Evaluation of Amplified Fragment Length Polymorphism for Differentiation of Avian Mycoplasma Species

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Amplified fragment length polymorphism (AFLP) was used for typing avian mycoplasma species. Forty-four avian mycoplasma strains were successfully typed into eight distinct groups, with each representing a different species. Homology of AFLP patterns of 35% or less was used as a cutoff value to differentiate avian mycoplasma strains into different species.

Some species of avian mycoplasma are important disease factors which adversely affect the commercial poultry industry in the United States. Avian mycoplasmas are very small prokaryotes devoid of cell walls. They tend to grow very slowly on a protein-rich medium containing 10 to 15% added animal serum and are rather resistant to certain antibiotics which are frequently employed in medium to retard growth of contaminant bacteria and fungi (4). Mycoplasma gallisepticum is the most pathogenic avian mycoplasma species, causing chronic respiratory diseases in chickens and infectious sinusitis in turkeys (11). When the infection becomes systemic, M. synoviae can also cause infectious synovitis in chickens and turkeys (5). M. iowae infection leads to reduced hatchability and embryo mortality in turkeys.

Condemnation of the infected flocks and reduction in feed conversion and egg production are the major factors related with economic losses. However, nonpathogenic species such as M. gallinarum and M. gallinaceum are often isolated and must be differentiated from pathogenic species. Amplified fragment length polymorphism (AFLP) has been extensively tested by Kokotovic et al. (7). Restriction endonucleases BglII and MfeI were used in the digestion of genomic DNA. The MFE1 primer used in this study, as in Kokotovic’s study, was modified by adding a selective nucleotide A at its 3’ end to increase the selectivity of the amplification reaction and to obtain better banding pattern resolution.

Fragment detection was carried on an ABI 310 automatic sequencer (ABI Applied Biosystems, Foster City, Calif.). A mixture consisting of 2.0 µl of PCR products, 12.0 µl of 100% deionized formamide, and 0.5 µl of GeneScan 500 ROX size standard (ABI Applied Biosystems, Foster City, Calif.) was heated to 95°C for 5 min and quickly chilled on ice before electrophoresis on the machine. Fragment size determination and pattern analysis were performed by using GeneScan 3.1 fragment analysis software (ABI Applied Biosystems). ABI chromatograms were converted into schematic gel images with GelCompar II 3.5 (Applied Math Inc., Austin, Texas). Background subtraction and data normalization were subsequently conducted. Cluster analysis was performed using the Pearson correlation and unweighted pair group methods with average linkages.

AFLP analysis in this study provided an optimal separation and a uniform sizing of the amplified fragments. Fragments of between 75 and 500 bp were used in numerical and cluster analysis for species differentiation. We found that M. gallisepticum strains had the highest banding pattern complexity, consisting of about 90 AFLP fragments, while M. meleagridis profiles had the lowest complexity, having approximately only 20 amplified fragments. Both M. gallinarum and M. iowae strains contained about 50 fragments. About 60, 40, and 30 fragments were generated for M. gallinaceum, M. pullorum, and M. synoviae, respectively. M. imitans had 70 fragments in its AFLP banding pattern. Reproducibility of AFLP analysis has been extensively tested by Kokotovic et al. (7). In this study, AFLP

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procedure was repeated three times on three randomly chosen strains from each species and highly reproducible results were obtained. Minor changes in band intensities existed but were insignificant for determining identity of strains (data not shown).

On the basis of cluster analysis, we chose the 35.0% linkage level (percent homology) as a cutoff value for discriminating mycoplasma strains at the species level (Fig. 1). AFLP data revealed eight distinct groups (I to VIII), each consisting of strains belonging to a single species. All strains within each group had different AFLP patterns, some of which were nearly identical. To validate this clustering method, we calculated error flags (not shown in the dendrogram) representing the value of the mean linkage level plus standard deviation for those groups of strains as indicated in the following paragraphs. The 35.0% cutoff line did not cross any of those error flags.

*M. pullorum*, *M. gallinarum*, and *M. gallinaceum* strains showed high AFLP pattern heterogeneity, with linkage levels of 47.8% ± 4.5% (mean plus standard deviation) (group III), 48.0% ± 9.0% (group VI), and 51.4% ± 6.8% (group IV), respectively. Our study is the first to report the genetic heterogeneity among those saprophytic avian mycoplasma species, though a larger number of strains, as well as other restriction enzymes, needs to be tested to make final conclusions.

On the other hand, *M. iowae* and *M. meleagridis* strains...
FIG. 1. AFLP fingerprints of avian mycoplasma species. The dendrogram was constructed using Pearson correlation and the unweighted pair group method with average linkages. The eight avian mycoplasma species groups generated at a 35.0% linkage level cutoff point are indicated.
showed high homogeneity in their AFLP profiles and clustered at linkage levels of 73.2% ± 6.2% (group VII) and 68.0% ± 7.8% (group VIII), respectively. Previous studies revealed that although heterogeneity in serological responses was observed for *M. iowae* (10), less-variable protein profiles and random amplified polymorphic DNA (RAPD) patterns were obtained for different strains (3, 14). Among pathogenic avian mycoplasmas, *M. gallisepticum* strains revealed the widest intraspecies heterogeneity by AFLP analysis, with a linkage level of 59.2% ± 2.2% (group I). The genetic variation of this species has been documented by several other molecular typing techniques, such as pulse field gel electrophoresis (12), random amplified polymorphic DNA (2, 12), restriction fragment length polymorphism (13), and Southern blotting (17). *M. synoviae* strains, with a linkage level of 66.1% ± 3.0% (group V), exhibited more genetic homogeneity than *M. gallisepticum*, and the same conclusion was made using an rRNA gene hybridization test conducted by Yogev et al. (16).

*M. imitans* shared many phenotypic properties with *M. gallisepticum* but had low genetic homology with *M. gallisepticum* in a DNA-DNA hybridization study (1). In this study, the two species were typed into related groups and linked at the 25.3% homology level.

As determined on the basis of these results, AFLP can be used as an additional confirmatory tool for identification of avian mycoplasma species. This can be achieved by setting up a database for reference strains; such a database was partially created in this study and can be easily expanded. Our follow-up studies will focus on using AFLP for typing avian mycoplasma strains within each species.

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