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Short communication

Detection of *Ehrlichia muris* DNA from sika deer (*Cervus nippon yesoensis*) in Hokkaido, Japan

Chie Tamamoto [a], Nobutaka Seino [a], Masatsugu Suzuki [b], Koichi Kaji [c], Hiroshi Takahashi [d], Hisashi Inokuma [a,*]

[a] *Department of Veterinary Clinical Science, Obihiro University of Veterinary Medicine & Agriculture, Obihiro 080-8555, Japan*
[b] *Faculty of Applied Biological Sciences, Gifu University1-1 Yanagido, Gifu 501-1193, Japan*
[c] *Faculty of Agriculture, Tokyo University of Agriculture and Technology, Tokyo 183-8509, Japan*
[d] *Kansai Research Center, Forestry and Forest Products Research Institute, Kyoto 612-0855, Japan*

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Abstract

Ehrlichia muris DNA was detected in the blood of sika deer (*Cervus nippon yesoensis*) by species-specific PCR based on the citrate synthase gene, which was shown to be more sensitive than species-specific PCR based on the 16S rRNA gene. Among 102 deer examined, one deer was positive. Deer may be a possible mammalian reservoir of *E. muris*.

Keywords: *Ehrlichia muris*; *Cervus nippon yesoensis*; Species-specific PCR

1. Introduction

Ehrlichia are small, gram-negative, pleomorphic, obligatory intracellular bacteria that primarily infect leukocytes (Ristic and Huxsoll, 1984; Rikihisa, 1991) and cause disease of varying severity in humans and in some domestic and wild animals. Based on genetic analyses of 16S rRNA gene sequences, the genus *Ehrlichia* includes *E. canis*, *E. chaffeensis*, *E. ewingii*, *E. ruminantium*, and *E. muris* (Dumler et al., 2001). An *Ehrlichia* species isolated from *Ixodes ovatus*, which has the highest degree of homology to *E. chaffeensis* and the second highest degree of homology to *E. muris*, is also a member of the genus (Shibata et al., 2000). *E. muris*, a relatively recently described *Ehrlichia* species found in murine hosts, is known to induce mild non-specific clinical signs and splenomegaly in experimentally infected mice (Kawahara et al., 1993). *E. muris*, which is closely related to *E. chaffeensis*, is thought to be transmitted by *Haemaphysalis flava* and *Ixodes persulcatus*, and is widely distributed in mainland Japan (Kawahara et al., 1999; Muramatsu et al., 2005; Inokuma et al., 2007). Recently the pathogen has also been detected from ticks and mice in Russia (Shapynov et al., 2004, 2006; Rar et al., 2005) and Slovakia (Smetanova et al., 2007). Although antibodies against *E. muris* have been detected in various domestic and wild animals in Japan, including mice, deer, monkeys, boars, bears and dogs, the pathogenicity of *E. muris* to these animals is still unknown (Kawahara et al., 1999; Watanabe et al. (2004)). The animal reservoir for *E. muris* is also unclear, although the pathogen has repeatedly been detected in mice.

As deer are often infested with ticks in the wild (Inokuma et al., 2002b), they can be an important animal reservoir of tick-borne pathogens. For example, deer are considered to be an important reservoir of

* Corresponding author. Tel.: +81 155 49 5370; fax: +81 155 49 5370.
E-mail address: inokuma@obihiro.ac.jp (H. Inokuma).

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E. chaffeensis, the causative agent of human monocytic ehrlichiosis (Lockhart et al., 1997). However, the relationship between *E. muris* and deer is unclear. In this study, to clarify the role of sika deer as an animal reservoir of *E. muris*, we attempted to detect *E. muris* DNA from peripheral blood obtained from wild sika deer (*Cervus nippon yesoensis*) in Hokkaido using PCR based on the citrate synthase gene (*gltA*).

2. Materials and methods

2.1. Sika deer and E. muris

A total of 102 sika deer (*Cervus nippon yesoensis*) were taken by hunting on Nakanoshima Island of Lake Toya, Hokkaido, Japan, from March 2004 to January 2005. Blood samples were collected from each deer as soon as possible after death and kept at −20 °C until further analysis. The QIAamp DNA Mini Kit was used to extract DNA from whole blood samples. DNA samples extracted from deer were stored at −20 °C in 200 ml of TE buffer until further use. DH82 cells infected with *E. muris* were kindly supplied by Dr. M. Kawahara, and DNA extracted from these cells was used as a positive control.

2.2. E. muris-specific PCR

Detection of DNA fragments of *E. muris* was attempted using two species-specific PCR reactions, the first based on the 16S rRNA gene, and the second on the *gltA* gene. The primer set MURIS and GA1UR, previously reported to be *E. muris*-specific (Inokuma et al., 2001a), was used to amplify the *E. muris* 16S rRNA gene. This PCR reaction produces a 413-bp fragment of the 16S rRNA gene with E. muris DNA. A set of primers was designed to amplify the *E. muris gltA* gene, EmCS638F (TAC-AGA-TTT-CTC-AAG-AAT-ATA-CA) and EmCS1349R (AAT-GCA-ATG-TTT-TCT-AAT-TCT-AC). The PCR amplification was performed in a 25-ml reaction mixture containing 5 ml of DNA template. The PCR was carried out under the following conditions: 35 cycles of denaturation (94 °C, 60 s), annealing (55 °C for 16S rRNA and 57 °C for *gltA*, 60 s) and extension (72 °C, 90 s). PCR products were electrophoresed at 100 V in 1.5% agarose gels (Wako Chemicals Ltd., Japan) for 30 min, stained with ethidium bromide, and observed by UV illumination. The specificity of the two PCR reactions was tested using DNA extracted from the following related *Ehrlichia* and *Anaplasma* species: *E. muris*, *Ehrlichia* detected from *Ixodes ovatus* (supplied by Dr. H. Fujita), *E. canis* (supplied by Dr. S. Harrus), *E. chaffeensis* (supplied by Dr. P. Brouqui), *A. platys* (Inokuma et al., 2002a), *A. centrale* (Inokuma et al., 2001b), *A. phagocytophilum* (supplied by Dr. P. Brouqui) and *Neorickettsia sennetsu* (supplied by Dr. P. Brouqui). The sensitivity of the two PCR reactions was evaluated using a dilution of *E. muris* DNA in distilled water (DW).

2.3. Analysis of PCR products

To confirm PCR results, the PCR product was purified using the Qiaquick PCR purification kit (QIAGEN) for direct sequence analysis using the method previously reported (Inokuma et al., 2007). The nucleotide sequences were confirmed by additional two independent PCR and sequence analysis. The sequence obtained was compared with other related sequences registered in GenBank.

3. Results and discussion

Based upon alignment data of the *gltA* gene from *Ehrlichia* and *Anaplasma* species, a PCR primer set was designed to specifically amplify *E. muris* DNA. The PCR reaction produced a 712-bp fragment of the *gltA* gene with positive control *E. muris* DNA. No amplification was observed using DNA from other related *Ehrlichia* and *Anaplasma* species, including the most closely related species *E. chaffeensis* and *Ehrlichia* sp. detected from *I. ovatus* (Fig. 1A). With this set of primers, *E. muris* DNA diluted at 1:10,000 with DW was detected (Fig. 1B). This was 100 times more sensitive than the 16S rRNA gene-based PCR (Fig. 1C) that was previously reported (Inokuma et al., 2001a). These findings suggest that the PCR may be used to detect *E. muris* DNA specifically. Because only one strain of *E. muris* was used in the present study, more strain variants should be examined to evaluate the specificity of the PCR. However, the species-specific PCR based on the citrate synthase gene, which was shown to be more sensitive than species-specific PCR based on the 16S rRNA gene, could be a useful tool for epidemiological study for *Ehrlichia* species.

Among 102 deer examined, one sample showed a positive band with *gltA*-based PCR, and no positive bands were seen with 16S rRNA-based PCR. Analysis of the sequence of the positive PCR product (excluding the primer region) revealed that it was most closely related to the *E. muris* sequence (AF 304144) that was used as a positive control in this study, with two nucleotide differences out of the 644 base pair sequence