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Isolation and Characterization of Unusual *Mycoplasma* spp. from Captive Eurasian Griffon (*Gyps fulvus*) in Sicily

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ABSTRACT: Mycoplasmas have been isolated from birds of prey during clinical examinations, but their significance to the health of raptors is unclear. We report the isolation and characterization of four mycoplasmas found in the upper respiratory tract of four sick Eurasian Griffon (*Gyps fulvus*) that were housed in a Sicilian rehabilitation center at Ficuzza, near Palermo in Sicily, before reintroduction into the wild. These included *Mycoplasma gallinarum*, an unidentified mycoplasma highly similar to *Mycoplasma glycophilum*, and two unidentified mycoplasmas with similarities to *Mycoplasma falconis and Mycoplasma gateae*.

Key words: Griffon, mycoplasma, PCR/DGGE, vultures, 16S rDNA sequencing.

The Eurasian Griffon (*Gyps fulvus*) is the largest vulture in the countries surrounding the Mediterranean, including Spain, Croatia, Albania, Turkey, and Italy. Although numbers of griffons have increased in Spain over the last few years, with 22,445 breeding pairs reported in 1999 (Parra and Telleria, 2004), their numbers across the rest of southern Europe have declined significantly (Sarrazin et al., 1994), and the species is considered threatened. This problem is exacerbated by a low breeding rate, both under natural and captive conditions; griffons enter the breeding population at the age of 4–5 yr and lay only one egg per nesting season. In Italy, the griffon is present in very limited numbers and is restricted to a small colony on Sardinia; reintroduction programs have been started in Italian national parks in Abruzzo, Sicily, and Udine using vultures translocated from Spain.

There are few reports on the causes of death in vultures, but poisoning (Shlosberg et al., 1994; Oaks et al., 2004a) and diseases (Merino et al., 2002; Oaks et al., 2004a) have been described. Vultures have a peculiar ecology in that they are part of an efficient and natural system to recycle organic material in the environment, and they can feed on and eliminate infected carcasses even in presence of pathogens like *Bacillus anthracis* or *Brucella melitensis* (Lobo Vilela et al., 1997).

The potential role of mycoplasmas as pathogens of birds of prey is unclear, but several have been isolated from clinical cases, including *Mycoplasma gallinarum*, *Mycoplasma gypis*, *Mycoplasma vulturii*, *Entoplasma* sp., *Mesoplasma* sp., and *Acholeplasma* sp. (Bölske and Mörner, 1982; Poveda et al., 1990a, b, 1994; Lierz et al., 2000; Oaks et al., 2004b). Captivity can further increase potential transmission of opportunistic pathogens, such as mycoplasmas, that can be present in healthy hosts; this can result in the emergence of new diseases or new pathologies. Although mycoplasmas are not particularly infectious, close contact among birds in breeding farms can facilitate horizontal transmission (Gerlach, 1994). We report the isolation and characterization of four mycoplasmas found in the upper respiratory tract of four sick Eurasian Griffons that were housed in a rehabilitation center in Ficuzza, near Palermo, Sicily, prior to their reintroduced into the wild in 2005.

All of the griffons originated from Spain as part of a project to reintroduce them into Italy. They were kept for 12 wk at the Lega Italiana Protezione Uccelli (LIPU) rehabilitation center in the Palermo dis-

trict of Sicily and were fed whole carcasses of chicken and lamb meat. Three days before sampling, the birds were lethargic and refused to feed. One week later, all the birds showed anorexia, poor body condition, and appeared pale around the mucous membrane of the eyes. The day after sampling, one bird was found on the ground of the cage in a lethargic state. Daily treatments with 100 mg/kg of Fenbendazolo (Panacur®) per os via the oral route were initiated, but the therapy was unsuccessful, and the bird died two days later.

Tracheal swabs were collected using standard methods (Nicholas and Baker, 1998; Lierz et al., 2000); swabs were placed in a tube with prewarmed mycoplasma broth medium (Friis, 1975). After 48 hr incubation in broth cultures at 37 C in an atmosphere containing 5% CO_2, 25 µl of broth was subcultured on solid media and monitored every 24 hr for up to 7 days for evidence of typical "fried-egg" colonies (Nicholas and Baker, 1998).

Swab samples were also inoculated on 5% sheep blood agar (Merck, Darmstadt, Germany) and Tryptic Soy Agar (DIFCO Laboratories, Detroit, Michigan, USA). These were incubated at 37 C under both aerobic and anaerobic conditions to detect other bacterial infections. Swabs also were tested for viruses by completing three passages in chicken embryo fibroblasts cell cultures as described in Docherty and Slota (1988).

Culturing from the tracheal swabs from four birds showed the presence of typical mycoplasma colonies after 3–4 days on solid media. Heavy growth was observed in broth after 2 days, and typical "fried egg" colonies were visible in agar media 3 days after the broth was inoculated. There was no significant growth of other bacteria on blood agar or Tryptic Soy Agar, and no cytopathic effects were observed in cell cultures.

Positive cultures were tested using denaturing gradient gel electrophoresis (DGGE; McAuliffe et al., 2005), which was performed using the Ingeny phorU 2×2 apparatus (Ingeny, Goes, The Netherlands). Samples (20 µl) were loaded onto 10% polyacrylamide/bis (37-5:1) gels with denaturing gradients from 30% to 60% (where 100% is 7 M urea and 40% v/v deionized formamide) in 1X TAE electrophoresis buffer (Severn Biotech, Kidderminster, UK). Electrophoresis was performed at 100 V at a temperature of 60 C for 18 hr. Gels were then stained with SBYR Gold (Cambridge BioScience, Cambridge, UK) in 1X TAE for 30 min at room temperature and visualized under ultraviolet (UV) illumination. Based on DGGE results, one isolate was identified as *Mycoplasma gallinarum*. For the remaining three mycoplasma isolates, the bands observed in the DGGE did not match any of the known mycoplasma species.

The three unidentified isolates were subjected to 16S rDNA sequencing. *Mycoplasma* DNA was extracted from a 1 ml aliquot of stationary-phase culture using the Genelute genomic DNA kit according to the manufacturer's instructions (Sigma, Poole, UK). The DNA was extracted from swabs by swirling the swab in 1 ml of phosphate buffered saline, removing the swab, and then using the Genelute kit (Sigma). Amplification of the V3 region of the 16S rDNA gene was performed according to the method of Muyzer et al. (1993) with minor modifications using the universal bacterial primer GC- 34I F: 5′CGCCCGCCGCGCGCGGCGGGCG-GGGCGGGCCCACGGGGCGCCTAC-GGGAGGCAGCAG and the mollicute-specific primer R543 (McAuliffe et al., 2005); for the PCR, 1 µl lysate was added as a template to 49 µl of a reaction mixture containing 10 mM Tris/HCI (pH 9.0), 1.5 mM $MgCl_2$, 50 mM KCI, 0–1% Triton X-100, 0.2 mM of each deoxynucleoside triphosphate and 0.5 U Taqgold (Applied Biosystems, Warrington, UK). The cycling conditions were: denaturation at 94 C for 5 min, followed by 30 cycles of 95 C for 1 min, 56 C for

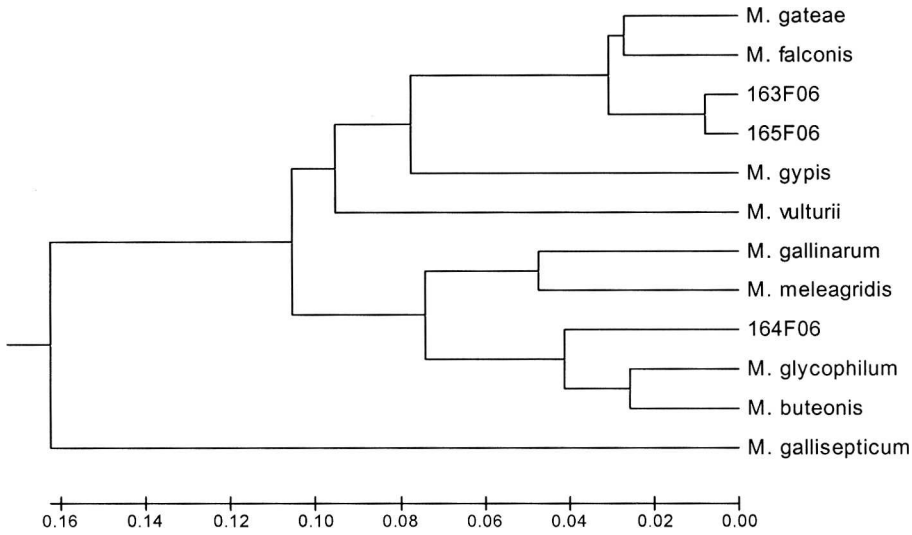

FIGURE 1. Comparison of 16S rDNA sequences of griffon vulture isolates with other known bird of prey mycoplasma species as determined using unweighted pair group mean analysis (UPGMA) analysis.

45 sec and 72 C for 1 min, and a final extension step of 72 C for 10 min, and samples were kept at 4 C until analysis. Aliquots were checked for correct amplification by electrophoresis on a 2% agarose gel followed by visualization with ethidium bromide under UV illumination.

Almost full-length 16S rDNA genes were amplified from all three isolates that were unidentifiable by DGGE, and these were sequenced in both the forward and reverse direction; contigs were assembled using the CAP Contig program in Bioedit (www.mbio.ncsu.edu/BioEdit/bioedit.html). These sequences were aligned with the closest matches found in the Ribosomal Database Project using the ClustalW function of the BioEdit package. Neighbor-joining phylogenetic trees were constructed with the Molecular Evolutionary Genetics Analysis package (MEGA version 2.1; http://www.megasoftware.net/) using unweighted pair group mean analysis (UPGMA), and the robustness of the phylogeny was tested by bootstrap analysis with 500 iterations. Based on sequence analyses, isolate 163F06 was found to be 96% similar to *Mycoplasma gateae* and 95% similar to *Mycoplasma falconis*. Isolate 164F06 was 94% similar to *Mycoplasma glycophilum*, and isolate 165F06 was identical to 163F06 (Fig. 1).

Results from DGGE and 16S rDNA sequencing confirmed the presence of *M. gallinarum*, an additional mycoplasma that was highly similar to *M. glycophilum*, and two identical mycoplasmas that were most closely related to *M. falconis and M. gateae.* The potential impact of *M. gallinarum* on avian health is largely unknown, although it has been associated with suboptimal hatchability of eggs in chickens (Martin de Las Mulas et al., 1990). To our knowledge, the pathogenicity of *M. gallinarum* for vultures is unknown, and, in this case, it is not possible to establish whether it was the cause of mortality; the other three vultures returned to normal health after ten days from the first clinical observation.

Mycoplasma glycophilum was originally isolated from chicken oviducts (Forrest and Bradbury, 1984), and, more recently, it has been associated with infraorbital sinusitis in pheasants in conjunction with *Pasteurella multocida* infection (Chin and Goshgarian, 2001), but it has never previously been identified in birds of prey. This study has also led to the identification of a potentially new mycoplasma species

with similarity to *M. falconis* and *M. gateae*. *Mycoplasma falconis* has previously been isolated from falcons (Poveda et al., 1994), but *M. gateae* is usually found in cats; obviously, further characterization by biochemical and serologic testing would be necessary to determine whether this is indeed a new species.

There are no previous reports of mycoplasma isolations from any griffon species, but earlier studies on black vultures (*Coragyps atratus*) in the USA described the isolation of *Mycoplasma corogypsi* from a footpad abscess (Panangala et al., 1993). Studies of the Indian white-backed vulture (*Gyps bengalensis*) found that an unusual intracellular mycoplasma with the proposed name *Mycoplasma vulturii* was present in pooled lung and spleen samples (Oaks et al., 2004b).

Further investigations including histology, immunohistochemistry, and biochemistry are needed to confirm pathogenicity in these mycoplasma species; nevertheless, the identification of *M. gallinarum, M. glycophilum*, and a new species with similarity to *M. falconis* from the dead and sick Eurasian Griffons suggests a potential role for these mycoplasmas as pathogens for griffons and other species of large vultures.

Due to the difficulties in isolating mycoplasmas from field samples, DGGE followed by 16S rDNA sequencing represented a very suitable tool, not only to identify the mycoplasma species involved, but also to confirm the pathogen directly in clinical material. This test is also capable of detecting mixed infections of mycoplasmas, which is a common occurrence in birds. In this study, DGGE confirmed its high efficiency in genetic identification of unknown mycoplasma strains.

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