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Evaluation of Sensitivity and Specificity of a *Mycoplasma haemomuris*-Specific Polymerase Chain Reaction Test

Chunbin Zhang and Yasuko Rikihisa, PhD*

***Background and Purpose*: *Mycoplasma haemomuris,* a small pleomorphic bacterium parasitic of red blood cells, often causes chronic and subclinical infection of rodents. *Mycoplasma haemomuris* is uncultivable, and a serologic testing method is not readily available. The purpose of the study reported here was to develop a sensitive and specific polymerase chain reaction (PCR) test for detection of *M. haemomuris* in blood samples.**

***Methods*: On the basis of the regions of the *M. haemomuris* 16S rRNA gene most divergent from corresponding regions of related bacteria, *M. haemomuris*-specific primers were designed so that these primers could selectively amplify *M. haemomuris* DNA. A PCR test was performed, using blood samples from BALB/c mice infected with *M. haemomuris* strains TR 8564, TR 8563, and TR 8556.**

Results*: Use of the PCR test enabled detection of *M. haemomuris* DNA in a minimum of 0.0001 μl of infected mouse blood. The test also was specific for *M. haemomuris* and did not amplify closely related species, such as *M. haemofelis*, *M. haemosuis, M. orale*, or *Anaplasma marginale.

***Conclusion*: This method is sensitive and specific for detection of *M. haemomuris*.**

Mycoplasma haemomuris is a small, uncultivable, pleomorphic, parasitic bacterium that lacks a cell wall and attaches to the surface of red blood cells of wild and laboratory rodents (1-3). The organism appears as dark blue or purple dots in Romanowsky-stained blood smears. When the infection level is low, it is difficult to differentiate these hemotrophic mycoplasmas from other basophilic structures, such as contaminated particles or cellular debris, in or on the erythrocytes. Previously, *M. haemomuris* was called *Haemobartonella muris.* Recent analysis of 16S rRNA gene sequences of *H. muris* along with *Haemobartonella felis, Eperythrozoon suis,* and *Eperythrozoon wenyonii* has led to their renaming and reclassification into the genus *Mycoplasma* (3, 4). *Mycoplasma haemomuris* is found worldwide; however, because it is uncultivable, only a few isolates have been maintained in the laboratory and molecularly and antigenically analyzed (3). Therefore, the molecular relatedness of *M. haemomuris* is visually recognized in rodents, and thus, its true geographic distribution remains unknown. *Mycoplasma haemomuris* is underrecognized because it generally does not cause overt clinical disease, and its numbers in the blood of healthy animals are below visual detection. However, once such animals are stressed, coinfected, splenectomized, or immunosuppressed, systemic disease, characterized by fever, prostration, anemia, and icterus, which sometimes may be refractory to conventional antibiotic therapy, manifests itself. The host range, mode of transmission, and antigenic and molecular characteristics of this organism are largely unknown. The availability of sensitive molecular diagnostic procedures will definitely improve identification of *M. haemomuris* infections, especially those that are latent. In the study reported here, we designed primers specific to *M. haemomuris*, and evaluated the sensitivity and specificity of the test.

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Department of Veterinary Biosciences, College of Veterinary Medicine, The Ohio State University, 1925 Coffey Road, Columbus, Ohio 43210.
**Corresponding author.*

Table 1. Sequences of primers used in the polymerase chain reaction (PCR) test

Primer name	DNA sequence (5'-3')
HM16S-1 (forward)	AGC GGA CCT CTA GCA ATA G
HM16S-2 (reverse)	AGC TAC AAC GCT GAG ACT C
HM16S-3 (forward)	TTC CTC ACA TGA GGT TGG C
HM16S-4 (reverse)	GAG CCT AAG CGT CAA TAT C

Materials and Methods

The 16S rRNA genes of *M. haemomuris* and eight species and strains most closely related to *M. haemomuris* (strain names are shown with GenBank numbers at the end of this subsection) were aligned, using DNASTAR software (DNASTAR Inc., Madison, Wis.) to compare conserved and variable regions. As shown in Table 1 and Fig. 1A, four primers, HM16S-1, HM16S-2, HM16S-3, and HM16S-4, were designed on the basis of the regions of the *M. haemomuris* 16S rRNA gene most divergent from corresponding regions of related bacteria so that these primers could selectively amplify *M. haemomuris*. Primers were synthesized at GIBCO-BRL (Gaithersburg, Md.)

Blood samples from 8-week-old BALB/c male mice infected with *M. haemomuris* strains TR 8564, TR 8563, and TR 8556 (Harlan, Indianapolis, Ind.) (3) were analyzed. The source colonies were specified to be free of mouse parvovirus, minute virus of mice, mouse hepatitis virus, Sendai virus, pneumonia virus of mice, Theiler's meningoencephalitis virus, reovirus 3, epizootic diarrhea of infant mice agent, ectromelia virus, lymphocytic choriomeningitis virus, mouse adenovirus, polyoma virus, mouse cytomegalovirus, Hantaan virus, lactate dehydrogenase-

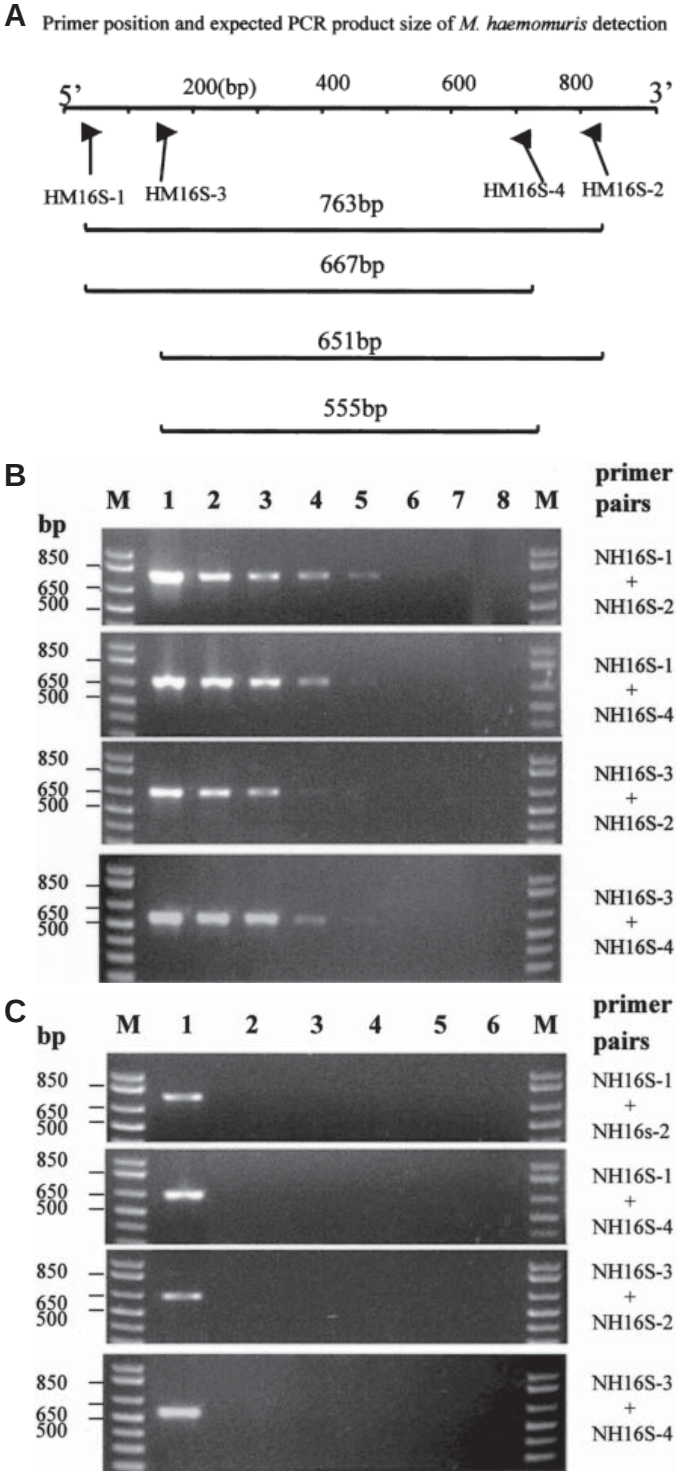

Figure 1. (A) Analyses of *Mycoplasma haemomuris*-specific polymerase chain reaction (PCR) primer positions and expected product sizes. (B) Sensitivity of *M. haemomuris*-specific PCR analysis gel electrophoresis of PCR products stained with ethidium bromide. Lanes: 1–7, serially diluted blood samples from mice infected with *M. haemomuris* TR 8564 (undiluted, 1:10, 1:100, 1:1,000, 1:10,000, 1:100,000, and 1:1,000,000 dilutions, respectively); 8, negative control (distilled water). M is a 1-Kb Plus DNA Ladder (GIBCO-BRL, Gaithersburg, Md.). (C) Specificity of *M. haemomuris*-specific PCR analysis. Lanes: 1–5, *M. haemomuris, M. haemosuis*, *M. haemofelis*, *Anaplasma marginale,* and *M. orale* DNA, respectively; 6, negative control (distilled water).

elevating virus, K virus, mouse thymic virus, *M. pulmonis,* other known pathogenic bacteria, and endo- and ectoparasites. All mice were housed in a biocontainment barrier facility at a temperature of 22 to 24°C and humidity of 40 to 60%, with 12 to 15 air exchanges/h, and a 12/12-h light/dark cycle. Use of mice for this study was approved by the Ohio State University Institutional Animal Care and Use Committee.

For each sample, 200 µl of blood was used to extract total DNA by use of a QIAamp blood kit (QIAGEN Inc., Valencia, Calif.) according to the manufacturer's protocol. The DNA was solubilized in 200 µl of TE buffer (10 m*M* Tris-HCl buffer, pH 8.4, and 1 m*M* EDTA). To determine the detection limitation of the PCR test, 1 µl each of 10-fold serially diluted DNA samples was used to perform the test. One microliter of DNA derived from 1 µl of blood containing *Anaplasma marginale* Florida (kindly provided by R. Stich, The Ohio State University, Columbus, Ohio), *M. haemofelis* Ohio, and *M. haemosuis* Taiwan (3) and 1 µl of *M. orale* (Stratagene, La Jolla, Calif.) were used to evaluate the specificity of the method.

The PCR test was carried out in a volume of 50 µl, using four primer pairs: HM16S-1/HM16S-2, HM16S-1/HM16S-4, HM16S-3/HM16S-2, and HM16S-3/HM16S-4. The 50-µl PCR mixture contained 1 µl of template DNA, 5 µl of 10X PCR buffer (10 m*M* Tris-HCl [pH8.4], 50 m*M* KCl), 2 µl of 50 m*M* $MgCl_2$, 1 µl of 10 m*M* deoxynucleoside triphosphate mixture, 1.5 U of *Taq* polymerase (GIBCO-BRL), and 8 pmol of each primer. Amplification was performed in a GeneAmp PCR system 9700 thermal cycler (Perkin-Elmer Applied Biosystems, Norwalk, Conn.), using a three-step program (94°C for three minutes; then 35 cycles of 94°C for one minute, 54°C for one minute, and 72°C for two minutes; and finally, 72°C for seven minutes). The PCR products were electrophoresed in 1% (wt/vol) agarose gel, stained with ethidium bromide, observed under UV light, and photographed by use of a still video photodocumentation system, Gel Print 2000I (Biophotonics Corp., Ann Arbor, Mich.). A 1-kb DNA ladder (GIBCO-BRL) was used as molecular size standard.

The GenBank accession numbers of the 16S rRNA gene sequences used for this study were *M. haemomuris* (U82963), *M. haemosuis* Illinois (U88565), *M. haemosuis* Zachary (AF029394), *M. wenyonii* (AF016546), *M. haemominutum* California (U88564), *M. erythrodidelphis* Illinois (AF178676), *M. haemofelis* Ohio-Florida (U88563), *M. haemofelis* Oklahoma (AF178677), and *M. haemofelis* (U95297).

Results and Discussion

On the basis of results of the GenBank search, the four primers would not anneal with any gene sequences other than the 16S RNA gene of *M. haemomuris*, including *M. pulmonis*. The PCR test using primer pairs HM16S-1/HM16S-2, HM16S-1/HM16S-4, or HM16S-3/HM16S-4 could detect up to a 1:10,000 dilution of the blood from mice infected with *M. haemomuris* TR 8564. The PCR test with primer pair HM16S-2/HM16S-3 could detect up to a 1:1,000 dilution (Fig. 1B). Because DNA was extracted from 200 µl of blood and solubilized in 200 µl of buffer, then 1 µl of the DNA was used for PCR analysis, this test could detect *M. haemomuris* in as little as 0.0001 µl of the blood. Two other strains of *M. haemomuris* gave the same results (data not shown). The four primer pairs used in the PCR test were specific for *M. haemomuris* and did not amplify closely related *M. haemofelis, M. haemosuis, M. orale,* or *A. marginale* (Fig. 1C). Blood samples from cats in-

fected with *M. haemofelis* and from pigs infected with M. haemosuis were previously used to sequence these organisms (3). *Anaplasma marginale* was visually detected in a smear of the bovine blood used in this study. *Mycoplasma orale* was used as the positive control in a Stratagen PCR test kit.

Mycoplasma haemomuris has so far been detected by microscopic observation of blood smears treated with Giemsa stain (1-3). Because microscopic observation requires fresh blood, it is subjective, has poor specificity for *M. haemomuris*, and cannot distinguish it from similar erythrocytotropic bacteria, such as *M. haemofelis, M. haemosuis, M. wenyonii*, and *Anaplasma* spp. The PCR test that requires small amounts (< 1 μl) of frozen or fresh blood samples, and was developed in the study reported here should help *M. haemomuris* diagnosis in laboratory and wild rodents.

After exposure to the infective agent, either patent infection or a carrier state might be induced; one of the determining factors is which strain of *M. haemomuris* is present. Strains recently isolated from wild mice in Japan and used for this study are extremely virulent (3). Other strains, however, have begun to be maintained in laboratories (unpublished data); therefore, comparative molecular studies of pathogenesis may become possible in the future. Availability of the *M. haemomuris*-specific PCR test would help researchers in finding various strains of *M. haemomuris. Eperythrozoon coccoides*, which infects mice and/or rats like *M. haemomuris*, was described (2). So far, the 16S rRNA gene of *E. coccoides* has not been sequenced; thus, whether this is a species distinct from *M. haemomuris* is unknown. Phylogenetically *Eperythrozoon* spp. and *Haemobartonella* spp. do not make separate clusters based on 16S rRNA gene sequence comparison (3, 4). At least two distinct species, *M. haemofelis* and *M. haemominutum* (smaller-form) of different pathogenicity are known to infect cats (4).

Although methods such as indirect fluorescent antibody tests and immunoblot (western) analysis have been developed using *M. haemomuris*-infected mouse red blood cells and red blood cell-free pure *M. haemomuris*, respectively, as antigens (3), it is not easy to make a large amount of antigens of consistent quality, because *M. haemomuris* has not been cultivated. Furthermore, because such serologic tests have not been developed for other closely related hemotrophic *Mycoplasma* spp., the specificity of the tests is unknown.

Recently, PCR methods have been developed for *M. haemofelis* and *M. haemosuis* on the basis of their 16S rRNA gene sequences (5-8). However, cross-reactivity with *M. haemomuris* was not evaluated for either PCR test. Availability of *M. haemomuris*-specific PCR test would enhance PCR diagnosis of hemotrophic *Mycoplasma* spp. in general and should help in obtaining needed information on the host ranges. Hemotrophic *Mycoplasma* spp. have been described in various nonhuman primate species (9-11). The partial 16S rRNA gene sequence of hemotrophic *Mycoplasma* sp. in squirrel monkeys recently was deposited at GenBank (AF338269), indicating that this organism is most closely related to *M. haemolama* from alpaka (GenBank No. AF306346). An ultrastructural observation has been made of *Haemobartonella*-like organisms infecting six patients with acquired immune deficiency syndrome in Brazil (12). Molecular identification of these organisms in humans has not been made. The PCR tests developed for various hemotrophic *Mycoplasma* sp. are expected to clarify whether any of these hemotrophic *Mycoplasma* sp. infecting animals cause infection in humans or there is a human-specific species or strains of hemotrophic *Mycoplasma* sp.

In the study reported here, four primer pairs were tested, and all of them were specific and reasonably sensitive, thus requiring a small amount of sample volume. For important specimens, the reliability of the *M. haemomuris* detection methods can be enhanced when two or more of the afore-described primer pairs are used and when PCR products of the expected sizes are obtained.

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