‘Candidatus Mycoplasma haemomacaque’ and *Bartonella quintana* bacteremia in cynomolgus monkeys

**Running Title**: New *Mycoplasma* species in cynomolgus monkeys

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Abstract

We report latent infections with *Bartonella quintana* and hemotropic *Mycoplasma* spp in a research colony of cynomolgus monkeys (*Macaca fascicularis*). Sequence alignments, evolutionary analysis, and signature nucleotide sequence motifs of the hemotropic *Mycoplasma* 16SrRNA and *RNase P* genes indicate the presence of a novel organism.

**Keywords:** cynomolgus, monkeys, *Candidatus* Mycoplasma, haemomacaque

Introduction

Hemotropic *Mycoplasma* spp. (hemoplasmas) are obligate erythrocytic bacteria that infect numerous animal species, including *Homo sapiens*. Infections are often chronic and sub-clinical; however, some animals and humans develop a hemolytic anemia, particularly when stressed or immunosuppressed[1, 2]. Phylogenetic analyses of 16S rRNA gene sequences have defined two major subclusters of hemoplasmas, namely the *haemosuis* and *haemofelis* groups [3-7].

Historically, diagnosis of hemoplasma infections has relied upon cytological examination of stained blood smears. In 1994, Dillberger and colleagues described *Haemobartonella*-like parasites in five wild-caught, anemic cynomologous monkeys (*Macaca fascicularis*) that originated from the Philippines; however, the organisms were not characterized phylogenetically [8]. For some animal species, the diagnostic sensitivity of blood smear examination is very poor and unspecific [3, 4, 9]. The development of molecular assays, primarily targeting the 16SrRNA
and the ribonuclease P genes (RNase P) of these cell wall-deficient, uncultivable microbes, has resulted in recent recognition of several novel animal hemoplasmas [4, 5, 10-13].

*Bartonella* spp. are facultative intracellular bacteria that also infect erythrocytes in numerous animal species, including *Homo sapiens*. Previously, *Bartonella quintana* DNA was amplified, cloned and sequenced from lysed erythrocytes and cultured colonies grown from peripheral blood collected from a captive-bred cynomolgus monkey (*Macaca fascicularis*) [14]. *Bartonella quintana* was subsequently isolated from 2 of 36 captive rhesus macaques in China, of which 33 of 33 were *B. quintana* seroreactive [15].

Hemotropic *Mycoplasma* and *Bartonella* organisms often cause persistent, occult infection in immunocompetent hosts. The extent to which infection with these bacteria in cynomolgus monkeys involved in a research study might influence assessments or outcomes associated with drug development studies is poorly characterized. This manuscript describes PCR amplification and DNA sequence characterization of a novel hemotropic *Mycoplasma* found in the blood of 44 of 52 cynomolgus research monkeys (*Macaca fascicularis*), as well as isolation of *Bartonella quintana* from one monkey. These animals were in a chronic toxicity study, and data from the pretest phase of the study are presented. Animals were tested for *Mycoplasma* and *Bartonella* on the basis of findings in a previous toxicity study that raised the possibility of latent infections. Based on the analysis of the 16S rRNA and RNase P gene sequences, we propose 'Candidatus Mycoplasma haemomacaque' as the name for the novel hemotropic *Mycoplasma* identified in this study.
Materials and Methods

Blood from 52 cynomolgus monkeys (Macaca fascicularis) was analyzed prior to the initiation of dosing in a toxicity study for the presence of hemotropic Mycoplasma spp. and Bartonella spp. Monkeys were considered healthy on the basis of multiple pretest physical clinical evaluations, including Coomb’s test and microscopic blood smear evaluation.

Blood samples were collected in EDTA-containing vacutainers and shipped overnight to Galaxy Diagnostics, Inc. for Mycoplasma spp., Bartonella spp. and testing. Blood samples were analyzed for the presence of Mycoplasma DNA by PCR testing targeting the 16S rRNA (a 1200 bp fragment) and RNase P (a 160 bp fragment) genes as previously reported [16]. Similarly, blood samples were analyzed for the presence of Bartonella spp. using the BAPGM enrichment PCR as described previously [16-18]. DNA of naïve dog and human blood extracted at the same time and manner, were used as negative controls for PCR testing.

Results

All animals were considered healthy on the basis of pretest screening. In particular, there was no evidence of anemia, hyperbilirubinemia, or bilirubinuria. By targeting the 16S rRNA and RNase P genes, DNA of a novel hemotropic Mycoplasma was amplified from 44 of 52 (84.6%) cynomologus monkey blood samples but not from any negative controls tested. Sequence analyses of both genes identified a distinct genotype when compared with other Mycoplasma spp. sequences deposited in GenBank. When the 1164 bp nucleotide sequence of the partial 16S rRNA gene (deposited as KC512401) was compared with M. coccoides (AY171918), M. turicensis (EU789559), M. haemofelis (EU442639), M. haemocanis (AY529641), M. haemovis (EU828581), M. haematoparvum (CQ129113), and M. haemominutum
(AM691834), the novel haemoplasma from cynomolgus monkeys shared 90.9% (1058/1164) homology with ‘Candidatus M. turicensis’, followed by 90.4% (1052/1164) homology with *M. coccoides*. The nucleotide sequence homology was lower with *M. haemocanis* (87.3%), *M. haemofelis* (85.8%), *M. kahanei* (79.6%) found in squirrel monkeys [19] (AF338269), and with *M. pneumoniae* (NC_016807) (76.5%). The bootstrap percentage values are given at the nodes of the phylogenetic tree shown in Figure 1.

Similarly, when the nucleotide sequence of the partial RNase P gene obtained from *Macaca fascicularis* was compared with *M. coccoides* (GenBank accession EU078619), ‘Candidatus Mycoplasma aoti’ (HM123756), *M. iowae* (EU078608), *M. pirum* (EU078607), ‘Candidatus M. turicensis’ (EF212003), *M. haemofelis* (EU078617), *M. haemocanis* (AF407211), *M. haemovis* (EU078612), *M. haematoparvum* (AY380803), and *M. haemominutum* (AY150990), there was very low homology with other reported *Mycoplasma* spp. ‘Candidatus Mycoplasma haemomacaque’ shared 78% with ‘Candidatus Mycoplama aoti’, 74.8% homology with *M. haemofelis* and *M. haemocanis*, followed by ‘Candidatus M. turicensis’ (72.8%), *M. coccoides* (68%), *M. pirum* (66%), *M. haematoparvum* (62%), and *M. iowa* (59.2%). Phylogenetic analysis of the partial RNase P gene, including comparisons with sequences available for hemotropic *Mycoplasma* spp. are shown in Figure 2.

In addition to the novel *Mycoplasma, Bartonella quintana 16S rRNA-23S rRNA intergenic spacer region DNA* was sequenced from extracted blood, from 7 and 14 day BAPGM enrichment cultures, and from a subculture isolate [20-23] from one monkey. Sequence analysis
of Bartonella ITS region revealed a homology of 420/420 (100%) bp with Bartonella quintana (Genbank accession number L35100).

Discussion

Infection with a novel hemotropic Mycoplasma sp. was documented in 44/52 (84.6%) monkeys and B. quintana was isolated from 1/52 (1.9%) cynomolgus monkeys in a research colony.

Analysis of the haemoplasma 16S rRNA gene sequences derived from Macaca fascicularis in this study identified a 92.3% similarity to M. coccoides and 90.6% similarity to ‘Candidatus M. turicensis’. The RNase P sequence, used to discriminate among hemotropic Mycoplasma organisms, as described by Birkenheuer et al. in 2002 and Tasker et al. in 2003, revealed low similarities with other hemotropic Mycoplasma spp., including M. haemocanis and M. haemofelis, ‘Candidatus M. turicensis’, and M. coccoides. Based upon the phylogenetic analysis of DNA sequences found in these cynomolgus monkeys with other hemoplasma and non-hemotropic Mycoplasma spp., the low percentage similarities of this bacteria supports its designation as a novel hemoplasma. Based upon differences in 16S rRNA and partial RNase P gene homologies, and according to the guidelines for naming uncultivated prokaryotes [24, 25], we propose a Candidatus designation for this newly recognized macaque hemoplasma, and recommend that it be named ‘Candidatus Mycoplasma haemomacaque’.

This report represents the third time that B. quintana has been isolated from non-human primate raised in research facilities [14, 15]. Infections resulting in chronic bacteremia have also been established experimentally in rhesus macaque monkeys (Macaca mulatta) inoculated with B.
quintana isolates derived from infected humans [26, 27], supporting that non-human primates may be able to acquire *B. quintana* from humans or from other monkeys.

Hemotropic *Mycoplasma* spp. (‘hemoplasmas’, formerly classified as *Haemobartonella* and *Eperythrozoon* spp.) [4, 12, 28, 29] appear to have co-evolved with animals, including dogs, cats, humans, alpacas, capybaras, and sea lions [1, 10, 13, 30-40]. The development of molecular assays, primarily targeting the 16S rRNA gene of these microbes, has resulted in the more recent recognition of several novel animal hemoplasmas [5, 12, 37, 41]. Hemoplasmas are obligate epierythrocytic organisms that attach to erythrocytes, appear to be relatively non-pathogenic and are more often visualized on blood smears during periods of stress, hard work or concurrent infection [1, 2, 7, 10, 42, 43]. In some animals, hemoplasma infection is associated with hemolytic anemia of variable severity, ranging from non-clinical hemolysis to severe anemia [7, 40, 44]. There were no pre-test hematological or serum biochemical abnormalities associated with the novel hemotropic *Mycoplasma* or *B. quintana* in the cynomolgus monkeys in this study.

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References


Figure Legends:

Figure 1: Phylogenetic tree based on 16S rRNA gene sequences, showing the position of *Candidateus M. haemomacaque* from other hemotropic *Mycoplasmas*. Bootstrap percentages are given at the nodes of the tree.

Figure 2: Phylogenetic tree based on RNase P gene sequences, showing the position of *Candidateus M. haemomacaque* from other hemotropic *Mycoplasmas*. Bootstrap percentages are given at the nodes of the tree.
Figure 1: Phylogenetic tree based on 16S rRNA gene sequences, showing the position of ‘Candidatus M. haemomacaque’ from other hemotropic Mycoplasmas. Bootstrap percentages are given at the nodes of the tree.
Bootstrap

RNase P