

Genotyping *Mycoplasma gallisepticum* Isolates from Songbirds by Random Amplification of Polymorphic DNA and Amplified-Fragment Length Polymorphism

David Ley¹, Sonia Altizer², John Cherry²

¹Department of Population Health and Pathobiology, College of Veterinary Medicine, NC State University, Raleigh, NC 27606 USA; ²Department of Environmental Studies, Emory University, Atlanta, GA 30322 USA

ABSTRACT

Mycoplasma gallisepticum has recently emerged as a cause of conjunctivitis and significant declines in house finch populations throughout eastern North America (2,5,6). *M. gallisepticum* has also been isolated from other songbirds with conjunctivitis including American goldfinches, purple finches, evening grosbeaks and pine grosbeaks (2,6,7). Random amplification of polymorphic DNA (RAPD) fingerprinting analyses demonstrated the presence of what appeared to be a single, unique RAPD profile among house finch and other songbird *M. gallisepticum* isolates, suggesting a single point source of origin and one common 'strain' that appeared to spill over from house finches and involve some other songbird species (6). However, genomic variability of *M. gallisepticum* house finch isolates has recently been demonstrated using polymerase chain reaction (PCR)-restriction fragment length polymorphism (RFLP) analysis and nucleotide sequencing of the *pvpA* gene (8). RAPD patterns of *M. gallisepticum* isolates from songbirds (over 100 isolates from 12 states, made from 1994 to 2003) showed notable homology among banding patterns regardless of temporal, geographic or host origin, until 2001 when some variability among banding patterns was noticed. These findings suggest that *M. gallisepticum* isolates from songbirds may be more polymorphic than previously recognized and/or evidence of *M. gallisepticum* molecular evolution. To explore the possibility of genomic variability among songbird isolates of *M. gallisepticum*, we selected samples from our archive of isolates to analyze by amplified-fragment length polymorphism (AFLP) and RAPD. Analyses of *M. gallisepticum* songbird isolates by AFLP and RAPD allows us to more definitively explore the genomic variability of these isolates and their molecular epidemiology, especially with respect to temporal, geographic, and host species relationships. These analyses also generate comparative data between RAPD and AFLP methodologies, providing an opportunity to evaluate the utility of AFLP for *M. gallisepticum* typing.

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. Also included were MG isolates from 11 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 5 house finches captured in 2001 (Table 1). Mycoplasmas isolated from songbirds by NCSU were from conjunctival swabs cultured in Frey's broth medium with 15% swine serum. Mycoplasma colonies on agar plates were identified as MG by direct immunofluorescence using fluorescein-conjugated rabbit antiserum provided by SH Kleven (Department of Avian Medicine, University of Georgia, Athens, GA). In preparation for RAPD and AFLP analyses mycoplasmas were grown in broth 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 one (or sometimes more) short arbitrary primers and subsequent 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, RAPD tests are known to have problems with reproducibility because they are sensitive to alterations in PCR conditions, and interpretation of banding pattern 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 (6), and uses the primer sets described by Fan (1) and Geary (3).

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 banding patterns covering a large number of loci with a single amplification, but is more time consuming than RAPD and requires a DNA sequencing gel. Our procedure for AFLP fingerprinting of MG has been adapted from Kokotovic (4).

Unfortunately, we had technical difficulties with instrumentation necessary for AFLP, and the results we had planned to present were not available for inclusion in this poster.

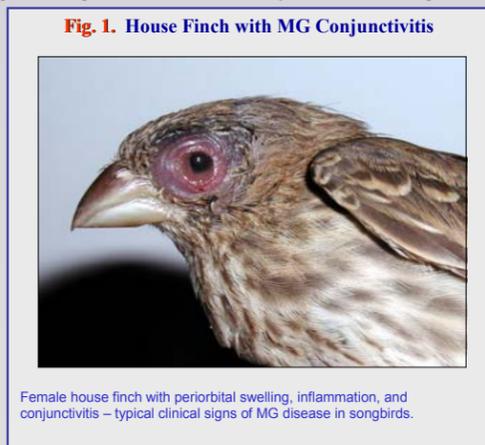


Table 1. MG Isolates from Songbirds 1994-2001

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
2001.035-1	04/01	House finch	New York	NCSU
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. Color-coding reflects the RAPD results shown in Figs. 2 and 3. 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. 2. RAPD Fingerprints of MG Vaccines, Reference Strains, and House Finch Isolates

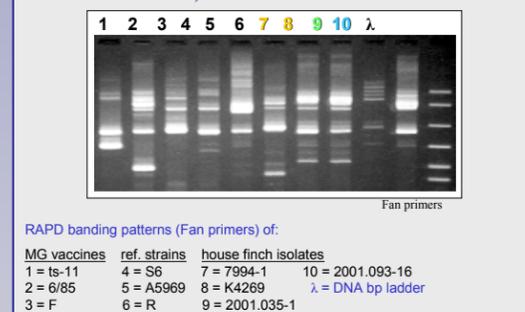
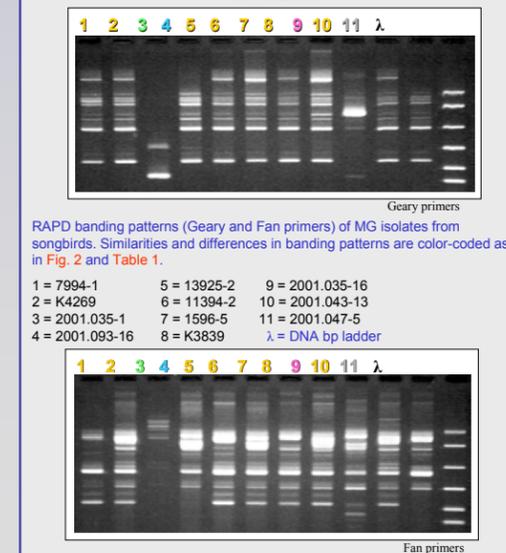


Fig. 3. RAPD Fingerprints of MG Isolates from Songbirds 1994-2001



RESULTS AND DISCUSSION

Fig. 2 shows RAPD banding patterns of MG vaccines (ts-11, 6/85, F), reference strains (S6, A5969, R), and four selected house finch isolates. Each of the vaccine and reference strains each have unique banding patterns, and can be easily distinguished from one another, and the house finch isolates. Two of the house finch isolates have similar banding patterns, and this is indicated by color-coding their lane numbers (7 and 8). The other two house finch isolates have different banding patterns, so their lane numbers each have a different color.

These results demonstrate the ability of RAPD to differentiate among known strains of MG, and potential utility to type unknown or field isolates of MG for the purposes of genotype identification and molecular epidemiology. These results also indicate that there may be significant genotypic variability among MG isolates from house finches.

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). Lane numbers are color-coded according to similarities and differences among the RAPD banding patterns, as in Fig. 2. Isolates with similar RAPD banding patterns have lane numbers of the same color and are interpreted as being the same or a closely related strain. Isolates with different RAPD banding patterns have lane numbers of different colors, and may represent different genotypes.

Seven of the eleven isolates analyzed have similar RAPD banding patterns suggesting a common and/or similar genotype. This 'dominant' RAPD-genotype includes the first isolate made (7994-1) from a house finch with conjunctivitis in 1994, each of the other selected isolates made to 07/96, and an isolate made 05/01. Additionally, these isolates were made from three songbird species in five states.

These results confirm previous observations that during the initial stages of the MG epidemic in songbirds, isolates had essentially identical RAPD banding patterns. This indicated that the outbreak in various songbird species and geographic locations was caused by the same strain of MG, suggesting a single point-source of origin (6).

However, analysis of more contemporary MG isolates made in 2001 shows four banding patterns that differ from the dominant RAPD-genotype. This finding is in agreement with the recent work of others (8) demonstrating apparent genotypic differences among MG isolates from songbirds. It is interesting to note that all four of the unique MG RAPD-genotypes were isolated from house finches, and three of four were recovered from New York.

These differences could arise from 'molecular evolution', changes occurring in the genome of the original/dominant MG strain, or they could be evidence of new introductions to house finches from some 'external' source(s). More extensive analyses of historical and contemporary isolates of MG from house finches and other songbirds, perhaps with improved genotyping techniques such as AFLP, may help answer this and other questions about the epidemiology of MG conjunctivitis in songbirds.

ACKNOWLEDGEMENTS

This research was supported in part by the State of North Carolina, National Science Foundation Grant No. DEB-0094456 to André Dhondt, and an Emory University Research Committee grant to S. Altizer.

REFERENCES

- Fan, H. H., et al. Studies of intraspecific heterogeneity of *Mycoplasma synoviae*, *M. meleagridis*, and *M. iowae* with arbitrarily primed polymerase chain reaction. Avian Dis 39: 766-77. 1995.
- Fischer, J. R., et al. Mycoplasma conjunctivitis in wild songbirds: the spread of a new contagious disease in a mobile host population. Emerg Infect Dis 3:69-72. 1997.
- Geary, S. J., et al. *Mycoplasma gallisepticum* strain differentiation by arbitrary primer PCR (RAPD) fingerprinting. Molecular & Cellular Probes 8:311-6. 1994.
- Kokotovic, B., et al. Amplified-fragment length polymorphism fingerprinting of *Mycoplasma* species. J Clin Microbiol 37:3300-07. 1999.
- Ley, D. H., et al. *Mycoplasma gallisepticum* isolated from house finches (*Carpodacus mexicanus*) with conjunctivitis. Avian Dis 40:480-3. 1996.
- Ley, D. H., et al. Molecular epidemiologic investigations of *Mycoplasma gallisepticum* conjunctivitis in songbirds by random amplified polymorphic DNA analyses. Emerg Infect Dis 3:375-80. 1997.
- Mikaelian, I. D., et al. Mycoplasmosis in evening and pine grosbeaks with conjunctivitis in Quebec. J Wildlife Dis 37:826-30. 2001.
- Pillai, S. R., et al. Molecular variability of house finch *Mycoplasma gallisepticum* isolates as revealed by sequencing and restriction fragment length polymorphism analysis of the *pvpA* gene. Avian Dis 47:640-8. 2003.