

Infection with Hemotropic *Mycoplasma* Species in Patients with or without Extensive Arthropod or Animal Contact

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PCR amplification targeting the 16S rRNA gene was used to test individuals with and without extensive arthropod and animal contact for the possibility of hemotropic mycoplasma infection. The prevalence of hemotropic mycoplasma infection (4.7%) was significantly greater in previously reported cohorts of veterinarians, veterinary technicians, spouses of veterinary professionals, and others with extensive arthropod exposure and/or frequent animal contact than in a previously reported cohort of patients examined by a rheumatologist because of chronic joint pain or evidence of small-vessel disease (0.7%). Based upon DNA sequence analysis, a *Mycoplasma ovis*-like species was the most prevalent organism detected; however, infection with “*Candidatus Mycoplasma haematoparvum*” and a potentially novel, but incompletely characterized, hemotropic *Mycoplasma* species was also documented. Historical exposure to animals and arthropod vectors that can harbor hemotropic *Mycoplasma* spp. should be considered during epidemiological investigations and in the evaluation of individual patients.

Hemotropic mycoplasmas (hemoplasmas) are obligate epierythrocytic, cell wall-deficient bacteria that cause not-yet-curable infections in numerous animal species, including human beings. In general, these bacteria induce persistent asymptomatic intravascular infections in domestic and wild animals and are not considered to be highly pathogenic. Therefore, hemotropic mycoplasma infections are often chronic and occult in nature; however, hemolytic anemia of variable severity, often in association with other infectious or noninfectious diseases, has been reported in animals (1–5). Disease manifestations in animals are most often reported in association with drug- or retrovirus-induced immunosuppression, with stressors such as poor nutrition, pregnancy, or lactation, or with concurrent infection with another, more virulent pathogen (6–11).

Historically, cytological examination of stained blood smears by light microscopy has been the traditional methodology used for hemoplasma detection in veterinary medicine, especially during the acute phase of infection when marked bacteremia can be observed (with up to 90% of red blood cells being parasitized) (12). However, the diagnostic sensitivity of a blood smear examination is generally less than 20% in animals that are chronically infected, and the specificity of a blood smear examination is hampered by artifacts, such as stain precipitates and Howell-Jolly bodies (3, 13, 14). The relatively recent development of PCR assays primarily targeting the 16S rRNA gene has resulted in the recognition of several novel hemoplasmas, some of which can infect humans (3, 9, 15).

Phylogenetic analyses of 16S rRNA gene sequences have identified two major subclusters of hemoplasmas, namely, the *Mycoplasma suis* and *Mycoplasma haemofelis* groups (3, 11, 13, 15, 16). The *M. suis* subcluster includes “*Candidatus Mycoplasma haemominutum*,” *Mycoplasma suis*, *Mycoplasma wenyonii*, “*Candidatus Mycoplasma haemolamae*,” and “*Candidatus Mycoplasma haemodidelphidis*,” which have been reported among cats, pigs, cattle, alpacas, and opossums, respectively. The *M. haemofelis* subcluster includes *M. haemofelis*, *M. haemomuris*, and *M. haemocanis*, reported among cats, mice, and dogs, respectively. Because of the high 16S rRNA gene sequence similarity among members of

these two major subclusters, differences in the species identity and host tropism can be more accurately detected by partial sequencing of the RNase P RNA gene (15, 17, 18).

Although the pathogenic potential of hemotropic mycoplasmas as a cause of human disease has not been clearly defined, these emerging zoonotic pathogens may pose a more serious public health concern than is currently appreciated. Based upon the visualization of organisms on stained blood smears only, researchers in Inner Mongolia, China, reported regional human hemotropic mycoplasma infection rates as high as 35.3% (population tested, 1,529 people), with infection rates of up to 57.0% in local pregnant women and 100% in newborns from infected mothers (19, 20). Similarly, a high prevalence of infection was found among farmers and veterinarians working with swine in China, most specifically in Shanghai, where 32 of 65 workers (49%) were PCR positive for an *M. suis*-like infection (21). Importantly, all three of these published reports from China support a greater risk of hemotropic mycoplasma infection among farmers, veterinarians, and other individuals who have frequent and close contact with domestic animals that serve as reservoir hosts. In addition to the evidence supporting vector transmission, poor sanitary settings appear to increase the risk of transmission (19–21).

Previously, based upon molecular confirmation, only five hemotropic *Mycoplasma* spp. were described as causes of human infections: *Mycoplasma haemofelis*-like (6), *M. suis*-like (21), *M. ovis* (9), “*Candidatus Mycoplasma haemohominis*” (22), and “*Candidatus Mycoplasma haematoparvum*” (23) organisms. The objective of this study was to determine the molecular prevalence of hemotropic mycoplasma infections in two previously pub-

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TABLE 1 Group, age, gender, occupation, state of residence, and hemotropic *Mycoplasma* sp. amplified and sequenced from each patient's blood sample

Patient group/no.	Age (yr)	Gender	Occupation	State or country of residence ^a	<i>Mycoplasma</i> sp. ^b
A/1	35	M	Insurance agent	North Dakota	<i>M. ovis</i>
A/2	31	F	Researcher	Georgia	<i>M. sp.</i>
B/1	49	M	Veterinarian	Texas	<i>M. ovis</i>
B/2	24	F	Veterinarian	Maryland	<i>M. ovis</i>
B/3	51	F	Veterinarian	North Carolina	<i>M. ovis</i>
B/4	44	F	Veterinarian	Colorado	<i>M. ovis</i>
B/5	30	F	Veterinary student	North Carolina	<i>M. ovis</i>
B/6	53	F	Veterinarian's wife	California	<i>M. ovis</i>
B/7	44	F	Wildlife manager	Minnesota	<i>M. ovis</i>
B/8	54	F	Veterinarian	North Carolina	<i>M. ovis</i>
B/9	27	F	Veterinarian	Grenada	" <i>Candidatus Mycoplasma haematoparvum</i> "

^a Many of these individuals had extensive international travel histories.

^b *M. sp.*, partially characterized hemotropic mycoplasma species.

lished cohorts of people who had been tested for serological and molecular evidence of infection from *Bartonella* spp. (24, 25).

MATERIALS AND METHODS

Patient populations. The two cohorts tested in this study consisted of individuals who had been previously tested for *Bartonella* infection as a component of an institutional review board (IRB)-approved research study entitled Detection of *Bartonella* Species in the Blood of People with and without Extensive Animal Contact (North Carolina State University IRB no. 64-08-05) (24, 25). Group A consisted of 296 patients examined (by B. R. Mozayeni, a rheumatologist) due to evidence of small-vessel disease manifested most often as a peripheral or central neurological deficit and/or chronic joint pain (25). Group B consisted of 193 patients with extensive arthropod exposure and/or frequent animal contact, many of whom were veterinarians, veterinary technicians, or spouses of veterinary professionals (23, 24). Group B patients self-reported histories of chronic poorly defined illnesses that contributed to their willingness to participate in this survey. For both cohorts, demographic information, animal and arthropod exposure histories, histories of visiting specialists, outdoor activities, self-reported clinical symptoms, and comorbid conditions were collected and tabulated using a standardized 5-page survey instrument. Questionnaires were mailed to each study participant for self-report, and the data were entered into an electronic database upon return to the Intracellular Pathogens Research Laboratory (IPRL). Demographic details for each cohort were provided in previously published articles (23–25).

DNA extraction. DNA samples from blood collected in EDTA-anticoagulated Vacutainers were extracted using the Qiagen DNA blood minikit (Qiagen) according to the manufacturer's instructions. The quantity and quality of each DNA sample were measured spectroscopically using absorbances at 260 nm and 260/280 nm, respectively (NanoDrop, Thermo Scientific, USA).

PCR assays. DNA from extracted blood samples was tested for the presence of hemotropic mycoplasmas by PCRs targeting the 16S rRNA and RNase P genes, as previously reported (26); a 620-bp fragment of the 16S rRNA gene was amplified using oligonucleotides Myco16S-322s (5'-GCC CAT ATT CCT ACG GGA AGC AGC AGT-3') and Myco16S-938as (5'-CTC CAC CAC TTG TTC AGG TCC CCG TC-3'), and a 160-bp fragment of the RNase P gene was amplified using oligonucleotides HemoMycoRNaseP30s and HemoMycoRNaseP200as (5'-GAT KGT GYG AGY ATA TAA AAA ATA AAR CTC RAC-3') and HemoMycoRNaseP200as (5'-GMG GRG TTT ACC GCG TTT CAC-3'). In addition, for all the samples that were initially PCR positive, sequential amplification of a 1,380-bp region of the 16S rRNA gene was performed using two sets of oligonucleotides: Myco16S-938as and HemMycop16S-41s (5'-

GYA TGC MTA AYA CAT GCA AGT CGA RCG-3') and Myco16S-938as and HemMycop16S-1420as (5'-GTT TGA CGG GCG GTG TGT ACA AGA CC-3'). The sequence span for each amplicon overlaps 616 bp, which was used to align the 16S rRNA genes for each hemotropic *Mycoplasma* sp. in order to better characterize the initial PCR result.

PCRs were performed in a 25- μ l reaction mix containing 12.5 μ l of Tak Premix Ex (Fisher Scientific, USA), 0.2 μ l of 100 μ M (each) forward and reverse primers (Integrated DNA Technologies), 7.3 μ l of molecular grade water, and 5 μ l of template DNA. Five microliters of RNase-free water was used as a PCR-negative control. Amplification was performed in an Eppendorf Mastercycler EP gradient. For 16S rRNA gene PCR, the initial denaturation was performed at 95°C for 2 min, followed by 55 cycles at 94°C for 15 s, 68°C for 10 s, and 72°C for 15 s, with a final extension at 72°C for 30 s. Similarly, for the RNase P gene, the initial denaturation was performed at 95°C for 2 min, followed by 55 cycles at 94°C for 15 s, 61°C for 15 s, and 72°C for 15 s, with a final extension at 72°C for 30 s. The amplicons were electrophoresed in 2% agarose gel incorporated with ethidium bromide and visualized using a UV transilluminator.

DNA sequencing and analyses. The positive PCR amplicons were sequenced to identify the *Mycoplasma* sp. (Eton Bioscience, USA). The chromatogram analysis and sequence editing were performed using ContigExpress software (Vector NTI v10; Invitrogen, USA). The sequence alignments and phylogenetic trees were measured as distance matrices using the neighbor-joining method, and the data set was resampled 1,000 times using MEGA3 software.

Statistical analysis. Statistical analysis was performed using SAS/STAT 9.2 for Windows (2008) (SAS Institute Inc., Cary, NC). The Fisher exact test was used to assess differences in the proportions of positive patients by group. The level of statistical significance was set at 0.05.

Nucleotide sequence accession numbers. The sequences determined in this study were deposited in GenBank under accession numbers KF313922 and KF366443.

RESULTS

The age, gender, occupation, state of residence, and hemotropic *Mycoplasma* sp. identified in each PCR-positive individual from each cohort in this study are listed in Table 1. Only 2 of 296 (0.67%) patient samples from group A and 9 of 193 (4.66%) patients from group B were PCR positive for amplification of hemotropic *Mycoplasma* species 16S rRNA genes (692 patient samples were PCR negative). A species resembling *Mycoplasma ovis* was the most prevalent organism that was amplified and sequenced from 9 of 11 patients in the two study groups. The prevalence of

hemoplasmas in patients with extensive arthropod exposure and/or frequent animal contact, predominantly veterinarians, veterinary technicians, and spouses who assist veterinarians with animal restraint and care (23, 24), was significantly higher than that in patients with less animal and vector contact (25) ($P < 0.0003$). The two group A *Mycoplasma*-infected patients did not report exposure to sheep, goats, or cattle, whereas all group B patients reported exposure to sheep and variable degrees of exposure to cattle, goats, wildlife, cats, and dogs.

For the two group A *Mycoplasma*-infected patients, analysis of the 16S rRNA gene sequences identified *Mycoplasma ovis*-like organisms (with 100% homology with GenBank accession number AF338268 and 99.9% homology with GenBank accession numbers GU230144 and GU230143) in patient A1 and a previously uncharacterized *Mycoplasma* sp. in patient A2. The 295-bp 16S rRNA gene sequence from the second patient shared only 93% homology with a *Mycoplasma* sp. detected from a capybara from Brazil (GenBank accession number FJ667774) and 92.2% homology with *Mycoplasma coccoides* (GenBank accession number AY171918.1). Based upon DNA sequence analysis, eight group B patients were infected with *Mycoplasma ovis*-like organisms (with 100% homology with GenBank accession number AF338268 and 99.9% homology with GenBank accession numbers GU230144 and GU230143), and one patient was infected with “*Candidatus Mycoplasma haematoparvum*” (99.8% homology with GenBank accession number GQ129113).

All 9 patients infected with *M. ovis*-like organisms identified on the basis of the 16S rRNA gene sequence (deposited in GenBank as accession number KF313922) were also shown to be infected with *M. ovis*-like organisms based upon their RNase P sequences, which were highly similar (99.2%) to that of *M. ovis* (GenBank accession number EU078612). Unfortunately, despite repeated attempts, we were not able to obtain an RNase P gene sequence from the previously uncharacterized *Mycoplasma* sp. (as identified by a 16S rRNA gene sequence) from patient A2. The 16S rRNA gene sequence from the group B patient infected with “*Candidatus Mycoplasma haematoparvum*” (deposited in GenBank as accession number KF366443) had 100% RNase P homology with “*Candidatus Mycoplasma haematoparvum*” (GenBank accession number AY380803).

Bartonella henselae infection was previously confirmed by PCR amplification and DNA sequencing from blood, serum, or *Bartonella Alphaproteobacteria* growth medium (BAPGM) enrichment blood cultures (23, 24) in 7 of the 8 veterinary professionals found to be infected with *M. ovis* during this study. One of these seven individuals, a veterinarian’s wife, was coinfecting with *B. henselae*, *Bartonella vinsonii* subsp. *berkhoffii* genotype II, and *M. ovis*. Despite obtaining several *Bartonella* species amplicons from a blood or enrichment culture from the eighth *M. ovis*-infected veterinarian, the available DNA sequence was not adequate to determine the infecting *Bartonella* sp. Also, although testing by a BAPGM enrichment blood culture was performed in an identical manner for each of the group A patients, PCR confirmation of coinfection with a *Bartonella* sp. was not obtained for either of them (25). However, patient A1 was *B. vinsonii* subsp. *berkhoffii* genotype I seroreactive (indirect immunofluorescence assay [IFA] titer, 1:128), and patient A2 was *B. henselae* and *B. vinsonii* subsp. *berkhoffii* genotypes I and III seroreactive (titer to all three antigens, 1:64). Therefore, based upon serology, it appeared that both group A *Mycoplasma*-infected patients had been exposed to a *Bar-*

tonella sp., but active infections were not confirmed with BAPGM enrichment blood culture PCR.

DISCUSSION

Several important findings were derived from this study. First, it appears that people in the United States with extensive arthropod and animal contact are at occupationally greater risk for hemotropic *Mycoplasma* species infections, particularly with *M. ovis*, than those who are infrequently exposed to arthropods or animals. Also, compared to reports from China (19–21), the prevalences of infections in both the high-risk and low-risk populations investigated in this study were low. Currently, the source(s) and mode(s) of transmission of *M. ovis*-like organisms in U.S. veterinary professionals are unknown. Second, a veterinarian from Grenada was infected with “*Candidatus Mycoplasma haematoparvum*,” *Anaplasma platys*, and *B. henselae* (23), suggesting that concurrent infections, potentially contributing to immune suppression, may facilitate PCR detection of hemotropic *Mycoplasma* spp. “*Candidatus Mycoplasma haematoparvum*” was originally detected and characterized in the blood of a splenectomized dog from the United States with hemic neoplasia (27). Subsequently, the same “*Candidatus*” species was found in other dogs in the United States (27) and in canids from Brazil (28), Europe (8, 29–32), and Australia (33–35). Although ticks, in particular *Rhipicephalus sanguineus* (the brown dog tick), have been suggested as vectors for canine hemotropic mycoplasma transmission, there are no experimental data to support vector competence by any tick species (8, 28, 30, 32, 33, 35, 36). Interestingly, in this study, *Mycoplasma ovis*-like DNA was amplified only from blood samples, whereas “*Candidatus Mycoplasma haematoparvum*” was amplified from both blood and serum samples. Third, we obtained PCR evidence by sequencing the 16S rRNA gene to support human infection with a novel hemotropic *Mycoplasma* sp. Regarding this partially characterized *Mycoplasma* sp., additional work is necessary to determine if the bacterium is associated with a specific reservoir host and to identify the mode(s) of transmission to humans.

Interestingly, the most frequently identified *Mycoplasma* sp. in this study resembled *M. ovis*, a species that has been detected most often in ruminants, including deer, goats, sheep, and Japanese serows (37–42). Human infection with an *M. ovis*-like organism (in one of the group B patients included in this study, a veterinarian who was coinfecting with *B. henselae*) was first reported by Sykes and colleagues (9). Using newly designed PCR primers and freshly extracted DNA from that patient’s stored blood sample, we reconfirmed infection with the same *M. ovis*-like strain during this study (1,265- and 1,266-bp 16S rRNA gene identity with GenBank accession numbers GU230143 and GU230144, respectively). Unfortunately, we were not able to detect the second *M. ovis*-like strain reported by Sykes et al. (GenBank accession number GU383115) in the blood sample dated 16 August 2005, as a fresh aliquot of DNA from this blood sample was no longer available. Coinfections with an *M. ovis*-like organism and *B. henselae* were also confirmed in six additional veterinary professionals in this study (23), supporting the possibility of a common vector or environmental source of infection (24). Coinfection with *B. henselae* and *M. haemofelis* was reported previously in an HIV patient from Brazil (6). As the cat flea is vector competent for the transmission of *B. henselae* and is the proposed vector for *M. haemofelis*, studies using a highly sensitive *M. ovis*-specific PCR assay to test fleas

appear warranted. Regardless of the source or mode of transmission, veterinary professionals in the United States appear to be at risk for coinfections with *M. ovis* and *B. henselae*. Interestingly, in the previous reports involving people from other nations, the prevalences of hemotropic mycoplasma infection seem to be associated with sanitary conditions and the types of occupational groups analyzed (20, 21). Poor sanitary conditions provide a good environment for breeding of mosquitoes (suspected to play an important role in the transmission of *M. suis* among swine) and other environmental situations potentially conducive to transmission, such as contact with contaminated blood from infected swine (21). A large-scale investigation of the hemotropic mycoplasma infection prevalence in China (where 1,529 people were tested) indicated that infection rates for people with animal contact were markedly different from those for people who did not routinely work with animals (55.3% versus 16.8%, respectively) (20). Because infections with a hemotropic *Mycoplasma* sp. were documented in our study several years after blood samples were collected, because most individuals were coinfecting with *B. henselae*, and because infections were confirmed in only a small number of individuals, we could not determine if these bacteria contributed to the symptoms reported in the patient questionnaires.

Hemotropic *Mycoplasma* spp. represent emerging, zoonotic pathogens that pose poorly defined health risks for animals and humans throughout the world. The lack of *in vitro* cultivation systems for the isolation of these cell wall-deficient bacteria has hampered and delayed their detection and characterization in humans and animals. The development of new and improved molecular techniques, particularly PCR amplification and DNA sequencing, has facilitated improved diagnostic detection, as well as the recognition of several zoonotic animal hemoplasmas. This study emphasizes the need for additional research and the importance of documenting exposures to vectors and to the animals that harbor arthropods and their associated bacteria, both in individual patients and during epidemiological investigations.

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