



HAL
open science

Development, evaluation of an improved diagnostic PCR for *Mycoplasma synoviae* using primers located in the haemagglutinin encoding gene *vlhA* and its value for strain typing

P.P. Hammond, A.S. Ramírez, C.J. Morrow, J.M. Bradbury

► To cite this version:

P.P. Hammond, A.S. Ramírez, C.J. Morrow, J.M. Bradbury. Development, evaluation of an improved diagnostic PCR for *Mycoplasma synoviae* using primers located in the haemagglutinin encoding gene *vlhA* and its value for strain typing. *Veterinary Microbiology*, 2009, 136 (1-2), pp.61. 10.1016/j.vetmic.2008.10.011 . hal-00532523

HAL Id: hal-00532523

<https://hal.science/hal-00532523>

Submitted on 4 Nov 2010

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

Accepted Manuscript

Title: Development, evaluation of an improved diagnostic PCR for *Mycoplasma synoviae* using primers located in the haemagglutinin encoding gene *vlhA* and its value for strain typing

Authors: P.P. Hammond, A.S. Ramírez, C.J. Morrow, J.M. Bradbury

PII: S0378-1135(08)00486-0
DOI: doi:10.1016/j.vetmic.2008.10.011
Reference: VETMIC 4239

To appear in: *VETMIC*

Received date: 11-8-2008
Revised date: 6-10-2008
Accepted date: 13-10-2008

Please cite this article as: Hammond, P.P., Ramírez, A.S., Morrow, C.J., Bradbury, J.M., Development, evaluation of an improved diagnostic PCR for *Mycoplasma synoviae* using primers located in the haemagglutinin encoding gene *vlhA* and its value for strain typing, *Veterinary Microbiology* (2008), doi:10.1016/j.vetmic.2008.10.011

This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.



1 **Development and evaluation of an improved diagnostic PCR for**
2 ***Mycoplasma synoviae* using primers located in the haemagglutinin encoding**
3 **gene *vlhA* and its value for strain typing**

4
5 P.P. Hammond¹, A.S. Ramírez², C.J. Morrow³ and J.M. Bradbury^{2*}

6
7 ¹Royal Veterinary College, Hawkshead House, Hawkhead Lane, North Mymms, Hatfield,
8 Herts. AL9 7TA. UK; present address: Crowshall Veterinary Services

9 1 Crows Hall Lane, Attleborough, Norfolk NR17 1AD

10 ²Department of Veterinary Pathology, University of Liverpool Leahurst Campus, Neston,
11 CH64 7TE, UK; present address: Unit of Epidemiology and Preventive Medicine, Veterinary

12 Faculty, University of Las Palmas de G.C. Trasmontaña s/n, Arucas. 35416. Las Palmas,
13 Spain

14 ³Aviagen Limited, Newbridge, Midlothian, EH28 8SZ, Scotland, UK; present address:

15 Bioproperties Pty Ltd, 36 Charter St., Ringwood, Victoria 3134 Australia

16

17

18

19

20

21 * Corresponding author at: Department of Veterinary Pathology, University of Liverpool

22 Leahurst Campus, Neston, CH64 7TE, UK

23 E.mail: jmb41@liverpool.ac.uk (J.M> Bradbury) Tel: + 44 (0)151 794 6115, Fax: + 44

24 (0)151 794 6005

25

1 **Abstract**

2 Using published primers, detection of *Mycoplasma synoviae* and strain identification
3 using the *vlhA* gene sequence was attempted. However, of 21 *M. synoviae* strains examined,
4 three could not be amplified, so a new reverse primer was designed with a target in the
5 conserved region of the *vlhA* gene. This allowed all 21 *M. synoviae* strains, a further nine
6 strains and also material from 11 swab samples from *M. synoviae*-positive birds, to produce a
7 PCR product, suggesting that the method could also be suitable for clinical specimens. The
8 protocol was then tested on the type strains of *M. synoviae* and the other 22 recognised avian
9 *Mycoplasma* species, with amplification of *M. synoviae* only. Further testing demonstrated
10 that this PCR was equally or more sensitive than other PCR tests used to detect *M. synoviae*.
11 Subsequent DNA sequence analysis of the PCR product based on percent similarity and
12 evolutionary relationship appeared to be a useful tool for strain differentiation.

13

14 Keywords: *Mollicutes*, *Mycoplasma*, *Mycoplasma synoviae*, diagnosis, polymerase chain
15 reaction, *vlhA*.

16

1 1. Introduction

2 *Mycoplasma synoviae* is a chicken and turkey pathogen causing economic loss in
3 intensive production. Reliable, rapid diagnosis is needed to prevent dissemination of infection
4 and has traditionally been achieved by serological screening for antibodies or by culture of the
5 mycoplasma. Serological screening is still in widespread use but may not detect subclinical
6 *M. synoviae* infections, and monitoring programmes that depend solely on detecting
7 seroconversion may be inadequate (Ewing et al., 1998; Kleven et al., 2001). Culture can be
8 costly and time consuming, and can also be inconclusive (Ewing et al., 1998) so PCR-based
9 tests are now routinely used for detecting pathogenic avian mycoplasmas. Some PCRs are
10 based on the 16S rRNA gene (Garcia et al, 1996; Lauerman et al., 1993), some are in-house
11 tests (e.g. Lauerman, 1998), and others are commercially-produced kits.

12 *M. synoviae* PCRs have also been based on the *vlhA* haemagglutinin (HA) gene
13 (Benčina et al., 2001; Hong et al., 2004). Ben Abdelmoumen Mardassi et al. (2005) used a
14 duplex PCR targeting the HA genes to differentiate *M. synoviae* and *M. gallisepticum* while
15 Jeffery et al. (2007) typed *M. synoviae* strains by single-strand conformation polymorphism or
16 high resolution melting curve analysis of the conserved 5' end of the *vlhA* gene. The gene
17 product is an abundant immunodominant surface lipoprotein with a conserved and variable
18 region (Noormohammadi et al., 2000). The N-terminal (conserved) region exists as a single
19 copy whose sequence varies among strains. The gene includes tandem repeats that encode
20 proline rich repeats (PRR) and also a region that is highly polymorphic (RIII) (Fig. 1). These
21 properties suggested it as a suitable target for molecular strain typing (Benčina et al., 2001).

22 Our aim was to test the PCR assay of Benčina et al. (2001) on *M. synoviae* strains
23 from different hosts, locations and years. Since some of our strains were not amplified an
24 alternative reverse primer, located in the conserved region of the *vlhA* gene (Fig. 1) was used.

1 The specificity and sensitivity of this PCR and its ability to detect *M. synoviae* in field isolates
2 and swabs were established and sequence analysis was evaluated for strain typing.

3

4 **2. Material and methods**

5

6 *2.1. Organisms and culture conditions*

7

8 The type strains of avian *Mycoplasma* species used were as follows: *M. anatis* 1340^T, *M.*
9 *anseris* 1219^T, *M. buteonis* BbT2g^T, *M. cloacale* 383^T, *M. columbinasale* 694^T, *M.*
10 *columbinum* MMP/1^T, *M. columborale* MMP/4^T, *M. corogypsi* Bv1^T, *M. falconis* H/T1^T, *M.*
11 *gallinaceum* DD^T, *M. gallinarum* PG16^T, *M. gallisepticum* PG31^T, *M. gallopavonis* WR1^T, *M.*
12 *glycophilum* 486^T, *M. gypis* B1/T1^T, *M. imitans* 4229^T, *M. iners* PG30^T, *M. iowae* 695^T, *M.*
13 *lipofaciens* R171^T, *M. meleagridis* 17529^T, *M. pullorum* CKK^T, *M. sturni* UCMF^T and *M.*
14 *synoviae* WVU1853^T. The details of the *M. synoviae* field strains and swabs are given in
15 Table 1.

16 The mycoplasmas were cloned once by filtration and their identity was confirmed by
17 the indirect fluorescent antibody test (Rosendal and Black, 1972). Then they were cultivated
18 in mycoplasma broth (Bradbury, 1977) at 37 °C for between one and five days, in a CO₂ rich
19 atmosphere.

20 Eleven swabs collected earlier from the trachea or choanal cleft of *M. synoviae*-
21 positive chickens were also included in these tests. The five swabs from year 2003 had proved
22 to be IDEXX PCR positive but culture negative.

23

24 *2.2. Sample preparation*

25

1 DNA extraction was done using Chelex resin for broth cultures and heat treatment for
2 swabs as described elsewhere (Ramírez *et al.*, 2006).

3

4 2.3. Testing the primer pairs

5

6 The primers used in the reaction are shown in Table 2. PCR amplification using *vlhAF*
7 and *vlhAR1* primers was performed on 21 isolates. The reaction mixture contained 0.5 μ M of
8 each primer (*vlhAF* and *vlhAR1*); 0.2mM dNTPs; 2.5 mM $MgCl_2$; 1X reaction buffer
9 (Abgene, AB-0192); 0.5 U Taq DNA polymerase (Abgene) made up to 49.5 μ l with water
10 (Sigma W4502).

11 DNA template (1 μ l) was added per tube and Sigma water (1 μ l) was added to another
12 tube to act as a negative control. Mineral oil (50 μ l) was layered on the surface of each
13 mixture. The thermal cycle, using Perkin Elmer PCR machine, was 94 °C for 5 min; 36 cycles
14 of 94°C for 1 min, 52°C for 1 min and 72°C for 1 min, then a final extension step of 72°C for 2
15 min.

16 Following the failure of these primers to amplify three of these strains, the combination
17 of *vlhAF* and *vlhAR2* was tested on the same 21 strains and also on nine others and on the 11
18 swabs indicated in Table 1 following the same PCR protocol as before.

19

20 2.4. Assay specificity and sensitivity

21

22 The specificity and sensitivity studies were carried out using the *vlhAF-vlhAR2* PCR
23 assay. Specificity was tested with the type strains of all the 23 avian *Mycoplasma* species
24 listed above. Sensitivity was determined by analysing serial 10-fold dilutions to 10^{-9} of broth
25 cultures from the *M. synoviae* type strain (WVU1853^T) and from a field strain (B155/02).
26 Viable counts were conducted for each culture and the numbers of colony-forming units

1 (CFU) per 100µl were calculated (Bradbury, 1977). The DNA was measured using a
2 fluorometer (DyNA QuantTM 200, Hoefer, Pharmacia Biotech Inc.), following the
3 manufacturer's instructions. PCR was carried out on each dilution.

4

5 *2.5. Comparison of the vlhAF-vlhAR2 PCR with three other diagnostic M. synoviae PCRs*

6

7 To compare the results of our PCR assay with other described methods, three other *M.*
8 *synoviae* PCR detection protocols were conducted with the type strain (WVU1853^T) and a
9 field strain (B155/02). These were the Lauerman method (Lauerman, 1998), a modified
10 Lauerman procedure (Ramirez et. al., 2006) in which DNA extraction incorporated diethyl
11 pyrocarbonate (DEP) and a commercial kit (FlockChek® *Mycoplasma synoviae* DNA probe
12 kit, IDEXX Laboratories Inc., Westbrook, Maine, USA).

13

14 *2.6. Sequencing*

15

16 Sequencing was conducted by Imperial College, London, and sequences were
17 determined for both strands of DNA. The resulting chromatograms were examined in
18 Chromas (version 1.45; School of Health Science, Griffith University, Australia). The
19 forward sequence and reverse complemented sequences were compared to produce a
20 consensus sequence using Generunner (version 3.05; Hastings Software, Inc.). Bioedit 7.0.0
21 (Hall, 1999) program was used for aligning all the sequences manually, and for deducing the
22 amino acid sequences. Nucleotide and amino acid sequences of all 41 *M. synoviae* strains (30
23 from cultures and 11 from swabs) were trimmed from nucleotide 49 to 421 corresponding to
24 the numbering of Benčina et al. (2001) for strain WVU1853^T. They were assigned in the
25 Genbank with accession numbers AM998371, AJ580981 to AJ580983, AJ580985,
26 AJ58098187 to AJ580989, AJ58091 and FM164342 to FM164373.

1

2 *2.7. Percent DNA similarity*

3

4 The percent similarity of all the *M. synoviae vlhA* gene sequences was determined using
5 the program Bioedit version 7.0.0 (Hall, 1999). The strains were then grouped into those
6 showing 100% similarity and a representative of each group was selected for subsequent
7 phylogenetic analysis. The percent similarity between the representatives was calculated
8 including and omitting insertions/deletions.

9

10 *2.8. Analysis of sequences*

11

12 Dendrograms were constructed from alignments of the representative strains by the
13 neighbour-joining method (Saitou and Nei, 1987) with 1000 bootstrap replicate analyses
14 (Felsenstein, 1985), using the Molecular Evolutionary Genetic Analysis (MEGA) software
15 (Center for Evolutionary Functional Genomics, Tempe, AZ, USA;
16 <http://www.megasoftware.net>) for sequence alignments (Tamura et al., 2007). The
17 evolutionary distances were computed, with the same program, using the Maximum
18 Composite Likelihood method (Tamura et al., 2004).

19 The method described by Benčina et al. (2001) was used initially to classify the *M.*
20 *synoviae* strains into types and subtypes. The types were assigned according to the length of
21 the *vlhA* gene PRR-coding region and the subtypes according to the sequence of the RIII
22 region. However after further examination of the percent similarity and of the relationships
23 revealed by the dendrogram a modified grouping was derived.

24

25 **3. Results**

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26

3.1. Testing the primer pairs

Using the *vlhAF-vlhAR1* primer combination three *M. synoviae* isolates (B27/00, B142/02 and B154/02) failed to produce the expected fragment of around 621 bp but with primer set *vlhAF-vlhAR2* a PCR product was obtained with all 41 samples tested. This included the previous three failed strains and the DNA extracted from the 11 swabs. The amplicon sizes varied between 315 and 372 bp (Fig. 2).

3.2. Specificity and sensitivity of the *vlhAF-vlhAR2* *M. synoviae* PCR and its comparison with three other diagnostic *M. synoviae* PCRs

The *vlhAF-vlhAR2* PCR was specific, producing an amplicon only with *M. synoviae* template and with no amplified PCR products from the other 22 avian *Mycoplasma* species tested (data not shown).

The PCR detected DNA template from strains WVU1853^T and B115/02 up to the dilution 10^{-5} , which corresponded to an amount of DNA close to 1 pg for both strains (data not shown). No colonies grew at this dilution. For the type strain the number of colony-forming units per 100 μ l was only 1 at a dilution of 10^{-3} , while for the field strain eight colonies were countable at a dilution of 10^{-4} . The PCR was more sensitive than the other three methods as none of them allowed detection of DNA from either strain at a dilution of 10^{-5} . The next most sensitive was the modified Lauerman method which detected *M. synoviae* in both strains to dilutions of 10^{-4} . The commercial kit detected *M. synoviae* DNA to a dilution of 10^{-3} in both strains while the unmodified Lauerman protocol detected the type strain only at 10^{-1} dilution and the field strain at 10^{-2} .

1 3.3. Sequence analysis and strain typing

2

3 Table 1 shows the sizes of amplicons and PRRs in nucleotides and amino acids
4 respectively, while Fig. 3 shows the point mutations and insertions/deletions in the nucleotide
5 alignments of the group representatives. The corresponding amino acid alignments are shown
6 in Supplementary Fig. 1.

7 Strain typing was initially based on the size of the PRR region and point mutations of
8 the RIII region (Benčina et al., 2001). Using the length of the PRR the strains were assigned
9 to Benčina's types A to E. Most strains were assigned to type C (Table 1). There were none
10 belonging to type B but a further type F was added to accommodate two strains whose PRR
11 length was 108 nt (36 amino acids). After this, the strains were subdivided numerically based
12 on the RIII region and most of those in type C fell into subtype C₃. However, while 21 of the
13 23 strains of this subtype had 100% similarity, the two remaining (identical) strains had only
14 99.6% similarity with the others. Percent similarity between the representatives of each group
15 with and without insertions/deletions is presented as Supplementary Table 1.

16 Grouping based on 100% similarity placed the strains into 12 groups (Table 1). It was
17 apparent some groups had identical *vlhA* gene sequences except for a single
18 insertion/deletion, as for example strains B11/85 and J26/85 in Supplementary Fig. 2. The
19 same occurred between strains B142/02 and B95/04/K261, between strains B91/96/798 and
20 B133/99/05, and between strains B48/05/1 and B38/96/1704. Based on that information a
21 dendrogram was derived with the representative strain of each group (Fig. 4) that revealed the
22 relationship between these groups.

23 When groups 2 to 12 were compared with group 1 which contained the *M. synoviae*
24 type strain WVU1853^T (Table 1 and Fig. 3) all the other strains had a deletion at nt 112
25 corresponding to nt 148 of Benčina et al. (2001). Of these, 32 strains (the members of groups

1 2 to 7) had a deletion of 18 nt/6 aa and seven (the members of groups 8 to 12) a deletion of 57
2 nt/19 aa, however only five strains (the members of groups 2, 3 and 8) had an insertion
3 between nt 64 and 65 (nt 111 and 112 of Benčina et al. 2001).

4 UK pheasant *M. synoviae* strains comprised a unique group (group 2 in Table 1).
5 These two strains plus a strain isolated from an Italian turkey (B38/96/1704) and two strains
6 from a UK chicken company (B48/05 and B57/05), contain the same insertion (5'
7 ACTCCAACACCT 3') in the PRR region (Fig. 3). Strains B133/99/5 and B133/99/12 which
8 were from the same Hungarian farm were homologous except that B133/99/5 exhibited a 57
9 nt deletion. Strains J15/ 85 and J26/85 from the mid 1980s exhibited a 57 nt deletion and were
10 identical except for one base change from G to A at nt 244 in J15/85. B7/86 and B11/85
11 isolated in the UK around the same time are identical to J26/85 except there was only an 18
12 bp deletion in the first two compared to a 57 bp deletion.

13

14 Discussion

15

16 PCR assays for detection of *M. synoviae* have been reported earlier (Garcia et al, 1996;
17 Lauerman et al., 1993 and 1998; Benčina et al., 2001; Hong et al., 2004, Ben Abdelmoumen
18 Mardassi et al, 2005; Ramírez et al., 2006; Jeffery et al., 2007). Four of them target the
19 haemagglutinin-encoding *vlhA* gene (Benčina et al., 2001; Hong et al., 2004; Ben
20 Abdelmoumen Mardassi et al, 2005; Jeffery et al., 2007). In our hands the primers used by
21 Benčina et al. (2001) did not amplify three of our field isolates. All emanated from Hungary
22 and, after amplification with the new reverse primer, could be placed in Benčina subtype C₃
23 and our groups 5 and 6. This lack of amplification can be explained by the fact that the
24 reverse primer of these authors, *VlhAR1*, targets the variable region of the *vlhA* gene (Fig. 1).
25 Hong et al. (2004) described a PCR with the PRR region as the target, excluding the RIII

1 region. Our primer *vlhAR2* was designed to target the 3' end of the conserved region,
2 including the RIII region. With the new reverse primer the DNA from all 21 isolates plus nine
3 more was amplified.

4 The results showed good specificity. This was demonstrated by no reactions with any of
5 22 other avian *Mycoplasma* species, including nine that fall within the *M. synoviae* cluster of
6 the 16S rDNA-based hominis phylogenetic group (Pettersson *et al.*, 2000), and when used
7 successfully on DNA extracted from 11 swabs, no nonspecific bands appeared. Thus our PCR
8 assay appears also to have sufficient sensitivity and specificity for clinical use.

9 The sensitivity was as good, if not better than several other PCR tests. The method
10 detected *M. synoviae* DNA of two strains when no colonies could be cultivated, while Ben
11 Abdelmoumen Mardassi *et al.* (2005) needed at least 90 CFUs for detection of *M. synoviae*
12 with their duplex PCR assay based in the haemagglutinin genes. Hong *et al.* (2004) could
13 detect 4.7×10^2 colour-changing units/ml and Lauerman *et al.* (1993) found a sensitivity of
14 100 CFUs for their *M. synoviae* PCR based on the 16S rRNA gene. The amount of DNA that
15 our PCR can detect is similar to the 1pg detected by a *Mycoplasma synoviae* PCR included in
16 a multiplex PCR described by Wang *et al.* (1997) and the PCR described by Ramírez *et al.*
17 (2006). These results agree with Razin's observation that "sensitivity of PCR assays is usually
18 in the order of magnitude of a few femtograms of mycoplasmal DNA. When translated into
19 numbers of organisms, 1fg of mycoplasmal DNA is approximately equivalent to the genomic
20 DNA of a single mycoplasma cell, based on a genome size of about 1000 kb" (Razin, 2002).

21 When the four *M. synoviae* diagnostic PCRs were compared, the *vlhAF-vlhAR2* PCR
22 described in this study performed best, detecting a lower amount of DNA than the other
23 methods. This could be due to the use of the resin Chelex in the DNA extraction, because
24 similar results were seen by Ramírez *et al.* (2006) when the same method was used for
25 extracting DNA for a PCR based on the 16S-23S rRNA intergenic spacer region.

1 PCR and partial *vlhA* gene sequencing may also be useful in strain identification.
2 Benčina et al., (2001) compared the 5' end haemagglutinin (*vlhA*) gene sequences of 30 *M.*
3 *synoviae* strains from chickens and turkeys and demonstrated 11 different types of *vlhA*
4 sequences, while Hong et al. (2004) found 14 different groups among 43 strains from the
5 same species. The majority of strains in both studies came from the USA and Slovenia.
6 Jeffery et al. (2007) examined 35 different isolates from Australia and the USA (from
7 chickens and one turkey) and revealed 10 distinct profiles by single-strand length
8 polymorphism. These corresponded to the 10 genotypes derived by high-resolution melting-
9 curve analysis. The 41 strains in our study were mainly from the UK and Hungary and
10 comprised chicken and turkey strains plus two from pheasants. They were assigned to 12
11 groups and showed considerable overlap with the groupings of Benčina et al. (2001) although
12 four strains, including the two pheasant strains did not fit into any of the Benčina types.

13 The *vlhA* gene has considerable divergence at the 3' end of the gene due to
14 recombination with pseudogenes (presumably as a mechanism to evade the host immune
15 response), but relative conservation at the 5' end (Noormohammadi et al., 2000). The 5' *vlhA*
16 region is present in the *M. synoviae* genome as a single copy and does not change its sequence
17 in clonal populations of *M. synoviae* (Noormohammadi et al., 2000). This observation is
18 crucial to strain identification as downstream of this region the sequence can change even in
19 clonal populations of *M. synoviae* and therefore it cannot be considered a conserved sequence
20 that characterises individual strains (Noormohammadi et al, 2000). The test evaluated by
21 Hong et al. (2004) combined PCR and sequencing of the PRR repeat region of the *vlhA* gene
22 to detect and type *M. synoviae* strains without the need for isolation. However, it excluded the
23 RIII region (positions 343/400), which Benčina et al. (2001) found very useful for subtyping
24 *M. synoviae* strains. The PRR region is useful mainly due to insertions/deletions and the RIII
25 region is useful because of polymorphisms and our findings have confirmed this, as have

1 those of Jeffrey et al. (2007) who, by chance, designed a reverse primer almost identical to
2 ours.

3 Nucleotide and amino acid sequence analyses of *M. synoviae* strains support the
4 observation that they are useful for strain identification and as a preliminary typing method.
5 Benčina et al. (2001) suggested that the polymorphisms in 5' end of the *vlhA* gene might be
6 very useful for epidemiological analysis of *M. synoviae* isolates and it is of interest that the
7 two strains from UK pheasants comprised a unique group, (named group 2 in Table 1).

8 Insertions/deletions were observed in all the strains examined and may be related to
9 pathogenicity (Benčina et al., 2001). Although isolates B133/99/5 and B133/99/12 were from
10 the same farm, B133/99/5 exhibited a 57 nt deletion. These could be the same strain, with
11 strain B133/99/5 having undergone a deletion during passage on the farm but it is also
12 possible to have more than one strain on a farm.

13 Isolates from the mid 1980s demonstrated greater similarity with the type strain
14 WVU1853^T than the present isolates, apart from strain B95/04/K4412 whose sequence was
15 exactly the same as the *M. synoviae* type strain. These two strains from Germany were from
16 the same sender but no further information about their origin was available although we
17 assumed both to be field strains. Strains included in group 5 (Table 1) appeared in isolates
18 from Hungary, Holland and the UK and confirmed earlier observations by Hazel (PhD, 2002)
19 using RAPD analysis.

20 DNA sequence analysis was used to determine the phylogenetic relationships among the
21 *M. synoviae* strains and the tree showed which species were more closely related. For
22 example using the typing system of Benčina et al. (2001) it appears that strains J26/85 and
23 J15/85 are the same type but different to B11/85 whereas the phylogenetic tree reveals that
24 J26/85 is more closely related to B11/85 than to J15/85. This is reinforced by the DNA
25 homologies (Supplementary table 1) because comparison of J26/85 with B11/85 with the

1 insertion/deletion the similarity is 88.9% but without the insertion/deletion is 100%. In
2 contrast, when comparing J26/85 with J15/85 the homologies are 99.6% with or without the
3 insertion/deletion.

4 This study confirms that DNA extracted from cultures as well as directly from swabs
5 can be successfully amplified with our primers and used in the diagnosis and strain typing of
6 *M. synoviae*. It also confirms the potential value of strain typing for epidemiological purposes
7 and suggests that the addition of phylogenetic studies is essential to understand the true
8 relationships between strains.

10 **Acknowledgements**

11 The authors wish to thank to Aviagen Ltd for sponsoring PPH and Christine Yavari and
12 Cynthia Dare for their helpful assistance in the laboratory.

14 **References**

- 16 Ben Abdelmoumen Mardassi, B., Ben Mohamed, R., Gueriri, I., Boughattas, S., Mlik, B.,
17 2005. Duplex PCR to differentiate between *Mycoplasma synoviae* and *Mycoplasma*
18 *gallisepticum* on the basis of conserved species-specific sequences of their hemagglutinin
19 genes. J. Clin. Microbiol. 43, 948-958.
- 20 Benčina, D., Drobnič-Valič, M., Horvat, S., Narat, M., Kleven, S.H., Dovč, P., 2001.
21 Molecular basis of the length variation in the N-terminal part of *Mycoplasma synoviae*
22 hemagglutinin. FEMS Microbiol. Lett. 203, 115-123.
- 23 Bradbury, J.M., 1977. Rapid biochemical tests for characterisation of the *Mycoplasmatales*. J.
24 Clin. Microbiol. 5, 531-534.

- 1 Ewing, L., Cookson, K.C., Phillips, R.A., Turner, K.R., Kleven, S.H., 1998. Experimental
2 infection and transmissibility of *Mycoplasma synoviae* with delayed serological response
3 in chickens. *Avian Dis.* 42, 230-238.
- 4 Felsenstein, J., 1985. Confidence limits on phylogenies: An approach using the bootstrap.
5 *Evol.* 39, 783-791.
- 6 Garcia, M., Jackwood, M.W., Head, M., Levisohn, S., Kleven, S.H., 1996. Use of species-
7 specific oligonucleotide probes to detect *Mycoplasma gallisepticum*, *M. synoviae* and *M.*
8 *iowae* PCR amplification products. *J. Vet Diagn. Investig.* 8, 56-63.
- 9 Hall, T.A., 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis
10 program for Windows 95/98/NT. *Nucl. Acids. Symp. Ser.* 41, 95-98.
- 11 Hazel, K.A., 2002. The risk factors for mycoplasma infection in UK broiler breeder and
12 turkey breeder flocks. Thesis. University of Liverpool.
- 13 Hong, Y., Garcia, M., Leiting, V., Benčina, D., Dufour-Zavala, L., Zaval, G., Kleven, S.H.,
14 2004. Specific detection and typing of *Mycoplasma synoviae* strains in poultry with PCR
15 and DNA sequence analysis targeting the hemagglutinin encoding gene *vlhA*. *Avian Dis.*
16 48, 606-016.
- 17 Jeffery, N., Gasser, R.B., Steer, P.A., Noormohammadi, A.H., 2007. Classification of
18 *Mycoplasma synoviae* strains using single-strand conformation polymorphism and high-
19 resolution melting-curve analysis of the *vlhA* gene single copy region. *Microbiol.* 153,
20 2679-2688.
- 21 Kleven, S.H., Rowland, G.N., Kumar, M.C., 2001. Poor serologic response to upper
22 respiratory infection with *Mycoplasma synoviae* in turkeys. *Avian Dis.* 48, 719-723.
- 23 Lauerman, L.H., 1998. *Mycoplasma* PCR assays. In: Lauerman, L.H. (Ed), *Nucleic acid*
24 *amplification assays for diagnosis of animal diseases.* American Association of Veterinary
25 *Laboratory Diagnosticians*, Auburn, Alabama, pp. 41-42.

- 1 Lauerman, L.H., Hoerr, F.J., Sharpton, A.R., Shah, S.M., Van Santen, V.L., 1993.
2 Development and application of a polymerase chain reaction assay for *Mycoplasma*
3 *synoviae*. Avian Dis. 37, 829-834.
- 4 Noormohammadi, A.H., Markham, P.F., Kanci, A., Whithear, K.G., Browning G.F., 2000. A
5 novel mechanism for control of antigenic variation in the haemagglutinin gene family of
6 *Mycoplasma synoviae*. Mol. Microbiol. 35, 911-923.
- 7 Pettersson B., Tully, J.G., Bölske, G., Johansson, K.E., 2000. Updated phylogenetic
8 description of the *Mycoplasma hominis* cluster (Weisburg et al. 1989) based on 16S rDNA
9 sequences. Int. J. Syst. Evol. Microbiol. 50, 291-301.
- 10 Ramírez, A.S, Naylor C.J., Hammond P.P., Bradbury J.M., 2006. Development and
11 evaluation of a diagnostic PCR for *Mycoplasma synoviae* using primers located in the
12 intergenic spacer region and the 23S rRNA gene. Vet. Microbiol. 118, 76-82.
- 13 Razin, S., 2002. Diagnosis of Mycoplasmal infections. In: Molecular Biology and
14 Pathogenicity of Mycoplasmas. Razin, S. and Herrmann, R. (Eds.) Kluwer
15 Academic/Plenum Publishers, New York, pp. 531-544.
- 16 Rosendal, S., Black, F.T., 1972. Direct and indirect immunofluorescence of unfixed and fixed
17 mycoplasma colonies. Acta Pathol. Microbiol. Scand. 80, 615-622.
- 18 Saitou, N., Nei, M., 1987. The neighbor-joining method: A new method for reconstructing
19 phylogenetic trees. Mol. Biol. Evol. 4, 406-425.
- 20 Tamura, K., Dudley, J., Nei, M., Kumar, S., 2007. MEGA4: Molecular Evolutionary Genetics
21 Analysis (MEGA) software version 4.0. Mol. Biol. Evol. 10.1093/molbev/msm092.
- 22 Tamura, K., Nei, M., Kumar, S., 2004. Prospects for inferring very large phylogenies by using
23 the neighbour-joining method. PNAS. 101, 11030-11035.
- 24 Wang, H., Fadl, A.A., Khan, M.I., 1997. Multiplex PCR for avian pathogenic mycoplasmas.
25 Mol. Cell. Probes. 11, 211-216.

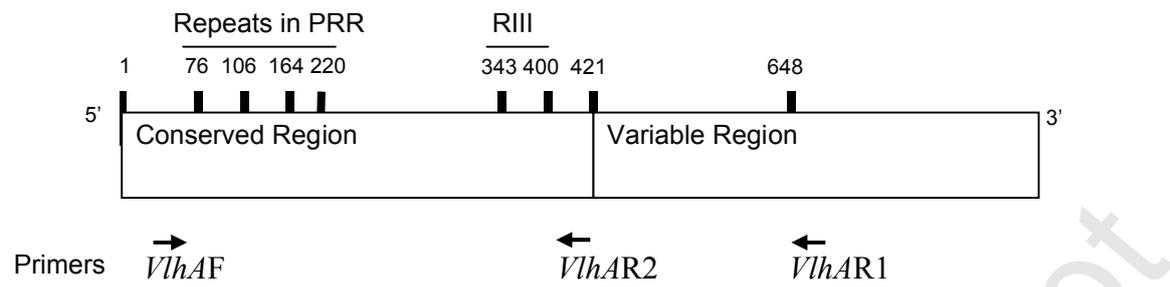
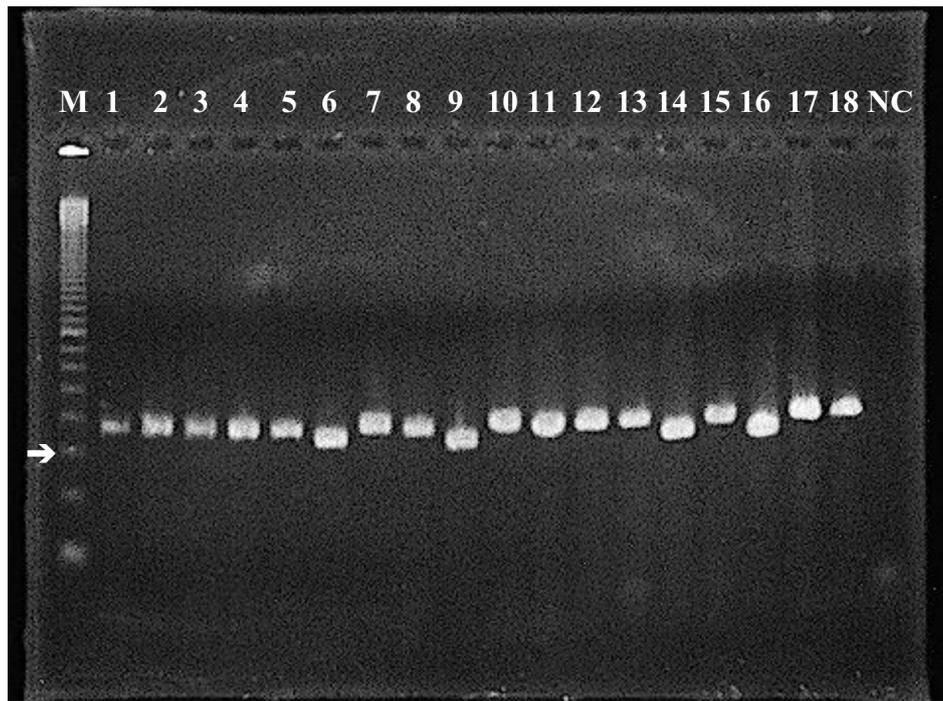


Fig. 1. Diagrammatic representation of the *M. synoviae vlhA* gene (based on Benčina *et al.*, 2001) and the primer locations.

1



2

3

4 Fig. 2. PCR Electrophoresis gel demonstrating *M. synoviae vlhA* gene amplification with new
5 reverse primer (*vlhAR2*). M. 100bp DNA marker ladder, the arrow indicates 300 bp; Lanes: 1.
6 B55/97/2a; 2. B138/96; 3. B91/96/801; 4. B110/02; 5. B91/96/798; 6. B38/96/1704; 7.
7 B13/96/P2; 8. B133/99/12; 9. B133/99/5; 10. B13/96/P1; 11. B3/98; 12. B85/99/3; 13. B7/86;
8 14. J26/85; 15. B55/97/1c; 16. J15/85; 17. B6/99; 18. B11/85; NC. Negative control.

9

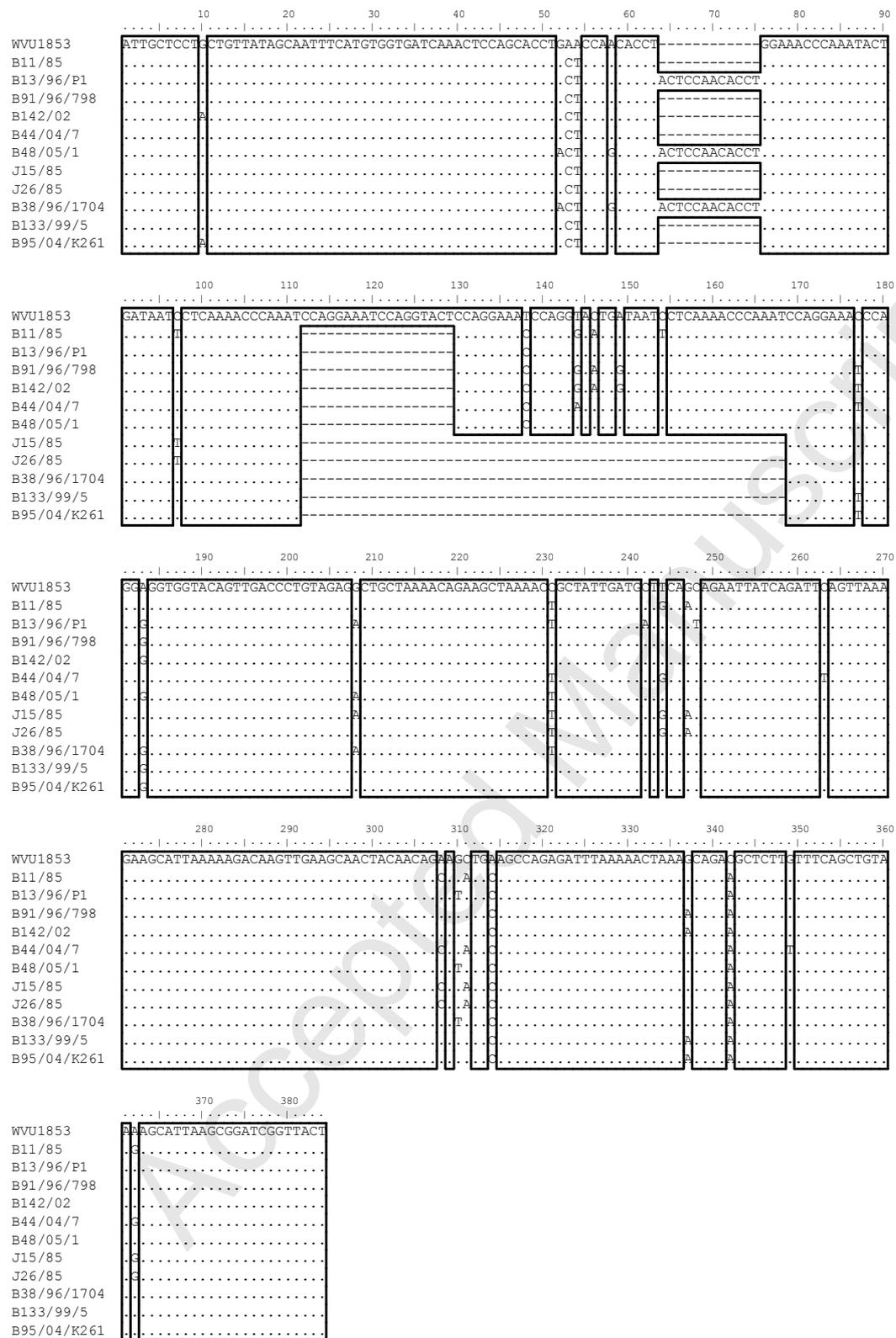


Fig. 3. Alignment of partial *vlhA* sequences of the 12 representative *M. synoviae* strains including the RIII region. Dots are used where the sequence is identical to the WVU1853^T sequence. Dashes are used to indicate a nucleotide deletion; only nucleotide substitutions are shown.

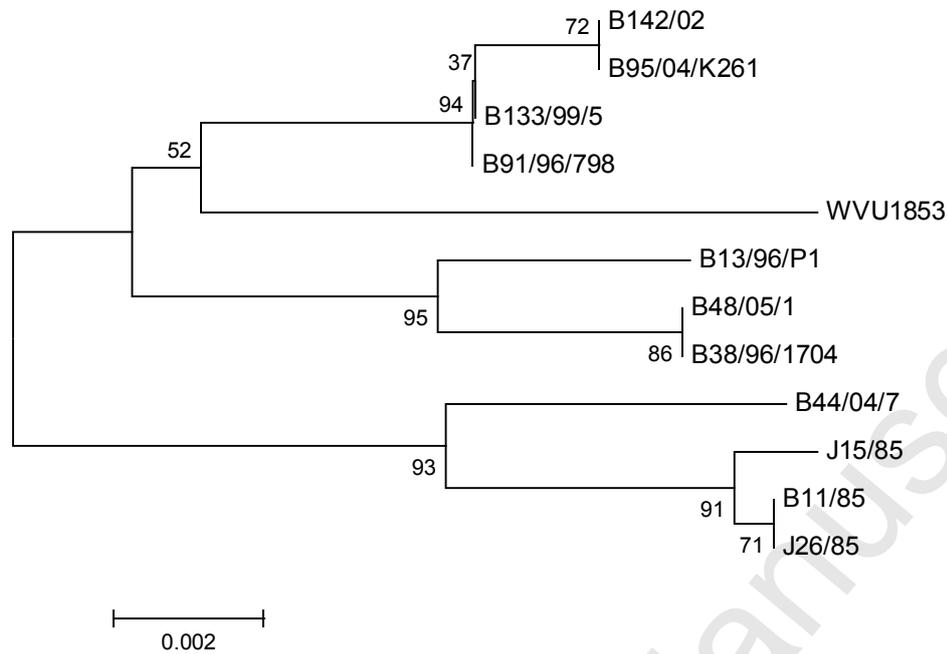


Fig. 4. Dendrogram constructed from partial *vhhA* gene sequences of the 12 representative *M. synoviae* group strains (Table 3). There were a total of 315 positions in the final dataset. Bootstrap values (expressed as percentages of 1000 replications) are shown at the branch points. Bar, 2 nucleotide substitutions per 1000 nucleotides.

1 Table 1: Details of *M. synoviae* strains and comparison of their *vlhA* gene nucleotide sequences^a using primers *vlhAF* and *vlhAR2*

Group ^b	Strain ^c	Host Species ^d	Country of origin	Year of isolation	Additional information ^e	PCR amplicon size (bp)	<i>vlhA</i> gene PRR (nt) ^f	Size of PRR (aa) ^g	Type & subtype ^h	GenBank accession no.	
1	WVU1853ⁱ	Ch	USA	1955		372	114	38	A	AM998371 ⁱ	
	B95/04/K4412	?	Germany	2004		372	114	38	A	FM164342	
2	B13/96/P1	Ph	UK	1996	}	Same location	366	108	36	F	AJ580987
	B13/96/P2	Ph	UK	1996			366	108	36	F	FM164343
3	B48/05	Ch	UK	2005	}	SC, DF	366	108	36	F	FM164344
	B57/05	Ch	UK	2005			“ “	366	108	36	F
4	B11/85	Tu	UK	1985	}	SC as B103/07/3, DF	354	96	32	C ₁	FM164346
	B7/86	Ch	UK	1986			354	96	32	C ₁	FM164347
5	B103/07/2(sw)	Ch	UK	2007	}	SF, DH	354	96	32	C ₁	FM164348
	B91/96/798	Tu	UK	1996			354	96	32	C ₃	FM164349
	B91/96/801	Tu	UK	1996	}	“ “	354	96	32	C ₃	FM164350
	B138/96/H1	Ch	UK	1996			354	96	32	C ₃	FM164351
	B55/97/1c	Ch	UK	1997	}	SH	354	96	32	C ₃	FM164352
	B55/97/2a	Ch	UK	1997			“ “	354	96	32	C ₃
	B3/98	Ch	Holland	1998	}	SF	354	96	32	C ₃	FM164354
	B6/99	Ch	Hungary	1999			354	96	32	C ₃	FM164355
	B85/99/2 (sw)	Ch	UK	1999	}	“ “	354	96	32	C ₃	FM164356
	B85/99/3 (sw)	Ch	UK	1999			354	96	32	C ₃	FM164357
	B133/99/12	Ch	Hungary	1999	}	SF as 133/99/5, DH	354	96	32	C ₃	FM164358
	B27/00 ^j	Ch	Hungary	2000			354	96	32	C ₃	AJ580988
	B110/02	Ch	Hungary	2002	}	SF, DH	354	96	32	C ₃	FM164359
	B155/02	Ch	UK	2002			354	96	32	C ₃	FM164360
	B9/03/11s (sw)	Ch	UK	2003	}	“ “	354	96	32	C ₃	FM164361
	B9/03/13s(sw)	Ch	UK	2003			354	96	32	C ₃	FM164362
	B9/03/4s (sw)	Ch	UK	2003			354	96	32	C ₃	FM164363
	B9/03/9s(sw)	Ch	UK	2003			354	96	32	C ₃	FM164364
	B35/03/2(sw)	Ch	UK	2003	}	SC, DF	354	96	32	C ₃	FM164365
	B115/03	Ch	UK	2003			354	96	32	C ₃	FM164366
	B103/07/3(sw)	Ch	UK	2007	}	“ “	354	96	32	C ₃	FM164367
	B103/07/4(sw)	Ch	UK	2007			354	96	32	C ₃	FM164368
6	B142/02^j	Ch	Hungary	2002	}	SC, DF	354	96	32	C ₃	AJ580989
	B154/02 ^j	Ch	Hungary	2002			354	96	32	C ₃	AJ580991

7	B44/04	Ch	UK	2004	}	SC	354	96	32	C ₆	FM164369
	B45/04	Ch	UK	2004			"	354	96	32	C ₆
8	B38/96/1764	Tu	Italy	1996			327	69	23	D	AJ580985
9	J15/85	Ch	UK	1985			315	57	19	E	AJ580981
10	J26/85	Ch	UK	1985			315	57	19	E	AJ580982
11	B133/99/5	Ch	Hungary	1999	}	SF as B133/99/12, DH	315	57	19	E	AJ580983
	B103/07/1(sw)	Ch	UK	2007			SC as B103/07/2, DF	315	57	19	E
12	B95/04/K261	?	Germany	2004			315	57	19	E	FM164372
	B47/06	Tu	Slovakia	2006			315	57	19	E	FM164373

1

2 ^aCorresponding to nucleotide numbers 49-421 in strain WVU1853^T (Benčina et al., 2001). This region includes the PRR and the RIII regions.

3 ^bGrouping based on sequence similarity.

4 ^cBold typeface indicates strains selected as group representatives; all were sequenced from cultures except those labelled (sw), which were
5 amplified for sequencing from swab samples.

6 ^dCh = chicken; Ph = pheasant; Tu = turkey; ? = unknown.

7 ^eKnown connections between samples: SC = same company; SF = same farm; DF = different farm; SH = same house; DH = different house.

8 ^fNumber of nucleotides in the *vlhA* sequence encoding PRR (after Benčina et al., 2001).

9 ^gNumber of amino acids in the *vlhA* sequence encoding PRR (after Hong et al., 2004).

10 ^hType and subtype classification according to the 5' end *vlhA* sequence and following the scheme of Benčina et al. (2001). Types A to F based on
11 length of PRR. Subtypes (e.g. C₃) based on sequence of RIII region.

12 ⁱSequence of WVU1853^T determined in this study was compared with GenBank sequence no. AF035624, complete cds of the *M. synoviae*
13 WVU1853^T phase-variable haemagglutinin (*vlhA*) gene as a control. No discrepancies were seen.

14 ^jThese three isolates failed to amplify with the combination of primers *vlhAF* and *vlhAR1*.

1 **Table 2.** PCR Primers for partial sequencing of 5' *M. synoviae vlhA* gene

<i>VlhAF</i>	Forward	5' ATTAGCAGCTAGTGCAGTGGCC 3'	Benčina et al. 2001
<i>VlhAR1</i>	Reverse	5' <u>C</u> AGCGCTAGTTTTGTTTTT <u>I</u> GG 3' ^a	Benčina et al. 2001
<i>VlhAR2</i>	Reverse	5' AGTAACCGATCCGCTTAATGC 3'	This study

2 ^a Bold letters are corrections from Benčina et al. (2001) (D Benčina: personal
3 communication).

Accepted Manuscript