

ABSTRACT

KIDD, LINDA BENJAMIN. Molecular characterization of rickettsial diseases in dogs.

(Under the direction of Dr. Ed. Breitschwerdt.)

Spotted Fever Group *Rickettsia* are important causes of morbidity and mortality worldwide. These arthropod borne, obligately intracellular organisms are notoriously difficult to detect in blood samples from infected patients. Standard diagnostic techniques do not differentiate among SFG members, thus the species of infecting *Rickettsia* is presumed based on geographic location. With the advent of molecular biology, newly discovered and previously known pathogenic and “non-pathogenic” SFG *Rickettsia* have been associated with disease in people in expanding regions of the world.

R. rickettsii, the cause of Rocky Mountain Spotted Fever, is arguably the most well known and well characterized SFG *Rickettsia*. The manifestations of this disease are similar in dogs and people, and because infection can precede or occur simultaneously with infection in their human companions, dogs are considered sentinels for the disease. Despite this, the species of *Rickettsia* infecting dogs with RMSF had never been characterized using molecular techniques. Using PCR, we amplified and sequenced portions of three genes from SFG *Rickettsia* isolated from dogs and people with RMSF in an endemic region. Gene sequence based criteria were applied and the isolates were identified as *R. rickettsii*. This study provides support that naturally occurring RMSF in dogs is comparable to the disease in people. *Rickettsia rickettsii* has been declared a Select Agent by the CDC and a Category C priority pathogen by the NIAID due to concerns that it is amenable for use in a bioterrorist attack. As a result of this study, 15 consensus sequences of genes amplified from *R. rickettsii*

naturally infecting dogs and people in North Carolina have been deposited in GENBANK (Accession numbers DQ15680-DQ15694). Knowledge of sequences of naturally occurring isolates may help identify aberrant strains intentionally released as a result of an act of bioterrorism.

Amplifying DNA from cultures of *Rickettsia* does not require a particularly sensitive PCR. Due to the nature of these organisms, sensitive assays are required in the clinical setting. No diagnostic tool that is sensitive, and can differentiate among species of infecting *Rickettsia* has been validated for use in infected dogs. We have created a PCR that amplifies a portion of the *ompA* gene from infected dog blood with a limit of detection of 1.5-30 copies of SFG *Rickettsia*. Sequencing of the product differentiates a number of different species. Using this tool we documented the presence of *R. conorii ssp conorii* DNA in the blood of three male Sicilian Yorkshire Terriers. These dogs had clinical illness compatible with acute rickettsiosis. This provides unique evidence that *R. conorii* may infect dogs and cause disease in this host. Future studies should investigate the role of *R. conorii* and other SFG *Rickettsia* as disease causing agents in dogs. Identifying SFG *Rickettsia* in naturally infected dogs may have implications for their human companions due to their potential role as sentinels for this group of illnesses.

**MOLECULAR CHARACTERIZATION OF
RICKETTSIAL DISEASES IN DOGS**

By

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Dedication

This dissertation is dedicated to my Mom, Lorna S. Benjamin, for being the most amazing role model, and my “green light”; to my Dad, Robert M. Benjamin, and Nancy Benjamin for their unwavering love and support; to my sister; Laurie Buffo for being my rock and best friend; to Jane Dewey and Lisa O’Brien, for being my forever friends; to Lauren Trepanier and Rebecca Stepien, professional role models and good friends; to Christian Osmond, for loving me and teaching me how to let go; to Velvet, Jenny, Carin, Swajour, The Count, Jasmine, Casey, Remy, Rosie, Fletchie, The Spot, Taz, the curly tailed wonder, and all before them, for making me smile; to all my patients and their human companions, and to God, for everything.

Biography

Linda Kidd was born in Madison, Wisconsin and always wanted to be a veterinarian. She went to nursery school at University of Wisconsin, Madison, left the institution to attend Philip Falk elementary school and Edgewood high school, and then came back to campus to earn a B.S. in Bacteriology, graduating in 1987. Fulfilling her lifelong dream of becoming a veterinarian, Linda graduated from the University of Wisconsin, School of Veterinary Medicine in (UWSVM) in 1991. After several years in private small animal practice, she again returned to the UWSVM in 1997 to obtain specialty training in Small Animal Internal Medicine. She completed the residency in July of 2000, and achieved Board Certification by the American College of Veterinary Internal Medicine at that time. She stayed on at the UWSVM as Clinical Instructor of Small Animal Internal Medicine until December of 2002, when she left to pursue a PhD degree in Immunology at North Carolina State University's College of Veterinary Medicine. There she discovered that Madison isn't the only nice place to live, but it will always be home.

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1) INTRODUCTION

Spotted fever rickettsioses are vector borne diseases caused by bacteria in the genus *Rickettsia*, family *Rickettsiaceae*, and order *Rickettsiales*. The organisms are small, rod shaped, vector borne, obligately intracellular bacteria. Most of the Spotted Fever Group (SFG) *Rickettsia* are tick transmitted. They primarily infect endothelial cells, which can result in widespread pathology and rather characteristic clinical signs. The obligate intracellular and endotheliotropic nature of these organisms, their complex relationship with the tick host, and their inherent virulence, has historically made these organisms difficult and dangerous to isolate, characterize and study. Standard diagnostic techniques such as serology, culture and most Polymerase Chain Reaction (PCR) assays do not differentiate among species, and simply document infection with a SFG *Rickettsia*. Consequently, until recently, it was thought that the SFG *Rickettsia* pathogenic to people were limited to *R. rickettsii* the cause of Rocky Mountain Spotted Fever in the Western Hemisphere, and *R. conorii*, the cause of Mediterranean or boutonneuse spotted fever in Europe, the Mediterranean and other parts of the world.^{1,2}

Advances in molecular techniques have contributed to the recognition and characterization of classic and novel rickettsial species as disease causing agents in expanding geographic locales.¹⁻⁴ There are now at least 20 recognized species in the SFG *Rickettsia*, over half of which were characterized in the last 10 years.² Characterizing species of *Rickettsia* in a given region has important implications for local residents, travelers and even global biodefense. Spotted Fever rickettsioses are important causes of morbidity and mortality in many countries around the world. *R. rickettsii* has been identified as a Select Agent by the

Centers for Disease Control and Prevention, and as a category C priority pathogen by the National Institute of Allergic and Infectious Disease (NIAID) due to concerns that it is amenable to use as a biological weapon.⁵ Characterization of naturally occurring species would also be important to differentiate naturally occurring tick transmitted disease from intentional release of an agent. A sensitive, routinely used diagnostic test that can differentiate among species of infecting SFG *Rickettsia* has not been developed.

Dogs are commonly exposed to tick transmitted diseases due to their proximity to vectors. It is well accepted that *R. rickettsii* infects dogs and causes disease quite similar to RMSF in people.⁶⁻¹⁰ Dogs are considered sentinels for RMSF, as disease often occurs before or in conjunction with disease in their human companions.¹¹⁻¹³ However, definitive confirmation of the species of infecting *Rickettsia* in dogs with RMSF has not been documented using molecular techniques.

Dogs are commonly exposed to *R. conorii* in endemic regions.¹⁴⁻²⁰ Data regarding clinical signs and infection with *R. conorii* in dogs is scarce, but living near a seropositive dog is a risk factor for disease in people.²¹ With advances in molecular techniques, further characterization of these and other SFG rickettsiosis in dogs will occur as it has in people. Beyond the role of mans best friend, the role of dogs as sentinels or reservoirs for disease outbreaks in people warrants further investigation into SFG rickettsioses in dogs.

This dissertation will investigate Spotted Fever Rickettsiosis in dogs and people by addressing the following:

- 1) Determine if the agent that causes RMSF in people is genetically similar to the agent that causes RMSF in dogs.
- 2) Develop a sensitive and specific polymerase chain reaction that can differentiate species of Spotted Fever Group (SFG) *Rickettsia* and amplify *Rickettsia* DNA from dog blood.
- 3) Demonstrate natural infection with *R. conorii* in dogs using molecular techniques.

2) LITERATURE REVIEW

Spotted Fever Rickettsioses in Dogs and People

Rocky Mountain Spotted Fever (RMSF) is caused by *R. rickettsii*. The disease was first described in 1899.² It is the most severe rickettsial illness in the United States. The disease is reportable, and, according to the CDC approximately 250 to 1200 cases occur each year. The majority of cases occur in the southeast, with North Carolina and Oklahoma having the highest prevalence.²² However, underreporting may severely underestimate incidence and mortality.²³ Infection also occurs in other countries in the Western Hemisphere. In Brazil it is known as “Brazilian Spotted Fever.”² The disease is caused by *R. rickettsii*. This obligately intracellular organism is transmitted primarily by *Dermacenter variabilis* and *Dermacenter andersoni* ticks in the United States, although there is strong evidence that a recent outbreak in Arizona was transmitted by *Rhiphicephalus sanguineus*.^{22,24} Amblyomma ticks are thought to be the primary vector in South America. Many other species of tick have been shown to be infected and may be potential vectors.² *Rickettsia rickettsii* is transstadially and transovarially transmitted in and thereby maintained by ticks in nature. Rodents and small mammals serve as reservoirs.^{25, 26} Infection can also occur by aerosolization, and laboratory

deaths have occurred in personnel working with this agent.²⁷ Direct transmission from crushing or damaging ticks during removal is also possible.²² The disease incidence is seasonal, and reflects tick activity, with the majority of cases in the United States being reported between the months of April and September. Children and males may be at increased risk for infection, and people with glucose 6-phosphate dehydrogenase deficiency and alcoholics are at risk for more severe disease. Clinical signs of infection are associated with systemic vasculitis. Signs occur within 5-10 days of a tick bite, although there is not always a history of tick exposure. Clinical signs include but are not limited to fever, gastrointestinal abnormalities, myalgia, headache, arthralgia, ocular and central nervous system abnormalities. Acute renal failure, respiratory failure, myocarditis shock and death can also occur. The characteristic rash of RMSF occurs in 35-60% of patients, most commonly affecting the soles of the feet and the palms of the hand. The rash occurs relatively late in infection. Hematologic abnormalities include, but are not limited to thrombocytopenia, hyponatremia and elevated liver enzymes, hypoalbuminemia, azotemia and coagulation abnormalities.^{22,28} Because clinical signs such as fever, gastrointestinal disturbances and headache are non-specific, and can occur without the characteristic rash, or history of a tick bite, patients are often misdiagnosed. In addition to the lack of characteristic clinical signs in some patients, the diagnosis of RMSF is hampered early on by a lack of rapid, specific and sensitive diagnostic techniques (see “Laboratory Diagnosis” below). Early diagnosis and treatment depends on a high index of suspicion in attending clinicians. The species of infecting *Rickettsia* is usually presumed based on geographic locale. Misdiagnosis and failure to treat with appropriate antibiotics early in the course of the disease is associated with poor outcome and death.^{24,28} Clinical signs usually resolve rapidly with

doxycycline treatment and supportive care, although chronic sequelae can occur.²⁹

Importantly, *R. rickettsii* has been identified as a Select Agent by the Centers for Disease Control and Prevention, and as a category C priority pathogen by the National Institute of Allergic and Infectious Disease (NIAID) due to concerns that it is amenable to use as a biological weapon.⁵

Rickettsia rickettsii also causes RMSF in dogs. Infection induced in experimentally and naturally infected dogs has been verified using culture, PCR, and serologic methods, although one should note that none of these techniques definitively verify the species of infecting SFG *Rickettsia*. Some, but not all studies show purebred dogs are at increased risk for infection.^{8,25,30} Severe disease has been reported in English Springer Spaniels with suspected phosphofructokinase deficiency.²⁵ The clinical and hematologic manifestations reflect consequences of vasculitis and are very similar to those found in people. Signs include but are not limited to lethargy, anorexia, fever, ocular signs, lymphadenomegaly, gastrointestinal abnormalities, edema and hyperemia, joint pain, lameness, central nervous system abnormalities, dyspnea, weight loss and cutaneous lesions such as petechiae, ecchymoses and cutaneous necrosis.⁶⁻¹⁰ History of tick exposure is not always present⁸. Laboratory findings can include but are not limited to anemia, thrombocytopenia, leucocytosis, hypoalbuminemia, and coagulation abnormalities.⁶⁻¹⁰ Experimental studies in sub-lethally infected dogs show clinical signs begin to resolve at the time organisms can no longer be cultured, around post infection day (PID) 10-13.^{10,31,32} This reflects the acute nature of the disease. The duration of infection using PCR has not been determined, although *Rickettsia* DNA could be demonstrated in experimentally infected dogs as early as day 3 and

was still present at PID 21 in one study. Although well characterized, clinical signs are nonspecific and can occur in the absence of a history of tick bite. Thus the diagnostic challenges that physicians face are identical for veterinarians, and the consequences of misdiagnosis can be equally grave.^{25,26} The disease is usually rapidly responsive to doxycycline and supportive therapy if treated early. It is important for veterinarians to communicate with dog owners and their physicians when RMSF is suspected. Illness in dogs can precede or coincide with illness in people in the same household, although dogs do not serve as reservoirs for the disease.^{11-13,32} The clinical and temporal relationship of the naturally occurring disease in dogs and people suggest that dogs can serve as sentinels for natural infection and perhaps bioterrorist attacks using this organism.

Mediterranean spotted fever, also known as Boutonneuse Spotted Fever, is another well characterized spotted fever rickettsiosis. It is caused by *R. conorii ssp conorii*, and occurs in many countries in the Mediterranean, hence its name. *R. conorii* is transmitted to humans by *R. sanguineus*, the brown dog tick. This tick prefers to feed on dogs, but will also feed on humans. Like RMSF, the disease is seasonal, reflecting activity of the tick. Although the disease is usually less severe than RMSF, severe infection can result in death.³³ Clinical signs are similar to RMSF, and include but are not limited to, fever, headache, myalgia, arthralgia, eschar, gastrointestinal signs. More severe clinical findings include cardiac abnormalities, acute renal failure, gastrointestinal hemorrhage, seizures and acute respiratory distress syndrome.^{1,2,33} Unlike RMSF, a tache noire is often present, and the rash is classically papular rather than macular. Common hematologic abnormalities can include anemia and thrombocytopenia. Hypoalbuminemia and changes in renal and liver values can

also occur. As is the case in RMSF, more severe disease is seen in individuals with glucose 6-phosphate dehydrogenase.^{2,34} Laboratory diagnostic criteria are reviewed below. As is the case for RMSF, commonly used diagnostics are group and not species specific, response to doxycycline therapy is rapid, and failure to treat early is associated with a worse prognosis is associated with a more severe or fatal outcome.³³

The division of *R. conorii* into four subgroups has recently been proposed based on phenotypic, genotypic and clinical criteria. Proposed subspecies include *R. conorii ssp conorii*, the cause of MSF, *R. conorii ssp israelensis*, the cause of Israeli Spotted Fever, *R. conorii ssp indica*, the cause of Indian Spotted Fever, and *R. conorii ssp caspia*, the cause of Astrakhan Fever.³⁵ Recently, *R. conorii ssp israelensis* has been described in patients from Sicily diagnosed with MSF, and was associated with more severe clinical signs than *R. conorii ssp conorii*.³⁶

Dogs living in endemic areas are commonly exposed to *R. conorii*. Most surveys do not test for more than one SFG *Rickettsia* or subspecies. However the difference in seroprevalence to two strains of *R. conorii* was assessed in Israeli dogs. Seroprevalence to *R. conorii*²¹ *ssp conorii* (*R. conorii* Moroccan) was 8% while seroprevalence to *R. conorii ssp israelensis* (*R. conorii* Israel) was 28%.¹⁴ Seroprevalence to *R. conorii* in dogs in Spain ranges from 36.8% to 58.6% depending on the region surveyed.^{15,16,19} In Portugal 85.5% of dogs tested had antibody to *R. conorii*.¹⁷ In Croatia 62.4%¹⁸, and in Zimbabwe 82% of dogs were seropositive²⁰. Seroprevalence in dogs was higher in summer than winter months in one, Delgado, 1995 #2440} but not another study.²¹ Naturally infected dogs can remain

seropositive for more than 240 days³⁷. Studies have shown that seroprevalence in people and dogs are associated^{15,20}, and living near dogs is a risk factor for MSF in people.²¹ Recently, 16s rDNA from *Rickettsia* species has been amplified from the blood of Italian dogs using PCR.³⁸

The relationship between infection with *R. conorii* and clinical illness in dogs is not as clear as is the case for *R. rickettsii*. No association between illness in dogs and seroreactivity was shown in one study.¹⁹ Clinical illness has been associated with infection in dogs in only two reports since 1932. Dogs have been experimentally infected with a Zimbabwean strain of *R. conorii*. Needle inoculated dogs seroconverted and organisms were re-isolated between day 3 and day 10 after inoculation. No hematologic or physical abnormalities other than regional lymphadenopathy and local erythema occurred.³⁹ The lack of clinical signs in experimentally infected or naturally exposed dogs suggests that dogs may not be as susceptible to the disease as people, perhaps a reflection of adaptation of the organism to its vector and its preferred host. However, it is possible that clinical signs may be more readily induced in dogs if a tick is used as the vector, as has been suggested for infection with *B. burgdorferi*.⁴⁰ It is also possible that metabolic or immune compromise would be necessary for an individual dog to exhibit clinical signs. In that case, the high rate of seroprevalence in healthy animals, combined with a lack of other feasible diagnostic techniques, and a low index of suspicion by veterinarians, may have precluded associating disease with clinical signs of infection.

Although *R. rickettsia* and *R. conorii* are the most well described Spotted Fever Rickettsioses in people and in dogs, advances in molecular techniques have contributed to the

recognition and characterization of classic and novel rickettsial species as disease causing agents in expanding geographic locales.⁴¹ Many other spotted fever rickettsioses have been described and characterized in people worldwide in recent years. Infection with only a few other SFG *Rickettsia* has been described in dogs.

First recognized in 1984, Japanese Spotted Fever is a spotted fever rickettsiosis in people caused by *R. japonica*.⁴² Clinical signs include fever, exanthema and eschar. Neurological signs and shock associated with hypercytokinemia have also been described.^{43,44} Studies have shown 12% of pet dogs and 45% of stray dogs in Okinawa, and 12.2% dogs in the Philippines had antibodies to *R. japonica* antigen.^{45,46} DNA from *R. japonica* has been amplified from dog blood using PCR.⁴⁵ No studies investigating whether infection causes clinical disease have been reported.

Antibodies to *R. australis* the cause of Queensland Tick Typhus in people, were found in 11.2% of dogs surveyed in new South Wales, Victoria, Flinders Island and Tasmania. Experimental infection of two dogs failed to induce any physical, hematologic or histopathologic abnormalities. Although they seroconverted, *R. australis* could not be re-isolated from the dogs blood.⁴⁷ Although it does not appear that *R. australis* causes significant infection in dogs, only two needle intraperitoneally and intramuscularly inoculated dogs were studied. The failure to produce clinical signs may be related to the route or means of inoculation or may only occur in immunocompromised individuals.

Rickettsia akari causes illness in people that is quite different than other members of the SFG. Unlike most SFG *Rickettsia*, *R. akari* is transmitted by a vector other than ticks. This agent is carried by the house mouse mite (*Liponyssoides sanguineus*). It is endemic to New York City but has been found in other parts of the United States and the world. Clinical signs include eschars, fever and papular or vesicular rashes. Originally described in 1946, the number of reported cases had decreased for several years, until 2001, when it was recognized with much greater frequency. Interestingly, anthrax associated bioterrorism has been credited with providing the increased incentive for pursuing and reporting a definitive diagnosis in patients with cutaneous lesions since that time.⁴⁸ In a serosurvey of New York dogs, 6/311 dogs were seropositive to *R. akari* antigen, and sera remained positive after cross adsorption with *R. rickettsii*, suggesting crossreactivity to *R. rickettsii* was not responsible for the positive titer.⁴⁹ Experimental or natural infection with *R. akari* in dogs has not been reported.

It is important to note that in studies that do not corroborate serological titers with cross absorption studies or DNA evidence of infection, only measure exposure to Spotted Fever *Rickettsia* because there is a high degree of crossreactivity among SFG *Rickettsia*. Thus presuming the species of infecting *Rickettsia* is based on geographic locale can be erroneous. Recently *R. honei* (Rather than *R. australis* or *R. conorii*) has been described as a cause of SFR in Flinders Island and *R. africae* as cause of African spotted Fever in Africa (rather than *R. conorii*).⁵⁰ Whether the antibodies in the dogs serosurveys represent exposure to the infectious agent in question or to another SFG *Rickettsia* cannot be determined.

Numerous *Rickettsia* species are found in various tick species throughout the world. Many are considered “nonpathogenic” due to a lack of association with clinical disease in people,

and failure to cause disease in laboratory animals. However, because diagnostic techniques are not species specific, and pathogenicity may differ among hosts, such generalizations may also result in misclassification of a rickettsial agent as “non-pathogenic.”^{51,52} For example, *R. parkerii*, thought to be a nonpathogenic endosymbiont of *A. maculatum* for many years, was recently demonstrated to cause disease in a man in Virginia.⁵³ It has been suggested that any rickettsial organisms identified in an arthropod that can act as a vector be considered potentially pathogenic.¹ *R. rhipicephali*, *R. canada(ensu)* and *R. montana(ensu)* are commonly found in ticks in North America and are considered non-pathogenic in people. Similarly, dogs experimentally infected with *R. canada*, *R. montana* or *R. rhipicephali* do not exhibit clinical disease.^{10,32,54} Whether these species can cause disease in the natural setting or in immunocompromised individuals has not been investigated.

Mechanisms of pathology and immunity induced by SFG *Rickettsia*.

The fate of *Rickettsia* after tick inoculation has not been studied, although it has been hypothesized that direct drainage by capillaries could introduce *Rickettsia* directly into the circulation, while entry into the subcutaneous tissues may result in capture by dendritic cells or direct lymphatic drainage followed by travel to local lymph nodes.⁵⁵ SFG *Rickettsia* primarily infect endothelial cells, and clinical signs are believed to result from systemic vasculitis.

The mechanism of entry into endothelial cells is not known, but outer membrane protein A and B are likely to be involved. Antibodies to Outer membrane protein A (OmpA) blocked adherence of *R. rickettsii* in an in vitro endothelial model of infection⁵⁶ and recombinant *E*

coli expressing outer membrane protein B (OmpB) acquired the ability to invade endothelial cells^{56, 57} Interestingly, although antibody directed against OmpA and OmpB protects SCID mice against infection with *R. conorii*, the protection is Fc dependent. and may be mediated through opsonization and impaired phagosomal escape of bacteria within endothelial cells, although this effect is mitigated by normal serum.^{58,59} Thus the mechanism of F_c mediated impaired phagosomal escape may not be the mechanism of protection that is operable in vivo.

After endothelial cell entry, *Rickettsia* escape the phagosome and enter the cytosol.⁶⁰ Movement within the cell is through rearrangement of actin. Actin rearrangement also allows SFG *Rickettsia* to move from cell to cell without rupture.^{61,62} Changes in the adherens junctions occur in human umbilical vein endothelial cells (HUVEC) infected with *R. conorii* results in interendothelial gaps. This may be one mechanism that SFG *Rickettsia* induce vasculitis. Some endothelial cells detach in this model. Circulating infected endothelial cells occur in people with MSF,⁶³ and infected endothelial cells can be separated using magnetic beads from human blood.⁶⁴ Detachment is likely another mechanism of systemic spread of the organism, evasion of the immune system, and induction of vascular leakage.

Vascular leakage may also occur due to lipid peroxidation of endothelial cell membranes, resulting from oxidative damage induced by *Rickettsia*.⁶¹ An inflammatory response is also induced. Infected endothelial cells produce cytokines such as IL-6 and IL-8,⁶⁵ and chemokines such as CXCL9 and CXCL10.⁶⁶ Expression of adhesion molecule 1 and vascular cell adhesion molecule also occurs.⁶⁷ Surprisingly, blocking this chemokine system did not

affect clearance of the organism in a mouse model, so the role of chemokines in recruitment of lymphocytes in vivo has not been fully elucidated.⁶⁷

Infection with *R. conorii* also induces tissue factor expression in endothelial cells in vitro.⁶⁸

A rise in tissue plasminogen activator inhibitor and a decrease in tissue plasminogen is associated with a lethal outcome in mice, and endothelial responses to infection likely contribute to thrombosis seen in severe disease.⁶⁹

Endothelial cells may mediate innate immunity to *Rickettsia*. γ -IFN and TNF α are important for the clearance of *Rickettsia*, possibly through TNF α and IFN γ activation of endothelial cells and NO production.^{58 70} NK cells play an important role in the production of γ -IFN and clearance of *Rickettsia* within few days of infection.⁷¹

Adaptive immunity to *Rickettsia* is also important and appears to be long lasting. Re-infection with *R. rickettsii* could not be induced in experimentally infected dogs, and has not been documented in naturally infected people or dogs.⁷² Cell mediated immunity has been shown to be an important component of adaptive immunity in mouse models of infection. A DNA /subunit vaccine using rickettsial outer membrane protein A (OmpA) and outer membrane protein B (OmpB) subunits resulted in antigen specific in-vitro lymphocyte proliferation and γ -IFN production. The vaccine also provided protection to lethal challenge in the absence of detectable antibody.⁷³

Mouse models of infection have shown the importance of CD8+T cells in eliminating endothelial cell infection with spotted fever group *Rickettsia*.^{61,74-76} CD8+ T cell epitopes have been identified in the outer membrane proteins of *Rickettsia* and are protective against infection.^{77,78} Depletion of CD8+ lymphocytes or CD4+ and CD8+ lymphocytes, but not CD4+ lymphocytes resulted in death or persistent endothelial cell infection (15 days versus 10 days) when CH3HEN mice were inoculated with a sublethal dose of *R. conorii*. CD8+ T cells localized to infected endothelial cells at the time of clearance in non-depleted mice. Adoptive transfer of immune CD8+ or CD4+ T cells provides immunity to a lethal inoculum injected into naïve mice.⁷⁵ CD8+ T cells demonstrate MHCI restricted cytotoxic activity against *R. conorii* infected endothelial cells and *R. australis* infected macrophages. C57BL6 MHCI knock out (KO) mice are unable to clear infection with *R. australis*.⁷⁶

The role of CD8+T cells extends beyond γ -IFN production. Although γ -IFN (KO) C57BL6 mice are more susceptible to infection than wild type mice, adoptively transferred γ -IFN KO CD8+ T lymphocytes can restore resistance. Induction of apoptosis by CD8+ T cells may be one important mechanism of clearance, as perforin KO mice are more susceptible to infection with *R. australis* than γ -IFN knockout mice.⁷⁶ Additionally, NF κ B mediated inhibition of apoptosis is important for survival of *Rickettsia* in vitro.⁷⁹ Studies examining the direct role of CD8+ T cells in clearing infection with *R. rickettsii* in people and dogs have not been performed. However, CD8+ T cell numbers are increased acutely and then normalize in human patients with SFG *Rickettsia* infection.⁸⁰ These data suggest that though γ -IFN production, specific cytotoxicity, and induction of apoptosis, CD8+ T cells help clear infection with SFG *Rickettsia*.

Although passive transfer of immune sera is not protective in animals, humoral immunity may also play an important role in clearance of *Rickettsia* or in protection from re-infection.⁵⁵ Anti-OmpA and OmpB antibodies enhance F_c-mediated phagocytosis by endothelial cells and macrophages.⁸¹ Infection with *R. rhipicephali* induces humoral and cellular responses in guinea pigs that cross react with *R. rickettsii* in vitro. These animals are protected against challenge with *R. rickettsii*⁸² However, infection with *R. montana(ensus)* did not protect dogs against challenge with *R. rickettsii* despite induction of a strong crossprotective antibody response.¹⁰ Although crossreacting antibody is produced by heterologous infection in these studies, the role of crossprotective cell versus humoral immunity was not separately addressed. Interestingly, cross protection between *R. australis* and *R. conorii* occurs in mice, but only homologous immune sera and not heterologous immune sera protect against homologous challenge.⁸³

Although much remains to be learned with regard to pathophysiology and immunity to *Rickettsia*, it appears that the target of infection, the endothelial cell, plays an active and important role in both, and that innate and both arms of the adaptive immune response are important in recovery from infection.

Select Laboratory Tests Used for Diagnosis and Characterization

Serology

Microimmunofluorescence is the serologic method of choice currently used to diagnose SFG rickettsiosis. However a positive titer does not equate to acute exposure. Healthy dogs and people have antibodies to SFG *Rickettsia* . Antibody to *R. rickettsii*

persisted at least 240 days after inoculation and declined by day 1024 in experimentally infected dogs.^{10,72} Serosurveys show people and dogs have antibodies to SFG *Rickettsia* in *R. rickettsii* endemic areas with no history of illness.^{2,84} In a study of people in Barcelona, 56.2% of individuals who had MSF 10 years earlier remained seropositive. Titers to *R. conorii* appear to persist in dogs as they do in humans. In a serosurvey of Spanish dogs sampled multiple times, antibody to *R. conorii* persisted more than 240 days.³⁷ Whether persistent titers after infection and positive titers in individuals without a history of illness represents repeated exposure, exposure to other “nonpathogenic” SFG *Rickettsia*, or long lived plasma cells is unknown. Persistent titers, coupled with the fact that dogs and people can be seronegative early in infection, but after disease onset makes documentation of seroconversion necessary to substantiate acute infection. Although a positive IgM titer is considered strong evidence for acute exposure to SFG *Rickettsia* in people,⁴ dogs naturally infected with *R. rickettsii* have detectable serum IgM without associated seroconversion of IgG on acute and convalescent samples. Thus the presence of IgM supports, but does not prove acute infection with SFG *Rickettsia* in dogs.⁷² Importantly, serologic crossreactivity among SFG *Rickettsia* has been demonstrated in many host species including dogs and people.^{82,85} In fact, cross reactivity between *R. rickettsii* and *R. conorii* is so extensive that antibody one agent can be used to detect exposure to the other.⁸⁵ Therefore without testing multiple antigens and/or immunoadsorption, the species of infecting SFG *Rickettsia* cannot be presumed based on serology alone.

Immunohistochemistry and Histochemistry

Immunohistochemistry and Gimenez staining can also be used to demonstrate SFG *Rickettsia* in biopsy specimens. For immunohistochemistry, crossreactivity among SFG

Rickettsia occurs, depending on the antibody used for detection. Gimenez staining also does not differentiate among SFG species.⁵⁰

Cell Culture

During infection with Spotted Fever Group (SFG) *Rickettsia*, dislodgement and hematogenous circulation of organisms within endothelial cells occurs.⁶³ Therefore, it is possible to demonstrate the organism in the whole blood of patients and experimentally infected animals. Obtaining an isolate from a patient confirms infection with SFG *Rickettsia* and allows further characterization of the infecting *Rickettsia* once an isolate is obtained. Vero and other cell lines are used. Gimenez, Giemsa or IFA staining confirms intracellular growth of SFG *Rickettsia*. A shell vial technique is also commonly used to culture SFG *Rickettsia*. With this technique, samples (blood, tissue or circulating endothelial cells) are centrifuged in a manner that adheres organisms onto a slide coated with human embryonic lung fibroblasts. The assay is performed in triplicate, and slides are stained using IFA at set intervals to detect growth.^{50,64} Using the shell vial technique, *R. conorii* was detected from the blood and skin of 59% of samples from patients with MSF if sampled early (when the antibody titer was negative), before antibiotic therapy. Twenty-nine point eight percent of samples overall were positive. No samples held at room temperature or at 4°C for 24 hours was positive using this technique.^{4,64} Once an isolate is obtained, species specific monoclonal antibodies, SDS PAGE and molecular techniques can be used identify a *Rickettsia* species or characterize new species. However it is important to note that these latter differentiating techniques are not routinely used in the diagnostic setting. Culturing these organisms can pose serious hazards to laboratory workers. *R. rickettsii* is a Select Agent and has caused fatal

infection through aerosolization under laboratory conditions.²⁷ Isolation procedures require specialized training and BSL 3 level certification.

Polmerase Chain Reaction

Advances in molecular techniques have revolutionized the ability to detect and characterize infection with SFG *Rickettsia* in people. Many genes have been sequenced from *Rickettsia* isolates, and as a consequence species designations of various isolates, and phylogeneic relationships between species, have shifted rapidly in recent years. The criteria for the definition of a species using gene sequencing techniques has come under scrutiny for bacteria in general, and *Rickettsia* in particular.⁵⁰ The number of base pair differences that defines a strain or species has not been determined. Substantiating strains or species designations based on genotype with identifiable phenotypic differences has been advocated, along with sequencing at least five different genes. Sequences from the genes *gltA* (citrate synthetase) *rrs* (16s r RNA), and the 5' region of *ompA* (outer membrane protein A), *gene D* and *ompB* (outer membrane protein B) have been used to establish gene sequence based criteria for the identification of new *Rickettsia* species. In this study sequences from members of the order *Rickettsiales* available in GENBANK were analyzed, and the degree of conservation of these gene sequences was determined among species. Criteria were set for cutoffs for degrees of pairwise nucleotide sequence homologies among these bacteria. Although there is a high degree of homology in the *rrs* and *gltA* genes for members of this genus, the 5' region of the outer membrane protein genes *ompA* and *ompB* exhibits a higher degree of variability between SFG species.⁸⁶

The description of a new *Rickettsia* species requires an isolate. In the clinical setting it is sometimes difficult to obtain an isolate from patient samples for reasons mentioned above. PCR has greatly facilitated the detection and characterization of SFG rickettsiosis in people. Like cell culture techniques, the sensitivity of PCR using blood specimens is potentially limited by a low level of circulating organisms. Serology is not limited by this constraint, but titers can be negative at presentation. PCR can demonstrate *Rickettsia* infection in seronegative patients, making it a potentially valuable tool in the acute diagnosis of disease.⁸⁷ A number of PCR assays, targeting a number of different genes, have been used to detect *Rickettsia* in clinical specimens. Some PCR assays have been shown to be more sensitive than cell culture.⁸⁸ Nested PCR (reamplification of previously amplified DNA using primers targeted to an internal sequence) increases sensitivity. Because reaction tubes have to be opened to add the second set of primers and additional polymerase, laboratory contamination is a major disadvantage of this technique. Suicide nested PCR utilizes nested PCR, but the primers are used only on a limited number of assays in a laboratory and then retired. This decreases the risk of contamination. A suicide nested PCR detecting sequences from several different genes was more sensitive than cell culture for detecting *Rickettsia* in skin biopsy specimens.⁸⁸ A nested suicide PCR targeting non-overlapping portions of the *ompA* gene was also found to be more sensitive than culture for skin biopsy specimens but not for serum.⁸⁹ One study in dogs experimentally infected with *R. rickettsii* showed a seminested PCR detected *Rickettsia* earlier and for longer than did cell culture.³¹ A PCR that targets DNA using primers Rr190.70 and Rr190.701, amplifies a 632 bp portion of the *ompA* gene. Recently, a PCR using these primers has been shown to detect 160 copies of *Rickettsia*.⁸⁸ By

comparison, the limit of detection of several recently developed real time, nested and conventional PCR assays ranges from 0.4-7 organisms^{87,88,90,91}.

Polymerase chain reaction combined with DNA sequencing, Restriction Fragment Length Polymorphism, or other analysis offer the potential advantage of identifying the species of the infecting SFG *Rickettsia* even without obtaining an isolate. Theoretically, the ideal target for diagnostic PCR would be a gene that would differentiate among species and would be variable enough that it would correlate with important phenotypic differences, and yet sustain enough selective pressure that extreme variation among individual members of the same strain or species would not occur. Because the protein OmpA is an antigen that contributes to the serologic differences used to phenotypically differentiate *Rickettsia* species,⁹² and its genotypic variability also differentiates among species,^{86,93} it is a logical target for PCR for clinical specimens. In fact, sequencing of the 632 bp amplicon using primers Rr190.70 and Rr190.70, shown to differ by 98.8% among *Rickettsia* species,⁸⁶ has been routinely used to identify the species of infecting SFG *Rickettsia* in clinical specimens.¹ A disadvantage of this PCR is a lack of sensitivity. Most other PCR assays with a demonstrated limit of detection superior to the assay using the Rr190.70 and Rr190.70 primers, either have not been developed for the purpose of differentiating SFG species, or they differentiate a limited number of species.^{87,90,91,94} No PCR with an established limit of detection or the ability to differentiate species of infecting SFG *Rickettsia* has been validated for use in the dog.

Treatment

Certain antibiotics that are effective against intracellular organisms have documented efficacy against *Rickettsia*. The treatment of choice for *R. rickettsii* in dogs and people and *R. conorii* in people is doxycycline. Chloramphenicol has been used to treat *R. rickettsii* in dogs and people is reported to be effective, but side effects can preclude its use.^{26,28,50} One study suggested that chloramphenicol alone was not as effective as doxycycline.⁹⁵ Enrofloxacin has also shown to be effective in treating experimentally infected dogs,⁹⁶ although azithromycin may be less effective.³¹ *Rickettsia* are resistant to many other routinely prescribed antibiotics. Trimethoprim sulfa may actually worsen disease. Response to antibiotics is rapid, within 24-48 hours in dogs. Other supportive care is provided as needed. Delay in diagnosis and appropriate antibiotic treatment is associated with increased morbidity and mortality in people and in dogs.^{25,26,28,33,41}

Summary

Rickettsia are important causes of morbidity and mortality in people worldwide. Some species are known to infect dogs, and dogs can act as sentinels for disease in people. Limitations of conventionally used diagnostic techniques used in dogs and people include a lack of sensitivity and inability to differentiate among infecting species. Developing such a diagnostic test in dogs will facilitate investigations into the spectrum of species that can infect dogs, and the disease spectrum associated with SFG *Rickettsia* in dogs. Because of their role as sentinels for disease for RMSF and MSF, such investigations will have implications for their human companions as well.

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3) MOLECULAR CHARACTERIZATION OF *RICKETTSIA RICKETTSII* INFECTING DOGS AND PEOPLE IN NORTH CAROLINA

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Abstract

Rocky Mountain spotted fever (RMSF) is an important cause of morbidity and mortality in people and dogs in the United States. Disease manifestations are strikingly similar in both species. Illness in dogs can precede illness in people in the same household. *R. rickettsii* has been identified as Select Agent by the Center for Disease Control and as a Category C priority pathogen by the National Institute of Allergic and Infectious Diseases (NIAID) because it is amenable to use as a bioterrorist agent. The clinical and temporal relationship of naturally occurring disease in dogs and people suggest dogs could serve as sentinels for natural infection and bioterrorist attacks using this organism. Recognizing genetic modifications in naturally occurring disease agents in order to distinguish them from intentionally released agents are priorities put forth by the NIAID. In order to determine if *Rickettsia* naturally infecting dogs are the same as those that infect people in a given geographical region, we characterized rickettsial isolates obtained from three dogs and two people diagnosed with RMSF in North Carolina.

Portions of three genes (*ompA*, *rrs*, and *gltA*) amplified by PCR were cloned and sequenced or directly sequenced. Reactions were run in duplicate in forward and reverse directions. Gene sequences were aligned with known sequences deposited in GENBANK and with each other.

Sequences of the 5' region of the *ompA* gene were 100% homologous with a tick strain (BITTERROOT) of *R. rickettsii* for all five isolates. Sequences of the *rrs* gene were 99.8-99.9 % homologous with a tick strain (SAWTOOTH) of *R. rickettsii*. *rrs* gene sequences

from one dog and the two people were identical. Sequences one dog isolate differed from these by one base pair. Sequences from another dog isolate differed by two base pairs. Sequences of the *gltA* gene are pending.

This confirms on a molecular level that *R. rickettsii* causing naturally occurring RMSF in North Carolina dogs is highly homologous to *R. rickettsii* that causes the disease in people in the same region. Sequence data will be deposited in GENBANK, thereby providing genetic information regarding naturally occurring *R. rickettsii*.

Introduction

Diseases caused by organisms in the Genus *Rickettsia* are heralded as paradigms of emerging infectious disease.^{1,2} In addition to the clinical features of rickettsial disease, diagnosis typically utilizes methods such as serological testing that do not differentiate among species, so the species of the infecting *Rickettsia* is generally presumed, based upon typical disease manifestations and the geographic location in which the tick bite was encountered. Advances in molecular techniques have contributed to the recognition and characterization of classic and novel rickettsial species as disease causing agents in expanding geographic locales.¹⁻³

Characterizing species of *Rickettsia* in a given region has important implications for local residents, travelers and even global biodefense. Rocky Mountain Spotted Fever (RMSF) is an important cause of morbidity and mortality in people and dogs in the United States.^{4,5,6} North Carolina has one of the highest reported incidences of the disease in people.^{5,6} Disease manifestations are strikingly similar in people and in dogs.^{1,2,5,7-9} Illness in dogs can precede illness in people in the same household.^{4, 10-12} *R. rickettsii* has been identified as a Select Agent by the Centers for Disease Control and Prevention, and as a category C priority pathogen by the National Institute of Allergic and Infectious Disease (NIAID) due to concerns that it is amenable to use as a biological weapon.¹⁴ The clinical and temporal relationship of the naturally occurring disease in dogs and people suggest that dogs can serve as sentinels for natural infection and perhaps bioterrorist attacks using this organism. Recognizing genetic modifications in naturally occurring disease agents in order to distinguish them from intentionally released agents are priorities recently put forth by the

NIAID. In addition, molecular characterization of *R. rickettsii* isolated from dogs has not been reported.

The purpose of this study was to characterize *Rickettsia* isolates obtained from naturally infected dogs and people in North Carolina by gene sequencing in order to determine if *Rickettsia* species infecting dogs are the same as those that infect people in a given geographical region, and to provide sequence information regarding this important infectious agent.

Materials and Methods

Rickettsia were cultured from the blood of three dogs and two people¹⁴ presenting with clinical findings consistent with RMSF using Vero or DH82 cells as previously described.¹⁵ DNA was extracted using the Qiaamp® DNA Blood mini kit following the manufacturer's instructions (bacterial culture suspension and tissue protocol). An extraction control was included in the procedure. PCR was performed in order to amplify portions of three genes [*gltA rrs*, *ompA* (5' region)] using primers shown in Table 1. Negative (water) controls were included in all assays, and extraction controls were included in the *rrs* and *ompA* PCR's. Both the 5' and 3' portions of the *gltA* gene were amplified using a modification of a previously described protocol.¹⁶ The reaction mixture contained forward and reverse primers at a final concentration of 1µM, 25 µl of PCR Master Mix (Promega), 1-2 µl of template DNA and H₂O to a final reaction volume of 50 µl. Thermocycler conditions included a denaturation step for 2 minutes at 95°C, then 40 cycles of denaturation (95°C for 30s), annealing (45°C for 30 s), and extension (72°C for 1 minute), and a final extension at 92°C

for 5 minutes. The *rrs* gene was amplified using the 8F and 1492R primers (Table 1) under the following conditions for all but one isolate. For each reaction, 27.5 ul of H₂O, 6 ul of 25mM MgCl₂ (3mM final concentration), 5 ul 10X Buffer, and 0.75 ul AmpliTaqGold® (Applied Biosciences) were incubated with 1 ul of a 1:10 dilution of DNase I (Rouche Diagnostics) at room temperature for 30 minutes, and then 95°C for 15 minutes. This was then added to 7 ul of a mixture of 2 mM of each dNTP's (final concentration of 280 uM), 1 ul of 25pmol/ul (final concentration of 0.5uM) of each primer, and 1-2 ul DNA template to make a final reaction volume of 50 ul. The DNase treatment was adapted from a previously described protocol, and utilized to overcome nonspecific amplification of residual bacterial DNA in the AmpliTaqGold® polymerase, and/or the water.¹⁸ Thermocycler conditions included a denaturation step at 94° C for 5 minutes, then 40 cycles of denaturation (94°C for 45 seconds), annealing (55°C for 30 sec), and extension (72°C for 1 minute), and a final extension of 72°C for 10 minutes. For one isolate , Rickettsial specific *rrs* primers (20F and 1455R, Table 1) were used under the following conditions to amplify 16s rDNA. 25 ul of PCR Master Mix (Promega), forward and reverse primers at a final concentration of 1uM, 2ul of DNA template, and water were combined to make a final reaction volume of 50 ul. Thermocycler conditions consisted of an initial denaturation step of 94°C for 5 minutes, and 40 cycles of denaturation at 95°C for 45s, annealing at 58°C for 45s, extension at 72°C for 1.5 minutes, and a final extension of 72°C for 5 minutes. The 5' region of the *ompA*, gene was amplified using a methods adapted from a previously described protocol.²⁰ A reaction mixture containing primers at a final primer concentration of 0.3uM, 5 ul of 10X Accuprime *Pfx* reaction mix, 1-2 ul of DNA template, 1ul of AccuPrime™ *Pfx* DNA polymerase, (2.5 U/ul) and H₂O to a final reaction volume of 50ul. Thermocycler conditions consisted of an

initial denaturation step at 95C for 2 minutes, then 35 cycles of denaturation (95C for 15 seconds), annealing (50C for 30 seconds), and extension (68C for 1 minute) with a final extension of 72C for 5 minutes. PCR products were purified using the Qiaquick® PCR purification kit or the Qiaquick® gel extraction kit according to manufacturers instructions. Products were cloned and sequenced, or subjected to direct sequencing. Cloning reactions were performed using the vector PGEM T easy System I® or the TOPO TA Cloning® kit according to manufacturers instructions. Sequencing reactions were performed on the LICOR 4200 DNA sequencer according to manufacturer's instructions, or through Davis Sequencing (www.davissequencing.com). Reactions were performed in duplicate, on separate clones or PCR reactions, in forward and reverse directions. Consensus sequences were created for each gene for each isolate. Regions of local sequence similarity were initially assessed using the Basic Local Alignment Search Tool (BLAST) for sequences deposited in GENBANK (<http://www.ncbi.nlm.nih.gov/BLAST>). Because sequences deposited in GENBANK for a given gene can differ in length, individual base pair variations that exist among members of the same species may not be accounted for when assessing homology using BLAST. To account for these differences, consensus sequences were created if more than one sequence was available in GENBANK for a given species of *Rickettsia*. GENBANK sequences for members of the genus *Rickettsia* with a species designation were downloaded and aligned using the CLUSTAL W multiple alignment tool (Bioedit version 5.0.6). Partial sequences were included only if a complete sequence for the same species was available in another deposit. *Rickettsia* species and GENBANK accession numbers used in the creation of the consensus sequences are shown in Table 2. To make comparisons, sequences were aligned and edited to the same length for each gene for each isolate. The GENBANK sequences were

similarly aligned and trimmed so that sequences were the same length as the isolates. Consensus sequences for the isolates and the GENBANK *Rickettsia* consensus sequences were then aligned simultaneously using CLUSTAL W multiple alignment tool (Bioedit version 5.0.6). Differences were enumerated manually, and also evaluated using a sequence identity matrix program (Bioedit Version 5.0.6).

Results

PCR water controls and DNA extraction controls were negative. Contiguous sequences resulting from amplification of the two portions the *gltA* gene resulted in amplicons ranging from 1181 to 1196 base pairs in length, corresponding to base pair -26 to 1156 in relation to the *gltA* gene of *R. conorii* (GENBANK accession number AE008677). *Rickettsia* species in GENBANK with available sequence in the -26 to +21 region of the *gltA* gene are limited to *R. conorii*, *R. typhi* and *R. prowazekii*. Therefore, utilizing the BLAST program, sequence homology for the amplified *gltA* gene for dog and human isolates described in this study matched most closely to *R. conorii* (99.1%- 99.3%). Known sequences for the *R. rickettsii* *gltA* gene begin at base pair 21, therefore sequence homology estimates in relation to *R. rickettsii* could only be made on sequences trimmed as described in Materials and Methods. The length of the trimmed sequences and the percent homology to the *R. rickettsii* consensus sequence is shown in Table 3. The trimmed *gltA* consensus sequences for the dog and human isolates had a calculated sequence identity of 0.996-0.998 to *R. rickettsii*. The isolates of dog origin were identical to each other, while the isolates of human origin shared 0.997 identity. The sequence identity for dog isolates compared to human isolates ranged from 0.997 to 0.998. The sequence identity between the dog isolates and the *R. rickettsii* consensus

sequence was not equal to 1.00 because of base pair variability at two sites in the *R. rickettsii* consensus sequence. When this variation was taken into account and homology was calculated manually, the dog isolate *gltA* sequences were 100% homologous with *R. rickettsii*, and homology of the human isolates to *R. rickettsii* ranged between 99.8 and 99.9% (Table 3). The isolate sequences were most homologous to the *R. rickettsii* consensus sequence. Sequences from *R sibirica* (0.992-0.994 sequence identity) and *R parkeri* (0.992-0.993 sequence identity) were the next most similar to the *gltA* sequences for the dog and human isolates. When compared to consensus sequences for known species of *Rickettsia*, *R rickettsii* was most homologous to *R parkeri*, *R conorii* and *R slovaca*, (0.992 sequence identity). *R parkeri* and *R sibirica* shared the highest identity for all sequences compared (0.999 sequence identity).

Sequences resulting from the amplification of the 16S rRNA gene for four of the isolates using the 8F and 1492R eubacterial primers ranged from 1270 to 1438 base pairs in length, corresponding to positions 11 to 1455 of the *R. rickettsii* (GENBANK accession number L36217) *rrs* gene. Using these primers resulted in amplification of a 16S DNA sequence from a *Mycoplasma* species in addition to *Rickettsia* for two dog isolates. Preliminary analysis showed 16S sequences matched most closely with *M. orale* (GENBANK accession number AY796060.1, strain NC10112) and *M timone* (GENBANK accession number AY050170.1). *Rickettsia* specific 16SDNA primers were designed in order to efficiently amplify rickettsial 16s DNA from one of the *Myocplasma* containing isolates (Table 1). This resulted in an amplicon of 1221 base pairs in length corresponding to positions 69 to 1290. Using the BLAST program, all *Rickettsia* 16S DNA amplicons from the isolates matched

most closely with *R. rickettsii* (strain SAWTOOTH GENBANK accession number RRU11021). Homology was 99.9 for all isolates. Consensus sequences for the *rrs* gene created from each isolate were 99.8-99.9% homologous with a consensus sequence created for the *rrs* gene sequences for *R. rickettsii*. (Table 2). Consensus *rrs* sequences from dog and human isolates were almost identical to each other (sequence identity 0.998-1.0 between dog and human isolates). The sequence identity with the *R. rickettsii* consensus sequence for one dog isolate was 0.998 while the rest of the dog and the human isolates had a sequence identity of 0.999 with *R. rickettsii*. The species with the next closest sequence match to the dog and human isolates were *R. conorii* and *R. slovaca*, (sequence identity ranging from 0.995-0.996). The species with the most homologous gene sequences (other than the isolates and *R. rickettsii*) were *R. heilonjiangii* to *R. japonica*, and *R. conorii* to *R. astrakhan* (sequence identity 0.999).

Sequences resulting from the amplification of the 5' region of the *ompA* gene ranged from 516-590 base pairs in length corresponding to base pair 91 to 680 of the *ompA* (190 KD) gene for *R. rickettsii* (GENBANK accession number M31227). These sequences were 100% homologous to *R. rickettsii* strain BITTERROOT (GENBANK accession numbers RRU43804 and M31227). Trimmed sequences of the 5' region of the *ompA* gene for the isolates were 100% homologous to each other (Table 2). These were also 100% identical to strain BITTERROOT of *R. rickettsii* (GENBANK accession number RRU43804). When consensus sequences were used for comparison, the identity between isolates and GENBANK sequences was highest for the isolates with regard to each other (sequence identity of 1.00), and the consensus sequence for *R. rickettsii* (sequence identity 0.994). The

identity between isolates and the consensus sequence of *R. rickettsii* was not 1.00 due to two variable base pairs in the *R. rickettsii* consensus sequence. *R. slovaca* and *R. sibirica* (sequence homology of 0.958 and 0.962) represented the next most homologous species to the isolates. The *Rickettsia* species' most homologous to *R. rickettsii* were also *R. slovaca* and *R. sibirica* (sequence identity 0.958 for both). Using these consensus sequences, the species that shared the most identity for *ompA* (other than *R. rickettsii* and the isolates described in this study) were *R. astrakhan* and *R. sibirica* (sequence identity of 0.976).

Discussion

Sequences from the genes *gltA*, *rrs*, and the 5' region of *ompA* have been utilized, along with those of *gene D* and *ompB*, in establishing gene sequence based criteria for the identification of new *Rickettsia* species. Although there is a high degree of homology in the *rrs* and *gltA* genes for members of this genus, the 5' region of the *ompA* gene is highly variable and can be used to help assign an isolate to a particular species.^{2, 21} The results in this study identified a high degree of homology among the dog and human isolates, and between these isolates and *R. rickettsii*. The BLAST analysis program cannot always adequately demonstrate homology because several sequences that vary in length are available for each *Rickettsia* gene. Therefore assigning homology based on the number of matching bases using BLAST is not adequate. In this study, a consensus of GENBANK sequences were generated and used for comparison among the dog and human isolates. Two variable base pairs occurred in the *R. rickettsii* consensus sequences for the *gltA* and *ompA* genes due to corresponding base pair differences in the available Gen Bank sequences. Base pairs from the isolates matched one of the base pairs from a GENBANK *R. rickettsii* sequence in all instances, and thus their

homology to *Rickettsia rickettsii* was higher when calculated manually than when calculated using the sequence identity matrix program (Bioedit version 5.1.0). Additionally, the primer used to amplify the 5' region of the *gltA* gene (Table 1) was designed using a *Rickettsia conorii* sequence, because sequence for this region of the *gltA* gene for *R. rickettsii* has not been described previously. Amplifcons from the 5' region of the *gltA* gene of the isolates was 46 base pairs longer than most *Rickettsia gltA* sequences deposited in GENBANK and therefore trimming was necessary to determine homology to other known species. Regardless of the method utilized to assess homology, all gene sequences for both the dog and the human isolates were more similar to *Rickettsia rickettsii* than any other available species consensus sequence, and differences were not large enough to assign the isolates to a new species, according to established criteria.²¹

Mycoplasma species DNA was amplified from two isolates when universal *rrs* (16SrDNA) eubacterial primers were used. This was presumed to be a cell line contaminant, as *Mycoplasma* was found in two additional isolates using the same cell line to culture blood from one dog and one human patient not included in this study. Preliminary sequence analysis showed highest degrees of homology with *M. orale* (GENBANK accession number AY796060.1 NC10112) and *M. timone* (GENBANK accession number AY050170.1). *Mycoplasma* contamination is a common problem in cell lines.²² However, coinfection with *Mycoplasma* and *Rickettsia* in these dogs and human patients cannot be ruled out as the original uninoculated cell lines were not available for analysis.

These results confirm on a molecular level that *R. rickettsii* isolated from dogs with clinical manifestations consistent with RMSF in North Carolina is highly homologous to *R. rickettsii* that causes the disease in people in the same region. These are the first sequences reported for *R. rickettsii* isolated from dogs. Complete (untrimmed) sequence data for the isolates have been deposited in GENBANK (accession numbers DQ150680 through DQ150694) thereby providing genetic information regarding naturally occurring *R. rickettsii*.

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Table 1 Primers used to amplify *Rickettsia* DNA.

Primer pair	Gene	Sequence	Reference
5' <i>gltA</i> Cs890R	<i>gltA</i>	5' ATTAGGGTTATACTTACTG3' 5' GCTTTAGCTACATATTTAGG3'	16
877p 1258n	<i>gltA</i>	5' GGGGACCTGCTCACGGCGG3' 5' ATTGCTAAAAGTACAGTGAACA3'	16 16
8F 1492R	<i>rrs</i>	5' AGAGTTTGATCCTGGCTCAG3' 5'-GGTTACCTTGTTACGACTT-3'	17* 17*
20F 1455R	<i>rrs</i>	5'TGGCTCAGAACGAACGCTATC3' 5' TTTACCGTGGTTGGCTGCCT3'	
RR190.70F RR190.701R	<i>ompA</i>	5' ATGGCGAATATTTCTCCAAAA3' 5' GTTCCGTTAATGGCAGCATCT3'	19 20

Table 2 *Rickettsia* species and accession numbers used for sequence comparison

Species	GENBANK accession number for each gene		
	<i>gltA</i>	<i>rrs</i>	<i>ompA</i>
<i>Israeli tick typhus</i>	U59727		AY197564, AY197563, AY197566, U43797
<i>R. aeischlimannii</i>	AY259084	U74757	
<i>R. africae</i>	U59733	L36098	
<i>R. akari</i>	U59717	L36099, U12958	
<i>R. amblyomma(mii)</i>		U11012	
<i>R. astrakhan</i>			U43791
<i>R. australis</i>	U59718	L36101, U17644, U12459	
<i>R. bellii</i>	U59716, AY362703, 375161	L36103, U11014	
<i>R. Canada</i>	U20241, U59713	AF503168, U15162, L36104	
<i>R. conorii</i>	U59730	U12460, L36105, L36107	AY346453, U43794, U43806, U45244, U46918
<i>R. heilongjiangii</i>	AF178034, AY285776	AF178037	AY280711, AF179362
<i>R. Helvetica</i>	AJ427878, U59723		
<i>R. honei</i>	U59726, AF022817		AF018075
<i>R. japonica</i>	U59724	L26213	
<i>R. limoni</i>		AF322442	
<i>R. marmionii</i>		AY737685	
<i>R. massiliae</i>	U59719	L36106, L36214	U43793, U43799
<i>R. montana(ensis)</i>	U74756	L36215, U11016	U43801
<i>R. parkerii</i>	U59732	L36673, U12461	
<i>R. peacockii</i>			AY319292, AH013412, AY357765, AY357766, AH0134413
<i>R. prowazekii</i>	AY730679, AY570300, M17149	M21789	
<i>R. rhipicephali</i>	U59721	L36216, U11019	U43803
<i>R. rickettsii</i>	AY189819, U59729	AY573599, L36217, M21293, 411021	AY319293, U43804
<i>R siberica</i>	U59734	D38628, U12462	U43807
<i>R. slovac</i>	AY129301, U59725, AF484070	L36221	U43808
<i>R. typhi</i>	U20245, U59714	L36221, U12463,	
<i>Thai tick typhus</i>			U43809

Table 3 Homology of isolate consensus sequences to consensus sequences of *R. rickettsii*

Isolate#	Origin	<i>gltA</i>	<i>rrs</i> *	<i>ompA</i>
1989CO1	Dog	100% (1141/1141)	99.8% (1223/1225)	100% (509/509)
1994CO2	Dog	100% (1141/1141)	99.9% (1224/1225)	100% (509/509)
1991CO3	Dog	100% (1141/1141)	99.9% (1224/1225)	100% (509/509)
1995HO1	Human	99.8% (1139/1141)	99.9% (1224/1225)	100% (509/509)
1995HO2	Human	99.9% (1140/1141)	99.9% (1224/1225)	100% (509/509)

*Final length includes gaps created in the alignment of all species.

**4) EVALUATION OF A PCR ASSAY FOR DETECTION OF SPOTTED FEVER
GROUP *RICKETTSIA* IN DOG BLOOD**

Abstract

Spotted Fever Group (SFG) *Rickettsia* are important causes of morbidity and mortality world-wide. *Rickettsia* are heralded as paradigms of emerging infectious disease as many new species and disease syndromes have been described with recent advances in molecular biology. Both dogs and people are infected by SFG *Rickettsia*. Dogs can serve as sentinels of disease in people in endemic areas. Due to their obligately intracellular and endotheliotrophic nature, these bacteria are present in low numbers in the blood of infected individuals, and therefore demonstrating infection using culture and PCR can be challenging. Recently, sensitive PCR techniques have been developed that can detect 0.4-7 organisms per reaction in vitro. The sensitivity of these assays may actually be somewhat lower diagnostically, as sensitivity was not determined in the presence of host DNA for most assays. In addition, most diagnostic techniques including serology, culture and most PCR's do not differentiate among infecting species of *Rickettsia*. Here we describe a PCR that has been developed to detect infection with SFG *Rickettsia* in dog blood that can detect 15-30 copies of *Rickettsia* 100% of the time and 1.5-3.0 copies 45% of the time. We also show that this PCR amplifies a portion of the *ompA* gene that when sequenced, is adequate to differentiate the species of most SFG *Rickettsia*. Such a diagnostic tool may assist in the accurate clinical diagnosis of early infection with *Rickettsia*, and in identifying infection with novel rickettsial agents. In addition, it may facilitate answering many as yet unanswered questions about infection with *Rickettsia* such as how long antibodies persist in relation to the organism.

Introduction

Spotted Fever Group *Rickettsia* are important causes morbidity and mortality in people worldwide.^{1,2} These arthropod borne, obligately intracellular organisms, primarily infect endothelial cells, and are present in low copy number in the blood.²⁻⁵ Therefore, they are notoriously difficult to detect in blood samples from infected patients. It is well established that *R. rickettsii* infects dogs, and the role of canine infection with other species of SFG *Rickettsia* is beginning to be elucidated.⁶⁻²³

Rickettsia rickettsii is a small, pleomorphic gram negative rod. This fastidious organism infects mammalian endothelial cells after transfer through feeding by a tick vector. Resulting disease manifestations are acute and severe and are quite similar in dogs and people.^{1,6,7,9,24-26} Mortality can be high if appropriate antibiotic treatment is not instituted soon after infection.^{27,28} Infection in dogs can precede or coincide with infection in people. Therefore, dogs are considered sentinels for this disease.²⁹⁻³³ Clinical signs in both species typically include (but are not limited to) fever, vasculitis, gastrointestinal signs, anemia central nervous system abnormalities and thrombocytopenia. Experimental studies in dogs show clinical signs begin to resolve at the time organisms can no longer be cultured, around post infection day (PID) 10-13.^{9,10,34} This reflects the acute nature of the disease. The duration of infection using PCR has not been established, although experimentally infected dogs can be PCR positive at least 21 days after infection.³⁴ Although well characterized, clinical signs of RMSF are not specific and can occur in the absence of a history of tick bite in both dogs and people. Therefore, diagnosis can be challenging, and the consequences of delayed or misdiagnosis are severe morbidity and death.^{7,35-38}

R. conorii causes Mediterranean Spotted Fever (MSF) in people. Although sometimes considered to be a milder disease than RMSF, the outcome can be fatal.³⁹ Recently, it has been proposed that *R. conorii* be divided into four subspecies, including *R. conorii subsp conorii*, *R. conorii subsp. indica*, *R. conorii subsp israelensis* and *R. conorii subsp caspia* based on phenotypic and genotypic dissimilarity.⁴⁰ These subspecies cause Mediterranean Spotted Fever, Israeli Spotted Fever, Indian Spotted Fever, and Astrakhan Spotted Fever respectively. However, because commonly used diagnostic tests do not differentiate among species and subspecies, the geographic distribution and clinical syndromes associated with infection with the different subspecies have yet to be completely defined. Importantly, infection with different subspecies (*R. conorii ssp israelensis* versus *R. conorii ssp conorii*) may account for differences in severity of clinical signs of MSF.^{41,42} MSF occurs primarily in Mediterranean countries, sub-Saharan Africa, and Croatia.^{1,43} It is transmitted by the brown dog tick, *Rhipicephalus sanguineus*, and living near seropositive dogs appears to be a risk factor for disease in people.¹⁷ Experimentally infected dogs develop minimal clinical signs, but organisms can be re-isolated from blood between post infection day 3-10¹⁹ Recently we demonstrated natural infection in a Yorkshire Terrier dogs in Italy using PCR and gene sequencing (Chapter 5).

Initial diagnosis of rickettsiosis in dogs and people is made based on presenting history and clinical signs or symptoms, respectively, and knowledge of rickettsial epidemiology, as species historically have been geographically segregated. Serology and cell culture help confirm the diagnosis of pathogenic SFG *Rickettsia* infecting human and canine patients. These tests have several limitations. Serology does not differentiate among species of SFG

Rickettsia, can be negative early in disease, and unless seroconversion is demonstrated, previous infection or exposure to nonpathogenic *Rickettsia* cannot be differentiated from active infection.^{2,9,18,44-46} In vitro cell culture requires specialized facilities. Cultivation of some species can pose a health threat to laboratory workers.⁴⁷⁻⁴⁹ Stains used to detect organisms in cell culture (Gimenez) do not differentiate among species within the spotted fever group.⁵⁰

Technological advancements in molecular biology and the ability to share data on a global level have led to unprecedented capabilities regarding the discovery and understanding of emerging or previously undiscovered infectious diseases. Such advances in technology have resulted in recent dramatic paradigm shifts for rickettsioses, diseases caused by organisms that in the past have been very difficult to study and describe due to their complex and highly specialized life cycles.^{1,2} There are many examples in the literature of how recognition of an atypical disease syndrome in a patient or set of patients by an observant clinician has led to the discovery of a new species that can cause disease in an individual.¹ With the increase in travel of people and their pets to many parts of the world, the geographical distribution of SFG *Rickettsia* cases is shifting.⁵¹ With the advancement of techniques to isolate and sequence DNA a large number of rickettsial species have been identified in arthropods.¹ Such organisms may be endosymbionts in the tick or may be pathogens to mammals. It has been recommended that any *Rickettsia* species found in a tick that can act as a vector, should be considered potentially pathogenic.¹ It is likely that with the advancement of molecular biological techniques other rickettsiosis will be identified in dogs just as they have in people, and dogs may serve as sentinels or reservoirs for disease in their human companions.

Sequences of the complete genome of *Rickettsia conorii* and of several genes from many different species in the spotted fever group of *Rickettsia* are now available in GENBANK. Comparison analysis of gene sequences has facilitated development of gene based criteria to establish what constitutes an existing or new species of *Rickettsia*. In order to define a new species, phenotypic characteristics and sequences of the genes *gltA* (citrate synthetase), *rrs* (16srRNA) *ompA* (outer membrane protein A), *ompB* (outer membrane protein B), and *gene D* are analyzed.⁵² Although a high degree of homology is shared among SFG *Rickettsia rrs* and *gltA* genes, the rickettsial *ompA* gene exhibits consistent variability among species.^{52,53} Its protein product is an outer membrane protein that contributes to serologic differences used for phenotypic species classification.⁵⁴ Therefore sequencing this gene provides a rational link between phenotypic and genotypic species differentiation. The 632 base pair hypervariable region of *ompA* amplified by primers RR 190.70F and RR190.701R has been shown to contain enough differences to successfully differentiate known species based on sequencing or RFLP.^{1,52,53,55} Sequence information from this gene product alone has been used to determine the species of *Rickettsia* infecting human patients.^{41,55} By determining species and strain differences, these techniques have help explained unusual clinical signs in patients presenting for a *Rickettsia*-like illness. For example, sequencing the 632 bp region of the *ompA* gene from cell cultures of *Rickettsia* taken from the blood of people with MSF has shown *R. conorii ssp israelensis*, may be responsible for a more severe form of the disease than *R. conorii ssp conorii*.⁴²

A validated diagnostic PCR that can amplify *Rickettsia* DNA directly from a patient's blood sample, and differentiate among species of infecting *Rickettsia* is not readily available for

people or dogs. The limit of detection for the *ompA* PCR using RR.190.70F and RR.190.701R was recently tested, and shown to be 160 copies of SFG *Rickettsia*.⁵⁶ However, the calculation for the limit of detection was not performed in the presence of host DNA, and thus sensitivity is likely to be diagnostically lower due to a high rate of non-targeted amplification by these primers.⁵⁷ Often, this PCR is performed on cultures of *Rickettsia* from patient samples. Cultures increase *Rickettsia* number by several orders of magnitude within a few days,⁵⁷ suggesting this PCR is usually utilized in the presence of an abundance of target DNA. The non-targeted amplification of DNA sometimes results in an amplicon close to the 632 bp target size that is not of rickettsial origin (personal experience, unpublished data), making recognition of a positive sample based on size alone difficult.

The sensitivity of PCR can theoretically be increased by decreasing the size of the targeted amplicon, as the length of time for amplification step can be shortened, thus preserving polymerase enzyme activity. Nested techniques, (reamplification of a previously amplified DNA fragment using primers targeted to an internal sequence) also increase sensitivity. Because reaction tubes have to be opened to add the second set of primers and additional polymerase, laboratory contamination is a major disadvantage of this technique. Suicide nested PCR utilizes nested PCR but the primers are used for a limited number of assays within a laboratory.⁵⁶ This theoretically decreases the risk of laboratory contamination as compared to nested techniques. Recently, the limit of detection for PCR using conventional, nested, suicide and real-time techniques has been determined. Sensitivity ranges from 0.4-7 organisms.^{5,56-58} However, for all but one assay,⁵⁷ the limit of detection was not determined in the presence of host DNA, which can decrease the sensitivity of the reaction. Also,

species differentiation by sequencing the products was not performed, or only determined for a limited number of SFG *Rickettsia*. The use of a nested diagnostic PCR that amplifies a region of the *rrs* gene for members of the genus *Rickettsia* has been described by our laboratory for use in dogs.³⁴ However, the sensitivity and repeatability of the assay has not been established, and because the 16sr RNA gene is highly conserved among members of the genus *Rickettsia*, product sequencing cannot differentiate members of the SFG.

The purpose of this study was to design a sensitive specific PCR that could detect and differentiate species of SFG *Rickettsia* from the blood of infected dogs.

Materials and Methods

Bacterial strains

Several tissue culture cell lines infected with *Rickettsia* and stored -70° C were available for analysis. *R. bellii* OSU 85-1034, *R. canada* McKiel LAA1, *R. montana* OSU 85-930, *R. parkeri* HmacA, *R. prowazekii* F-16 strain (flying squirrel isolate) L3B1, *R. prowazekii* strain Breinl, *R. rhipicephali* CA871 V4P13, *R. rickettsii* strain HLP#2, *R. rickettsii* strain Bitterroot, and *R. typhi* strain RTW were kindly provided by Dr. Gregory Dasch. *R. cmblyomma* BN8.2, *R. rickettsia* strain Shelia Smith, *R. conorii* Israel 2, *R. conorii* Mor VR141 were kindly provided by Dr. David Walker.

DNA template

DNA was extracted from *Rickettsia* cell cultures using the QIAamp® DNA blood mini kit (bacterial liquid culture protocol) according to manufacturer's instructions. DNA

from dogs naturally infected with *R. rickettsii* was extracted from banked frozen whole blood in EDTA using the QIAamp® DNA blood minikit (whole blood protocol), or the BIOROBOT M48 DNA Robot using the Qiagen MagAttract® DNA extraction kit. DNA was extracted from the blood of dogs naturally infected with *R. conorii* as described (Chapter 5). For sequencing reactions, PCR products were directly purified or gel extracted and purified using the QIAquick® PCR purification kit or the QIAquick® gel extraction kit according to manufacturers instructions.

Quantitation of DNA extracted from whole blood

Genomic DNA was quantitated using the Nanodrop ND1000 Spectrophotometer® and Genom-48 Version 2® software.

Detection of PCR products

10 ul of reaction products were subjected to electrophoresis in 1X TBE using 2% agarose gels stained with 1-2 ul of 1% ethidium bromide and transilluminated with UV light.

DNA sequencing

All purified PCR products were directly sequenced in forward (5') and reverse (3') directions using primers 107F and 299R (see below). All sequencing reactions were performed through Davis Sequencing (www.davissequencing.com). Sequence obtained in the forward and reverse directions were downloaded, analyzed, and aligned using the BIOEDIT version 7.0 alignment program. Chromatograms were evaluated to assist in resolving any ambiguous base calls. Primers sequences were removed and consensus sequences were

aligned with known sequences in GENBANK using the Basic Local Alignment Search Tool (BLAST) (NCBI Bethesda MD; <http://www.ncbi.nlm.nih.gov/BLAST>). Matches with uncharacterized (nonspecified) *Rickettsia* were ignored, unless homology with a known *Rickettsia* species was less than that with an uncharacterized isolate.

Serology

Immunofluorescence testing was performed as previously described.⁵⁹

Dogs naturally infected with *R. rickettsii*

10,000 records in the sample submission database at the Vector Borne Disease Diagnostic Laboratory North Carolina State University were searched for the following criteria: Two serum samples taken 20-50 days apart demonstrating a four fold increase in IFA titer to *R. rickettsii*, and availability of banked EDTA whole blood from the first (acute) submission date. One additional sample, not in the original search, met the same serological and sample criteria and was therefore included.

Dogs experimentally infected with *R. rickettsii*

Samples of DNA, extracted from the blood of three dogs previously experimentally infected with *R. rickettsii*, (Breitschwerdt 1997) and kept at -20°C were available for analysis. Dog 1 was inoculated with 5×10^5 CFU/ml of *R. rickettsii* strain Domino intradermally over two sites. DNA samples were available from day 0 (before inoculation), and post inoculation day (PID) 3, 6, 7, and 18. Dog 2 was inoculated similarly, but was treated with 5.1 mg/kg of doxycycline hyclate orally every 12 hours beginning on day 7 and

continuing for 7 days. Samples were available from this dog from day 0 (preinoculation), and PID 3, 7, 10, 13, and 18. Dog 3 was infected with *Rickettsia* and received doxycycline as above, but also received prednisone at 2.0 mg/kg orally every 12 hours for the same treatment interval. Samples from this dog were available from day 0 (preinoculation), and PID 3, 6, 7, 10, and 18. All samples were assayed using the PCR described below three times. Amplicons of the appropriate size were purified and directly sequenced as above.

Dogs naturally infected with *R. conorii*

DNA extracted from whole blood taken from three Sicilian intact male Yorkshire Terrier dogs naturally infected with *R. conorii* (Chapter 4) were assayed in duplicate as described⁶⁰. Amplicons of appropriate size from two dogs were sequenced as described above.

Primer design and selection

The primers 107F 5'GCTTTATTCACCACCTCAAC3' and 299R 5'TRATCACCACCGTAAGTAAAT3' were derived based on the gene sequence of the 5' hypervariable region of known *ompA* sequences using the following sequences deposited in GENBANK (with GENBANK accession numbers); Astrakhan *Rickettsia* (U43791), *R africae* (U43790), *R amblyomma* (AY062007), *R. australis* (AF149108), *R cooley* (AF031535), *R cooperii* (AY362706), *R conorii* (U45244), *R. conorii* (U438060, *Israelei tick typhus* (AY197565), *Israelei tick typhus* (U43797), *R. heilongjiangensis* (AF179362), *R heilongjiangensis* (AY280711), *R honei* (DQ309096), *R. japonica* (U43795), *R. massilae* (U437999), *R. montana* (U43801), *R. montana* (AF045223), *R. parkeri* (U43802), *R*

peacockii (U55821), *R. rhipicephali* (U43803), *R. rickettsii* (DQ164838), *R. rickettsii* (DQ150687), *R. slovaca* (U43808), *R. sibirica* (U43807), and *Thai tick typhus* (U43809). Using the CLUSTAL W alignment program (BIOEDIT Sequence Alignment Editor Version 7.0), two conserved regions were chosen within the gene fragment that would result in amplification of a small target sequence (209-215 bp), while maintaining heterogeneous sequence between the conserved regions that would allow speciation based on gene sequencing. The latter was determined by comparing the target sequence (including primers) for each species using the Sequence Identity Matrix Program function in the BIOEDIT Sequence Alignment Editor 7.0 software. Primers were manufactured by Integrated DNA Technologies.

Plasmid standard for absolute quantitation of DNA copy number

A plasmid standard for template quantitation was prepared as follows: Plasmid clones were prepared from the amplicon of the 5' hypervariable region of the *ompA* gene of *R. conorii* that was amplified from the blood of a naturally infected dog using the primer pair RR190.70F and RR190.701R as described (CHAPTER 5). The presence of the target DNA sequence within the plasmid was verified by sequencing as above. Plasmid copy number was estimated in duplicate by estimating DNA concentration of a restriction digested (EcoR1) cloned plasmid preparation and subjecting the preparation to electrophoresis using 2% agarose gel stained with ethidium bromide as above. A DNA standard for comparison (Hyladder™ 1000 bp Denville Scientific Inc) was used to determine copy number. Ten fold serial dilutions of plasmids were made in Buffer AE and kept at 4 or -20°C.

Optimization of Polymerase chain reaction

Optimal conditions for amplification were determined over a range of annealing temperatures (45-55°C) and primer concentrations (3-5 ul of 10 uM per 50 ul reaction) using 25 ul Promega PCR Master Mix.[®] For optimization, DNA template consisted of 5 ul of 30-60 copies/ul of plasmid template per reaction. 5 ul of DNA extracted from the blood of a dog free of rickettsial infection (22-30 ng DNA/ul) was included in each reaction mixture, as the presence of dog DNA decreased the sensitivity of the assay and would mimic the clinical diagnostic situation. Molecular grade water was added to make a 50 ul reaction mixture.

Determining the Limit of Detection

To determine the limit of detection of the PCR, serial 10-fold dilutions of a known concentration of *ompA* plasmid template (5ul) were assayed in the presence of 5 ul of control canine DNA (18-40 ng/ul). The limit of detection was determined 10 times in duplicate. Amplification of the desired target was verified by direct sequencing of purified product as described above.

Specificity

To determine the specificity of the PCR, DNA extracted from cultures of several Spotted Fever group and typhus group *Rickettsia* was assayed (see Materials and Methods for the list of *Rickettsia* species) Amplicons were purified and directly sequenced once in forward and reverse directions as described above.

To determine if false positive results would occur in dogs infected with commonly occurring vector borne agents that can cause clinical signs similar to those caused by *R. rickettsii*,

samples and cultures used as positive controls for routine diagnostic PCR in our laboratory were assayed in triplicate. This included DNA extracted from cell cultures of *Ehrlichia canis*, *E. ewingii* and *Bartonella henselae*, and from the blood of dogs infected with *A. platys*, *A. phagocytophylum*, and *B. gibsonii*. To determine if amplification from annealing of primer's to themselves or non-targeted amplification of dog DNA results in an amplicon the size of the targeted gene segment, negative controls in all assays included 5 ul of control dog DNA (17-40 ng/ul) and an additional reagent control with no DNA template.

Detection of infection in dogs

To determine whether *Rickettsia* DNA could be amplified from infected dogs, DNA extracted from the blood of dogs experimentally or naturally infected with *R. rickettsii* and dogs naturally infected with *R. conorii* were assayed. Amplicons from two positive dogs infected with *R. conorii* were purified and sequenced as described above. For one dog infected with *R. conorii*, neither the product from the PCR that amplifies a 212 bp region of the *ompA* gene described here nor a PCR that amplifies a 632 bp segment of the *ompA* gene (19070F and RR190701R) that contains the 212 bp region was present in high enough concentration for direct sequencing. Therefore, the larger amplicon was used a template for the PCR that amplifies the 212 bp region, and that product was directly sequenced.

The integrity of the DNA extracted from the blood of dogs was tested using a PCR that amplifies the glyceraldehyde-3-phosphate dehydrogenase pseudogene. The primer pairs GAPDH-A 5'CCTTCATTGACCTCAACTACAT3' and GAPDH-B 5'CCAAACTTGTCATGGATGACC3' were used in a 25 ul reaction mixture containing 1.5mM MgCl₂ (1.5 ul 25uM mgCl₂), 0.2 mM of dNTP (0.5uL of 10mM dNTP), 25 pmol of

each primer (0.5 ul of 50 pmol/ul), 0.625 ul of AmpliTaq Gold® and 5ul template DNA.

Thermocycler conditions consisted of initial denaturation of 95°C for 5 minutes, 40 cycles of 95°C for 45 seconds, 55°C for 45 seconds, 72°C for 45 seconds, and a final extension time of 72°C for 5 minutes.

Results

Optimization of PCR

The following conditions were determined to be optimal for amplifying the 212 bp fragment of *Rickettsia ompA* in the presence of DNA extracted from dog blood: The reaction mixture consisted of 25 ul of Promega PCR mastermix®, 3 ul of 10uM (30 picomoles) of each primer, 5 ul of target DNA and 14 ul of water per 50 ul reaction mixture. Thermocycler conditions consisted of an initial denaturation at 95°C for 2 minutes followed by 40 cycles of 95 °C for 30 seconds, 58.4 °C for 45 seconds, and 72°C for 30 seconds, followed by a final extension occurred at 72 °C for 7 minutes.

Limit of Detection

This protocol could detect 1.5-3 copies of target DNA per 50 ul reaction 50% of the time (10/20 reactions), and 15-30 copies of target DNA per 50 ul reaction 100% of the time (20/20) reactions (Figure 1). A template mixture containing 7-15 copies of plasmid was also tested, and was positive in three of five reactions. Direct sequencing verified the desired target was amplified at both plasmid concentrations. Therefore, a valid positive control for the assay was determined to be of 4.5 ul of control dog DNA (17-40 ng DNA/ul), and 0.5 ul

of 30-60 copies of *R. conorii* target sequence containing plasmid/ul per reaction mixture (3-6 copies/ul) which amplifies the target sequence 100% of the time.

Specificity

Sequence Identity

Sequence identity for aligned sequences available in GENBANK for the region of the *ompA* gene amplified by primers 107R and 299R ranged from 0.797-0.995 with the exception of *R. honei* and Thai tick typhus were identical in this region (Table 1 part a-d). This is consistent with a previous report that these organisms have identical *ompA* gene sequences.⁶¹ Two separate sequences were available from GENBANK for *R. heilonjiangensis*, *R. conorii*, Israeli Tick Typhus, and *R. montana*. The sequence identity in this region of the *ompA* gene for members of the same species was identical with the exception of *R. montana*, whose sequences differed by one base pair. The average identity shared among *Rickettsia* species was 0.90816. When identical duplicate sequences for members of the same species were removed, the average identity between all species was 0.9045483. This analysis indicates that sequencing this region of the *ompA* gene amplified by primers 107F and 299R can differentiate most known species and subspecies of Spotted Fever Group (SFG) *Rickettsia*.

Amplification of DNA from SFG *Rickettsia*

An amplicon of appropriate size (209-215 base pairs) was obtained when DNA extracted from cultures of Spotted Fever group *Rickettsia* was tested. Identification of organisms was verified by sequencing. When subjected to BLAST

analysis, amplicons from all isolates were most homologous with sequences for their respective species deposited in GENBANK with the exception of *R. amblyomma* which was a 100% (168/168) match with *Rickettsia* strain AaR/SoCarolina (GENBANK accession number AF453408) and 99.4% identical (167/168) with *R. amblyomma* (GENBANK accession number AY0620007.1), and *R. rhipicephali*, which was 99.4% (170/171) homologous with *Rickettsia*. species R300 and 98.8% homologous (169/171) with *R. rhipicephali* (GENBANK accession number RRU43803). The species identity of AF45308 and *Rickettsia* species R300 have not been determined to our knowledge. Different *R. rickettsii* strains (Sheila Smith, HLP and Bitterroot) could not be differentiated as sequences from this region of *ompA* in GENBANK are 100% homologous. *Rickettsia conorii ssp. israelensis* (Israeli tick typhus) could be differentiated from *R. conorii ssp conorii* however, as they share only 95.3% homology in this region. Sequences of *R. bellii* or *R. canadensis* for this region of the *ompA* gene were not found in GENBANK for comparison. The sequence for *R. canadensis* culture did not match closely with any other *Rickettsia* species *ompA* sequence in GENBANK. Results were verified by repeating the sequencing reactions twice, in forward and reverse directions both times and used to create a consensus sequence for further comparison. Upon comparing the *ompA* sequence obtained for the *R. canadensis* culture with the sequences from the *Rickettsia* species used to make the sequence identity matrix in the manuscript, the *R. canadensis* sequence of 171 base pairs aligned most closely with *R. afticae* and both *R. rickettsia* sequences, sharing only 0.627 identity with these species. Sequences for *R. belli* matched 100% with *Rickettsia* strain AARSoCA and 99% (168/169 bp) with

R. amblyomma AYO62007.1, making contamination with *R. amblyomma* a possibility. As expected, no amplicons were obtained when the template DNA was from members of the typhus group *Rickettsia* (*R. typhi* and *R. prowazekii*) as these *Rickettsia* lack the *ompA* gene.

Non-targeted Amplification

Non-targeted amplification of bands approximately 70, 120 and 1000 base pairs (bp) occurred when the targeted *ompA* gene was present at low copy number, with or without the presence of dog DNA. The presence of dog DNA resulted in amplification of faint bands 1000 bp, 120 bp and 70 bp, while water controls contained bands 120 bp or 70 bp (FIGURE 2). On one occasion a faint band 170 base pairs in length was amplified from a sample of dog blood. Sequencing of the 120 and 170 base pair band shared little homology to any sequences in GENBANK, suggesting nontargeted amplification of primer pairs or host DNA. These amplicons could be differentiated from the specific approximately 209-215 bp *Rickettsia* target amplicon based on size.

Other organisms

No amplification occurred when cultures of *Bartonella*, *E. canis*, *E. chaffeensis* or DNA extracted from the blood of dogs infected with *E. ewingii*, *E. platys*, *A. phagocytophylum* and *B. gibsonii* were assayed in triplicate.

Experimentally infected dogs

DNA extracted from the blood of Dog 1 (untreated control dog) was positive on day 7 for two out of three assays. Sequencing of the PCR product verified amplification of the *R.*

rickettsii ompA gene. DNA extracted from the blood of dog 2 (Doxycycline treated dog) was positive on day 3 and day 7 for one assay and was negative for all time points for two other assays. The positive amplicon from day 7 was purified and sequenced and was 100% homologous with the *R. rickettsii ompA* gene fragment. Not enough sample could be extracted for direct sequencing of the amplicon from day 3 for direct sequencing. DNA extracted from the blood of Dog 3 (doxycycline and prednisone treated) was negative at all time points for all assays. Samples from all dogs were positive when using a sample quality control PCR that amplifies the GAPDH pseudogene, except for dog three whose samples were negative or very faintly positive.

Naturally infected dogs

Rickettsia rickettsii

Samples were available from five dogs who met the criteria for natural infection. Samples from two dogs were PCR positive for three of three assays, while samples from two other dogs were PCR negative for three of three assays. One dog's samples were PCR positive two out of three times. Results are shown in Table 1 and Figure 4. Sequencing verified 100% homology of 171 bp product (212 bp amplicon with primer removed) with *R. rickettsii* for all 3 positive dogs. No history regarding how long the dogs had been ill or whether they were receiving treatment at the time the first sample was drawn was available.

Rickettsia conorii

All three dogs previously determined to be naturally infected with *Rickettsia* (Chapter 5) were positive using this assay on each of two replicate tests. Sequencing

of 171 bp amplicon for Dog 2 showed 100 % homology with *R. conorii ssp conorii* MALISH 7 AE0086741 and *R. conorii ompA* AE008674.1. For Dog 3 not enough product was present for direct sequencing using either this region of the *ompA* gene or a 632 bp amplicon obtained by using RR190.70F and RR190.701R primers as described (Chapter 3). Therefore, 2 ul of the larger *ompA* PCR product was used as DNA template for the PCR described in this paper, as the target sequence for the latter PCR is contained within this larger fragment. . An appropriately sized amplicon (212 bp) was amplified and sequence was identical to *R.conorii* sequences deposited in GENBANK as above (Figure 4).

Discussion

DNA sequences from a 632 base pair hypervariable region of the 5' end of the *ompA* gene can be used to differentiate species of SFG *Rickettsia*.^{1,52,53} However, the PCR used to amplify this region is relatively insensitive, and non-targeted amplification occurs^{56,57} We have designed a conventional PCR that amplifies a product within this region that ranges in size from 209-215 bp, depending on the species tested. The primers target sequences that are conserved among many members of the spotted fever group *Rickettsia*. Accordingly, we found this PCR amplifies DNA from cultures of several species of SFG *Rickettsia*. Like the larger *ompA* gene fragment from which this PCR was designed, there appears to be enough variability in this region to distinguish species based on sequencing. This conclusion was supported both by sequence identity analysis of the expected product using known sequences of *Rickettsia* species deposited in GENBANK (Table 1), and by sequencing of DNA amplified from several rickettsial cultures and comparing those sequences to those in

GENBANK. For these samples, sequence homology was greatest for the GENBANK sequence that matched the culture's respective species for all but two isolates, which matched more closely (one base pair more) to uncharacterized strains of *Rickettsia* as compared to sequences from their designated species. It is possible the sequences in GENBANK from these non-characterized species actually are strains of the species in question. It is also possible single base pair differences were due to PCR amplification or sequencing errors. Sequencing of the entire 632 bp fragment of the *ompA* gene may be necessary for species differentiation in some cases. Performing the reactions in duplicate (having four copies of each basepair for each sample) would also verify the fidelity of the sequence.

Several primer pairs targeting the *ompA* gene of SFG *Rickettsia* do not amplify *ompA* DNA from *R. bellii*.^{53,57,62} or *R. canadensis*.⁵⁷ Antigenically, *R. canadensis* does appear to have an outer membrane protein A.⁵⁴ Interestingly, our PCR amplified what appears to be *ompA* DNA from this species. Sequences for *R. canadensis* did not match with greater than 62.7% homology with any *Rickettsia* sequences deposited in GENBANK. Such differences in the *ompA* sequence may explain the failure of other primers to amplify different regions of this gene. This is the first sequence obtained for the *ompA* gene from this species.

We have also shown that this PCR is sensitive. It can detect only a few copies of *Rickettsia* DNA in the presence of dog DNA. *Rickettsia* DNA from both experimentally and naturally infected dogs can be amplified and sequencing of the desired product identifies the infecting *Rickettsia* species. Furthermore, DNA can be detected before dogs develop antibodies (Table 1). It is not unexpected that some of the dogs defined as having natural or experimental

infection tested negative, as the copy number of circulating *Rickettsia* may be beyond the limit of detection.

Data regarding the experimentally infected dogs from the original study was available for comparison. At the time of the previous study, all dogs inoculated with *R. rickettsii* seroconverted and showed signs of illness suggesting they all became infected. At that time, blood was cultured for SFG *Rickettsia* on day 6 and day 10 after infection. The blood cultured from Dog 1 (infected dog no treatment) on PID 6 was positive for SFG *Rickettsia*, while blood from day 10 was negative. The PCR was positive for samples from day 7 on two of three occasions. Blood drawn from Dog 2 (doxycycline treated) was negative for growth on PID 6 and 10, but samples from day 3 and day 7 were positive using PCR on one of three occasions. Blood samples drawn from Dog 3 (doxycycline and immunosuppressive prednisone treatment) on PID 6 and 10 were positive for SFG *Rickettsia* using cell culture, however, the PCR was negative at all time points. Organism counts for cell culture calculated at the time of the study ranged from 10-100 *Rickettsia*/ml of dog blood. This equates to 0.1-0.1 organisms per microliter of dog blood. 200 ul of whole blood is used to obtain 200 ul of DNA during the extraction procedure, and 5 ul of DNA is used per PCR reaction. Thus, if the *R. rickettsii* cell culture count is correct, 0.05-0.5 (viable) organisms per were present in each PCR assay. Thus, negative PCR results may be because the number of organisms present was beyond the limit of detection of the assay. The PCR was performed on samples of DNA extracted from blood and stored at -20 °C several years after the cell cultures were performed. It is possible that the DNA may have degraded, or inhibitors of PCR have accumulated during storage. This is likely true at least for Dog 3 whose genomic DNA could

not be amplified robustly. It is interesting to note that the samples from the doxycycline treated, non-immunosuppressed dog were PCR positive at two different time points (day 3 and day 7), despite being cell culture negative on day 6 and day 10. Cell culture detects live organisms whereas PCR can theoretically detect circulating non-viable organisms. This may explain the amplification of *R. rickettsii* DNA in the doxycycline treated dog while tissue culture was negative during a similar time frame.

In addition to being sensitive, the *ompA* PCR described in this study is specific, as it does not amplify DNA from other species of vector borne organisms that cause clinical signs similar to *R. rickettsii*, or members of the genus *Rickettsia* (typhus group) that do not possess the *ompA* gene. Non-targeted amplification of DNA of the wrong size occurs, but can be differentiated from the appropriately sized product. However, researchers and diagnosticians need to be aware that confusion could result if gels are not carefully run and analyzed. The effect of DNA of hosts other than dogs was not tested, and needs to be evaluated before applying the PCR to other host species..

A sensitive diagnostic assay that can differentiate among numerous SFG species (with sequencing of the product) does not exist for the diagnosis of *Rickettsia* infection in dogs or people to date. This assay may assist in the accurate clinical diagnosis of early infection with *R. rickettsii* or *R. conorii*, and may also assist in identifying infection with novel rickettsial agents in dogs with atypical signs of illness. It will also help to address many as yet unanswered questions about infection with *Rickettsia* such as the natural duration of infection, and how long antibodies persist in relation to persistence of the organism.

Answers to these questions will have profound implications for the diagnosis and treatment of *Rickettsia* infection in dogs. Because dogs are sentinels of disease in people, the use of this assay may also impact the health status of their human counterparts as well.

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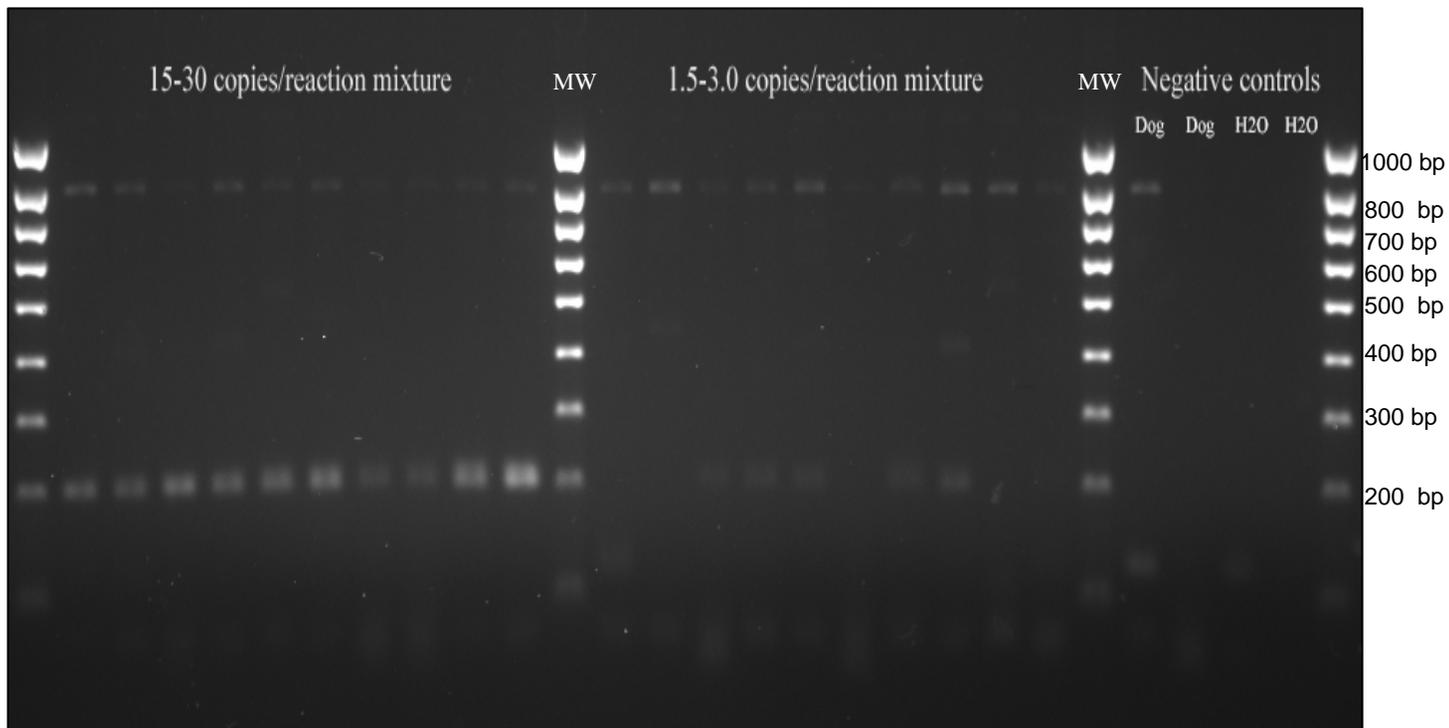


Figure 1 Limit of detection of a PCR that amplifies a 209-215 bp fragment of the *Rickettsia ompA* gene using primers 107F and 277R. Plasmids with a 632 bp gene fragment of the *R. conorii ompA* gene that contain the target sequence were quantitated and used as DNA template. Plasmid copy number is as indicated. Negative controls contained reaction mixtures with and without DNA extracted from the blood of a dog without *Rickettsia* infection. Size of bands in base pairs (bp) are based on the molecular weight marker (MW)

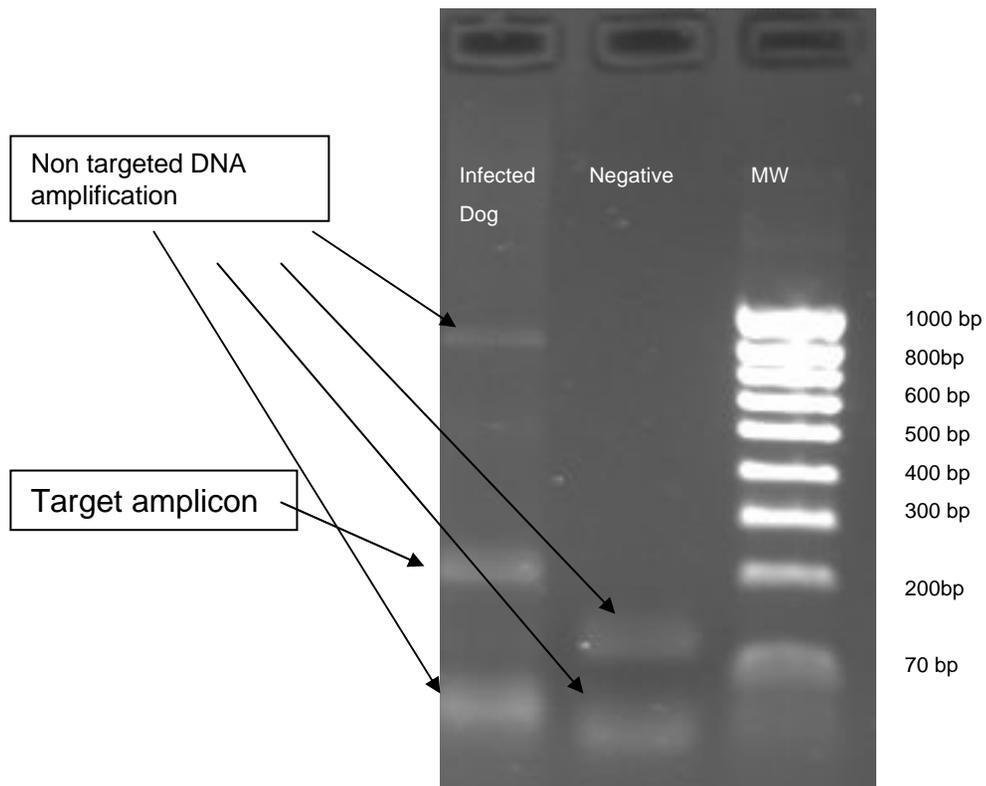


Figure 2 Non-targeted amplification that occurs with and without the presence of DNA from an 107F and 277R. The size of bands in base pairs (bp) is based on the molecular weight marker (MW).

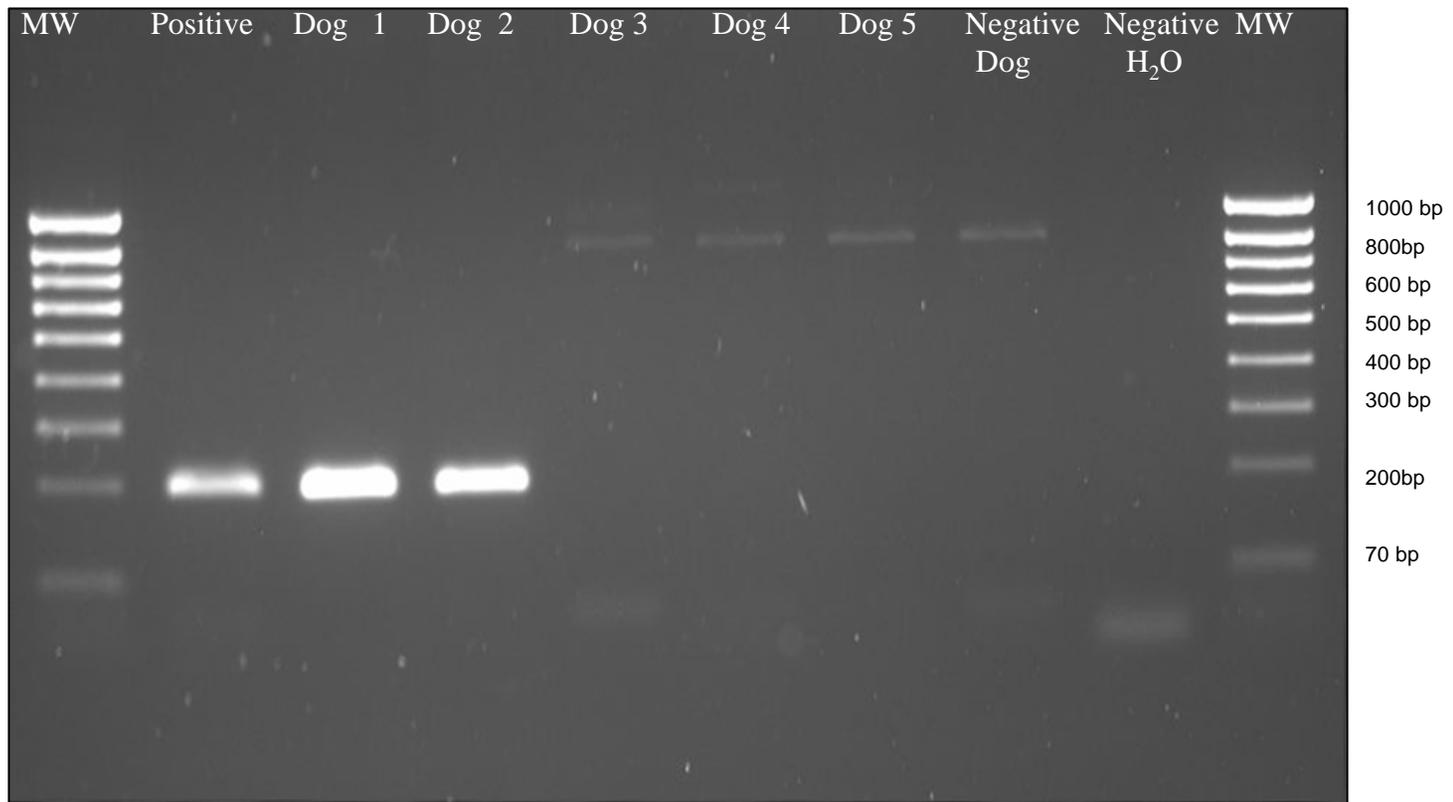


Figure 3 A 212 bp fragment of the *Rickettsia rickettsii ompA* gene amplified from DNA extracted from naturally infected dogs using primers 107F and 277R. Infection with *R. rickettsii* was verified by direct sequencing of the product. Negative controls contained reaction mixtures with and without DNA extracted from the blood of a dog without *Rickettsia* infection. Size of bands in base pairs (bp) are based on the molecular weight marker (MW).

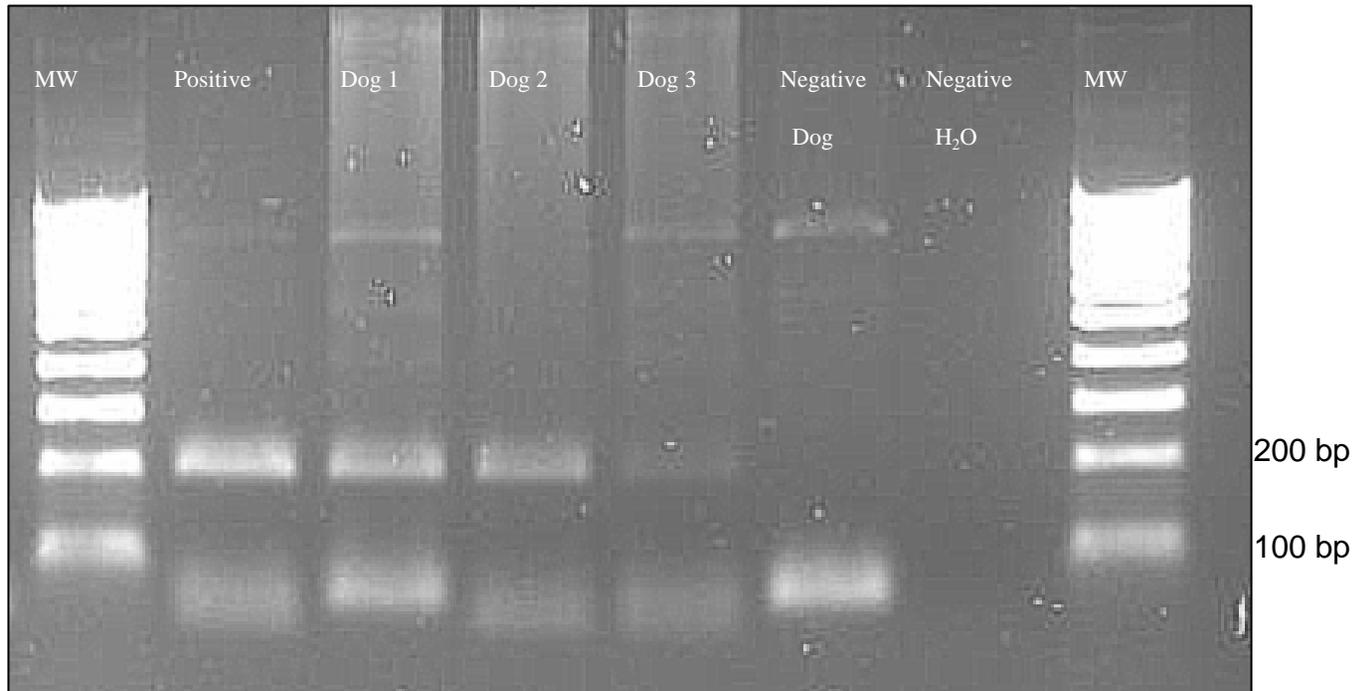


Figure 4 A 212 bp fragment of the *Rickettsia conorii ompA* gene amplified from DNA extracted from naturally infected dogs using primers 107F and 277R. Infection with *R. conorii* was verified by sequencing of the product. Negative controls contained reaction mixtures with and without DNA extracted from the blood of a dog without *Rickettsia* infection. Size of bands in base pairs (bp) are based on the molecular weight marker (MW).

Table 1 Part A Sequence identity matrix calculated for the targeted 209-215 base pair region of the *Rickettsia ompA* gene using Bioedit® Version 7.0. Numbers indicate degree of homology among indicated sequences with identical sequences having a value of 1.0.

Sequence (GENBANK accession number and species)	U43790	AY062007	AF149108	U4379	AY362706	AF031535	U45244	U43806
	<i>R. africa</i>	<i>R. amblyomma</i>	<i>R. australis</i>	Astrakhan fever <i>rickettsia</i>	<i>R. sp. cooperii</i>	<i>R. cooley</i>	<i>R. conorii</i>	<i>R. conorii</i>
U43790 <i>R. africa</i>	ID	0.896	0.844	0.99	0.985	0.83	0.953	0.953
AY062007 <i>R. amblyomma</i>	0.896	ID	0.885	0.896	0.886	0.889	0.874	0.874
AF149108 <i>R. australis</i>	0.844	0.885	ID	0.834	0.83	0.894	0.809	0.809
U43791 Astrakhan fever <i>rickettsia</i>	0.99	0.896	0.834	ID	0.981	0.83	0.953	0.953
AY362706 <i>R. sp. cooperii</i>	0.985	0.886	0.83	0.981	ID	0.82	0.944	0.944
AF031535 <i>R. cooley</i>	0.83	0.889	0.894	0.83	0.82	ID	0.804	0.804
U45244 <i>R. conorii</i>	0.953	0.874	0.809	0.953	0.944	0.804	ID	1
U43806 <i>R. conorii</i>	0.953	0.874	0.809	0.953	0.944	0.804	1	ID
AY280711 <i>R.</i> <i>heilonjiangensis</i>	0.929	0.9	0.863	0.919	0.915	0.844	0.893	0.893
AF179362 <i>R.</i> <i>heilonjiangensis</i>	0.929	0.9	0.863	0.919	0.915	0.844	0.893	0.893
DQ309096 <i>R. honei</i>	0.99	0.886	0.839	0.981	0.976	0.825	0.944	0.944
AY197565 Israeli tick typhus	0.981	0.896	0.839	0.981	0.971	0.834	0.953	0.953
U43797 Israeli tick typhus	0.981	0.896	0.839	0.981	0.971	0.834	0.953	0.953
U43795 <i>R. japonica</i>	0.919	0.886	0.863	0.91	0.905	0.844	0.883	0.883
U43799 <i>R. massilae</i>	0.882	0.91	0.849	0.882	0.872	0.853	0.855	0.855
AF045223 <i>R. montana</i>	0.858	0.909	0.89	0.867	0.858	0.885	0.832	0.832
U43801 <i>R. montana</i>	0.863	0.914	0.895	0.872	0.863	0.885	0.837	0.837
U43802 <i>R. parkerii</i>	0.971	0.882	0.825	0.966	0.976	0.816	0.93	0.93
U55821 <i>R. peacockii</i>	0.929	0.858	0.797	0.938	0.919	0.811	0.893	0.893
U43803 <i>R. rhipicephali</i>	0.891	0.919	0.858	0.891	0.882	0.863	0.865	0.865
DQ164838 <i>R. rickettsii</i>	0.981	0.896	0.844	0.981	0.971	0.83	0.953	0.953
DQ150687 <i>R. rickettsii</i>	0.981	0.896	0.844	0.981	0.971	0.83	0.953	0.953
U43807 <i>R. sibirica</i>	0.981	0.896	0.834	0.981	0.971	0.839	0.953	0.953
U43808 <i>R. slovacae</i>	0.99	0.9	0.844	0.99	0.981	0.839	0.953	0.953
U43809 Thai tick typhus	0.99	0.886	0.839	0.981	0.976	0.825	0.944	0.944

* ID= Identity

Table 1 continued Part B Sequence identity matrix calculated for the targeted 209-215 base pair region of the *Rickettsia ompA* gene using Bioedit® Version 7.0. Numbers indicate degree of homology among indicated sequences with identical sequences having a value of 1.0.

Sequence (GENBANK accession number and species)	AY280711	AF179362	DQ309096	AY197565	U43797 Israeli tick typhus	U43795	U43799
	<i>R. heilonjiangensis</i>	<i>R. heilonjiangensis</i>	<i>R. honei</i>	Israeli tick typhus		<i>R. japonica</i>	<i>R. massilae</i>
U43790 <i>R. africa</i>	0.929	0.929	0.99	0.981	0.981	0.919	0.882
AY062007 <i>R. amblyomma</i>	0.9	0.9	0.886	0.896	0.896	0.886	0.91
AF149108 <i>R. australis</i>	0.863	0.863	0.839	0.839	0.839	0.863	0.849
U43791 Astrakhan fever rickettsia	0.919	0.919	0.981	0.981	0.981	0.91	0.882
AY362706 <i>R. sp. cooperii</i>	0.915	0.915	0.976	0.971	0.971	0.905	0.872
AF031535 <i>R. cooley</i>	0.844	0.844	0.825	0.834	0.834	0.844	0.853
U45244 <i>R. conorii</i>	0.893	0.893	0.944	0.953	0.953	0.883	0.855
U43806 <i>R. conorii</i>	0.893	0.893	0.944	0.953	0.953	0.883	0.855
AY280711 <i>R. heilonjiangensis</i>	ID	1	0.919	0.919	0.919	0.966	0.882
AF179362 <i>R. heilonjiangensis</i>	1	ID	0.919	0.919	0.919	0.966	0.882
DQ309096 <i>R. honei</i>	0.919	0.919	ID	0.971	0.971	0.91	0.872
AY197565 Israeli tick typhus	0.919	0.919	0.971	ID	1	0.91	0.882
U43797 Israeli tick typhus	0.919	0.919	0.971	1	ID	0.91	0.882
U43795 <i>R. japonica</i>	0.966	0.966	0.91	0.91	0.91	ID	0.877
U43799 <i>R. massilae</i>	0.882	0.882	0.872	0.882	0.882	0.877	ID
AF045223 <i>R. montana</i>	0.886	0.886	0.849	0.858	0.858	0.882	0.867
U43801 <i>R. montana</i>	0.891	0.891	0.853	0.863	0.863	0.877	0.872
U43802 <i>R. parkerii</i>	0.915	0.915	0.962	0.957	0.957	0.905	0.867
U55821 <i>R. peacockii</i>	0.872	0.872	0.919	0.919	0.919	0.858	0.839
U43803 <i>R. rhipicephali</i>	0.896	0.896	0.882	0.891	0.891	0.891	0.985
DQ164838 <i>R. rickettsii</i>	0.929	0.929	0.981	0.981	0.981	0.919	0.882
DQ150687 <i>R. rickettsii</i>	0.929	0.929	0.981	0.981	0.981	0.919	0.882
U43807 <i>R. sibirica</i>	0.919	0.919	0.971	0.981	0.981	0.91	0.882
U43808 <i>R. slovac</i>	0.929	0.929	0.981	0.981	0.981	0.919	0.886
U43809 Thai tick typhus	0.919	0.919	1	0.971	0.971	0.91	0.872

* ID= Identity

Table 1 continued Part C Sequence identity matrix calculated for the targeted 209-215 base pair region of the *Rickettsia ompA* gene using Bioedit® Version 7.0. Numbers indicate degree of homology among indicated sequences with identical sequences having a value of 1.0.

Sequence (GENBANK accession number and species)	AF045223	U43801	U43802	U55821	U43803	DQ164838	DQ150687	U43807
	<i>R. montana</i>	<i>R. montana</i>	<i>R. parkerii</i>	<i>R. peacockii</i>	<i>R. rhipicephali</i>	<i>R. rickettsii</i>	<i>R. rickettsii</i>	<i>R. sibirica</i>
U43790 <i>R. africa</i>	0.858	0.863	0.971	0.929	0.891	0.981	0.981	0.981
AY062007 <i>R. amblyomma</i>	0.909	0.914	0.882	0.858	0.919	0.896	0.896	0.896
AF149108 <i>R. australis</i>	0.89	0.895	0.825	0.797	0.858	0.844	0.844	0.834
U43791 Astrakhan fever rickettsia	0.867	0.872	0.966	0.938	0.891	0.981	0.981	0.981
AY362706 <i>R. sp. cooperii</i>	0.858	0.863	0.976	0.919	0.882	0.971	0.971	0.971
AF031535 <i>R. cooley</i>	0.885	0.885	0.816	0.811	0.863	0.83	0.83	0.839
U45244 <i>R. conorii</i>	0.832	0.837	0.93	0.893	0.865	0.953	0.953	0.953
U43806 <i>R. conorii</i>	0.832	0.837	0.93	0.893	0.865	0.953	0.953	0.953
AY280711 <i>R. heilonjiangensis</i>	0.886	0.891	0.915	0.872	0.896	0.929	0.929	0.919
AF179362 <i>R. heilonjiangensis</i>	0.886	0.891	0.915	0.872	0.896	0.929	0.929	0.919
DQ309096 <i>R. honei</i>	0.849	0.853	0.962	0.919	0.882	0.981	0.981	0.971
AY197565 Israeli tick typhus	0.858	0.863	0.957	0.919	0.891	0.981	0.981	0.981
U43797 Israeli tick typhus	0.858	0.863	0.957	0.919	0.891	0.981	0.981	0.981
U43795 <i>R. japonica</i>	0.882	0.877	0.905	0.858	0.891	0.919	0.919	0.91
U43799 <i>R. massilae</i>	0.867	0.872	0.867	0.839	0.985	0.882	0.882	0.882
AF045223 <i>R. montana</i>	ID*	0.995	0.844	0.83	0.877	0.867	0.867	0.858
U43801 <i>R. montana</i>	0.995	ID	0.849	0.834	0.882	0.872	0.872	0.863
U43802 <i>R. parkerii</i>	0.844	0.849	ID	0.905	0.877	0.957	0.957	0.957
U55821 <i>R. peacockii</i>	0.83	0.834	0.905	ID	0.849	0.919	0.919	0.919
U43803 <i>R. rhipicephali</i>	0.877	0.882	0.877	0.849	ID	0.891	0.891	0.891
DQ164838 <i>R. rickettsii</i>	0.867	0.872	0.957	0.919	0.891	ID	1	0.981
DQ150687 <i>R. rickettsii</i>	0.867	0.872	0.957	0.919	0.891	1	ID	0.981
U43807 <i>R. sibirica</i>	0.858	0.863	0.957	0.919	0.891	0.981	0.981	ID
U43808 <i>R. slovaca</i>	0.867	0.872	0.966	0.933	0.896	0.981	0.981	0.981
U43809 Thai tick typhus	0.849	0.853	0.962	0.919	0.882	0.981	0.981	0.971

* ID= Identity

Table 1 continued Part D Sequence identity matrix calculated for the targeted 209-215 base pair region of the *Rickettsia ompA* gene using Bioedit® Version 7.0. Numbers indicate degree of homology among indicated sequences with identical sequences having a value of 1.0.

Sequence (GENBANK accession number and species)	U43808 <i>R. slovaca</i>	U43809 Thai tick typhus
U43790 <i>R. africa</i>	0.99	0.99
AY062007 <i>R. amblyomma</i>	0.9	0.886
AF149108 <i>R. australis</i>	0.844	0.839
U43791 Astrakhan fever rickettsia	0.99	0.981
AY362706 <i>R. sp. cooperii</i>	0.981	0.976
AF031535 <i>R. cooley</i>	0.839	0.825
U45244 <i>R. conorii</i>	0.953	0.944
U43806 <i>R. conorii</i>	0.953	0.944
AY280711 <i>R. heilonjiangensis</i>	0.929	0.919
AF179362 <i>R. heilonjiangensis</i>	0.929	0.919
DQ309096 <i>R. honei</i>	0.981	1
AY197565 Israeli tick typhus	0.981	0.971
U43797 Israeli tick typhus	0.981	0.971
U43795 <i>R. japonica</i>	0.919	0.91
U43799 <i>R. massilae</i>	0.886	0.872
AF045223 <i>R. montana</i>	0.867	0.849
U43801 <i>R. montana</i>	0.872	0.853
U43802 <i>R. parkerii</i>	0.966	0.962
U55821 <i>R. peacockii</i>	0.933	0.919
U43803 <i>R. rhipicephali</i>	0.896	0.882
DQ164838 <i>R. rickettsii</i>	0.981	0.981
DQ150687 <i>R. rickettsii</i>	0.981	0.981
U43807 <i>R. sibirica</i>	0.981	0.971
U43808 <i>R. slovaca</i>	ID	0.981
U43809 Thai tick typhus	0.981	ID

* ID= Identity

Table 2 PCR and serological results for dogs naturally infected with *Rickettsia rickettsii*. PCR amplification of a fragment of the *ompA* gene was performed as described in the text using primers 107F and 277R on EDTA anti-coagulated blood collected at the time the acute serological sample was obtained. A positive titer is considered greater than or equal to 1:64, and seroconversion is considered a four-fold increase in titer on acute and convalescent samples. PCR reactions were performed in three separate reactions. The number of positive or negative PCR results is indicated.

Dog	Acute <i>R. rickettsii</i> IFA titer	Convalescent <i>R. rickettsii</i> IFA titer (day after initial sample)	PCR result from acute sample*
1	< 1:16	1: 2048 (20)	Positive (3/3)
2	1:512	1:2048 (47)	Positive (3/3)
3	<1:16	1: 8192 (28)	Positive (2/3)
4	1:64	1:1024 (22)	Negative (3/3)
5	1:64	1:1024 (34)	Negative (3/3)

* Sequencing of the amplicon confirmed infection with *R. rickettsii* in PCR positive dogs.

**5) MEDITERRANEAN SPOTTED FEVER ASSOCIATED WITH *R. CONORII*
INFECTION IN THREE SICILIAN YORKSHIRE TERRIER DOGS**

Abstract

Mediterranean Spotted Fever is an important cause of morbidity and mortality in people. Individuals with as glucose-6-phosphate dehydrogenase deficiency appear to be at risk for more severe disease. The etiologic agent, *Rickettsia conorii* is transmitted by the brown Dog Tick, *Rhipicephalus sanguineous*. Seroprevalence studies show dogs are commonly exposed to *R. conorii* in endemic regions, and proximity to seropositive dogs is a risk factor for the disease in people. A recent study demonstrated *Rickettsia ssp* DNA in the blood of dogs in a MSF endemic region. However, evidence that *R. conorii* infection causes clinical signs in dogs is lacking. In this report, we document acute infection with *R. conorii* in three naturally infected male Yorkshire terriers with Spotted Fever like illness using molecular techniques. Whether clinical illness is unique to this breed and/or sex requires further investigation. The finding that MSF can occur in dogs should alert veterinarians and physicians to the possibility that clinically ill dogs could serve as sentinels for MSF as they do for RMSF in *R. rickettsii* endemic regions.

Introduction

Spotted fever rickettsiosis are important vector borne illnesses in people worldwide, and recently have been heralded as paradigms of emerging infectious disease.¹ These obligately intracellular organisms primarily infect endothelial cells, resulting in clinical signs that are a reflection of systemic vasculitis. *Rickettsia* are bacteria that are notoriously difficult to culture. Standard serological characterization is limited to differentiation at the Spotted Fever Group (SFG) level. Historically, the species of infecting *Rickettsia* was presumed based on presenting clinical findings and knowledge of rickettsial epidemiology, as different species appeared to be relatively geographically restricted. Advances in molecular techniques coupled with recognition of atypical disease patterns by astute clinicians has contributed to the recognition and characterization of classic and novel *Rickettsia* at the species level. This in part, has contributed to the recent phenomenon of disease emergence in expanding geographic locales, and the recognition of new species pathologic for humans.^{1,2}

The oldest and arguably the most well characterized spotted fever rickettsiosis is Rocky Mountain Spotted Fever (RMSF), caused by *R. rickettsii*. This disease is found in Canada, the United States, and Central and South America. The primary tick vectors in the United States are *Dermacentor variabilis* and *D. andersonii*, although recently an outbreak of RMSF in people in Arizona was attributed to transmission by *R. sanguineus*.^{2,3} Natural and experimental infection with *R. rickettsii* in dogs has been well characterized.⁴⁻⁸ The clinical signs of infection and pathophysiology of RMSF are quite similar in dogs and people, and dogs serve as sentinels for the disease in their human companions, with illness in dogs preceding or coinciding with RMSF in people in the same household.⁹⁻¹¹ When infected with

R. rickettsii, Dogs with suspected phosphofructokinase deficiency and humans with glucose-6-phosphate dehydrogenase deficiencies may be more severely affected.^{2,12,13} German Shepard and other purebred dogs were at increased risk for RMSF in one¹⁴ but not another⁶ study.

Rickettsia conorii, transmitted by *R. sanguineous*, is also a well characterized tick borne SFG *Rickettsia* that causes Mediterranean Spotted Fever (MSF) in people. *R. conorii* is transmitted by *R. sanguineous*, the brown dog tick. Mediterranean Spotted Fever occurs in Mediterranean countries, Sub Saharan Africa, and Croatia.^{1,2,15} The exact distribution of Mediterranean spotted fever is difficult to assess, as diagnostic tools such as serology typically do not differentiate among species or subspecies of SFG *Rickettsia*. It has recently been proposed that *R. conorii* be divided into four subspecies based on genetic and phenotypic criteria.¹⁶ Using this differentiation, *R. conorii ssp conorii* is the cause of MSF, while *R. conorii ssp israelensis* causes Israeli Spotted Fever, *R. conorii ssp caspia* causes Askaratan Spotted fever, and *R. conorii ssp indica* causes Indian Spotted Fever. Although sometimes considered to be a milder disease than RMSF, the outcome of MSF can be fatal.¹⁷ A recent report associated a more severe form of MSF to infection with *R. conorii ssp israelensis*.¹⁸ Host factors also play a role as people with glucose-6-phosphate dehydrogenase deficiency are more severely affected.^{2,19}

Numerous studies have shown that *R. conorii* seroprevalence and in people are dogs are associated, and living near dogs is a risk factor for the disease in people.²⁰⁻²² Seroprevalence in dogs is high in *R. conorii* endemic regions, ranging from 36.8-85.5% overall.^{20,23-27}

Despite the high rate of exposure to *R. conorii* in dogs, clinical illness has been associated with infection in only two dogs since 1932.²⁸ There is only one study investigating clinical manifestations of experimental infection in dogs in the current literature. Dogs were inoculated by needle injection intradermally over the triceps muscle with 1000 or 20,000 organisms. Dogs seroconverted and organisms were re-isolated between day 2-day 10 after inoculation.²⁸ No hematologic or physical abnormalities occurred other than regional lymphadenopathy and local erythema. The lack of clinical signs in experimentally infected or naturally exposed dogs suggests that dogs may not be as susceptible to development of disease manifestations as people, perhaps a reflection of adaptation of the organism to its vector (*R. sanguineus*) and its preferred host, the dog. Organisms in this study were virulent when inoculated into guinea pigs. It is possible that metabolic or immune compromise would be necessary for an individual dog to develop clinical signs. In that case, the high rate of *R. conorii* seroprevalence in healthy animals, combined with a lack of specific diagnostic techniques, and a low index of suspicion by veterinarians, may have precluded associating clinical signs of MSF with infection. In this report, we document infection with *R. conorii ssp. conorii* in three acutely ill, febrile Yorkshire Terrier dogs using PCR, DNA sequencing and serology.

Case presentation

Dog 1

On May 31st, 2005, a six-year-old male Yorkshire terrier dog from Catania (Sicily, Italy) presented to a local veterinarian with a history of anorexia of two days duration. The dog ate a homemade diet, lived indoors and its vaccination status was current. The dog had

no history of previous illness with the exception of sporadic vomiting and colitis which was treated with changing the diet. Ticks had been removed from the dog two weeks earlier, and a tick control product had been applied. Abnormalities found on physical examination included fever (40.1°C), tachycardia, a mildly enlarged right popliteal lymph node, blepharitis, a hunched posture possibly associated with abdominal pain, and a stiff gait. Hematological abnormalities included mature neutrophilia, (11,700/ μ L, reference interval 3,900-8,000/ μ L), band neutrophilia (468/ μ L, reference interval 0-300/ μ L) and mild thrombocytopenia (112,000/ μ L, reference interval 150,000-350,000/ μ L). Serum biochemistry identified a slight increase in alanine amino transferase (ALT) (112 IU/L, 10-85 IU/L) and mild hypoproteinemia (5 g/dL, 5.5-7.5 g/dL). Serum protein electrophoresis identified a mild hypoalbuminemia (40.4%, reference interval 52-66 %), mildly increased beta globulin (13.9%, reference interval 4.5-6.8 %) and alpha₂-globulin (16.5%, reference interval 4.8-11 %). No abnormalities were seen on abdominal radiographs. The dog was treated with doxycycline (10mg/kg/SID/PO for a month) and supportive therapy including intravenous fluids. Clinical signs resolved completely within 48 hours.

EDTA-anticoagulated whole blood samples were obtained prior to treatment and were submitted to Laboratorio Veterinario Privato "San Marco" (Padova, Italy) for *E. canis*, *Rickettsia* genus, *Leishmania infantum*, *Babesia* genus, *Borrelia burgdorferi* and *Anaplasma phagocytophilum* polymerase chain reaction (PCR) testing. The PCR for *Rickettsia* genus was positive while the rest were negative.

One week later the dog re-presented at the referring veterinarian for further sample collection. The dog was clinically normal. Blood and lymph node aspirates were obtained. Complete blood count revealed a mild leukocytosis with mature neutrophilia (16,400/ μ L, reference interval 3,800-8,800/ μ L). The thrombocytopenia had resolved. Serum biochemistry revealed slight hyperglobulinemia (4.3 g/dL, reference interval 2.6-4.0 g/dL), increased C-reactive protein (2.31 mg/dL, reference interval 0.0-0.15 mg/dL), a slight increase in creatine kinase (CK) (319 IU/L, reference interval 40-150 IU/L), alkaline phosphatase (ALP) (177 IU/L, reference interval 20-120 IU/L) and gamma glutamyl transpeptidase (GGT) (10.4 IU/L, reference interval 2-8 IU/L). Serum electrophoresis revealed an increase of alpha₂-globulin (18.5 %, reference interval 8.0-14.0 %) and gammaglobulin (23.3 %, reference interval 6.0-15.0 %) and a slight decrease in albumin (40.9 %, reference interval 53.0-65.0 %). Blood and lymph node samples were subjected to PCR for *Rickettsia* genus, *E. canis*, *A. phagocytophilum* and *L. infantum*. All PCR results were negative from both blood and lymph nodes. Serum was submitted from the same date for serology (IFA) to detect antibody to *R. rickettsii*, *R. conorii*, *E. canis*, *A. phagocytophilum*, *B. henselae*, *B. vinsonii* subsp. *berkoffii*, *Borrelia burgdorferi*, *Babesia canis* and *L. infantum* antigens. (Table 1).

DNA extracted from samples from blood (day 1 and day 7) and lymph node (day 7) was sent to The Intracellular Pathogens Research Laboratory in the United States. These DNA samples were subjected to PCR that amplifies portions of the *Rickettsia ompA* gene to determine the species of infecting *Rickettsia* through gene sequencing. The person who performed the PCR was blinded to sample date, source, and previous *Rickettsia* genus PCR results. Only the blood sample from day 1 was positive. (See Results).

Two months later, the dog remained clinically healthy. Blood and lymph node samples were submitted for molecular and serological testing as above. Complete blood count and serum biochemistry revealed no abnormalities. All PCRs tested were negative. The results of serological tests are presented in Table 1.

Dog 2

On September 19, 2005, a five-year-old male Yorkshire Terrier from Catania (Sicily, Italy) presented to the referring veterinarian for anorexia and ptyalism. He was an indoor dog but had a history of exposure to ticks recently. The dog was current on vaccinations and had not been ill previously with the exception of sporadic vomiting for one year). Abnormalities found on physical examination included fever (41°C), joint pain, and right rear limb lameness. Complete blood count revealed a mild normocytic-normochromic anemia (25%, reference interval 37-55) and slight mature neutrophilia (11,680/ μ L, reference interval 3,900-8,000/ μ L). EDTA whole blood and serum was submitted to Laboratorio Veterinario Privato “San Marco” (Padova, Italy) for testing as above. *Rickettsia* genus PCR was positive, and DNA extracted from whole blood was submitted to the Intracellular Pathogens Research Laboratory. PCR was performed to determine the species of infecting *Rickettsia*. (see Materials and Methods). Treatment with doxycycline (10mg/kg/SID/PO for a month) and supportive therapy including intravenous fluids, resulted in rapid clinical improvement of the dog after 48 hours. EDTA anticoagulated whole blood and serum samples were submitted one week and one month later for serologic and PCR testing as before. All PCR results were negative at one week. Serum biochemistry revealed slight hyperglobulinemia (4.3 g/dL,

reference interval 2.6-4.0 g/dL), increased C reactive protein (0.64 mg/dL, reference interval 0.0-0.15 mg/dL). Serum electrophoresis revealed an increase of alpha₂-globulin (17.5 %, reference interval 8.0-14.0 %) and gammaglobulin (24.6 %, reference interval 6.0-15.0 %) and a slight decrease in albumin (41.1%, reference interval 53.0-65.0 %) . Complete blood count, serum biochemistry and serum electrophoresis revealed no abnormalities one month after presentation.

Dog 3

On September 17th 2005 An intact 2-year-old Yorkshire Terrier from Catania (Sicily, Italy) presented to the referring veterinarian with a history of intermittent vomiting, abdominal pain and anorexia. The dog had a history of exposure to ticks, was current on vaccinations and had not been ill previously. Physical examination abnormalities included fever (41 °C), abdominal pain, dehydration, peripheral lymphadenomegaly (popliteal and preescapular lymph nodes) and conjunctivitis. Complete blood count revealed mild thrombocytopenia (112,000/ μ L, reference interval 150,000-350,000/ μ L) . Serum biochemistry identified slight hypoproteinemia (5.3 g/dL, reference interval 5.5-7.5). Serum protein electrophoresis identified hypoalbuminemia (28.6%, reference interval 52-66 %), increased alpha₂-globulin (14.8%, reference interval 4.8-11 %), beta₁ globulin (13%, reference interval 4.5-6.8 %), beta₂ globulin (15.6%, reference interval 8.8-12.5%) and gammaglobulin (23%, reference interval 8.8-22%). Whole blood EDTA and serum samples were submitted to Laboratorio Veterinario Privato “San Marco” (Padova, Italy) for testing as above. PCR for *Rickettsia* genus was positive. DNA extracted from whole blood was submitted to the Intracellular Pathogens Research Laboratory. PCR and DNA sequencing

were performed to determine the species of infecting *Rickettsia*. (see Materials and Methods). The dog was treated with ceftriaxone (30mg/kg/BID/IV for 5 days) and supportive therapy including intravenous fluids. By day four the dog had improved dramatically. After six days, therapy was switched to doxycycline based on the positive *Rickettsia* genus PCR. *Rickettsia* genus PCR performed on EDTA anticoagulated blood was negative six days after clinical presentation and before starting treatment with doxycycline. The dog was healthy at that time and one month later when serum samples were submitted for serology and PCR as before. All PCRs tested were negative. Complete blood count performed at six days revealed lymphocytosis (5,594/ μ L, reference interval 1,300-4,100/ μ L). The thrombocytopenia had resolved. Serum biochemistry revealed slight hyperglobulinemia (4.1 g/dL, reference interval 2.6-4.0 g/dL) and increased C-reactive protein (2.70 mg/dL, reference interval 0.0-0.15 mg/dL). Serum electrophoresis revealed an increase of alpha₂-globulin (19.3 %, reference interval 8.0-14.0 %) and a slight decrease in albumin (44.9 %, reference interval 53.0-65.0 %). Complete blood count, serum biochemistry and serum electrophoresis revealed moderately elevated blood urea nitrogen (BUN) at six days (BUN 75 (mg/dL), reference interval 18-43 mg/dL), and one month after initial presentation (BUN 54 mg/dL, reference interval 18-43 mg/dL). Creatinine was normal at both time points (creatinine: 0.83 mg/dL and 0.87 (mg/dL), respectively, reference interval 0.70-1.30 mg/dL), suggesting gastrointestinal hemorrhage as a cause of the azotemia. Renal dysfunction cannot be ruled out as a urinalysis was not obtained at the time of blood sampling. Dehydration seems a less likely cause of the azotemia as the dog was clinically normal.

Materials and Methods

Rickettsia strain for *ompA* PCR control

DNA extracted from *R. rickettsii* strain Domino, grown in Vero cells and frozen at -80°C was used as a positive control for the 632 bp *ompA* PCR assay (see below).

Leishmania strains for PCR

DNA extracted from cultured *L. infantum* (MCAN/FR/96/LEM3227) zymodeme MON-1, *L. tropica* (MCAN/MA/90/LEM2007) zymodeme MON-102 and *L. braziliensis* (MCAN/BR/81/RICO) zymodeme MON-43 reference strains were provided by Laboratoire de Parasitologie, Centre National de référence des leishmanioses, Université of Montpellier (Montpellier, France).

Serologic techniques

Immunofluorescent assays (IFA) to detect antibodies against *Ehrlichia. canis*, *Leishmania infantum*, *Babesia canis*, *Bartonella henselae*, *Bartonella vinsonii ssp berkhoffi* and *R. rickettsii* antigens were performed at the Intracellular Pathogens Research Laboratory in Raleigh (IPRL), North Carolina, USA as previously described.^{29,30} with the exception that *R. rickettsia* antigen used was supplied by Panbio diagnostics.

Immunofluorescent assays to detect antibodies specific for *Borrelia burgdorferi*, sensu stricto *E. canis*, *B. canis*, *Anaplasma phagocytophilum*, *R. rickettsii* and *R. conorii* were performed at the Laboratorio d'Analisi Veterinarie "San Marco", Padova (Italy). *R. rickettsia* antigen used was supplied by VMRD, Inc. *Ehrlichia canis* and *Anaplasma phagocytophilum* antigen

were supplied by Panbio diagnostics. *Babesia canis canis* antigen was supplied by BOSE. *Leishmania infantum* antigen was supplied by BVT Bio Veto Test. *Borrelia burgdorferi sensu stricto* and *R. conorii* were supplied by Fuller laboratories. Two twofold serial dilutions of sera (1:80, 1:160) in PBS pH 7.4 were made in microtiter plates. Thirty microliters of each dilution was applied per well, and slides were incubated at 37°C for 30 min. Then, fluid was discharged and washed in PBS with agitation for 5 min and air-dried. Thirty microliters of fluorescein conjugated goat anti-dog IgG (heavy and light chain, catalog n. 035-10, VMRD, Inc) or fluorescein conjugated goat anti-dog IgM (affinity purified, heavy chain specific, n. 036-10, VMRD, Inc) were applied per well. Fluorescein conjugated goat anti-dog IgM was only used for *R. conorii* and *R. rickettsia*. Slides were incubated for 30 min at 37°C and washed again in PBS with agitation for 5 min, rinsed with deionized water and air dried. A cover slip was applied using mounting medium (50% glycerol and 50% buffer, pH 9.0-9.6, VMRD, inc) and viewed with a fluorescence microscope (magnification, X40). Samples with an IFA titer $\geq 1:160$ were retested with serial dilutions from 1:80 to 1:40960. End point titers were determined as the last dilution at which brightly stained organisms could be detected on a fluorescence microscope with exciter and barrier filters. For all antigens, a reactive serum was defined as a titer of $\geq 1:80$. Positive and negative sera controls were used for all IFA testing.

DNA extraction from *Rickettsia* culture

DNA was extracted from a culture of *R. rickettsii* NCSU strain Domino was performed using the Qiagen[®] DNA blood mini-kit, (bacterial cell culture/tissue protocol) according to manufacturers instructions.

DNA extraction from dog blood and *Leishmania* culture

DNA extraction was performed from whole blood samples by the High Pure PCR Template Preparation Kit (Roche Applied Science) in accordance with the manufacturer's protocol with some modifications. 200 µl of Blood samples were incubated with 40 µl of Proteinase K and with 200 µl of Binding Buffer at 65°C overnight. Subsequent steps were carried out according to the manufacturer's instructions. DNA was eluted in 50 µl of elution buffer at 72°C.

Polymerase Chain Reaction

Polymerase chain reaction for the detection of *Rickettsia* genus, *A. phagocytophilum*, *E. canis*, *Babesia*, *Borrelia burgdorferi* and *Leishmania infantum* DNA were performed at *Laboratorio d'Analisi Veterinarie "San Marco"* (Padua, Italy) A qPCR for detection of *Rickettsia* DNA in whole blood samples was performed using Light Cycler instrument (Roche). Commercial *Rickettsia* spp. primers Rr-prim3 (5'GAAACCGAAAGAGAATCTTCCGAT-3') and Rr-prim4 (5'TCCTAGTGTAGAGGTGAAATTCTTA-3')³¹ and hybridization probes LC sets (TIB MOLBIOL) that amplified a fragment of 16S rRNA gene, were used. PCR amplifications were carried out in a final volume of 10 µL, including 2.5 µL of DNA template, 2 µL of LC fast start DNA master^{PLUS} (Roche Applied Science) and 0.5 µL of *Rickettsia* LCSet primers and probes following manufacturer's instructions (TIB MOLBIOL) with minor modifications. Amplifications were conducted in sealed 20 µL LightCycler glass capillaries. Thermal cycling in a Roche LightCycler, version 3.5.17, was comprised of an initial

denaturation step at 95°C for 8 min, followed by 55 cycles of a denaturation at 95°C for 5s, annealing at 59°C for 7s, and extension at 72°C for 7s, with quantification of the fluorescence signal. A final melting curve analysis was performed at an initial temperature of 72°C for 30s, followed by 95°C for 20s, 50°C for 20s, continuous heating from 0.5°C/s to 85°C for 1s and a cooling step of 40°C for 5s. A reaction was considered positive when the normalized fluorescence signal (ratio of the signal from detection channel F2 [640 nm] to the signal from detection channel F1 [495 nm] at the end of the annealing step) showed an exponential increase in fluorescence. The melting temperatures of probe-template hybrids were automatically determined by the software. *Rickettsia rickettsii* had a melting temperature of approximately 65°C while other *Rickettsia spp* such as *Rickettsia conorii* had a melting temperature of approximately 59°C. Negative (water instead of DNA) and *R. rickettsii* positive controls (*Rickettsia* LCSet positive control, TIB MOLBIOL) were added in each qPCR run.

Polymerase chain reaction for the detection of *Ehrlichia canis* (*Ec*) and *Anaplasma phagocytophylum* (*Ap*) was performed using quantitative real-time PCR (qPCR) using a LightCycler® instrument (Roche) PCR amplification was carried out in a final volume of 10 µL, including 2.5 µL of DNA template, 2 µL of LC fast start DNA master^{PLUS} (Roche) and 0.4 µL of *Ec* or *Ap* LCSet primers and probes following manufacturer's instructions (TIB MOLBIOL). Thermal cycling comprised an initial denaturation step at 95°C for 8 min, followed by 60 cycles for *Ec* and 45 cycles for *Ap* of a denaturation at 95°C for 5s, annealing at 61°C for 10s, and extension at 72°C for 11s, with quantification of fluorescence signal. A final melting curve analysis was performed using an initial temperature of 72°C for 30s,

followed by 95°C for 20s, 40°C for 30s, continuous heating at 0.5°C/s to 85°C and a cooling step of 40°C for 1s. Negative and positive controls (*Ec* and *Ap* LCSet positive controls, TIB MOLBIOL) were added in each qPCR run. ³²

A PCR for detection and quantification of *L. infantum* DNA in blood samples was developed using Light Cycler instrument (Roche Applied Science). Commercial *L. infantum* primers and hybridization probes LC set (TIB molbiol) that amplified a fragment of the kinetoplast minicircle were used for accurate sensitivity. PCR amplification was carried out in a final volume of 10 µL, including 2.5 µL of DNA template, 2 µL of LC fast start DNA master^{PLUS} (Roche Applied Science) and 0.5 µL of *L. infantum* LCSet primers and probes following manufacturer's instructions (TIB molbiol). Amplifications were conducted in sealed 20 µL LightCycler glass capillaries. Thermal cycling in a Roche LightCycler, version 3.5.17, comprised an initial denaturation step at 95°C for 8 min, followed by a first PCR step with 15 cycles of 95°C for 5s, 62°C for 9s, and 72°C for 4s, with no quantification of fluorescence signal, followed by a second PCR step consisting in 40 cycles of 95°C for 5 s, 50°C for 9s, and 72°C for 4s. A final melting curve analysis was performed by an initial temperature of 72°C for 30 s, followed by 95°C for 20 s and 40°C for 30 s and continuous heating at 0.5°C/s to 85 °C and a cooling step of 40°C for 1 s. A reaction was considered positive when the normalised fluorescence signal (ratio of the signal from detection channel F2 [640 nm] to the signal from detection channel F1 [495 nm] at the end of the annealing step) showed an exponential increase in fluorescence. The melting temperature of probe-template hybrids were automatically determined by the software. A negative control consisting of the reaction

mixture and water instead of template DNA and a positive control were added in each qPCR run.

Polymerase chain reaction for speciation of *Rickettsia* using the *ompA* gene was performed at the IPRL Raleigh, North Carolina, USA. A PCR (referred to here as “large *ompA*”) that amplifies a 632 bp 5’ hypervariable region of the outer membrane protein A gene (*ompA* gene) of spotted fever group *Rickettsia* was performed as described³³ on samples of DNA extracted from blood and lymph nodes. 10 ul of reaction products were subjected to electrophoresis in 1X TBE using 2% agarose gels stained with 1-2 ul of 1% ethidium bromide and transilluminated with UV light. For Dog 1, a 632 bp amplicon from this reaction was gel purified using the QIAquick® gel extraction kit, and cloned using the TOPO TA Cloning® kit according to manufacturers instructions. Two clones were sequenced in forward and reverse directions using primers M13F and M13R. All sequencing reactions were performed through Davis sequencing (www.davissequencing.com). For Dog 2 and Dog 3, not enough PCR product resulted from the large 632 base pair product *ompA* PCR for direct sequencing. A highly sensitive PCR, referred to here as “small *omp PCR*” was used to amplify a 212 bp portion of the 5’ hypervariable region of the *ompA* gene from all three dogs as previously described³⁴ The species of infecting SFG *Rickettsia* can be determined from sequencing the PCR products.³⁴ For Dog 2 PCR products were directly purified using the QIAquick® PCR purification kit according to manufacturers instructions and subjected to direct sequencing in the forward and reverse directions using primers 107F GCTTTATTCAACCTCAAC3’ and 299R 5’TRATCACCACCGTAAGTAAAT as described.³⁴ For dog 3, not enough product from the small *ompA* PCR was available for

direct sequencing. Therefore 2ul of gel purified PCR product from the large *ompA* PCR was used as DNA template, and the small *ompA* PCR was performed again. PCR products resulting from this reaction were directly purified and sequenced in duplicate in forward and reverse directions.

Babesia PCR was performed as described previously by conventional PCR.³⁵ *Borrelia burgdorferi sensu lato* PCR was performed by Scanelis Laboratory (WWW.scanelis.com, France).

Sequence analysis

Sequence obtained in the forward and reverse directions were downloaded, analyzed, and aligned, and consensus sequences were created for each dog using the BIOEDIT version 7.0 alignment program (CLUSTAL W). Chromatograms were visually examined to assist in resolving any ambiguous base calls. Primer sequences were removed and consensus sequences were aligned with known sequences in GENBANK using the Basic Local Alignment Search Tool (BLAST) (NCBI Bethesda MD; <http://www.ncbi.nlm.nih.gov/BLAST>).

Results

Serology

Serologic results are presented in Table 1. Dog 2 and 3 seroconverted to *R. conorii*. Dog 1 had high titers to *R. conorii* at the time of sampling (one week after clinical presentation). Dog 1 had low titers to *B. canis*, *A. phagocytophilum*, and *B. burgdorferi*. Dog

2 had a low titer to *E. canis* on one date. No dogs were seropositive to *Bartonella henselae*, *Bartonella vinsonii ssp berkhoffi*, or *Leishmania infantum*.

PCR

Rickettsia DNA was detected using real time q PCR for *Rickettsia* genus on all initial blood samples from all dogs. Melt curve analysis was consistent with *Rickettsia* species other than *R. rickettsii*. Initial (acute) blood samples were also positive for all dogs using both the large (632 bp) (Figure 1) and small (212 bp) (Figure 2) *Rickettsia ompA* PCR assays. PCR results for the remaining organisms were negative. All PCR results were negative using blood and lymph node samples obtained from Dog 1 seven days after initial presentation, and for blood samples from Dog 2 and 3 at six or seven days and one month after treatment.

Sequencing of the *ompA* gene fragments:

The sequence obtained from all three dogs was 100% homologous to a portion of the complete genome sequence corresponding to the *ompA* gene from *R. conorii ssp conorii* (Malish 7) AE0086741. The alignments of consensus sequences from the three dogs with those of *R. conorii* are shown in Figure 3.

Discussion

Infection with *R. rickettsii* in dogs and people, and infection with *R. conorii* infection in people causes an acute, doxycycline responsive, illness with clinical abnormalities include but are not limited to fever, rash, vomiting, diarrhea, central nervous system dysfunction, joint pain and thrombocytopenia, mild liver enzyme elevation, CK elevation and

hypoalbuminemia.^{2,4,6-8,17,36,37} Although there is abundant serologic evidence that dogs are commonly exposed to *R. conorii* or related SFG rickettsiae, culture or molecular evidence for natural infection or that infection causes clinical signs in dogs is lacking.^{20,21,23-25,27,28} To our knowledge, this is the first report documenting amplification of *R. conorii* DNA from dogs naturally infected with *R. conorii*, although amplification of *Rickettsia* DNA from dogs naturally infected with a *Rickettsia* species in Italy, and *R. conorii* DNA from dogs experimentally infected through inoculation with naturally infected ticks have recently been described.^{32,38} We also provide clinical, hematological and serological evidence that acute *R. conorii* infection can be associated with naturally-occurring illness in dogs.

At the time of presentation, clinical abnormalities detected on routine physical examination and blood work (acute onset fever, lethargy, thrombocytopenia, mild liver enzyme elevation, hypoalbuminemia) in these dogs were consistent with a Spotted Fever rickettsiosis. Interestingly, C-reactive protein was elevated in all three dogs. C-reactive protein, a non-specific marker of inflammation, has been shown to be increased in patients with spotted fever rickettsioses.³⁹⁻⁴¹ We amplified DNA from portions of two genes (*rrs* and *ompA*), and sequences from the *ompA* gene fragments were 100% homologous with *R. conorii ssp conrorii*. Recently, a more virulent form of MSF in people in Sicily has been associated with *R. conorii ssp israelensis*.¹⁸ Sequence analysis of fragments of the *ompA* gene sequences shown to differentiate SFG *Rickettsia* species and subspecies suggested infection with this potentially more pathogenic strain of *R. conorii ssp israelensis* was not the cause of this unprecedented disease in these dogs.

It is very unlikely that PCR contamination accounted for the detection of *R. conorii* DNA in these three dogs. Two different genes were amplified in two different laboratories at different times. Furthermore, the positive control for the large 632 base pair *ompA* PCR was *R. rickettsii*, therefore sequencing definitively confirmed that no contamination from the positive control occurred for Dog 1. Laboratory contamination with *R. conorii* DNA was highly unlikely for Dog 1 (the first dog affected and tested) as the laboratory is in the United States and was recently built. Therefore, PCR had not been performed on any samples containing *Rickettsia* species other than *R. rickettsii* at the time samples from Dog 1 were received. The plasmid clones of the large *ompA* (632 bp) fragment were quantified and used as positive controls for the small *ompA* assay ensure the assay always performs at the limit of detection.³⁴ The plasmid could conceivably act as a source of contamination for this PCR. However this is unlikely, as plasmid DNA is always added to the positive control tube in a separate room, after the PCR reactions containing master mix and patient DNA template are prepared. The positive control tube is therefore the only tube opened in the presence of the plasmids while the other tubes remain closed. Additionally, all negative DNA and reagent controls were consistently negative for all assays.

It is likely that the clinical signs observed in these dogs can be attributed to infection with *R. conorii*. Infection with *R. rickettsii* in dogs and people, and *R. conorii* in people causes acute transient clinical signs like those described in the dogs in this case, including fever, thrombocytopenia, polyarthropathy, and gastrointestinal abnormalities. The diagnostic evidence in this report suggests that *R. conorii* infection was temporally associated with the clinical abnormalities. Antibodies produced due to SFG *Rickettsia* infection in animals and

people demonstrate a high degree of serological crossreactivity, and IFA titers to *R. rickettsii* are representative of exposure to *R. conorii* in people⁴² This report documents similar crossreactivity to the two agents in dogs. The fourfold increase in IgG in Dogs two and three indicates acute infection⁴³ IgM indicates acute exposure to SFG *Rickettsia* in people.⁴⁴ IgM titers rise acutely and disappear by day 35 and 80 in dogs experimentally infected with *R. conorii* and *R. rickettsii* respectively.^{28,43} However, the diagnostic utility of IgM titers to *R. rickettsii* for confirming RMSF in dogs is unclear, since high *R. rickettsii* IgM titers are found in dogs that do not seroconvert.⁴³ Thus the presence of IgM supports but does not prove acute infection with SFG *Rickettsia* in dogs. Further evidence for *R. conorii* infection as a cause of the associated clinical signs was provided by the initial detection of *R. conorii* DNA followed by the failure to detect DNA in Dog 1 after one week with doxycycline and resolution of clinical signs two days after initiating doxycycline therapy. In Dog 2, clinical signs resolved rapidly with doxycycline therapy and the *Rickettsia* genus PCR was negative one week after clinical presentation and specific therapy. Interestingly, clinical signs in Dog 3 also resolved rapidly, while receiving a cephalosporin antibiotic for the first six days, which has no known anti-rickettsial efficacy.² Immune clearance likely accounted for the resolution of clinical signs in this case, as clearance occurs by day 10 in experimentally infected untreated dogs.²⁸ Systemic infection with a ceftriaxone responsive bacteria is a possible alternative explanation for the clinical observations in Dog 3 but the rapid resolution of the illness is not consistent with severe sepsis.

Coinfection with *A. phagocytophilum*, *E. canis* or *B. burgdorferi* or *B. canis* could have contributed to clinical signs observed in these dogs. Coexposure to the other agents and an

association between antibodies to *R. conorii*, *E. canis* and *A. phagocytophilum* has been documented in Mediterranean dogs²⁴ DNA from organisms other than *R. conorii* was not found in any dog. However Dog 1 had 1:160 titer to *A. phagocytophilum* at initial presentation. *A. phagocytophilum* causes an acute illness within a few days tick inoculation in dogs, and clinical signs are similar to those described here. Low titers to *E. canis* were detected in one laboratory (LAVS, Italy) but not in another (IPRL, USA) for Dog 1 and 2. Inter-laboratory variation in detection of *E. canis* antibodies may be attributed to variation in technique. Whether the dog has truly been exposed to *E. canis* is not known. Clinical signs of acute *E. canis* infection can be similar to those described here. Chronic subclinical infection also occurs, and antibodies remain for extended periods of time despite treatment.⁴⁵ Although direct evidence for acute infection with *E. canis* is lacking in this dog, this agent cannot be ruled out as a contributor to clinical signs. Similarly, exposure to *B. canis* was documented in one (LAVS, Italy) but not another (IPRL, USA) laboratory. Again PCR was negative, and the dogs clinical signs resolved without anti-protozoal treatment for *B. canis*. Dog 1 also seroconverted to *B. burgdorferi*. Experimentally, *B. burgdorferi* does not cause clinical signs in dogs until 60-150 days after infection.⁴⁶ Because seroconversion was documented, acute *Borrelia spp.* exposure was likely, making it difficult to directly attribute clinical signs to infection with *B. burgdorferi*. *Borrelia burgdorferi* PCRs were negative for all three dogs. If present, coinfection with other tickborne organisms may have also contributed to the clinical signs in these dogs indirectly, as coinfection with one organism can alter susceptibility to another organism in an individual host.⁴⁷⁻⁴⁹

In addition to the possibility of coinfection, individual differences can alter susceptibility to SFG *Rickettsia*. All three dogs in this report were intact male Yorkshire terriers. Purebred dogs may be at increased risk for RMSF, although an increased risk in Yorkshire Terriers has not been reported.^{12,14} Male dogs and humans may be at increased risk for infection with *R. rickettsii*, and male dogs are more likely to be seropositive to *R. conorii*^{14,24,50} It has been suggested that English Springer Spaniels with suspected phosphofructokinase deficiency may have more severe illness (dermal necrosis) when infected with *R. rickettsii*.¹² Decreased ability to release hemoglobin and increased red blood cell fragility in these dogs would theoretically predispose them to more severe hypoxic damage during infection. People with glucose 6-phosphate dehydrogenase deficiency are more severely affected by both *Rickettsia*.^{2,13,19} Glucose 6-phosphate dehydrogenase reduces nicotinate adenine dinucleotide phosphate (NADP) to its reduced form NADPH. NADPH protects cells against oxidative injury. *Rickettsia* induce oxidative injury upon infection⁵¹⁻⁵³, and a reduced ability to protect against oxidative injury may explain the more severe illness associated with infection in these individuals. Although all three dogs in this study were male Yorkshire terriers, they were not directly related. Metabolic or immunologic defects cannot be ruled out as they were not tested for, although all dogs were reported to be previously healthy. Although inherited immunodeficiencies have not been reported in Yorkshire terriers, they have been shown to be at increased risk for infection with *Babesia canis*.⁵⁴ The relationship of infection with *R. conorii* in Yorkshire terriers requires further investigation.

In this report we describe acute infection with *R. conorii* in unrelated intact male Yorkshire Terriers, which was confirmed using molecular techniques. Clinical signs were associated

with evidence of acute *R. conorii* infection, although infection with other organisms contributing to clinical signs cannot be completely ruled out. With increased awareness of the possibility that *R. conorii* can cause illness in dogs in *R. conorii* endemic regions, and with the accompanying advances in molecular biology that improve the ease and accuracy of diagnostic testing, further evidence can be gathered regarding the extent to which *R. conorii* causes clinical disease in dogs. The potential relationship of *R. conorii* infection to the signalment of intact male Yorkshire Terrier also requires further investigation. This discovery should alert human physicians that clinically ill, as well as seropositive dogs may serve as active sentinels for MSF as they do for RMSF in Central North and South America.

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Table 1 Reciprocal IFA titers for three dogs with clinical and molecular evidence of natural *R. conorii* infection.

Dog	Date	<i>B. canis</i> IgG	<i>E. canis</i> IgG	<i>Ap</i> * IgG	<i>R. rickettsii</i> IgM	<i>R. rickettsii</i> IgG	<i>R. conorii</i> IgM	<i>R. conorii</i> IgG	<i>B. burgdorferii</i> IgG
1	6/8/06	neg	160 (neg)	160	1280	20480	280	20480	neg
1	8/3/05	80 (neg)	neg	neg	80	320	80	320	160
2	9/19/05	neg	neg	neg	640	80	640	80	neg
2	9/28/05	neg	80 (neg)	neg	640	20480	1280	40960	neg
2	10/25/05	neg	160 (neg)	neg	640	5120	640	10240	neg
3	9/17/05	neg	neg	neg	2560	neg	1280	80	neg
3	9/23/05	neg	neg	neg	10240	5120	10240	10240	neg
3	10/25/05	neg	neg	neg	5120	5120	2560	5120	neg

All dogs were seronegative for *L. infantum*, *B. henselae* and *Bartonella vinsonii ssp berkhoffi* at all time points. *B. canis* and *E. canis* titers were performed at both LAV and IPRL. Titers from the IPRL are indicated in parentheses where results differed.

**Ap*=*Anaplasma phagocytophylum*

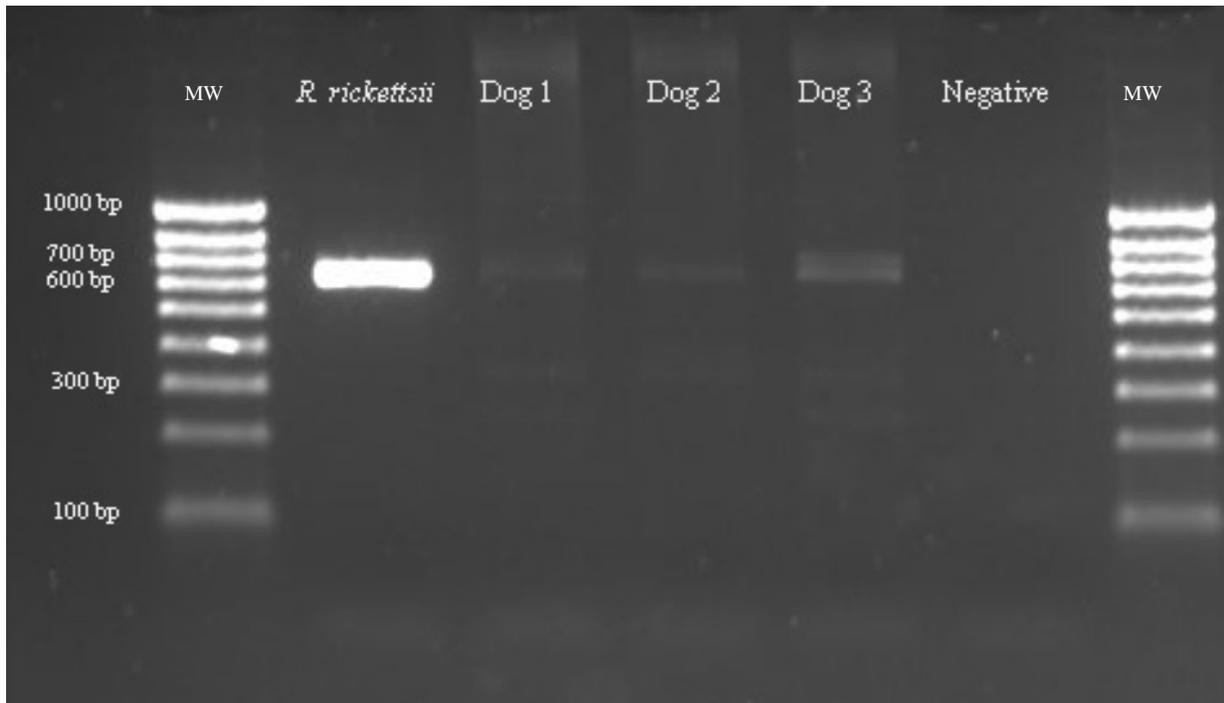


Figure 1 Fragment of the *ompA* gene amplified from the blood of three dogs infected with *R. conorii*, using primers RR190.70F and RR190.701R. Size of bands in base pairs (bp) are based on the molecular weight marker (MW)

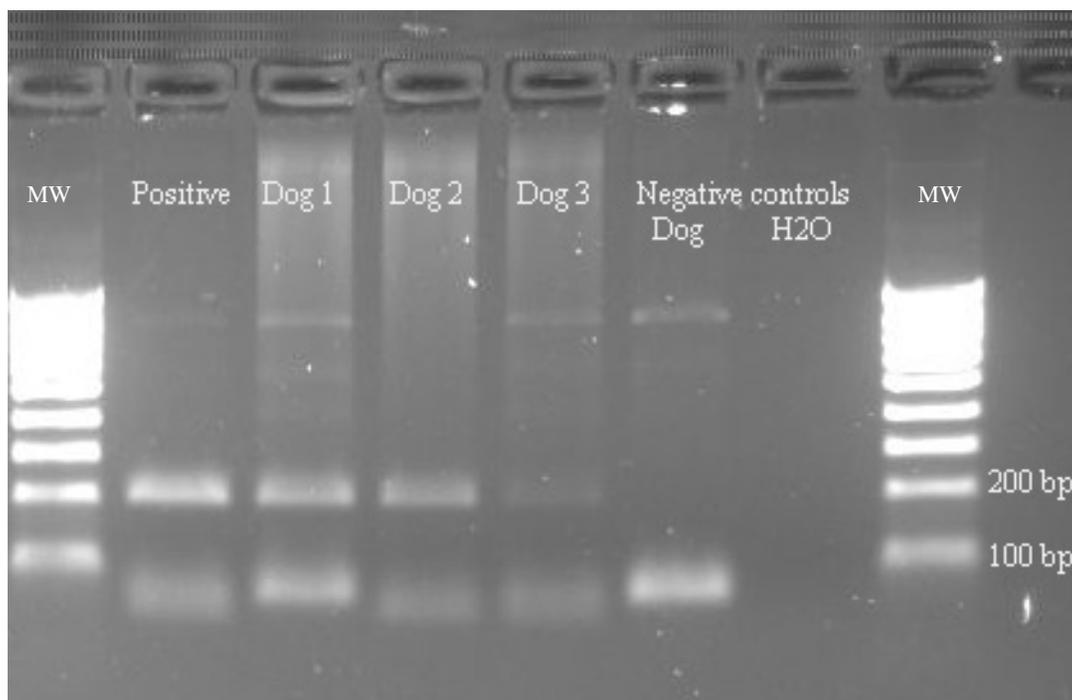


Figure 2 212 bp fragment of the *ompA* gene amplified using primers 107F and 299R (see text) from dogs naturally infected with *R. conorii*. Negative controls contained reaction mixtures with and without DNA extracted from the blood of a dog without *Rickettsia* infection. Size of bands in base pairs (bp) are based on the molecular weight marker (MW)

Figure 3 Alignment of *R. conorii ssp conorii ompA* gene consensus sequences amplified from the blood of three dogs. Primers Rr190.70F and RR190.701R were used for sequences obtained from the blood of Dog 1, and primers 107F and 277R were used for samples from Dog 2, and Dog 3 (see text). The *R. conorii* sequence corresponds to GENBANK accession number AE0086741.

Figure 3 continued

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          .....|.....| .....|.....| .....|.....| .....|.....| .....|.....|
          10      20      30      40      50
Dog 1      TTATTTCAAA AAGCAATACA ACAAGGTCTT AAAGCCGCTT TATTCACCAC
Dog 2      -----
Dog 3      -----
R. conorii TTATTTCAAA AAGCAATACA ACAAGGTCTT AAAGCCGCTT TATTCACCAC

          .....|.....| .....|.....| .....|.....| .....|.....| .....|.....|
          60      70      80      90     100
Dog 1      CTCAACCGCA GCGATAATGC TGAGTAGTAG CGGGGCACTC GGTATTGCTG
Dog 2      -----CGCA GCGATAATGC TGAGTAGTAG CGGGGCACTC GGTATTGCTG
Dog 3      -----CGCA GCGATAATGC TGAGTAGTAG CGGGGCACTC GGTATTGCTG
R. conorii CTCAACCGCA GCGATAATGC TGAGTAGTAG CGGGGCACTC GGTATTGCTG

          .....|.....| .....|.....| .....|.....| .....|.....| .....|.....|
          110     120     130     140     150
Dog 1      TTTCAGGTGT TATTGCTACT AATAATAATG CAGCATTTAG TGATAATGTT
Dog 2      TTTCAGGTGT TATTGCTACT AATAATAATG CAGCATTTAG TGATAATGTT
Dog 3      TTTCAGGTGT TATTGCTACT AATAATAATG CAGCATTTAG TGATAATGTT
R. conorii TTTCAGGTGT TATTGCTACT AATAATAATG CAGCATTTAG TGATAATGTT

          .....|.....| .....|.....| .....|.....| .....|.....| .....|.....|
          160     170     180     190     200
Dog 1      GGCAATAATT GGAATGAGAT AACGGCTGCA GGGGTAGCTA ATGGTACTCC
Dog 2      GGCAATAATT GGAATGAGAT AACGGCTGCA GGGGTAGCTA ATGGTACTCC
Dog 3      GGCAATAATT GGAATGAGAT AACGGCTGCA GGGGTAGCTA ATGGTACTCC
R. conorii GGCAATAATT GGAATGAGAT AACGGCTGCA GGGGTAGCTA ATGGTACTCC

          .....|.....| .....|.....| .....|.....| .....|.....| .....|.....|
          210     220     230     240     250
Dog 1      TGCTCGCGGT CCTCAAACA ATTGGGCATT TACTTACGGT GGTGATTATA
Dog 2      TGCTCGCGGT CCTCAAACA ATTGGGC--- -----
Dog 3      TGCTCGCGGT CCTCAAACA ATTGGGC--- -----
R. conorii TGCTCGCGGT CCTCAAACA ATTGGGCATT TACTTACGGT GGTGATTATA

          .....|.....| .....|.....| .....|.....| .....|.....| .....|.....|
          260     270     280     290     300
Dog 1      CTATCACTGC AGATGTAGCC GATCATATTA TTACGGCTAT AAATGTTGCG
Dog 2      -----
Dog 3      -----
R. conorii CTATCACTGC AGATGTAGCC GATCATATTA TTACGGCTAT AAATGTTGCG

          .....|.....| .....|.....| .....|.....| .....|.....| .....|.....|
          310     320     330     340     350
Dog 1      GATACTACTC CCATAGGTCT AAATATTGCT CAAAATACCG TCGTTGGTTC
Dog 2      -----
Dog 3      -----
R. conorii GATACTACTC CCATAGGTCT AAATATTGCT CAAAATACCG TCGTTGGTTC

          .....|.....| .....|.....| .....|.....| .....|.....| .....|.....|
          360     370     380     390     400
Dog 1      GATTGTAACG GGAGGTAACT TGTTCCTGT TACTATTACT GCCGGCAAAA
Dog 2      -----
Dog 3      -----
R. conorii GATTGTAACG GGAGGTAACT TGTTCCTGT TACTATTACT GCCGGCAAAA

          .....|.....| .....|.....| .....|.....| .....|.....| .....|.....|

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Figure 3 continued

	410	420	430	440	450
Dog 1	GCTTAACTTT	AAACGGTAAT	AATGCTGATG	CTGCAAATCA	TGGTTTGGT
Dog 2	-----	-----	-----	-----	-----
Dog 3	-----	-----	-----	-----	-----
<i>R. conorii</i>	GCTTAACTTT	AAACGGTAAT	AATGCTGATG	CTGCAAATCA	TGGTTTGGT

		460	470	480	490
Dog 1	GCTCCTGCCG	ATAATTATAC	AGGTTTAGGA	AATATAGCTT	TAGGGGGAGC
Dog 2	-----	-----	-----	-----	-----
Dog 3	-----	-----	-----	-----	-----
<i>R. conorii</i>	GCTCCTGCCG	ATAATTATAC	AGGTTTAGGA	AATATAGCTT	TAGGGGGAGC

	510	520	530	540	550
Dog 1	GAATGCTGCA	CTAATTATAC	AATCTGCAGC	CCCGCAAAG	ATAACACTTG
Dog 2	-----	-----	-----	-----	-----
Dog 3	-----	-----	-----	-----	-----
<i>R. conorii</i>	GAATGCTGCA	CTAATTATAC	AATCTGCAGC	CCCGCAAAG	ATAACACTTG

	560	570	580	590	
Dog 1	CAGGCAATAT	AAATGGAGGA	GGTATAATAA	CTGTCAAGAC	
Dog 2	-----	-----	-----	-----	
Dog 3	-----	-----	-----	-----	
<i>R. conorii</i>	CAGGCAATAT	AAATGGAGGA	GGTATAATAA	CTGTCAAGAC	

6) SUMMARY AND CONCLUSIONS

This study addressed the following aims:

- 1) Determine if the agent that causes RMSF in people is genetically similar to the agent that causes RMSF in dogs.
- 2) Develop a sensitive and specific polymerase chain reaction that can differentiate species of Spotted Fever Group *Rickettsia* (SFG) and amplify SFG *Rickettsia* DNA from dog blood.
- 3) Demonstrate natural infection with *R. conorii* in dogs using molecular techniques.

These questions were answered as follows:

- 1) The agent that causes RMSF in people is genetically very similar to the agent that causes RMSF in dogs.
- 2) A PCR that can amplify 15-30 copies of SFG DNA in the presence of dog blood 100% of the time, and 1.5-3.0 copies 50% of the time was developed. Sequencing of the produce accurately differentiates among SFG species.
- 3) Using a conventional PCR for the *ompA* gene and the PCR developed in specific aim 2, DNA 100% homologous to *R. conorii ssp conorii* was amplified from the blood of three clinically ill intact male Yorkshire Terriers from an endemic area.

Spotted fever rickettsiosis are important causes of morbidity and mortality in people. Dogs are exposed to the same vectors as their human companions, and are sentinels for RMSF and

MSF. Increased awareness in clinicians and advances in molecular biology have facilitated an increased recognition in the variety and type of diseases caused by SFG *Rickettsia* in people. This study has reinforced the observation that RMSF in dogs and people is similar, and caused is by the same organism during natural infection. We have also demonstrated that through the use of a sensitive and specific PCR, infection with MSF may cause clinical signs in dogs. The fact that these dogs were all the same breed and sex is striking, and suggests as in people, certain individuals may be more susceptible to disease than others. This work raises many questions regarding whether *R. conorii* causes disease in dogs as it does in people, whether individual traits predispose to infection as is the case for people, whether other SFG *Rickettsia* can cause disease in dogs, and whether dogs could be used as sentinels for other SFG *Rickettsia* or even bioterrorist attacks using these agents.