Experimental inoculation of Beagle dogs with *Ehrlichia* species detected from *Ixodes ovatus*

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Abstract

Three beagle dogs were inoculated with mice spleen/liver homogenate infected with *Ehrlichia* species detected from *Ixodes ovatus* (EIO) and one dog was used as a control. All three infected dogs did not show clinical signs of disease except for mild pyrexia throughout the 41-day study period. Splenomegaly was observed from day 7 post-inoculation (p.i) in two of the dogs. Hematological and biochemical abnormalities included mild thrombocytopenia, hypoproteinaemia, hypoalbuminaemia and increased C-reactive protein values. One of the dogs' splenic aspirate sample was PCR-positive for *Ehrlichia* day 7 p.i and another dogs' blood and bone marrow aspirate sample was PCR-positive day 41 p.i. Sequence analysis of the PCR products showed 100% homology with the 16SrRNA partial gene sequence of *Ehrlichia* sp. HF565. Antibody titers to EIO were observed in all three experimentally infected dogs starting from the first week p.i and cross-reactivity with *Ehrlichia canis* was detectable in one of the dogs starting day 7 p.i. These data suggest that infection of dogs with EIO is possible, though is probably of low pathogenic importance. Cross-reactivity of EIO infected dog serum with *E. canis* raises the likelihood of false *E. canis* seropositive dogs.

1. Introduction

Ehrlichia are small, gram-negative, pleomorphic, obligatory intracellular bacteria that primarily infect leukocytes (Ristic et al., 1984; Rikihisa, 1991) and cause disease of varying severity in humans, some domestic and wild animals. The genus *Ehrlichia,* based on the 16S rRNA gene sequence, includes *Ehrlichia canis, Ehrlichia chaffeensis, Ehrlichia ewingii, Ehrlichia ruminantium* and *Ehrlichia muris* (Dumler et al., 2001). *Ehrlichia* species detected from *Ixodes ovatus* (EIO) which was first isolated in Japan in 1994 (Fujita and Watanabe, 1994) has not yet been included in this genus though analysis of the 16SrRNA gene sequence revealed greatest homology to *E. chaffeensis* and then to *E. muris* (Shibata et al., 2000). *E. canis* is a well recognized pathogen of dogs, but in Japan there has only been one confirmed case of *E. canis* (Suto et al., 2001). *E. chaffeensis* is the aetiologic agent of human monocytotropic ehrlichiosis, a severe, life-threatening, emerging infectious disease of humans which was first reported in the United States in 1987 (Maeda et al., 1987). *E. muris,* a relatively new *Ehrlichia,* is a pathogen of mice and induces mild non-specific clinical signs and splenomegaly in experimentally infected mice (Kawahara et al., 1993). The EIO is more pathogenic and causes mortality in experimentally infected mice, 8-10 days post infection (Shibata et al., 2000). Both *E. muris* and the EIO, are as of yet not known to cause disease in dogs, however since their suspected tick vectors, *Haemaphysalis flava* and *Ixodes ovatus,* respectively, are widespread throughout mainland Japan (Yamaguchi et al., 1971), the possibility of infection is very high. Dogs seropositive for *E. muris* in Gifu, Ibaraki (Kawahara et al., 1993) and Yamaguchi prefectures (Watanabe et al., 2004) have been previously reported. So far, only one EIO PCR positive dog has been identified in Japan (Inokuma et al.,

2001). However a number of EIO seropositive dogs were detected in Yamaguchi prefecture and its surrounding areas (Watanabe et al., 2004). In the study conducted by Watanabe et al (2004), considerable cross-reactivity was seen and a lot of samples reacted with *E. canis.* This could create confusion when trying to make a definitive diagnosis and when trying to pinpoint the aetiological agent in ehrlichiosis cases. Since the possibility of infection with the domestic *Ehrlichia* has been established, it is now necessary to clarify the pathogenesis in dogs. Experimental infection of dogs with *E. muris* did not produce any disease (Wen et a., 1995), however there have been no similar experiments conducted with EIO. It is also imperative to ascertain the zoonotic factor as EIO is closely related to *E. chaffeensis.* The aim of this study was to determine the pathogenesis of EIO in experimentally infected dogs and to measure antibody titers and assess cross-reactivity with *E. canis.*

2. Materials and methods

2.1. Ehrlichia

Four BALB/c mice were inoculated intraperitoneally with 0.2ml of mice spleen infected with EIO (strain HF 652, supplied by Dr. Hiromi Fujita, Ohara Hospital, Fukushima Prefecture) homogenized in 10% w/v sucrose-phosphate-glutamate (SPG) buffer (0.218 mol/L sucrose, 0.0038 mol/L KH_2PO_4, 0.0072mol/L K_2HPO_4, 0.0049 mol/L monosodium glutamic acid, pH 7.0). The mice were sacrificed on day 8 post-inoculation and the livers and spleens were harvested. The organs were weighed and ground *in toto* at a 10% w/v concentration in SPG on ice and inoculated into the dogs on the same day.

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2.2. Inoculation of dogs with EIO

Four mature beagle dogs, one male and three females (1 to 2 years of age, 9 to 12 kg) were purchased. They were housed indoors and maintained according to the Yamaguchi University Animal Care and Use Committee regulations. A one week acclimatization period was given during which time clinical signs, food consumption and rectal temperatures were monitored daily. One week prior to inoculation and on the day of inoculation each dog was subjected to a physical examination and chest and abdominal radiographs. Three milliliters of blood were obtained from the cephalic vein of each dog. Complete blood counts and blood chemistry analysis were performed using the heparin anti-coagulated blood and EDTA anti-coagulated blood was used for PCR. Serum obtained was subjected to the indirect immunofluorescence assay (IFA) to test for antibodies to EIO and *E. canis.* All dogs were clinically healthy, PCR negative for *Ehrlichia,* and sero-negative for EIO and *E. canis* antibodies. All hematological and clinical chemistry parameters fell into the normal range (Aeillo et al., 1998).

After the acclimatization period, 3 dogs were inoculated intraperitoneally with 2ml/kg of 10% w/v EIO infected fresh mice spleen/liver homogenate in SPG. One control dog was inoculated with 2ml/kg of 10% w/v uninfected mice spleen/liver homogenate in SPG. Clinical signs and food consumption were monitored daily and rectal temperatures were recorded each time blood was collected. Blood from the cephalic vein was collected in EDTA tubes daily for the first week, then twice a week till day 41. Each blood sample was subjected to hematological and biochemical analysis (C-reactive protein) and PCR. Peripheral blood smears were examined for evidence of platelet aggregation. Sera were collected and IFA was conducted on each serum sample.

Serum albumin was measured once a week. The spleen was aspirated one week prior to inoculation and then on day 7, 24 and 41 post-inoculation (p.i). Bone marrow aspiration was conducted one week prior to inoculation and then day 41 p.i. The aspirated material was then subjected to PCR. Abdominal radiographs were taken day 7, 13, 17, 24, 31 and 38 p.i.

2.3. DNA Extraction and PCR Amplification

Total DNA was extracted from each blood, bone marrow and spleen sample using the QIAamp DNA Mini kit (QIAGEN GmbH, Hilden, Germany), placed in 200 µl of TE (Tris-EDTA) buffer, and stored at -20ºC until further use. For screening purposes PCR amplification was performed with a 25-µl reaction mixture containing 5 µl of each DNA template with a set of primers, EHR16SD (5´-GGT-ACC-YAC-AGA-AGA-AGT-CC-3´) and EHR16SR (5´-TAG-CAC-TCA-TCG-TTT-ACA-GC-3´). This primer set amplifies a 345-bp fragment of the 16SrRNA gene of bacteria within the family *Anaplasmataceae* including the genera *Anaplasma, Ehrlichia, Neoricketssia* and *Wolbachia* (Parola et al., 2000). In each test, distilled water and *Anaplasma platys* DNA were used as the negative and positive control, respectively. The amplification was performed in a Gene Amp PCR system 9700 (Applied Biosystems, CA, U.S.A) with the following program: an initial 5 min denaturation at 95ºC; 34 repeated cycles of denaturation (95ºC for 30s), annealing (55ºC for 30s), and extension (72ºC for 90s); followed by a 5 min extension at 72ºC. The amplification products were visualized on a 2% agarose gel after electrophoretic migration of 7 µl of amplified material.

2.4. Sequence determination

Samples positive in the screening PCR were then subjected to another PCR using the primer set of universal fD1 (5'-AGA-GTT-TGA-TCC-TGG-CTC-AG-3') (Weisburg et al., 1991) and *Ehrlichia* genus-specific EHR16SR (5´-TAG-CAC-TCA-TCG-TTT-ACA-GC-3´) (Parola et al., 2000). These primers amplify 760bp of the partial sequence of ehrlichial 16SrRNA gene, including the divergent region. Five microliters of each sample was used as the template DNA in a final volume of 25 µl and amplification was performed using the same method as described for the screening PCR. The primers for the second step PCR were fD2 (5'-ATC-CTG-GCT-CAG-AAC-CGA-ACG-3') and the *Ehrlichia* genus-specific reverse primer of GA1UR (5'-GAG-TTT-GCC-GGG-ACT-TCT-TCT-3') (Warner et al., 1996). These primers amplify a 400bp fragment of the ehrlichial 16SrRNA gene, including the divergent region. The first PCR product from each sample was diluted 1 to 10 with distilled water and 5 µl of the diluted solution was used as the template DNA for the second PCR in a final volume of 25 µl. The amplification parameters were the same as for the first PCR except that the number of cycles was set at 40. For both the first and second PCR, distilled water and *Anaplasma platys* DNA were used as the negative and positive control, respectively.

The amplification products were then extracted with the QIAPCR purification kit (QIAGEN) for sequence analysis. Fluorescence-labeled dideoxynucleotide technology was used for the DNA sequencing reaction (Perkin-Elmer, Applied Biosystems Division, Foster City, CA, USA). Samples were then sequenced using a Perkin-Elmer ABI Prism 377 automated DNA sequencer at the DNA Core Facility of the Center for Gene

Research, Yamaguchi University. The sequence data of the PCR products were analyzed by BLAST for the homology search.

2.5. Indirect IFA

IFA antigen slides were routinely prepared (Brouqui et al., 1994) using DH82 cells infected with *E. canis* (Israel strain, supplied by Dr. Harrus, The Hebrew University of Jerusalem) and mice spleen infected with EIO (strain HF 652, supplied by Dr. Hiromi Fujita, Ohara Hospital, Fukushima Prefecture). Sera were screened at a 1:20 dilution in phosphate-buffered saline (pH 7.4) Tween 0.5% (PBST) and an optimized dilution (1:200) of fluorescein isothiocyanate-labelled IgG conjugate in PBST was used as the secondary antibody. Reactive antibodies were then detected using a fluorescence light microscope. Those samples that reacted with *E. canis* or EIO antigen at the screening dilution were then titrated using serial twofold dilutions to determine end titers. The positive and negative controls used were *E. canis* positive sera from a naturally infected dog, EIO positive sera from an experimentally infected mouse and PBST, respectively.

3. Results

3.1. Clinical manifestation and radiological examination

Except for mild transient pyrexia, no clinical signs were detected in all three dogs throughout the 41 day observation period. A febrile response was noted Days 20 (39.7ºC) and 24 (39.6ºC) p.i in Dog 3, Days -7 (39.6), 24 (39.5ºC) and 31 (39.7ºC) p.i in Dog 2 and Day 24 (39.8ºC) p.i in Dog 1. The control dog was clinically normal and had

a normal rectal temperature throughout the study period. Abdominal radiographs revealed splenomegaly in two of the three dogs infected with EIO. Dogs 2 and 3 showed splenomegaly on Days 7, 13 and 17 p.i from which point on Dog 3 had a normal spleen size whereas Dog 2 continued to show splenomegaly even on the final radiological examination. Dog 1 and the control dog had a constant spleen size throughout the study period.

3.2. Hematology and Clinical Biochemistry

Thrombocytopaenia was observed in Dog 3 beginning from Day 1 till Day 17 p.i, Day 17 and 38 p.i in Dog 1 and Dog 2 showed a low platelet count on Day -7 p.i (Figure 1A). As can be seen in Figure 1B, increased CRP values were consistently observed in all experimentally infected dogs during the first five days p.i and further high CRP values were seen during the course of the experiment. Other notable biochemical abnormalities included a mild hypoproteinaemia, that was observed in Dog 3 on Days 1 (5.8g/dl), 3 (5.8g/dl), 24 (5.6g/dl), 27 (5.6g/dl) and 38 (5.9g/dl) p.i., and a mild hypoalbuminaemia also in Dog 3 on Day 24 (2.5g/dl) p.i. The other two dogs and the control, however, did not have abnormal plasma protein or serum albumin levels at any point during the study.

3.3. PCR amplification of EIO DNA from dog blood

EIO was amplified from dog-blood, spleen and bone marrow samples by screening PCR (Table 1). Splenic aspiration sample for Dog 3 was PCR-positive Day 7 p.i but the following splenic aspiration sample was negative. Blood and bone marrow aspiration samples of Dog 1 were PCR-positive Day 41 p.i. Due to this finding on the

last day of the study, a subsequent blood sample was taken from Dog 1, Day 51 p.i which was PCR-negative. Dog 2 and the control dog were negative throughout the study period.

3.4. Sequence determination

Sequence analysis of all three PCR positive samples showed 100% homology with the 16SrRNA partial gene sequence of *Ehrlichia* species HF565 (GenBank accession number AB024928).

3.5. Indirect IFA

The control dog lacked detectable titers (titer < 1:20) to both EIO and *E. canis* antigen throughout the study period. Positive titers to EIO were detected in all three experimentally infected dogs, starting from Day 5 p.i (1:20) in Dog 1 and Day 6 p.i (1:20) in Dogs 2 and 3 (Table 1). Anti-EIO antibodies were observed in all three dogs up until Day 41, the last day of the study. The highest titers measured were 1:40 in Dogs 1 and 2, and 1:80 in Dog 3. Positive titers to *E. canis* were only detected in Dog 3 starting from Day 7 p.i up till Day 41 p.i. The highest measured titer was 1:40 which was obtained Day 10 p.i and this titer remained constant till Day 41 p.i, the last day of the study (Table 1).

4. Discussion

There is very little information about the pathogenicity of the recently discovered ehrlichial organisms, *E. muris* and EIO, to other animals apart from mice. Only one study has been conducted to determine the pathogenicity of *Ehrlichia muris* to dogs,

which showed the inability of *Ehrlichia muris* to establish an infection in experimentally infected German Shepard dogs (Wen et al., 1995). In this same study IFA test titers were negative throughout the 5 weeks of the experiment except at 3 weeks post-infection. However, there is no information about the effect of EIO in experimentally infected dogs. This is the first time the pathogenesis of EIO in dogs has been studied.

In this study, dogs that were experimentally infected with EIO did not show any clinical signs of disease except for mild pyrexia. Splenomegaly, often seen in acute cases of canine monocytic ehrlichiosis (Lappin, 1997; Kelly, 1999; Castro et al., 2004; Harrus et al., 2004) was observed in two of the experimentally infected dogs. Mild, transient thrombocytopenia was evident in two out of the three experimentally infected dogs but was prominent in only one of the dogs. Thrombocytopenia is considered to be the most consistent and common laboratory abnormality of dogs infected with *E. canis* (Harrus et al., 1999; Kelly, 1999) and mild thrombocytopenia is one of the most reliable parameters to judge possible subclinical ehrlichial infection (Waner et al., 1997). In chronic *E. canis* infections, it is believed that thrombocytopenia is most probably due to bone marrow hypoplasia but in other stages, possible reasons include the production of anti-platelet antibodies (Harrus et al., 1996) and decreased half-life of platelets and a subsequent increase in platelet destruction by the spleen (Smith et al., 1975; Pierce et al., 1977).

Common biochemical abnormalities in canine ehrlichiosis cases include; hypoalbuminemia, hyperglobulinemia and hypergammaglobulinemia (Harrus et al., 1996; Harrus et al., 1997; Harrus et al., 1999). In this experiment, however, these abnormalities were not apparent as only one dog showed very mild hypoproteinaemia and hypoalbuminaemia around week 2 post-inoculation. Previous studies have shown that C-

reactive protein, a β-globulin, increased gradually from day 6 post-infection to reach a peak before declining in dogs experimentally infected with *E. canis* (Shimada et al., 2002). We observed high CRP values in all dogs the first five days after inoculation with EIO, and though the control dog also showed an increase in CRP, the value was not as high and did not persist for as long. High CRP values were detected in an inconsistent manner in one of the dogs (Dog 3).

Interestingly, one of the dogs' splenic aspirate PCR was positive on Day 7 p.i at which point antibodies against EIO and *E.canis* were also seen. It has been shown that splenic aspirate PCR is superior to blood PCR for the evaluation of ehrlichial elimination as ehrlichial DNA could still be detected in dogs, experimentally infected with *E. canis* and then treated with doxycycline, when blood samples were negative (Harrus et al., 2004). Therefore the fact that subsequent splenic aspirate samples from the dog were negative may indicate successful elimination of the ehrlichial organism. Another dog's (Dog 1) blood and bone marrow aspiration sample were PCR positive on Day 41 p.i but a blood sample taken 10 days later was PCR negative. The result of the IFA test conducted on this sample was negative. The reason for this late detection of ehrlichial DNA requires further investigation.

Sequence analysis was performed to confirm that the PCR positive samples were indeed EIO. The results revealed 100% homology with the determined partial sequence of the 16SrRNA gene of *Ehrlichia* species HF565 (GenBank accession number AB024928). Of the 1,449-bp 16SrRNA gene sequences compared in a study by Shibata et al (2004), all HF strains, including strain HF652 used in this study, had identical base sequences.

All three experimentally infected dogs developed antibodies against EIO, that were first detectable in the first week following inoculation and persisted till the last day of the study. More interestingly, however, was the cross-reactivity with *E. canis* observed via IFA in one dog (Dog 3) starting from Day 7 p.i (titer of 1:20). The following test day, the IFA titer was 1:40 and sera remained consistently at this titer till the last day of the study. In this experiment *E. canis* antigen was used because it is common practice to test for antibodies against *E. canis* when canine ehrlichiosis is suspected. Antibodies against *E.canis* have been detected in dogs in Japan (Inokuma et al., 1998, Inokuma et al., 1999 and Yamamoto et al., 1994) but only one clinical case of *E.canis* infection in a dog has been reported (Suto et al., 2001). Therefore rather than the possibility of *E. canis* having established itself in Japan, it is more likely that the domestic *Ehrlichia* are causing cross-reactivity with *E. canis* antigen. This report confirms that serum of dogs infected with EIO will react and show positive seroreactivity against *E. canis* which may lead to false seropositives. It is vital now in Japan to suspect possible infection with the domestic ehrlichia when IFA results indicate *E.canis* seropositive dogs.

It was observed that results varied between the experimentally infected dogs. This may be due to individual susceptibility of the dogs as well as differences in the quantity of ehrlichial organisms inoculated into each dog. A cell line infected with EIO has, as of yet, not been established and thus quantification of the ehrlichial organisms is difficult.

From the results it can be concluded that EIO is of low pathogenic importance to dogs, however the findings suggest that subclinical infection may be possible and thus

infections with this new ehrlichia in dogs may occur more frequently in Japan than recognized. Most importantly this study has shown that dogs infected with EIO will seroreact with *E. canis* antigen in IFA and therefore is likely to cause confusion in diagnosis making. Clinicians in Japan should be aware of this new ehrlichia and the role it may play in disease dynamics.

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Figure captions

Fig. 1 Platelet count (A) and C-reactive protein values (B) of the dogs throughout the 41-day study period. The solid horizontal line represents the lower limit for normal blood platelet concentrations (i.e., 200,000 per μl) in A, and the solid horizontal line represents the upper limit for normal C-reactive protein concentrations (i.e., 1 mg/dl) in B.

Table 1.
Results of antibody titers against EIO and *Ehrlichia canis* and genus-specific PCR for *Ehrlichia* in dogs inoculated with *Ehrlichia* species detected from *Ixodes ovatus*

ID	Days Post-Inoculation																		
	-7	0	1	2	3	4	5	6	7	10	13	17	20	24	27	31	34	38	41
IFA Titers to EIO Ag																			
3	-	-	-	-	-	-	-	20	40	40	40	40	40	40	80	80	80	40	40
2	-	-	-	-	-	-	-	20	40	40	40	40	20	20	20	20	20	20	20
1	-	-	-	-	-	-	20	40	40	40	40	20	20	20	20	20	20	20	20
Con	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
IFA Titers to *E.canis* Ag																			
3	-	-	-	-	-	-	-	-	-	20	40	40	40	40	40	40	40	40	40
2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Con	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
PCR																			
3	-	-	-	-	-	-	-	-	-	+a	-	-	-	-	-	-	-	-	-
2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+b
Con	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

a: positive from spleen aspirate
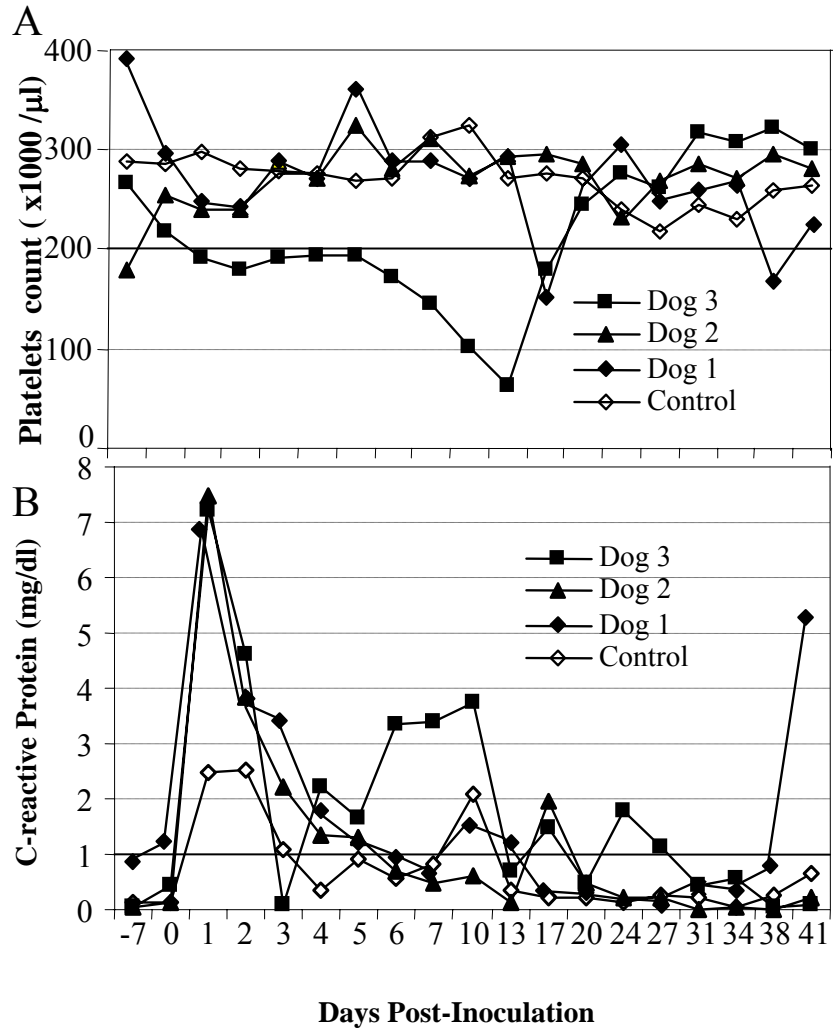b: positive from peripheral blood and bone marrow aspirate

Fig.1