Blood Meal Analysis for the Detection and Identification of Host DNA in the Gut Contents of *Ornithodoros coriaceus*

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in Animal Science

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ABSTRACT

Ornithodoros coriaceus ticks are the vector for the disease epizootic bovine abortion, an abortive disease of cattle that can cause significant revenue losses in the beef cattle industry, in the western United States. The transmission cycle of the etiologic agent is unknown and it has not been determined whether there is a vertebrate reservoir host for the disease. Research into the composition of blood meals of arthropod vectors has been shown to provide evidence to link vector species to specific hosts which can be used to determine possible disease reservoirs. To determine if host identification through blood meal analysis could be performed on field caught Ornithodoros coriaceus, markers from three mitochondrial genes, 18S rDNA, 12S rDNA, and cytochrome b, were used to detect host DNA in the gut contents of the soft tick vector. Polymerase chain reaction in combination with reverse line blot showed that the cytochrome b marker could accurately identify host DNA in ticks, fed experimentally on a known host blood, on day 175 post-feeding in 92.9% of ticks, in 40% of ticks on day 196, and only 26.7% of ticks at day 207. The cytochrome b marker was not useful in identifying host DNA from ticks caught in the field. The 12S markers could discriminate between vertebrate DNA to the genus level but probes, designed for identification in reverse line blot, did not differentiate between species. The 18S marker was non-discriminatory and could not be used for further analysis.
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LITERATURE REVIEW

Tick Borne Disease

Complex relationships have developed between specific tick species, the organisms they transmit, and the susceptible host populations these ticks feed upon. It is necessary to develop a complete understanding of these relationships before developing control management techniques or disease prevention programs (Oliver 1996). After mosquitoes, ticks are the second most important arthropod vectors of disease to both humans and livestock; surpassing all other arthropods in the variety of pathogens they transmit (Sonenshine 1991; Pichon et al. 2003).

Ticks are hematophagous parasites of mammals, birds, reptiles, and amphibians (Sonenshine 1991). Tick borne diseases are generally found in specific geographic regions where the tick vector is distributed and can be caused by diverse pathogenic organisms, including a variety of bacteria, protozoa, and viruses (Oliver 1996). Tick borne diseases have an impact on both human and veterinary health. On an annual basis billions of dollars are spent on control and protection of livestock from tick borne diseases (USDA 2003).

Of the 850 described tick species only 10% cause a significant impact to humans or livestock (Oliver 1996). Some tick borne diseases of epidemiologic significance are Lyme disease; tick borne encephalitis, Rocky Mountain spotted fever, relapsing fever, tularemia, babesiosis, ehrlichiosis, anaplasmosis, theileriosis, Colorado tick fever, and epizootic bovine abortion (Piesman and Eisen 2008). Disease causing pathogens are often associated with a specific tick genus and sometimes a particular species meaning that the pathogen can only be transmitted by that specific type of tick. Other disease
causing pathogens have the ability to be transmitted by multiple tick genera such as *Anaplasma marginale*, a rickettsial pathogen that can be vectored by multiple species of ticks as well as flies (Scoles et al. 2008). Of the diseases listed above, Relapsing fever, epizootic bovine abortion, and African swine fever are all diseases transmitted by soft ticks belonging to the genus *Ornithodoros* (Rebaudet and Parola 2006; Schmidtmann et al. 1976; Schwann et al. 1991).

An overview of soft tick diseases show patterns between the tick species that transmits the pathogen and the hosts that each tick associates with, such as with relapsing fever and African swine fever. Relapsing fever is an illness caused by several spirochetes in the genus *Borrelia* that causes reoccurring fever, fatigue, and aching muscles. The disease is known as tick-borne relapsing fever (TBRF) and is a zoonotic disease found in North and South America, Africa, Asia, and Europe. Within each region there exists a specific relationship between the *Ornithodoros* spp. vector, the particular *Borrelia* transmitted and their reservoir hosts within the geographical region where these three overlap (Rebaudet and Parola 2008). African swine fever is an *Iridovirus* virus that is highly contagious and usually a fatal disease of pigs (Basto et al. 2006; Vial et al. 2007). The disease is transmitted by host to host contact as well as through the primary tick vector, *Ornithodoros moubata* but can be harbored and transmitted by other *Ornithodoros* species (Basto et al. 2006). African swine fever is endemic in eastern and southern Africa and is maintained by the reservoir hosts, warthogs or bushpigs, or by the vectors themselves, *Ornithodoros moubata* or *O. erraticus* (Vial et al. 2007). In western North America, epizootic bovine abortion is a disease of importance in the beef cattle
industry. The soft tick vector that transmits the disease is of interest due to the lack of knowledge about the maintenance of the pathogen in the wild.

The soft tick *Ornithodoros coriaceus* transmits epizootic bovine abortion, a disease of cattle causing late term abortion. Epizootic bovine abortion is caused by a deltaproteobacteria (King et al. 2005) but efforts to grow the causative agent in culture have proved unsuccessful. The epidemiology of this disease is not completely understood due to the rapid feeding habits of the tick vector, researchers are unable to find the tick attached to a host in the wild. This results in difficulty determining preferred hosts of the vector. Although the disease can be duplicated in vitro, it is time consuming and costly. Due to the difficulty of studying the disease by duplicating it, in vitro (Schmidtmann et al. 1976; King et al. 2005), and the lack of complete understanding of the transmission cycle, studying the tick vector may provide a link to the preservation of the pathogen in the wild. With the advent of more advanced molecular techniques it is possible to detect small amount of host DNA in the gut of arthropod vectors to determine the preferred hosts of the species under study (Kirstein et al. 1996; Pichon et al. 2003; Kent et al. 2005). These techniques may provide an indirect means to determine reservoir hosts of the disease.

Most modern techniques used to detect vertebrate DNA in the midgut of ticks has yet to be tested in soft tick species. Precipitin testing, which has some drawbacks, has been shown to provide results when tested in *Ornithodoros coriaceus*, but with more sensitive techniques available we can explore new methods of blood meal analysis (Garcia 1957). Polymerase chain reaction (PCR) using mitochondrial markers are sensitive enough to identify small degraded fragments of DNA in other tick vector
species (Kirstein et al. 1996; Parson et al. 2000; Oshaghi et al. 2006). This method has been used to identify host DNA and elucidate reservoir hosts when studying other tick borne disease such as Lyme disease (Kirstein et al. 1996; Pichon et al. 2003; Humair et al. 2007). Due to the lack of information about preferred hosts for the tick vector of epizootic bovine abortion, *Ornithodoros coriaceus*, this technique may prove useful in discovering the complex relationship between this disease and the vector. Once a detection system is tested and shown to provide host identification results it can be applied to wild caught ticks, which is a valuable tool in studying, not only EBA, but other soft tick diseases as well. Specifically, by determining preferred host species for the EBA vector we can get an idea about what wild species are possible carriers for the disease. Host determination can provide a list of possible reservoirs for the disease, which can be used to determine if these carriers are important in the life cycle of the EBA pathogen.

**Epizootic Bovine Abortion**

Epizootic bovine abortion (EBA) is a tick-transmitted disease affecting beef cattle in the western United States. The disease has been described in California since 1923 as an important cause of abortion in cattle grazed in the foothill regions of the Sierra Nevada Mountains (Kennedy et al. 1960). Historically, these areas served as summer pastures for many California beef herds (Howarth et al. 1958). The presence of the disease was suspected in other western states y Howarth et al. 1958 but was not confirmed until the last decade (BonDurant and Anderson 1997; Hall et al. 2002). Due to an absence of suitable vector habitat in areas where dairy cattle are raised, the disease is rarely diagnosed in dairy herds (Kennedy et al. 1960; Storz et al. 1960). Epizootic bovine
abortion has been diagnosed in cattle herds in northern Nevada, southern Idaho, and the Klamath Basin of Oregon (BonDurant and Anderson 1997; Hall et al. 2002; Howarth et al. 1956). Diagnosis of the disease is confirmed post mortem, by pathologic and histological changes in the fetus that are characteristic of reported EBA pathology (Anderson et al. 2006).

Pathology

Epizootic bovine abortion causes late term abortion in cattle at 6 to 8 months of gestation (Howarth et al. 1958; Storz et al. 1960). The disease is primarily diagnosed by characteristic gross lesions present in aborted fetuses, that develop progressively over a period of many months (Kennedy et al. 1960; Hall et al. 2002; Stott et al. 2002; Anderson et al. 2006). The general pathology of the disease includes gross lesions that are characterized by petechiation in the oral cavity, conjunctiva, and thymus, by marked enlargement of the lymph nodes and spleen, and by hepatomegaly (Kennedy et al. 1960). Histologic examination of the tissues is required for a confirmation of an EBA diagnosis (Kennedy et al. 1960; Hall et al. 2002). Lesions found in the thymus are typical for EBA and develop in the late stages of the disease; they are characterized by a loss of cortical thymocytes and macrophage infiltration (Anderson et al. 2006). An immune mediated response causes acute vasculitis in many organs and acute necrosis in lymphoid organs (Anderson et al. 2006).

Further changes include gross enlargement of the lymph nodes, hyperplasia and macrophage presence in the most commonly affected organs; the liver, thymus, spleen, and lymph nodes (Kennedy et al. 1960; Anderson et al. 2006). The kidneys, pancreas, and heart are often affected as well, showing enlargement of the organs and
immunological cellular infiltration (Kennedy et al. 2006). The infected fetus will often show a distended abdomen due to ascites and enlargement of the liver (Kennedy et al. 1960; Hall et al. 2002). There can be a substantial spike in serum immunoglobulin concentrations in the blood of affected fetuses (Kimsey et al. 1983; King et al. 2005). Of the EBA infected calves those that are not expelled as a dead fetus can be born premature and are often weak, usually resulting in death (Kimsey et al. 1983).

**Epidemiology**

Abortions occur after cattle are grazed in EBA enzootic areas where the tick vector, *Ornithodoros coriaceus* is found. Calf losses due to EBA are typically seen in first year heifers more often than in older cattle, although any age cow can develop the disease (Kennedy et al. 1960). Anecdotal evidence suggests that previously affected cows do not exhibit repeated abortions if there is continued exposure to the pathogen in EBA endemic areas (Howarth et al. 1958; Kennedy et al. 1960). Susceptible animals include pregnant cows, particularly heifers, in the late first trimester or early second trimester, exposed for the first time to *Ornithodoros coriaceus* ticks carrying the pathogen (Kennedy et al. 1960; Storz et al. 1960; Schmidtmann et al. 1976).

**Economic Importance**

Efficient production is critical to sustaining the market for beef cattle (Blezinger 2002). The percentage of live calves born each year contributes to the overall efficiency of a cattle producers input resources (Blezinger 2002). Profits are dictated by the weight and number of animals sent to market and disease control plays a large role in maintaining the profitability of this industry. Diseases that affect the reproductive health of cattle often result in considerable economic loss; it was found that low pregnancy rates
cost $198 million in lower production efficiency annually (Smith 2001). Epizootic bovine abortion is acknowledged as a significant deterrent to maximum calf production in California; it is estimated that greater than 90,000 calves are lost annually to epizootic bovine abortion (Maas 1995). Abortion rates following infection with the agent of EBA have been reported to be as high as 75% in susceptible populations, causing considerable reductions in cattle productivity (McKercher et al. 1965).

**Etiology**

A major obstacle to the study of EBA has been the lack of knowledge concerning the etiologic agent of the disease and how it may be transmitted in nature. Over the history of the study of epizootic bovine abortion many microbes have been suspected as the etiologic agent responsible for this disease. The pathogen was assumed to be infectious because researchers noticed that once an EBA calf had aborted, if continuously exposed to EBA endemic areas, they did not seem to suffer from repeated abortions, suggestive of an immune response (Kennedy et al. 1960). Also, tissue changes that took place in affected fetuses were characteristic of other infectious diseases (Kennedy et al. 1960). Suspected microorganisms have included; a *Chlamydia* organism, several viruses, and a member of the spirochete family, but all these microbes have been eliminated as the pathogen that causes EBA (Storz et al. 1960; McKercher et al. 1965; McKercher et al. 1980; Lane et al. 1985).

Initially, the causative agent was thought to be a viral organism isolated from EBA aborted fetuses. A cell culture system was developed from multiple tissues and fluids including visceral organs, stomach contents, and peritoneal fluid obtained from EBA aborted fetuses (Storz et al. 1960). The cultures resulted in the growth of an
organism identified as a member of the psittacosis-lymphogranuloma-venereum group of viruses. Continued research proved inconclusive as to whether the recovered agent was responsible for abortions in cattle but similar viruses were identified as a cause of abortion in ruminants in Germany (Storz et al. 1960). The unidentified virus from the Storz, 1960 study was used to develop a vaccine but it did not provide protection against subsequent abortions (McKercher et al. 1966). It was suggested that *Chlamydia* organisms could also be the cause of EBA but protection from abortions caused by the EBA pathogen failed when cows were inoculated with a vaccine made against the organism (Storz et al. 1960; McKercher et al. 1966; McKercher et al. 1969; Schmidtmann et al. 1976; Kimsey et al. 1983).

Since the disease is restricted to warmer months of the year and occurs in defined geographic areas within specific habitats, an arthropod vector was suspected (Kennedy et al. 1960; Storz et al. 1960; Schmidtmann et al. 1976). Along with the failure to identify an etiologic agent for EBA, researchers were led to further explore the possibility of vector transmission. Rangeland populated by the tick, *Ornithodoros coriaceus* has an overlapping coincidence with EBA endemic areas therefore EBA is present only where the tick vector is found (Schmidtmann et al. 1976; Loomis 1974). The tick is associated with deer and cattle bedding sites and was also active during the warm season (Loomis, 1974; (Schmidtmann et al. 1976). Based on specific criteria, which is explained in more detail later, *O. coriaceus* was suspected to be the vector of EBA. When the tick was fed on susceptible pregnant heifers, it resulted in abortions with the characteristic pathology of EBA (Schmidtmann et al. 1976).
With this knowledge, a viral agent, the “74 isolate” was implicated when isolated from the salivary glands of *Ornithodoros coriaceus*. When investigator experimentally challenged susceptible cattle the virus did not induce abortion (Wada et al. 1976). A similar organism the “76 isolate” isolated from the tick also failed to cause abortion (McKercher et al. 1980). After Stott et al., 2002 found that antibiotic treatment, at the time of experimental challenge, could eliminate the agent of EBA; viral agents were considered a less likely causative agent of EBA. A challenge system was developed for the inoculation of EBA-infected fetal thymus into pregnant heifers at 90-120 days of gestation (Stott et al. 2002). The system produced consistent and predictable transmission of the pathogen and when concurrently treated with antibiotics, the infection was eliminated (Stott et al. 2002).

In a later experiment, fetal necropsy tissues were obtained from EBA infected fetuses and subjected to a PCR, amplifying a bacterial 16s rRNA fragment (King et al. 2005). Sequencing and phylogenetic analysis was performed on the PCR amplicons, of the isolated agents, and although other bacterial agents were isolated and found in PCR, a novel bacterium was the most frequently recognized bacteria. The experiment was repeated three times and this bacterium was the only agent seen in multiple experiments (King et al. 2005). They determined that the bacterium responsible for EBA was a deltaproteobacteria, most closely related phylogenetically to the order *Myxococcales*. Furthermore, the same bacterium (aoEBA) was also detected in nymph and adult *O. coriaceus* ticks from EBA endemic regions (King et al. 2005; Chen et al. 2006). The PCR developed for the detection of aoEBA was used to determine that the majority of infected ticks were carrying low numbers of the pathogen and artificial feeding did not
stimulate further replication of the bacteria (Chen et al. 2006). Tick salivary glands were removed from 6 ticks and analyzed for the presence of aoEBA and found to be carrying the bacteria. The remaining tissues of all 6 ticks were also analyzed and no aoEBA was detected (Chen et al. 2006). From this data it is likely the bacteria is most commonly located in the tick salivary gland. Although, the identified bacteria has been found in both EBA infected fetuses and the tick vector, efforts to culture this pathogen, outside of a living host, have been unsuccessful (King et al. 2005).

**Ornithodoros coriaceus**

Epizootic bovine abortion was originally thought to be a venereal disease due to the involvement of the reproductive tract, but sexual contact was later ruled out as the route of transmission with artificial insemination by eliminating the role of the bull in impregnating the cow (Mckercher et al. 1965). Without contact with an infected bull, heifer cows, which had never been bred, could not have contracted EBA without sexual experience. Nasal and oral routes of transmission were also eliminated as potential routes of infection (Schmidtmann et al. 1976). Although the methods were not described in detail, the disease could not be reproduced when pregnant heifers were exposed to aerosol and fomite transmission from cows that had subsequently aborted EBA fetuses (Schmidtmann et al. 1976). In early studies, vector and/or reservoir host involvement was suspected of EBA due to the limited geographic distribution of the disease, and after eliminating some of the probable transmission routes, researchers began to focus on determining if a vector was involved in the spread of this disease.

Several findings appeared to implicate vector involvement in the transmission of epizootic bovine abortion. Reports indicated that the disease had a very specific
geographic limitation, even though the exact distribution was unknown (Kennedy 1960). To be exposed to an arthropod vector the cattle would have to be exposed at the appropriate time of pregnancy to be susceptible to the pathogen. Cattle that had EBA induced abortion were typically grazed on foothill pastures in California, providing an opportunity for the cows to have been exposed to an arthropod vector. Outbreaks of epizootic bovine abortion also appeared to be restricted to the warmer months of the year, particularly July through October, under California conditions, which is an active time for arthropod species (Howarth 1958). Although infectious, the disease could not be reproduced by droplet contact or direct transmission, supporting the claims that the disease was transmitted by other means. There was sufficient evidence to assume an arthropod vector was responsible for EBA transmission (Schmidtmann et al. 1976).

To test this hypothesis, hematophagous arthropods, found in EBA enzootic habitats, were investigated on hypothetical competence parameters to determine their roles as possible vectors for EBA (Schmidtmann et al. 1976). Twenty-nine species of Diptera and 3 species of Acari were captured in EBA enzootic pastures, on or near a bovine host during EBA exposure periods. During this collection time, 2 of the 5 susceptible cows aborted fetuses with EBA characteristic lesions. Captured arthropod species were tested under controlled conditions, to determine if they would take a blood meal on a bovine host. After eliminating arthropod species that did not fit competence parameters and controlled feeding conditions, cows were then placed on an elevated platform, to exclude wingless arthropods, such as ticks. It was demonstrated that cows were not exposed to EBA while on the platforms, implicating a tick vector (Schmidtmann et al. 1976). The argasid tick, Ornithodoros coriaceus was then selected as the most
likely vector species based on the following criteria: 1) geographic coincidence with EBA; 2) the tick readily feeds on cattle; and 3) the tick is active during the summer months which was considered a time of maximum exposure for EBA. *Ornithodoros coriaceus* ticks were then experimentally fed on California resident pregnant heifers, from non-EBA enzootic areas, to determine the ticks’ ability to transmit the disease (Schmidtmann et al. 1976).

Schmidtmann et al. 1976, acquired test and control heifers from non-enzootic areas and each cow was determined to be pregnant by rectal palpation. *Ornithodoros coriaceus* ticks were collected and left to engorge on the test heifers in a chamber attached to the dorsal surface of the cow. Of the 12 exposed individuals, 5 cows aborted calves with pathological lesions consistent with EBA and 4 other cows gave birth to premature small calves. The calves that were born premature exhibited some abnormal symptoms such as; lymphadenopathy, elevated IgG levels, and incomplete eruption of their incisor teeth, but there was no conclusive evidence to determine if this was due to EBA infection (Schmidtmann et al. 1976). Although the tick vector had been linked with the EBA pathogen at the time of this study, there were not techniques sensitive enough to be able to directly quantify the EBA pathogen within the tick.

A Taqman real-time polymerase chain reaction was developed to detect the deltaproteobacterium that causes EBA and tested on field collected ticks throughout California, Nevada, and Oregon (Teglas et al. 2006). The Taqman probe was designed using the 16s-rRNA gene sequence previously described by King et al. (2005) (Teglas et al. 2006). When tested on field collected ticks from sites in California, Oregon, and Nevada the study showed that 9 of the 20 sites had ticks that carried the EBA bacterium
with an overall prevalence of 5.9%, although it varied by region (Teglas et al. 2006). To better define the relationship between the tick and the EBA agent, the 16s rRNA gene was used to detect the bacterium in EBA tissues (King et al. 2005). The developed primers were also used to detect the same bacterium in *Ornithodoros coriaceus* from sites in California, Nevada, and Oregon (Chen et al. 2007). Chen et al. (2007) showed that 1.6% of ticks were carrying the aoEBA but when PCR was used in conjunction with southern blotting the percentage increased to 10.6%. *Ornithodoros coriaceus* was found to be infected with the bacterium that causes EBA in all three geographic locations (CA, NV., OR.) with variable infection rates (5-20%) (Chen et al. 2007). The identification of the bacterium within both EBA infected tissues and *Ornithodoros coriaceus* confirmed the association between the disease and the recognized vector.

**Life history**

Ticks belong in the class Arachnida and can be distributed into three families: Ixodidea (hard ticks), Argasidea (soft ticks), and Nuttalliellidae. All ticks undergo four life stages: egg, larva, nymph, and adult with the larva having three legs and a fourth limb bud which will develop with the molt to the nymphal instar (Sonenshine 1991). Ticks are sexually dimorphic but these differences are less visible in Argasidae which are distinguished by the shape and size of the sexual aperture and the size differentiation, females being much larger than males (Furman and Loomis 1984).

The tick *Ornithodoros coriaceus* (Koch), also known as the pajahuello tick, was first described in 1844 from a single female individual that originated from Mexico (Hermes 1916). Superstition about the tick made it infamous in the mountains of Santa Clara and San Benito Counties of California, but an accurate identification had not been
published, prior to the description of the female found in Mexico. The ticks were believed to have originated in Mexico and later dispersed to the mountain regions of California (Hermes 1916). The ticks known geographic distribution includes areas surrounding California, northern Nevada, southern Idaho, southern Oregon, and Mexico (BonDurant and Anderson 1997; Hall et al. 2002; Hermes 1916; Howarth et al. 1956). It is unknown as to whether the tick was historically found in these areas or if *O. coriaceus* has dispersed northeast from Mexico, where it was initially described. Important observations of the pajahuello tick began when awareness of the severity of the tick bite, which results in a slow healing lesion in humans, became evident (Hermes 1916).

*Ornithodoros coriaceus* ticks were collected in the summer of 1913 and a female tick was allowed to lay eggs, after engorging on monkey blood (Hermes 1916). From this female, multiple larval hatchings were recorded and information was inferred from each hatching. Larval ticks were fed on the ear of a rabbit between molts, and Hermes (1916) documented an incubation period, the time from egg to larva, of 21 days (26.3°C), a complete developmental cycle (from hatching from the egg to the time the tick produces her own egg) of 15 months, and a developmental stage from egg to adult of 159 days for males and 343 days for females. He found that the developmental cycle can be reduced by applying the ticks to host animals at shorter time intervals between feedings. Hermes (1916) noted that under natural conditions it would be likely that a single generation could be produced in a year but a second year may be necessary. The time to develop between nymph and adult varied considerably based on the interval between tick feedings and whether nymphal ticks were developing into females or males, females requiring more molts to mature than males. The minimum larval molting period was 19
days post-feeding and molts could vary between 4 to 7 instars for a single individual. A single female was shown to remain fertile for up to two years following a single feeding and could lay over a thousand eggs in a season. Hermes (1916) found that *Ornithodoros coriaceus* required a short feeding period from 15-30 minutes for nymphs and adult ticks, and that they would feed on multiple species, in the lab. He accounted for many eggs being laid by females multiple times in a season and also for first stage nymphs to molt without feeding when held at 26°C with varying humidity, which was reconfirmed by Smith, 1944. Although, Hermes was the first to explain the feeding behavior and life history of this tick, his observations were based on only a few records and a limited number of samples. Further research and additional observations was desirable to develop the understanding of the life cycle of this tick. (Hermes 1916)

A more comprehensive study was performed by Smith (1944) with a larger sample size and each tick life stage was analyzed individually. Approximately 20 lots of eggs were incubated at various temperatures between 25.7 °C and 30 °C. Incubation periods were between 16-18 days on average but ranged between 10 days to 29 days and fluctuated with varying temperatures. Incubation times were the longest at the lowest and highest temperatures. Larval ticks were shown to engorge on blood approximately 63 days after hatching and if left unfed would only live between 2-5 months. Larva attached to a host for 9.2 days on average, with a median of 7 days within a range of 5-18 days. After dropping off a host, the larva would then molt, over several days. The period of time from engorgement to molt varied depending on the incubation temperature. The average time to molting, for larva, was 10.7 days but could be as great as 21 days before becoming a first stage nymph (Smith 1944). Nymphs also appeared to fast for extensive
periods of time, sometimes not accepting a meal for 19-301 days. The variation in molting time for each instar was extensive and could range from less than a month to over a year. The adult tick was shown to behave much like the nymph but often had an even longer duration between feedings. The adult ticks were shown to fast for as long as 3 years and four months after molting before taking a meal. Engorgement would take 10-34 minutes for males and 5-51 minutes for females. Mating behavior was shown to take place off of the host after single or multiple feedings. Oviposition occurred between two weeks to seven months after mating. Females would lay multiple lots of eggs, having between 28-1,424 eggs in a lot (Smith 1944). Besides feeding and mating other behaviors of the tick is very important to the understanding of the species.

Soft ticks seek hosts by responding to external stimulus cues such as emissions during respiration (Garcia 1962). *Ornithodoros coriaceus* are often found associated with the bedding sites of deer and cattle and have been documented to be found in the leaf litter, where they spend most their lives (Schmidtmann et al. 1976; Garcia 1957). Although soft ticks may associate with preferred host species they feed quickly and do not associate with only a single host throughout their lifetime even if they tend to feed on a specific species. Due to their life cycle there are several developmental stages at which point they find a host. *Ornithodoros coriaceus* have specific anatomical and physiological features that make them good vectors such as multiple host feeding, long life cycle, extensive digestion and starvation.

**Tick Anatomy**

The cuticle, or outer surface of the exoskeleton, has a lipid bilayer that prevents water loss, providing ticks with an efficient tool to withstand desiccation (Sonenshine
The cuticle also facilitates rapid engorgement and can accommodate engorgement of a large volume of blood (Furman and Loomis 1984; Sonenshine 1991). This could play a role in the extended periods of starvation *O. coriaceus* can withstand between meals and influence their ability to harbor disease for long periods of time as a dormant reservoir (Furman and Loomis 1984). A more detailed review of the alimentary system including the salivary glands and the digestive tract is necessary to get a more complete picture of the functionality of the tick as a vector.

**Alimentary system**

Unlike vessel feeder such as mosquitoes, the mouthparts of ticks create hollows in the host skin, creating a lesion in the epidermis from which they feed upon and are therefore called pool feeders (Nuttall et al. 1999). The mouthparts, or chilercerae, tear at the host skin and penetrate the wound site with the hypostome, which is a barb-like anchor to facilitate attachment to the host. Saliva enters via the preoral canal between the dorsal hypostomal gutter and the cheliceral shafts, which initiate a response that enlarges the feeding pool. Fluid is then drawn into the preoral canal by the sucking action of the pharynx. The pharynx alternates between contraction and relaxation providing pulses of blood flow or ejection of salivary fluids.

In ticks, the salivary glands are the site of osmoregulation and fluid secretion and are also of major importance in pathogen transmission (Bowman et al. 2004). While feeding, salivary secretions from the tick prevent coagulation of the host blood and fluid is exchanged between both the host and the tick. The paired acinar are located anterolaterally on both sides of the tick’s hemocoel. Paired salivary ducts enter the salivarium, a shallow tube above the pharynx, before emptying into the buccal cavity.
where the outward flowing saliva alternates with host fluid flowing inward. This creates an opportunity for pathogens located within the salivary glands to be passed to the host species. For many pathogens, the salivary glands are the site for replication and reproduction (Bowman et al. 2004).

The functional morphology of the arthropod digestive tract is important to understanding the host-parasite relationship because the pathogen must be able to survive the digestive process and reproduce before being transmitted back to another host. The digestive tract is separated into the foregut, midgut, and hindgut and absorption is confined to the midgut and tubules of the digestive gland. The esophagus of fluid sucking arthropods is a muscular pharynx located in the foregut region along with the crop for storage, and the proventriculus, which regulates food passage to the midgut. The salivary glands containing digestive and anticoagulant enzymes are found beneath the midgut. The midgut functions as the site of absorption and digestion of the blood meal. The hindgut is split into the intestine and rectum both important for waste elimination and regulation of water and ions. During the initial phase of digestion the blood meal is concentrated in the gut lumen and very little digestion occurs. The second phase is a more intensive intracellular digestion by the digestive cells in the midgut (Melhourn 2001). The third phase encompasses slow digestion and low activity, where the rupturing of red blood cell membranes and the release of hemoglobin is gradual (Melhourn 2001).

The tick digestive process is intracellular and slow therefore meals can remain undigested for long periods of time (Sonenshine 1991). The slow digestive process allow the tick to endure a long period of starvation unlike other blood feeding arthropods whose extracellular digestion is rapid, meaning they will have to feed more often. Also,
ingested pathogens are protected against rapid digestion within the tick gastrointestinal tract due to the slow digestive process, giving them the opportunity to migrate and penetrate other tissues within the host (Sonenshine 1991).

Due to the rapid engorgement, short feeding time, and long starvation periods, *Ornithodoros coriaceus* is rarely found attached to a vertebrate host. Therefore, little is known about host selection. The information available regarding the feeding behavior of arthropod vectors is essential to the comprehensive understanding of the associations between a host and vector, and their role in disease transmission (Clausen et al. 1998).

**Host Identification**

Host selection by a vector species plays a significant role in the epidemiology of arthropod borne diseases, and is often the key to the continued transmission of a particular pathogen (Oliver 1996; Gubler 1998; Piesman and Eisen 2008). Vectors become infected with disease causing pathogens through blood feeding and a host animal can serve as a source of infection to the vector. These reservoir hosts can serve to maintain a disease and facilitate the transmission of a pathogen to new vectors, which then serve to infect other host species they feed upon. Most pathogens are host generalists, meaning they are able to infect multiple host species and are important to disease dynamics and pathogen transmission (Brunner et al. 2008). Host infection rate and prevalence determine the pattern of pathogen availability and the opportunity for infection, which influence the process of transmission between susceptible species (Daniels et al. 2007). Host preference by a vector species has been shown to play a key role in the maintenance of a disease as well as reservoir competence (Brunner et al. 2008). Reservoir competence is the product of the probability the host is infected or the
prevalence and the probability it will transmit the pathogen to the vector, if infected, known as infectivity (Brunner et al. 2008). In the case of Lyme disease the pathogen (Borrelia burgdorferi) is maintained and transmitted by small mammals and birds, causing a large repertoire of animal in which the tick vector can acquire the pathogen and pass it to other hosts (Matuschka et al, 1992). The cyclic nature of pathogenic transmission often creates a pattern that must be interrupted in order to protect susceptible animals of economic interest. To halt further transmission and gain control of a disease the life cycle of the pathogen and the cycle of transmission must be completely understood. Due to the difficulty of finding vectors on a host at the time of feeding and the inconvenience of trapping large numbers of animals, using direct sampling for host identification studies, is impracticable. Molecular techniques have been employed to indirectly determine a blood meal source in hematophagous vectors to establish vector/host associations.

Biological techniques, such as identifying antibodies or looking for host DNA within the vector, have been used to determine the host source of a blood meal in arthropod vectors. Serological tools such as enzyme-linked immunosorbent assays (ELISA), precipitin testing, and latex agglutination have their drawbacks because they require relatively un-degraded DNA and a priori assumptions to be made as to the origin of an arthropod vector’s blood meal. Serological techniques require the creation of specific antibodies against known animal species in order for these methods to be of use. The ELISA test is able to detect antibodies or antigens that give off a signal when the antibody present within the antisera interacts with the antigen. In a study of Glossina fly species, the vector of trypanosomiasis, host specific antibodies were needed for ELISA
testing and required identification of vertebrate sources of blood meals, prior to its
detection (Clausen et al. 1998). Previous findings showed a very characteristic host
selection for each species of *Glossina* fly (Weitz 1963). Based on previously recorded
host selection data, host specific antisera was developed against each suspected host
species and tested on 29,245 flies of eleven *Glossina* species (Clausen et al. 1998). The
study reported that the source of the blood meal could be identified to the genus level in
62.8% of the flies tested and of those flies 44.9% could be identified to the species level
(Clausen et al. 1998). Without previously published data on host preferences for
*Glossina*, researchers would not have known which host antisera to develop.

In a similar study, Zimmerman et al, (2006) tested anopheline mosquitoes host
preferences using the same ELISA methods for a more detailed account of malaria
transmission. Engorged anopheline mosquitoes (2,046) were captured from the wild and
tested using ELISA with commercially available IgG (antibody) antisera for humans,
pigs, bovine, dog, chicken, and rat. When surveying mosquitoes, only those with a fresh
blood meal (within 48 hrs) were useful for identification of host species. Each mosquito
was tested for all host species described above and 58% had a positive reaction to at least
one of these host species IgG molecules (Zimmerman et al. 2006). Another study of
mosquitoes, using ELISA detection methods, has shown over 99% reactivity to at least
one host IgG antibody, finding that blood meal volume, species, and temperature can
affect the period of detection of host proteins and can create differences in the number of
positive results in each study (Greica et al 2002). Although this technique showed
significant results in mosquitoes, it had to be tested within a very short window of time
after feeding, to accurately identify a host.
Precipitin testing has similar drawbacks when it comes to developing specific antibodies against species-specific antigens. A precipitation reaction occurs as the result of combining a solution of antibodies with an antigen dilution, which will create a visible accumulation of particles if the antibody reacts to the antigen. Host specific antisera must be developed containing antibodies that are specific to one or more antigens and when combined in solution, the positive reaction identifies the specific host species. Precipitin testing has been shown to accurately identify host species, to the genus level, in both Phlebotomine sandflies and anopheline mosquitoes that had recently acquired a meal (Tesh et al. 1971; Flores-Mendoza et al. 1996). Serological techniques are valuable when tested on arthropod species that have taken a fresh blood meal, but tick species can go for long periods of time without feeding. To determine the practicality of using serological methods to detect host DNA in ticks, ELISA was used to detect the host blood meal in Ixodes ticks.

Kirstein et al. (1999) utilized an inhibition ELISA to detect host specific IgG molecules in the gut contents of Ixodes ricinus nymphal ticks by incubating them with biotinylated anti-host immunoglobulin G (IgG), designed to detect possible reservoir hosts of Borrelia burgdorferi. Immunoglobulin G in the tick gut was bound to anti-IgG in the incubation mixture and identified by aliquoting the solution across microtitre wells coated with IgG of known reservoir host species. The inhibition ELISA was able to detect very small amounts of host IgG in the tick and was able to accurately identify hosts. The detection ability of the ELISA decreased significantly within 6 weeks after nymphs molted to the adult stage due to degradation of IgG molecules. Ticks do not feed as often as other hematophagous arthropod species and digest their blood meal over long
periods of time before taking another meal. Due to the extensive digestive periods of tick arthropods, the blood meal may be several months old and degraded by enzymatic activity, leaving only small amount of recoverable IgG, making accurate host identification impossible using methods such as ELISA (Kirstein 1999). Serological techniques that require a priori assumptions to be made, can be expensive and laborious, and are ineffective with degraded DNA. Polymerase chain reaction, a technique used to amplify DNA, is a more reliable and suitable technique for targeting degraded DNA because small enough amplicons can be selected to allow for successful amplification even from degraded DNA. This feature couple with the fact that DNA is more stable in blood than IgG molecules could improve the success of host identification experiments.

Blood meal analysis of hematophagous arthropods has become more accurate and reliable with the development of more precise molecular techniques such as polymerase chain reaction (Kirstein et al. 1996, Pichon et al. 2003, Humair et al. 2007). Techniques utilizing the polymerase chain reaction allow for the rapid amplification of target sequences of DNA with the ability to replicate gene sequences up to several orders in magnitude. This technology made finding small amounts of recoverable DNA in the gut contents or arthropods possible by targeting specific markers used to detect vertebrate DNA (Kirstein et al. 1996, Kirstein et al. 1999, Parson et al. 2000). Polymerase chain reaction using mitochondrial markers has been shown, in ticks, mosquitoes, and flies, to detect DNA and identify the source of a host blood meal (Kirstein et al. 1996, Pichon et al. 2003, Humair et al. 2007). One drawback to standard PCR is that many of the samples in arthropod studies do not show strong bands on a gel, indicating a positive result, because of very low quantities of quality DNA. Further blood meal analysis
research coupled PCR with various other molecular techniques; such as restriction length polymorphism and reverse line blot (RLB) to increase the sensitivity of DNA detection and the specificity of vertebrate species identification (Kirstein et al. 1996, Oshaghi et al. 2006, Steuber et al. 2005, Humair et al. 2007).

The most widely used molecular markers to detect vertebrate DNA are designed from conserved regions of the cytochrome b gene (Kirstein et al. 1996; Pichon et al. 2005; Van Den Hurk et al. 2007; Oshaghi et al. 2006; Steuber et al. 2005). Cytochrome b is a well-characterized protein from complex III of the mitochondrial phosphorylation system and is encoded in the mitochondrial genome (Hatefi 1985). The cytochrome b gene is found in high numbers in each cell and has a wide variety of published sequences available for different species (Luyo-Acero et al. 2004; Kent et al. 2005). Mitochondrial DNA is preferred for the identification of host species because it contains high proportions of evolutionary caused nucleotide substitutions as an invaluable tool in differentiating closely related species (Streuber et al. 2005). A large proportion of mitochondria, each with their own DNA is also found in each cell, making it ideal for identification purposes using small amounts of DNA (Kirstein et al. 1996). In the feeding and digestion process of a tick, the biochemical composition of the blood is altered. When a blood meal containing, lymph, leukocytes, erythrocytes, and tissue enter the tick gut, secretory cells are released and pinched from the lumen of the gut wall (Schwemmler et al. 1989). These cells contain a hemolysin, which causes lysis of erythrocytes and leukocytes. Hemolysis of the blood is rapid and few intact erythrocytes or leukocytes are found in the lumen of the midgut and although complete digestion is slow, initial breakdown causes partial degradation of the molecules (Ribiero et al. 1988). Mammalian
red blood cells are anucleate when mature and therefore do not contain DNA and are not valuable for amplification. The limited numbers of leukocytes (white blood cells) are the only accessible DNA available for amplification in PCR. Although the initial stages of tick digestion are rapid, the breakdown of the entire blood meal occurs over a long period of time in soft ticks (Sonenshine 1991). This can be utilized to detect small fragments of DNA in the tick gut before complete degradation (Kirstein et al. 1996). Thus, when DNA is partially degraded, the primers amplify smaller fragments of DNA more successfully. Multiple molecular markers used in PCR, targeting small regions of a specific gene for amplification, have been used to identify the source of host blood meals in ticks, flies, and mosquitoes (Kirstein et al. 1996; Pichon et al. 2003; Humair et al. 2007).

Ixodid tick specimens from a museum collection, that had been engorged on vertebrate blood of known animals, and preserved in 70% alcohol, were used in a study to determine if the cytochrome b gene could be used as an accurate molecular marker for amplification in PCR. The DNA from the tick samples was extracted and amplified in PCR using a 400-bp fragment of the vertebrate specific cytochrome b gene. Post PCR, all known hosts were accurately identified by sequencing, but the museum specimens had been specifically selected because they had been engorged prior to preservation. In the field, questing ticks typically have not fed for long periods of time and have digested the blood meal enough to degrade the DNA within the gut (Tobolewski et al. 1992).

Polymerase chain reaction using cytochrome b as a marker, in combination with restriction fragment length polymorphism (RFLP) has accurately detected host DNA in *Anopheles* mosquitoes and *Glossina* flies (Steuber et al. 2005, Oshaghi et al. 2006). The
gene was amplified in bovidae species with cytochrome b primers specific for vertebrate species. The resulting fragments were sequenced and restriction sites were identified (Steuber et al. 2005). Restriction profiles for the potential bovidae hosts of tsetse flies were developed using multiple restriction enzymes. Once developed, the restriction profiles accurately identified host DNA from fed tsetse flies. Host DNA detection was 100% after 24 hours post-feeding, 40% after 96 hours, and the signal disappeared after 120 hours post-feeding (Steuber et al. 2005). Similar results were found using cytochrome b primers in a PCR to study the blood meal of fed Culex quinquefasiatus and Anopheles stephensi mosquitoes (Oshaghi et al. 2006). DNA extracted from a blood meal of fed mosquitoes provided an efficient template for up to 33 hours post ingestion. This template DNA could then be used for further analysis in RFLP and direct sequencing. After 36 hours post feeding the extracted DNA did not act as a sufficient template for PCR amplification and therefore could not be used in further analysis. It was shown that the duration of time since the last feeding was associated with successful amplification, rather than temperature or the size of the blood meal (Oshaghi et al. 2005). Due to the differences of blood meal acquisition and the digestion rates between other arthropod vectors and ticks, it is difficult to evaluate the feasibility of utilizing particular molecular techniques unless they have been tested on a species more closely related to Ornithodoros coriaceus.

Kirstein et al. (1996) were examining the discriminatory ability of the cytochrome b gene for host vertebrate DNA in Ixodes ricinus, the tick carrier of Lyme disease in Europe. Degenerate PCR primers were developed that amplified a 638-bp region of the cytochrome b gene from vertebrate species. After amplification, the products were
analyzed with RFLP using HaeII and DdeI restriction enzymes run on a 1% agarose gel, which produced recognizable banding patterns for each particular vertebrate species being studied. All species could be distinguished from one another except for Cervus species which all produced the same banding pattern when tested. For engorged larval ticks the detection window was only 10 days post-engorgement. A nested PCR was developed to amplify regions within the 638-bp amplicon and the results showed detection at 90 days post-engorgement with a 116-bp region, 200 days with a 134-bp and 95-bp regions, and could detected host DNA 40% of the time at day 280 when amplified with the 95-bp primer. To increase detection, a species-specific reverse line blot was developed to facilitate specific identification of host animal species. A probe to detect Mus musculus was designed and tested on larval ticks fed on Mus musculus. Hybridization occurred in larval ticks up to 280 days post-engorgement (Kirstein et al. 1996). The cytochrome b gene is variable and it is difficult to design universal primers to cover a more complete range of possible hosts. Therefore, after determining the utility of this technique an additional screening, using an 18S universal primer was added to the process to limit variability (Pichon et al. 2003).

The 18S rDNA gene, that codes for a small ribosomal subunit and is highly conserved in vertebrates and does not amplify tick DNA, was used to develop universal primers for host DNA amplification in ticks. Larval Ixodes ricinus ticks fed in the lab and field collected nymph DNA was extracted and amplified with universal 18S rDNA primers (Pichon et al., 2003). The primers amplified a 150-bp gene fragment that was analyzed with a reverse line blot technique. Specific probes for reverse line blot were developed from sequencing data from the 18S rDNA gene of 15 vertebrate species. Ten
nymphal ticks were fed on mice and rabbit blood and analyzed using PCR and RLB. All specimens tested positive for host DNA for up to 7 months and 40% tested positive for up to 9 months. Field collected ticks were tested with the same technique and 26 nymphs out of 49 (53%) were positive for host DNA (Pichon et al. 2003).

A 12S rDNA genetic marker has also been used in combination with reverse line blot to identify host blood meal sources in *Ixodes ricinus* (Humair et al. 2007). The technique uses a single run PCR with a marker that amplifies a 145-bp region of the 12S rDNA gene. Primers were developed in a conserved region of the gene flanking a variable region. External primers were also developed for the sequencing in suspected vertebrate host species. Probes were designed from sequencing data to be species specific and used in a reverse line blot. Once the DNA in the tick gut contents was amplified, the target sequence was run on RLB, and if present species specific probes annealed to the respective host sequences. Host DNA was detected in 48.6% of questing *Ixodes ricinus* ticks. Of the positive specimens, 62% could be identified to species level and all others could only be identified to group level (small rodent, birds, artiodactyl). A probe for the detection of *Borrelia* species bacteria was used in the reverse line blot and was found in 21.1% of the ticks and the host blood was detectable in 65.2% of these ticks (Humair et al. 2007). This technique is valuable because of the ability to test multiple species and pathogens simultaneously with a high level of sensitivity.

Reverse line blot utilizes species specific probes that can bind recognizable DNA sequences to a blotting membrane. Using a streptavidin complex, the source DNA will bind to a probe and emit a glowing light that can be visualized on x-ray film. The advantage of this technique is that multiple samples can be tested against multiple probes
in a single experiment. Reverse line blot is becoming a standard molecular tool for
diagnostic and epidemiological studies especially in the field of tick borne disease.
Reverse line blot has been used primarily in studies involving identification or typing of
microorganisms including bacteria, viruses, and parasites. In tick borne disease this
technique has been utilized for research into blood meal analysis when studying Lyme
disease as well as pathogen typing within a single tick to detect the host source of an
infected tick. The reverse line blot method involves covalent coupling of
oligonucleotide probes that have an amine label that interacts with the carboxyl-activated
membrane. This is followed by the hybridization of PCR products to the corresponding
probe which is detected by a streptavidin labeled peroxidase and chemiluminescent
substrate (Agtervels and Zwart, 2002; Kong et al. 2006). The membrane can facilitate
the conjugation of 43 probes with 43 samples in a single blot and each blot can be
stripped and reused up to 20 times (Kong et al. 2006). The system, once established, is
more flexible and inexpensive as compared to other systems (Kong et al. 2006). No
technique satisfies every diagnostic requirement for an ideal system. Sensitivity,
specificity, discriminatory power, turn-around time, reproducibility, performance ease,
and cost are all components that have variable weight with each molecular tool.

**Artificial Feeding**

Without knowing the source of a host blood meal we cannot be confident that our
detection methods are accurate. Artificial feeding of ticks in the laboratory provides a
means for investigators to reliably know what host species the tick fed upon last and
when they became engorged. Using PCR on ticks that have been artificially fed can
provide a positive control for blood meal analysis in *Ornithodoros coriaceus*. Also
artificial feeding can be utilized to determine how long host DNA can be accurately
detected within the tick gut.

Maintaining ticks in the laboratory is well documented for *Ornithodoros* species. In the study of African swine fever (ASF), *Ornithodoros moubata*, the vector for ASF, has been successfully maintained in the laboratory and have been artificially fed through a variety of methods. Ticks have been maintained in vials, bell jars, Petri dishes, and other holding chambers, often kept in an incubator to maintain humidity and temperature levels preferable to the specific tick species (Endris et al. 1986).

Temperature and humidity are critical components to tick maintenance due to the rapid desiccation of the tick body at higher temperatures and because of the specific temperature and humidity requirements for egg laying in females. Ticks are able to maintain body fluids and live for long periods of time when kept at temperatures below 50 ºC but rapidly desiccate at temperatures at or above 60 ºC (Garcia 1957). High humidity is critical for egg development because the eggs desiccate easily at relative humidity levels of less than 75% (Osborne et al. 1985). In the maintenance of *Ornithodoros moubata*, the ticks were maintained at 27ºC and a relative humidity of 85+/-5%, which was found to avoid tick desiccation and still facilitate egg laying. Using incubators to keep the temperature around 27ºC and 85% humidity has become a standard when maintaining *Ornithodoros* species (Osborne et al. 1985; Schwan et al. 1991).

Ticks have been fed in a laboratory setting on live animals such as mice, hamsters, rabbits and monkeys (Herms 1916; Endris et al. 1986; Osborne et al. 1985). Artificial methods utilizing membranes have been used to eliminate the need for live hosts. Membrane feeding techniques provide the ability to feed in vitro with controlled
host selection, at known time intervals. Feeding methods using animal skins, gut tissue, parafilm, or silicone membranes have been developed (Galun et al. 1965; Osborne et al. 1985; Endris et al. 1986). The use of silicone membranes over other procedures has been shown to be advantageous for sustainability, cost effectiveness, and convenience (Osborne et al. 1985). When using silicone membranes, to feed *Ornithodoros moubata* investigators found that up to 85% of ticks fed to engorgement (Osborne et al. 1991).

Schwan et al, 1991 found feeding *O. moubata* on live animals to be expensive and did not obtain satisfactory results but when they used heparinized bovine blood, not only could hundreds of ticks be fed simultaneously, the average feeding rate was between 80-100% feeding to repletion. Heparinized, defibrinated, and haemolyzed blood obtained from multiple host species did not appear to influence tick feeding behavior or reproduction (Schwan et al. 1991). The methods used by Osborne et al., 1985 were adapted to artificially feed *Ornithodoros coriaceus* by using organdy cloth and a silicone membrane matrix (Chen et al. 2007). By spreading silicone over the cloth and placing rabbit hair onto the membrane prior to drying, the apparatus was used to feed *O. coriaceus* by simulating a live animal, using bovine blood collected from cattle. The blood was warmed to 37°C prior to feeding and was performed in a humidified incubator containing 5% CO₂. *Ornithodoros coriaceus* showed efficient attachment and engorgement using this method (Chen et al. 2007).

**Conclusion**

To completely understand a disease, the life cycle of the causative pathogen is essential for future planning and control of that disease. Due to the lack of knowledge concerning the etiologic agent of epizootic bovine abortion a more complete picture is
necessary to direct future research efforts regarding this disease. The only identified vector for the disease is *Ornithodoros coriaceus*. The tick vector is known to feed quickly and preferred hosts are only speculative. Research into the feeding habits of other arthropod vectors has provided some insight into other tick borne diseases, such as Lyme disease. Molecular techniques provide the opportunity to indirectly sample host DNA from the undigested blood meal of tick vectors. By utilizing these techniques, we may clarify *Ornithodoros coriaceus’* host associations and provide a future direction into researching epizootic bovine abortion.
INTRODUCTION

The disease epizootic bovine abortion is a source of economic concern for the beef cattle industry in the western United States. Epizootic bovine abortion (EBA) is a tick borne disease that causes late term abortion in cattle resulting in the loss of an estimated 5-10% of the beef cattle crop annually from one study in California (Maas 1996). The only identified vector for the EBA pathogen is the soft tick, *Ornithodoros coriaceus* (Schmidtmann et al. 1976, King et al. 2005). *Ornithodoros coriaceus* is found in habitats within the Sierra Nevada Mountains in California and Nevada, in southern Oregon and has been reported in Mexico and Idaho (BonDurant and Anderson 1997; Hall et al. 2002; Howarth et al. 1956; Traum and Hart 1923). The geographic distribution of the tick appears to mirror that of the disease (Kennedy et al. 1960; Kimsey et al. 1983). Little is known about the persistence of the pathogen in the wild, except for the ability of the tick vector to transmit the bacteria and the capacity for infectivity in cattle, although it is unknown if cows can serve as reservoir host (Schmidtmann et al. 1976; Stott et al. 2002; Chen et al. 2007).

*Ornithodoros coriaceus* is a hematophagous arthropod that feeds very rapidly, usually feeding for less than an hour on a particular host before dropping off into the environment to molt or lay eggs. The preference of a host by a vector species has been shown to play a key role in the maintenance of a disease as well as playing a role in that host’s competence as a reservoir (Brunner et al. 2008). Determining the preferred hosts of *Ornithodoros coriaceus* has been complicated by the limited time a tick is attached to a host for feeding. Due to the difficulty of finding engorged ticks on a host animal, there is little known about the host preferences of this tick species except through its
association with deer and cattle bedding sites and inferences have been made to suggest the tick feeds primarily on these species (Furman and Loomis 1984; Garcia 1963; Smith 1944). Although these speculations exist, no conclusive evidence has been collected to test this hypothesis.

A variety of molecular techniques such as enzyme immunoassays, precipitin tests, and polymerase chain reaction have been used to detect host DNA in fresh arthropod blood meals in which the host DNA has not been broken down by the digestive system (Tesh et al. 1971; Clausen et al. 1998; Van Den Hurk et al. 2007). In host seeking ticks, often the blood meals are old and degraded due to intermittent feeding periods. In hard ticks, the tick molts between feedings, and can go for an extensive periods without a meal. Although this is also the case in soft ticks, multiple blood meals can be taken between molts and DNA may be less degraded due to phagocytosis by the digestive cells and their intracellular digestive process (Kent 2009). The soft tick digestive tract has evolved to facilitate long periods of starvation, when hosts are not available. Therefore, the tick digestive system relies on slow, intracellular processes and blood can remain undigested for extensive periods of time (Sonenshine 1991). Consequently, the host blood is partially digested and small fragments of the DNA may remain intact as targets for amplification. Mitochondrial genes have been reliable targets for vertebrate identification in blood meal analysis in arthropod vectors (Kirstein et al. 1996; Van Den Hurk et al. 2007; Humair et al. 2007). Mitochondrial genes are a robust tool when working with degraded DNA from a blood meal because mitochondrial DNA (mtDNA) is found in high concentration in cells (Tobe et al. 2008). Depending on the tissue type, an average of 107 mitochondria per cell and 1 to 15 mtDNA per mitochondrion may exist
(Satoh et al. 1991) and mitochondria compromise between 15-20% of the total cell volume (Tyler 1992). Additionally, mitochondrial DNA evolves 5-10 % times more quickly than nuclear DNA (Castro et al. 1998) and therefore has a higher resolving power between taxonomic categories making mitochondrial markers useful for host identification purposes (Kent 2009).

In previous research, polymerase chain reaction in combination with southern blots, restriction fragment length polymorphism (RFLP), and reverse line blot has been used to identify host DNA in tick species. The European vector for Lyme disease, *Ixodes ricinus* has been studied using mitochondrial markers from the 12s rDNA, 18s rDNA, and cytochrome b genes to detect host DNA in questing nymphal ticks (Kirstein et al. 1996; Pichon et al. 2003; Humair et al. 2006).

Three genes with published primers were selected from previous blood meal identification tick studies to test in *Ornithodoros coriaceus*. Degenerate cytochrome b primers, nondegenerate 12S primers, and a 18S universal primer were used to amplify vertebrate DNA within a tick host. These techniques have not been previously tested in soft ticks and its use could elucidate the host species preferences of *Ornithodoros coriaceus*, as well as other ticks in the genus *Ornithodoros*. This study was designed to determine the length of time host DNA can be detected and accurately identified in the gut remnants of *Ornithodoros coriaceus* to be used in a system for host identification studies of field caught ticks. The objectives of this study were to determine if mitochondrial primers can target and amplify host DNA and be used to identify host animals to the species level from collected *Ornithodoros coriaceus*. 
MATERIALS AND METHODS

Tick Collection and Maintenance

*Ornithodoros coriaceus* Koch ticks were collected, to be used in host DNA studies, using dry ice bait traps as previously described (Garcia et al. 1963) during the summer months (May-September 2006-2008) in the foothill regions of California and Nevada (Table 1). Cubes of dry ice were placed on a patch of cleared ground to provide a source of carbon dioxide (CO$_2$) as an attractant. Dry ice baits were placed beneath trees that could serve as potential bedding sites for animals. Ticks were collected with forceps while they approached the ice and then placed into conical tubes until transferred into Petri dishes in the lab. Species identification was confirmed in the laboratory by visualization under a microscope. *Ornithodoros coriaceus* tick species were confirmed by the presence of two pairs of eyes, as it is the only species in this genus in the western hemisphere with this feature, and identified to sex and life stage by observance of the genital pore, which is distinguishable between males and females and undeveloped in nymphs (Furman and Loomis 1984; Teglas 2005). The ticks were stored in clear plastic containers, filled with autoclaved sand, at 28°C and 80% relative humidity until the time of DNA extraction.

Artificial Feeding

Field collected ticks used in host identification and duration studies were fed on bovine blood using modified methods from Chen et al. (2007). A square piece of tulle material was cut to fit over a 100 ml glass beaker. The tulle was placed onto parafilm and a thin layer of silicon sealant was spread evenly over an 8 cm diameter region. Yak, rabbit, or sheep fur was placed on the wet silicon and left to dry overnight. To create the
feeding apparatus, defibrinated bovine blood (Haemostat Laboratories, Dixon, CA) was warmed to 37 °C and placed in a 10 centimeter Petri dish. The ticks were placed into a 250 ml beaker, the tulle was secured onto the top of the beaker using a rubber band, with the fur facing the ticks and the beaker was inverted on top of the warmed defibrinated blood in the Petri dish. Parafilm was wrapped around the edges to prevent blood from leaking into the apparatus. The apparatus was then placed in an environmental chamber at 28° C and 80-85% humidity for 2 hours, to allow the ticks’ time to feed. Post-engorgement, ticks were placed into a Petri dish lined with filter paper for the initial coxal fluid release, then placed into storage containers in the incubator until the time of DNA extraction. At the initial stages of the experiment, ticks were weighed before and after feeding to determine if they were taking a blood meal. Once it was established that the ticks were willing to feed with this method, weight change was no longer taken and only visual conformation of engorgement was used to include a fed tick in the study.

**Duration Study**

To determine the length of time a blood meal can be detected in *Ornithodoros coriaceus* ticks, field collected ticks were separated into sex and life stage after completing a blood meal, and stored in an incubator until the time of extraction. DNA was extracted from fed ticks at intervals from day 1 to day 325 post engorgement (Table 2). Ticks of both sexes and all life stages were not included during each sampling date due to tick death or because fed ticks were not equally represented for that sampling time. Nymph ticks were in greater abundance during collection and therefore have a higher representation in the study. Ticks were only included if they took a blood meal, and with
a low number of males and female ticks attempting to feed, we did not have a high sample number of adults, that actually took a blood meal, to be used in the study.

**Genomic DNA Extraction**

Prior to DNA extraction, live ticks were washed in phosphate buffered saline (PBS) and brushed to remove excess dirt three times and then frozen for 20 minutes at -20°C. Genomic extractions were performed using the DNeasy tissue kit according the manufacturer’s instructions (Qiagen, Chatsworth, CA). Ticks were cut in half from anterior to posterior end and placed into a tube for digestion. Ticks were digested with Buffer ATL with 2 mg/ml final concentration of proteinase K at 56°C overnight. Ticks were left to digest overnight to lyse all internal tissues, including the gut. The lysis digestion does not break down the exoskeleton, which was left in the original tube when the DNA suspension was transferred to the spin column.

Vertebrate DNA was collected from local species within the Sierra Nevada Mountains either from fresh tissues of deceased animals or from frozen samples in storage at the University of Nevada, Reno. Tissue was collected from organs from fresh samples and from the ear or tail of frozen samples. Genomic extractions were performed using the DNeasy tissue kit according the manufacturer’s instructions (Qiagen, Chatsworth, CA). Tissues were left to incubate in lysis buffer overnight to ensure proper digestion. Vertebrate DNA was used for sequencing and probe design for reverse line blot. For mammal DNA extraction, ~200 µl of whole blood or ~25 ng of tissue were treated with the DNeasy tissue/blood kit according to manufacturer’s instructions (Qiagen, Chatsworth, CA). Extractions were performed according to the DNeasy standard protocol and DNA extracts were eluted in 200 µl of elution buffer. Double
stranded DNA was quantified using a fluorescent nucleic acid stain (PicoGreen®) and read on a Labsystems Fluoroskan Ascent fluorescence plate reader. Extracted genomic DNA was stored at -20º C until PCR analysis.

**DNA Amplification**

Vertebrate DNA was amplified with polymerase chain reaction using three vertebrate mitochondrial markers to assess the discriminatory ability of each marker to be used as an identifier for host DNA within the tick gut (Table 3). DNA from ticks fed on bovine blood were also amplified with PCR, using each gene to determine if the markers could identify the source of the blood meal after ingestion. Published primer sets from (Kirstein et al., 1996-cytb, Pichon et al., 2003-18s, and Humair et al., 2007-12s) each gene was used to amplify vertebrate DNA and host DNA in tick guts (Table 3). Primer sets cyt1 and cyt3 (638-bp), cyt1 and 2cyt6 (236-bp), and 2cyt1 and 2cyt6 (134-bp) were used to amplify regions of the cytochrome b gene. Primer set 18s-0033 and 18s-0049 (150-bp) were used to amplify regions of the 18s rDNA gene and primer sets 12s-12F and 12s-13R (~600-bp) and 12s-6F and 12S-9R (~145-bp) were used to amplify regions of the 12s rDNA gene. For each reaction, ten to twenty ng of DNA was used in a 30 µl PCR reaction which included 2 µM of each primer, 200 µM dNTP, and 1 U Titanium taq and 1X Titanium buffer containing 3 mM MgCl₂. PCR parameters for cytochrome b were 95 ºC for one minute followed by 33 cycles of 95ºC for 30 seconds, 55 ºC for 30 seconds and 72 ºC for 30 seconds followed by a 5-minute final extension at 72 ºC. PCR parameters for 18s were 94ºC for four minutes followed by 50 cycles of 94ºC for 30 seconds, 59 ºC for 30 seconds and 72 ºC for 30 seconds followed by a 5-minute final extension at 72 ºC. PCR parameters for 12s-12F and 12s-13R were 94 ºC
for one minute followed by 25 cycles of 94°C for 30 seconds, 60°C for 30 seconds and 72°C for 30 seconds followed by a 5-minute final extension at 72°C. A touch down PCR was used for 12s-6F and 12s-9R: with an initial denaturation for 3 minutes at 94°C, the extension is a single cycle at 94°C for 20s, 60°C for 30s, and 72°C for 30 s, followed by 20 cycles of 94°C for 30 seconds, with the annealing temperature decreasing from 60°C by 1°C for 8 cycles until the temperature reaches 52°C at which point the annealing temperature will hold for the remaining 12 cycles, then 72°C for 30 seconds followed by a 7-minute final extension at 72°C. Amplification products were visualized using a 1.5% agarose gel, containing ethidium bromide.

A nested PCR was used to increase the sensitivity for the detection of host DNA in tick blood meals for use in a reverse line blot. Nested reactions were performed in a total volume of 30 µl including 2 µM of each primer, 200 µM dNTP, 5 µl of product from the initial PCR, and 1 U Titanium taq and 1X Titanium buffer containing 3 mM MgCl₂. Thermocycling conditions were the same for nested PCR reactions as described for the initial PCR. Primers 2cytb1 and 2cytb3 were used to amplify a 368-bp sequence from the 638-bp PCR product, 2cytb1 and 2cytb5 were used to amplify a 116-bp sequence from the 236-bp PCR product, and 3cytb1 and 2cytb5 were used to amplify a 95-bp sequence from the 134-bp PCR product. All primers were biotinylated for use in reverse line blot (Pall, Ann Arbor, MI). A positive control of both bovine and deer DNA were included for each PCR run and RLB. Contamination was monitored by inclusion of a negative control of sterile water that did not contain DNA but was subjected to the same protocol as the other samples. To eliminate contamination DNase and bleach was used to clean the workspace after each PCR, filter tips and tubes were used, and PCR water was
used in each PCR run. Amplification products were visualized via electrophoresis using 1% agarose gel.

**Sequencing**

Amplified PCR products from the 638-bp fragment of the cytochrome b gene (cytb1, cytb3), the 150-bp product of the 18s gene (18s-0033, 18s-0049), and 600-bp product of the 12s gene was purified using a Qiagen MinElute filter plate on the Qiagen BioRobot 3000 then sequenced using the ABI Bigdye Terminator Cycle Sequencing Ready Reaction Kit v3.1 and run on the ABI 3730 DNA Analyzer at the Nevada Genomics Center, Reno NV. Sequences were accepted if over 80% of the nucleotides had a Phred quality rank above the q20 reference line. Sequences were compared to matching results on the GenBank database using FASTA and BLAST programs to identify source DNA.

**Probe Design**

Vertebrate DNA from known animal sources was sequenced using the cytb1 and cytb3 primers and the 12S-12F and 12S-13R primers. Forward and reverse sequences were obtained for vertebrate DNA for bovine, deer, and small rodents. DNA sequences were aligned with Geneious software (Biomatters Ltd., Auckland, New Zealand) and probes were designed from species-specific variable regions of the cytochrome b gene and 12S gene, within the target amplicon (Table 4, Table 5). Applicable probes from a published source were also used for the 12S gene (Humair et al. 2007). Each probe was made from a unique fragment, ranging from 16-26 bp in length with 40-50% GC content. Each probe was modified with a 5’ amine group to facilitate covalent linkage to the
biodyne C membrane. Both sense and antisense probes were developed and tested to increase specificity and sensitivity.

**Reverse Line Blot Hybridization**

The reverse line blot technique was used for the identification of host DNA based on the RLB protocol developed by Agterveld and Zwart (2004). The membrane was washed in 16% 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDAC) for 10 minutes to activate the membrane then rinsed in tap water. Species specific probes were diluted in 500mM NaHCO₃ to 1000 pmol/µl then they were covalently linked to an activated Biodyne C membrane (Pall, Dreieich, Germany) by a 5’ amino linked modification. The membrane was placed into a miniblotter system (Immunetics, Cambridge, NA), and 150 µl of the biotinylated probes were filled in line slots and left for a brief incubation (1 minute at room temperature). The oligonucleotide solutions were removed from the miniblotter in the same order they were loaded in, and the blot was incubated in 100mM NaOH for 8 minutes at room temperature. The membrane was then washed in 250 ml of 2x SSPE/0.1% SDS for 5 minutes at 60°C in a rolling bottle incubator. The membrane was placed into the miniblotter and rotated perpendicular to the placement of the oligonucleotide probes after a second wash for 5 minutes at room temperature in 250 ml of 2x SSPE/0.1% SDS. The slots were then filled with biotinylated heat denatured (99°C for 10 minutes) PCR products, brought up to 150 µl in 2x SSPE/0.1% SDS, then incubated for 60 minutes at 42°C on a horizontal service. The PCR products were removed and the membrane was washed twice in 250 ml of 2x SSPE/0.5% SDS for 10 minutes at 52°C in a rolling bottle. The membrane was then washed in a 1:4000 diluted streptavidin-peroxidase conjugate (Roche diagnostics, Rotkreuz, Switzerland) in 2x
SSPE/0.5% SDS for 45 min. at 42°C. The membrane was washed twice in 250 ml of 2x SSPE/0.5% SDS for 10 minutes at 42°C then twice in 250 ml of 2xSSPE buffer for 5 minutes at room temperature. The membrane was then incubated for 1-2 minutes with enhanced chemiluminescence detection liquid (Roche diagnostics, Rotkreuz, Switzerland) and exposed to X-ray film in an X-ray cassette for 1 to 30 minutes depending on the signal. Films were developed in an automated film processor and results were recorded by visual observation of the films. A positive result was indicated by a solid black dot on the x-ray film, in the appropriate location from chemiluminescence caused by the DNA adhering to the probe on the blotting substrate.
RESULTS

Tick Collection

The presence and abundance of *Ornithodoros coriaceus* has not been thoroughly studied in Nevada and the exact distribution of tick populations throughout the state is still unknown. The distribution of *Ornithodoros coriaceus* has been better documented in California and has been reported in studies of epizootic bovine abortion in Nevada. Twelve sites were selected that fit the approximate elevation and vegetation standards for *O. coriaceus* habitat, and each site was surveyed for the presence of *Ornithodoros coriaceus* (Figure 1). Of the twelve sites, ticks were collected from six sites along the Nevada/California border, totaling 4 sites in California and two sites in Nevada (Table 1). In total, 282 ticks were analyzed for the detection and identification of host DNA.

Of the laboratory fed ticks, 98 were analyzed to determine the length of time host DNA could be detected and identified with polymerase chain reaction, using three mitochondrial markers, and reverse line blot. The remaining ticks (184 field-collected) (Table 6) were analyzed for the detection and identification of bovine and deer DNA amplifying the cytochrome b gene with polymerase chain reaction and reverse line blot.

Artificial Feeding

*Ornithodoros coriaceus* ticks readily took a blood meal through the parafilm membranes and required no further tactile stimuli beyond animal fur attached to the feeding membrane. When weighed, the mean weight gain for female ticks was 0.264 g (SD = 0.235 g), male ticks was 0.032 g (SD = 0.022 g), and for nymphs mean weight gain was 0.044 g (SD = 0.048 g) (Table 7).
Vertebrate DNA Amplification and Characterization of Molecular Markers

Three mitochondrial markers were chosen from previous blood meal analysis studies of host DNA in tick vectors, to find a mitochondrial gene that is sufficiently heterologous to be used as a discriminatory marker for RLB probe design in host DNA identification studies in *Ornithodoros coriaceus*. Polymerase chain reaction products of the cytochrome b gene, 12S rDNA gene, and the 18S rDNA gene were amplified for 22 mammals, 1 bird, and 1 reptile, and when possible, samples were taken from multiple individuals from a single species (Table 8). Due to the presumption that *O. coriaceus* prefers cattle and deer hosts, multiple individuals were sequenced and used for probe design of *Bos taurus* (bovine) and from the *Odocoileus* (deer) genus, to account for possible intraspecies genetic variability. All three vertebrate primers amplified DNA from each species except for the horse, which was from poor quality DNA. Each sample that had a strong banding pattern on gel electrophoresis was sequenced. The 18S marker was non-discriminatory and sequences could only be characterized to the phylogenetic class level using a BLAST search. Both cytochrome b (638-bp) and 12S (~600-bp) genetic markers could accurately identify each sample to the species level in a BLAST search. Although analysis of the 12S marker determined the correct species within the search results, a BLAST search also returned results of many closely related species, with either the same or close to the same percentage of matching nucleotides. Therefore, the 12S marker could only be used to accurately identify unknown DNA to the family level. Sequences for cytochrome b and 12S were used to design probes for reverse line blot.

Amplification of the smaller DNA fragments of the cytochrome b gene were analyzed with PCR and gel electrophoresis to determine if a smaller gene fragment target
provided better amplification of host DNA in the ticks. DNA extractions from artificially fed ticks were tested on each cytochrome b primer pair. Nested PCR of the cytochrome b gene was consistently contaminated and did not provide good DNA amplification for any of the smaller fragments (368-bp, 116-bp, and 134-bp). The 236-bp (cytb1, 2cytb6) fragment of the cytochrome b gene was small enough to amplify the fragmented host DNA but large enough to eliminate contamination issues. Primers cytb1 and 2cytb6 were used for amplification of host DNA within the fed ticks and was the region used for probe design.

**Design of the Vertebrate Specific Probes**

DNA from 4 deer and 8 bovine animals were amplified for sequencing along with many other native species from the western U.S. for comparison to bovine alignments in the probe design. For each species, a unique PCR amplicon of ~600-bp was sequenced and aligned to design species-specific probes. Twenty six cytochrome b probes were designed; fifteen bovine probes and 11 deer probes were tested for hybridization in bovine, deer, sheep, pig, reptile, squirrel, and mouse by reverse line blot (Figure 2). The probes that demonstrated hybridization were B17, B27, B18, B19, D3, D4, and D5. Probe B17 hybridized to bovine species only, B27 hybridized to bovine and showed slight hybridization to sheep, and B18 and B19 were nonspecific and hybridized to bovine, sheep, deer, reptile, squirrel, and mouse DNA. Probe D3 hybridized to deer, squirrel, and mouse. D4 was the only deer probe to hybridize with deer only. D5 was nonspecific and hybridized to all mammals, as well as the negative control (water), and was eliminated from the experiment. Probes for the 12S gene were designed from previously published probes for mammal, rodent, artiodactyls, bird, and lizard (Humair et
Probes for *Odocoileus spp.*, *Bos spp.*, and *Canis spp.* were designed for this study from collected samples and sequences aligned with the Geneious software (Table 5). Samples were run in a PCR with the 145-bp 12S gene primers and run on reverse line blot against the 12S probes. The samples included bovine, dog, rat, pocket mouse, jack rabbit, quail, ovine, and deer and were run against all 9 probes (Figure 3). The *Bos*, bird, artiodactyl, *Odocoileus*, and lizard probe did not show hybridization to any samples. The *Lepus* probe hybridized to all 8 vertebrate samples. The mammal probe hybridized to bovine, rat, mouse, rabbit, and sheep DNA but not to dog, deer, or quail DNA. The rodent probe hybridized to rat DNA only. To test the 12S reverse line blot system on tick extractions, the probes were run with 4 concentrations against 43 samples of bovine fed ticks (Figure 4). The mammal probe hybridized at 125 pmol/µl and 500 pmol/µl better than 1000 pmol/µl or 200 pmol/µl but only hybridized to 10 samples. The rodent probe hybridized to 3 samples at 1000 pmol/µl and 2000 pmol/µl. The artiodactyl probe hybridized to all samples at 125 pmol/µl and at the highest concentration, 2000 pmol/µl but seemed to have bled over because there were 49 blot markings when there were only 45 samples. The bird, *Bos taurus*, lizard, *Odocoileus*, and IDT Rodent probes showed no hybridization. The *Canis* probe showed hybridization to 24 samples at all concentrations. The *Lepus* probe hybridized strongly to 10 samples and had weak hybridization of 3 samples at all concentrations. The IDT artiodactyls probe hybridized to 28 samples at all concentrations.

**Tick Host DNA Identification**

Following validation of the efficacy of cytochrome b as a discriminatory molecular marker for species identification, the utility of each marker was tested by PCR
amplification of host DNA from *Ornithodoros coriaceus* gut contents, fed on a known host, *Bos taurus*. A product from the cytochrome b gene (236-bp) was amplified from adult and nymphal *O. coriaceus* ticks which had engorged on bovine blood. Ticks were analyzed for host DNA using bovine and deer specific probes in a reverse line blot (Figure 5). To determine how long a host blood meal can be detected, ticks were analyzed from day 7 post-feeding to day 325. Host DNA could be detected and correctly identified in RLB in all ticks from day 7-133. Bovine DNA could be detected and identified in 92.9% of the ticks tested 175 days post feeding and in and in 40% of ticks on day 196. By day 207 the percentage of ticks that contained identifiable host DNA dropped to 26.7% and by day 215 the signal could no longer be detected. The signal from probe B17 became much fainter after day 84 post feeding and was consistently faint from day 133 to day 207, until the signal faded completely. The signal from probe B27 was stronger due to greater luminescence until day 84 and day 133, at which point the signal grew fainter than previous samples but still showed greater luminescence than probe B17. The signal was strong in 9 out of 14 individuals for day 175 but only for half of individuals from day 196. By day 207 the signal was either represented by a strong positive or a negative signal.

Field collected ticks were amplified with the cytochrome b gene and bovine and deer probes were hybridized to the samples with reverse line blot. Ticks from Wellington Range, Mono Valley, and Bodie did not show hybridization to any of the probes. The bovine positive control showed strong hybridization to both B17 and B27 but the D4 probe did not hybridize to the deer control and therefore the tick samples may not have hybridized due to the probe binding nonspecifically (Figure 6). The ticks collected from
Alkali Lake, Hallelujah Junction, and Virginia City showed some hybridization but the positive controls did not consistently hybridize to the correct species and the negative water control faintly hybridized to the B17 probe and stronger to the B27 and D4 probes (Figure 7a, Figure 7b). The 6 blots that tested the probes for the plate containing samples for Alkali Lake, Hallelujah Junction, and Virginia City showed strong hybridization to all samples in a single blot and then another blot showed no hybridization (Figure 7c, Figure 7d).
DISCUSSION

This paper evaluates the use of three vertebrate mitochondrial markers for the amplification of host DNA in the gut contents of the soft tick, *Ornithodoros coriaceus* the vector for epizootic bovine abortion. The methods consisted of targeting mitochondrial genes used in previous tick studies, to assess the applicability of these markers in host blood meal analysis of *O. coriaceus*, which has not been previously tested. Of the three genes (18S rDNA, 12S rDNA, cytochrome b), the 638-bp cytochrome b marker provided the most valuable sequences, from vertebrate species, for designing probes for reverse line blot. Molecular identification of host DNA in the tick gut contents was achieved by targeting the 236-bp cytochrome b gene in ticks fed in a laboratory setting on a single host source. The system could detect bovine DNA in all ticks fed on bovine blood, with a strong luminescent signal for 77 days post feeding. Although the luminescent signal was faint, the system could detect host DNA in most ticks for 175 days post-feeding before decreasing to only 40% of the samples on day 196. Host identification of fed ticks was similar to other studies that showed detection levels between 90-200 days post-feeding, with similar sized markers that amplified fragments between 145-236-bp (Kirstein et al. 1996, Pichon et al., 2003). The 18S rDNA mitochondrial marker appeared to be too highly conserved and lacked enough variability be used as a discriminatory marker for species identification, which is consistent with previous studies, and could only detect host subgroups to either the suborder or the family but not genera or species (Pichon et al. 2003). There was a low level of sequence variability of the 18S marker and the sequences did not provide enough variation for effective probe design for species identification for use in this study. The 12S mitochondrial marker was
heterologous enough to provide species differentiation but due to the small amplicon size (145-bp) there were limited regions of variability for probe design. Genetic amplification using published probes of the 12S gene could not be duplicated in *O. coriaceus* and therefore did not provide host DNA identification in reverse line blot.

Each marker amplified a different size fragment of the target gene, which influenced the ability of the primer to provide enough sequence template to be used to design species specific probes for identification purposes in RLB. The 18S universal primers amplified a 150-bp fragment which included a variable region specific to subgroups of vertebrates, and could have been useful if the sequencing included the variable region. Although the small amplicon was very sensitive, it did not always include enough sequence template and would often exclude the variable region. A BLAST search was unable to differentiate between species from the 18S nucleotide sequences. External primers to this region could be helpful in utilizing these markers as an initial detection of host families in studies of ticks, like *O. coriaceus*, where very little is known about the host source. Unlike the 18S marker, the 12S marker amplified a larger 600-bp fragment of the 12S rDNA gene, which was external to the 145-bp fragment that was used to amplify the degraded host DNA in the tick extractions for both artificially fed ticks and field caught ticks. When the 600-bp 12S amplicon was sequenced the internal 145-bp region was always within the nucleotide sequence and probes were designed within that 145-bp region. The 145-bp fragment included multiple variable regions within the amplicon which provided useful sequence template for probe design. Although the 12S marker provided enough template, the 145-bp 12S fragment was difficult to work with because of periodic contamination issues with the PCR
reactions, which could be due to the small amplicon size. Contamination was often the case in previous studies utilizing the 12S primers that amplified the small 145-bp fragment and mammalian and human probes were eliminated from the RLB to control for human contamination (Humair et al. 2007). The cytochrome b primers also amplified a larger 638-bp fragment of different vertebrate species DNA which was used for sequencing. A variety of primers amplified smaller fragment (368-bp, 236-bp, 134-bp, 116-bp) within the 638-bp sequence which would have provided different regions for probe design from various amplicon sizes but due to contamination only the 368-bp and the 236-bp fragment could be used for probe design. Amplifying large fragments of DNA for vertebrate species, containing internal primers that amplified small fragment with variable regions, was effective for provided sequence template for probe design for the 12S gene and the cytochrome b gene.

Amplicon size is important in systems that utilize PCR markers for host detection in arthropod blood meals. An inverse relationship between the size of the amplicon and the effectiveness of the PCR exists in studies where degraded DNA is used as the template in PCR (Kirstein et al. 1996). Although this has been shown to be the case, contamination can become a problem when the marker amplifies a very small target, as was shown with the nested cytochrome b primers in both this study as well as in previous research (Pichon et al. 2003, Humair et al. 2007). Amplicon size is crucial to PCR success when applied to field caught Ornithodoros ticks. Generally, ticks can only be collected when they are searching for a host, and therefore are often depleted and in need of a blood meal, leaving very little intact DNA for analysis. Although Ornithodoros ticks can go months without feeding, host DNA is present longer than in other arthropods
because the digestive cells in the midgut phagocytize the blood meal and digestion occurs intracellularly (Tarnowski et al. 1989). Gradual digestion results in a low quantity of template DNA that is damaged and fragmented and primers can amplify only small fragments of DNA (Kent et al. 2005; Anderung et al. 2008). In this study, primers that amplified host DNA fragments that were smaller than 200-bp in length resulted in amplification of contaminating DNA in the PCR products. Nested PCR for the cytochrome b markers could not be used because even when the fragments were greater than 200-bp the second PCR was also consistently contaminated. Bands from contaminated PCR negative water controls were sequenced and run in a BLAST search. The contaminated sequences returned BLAST results showing either human, bovine, or junk DNA meaning that the BLAST search did not return any specific species identification. This showed that there was either human contamination from human error, possible contamination from previous experiments or other templates at the workbench. To eliminate contamination new PCR water was used for every experiment, filter tubes were used, new tips and tubes for each PCR run, each area was cleaned with bleach and UV light prior to the experiment, and new aliquots were used for each PCR. When this did not eliminate contamination of the PCR amplifying the 116-bp and nest 95-bp fragment of the cytochrome b gene, PCR of the 134-bp fragment was run using the same protocol. Contamination was eliminated but there was no amplification of host DNA within either field collected ticks or artificially fed ticks. When using larger fragments of the cytochrome b gene there was problems with amplification in the ticks. The primers that amplified the 638-bp and 368-bp fragments of the cytochrome b gene showed little or no amplification in the tick extractions from both artificially fed ticks and
field collected ticks. The primers amplifying the 236-bp fragment was the only PCR products that were not contaminated and still provided amplification although the PCR amplifying the 134-bp fragment eliminated contamination while not amplifying host DNA within the ticks. There was a fine line between finding a marker that would amplify fragmented host DNA within the tick while still eliminating contamination. Each primer set had to be small enough to amplify fragmented DNA and be large enough to eliminate contamination while working within the optimal condition to obtain maximum specificity and yield (Robertson et al. 1998).

PCR sensitivity is crucial when analyzing DNA in the gut contents of ticks because of the extensive time periods between feeding, which leaves the blood meal partially digested and the DNA severely degraded (Humair et al. 2007). Both degenerate and nondegenerate primers were used to provide flexibility in the adherence of the primer to the target sequence. Nondegenerate primers, such as those used to amplify the 12S gene fragments, were used to avoid mispriming and increase specificity (Humair et al. 2007). On the other hand, degenerate primers, like the cytochrome b primers, provided a means to decrease specificity while increasing sensitivity. The primers currently used in this study were selecting for either sensitivity or specificity but a sacrifice was made for one or other depending on the primer used.

Effective probe design needs to assure sensitivity, specificity, and consistency in order to hybridize appropriately to host DNA in blood meal analysis studies (Feng et al. 2007). By designing sense and antisense probes along the target gene sequence and extending the base pair length of the probes, we increased sensitivity and specificity. The initial probes designed for the cytochrome b gene were between 16-18 bp in length and
by extending the probe out to be between 20-24 bp in length we were able to detect host DNA using the B17 and B27 probes (Figure 9). The original shortened probes were B1 and B2 which did not show hybridization in screening experiments. With the 12S probes hybridization with multiple species DNA occurred indicating low specificity even though the primers were designed to increase specificity. Sequencing results from the 12S markers showed less variability between species which could attribute to the non-specific binding of probes designed from those sequences. The inability to repeat the results from the published probe sets from previous 12S experiments could be due to technique differences although the experimental guidelines were followed according to the authors published methods (Humair et al., 2007). The probes developed from this experiment were designed for many of the same species as in the current experiment and probes were tested against the same species except for DNA from the representative deer and bird species to test the Artiodactyl and bird probes. Targets within the amplicon, that provided enough variation to be species specific, were limited, and host identification studies would greatly benefit from further primer design of the 12S and cytochrome b mitochondrial markers to increase areas for probe design. Most blood meal analysis studies use primers that amplify the same region within the gene of interest limiting flexibility in primer and probe design. Designing primers, from mitochondrial genes, that amplify a larger portion of the vertebrate genome offer the possibility of finding variable regions flanked by conserved regions which provide sites for probe design.

Proper probe design is imperative for the reverse line blot technique to effectively identify vertebrate host DNA after PCR amplification and be of practical use in host identification studies. Whether vertebrate DNA is being tested directly or within
an arthropod vector, the entire process is worthless without efficient probes. The most challenging aspect to probe design is having template sequences from which to design probes that offer enough variability to be species specific. Due to digestion and degradation of DNA, amplicon sizes were small and provided limited sequence template for probe design for RLB, in this study. Designing probes for RLB requires consideration of reaction kinetics, base pair length, melting temperature, GC content, competition factors, and mutation (Kong et al. 2006). Probes should be between 18-30 bp in length, have similar reaction kinetics to your primers and other probes, possess high GC content, have a $T_m$ between 55-65 °C and be close to the RLB hybridization temperature, which was only 42 °C for RLB in this study. The $T_m$ of the primers and probes were not taken into account and the $T_m$ of the probes was not the same as the hybridization temperature in the RLB which could account for some of the problems with hybridization of the probes to the blotting membrane. Both antisense and sense probes should be designed to account for mutation and amplicon size of the target should be less than ~400-bp to limit competition with similar sequences up or downstream (Kong et al. 2006). By using previously published primers we were limited by the range of the amplification site of each primer, which was not designed primarily with probe design in mind. By having a larger sequence to work with it may be possible to meet the guidelines suggested by Kong et al. 2006. Probes should be designed to the family or genus level as a screening tool prior to developing species specific probes. With a broader taxonomic category, probe design is more feasible because the necessity for species variability is less stringent. Once a family or genus is identified then species specific primers can be developed to eliminate nonspecific binding of probes.
The reverse line blot technique has the potential to be a powerful tool for host identification in disease studies. The ability to design 43 different probes, for both vertebrate and pathogen DNA, and run them against 43 tick lysates, all in a single experiment is invaluable. It is also less expensive and more flexible than other methods, such as direct sequencing making it a good tool for epidemiology and diagnostic applications (Kong et al. 2006). We found the biggest drawback of this technique to be repeatability. Wash steps greatly influence the adherence of DNA to probes and variability of the chemiluminescent signal created differences in band appearance on X-ray film. Depending on exposure time, as was seen in the field caught ticks for Alkali Lake, Virginia City, and Hallelujah Junction, each blot had either complete hybridization or the lack thereof creating results that could not be accurately interpreted (Figure 7c, Figure 7d). DNA was placed in the miniblotter system and left to bind to the probes prior to multiple wash steps. Wash steps were meant to eliminate nonspecific binding but if hybridization of the species specific probes was weaker than other probes, bound DNA was likely to wash away and show a negative result in samples where a positive result was found in a previous run of the same experiment. Nonspecific DNA adherence to the blotting membrane became a problem as well, showing a positive result where there was a negative previously. Probes and samples did not always exhibit the same hybridization from one test to the next. Experiments were run multiple times with the same samples and never provided the exact same results from one run to the next.

Variation in exposure times could also have led to the differences in the visibility of a positive signal on the film. Each blot was exposed to X-ray film for multiple time periods to visualize both strong and weak luminescence and develop a
picture that could be used to analyze all probes and samples being tested. Times varied between 30 seconds to 30 minutes. The same blot would be exposed for an initial 30 seconds then for 30 minutes and depending on the signal, would be exposed to times in between to get a decent picture. Probe B27 had a much stronger signal indicating stronger hybridization than B17. When the blot was exposed for longer time periods to acquire a stronger signal from B17 then the signal from B27, which was next to B17 on the blot (Figure 5), would bleed into the other lanes creating an incoherent signal from both probes.

When fed ticks were maintained after engorging on bovine blood, DNA was shown to be too degraded for identification after 7 months and provided accurate identification only 40% of the time after 6 months post feeding. Hybridization decreased greatly after only three months and often times, only a faint signal could be detected. It is possible that greater exposure times may have increased signal detection but due to increased signal strength of other probes on the same blot, longer exposure caused over exposure of the strong signal which blurred the signal from other probes.

Field collected ticks were analyzed with PCR, amplifying the 236-bp cytochrome b marker, and cytochrome b probes B17, B27, and D4 in RLB for host identification of bovine and deer species. In the experiments that were not discarded due to contamination of the negative control or inefficient binding of the probe to the positive control, the tick samples did not show any hybridization to the bovine probes. In all experiments the RLB blots indicated that deer probes did not demonstrate proper hybridization and were not useful in determining whether the ticks contained vertebrate DNA from deer hosts. When extracted, most ticks were depleted and had probably not fed for a long period of
time. We cannot determine whether that time frame is beyond the level of detection of the cytochrome b probes used in the RLB, as demonstrated by the artificially fed ticks (Figure 5). Due to the inability to study these ticks in their natural habitat we are not aware of how long these ticks would typically go without a meal in a natural setting. Data from studies of artificially fed ticks survived without a blood meal for a year and anecdotal evidence has suggested that the ticks can live even longer without sustenance (Smith 1944; Garcia 1963).

Although we can not determine whether deer or cattle are a primary food source, it is possible the ticks are utilizing other sources of food from species that have not been thought of, as a host for O. coriaceus, in the past. In an unreported preliminary study to determine if we could detect the host DNA source by direct sequencing of a large fragment (638-bp) of the cytochrome b gene, we ran four 96 well plates of ticks collected from various sites in California and only 8 ticks amplified sufficient DNA to be sequenced. The sequencing results indicated that the ticks had been feeding on bovine blood and a rodent that was identified as Rattus rattus using a BLAST search. Due to the lack of hybridization of the bovine probes to the field collected ticks and the possible rodent association it is possible that O. coriaceus are not only feeding on large mammals but other host sources including small mammals, possible from the Muridae family or Rodentia order.

Polymerase chain reaction amplification of vertebrate host DNA with vertebrate mitochondrial genes is possible in Ornithodoros coriaceus ticks. Mitochondrial markers are capable of vertebrate DNA detection in soft ticks up to 6 months post feeding. Although host DNA can be identified for a relatively extended period of time, further
primer design may increase the sensitivity, which is necessary for host DNA detection in field caught ticks. Mitochondrial genes provide decent options for sequence variability of vertebrate species in which to design probes for use in reverse line blot and still have conserved regions for PCR primer design. Although the mitochondrial markers occasionally amplified host DNA in the tick gut there was considerable variability in both the sensitivity and specificity of each marker. Reverse line blot identified vertebrate DNA from artificially fed ticks for 207 days post feeding but was unable to provide consistent results. With minor modification and successful probe design this technique can be a valuable tool in future host identification studies but may not be as consistent as other techniques such as RFLP (Kirstein et al. 1996). Future studies combining PCR with RLB would greatly benefit from primer development offering greater areas for probe design as well as a thorough analysis of the effect of wash temperature on the hybridization in reverse line blot wash steps.
**Table 1.** Elevation and geographic coordinates for tick collection sites throughout Nevada and California where *Ornithodoros coriaceus* were collected and used in blood meal analysis and duration studies.

<table>
<thead>
<tr>
<th>Collection Site</th>
<th>Latitude</th>
<th>Longitude</th>
<th>Elevation (m)</th>
<th>County</th>
<th>State</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alkali Lake</td>
<td>41° 44.512</td>
<td>120° 12.223</td>
<td>1527</td>
<td>Modoc</td>
<td>CA</td>
</tr>
<tr>
<td>Bodie</td>
<td>38° 36.313</td>
<td>119° 27.259</td>
<td>2552</td>
<td>Mono</td>
<td>CA</td>
</tr>
<tr>
<td>Hallelujah Junction</td>
<td>39° 42.193</td>
<td>120° 01.256</td>
<td>1598</td>
<td>Sierra</td>
<td>CA</td>
</tr>
<tr>
<td>Mono Valley</td>
<td>38° 36.464</td>
<td>119° 27.797</td>
<td>2210</td>
<td>Mono</td>
<td>CA</td>
</tr>
<tr>
<td>Virginia City</td>
<td>39° 38.083</td>
<td>119° 64.278</td>
<td>1827</td>
<td>Storey</td>
<td>NV</td>
</tr>
<tr>
<td>Wellington Range</td>
<td>38° 36.217</td>
<td>119° 27.246</td>
<td>2438</td>
<td>Lyon</td>
<td>NV</td>
</tr>
</tbody>
</table>
Table 2. Numbers of ticks tested by sex, life stage, and extraction date following artificial feeding

<table>
<thead>
<tr>
<th>Extraction Day-Post Feeding</th>
<th>Nymph</th>
<th>Male</th>
<th>Female</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>15</td>
<td>5</td>
<td>1</td>
<td>0</td>
<td>6</td>
</tr>
<tr>
<td>21</td>
<td>2</td>
<td>1</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>28</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>30</td>
<td>4</td>
<td>0</td>
<td>1</td>
<td>5</td>
</tr>
<tr>
<td>35</td>
<td>3</td>
<td>2</td>
<td>0</td>
<td>5</td>
</tr>
<tr>
<td>42</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>70</td>
<td>3</td>
<td>1</td>
<td>0</td>
<td>4</td>
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<td>77</td>
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<td>3</td>
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<tr>
<td>84</td>
<td>2</td>
<td>0</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>133</td>
<td>2</td>
<td>0</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>175</td>
<td>7</td>
<td>9</td>
<td>1</td>
<td>17</td>
</tr>
<tr>
<td>196</td>
<td>1</td>
<td>2</td>
<td>2</td>
<td>5</td>
</tr>
<tr>
<td>207</td>
<td>7</td>
<td>7</td>
<td>1</td>
<td>15</td>
</tr>
<tr>
<td>215</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>225</td>
<td>2</td>
<td>2</td>
<td>1</td>
<td>5</td>
</tr>
<tr>
<td>247</td>
<td>4</td>
<td>0</td>
<td>1</td>
<td>5</td>
</tr>
<tr>
<td>273</td>
<td>3</td>
<td>2</td>
<td>1</td>
<td>6</td>
</tr>
<tr>
<td>280</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>287</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>325</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>1</td>
</tr>
</tbody>
</table>
Table 3. Oligonucleotide sequences of the primers used for PCR amplification of the cytochrome b, 18S rDNA, and 12S rDNA genes for detection of vertebrate DNA.

<table>
<thead>
<tr>
<th>Primer ID</th>
<th>Nucleotide sequence (5’-3’)</th>
<th>Gene Amplified</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>cytb1</td>
<td>b-CCATGAGGACAATACTCATTTCTG</td>
<td>Cytochrome b</td>
<td>Kirstein et al. 1996</td>
</tr>
<tr>
<td>2cytb1</td>
<td>b-GGMMTYTCAGTAGACAAAGC</td>
<td>Cytochrome b</td>
<td>Kirstein et al. 1996</td>
</tr>
<tr>
<td>3cytb1</td>
<td>b-ACCYTNACCYCATTTCGC</td>
<td>Cytochrome b</td>
<td>Kirstein et al. 1996</td>
</tr>
<tr>
<td>2cytb6</td>
<td>b-AWTCCCTGKGGGTTRT</td>
<td>Cytochrome b</td>
<td>Kirstein et al. 1996</td>
</tr>
<tr>
<td>2cytb5</td>
<td>b-GAKCCTGTYTCTGTGGAGGA</td>
<td>Cytochrome b</td>
<td>Kirstein et al. 1996</td>
</tr>
<tr>
<td>cytb3</td>
<td>b-GGGTTACTGTTTCCCGTCYCGGG</td>
<td>Cytochrome b</td>
<td>Kirstein et al. 1996</td>
</tr>
<tr>
<td>12s-6F</td>
<td>b-CAAACTGGATTAGATACC</td>
<td>12S rDNA</td>
<td>Humair et al. 2007</td>
</tr>
<tr>
<td>12s-9R</td>
<td>b-AGAATCCCTGTGTCTAG</td>
<td>12S rDNA</td>
<td>Humair et al. 2007</td>
</tr>
<tr>
<td>12s-12F</td>
<td>b-TGCCAGCCACCGCGGTCA</td>
<td>12S rDNA</td>
<td>Humair et al. 2007</td>
</tr>
<tr>
<td>12s-13R</td>
<td>b-AGGAGGGTGACCGGGCGGT</td>
<td>12S rDNA</td>
<td>Humair et al. 2007</td>
</tr>
<tr>
<td>18s-0033</td>
<td>TTCTAGAGCATTACATGCAGGA</td>
<td>18S rRNA</td>
<td>Pichon et al. 2003</td>
</tr>
<tr>
<td>18s-0049</td>
<td>YCGAGGGTTATCTAGAGTGCA</td>
<td>18S rRNA</td>
<td>Pichon et al. 2003</td>
</tr>
</tbody>
</table>
Table 4. Oligonucleotide sequences of cytochrome b probes used in RLB for
detection and identification of amplified vertebrate DNA.

<table>
<thead>
<tr>
<th>Probe ID</th>
<th>Sequence of 5' primary-amined oligonucleotides</th>
<th>Target Species</th>
</tr>
</thead>
<tbody>
<tr>
<td>B12R</td>
<td>GAGGCGGATTCTCAGTAGAC</td>
<td>Bovine</td>
</tr>
<tr>
<td>B17F</td>
<td>GATTCTCAGTAGACAAAGCAACCCTT</td>
<td>Bovine</td>
</tr>
<tr>
<td>B27F</td>
<td>CGGATTCTCAGTAGACAAGCAAC</td>
<td>Bovine</td>
</tr>
<tr>
<td>B18R</td>
<td>CCTTACCGATTCTTCCGTTTCCAT</td>
<td>Bovine</td>
</tr>
<tr>
<td>B19F</td>
<td>AAGGGTTGCTTTTGTCTACTGAG</td>
<td>Bovine</td>
</tr>
<tr>
<td>BR14F</td>
<td>AGAGAAGGGCTAGGGCTAGTAC</td>
<td>Bovine</td>
</tr>
<tr>
<td>B11F</td>
<td>GTCTACTGAGAATCCGCTCAG</td>
<td>Bovine</td>
</tr>
<tr>
<td>B1F</td>
<td>GTCTACTGAGAATCCGACC</td>
<td>Bovine</td>
</tr>
<tr>
<td>B2R</td>
<td>CGGATTCTCAGTAGAC</td>
<td>Bovine</td>
</tr>
<tr>
<td>B4R</td>
<td>CTTACCGATTCTTCGCT</td>
<td>Bovine</td>
</tr>
<tr>
<td>BR4</td>
<td>AGCGAAGAATCGGGTAAG</td>
<td>Bovine</td>
</tr>
<tr>
<td>B6R</td>
<td>CCATTATTCTCCATC</td>
<td>Bovine</td>
</tr>
<tr>
<td>BR6F</td>
<td>GGAAGGATAAAAATGG</td>
<td>Bovine</td>
</tr>
<tr>
<td>B7R</td>
<td>CTCAGTAGACAAAGCAACCCTT</td>
<td>Bovine</td>
</tr>
<tr>
<td>B8F</td>
<td>TACCGATTCTTCGTTTCCAT</td>
<td>Bovine</td>
</tr>
<tr>
<td>B9R</td>
<td>AAGGGTTGCTTTTGTCTACTG</td>
<td>Bovine</td>
</tr>
<tr>
<td>DF1</td>
<td>CTAACCCGATTCTTCGCCCTCCAC</td>
<td>Deer</td>
</tr>
<tr>
<td>DF2</td>
<td>CTAACCCGATTCTTCGCC</td>
<td>Deer</td>
</tr>
<tr>
<td>DF3</td>
<td>CCCATTATCTCAAGAC</td>
<td>Deer</td>
</tr>
<tr>
<td>DF4</td>
<td>CTATAGTCCATTACTCTTCAC</td>
<td>Deer</td>
</tr>
<tr>
<td>DF5</td>
<td>GATCTAACAACCCAACAGGAATTCC</td>
<td>Deer</td>
</tr>
<tr>
<td>DF6</td>
<td>CAGACAAAAATTCATTCCACC</td>
<td>Deer</td>
</tr>
<tr>
<td>DR7</td>
<td>CAGGAAATATCATCCAAGGTGTTAATA</td>
<td>Deer</td>
</tr>
<tr>
<td>DR8</td>
<td>CAGGAAATATCATCAGGTTTAATAG</td>
<td>Deer</td>
</tr>
<tr>
<td>DR9</td>
<td>GTTTAATATGGAGGGAGGGGTATTG</td>
<td>Deer</td>
</tr>
<tr>
<td>DR10</td>
<td>CTGGTGGAATATCTACTAATAGC</td>
<td>Deer</td>
</tr>
<tr>
<td>DR11</td>
<td>GTAGTAGGGGTGAATAGGAATTG</td>
<td>Deer</td>
</tr>
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</table>
Table 5. Oligonucleotide sequences of 12S probes used in RLB for detection and identification of amplified vertebrate DNA.

<table>
<thead>
<tr>
<th>Probe ID</th>
<th>Sequence of 5’ primary-amined oligonucleotides</th>
<th>Target</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mammal</td>
<td>AAAACTCAAGGACTTGGC</td>
<td>12S</td>
<td>Humair et al. 2007</td>
</tr>
<tr>
<td>Artiodactyl</td>
<td>TATTCCGAGGACTACTAC</td>
<td>12S</td>
<td>Humair et al. 2007</td>
</tr>
<tr>
<td>Bird</td>
<td>TACGAGCACAAACGCTTTAA</td>
<td>12S</td>
<td>Humair et al. 2007</td>
</tr>
<tr>
<td>Rodent</td>
<td>GGCGGTACTTTATATCCAT</td>
<td>12S</td>
<td>Humair et al. 2007</td>
</tr>
<tr>
<td>Lizard</td>
<td>GAGAAGTCAAGTGAAAAACT</td>
<td>12S</td>
<td>Humair et al. 2007</td>
</tr>
<tr>
<td>Lepus</td>
<td>ACCCGCCTAGAGGAGCCTG</td>
<td>12S</td>
<td>Humair et al. 2007</td>
</tr>
<tr>
<td>Canis</td>
<td>CGCCAGGACTACTAGCAAT</td>
<td>12S</td>
<td>Developed for this study</td>
</tr>
<tr>
<td>Odocoileus</td>
<td>TAAACATAATAGTTATAT</td>
<td>12S</td>
<td>Developed for this study</td>
</tr>
<tr>
<td>Bovine</td>
<td>GATAATTACATAACAAAAT</td>
<td>12S</td>
<td>Developed for this study</td>
</tr>
</tbody>
</table>
**Table 6.** Number of field collected ticks separated by sex, life stage, and collection site amplified with the cytochrome b gene for tested to detect bovine and deer host DNA.

<table>
<thead>
<tr>
<th>Collection Site</th>
<th>Male</th>
<th>Female</th>
<th>Nymph</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alkali Lake</td>
<td>0</td>
<td>4</td>
<td>12</td>
<td>16</td>
</tr>
<tr>
<td>Hallelujah Junction</td>
<td>3</td>
<td>5</td>
<td>20</td>
<td>28</td>
</tr>
<tr>
<td>Virginia City</td>
<td>3</td>
<td>3</td>
<td>22</td>
<td>44</td>
</tr>
<tr>
<td>Wellington Hills</td>
<td>12</td>
<td>7</td>
<td>48</td>
<td>67</td>
</tr>
<tr>
<td>Mono Valley</td>
<td>3</td>
<td>3</td>
<td>17</td>
<td>23</td>
</tr>
<tr>
<td>Bodie</td>
<td>0</td>
<td>0</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td><strong>Total Field collected ticks</strong></td>
<td></td>
<td></td>
<td></td>
<td>184</td>
</tr>
</tbody>
</table>
**Table 7.** Weight gain mean, median, and standard deviation, in grams for artificial feeding of *Ornithodoros coriaceus* ticks, separated by sex and life stage.

<table>
<thead>
<tr>
<th>Weight Gain</th>
<th>Female (N=4)</th>
<th>Male (N=5)</th>
<th>Nymph (N=28)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Average</td>
<td>0.264</td>
<td>0.032</td>
<td>0.044</td>
</tr>
<tr>
<td>Median</td>
<td>0.283</td>
<td>0.03</td>
<td>0.028</td>
</tr>
<tr>
<td>Standard Deviation</td>
<td>0.235</td>
<td>0.022</td>
<td>0.048</td>
</tr>
</tbody>
</table>
Table 8. Vertebrate species used for DNA extraction for sequencing and probe design.

<table>
<thead>
<tr>
<th>Scientific name</th>
<th>Common name</th>
<th># of individuals</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Bos taurus</em></td>
<td>Bovine</td>
<td>8</td>
</tr>
<tr>
<td><em>Canis familiaris</em></td>
<td>Dog</td>
<td>1</td>
</tr>
<tr>
<td><em>Coturnix coturnix</em></td>
<td>Quail</td>
<td>1</td>
</tr>
<tr>
<td><em>Dipodomys merriami</em></td>
<td>Kangaroo rat</td>
<td>4</td>
</tr>
<tr>
<td><em>Equus caballus</em></td>
<td>Horse</td>
<td>1</td>
</tr>
<tr>
<td><em>Lepus californicus</em></td>
<td>Jack Rabbit</td>
<td>2</td>
</tr>
<tr>
<td><em>Microdipodops</em></td>
<td>Kangaroo mouse</td>
<td>2</td>
</tr>
<tr>
<td><em>Mus musculus</em></td>
<td>Mouse</td>
<td>1</td>
</tr>
<tr>
<td><em>Odocoileus hemionus</em></td>
<td>Black tailed deer</td>
<td>3</td>
</tr>
<tr>
<td><em>Odocoileus virginianus</em></td>
<td>White tailed deer</td>
<td>1</td>
</tr>
<tr>
<td><em>Oryctolagus cuniculus</em></td>
<td>Cottontail rabbit</td>
<td>2</td>
</tr>
<tr>
<td><em>Ovis aries</em></td>
<td>Sheep</td>
<td>4</td>
</tr>
<tr>
<td><em>Perognathus longimembris</em></td>
<td>Pocket mouse</td>
<td>2</td>
</tr>
<tr>
<td><em>Permomyscus maniculatus</em></td>
<td>Deer mouse</td>
<td>2</td>
</tr>
<tr>
<td><em>Pituophis melanoleucus</em></td>
<td>Bull snake</td>
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</tr>
<tr>
<td><em>Procyon lotor</em></td>
<td>Raccoon</td>
<td>1</td>
</tr>
<tr>
<td><em>Puma concolor</em></td>
<td>Mountain Lion</td>
<td>1</td>
</tr>
<tr>
<td><em>Rattus norvegicus</em></td>
<td>Rat</td>
<td>1</td>
</tr>
<tr>
<td><em>Sciurus griseus</em></td>
<td>Western gray squirrel</td>
<td>2</td>
</tr>
<tr>
<td><em>Spermophilus beecheyi</em></td>
<td>California ground squirrel</td>
<td>2</td>
</tr>
<tr>
<td><em>Tamias dorsalis</em></td>
<td>Cliff chipmunk</td>
<td>1</td>
</tr>
<tr>
<td><em>Tamias quadrrimaculatus</em></td>
<td>Long eared chipmunk</td>
<td>1</td>
</tr>
<tr>
<td><em>Tamiasciurus douglasii</em></td>
<td>Douglas squirrel</td>
<td>1</td>
</tr>
<tr>
<td><em>Vulpes vulpes</em></td>
<td>Fox</td>
<td>1</td>
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</table>
Figure 1. Tick collection sites throughout Nevada and California. Tick collection sites are recognized by the circle or square markers, circles representing sites where *Ornithodoros coriaceus* ticks were found and square markers representing sites where *Ornithodoros coriaceus* were not found at the time of the survey. Due to the closeness of the Bodie collection site and the Wellington Range site only one circle represents both locations.
Figure 2. Amplification and RLB hybridization of species-specific probes, targeting the cytochrome b gene of 15 vertebrate species; lanes 1-6: bovine; lanes 7-8: ovine; lanes 9-12: deer; lanes 13-15 reptile, squirrel, mouse; 16: water. Probes bov1-bov19 and Deer1-5 were run in two concentrations; 500 pmol/µl and 1000 pmol/µl. Probes B17, B27, B18, and B19 showed hybridization with bovine DNA. Probes B27, B18, and B19 showed hybridization with ovine DNA and probes B18 and B19 also showed hybridization with squirrel, mouse, and reptile. Probes deer 1 showed hybridization with deer, squirrel and mouse. Probe deer4 showed hybridization with deer DNA.
**Figure 3.** 12S reverse line blot with vertebrate DNA along the y-axis and 12S probes along the x-axis. The probe order is 1) *Bos taurus* 2) *Canis* 3) Rodent 4) *Lepus* 5) Bird 6) Artiodactyl 7) *Odocoileus* 8) Lizard 9) Mammal 10) IDT Artiodactyl 11) IDT Rodent. *Bos taurus* probe did not show hybridization, the dog probe showed hybridization to bovine DNA, and the rodent probe showed hybridization with rat DNA. The *Lepus* probe hybridized to all vertebrate DNA and the mammal probe hybridized to everything but deer, quail and dog DNA.

1 2 3 4 5 6 7 8 9 10 11

Deer
Sheep
Quail
Jackrabbit
Pocket mouse
Rat
Dog
Bovine
Water

**Figure 4.** 12S reverse line blot with duration study tick extractions fed on a bovine host along the y-axis and 12S probes along the x-axis. The probe order is 1) Mammal 2) Rodent 3) Artiodactyl 4) Bird 5) *Canis* 6) *Bos taurus* 7) Lizard 8) *Odocoileus* 9) *Lepus* 10) IDT Rodent 11) IDT Artiodactyl.

1 2 3 4 5 6 7 8 9 10 11

Bovine DNA
Water
Figure 5. Reverse line blot imaging of cytochrome b probes B17 and B27 run against artificially fed ticks in the duration study. a) RLB of cytochrome b probes run against samples day 15-day 20 showing hybridization to all samples for both probes B17 and B27. b) RLB of cytochrome b probes run against samples day 30-day 84 showing hybridization to all samples for both probes B17 and B27. c) RLB of cytochrome b probes run against samples day 84-day 175 showing hybridization for all samples with probe B27 and faint hybridization to probe B17 for all samples except D175N6. d) Duration plate 4 is the RLB of cytochrome b probes run against samples day 175-day 325. e) RLB of cytochrome b probes run against samples day 207-day 247 showing hybridization of all samples with B18 and hybridization to ticks from day 175, 196, and 207 post feeding with probes B17 and B27. f) RLB of cytochrome b probes run against samples day 247-day 287 showing a lack of hybridization to B17 and B27 for day 207 post feeding.
Figure 6. Reverse line blot imaging of cytochrome b probes B17 and B27 run against field collected ticks from Wellington Range, Bodie, and Mono Valley (WRBMV) sites. RLB of cytochrome b probes run against WRBMV samples; lane 1 is the negative water control, lane 2-16 are field collected ticks, and lane 17 is a bovine positive control. The positive bovine control showed hybridization to both bovine probes B17 and B27.
**Figure 7.** Reverse line blot imaging of cytochrome b probes B17, B27, B18, B19, and D4 run against field collected ticks from Alkali Lake, Virginia City, and Hallelujah Junction (ALVCHJ) collection sites.  

a) RLB of cytochrome b probes run against ALVCHJ samples; lane 1-2 are field collected tick samples, lane 3 is the water negative control which has hybridized to all probes, lane 4-7 are bovine DNA, bovine DNA, deer DNA, and deer DNA, and lane 8-23 are field collected ticks. Due to contamination of the negative control all other results were negated for blot 7a and blot 7b.  
b) RLB of cytochrome b probes run against ALVCHJ samples; lane 1-19 are field collected ticks.  
c) RLB of cytochrome b probes run against ALVCHJ samples; lane 1-17 are field collected ticks showing complete hybridization of all samples to both B17 and B27 probes.  
d) RLB of cytochrome b probes run against ALVCHJ samples; lane 1-17 are field collected ticks showing a lack of hybridization for all probes.
LITERATURE CITED


