Use of Real-Time PCR To Detect and Quantify Mycoplasma haemofelis and “Candidatus Mycoplasma haemominutum” DNA

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A real-time PCR assay using Taqman probes was developed to detect and quantify Mycoplasma haemofelis and “Candidatus Mycoplasma haemominutum” in feline blood samples. The assay was rapid and sensitive and was successfully used to monitor the in vivo kinetics of cats experimentally infected with each species.

The feline hemoplasmas, Mycoplasma haemofelis and “Candidatus Mycoplasma haemominutum,” are hemotropic bacterial species that can induce anemia in cats (4, 6, 7). Conventional PCR assays are known to be more sensitive than cytotology for diagnosis of infection (3, 9). The aim of this study was to develop a real-time PCR assay to detect and accurately quantify feline hemoplasma DNA in blood samples from naturally and experimentally infected cats.

EDTA anticoagulated blood samples were obtained both from naturally and experimentally infected cats known to be infected with M. haemofelis and/or “Candidatus M. haemominutum” (n = 30) or from uninfected cats (n = 20), based on cytological examination of blood smears or the results of a previously described specific conventional PCR assay (8). DNA was extracted from 100 μl of blood using the DNeasy Tissue Kit (Qiagen, Crawley, United Kingdom) and was eluted into 200 μl of buffer AE, in accordance with the manufacturer’s instructions. Tenfold serial dilutions of genomic DNA template positive for either M. haemofelis or “Candidatus M. haemominutum” were prepared. Plasmids containing cloned 16S ribosomal DNA (rDNA) PCR products from M. haemofelis or “Candidatus M. haemominutum” were also generated. The plasmid copy number was determined by spectrophotometry and serial 10-fold dilutions prepared for PCR following linearization with EcoRI. Primers (Hf Forward, 5’-ACGAAA GTCTGATGGAGCAATA-3’; and Hf Reverse, 5’-ACGCCC AATAATTCGGRATA-3’) (Life Technologies, Paisley, United Kingdom) known to be specific for feline hemoplasmas (5, 8) were used. Taqman probes specific for either M. haemofelis (hexachloro-fluorescein-TACGAGGGATTTATG ATAGTACTCTGGTA-black hole quencher 1) or “Candidatus M. haemominutum” (6-carboxyfluorescein-AGCTTGGATA GGAAATGATTAGCCTTTGA-black hole quencher 1) were designed (purchased from Cruachem Ltd., Glasgow, United Kingdom). PCRs comprised 12.5 μl of 2× Platinum Quantitative PCR SuperMix-UDG (Invitrogen Ltd., Glasgow, United Kingdom), a 260 nM concentration of each primer, 300 nM “Candidatus M. haemominutum” probe, 200 nM M. haemofelis probe, 6 mM MgCl₂ (final concentration), 1 U of additional Platinum Taq polymerase (2.2 U per 25-μl reaction), and 2 μl of template DNA, made up to a final volume of 25 μl with water. PCR was performed using an iCycler IQ system (Bio-Rad Laboratories Ltd., Hemel Hempstead, London, United Kingdom) with an initial incubation at 50°C for 3 min and then 95°C for 2 min followed by 45 cycles of 95°C for 5 s and 58°C for 30 s. Fluorescence was detected at 530 and 570 nm at each annealing step. DNA samples from noninfected cats and water were subjected to PCR as negative controls. All samples were run in triplicate. Intra-assay precision was measured by performing 10 replicates of each of seven hemoplasma-positive genomic DNA templates in a single PCR run. Interassay precision was measured by performing eight replicates of each of three hemoplasma-positive genomic DNA templates in separate PCR runs. Coefficients of variation were calculated. To enable standardization of hemoplasma DNA to an internal control (2), a real-time PCR assay for 28S rDNA was used. Briefly, forward (5’-CGCTAATAAGGGAATGTAGCTAG G-3’) and reverse (5’-TGTCGAACCTCCAGTTCTCTG G-5’) primers were used together with a molecular beacon (Texas red-CGCGCACCCTACTGATGATGTGTTGTTGC CGCGCG-DABCYL, where stem sequences are underlined) to amplify feline 28S rDNA. PCRs consisted of 25-μl reaction mixtures containing 12.5 μl of 2× Platinum Quantitative PCR SuperMix-UDG, a 300 nM concentration of each primer, 170 nM 28S rDNA molecular beacon, 2 μl of template, and water to a final volume of 25 μl, with fluorescence data collected at 620 nm.

To assess the kinetics of M. haemofelis and “Candidatus M. haemominutum” infection in vivo, two healthy female specific-pathogen-free adult cats were inoculated intravenously with blood collected from cats chronically infected with either M. haemofelis or “Candidatus M. haemominutum.” EDTA blood samples (0.2 ml) were collected before inoculation (day 0) and at intervals of 1 to 9 days postinoculation (p.i.) and subjected to DNA extraction. Blood smears were also prepared for cytological examination with Giemsa staining, and hematocrit (HCT) was measured at defined time points (Fig. 1 and 2).
DNA samples were analyzed by both hemoplasma and 28S rDNA real-time PCR. The latter was used to standardize hemoplasma PCR threshold cycle values to a specified amount of 28S rDNA. Quantification of hemoplasma DNA was then achieved using the standard curve generated by serial dilutions of plasmid DNA template containing known copy numbers of either *M. haemofelis* or *Candidatus M. haemominutum*.

The GenBank accession numbers of the target DNA sequences used for the development of the real-time assays described above were U88564, U95297, AF178677, AF271154, and AF353617.

The assay was able to specifically detect *Candidatus M. haemominutum* and/or *M. haemofelis* in all genomic DNA samples extracted from cats known to be infected with both or each of the organisms (*n* = 30). Control samples from uninfected cats (*n* = 20) and no template water reactions were all negative. In genomic DNA samples, the efficiency of amplification of *Candidatus M. haemominutum* was 99.7% over a

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**FIG. 1.** Graph plotting standardized log$_{10}$ of *M. haemofelis* (Mhf) copy number of DNA template present per microliter of blood versus day postinoculation in a cat experimentally infected with *M. haemofelis*. Standardization was to 28S rDNA. Hematocrit (HCT) values are also shown.

**FIG. 2.** Graph plotting standardized log$_{10}$ of *Candidatus M. haemominutum* (CMhm) copy number of DNA template present per microliter of blood versus day postinoculation in a cat experimentally infected with *Candidatus M. haemominutum*. Standardization was to 28S rDNA. Hematocrit (HCT) values are also shown.
with a correlation of 0.992 and the of starting templates with a correlation of 0.994. Using plasmid DNA as template, amplification of “Candidatus M. haemominutum” showed 99.7% efficiency over a 10^8 range of starting templates with a correlation of 0.993, while M. haemofelis amplification was 103.6% efficient over a 10^9 range of starting templates with a correlation of 0.997. Thus, the efficiencies of hemoplasma DNA amplification in reactions with genomic and plasmid DNA templates are very similar, allowing accurate quantification of unknown DNA samples from the standard curves generated by amplification of plasmid DNA dilutions. The assay was able to detect 3.63 and 1.67 copies of “Candidatus M. haemominutum” and M. haemofelis template, respectively, in triplicate reactions. The intra-assay and interassay coefficients of variation ranged from 1.5 to 2.7% and 1.4 to 2.2%, respectively.

Measurement of the copy number of hemoplasma DNA in experimentally infected cats is shown in Fig. 1 and 2. The hemoplasma copy number increased rapidly following inoculation, reaching a maximum on day 18 p.i. for M. haemofelis and day 45 p.i. for “Candidatus M. haemominutum.” The M. haemofelis-infected cat developed severe hemolytic anemia (with HCT values falling to below the reference range of 0.25 to 0.45 liter/liter) on two occasions, necessitating antibiotic treatment with doxycycline. On both occasions treatment was associated with a fall in the M. haemofelis copy number and rise in HCT. The “Candidatus M. haemominutum”-infected cat showed some fluctuation in HCT values during the 1st month p.i., but values remained within the reference range throughout the study. Definitive cytological evidence of infection was seen only on blood smears collected between days 25 and 29 from the M. haemofelis-infected cat.

The real-time PCR assay described provides a reliable method for diagnosis of infection and quantification of feline hemoplasma DNA in blood samples and offers the advantages of greater speed, increased throughput, and decreased risk of false-positive results over conventional PCR. It is hoped that quantification may help the veterinarian determine the significance of a positive PCR result, which can be problematic with infections such as “Candidatus M. haemominutum” and M. haemofelis, which do not always result in disease in the host. The assay can also be used to monitor response to treatment. The differing pathogenicity of these species previously reported (3, 9) was evident in the present study by the fall in HCT that occurred in association with rises in the M. haemofelis copy number, whereas the HCT values of the “Candidatus M. haemominutum”-infected cat remained within the reference range.

To relate accurately the copy number detected in PCR to the number of organisms present per microliter of blood, the amplification efficiency of the PCR assay and the number of 16S rDNA sequences present in the genome being amplified should be considered. The M. haemofelis copy number of 3.81 × 10^6 organisms per μl of blood, suggesting that the copy number provides a good indication of the number of organisms present. True correlation between copy number and the number of organisms per microliter of blood requires quantitative culture of organisms. This is not possible for these feline hemoplasma species since they have not yet been successfully cultured.

This real-time PCR assay should provide a valuable molecular tool for the diagnosis and quantification of M. haemofelis and “Candidatus M. haemominutum” DNA in feline blood samples.

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REFERENCES