INVESTIGATION OF SPOTTED FEVER GROUP *Rickettsia* IN DOGS AND TICKS IN NORTHERN CALIFORNIA

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Jeremy Corrigan

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ABSTRACT

INVESTIGATION OF SPOTTED FEVER GROUP *Rickettsia* IN DOGS AND TICKS IN NORTHERN CALIFORNIA

Jeremy Corrigan

Spotted Fever Group (SFG) *Rickettsiae* are important zoonoses affecting both people and dogs. Rocky Mountain spotted fever (RMSF) caused by *Rickettsia rickettsii*, is the most well-known zoonotic SFG; historically in California cases were identified from Modoc plateau area but lately the annual number of cases rarely exceeds three. More recently, a distinct but related serotype and genotype to *R. rickettsii*, *Rickettsia* spp., strain 364D (proposed name *R. philipii*), was identified from a tick-bite associated eschar from a northern Californian human that brought attention to SFG *Rickettsia* in northwestern California. Historic cases of RMSF and this recent case of 364D raise the public health question as to whether SFG *Rickettsia* are more prevalent in northern California than previously reported. Also, dogs may play a role in bringing humans in contact with SFG *Rickettsia* or serve as sentinels for human disease. The aim of this study was to conduct a large scale survey of Lake and Mendocino County’s dog populations for infection with SFG *Rickettsia* where both 364D and *R. rickettsii* has been shown to be endemic in the local tick population. Serologic assessment using an indirect immunofluorescence assay was used to evaluate past exposure of dogs to *Rickettsia rickettsii*. Real-time quantitative Polymerase Chain Reaction (qPCR) was used to molecularly detect the presence of the pathogen in the blood. It targeted the *ompA* gene that codes for an outer membrane protein A (*ompA*), present on all SFG *Rickettsia*. This
gene allowed us to target all potential SFG *Rickettsia* that might potentially infect the local canine population. Any positive SFG *Rickettsia* identified were further classified by a nested PCR assay targeting an intergenic region *RR0155-rpmB* and was confirmed by sequencing. From June to September 2010, a total of 242 individual blood samples and 36 ticks were collected from dogs by veterinarians and county shelter personnel in northern California. 45 out of 242 (19%) serum samples tested were positive with reactive titers of 1:64 or greater to *R. rickettsii*. The highest seropositivity occurred in July when compared to the other three collection months (p<0.05). Of all the dog samples tested, two out of 240 (0.83%) had detectable *R. rickettsia* DNA. In addition, we identified one *Dermacentor occidentalis* tick with SFG *Rickettsia* and one *Dermacentor variabilis* tick with *R. rickettsii*. This is the first time that *R. rickettsii* has been detected in a *D. variabilis* tick in California thereby adding to the known *R. rickettsii* positive tick species in California. Additionally, we show that *R. rickettsia* occurs in dogs and ticks in northern California, expanding the known geographic and host species distribution of documented *R. rickettsii* infections in California. This may be of public health concern given the close proximity of owners to their pets and shared peri-domestic environments. This research shows that even though the incidence of RMSF is low in California the potential risk of infection still exists and residents need to be aware of the risks in order to protect themselves and their pets.
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INTRODUCTION

Tick-borne rickettsioses can cause serious diseases in humans and animals (Nicholson et al. 2006, Nicholson et al. 2010). Rickettsioses are considered zoonotic diseases because they are transmissible from animal to humans and are considered vector-borne zoonoses because they are transmitted by the bite of various arthropods including ticks, lice, fleas and mites (Fournier and Raoult 2009). Currently there are 25 validated species of the genus Rickettsia which 16 are recognized human pathogens (Fournier and Raoult 2009, Goddard 2001). In addition to pathogenic Rickettsia spp. there are numerous non-pathogenic Rickettsia spp. that reside within ticks (Azad and Beard 1998). Rickettsiae are obligate intracellular bacteria and are classified into two groups: the typhus group and spotted fever group (SFG) (Fournier and Raoult 2009). This research will focus on tick transmitted SFG Rickettsia in northern California.

Rocky Mountain spotted fever (RMSF); caused by Rickettsia rickettsii a member of the SFG Rickettsia, is the most common human disease transmitted by Dermacentor species in the United States (Warner and Marsh 2002). RMSF is found throughout all of the United States but 60% of human cases are reported from five states: North Carolina, Oklahoma, Arkansas, Tennessee and Missouri (Centers for Disease Control 2011). RMSF is a reportable disease with an average of 1500 cases reported each year (2008) to the Centers for Disease Control and Prevention (CDC) and has a peak occurrence in June and July (Centers for Disease Control 2011, Dumler and Walker 2005), coinciding with D. variabilis adult tick activity (Goddard 1996). Incidence has increased since 1920 from
less than 2 cases per million people to more than 8 cases per million people in 2008; however the fatality rate has drastically dropped in that same time period (Centers for Disease Control 2011).

RMSF is difficult to diagnose in humans and can progress rapidly (<8 days) if left untreated. Mortality can be as high as 23% in untreated cases even in otherwise healthy individuals (Mandell et al. 2005). This disease presents as non specific fever, fatigue, headache, muscle pain, nausea and vomiting escalating quickly to systemic vasculitis and septic shock and can present with or without a rash (Warner and Marsh 2002). Diagnosis is often made based on patient history, including a tick bite or exposure (Everett and Rham 1970, Gordon et al. 1983).

In North America, the primary invertebrate vector for tick-borne SFG *Rickettsiae* is ticks (Mandell et al. 2005). Ticks are blood sucking arthropods of the class Arachnida with three recognized families: Ixodidae (hard ticks); Argasidae (soft ticks); and Nuttalliellidae (found only in Africa) (Mandell et al. 2005). *R. rickettsii* circulates in a zoonotic cycle that includes transtadial and transovarial maintenance by Ixodidae ticks and transmission to vertebrate hosts during feeding, including rodents, small mammals, and occasionally dogs and humans (Eremeeva et al. 2003, Pacheco et al. 2011). Transovarial transmission occurs when the bacterium is transmitted from parent to offspring, an example is when *R. rickettsii* infected female ticks lay their eggs and the resulting larvae are infected with this bacterium (Labruna 2009). Transtadial transmission occurs when an infection is picked up by one stage in the vector's life cycle and transmitted to succeeding stages during metamorphosis, for example when ticks transmit
the bacterium from larva to nymphs and then to adults (Labruna 2009). Therefore ticks act as reservoir hosts for *R. rickettsii* because they maintain the infection through all life stages and serve as a potential source for human disease.

The principal vectors of RMSF in the United States are the Rocky Mountain wood tick (*D. andersoni*) and the American dog tick (*D. variabilis*) (Mandell *et al.* 2005, Warner and Marsh 2002). *D. variabilis* is the best characterized species of tick responsible for transmitting *R. rickettsii* (Carmichael and Fuerst 2010, Reese *et al.* 2011), and is widely distributed east of the Rocky Mountains and in limited areas of the Pacific Coast. Adult American dog ticks overwinter in the soil and are most active from around mid-April to early September. Larvae are active from about March through July and nymphs are usually found from June to early September (Goddard 1996). Larvae and nymphs mainly feed on small rodents, adult ticks prefer medium and large sized mammals including domestic dogs and have been known to feed on humans and larger mammals (Centers for Disease Control 2011, Cooney *et al.* 2005). A study in Maryland revealed 3.8% prevalence (n=392) of SFG *Rickettsia* in *D. variabilis*, another study reports 4% prevalence (n=308) for SFG *Rickettsia* (Ammerman *et al.* 2004, Stromdahl *et al.* 2001). A study of prevalence in South Carolina of SFG *Rickettsia* in ticks removed from human hosts revealed that 11 of 51 persons from whom *Rickettsia*-positive *D. variabilis* were removed reported symptoms of RMSF, underscoring the role of *D. variabilis* in the transmission of *R. rickettsii* to humans (Loving *et al.* 1978).

*Dermacentor andersoni* can transmit RMSF and is commonly found in the Rocky Mountain States (Colorado, Idaho, Montana, Nevada, Utah, and Wyoming) with small
areas in upper northeast of California (Centers for Disease Control 2011, Furman and Loomis 1984). Peak activity for these ticks occurs from April to September when adults and nymphs are most active. Larvae and nymphs feed on small rodents and adults primarily feed on large mammals (Centers for Disease Control 2011).

Other emerging tick vectors of disease in the United States include the Pacific Coast tick (*D. occidentalis*) and *Rhipicephalus sanguineus*, commonly called the brown dog tick. *D. occidentalis* is known to transmit a variety of pathogens including: bovine anaplasmosis, Colorado tick fever virus, *Francisella tularensis*, and SFG *Rickettsia* (Philip *et al.* 1981, Wikswo *et al.* 2008). This tick is limited to California, Oregon and Northern Baja (Mexico) with adults found year round with peak activity in April and May. Adults primarily feed on cattle, horses, deer and humans but are rare on dogs and bears (Wikswo *et al.* 2008). Nymphs are common during the spring and summer and feed on rodents and small mammals (Centers for Disease Control 2011). *Rh. sanguineus* is capable of transmitting the agents that cause RMSF and Tularemia (Beeler *et al.* 2011, Dantas-Torres 2008, Demma *et al.* 2005, Piranda *et al.* 2011). This tick is widely distributed throughout the U.S. and the world and follows a similar season activity pattern as *Dermacentor* species ticks (Dantas-Torres 2008). Dogs are the primary host for all life stages but under certain circumstances (such as large infestations); they may feed on humans and other large mammals (Dantas-Torres 2008, Pacheco *et al.* 2011).

The ecology of *Rickettsia* species in terms of hosts has been characterized in others areas of the United States. Rodents serve as amplifying hosts, where the *Rickettsia* multiplies in the rodent allowing new lineages of ticks to feed upon them and become
infected (Dumler and Walker 2005, Labruna 2009, McDade and Newhouse 1986). Some tick-borne diseases also affect companion animals who may act as reservoirs or at the least sentinels for human disease, e.g. one that is infected with a whereas a sentinel host is one that is infected with a disease and it occurs in such a manner that it warns us before the disease spreads to another species, due to the peridomestic environment shared by dogs, ticks and humans (Demma et al. 2005, Elchos et al. 2003, Feng et al. 1979, Kidd et al. 2006, Paddock et al. 2002). Clinical illness in dogs caused by *R. rickettsii* has been reported in the United States and Brazil (Gasser et al. 2001, Grindem et al. 1999, Labruna et al. 2009). Often, illness in dogs can precede illness in people who reside within the same household (Elchos and Goddard 2003, Gordon et al. 1983, Paddock et al. 2002) and can progress rapidly resulting in death (Keenam et al. 1977). Norment and Burgdorfer et al. 1984 report that dogs are believed to be comparatively inefficient reservoirs for *R. rickettsii*. However a recent study done in Brazil reported that a Brazilian strain of *R. rickettsii* was maintained and pathogenic in dogs (Piranda et al. 2008).

Recently an outbreak of RMSF in Arizona where there were 16 confirmed human cases with two deaths associated with *Rh. sanguineus* ticks (Nicholson et al. 2006) has brought attention to this new vector and expanded geographic region of RMSF. Additionally, an outbreak in Mexicali, Mexico where there were over 1200 suspected human RMSF cases with 217 lab confirmed cases with six deaths also associated with *Rh. sanguineus* ticks has increased awareness of the expanding distribution of this disease in the United States (Sanchez et al. 2009) A recent study of *Rh. sanguineus* ticks
collected off of dogs and horses in Juiz de Fora, southeast Brazil, showed that 13.1% of collected ticks were positive for *R. rickettsii*, highlighting this tick’s ability to harbor this bacterium and potentially transmit it to another host during feeding (Pacheco et al. 2011). Other research has also identified *R. rickettsii* in *Rh. sanguineus* ticks in California suggesting that there are possibly additional vectors of *R. rickettsii*, effectively expanding its known geographical distribution (Nicholson et al. 2006, Wilswo et al. 2007). *Rh. sanguineus* ticks may be very efficient at transmitting the agent transstadially since a recent study suggests that *Rh. sanguineus* are not capable of maintaining *R. rickettsii* through successive generations due to low filial infection rates (Piranda et al. 2011).

Another emerging SFG Rickettsial disease that has been designated *Rickettsia* 364D was isolated from a *D. occidentalis* tick in 1973 in Mendocino County (Cory et al. 1975). Further, microimmunofluorescence analysis of this isolate and another acquired from southern California in 1966 revealed that these isolates were a distinct but related serotype to *R. rickettsii* (Philip et al. 1978). Molecular confirmation of this serotype data also suggests *Rickettsia* 364D represents a genotype distinct from *R. rickettsii* (Karpathy et al. 2007). This novel *Rickettsia* 364D strain has been shown to elicit minimum to moderate scrotal reaction when inoculated into guinea pigs and convalescing animals develop antibody titers to this agent ranging from 1:256 to 1:2,048 as well as terminated 5-day old chick embryos (Philip et al. 1978). These data indicate that it is possible that *Rickettsia* 364D can cause illness in animals and humans and suggest that there are other causes of tick-borne spotted fever in the United States and California for which *Rickettsia* 364D is a candidate pathogen (Paddock 2005). In addition, data from antibody
absorption tests of convalescent-phase serum specimens of patients in northern California with presumed RMSF suggest an infection with *Rickettsia* 364D (Lane *et al.*1981). Due to the non specific symptoms, it may be possible that *Rickettsia* 364D infections are being mis-diagnosed as RMSF infections and treated without more specific laboratory confirmation. *Rickettsia* 364D is very similar yet distinct from *R. rickettsii* and it is possible it causes similar illness like RMSF (Shapiro *et al.* 2010).

*Rickettsia* 364D has only recently been documented as a cause of human illness (Shapiro *et al.* 2010). A resident in Lake County California presented with an eschar on his forearm that was initially suspected to be cutaneous anthrax (Shapiro *et al.* 2010). A skin punch biopsy of the lesion was tested by immunohistochemistry and molecular analysis; acute and convalescent serum serology was also performed on this patient by the CDC as well as in three other patients identified retrospectively with eschars. High IgG titers were detected to *R. rickettsii, R. rhipicephali* and *Rickettsia* 364D antigens ranging from 1:1,024 to 1:2,048 as well as molecular confirmation of *Rickettsia* 364D by sequencing the PCR products from blood. This is the first time *Rickettsia* 364D has been clearly associated with human disease (Shapiro *et al.* 2010).

In addition, ticks collected near the patients’ home had detectable SFG *Rickettsia* in three *D. occidentalis* ticks: *Rickettsia* 364D (1 male), *R. rhipicephali* (1 male) and an unidentified *Rickettsia* (1 female). This prompted the CDC to begin an investigation of the local tick species in Lake County, of which most species found were *D. occidentalis*. In April of 2008 the CDC collected 33 *D. occidentalis* of which 3 (9.1%) were positive for *Rickettsia* 364D by PCR consistent with a 1981 study reporting a 3.7% prevalence
(n=215) (Bonilla 2008). Recent additional studies performed in southern California found a *Rickettsia 364D* prevalence in *D. occidentalis* tick of 7.7% (n=365) (Wikswo et al. 2008) and 7.7% (n=52) (Bonilla et al. 2009), respectively.

Since *Rickettsia 364D* is closely related to *R. rickettsii*, it would be possible that dogs may also serve as reservoirs or at least sentinels for *Rickettsia 364D*, since dogs and people can both be bitten by *Dermacentor* spp. ticks (McQuiston et al. 2011). The purpose of this research is to investigate what *Rickettsia* species are infecting dogs in northern California to both expand knowledge on *Rickettsia* distribution and investigate if the dogs are infected with *Rickettsia 364D*. Reported cases of RMSF in California dogs is not known since there is no official reporting mechanism for these veterinary cases. However, according to a 2007 California Department of Public Health (CDPH) survey of canine RMSF there were 25 cases reported in California in that past year. There are a few serological SFG *Rickettsia*-canine surveys, which investigate prevalence of SFG *Rickettsia* in domestic dogs, but most are not in the United States. In addition, most prevalence studies are only done in response to documented human or domestic animal rickettsial infection. Prevalence among dogs in areas where human infections occurred ranged from 4.4% (16/364) in Brazil, 23% (36/153) in Croatia, 45% (216/481) in France, 58.6% (34/58) in Spain to 82% (150/184) in Zimbabwe (Fortes et al 2010, Kelly and Mason 1991, Herrero et al. 1992, Punda-Polic et al. 1995, Raoult et al. 1985). In the United States additional canine serosurveys have been performed in response to human SFG *Rickettsia* outbreaks. The prevalence of SFG *Rickettsia* in canines in these studies ranged from 45.4% (33/73) in Ohio, 75% (12/16) in another location in Ohio, to 77%

Recently in southern California, four dogs were tested as part of a follow up investigation to a reported increased number of human cases of *R. rickettsii* infections. All four were seropositive to SFG *Rickettsia* (Beeler et al. 2011). This high prevalence is intriguing but the small sample size makes it difficult to assess the significance of this finding.

The aim of this study is to conduct a large scale survey of Lake and Mendocino County’s dog populations for infection with SFG *Rickettsia* where *Rickettsia* 364D has been shown to be endemic in the local tick populations. We suspect that dogs that have been exposed to SFG *Rickettsia* naturally will have robust antibody titers (greater than 1:64). Verification of current infection can be accomplished through PCR of whole blood from these dogs. Understanding the prevalence of exposure through serology and the prevalence in active infection may provide insight into the role domestic dogs may play in maintaining or transmitting *Rickettsia* species in this northern California environment.

Due to improved diagnostic methods and increased interest in SFG *Rickettsia*, methods have expanded from serology to include molecular techniques. Molecular techniques are highly specific and sensitive and able to identify an active infection rapidly but are still limited by its dependence on circulating organisms for detection. It takes anywhere between 4-48 hours to transfer the SFG *Rickettsia* bacterium to the host during a tick bite. Culture techniques are only successful early in the disease progression, while molecular techniques like polymerase chain reaction (PCR) are successful during the late subclinical infection phase and symptomatic phase. Serology detection is very useful in identifying a past infection, usually 22-28 days past initial infection (Figure 1).
Figure 1. Overview of the disease progression of Rocky Mountain spotted fever and comparison of the different accepted diagnostic laboratory assays.
Serology, using immunofluorescence assay (IFA) has the advantage of not being dependent on the amount of circulating organisms, which is often very low in the blood of dogs (Parola et al. 2005). It has been shown that clinical signs of *R. rickettsii* illness in dogs resolve when the organism can no longer be cultured, usually 10-13 days post infection (Breitschwerdt et al. 1999), emphasizing the acute nature of this disease. Serology has the distinct disadvantage of not being able to differentiate SFG *Rickettsia* and can often be negative in an early, active infection. In addition, unless paired acute and convalescent samples are available, at best IFA titers demonstrate exposure, but not necessarily active infection, to *Rickettsia* species.

Many polymerase chain reaction (PCR) protocols have been utilized (Fournier and Raoult 2004, Karpathy et al. 2007, Kidd et al. 2008, Roux et al. 1996, Shapiro et al. 2010) to identify SFG *Rickettsia* isolates targeting several different gene targets. There is a high degree of homology among SFG *Rickettsia* in the 16S rRNA and citrate synthase (*gltA*) genes; however, the outer membrane protein A (*ompA*) gene exhibits variability among species, especially the 5’ hypervariable region (Kidd et al. 2008, Roux et al. 1996). Most research using the *ompA* gene has utilized the 632 bp fragment amplified using the RR190.70 and RR190.701 primers, which is capable of differentiating most *Rickettsia* species by sequencing (Kidd et al. 2008).

Recently, a new qPCR approach was developed using primers called 107F and 299R that target a smaller portion (209-215 bp) of the above *ompA* gene 632 base pair fragment which has been shown to be more sensitive and specific (Kidd et al. 2008). The sensitivity has been shown to detect 15-30 copies of SFG *Rickettsia* DNA 100% of the
time and 1.5-3.0 copies 45% of the time (Kidd et al. 2008). In addition, the specificity
was tested with other commonly occurring vector-borne diseases such as *Ehrlichia*
species, *Bartonella henselae* and *A. phagocytophilum* and it was shown to not amplify
these bacterial species (Kidd et al. 2008). Sequencing of the 209-215 bp fragment of the
5’ hypervariable region of the *ompA* gene allows for SFG *Rickettsia* species identification
(Kidd et al. 2008). The one drawback to this approach is that this region
cannot distinguish *R. rickettsii* isolates from *Rickettsia* 364D. Despite this issue, there are
other molecular targets within that 632 base pair fragment that have been successfully
used to identify SFG *Rickettsia* in ticks and humans (Shapiro et al. 2010).

Targeting the *ompA* gene allows for identification of SFG *Rickettsia* but cannot
differentiate *R. rickettsii* isolates from *Rickettsia* 364D isolates (Shapiro et al. 2010). To
mitigate this problem there have been PCR assays developed that target intergenic
regions; RR0155-rpmB, RR1240-tlc5 and several others, that are used to differentiate
*Rickettsia* 364D from known *R. rickettsii* isolates (Karpathy et al. 2007, Shapiro et al.
2010). A common strategy for identifying SFG *Rickettsia* uses qPCR targeting the *ompA*
gene to first identify SFG *Rickettsia*, and then use nested PCR targeting an intergenic
region with direct sequencing of the PCR products which assists in species identification
(Karpathy et al. 2007, Shapiro et al. 2010).

In this study, a combination of serology and PCR was used on canine samples
collected from canines from rural Lake and Mendocino Counties. The purpose of using
these two methods together was to identify evidence of SFG *Rickettsia* infections and
possibly a *Rickettsia* 364D infection in the local tick and canine population. This study
will provide insight to better understand the ecology and epidemiology of SFG *Rickettsia* in northern California.
MATERIALS AND METHODS

Sample Collection

This study involved the use of dogs, requiring a Humboldt State University (HSU) animal care and use protocol which was approved by the HSU IACUC committee (09/10.B35.A). This study was conducted at several sites in Lake and Mendocino Counties California where previous data suggest that \textit{Rickettsia} 364D occurs naturally (Bonilla 2008, Bonilla 2009). Prevalence of \textit{Rickettsia} 364D in canines is not known and is expected to vary by location sampled due to vector and pathogen distribution (Otranto \textit{et al.} 2009). The infection prevalence of \textit{Rickettsia} 364D in \textit{D. occidentalis} ticks from Lake County was 7.7\% in one study (Bonilla 2008). With a small tick infestation prevalence and a likely smaller canine prevalence, a sample size of 141 was determined to be the minimum sample size required to detect a positive dog using the formula: 

\[ n(p)(1-p) > 10 \]  

\( p = \text{infection prevalence (7.7\%)} \), \( n = \text{desired sample size} \) (Bluman and Hill 2010), however we aimed to collect at least 200 canine samples. Since Mendocino and Lake Counties were the two locations of interest, the managers of both County shelters were asked to participate. In addition, letters, phone calls and personal visits were made to get in contact with all known veterinary services provided by each county asking for participation in the study. Shelter workers and veterinarians that accepted our offer to participate assisted in blood draws of the canine samples. Collection visits were conducted from June 2010 to late September 2010 during which time both nymphal and adult \textit{D. occidentalis} ticks can be active (personal communication Denise Bonilla, CDPH).
Lake and Mendocino County Shelter Canine Blood and Tick Collection

The veterinarian and other shelter workers carried out blood draws by restraining the canines and taking approximately three ml of whole blood. The blood was collected in a red top tube with no EDTA (Becton Dickson, Franklin Lakes, NJ) and immediately placed on ice. After allowing the blood to sit for at least one hour the blood was centrifuged at 10,000 rpm for 10 minutes. The serum was removed and placed in a sterile microcentrifuge tube and stored at -20°C until processed. The remaining blood clot was stored at -20°C until processed.

A specimen log sheet was created and used to record a variety of data and demographic information during shelter collection visits including: HSU ID, veterinarian canine ID, location where the dog was found or came from, any ticks associated with them, shelter intake date and breed. Unique consecutive identifiers or HSU ID’s were given to samples corresponding to the collection site; Mendocino County Shelter (MCS), Lake County Shelter (LCS), Animal Hospital of Lake County (AHLC), Main Street Veterinary Clinic (MSVC), Clearlake Veterinary (CV), Wasson Memorial Veterinary Clinic (WMVC), Yokayo Veterinary Clinic (YVC) and Mendocino County Shelter (MCS).

Any ticks collected from a dog were labeled with a unique consecutive identifier and placed into a sterile container. Each tick was described as engorged or non-engorged when removed from the canine and the stage of the tick was also noted, i.e. nymph or adult, identified using several keys and a dissecting microscope and then stored in 70%
ethanol until processed (Furman and Loomis 1984, Misurelli 2010). Briefly, *Ixodes* spp. ticks were identified by the presence of the anal groove, which is anterior to the anus (Misurelli 2010). The following characteristics were used to identify to the genus *Dermacentor*: anal groove posterior, second segment of the palps do not extend laterally, mouthparts the same length as basis capituli, basis capituli do not extend laterally and finally, and the festoons number 11. Once determined to be *Dermacentor* species, the following characteristics were used to distinguish *D. occidentalis* from *D. variabilis*: basis capitulum longer than wide, cornua as long as or longer than wide, sizes of scutal punctuations not generally disparate, pearl grey coloration of scutum more extensive than brown and known species range (Furman and Loomis 1984, Misurelli 2010).

**Lake and Mendocino County Veterinary Canine Blood and Tick Collection**

Veterinarians in Lake (n = 4) and Mendocino (n = 2) counties were asked to collect samples for this study. Blood was collected from dogs that either had a fever and appeared sick or were high risk for tick transmitted diseases, based on veterinarian assessment of patient history. Whole blood samples were collected and handled as described above. A survey was supplied with the intent that it be completed with every sample submitted. The survey contained a variety of demographic information and data used for further epidemiological analysis such as: date drawn, breed, sex, age, temperature, tick history, tick control and comments. Any ticks submitted were labeled, put in containers (Fisher Scientific, Pittsburg, PA) and identified as described earlier.

**Serological Analysis of Canine Blood**

An indirect immunofluorescence assay (IFA) was performed to screen all samples
for antibodies to *R. rickettsii* and other SFG *Rickettsia* detecting past exposure to the agent using an IFA kit purchased from Veterinary Medical Research and Development (VMRD, Pullman, Washington). Each sample was screened in duplicate at dilutions 1:32 and 1:64. If the sample was deemed reactive at any tested dilution, then the endpoint titer was determined using two-fold dilutions and a reactive result was recorded if the sample was reactive at a titer of 1:64 or greater as described at the VMRD website (VMRD 2010).

Dog whole blood samples were prepared as described earlier and serum was removed and diluted using serum diluting buffer, pH 7.2 to the 1:32 and 1:64 dilutions. Diluted serum and controls were added to designated wells and run in duplicate along with a positive and negative control. The slides were then incubated at 37°C for 30 minutes. Using a wash bottle, the slides were rinsed using FA rinse buffer, pH 9.0 and then soaked for ten minutes in FA rinse buffer. Slides were then blotted gently and allowed to air dry. Anti-canine IgG FITC labeled conjugate was added to each well. The slides were then incubated again in a humid chamber at 37°C for 30 minutes and rinsed as described earlier and mounting fluid was added to each well.

The slides were viewed and scanned using a fluorescence microscope (Carl Zeiss, Thornwood, NY) at 200X. Confirmation was determined at 400X. Positive wells were recorded using the SPOT-RT Slider digital camera controlled by SPOT Advanced v3.1 software for Windows 95/98/NT (Diagnostic Instruments, Inc., Sterling Heights, MI) with user defined settings. Exposures were set separately for each RGB channel. A FITC filter was used with the following settings; Gain = 2, Pixel Bit Depth = 24 (RGB), Use =
Green, Exposure Time = 15 seconds, Chip Imaging Area = Full Chip, Binning = None, Chip Defect Correction = ON, Noise Filter = OFF, Background Subtraction = Off, and Flatfield Correction = OFF. A positive reaction was determined when the sample well and positive control organisms had observed positive fluorescence and negative on negative organisms without background. Each run had a *R. rickettsii* positive control (purchased from VMRD) and negative control (negative canine serum purchased from VMRD).

Samples were observed for intensity of apple-green fluorescence (brightness) of apple-green fluorescence present in each well and recorded as 0, 1+, 2+, 3+, and 4+. For intensity; 0 = no fluorescence, 1+ = very dim but detectable apple-green fluorescence, 2+ = dull apple-green fluorescence, 3+ = bright apple-green fluorescence and 4+ = brilliant apple-green fluorescence. A test was negative if the sample showed 0 fluorescence intensity. A test was positive if the sample showed 3+ to 4+ fluorescence intensity. All other results were considered unsatisfactory and repeated. If a sample was reactive during the screening test, then the sample was further processed and an endpoint titer was determined.

The IFA end point titer was determined using the procedure described earlier with the addition of multiple dilutions for each sample to determine the endpoint titer. The samples were diluted in two-fold increments beginning at 1:32 going out to the 1:256 dilutions (1:32, 1:64, 1:128 and 1:256). The endpoint titer was recorded as the well that was two-fold less than the greatest reacting dilution. If the sample was reactive at 1:256 the result was recorded as >1:256. A typical IFA panel is shown in Figure 2.
Figure 2. A typical reactive IFA panel obtained. a. View of a negative well. b. View of a positive well. c. View of sample LCS-33 that had a reactive titer of >1:256.
Extraction of Canine Blood and Ticks

DNA from all whole blood samples and ticks were extracted by the DNeasy Blood and Tissue Kit (Qiagen, Valencia, CA). A mock extraction consisting of Phosphate Buffered Solution (PBS) was done with every 10 extraction as an extraction control.

Ticks were washed three times with 95% ETOH and then placed in a 1.5 mL microcentrifuge tube with a plastic DNA and RNA free pestle. The tubes with the ticks and the pestles were placed in liquid nitrogen for five minutes to freeze the ticks. The ticks were then ground up into powder form using the pestle. DNA was then extracted from the powder of each crushed tick using the Qiagen DNeasy kit as described by manufacture (Qiagen, Valencia, CA).

Extracted DNA Quality Check Using Conventional PCR Targeting the GADPH Gene

All samples extracted were quantitatively and qualitatively evaluated spectrophotometrically using the NanoDrop ND-1000 system and analyzed using v 3.7 software (ThermoScientific, Rochester, NY). The extracted DNA was submitted to a conventional PCR assay that amplified the GADPH in canines, which is commonly used as a canine housekeeping gene and PCR target (Kidd et al. 2008) to verify successful extraction before moving on for further PCR analysis. PCR primers targeting the GADPH gene in canines were chosen from Kidd et al. 2008 (Table 1). The size of the expected amplicon was 200 base pairs in size and was used as an extraction control and screened the samples for inhibition (Table 1). These primers were used in a total reaction volume of 25 µl containing: 12.5 µl of 2x EconoTaq 2x Master mix (Lucigen, Middletown, WI),
Table 1. Primers targeting the *GADPH*, *ompA* and *RR0155-rpmB* genes used to identify SFG *Rickettsia* in canine blood samples.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Primer Sequence 5’-3’</th>
<th>Application</th>
<th>Amplicon Size</th>
<th>Melting Temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Canine GADPH Gene</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GADPH-A F</td>
<td>5’- CCTTCATTGACCTCAACTACAT-3’</td>
<td>Regular PCR</td>
<td>180 bp</td>
<td>52.4°C</td>
</tr>
<tr>
<td>GADPH-B R</td>
<td>5’- CCAAACTTTGTCATGGATGACC-3’</td>
<td></td>
<td>53.9°C</td>
<td></td>
</tr>
<tr>
<td><strong>Outer membrane protein A gene (ompA)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RR190-547F</td>
<td>5’-CCTGCCGATAATTATACAGTTTA-3’</td>
<td>SYBR Green</td>
<td>157 bp</td>
<td>52.4°C</td>
</tr>
<tr>
<td>RR190-701R</td>
<td>5’-GTTCCGTTAATGGCAGCATCT-3’</td>
<td>qPCR</td>
<td></td>
<td>55.6°C</td>
</tr>
<tr>
<td><strong>RR0155-rpmB intergenic region</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RR0155-PF</td>
<td>5’-GGATTTCTAATACAAGCGATTAGG-3’</td>
<td>Nested PCR</td>
<td></td>
<td>52.3°C</td>
</tr>
<tr>
<td>RR0155-PR</td>
<td>5’-GAAAAGATAGGCACTGATCACATCT-3’</td>
<td>(1°)</td>
<td></td>
<td>54.0°C</td>
</tr>
<tr>
<td>RR0155-FN</td>
<td>5’-TTTCTAGCAGCGTTGTTTTATCC-3’</td>
<td>Nested PCR</td>
<td>290 bp</td>
<td>55.7°C</td>
</tr>
<tr>
<td>RR0155-RN</td>
<td>5’-TTAGCCCATGTTGACAGGTACT-3’</td>
<td>(2°)</td>
<td></td>
<td>56.4°C</td>
</tr>
</tbody>
</table>
0.5 µl’s of 5 µM forward and reverse primer, 9.5 µl of deionized sterile water and 2 µl (50-100 ng/µl) of sample DNA. DNA was amplified using the Applied Biosystems 2720 Thermal cycler (Applied Biosystems Inc., Carlsbad CA) using the following conditions; denaturing at 95°C for five minutes, followed by 35 cycles of 95°C for 45 seconds, 55°C for 45 seconds, 72°C for 45 seconds, and then a final extension at 72°C for five minutes. Deionized sterile water was used as a negative control and DNA successfully extracted from a canine blood sample and verified was used as a positive control. Samples that yielded a visualized band of the correct size on a 2% agarose gel were considered positive.

**Molecular Analysis of Canine Blood and Ticks**

Real-time, conventional, and nested PCR followed by direct sequencing of the PCR products were used to analyze each sample. Figure 3 illustrates the molecular workflow of this project.

**Standard preparation of positive controls for the qPCR and nested PCR assays**

*Rickettsia rickettsii* cell culture was donated from Fuller Labs (Fuller Labs, Fullerton, CA) and the DNA was extracted using the QIAamp DNA blood and tissue kit (Qiagen, Valencia CA), bacterial liquid culture protocol, according to manufacturer’s instructions. The extracted DNA was stored at -20°C in four small aliquots. This DNA was used as the positive control for the SYBR Green qPCR assay as well as the nested PCR which targeted the intergenic region RR0155-rpmB.

To quantitatively and qualitatively detect SFG *Rickettsia* in dog and tick samples, 10-fold serial dilutions of clones from the *ompA* gene were used to make a standard curve to
Figure 3. Molecular workflow diagram of the study. DNA is extracted from both ticks and canine blood samples (A). The samples are then processed by conventional PCR targeting the *GADPH* gene to check the quality of the DNA (B). Once verified each sample is screened using a SYBR- green qPCR assay targeting a portion of the *ompA* gene for SFG *Rickettsia* identification (C). Each sample that is positive is submitted to a nested PCR targeting the RR0155-rpmB intergenic region to allow for species identification (D). Each PCR product is gel electrophoresis confirmed or directly sequence for identification (E).
measure the DNA copy number. Clones were prepared from the 5’ hypervariable region of the ompA gene of *R. rickettsii* that was amplified directly using the primer pair RR190-547F and RR190-701R, and primer set, RR0155-P, RR0155-PR and RR0155-FN, RR0155-RN (Karpathy *et al.* 2007). The PCR products were verified via 2% agarose gel electrophoresis to confirm that the PCR was successful and the product was the correct size. The PCR product was then cloned using the Strataclone PCR Cloning Kit (Agilent Technologies, Santa Clara CA) and transformed into Stratoclonne competent cells (Agilent Technologies, Santa Clara CA). Clones were analyzed using standard blue/white screening on LB–ampicillin (100 μg/ml) agar plates with 40 μl of 2% X-Gal spreading on the surface of the plates. White colonies were re-streaked on ampicillin plates with 2% X-Gal to ensure a pure culture. White colonies were selected and grown in LB broth with 100 μg/ml ampicillin medium overnight. DNA was extracted from these bacteria using the Wizard Plus SV Minipreps DNA Purification System (Promega, Madison WI) according to manufactures instructions. Once plasmids were isolated, restriction enzyme analysis was performed using an Eco R1 digest (Fermentas Life Sciences, Maryland) to cut the DNA insert out. An additional 2% DNA gel verified the correct size fragment. The plasmid DNA concentrations of the clones were recorded using the NanoDrop ND-1000 system and analyzed using v 3.7 software (ThermoScientific, Rochester, NY). Clones were then sent to Elim Biopharmaceuticals Inc. (Hayward, CA) for sequencing to verify that we had the expected sequence of the inserts.

Known quantities of standards were individually run by qPCR and Ct values of each dilution were calculated and recorded by the Applied Biosystems 7300 Real Time
PCR System using Sequence Detection Software (SDS) Version 1.3.1 (Applied Biosystems Inc., Carlsbad, CA). Clones were prepared to quantify sample DNA copy number based on sample Ct values compared to the standard curve values.

**Specificity and Sensitivity of qPCR and nested PCR**

The specificity of each molecular assay was assessed using the BLAST search analysis of each primer set showing unique identification and amplification. The sensitivity of each assay was determined by using the known quantity prepared clones, making 10-fold serial dilutions down to a single copy, and processed in duplicate with each PCR assay. Sensitivity is defined as the lowest detectable copy number. The Applied Biosystems 7300 Real Time PCR System and SDS Version 1.3.1 software (Applied Biosystems Inc., Carlsbad, CA) recorded and calculated the efficiency of each assay. Contamination and nonspecific amplification of each assay was assessed by analyzing a mock extraction control and no template control (NTC).

**SYBR Green qPCR Detection of SFG Rickettsia**

Once extracted DNA was quality-checked, a sample of it was submitted to a SYBR Green qPCR assay targeting the *ompA* gene, to identify SFG *Rickettsia*. The primers RR190-547F and RR190-701R used in the SYBR Green qPCR assay targeted the *ompA* gene of SFG *Rickettsia*, which results in amplification of a small sequence of 157 bp in size (Table 1) (Wikswo et al. 2008). The primers target a conserved region of the gene and amplify a heterogenous region. The product can be isolated and sequenced and then compared against the known validated SFG *Rickettsia* species using Basic Local Alignment Search Tool (BLAST). Note that three SFG *Rickettsia* species (*R. helvetica*, *R.
asiatica and R. felis) cannot be amplified by this primer set but this is not detrimental to
the analysis because two of the species are not known in this country (R. helvetica and R.
asiatica) and the other is not epidemiologically relevant (R. felis) because it is
uncommon, and is flea transmitted (Reif et al. 2011). The ompA primer pairs RR190-547F
and RR190-701R described were used in a total reaction volume of 25 µl containing 12.5
µl of Power SYBR Green 2x Master mix (Applied Biosystems, Carlsbad, CA), 1 µl of 5
µM forward and reverse primer, 2.5 µl 0.04% BSA, 3.0 µl of deionized sterile water and
5 µl (50-100 ng/µl) sample DNA. DNA was amplified using the Applied Biosystems
7300 Real Time PCR System using the following thermocycler conditions: denaturing at
95°C for three minutes, followed by 40 cycles of 95°C for 30 seconds, 57°C for 20
seconds, 65°C for one minute, followed by a melt curve cycle to assess dissociation
characteristics of our products. Deionized sterile water was used as a negative control and
clone standards of R. rickettsii DNA as described earlier was used as a positive control.
Amplification was detected by SYBR Green fluorescence with an emission at 522 nm.

A melt curve analysis was performed to check that the correct fragments were
amplified, that there was no non-specific amplification or primer dimer contamination
and ensured that all samples had similar melting temperatures. Samples that yielded at
least one reactive well during the SYBR Green qPCR assay with characteristic
amplification curve and expected melting temperature were considered “suspicious” or
“preliminarily positive” and were further characterized by purification and sequencing
(described below). An aliquot of the original isolated DNA was further analyzed by
nested PCR to confirm the species. The software analyzed the data with the following
settings: manual cycle threshold (Ct) with threshold setting at 0.200, manual baseline with start cycle set at 3 and end cycle set at 15.

**Nested PCR for Species Identification**

The nested PCR targeted the variable intergenic region \textit{RR0155-rpmB} and was used to further characterize \textit{ompA} “suspicious” or “preliminarily positive” samples. The primers (Table 1) followed Karpathy \textit{et al.} 2007 and targeted the intergenic region RR0155-rpmB, which yielded an amplicon approximately 290 bp in size. This region has small nucleotide differences that allows for species differentiation of \textit{R. rickettsii} from \textit{Rickettsia 364D} based on sequencing. These primers were developed using \textit{R. rickettsii} Sheila Smith complete genome sequence and have been validated on 38 different \textit{R. rickettsii} isolates (Karpathy \textit{et al.} 2007). Table 2 summarizes the important nucleotide distinctions of a \textit{Rickettsia 364D} isolate from the other 37 \textit{R. rickettsii} isolates tested using the RR0155-rpmB intergenic region.

The primary target of the \textit{RR0155-rpmB} gene was amplified using the primer pairs described above (Table 1) in a total reaction volume of 25 µl containing: 12.5 µl of 2x EconoTaq 2x Master mix (Lucigen, Middletown, WI), 0.5 µl of 5 µM forward and reverse primer, 9.5 µl of deionized sterile water and 2 µl (50-100 ng/µl) sample DNA. DNA was amplified using the Applied Biosystems 2720 Thermal cycler using the following thermocycler conditions; denaturing at 95°C for five minutes, followed by 25 cycles of 95°C for 30 seconds, 59.5°C for 30 seconds, 68°C for one minute, and then a final extension at 72°C for 10 minutes. This PCR product was then submitted to a secondary PCR targeting the same intergenic \textit{RR0155-rpmB} gene amplified using internal
Table 2. Genotypic differences of *Rickettsia rickettsii* isolates using a nested PCR assay targeting the intergenic region RR0155-rpMB. Genotypes A2, B2, C2 and E2 were *R. rickettsii* isolates which were all distinct from Genotype D2, which was a *Rickettsia* 364D isolate allowing differentiation of *R. rickettsii* from *Rickettsia* 364D (Karpathy *et al.* 2007).

<table>
<thead>
<tr>
<th>Nucleotide # 297</th>
<th>nt. 63-64</th>
<th>nt. 81-87</th>
<th>nt. 158</th>
<th>nt. 170</th>
<th>nt. 192</th>
<th>nt. 216</th>
</tr>
</thead>
<tbody>
<tr>
<td>Genotype A2 (5)</td>
<td>TA</td>
<td>- - - - - -</td>
<td>C</td>
<td>A</td>
<td>T</td>
<td>T</td>
</tr>
<tr>
<td>Genotype B2 (4)</td>
<td>TA</td>
<td>CCTTGTGTC</td>
<td>C</td>
<td>A</td>
<td>T</td>
<td>T</td>
</tr>
<tr>
<td>Genotype C2 (27)</td>
<td>TA</td>
<td>CCTTGTGTC</td>
<td>C</td>
<td>G</td>
<td>T</td>
<td>T</td>
</tr>
<tr>
<td>Genotype D2 (1)-364D</td>
<td>--</td>
<td>CCTTGTGTC</td>
<td>T</td>
<td>G</td>
<td>C</td>
<td>C</td>
</tr>
<tr>
<td>Genotype E2 (1)</td>
<td>TA</td>
<td>CCTTGTGTC</td>
<td>C</td>
<td>G</td>
<td>T</td>
<td>C</td>
</tr>
</tbody>
</table>
primer pairs RR0155-FN and RR0155-RN (Table 1) in a total reaction volume of 25 µl containing: 12.5 µl of 2x EconoTaq 2x Master mix (Lucigen, Middletown, WI), 0.5 µl of 5 µM forward and reverse primer, 9.5 µl of deionized sterile water and 2 µl (50-100 ng/µl) template DNA. DNA was amplified using the Applied Biosystems 2720 Thermal cycler using the following conditions; denaturing at 95°C for five minutes, followed by 45 cycles of 95°C for 30 seconds, 59.5°C for 30 seconds, 68°C for one minute, and then a final extension at 72°C for 10 minutes. Deionized sterile water was used as a negative control and clone standards of R. rickettsii DNA as described earlier was used as a positive control. The final PCR products were verified and analyzed using gel electrophoresis and the concentration was determined using the NanoDrop ND-1000 system and analyzed using v 3.7 software (ThermoScientific, Rochester NY). Samples with a band of the correct size on a 2% agarose gel were considered positive. Positive samples were gel purified and mailed to Elim Biopharmaceuticals Inc. (Hayward, CA) for sequencing.

**Detection of PCR Products**

2% agarose gels were prepared, 10 µl of the reaction products were added to individual wells as well as 10 µl of a 100 KB ladder which aided in estimation of the PCR fragment size. The gels were then submitted to gel electrophoresis in 1X TBE for one hour at 99 volts. The gels were then stained with ethidium bromide and then trans-illuminated with UV light using the Alpha Innotech - Alpha Imager HP (Protein Simple, Santa Clara CA). Images were captured and stored in digital format.
Sequencing of PCR Products for Confirmation

The PCR products were directly purified using the QIAquick PCR purification kit (Qiagen, Valencia, CA) according to manufactures instructions before sequenced. The purified PCR products yielded from the primer pairs’ RR190-547F and RR190-701R and RR0155-FN and RR0155-RN were sequenced directly in the forward (5’) and the reverse (3’) directions as were the PCR products yielded from the qPCR assay targeting the \textit{ompA} gene. Sequencing was performed by Elim Biopharmaceuticals, Inc. (Hayward, CA), SYBR Green was negligible and was not a problem when sequencing. The amount of DNA required was between 3-5 ng of DNA per sequence reaction.

Bioinformatics Analysis

Sequences were downloaded online from Elim Biopharmaceuticals, Inc. (Hayward, CA) and analyzed with CodonCode Aligner V. 3.71 (CodonCode Corporation, Dedham MA) with the following steps: load the sequences, view traces, analyze chromatograms and keep base calls with quality scores greater than 20, crop the ends of the sequence that are poor quality and export to a text file. The text files were created to aide in analysis using BLAST on the National Center for Biotechnology Information (NCBI) website (NCBI, 2012). Sequences were copied and a BLAST search was performed to find homologous sequences. If the sequence were 100% homologous it was considered the same species or genotype for purpose of identification. Sequences were then aligned using ClustalX (Clustal, Dublin, Ireland) using the cropped sequences by CodonCode Aligner (CodonCode Corporation, Dedham, MA).
Statistical Analysis

All demographic, epidemiological and collection information from sampled dogs was entered into an Excel spreadsheet and manipulated by Epi Info v7 (Centers for Disease Control, Atlanta, GA) for data analysis. Statistical significance for proportions and unadjusted odds ratios were determined by normal 95% confidence intervals (CI). For IFA-seropositive results with a numerator >10, Fisher’s exact test and gamma-distributed 95% CI were used to test for significance. A P value < 0.05 was considered statistically significant. If the denominator was <10 or if there were zero IFA-seropositive results, no statistical tests were conducted.

A univariate analysis of the following factors was conducted with seropositive status as the outcome: breed, age (when noted), sex (when noted), county, type of animal facility, and length of time in shelter (for shelter dogs). The 44 breeds represented were classified into eight groups based on American Kennel Club (AKC) classification (AKC, 2012): herding, hound, non-sporting, sporting, terrier, toy, working and mix breeds. A separate analysis for the Great Pyrenees breed versus all other breeds was conducted because samples from this breed came from a specific population of dogs (livestock guardian dogs) from the MSVC and it was known that these dogs were constantly in the fields, in contact with ticks.
RESULTS

Sample Collection

Samples were drawn from dogs during the months of June-September, 2010, when tick activity was at its peak (Goddard 1996) (Table 3). A total of 242 samples were collected from both counties. 135 samples were collected from Mendocino County: 114 from Mendocino County Shelter (MCS), 16 from Mendocino Animal Hospital (MAH), and five from Yokayo Veterinary Center (YVC). 107 samples were collected from Lake County: 76 from Lake County Shelter (LCS), 22 from Main Street Veterinary Clinic (MSVC), four from Wasson Memorial Veterinary clinic (WMVC), two from Animal Hospital of Lake County (AHLC) and three from Clearlake Veterinary (CV). MSVC submitted an interesting population of dogs: livestock guardian dogs (n = 14) which, according to the veterinarian, worked in the fields all day long in heavily infested tick areas. These dogs were well protected and usually had more than one tick preventative at all times including Biospot and a Preventic Collar. Among the 52 veterinary dogs, 30 (58%) were male, and the mean age was 5.4 years.

A total of 35 ticks were collected and removed from dogs in this project; 13 were identified as *Ixodes* spp., nine were identified as *D. occidentalis* and 13 were identified as *D. variabilis*. Only adult ticks were found and seven of them were engorged. All were collected in June and July. A total of 13 (5%) dogs had ticks on them at the time of blood draw. *Ixodes* spp. ticks were not likely to be positive for *R. rickettsia* (Foley et al. 2008, Lane et al. 2005), therefore only 22 *Dermacentor* species ticks were processed.
Table 3. Summary table of each collection site in Lake and Mendocino County that was used in this study. Data was compiled and separated by collection month. The seroprevalence in the month of July was significantly higher (39.62%) than any other collection month (p<0.05).

<table>
<thead>
<tr>
<th>Month</th>
<th>Collection Site</th>
<th># of Dogs</th>
<th>IFA Positive(^a)</th>
<th>% IFA Positive</th>
<th>95% CI (LB, UB)</th>
</tr>
</thead>
<tbody>
<tr>
<td>June</td>
<td>Mendocino County - Total</td>
<td>32</td>
<td>5</td>
<td>15.63%</td>
<td>(5.21%, 36.46%)</td>
</tr>
<tr>
<td></td>
<td>Mendocino County Shelter</td>
<td>29</td>
<td>5</td>
<td>17.24%</td>
<td>(5.74%, 36.46%)</td>
</tr>
<tr>
<td></td>
<td>Mendocino Animal Hospital</td>
<td>3</td>
<td>-----</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Lake County - Total</td>
<td>17</td>
<td>1</td>
<td>5.88%</td>
<td>(0.16%, 32.77%)</td>
</tr>
<tr>
<td></td>
<td>Lake County Shelter</td>
<td>13</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>AHLC</td>
<td>2</td>
<td>1</td>
<td>50.00%</td>
<td>-----</td>
</tr>
<tr>
<td></td>
<td>WMVC</td>
<td>2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>July</td>
<td>Mendocino County - Total</td>
<td>24</td>
<td>8</td>
<td>33.33%</td>
<td>(14.66%, 65.68%)</td>
</tr>
<tr>
<td></td>
<td>Mendocino County Shelter</td>
<td>15</td>
<td>4</td>
<td>26.67%</td>
<td>(7.49%, 68.28%)</td>
</tr>
<tr>
<td></td>
<td>Mendocino Animal Hospital</td>
<td>4</td>
<td>4</td>
<td>100.00%</td>
<td>-----</td>
</tr>
<tr>
<td></td>
<td>Yokayo Veterinary Center</td>
<td>5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Lake County - Total</td>
<td>29</td>
<td>13</td>
<td>44.83%</td>
<td>(26.73%, 62.93%)</td>
</tr>
<tr>
<td></td>
<td>Lake County Shelter</td>
<td>10</td>
<td>1</td>
<td>10.00%</td>
<td>(0.25%, 55.72%)</td>
</tr>
<tr>
<td></td>
<td>AHLC</td>
<td>17</td>
<td>12</td>
<td>70.59%</td>
<td>(48.93%, 92.25%)</td>
</tr>
<tr>
<td></td>
<td>WMVC</td>
<td>2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>August</td>
<td>Mendocino County - Total</td>
<td>21</td>
<td>2</td>
<td>9.52%</td>
<td>(1.21%, 34.40%)</td>
</tr>
<tr>
<td></td>
<td>Mendocino County Shelter</td>
<td>16</td>
<td>2</td>
<td>12.50%</td>
<td>(1.59%, 45.15%)</td>
</tr>
<tr>
<td></td>
<td>Mendocino Animal Hospital</td>
<td>5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Lake County - Total</td>
<td>22</td>
<td>2</td>
<td>9.09%</td>
<td>(1.16%, 32.84%)</td>
</tr>
<tr>
<td></td>
<td>Lake County Shelter</td>
<td>18</td>
<td>1</td>
<td>5.56%</td>
<td>(0.15%, 30.95%)</td>
</tr>
<tr>
<td></td>
<td>MSVC</td>
<td>2</td>
<td></td>
<td></td>
<td>-----</td>
</tr>
<tr>
<td></td>
<td>Clearlake Veterinary</td>
<td>2</td>
<td>1</td>
<td>50.00%</td>
<td>-----</td>
</tr>
<tr>
<td>September</td>
<td>Mendocino County - Total</td>
<td>54</td>
<td>10</td>
<td>18.52%</td>
<td>(8.16%, 38.88%)</td>
</tr>
<tr>
<td></td>
<td>Mendocino County Shelter</td>
<td>54</td>
<td>10</td>
<td>18.52%</td>
<td>(8.16%, 28.88%)</td>
</tr>
<tr>
<td></td>
<td>Lake County - Total</td>
<td>39</td>
<td>5</td>
<td>12.82%</td>
<td>(4.27%, 29.92%)</td>
</tr>
<tr>
<td></td>
<td>Lake County Shelter</td>
<td>35</td>
<td>5</td>
<td>14.29%</td>
<td>(4.76%, 33.34%)</td>
</tr>
<tr>
<td></td>
<td>MSVC</td>
<td>3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Clearlake Veterinary</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\(^a\) Titors ≥ 1:64 are considered positive for this study.
Serological Analysis of Canine Blood

Sixty-six samples were reactive at >1:32 and end point titer determined for these. 46 (19%) of these had reactive titers of 1:64 or greater and were considered positive. Sex, age, or use of tick control were not related to seropositivity (p>0.05). Among shelter dogs, days in shelter was not related to seropositivity. Dogs from veterinary practices were three times more likely to be seropositive than dogs from shelters (95% CI = 1.6 – 6.1). There was no difference in seropositivity in dogs from Mendocino and Lake Counties (18.5% and 19.6% respectively, p>0.05).

Working dogs had the highest percentage positive (47%) followed by sporting (29%), mix (25%), and terrier (14%) (Table 4). While no breed group was more likely to be seropositive than another group, the specific population of Great Pyrenees (livestock guardian dogs) which were known working herding dogs in heavily tick-infested areas (n =14) was 13.3 times more likely than all other dogs to be seropositive (95% CI = 3.96 – 44.9). Six of the dogs from the Mendocino County shelter were imported directly from Mexico; one of these dogs had a very high IFA titer of >1:256.

A significantly greater proportion of positive samples were detected in July 2010, compared to the other three collection months (36% IFA reactive in July 2010, 95% CI (23%, 49%); p < 0.001, x² = 12.1; (Table 3 and Figure 4). Comparing the other three collection months (June, August and September) to each other, there was no difference in IFA positive dogs (p>0.05, x² = 15.7).
Table 4. Summary table breakdown of canine breeds collected at Lake and Mendocino counties. A total of 242 breeds were collected with 46 (19%) of the dogs seropositive for *Rickettsia rickettsii* with a seroprevalence ranging from 7.14% to 47.22%. Two dogs from Mendocino County Shelter were PCR positive and were from the following breed groupings; herding and terrier.

<table>
<thead>
<tr>
<th>Breed Grouping</th>
<th>Total Collected</th>
<th>Total IFA positive</th>
<th>Seroprevalence %</th>
<th>Molecular Confirmation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Herding</td>
<td>66</td>
<td>8</td>
<td>12.12%</td>
<td>1 SFG <em>Rickettsia</em></td>
</tr>
<tr>
<td>Hound</td>
<td>11</td>
<td>0</td>
<td>0.00%</td>
<td></td>
</tr>
<tr>
<td>Mix</td>
<td>8</td>
<td>2</td>
<td>25.00%</td>
<td></td>
</tr>
<tr>
<td>Non-sporting</td>
<td>10</td>
<td>0</td>
<td>0.00%</td>
<td></td>
</tr>
<tr>
<td>Sporting</td>
<td>27</td>
<td>8</td>
<td>29.63%</td>
<td></td>
</tr>
<tr>
<td>Terrier</td>
<td>70</td>
<td>10</td>
<td>14.29%</td>
<td>1 <em>R. rickettsia</em></td>
</tr>
<tr>
<td>Toy</td>
<td>14</td>
<td>1</td>
<td>7.14%</td>
<td></td>
</tr>
<tr>
<td>Working</td>
<td>36</td>
<td>17</td>
<td>47.22%</td>
<td></td>
</tr>
</tbody>
</table>
Figure 4. Percent IFA reactive by month and year of collection. Samples were drawn from dogs during the summer months of June to September, 2010, when tick activity is at its peak.
Molecular Analysis of Canine Blood and Ticks

Clone Standard Preparation and Sensitivity of qPCR and Nested PCR

The *ompA* and *RR0155-rpmb* clones were successfully constructed and verified via gel electrophoresis after restriction enzyme digest. 10-fold serial dilution standards were successfully prepared from clones and processed in duplicate with each SYBR Green qPCR as a positive control and used to quantify positive samples with $R^2$ value = 0.998. The PCR efficiency was calculated using the slope of -3.23 produced from the clone standards and the formula, $E = -1 + 10^{(-1/slope)}$ yielding a qPCR reaction efficiency of 103%. In addition, a melting curve verified that the correct fragments were amplified and that non-specific amplification or primer dimers did not contaminated our results. The standards dissociation melting temperature (Tm) ranged from 76.2°C to 77.2°C with a mean of 76.9°C. The clone standards synthesized for the SYBR Green qPCR assay yielded a limit of detection of three copies and detected all concentrations down to 30 copies 100% of the time (16/16 reactions) and three copies 64% of the time (18/28 reactions) and was verified additionally via gel electrophoresis. Direct sequencing of the three and 30 concentrations verified that the desired target was amplified with 100% sequence identity with the *ompA* gene of SFG *Rickettsia* via BLAST analysis. The standards produced for the nested PCR assay were detected at all concentrations down to 32 copies 100% of the time (10/10) and 3.2 copies 60% of the time (6/10 reactions). A 10-fold dilution series was prepared and a gel was processed to show the sensitivity of this assay, 3.2 copies. Direct sequencing of the 3.2 and 32 concentrations verified that the
desired target was amplified with 100% sequence identity with the RR0155-rpmB gene of
*R. rickettsii* via BLAST analysis.

**Validation of Quality of DNA Extracted from Canine Blood Clots**

All 240 canines blot clots and 22 collected *Dermacentor* species tick samples were successfully extracted. The canine extracts were 100% amplified and verified by regular PCR targeting the canine gene, *GADPH*. Each sample was gel confirmed by producing a band of the appropriate size, 180 bp. All of the NTC’s tested did not produce a detectable band; the positive control produced a 180 bp size band, and each mock extraction also did not produce a band.

**Detection of SFG Rickettsia by SYBR Green qPCR**

The initial results of the SYBR Green qPCR produced 16 canine samples and 10 tick samples that yielded at least one reactive well with characteristic amplification curve and expected melting temperature. Upon duplicate analysis, of the 16 suspicious canine samples, two samples from MCS were *ompA* PCR positive and demonstrated reproducible results. Of the 10 suspicious tick samples, only two ticks demonstrated reproducible results.

Two of the total 22 (9%) processed ticks had detectable SFG rickettsial DNA; Tick-31 a female *D. occidentalis* tick collected off canine sample MSVC-04 on July 8, 2010, which was a 7 year old, male border collie, categorized as a herding dog. Tick-32 was a *D. variabilis* tick collected off canine sample AHLC-02 on June 29, 2010, which was a 7 year old, male beagle, categorized as a hound. Tick-31 produced a Ct value of 33.03 in qPCR, which corresponds to 15.45 copies of SFG *Rickettsia* DNA with a
melting temperature of 76.9°C (Figure 5). Tick-32 also produced a reproducible Ct value of 34.88 in qPCR which corresponds to 4.13 copies of SFG *Rickettsia* DNA with a melting temperature of 76.9°C (Figure 5). Of the 240 Canine samples tested only two (1.9%) were positive in qPCR. Sample MCS-25 was a pit-bull mix (terrier) that produced a Ct value of 33.92 in qPCR which corresponds to 8.22 copies of SFG *Rickettsia* DNA with a melting temperature of 76.9°C (Figure 5). MCS-27 was a German Sheppard mix (herding) that produced a reproducible Ct value of 35.64 in qPCR that corresponds to 2.42 copies of SFG rickettsial DNA with a melting temperature of 76.9°C (Figure 5). Neither MCS-25 nor MCS-27 was seropositive by IFA. The size of each PCR amplicon was confirmed via gel electrophoresis.

*Rickettsia* Species Identification by Nested PCR

Each “suspicious” or “preliminary positive” canine sample (n=16) and tick samples (n=10) were processed in duplicate by this nested PCR assay. Tick samples (Tick-31 and Tick-32) as well as canine samples (MCS-25 and MCS-27) produced a 290 bp size band indicating that the sample contained a SFG *Rickettsia*. The molecular results are summarized in table 5.

Bioinformatics Analysis and Sequence Confirmation

The *ompA* and *RR0155-rpmB* PCR amplicons were purified and their sizes were confirmed by gel electrophoresis. The sequence from the dog sample MCS-25 (nt = 109) *ompA* amplicon shared 99% nucleotide sequence identity with *Rickettsia* str. 364D (CP003308.1) and *R. rickettsii* str. Sheila Smith (CP000848.1) (Table 6). Based on the alignment there was a deletion of the nucleotide A at position 16 (Figure 6). The
A

Delta R in vs Cycle

1.0*10^{-11}

3.0 x 10^2

3.0 x 10^1

Tick-32

Tick-31

MCS-25

5.0 Copies

MCS-27

Cycle Number

Delta R in

B

Dissociation Curve

0.35

0.30

0.25

0.20

0.15

0.10

0.05

Temperatures

-0.15

-0.10

-0.05

0.00

0.05

0.10

0.15

0.20

0.25

0.30

0.35

63

64

65

66

67

68

69

70

71

72

73

74

75

76

77

78

79

80

81

82

83

84

85

86

87

88

89

90

91

92

93

94

95

76.9°C
Figure 5. An amplification plot and dissociation curve created during the SYBR Green qPCR assay targeting the \textit{ompA} gene. \textbf{a.} An amplification plot showing standard curve replicates; 3.0 \times 10^2, 3.0 \times 10^1, and 3.0 copies for reference, as well as the positive samples in this study (all other standards produced a similar Tm; MCS-25 (8.22 copies), MCS-27 (2.42 copies), Tick-31 (15.45 copies) and Tick 32 (4.13 copies). \textbf{b.} A dissociation curve graph showing dissociation peaks of each sample; 3.0 \times 10^2 (76.9°C), 3.0 \times 10^1 (77.2°C), 3.0 copies (76.9°C), MCS-25 (76.9°C), MCS-27 (77.2°C), Tick 31 (76.9°C), and Tick 32 (76.9°C); each sample only exhibited one PCR product.
Table 5. Molecular results of Lake and Mendocino County dog samples. A total of 240 blood samples were collected and tested with the PCR assay targeting the *ompA* gene and the intergenic region *RR0155-rpmB*. Only two dogs were PCR positive and both were collected from Mendocino County Shelter.

<table>
<thead>
<tr>
<th>Submitter</th>
<th>Total Dogs Collected</th>
<th>Preliminary PCR <em>ompA</em> (+)*</th>
<th>Confirmation of PCR <em>ompA</em> (+)</th>
<th>PCR of <em>RR0155-rpmB</em></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Lake County</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lake County Shelter</td>
<td>76</td>
<td>9</td>
<td>0/76</td>
<td>0/9</td>
</tr>
<tr>
<td>Main Street Veterinary Clinic</td>
<td>22</td>
<td>3</td>
<td>0/22</td>
<td>0/3</td>
</tr>
<tr>
<td>Wasson Memorial Veterinary Clinic</td>
<td>4</td>
<td>0</td>
<td>0/4</td>
<td>Not Tested</td>
</tr>
<tr>
<td>Animal Hospital of Lake County</td>
<td>2</td>
<td>0</td>
<td>0/1</td>
<td>Not Tested</td>
</tr>
<tr>
<td>Clearlake Veterinary</td>
<td>3</td>
<td>1</td>
<td>0/2</td>
<td>0/1</td>
</tr>
<tr>
<td><strong>TOTAL</strong></td>
<td>107</td>
<td>13</td>
<td>0/105 (0%)</td>
<td>0/13 (0%)</td>
</tr>
<tr>
<td><strong>Mendocino County</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mendocino County Shelter</td>
<td>114</td>
<td>3</td>
<td>2/114 (2%)</td>
<td>2/3 (66%)</td>
</tr>
<tr>
<td>Yokayo Veterinary Center</td>
<td>5</td>
<td>0</td>
<td>0/5</td>
<td>Not Tested</td>
</tr>
<tr>
<td>Mendocino Animal Hospital</td>
<td>16</td>
<td>0</td>
<td>0/16</td>
<td>Not Tested</td>
</tr>
<tr>
<td><strong>TOTAL</strong></td>
<td>135</td>
<td>3</td>
<td>2/135 (1.5%)</td>
<td>2/3 (66%)</td>
</tr>
</tbody>
</table>

*Note.* IFA, Immunofluorescence assay; IgG, immunoglobulin G

* Produced at least one characteristic amplification curve and appropriate Tm and were considered suspicious or preliminary positive.
Table 6. BLAST analysis of the positive sequences obtained in this study targeting the \textit{ompA} gene using SYBR Green qPCR; MCS-25, MCS-27, Tick-31 and Tick-32. The sequences were compared to 14 common SFG \textit{Rickettsia} species. Only top two matches are shown.

<table>
<thead>
<tr>
<th>Sample</th>
<th>GenBank Name</th>
<th>Accession #</th>
<th>E Value</th>
<th>Identities</th>
<th>Gaps</th>
</tr>
</thead>
</table>
| **MCS-25** bp=109
  \textit{Rickettsia} str. 364D | CP003308.1 | 6.00E-52    | 109/110 (99%) | 1/110               |
  \textit{R. rickettsii} str. S.S. | CP000848.1 | 6.00E-52    | 109/110 (99%) | 1/110               |
| **MCS-27** bp=108
  \textit{R. conorii} | AE006914.1 | 5.00E-53    | 108/108 (100%) | 0/108               |
  \textit{R. africae} | CP001612.1 | 5.00E-53    | 108/108 (100%) | 0/108               |
| **Tick-31** bp=111
  \textit{Rickettsia} str. 364D | CP003308.1 | 7.00E-52    | 109/110 (99%) | 1/110               |
  \textit{R. rickettsii} str. S. S. | CP000848.1 | 6.00E-52    | 109/110 (99%) | 1/110               |
| **Tick-32** bp=109
  \textit{Rickettsia} str. 364D | CP003308.1 | 1.00E-53    | 109/109 (100%) | 0/109               |
  \textit{R. rickettsii} str. S. S. | CP000848.1 | 1.00E-53    | 109/109 (100%) | 0/109               |
Figure 6. ClustalX alignment file of cropped and edited sequences obtained from the SYBR Green qPCR assay targeting the \(ompA\) gene. File includes sequences from Tick-31, Tick-32, MCS-25, MCS-27, known copies of cloned DNA, 30 copies and 3.0 copies, as well as 14 known SFG \textit{rickettsia} including, \textit{Rickettsia philipi}, strain 364D (\textit{Rickettsia philipi} 364D). Base pair differences are highlighted above.
Table 1: ClustalX alignment file of cropped and edited sequences obtained from the SYBR Green qPCR assay targeting the *ompA* gene. File includes sequences from Tick-31, Tick 32, MCS-25, MCS-27, known copies of cloned DNA, 30 copies and 3.0 copies, as well as 14 known SFG *rickettsia* including, *Rickettsia philipi*, strain 364D (*Rickettsia 364D*. Base pair differences are highlighted above.

<table>
<thead>
<tr>
<th>Sequence</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>MCS-25</td>
<td>AAAAGCTTATTTTCTAAAACCTGTTATATAATTACGCAGG</td>
</tr>
<tr>
<td>Tick-31</td>
<td>AAAAGCTTATTTTCTAAAACCTGTTATATAATTACGCAGG</td>
</tr>
<tr>
<td>30 Copies</td>
<td>AAAAGCTTATTTTCTAAAACCTGTTATATAATTACGCAGG</td>
</tr>
<tr>
<td>3 Copies</td>
<td>AAAAGCTTATTTTCTAAAACCTGTTATATAATTACGCAGG</td>
</tr>
<tr>
<td>R. sibirica</td>
<td>AAAAGCTTATTTTCTAAAACCTGTTATATAATTACGCAGG</td>
</tr>
<tr>
<td>R. parkeri</td>
<td>AAAAGCTTATTTTCTAAAACCTGTTATATAATTACGCAGG</td>
</tr>
<tr>
<td>R. honei</td>
<td>AAAAGCTTATTTTCTAAAACCTGTTATATAATTACGCAGG</td>
</tr>
<tr>
<td>R. africae</td>
<td>AAAAGCTTATTTTCTAAAACCTGTTATATAATTACGCAGG</td>
</tr>
<tr>
<td>R. conori</td>
<td>AAAAGCTTATTTTCTAAAACCTGTTATATAATTACGCAGG</td>
</tr>
<tr>
<td>R. slovaca</td>
<td>AAAAGCTTATTTTCTAAAACCTGTTATATAATTACGCAGG</td>
</tr>
<tr>
<td>R. rhipicephali</td>
<td>AAAAGCTTATTTTCTAAAACCTGTTATATAATTACGCAGG</td>
</tr>
<tr>
<td>R. massiliae</td>
<td>AAAAGCTTATTTTCTAAAACCTGTTATATAATTACGCAGG</td>
</tr>
<tr>
<td>R. slovaca</td>
<td>AAAAGCTTATTTTCTAAAACCTGTTATATAATTACGCAGG</td>
</tr>
<tr>
<td>R. philipi, 364D</td>
<td>AAAAGCTTATTTTCTAAAACCTGTTATATAATTACGCAGG</td>
</tr>
<tr>
<td>R. rickettsii</td>
<td>AAAAGCTTATTTTCTAAAACCTGTTATATAATTACGCAGG</td>
</tr>
<tr>
<td>MCS-27</td>
<td>------------------------------------------</td>
</tr>
<tr>
<td>Tick-32</td>
<td>------------------------------------------</td>
</tr>
<tr>
<td>R. peacockii</td>
<td>AAAAGCTTATTTTCTAAAACCTGTTATATAATTACGCAGG</td>
</tr>
</tbody>
</table>

Figure 6. ClustalX alignment file of cropped and edited sequences obtained from the SYBR Green qPCR assay targeting the *ompA* gene. File includes sequences from Tick-31, Tick 32, MCS-25, MCS-27, known copies of cloned DNA, 30 copies and 3.0 copies, as well as 14 known SFG *rickettsia* including, *Rickettsia philipi*, strain 364D (*Rickettsia 364D*. Base pair differences are highlighted above.
sequence from the other dog sample MCS-27 (nt = 108) ompA amplicon shared 100% nucleotide sequence identity with \textit{R. conorii} (AE006914.1) and \textit{R. africae} (CP001612.1) (Table 6); however, the sequence quality was poor with low confidence. The sequence of \textit{ompA} from the \textit{D. occidentalis} tick sample Tick-31 (nt = 111) shared 99% nucleotide sequence identity with \textit{ompA} from \textit{Rickettsia} str. 364D (CP003308.1) and \textit{R. rickettsii} str. Sheila Smith (CP000848.1). Based on the alignment, at position two there was a substitution of a C for an A and at position 16 the nucleotide A was deleted (Figure 6). The sequence from the last positive \textit{D. variabilis} tick sample Tick-32 (nt = 109) \textit{ompA} amplicon shared 100% nucleotide sequence identity with \textit{Rickettsia} str. 364D (CP003308.1) and \textit{R. rickettsii} str. Sheila Smith (CP000848.1). An alignment file was created with each positive sample and 14 other SFG \textit{Rickettsia} species to show similarities and conserved regions (Figure 6).

The nested PCR \textit{RR0155-rpmB} positive samples: MCS-25 (Pit-bull mix), MCS-27 (German sheppard mix), Tick-31 (\textit{D. occidentalis}) and Tick-32 (\textit{D. variabilis}) provided quality sequences to analyze. The sequence from the dog sample MCS-25 (nt = 232) \textit{RR0155-rpmB} amplicon shared 100% nucleotide sequence identity with \textit{R. rickettsii} str. Sheila Smith (CP000848.1) (Table 7), the next closest relative was \textit{R. peacockii} (96%) (CP003308.1). The sequence from the other dog sample MCS-27 (nt = 218) \textit{RR0155-rpmB} amplicon yielded a poor sequence which shared 90% nucleotide sequence identity with \textit{R. rickettsii} str. Sheila Smith (CP000848.1), the next closest relative was \textit{R. peacockii} (86%) (CP003308.1). Based on the alignment there were several nucleotide substitutions; position 6 an A to a G, position 16 a G to an A, position 18 a T to a G,
Table 7. BLAST analysis of the positive sequences obtained in this study targeting the \textit{RR0155-rpmB} gene using nested PCR; MCS-25, MCS-27, Tick-31 and Tick-32. The sequences were compared to 9 common SFG \textit{Rickettsia} that had complete genomes sequenced. Only top three matches are shown.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Gene bank Name</th>
<th>Accession #</th>
<th>E Value</th>
<th>Identities (%)</th>
<th>Gaps</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>MCS-25</strong> bp=232</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>\textit{R. rickettsii} str. S. S.</td>
<td>CP000848.1</td>
<td>1.00E-118</td>
<td>232/232 (100%)</td>
<td>0/232</td>
<td></td>
</tr>
<tr>
<td>\textit{R. peacockii}</td>
<td>CP001227.1</td>
<td>2.00E-110</td>
<td>230/239 (96%)</td>
<td>7/239</td>
<td></td>
</tr>
<tr>
<td>\textit{Rickettsia} str. 364D</td>
<td>CP003308.1</td>
<td>4.00E-106</td>
<td>225/236 (95%)</td>
<td>7/236</td>
<td></td>
</tr>
<tr>
<td><strong>MCS-27</strong> bp=218</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>\textit{R. rickettsii} str. S. S.</td>
<td>CP000848.1</td>
<td>2.00E-91</td>
<td>205/219 (94%)</td>
<td>1/219</td>
<td></td>
</tr>
<tr>
<td>\textit{R. peacockii}</td>
<td>CP001227.1</td>
<td>2.00E-83</td>
<td>203/226 (90%)</td>
<td>8/226</td>
<td></td>
</tr>
<tr>
<td>\textit{Rickettsia} str. 364D</td>
<td>CP003308.1</td>
<td>2.00E-78</td>
<td>200/226 (88%)</td>
<td>10/226</td>
<td></td>
</tr>
<tr>
<td><strong>Tick-31</strong> bp=228</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>\textit{R. rickettsii} str. S. S.</td>
<td>CP000848.1</td>
<td>2.00E-116</td>
<td>228/228 (100%)</td>
<td>0/228</td>
<td></td>
</tr>
<tr>
<td>\textit{R. peacockii}</td>
<td>CP001227.1</td>
<td>3.00E-108</td>
<td>226/235 (96%)</td>
<td>7/235</td>
<td></td>
</tr>
<tr>
<td>\textit{Rickettsia} str. 364D</td>
<td>CP003308.1</td>
<td>2.00E-102</td>
<td>222/235 (94%)</td>
<td>9/235</td>
<td></td>
</tr>
<tr>
<td><strong>Tick-32</strong> bp=185</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>\textit{R. rickettsii} str. S. S.</td>
<td>CP000848.1</td>
<td>2.00E-90</td>
<td>183/185 (99%)</td>
<td>0/185</td>
<td></td>
</tr>
<tr>
<td>\textit{R. peacockii}</td>
<td>CP001227.1</td>
<td>2.00E-82</td>
<td>181/192 (94%)</td>
<td>7/192</td>
<td></td>
</tr>
<tr>
<td>\textit{Rickettsia} str. 364D</td>
<td>CP003308.1</td>
<td>1.00E-78</td>
<td>177/190 (93%)</td>
<td>7/190</td>
<td></td>
</tr>
</tbody>
</table>
position 19 an A to a C, position 22 a T to a C, position 24 a T to a C, position 25 a T to a C, position 37 a T to a G, position 39 a T to a G, position 62 an A to a G, position 63 a T to a G, position 64 an A to a G and position 92 an A to a G, which made this sequence different from *R. rickettsii* str. Sheila Smith (CP000848.1). Based on alignment at position 57 there was a seven nucleotide deletion when compared to *Rickettsia* str. 364D (CP003308.1) (Figure 7). The sequence from the tick sample Tick-31 (nt = 228) RR0155-rpmB amplicon shared 100% nucleotide sequence identity with *R. rickettsii* str. Sheila Smith (CP000848.1) (Table 7), the next closest relative was *R. peacockii* (96%) (CP003308.1). The sequence from the last positive tick sample Tick-32 (nt = 185) RR0155-rpmB amplicon shared 99% nucleotide sequence identity with *R. rickettsii* str. Sheila Smith (CP000848.1), the next closest relative was *R. peacockii* (94%) (CP003308.1) (Table 7). Based on the alignment file there were two nucleotide substitutions at positions 97 and 145 where an A was substituted for a G (Figure 7). An alignment file was created with each positive sample and 9 other SFG *Rickettsia* species to show similarities and conserved regions (Figure 7). Only SFG *Rickettsia* that have whole genome sequences deposited in GenBank were used for analysis.

Taking all the data together Tick-31 showed 99% homology with *Rickettsia* 364D and *R. rickettsii* in the ompA region but the intergenic region RR0155-rpmB showed 100% homology with *R. rickettsii* indicating it was *R. rickettsii*. Tick-32 also showed 99% homology with *Rickettsia* 364D and *R. rickettsii* in the ompA region and 100% homology in the intergenic region RR0155-rpmB with *R. rickettsii* indicating it was *R.
Figure 7. ClustalX alignment file of cropped and edited sequences obtained from the nested PCR assay targeting the *RR0155-rpmB* gene. File includes sequences from Tick-31, Tick-32, MCS-25, MCS-27, known copies of cloned DNA, 30 copies and 3.0 copies, as well as 9 known SFG *rickettsia* including, *Rickettsia philipi*, strain 364D. Base pair differences are highlighted above.
Figure 7. ClustalX alignment file of cropped and edited sequences obtained from the nested PCR assay targeting the RR0155-rpmB gene. File includes sequences from Tick-31, Tick-32, MCS-25, MCS-27, known copies of cloned DNA, 30 copies and 3.0 copies, as well as 15 known SFG *rickettsia* including, *Rickettsia philipi*, strain 364D. Base pair differences are highlighted above.
*rickettsii*. Canine sample MCS-25 showed 99% homology with *Rickettsia* 364D and *R. rickettsii* in the *ompA* region but the intergenic region RR0155-rpmB showed 100% homology with *R. rickettsii* indicating it was *R. rickettsii*. MCS-27 showed 100% homology with *R. conorii* and *R. africae* in the *ompA* region but the intergenic region RR0155-rpmB showed 94% homology with *R. rickettsii*, indicating that SFG *Rickettsia* was detected. Table 8 summarizes this molecular data.
Table 8. Molecular summary data table of each positive sample in this study. Two ticks and one dog sample were positive for *R. rickettsii* (Tick-31, Tick-32 and MCS-25) while one dog sample was positive for SFG *rickettsia* (MCS-27).

<table>
<thead>
<tr>
<th>Sample</th>
<th>ompA Copy #</th>
<th>ompA qPCR</th>
<th>RR0155-rpmB PCR</th>
<th>Final Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>MCS-25</td>
<td>8.22</td>
<td><em>Rickettsia</em> 364D (99%)</td>
<td><em>R. rickettsii</em></td>
<td><em>R. rickettsii</em></td>
</tr>
<tr>
<td>(Terrier)</td>
<td></td>
<td><em>R. rickettsii</em> (99%)</td>
<td>(100%)</td>
<td></td>
</tr>
<tr>
<td>MCS-27</td>
<td>2.42</td>
<td><em>R. conorii</em> (100%)</td>
<td><em>R. rickettsii</em></td>
<td>SFG <em>Rickettsia</em> most closely related to <em>R. rickettsii</em></td>
</tr>
<tr>
<td>(Herding)</td>
<td></td>
<td><em>R. africae</em> (100%)</td>
<td>(94%)</td>
<td></td>
</tr>
<tr>
<td>Tick-31</td>
<td>15.45</td>
<td><em>Rickettsia</em> 364D (99%)</td>
<td><em>R. rickettsii</em></td>
<td><em>R. rickettsii</em></td>
</tr>
<tr>
<td>(D. variabilis)</td>
<td></td>
<td><em>R. rickettsii</em> (99%)</td>
<td>(100%)</td>
<td></td>
</tr>
<tr>
<td>Tick-32</td>
<td>4.13</td>
<td><em>Rickettsia</em> 364D (100%)</td>
<td><em>R. rickettsii</em></td>
<td><em>R. rickettsii</em></td>
</tr>
<tr>
<td>(D. occidentalis)</td>
<td></td>
<td><em>R. rickettsii</em> (100%)</td>
<td>(99%)</td>
<td></td>
</tr>
</tbody>
</table>
DISCUSSION

In this study we looked for SFG *Rickettsia* in canines and found principally evidence for RMSF, not *Rickettsia* 364D. However, we provide a dependable large scale canine seroprevalence study reporting a seropositivity of 19% to *R. rickettsii* which is comparable to other seroprevalence studies: 16 out of 364 (4.4%) in Brazil, 36 out of 153 (23%) in Croatia, 216 out of 481 (45%) in France, 34 out of 58 (58.6%) in Spain and 150 out of 184 (82%) in Zimbabwe (Fortes et al 2010, Kelly and Mason 1991, Herrero et al. 1992, Punda-Polic et al. 1995, Raoult et al. 1985). It is also similar to other areas in southwest United States considered “endemic” for RMSF (McQuiston et al. 2011). These data indicate that canines are naturally exposed to SFG *Rickettsia* in northern California. This is the first large scale survey of seroprevalence and prevalence in canines in California which helps us better understand the epidemiology of SFG *Rickettsia* in northern California.

Overall, there was no difference in the prevalence of IFA-reactive dogs between Lake and Mendocino Counties; p>0.05. However, differences between IFA prevalence detected in Lake vs. Mendocino shelters and veterinary clinics included in this study did approach 95% statistical significance. Seropositivity did not relate to breed but more related to breed lifestyle as shown by the livestock guardian dogs (71%), which are breeds of dogs that have been bred for centuries to protect livestock from predators (Livestock Guardian Dogs, 2011). This population of dogs in our study spend the majority of their time in pastures containing blades of grass that ticks often sit and quest;
increasing their risk of becoming infested by ticks and therefore increasing their risk for acquiring common tick pathogens like SFG *Rickettsia* (Crosbie and Boyce 1998, Wright *et al.* 2000). Despite the use of two tick and flea preventative measures, Biospot and a Preventic collar, this population of dogs had a high seroprevalence, which is not unexpected.

The IFA targeted the canine IgG antibody which indicates that these dogs have been exposed to *R. rickettsii* or some other SFG *Rickettsia*. Since many known SFG *Rickettsia* cross react with each other we can only conclude that these dogs have been exposed to SFG *Rickettsia* in the past, not necessarily indicating an active infection. The known SFG *Rickettsia* to cross react with *R. rickettsii* are: *R. massiliae, R. rhipicephali,* and *Rickettsia* 364D (Beeler *et al.* 2011, Scola and Raoult 1997, Wodeka *et al.* 2008) and are the most likely candidates responsible for the seroconversion. For example, a recent study investigating a *Rh. sanguineus* infestation reveals the weakness of serology: a dog was reported as seropositive for SFG *Rickettsia*, however the serum samples contained antibodies reactive with *R. massiliae, R. rhipicephali, R. rickettsii* and *Rickettsia* 364D. (Beeler *et al.* 2011). The group also collected *Rh. sanguineus* ticks associated with the dog and on the property and found only detectable *R. massiliae* DNA by regular PCR, indicating that it was the most likely SFG *Rickettsia* responsible for the seropositivity (Beeler *et al.* 2011). Interestingly, they did not detect any SFG rickettsial DNA in any of the dog samples; an apparent common phenomenon in canine seroprevalence studies (Beeler *et al.* 2011).
IFA is more sensitive to exposure than qPCR (Scola and Raoult 1997) and determines whether the canine has had a previous immune response. The IgG antibody response was targeted because it persists longer and identifies canines that have been exposed to *R. rickettsii* or other SFG *Rickettsia* (Breitschwerdt *et al.* 1990). In a normal immune response *IgM* will come up first but will quickly dissipate as *IgG* antibodies rise and remain for a long period of time. According to previous studies of experimentally and naturally infected dogs, *IgG* antibodies became detectable between post infection (PI) days 22 and 28, peaked by PI day 42, and decreased gradually through PI day 130 (Breitschwerdt *et al.* 1998). A more recent study of *R. rickettsii* experimentally infected dogs showed that *IgG* antibodies can persist to PI day 240 and slowly declined by PI day 1024 (Breitschwerdt *et al.* 1990). Determining endpoints of reactive samples and qPCR testing provided more insight on active infection (Breitschwerdt *et al.* 1990).

The 240 blood clots analyzed for SFG rickettsial DNA by PCR indicated presence of SFG rickettsial DNA in only two (0.8%) canine samples, providing support that titers reflect exposure to RMSF rather than active infections. Very few large scale studies have been done in the United States to describe prevalence of *R. rickettsii* DNA in canines; however, a study done in Japan reports that 2.4% of their samples had detectable SFG Rickettsial DNA (Satoh *et al.* 2002). Thus, it appears that while dogs may be at risk for exposure to SFG *Rickettsia*, clinical disease prompting testing is uncommon and actual infection is therefore very difficult to detect.

Mendocino County Shelter produced the only two canine confirmed SFG rickettsial infections by PCR. MCS-25 (8.22 copies), a pit-bull mix from Covello, was
drawn at the MCS on June 4, 2010 and had no known flea and tick prevention. The IFA for this dog was negative, which is not unprecedented because we targeted the IgG antibody which is not present in the early stages of an acute infection. The ompA gene showed 99% sequence identity to both Rickettsia str. 364D and R. rickettsii and the nested PCR was necessary for species confirmation. The nested PCR assay targeting the RR0155-rpmB gene confirmed that the SFG Rickettsia detected was a R. rickettsii strain showing 100% identity to sequences of R. rickettsii.

The other PCR positive sample was MCS-27 (2.42 copies), which was collected at the MCS on June 4, 2010 from a German shepherd mix from the Hopland Indian Reservation. The serology on this dog was also negative. The SYBR Green qPCR assay detected SFG Rickettsial DNA at low levels and was also confirmed by gel electrophoresis. Despite the low level of DNA in the sample, the nested PCR assay also produced a band of the desired size. In this case sequence data showed 94% identity to R. rickettsii, indicating that this sample was a close relative of R. rickettsii. We provide molecular evidence that MCS-27 is infected with low levels of SFG Rickettsia DNA even though the quality of the DNA sequence was poor.

In this study neither of the SFG Rickettsia DNA positive dogs showed clinical signs consistent with a naturally occurring RMSF infection. Several of the dogs presented with fever or lethargy, however, presence of clinical signs were not associated with seropositivity or being PCR positive. Therefore low DNA recovery may be due to the fact that we were testing dogs at a time well after any rickettsemia and the IgG antibody simply detected past exposure (Figure 1).
Often presence and prevalence of ticks on domestic dogs are used as a potential risk factor for RMSF (Fritz et al. 2011). *Dermacentor* spp. ticks would be the likely vector to both dogs and humans in this environment. We collected 32 ticks that were directly associated with the dogs included in this study. Only 2 (9.1%) of the tested *Dermacentor* spp. ticks were confirmed positive by molecular analysis, similar to other previously reported prevalence rates of SFG *Rickettsia* in California: 1.6% from 62 collected *Rh. sanguineus* ticks (Wikswo et al. 2007), 10.3% from 800 collected *D. occidentalis* ticks (Philip et al. 1981) and *Rickettsia* 364D in California of 7.7%. Even though the common vectors of RMSF in the United States include: the American dog tick (*D. variabilis*), Rocky Mountain wood tick (*D. andersoni*), and the brown dog tick (*Rh. sanguineus*), *R. rickettsii* has only been molecularly detected in California in *Rh. sanguineus* ticks in Baja California (Eremeeva et al. 2011) and Riverside County (Wikswo et al. 2007) and in *D. occidentalis* in southern California (Wikswo et al. 2008). Despite the low prevalence of molecular detection, 2 out of 22 ticks (8.7%; Gamma Distribution Confidence Interval 1.1%, 31.4%), the tick results are important to our understanding of *R. rickettsii* ecology.

Tick-31 (15.45 copies), a female *D. occidentalis*, was eventually identified as infected with *R. rickettsii* and was collected from dog MSVC-04. MSVC-04 (Border collie) was determined to be serologically positive with a weak titer of 1:64 but was negative by qPCR, indicating that this dog simply had been exposed in the past and did not have an active infection. The tick was attached to the dog but clearly not the current source of the dog’s exposure since she was not engorged, which indicates that it may not
have had sufficient time to transmit the bacterium to the canine (Piranda et al. 2011), or transmitted but not amplified.

Tick-32 (4.13 copies), collected from canine AHLC-02 on June 29, 2010, was a *D. variabilis* adult non-engorged tick, molecularly confirmed to be infected with *R. rickettsii* via SYBR Green qPCR and intergenic spacer PCR. The sequence data was poor compared to the other sequences which may be due to the low amount of detectable DNA in the sample (4.13 copies). The canine AHLC-02 (Beagle) was both serologically and molecularly negative for SFG *Rickettsia*. Given that this non-engorged tick was removed at the time of blood draw, it is unlikely that it transmitted the bacterium to the dog.

We have detected only the third and fourth ticks positive for *R. rickettsii* in California and the first ever detection of *R. rickettsii* in *D. variabilis* ticks in California (though it is a known tick vector of *R. rickettsii* in other endemic areas of the United States), thereby adding to the known *R. rickettsii* positive tick species in California (Wikswo et al. 2007). Additionally, we add to growing evidence that *D. occidentalis* is an important vector of RMSF in California (Wikswo et al. 2008).

Our detection of *R. rickettsii* and SFG *Rickettsia* by qPCR in two canines drawn from Mendocino County confirms that canines can serve as sentinels for *R. rickettsii* transmission in northern California. Their role as propagator of the agent is less clear. In vertebrate hosts, *R. rickettsii* causes acute infection lasting for only a few days or weeks, with no persistent maintenance of the agent (Burgdorfer 1988). Thus, vertebrate hosts cannot be considered reservoirs of *R. rickettsia* in nature (Labruna 2009). For dogs to serve as a significant amplifier of *R. rickettsii* in nature, they would have to be fairly
consistently infested with ticks. This scenario occurred in the RMSF outbreak in Arizona where *Rh. sanguineus* ticks heavily infested dogs and the environment where they and their owners lived. However, in this environment, dogs are the more likely to be opportunistically fed upon by *Dermacentor* spp. ticks. Therefore, it is likely that canines act as sentinel hosts for *R. rickettsii* in northern California (Elchos and Goddard 2003). However, caution should be taken in this area to avoid infestations with *Rh. sanguineus* in the peridomestic environment because infected dog could switch from being a sentinel to being a reservoir.

This project started out as a result of a recent increased awareness of the newly recognized pathogenic *Rickettsia* 364D in northern California (Shapiro *et al.* 2010). *R. rickettsii* has been confirmed to cause RMSF in dogs, putting humans at greater risk for infection (Elchos and Goddard 2003, Kelly 1992, Weiser and Greene 1989). According to Sharon Messenger of the Viral and Rickettsial Diseases Laboratory in Richmond CA, SFG *Rickettsia* are becoming an increasing risk for California’s population and that better diagnostic tests by Public Health need to be developed for increased rapid response to these pathogens (Messenger, 2011). Often delayed diagnosis and treatment can result in death up to 25% of the time in humans due to RMSF (Centers for Disease Control 2011). In the past, rare occurrence of RMSF in California has lead to delayed diagnosis of a rather classical presentation of RMSF (Everett and Rham 1970).

Usually RMSF is diagnosed by a combination of methods such as seroconversion, molecular analysis and resolution after doxycycline therapy (Breitschwerdt *et al.* 1999, Labruna *et al.* 2009). Often diagnosis is difficult due to low circulating detectable DNA
in sample. Moreover, it is very difficult to obtain a PCR positive sample mainly due to
the acute nature of the disease kinetics of the serological response (Piranda et al. 2008).
Though differentiation of SFG Rickettsia is of little clinical value and speciation is very
labor intensive and expensive; it is useful in epidemiological studies to understand the
origin of infection and the potential risk posed to humans. For these kinds of studies,
there is great need for the identification of a single qPCR gene target that is capable of
identifying all SFG Rickettsia and also differentiates among SFG isolates.

RMSF and SFG rickettsial infections remain of public health concern as five
additional Rickettsia 364D human cases were reported from northern California in
humans in 2011 (personal communication Anne Kjemtrupt, CDPH biologist). R.
rickettsii ecology in California remains poorly characterized but this study verified that
D. variabilis and D. occidentalis ticks may carry the organism. R. rickettsii has expanded
its distribution and has been reported in North, Central and South America where
different tick species serve as vectors (Chapman et al. 2006, Labruna et al. 2009). This
expanded distribution of R. rickettsia has raised awareness among public health officials
that California residents may be at greater risk than previously thought for SFG rickettsial
infections.

It may be because of increased surveillance and better testing that these bacteria
have recently become more apparent (Messenger, 2011). For example, there was a recent
outbreak of RMSF on the Western Mexico and US Border Region from 2008-2010 that
had more than 118 cases and at least nine deaths associated with an outbreak of RMSF
(Arredondo-Jimenez 2010). This case was highlighted by: suitable environmental
conditions, poverty and migration, careless dog ownership and abundant street dogs, infestation of *Rh. sanguineus* ticks, poor sanitation and waste disposal which created the perfect environment for a sustained outbreak that the local community was not prepared to handle (Arredondo-Jimenez 2010). The need to control the stray dog population in Mexico is especially important in the USA border region as the potential risk exists for transfer of infected vector ticks to the USA region via stray dogs (Fritz *et al.* 2011). Therefore, it is important to know that dogs can be infected in this area because epidemiology could quickly change if *Rh. sanguineus* infestation occurred among infected dogs. This tick persists happily near dwellings and can bite people and could shift the role of the dog from sentinel to reservoir (Eremeeva *et al.* 2011, Sanchez *et al.* 2009).

Our finding of SFG *Rickettsia* in *Dermacentor* spp. ticks is important; however tick identification was done using morphological keys that did not emphasize the spiracular plate morphology or goblet cell morphology which is an important differentiating feature of *Dermacentor* ticks (Furman and Loomis 1984). This could have led to mis-identification of *D. occidentalis* as *D. andersoni* or *D. variabilis* as *D. andersoni*; the obvious “measled” appearance of *D. occidentalis* facilitates its clear differentiation between *D. occidentalis* and *D. variabilis*. The difference between *D. variabilis* and *D. andersoni* (another potential vector for RMSF) is less obvious without spiracular plate evaluation; however, *D. andersoni* has been collected primarily in the eastern part of the state, from Modoc County down to the eastern range of the northern Sierras. There are no records of *D. andersoni* in north coastal California Counties.
(Furman and Loomis, CDPH tick database) to author’s knowledge, so identification of *D. variabilis* is most likely correct. Further PCR analysis will be performed on remaining DNA to verify species.

A point should be made about the source of the samples in this study as 190 (78%) of the samples were collected from shelters. Shelter dogs are often used in studies for ease of use and because they are a great source for a large number of samples in a short amount of time (Dantas-Torres *et al.* 2011, Skotarczak *et al.* 2005, Tsai *et al.* 2011). One disadvantage is that these dogs are theoretically exposed to ticks and their pathogens at higher rates than domestic dogs are which would tend to give an overestimate of the overall prevalence. In addition, it is difficult to coordinate a large scale seroprevalence study and shelters provide the best opportunity to acquire a sufficient volume of samples in a short amount of time to give an accurate picture of seroprevalence. Interestingly, July had a significantly higher positive seroprevalence when compared to the other three collection months, perhaps due to increased tick and host activity (Goddard 1996). Perhaps, future seroprevalence studies should be yearlong to better assess seasonality of the disease.

The veterinarians collected demographic information on the submitted samples; however, several spots on the forms were left incomplete. More demographic information would have been helpful (e.g. sex of dogs from shelter) which would have provided a more thorough analysis of risk factors. Drawing blood from many dogs in one sitting, as our study was designed to do at the shelters, as well as collect demographic information, proved difficult. The shelters graciously allowed us to enter their establishment and
offered their assistance several times throughout the summer and minimal disruption of
their normal workflow was a primary goal. In addition, the population of dogs we chose
for this study; shelter dogs, often did not have accompanying demographic or history
making our epidemiological study straightforward.

It was shown that SFG *Rickettsia* circulates in dogs and ticks in northern
California, thereby expanding the known geographic and species distribution of
documented *R. rickettsii* infections in California. This may be of a significant public
health concern as northern California residents share a peri-domestic environment with
dogs and also spend more time outdoors, significantly increasing their risk of being
infected with RMSF. This research shows that even though RMSF is rare in California
the potential risk of infection still exists and residents need to be aware of the risks in
order to protect themselves and their pets.

Although *Rickettsia* 364D was not detected in the canine samples it does not rule
out the possibility that *Rickettsia* 364D circulates in the northern California tick and dog
population. Lack of evidence of a *Rickettsia* 364D infection in canines suggests dogs may
not be infected with this agent. Since *Rickettsia* 364D has been recovered from *D.
occidentalis* ticks in California and Lake County most recently (CDPH current data),
small mammal reservoirs (which provides blood meals for immature *D. occidentalis*
ticks) (Philip *et al.* 1981, Wikswo *et al.* 2008) may be the next logical reservoir to
investigate.
LITERATURE CITED


Arredondo-Jimenez, JA. 2010. Rocky Mountain spotted fever in the Western Mexico – U.S. border region. Paper presented at American Society of Tropical Medicine and Hygiene 59th Annual Meeting; November 2010; Atlanta, GA.


APPENDICES

Appendix 1 - Collection Photograph

Mendocino County Shelter employees demonstrate the collection procedure on the extremities of the resident canines used in this study.
Appendix 2 – Electrophoresis Gel Pictures

Electrophoresis gel confirming that Lake County Shelter (LCS) samples 1-11 was successfully extracted by producing an expected band when amplifying the GADPH gene.
Electrophoresis gel confirming the PCR products obtained from the SYBR Green qPCR assay targeting the *ompA* gene using the RR190.547 and RR190.701 primers. Lanes 2-9 are standards, lanes 11-12 are the positive dog samples and lanes 13-14 are the positive tick samples.
Electrophoresis gel confirming the PCR products obtained from the nested PCR assay targeting the \textit{RR0155-rpmB} gene using the RR0155-PF, RR0155-PR, RR0155-FN and RR1055-RN primers.
Appendix 3 – Sequence Data

a. >MCS-25 *ompA* bp=109
   TCTATATTTCTGCAGTTATCTTTGACGGAGCTGCAGATTGTATAATTAGTGACATTCTCCTCCCCCTAAAGCTATATTTCCTTACCTGTATAATTATCGGCAGG

b. >MCS-27 *ompA* bp=108
   TTCCGTTAATGGCAGCATCTGTCTTGACAGTTATTATACCTCCTCCATTTATATTGGCATCTGTCTTGACAGTTATTATACCTCCTCCATTTATAT
   GCCTGACAGCATCTGTCTTGACAGTTATTATACCTCCTCCATTTATACCTGACAGCATCTGTCTTGACAGTTATTATACCTCCTCCATTTATAT
   GCCTGACAGCATCTGTCTTGACAGTTATTATACCTCCTCCATTTATAT


c. >Tick-31 *ompA* bp=111
   CCTCTATATTTCTGCAGTTATCTTTGACGGAGCTGCAGATTGTATAATTAGTGACATTCTCCTCCCCCTAAAGCTATATTTCCTTACCTGTATAATTATCGGCAGG


d. >Tick-32 *ompA* bp=109
   GTTCCGTTAATGGCAGCATCTGTCTTTGACAGTTATTATACCTCCTCCATCTATA
   GTTCCGTTAATGGCAGCATCTGTCTTTGACAGTTATTATACCTCCTCCATCTATA
   GTTCCGTTAATGGCAGCATCTGTCTTTGACAGTTATTATACCTCCTCCATCTATA
   GTTCCGTTAATGGCAGCATCTGTCTTTGACAGTTATTATACCTCCTCCATCTATA
   GTTCCGTTAATGGCAGCATCTGTCTTTGACAGTTATTATACCTCCTCCATCTATA
   GTTCCGTTAATGGCAGCATCTGTCTTTGACAGTTATTATACCTCCTCCATCTATA
   GTTCCGTTAATGGCAGCATCTGTCTTTGACAGTTATTATACCTCCTCCATCTATA

Sequence obtained from the SYBR Green qPCR assay using the primer pair sets; RR190-547 and RR190-701. **a.** Sequence obtained from dog sample MCS-25, bp = 109. **b.** Sequence obtained from dog sample MCS-27, bp = 108. **c.** Sequence obtained from tick sample Tick-31, bp = 111. **d.** Sequence obtained from tick sample Tick-32, bp = 109.
Sequence obtained from the nested PCR assay using the primer pair sets; RR0155-PF and RR0155-PR, and RR0155-FN and RR0155-RN. 

**a.** Sequence obtained from dog sample MCS-25, bp = 232. 

**b.** Sequence obtained from dog sample MCS-27, bp = 218. 

**c.** Sequence obtained from tick sample Tick-31, bp = 228. 

**d.** Sequence obtained from tick sample Tick-32, bp = 185.