Original Article

Serologic responses to peptides of Anaplasma phagocytophilum and Borrelia burgdorferi in dogs infested with wild-caught Ixodes scapularis

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Anaplasma phagocytophilum and Borrelia burgdorferi are both transmitted by Ixodes spp. and are associated with clinical illness in some infected dogs. This study evaluated canine antibody responses to the A. phagocytophilum p44 peptides APH-1 and APH-4 as well as the B. burgdorferi C6 peptide before and after doxycycline treatment. A total of eight dogs were infested with wild-caught I. scapularis for 1 week. Blood was collected prior to tick attachment and from Days 3–77 to 218–302 with doxycycline treatment beginning on Day 218. Blood was assayed for A. phagocytophilum DNA by PCR assay. Sera was assessed for antibodies by immunofluorescent antibody (IFA) test and ELISA. Anaplasma phagocytophilum DNA was amplified from blood of all dogs by Day 7. Antibodies to APH-4 were detected in serum as early as 14 days after tick exposure and six dogs had APH-4 antibodies detected 3–7 days before antibodies against APH-1. All dogs were seropositive for A. phagocytophilum from Days 218 to 302. Antibodies to B. burgdorferi were detected in 6/8 dogs beginning 21 days after I. scapularis infestation. Among the five dogs that remained seropositive at Day 218, C6 antibody levels declined on average 81% within 84 days of initiating treatment. The results suggest that the APH-4 peptide may be more useful than APH-1 for detecting antibodies earlier in the course of an A. phagocytophilum infection. After doxycycline administration, C6 antibody levels but not APH-1 or APH-4 antibody levels decreased, suggesting a treatment effect on C6 antibody production.

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Introduction

Anaplasma phagocytophilum and Borrelia burgdorferi share Ixodes spp. as vectors and both are sometimes associated with clinical illness in infected dogs (Carrade et al., 2009; Krupka and Straubinger, 2010). A. phagocytophilum primarily infects neutrophils. PCR assays can be used to amplify A. phagocytophilum DNA in blood (Kirtz et al., 2005). In contrast, B. burgdorferi is a spirochetal bacterium that primarily resides in the subcutaneous tissues and synovial membranes of infected dogs (Little et al., 2010) and may be found in other organs such as bladder and heart (Dorward et al., 1991; Hovius et al., 1999). PCR testing for B. burgdorferi DNA may be performed on skin biopsies from the site of tick attachment or distal tissue sites (Hovius et al., 1999; Straubinger, 2000), however PCR testing on blood samples is insensitive since the organism is not in the peripheral circulation.

Both A. phagocytophilum and B. burgdorferi induce robust humoral immune responses that can be measured by a number of different assays. One commercially available test kit (SNAP 4Dx Plus, IDEXX Laboratories, Inc.) utilizes a B. burgdorferi peptide (C6) to differentiate antibodies induced by natural infection from those induced by vaccination (O’Connor et al., 2004; Stillman et al., 2014). The C6 peptide is derived from the immunodominant VlsE protein which is encoded by a linear DNA plasmid and expressed approximately 7–10 days after the spirochete infects a mammalian host (Crotzer et al., 2004). B. burgdorferi uses mechanisms of gene conversion as a strategy for immune evasion similar to Treponema pallidum and A. marginale to allow for persistent bacterial infections (Palmer et al., 2009; Rikihisa, 2011). Antibodies against the C6 peptide of B. burgdorferi have also been measured quantitatively by ELISA (Lyme Quant C6 Test, IDEXX Laboratories, Inc.) and the concentration of C6 antibodies in serum has been shown to decrease in dogs after the administration of drugs, like doxycycline, that have anti-B. burgdorferi effects (Philipp et al., 2001; Levy et al., 2008; Wagner et al., 2015). Furthermore, the
antibody response to the C6 peptide has been shown to correlate with the clinical response to treatment in human patients infected with *B. burgdorferi* (Philipp et al., 2001, 2003, 2005).

The same commercially available test kit for C6 antibody also detects antibodies to an invariant peptide of the immunodominant p44 (msp2) protein of *A. phagocytophilum* (Stillman et al., 2014). The family of p44 outer membrane glycol-proteins enables antigenic variation and immune evasion by the bacteria thereby favoring persistence within the host (Rikihisa, 2011). With over 100 pseudogenes giving rise to multiple variants of p44, a broad repertoire of antibody responses by the host is possible (Rejmanek et al., 2012). Due to the acute nature of granulocytic anaplasmosis, PCR testing on peripheral blood may be a more sensitive diagnostic tool in acutely infected dogs compared to serology (Scorpio et al., 2011; Moroff et al., 2014). However, it has been reported that some clinically normal dogs may continue to have DNA evidence of *A. phagocytophilum* in their blood beyond 60 days post-infection (Scorpio et al., 2011). Serologic responses may be monitored by immunofluorescent antibody (IFA) titer but a commercially available, quantitative ELISA test is not available for *A. phagocytophilum* (Scorpio et al., 2011; Nair et al., 2016).

To date, the effect of doxycycline administration on antibody responses to p44 in *A. phagocytophilum* infections has not been extensively studied (Gaunt et al., 2010; Moroff et al., 2014). The objective of this study was to compare serologic responses against two p44 peptides and the C6 peptide of *B. burgdorferi* in the same dogs, pre- and post-treatment, using an experimental infection model favoring co- transmission of *A. phagocytophilum* and *B. burgdorferi*.

Materials and methods

**Dogs**

A total of eight young-adult, mixed sex, laboratory reared beagle dogs were purchased from a research facility (High Quality Research). The dogs were shown to be negative for *A. phagocytophilum* DNA by a PCR assay (IDEXX Laboratories) and antibodies against *A. phagocytophilum* and *B. burgdorferi* by a commercially available test kit (SNAP 4DX, IDEXX Laboratories). Dogs were housed in compatible groups with enrichment provided by the research facility except during the I. scapularis attachment period when the dogs were individually housed. The protocol was approved by the Institutional Animal Care and Use Committee by the research facility (#187004) on 8 January 2012.

**Ixodes scapularis infestation**

Co-transmission of *A. phagocytophilum* and *B. burgdorferi* was performed with wild-caught adult *I. scapularis* from a region of the USA where both pathogens are now endemic. Field-collected, adult *I. scapularis* from Rhode Island were purchased for use in this study (Tick Encounter, University of Rhode Island). *A. phagocytophilum* and *B. burgdorferi* infection rates in the ticks, as determined by a representative aliquot of adult female ticks (n = 30) from the capture area that year, were approximately 15% and 50%, respectively. Total DNA was extracted from an aliquot of ticks and infection rates estimated using PCR as previously described (McCall et al., 2011). A total of 12 male and 13 female *I. scapularis* were placed on each of the eight beagles in a tick chamber on Day 0 and allowed to feed for seven days. On the eighth day, the ticks and chambers were removed, between three and 10 ticks were noted to have fed per dog, and the dogs were treated with an acaricide (Frontline, Merial Ltd.).

**Observations**

The dogs were observed daily for signs of inappetence, lameness, or depression. The body temperature was estimated by a temperature sensing microchip to monitor for fever (Quinby et al., 2009). In the doxycycline treatment period, the dogs were also observed for regurgitation, vomiting, or diarrhea.

**Sample collection and assays**

In the pre-treatment part of the study, a total of 12 mL of blood was collected from the jugular vein of the dogs prior to tick placement on Day 0 and post tick placement from Days 3 to 77 (on days 3, 7, 10, 14, 17, 21, 24, 28, 35, 42, 49, 56, 63, 70, 77). An aliquot of anticoagulated whole blood was assessed by *A. phagocytophilum* PCR assay. The remaining blood was processed to serum and stored at −80°C until assessed in the serologic assays. Dogs were observed from Day 77 to Day 218 at which point doxycycline administration was administered to all dogs at approximately 10 mg/kg, PO, once daily for 28 days after the morning feeding to lessen gastrointestinal side effects. Additional blood samples were collected weekly and processed as described above between Day 218 (prior to doxycycline) and Day 302.

**PCR**

Real-time PCR performed by IDEXX using a proprietary hybridization probe assay was used to detect an *A. phagocytophilum* p44 polynucleotide (Scorpio 2010). Genomic DNA was extracted from canine whole blood using a commercially available kit (High Pure PCR Template Preparation Kit, Roche Applied Science) according to the manufacturer’s instructions. Real-time PCR was performed using a LightCycler 480 Genotyping Master mix (Roche Applied Science) in a total 20 μL reaction volume with 5 μL of template DNA. Primer and probe concentrations used were: 0.3 μM of the forward primer and 0.6 μM of the reverse primer and 0.3 μM of each probe. PCR was performed under the following conditions: a single hot-start cycle at 95°C for 10 min followed by 50 cycles of denaturation at 95°C for 20s, annealing at 60°C for 30 s with a single data acquisition and extension at 72°C for 20s. A melting curve was performed by heating the PCR product to 95°C for 1 min, cooling to 45°C for 1 min and gradually heating to 80°C with a ramp rate of 0.4°C per second and 4 data acquisitions per C. A. *phagocytophilum* positive samples were detected by the presence of a crossing point value indicating the cycle number where the amplicon fluorescence was detectable above background fluorescence. Confirmation of the detection of *A. phagocytophilum* amplicons was based on the Tm values (63.5°C ± 0.5°C) obtained from analysis of the melting curves.

**Serology**

Serology was performed using a multi-analyte research ELISA assembled within a SNAP device (SNAP M-A) (Quirillo et al., 2014; Hegarty et al., 2015). Like other SNAP tests, SNAP M-A uses a reversible chromatographic flow of sample and automatic sequential flow of wash solution and enzyme substrate (Stillman et al., 2014). SNAP M-A is designed to detect antibodies against six different tick borne pathogens, *A. phagocytophilum*, *A. platys*, *B. burgdorferi*, *Ehrlichia canis*, *E. chaffensis* and *E. ewingii*. This ELISA utilizes the same reagents on individual spots for *B. burgdorferi* (IS) and *A. phagocytophilum* (APH-1) as the commercially available test and is performed in the same way (Stillman et al., 2014). SNAP M-A, however, has an additional peptide (APH-4) deposited in a separate spot to detect specific antibodies to *A. phagocytophilum* (Luo et al., 2008). Both the APH-1 and APH-4 peptides are derived from the immunodominant p44 protein of *A. phagocytophilum*.

The APH-1 peptide detects specific and cross-reactive antibodies against *A. phagocytophilum* and *A. platys*, while the APH-4 peptide only detects antibodies to *A. phagocytophilum* (O’Connor and Chandrashekar, 2005; Liu et al., 2012). Densigraph measurements of spot intensity on the SNAP M-A test were used as an estimate of antibody concentration. The optical density of the background was measured in the same location on each device and subtracted from that of the spot being evaluated. Quantitative serology for *B. burgdorferi* C6 antibody levels and *A. phagocytophilum* IFA titers (treatment phase only) was performed on aliquots of serum that were sent to a commercial laboratory (IDEXX Laboratories). *A. phagocytophilum* peptide-specific antibody levels were measured using an indirect ELISA formatted for a microtiter plate. The two *A. phagocytophilum* p44 peptides (APH-1 and APH-4) were coated (100 μL/mL) onto separate microtiter plates (Thermo Scientific) in 0.05 M sodium carbonate buffer (pH 9.6) at concentrations of 0.25 μg/mL. The plates were washed (phosphate buffered saline (PBS) based buffer with detergent) and blocked using a Tris buffered solution (pH 7.4) containing Tween-20 and sucrose (300 μL/well). Plates were stored desiccated in Mylar bags at 2–7°C until use.

Samples were serially diluted 2-fold 1:100 to 1:204,800 in buffered solution containing detergent and blocking protein, added to the microtiter plate (100 μL). The plate was incubated 30 min at room temperature (50 μL). The plate was washed (5% Tween-20, 0.05% Nonidet P-40 detergent with detergent). Horseradish peroxidase-labeled anti-canine (heavy and light chain) antibody (Jackson ImmunoResearch Laboratories) was diluted 1:3000 (for APH-1) or 1:2000 (for APH-4) in buffered solution containing detergent and blocking protein, added to the microtiter plate (100 μL/well) and incubated 30 min at room temperature. The plate was washed and 3,3′,5,5′-tetramethylbenzidine substrate solution was added (50 μL/well). Optical density was measured at 650 nm. A cut-off value for the assays was established based on the mean optical density plus 3 standard deviations (SDs) of the Day 0 samples. The reciprocal antibody titer was designated as the last dilution with an optical density of >0.05.

**Statistics**

The data were evaluated using the Shapiro–Wilk test and followed a normal distribution. A paired t test, using results from individual dogs, was employed to assess the change in optical density for *A. phagocytophilum* and *B. burgdorferi* relative to change between the treatment and pre-treatment phases was assessed at P < 0.05 using commercially available software (GraphPad Prism v6.0).
Results

Clinical findings

None of the dogs developed detectable lameness, inappetence or depression consistent with anaplasmosis or borreliosis over the course of the study. Body temperatures were only rarely out of the normal range and never associated with other clinical signs of illness. Doxycycline was well tolerated by all dogs in the study.

Real-time PCR

A. phagocytophilum real-time PCR was performed with whole blood samples collected from Day 0 through Day 77. A. phagocytophilum DNA was amplified by PCR in all dogs by Day 7 and 6/8 dogs remained PCR positive through Day 77 (Fig. 1). Prior to the initiation of doxycycline treatment at Day 218, all eight dogs were PCR negative for A. phagocytophilum DNA and remained negative through Day 302.

Serology

For serology, samples from all eight dogs were tested before and then for 11 weeks after I. scapularis infestation using the SNAP M-A assay. Antibodies to APH-1 and APH-4 peptides were detected in serum of all dogs between Days 14 and 28. The species-specific peptide (APH-4) was able to detect antibodies to A. phagocytophilum 3–7 days earlier than the cross-reactive peptide (APH-1) in six of the eight dogs (Fig. 1). Antibodies to APH-1 and APH-4 persisted through Day 77 in eight dogs and seven dogs, respectively (Figs. 1, 2A).

While all eight dogs were seropositive for antibodies to A. phagocytophilum, antibodies to B. burgdorferi were only detected in 6/8 dogs. Using the SNAP M-A device, antibodies to the C6 peptide were first detected between 21 and 42 days after I. scapularis infestation, with four of the six seropositive by day 28 (Fig. 2C). Quantitative C6 antibody levels were first detected at 21 or 28 days after I. scapularis infestation (Fig. 3A). All six dogs remained seropositive on both tests through Day 77. Quantitative antibody levels to C6 remained elevated in three dogs (AO710, SQQO, TIRO) while three dogs (AO110, AO610, SLRO) demonstrated fluctuations in antibody levels over this period (Fig. 3A).

At Day 218, all dogs were administered doxycycline as described in the Methods. A. phagocytophilum IFA titers were positive for all eight dogs from Day 218 through Day 302. Although some individual titers fluctuated by as much as a four-fold change over this time, individual titers at Day 218 compared to Day 302 remained unchanged in four dogs, increased by a single dilution in two dogs, and decreased by a single dilution in two dogs (Table 1). When comparing each dog’s titer at Day 218 to the paired titer at Day 302, A. phagocytophilum antibody titers measured using the APH-1 or APH-4 peptide ELISA either remained unchanged or demonstrated a two-fold reduction in most dogs (Table 1). Only one dog had a four-fold decrease in titer on the APH-4 peptide ELISA. Seroreactivity evaluated using densigraph measurements of spot intensity on SNAP M-A (APH-1) as a general indicator of Anaplasma antibody concentration decreased significantly between Day 218 and Day 302 (paired t test P < 0.05; Fig. 2B).

Serum samples collected from Day 218 to Day 302 were also tested by the quantitative C6 assay. On day 218, three of the eight dogs had no detectable C6 antibody levels and this included the two dogs that had not previously seroconverted to the C6 peptide (AO510, AO610, THRO). The third dog had the lowest peak antibody concentration during the pre-treatment period (AO610). Of the remaining five dogs that were seropositive, C6 antibody levels ranged from 5 to 51 U/mL (Fig. 3B). Following doxycycline treatment, all dogs demonstrated a decrease in C6 antibody levels that ranged from 63 to 92% with a mean decline of 81% relative to pre-treatment values in the 12 weeks they were monitored (Fig. 3B). C6 antibody levels on the SNAP M-A test, based on densigraph measurements of spot intensity, decreased significantly between Day 218 and Day 302 (paired t test P < 0.05; Fig. 2D). While both APH-1 and C6 optical density readings on SNAP M-A demonstrated significant decreases between pre- and post-treatment, the magnitude of the difference was significantly greater for C6 (P < 0.05).

Discussion

In this study, we evaluated several diagnostic tools for use in dogs experimentally infected with ticks harboring A. phagocytophilum and B. burgdorferi, particularly focusing on the serological profile of the dogs after doxycycline therapy. While there were no visible clinical signs observed in any of the infected dogs, prior studies have reported mixed observations with experimental infections of either A. phagocytophilum or B. burgdorferi; some studies reported severe clinical signs in experimentally infected dogs like fever, lethargy, and lameness, while in other studies, the dogs developed subclinical infections (Straubinger et al., 2000; Carrade et al., 2009; LaFleur et al., 2009; Kriemer et al., 2011; Scorpio et al., 2011; Wagner et al., 2015). Age of the dogs at the time of experimental infection may be factor whereby younger dogs are more likely to demonstrate severe clinical disease (Straubinger et al., 2000; Summers et al., 2005).

All dogs infected with I. scapularis were positive for circulating A. phagocytophilum DNA by PCR on day 7 suggesting establishment of active infections. In addition, all developed antibodies to the p44-derived peptides which was similar to previously reported studies (Scorpio et al., 2011; Moroff et al., 2014). However, only six of the eight dogs developed anti-Borrelia C6 antibodies. The two Borrelia C6 antibody negative dogs most likely did not become infected with Borrelia even though there was tick transmission of A. phagocytophilum. Interestingly, these two dogs did not seroconvert for B. burgdorferi infection even though 50% of the ticks, on average, contained B. burgdorferi DNA. In a similar study, it was observed that four immature beagle dogs exposed to ticks had negative results on all serological and molecular tests for B. burgdorferi (Moroff et al., 2014). These results suggest that some dogs may be able to naturally eliminate B. burgdorferi before systemic immune responses develop.

The level of C6 antibody has not been shown to predict clinical illness. However, C6 antibody levels do correlate with the number...
of viable spirochetes and quantity of *B. burgdorferi* DNA detected within the skin at the site of tick attachment (Straubinger, 2000; Straubinger et al., 2000; Philipp et al., 2001). Skin biopsies from dogs experimentally infected with *B. burgdorferi* contained viable spirochetes during the first 120 days post tick infection (Straubinger et al., 2000). However, after antibiotic treatment skin samples were rarely PCR positive compared to those from untreated controls (Straubinger et al., 2000). Thus, the organisms were greatly reduced in antibiotic treated dogs. The reduction in spirochetes coincided with a decrease in C6 antibody levels to almost background levels even though antibody levels remained high in untreated controls (Philipp et al., 2001). Likewise, measurement of antibody responses to whole cell extracts or p39 have not demonstrated the same type of response following treatment (Philipp et al., 2001, 2003, 2005; Marangoni et al., 2006). This suggests that the production of antibodies to the C6 peptide depends upon the presence of viable organisms in the infected animal and the potential role VlsE has in evading the host immune response (Philipp et al., 2001). This appears to differ from other *B. burgdorferi* antigens which may become sequestered and stimulate plasma cell differentiation and a long-lived antibody response.

**Fig. 2.** Optical density measurements from the APH-1 and C6 peptides on SNAP M-A for the eight dogs included in the study. (A) APH-1 results post-tick exposure and prior to antibiotic therapy. (B) APH-1 results post-treatment. (C) C6 results post-tick exposure and prior to antibiotic therapy. (D) C6 results post-treatment.

**Fig. 3.** Quantitative C6 antibody levels post-tick exposure and prior to antibiotic therapy (A) and post-treatment (B) for the eight dogs included in the study.

<table>
<thead>
<tr>
<th>Dog ID</th>
<th>IFA</th>
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<th>APH-4</th>
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<td></td>
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<td>Day 302</td>
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<td>1:200</td>
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<tr>
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<td>1:100</td>
<td>1:200</td>
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<td>1:800</td>
<td>1:800</td>
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<tr>
<td>SQQQ</td>
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<tr>
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<td>1:800</td>
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<tr>
<td>TIRO</td>
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Results for *A. phagocytophilum* IFA and the quantitative peptide ELISA plates for APH-1 and APH-4 before (Day 218) and after (Day 302) antibiotic therapy.
Likewise, it has been proposed that decreasing C6 antibody levels correlate with a reduction in spirochetal load and perhaps dormancy of the organisms which reduces VlsE transcription and subsequent antigenic stimulation (Embers et al., 2012). While this has been shown to occur following antibiotic treatment in either experimentally or naturally infected dogs (Philipp et al., 2001; Levy et al., 2008; Wagner et al., 2015), experimental infections have also shown that C6 antibody levels can to return to baseline in the absence of antibiotic therapy suggesting effective immune control of the infection (Embers et al., 2012). In the present study, three of six dogs demonstrated a decline in C6 antibody levels prior to antibiotic treatment (between Day 77 and Day 218). Only one of these dogs had antibody concentrations below the level of detection (<10 U/mL) at Day 218. However, the other three dogs had increasing concentrations of C6 antibodies during the 20-week observation period, including one dog with a greater than 200% increase. Although an untreated, control group was not included in this study, this 20-week observation period permitted assessment of C6 antibody levels in the absence of tick exposure and allowed these dogs to serve as their own controls. After this five-month time period, only one of the six dogs had a reduction in the C6 antibody level below a value of 30 U/mL and presumably a reduction in organism load and viability. Following antibiotic treatment, C6 antibody levels decreased, on average, 93% for the five seropositive dogs within 12 weeks of initiating treatment, and all but one had values less than 30 U/mL. The rapid decline of antibody levels to C6 following doxycycline therapy suggests that measuring C6 antibody levels may serve to assess whether a B. burgdorferi infection has been brought under control by the animal’s immune system. Whether this decrease has clinical implications has not been proven and does not necessarily indicate that the organisms have been cleared, disease has been prevented, or all pathologic antibodies have been resolved. The C6 peptide does, however, serve as a unique and valuable marker for monitoring the production of antibodies to a protein that has been proposed to be utilized by B. burgdorferi to actively evade the immune response in infected hosts (Philipp et al., 2001). Studies assessing for associations between antibody levels and clinical outcomes in dogs need to be performed. While this type of prospective field study would be beneficial, it would nevertheless be challenging to perform given the relatively low incidence of overt clinical disease in infected dogs (estimated between 5 and 10%) and the requirement that infected dogs not be treated with antibiotics while monitored for appearance clinical signs.

Both A. marginale and A. phagocytophilum are well known chronic infections in cattle and small ruminants, respectively, while persistent infections in dogs with A. phagocytophilum have been suggested (Scorpio et al., 2011; Brown, 2012; Nair et al., 2016). Consistent with previous studies, A. phagocytophilum DNA was detectable in the circulation of infected dogs beyond the acute infection and up to 77 days in 6/8 dogs. Although the DNA was no longer detectable at Day 218 and beyond, all dogs remained seropositive by IFI and ELISA through the end of the study. Antibiotic treatment did not show an association with a reduction in serum antibody levels by IFI and the APH peptide ELISA. The specific reasons for this were not evident in this study however several hypotheses could be considered. First, the targets evaluated for assessing antibody response may not have been suitable despite their proposed role in antigenic variation and immune evasion like that of VlsE. Second, the timing of antibiotic therapy, while suitable for the study of the B. burgdorferi response, might not have been appropriate for the A. phagocytophilum infection to detect a post-treatment decrease in antibody levels. Third, some infections may be associated with long-lived plasma cells and persistent antibody does not necessarily reflect persistent infection. Finally, it may be that if dogs do establish low level chronic infections with A. phagocytophilum, antibiotic therapy could be less efficacious during these later stages. Further studies are needed to fully understand the post-treatment antibody response to A. phagocytophilum in the dog.

Conclusions

This study evaluated antibody responses to peptides from VlsE of B. burgdorferi and p44 of A. phagocytophilum. Both of these proteins are associated with mechanisms of immune evasion for each bacterium and may help to facilitate persistent infections in dogs. Antibiotic treatment had a more dramatic effect on reducing C6 antibody levels than what could be measured quantitatively for antibodies directed against peptides of p44. Doxycycline treatment at 218 days post-infection did not markedly alter p44 antibody responses to the peptides tested. However, antibodies to the APH-4 peptide of p44 could be detected earlier in infection, and most A. phagocytophilum infected dogs had DNA amplified by PCR on peripheral blood through 77 days post-infection.

Conflict of interest statement

MR Lappin has received funding from IDEXX Laboratories for research studies. All other authors are employees of IDEXX Laboratories. None of the authors has any other financial or personal relationships that could inappropriately influence or bias the content of the paper.

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All authors were involved in the design of the study, developing the methods, performing the analysis, and reviewing the manuscript. RC, MJB and LAP wrote the manuscript. LAP performed the animal studies as indicated in the manuscript with all dogs being adopted following treatment.

Preliminary results were presented as an Abstract at the ACVIM Forum, Indianapolis, IN 3–6 June 2015.

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