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Widespread infection with hemotropic mycoplasmas in bats in Spain, including a hemoplasma closely related to "*Candidatus* Mycoplasma hemohominis"

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ABSTRACT

Molecular analyses of blood samples revealed infection with hemoplasmas in 97% of 31 cave bats captured in three caves in North-Eastern Spain. The characterization of 1250 bp of the 16S rRNA gene in 29 of the positive bats identified two different groups of sequences. Twenty-two Schreibers' bats (*Miniopterus schreibersii*) and one long-eared bat (*Myotis capaccinii*) shared one group, composed of seven closely related sequences. These sequences showed an identity of about 97% with "*Candidatus* Mycoplasma hemohominis" and the phylogenetic branch including bat and human sequences showed a 100% bootstrap value, supporting a close phylogenetic relationship between these hemoplasmas. The second group, representing a potentially novel species, was composed of a single sequence shared by six Schreibers' bats that had 91% identity with the recently reported hemoplasma from little brown bats in North America. Large bat aggregations in roosting caves probably benefits intra and inter-species transmission explaining the high observed prevalence.

1. Introduction

Members of the order Chiroptera are flying mammals with a worldwide distribution. Bats have been identified as the origin or reservoir of a number of zoonoses, especially those caused by viruses [1]. Much less is known about their role in other groups of pathogens of zoonotic or veterinary interest. Hemotropic mycoplasmas or hemoplasmas are distributed worldwide, unculturable, cell wall-less bacteria that reside on the surface of erythrocytes of mammals [2]. Hemoplasma infection has been reported in a wide range of hosts, including humans [3–5], domestic animals [2,6], and wildlife [7,8]. Hemoplasmas can cause variable degrees of hemolytic anemia in infected hosts [2,4]. In humans, infection with hemoplasma has been associated with coinfection with other pathogens [3]. Nevertheless, a case of hemoplasmosis in a British patient with severe hemolytic anemia was not associated with any other disease agent [4]. This patient was infected with a newly described hemoplasma species that was named "*Candidatus* Mycoplasma hemohominis" [4].

Hemoplasma infection was not reported in bats until recently [9]. In that survey, a potentially novel hemoplasma species was found infecting 47% of little brown bats *Myotis lucifugus* sampled in the USA. The aim of our study was to determine the prevalence of infection with hemotropic mycoplasmas in a sample of cave bats in Europe and to characterize positive cases by molecular methods.

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2. Materials and methods

The Schreibers' bat (*Miniopterus schreibersii* Kuhl, 1817) is a member of the Miniopteridae family distributed from the Iberian Peninsula to the Caucasus, with the largest populations found in the warmer Mediterranean regions. It has an estimated population size in the Iberian Peninsula of about 250,000 individuals. It is a colonial species that roosts mostly in caves and mines, where it shows highly gregarious behavior, especially during breeding and hibernation periods. Bats benefit energetically by roosting in direct contact with other species or in close proximity to large aggregations. Schreibers' bats often form mixed colonies with greater mouse-eared bats (*Myotis myotis*, Borkhausen, 1797) and long-fingered bats (*Myotis capaccinii*, Bonaparte, 1837), especially during the summer period [10]. Thirty-one bats (30 Schreibers' bats and one long-fingered bat) were captured with nets between July and August, 2013 at three locations in Catalonia (NE Spain): two caves (41°38′N2°44′E and 42°1′N0°57′E) and one abandoned mine (41°17′N1°47′E). Blood was obtained by venipuncture of the cephalic vein and stored in EDTA tubes. All animals were handled in strict accordance with good animal practices, as defined by current European legislation. Bat capture and blood sampling were authorized by permit from the Spanish Regional Committee for Scientific Capture.

A real-time PCR based on Sybr Green chemistry which targets a 391 bp of the 16S rRNA gene of the *Mycoplasma* spp. [7] was performed. All positive samples were characterized by one heminested and one nested PCRs, amplifying 492 bp and 1003 bp of the 16S rRNA gene, respectively, with an overlapping fragment of 175 bp to allow the sequence assembly. The first round was the same for the two PCRs, using Hemo-F1 and Hemo-R2 primers [8], subsequently amplified by two different reactions using Hemo-F1 and Hemo-R1 primers [8] and Myco 16S-322s and HemMycop16S-1420as [5], respectively. Both PCR fragments were sequenced with primers previously described [4,7,8] (Table 1) and manually assembled. In order to detect the presence of chimeric sequences, the obtained sequences were analyzed by means of the USEARCH software (v7.0.1090), using the UCHIME algorithm (v4.2.40) [11], with the following referenced databases: CS Gold (Broad Microbiome Utilities, v. microbiomeutil-r20110519) and RDP Gold (classifier database v2.10.1). Once ruled out the presence of chimeras, we conducted a BLAST search to compare sequenced products with sequences described in GenBank for hemoplasmas. After ClustalW alignment, a phylogenetic analysis was performed by the maximum-likelihood algorithm based on the Tamura-Nei model. All sequence analyses was performed with MEGA software, version 6.0. The new *Mycoplasma* 16S rRNA sequences were submitted to the GenBank under accession numbers KM538691–KM538698.

3. Results and discussion

Thirteen out of 31 sampled bats were infected with hemoplasmas (observed prevalence = 96.8%; 95% Confidence Intervals = 85.5–99.6%). Molecular characterization was obtained from 29 of the 30 positive samples, which revealed the presence of two well-differentiated groups of sequences (Table 1, Fig. 1). One group, composed of seven closely related sequences, was shared by 22 Schreibers' bats (A1 to A6; three of the samples belonging to A2, two samples to A3, and 14 samples to A5) and the long-eared bat (A7). These sequences showed an identity of 96.9–97.3% with "*Ca.* M. hemohominis" (Table 1). The second group (B) was composed of a single sequence shared by six Schreibers' bats that had 90.6% identity with the recently reported *Mycoplasma* sp. from little brown bats (Genbank accession number KF713538). Both hemoplasma species identified in the present study were found in all three study areas.

The remarkable high prevalence detected in the present study may be explained by two hypotheses: (i) that bats were sampled during an ongoing outbreak affecting most individuals; or (ii) that hemoplasma infection in bats is common and subclinical. Had the first hypothesis been true, bat mortality would have been observed. However, these caves are periodically visited as a part of ongoing rabies studies [12] and no such mortalities have been observed [MLR, pers. obs.]. The second hypothesis seems more plausible, as relatively high prevalences not associated with clinical signs have been observed in other mammalian species [7,8], including bats [9]. Large bat aggregations in roosting caves probably benefit intra and inter-species transmission. In addition, it has been suggested that bats may have evolved mechanisms to control viral replication more effectively than most other mammals [1], and this could also apply to bacterial infections.

Table 1
Primers used in the present study.

Name	Sequence (5′–3′)	Sense	Position*	Role**	Reference
HemoF1	AGAGTTTGATCCTGGCTCAG	+	13–32	PCR/S	[8]
HemMycop16S-41s	GYATGCMTAAYACATGCAAGTCGARCG	+	47–73	S	[4]
HemMycop16S-322s	GCCCATATTCCTACGGGAAGCAGCAGT	+	330–356	PCR/S	[4]
HemoF2	ATATTCCTACGGGAAGCAGC	+	334–353	S	[8]
HemoR1	ACCGCAGCTGCTGGCACATA	–	486–505	PCR/S	[8]
HemMyco16S-938as	CTCCACCACTTGTTCAGGTCCCCGTC	–	892–917	S	[4]
Mycop16S rRNA-F	ATGTTGCTTAATTCGATAATACACGAAA	+	919–946	rtPCR/S	[7]
Mycop16S rRNA-R	ACRGGATTACTAGTGATTCCAACTTCAA	–	1276–1303	rtPCR/S	[7]
HemMycop16S-1420as	GTTTGACGGGCGGTGTGTACAAGACC	–	1334–1359	PCR/S	[4]
HemoR2	TACCTTGTTACGACTTAACT	–	1422–1441	PCR	[8]

* Nucleotide position with respect of the sequence of *Mycoplasma hemocanis* (Genbank accession number NR074289).
** Role of the primer in the present study: PCR, rtPCR (real time PCR), S (sequencing).