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Identification of a Novel Mycoplasma Species from an Oriental White-Backed Vulture (*Gyps bengalensis*)

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An intracellular organism was isolated from the tissues of an Oriental white-backed vulture (*Gyps bengalensis*) in chicken embryo fibroblast cell cultures. Biochemical and physical properties, ultrastructural features, and 16S ribosomal DNA sequencing classified this organism as a new taxon of mycoplasma, for which the name "*Mycoplasma vulturii*" is proposed.

Dramatic population declines of Oriental white-backed vultures (*Gyps bengalensis*) have occurred in Pakistan due to high levels of renal failure-associated adult mortality as a result of poisoning by livestock residues of the anti-inflammatory drug diclofenac (6, 13). The associated diagnostic investigation included attempts to isolate viruses or other intracellular pathogens. As a result, a mycoplasma, designated Gb-V33, was grown from a tissue pool of lung and spleen from one of these cases (vulture 33). This vulture was found dead at the Changa Manga forest plantation at approximately 31°04′10″N, 73°58′39″E. The most apparent gross and microscopic lesions in this vulture were those of renal failure and visceral gout related to diclofenac intoxication (13). However, this vulture also had mild heterophilic inflammation in the trachea and bronchi. In this report we describe the isolation of Gb-V33; its characterization as a mycoplasma based on biochemical properties, ultrastructural features, and 16S ribosomal DNA (rDNA) sequencing; and its prevalence in the vulture population.

Culture and biochemical characterization. Gb-V33 was initially detected in primary chicken embryo fibroblasts (CEFs) on the third passage of a pool of lung and spleen samples from vulture 33, in which it caused detectable cytopathology characterized by pronounced vacuolation and occasional syncytium formation at approximately 6 days postinfection. The CEF cells were prepared as previously described (4) and maintained in Eagle's minimal essential medium supplemented with 10% (vol/vol) fetal bovine serum, 2 mM L-glutamine, 100 U of penicillin ml^{-1}, 0.1 mg of streptomycin ml^{-1}, and 2.5 μg of amphotericin B ml^{-1}. To maintain and passage Gb-V33, it was harvested by mechanically scraping free infected cell monolayers, collecting aliquots of pooled supernatants and cells, and freezing them at −80°C until needed. In subsequent passages cytopathology was detectable at approximately 3 to 5 days postinfection.

Gb-V33 was initially classified as a bacterium based on biochemical and physical properties. The presence of a DNA genome was demonstrated by sensitivity to 5-bromo-2′-deoxyuridine, an inhibitor of DNA replication. Tenfold serial dilutions of Gb-V33 were made in media with and without 50 μg of 5-bromo-2′-deoxyuridine ml^{-1}, inoculated onto confluent CEFs in 24-well plates, and observed daily for cytopathology. Gb-V33 was also shown to be sensitive to tetracycline in a similar fashion by using medium with tetracycline hydrochloride at a final concentration of 10 μg ml^{-1}. Both treatments decreased the infectivity of the stock cultures by at least 1 $\log_{10}$ order of magnitude (data not shown). The size of Gb-V33 was shown to be between 0.2 and 0.45 μm based on the ability of 0.2 (but not 0.45)-μm-diameter-pore filters to remove infectivity from an infectious stock of organism. However, no bacterial growth was detected with standard aerobic or microaerophilic cultures by using nonselective agars and broths. Cell-free cultivation for mycoplasmas by using pleuropneumonialike organism broth and agar (Remel TI no. 20360-A and 20260-A; Remel Inc., Lenexa, Kans.) and SP4-glucose broth and agar (Remel TI no. 20376 and 20276) did not detect growth after 15 and 45 days of incubation, respectively.

Ultrastructural features. Ultrastructural studies of Gb-V33 were performed on infected CEF cell monolayers fixed for 4 h with 2.5% glutaraldehyde in 0.1 M cacodylate buffer, followed by rinsing with 0.1 M cacodylate buffer in 3.5% sucrose. The fixed cell pellet was embedded in Epon resin and thin sectioned for transmission electron microscopy. Cells infected with Gb-V33 revealed organisms within intracytoplasmic vacuoles (Fig. 1A). The salient ultrastructural features of Gb-V33 were a nonnucleated organism that lacked a cell wall, a coccoid to oval shape, and an approximate diameter of 0.5 μm. These features were characteristic of mycoplasmas (7, 8). Another feature was the organization of electron-dense granules, presumably ribosomes, on round or oval membrane-bound structures approximately 0.1 to 0.3 μm in diameter (Fig. 1B), resulting in structures similar in appearance to the rough endoplasmic reticulum in the cytoplasm of the CEFs. One or two of these structures per organism were found in approximately half of the organisms observed. Some Gb-V33 organisms demonstrated what appeared to be a membrane-bound

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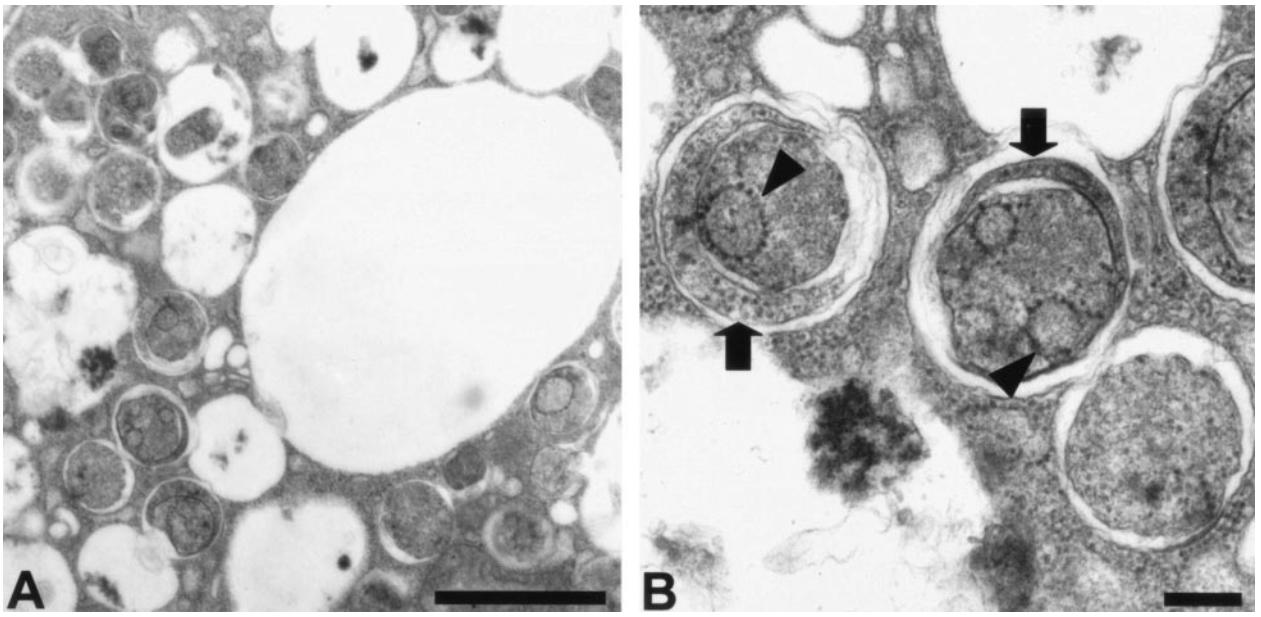

FIG. 1. Transmission electron micrographs of CEFs infected with Gb-V33. (A) Multiple coccoid organisms within intracytoplasmic vacuoles of an infected cell. Bar, 1 μm. (B) Higher magnification demonstrating lack of a cell wall, nap structures (arrows), and internal vacuoles (arrowheads). Bar, 200 nm.

structure that was compressed between the organism and the wall of the vacuole (Fig. 1B).

16S rDNA phylogenetic analysis. 16S rDNA sequencing was used for phylogenetic analysis of Gb-V33. Total cellular DNA was extracted from infected cell cultures by using a commercial DNA extraction kit (Puregene; Gentra Systems Inc., Minneapolis, Minn.), and PCR was performed with recombinant *Taq* DNA polymerase (Qiagen Inc., Valencia, Calif.). An approximately 1.5-kb fragment of the 16S rDNA gene was amplified using consensus primers corresponding to the U1 and U8 conserved sequences of the 16S rDNA gene (14). To control for adventitial bacterial contamination of the cell cultures, PCR for the 16S rDNA gene was performed on uninfected CEF cells, produced and cultured at the same time as the infected cell cultures, with negative results. Amplicons were cloned into the pCR2.1 sequencing vector (TOPO TA cloning kit; Invitrogen, Carlsbad, Calif.), and both strands were sequenced by automated dideoxy DNA methods.

Comparison of a 1,429-nucleotide segment of the 16S rDNA sequence to the GenBank database (National Center for Biotechnology Information) with BLASTN.2.2.3 software (1) classified Gb-V33 as a mycoplasma. The sequence of Gb-V33 did not completely match any available sequences but was most closely related to mycoplasmas in the *Mycoplasma neurolyticum* cluster of the *Mycoplasma hominis* group (9) with an identity of approximately 90%. Similarity analysis (AlignX program, Vector NTI 8; Informax, Inc., Frederick, Md.) showed that Gb-V33 had 87% similarity to *Mycoplasma collis*, *Mycoplasma cricetuli*, and *Mycoplasma hyorhinis* and 86% similarity to *Mycoplasma cloacale*, *Mycoplasma falconis*, *Mycoplasma lagogenitalium*, *Mycoplasma molare*, and *M. neurolyticum*. Gb-V33 also had 86% similarity to *Mycoplasma gypis*, a mycoplasma that has been isolated from griffon vultures (*Gyps fulvus*) in Europe (15, 16). In contrast, Gb-V33 was only 73 to 84% similar to other pathogenic mycoplasmas of birds, including *Mycoplasma gallisepticum*, *Mycoplasma meleagridis*, and *Mycoplasma sturni*.

Sequence alignment was performed with the CLUSTAL W (1.82) multiple sequence alignment program (European Bioinformatics Institute). The 1,429-nucleotide sequence of Gb-V33 was aligned with the GenBank sequences of 37 other species of mycoplasmas representing all of the major groups and clusters within the genus *Mycoplasma* (9, 15), and phylogenetic analysis was performed using 1,000 replicates of bootstrapping and 100 replicates of DNA parsimony with the PHYLIP package (5). A phylogenetic tree was constructed using strict majority rule consensus trees. The resulting tree (Fig. 2) had the same topology as previously published phylogenetic estimates based on 16S rDNA sequences (9, 15) and demonstrated that Gb-V33 is a member of the *M. neurolyticum* cluster in the *M. hominis* group and that it was also quite distinct from *M. gypis* and other avian mycoplasmas.

Clinical significance. A heminested PCR assay for the 16S rDNA gene of Gb-V33 was developed based on variable regions in the gene, utilizing forward primer 160 (5′-CTTAAA TAGAAGGCATCTTTT-3′) and reverse primers 529 (5′-AT AATTCCGGATAACGCTTGC-3′) and 455 (5′-AAGGTAC CGTCAAACTAATGTC-3′). Primers 160 and 529 are in conserved regions of the 16S rDNA sequence, while primer 455 is in a region unique to Gb-V33. The first round of PCR utilized primers 160 and 529, while the second round of PCR utilized primers 160 and 455. Reaction mixtures were analyzed with ethidium bromide-stained agarose gels. Amplification of the avian β-actin gene (17) was used to demonstrate that all negative samples had intact DNA and did not contain nonspecific PCR inhibitors.

The PCR assay directly detected Gb-V33 in both lung and spleen, but not kidney, tissue of the original case (vulture 33).

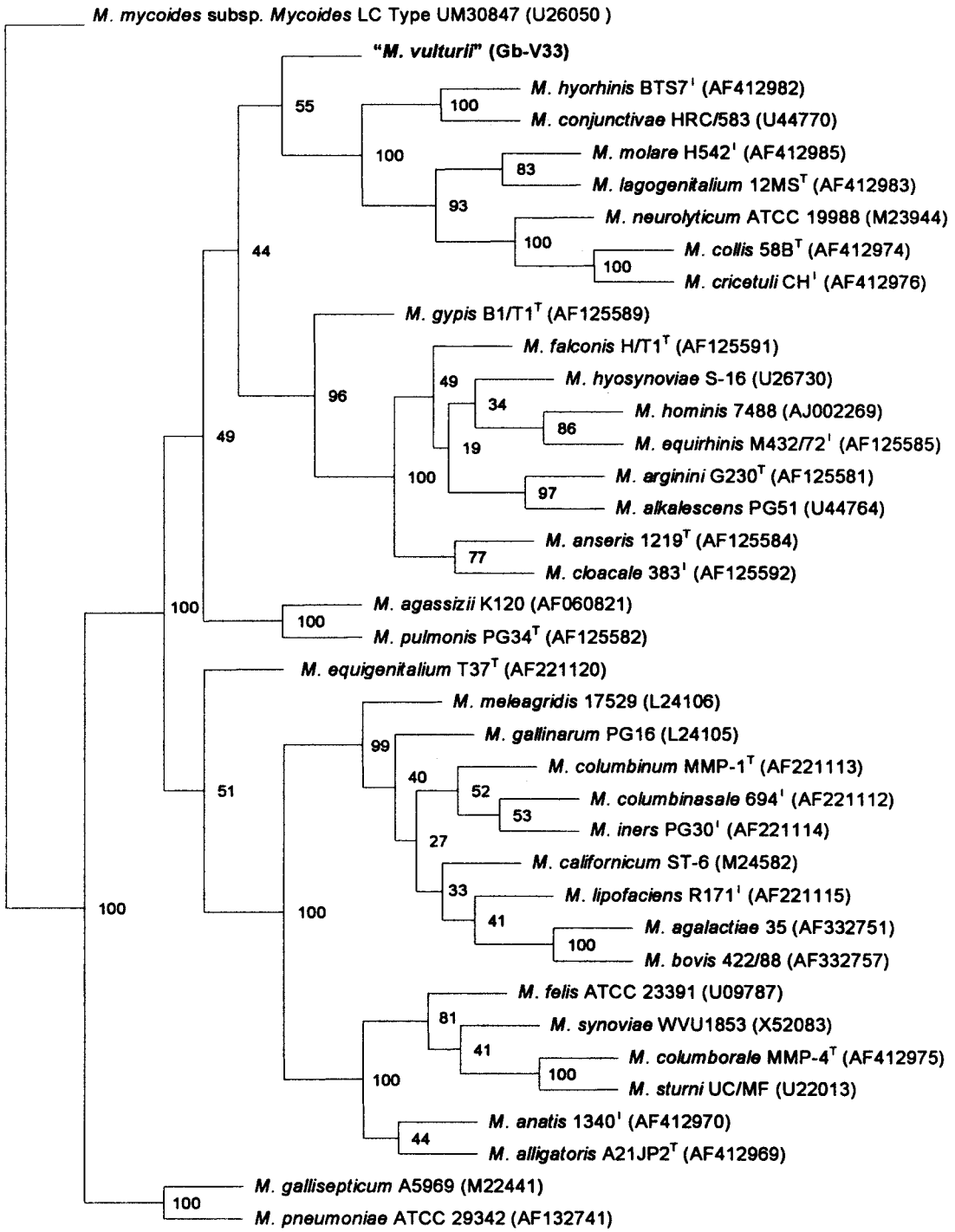

FIG. 2. Phylogenetic comparison of "*M. vulturii*" (Gb-V33) to other mycoplasmas based on 16S rDNA sequences. *Mycoplasma mycoides* subsp. *mycoides* was used to root the tree. Bootstrap percentage values from 1,000 resamplings of the data sets are shown at the nodes.

The amplicon was confirmed as identical to Gb-V33 by direct sequencing (data not shown), further indicating that Gb-V33 was not a cell culture contaminant. In addition, detection in the spleen suggested that Gb-V33 was causing a systemic infection. The strongest PCR amplification was from the lung (data not shown), indicating that this would be the optimal tissue to test in other vultures. Testing by PCR of DNA extracted from the lungs of 37 additional vultures indicated infection in eight of these birds and a crude estimated prevalence of ~24%. Histopathology of the other infected vultures did not detect lesions consistent with mycoplasmosis in the respiratory tract or other tissues.

Conclusions. Based on biochemical, ultrastructural, and genetic characteristics, we have identified Gb-V33 as a new mycoplasma taxon that infects Oriental white-backed vultures, and we propose that it be named "*Mycoplasma vulturii*." Pre-

liminary PCR testing indicates a prevalence of ~24% in the wild population of Oriental white-backed vultures. However, the data do not indicate that "*M. vulturii*" is an obligate pathogen. Although mild inflammation was noted in the respiratory tract of vulture 33, this vulture was ultimately shown to have died due to diclofenac poisoning (13). Respiratory or other lesions consistent with mycoplasmosis were not evident in the other infected birds examined (13). The requirement for nested PCR methods to consistently detect "*M. vulturii*" indicates that it was present in very low numbers in the infected birds, also suggesting that these infections were subclinical. Consequently, it is most likely that "*M. vulturii*" is a commensal organism of Oriental white-backed vultures and possibly an opportunistic pathogen.

"*M. vulturii*" is propagated in primary CEF cultures in which it causes vacuolating cytopathology and is present intracellularly. Intracellular replication has also been noted for other mycoplasmas including *M. gallisepticum*, *Mycoplasma genitalium*, *Mycoplasma penetrans*, and *Mycoplasma pneumoniae* (3, 11, 19). Initial attempts to cultivate "*M. vulturii*" on cell-free media were unsuccessful. Some mycoplasmas, such as the recently reclassified hemotrophic mycoplasmas formerly in the genera *Haemobartonella* and *Eperythrozoon*, appear to be obligate cellular parasites that cannot be propagated on cell-free media (12). Others, such as some strains of *M. gallisepticum* and *Mycoplasma synoviae*, appear to be uncultivable with present cell-free media (2). For "*M. vulturii*," more comprehensive attempts at cell-free cultivation will be required to determine if it is truly an obligate intracellular organism. The internal structures noted ultrastructurally in "*M. vulturii*" are unusual. Internal vacuoles have been described for *M. genitalium*, but no known function has been ascribed to them (8). The membrane-bound structures compressed between the organism and the wall of the host cell vacuole were another unusual feature of "*M. vulturii*." One possible explanation for this structure is that the organisms were elongated and folded over upon themselves. However, longitudinal sections showing elongated forms were not observed in the sections examined. Alternatively, this compressed structure could be a form of the electron-dense layer that has been previously described for the intracellular forms of *Mycoplasma fermentans* (11), a "nap" structure that has been described for *M. genitalium* (18), or part of the attachment organelle that has been described for *M. pneumoniae* (10) and the hemotrophic mycoplasmas (12).

Nucleotide sequence accession number. The GenBank accession number for the 16S rDNA gene sequence of Gb-V33 is AY191226. Gb-V33 has also been deposited with the American Type Culture Collection (ATCC BAA-882).

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