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AN EPIZOOTIOLOGICAL STUDY OF AVIAN MYCOPLASMAS IN SOUTHERN SPAIN

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SUMMARY

An epizootiological study was carried out by investigation of 562 birds for mycoplasmas. The birds belonged to 18 different domestic and wild avian species of the following orders: Galliformes, Anseriformes, Gruiformes, Passeriformes, Columbiformes, Falconiformes, Psittasiformes and Trogoniformes. Eighty nine (15%) of the trachea and oropharynx samples examined were mycoplasma positive and 108 mycoplasma isolates were obtained and identified by the growth inhibition test, using rabbit antisera against fifteen avian mycoplasma species.

The species most frequently detected were *Mycoplasma gallinarum* (27.7%), *M. gallinaceum* (17.5%), *M. iners* (14.8%), *M. pullorum* (7.4%), *M. columbinum* (7.4%), *M. anatis* (6.4%), *M. synoviae* (4.6%) and *M. gallisepticum* (3.7%). Four strains isolated from griffon vultures (*Gyps fulvus*) could not be identified with antisera against the 15 described avian mycoplasma species.

INTRODUCTION

Avian mycoplasmas have been frequently isolated from domestic poultry, including chickens, turkeys, ducks and pigeons. Shimizu *et al.* (1979) have reported a high incidence of *M. gallisepticum*, *M. gallinarum*, *M. iners*, *M. pullorum* and *M. gallinaceum* in domestic birds. Jordan (1983) has described *M. gallisepticum*, *M. gallinarum*, *M. iowae* and *M. synoviae* as the species most frequently isolated from fowl tissues. More recently, Bencina *et al.* (1987a,b) have reported *M. gallisepticum*, *M. gallinarum*, *M. pullorum*, *M. gallinaceum* and *M. iners* as the most frequently isolated species on a chicken farm. Few data are available, however, regarding wildfowl, largely because of sampling difficulties.

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The purpose of this paper was to establish the distribution of *Mycoplasma* species in poultry and wildfowl in southern Spain, and to characterise the isolated mycoplasmas according to the criteria recommended by the Subcommittee on the Taxonomy of Mollicutes (1979).

MATERIALS AND METHODS

Birds

A total of 562 birds belonging to 18 species of eight orders were investigated. From all birds, samples of trachea and oropharynx were obtained using cotton swabs. Sampling was carried out on 22 industrial chicken farms and eight turkey farms in southern Spain. Samples from wild Anseriformes and Gruiformes were taken from birds killed during hunts on the marshes of the River Guadalquivir. House sparrows were caught in towns, and sometimes on chicken farms around Córdoba. Other domestic and wild birds were referred to the Faculty of Veterinary Sciences Diagnostic Service in Córdoba between 1984 and 1987. The former came from several factories. Birds investigated are listed in Table 1.

Media for mycoplasmas

The base medium used contained: PPLO broth base (Difco), 21g; yeast extract (Difco), 10g; DNA (Sigma), 0.20g; Eagles vitamin solution 100X (Difco), 5 ml; thallium acetate (10%), 2.5 ml; β -nicotinamide adenine dinucleotide, (Sigma) 0.1 g; L-cysteine hydrochloride (Sigma), 0.1 g; penicillin G, 1,000,000 iu; swine serum, 150 ml and double-distilled water, 840 ml. For the solid medium, PPLO broth was replaced by PPLO agar (Difco) 34 g/l.

Mycoplasma isolation

Cotton swabs were inoculated into 2 ml of liquid medium. After 5 days incubation at 37°C, they were streaked on to solid media which were incubated for seven days at 37°C in a moist atmosphere. Single colonies were picked from the plates and inoculated into 2 ml of liquid medium. After an incubation of 5 to 7 days fluid cultures were filtered using Millipore filters of 0.45 μ m pore diameter and cloned three times. A total of 108 mycoplasma strains were obtained and stored until identification according to the method proposed by Leach (1983), agar blocks with young colonies being frozen at -40°C.

Preparation of antigens and antisera

The following reference strains of the avian *Mycoplasma* species were used: *M. anatis* (1340), *M. columbinasale* (694), *M. columbinum* (MMP-1), *M. columborale* (MMP-4), *M. gallinaceum* (DD), *M. gallinarum* (PG16), *M. gallisepticum* (PG31), *M. gallopavonis* (WR1), *M. pullorum* (CKK), *M. iners* (PG30), *M. meleagridis* (17529), *M. iowae* (695), *M. synoviae* (WVU 1853), *Acholeplasma laidlawii* (PG8) and *A. axanthum* (S743). Reference strains were provided by Professor Dr E.A. Freundt, Institute of Medical Microbiology, Denmark.

These strains were grown in a medium similar to that described above, but without thallium acetate, and with additional supplements: 1% glucose for those mycoplasmas fermenting glucose, and 0.2% arginine for the arginine-hydrolysing mycoplasmas. Phenol red (0.06%) was used as an indicator in both cases.

For the production of antisera, rabbits were immunised with each of the reference strains of the avian mycoplasma species (two rabbits per strain), using a modification of the method proposed by Senterfit (1983). Antigen was homogenised with incomplete Freund's adjuvant. The specificity of the antisera was assessed by the growth inhibition (GI) test (Clyde, 1964).

Biochemical and serological tests

The biochemical characteristics of the isolates were analysed using methods described by Aluotto *et al.* (1970). The following properties were investigated: growth in mycoplasma broth and agar medium without serum, sensitivity to digitonin, hydrolysis of urea, fermentation of glucose, hydrolysis of arginine, phosphatase activity, film and spots production, tellurite and tetrazolium reduction in aerobic and anaerobic conditions. Mycoplasma isolates were identified by the growth-inhibition (GI) test using filter-paper discs impregnated with the antisera.

RESULTS

Specificity of antisera

The antisera obtained against the reference strains were highly specific, reacting only with the colonies of the homologous species. Potency was measured by the GI test, the width of the inhibition zone ranged from 3 to 7 mm; *M. anatis* (4 mm), *M. columbinasale* (4 mm), *M. columbinum* (7 mm), *M. columborale* (6 mm), *M. gallinaceum* (5 mm), *M. gallinarum* (4 mm), *M. gallisepticum* (3 mm), *M. gallopavonis* (5 mm), *M. pullorum* (7 mm), *M. iners* (5 mm), *M. meleagridis* (3 mm), *M. iowae* (3 mm), *M. synoviae* (3 mm), *A. laidlawii* (3 mm), *M. axanthum* (3.5 mm).

Isolation and identification of mycoplasmas

Mycoplasmas were isolated from 89 of the 562 samples investigated. Most of the birds tested were battery hens originating from 22 farms in the provinces of Córdoba, Seville, Cadiz and Málaga. Forty-five (15%) of the 300 samples from these hens were positive. The mycoplasma species most frequently encountered were: *M. gallinarum* (42.1%) *M. gallinaceum* (21.8%), *M. iners* (20.3%) and *M. pullorum* (10.9%).

From the other birds investigated the following species yielded mycoplasmas: red-legged partridge, golden pheasant, turkey, Japanese quail, shoveler, coot, house sparrow, pigeon, peregrine falcon, griffon vulture and hoopoe. Table 1 shows the distribution of mycoplasmas isolated in each host species.

DISCUSSION

From the battery hens of the 22 farms, seven different species of avian mycoplasma were isolated. The most frequently encountered were *M. gallinarum*, *M. gallinaceum*, *M. iners* and *M. pullorum*, whilst *M. gallisepticum* and *M. synoviae* - which are considered to be pathogenic - showed a very low incidence. These results differ from those reported by other authors (Shimizu *et al.*, 1979; Jordan, 1983; Bencina *et al.*, 1987a,b), perhaps because samples in this study were taken from battery hens without clinical symptoms.

M. synoviae was isolated from a red-legged partridge 7 months after an outbreak of respiratory disease during which *M. synoviae* had already been isolated (Poveda *et al.*, 1986). *M. gallinaceum* and *M. iners* were isolated from the golden pheasant, and

Table 1. Birds examined and incidence of mycoplasmas

Birds	Source	No. birds positive/ no. tested	No of strains isolated	<i>M. gallisepticum</i>	<i>M. gallinaceum</i>	<i>M. gallinarum</i>	<i>M. synoviae</i>	<i>M. iners</i>	<i>M. columbinum</i>	<i>M. anatis</i>	<i>M. gallopavonis</i>	<i>M. iowae</i>	<i>M. meleagridis</i>	<i>M. pullorum</i>	<i>M. columbinasale</i>	<i>M. columborale</i>	Not identified
Galliformes																	
Chicken (<i>Gallus domesticus</i>)	Farm	45/300	60	1	12	25	1	13	-	-	-	1	-	7	-	-	-
	Factory	4/7	4	-	2	2	-	-	-	-	-	-	-	-	-	-	-
Red-legged partridge (<i>Alectoris rufa</i>)	Factory	1/15	1	-	-	-	1	-	-	-	-	-	-	-	-	-	-
Golden pheasant (<i>Chrysolophus pictus</i>)	Factory	2/16	2	-	1	-	-	1	-	-	-	-	-	-	-	-	-
	Farm	3/90	3	-	-	2	-	-	-	-	-	-	1	-	-	-	-
Turkey (<i>Meleagris gallopavo</i>)	Factory	2/5	2	-	-	-	-	-	-	2	-	-	-	-	-	-	-
Japanese quail (<i>Conturnix conturnix</i>)	Farm	1/1	1	1	-	-	-	-	-	-	-	-	-	-	-	-	-
Anseriformes																	
Mallard (<i>Anas platyrhynchos</i>)	Wild	0/10															
Shoveler (<i>Anas clypeata</i>)	Wild	3/10	3	-	-	-	-	-	-	3	-	-	-	-	-	-	-
Pochard (<i>Aythya ferina</i>)	Wild	0/4															
Teal (<i>Anas crecca</i>)	Wild	0/8															
Gruiformes																	
Coot (<i>Fulica atra</i>)	Wild	4/10	4	-	-	-	-	-	-	4	-	-	-	-	-	-	-
Passeriformes																	
House sparrow (<i>Passer domesticus</i>)	Wild	3/40	3	-	-	-	3	-	-	-	-	-	-	-	-	-	-
Canary (<i>Serinus canarinus</i>)	Shop	0/15															
Columbiformes																	
Pigeon (<i>Columba livia</i>)	Factory	15/32	15	-	2	-	-	1	8	-	-	-	-	1	1	2	-
Falconiformes																	
Peregrine Falcon (<i>Falco peregrinus</i>)	Wild	2/2	5	2	1	1	-	1	-	-	-	-	-	-	-	-	-
Griffon vulture (<i>Gyps fulvus</i>)	Wild	2/2	4	-	-	-	-	-	-	-	-	-	-	-	-	-	4
Psittaciformes																	
Budgerigar (<i>Melopsittacus</i> sp.)	Shop	0/4															
Trogoniformes																	
Hoopoe (<i>Upupa epops</i>)	Wild	1/1	1	-	1	-	-	-	-	-	-	-	-	-	-	-	-
Total			108	4	19	30	5	16	8	7	2	1	1	8	1	2	4

M. gallinarum, *M. gallopavonis* and *M. meleagridis* from turkeys. *M. gallisepticum* was isolated from one commercially-reared Japanese quail suffering from acute respiratory disease.

This is the first report describing the isolation of *M. anatis* from the shoveler and coot. *M. anatis* has however been isolated from other wild Anseriformes including the teal (Amin, 1977). Originally it was reported in domestic ducks (Roberts, 1964), and Peking ducks (Karpas and Fabricant, 1969).

M. synoviae was isolated from three house sparrows captured on commercial poultry farms with natural ventilation. Jain *et al.* (1977) found *M. gallisepticum* in the house sparrow, and Shimizu *et al.* (1979) isolated *M. synoviae* from a different sparrow species (*Passer montanus*), a finding which led them to suspect the epidemiological importance of sparrows. Experiments carried out by Kleven and Fletcher (1983) showed that the house sparrow was highly resistant to *M. synoviae* infection.

M. gallisepticum, *M. gallinarum*, *M. gallinaceum* and *M. iners* were isolated from two peregrine falcons (*Falco peregrinus*) at a recovery centre for wild birds. Both birds had respiratory disorders and infraorbital sinusitis. Furr *et al.* (1977) isolated three mycoplasma strains from falcons in captivity. (*Falco cherrug* and *Falco peregrinus*), but performed only a preliminary biochemical and serological identification. The mycoplasma species found in the falcons and others birds of prey in the same centre in the present study may have originated from carcasses of factory chickens, used for feeding the birds.

Four mycoplasma strains were isolated from wild griffon vultures (*Gyps fulvus*). Based on the sensitivity to digitonin, the filterability, the absence of growth in a solid medium without serum, and the inability to hydrolyse urea they were tentatively assigned to the genus *Mycoplasma*. These strains did not ferment glucose, but hydrolysed arginine, reduced potassium tellurite and triphenyl-tetrazolium chloride, produced films and spots and showed phosphatase activity. None of the four isolates reacted with any of the antisera against the reference strains, though antisera to four of the recognised avian mycoplasma species (*M. cloacale*, *M. glycophilum*, *M. lipofaciens* and *M. anseris*) were not used.

M. gallinaceum was isolated from the tracheal exudate of a hoopoe (*Upupa epops*) which showed nervous signs and high titres to Paramyxovirus 1 in the haemagglutination inhibition test.

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RESUME

Etude épidémiologique de la mycoplasmose aviaire dans le Sud de l'Espagne

Une étude épidémiologique a été réalisée sur 562 oiseaux afin de rechercher la présence de mycoplasmes. Ces oiseaux appartenant à 18 espèces domestiques ou sauvages appartenant aux ordres suivants: Galliformes, Anseriformes, Gruiformes, Passeriformes, Columbiformes, Falconiformes, Psittaciformes et Trogoniformes. Quatre vingt neuf échantillons de trachées et d'oropharynx se sont révélés positifs en mycoplasmes (15%) et 108 mycoplasmes ont été isolés et identifiés par les tests d'inhibition de croissance utilisant un antiserum de lapins vis-à-vis de 15 espèces aviaires. Les espèces les plus fréquemment rencontrées ont été *Mycoplasma gallinarum* (27,7%), *M. gallinaceum* (17,5%), *M. iners* (14,8%), *M. pullorum* (7,4%), *M. columbinum* (7,4%), *M. anatis* (6,4%), *M. synoviae* (4,6%) et *M. gallisepticum* (3,7%).

ZUSAMMENFASSUNG

Epizootologische Untersuchung von aviären Mykoplasmen in Südsanien

Im Rahmen einer epizootologischen Untersuchung wurden 562 Vögel auf Mykoplasmen untersucht. Die Vögel gehörten zu 18 verschiedenen Haus- und Wildvogelarten folgender Ordnungen: Galliformes, Anseriformes, Gruiformes, Passeriformes, Columbiformes, Falconiformes, Psittaciformes und Trogoniformes. Neunundachtzig (15%) der untersuchten Trachea- und Oropharynxproben waren mykoplasmapositiv. 108 Mykoplasmaisolatate wurden gewonnen und durch Wachstumshemmtest unter Verwendung von Kaninchen-antisera gegen fünfzehn aviäre Mykoplasmaspecies identifiziert.

Die am häufigsten gefundenen Species waren *Mycoplasma gallinarum* (27%), *M. gallinaceum* (17,5%), *M. iners* (14,8%), *M. pullorum* (7,4%), *M. columbinum* (7,4%), *M. anatis* (6,4%), *M. synoviae* (4,6%) und *M. gallisepticum* (3,7%).

Vier Stämme aus Gänsegeiern (*Gyps fulvus*) konnten mit den Antisera gegen 15 beschriebene aviäre Mykoplasmaspecies nicht identifiziert werden.

RESUMEN

**Estudio epizootiológico de los micoplasmas
aviares en el sur de España**

Se llevó a cabo un estudio epizootiológico de micoplasmas sobre una población de 562 aves. Los pájaros pertenecían a 18 especies diferentes de aves domésticas y salvajes de los órdenes siguientes: Galliformes, Anseriformes, Gruiformes, Passeriformes, Columbiformes, Falconiformes, Psittasiformes y Trogoniformes. Un total de 89 (15%) de las muestras de tráquea y orofaringe examinadas fueron positivas a micoplasma, aislándose 108 micoplasmas e identificándolos por la prueba de la inhibición del crecimiento empleando antisueros de conejo frente a 15 especies diferentes de micoplasmas aviares.

Las especies detectadas más frecuentemente fueron *Mycoplasma gallinarum* (27.7%), *M. gallinaceum* (17.5%), *M. iners* (14.8%), *M. pullorum* (7.4%), *M. Columbinum* (7.4%), *M. anatis* (6.4%), *M. synoviae* (4.6%) y *M. gallisepticum* (3.7%).