

Variability of *Mycoplasma gallisepticum* Isolates from House Finches Detected by Random Amplification of Polymorphic DNA (RAPD) and Amplified Fragment Length Polymorphism (AFLP)

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INTRODUCTION

Mycoplasma gallisepticum (MG) conjunctivitis emerged in 1994 as a disease of free-ranging house finches (*Carpodacus mexicanus*) in the eastern United States and has since spread to house finches throughout their entire eastern range (3,7,8). The resulting epidemic of MG conjunctivitis produced an unprecedented decline of eastern house finch populations, and the endemic disease remains associated with repeating seasonal peaks of disease and limitation of host populations (1,5). MG has also been isolated from other songbirds with conjunctivitis including American goldfinches, a blue jay, purple finches, and evening and pine grosbeaks (3,8,9). Random amplification of polymorphic DNA (RAPD) demonstrated the presence of what appeared to be a single, unique RAPD profile among house finch and other songbird MG isolates, suggesting a single point source of origin and one 'strain' common to the outbreak (8). However, genomic variability of MG house finch isolates has recently been identified by PCR-RFLP and nucleotide sequencing of the *pvpA* gene (10). These findings suggested that house finch MG isolates may be more polymorphic than previously recognized and provide evidence of molecular evolution.

We have seen some evidence of genomic variability among MG isolates by RAPD fingerprinting. However, RAPD fingerprints are prone to variability, and may be difficult to reproduce and standardize, making interpretation challenging and subjective. To explore the possibility of genomic variability among house finch isolates of MG we selected samples from our archive of isolates to analyze by RAPD and amplified-fragment length polymorphism (AFLP). The AFLP technique has been successfully used to explore the genomic variability of several *Mycoplasma* spp. (6). Analyses of MG isolates by RAPD and AFLP allows us to more definitively explore the potential genomic variability of these isolates and their molecular epidemiology especially with respect to possible host, temporal and geographic relationships. These analyses also generate comparative data between RAPD and AFLP methodologies, providing an opportunity to evaluate the utility of AFLP for MG genotyping.

MATERIALS AND METHODS

MG Strains and Isolates. MG strains analyzed included vaccine strains F, 6/85 (Intervet Inc., Millsboro, DE), and ts-11 (Select Laboratories, Gainesville, GA); and reference strains S6, R, and A5969 from domestic poultry. Also included were MG isolates from 10 wild-captured songbirds showing signs of conjunctivitis (Fig. 1). These included 6 birds captured between 1994-96 (1 blue jay, 1 American goldfinch, and 4 house finches), and 4 house finches captured in 2001 (Table 1). *Mycoplasmas* were grown in both cultures, and DNA was isolated using a DNeasy Tissue Kit (QIAGEN Inc., Valencia, CA).

RAPD. Random amplification of polymorphic DNA (RAPD) is a PCR-based method of DNA fingerprinting that results in amplification of 'anonymous' stretches of DNA with short arbitrary primers and visualization of the amplification products by agarose gel electrophoresis. Compared to other currently available methods of MG strain identification, RAPD is fast, relatively simple to perform and cost effective. However, there are disadvantages and limitations to RAPD fingerprinting. A pure culture of the mycoplasma isolate is required, and RAPD tests are known to have problems with reproducibility because they are sensitive to alterations in PCR conditions, and interpretation of banding patterns can be challenging. The challenges of reproducibility and interpretation can usually be overcome by using one or more additional primer sets to confirm apparent relationships or resolve ambiguous results. Our procedure for RAPD fingerprinting of MG has been published (8), and uses the primer sets described by Fan (2) and Geary (4).

AFLP. Amplified fragment length polymorphism (AFLP) is a selective restriction fragment amplification technique based on the ligation of adapters (linkers and indexers) to a digest of total genomic DNA, followed by a PCR-based amplification with adapter-specific primers. Like RAPD, AFLP allows simultaneous sampling of multiple loci distributed throughout the entire genome, but allows the researcher to control the number of bands generated by using increasingly specific primer sets. AFLP can generate consistent and reproducible banding patterns covering a large number of loci with a single amplification, but is more time consuming than RAPD and requires expensive automated genetic analyzers to visualize the banding patterns.

Our procedure for AFLP fingerprinting of MG was carried out according to Kokotovic (6). Amplification fragments were detected on a 310 Genetic Analyzer (Applied Biosystems, Foster City, CA) and initial data collection and preprocessing were performed using GenScan analysis software (Applied Biosystems, Foster City, CA). The preprocessed denatometric curve data were imported to GelCompar 2.0 (Applied Maths BVBA, St-Martens-Latem, Belgium) where similarity among samples was calculated using the band-based Dice similarity coefficient, and clustering of samples (dendrogram) was performed using the unweighted pair-group method with arithmetic averaging (UPGMA).

Fig. 1. House Finch with MG Conjunctivitis



Female house finch with periorbital swelling, inflammation, and conjunctivitis – typical clinical signs of MG disease in songbirds.

Fig. 2. RAPD Fingerprints of MG Vaccines, Reference Strains, and House Finch Isolates

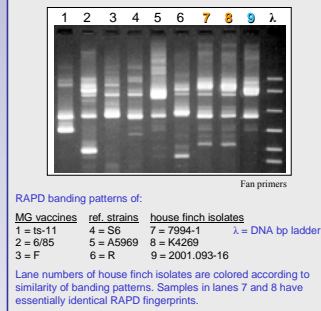


Table 1. MG Isolates from Songbirds 1994-2001

Isolate id.	Isolated (mo/yr)	Host species	Location	Isolated by
7994-1	06/94	House finch	Virginia	NCSU
11394-2	07/94	Blue jay	Virginia	NCSU
K3839	11/94	House finch	Maryland	SCWDS/UGA
13295-2	08/95	House finch	North Carolina	NCSU
1596-5	02/96	Am. goldfinch	North Carolina	NCSU
K4269	07/96	House finch	Ohio	SCWDS/UGA
2001.035-16	04/01	House finch	New York	NCSU
2001.043-13	05/01	House finch	Wisconsin	NCSU
2001.047-5	05/01	House finch	New York	NCSU
2001.093-16	10/01	House finch	Georgia	NCSU

Selected MG isolates from three songbird species made in 1994 to 2001 from seven states of the USA. Two isolates (SCWDS/UGA) were provided by P Luttrell and JF Fischer (Southeastern Cooperative Wildlife Disease Study) and SH Kleven (Dept. Avian Med., UGA).

Fig. 3. RAPD Fingerprints of MG Isolates from Songbirds 1994-2001

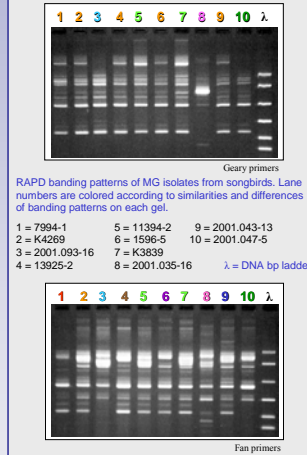
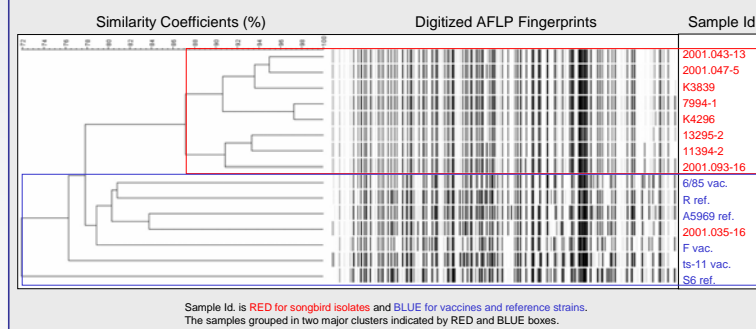


Fig. 4. AFLP analysis of MG Vaccines, Reference Strains, and Songbird Isolates



RESULTS AND DISCUSSION

Fig. 2 shows RAPD banding patterns of MG vaccines (ts-11, 6/85, F), reference strains (S6, A5969, R), and three house finch isolates. Each vaccine and reference strain has a unique banding pattern, and can be easily distinguished from one another and from the house finch isolates. Two of the house finch isolates have similar banding patterns - indicated by lane numbers (7 and 8) of the same color.

These results demonstrate the ability of RAPD to differentiate among known strains of MG, and potential utility to recognize unknown field isolates of MG for the purposes of genotype identification and molecular epidemiology.

Fig. 3 shows RAPD banding patterns of selected isolates (Table 1) from three songbird species (house finch, American goldfinch, and blue jay) made from 1994 to 2001 in seven states (VA, MD, NC, OH, NY, WI, GA). Isolates with different RAPD fingerprints have lane numbers of different colors. Fingerprints resulting from Geary primers show similar patterns for five of the ten isolates. Fingerprints resulting from Fan primers show more diversity among the isolates (only isolates in lanes 5 and 7 have identical banding patterns), although some of the pattern differences are subtle. Therefore, Fan primers appear to be more discriminatory than Geary primers.

These results indicate that while there may be considerable genotypic homology among MG isolates from songbirds, some variability is also detectable. The challenges of making subjective visual interpretations of RAPD banding patterns are also evident.

Fig. 4 shows AFLP results of MG vaccines (ts-11, 6/85, F), reference strains (S6, A5969, R), and 9 songbird isolates (Table 1, except 1596-5). AFLP analysis generated 50-80 bands per sample, which allows resolution of finer-scale quantitative variation among the samples.

AFLP can be used for genetic fingerprinting and molecular characterization of MG strains, and yields results that have better discriminatory power and are more reproducible than RAPD.

All but one of the songbird isolates were grouped together and have similarity coefficients of 91.5 to 97%, and cluster at a linkage level of 87%, indicating that they are closely related. The vaccine and reference strains have similarity coefficients of 72 to 85.5%, confirming that they are different strains. One house finch isolate (2001.035-16) was clearly different from the other songbird isolates and was grouped with the vaccine and reference strains, suggesting that substantial molecular evolution or a separate introduction of a 'new' MG strain occurred.

AFLP results support previous observations that during the initial stages of the MG epidemic in songbirds, isolates had genotypes that appeared to be closely related. This indicated that the outbreak in various songbird species and geographic locations was caused by the same or closely related strain of MG, suggesting a single point-source of origin (8).

More extensive analyses of historical and contemporary isolates of MG from house finches and other songbirds, using improved genotyping techniques such as AFLP, may help answer this and other questions about the epidemiology of MG conjunctivitis.

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