., 2010; Szczepanek et al., 2010). However the current study is the first study directly comparing these genes for their capacity, by nucleotide sequencing and HRM curve analysis, for MG strain identification. To evaluate the capacity of these genes in PCR-HRM curve analysis, a panel of 4 MG strains (ts-11, 6/85, F and S6) and 8 field isolates were examined. Comparison of the PCR-HRM curve analysis for these genes revealed that the *gapA*, *IGSR* and *mgc2* had rather limited differentiation capacity. However, *vlhA* and *pvpA* genes were found to provide a high differentiation power for MG strains/isolates. This was mainly due to high interstrain sequence variations of these genes which were highly correlated with HRM GCPs.
Using *vlhA* and *pvpA* genes in PCR-HRM, the melt curve profiles produced by ts-11 strain were distinct from all MG strains (6/85, F and S6) and field isolates with the exception of the ts-11 vaccine parent strain 80083 and 2 field isolates 69364 and 92032. Similarity of the HRM curves of the field isolates 69364 and 92032 to that of ts-11 was expected as these two isolates had been isolated from flocks previously vaccinated with ts-11, and therefore they

were most likely vaccine reisolates. The similarity of ts-11 to its parent strain 80083 was also expected due to their identical *vlhA* sequence. Currently these two strains can only be differentiated based on their differential growth at different temperatures as no genetic difference has been found so far between them.

The PCR-HRM using *pvpA* primer set failed to detect and amplify an amplicon from a MG field isolate in our laboratory even when lower annealing temperatures were used (data not shown). Also recently, during routine examination of clinical specimens submitted to our laboratory, the *vlhA* primer set was found to fail to generate an amplicon from another MG isolate (unpublished data). Further identification of these isolates was not possible mainly due to limited quantity of DNA available from clinical specimens submitted. Therefore it is suggested that these two assays should be used in conjunction with a primary/screening PCR system such as 16S rRNA PCR (Kempf et al., 1993) particularly when negative results are unexpectedly observed.

In brief, this study provides invaluable information on the usefulness of PCR-HRM curve analysis as a rapid and reliable technique for strain identification of MG.

Acknowledgement

Funding for this project was partly provided by the Poultry Cooperative Research Centre.

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

Strain/isolate	**Origin**	**Reference**
ts-11	Australia	(Whithear et al., 1990)
F	USA	(Carpenter et al., 1979)
S6	USA	(Adler et al., 1957)
6/85	USA	(Evans and Hafez, 1992)
80083	Australia	(Whithear et al., 1990)
87089	Australia	This study
86064	Australia	This study
69364	Australia	This study
86026	Australia	This study
86041	Australia	This study
E0006	Australia	This study
92032	Australia	This study

Table 2
Sequence of primers used in this study, and the size of PCR product generated.

Primer	Oligonucleotides Sequence (5'-3')	PCR product size range (bp)	Reference
ts-11-F	GTTTGGAGTTGGTGTATAGTTAG	226-352	(Ghorashi et al., 2010)
ts-11-R	TCTTCTTCGAAAACAAAAGG		
pvpA-F	GAAAATGTTGAAGCCACT	374-695	(Jiang et al., 2009)
pvpA-R	GGATTATTTGGTGTTGGA		
IGSR-F	GTAGGGCCGGTGATTGGAGTTA	811-815	(Raviv et al., 2007)
IGSR-R	CCCGTAGCATTTCGCAGGTTTG		
gapA-3F	TTCTAGCGCTTTAGCCCTAAACCC	332	(Ferguson et al., 2005)
gapA-4R	CTTGTGGAACAGCAACGTATTCGC		
mgc2-1F	GCTTTGTGTTCTCGGGTGCTA	791-857	(Ferguson et al., 2005)
mgc2-1R	CGGTGGAAAACCAGCTCTTG		

Table 3
Nucleotide divergence (%) of each MG strain against consensus sequence.

Strain	Target gene				
	vlhA	***pvpA***	***IGSR***	***mgc2***	***gapA***
ts-11	37.5	4	2.3	2.1	0.6
6/85	62.8	47.6	1.5	9.1	0.6
F	10.5	35.5	1.7	5.5	2.4
S6	0.0	12.8	2.3	29.7	2.7

Table 4
Nucleotide divergence of MG strains against ts-11 sequence.

Strain	**Target gene**				
	vlhA	***pvpA***	***IGSR***	***mgc2***	***gapA***
6/85	38.5	47.9	2.1	9.3	0.0
F	32.3	36.8	3.2	6.1	2.4
S6	37.5	12.4	3.7	2.9	2.7

Table 5
GCP values of MG strains against ts-11 genotype.

Strain	**ts-11**				
	vlhA	***pvpA***	***IGSR***	***gapA***	***mgc2***
6/85	0.3	0.0	35.9	99.2	53.5
F	0.0	1.5	53.9	82.0	80.4
S6	0.0	71.7	44.9	89.4	9.3

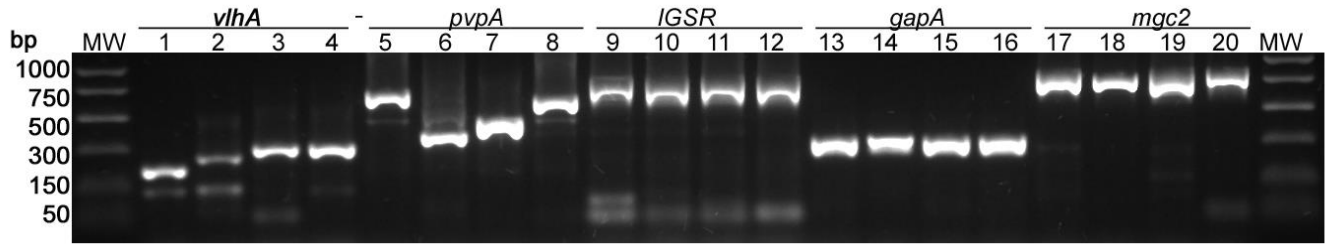

Fig. 1. Agarose gel electrophoresis of PCR products of the *vlhA*, *pvpA*, *IGSR*, *gapA* and *mgc2* genes from MG strains ts-11, 6/85, F and S6, MW, molecular weight marker (PCR Marker, Sigma).

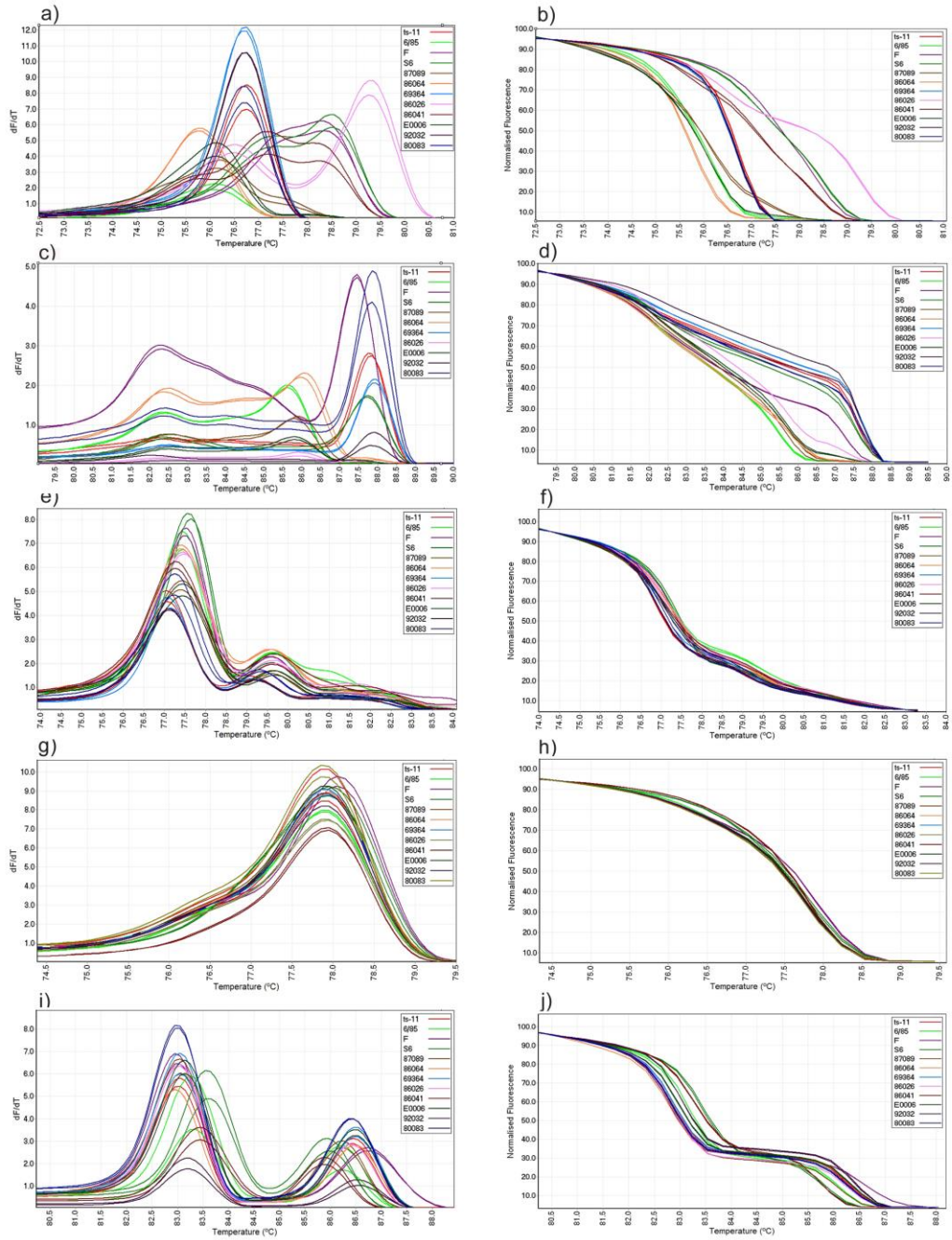

Fig. 2. Conventional and normalised melt curves analysis of PCR products of *vlhA* (a and b), *pvpA* (c and d), *IGSR* (e and f), *gapA* (g and h) and *mgc2* (i and j) genes from 4 MG strains and 8 field isolates.

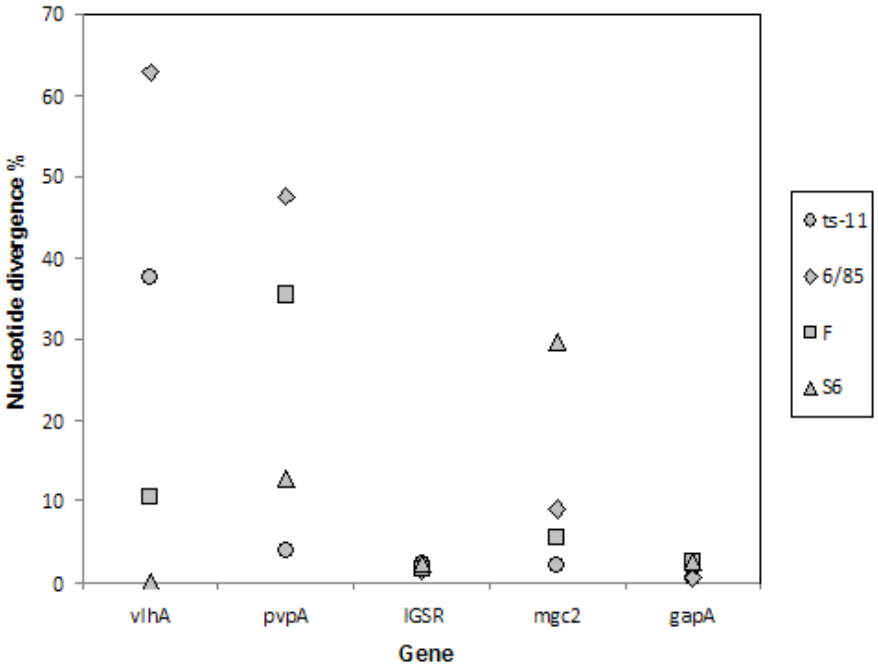

Fig. 3. Nucleotide divergence (%) of amplicon sequences against consensus sequence for different genes in selected MG strains.

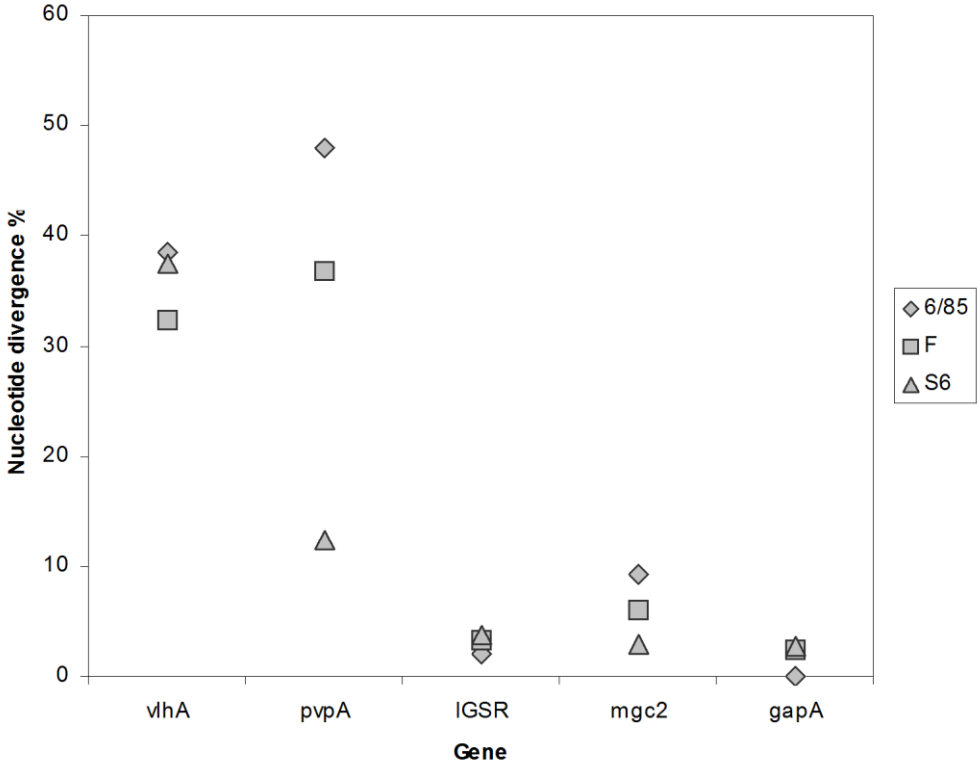

Fig. 4. Nucleotide divergence (%) of amplicon sequences against ts-11 nucleotide sequence for different genes in selected MG strains.

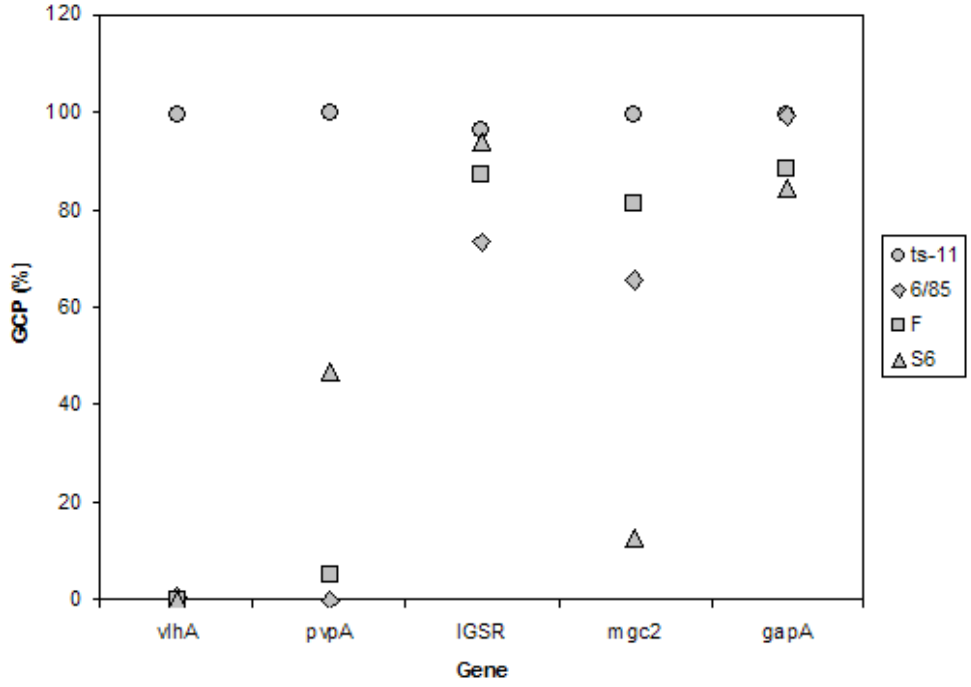

Fig. 5. Distribution of GCP values for MG strains when ts-11 strain is set as genotype.

Phase 2, Identification of *Mycoplasma gallisepticum* mixed infection using PCR and high-resolution melt curve analysis

Abstract

Differentiation of sole MG strains using molecular diagnostic methods including PCR and high resolution melt (HRM) curve analysis have been reported before but the potential of the tests for identification of more than one MG strain in a mixed population has not been evaluated. In the present study, the capability of PCR-HRM curve analysis technique, using *vlhA* and *pvpA* genes was assessed for identification of the involvement of two MG strains. Different ratios of of two MG strains from 1 to 10^{-4} were tested. Some mixtures generated conventional and normalized curves that were distinct from those of individual strains. Using genotype confidence percentages (GCP) generated from HRM curve analysis, it was also found that *vlhA* PCR-HRM was superior to *pvpA* PCR-HRM for detection of mixtures of ts-11 vaccine strain with one of the MG strains 6/85, F, S6 or a field isolate. The success of *vlhA* PCR-HRM to detect mixed infection was mainly DNA concentration-dependent. This is the first study examining the capacity of PCR-HRM technique for identification of mixed MG strain infection.

Key words: high resolution melting curve analysis, *Mycoplasma gallisepticum*, mixed infection

Introduction

Mycoplasma gallisepticum (MG) is an important poultry pathogen causing economic loss in many parts of the world. Eradication policy is the preferred method of MG control (Abd-el-Motelib and Kleven, 1993) with vaccination is used in areas when eradication is not feasible.

Isolation of more than one Mycoplasma species from a particular site of a host has been reported (Adler and Yamamoto, 1957; Jordan and Amin, 1980). But the paradigm is that usually one strain of each species infects the host at a given time. Nevertheless, recent reports indicate that birds vaccinated with a live MG vaccine and experimentally, challenged with a field strain may harbour both strains at the same time (Ferguson et al., 2005).

Identification of MG strains/isolates involved in an outbreak may be important in control of the disease and epidemiological investigations. A number of molecular diagnostic methods have been reported for differentiation of individual MG strains/isolates (Cherry et al., 2006; Ferguson et al., 2005; Ghorashi et al., 2010; Hong et al., 2005; Kiss et al., 1997; Raviv et al., 2008). However, limited reports are available for detection and differentiation of individual strains in a mixed strain infection. Conventionally, detection of individual MG strains in a mixed infection is carried out using culture and cloning. However, this might be difficult as faster growing strain/isolates may overgrow the other strain in the culture (Bradbury and McClenaghan, 1982) making it difficult to detect the slower growing strain. Mixture of two MG strains have been detected by using monoclonal antibodies in immunofluorescence (Bradbury and McClenaghan, 1982; Jordan and Amin, 1980; Levisohn and Dykstra, 1987) or immunofluorescence combined with immunoperoxidase (Bencina and Bradbury, 1992). These techniques however require culture of the organism that is the time consuming and carry the risk of dominant strain overgrowing the other strain. The mixtures of the live vaccine strain ts-11 and a field isolate have also been successfully detected using PCR-RFLP (Lysnyansky et al., 2005). However, this technique requires, gel purification of PCR-amplified

DNA, enzymatic digestion of DNA and gel electrophoresis which are time consuming and relatively expensive.

Previously we compared the differentiation power of PCR-HRM curve analysis using *vlhA*, *pvpA*, *mgc2*, *gapA* and *IGSR* genes for differentiation of individual MG strains/isolates (see phase 1 of this report).

The aim of this part of study was to assess the potential of PCR-HRM curve analysis of *vlhA* and *pvpA* genes for rapid and reliable detection of mixed MG strains.

Material and methods

MG strains

MG vaccine strains ts-11, F and 6/85 were cultured or harvested from a commercial vial of vaccine (Table 1). The S6 reference strain and an Australian MG field isolate (86026) were cultured in Mycoplasma broth (MB) as described before (Morrow et al., 1998).

DNA extraction

Total genomic DNA was extracted from mycoplasma cultures or from a commercial vial of vaccines using DNA extraction kit (QIAGEN) according to the manufacturer's instructions. Briefly, 0.5 ml of mycoplasma culture was pelleted by centrifugation at 20000 ***g*** for 5 min. The cells were washed twice in phosphate buffered saline (PBS) and resuspended in 500 µl RLT lysis buffer (QIAGEN) and incubated for two hours at room temperature or overnight at 4 ºC. Then 15 µl of Qiaex II matrix (QIAGEN) and 300 µl 70% ethanol were added and mixed, and the lysate was loaded into a multispin MSK-100 column (Axygen Inc., Hayward, CA, USA), centrifuged for 30 s at 10000 ***g*** and the flow-through discarded. The column was washed with 600 µl RW1 buffer (QIAGEN) and twice with 500 µl RPE buffer (QIAGEN) and

subjected to centrifugation at 18000 ***g*** for 90 s. The DNA was eluted from the matrix using 50 µl distilled water and the quantity of extracted DNA was measured by spectrophotometer and adjusted to 1 ng/µl for each specimen. Further DNA dilutions were prepared in dH_2O if required before PCR or stored at -20 ºC for future use.

PCR and High-resolution melt curve acquisition and analysis

Two sets of primers for *vlhA* (Ghorashi et al., 2010) and *pvpA* (Jiang et al., 2009) PCR, were used as described before. In order to assess the capability of PCR-HRM technique to identify mixed infections with two different MG strains in a single specimen, the ts-11 vaccine strain was used as a model and different combinations of ts-11 vaccine strain with a second MG strain were investigated. In each PCR the DNA concentration for ts-11 vaccine strain was kept constant but a series of 10 fold dilutions of DNA from the contaminant MG strain (F, 6/85, S6 strain or 86026 field isolate) was added.

PCR amplifications were performed in 25 µl reaction volume on I-Cycler thermal cycler (Bio-Rad). The reaction mixture contained 2 µl of genomic DNA (1 µl from each MG strain), 25 µM of each primer, 1.5 mM $MgCL_2$, 1250 µM of each dNTP, 5 µM SYTO® 9 green fluorescent nucleic acid stain (Invitrogen), 1× GoTaq® Green Flexi Reaction Buffer (Promega) and 1 U of Go Taq DNA polymerase (Promega). PCR condition for *vlhA* gene was one cycle of 94 ºC for 60 s, 40 cycles of 94 ºC for 10 s, 50 ºC for 10 s and 72 ºC for 10 s, and a final cycle of 72 ºC for one min. The optimal PCR conditions for amplification of *pvpA* gene was determined to be one cycle of 94 ºC for 2 min s, 40 cycles of 94 ºC for 30 s, 55 ºC for 30 s and 72 ºC for 30 s, and a final cycle of 72 ºC for 10 min.

HRM curve analysis was performed in a Rotor-Gene™ 6000 thermal cycler (Corbett Life Science Pty Ltd). The PCR products were subjected to two different rampings of 0.2 °C and 0.3 °C/s between 70 °C and 90 °C. All specimens were tested in triplicate and their melting profiles analysed using Roter Gene 1.7.27 software and the HRM algorithm provided. The

normalisation regions of 74.0-75.0 and 80.0-81.0 for *vlhA*-PCR and , 81.0-82.0 and 89.0-90.0 for *pvpA*-PCR were used for analysis of amplicons.

In order to be able to access and compare the performance of HRM curve analysis for detection of mixed MG strains in *vlhA*-PCR and *pvpA*-PCR, the ts-11 vaccine strain and the companion contaminant (F, 6/85, S6 or MG4 field isolate) in each experiment were set as (different) respective genotypes.

To validate each experiment, the cut off point for the tested MG strains was re-calculated. To calculate the cut-off points for ts-11 vaccine strain and each contaminant strain (F, 6/85, S6 and MG4 field isolate), genotype confidence percentage (GCP) values for all MG strains (ts-11, F, 6/85, S6 and MG4 field isolate) were averaged individually and the standard deviation (SD) calculated and used to establish the GCP range for respective MG strain cut off point in the *vlhA* and/or *pvpA* gene. The calculated cut off points (3 SD subtracted from mean GCP) were used to evaluate the differentiation power of the test to discriminate mixed specimens from un-mixed specimens.

Detection of mixed MG strains in comparison with un-mixed strains were accessed based on the average HRM-GCP value. Any GCP of less than cut off point was regarded as ‘variation’ or mixed infection.

Results

Detection of mixed infections by HRM curve analysis is concentration-dependent

The mean of genotype confidence values for the MG strains/isolate (ts-11, 6/85, F, S6 and 86026) in *vlhA* and *pvpA* genes were calculated. To establish a cut off point for GCP for each strain, a value of 3 SD was subtracted from the average of respective GCPs. The GCP cut off points for ts-11, 6/85, F, S6 and 86026 using *vlhA* gene were determined to be 72.9, 77.4,

60.2, 70.7 and 75.8, respectively. This means that, for ts-11 as an example, all mixed specimens with GCP < 72.9 are genotyped as variation (or mixed infection) while specimens with GCP ≥72.9 are genotyped as ts-11 vaccine strain.

The GCP cut off points for ts-11, 6/85, F, S6 and 86026 using *pvpA* gene PCR HRM curve analysis were also determined to be 75.6, 72.4, 69.0, 76.5 and 79.2, respectively.
Then calculated cut off points were applied for genotyping mixed-MG strains using PCR amplicons of respected genes.

Ten mixtures of ts-11 DNA with each of MG strains F, 6/85, S6 and 86026 at different concentrations (1-10^{-4} ng and reciprocal) were tested in *vlhA* PCR-HRM (Table 2). Mixed specimens of MG ts-11 and F were detected as variation (non-ts-11) only at one dilution (10^{-2} ng ts-11 and 1 ng F) with a mean GCP of 23 (Table 2.a). This mixture generated 2 peaks at 76.7 ± 0.01 and 78.4 ± 0.03 in conventional melt curve analysis and a distinct normalized curve compared to both ts-11 and F (Fig. 1a, b). Mixtures with equal DNA concentrations and all mixtures with constant DNA concentration of ts-11 (1 ng) and variable dilutions (10^{-1} – 10^{-4}) of F were genotyped as ts-11.

Mixtures of MG ts-11 and 6/85 were detected as variation at 2 dilutions of 10^{-3} and 10^{-4} ng of ts-11 and 1 ng of 6/85 with a mean GCP of 22.1 and 32.5, respectively (Table 2.b). The 2 mixtures each generated 2 peaks in conventional melt curve analysis at 76.2 ± 0.05, 78.1 ± 0.08 and 76.2 ± 0.2 and 78.3 ± 0.3 which were distinct to that of ts-11 and 6/85. These 2 mixtures also produced 2 distinct normalized melt curves to ts-11, 6/85 and to each other with a mean GCP of < 33 (Fig. 1c, d). All DNA concentrations of 1 ng ts-11 mixed with different DNA concentrations of 6/85 (1-10^{-4}) had a GCP > 83.0 and therefore were genotyped as ts-11.
Mixtures of MG ts-11 and S6 were detected as variation at 4 mixtures of ts-11 and S6 DNA concentrations of 1 and 10^{-1} ng, 1 and 10^{-2} ng, 10^{-2} and 1 ng and 10^{-3} and 1 ng, respectively (Table 2 c). The 2 mixtures of ts-11 (1 ng) and S6 (10^{-1} and 10^{-2} ng) each generated one

peak at 76.7 ± 0.4 in conventional melt curve analysis while the 2 mixtures of ts-11 (10^{-2} and 10^{-3} ng) and S6 (1 ng) each generated 3 peaks in conventional melt curve analysis. The mixture of ts-11 (10^{-2} ng) and S6 (1 ng) generated 3 peaks at 74.3 ± 0.07, 76.7 ± 0.1 and 78.4 ± 0.05 and mixture of ts-11 (10^{-3} ng) and S6 (1 ng) generated 3 peaks at 74.3 ± 0.03, 76.8 ± 0.07 and 78.3 ± 0.01. The 4 mixtures of MG ts-11 and S6, each generated distinct normalized curves to that of ts-11 and/or S6 (Fig 1e, f) with a GCP of < 72.0 and therefore identified as variation or mixture.

Mixtures of MG ts-11 and 86026 were detectable at only one dilution of ts-11 (10^{-1} ng) and 86026 (1 ng) with a mean GCP of 4.4 (Table 2 d). This mixture generated 2 peaks in conventional melt curve analysis at 76.7 ± 0.03 and 79.4 ± 0.01 which were different to that of ts-11 (76.6 ± 0.6) and 86026 (76.6 ± 0.01 and 79.3 ± 0.01). The mixture also produced a distinct normalized curve to ts-11 and 86026 (Fig 1g, h).

Using *pvpA* PCR-HRM, the ts-11 cut off point was found to be 75.6 which was applied for genotyping mixed strains.

All mixtures of MG ts-11 and F were not detectable as mixture and genotyped as ts-11 or F. The DNA mixture of ts-11 and F at 1/1 ng and $1/10^{-1}$ ng concentrations generated a conventional melt curve with 2 peaks similar to F strain while, mixtures of ts-11 and F with $1/10^{-2}$ ng, $1/10^{-3}$ ng and $1/10^{-4}$ ng of DNA concentrations produced 2 peaks similar to ts-11 (Fig 2a, b). All mixtures of ts-11 with different DNA concentrations (10^{-1}-10^{-4}) and constant concentration of F (1 ng) were genotyped as F (Table 3 a).

Mixtures of MG ts-11 and 6/85 were detectable at 2 dilutions of 1ng ts-11 and 1 and 10^{-1} ng of 6/85 with a mean GCP of 12.1 and 34.1, respectively (Table 3 b). The 2 detectable mixtures of ts-11 and 6/85 at 1 ng/1 ng and 1 ng/10^{-1} ng each generated 4 peaks in conventional melt curve analysis at 82.8 ± 0.1, 83.8, 85.5 ± 0.1, 87.7 and 82.4 ± 0.05, 83.8 ± 0.08, 85.7 and 87.8 ± 0.01 respectively, which were distinct to that of ts-11 and 6/85 (which each produced only 2 peaks). These 2 mixtures also generated 2 distinct normalized melt curves to ts-11 and 6/85 (Fig 2c, d). All other mixtures of ts-11 and 6/85 had a GCP > 77.0 and therefore were genotyped as ts-11 or 6/85.

All mixtures of MG ts-11 and S6 generated 2 peaks in conventional melt curve analysis similar to ts-11 or S6 and were not identified as a mixture. The normalized curves of mixed specimens were also similar to that of ts-11 or S6 (Fig 2e, f). All constant DNA concentrations of ts-11 (1 ng) and variable concentrations of S6 ($1\text{-}10^{-4}$ ng) were genotyped as ts-11 with a mean GCP > 81.0. Similarly, all constant DNA concentrations of S6 (1 ng) and variable concentrations of ts-11 ($1\text{-}10^{-4}$ ng) were genotyped as S6 with a mean GCP > 92.0 (Table 3 c).

Three mixtures of MG ts-11 and 86026 were detectable at dilutions of 10^{-1} and 1 ng, 10^{-2} and 1 ng and 10^{-3} and 1 ng of ts-11 and 86026 with a mean GCP of 26.6, 16.0 and 63.4, respectively (Table 3 d). Each detectable mixture generated 3 peaks in conventional melt curve analysis as 82.9 ± 0.03, 86.1 ±0.03 and 88.1 for ts-11 (10^{-1})and S6 (1 ng) mixture, 82.9, 86.1 ± 0.01 and 88 ± 0.04 for ts-11 (10^{-2}) and S6 (1 ng) mixture and 82.9 ± 0.1, 86.2 ± 0.03 and 88.0 for ts-11 (10^{-3}) and S6 (1 ng) mixture. The ts-11 and 86026 each generated only 2 peaks. The detectable mixtures also generated 3 distinct normalized curves compared to ts-11 and/or 86026 (Fig 2g, h). All other mixtures of ts-11 and 86026 had a GCP > 77.0 and therefore genotyped as ts-11 or 86026.

All mixtures of MG ts-11 and F and ts-11 and S6 at DNA concentrations had GCP values > 75.6 and genotyped as ts-11, F or S6. Therefore, *pvpA* PCR-HRM curve analysis failed to identify them as 'variation' or mixture (Figure 2a, b, e, f). However, two DNA mixtures of ts-11 and 6/85 (Figure 2c, d) and three DNA mixtures of ts-11 and 86026 (Figure 2g, h) were genotyped as 'variation' and identified as mixed MG specimens.

Discussion

The *vlhA* and *pvpA* PCR-HRM tests described in this study were developed to evaluate and compare the potential of HRM curve analysis to detect mixed MG strains in a single specimen. Since the ts-11 vaccine strain is widely used in poultry industry worldwide, we

used a combination of ts-11 and other MG strains as a model to evaluate *vlhA* and *pvpA* PCR-HRM tests.

Expansion of poultry industry and increased concentration of chickens have required continues and a reliable system for monitoring of infectious diseases such as MG. Increasing use of live MG vaccines such as ts-11 or 6/85 in global poultry industry has necessitated development of laboratory tests that can differentiate MG vaccine strains from field isolates. A number of molecular methods have been developed and reported that can differentiate individual MG strains and isolates. However the performance of these tests for detection of individual strains in a mixed infection, when an unknown strain is involved, has not been assessed. Recent reports have indicated that after experimental challenge of birds vaccinated with ts-11, a mixed population of ts-11 and challenge strain can be detected in respiratory system of the birds for some time after challenge. While identification of two known strains using strain specific probes is readily done, identification of unknown (field-) strains under field condition has been a challenge for diagnostic laboratories. In these situations, a method that can simultaneously detect and differentiate all strains would be a significant advancement. The PCR-HRM curve analysis for *vlhA* and *pvpA* genes described in this study was used to evaluate their potential to detect mixed infections compared to specimens containing single MG strain/isolate. The *vlhA* PCR-HRM curve analysis could identify, at least 1 or 2 (out of 4) mixed dilutions of ts-11 vaccine strain with F, 6/85, S6 or 86026. However, *pvpA* PCR-HRM curve analysis detected 2 to 3 (out of 4) dilutions of ts-11 with 6/85 and ts-11 with 86026 mixtures but failed to detect all 4 dilutions of ts-11 with F and ts-11 with S6 mixtures. This was further evidenced by higher nucleotide sequence variability of *vlhA* gene, as compared to *pvpA*, between ts-11 vaccine strain and other MG strains. Therefore it appears that where ts-11 is suspected to present (eg. In ts-11 vaccinated flocks), the *vlhA* PCR-HRM technique should be used as a preferred technique.

High GC content (>60%) can be a critical factor in obtaining optimal PCR-HRM results (van der Stoep et al., 2009). The amplicon size also plays a crutial role in HRM curve analysis. Amplicon sizes of 200-400 bp (or less) can increase the detection sensitivity of sequence

diversity in tested specimes (Chou et al., 2005; van der Stoep et al., 2009). The *vlhA* and *pvpA* amplicons all had a GC content of less than 50%. However, the amplicon size of *vlhA* gene for tested MG strains/isolate were smaller than of *pvpA* amplicons.More importantly, higher nucleotide sequence diversity in *vlhA* gene along with smaller PCR amplicon size compared to *pvpA* gene PCR amplicons can be responsible for better performance of *vlhA* PCR-HRM in identifying mixed specimens.

The success of the *vlhA* PCR-HRM to detect mixed infections was however dependent on the copy number DNA from strains involved. This is one of the limitations of the test. It is therefore suggested that ambiguous results (e.g. unusually low GCP in ts-11 vaccinated flocks) should be further examined by other techniques by gel electrophoresis and/or sequencing of the PCR amplicons and analysis of the sequences.

The simplicity, ease of use, low cost and high sensitivity of PCR-HRM curve analysis has made PCR-HRM a choice and multipurpose method for detection and differentiation of avian pathogens. Screening of clinical specimens for detection of two or more strains of one pathogen in a single specimen could be another application of this robust laboratory method.

Acknowledgements

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Table 1. *Mycoplasma gallisepticum strains/isolate used in this study and their origin*

Isolate/strain	**Origin**	**Reference**	**GenBank Accession No.**	
			vlhA	***pvpA***
ts-11	Australia	(Whithear et al., 1990)	FJ654144	JN001166
F	USA	(Carpenter et al., 1979)	FJ654142	JN001167
S6	USA	(Adler et al., 1957)	FJ654143	JN001168
6/85	USA	(Evans and Hafez, 1992)	FJ654146	JN001169
86026	Australia	This study	JN001165	JN001170

Table 2. The genotyping of mixed MG strains using *vlhA* PCR-HRM with different DNA concentrations

a)

Sample	DNA conc (ng)	Mean GCP	Genotype
			72.9 CP
ts-11	1	99.9	ts-11
F	1	99.7	F
ts-11 + F	1 + 1	95.2	ts-11
ts-11 + F	$1 + 10^{-1}$	99.6	ts-11
ts-11 + F	$1 + 10^{-2}$	99.1	ts-11
ts-11 + F	$1 + 10^{-3}$	99.7	ts-11
ts-11 + F	$1 + 10^{-4}$	99.7	ts-11
ts-11 + F	$10^{-1} + 1$	91	ts-11
ts-11 + F	$10^{-2} + 1$	23	Variation
ts-11 + F	$10^{-3} + 1$	99.1	F
ts-11 + F	$10^{-4} + 1$	99.7	F

b)

Sample	DNA conc (ng)	Mean GCP	Genotype
			72.9 CP
ts-11	1	99.6	ts-11
6/85	1	99.4	6/85
ts-11 + 6/85	1 + 1	97.8	ts-11
ts-11 + 6/85	$1 + 10^{-1}$	97.6	ts-11
ts-11 + 6/85	$1 + 10^{-2}$	83.3	ts-11
ts-11 + 6/85	$1 + 10^{-3}$	97.6	ts-11
ts-11 + 6/85	$1 + 10^{-4}$	96.4	ts-11
ts-11 + 6/85	$10^{-1} + 1$	85.0	6/85
ts-11 + 6/85	$10^{-2} + 1$	84.3	6/85
ts-11 + 6/85	$10^{-3} + 1$	22.1	Variation
ts-11 + 6/85	$10^{-4} + 1$	32.5	Variation

c)

Sample	DNA conc (ng)	Mean GCP	Genotype
			72.9 CP
ts-11	1	99.7	ts-11
S6	1	98.5	S6
ts-11 + S6	1 + 1	74.5	ts-11
ts-11 + S6	$1 + 10^{-1}$	71.2	Variation
ts-11 + S6	$1 + 10^{-2}$	46.4	Variation
ts-11 + S6	$1 + 10^{-3}$	99.8	ts-11
ts-11 + S6	$1 + 10^{-4}$	97.4	ts-11
ts-11 + S6	$10^{-1} + 1$	95.0	ts-11
ts-11 + S6	$10^{-2} + 1$	4.6	Variation
ts-11 + S6	$10^{-3} + 1$	60.5	Variation
ts-11 + S6	$10^{-4} + 1$	99.6	S6

d)

Sample	DNA conc (ng)	Mean GCP	Genotype
			72.9 CP
ts-11	1	99.2	ts-11
86026	1	98.5	86026

ts-11 + 86026	1 + 1	95.1	ts-11
ts-11 + 86026	$1 + 10^{-1}$	97.3	ts-11
ts-11 + 86026	$1 + 10^{-2}$	99.5	ts-11
ts-11 + 86026	$1 + 10^{-3}$	98.9	ts-11
ts-11 + 86026	$1 + 10^{-4}$	99.5	ts-11
ts-11 + 86026	$10^{-1} + 1$	4.4	Variation
ts-11 + 86026	$10^{-2} + 1$	79.4	86026
ts-11 + 86026	$10^{-3} + 1$	97.3	86026
ts-11 + 86026	$10^{-4} + 1$	99.1	86026

Table 3. The genotyping of mixed MG strains using *pvpA* PCR-HRM with different DNA concentrations

a)

Sample	DNA conc (ng)	Mean GCP	Genotype
			75.6 CP
ts-11	1	99.1	ts-11
F	1	99.9	F
ts-11 + F	1 + 1	98.5	F
ts-11 + F	$1 + 10^{-1}$	77.2	F
ts-11 + F	$1 + 10^{-2}$	90.5	ts-11
ts-11 + F	$1 + 10^{-3}$	98.5	ts-11
ts-11 + F	$1 + 10^{-4}$	98.5	ts-11
ts-11 + F	$10^{-1} + 1$	92.9	F
ts-11 + F	$10^{-2} + 1$	95.0	F
ts-11 + F	$10^{-3} + 1$	99.0	F
ts-11 + F	$10^{-4} + 1$	98.9	F

b)

Sample	DNA conc (ng)	Mean GCP	Genotype
			75.6 CP
ts-11	1	96.5	ts-11
6/85	1	99.9	6/85
ts-11 + 6/85	1 + 1	12.1	Variation
ts-11 + 6/85	$1 + 10^{-1}$	34.1	Variation
ts-11 + 6/85	$1 + 10^{-2}$	85.7	ts-11
ts-11 + 6/85	$1 + 10^{-3}$	96.4	ts-11
ts-11 + 6/85	$1 + 10^{-4}$	97.3	ts-11
ts-11 + 6/85	$10^{-1} + 1$	81.3	6/85
ts-11 + 6/85	$10^{-2} + 1$	77.0	6/85
ts-11 + 6/85	$10^{-3} + 1$	97.8	6/85
ts-11 + 6/85	$10^{-4} + 1$	98.0	6/85

c)

Sample	DNA conc (ng)	Mean GCP	Genotype
			75.6 CP
ts-11	1	99.4	ts-11
S6	1	97.7	S6
ts-11 + S6	1 + 1	81.2	ts-11
ts-11 + S6	$1 + 10^{-1}$	89.3	ts-11
ts-11 + S6	$1 + 10^{-2}$	96.2	ts-11
ts-11 + S6	$1 + 10^{-3}$	99.3	ts-11
ts-11 + S6	$1 + 10^{-4}$	98.8	ts-11
ts-11 + S6	$10^{-1} + 1$	97.1	S6
ts-11 + S6	$10^{-2} + 1$	95.8	S6
ts-11 + S6	$10^{-3} + 1$	92.8	S6
ts-11 + S6	$10^{-4} + 1$	93.5	S6

d)

Sample	DNA conc (ng)	Mean GCP	Genotype
			75.6 CP
ts-11	1	99.8	ts-11

86026	1	99.1	86026
ts-11 + 86026	1 + 1	77.3	ts-11
ts-11 + 86026	$1 + 10^{-1}$	99.1	ts-11
ts-11 + 86026	$1 + 10^{-2}$	99.9	ts-11
ts-11 + 86026	$1 + 10^{-3}$	99.6	ts-11
ts-11 + 86026	$1 + 10^{-4}$	99.8	ts-11
ts-11 + 86026	$10^{-1} + 1$	26.6	Variation
ts-11 + 86026	$10^{-2} + 1$	16.0	Variation
ts-11 + 86026	$10^{-3} + 1$	63.4	Variation
ts-11 + 86026	$10^{-4} + 1$	94.3	86026

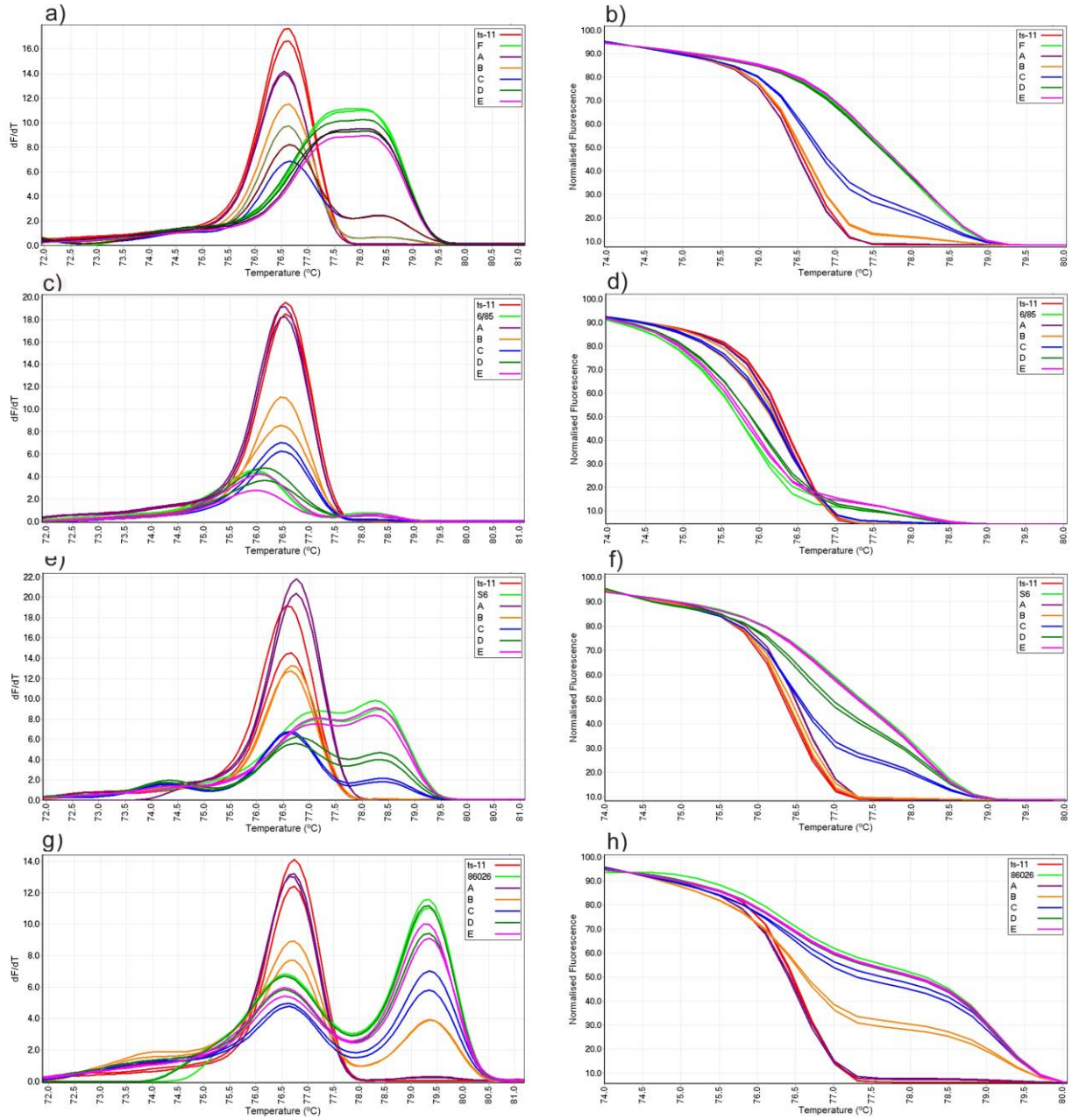

Fig. 1. Conventional and normalised melt curve analysis of mixed strains using *vlhA*-PCR. Mixture of ts-11 and F strain (a and b), ts-11 and 6/85 strain (c and d), ts-11 and S6 strain (e and f), ts-11 and 86026 field isolate (g and h). Specimens A, B, C, D and E each contains 1 ng of ts-11 DNA and 1-10^{-4} ng of contaminant MG DNA, respectively.

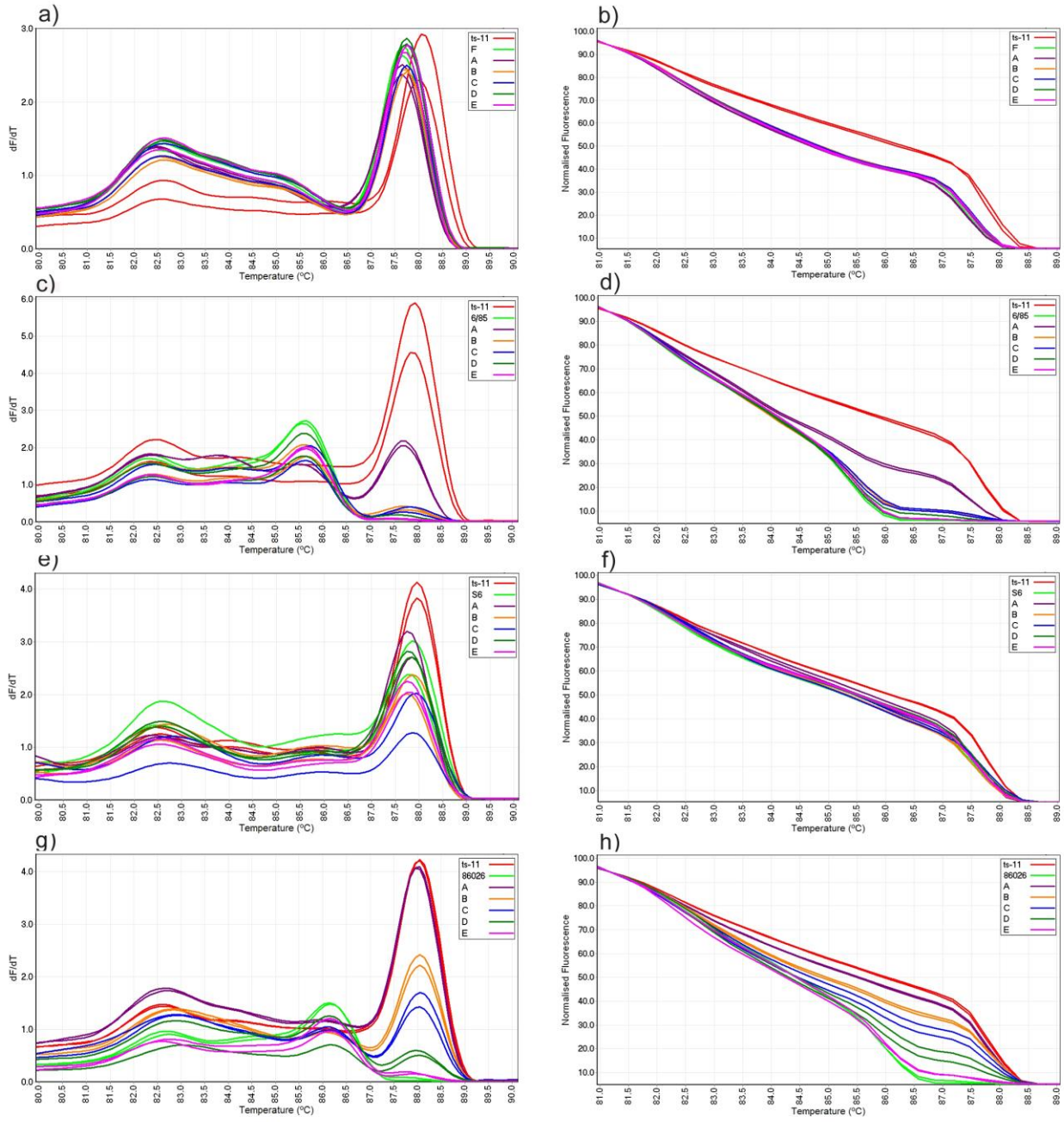

Fig. 2. Conventional and normalised melt curve analysis of mixed strains using *pvpA*-PCR. Mixture of ts-11 and F strain (a and b), ts-11 and 6/85 strain (c and d), ts-11 and S6 strain (e and f), ts-11 and 86026 field isolate (g and h). Specimens A, B, C, D and E each contains 1 ng of ts-11 DNA and 1-10^{-4} ng of contaminant MG DNA, respectively.

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