



Strain identification of avian mycoplasmas

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Why strain identification?

- Epidemiological investigations?
 - Origin of the infection within a highly globalised industry
 - Risk of transmission from non poultry avian species
- Development of prevention strategies?
- Regulatory and registration bodies → vaccine companies

The ideal test

- Reliable
- Reproducible
- Rapid
- Inexpensive
- Easily interpreted
- Amenable to conventional diagnostic laboratories
- Applicable for all countries

Methods

- Methods that require isolation and growing of the organism
- PCR based methods

Temperature sensitivity phenotype

33°C

39.5°C



Log₁₀ difference of 3 \geq means Ts+

MS-H

Field isolates

23.1

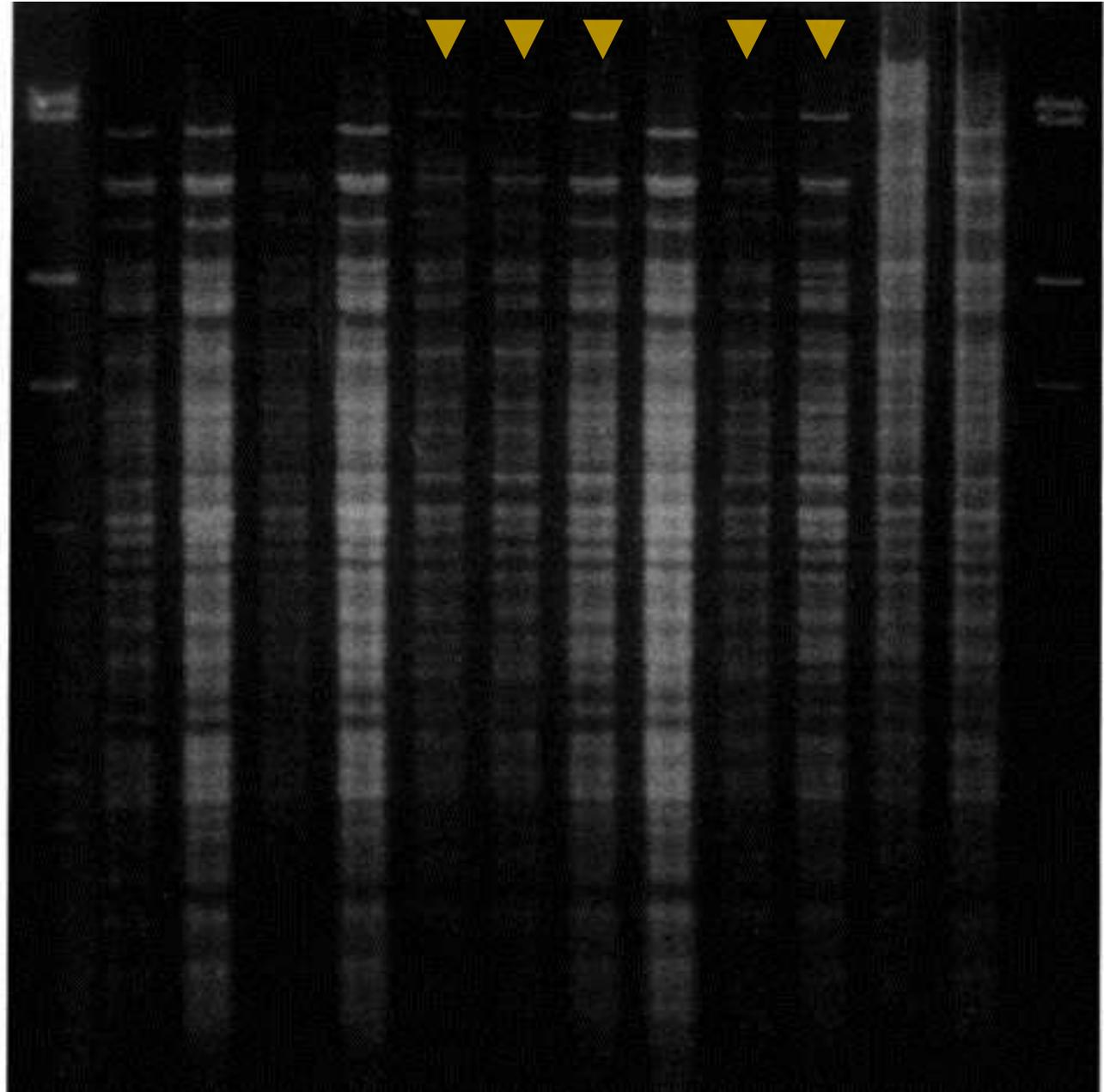
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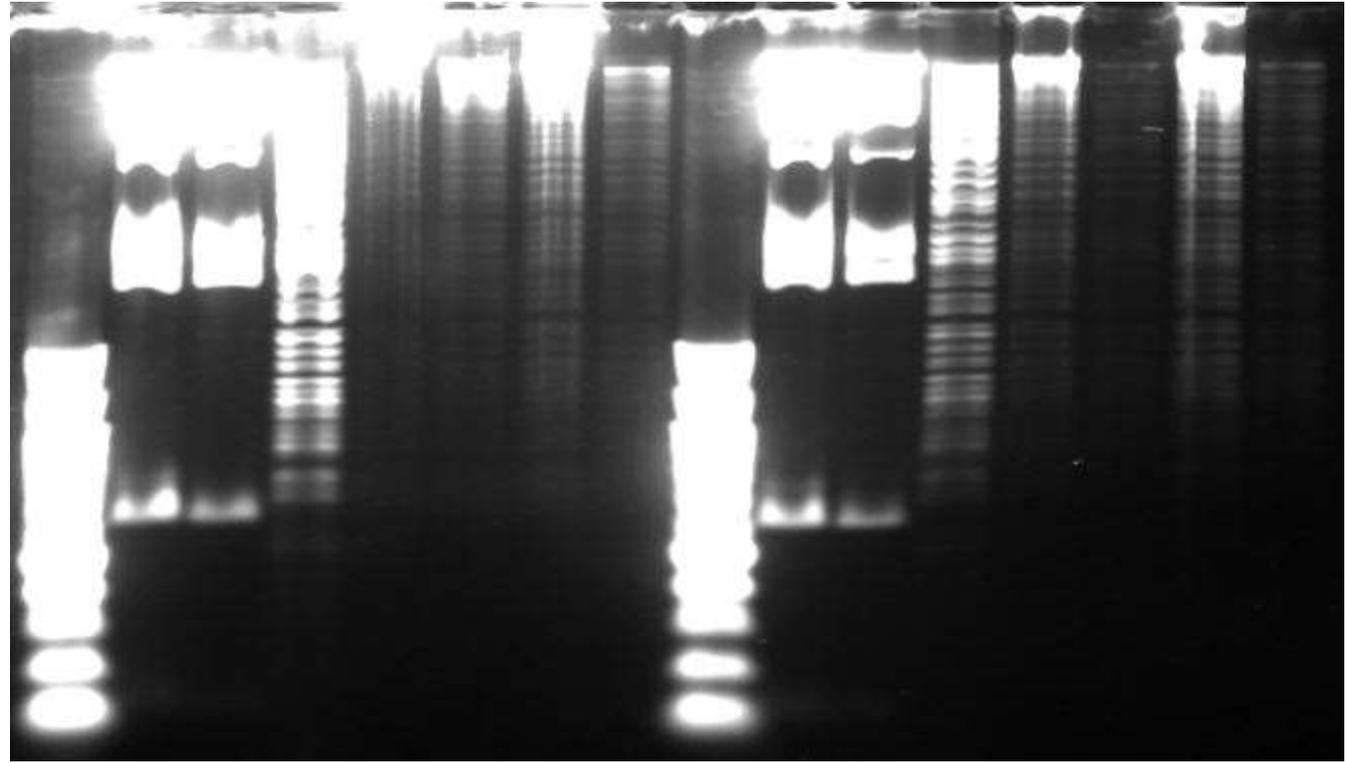
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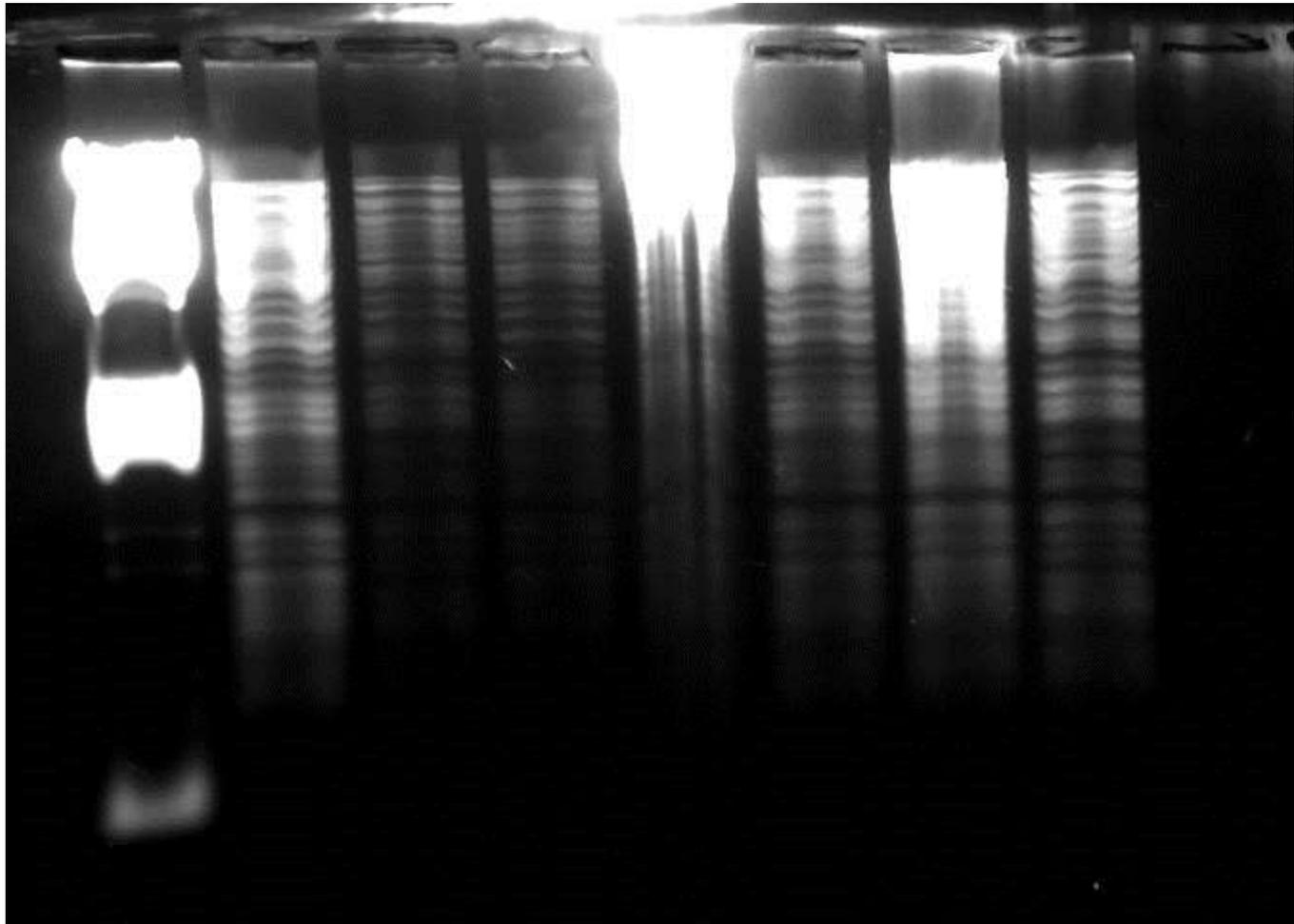
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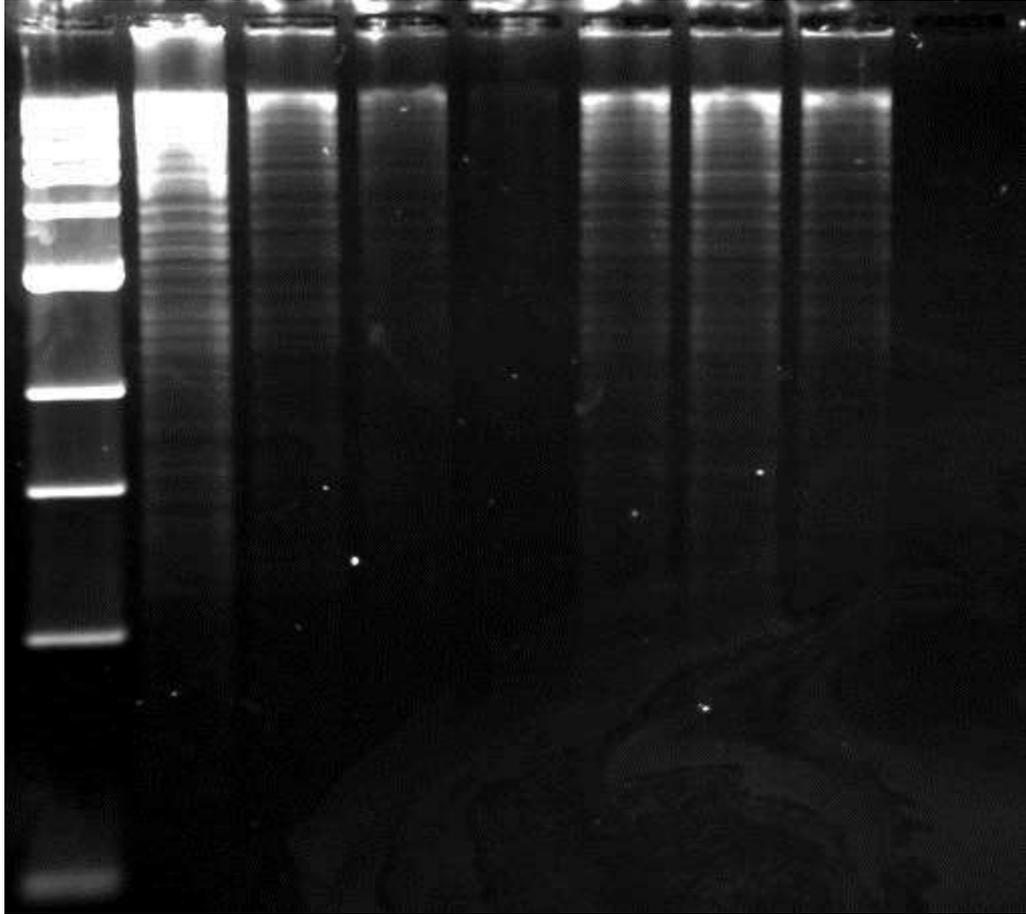
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2.0



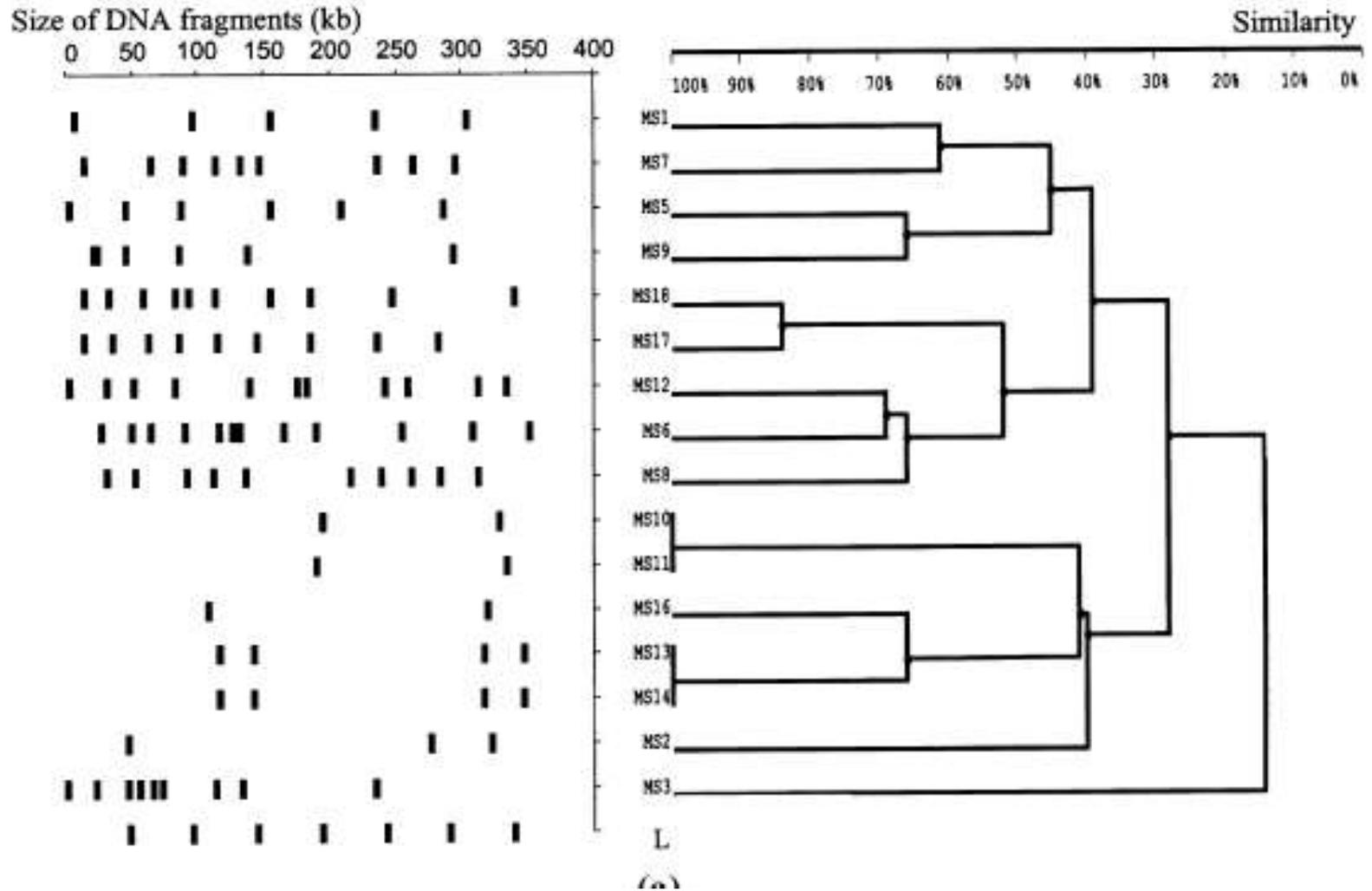






AFLP

C. Marois et al. / Veterinary Microbiology 79 (2001) 1-9



Methods

- RFLP of genomic DNA
- ts phenotype (for ts vaccines only)
- AFLP
- PCR based techniques
 - RAPD
 - SSCP
 - HRM
 - Targeted sequencing including MLST
- Whole genome sequencing

RAPD

Intraspecies heterogeneity of avian mycoplasmas

771

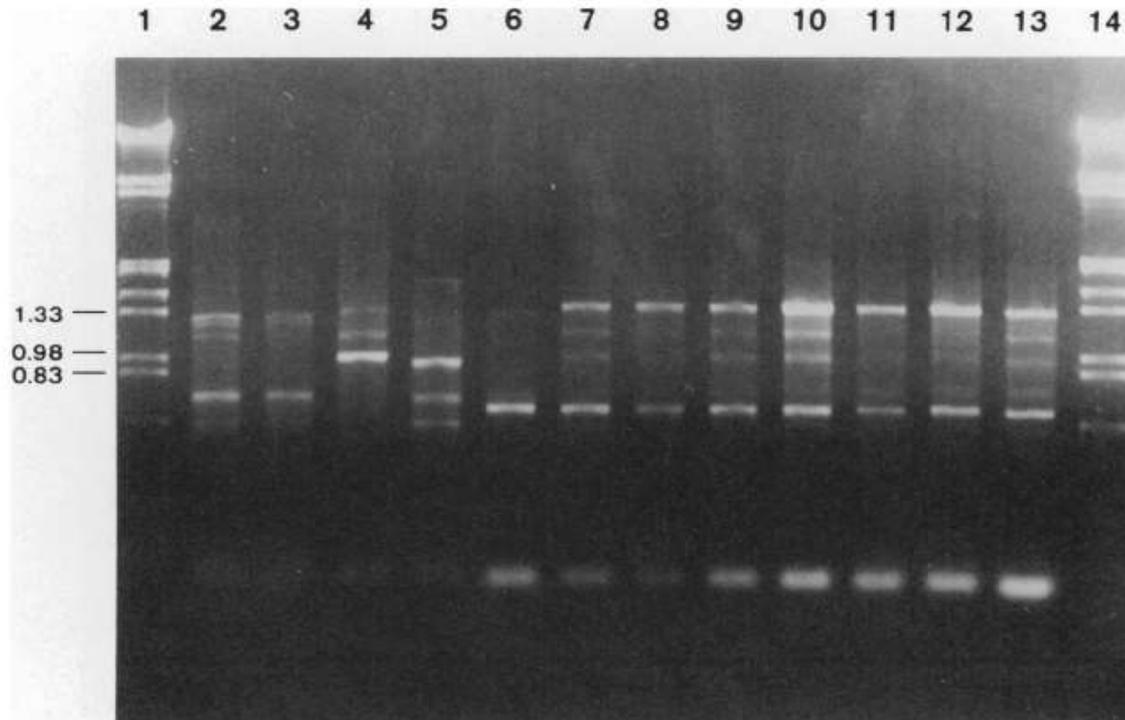


Fig. 3. AP-PCR fingerprints of 12 *M. iowae* isolates generated by three arbitrary primers. Lanes 1 and 14 = molecular size markers made from bacteriophage lambda DNA digested with *Hind*III and *Eco*RI. Numbers at left are molecular size markers in kb. Lane 2 = I; Lane 3 = K3146; Lane 4 = K; Lane 5 = K3495A; Lane 6 = PPAV; Lane 7 = C1; Lane 8 = B50/92; Lane 9 = B76/92; Lane 10 = B65/92; Lane 11 = B10/80; Lane 12 = m4/77/5T; Lane 13 = 90162.

Target for PCR based techniques

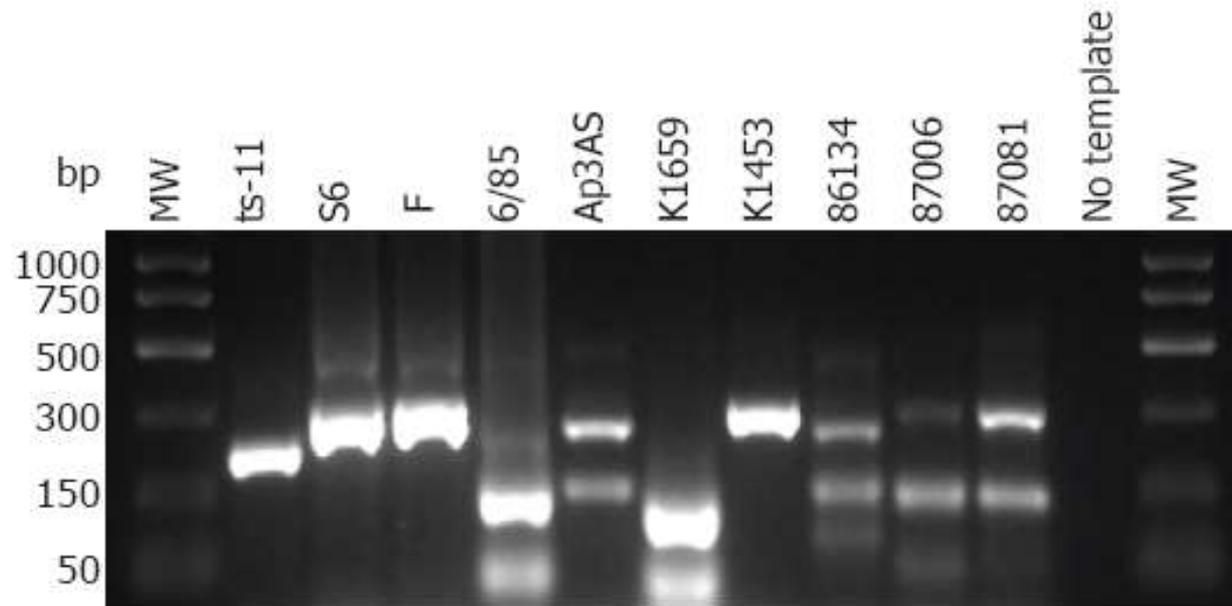
- Should vary between strains
- Should not vary after passage, etc
- Should have conserved flanking regions
- Should not have homologue in other mycoplasma species or bacteria



Size difference after PCR MS



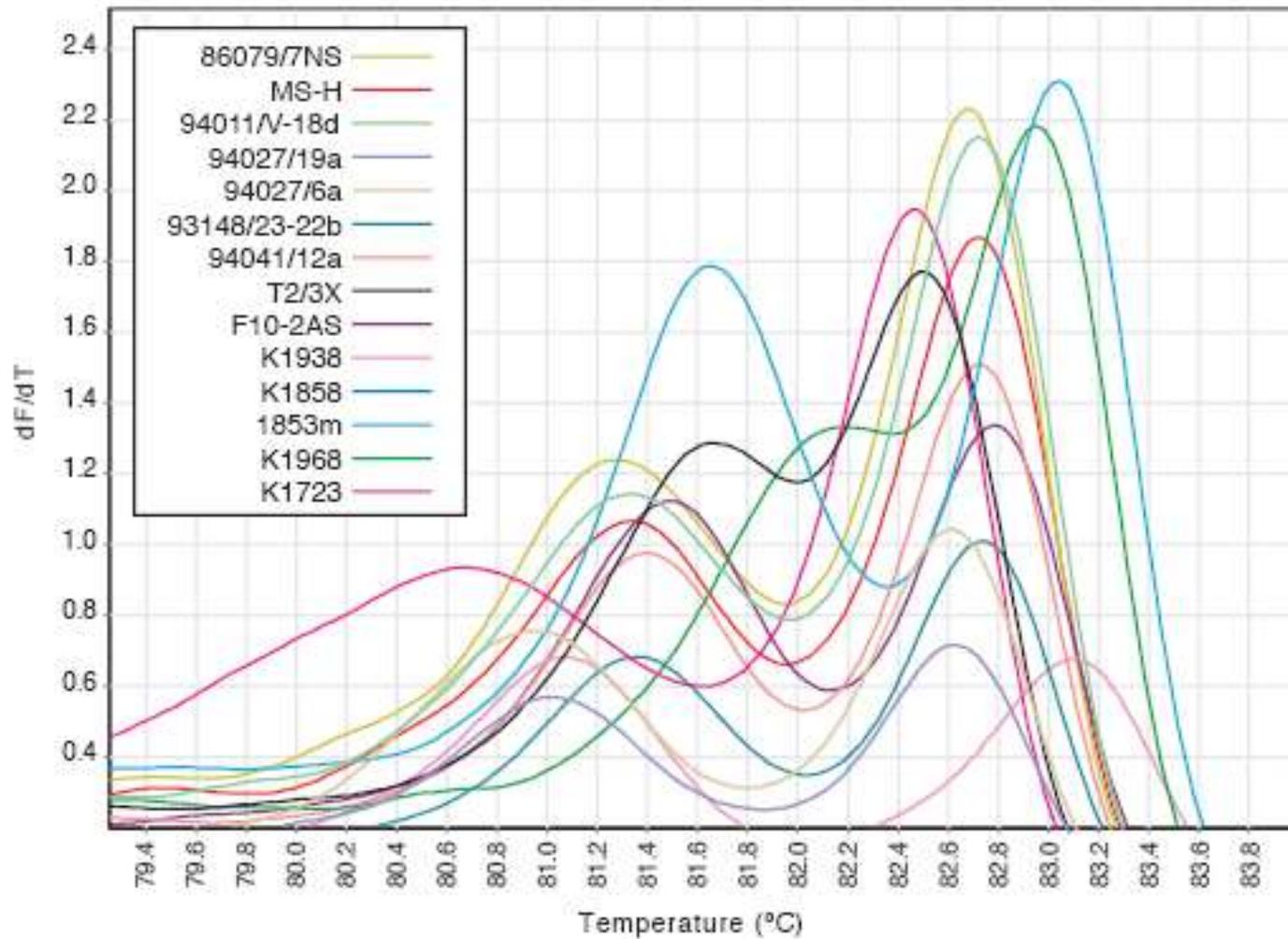
Size difference after PCR MG

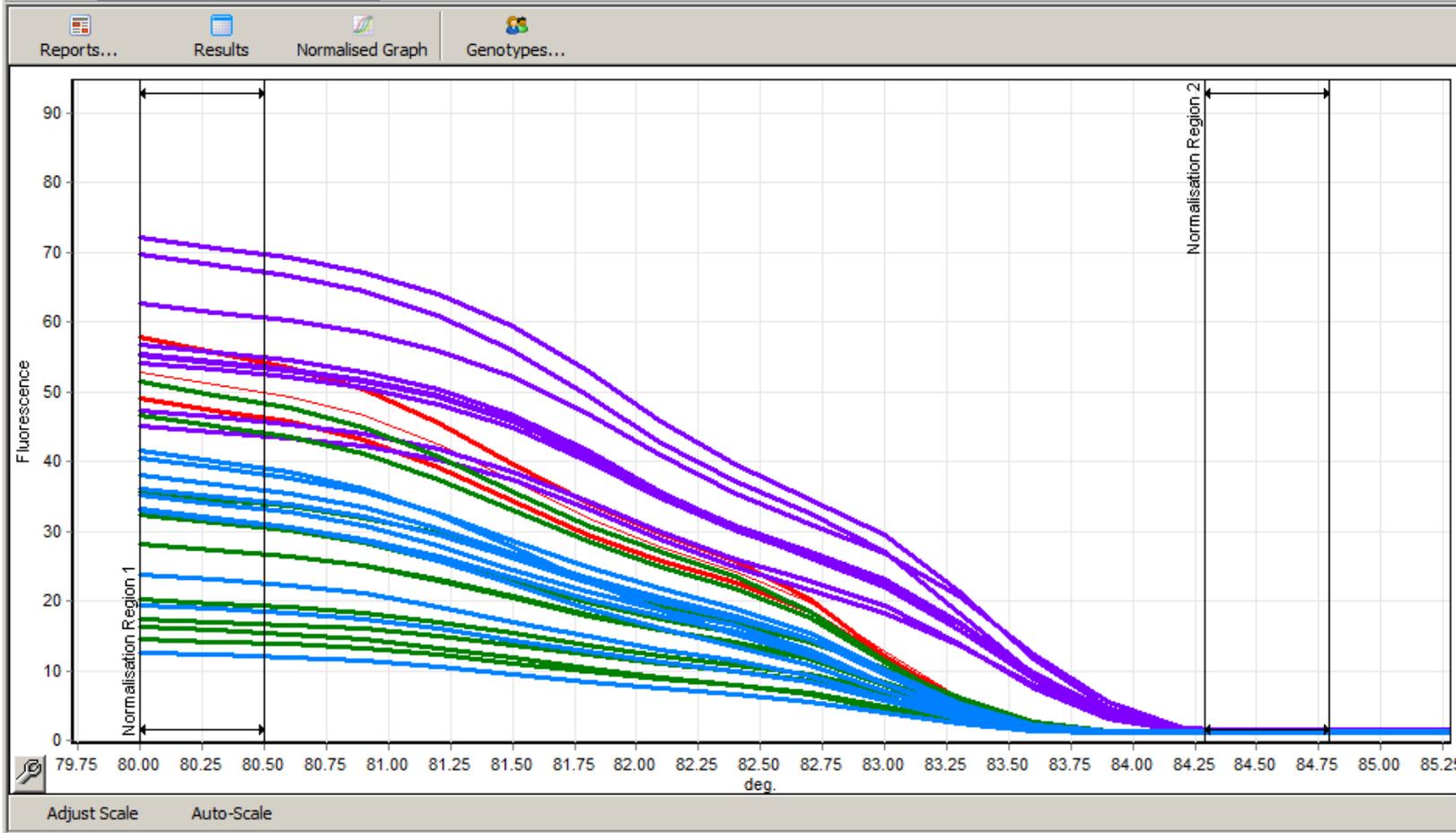


SSCP



HRM curve analysis





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1	MS-H
2	MS-H
3	MS-H
4	---
5	---
6	---
7	101548-1
8	101548-1

Bank On Bank Off
 Named On All On All Off
 Edit Samples...

Normalisation Regions

Leading Range
 Start: 80
 End: 80.5

Trailing Range
 Start: 84.29
 End: 84.79

Confidence Percentage
 Threshold: 80 %

Open Save Help View Settings Profile Temp. Samples Anal

Channels HRM A.HRM HRM B.HRM

No.	C	Name	Genotype	Confidence %
1		MS-H	Vaccine	99.66
2		MS-H	Vaccine	98.54
3		MS-H	Vaccine	99.56
7		101548-1	Variation	31.54
8		101548-1	Variation	11.62
9		101548-1	Variation	17.35
10		101548-2	Variation	19.87
11		101548-2	Variation	25.21
12		101548-2	Variation	18.29
13		101548-3	Variation	19.68
14		101548-3	Variation	17.48
15		101548-3	Variation	22.43
16		101546-3A	Vaccine	99.38
17		101546-3B	Variation	51.36
18		101546-3C	Vaccine	98.46
19		101546-3D	Vaccine	99.62
20		1015461-3E	Vaccine	93.67
21		101546-3F	Vaccine	99.40
22		101546-3G	Vaccine	88.24
23		101546-3H	Variation	67.11
24		101546-3I	Vaccine	85.50
25		101546-3J	Variation	77.80
26		101546-6A	Vaccine	99.01
27		101546-6B	Vaccine	94.17
28		101546-6C	Vaccine	98.71
29		101546-6D	Variation	76.37
30		101546-6E	Vaccine	90.81
31		101546-6F	Vaccine	99.16
32		101546-6G	Vaccine	99.62
33		101546-6H	Vaccine	90.78
34		101546-6I	Vaccine	89.45
35		101546-6J	Vaccine	99.38

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17	101546-3B
18	101546-3C
19	101546-3D
20	1015461-3E
21	101546-3F
22	101546-3G
23	101546-3H
24	101546-3I
25	101546-3J
26	101546-6A
27	101546-6B
28	101546-6C
29	101546-6D
30	101546-6E
31	101546-6F
32	101546-6G

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 Named On All On All Off
 Edit Samples...

Normalisation Regions

Leading Range

Start: 80

End: 80.5

Trailing Range

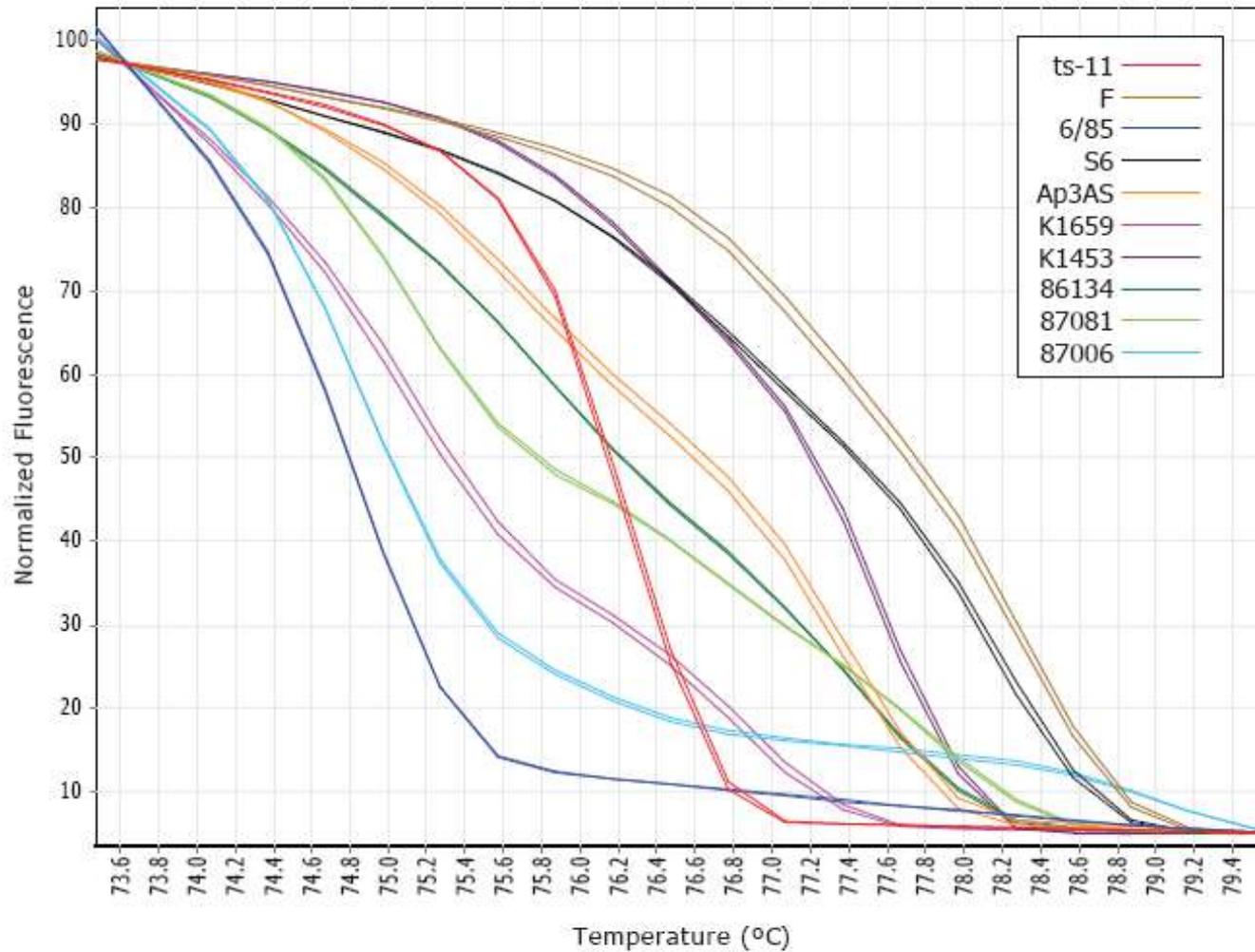
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End: 84.79

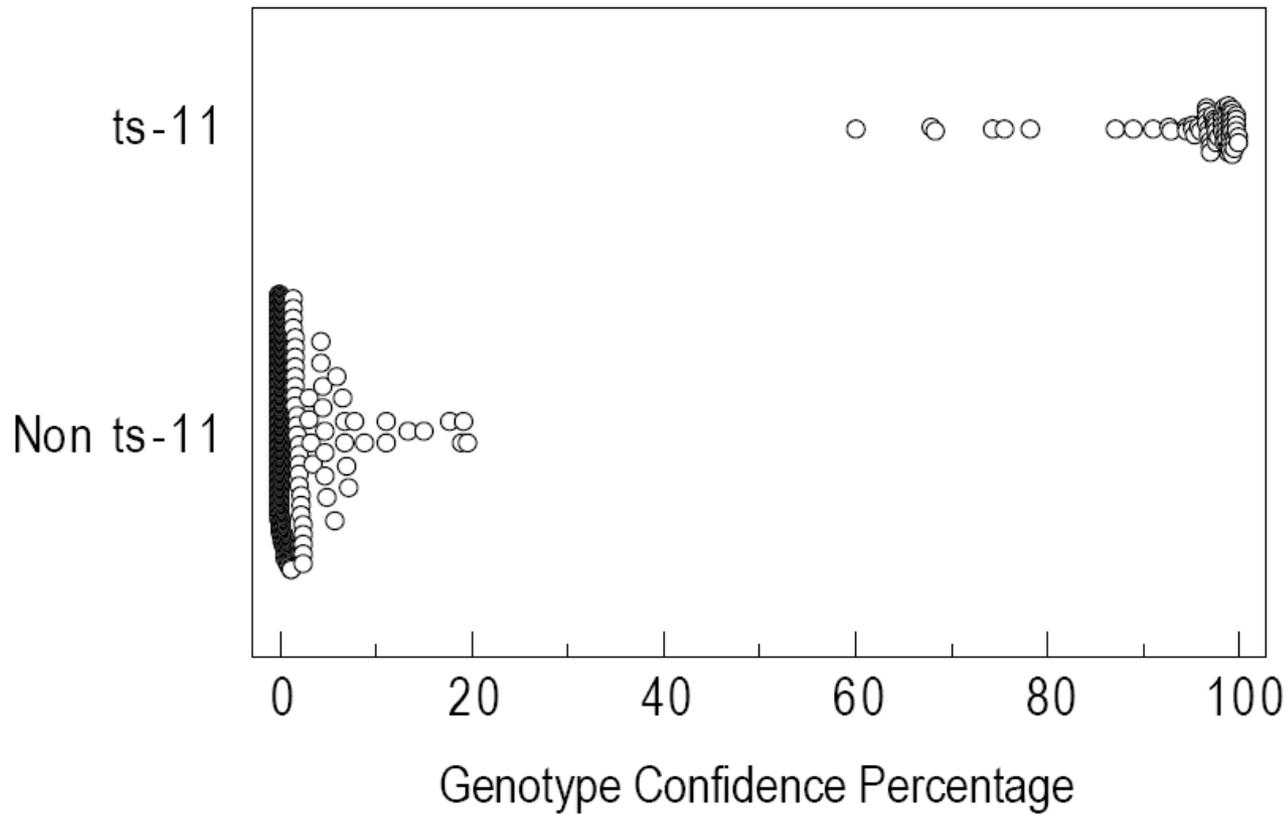
Confidence Percentage

Threshold 80 %

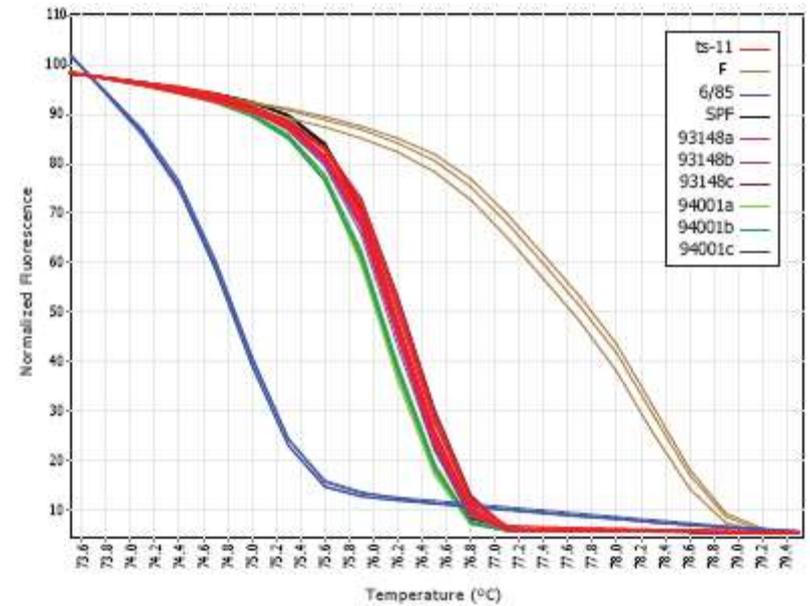
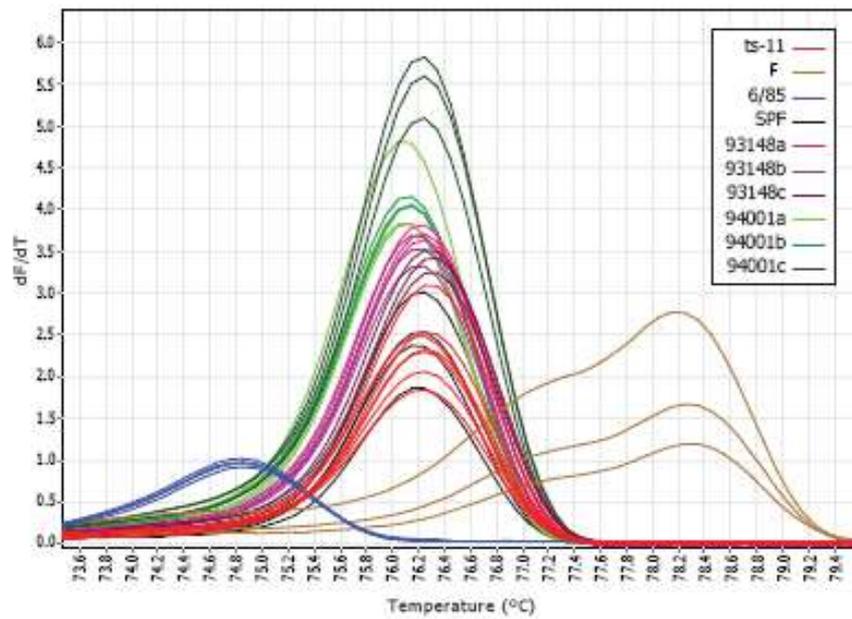
MG PCR-HRM curve analysis



Ts-11 against field strains



Consistency after passage *in vivo*



Challenges

1. Lack of universally accepted test for MG and MS

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	GGATTATTTGGTGTGGGA		
IGSR-F	GTAGGGCCGGTATTGGAGTTA	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		

Differentiation power of different targets

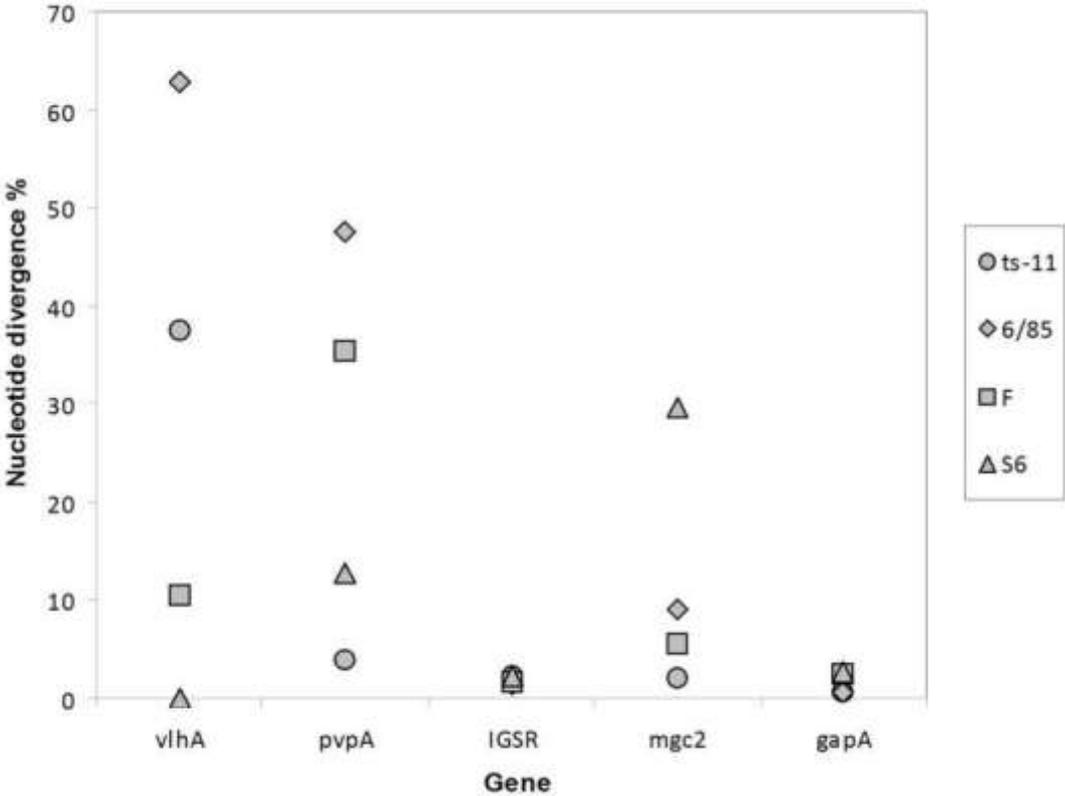


Table 3. Comparisons of RFLP patterns generated by restriction digestion of PCR products of selected genes from different ILTV isolates.

PCR product	ORFB-						Restriction endonuclease	Pattern combination ^A	Class ^B
	gE	gG	TK	ICP4	ICP18.5	TK			
Isolate/ strain	RFLP PATTERNS								
SA2	A	A	A	A	A	A	AAAAA	1	
A20	A	A	A	A	A	A	AAAAA	1	
CSW	A	B	B	C	C	B	BBCCB	4	
V1-99	A	B	B	B	B	B	BBBBB	2	
V2-02	A	B	B	B	B	B	BBBBB	2	
V3-02	A	B	B	A	C	B	BBACB	3	
V1-03	A	A	A	A	A	B	AAAAB	5	
V1-04	ND ^C	B	B	A	C	B	BBACB	3	
N1-03	A	B	B	B	B	B	BBBBB	2	
N1-04	A	B	B	B	B	B	BBBBB	2	
N2-04	ND	B	B	B	B	B	BBBBB	2	
N3-04	ND	B	B	B	B	B	BBBBB	2	
S1-04	A	B	B	A	C	B	BBACB	3	
S2-04	ND	B	B	A	C	B	BBACB	3	
S3-04	ND	B	B	A	C	B	BBACB	3	
S4-04	ND	B	B	A	C	B	BBACB	3	
Q1-95	A	A	A	A	A	A	AAAAA	1	
Q1-96	ND	A	A	A	A	A	AAAAA	1	
Q1-00	ND	A	A	A	A	A	AAAAA	1	
Q1-01	A	B	B	B	B	B	BBBBB	2	

MLST - Cizelj et al (2015)

Table 1. Genotypes of *M. synoviae* isolates

Genotype ^a	Strain	Year of isolation	Sequences of genes or loci								Reference
			5'- <i>vlhA</i> ^b	<i>vlhA</i> pseudogene		<i>cysP</i> ^c	<i>nanH</i>	<i>recA</i>	<i>lonB</i>	MS_0036	
				First	Last						
1.	WVU 1853	1955	A (114)	ST1	ST1	ST1 (-39)	ST1	ST1	ST1	ST1	Benčina <i>et al.</i> (2001)
	F10-2AS	1970	E1 (57)			ST1 (-39)	ST1		ST1	ST1	Hong <i>et al.</i> (2004)
2.	ULB 02/T6	2002	E1 (57)	ST2	ST2	ST2 (-39)	ST2	ST2	ST2	ST2	Slavec <i>et al.</i> (2011)
	ULB 08/T3	2008	E1 (57)	ST2	ST2	ST2 (-39)	ST2				Dušanić <i>et al.</i> (2009)
3.	FMT	1979	D (69)			ST3	ST3				Benčina <i>et al.</i> (2001)
	K2581	1988	E2 (57)	ST3	ST3	ST3	ST3	ST3			Benčina <i>et al.</i> (2001)
	K3344	1992	E2 (57)	ST3	ST3	ST3	ST3	ST3	ST3	ST3	Benčina <i>et al.</i> (2001)
4.	K2426D	1987	C2 (96)	ST4		ST4	ST4	ST4			Benčina <i>et al.</i> (2001)
	K3009/37	1990	E3 (57)			ST4	ST4	ST4	ST4	ST4	Benčina <i>et al.</i> (2001)
5.	ULB 9122	1991	C4 (96)	ST5		ST5	ST5	ST5	ST5	ST5	Benčina <i>et al.</i> (2001)
	ULB 07/P5	2007	C4 (96)			ST5	ST5				Berčič <i>et al.</i> (2011)
6.	PAA2	1994	C3 (96)			ST6 (-39)	ST6	ST6			Benčina <i>et al.</i> (2001)
	K4463B	1997	C3 (96)			ST6 (-39)	ST6	ST6	ST6	ST6	Benčina <i>et al.</i> (2001)
	B27/00	2000	C3 (96)			ST6 (-39)	ST6				Ramirez <i>et al.</i> (2011)
7.	K1968	1983	B (125)	ST6		ST7	ST7	ST7	ST7		Hong <i>et al.</i> (2004)
8.	ULB925	1993	C1 (96)			ST8	ST8	ST8			Benčina <i>et al.</i> (2001)
9.	K1723	1983	C5 (96)	ST7		ST9	ST9	ST9			Benčina <i>et al.</i> (2001)
10.	MS53	?	F (108)	ST8	ST4	ST10 (-30)	ST10	ST10	ST8	ST7	NC_007294.1

^aGenotype, according to all sequenced genes.

^bType of gene sequence and number of nucleotides in the PRR.

^cST, sequence type; the lengths of deletions (number of nucleotides) is presented in parentheses.

MLST - Dijkman et al (2016)

Gene	Nucleotide sequences						Aminoacid sequences						dN ^l	dS ^m	dN/ dS ⁿ
	Target size ^a	Single variants ^b	Proportion ^c	Parsim informative sites ^d	Proportion ^e	No. of alleles ^f	Target size	Single variants ^g	Proportion ^h	Parsim informative sites ⁱ	Proportion ^j	No. of alleles ^k			
<i>uvrA</i>	391	20	5.1%	17	4.4%	23	130	4	3.1%	3	2.3%	5	0.589	3.352	0.176
<i>ruvB</i>	429	27	6.3%	17	4.0%	27	142	11	7.8%	7	4.9%	15	0.801	1.048	0.764
<i>nanA</i>	524	42	8.0%	33	6.3%	35	174	20	11.5%	14	8.1%	27	1.974	3.017	0.654
<i>ugpA</i>	470	45	9.6%	30	6.4%	40	156	14	9.0%	6	3.9%	12	0.304	7.233	0.042
<i>lepA</i>	304	18	5.9%	11	3.6%	18	101	6	5.9%	3	3.0%	7	0.123	1.206	0.102
<i>vlhA</i>	279-381	64	16.8%-22.9%	37	9.7%-13.3%	50	93-127	39	30.7%-41.9%	21	16.5%-22.6%	47	5.876	2.355	2.495
ST	2118	152	7.2%	108	5.1%	76	703	55	7.8%	33	4.7%	55	8.771	10.371	0.846

Challenges

1. Lack of universally accepted test for MG and MS
2. Variations in targeted nucleotides



Research Note—

Revised *Mycoplasma synoviae* *vlhA* PCRs

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SUMMARY. *Mycoplasma synoviae* (MS) is an important pathogen of chickens and turkeys. In recent years sequence analysis of the partial MS variable lipoprotein and hemagglutinin A (*vlhA*) gene PCR product has been utilized routinely for MS strain genotyping. Several PCR assays have been proposed for the amplification of the conserved upstream region of the MS *vlhA* gene; however, in several clinical instances the published assays failed to generate *vlhA* PCR products from confirmed MS-positive cases. These occurrences hindered our capability to genotype those cases. *In silico* analysis of the published MS *vlhA* PCRs raised concerns, which were addressed by the design of revised MS *vlhA* PCRs. The published and revised assays were tested for their relative sensitivity and specificity with laboratory and clinical MS-positive samples. One of the revised MS *vlhA* PCRs (revised Hong) was demonstrated to be more sensitive and specific, and amplified all clinical samples analyzed in this study.

Table 2. Summary of MS *vlhA* PCR assays primer sequences and annealing temperatures.^A

Assay	Forward primer sequence 5'→3' (T _m)	Reverse primer sequence 5'→3' (T _m)	Annealing temperature (C) ^B	Amplicon size (bp) ^C
Hong <i>et al.</i> (4)	GATGCGTAAAATAAAAGGAT (52.2 C)	GCTTCTGTTGTAGTTGCTTC (58.3 C)	55	367
Revised Hong	CCATGCTCCTGCTGTTAT (58.0 C)	KMTKCTGTTGTAGTTGCTTCAA ^D (58.0 C)	55	295
Hammond <i>et al.</i> (3)	ATTAGCAGCTAGTGCAGTGGCC (64.5 C)	AGTAAACCGATCCGCTTAATGC (60.6 C)	52	388
Revised Hammond	GGCCATTGCTCCTRCTGTTAT (61.5 C)	AGTAAACCGATCCGCTTAATGC (60.6 C)	56	370

Challenges

1. Lack of universally accepted test for MG and MS
2. Variations in targeted nucleotides
3. Usefulness of the test depending on geographical location

<i>Mycoplasma</i> species	Isolate	Origin	GenBank accession no. (where available)	SSCP profile
<i>M. mycoides</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
	ZNS/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/S-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
	94042/8a	Australia, NSW, field isolate, palatine cleft (Melbourne University culture collection)	AY913823	A
	93148/24-12b	Australia, VIC, MS-H re-isolate, palatine cleft (Markham <i>et al.</i> , 1998)		A
	93198/1-12b	Australia, VIC, MS-H re-isolate, 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 & Cotter, 1974)	AY907704	D
	4GPH3	Australia, field strain, hock joint (Morrow <i>et al.</i> , 1990a)		D

Challenges

1. Lack of universally accepted test for MG and MS
2. Variations in targeted nucleotides
3. Usefulness of the test depending on geographical location
4. Required equipment and reagents do not exist in all laboratories
5. Current techniques are not necessarily related to virulence or tissue tropism
6. DIVA doesn't detect reversion to virulence?

Mutations in GTP Binding Protein Obg of *Mycoplasma synoviae* Vaccine Strain MS-H: Implications in Temperature-Sensitivity Phenotype

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Abstract

Mycoplasma synoviae strain MS-H, developed by chemical mutagenesis of the Australian field strain 86079/7NS, is a live temperature-sensitive (*ts*⁻) vaccine used for control of *M. synoviae* infection in poultry worldwide. Genetic basis of temperature sensitivity and attenuation of MS-H has not been revealed thus far. Comparison of the complete genome sequence of MS-H, its parent strain 86079/7NS and two non-temperature sensitive (*ts*⁻) reisolates of MS-H revealed a mutation in a highly conserved domain of GTP binding protein Obg of MS-H, with reversion in *ts*⁻ MS-H reisolates. Nucleotide change from G to A at position 369 of the *obg* gene resulted in an alteration of glycine to arginine at position 123 in Obg fold. Further analysis of the complete *obg* gene sequence in several MS-H reisolates revealed that a Gly123Arg substitution was associated with alteration in temperature sensitivity phenotype of MS-H. A second mutation, C to T at position 629, in *obg* gene was found in some of the MS-H reisolates and appeared to suppress the effects of the Gly123Arg substitution. *In silico* analysis of point mutations revealed that Gly123Arg has highly destabilizing effect on the MS-H Obg structure that can potentially abolish its biological functions *in vivo* especially at non-permissive temperature. Findings of this study implicate Obg alteration (Gly123Arg) as one of the possible causes of MS-H attenuation/temperature sensitivity and warrant further investigations into exploring the role of Obg-like proteins, an evolutionarily conserved protein from human to bacteria, in the biology of mycoplasmas.

Citation: Shahid MA, Markham PF, Markham JF, Marends MS, Noormohammadi AH (2013) Mutations in GTP Binding Protein Obg of *Mycoplasma synoviae* Vaccine Strain MS-H: Implications in Temperature-Sensitivity Phenotype. *PLoS ONE* 8(9): e73954. doi:10.1371/journal.pone.0073954

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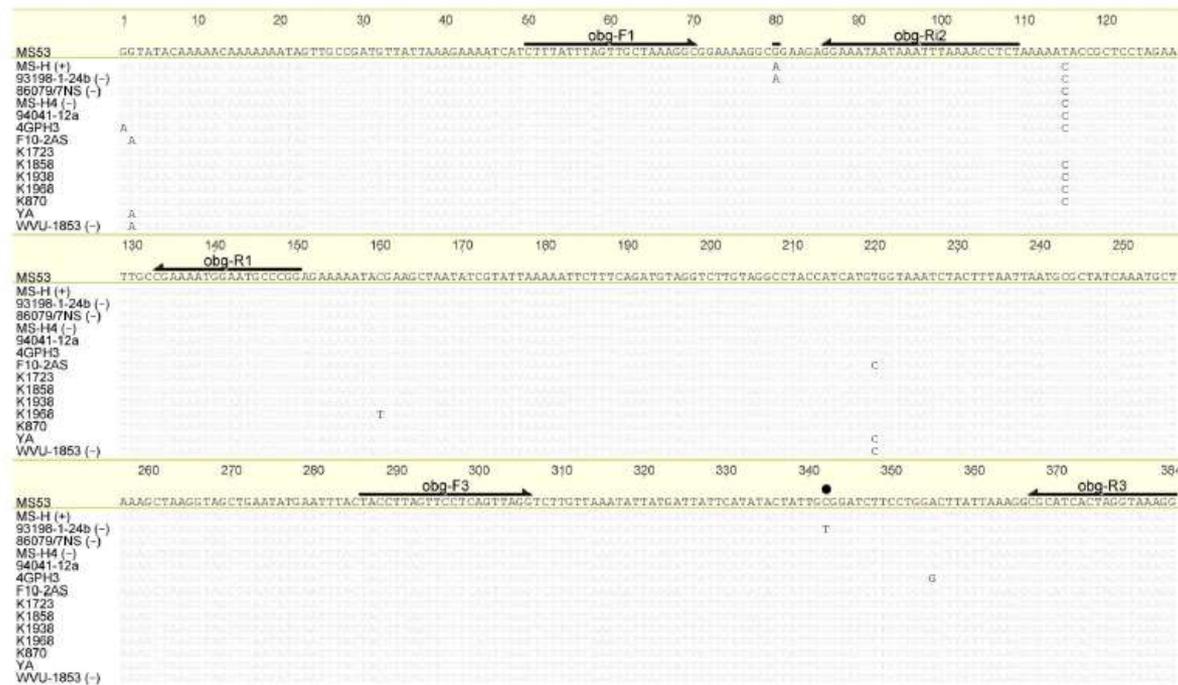
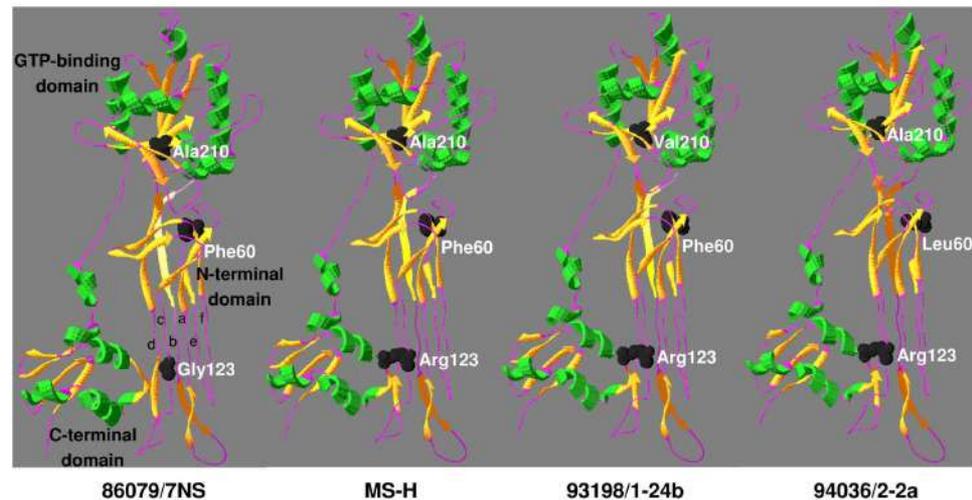
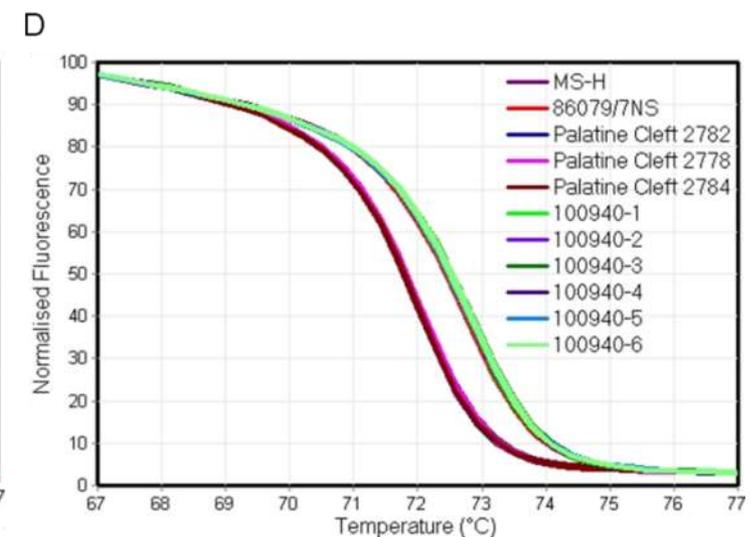
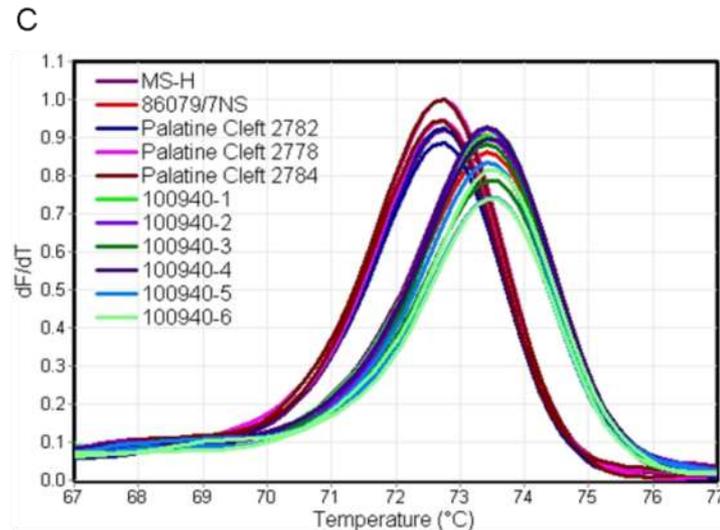
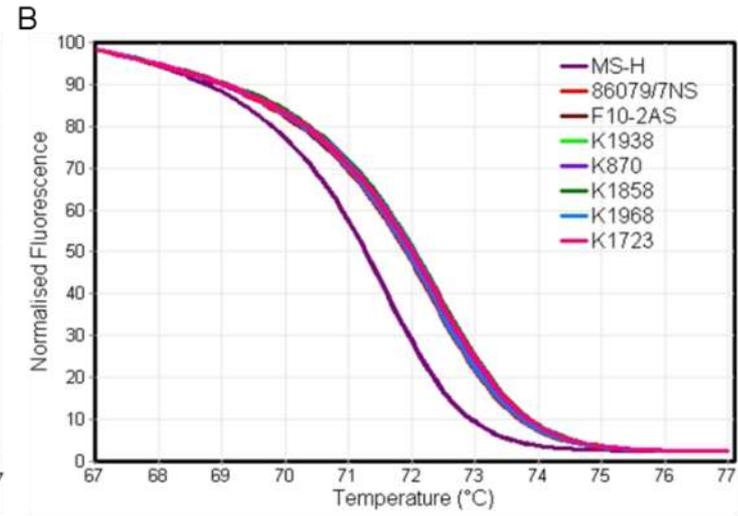
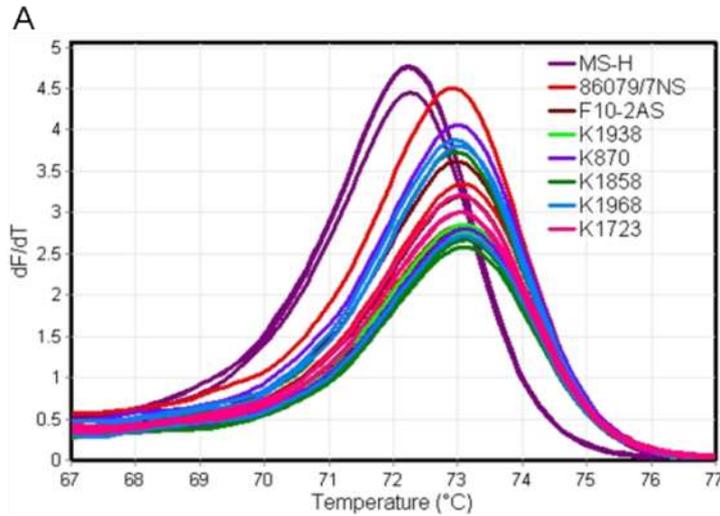


Figure 2. Comparison of partial *obg* nucleotide sequences (corresponding to nt 288-671 of MS53 *obg*, GenBank accession number no. AE017245) from selected *M. synoviae* strains/isolates. Nucleotide differences are highlighted keeping MS53 as reference. Location of primers used in *obg* PCRs as well as SNP G367A discovered in MS-H genome are highlighted with arrows and bar above the sequence, respectively. Location of SNP C629T, observed only in 93198/6-1a, 93198/1-24b, 94036/9-2a and 94036/2-1a [18], is also highlighted with a dot. doi:10.1371/journal.pone.0092215.g002



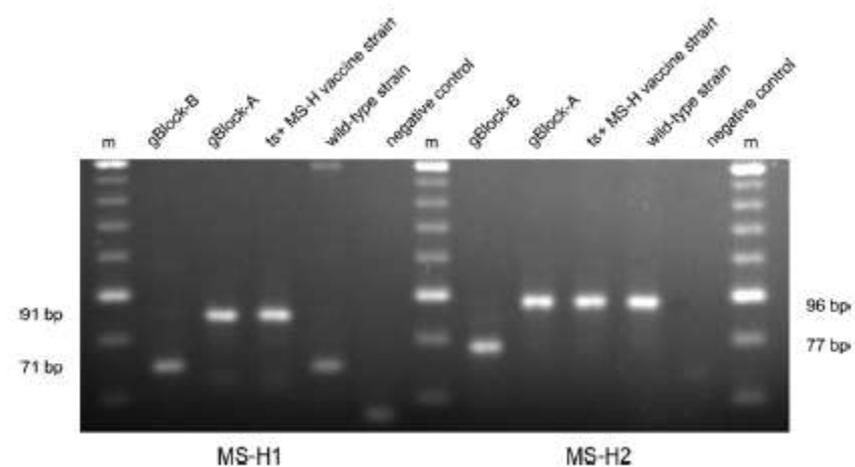
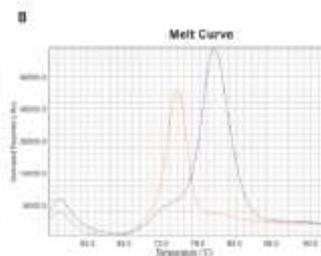
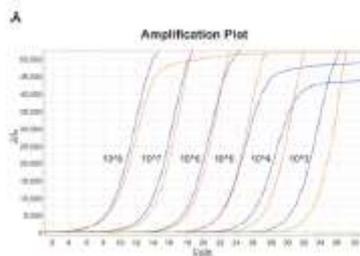
PCR-HRM for determination of ts phenotype



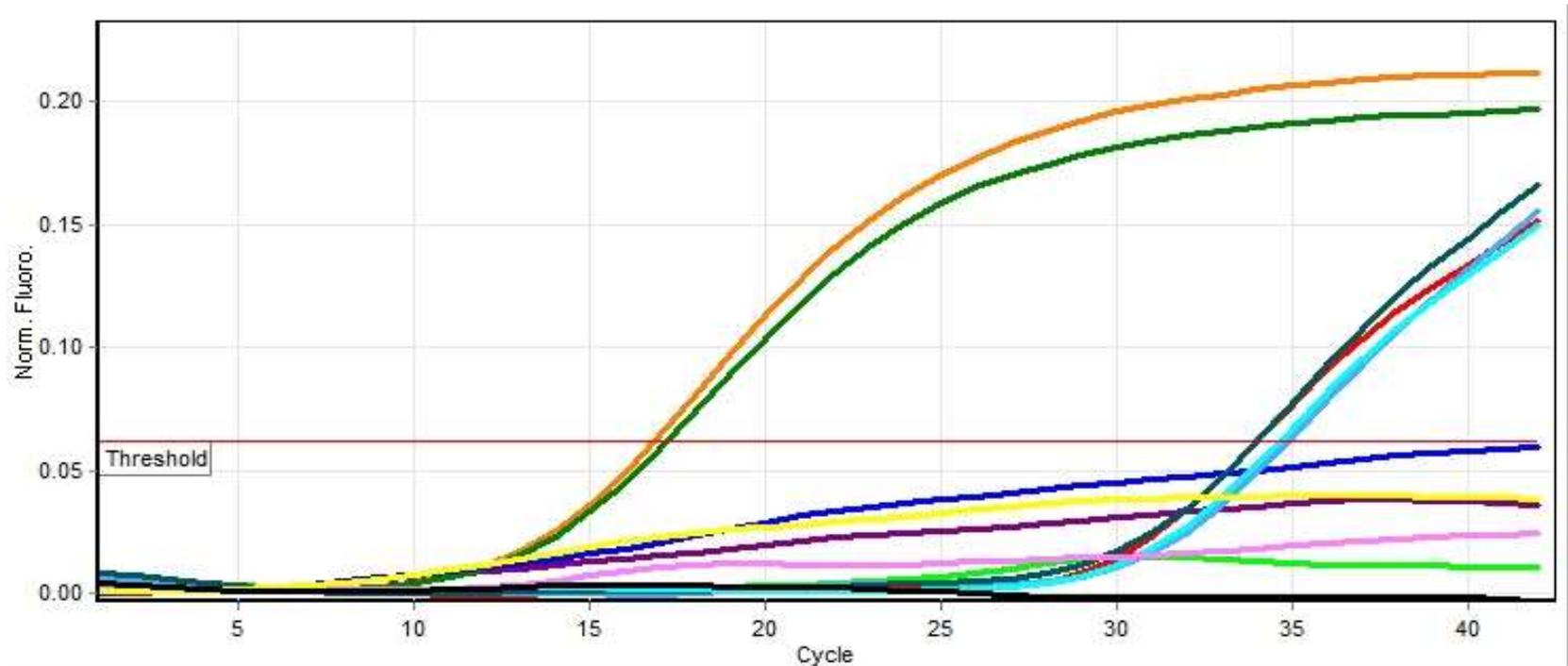
Obg HRM and agarose gel based MAMA (Kreizinger et al 2015)

Table 4. Matrix showing the SNP states, melt-MAMA melting temperatures (T_m) and agarose-MAMA PCR fragment sizes in the ts^+ MS-H vaccine strain genotype, ts^- MS-H re-isolate genotype and wild-type *M. synoviae* strain genotype.

Strain genotypes	nt367 SNP state	nt629 SNP state	MS-H1 melt-MAMA T_m ($^{\circ}C$)	MS-H2 melt-MAMA T_m ($^{\circ}C$)	MS-H1 agarose-MAMA PCR fragment size (bp)	MS-H2 agarose-MAMA PCR fragment size (bp)
ts^+ MS-H vaccine strain	A	C	80.1	76.8	91	96
ts^- MS-H re-isolate ^a	A	T	80.1	70.9	91	77
wild-type strain ^b	G	C	75.0	76.8	71	96

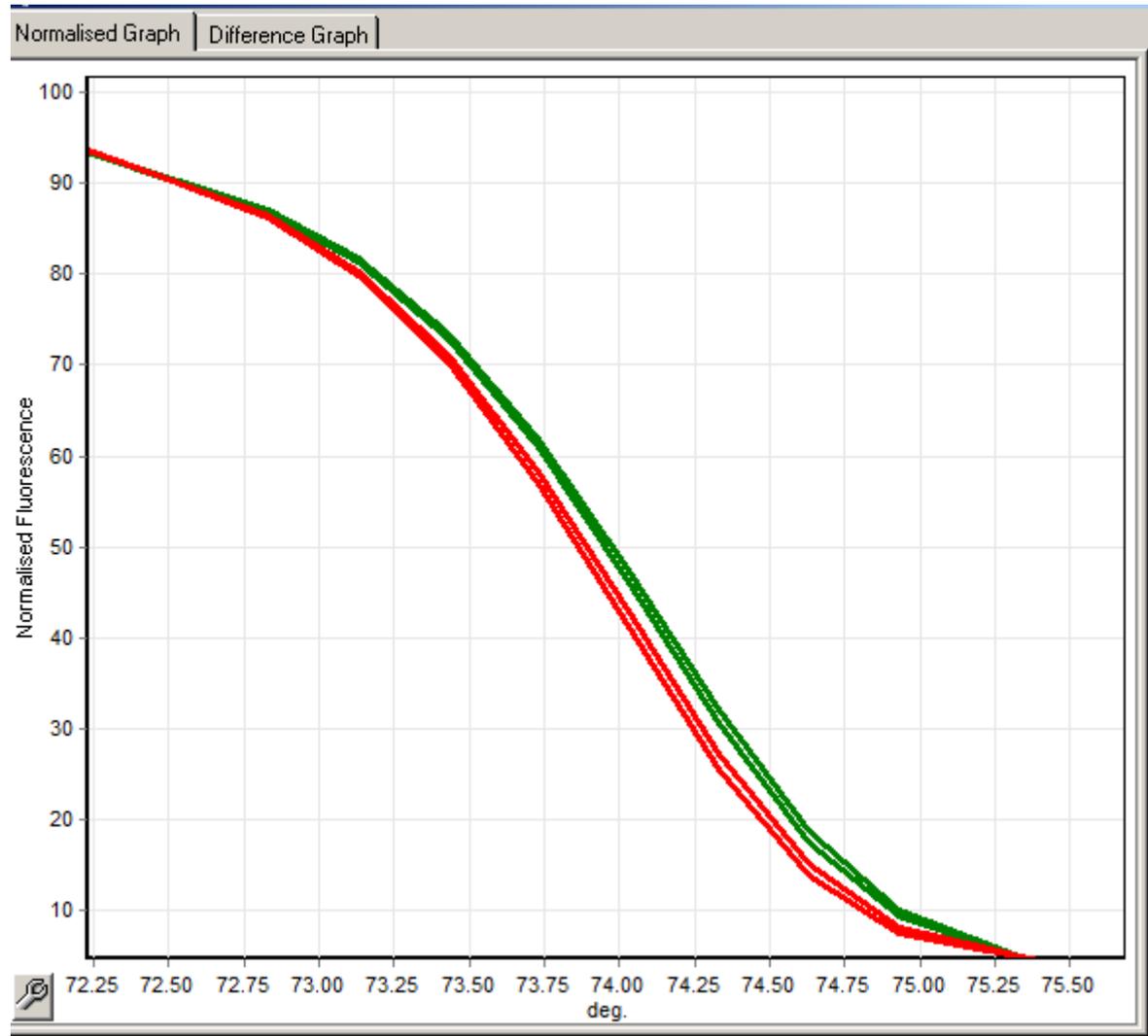


Probe based technique using *obg* gene



KyIt® MS-H DIVA
DNA Extraction and Real-Time PCR Detection Kit
for differentiation of *Mycoplasma synoviae*
live vaccine strain MS-H from field strains

A new DIVA test target



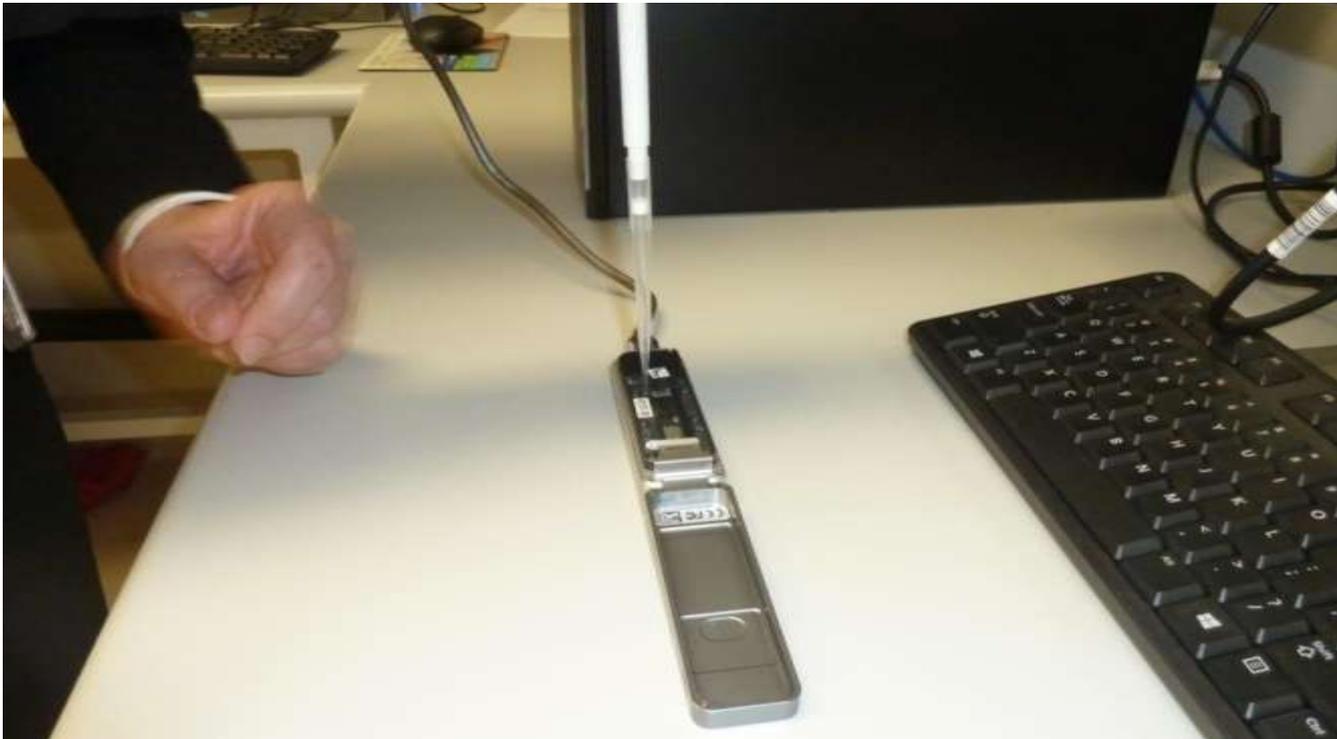


Nobilis MS live (MSD)

Challenges

1. Lack of universally accepted test for MG and MS
2. Variations in targeted nucleotides
3. Usefulness of the test depending on geographical location
4. Required equipment and reagents do not exist in all laboratories
5. Current techniques are not necessarily related to virulence or tissue tropism
6. DIVA doesn't detect reversion to virulence?
7. Current techniques are not be suitable for all vaccines

Future



Why DIVA?

- Are other avian vaccines (eg ILTV, IBV) subjected to the same level of scrutiny?
- Is there any evidence that mycoplasma vaccines failed to provide protective immunity against field challenge – if vaccination is done “by the book” and applied to mycoplasma free flocks?
- Are we expecting too much from mycoplasma vaccines? Is there a “perfect vaccine”?
- Should we be concerned if a field strain is detected in a “single” bird or flock?

Acknowledgements



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Australian Research Council

