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Short communication. Strain heterogeneity in *Mycoplasma pullorum* isolates identified by random amplified polymorphic DNA techniques

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Abstract

Mycoplasmas were isolated from chickens with respiratory problems during field investigations of a concentrated respiratory disease outbreak in western Cuba, 1997. A high percentage of mycoplasma cultures from tracheas and air-sac lesions yielded pure cultures of *Mycoplasma pullorum*. The aim of the present work was to investigate the heterogeneity among *M. pullorum* isolates from Cuba and strains from other countries using random amplified polymorphic DNA (RAPD) techniques**.** The results show that the RAPD method may be a useful identification tool for studying the epidemiology of poultry mycoplasmosis in Cuba.

Key words: avian mycoplasmosis, RAPD, chronic respiratory syndrome.

Resumen

Nota corta. Análisis de heterogeneidad entre aislados de *Mycoplasma pullorum* por medio de técnicas de amplificación de DNA polimórfico

En 1997 se aislaron micoplasmas en el curso de investigaciones de campo en pollos con un brote de enfermedad respiratoria localizada en la zona oeste de Cuba. Un alto porcentaje de los cultivos de micoplasmas procedentes de lesiones de tráquea y alveolos revelaron la presencia de *Mycoplasma pullorum.* El objetivo de este trabajo fue investigar la heterogeneidad de los aislados cubanos entre sí y con cepas de otros países, mediante la técnica RAPD (DNA polimórfico amplificado al azar). Los resultados muestran que la técnica RAPD puede ser una herramienta de identificación útil en el estudio de la epidemiología de la micoplasmosis aviar en Cuba.

Palabras clave: micoplasmosis aviar, RAPD, síndrome respiratorio crónico.

Mycoplasmas are of great concern in veterinary medicine since they are responsible for economic losses in animal production. The most important avian mycoplasmas that cause severe respiratory disease in chickens and turkeys are *Mycoplasma gallisepticum* and *M. synoviae. M. iowae* and *M. meleagrides* affect turkeys alone (Fan *et al*., 1995).

Chronic respiratory syndrome (CRS) in birds is a disease with a multifactorial aetiology. *M. gallisepticum* and *M. synoviae* are the most important pathogens in CRS, although Citti and Rosengarten (1997) indicate that other species may also participate in the disease.

In Cuba, CRS causes losses of some US$1 million per year. Recent data (Instituto Medicina Veterinaria, 2003) show that in the chicken-raising industry CRS is the second most important cause of death induced by an infectious agent, with an incidence of 12.67%.

In 1997, during field investigations of an outbreak of CRS in western Cuba, mycoplasmas were isolated from chickens with respiratory problems. Lobo (1998) showed the incidence of *M. gallisepticum, M. synoviae* and *M. pullorum* in these diseased birds to be 40%, 30% and 22% respectively.

For a long time *M. pullorum* was thought to be a saprophytic organism of the avian respiratory tract (Jordan, 1985), but since 1995 it has been isolated alone and with other mycoplasma species from birds with CRS (Bencina *et al.,* 1987). Two isolates with the biochemical and serological characteristics of *M. pullorum* have been isolated from adult turkeys and dead turkey embryos in France (Moalic *et al*., 1997).

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Mycoplasma species show genotypic variability induced by rearrangements and deletions of genetic material, as well as insertions of new elements into the genome (Geary *et al*., 1994). Compared to SDS-PAGE and RFLP analysis, RAPD detects more polymorphism and is technically simpler and faster (Bashiruddin, 1998); the technique has been successfully used to identify isolates of closely related organisms. A number of studies have indicated that RAPD is useful in genotype identification, population studies, phylogenetic studies and genetic mapping (Stakenborg *et al*., 2002). RAPD is an easier, more reliable, and faster tool for strain identification than RFPL (Shreekumar *et al*., 2002).

Due to the current scarcity of knowledge regarding these species, the genotypes of Cuban *M. pullorum* isolates were characterised by RAPD analysis and compared to those of strains from other countries.

Table 1 shows the mycoplasma isolates and reference strains studied. All the field isolates were previously identified by direct immunofluorescence using the method of Talkington and Kleven (1983), and grown in Frey's medium (Frey *et al.,* 1968) with 20% horse serum, 1.5% glucose supplemented with cysteine hydrochloride, thallium acetate, phenol red and penicillin (Frey *et al.*, 1968).

A volume of 100 µl of each mycoplasma culture was mixed with 500 µl of DNAzol reagent (Gibco) and 500 µl of 100% ethanol, and centrifuged in a microcentrifuge at 13,000 rpm for 8 min at room temperature. The cell pellets were suspended in 75% ethanol and centrifuged as above. Finally, the cell pellets were suspended in 35-50 µl of sterile water and stored at -20°C.

Three oligonucleotide primers (M16SPCR5', M13F and S1OLIGO3') described by Fan *et al*. (1995) were used for RAPD-PCR analyses. These primers were synthesized at the University of Georgia (Molecular Genetics Facility, Athens GA, USA).

Amplification reactions were prepared in a volume of 50 µl containing 5 µl of 10x PCR buffer (Promega), 6 µl of 25 mM $MgCl_2$ (Promega), 1 µl of 10 mM nucleotide mix (Pharmacia), 1 µl of 50 M concentrations of each of the three primers, 1 µl (5 units) of *Taq* DNA polymerase (Promega) and 5 µl of chromosomal DNA (100-1000 ng). The amplification conditions for all isolates were one cycle of 94ºC for 5 min, 28ºC for 2 min and 74ºC for 3 min followed by 3 cycles of 94ºC for 0.15 s, 28ºC for 2 min and 74ºC for 3 min. These were followed by 35 cycles of 94ºC for 0.15 s, 45ºC for 2 min and 74ºC for 3 min. All amplifications were performed in a PCR Express Thermocycler (ThermoHybaid).

Table 1. Mycoplasma isolates used in this work. All were isolated from chicken tracheas, except 6/85, HF51 and Ts-11, the sources of which were unknown

Species	Strain/isolate	Isolated by/reference	Properties
M. gallinaceum	S-594-TT	Unknown	Reference strain
M. gallinarum	26	Unknown	Reference strain
M. gallisepticum	F	R. Yamamoto	Vaccine strain
	R	D.J. Ritchie	Virulent strain
	6/85	Unknown	Virulent strain
	HF51	Univ Georgia (USA) strain collection	Field isolate
	Ts-11	K. Whithear. Univ Georgia (USA) strain collection	Field isolate
	A5969	H. Van Roekel	Reference strain
M. imitans	K4589	Unknown	Reference strain
M. pullorum	R63	Unknown	Reference strain, Germany
	10	Unknown. Univ Georgia (USA) strain collection	Field isolate
	MP17	This work. 1997	Field isolate. Caimito, Havana (west Cuba)
	MP24	This work. 2000	Field isolate. Artemisa, Havana (west Cuba)
	MP55	This work. 1998	Field isolate. Pinar del Río (west Cuba)
	MP137	This work. 2000	Field isolate. Guantánamo (east Cuba)
M. synoviae	F10-2AS	H.W. Yoder	Unknown

Figure 1 shows the heterogeneity among the Cuban *M. pullorum* isolates and the rest of the strains. Identical patterns were found in MP24 (lane 13) and MP17 (lane 15), isolated from two different regions of Havana in 2000 and 1997 respectively. Isolates MP R63 from Germany (lane 10), MP 10 from the USA (lane 12) and the Cuban MP 55 (lane 14) had different patterns.

Figure 2 shows the RAPD patterns generated for six *M. pullorum* isolates. Identical patterns were found for MP24 and MP17 (lines 5 and 6) from Havana (western Cuba). Isolate MP137 from Guantánamo (eastern Cuba, lane 4) had a different pattern compared to MP24, MP17 and MP55 (lane 3), this latter from Pinar del Río (western Cuba). Thus, three distinctive patterns was detected in the Cuban isolates.

After subjecting the RAPD products to electrophoresis, similarity coefficients (*F*) between pairs of isolates or strains of a species were calculated as described by Fan *et al*. (1995) using the equation:

$$F = (n_{ny} + n_{yx}) / (n_x + n_y) \times 100$$

where n_x and n_y are the number of major fragments in isolates X and Y respectively, n_{ny} is the number of major fragments in isolate X that match any fragments in isolate Y, and n_{yx} is the number of major fragments in isolate Y that match any fragments in isolate X.

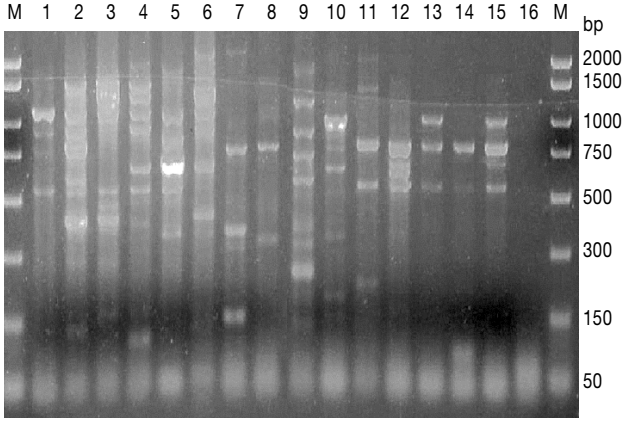

Figure 1. RAPD results of fifteen avian mycoplasma isolates and strains analysed with three arbitrary primers. Lanes: M, molecular weight marker 1kb (Sigma). Lanes 1-6, *M. gallisepticum*-isolates F (lane 1), R (2), 6/85 (3), HF51 (4), Ts-11 (5), A5969 (6); 7, *M. gallinaceum* S-594-TT; 8, *M. gallinarum* 26; 9, *M. imitans* K4589; 10, *M. synoviae* F10-2AS; 11, *M. pullorum* R63; 12, *M. pullorum* 10; Lanes 13-15, Cuban isolates of *M. pullorum*-MP24 (13), MP55 (14); MP17 (15); 16, negative control mixture with no DNA. Amplified products were analysed on 2% (w v^{-1}) agarose (Amresco, OH, USA) gels in TAE buffer (40 mM Tris, 2 mM EDTA, pH 8.0) containing 0.5 µg ml^{-1} ethidium bromide.

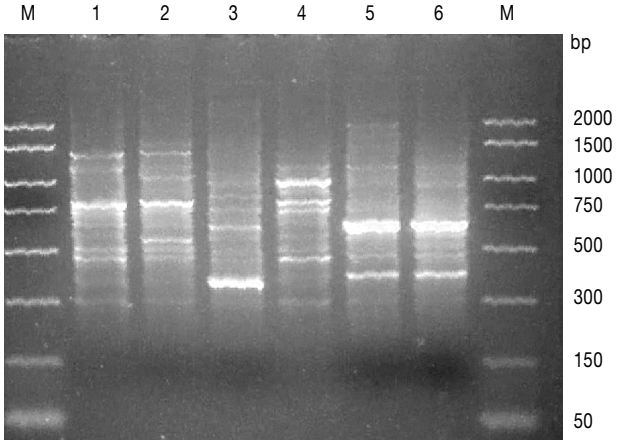

Figure 2. Six *Mycoplasma pullorum* isolates analysed with three arbitrary primers (M16SPCR5', M13F and S1OLIGO3'). Lanes: M, molecular weight marker 1kb (Sigma); 1, R63 (reference strain); 2, 10 (USA strain); 3, MP55; 4, MP137; 5, MP24; 6, MP17. Amplified products were analysed on 2% (w v^{-1}) agarose (Amresco, OH, USA) gels in TAE buffer (40 mM Tris, 2 mM EDTA, pH 8.0) containing 0.5 µg ml^{-1} ethidium bromide.

Table 2 shows the *F* values for *M. pullorum* pairs. When MP24 and MP17 (a pair of isolates from Havana) were compared, the *F* value was 100%. When MP24 was compared with the rest of the *M. pullorum* strains, *F* values of between 50% and 30% were found.

The diversity of the banding patterns of the isolates reflects the genetic diversity in the natural populations. Heldtander *et al*. (2002) showed that RAPD allows avian mycoplasma isolates to be distinguished with much more sensitivity than RFLP analysis. The simple and rapid DNA preparation associated with the technique should allow cost effective analyses of numerous field isolates, providing significant new

Table 2. Similarity coefficients of the combined data for *M. pullorum* isolates

	R63	MP10	MP55	MP137	MP24	MP17
R63	100%	67%	67%	67%	50%	50%
MP10		100%	40%	30%	40%	40%
MP55			100%	50%	50%	50%
MP137				100%	30%	30%
MP24					100%	100%
MP17						100%

insights into the epidemiology and infection mechanisms of avian mycoplasmas.

RAPD easily differentiated the *M. pullorum* isolates collected from different parts of Cuba in different years. The results show that Cuban *M. pullorum* isolates belong to different genotypes, which can be distinguished by the characteristic banding patterns of their amplified DNAs on agarose gels (Fig. 2).

Our results agree with those of Fan *et al*. (1995) and Minion (2002) who demonstrated the advantages of RAPD in the identification and analysis of genome diversity.

These results suggested that the sources of the outbreaks from Havana, Pinar del Río and Guantánamo were unrelated. The heterogeneity among *M. pullorum* isolates might be explained in that mycoplasmas have higher mutation rates than bacteria, as shown in the higher genotypic and phenotypic diversities of the organisms belonging to the class *Mollicutes* (Stakenborg, 2002; Mettifogo *et al.,* 2002). Kleven and Levisohn (1996) indicate that the confirmation of mycoplasmas in one individual predicts that all animals will be infected (an observation frequently made in the studied regions), suggesting that the incidence of *M. pullorum* in Cuba could be high.

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