

Classification of *Mycoplasma synoviae* strains using single-strand conformation polymorphism and high-resolution melting-curve analysis of the *vlhA* gene single-copy region

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Mycoplasma synoviae is an economically important pathogen of poultry worldwide, causing respiratory infection and synovitis in chickens and turkeys. Identification of *M. synoviae* isolates is of critical importance, particularly in countries in which poultry flocks are vaccinated with the live attenuated *M. synoviae* strain MS-H. Using oligonucleotide primers complementary to the single-copy conserved 5' end of the variable lipoprotein and haemagglutinin gene (*vlhA*), amplicons of ~400 bp were generated from 35 different *M. synoviae* strains/isolates from chickens and subjected to mutation scanning analysis. Analysis of the amplicons by single-strand conformation polymorphism (SSCP) revealed 10 distinct profiles (A–J). Sequencing of the amplicons representing these profiles revealed that each profile related to a unique sequence, some differing from each other by only one base-pair substitution. Comparative high-resolution melting (HRM) curve analysis of the amplicons using SYTO 9 green fluorescent dye also displayed profiles which were concordant with the same 10 SSCP profiles (A–J) and their sequences. For both mutation detection methods, the Australian *M. synoviae* strains represented one of the A, B, C or D profiles, while the USA strains represented one of the E, F, G, H, I or J profiles. The results presented in this study show that the PCR-based SSCP or HRM curve analyses of *vlhA* provide high-resolution mutation detection tools for the detection and identification of *M. synoviae* strains. In particular, the HRM curve analysis is a rapid and effective technique which can be performed in a single test tube in less than 2 h.

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INTRODUCTION

Mycoplasma synoviae is an economically important pathogen of poultry worldwide, causing respiratory infection and synovitis in chickens and turkeys (Kleven, 1997). Provisional diagnosis and monitoring of *M. synoviae* infection is usually made using serological assays, while definitive diagnosis is made by isolation and identification of the organism. However, the latter is generally expensive and time consuming (requiring 1–2 weeks to complete). Recently, PCR has been employed for identification of suspected cultures or for rapid detection of *M. synoviae* directly from clinical samples (Garcia *et al.*, 1995; Kiss *et al.*, 1997; Lauerman *et al.*, 1993; Silveira *et al.*, 1996; Wang *et al.*, 1997). Further identification of the *M. synoviae*

isolates (i.e. defining the strain involved) is very useful for epidemiological tracing and is of critical importance in countries in which poultry flocks are vaccinated with the live attenuated *M. synoviae* strain MS-H (Vaxsafe MS, Bioproperties Australia). RFLP has been described elsewhere (Morrow *et al.*, 1990b) for classification of *M. synoviae* strains and is currently in use in our laboratory for classification of new field *M. synoviae* strains. However, RFLP is time consuming, since it requires isolation and culture of the organism and extraction of genomic DNA, and can be unreliable, particularly because of genomic rearrangements that may occur in progenies of a single *M. synoviae* isolate (Noormohammadi *et al.*, 2000). Also, sequence variation in an individual isolate may go undetected, because only a relatively small number of restriction enzymes scans a subset of a putatively variable site. An arbitrarily primed PCR has also been described (Fan *et al.*, 1995) for interspecies classification of *M. synoviae*, but the reproducibility of this technique as a

Abbreviations: HRM, high-resolution melting; SSCP, single-strand conformation polymorphism.

A table of raw data from HRM curve analysis is available with the online version of this paper.

Table 1. *Mycoplasma* species and strains used in this study, and their origin and SSCP profiles

NA, Not applicable.

<i>Mycoplasma</i> species	Isolate	Origin	GenBank accession no. (where available)	SSCP profile
<i>M. synoviae</i>	86079/7NS	Australia, NSW, parent strain of the MS-H vaccine, palatine cleft (Morrow <i>et al.</i> , 1998)		A
	MS-H	Australia, vaccine strain derived from 86079/7NS (Morrow <i>et al.</i> , 1998)	AF464936	A
	88064/FP3	Australia, VIC, field isolate, foot pad (Morrow <i>et al.</i> , 1990a)		A
	94011/V-18d	Australia, VIC, field isolate, palatine cleft (Markham <i>et al.</i> , 1998)	DQ661614	A
	2NS/3X	Australia, NSW, field isolate, palatine cleft (Melbourne University culture collection)		A
	85099/303	Australia, NSW, field isolate, foot pad (Morrow <i>et al.</i> , 1990a)		A
	85099/MS 1F	Australia, NSW, field isolate, foot pad (Morrow <i>et al.</i> , 1990a)		A
	94050/T1B	Australia, NSW, field isolate, palatine cleft (Melbourne University culture collection)		A
	93220/C-27a	Australia, QLD, field isolate, palatine cleft (Markham <i>et al.</i> , 1998)		A
	93107/5-5b	Australia, NSW, field isolate, palatine cleft (Markham <i>et al.</i> , 1998)		A
	94050/L-1	Australia, NSW, field isolate, palatine cleft (Melbourne University culture collection)		A
	94042/6a	Australia, NSW, field isolate, palatine cleft (Melbourne University culture collection)	AY913823	A
	93148/24-12b	Australia, VIC, MS-H reisolate, palatine cleft (Markham <i>et al.</i> , 1998)		A
	93198/1-12b	Australia, VIC, MS-H reisolate, palatine cleft (Markham <i>et al.</i> , 1998)		A
	94003/C-22a	Australia, QLD, field isolate, palatine cleft (Markham <i>et al.</i> , 1998)		A
	94012/2a	Australia, QLD, field isolate, palatine cleft (Markham <i>et al.</i> , 1998)		A
	94046/W1B-17a	Australia, QLD, field isolate, palatine cleft, (Melbourne University culture collection)	AY913827	A
	94029/1a	Australia, NSW, field isolate, palatine cleft, (Melbourne University culture collection)	AY913822	A
	94027/10a	Australia, NSW, field isolate, palatine cleft, (Melbourne University culture collection)	AY907705	B
	94027/19a	Australia, NSW, field isolate, palatine cleft, (Melbourne University culture collection)		B
	94027/6a	Australia, NSW, field isolate, palatine cleft, (Melbourne University culture collection)		B
	93148/23-22b	Australia, VIC, field isolate, palatine cleft (Markham <i>et al.</i> , 1998)	AY907707	C
	94041/12a	Australia, NSW, field isolate, palatine cleft (Melbourne University culture collection)	AY907706	C
	T2/3X	Australia, VIC, field isolate, trachea (Gilchrist & Cottew, 1974)	AY907704	D
	4GPH3	Australia, field strain, hock joint (Morrow <i>et al.</i> , 1990a)		D
	F10-2AS	USA, NC, field strain, airsac (Yoder <i>et al.</i> , 1977; Yogev <i>et al.</i> , 1988)	AY913824	E
	K1938	USA, AR, field strain (Morrow <i>et al.</i> , 1990b)	DQ661613	F
	FMT	USA, MS, field strain, trachea (Vardaman & Drott, 1980; Yogev <i>et al.</i> , 1988)		F
	K870	USA, ME, field strain (Hong <i>et al.</i> , 2004)		F
	K1858	USA, field strain, trachea (Weinack <i>et al.</i> , 1983)	AY913826	G
	1853m	USA, WV, type culture (Olson <i>et al.</i> , 1956), via MN, joints		H
	WVU-1853-C+	USA, WV, type culture, joints (Olson <i>et al.</i> , 1956)	AF035624	H
YA	Source unknown (Noormohammadi <i>et al.</i> , 1997)	AY913828	H	
K1968	USA, CO, field strain, turkey, joints (Hong <i>et al.</i> , 2004; Morrow <i>et al.</i> , 1990b)	AF314230	I	
K1723	USA, AR, field strain, trachea (Hong <i>et al.</i> , 2004)	AY913825	J	
<i>M. gallisepticum</i>	K2320	USA, PA, field strain, trachea*		NA
	K2079	USA, OH, field strain, trachea*		NA

Table 1. cont.

<i>Mycoplasma</i> species	Isolate	Origin	GenBank accession no. (where available)	SSCP profile
	K435	USA, GA, field strain, trachea/sinus*		NA
	YS1A	Australia, VIC, field strain, chicken embryos (Melbourne University culture collection)		NA

*S. H. Kleven, personal communication.

routine procedure has not been evaluated. More recently, sequence analysis of the single-copy conserved region of the *M. synoviae* *vlhA* gene (Noormohammadi *et al.*, 2000) has been used for classification of *M. synoviae* strains and epidemiological investigations (Bencina *et al.*, 2001; Hong *et al.*, 2004). These studies have suggested that strain identification based on the *vlhA* PCR should be regarded as a preliminary typing method for *M. synoviae*. However, this procedure is considered time consuming (requiring sequencing of the PCR product and subsequent analysis) and is relatively expensive as a routine diagnostic tool.

PCR-based mutation detection techniques, such as single-strand conformation polymorphism (SSCP) and melting-curve analysis, provide useful and cost-effective alternatives for the direct analysis of genetic variation, particularly when large numbers of samples are to be analysed. Such techniques usually rely on the physical properties or the modification of DNA fragments for the separation of molecules of the same or very similar size (i.e. differing by only one or a few nucleotides). SSCP has been described extensively in the literature as a relatively rapid technique for the discrimination of variants of a given bacterium (Charvalos *et al.*, 1996; Guttman *et al.*, 1996; Speldooren *et al.*, 1998; Telenti *et al.*, 1993; Tokue *et al.*, 1994), but so far has not been assessed for discrimination of *M. synoviae* strains. Also, high-resolution melting (HRM) curve analysis without using a fluorescence hybridization probe has been introduced recently as a rapid technique for genotyping and mutation scanning (Gundry *et al.*, 2003). The introduction of new fluorescent dyes, such as LCGreen I (Wittwer *et al.*, 2003) and SYTO 9 green (Krypuy *et al.*, 2006; Monis *et al.*, 2005), has brought further improvement to HRM curve analysis for clinical (Reed & Wittwer, 2004; Wittwer *et al.*, 2003; Zhou *et al.*, 2005) and/or epidemiological (Cheng *et al.*, 2006; Odell *et al.*, 2005; Robinson *et al.*, 2006) investigations.

The main purpose of the present study was to compare SSCP and SYTO 9 green HRM curve analyses for the detection of inter-strain nucleotide variation in the *M. synoviae* *vlhA* gene, as alternatives to sequencing, and to assess their usefulness for routine diagnostic and epidemiological applications.

METHODS

M. synoviae and *Mycoplasma gallisepticum* strains/isolates.

Several *M. synoviae* isolates from a range of geographical locations and four different *M. gallisepticum* isolates were used in this study (Table 1). All cultures were grown in mycoplasma broth (MB) as modified (Morrow *et al.*, 1998) from the original formulation of Frey *et al.* (1968).

Extraction of genomic DNA. Total genomic DNA was extracted from mycoplasma cultures as described previously (Sykes *et al.*, 1998), with some modifications. Briefly, 0.5 ml of mycoplasma cells was harvested at late-exponential phase by centrifugation at 20 000 g, the pellet was washed two times in PBS, solubilized in 300 µl RLT lysis buffer (Qiagen) and incubated at 4 °C overnight. A 15 µl volume of Qiaex II matrix (Qiagen) and 300 µl 70% ethanol were added to the suspension, and the matrix was resuspended by vortexing. This suspension was loaded into a multispin MSK-100 column (Axygen). Columns were centrifuged for 30 s at 10 000 g in a microcentrifuge and the flow-through discarded. The matrix was washed once with 600 µl buffer RW1 (Qiagen) and twice with 500 µl buffer RPE (Qiagen). For each wash, columns were centrifuged for 30 s at 10 000 g. The columns were centrifuged for 90 s at 18 000 g to dry the matrix. The matrix was overlaid with 30 µl distilled water. After incubation at room temperature for 1 min, columns were centrifuged for 1 min at 10 000 g. The genomic DNA samples were used immediately or stored at -20 °C prior to use in the PCR.

PCR amplification. On the basis of the *vlhA*₁ gene sequence (the first characterised *M. synoviae* *vlhA* gene variant) (GenBank database accession no. AF035624), two oligonucleotide primers Link (5'-TACTATTAGCAGCTAGTGC-3') and MSCons-R (5'-AGTAACCGATCCGCTTAAT-3') were designed and used to amplify 350–400 bp of the single-copy conserved 5' end of the *vlhA* genes (Noormohammadi *et al.*, 2000) from different *M. synoviae* strains. PCR was performed using Platinum *Taq* DNA Polymerase High Fidelity (Invitrogen) with some modifications. A 50 µl reaction mixture contained 200 µM each of dATP, dCTP, dGTP and dTTP, 2 mM MgSO₄, 250 µM each primer, 1 U Platinum *Taq* DNA Polymerase High Fidelity, 5 µl 10 × Platinum *Taq* DNA polymerase buffer and 1 µl extracted *M. synoviae* genomic DNA. The reaction mixture was incubated at 94 °C for 2 min, then subjected to 35 cycles of 96 °C for 15 s, 54 °C for 15 s and 68 °C for 20 s.

In each set of reactions, chicken and *M. gallisepticum* DNA and distilled H₂O were included as negative controls. All PCR products were purified using the QIAquick PCR purification kit (Qiagen) prior to nucleotide sequencing or SSCP analysis.

Agarose gel electrophoresis and SSCP analysis. After PCR, individual amplicons were mixed with an equal volume of loading buffer (10 mM NaOH, 95% formamide, 0.05% of both bromophenol

blue and xylene cyanole) and the intensity of selected samples was verified on ethidium bromide-stained 2% agarose gels using TBE (65 mM Tris/HCl, 27 mM boric acid, 1 mM EDTA, pH 9; Bio-Rad) as the buffer and Φ X174-*Hae*III (Promega) as a size marker. After denaturation at 94 °C for 15 min and snap cooling on a freeze block (-20 °C), the samples were subjected to SSCP analysis, as recently described (Gasser *et al.*, 2004). In brief, samples (~12 μ l each) were loaded into the wells of precast GMA S-12x13 gels (96 \times 261 mm; product no. 3548, Elchrom Scientific) and subjected to electrophoresis for 16.5 h at 72 V and 7.2 °C (constant) in a horizontal SEA2000 apparatus (Elchrom Scientific) connected to a MultiTemp III (Pharmacia) cooling system. After electrophoresis, gels were stained for 15 min with ethidium bromide (0.5 μ g ml⁻¹), destained in water for the same time and then photographed (using 667 film, Polaroid) upon UV transillumination. SSCP profiles were demonstrated to be reproducible on different days using amplicons produced on different days (data not shown).

HRM curve analysis. Amplification of target sequences was carried out on a RotorGene thermal cycler (RG6000, Corbett Research). Each 20 μ l reaction consisted of 200 μ M each of dATP, dCTP, dGTP and dTTP, 1 mM MgCl₂, 250 μ M each primer (Link and MSCons-R), 5 μ M SYTO 9 green fluorescent nucleic acid stain (Invitrogen), 0.5 U *Taq* DNA polymerase (Promega), 2 μ l 10 \times *Taq* DNA polymerase buffer and 1 μ l extracted *M. synoviae* genomic DNA. The reaction mixture was incubated at 96 °C for 2 min and then subjected to 40 cycles of 96 °C for 15 s, 54 °C for 15 s and 72 °C for 20 s. Optical measurements in the green channel (excitation at 470 nm and detection at 510 nm) were recorded during the extension step. After completion of 40 PCR cycles, melting-curve data were generated by increasing the temperature from 70 to 99 °C at 0.2 °C s⁻¹ and recording fluorescence. HRM curve analysis was performed using the software Rotor-Gene 1.7.27 and the HRM algorithm provided. Normalization regions of 79.88–80.00 and 83.08–84.23 and a confidence threshold of 90% were applied, and profiles giving an identity of less than 95% to any of the existing profiles were considered as distinct profiles.

Sequencing and nucleotide sequence analyses. Amplicons were purified using the QIAquick PCR purification kit (Qiagen), eluted in 30 μ l H₂O and then subjected to automated sequencing (BigDye chemistry, Applied Biosystems) in both directions, using the same primers as for PCR. The nucleotide sequences were compared with each other and with those previously available in GenBank (accession nos AF464936, AF035624 and AF314230). Computer analysis of the nucleotide sequences was performed using the programs provided by the Australian National Genomic Information Service (<http://www.angis.org.au>). The computer program CLUSTALW-fast (Thompson *et al.*, 1994) was used to create multiple sequence alignments, and OldDistances was used to calculate pairwise similarities between sequences.

RESULTS

Ten distinct SSCP profiles represented the *M. synoviae* isolates

On the basis of the *vlhA* gene sequence available in GenBank, two oligonucleotide primers, Link and MSCons-R, were designed and used to amplify 350–400 bp of the single-copy conserved 5' end of the *vlhA* gene (Noormohammadi *et al.*, 2000) from genomic DNA extracted from 35 *M. synoviae* strains/isolates (Table 1). PCR products of the expected size range were generated from all *M. synoviae*

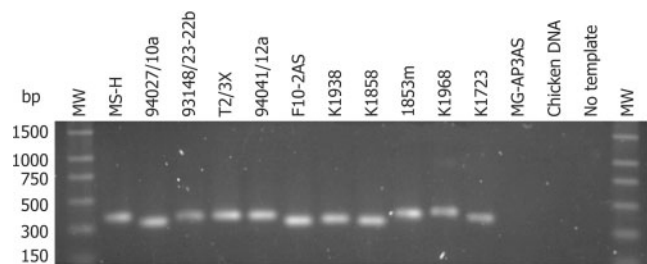


Fig. 1. Agarose gel electrophoresis of PCR products of the *vlhA* gene from different *M. synoviae* strains/isolates. MW, molecular weight marker (PCR Marker, Sigma).

strains/isolates, but not from *M. gallisepticum* or chicken DNA (Fig. 1). SSCP analysis of the PCR products resulted in 10 distinct profiles named A–J. An example of each profile is presented in Fig. 2. The number of bands per profile varied from two to four, considering both single-stranded and double-stranded DNA. Most Australian strains/isolates, including the vaccine strain MS-H and its parent strain 86079/7NS, produced profile A (see Table 1). Some other Australian strains/isolates, however, produced profiles B, C or D. All USA strains, but none of the Australian strains, produced one of the profiles E, F, G, H, I or J. Repeated examination of all isolates/strains by SSCP did not alter the profile produced by each strain/isolate.

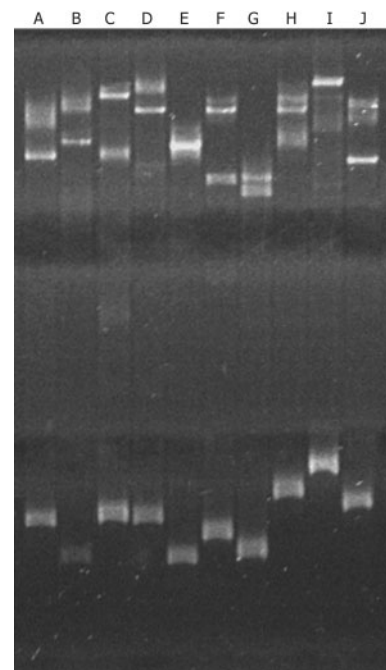


Fig. 2. SSCP profiles of the PCR products of the *vlhA* gene from different *M. synoviae* strains/isolates. Profiles: A, MS-H; B, 94027/10a; C, 93148/23-22b; D, T2/3X; E, F10-2AS; F, K1938; G, K1858; H, 1853m; I, K1968; J, K1723.

	1	11	21	31	41	51	61	71	81	91
Consensus	TACTATTAGCAGTAGTGCAGTGGCCATTGCTCCTGCTGTTATAGCAATTCATGTGGTGATCAAACCTCCAGCACCTGCTCCA	-----	-----	-----	-----	-----	-----	-----	-----	-----ACACC
94027/10aA.....
T2/3XT.....A.....
94041/12aT.....A.....
93148/23-22bT.....A.....
K1968G.....A.....
WVU-1853AA.....
YAAA.....
F10-2ASAA.....
MS-H
94046/W1B-17a
94011/V-18d
94042/6a
94029/1a
K1723
K1858G.....A.....
K1938A.....GCACCTACTCCA

	101	111	121	131	141	151	161	171	181	191
Consensus	TGGAAACCCAAATCTGATAATCCTCAAACCCAAATCCAGGAAA	CCAGG	ACT	-----	-----	-----	-----	-----	-----	-----GA-----TAAT
94027/10aG.A.GATAATCCTCAAACCCAAATCCAGGAAACCCAG.....
T2/3XC.....G.A.GATAATCCTCAAACCCAAATCCAGGAAACCCAG.....
94041/12aC.....A.....GATAATCCTCAAACCCAAATCCAGGAAACCCAG.....
93148/23-22bC.....A.....GATAATCCTCAAACCCAAATCCAGGAAACCCAG.....
K1968C.....A.....GATAATCCTCAAACCCAAATCCAGGAAACCCAG.....ACTGA.....
WVU-1853T.....T.....CCAGGAAATCCAGGTACT.....
YAT.....T.....CCAGGAAATCCAGGTACT.....
F10-2AS
MS-H
94046/W1B-17a
94011/V-18d
94042/6a
94029/1a
K1723
K1858
K1938

	201	211	221	231	241	251	261	271	281	291
Consensus	CTCAAACCCAAATCCAGGAAA	CCAGG	GGTGGTACAGTTGACCCGTGAGAGGCTGCTAAAACAGAAAGCTAAAACCGCTATTGATGCTTCAGCAGAAT	-----	-----	-----	-----	-----	-----	-----
94027/10aT.....G.A.....
T2/3XT.....G.A.....
94041/12aT.....G.A.....
93148/23-22bT.....G.A.....
K1968T.....A.....T.....G.....
WVU-1853C.....A.....
YAC.....A.....
F10-2ASC.....A.....
MS-HT.....G.....
94046/W1B-17aT.....G.....
94011/V-18dT.....G.....
94042/6aT.....G.....
94029/1aT.....G.....
K1723C.....A.....
K1858T.....G.....
K1938G.....A.....T.....A.....

	301	311	321	331	341	351	361	371	381	391
Consensus	TATCAGATTTCAGTTAAAGAAGCATTAAAGAGACAAGTTGAAGCAACTACAACAGAAAGCTGCAGCCAGAGATTTAAAACTAAAGCAGAAGCTCTTGTTTC	-----	-----	-----	-----	-----	-----	-----	-----	-----
94027/10aC.A.....
T2/3XC.A.....
94041/12aC.A.....
93148/23-22bC.A.....
K1968C.A.....
WVU-1853A.....C.....
YAA.....C.....
F10-2ASA.....C.....
MS-HA.....
94046/W1B-17aA.....
94011/V-18dA.....
94042/6aA.....
94029/1aA.....
K1723A.....A.....
K1858T.....
K1938T.....

	401	411	421
Consensus	AGCTGTAAAAGCATTAAAGCGGATCGGTTACT	-----	-----
94027/10a
T2/3X
94041/12a
93148/23-22b
K1968T.....
WVU-1853
YA
F10-2AS
MS-H
94046/W1B-17a
94011/V-18d
94042/6a
94029/1a
K1723
K1858
K1938

Fig. 3. Comparison of the partial nucleotide sequence of the *vlhA* gene amplified from different *M. synoviae* strains/isolates. The computer program CLUSTALW-fast was used for this comparison. Identical nucleotides and deletions are shown by ‘.’ and ‘-’, respectively.

Correlation between SSCP profiles and *vlhA* sequence data

In order to establish the extent of sequence variability in the *vlhA*-gene PCR products generated from *M. synoviae*

strains/isolates, a representative of each SSCP profile A–J (MS-H, 94027/10a, 93148/23-22b, T2/3X, F10-2AS, K1938, K1858, WVU-1853-C+, K1968 and K1723, respectively) was selected. Where available, the nucleotide sequence of the respective *vlhA* region was retrieved from GenBank;

Table 2. Percentage differences among sequences from *vlhA* PCR products of selected *M. synoviae* strains

Differences were calculated using the GCG program OldDistances.

Strain	Strain															
	94027/ 10a	T2/3X	94041/ 12a	93148/ 23-22b	K1968	WVU- 1853	YA	F10- 2AS	MS-H	94046/ W1B- 17a	94011/ V-18d	94042/ 6a	94029/ 1a	K1723	K1858	K193- 8
94027/10a	0.0															
T2/3X	0.3	0.0														
94041/12a	1.3	1.6	0.0													
93148/23-22b	1.3	1.6	0.0	0.0												
K1968	6.9	7.1	6.6	6.6	0.0											
WVU-1853	13.6	13.9	13.6	13.6	9.7	0.0										
YA	13.6	13.9	13.6	13.6	9.7	0.0	0.0									
F10-2AS	10.4	10.7	10.7	10.7	13.7	7.7	7.7	0.0								
MS-H	11.8	12.1	11.8	11.8	8.1	4.4	4.4	7.3	0.0							
94046/W1B-17a	11.8	12.1	11.8	11.8	8.1	4.4	4.4	7.3	0.0	0.0						
94011/V-18d	11.8	12.1	11.8	11.8	8.1	4.4	4.4	7.3	0.0	0.0	0.0					
94042/6a	11.8	12.1	11.8	11.8	8.1	4.4	4.4	7.3	0.0	0.0	0.0	0.0				
94029/1a	11.8	12.1	11.8	11.8	8.1	4.4	4.4	7.3	0.0	0.0	0.0	0.0	0.0			
K1723	12.4	12.6	12.4	12.4	8.1	4.4	4.4	7.6	1.6	1.6	1.6	1.6	1.6	0.0		
K1858	8.2	8.5	9.0	9.0	13.2	11.0	11.0	5.0	7.6	7.6	7.6	7.6	7.6	8.5	0.0	
K1938	9.7	10.0	10.5	10.5	14.5	12.4	12.4	6.6	9.1	9.1	9.1	9.1	9.1	10.0	1.7	0.0

otherwise the *vlhA* PCR product from the strain was subjected to nucleotide sequencing. In addition, a number of replicates from each profile, including 94011/V-18d, 94042/6a, 94046/W1B-17a and 94029/1a (profile A), 94041/12a (profile C) and YA (profile H), were selected and similarly examined. Comparison of the nucleotide sequences revealed that all *M. synoviae* strains/isolates with different SSCP profiles had different nucleotide sequences (Fig. 3). All *M. synoviae* strains/isolates with identical SSCP profiles (MS-H, 94011/V-18d, 94042/6a, 94046/W1B-17a and 94029/1a; 93148/23-22b and 94041/12a; WVU-1853-C+ and YA) had identical nucleotide sequences. In total, 10 different genotypes of *M. synoviae* were defined by sequencing. There was a complete concordance between SSCP profiles and the defined nucleotide sequence types. Differences between nucleotide sequences ranged from 0.3% (between 94027/10a and T2/3X) to 14.5% (between K1968 and K1938). Hence, the lowest level of nucleotide variation detectable by SSCP was 1 nt between strains 94027/10a and T2/3X (see Fig. 3 and Table 2).

HRM curve profiles of the *vlhA* PCR products from *M. synoviae* strains/isolates correlated with those of SSCP

PCR products from *M. synoviae* strains representative of each SSCP profile were selected and subjected to HRM curve analysis as described in Methods. All strains generated a major peak melting between 83.15 and 83.65 °C, and a smaller shoulder peak melting between 81.42 °C and 82.33 °C (Fig. 4a and Table 3). Analyses of the

normalized curves (Fig. 4b) revealed that *M. synoviae* strains with distinct SSCP profiles also represented distinct HRM genotypes (Table 3). *M. synoviae* strains with identical SSCP profiles (and an identical nucleotide sequence of the *vlhA* PCR product) generated identical genotypes (see Supplementary Table S1). For example, *M. synoviae* isolates 86079/7NS, MS-H and 94011/V-18d produced the same HRM curve profile (A). Similarly, isolates 94027/19a and 94027/6a, and 93148/23-22b and 94041/12a, also produced the same profiles, B and C, respectively. Several HRM runs (performed on different days) using at least one representative from each profile resulted in only minor variations in melting temperatures of the peaks, but following normalization of data, the profiles were consistent with those described above (Table 4). Further details of HRM runs are available in Supplementary Table S1.

DISCUSSION

This study provides a direct comparison between nucleotide sequencing, SSCP and HRM curve analysis using SYTO 9 for detection of differences in a relatively short stretch of the *M. synoviae vlhA* gene. In addition, to the best of our knowledge, this is the first study that describes the use of SYTO 9 in HRM curve analysis for typing of an infectious agent. The results showed that both SSCP and HRM curve analysis were capable of detecting variations of 1 bp in PCR products of approximately 400 bp (e.g. see profiles B and D). Nucleotide sequencing is now available

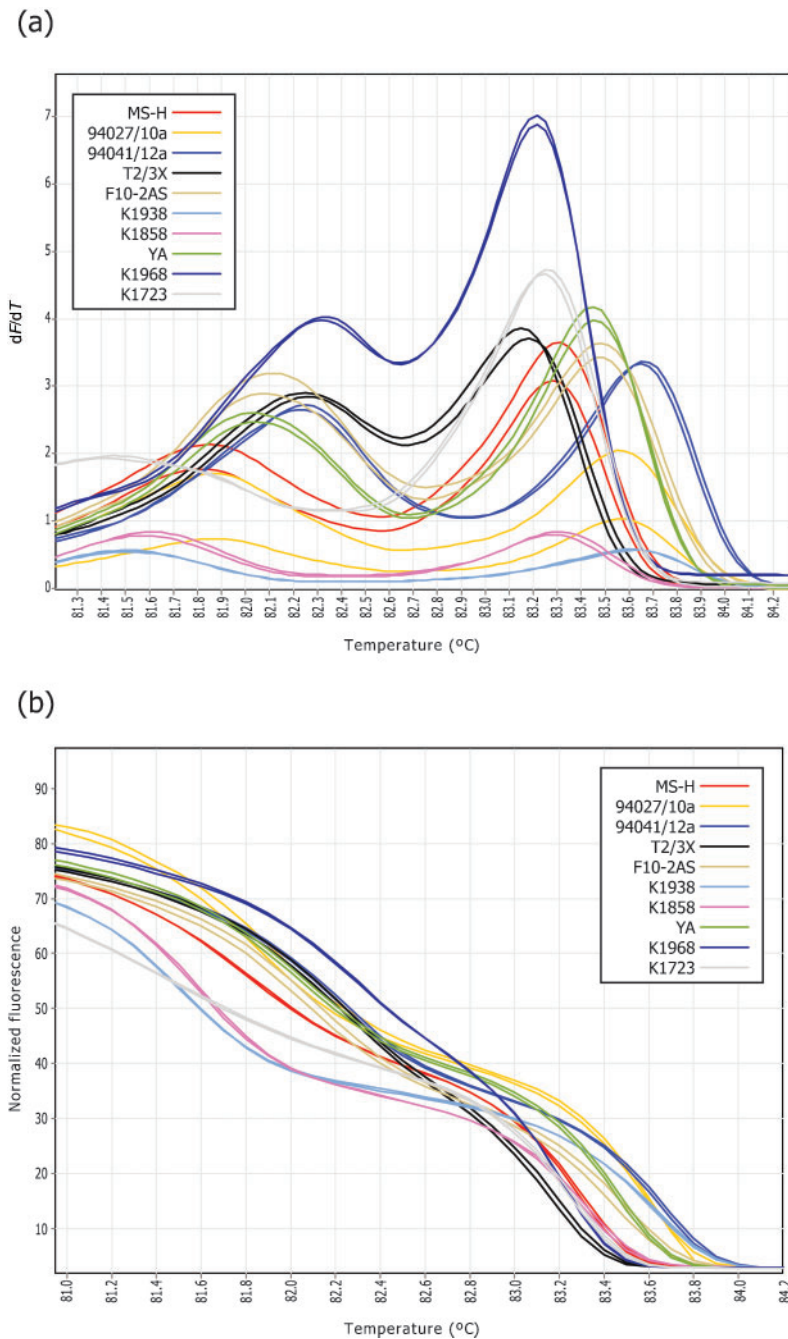


Fig. 4. (a) Melting-curve analysis of PCR products of the *vIhA* gene from different *M. synoviae* strains/isolates. (b) Normalized HRM curve analysis of PCR products of the *vIhA* gene from different *M. synoviae* strains/isolates.

in/to many diagnostic facilities at a low cost. SSCP is also relatively inexpensive (in terms of reagents) to perform and does not require sophisticated equipment. Two exceptional advantages of SSCP are its very high resolving capacity and the provision of the option of excising bands for further analysis, e.g. by nucleotide sequencing. This option is not possible for HRM curve analysis. However, nucleotide sequencing and SSCP are time-consuming procedures and require skill for interpretation of results. In contrast, the HRM curve analysis is rapid and convenient, and all relevant procedures including PCR and melting-curve analysis can be performed in a single tube. An additional

advantage of HRM curve analysis is that it can be performed in an automated module, obviating the need for extensive interpretation of results. Furthermore, with each unknown specimen, a library of prototype profiles can be used to facilitate determination of the identity of a profile. Research within our laboratory has shown that such a library of prototype PCR products (which includes SYTO 9) can be reused several times without detectable variation in the melting-curve temperature (results not shown). This is in agreement with other studies (Monis *et al.*, 2005), in which, in contrast to SYBR Green I, SYTO 9 has been found to produce highly reproducible DNA

melting and to be less selective in incorporation into particular amplicons.

All HRM curve profiles generated from *M. synoviae* strains in this study were found to have a major peak and a shorter shoulder peak. However, only one single DNA band could be detected when PCR products were subjected to agarose gel electrophoresis (see Fig. 1). It is unlikely that the shoulder peaks resulted from primer dimers, since such a peak was not detected in a no-template control that contained both primers. In addition, the melting temperature of the shoulder peak varied from one strain to another. It is also very unlikely that PCR products consisted of two variant sequences, since all *M. synoviae* cultures used in this study were grown from single colonies and all produced only one (and not more than one) shoulder peak. More importantly, within a single strain of *M. synoviae*, the *vlhA* single-copy gene region targeted for PCR in this study is known to be conserved (Bencina *et al.*, 2001; Hong *et al.*, 2004; Noormohammadi *et al.*, 2000, 2002). Examination of the nucleotide sequence of the targeted *vlhA* gene region revealed an uneven distribution of G/C nucleotides through the length of the region. In the type strain WVU-1853-C+, the 5' end (bp 1–240) of this

region has a G/C content of 46%, while the 3' end (bp 241–398) is AT rich and has a G/C content of only 36%. Thus it is likely that the presence of shoulder peaks is a result of uneven G/C distribution throughout the targeted DNA. However, irrespective of the cause, it seemed that the presence of the shoulder peak and its combination with the major peak contributed to the profiles generated (see normalized graphs in Fig. 4b), increasing the power of the HRM curve technique by adding further variation to the normalized melting curves.

This study describes, for what is believed to be the first time, the use of real-time PCR followed by melting-curve and SSCP analysis for detection and strain classification of *M. synoviae* isolates. An arbitrary-primed PCR has previously been described for detection of *M. synoviae* strain variations (Fan *et al.*, 1995), but the reproducibility of the method is still under question and the results are difficult to interpret. In addition, the approach does not determine whether the profile variation detected relates to genomic rearrangements that commonly occur within single isolates (Noormohammadi *et al.*, 2000). PCR followed by sequencing of the amplified product has also been described (Hong *et al.*, 2004) for detection of *M. synoviae* strain variations; however, this approach is considerably more time consuming and the results often require interpretation.

Due to identical sequences over the *vlhA* gene region examined in this study, the PCR HRM curve analysis was unable to discriminate between the vaccine strain MS-H (or its parent strain 86079/7NS or its reisolates after passage *in vivo*) and the Australian isolates 93220/C-27a and 93107/5-5b. The last two have been shown to be distinct from MS-H by RFLP of genomic DNA (Markham *et al.*, 1998). Recent studies in our laboratory have revealed that progenies of a single *M. synoviae* strain isolated after infection *in vivo* may exhibit different RFLP patterns

Table 3. Melting points for the peaks and HRM curve profiles of *vlhA* PCR products from selected *M. synoviae* strains

<i>M. synoviae</i> isolate	SSCP profile	Peak 1 melting point (°C)*	Peak 2 melting point (°C)*	HRM curve genotype	Confidence (%)
MS-H	A	81.82	83.28	A	99.79
MS-H	A	81.85	83.30	A	99.79
94027/10a	B	81.87	83.57	B	95.40
94027/10a	B	81.90	83.60	B	95.40
94041/12a	C	82.23	83.65	C	99.87
94041/12a	C	82.25	83.65	C	99.87
T2/3X	D	82.25	83.15	D	99.41
T2/3X	D	82.25	83.18	D	99.41
F10-2AS	E	82.12	83.48	E	99.97
F10-2AS	E	82.10	83.48	E	99.97
K1938	F	81.52	83.63	F	99.91
K1938	F	81.50	83.63	F	99.91
K1858	G	81.58	83.28	G	99.64
K1858	G	81.60	83.30	G	99.64
YA	H	82.03	83.45	H	99.88
YA	H	82.05	83.45	H	99.88
K1968	I	82.33	83.22	I	99.86
K1968	I	82.32	83.22	I	99.86
K1723	J	81.45	83.25	J	99.81
K1723	J	81.42	83.25	J	99.81

*Melting points are from an HRM run for which curves and normalized graphs are shown in Fig. 4(a) and Fig. 4(b), respectively.

Table 4. Mean \pm SD of the melting points for the two peaks of different profiles resulting from several runs of *vlhA* PCR followed by HRM curve analysis

Raw data are available in Supplementary Table S1.

Profile	No. of samples tested	Peak 1 (°C)	Peak 2 (°C)
A	19	81.94 \pm 0.32	83.44 \pm 0.34
B	14	81.97 \pm 0.51	83.68 \pm 0.50
C	15	82.35 \pm 0.40	83.80 \pm 0.40
D	16	82.41 \pm 0.33	83.37 \pm 0.33
E	13	82.21 \pm 0.38	83.61 \pm 0.38
F	25	81.67 \pm 0.33	83.78 \pm 0.31
G	16	81.70 \pm 0.27	83.41 \pm 0.25
H	13	82.24 \pm 0.29	83.66 \pm 0.29
I	13	82.49 \pm 0.34	83.39 \pm 0.34
J	13	81.66 \pm 0.36	83.43 \pm 0.36

(unpublished data). However, it is not known whether the isolates 93220/C-27a and 93107/5-5b are closely related to the MS-H vaccine (or its parent strain 76079/7NS), but have undergone genomic rearrangement, or whether they are indeed distinct strains but have an identical *vlhA* single-copy conserved 5' region. Hence, when an unknown Australian isolate exhibits SSCP or HRM curve patterns similar to those of the MS-H vaccine, confirmation of the results by conventional RFLP of genomic DNA may be useful.

PCR HRM curve analysis was able to discriminate between all the Australian vaccine/field isolates and the overseas (USA) strains examined in this study. This feature is particularly useful for discrimination of the vaccine strain from local field strains if the MS-H vaccine were to be introduced into countries such as the USA in which HRM curve profile A (MS-H type) is not found.

In summary, the combination of PCR and HRM curve analysis is a rapid and specific technique for the characterization of *M. synoviae* isolates. The entire process including extraction of DNA, PCR and HRM curve analysis can be completed within 2 h. Research in our laboratory on other avian pathogens has shown that a separately performed PCR step using conventional thermocyclers with SYTO 9 may be added immediately before HRM curve analysis. Studies are currently under way in our laboratory to adopt this assay for direct identification of *M. synoviae* in clinical specimens collected from diseased birds.

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