Haemotropic mycoplasma infection revealed by real-time PCR in specific pathogen-free rats

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Abstract

The presence of Mycoplasma haemomuris (haemoplasma) in blood samples collected from specific pathogen-free (SPF) laboratory rats bred in Japan was reported. Its presence was examined in Fischer 344, Sprague-Dawley (SD), and Wistar rat strains of both sexes by real-time PCR. All strains were positive for M. haemomuris infection. The 16S rRNA gene of M. haemomuris strain detected in the animals was amplified using end-point PCR. Only the entire nucleotide sequence of 16S rRNA gene of a mycoplasma strain detected in SD rats was determined and compared to those of other haemoplasmas. Our investigations suggest a wide M. haemomuris infection among the SPF rats purchased from commercial breeders in Japan.

Keywords: rat, specific pathogen free state, Mycoplasma haemomuris.

Introduction

It has long been recognised that most of the laboratory rats are carrying Mycoplasma haemomuris (formerly Bartonella muris or Haemobartonella muris). Latent infection of this haemotropic parasite (haemoplasma), an aetiological agent of infectious anaemia or splenomegaly in rodents, may undermine the validity of various animal experiments (2). Certain inbred rats, including Fischer 344 (F344), Sprague-Dawley (SD) and Wistar strains maintained in USA, Italy, and UK have been probably infected with M. haemomuris (3, 6, 8, 13). However, no examination has been documented in other countries up till now. Therefore, the presence of haemoplasma infection in specific pathogen-free (SPF) rats raised by commercial breeders in Japan was examined. Haemoplasma species have been identified solely on the basis of nucleotide sequences of the 16S rRNA or RNase P RNA genes, because of lack of appropriate means to cultivate them in vitro (7).

Material and Methods

Animals and specimens. Twenty-two anticoagulated blood samples were collected under ethyl ether anaesthesia from SPF rats of F344 (n = 2), SD (n = 18), and Wistar (n = 2) strains of both sexes. All animals were purchased from a commercial breeder in Japan, and subjected to experiments immediately after receipt, without housing in our facility. The protocol used in the present study was approved by the Animal Care and Use Committee of Iwate University (Morioka, Japan), and all animal experiments were performed in accordance with the Guidelines for Care and Use of Laboratory Animals established by the Committee. Total DNA was extracted from 200 μL of blood samples using the QIAamp DNA Blood Mini Kit (Qiagen, Germany).

Analysis. To detect haemoplasmas, haemoplasma specific PCR primers for the 16S rRNA gene were used, as described elsewhere (9). Real-time PCR was performed using a SmartCycler (Cepheid, USA) with SYBR Premix Ex Taq (TaKaRa Bio., Japan).
The reaction mixture contained: 0.2 μL of each primer (50 pmol/μL); 12.5 μL of 2X SYBR Premix Ex Taq; and distilled water to a volume of 23 μL. Finally, 200 pg of a DNA sample was added to this mixture as a template. Amplification was achieved with 40 cycles of denaturation at 95°C for 5 s, annealing at 57°C for 20 s, and elongation at 72°C for 15 s, after the initial denaturation at 94°C for 30 s.

Afterwards end-point PCR was performed to determine the nucleotide sequence of the 16S rRNA gene. The end-point PCR was conducted with 50 μL of reaction mixtures each containing: 1 μL (100 pg) of DNA solution; 0.8 μL (one unit) of Tks Gflex DNA polymerase (TaKaRa Bio., Japan); 25 μL of 2X Gflex PCR buffer; 0.2 μL (50 pmol/μL) each of the forward primer (5’-AGAGTTTGATCCTGGCTCAG-3’) and the reverse primer (5’-TACCTTGTTACGACTTA ACT-3’), and water to a final volume of 50 μL. After the initial denaturation at 94°C for 5 min, the reaction cycle was conducted 35 times with denaturation at 98°C for 10 s, annealing at 55°C for 60 s, extension at 68°C for 30 s, and final extension at 68°C for 5 min in a thermal cycler. The PCR product was fractionated on horizontal, submerged 1.0% agarose gels in TAE buffer (40 mM Tris, pH 8.0, 5 mM sodium acetate, 1 mM disodium ethylenediaminetetraacetate) at 100 volts for 30 min. After electrophoresis, the gels were stained in ethidium bromide solution (0.4 μg/mL) for 15 min and visualised under a UV transilluminator. DNA was extracted using a NucleoSpin Extract II kit (Macherey-Nagel, Germany) and was subjected to a direct sequencing in a 3500 Genetic Analyzer (Applied Biosystems, USA).

To identify the haemoplasma species, the 16S rRNA gene sequence of a haemoplasma strain detected in the SD rat was compared to other haemoplasma sequences from the DNA database using the Clustal W software (12). Phylogenetic tree generated using the neighbour-joining method (11) from a distance matrix was corrected for nucleotide substitutions using the Kimura two-parameter model (5).

Results

All the samples collected from the SPF rats were positive by the real-time PCR using haemoplasma-specific primers. Melting temperature of the PCR products was shown as a single peak at 85.92 ±0.16°C (Fig. 1). Using the end-point PCR, a band of approximately 1 kbp was produced from haemoplasmas detected in the SD rats by agarose gel electrophoresis (data not shown). The nucleotide sequences of the 16S rRNA gene from haemoplasma strains detected in SD rats of both sexes were successfully determined, though nucleotide sequencing of other strains was incomplete due to unknown reason. Both the nucleotide sequences from the two haemoplasma strains, SD001 detected in a male rat and SD002 in a female rat, were identical (100% similarity) in duplicate sequencings. No sex difference was noted.

The phylogenetic tree indicated that the haemoplasma strains detected in SD rats belonged to M. haemomuris (Fig. 2). The 16S rRNA gene nucleotide sequences of haemoplasma strains detected in the SD rat had 98.9% identity to that of M. haemomuris (10), which was supported by a high bootstrap value of 1000 in a phylogenetic tree. The SD002 sequence for the phylogenetic analysis was used because it is identical to SD001, and it identified them as M. haemomuris.
Fig. 2. Comprehensive neighbour-joining phylogenetic tree generated by the nucleotide sequences of 16S rRNA genes showing the evolutionary relationship among haemoplasmas and a strain (SD002) detected in a SD rat. The SD002 sequence was used because it is identical to that of SD001 in this tree. Accession numbers are shown in a parenthesis. Mycoplasma fermentans PG18, represented by FJ226561, was included as an out-group. The data was re-sampled 1000 times to generate bootstrap values indicated at the branch points. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances that were used to infer the phylogenetic tree (scale bar, 0.1 nucleotide substitutions per site).

Discussion

Haemoplasmas in SPF laboratory animals have been overlooked for a long time because they are uncultivable. Haemoplasma infection causes anaemia by immune-mediated haemolysis in host animals and alters host immune system by inducing autoimmune condition by mimicking the host cell membrane (3). Thus, it is necessary to examine haemoplasma infection prior to immunological study in SPF rats, but the investigation has been hampered by the lack of appropriate diagnostic procedures. Diagnosis of haemoplasma infection depended on cytological identification of the organisms on blood smears; however, this method has a low sensitivity and may misidentify the haemoplasmas as Howell-Jolly or Heinz bodies.

Although the infection route was unknown, blood-feeding arthropods, including a louse, have been considered to be the most probable source (4). However, the possibility of transplacental transmission remains as an open question because haemoplasma infections were reported in rat colonies free of ectoparasites (1, 3). In the current investigation, using haemoplasma-specific real-time PCR (9), SPF rats upon receipt from an accredited Japanese breeder facilitated with a strict barrier system, which can eliminate possible transmission by arthropod vectors, were examined.

To identify the haemoplasma species, the 16S rRNA gene sequence of a haemoplasma strain detected in the SD rat was compared to other haemoplasma sequences from the DNA database using Clustal W (12). A phylogenetic tree (Fig. 2) was generated using the neighbour-joining method (11) from a distance matrix corrected for nucleotide substitutions using the Kimura two-parameter model (5). The 16S rRNA gene nucleotide sequences of haemoplasma strains detected in the SD rat had 98.9% identity to that of M. haemomuris (10), which was supported by a high bootstrap value of 1000 in a phylogenetic tree. The SD002 in the tree was used, because SD001 and SD002 were considered to be the same strain derived from SPF inbred rats, which have been expected to be identical or no significantly different.

The real-time PCR to detect a haemoplasma infection in SPF rats bred in Japan, was applied. The phylogenetic analysis of the 16S rRNA gene demonstrated that a haemoplasma strain in SPF rats was identified as M. haemomuris. The nucleotide
sequence of the 16S rRNA gene of a haemoplasma strain detected in SD rats has been deposited to the DNA database under the accession number AB820289. Although, the determination of the nucleotide sequence of the 16S rRNA gene of a haemoplasma strain detected in the SD strain was only successful, F344 and Wistar strains may also be infected with the same strain due to almost the same Tm value obtained in the melting experiments. In conclusion, haemotropic mycoplasma infections in SPF laboratory rats, which may influence the results of animal experiments, were confirmed. The haemoplasma examination by real-time PCR prior to animal experiments using SPF inbred rats, is recommended.

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References