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

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Abstract

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

Keywords: *Mollicutes, Mycoplasma, Mycoplasma synoviae*, diagnosis, polymerase chain reaction, *vlhA*. 
1. Introduction

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

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

Our aim was to test the PCR assay of Benčina et al. (2001) on *M. synoviae* strains from different hosts, locations and years. Since some of our strains were not amplified an alternative reverse primer, located in the conserved region of the *vlhA* gene (Fig. 1) was used.
The specificity and sensitivity of this PCR and its ability to detect \textit{M. synoviae} in field isolates and swabs were established and sequence analysis was evaluated for strain typing.

2. Material and methods

2.1. Organisms and culture conditions

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

The mycoplasmas were cloned once by filtration and their identity was confirmed by the indirect fluorescent antibody test (Rosendal and Black, 1972). Then they were cultivated in mycoplasma broth (Bradbury, 1977) at 37 °C for between one and five days, in a CO\textsubscript{2} rich atmosphere.

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

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

2.3. Testing the primer pairs

The primers used in the reaction are shown in Table 2. PCR amplification using vlhAF and vlhAR1 primers was performed on 21 isolates. The reaction mixture contained 0.5 μM of each primer (vlhAF and vlhAR1); 0.2 mM dNTPs; 2.5 mM MgCl2; 1X reaction buffer (Abgene, AB-0192); 0.5 U Taq DNA polymerase (Abgene) made up to 49.5 μl with water (Sigma W4502).

DNA template (1 μl) was added per tube andSigma water (1 μl) was added to another tube to act as a negative control. Mineral oil (50 μl) was layered on the surface of each mixture. The thermal cycle, using Perkin Elmer PCR machine, was 94 °C for 5 min; 36 cycles 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 min.

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

2.4. Assay specificity and sensitivity

The specificity and sensitivity studies were carried out using the vlhAF-vlhAR2 PCR assay. Specificity was tested with the type strains of all the 23 avian Mycoplasma species listed above. Sensitivity was determined by analysing serial 10-fold dilutions to $10^{-9}$ of broth cultures from the M. synoviae type strain (WVU1853T) and from a field strain (B155/02).

Viable counts were conducted for each culture and the numbers of colony-forming units
(CFU) per 100μl were calculated (Bradbury, 1977). The DNA was measured using a fluorometer (DyNA Quant™ 200, Hoefer, Pharmacia Biotech Inc.), following the manufacturer’s instructions. PCR was carried out on each dilution.

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

To compare the results of our PCR assay with other described methods, three other M. synoviae PCR detection protocols were conducted with the type strain (WVU1853T) and a field strain (B155/02). These were the Lauerman method (Lauerman, 1998), a modified Lauerman procedure (Ramírez et. al., 2006) in which DNA extraction incorporated diethyl pyrocarbonate (DEP) and a commercial kit (FlockChek® Mycoplasma synoviae DNA probe kit, IDEXX Laboratories Inc., Westbrook, Maine, USA).

2.6. Sequencing

Sequencing was conducted by Imperial College, London, and sequences were determined for both strands of DNA. The resulting chromatograms were examined in Chromas (version 1.45; School of Health Science, Griffith University, Australia). The forward sequence and reverse complemented sequences were compared to produce a consensus sequence using Generunner (version 3.05; Hastings Software, Inc.). Bioedit 7.0.0 (Hall, 1999) program was used for aligning all the sequences manually, and for deducing the amino acid sequences. Nucleotide and amino acid sequences of all 41 M. synoviae strains (30 from cultures and 11 from swabs) were trimmed from nucleotide 49 to 421 corresponding to the numbering of Benčina et al. (2001) for strain WVU1853T. They were assigned in the Genbank with accession numbers AM998371, AJ580981 to AJ580983, AJ580985, AJ580987 to AJ580989, AJ580991 and FM164342 to FM164373.
2.7. Percent DNA similarity

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

2.8. Analysis of sequences

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

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

3. Results
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 µl 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\(^{-2}\).
3.3. Sequence analysis and strain typing

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

Strain typing was initially based on the size of the PRR region and point mutations of the RIII region (Benčina et al., 2001). Using the length of the PRR the strains were assigned to Benčina’s types A to E. Most strains were assigned to type C (Table 1). There were none belonging to type B but a further type F was added to accommodate two strains whose PRR length was 108 nt (36 amino acids). After this, the strains were subdivided numerically based on the RIII region and most of those in type C fell into subtype \( C_3 \). However, while 21 of the 23 strains of this subtype had 100% similarity, the two remaining (identical) strains had only 99.6% similarity with the others. Percent similarity between the representatives of each group with and without insertions/deletions is presented as Supplementary Table 1.

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

When groups 2 to 12 were compared with group 1 which contained the \( M. \ synoviae \) type strain WVU1853\( ^T \) (Table 1 and Fig. 3) all the other strains had a deletion at nt 112 corresponding to nt 148 of Benčina et al. (2001). Of these, 32 strains (the members of groups
2 to 7) had a deletion of 18 nt/6 aa and seven (the members of groups 8 to 12) a deletion of 57 nt/19 aa, however only five strains (the members of groups 2, 3 and 8) had an insertion between nt 64 and 65 (nt 111 and 112 of Benčina et al. 2001).

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

**Discussion**

PCR assays for detection of *M. synoviae* have been reported earlier (Garcia et al, 1996; Lauerman et al., 1993 and 1998; Benčina et al., 2001; Hong et al., 2004, Ben Abdelmoumen Mardassi et al, 2005; Ramírez et al., 2006; Jeffery et al., 2007). Four of them target the haemagglutinin-encoding *vlhA* gene (Benčina et al., 2001; Hong et al., 2004; Ben Abdelmoumen Mardassi et al, 2005; Jeffery et al., 2007). In our hands the primers used by Benčina et al. (2001) did not amplify three of our field isolates. All emanated from Hungary and, after amplification with the new reverse primer, could be placed in Benčina subtype C3 and our groups 5 and 6. This lack of amplification can be explained by the fact that the reverse primer of these authors, *VlhAR1*, targets the variable region of the *vlhA* gene (Fig. 1). Hong et al. (2004) described a PCR with the PRR region as the target, excluding the RIII...
region. Our primer vlhA R2 was designed to target the 3’ end of the conserved region, including the RIII region. With the new reverse primer the DNA from all 21 isolates plus nine more was amplified.

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

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

When the four M. synoviae diagnostic PCRs were compared, the vlhA F-vlhA R2 PCR described in this study performed best, detecting a lower amount of DNA than the other methods. This could be due to the use of the resin Chelex in the DNA extraction, because similar results were seen by Ramírez et al. (2006) when the same method was used for extracting DNA for a PCR based on the 16S-23S rRNA intergenic spacer region.
PCR and partial \( vlhA \) gene sequencing may also be useful in strain identification.

Benčina et al., (2001) compared the 5' end haemagglutinin (\( vlhA \)) gene sequences of 30 \( M.\) \( synoviae \) strains from chickens and turkeys and demonstrated 11 different types of \( vlhA \) sequences, while Hong et al. (2004) found 14 different groups among 43 strains from the same species. The majority of strains in both studies came from the USA and Slovenia.

Jeffery et al. (2007) examined 35 different isolates from Australia and the USA (from chickens and one turkey) and revealed 10 distinct profiles by single-strand length polymorphism. These corresponded to the 10 genotypes derived by high-resolution melting-curve analysis. The 41 strains in our study were mainly from the UK and Hungary and comprised chicken and turkey strains plus two from pheasants. They were assigned to 12 groups and showed considerable overlap with the groupings of Benčina et al. (2001) although four strains, including the two pheasant strains did not fit into any of the Benčina types.

The \( vlhA \) gene has considerable divergence at the 3' end of the gene due to recombination with pseudogenes (presumably as a mechanism to evade the host immune response), but relative conservation at the 5' end (Noormohammadi et al., 2000). The 5' \( vlhA \) region is present in the \( M.\) \( synoviae \) genome as a single copy and does not change its sequence in clonal populations of \( M.\) \( synoviae \) (Noormohammadi et al., 2000). This observation is crucial to strain identification as downstream of this region the sequence can change even in clonal populations of \( M.\) \( synoviae \) and therefore it cannot be considered a conserved sequence that characterises individual strains (Noormohammadi et al, 2000). The test evaluated by Hong et al. (2004) combined PCR and sequencing of the PRR repeat region of the \( vlhA \) gene to detect and type \( M.\) \( synoviae \) strains without the need for isolation. However, it excluded the RIII region (positions 343/400), which Benčina et al. (2001) found very useful for subtyping \( M.\) \( synoviae \) strains. The PRR region is useful mainly due to insertions/deletions and the RIII region is useful because of polymorphisms and our findings have confirmed this, as have
those of Jeffrey et al. (2007) who, by chance, designed a reverse primer almost identical to ours.

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

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

Isolates from the mid 1980s demonstrated greater similarity with the type strain WVU1853T than the present isolates, apart from strain B95/04/K4412 whose sequence was exactly the same as the *M. synoviae* type strain. These two strains from Germany were from the same sender but no further information about their origin was available although we assumed both to be field strains. Strains included in group 5 (Table 1) appeared in isolates from Hungary, Holland and the UK and confirmed earlier observations by Hazel (PhD, 2002) using RAPD analysis.

DNA sequence analysis was used to determine the phylogenetic relationships among the *M. synoviae* strains and the tree showed which species were more closely related. For example using the typing system of Benčina et al. (2001) it appears that strains J26/85 and J15/85 are the same type but different to B11/85 whereas the phylogenetic tree reveals that J26/85 is more closely related to B11/85 than to J15/85. This is reinforced by the DNA homologies (Supplementary table 1) because comparison of J26/85 with B11/85 with the
insertion/deletion the similarity is 88.9% but without the insertion/deletion is 100%. In contrast, when comparing J26/85 with J15/85 the homologies are 99.6% with or without the insertion/deletion.

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

**Acknowledgements**

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**References**


9 Tamura, K., Nei, M., Kumar, S., 2004. Prospects for inferring very large phylogenies by using the neighbour-joining method. PNAS. 101, 11030-11035.
Fig. 1. Diagrammatic representation of the *M. synoviae* *vlhA* gene (based on Benčina *et al.*, 2001) and the primer locations.
Fig. 3. Alignment of partial vha sequences of the 12 representative M. synoviae strains including the RIII region. Dots are used where the sequence is identical to the WVU1853T sequence. Dashes are used to indicate a nucleotide deletion; only nucleotide substitutions are shown.
Fig. 4. Dendrogram constructed from partial \textit{vihA} gene sequences of the 12 representative \textit{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.
### Table 1: Details of *M. synoviae* strains and comparison of their *vlhA* gene nucleotide sequences using primers *vlhAF* and *vlhAR2*

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<th>Group</th>
<th>Strain</th>
<th>Host Species</th>
<th>Country of origin</th>
<th>Year of isolation</th>
<th>Additional information</th>
<th>PCR amplicon size (bp)</th>
<th><em>vlhA</em> gene PRR (nt)</th>
<th>Size of PRR (aa)</th>
<th>Type &amp; subtype</th>
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1. aCorresponding to nucleotide numbers 49-421 in strain WVU1853<sup>T</sup> (Benčina et al., 2001). This region includes the PRR and the RIII regions.
2. bGrouping based on sequence similarity.
3. cBold typeface indicates strains selected as group representatives; all were sequenced from cultures except those labelled (sw), which were amplified for sequencing from swab samples.
4. dCh = chicken; Ph = pheasant; Tu = turkey; ? = unknown.
5. eKnown connections between samples: SC = same company; SF = same farm; DF = different farm; SH = same house; DH = different house.
6. fNumber of nucleotides in the vlhA sequence encoding PRR (after Benčina et al., 2001).
7. gNumber of amino acids in the vlhA sequence encoding PRR (after Hong et al., 2004).
8. 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 length of PRR. Subtypes (e.g. C<sub>3</sub>) based on sequence of RIII region.
9. iSequence of WVU1853<sup>T</sup> determined in this study was compared with GenBank sequence no. AF035624, complete cds of the <i>M. synoviae</i> WVU1853<sup>T</sup> phase-variable haemagglutinin (vlhA) gene as a control. No discrepancies were seen.
10. jThese three isolates failed to amplify with the combination of primers vlhAF and vlhAR1.
**Table 2.** PCR Primers for partial sequencing of 5’ *M. synoviae vlhA* gene

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<sup>a</sup> Bold letters are corrections from Benčina et al. (2001) (D Benčina: personal communication).