Mycoplasma neophronis sp. nov., isolated from the upper respiratory tract of Canarian Egyptian vultures (*Neophron percnopterus majorensis*)

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Six strains with the typical characteristics of mycoplasmas were isolated from the tracheae of six Canarian Egyptian vultures (*Neophron percnopterus majorensis*). The results of biochemical, serological and molecular genetic studies showed that the isolates were nearly identical and that they could be considered as representing a novel species of the genus *Mycoplasma*. Colonies possessed the typical fried-egg appearance and electron micrographs revealed a pleomorphic cellular morphology with the lack of a cell wall. The isolates hydrolysed arginine and required sterol for growth but did not ferment glucose or hydrolyse urea. We propose that the isolates be assigned to a novel species, *Mycoplasma neophronis* sp. nov. The type strain is G.A.^T (=DSM 24097^T=ATCC BAA-2157^T). The antiserum of strain G.A.^T has been deposited in the *Mollicutes* collection at Purdue University (Indiana, USA).

The Canarian Egyptian vulture, or guirre (*Neophron percnopterus majorensis*), is a highly endangered subspecies of Egyptian vulture found only in the eastern Canary Islands (Donázar *et al.*, 2002). Their population has been estimated to be just 30 breeding pairs (Palacios, 2004). The subspecies is presently listed as endangered on the International Union for Conservation of Nature Red List of Threatened Species. The causes of its decline are the loss of territory caused by human pressure, changes in livestock management, including the regular use of veterinary drugs, and intoxication by poison or lead ingestion (Lemus *et al.*, 2008).

Mycoplasmas have been previously isolated from vultures (Poveda *et al.*, 1990, 1994; Panangala *et al.*, 1993; Oaks *et al.*, 2004; Loria *et al.*, 2008; Ruder *et al.*, 2009; Lecis *et al.*, 2010) but their significance to the health of these birds is still unclear. A positive association between avian bacterial pathogens such as those of the genera *Chlamydophila* and *Salmonella* and the genus *Mycoplasma* has also been

identified (Gangoso *et al.*, 2009). In this paper, we describe the isolation of six *Mycoplasma* strains (G.A.^T, T80, T157, T185, T186 and T331) obtained from the tracheae of six apparently healthy guirres. We have followed the minimal standards for description of novel species of the class *Mollicutes* (division *Tenericutes*, order *Mycoplasmatales*) published by the Subcommittee on the Taxonomy of *Mollicutes* (Brown *et al.*, 2007).

Samples were taken from six Canarian Egyptian vultures on the day of their entry to the Wildlife Recovery Centre, Canary Islands, Spain. The causes of admission were electrocution (one guirre, from which strain T80 was isolated), intoxication by poison (three guirres, from which strains T157, T185 and T186 were isolated), a malformed beak (one guirre, from which strain T331 was isolated) and an accident with an electricity pylon that resulted in a leg injury (one guirre, from which strain G.A.^T was isolated). All samples were taken from the guirres' tracheae with sterile cotton swabs and incubated in liquid SP-4 II (Ramírez *et al.*, 1997) containing 20% horse serum at 37 °C for 24 h. After that, the cultures were filtered through 0.45-µm-pore sterile membranes and plated onto solid SP-4 II. Plates were incubated at 37 °C under aerobic conditions. The isolation and examination of mycoplasmas were then performed as described by

Abbreviation: ISR, intergenic spacer region.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain G.A.^T is FR682077 and those for the ISR sequences of strains G.A.^T, T157, T185, T186, T331 and T80 are HE572620–HE572625, respectively.

Fig. 1. Maximum-likelihood phylogenetic tree based on almost-complete 16S rRNA gene sequences and taken from a tree constructed using all members of the genus *Mycoplasma*, showing the position of strain G.A.^T in the genus *Mycoplasma*. Bootstrap values (%; 1000 replications) are shown at branch nodes. Bar, 5% sequence divergence.

Bradbury (1998). From each culture, one colony was isolated in pure culture by cloning (subculturing one single colony) three times following the procedure of Tully (1983). Colonies of strains G.A.^T, T80, T157, T185, T186 and T331 were easily visible on agar under a stereomicroscope within 3–5 days of incubation. They showed a typical fried-egg appearance at $\times 4$ magnification. Growth was inhibited in the presence of 1–5% digitonin, with zones of inhibition ranging from 6 to 10 mm, which indicated the biochemical requirement for sterols that is characteristic of members of the *Mycoplasmatales*, but not the *Acholeplasmatales* (Poveda, 1998).

In all phenotypic tests, positive and negative controls were used, following the procedures described by Poveda (1998). All isolates showed an identical phenotype: hydrolysis of arginine, but no fermentation of glucose and mannose, no reduction of tetrazolium chloride salts and no hydrolysis of urea. None of the isolates produced films or spots or adsorbed sheep erythrocytes (Gardella & Del Giudice, 1983).

Strain G.A.^T was designated as the reference strain for this work. Its almost-complete 16S rRNA gene sequence was determined as described by Johansson *et al.* (1998) and added to the updated and prealigned 16S rRNA gene database (release LTP_s104; <http://www.arb-silva.de/projects/living-tree/>), which contains all sequences of bacterial type strains for which an entry of high quality is found (Yarza *et al.*, 2010). The sequence was aligned using the ARB software (Ludwig *et al.*, 2004) (www.arb-home.de) and improved manually. All tree reconstructions were performed using the RAXML algorithm version 7.0 with the GTRGAMMA model (Stamatakis, 2006) using subsets of data corresponding to the type strains of species of

the genus *Mycoplasma*, using a 40% sequence conservation filter for all *Bacteria* (Yarza *et al.*, 2010). Fig. 1 shows a subtree of a larger reconstruction based on all members of the genus *Mycoplasma* available in LTP_s104. Strain G.A.^T was clearly affiliated with the *Mycoplasma hominis* cluster (Pettersson *et al.*, 2000), which comprises sequences with <90% 16S rRNA gene sequence similarity with the closest relatives outside the cluster and which, from the taxonomic point of view, is a putative new genus within the family *Mycoplasmataceae* (Yarza *et al.*, 2008). Strain G.A.^T clustered with *Mycoplasma spumans* PG 13^T and *Mycoplasma falconis* H/T1^T (Poveda *et al.*, 1994), with which it shared 98 and 97% 16S rRNA gene sequence similarities, respectively. Sequence similarities with other members of the *M. hominis* cluster are given in Table 1.

Comparison of intergenic spacer region (ISR) sequences has been demonstrated to have a higher resolution power in the genus *Mycoplasma* than comparison of 16S rRNA gene sequences (Harasawa, 1999). The ISR of all isolates was amplified using the forward primer 5'-CGTTCTCG-GGTCTTGACAC-3' and the reverse primer 5'-CGCAGGTTTGACGTCCTTCATCG-3', using the conditions as described by Ramírez *et al.* (2008). The ISR sequences were identical, in accordance with the isolates' high phenotypic similarity. Furthermore, 16S rRNA gene sequence similarity was studied by means of denaturing gradient gel electrophoresis (McAuliffe *et al.*, 2005), which suggested that the isolates' 16S rRNA gene sequences were also identical (data not shown).

Whole-cell protein samples from the isolates were separated on 10% polyacrylamide gel and subjected to SDS-PAGE (Laemmli, 1970) using a Mini-Protein Tetra Cell (Bio-Rad),

Table 1. Characteristics of strain G.A.^T and members of the *M. hominis* cluster

Strains: 1, *Mycoplasma neophronis* sp. nov. G.A.^T; 2, *M. alkalescens* PG 51^T; 3, *M. anseris* 1219^T; 4, *M. arginini* G230^T; 5, *M. arthritis* PG6^T; 6, *M. auris* U1A^T; 7, *M. buccale* CH 20247^T; 8, *M. canadense* 275C^T; 9, *M. cloacale* 383^T; 10, *M. equirhinis* M432/72^T; 11, *M. falconis* H/T1^T; 12, *M. faucium* DC 333^T; 13, *M. gateae* C8; 14, *M. hyosynoviae* S16^T; 15, *M. indienne* 3T^T; 16, *M. orale* CH 19299^T; 17, *M. phocicebrale* 1049^T; 18, *M. phocidae* 105^T; 19, *M. salivarium* PG 20^T; 20, *M. spumans* PG 13^T; 21, *M. subdolum* TB^T; 22, *M. hominis* PG21^T. All strains are negative for glucose fermentation and positive for arginine hydrolysis. Data from this study and Pettersson *et al.* (2000). A, Avian; B, bovine; C, caprine; E, equine; F, feline; H, human; M, murine; O, ovine; PH, *Phoca vitulina*; PO, porcine; PR, primate; +, positive; −, negative; ND, no data available.

Characteristic	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22
Principal host	A	B	A	C	M	C	H/PR	B	A	E	A	H/PR	F	PO	PR	H/PR	PH	PH	H	C	E	H
Biochemical properties																						
Tetrazolium chloride salt reduction	−	−	−	−	−	+	+	+	+	−	+	+	+	−	−	+	−	−	−	−	−	−
Film/spot production	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−
DNA G + C content (mol%)	31	ND	26	28.1	ND	26.6–27.0	25.0–26.4	29	26	ND	27.5	ND	28.5	ND	32	27	ND	ND	ND	28.4	ND	ND
16S rRNA gene sequence similarity with strain G.A. ^T (%) [*]	100	97	96	97	96	97	95	97	96	96	97	95	95	94	94	94	97	96	95	98	95	96
Inhibition of growth of strain G.A. ^T by antisera ^{*†}	+	− ^a	− ^a	− ^a	− ^b	− ^c	− ^a	− ^a	− ^d	− ^a	− ^c	− ^a	− ^a	− ^a	− ^c	− ^a	− ^b	− ^a	− ^b	− ^a	− ^a	− ^a

^{*}Data were taken from this study.

[†]The following antisera were used: *a*, rabbit, Institute of Medical Microbiology, University of Aarhus, Denmark; *b*, rabbit, Central Public Health Laboratory, Colindale, London, UK; *c*, rabbit, J. Bradbury, University of Liverpool, UK; *d*, rabbit, National Collection of Type Cultures, London, UK.

according to the manufacturer's instructions. Gels were stained with Coomassie R-250 and destained with 40 % methanol and 10 % acetic acid. All isolates showed similar protein profiles (Fig. 2).

Fig. 2. SDS-PAGE profiles of whole-extract proteins of the six isolates of *Mycoplasma neophronis* sp. nov. Lanes: PM, protein marker; 1, strain G.A.^T; 2, strain T80; 3, strain T157; 4, strain T185; 5, strain T186; 6, strain T331.

Because serology is still an essential test to differentiate species in the class *Mollicutes* (Brown *et al.*, 2007), a serological study between strain G.A.^T and members of the *M. hominis* cluster was performed. In addition, antiserum against strain G.A.^T was prepared in rabbits using a standard technique (Senterfit, 1983). No inhibition of the growth of the isolates (Poveda & Nicholas, 1998) was observed with a panel of heterologous antisera against members of the *M. hominis* cluster (Table 1). However, antiserum against strain G.A.^T gave a strong reaction with strains T80, T157, T185, T186 and T331 (inhibition zone size 5 mm). Altogether, the isolates were shown to have a serological pattern that was different from those of the other members of the *M. hominis* cluster. These results indicated that the isolates could be considered as representing a novel species within the cluster. This consistency in phenotypic pattern has not been found within any of the other phylogenetic groups of the *M. hominis* cluster (Pettersson *et al.*, 2000). The antiserum of strain G.A.^T has been deposited in the *Mollicutes* collection at Purdue University (Indiana, USA).

The cellular morphology of strain G.A.^T was assessed by transmission electron microscopy using cells harvested from SP-4 II broth. Cells were fixed in 2 % paraformaldehyde, washed with 0.2 M Tris-buffered saline, post-fixed with 1 % (w/v) osmium tetroxide, sequentially dehydrated in graded dilutions of ethanol and then embedded in vinyl cyclohexane dioxide epoxy resin. Thin sections were collected on copper grids, stained with 10 % uranyl acetate

Fig. 3. Electron micrograph of an ultrathin section of strain G.A.^T. Note the presence of a bilayered cell membrane (arrow) and absence of a cell wall. Bar, 100 nm.

in methanol and 0.4 % lead citrate and observed at 60 kV. Strain G.A.^T exhibited the bilayered cell membrane and the absence of a cell wall that is characteristic of mycoplasmas (Brown *et al.*, 2007) (Fig. 3).

The DNA G + C content of strain G.A.^T, determined using HPLC (Urdiain *et al.*, 2008), was 31 mol%.

Altogether, the six strains isolated from different Canarian Egyptian vultures were identical in their 16S rRNA gene sequences and phenotypic and serological profiles. Despite the phenotypic similarities between strain G.A.^T and its closest phylogenetic neighbours, *M. spumans* and *M. falconis*, there were significant differences (Table 1). These differences, combined with an absence of growth inhibition by heterologous antisera for *M. spumans* and *M. falconis*, indicate that the isolates can be considered as representing a hitherto unclassified species of the genus *Mycoplasma*, for which the name *Mycoplasma neophronis* sp. nov. is proposed.

Description of *Mycoplasma neophronis* sp. nov.

Mycoplasma neophronis [ne.o.phro'nis. N.L. gen. n. *neophronis* of *Neophron*, isolated from the Canarian Egyptian vulture (*Neophron percnopterus majorensis*)].

The species description is based on six strains and fulfils the revised minimal criteria for species descriptions of members of the class *Mollicutes*. No cell wall. Cells can be filtered through 450-nm-pore membranes. Produces 'fried-egg' colonies on solid media. Arginine is hydrolysed, but urea is not. Glucose and mannose are not fermented. No production of films or spots and no adsorption of sheep erythrocytes. Growth is inhibited by digitonin and requires serum or sterol. Contains conserved mycoplasmal genus-specific sequences in the 16S rRNA gene and is serologically

distinct from other members of the genus *Mycoplasma*. Pathogenicity has not been fully demonstrated.

The type strain is G.A.^T (=DSM 24097^T=ATCC BAA-2157^T), isolated from the upper respiratory tract of a Canarian Egyptian vulture at the Wildlife Recovery Centre, Canary Islands, Spain. The DNA G + C content of the type strain is 31 mol%.

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