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Experimental primary and secondary infections of domestic dogs with *Ehrlichia ewingii*

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ABSTRACT

In this study, the infection dynamics of *Ehrlichia ewingii*, causative agent of granulocytotropic ehrlichiosis in dogs and humans, was examined in experimentally infected dogs by using a combination of physical examination, hematologic and biochemical analyses, and molecular and serologic assays. For the experimental trials, blood from an *E. ewingii*-infected dog was inoculated intravenously into two naïve dogs and two dogs with prior experimental exposure to *E. ewingii* (both were negative for *E. ewingii* DNA by polymerase chain reaction (PCR) assay, but seropositive from initial infection 8 and 10 months prior to challenge). A negative control dog was inoculated with blood from a negative dog. The two primary infection dogs were positive for *E. ewingii* DNA on DPI 4, remained consistently positive until DPI 60, and were intermittently positive until the end of the study (DPI 144). The two primary infection dogs developed antibodies reactive to *E. ewingii* by DPI 28 and remained seropositive for the duration of the study. Primary infected dogs had intermittent fever, thrombocytopenia, and leukopenia and some dogs were hyperphosphatemic and/or had elevated ALP levels. The two challenge dogs were positive for *E. ewingii* DNA on DPI 4 and 18, which was similar to the primary infection dogs, but the duration of *E. ewingii* DNA detection was shorter. Also, the two challenged dogs did not develop pyrexia or show any hematologic or biochemical abnormalities. *E. ewingii* was successfully transmitted between dogs by *Amblyomma americanum*, but not *Rhipicephalus sanguineus*. This study provides data on the infection dynamics of *E. ewingii* in dogs during primary and challenge infections and suggests that prior exposure may lessen clinical disease during subsequent infections.

1. Introduction

Ehrlichia ewingii is a tick-transmitted pathogen of domestic dogs and humans (Anderson et al., 1992; Paddock et al., 2001; Masters et al., 2009; Gieg et al., 2009). Surveys in Missouri, Oklahoma, and Arkansas showed that *E. ewingii* was the predominant ehrlichial agent infecting dogs (Liddell et al., 2003; Little et al., 2010). The primary vector of *E. ewingii* is *Amblyomma americanum*, the lone star tick (Anziani et al., 1990), but PCR evidence of infection has been reported in *Dermacentor variabilis* and *Rhipicephalus sanguinius* (Murphy et al., 1998; Steiert and Gilfoy, 2002). Natural infections have been reported in white-tailed deer (*Odocoileus virginianus*)

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and *E. ewingii* has been transmitted between deer indicates that deer harbor infectious organisms (Yabsley et al., 2002; Arens et al., 2003; Liddell et al., 2003).

Although previous studies have investigated the course of *E. ewingii* infection in dogs (Anziani et al., 1990; Rikihisa et al., 1992), they were limited because either (1) inoculum was not confirmed as *E. ewingii*, (2) few clinical parameters were evaluated, (3) polymerase chain reaction (PCR) assay was not used to detect *E. ewingii*, or (4) serologic testing was not conducted. The objective of this project was to investigate the infection dynamics and clinical abnormalities of *E. ewingii* infection in experimentally infected dogs using a combination of physical examination (including rectal temperature), hematological and biochemical analyses, PCR assay, and an ELISA assay. This study contributes to the data on the infection dynamics of *E. ewingii* in terms of clinical presentation and clinical pathology, while uniquely combining serological data, namely development and persistence of antibodies, and PCR assays to establish a better understanding of the dynamics of this organism in the canine host. In addition, dogs were challenged with *E. ewingii* to determine if previous infection provided any immunity to infection or would lessen clinical signs.

2. Materials and methods

2.1. Experimental animals

Seven dogs (two beagles and five mongrels) were used in this study. Dogs 2, 3, 4, 8, and 9 were ~6 months old and dogs 135 and 913 were 6 and 2 years old, respectively. Dogs were fully vaccinated and dewormed and were kept in a climate-controlled, tick-free, animal housing facility at the College of Veterinary Medicine, University of Georgia (Athens, Georgia) for the duration of these studies. All experimental procedures were approved by the Institutional Animal Care and Use Committee. Animals were provided a diet of commercially available dry dog food and water ad libitum.

Prior to inoculation, the dogs were shown to be negative for infection with vector-borne disease agents by polymerase chain reaction (PCR) assay for *Ehrlichia chaffeensis*, *Ehrlichia canis*, *E. ewingii*, *Anaplasma phagocytophilum*, *Borrelia* spp., and *Bartonella* spp. (Dawson et al., 1996; Yabsley et al., 2002, 2005, 2008) and prior exposure by screening serum samples for anti-*E. ewingii* antibodies by a peptide-based ELISA assay as described (O'Connor et al., 2010) and for antibodies reactive against *E. chaffeensis*, *A. phagocytophilum*, and *Borrelia* spp. by indirect fluorescent antibody (IFA) (Moyer et al., 2006; Yabsley et al., 2008).

2.2. Sample collection and testing

Approximately 3 ml of blood was collected via aseptic cephalic venipuncture into EDTA Vacutainer tubes (2 ml) (Becton Dickinson, Rutherford, NJ) and plain glass tubes (1 ml). Whole blood was centrifuged at $1500 \times g$ for 10 min; plasma was removed and frozen at −20 °C for future serologic assays and remaining blood components stored at −20 °C for PCR assays. DNA was extracted from 300 μl of blood and tested for *E. ewingii* by PCR as described (Yabsley et al., 2002). Stringent protocols and controls were utilized in all PCR assays to prevent and detect contamination. DNA extraction, primary amplification, secondary amplification, and product analysis were performed in separate dedicated laboratory areas. A negative water control was included in each set of DNA extractions, and one water control was included in each set of primary and secondary PCR reactions. Plasma samples were tested for antibodies to *E. ewingii* using an ELISA that used a synthetic 21-mer peptide derived from the p28 protein (O'Connor et al., 2010).

Culture attempts from dogs were conducted in ISE6 tick cells as described (Munderloh et al., 1999, 2007). Tick cell cultures were maintained at 34 °C in closed flasks (Greiner America, Longwood, NC), with L15B300 supplemented with 10% tryptose phosphate broth (Difco Laboratories, Detroit, MI), 5% heat-inactivated fetal bovine serum (Harlan, Indianapolis, IN), and 0.1% bovine lipoprotein concentrate (ICN, Irvine, CA), pH 7.2. Medium for inoculated cultures was additionally supplemented with 25 mM HEPES and 0.25% $NaHCO_3$ (Sigma, St. Louis, MO), and the pH was adjusted to 7.5–7.7 with 1 N NaOH (Munderloh et al., 1999). Culture attempts to isolate organisms from experimentally infected dogs were made the day after the first day of PCR positivity and periodically thereafter (at least once per week until dogs became PCR negative). For culture, EDTA whole blood samples were collected aseptically and processed within 0.5 h. Culture attempts were conducted using either whole blood (100 μl) inoculated directly into flasks of ISE6 tick cells or with erythrocytes or buffy coat fractions which were obtained by centrifuging 0.5–1 ml whole blood at $1000 \times g$ for 20 min. The buffy coat or erythrocytes fractions were placed in 10 ml of L-15B300 media without supplements, mixed gently, and then centrifuged at $1000 \times g$ for another 20 min. The supernatant was removed and the pellet mixed with ISE6 cells freshly harvested from a single 5 ml flask suspended in L15-B media. An additional 10 ml of L-15B was added, the mixture incubated at room temperature for 15 min, and then centrifuged at $1000 \times g$ for 30 min. The supernatant was removed and the pellet was resuspended in 5 ml of L-15B300 medium (Munderloh et al., 1999), transferred to a new 5 ml flask, and incubated at 34 °C. All cultures were fed twice weekly with appropriate media. Each flask was monitored for development of cytopathic effect at each feeding and maintained for at least 90 days.

Samples of whole blood and serum were submitted to the College of Veterinary Medicine Clinical Pathology Lab for CBC and serum biochemistry analyses including ALP, ALT, electrolytes, calcium, phosphorus, total protein, and albumin. Thin blood smears were prepared, fixed in methanol for 10 min, and stained with Giemsa buffered to pH 6.8 at 37 °C for 30 min. At the completion of the study, dogs were treated with doxycyline (10 mg/kg PO BID for 14 days) and placed up for adoption or transfered to other studies.

2.3. Infection of dogs 135, 913, and 8

Dog 135 was inoculated with 2 ml of blood previously collected from an *E. ewingii* naturally infected in Missouri.