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University of Nevada, Reno

Transstadial Transmission and Transovarial Transmission of *Ornithodoros coriaceus* and Its Connection to Epizootic Bovine Abortion

A thesis submitted in partial fulfillment
of the requirements for the degree of

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by

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Abstract

Epizootic Bovine Abortion (EBA), also known as Foothill abortion, is a disease of beef cattle in the western U.S. that can result in late term abortion and significant economic losses (McKercher et al., 1963). *Ornithodoros coriaceus* is the only known vector that transmits the bacteria pathogen (typically referred to as the etiologic agent of EBA, aoEBA) to cattle hosts. The bacteria are a yet unnamed, intracellular organism, genetically most closely related to soil dwelling organisms (King et al., 2005). If an infected tick feeds on a susceptible pregnant cow, the bacterial pathogen can be transmitted to the fetus, where it slowly develops within the fetal calf lymphatic system (Hall et al., 2002). The pregnant cow often develops an asymptomatic infection and does not appear clinically ill while infection in the developing fetus usually results in abortion. In order to determine if *Ornithodoros coriaceus* can act as a biological vector of aoEBA (as compared to simply serving as a mechanical source of the pathogen), it was tested whether transstadial or transovarial transmission could be detected in experimentally infected ticks. Ticks were collected from an area where the prevalence of the disease was well documented; experimentally infected, fed, and allowed to reproduce. Then through DNA extraction and QPCR ticks were tested for the prevalence of aoEBA. It does not appear that the ticks have a transstadial or transovarial transmission.

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Important Terms and Concepts

EBA-Epizootic Bovine Abortion

aoEBA- etiologic agent of EBA

Ornithodoros coriaceus-tick vector of EBA

Transstadial transmission- tick maintains pathogen infection throughout its life

Transovarial transmission-transmission of pathogen from female to her eggs

Transstadial Transmission and Transovarial Transmission of *Ornithodoros coriaceus* and Its Connection to Epizootic Bovine Abortion

Introduction: Epizootic Bovine Abortion (EBA), also known as Foothill abortion, is a disease of beef cattle in the Western U.S. that can result in late term abortion and significant economic losses to cattle producers (McKercher et al., 1963). Epizootic Bovine Abortion (EBA) has been identified in cattle raised in California, Oregon, and Nevada and is caused by a unique bacterial pathogen, which has yet to be officially named (Hall et al., 2002). *Ornithodoros coriaceus* is the only identified vector shown to transmit the bacterial pathogen (typically referred to as the etiologic agent of EBA or aoEBA) to cattle hosts. The bacteria infect the cells of organs involved in the immune response such as the thymus, spleen, and lymph nodes, and is genetically most closely related to soil dwelling organisms (King et al., 2005). If an infected tick feeds on a susceptible pregnant cow, the bacterial pathogen can be transmitted to the fetus, where the pathogen slowly develops within the fetal calf lymphatic system (Hall et al., 2002). The pregnant cow develops an asymptomatic infection and does not appear clinically ill, while infection in the developing fetus can result in abortion or the birth of weak and unhealthy calves (Coker et al., 2012)

Little is known regarding the transmission dynamics of the agent of EBA in nature. The tick vector has been identified feeding on multiple mammalian hosts (including rodents, deer, and humans) as well as on birds, but it is unknown which, if any, of these species can serve as a reservoir host for the pathogen (Furman and Loomis, 1984). The life cycle of *Ornithodoros coriaceus* includes an egg stage, a larval

stage, and up to 5 nymphal instars before molting to become a sexually mature adult. Each of these stages is usually preceded by a blood meal with the exception of the first nymphal instar (Loomis et al., 1974). In other tick-borne diseases such as babesiosis and relapsing fever, the tick vector can serve as the host of the bacteria through transovarial transmission of the pathogen via transmission from an infected female to her progeny through the eggs. In other tick vectored diseases, for example Lyme disease, the tick maintains infection throughout its life via a process known as transstadial transmission and can serve to transmit the disease between vertebrate hosts but does not pass the pathogen along to its progeny (Magnarelli et al., 1987).

In order to determine if *Ornithodoros coriaceus* can act as a biological vector of aoEBA (as compared to simply serving as a mechanical source of the pathogen), it is necessary to determine whether transstadial or transovarial transmission can be detected in experimentally infected ticks. Previous investigators have shown evidence of transstadial transmission of the aoEBA as both infected nymphs and adults are found in the wild (Chen et al., 2007), but the transovarial route of transmission has yet to be tested. The aim of this project is to collect *O. coriaceus* ticks from an area where the prevalence of infection with the aoEBA is well documented and, then experimentally infect ticks in a laboratory setting, allow them to feed and molt to the next life stage as nymphs or to reproduce as adults. Ticks used in this experiment were tested for the presence of the EBA bacteria using a Quantitative Polymerase Chain Reaction (qPCR) detection system.

Literature Review:

Typically in domestic animals, abortion is the resorption of the fetus, premature expulsion of a dead fetus or a living one incapable of life. In cattle herds, the loss of a calf equates to an economic loss to the producer. A certain number of abortions may be expected annually. When there is an extreme increase in the number of abortions occurring in a herd, there is a need for investigation to occur (Smith 1990).

Non-infectious abortions in cattle can be caused by many different factors. Many abortions are attributed to genetic or chromosomal factors, inadequate nutrition, and toxic plants (Smith 1990). Nutrition plays a role in abortions; if an animal is not getting adequate nutrition it will not be able to supply the energy needs of the growing fetus. Energy restrictions of more than 10-20% start resulting in less than maximum cow reproductive traits and calf development (Fiems et al., 2009). Iodine deficiency is another cause of reproductive issues in cattle. Iodine is mainly present in the hormones of the thyroid, and the hormones affect fetal development, immunity, and reproduction. The severity of the reproductive problems is dependent on age, species, and environment. Selenium deficiency combined with iodine deficiency aggravates the symptoms (Lebreton et al., 2006). Toxic plants such as pine needles, broomweed, and locoweed, can also lead to abortion on cattle (Smith 1990).

Infectious agents are often implicated in bovine abortions. Infectious agents can be caused by bacteria in such diseases as: chlamydia, leptospirosis, brucellosis,

and Epizootic Bovine Abortion; and also in viral diseases such as bovine viral diarrhoea and infectious bovine rhinotracheitis. As compared to many of the other abortigenic diseases in cattle that are transmitted from animal to animal, Epizootic Bovine Abortion is unique among these diseases because the bacteria are vectored by the soft tick, *Ornithodoros coriaceus* (Hall et al., 2002).

The diagnosis of EBA in an aborted fetus is typically based on characteristic gross and histological lesions at the time of necropsy. Infected fetuses characteristically show enlarged lymph nodes, petechial hemorrhages of the oral mucosa and conjunctiva, ascites--free fluid in the abdomen, and spleno-hepatomegaly--enlargement of the spleen and liver (Kennedy et al., 1960). When tissues are examined under a microscope (histology), histologic thymic changes that are characteristic of EBA are observed. The histologic thymic changes include severe histocytic thymositis with depletion of thymocytes, inflammation of the thymus, which causes damage to the thymocytes, also known as the precursors to T-cells, and interlobular hemorrhage, which causes severe immune reaction in the vasculature of the tissues resulting in leakage and leading to fluid accumulation in the abdominal cavities. (Anderson et al., 2006)

Also found in the histological assessment are the gross enlargement of lymph nodes, the result of cortical follicular hyperplasia and histolytic lymphadenitis. Unlike a lymphoma or cancer of the lymph nodes, the enlarged lymph nodes are a heightened immune response state. The cells in the lymph nodes are polymorphous, meaning multiple varieties of cells from all over the body are trying to fight the

infection. The lymph nodes are enlarged due to hypertrophy of the cells, as they are stimulated by the immune response, and the recruitment of white blood cells from other parts of the body. In addition, during necropsy, widespread, predominately nonsuppurative histological lesions typical of EBA are observed in most organs, including the brain, lung, heart, liver, and spleen- indicating damage of the organs at the cellular level without presence of pus and exudate (Anderson et al., 2006)

The Argasid or soft tick, (which vectors the aoEBA), *Ornithodoros coriaceus* was first identified in Sonora, Mexico, and its distribution occurs through other western states of Mexico. In the U.S., *Ornithodoros coriaceus* has been identified in California, Nevada, and Southern Oregon (Furman & Loomis 1984). In a laboratory setting the lifecycle of *O. coriaceus* from egg to adult takes a little over 1 year although the adult ticks can continue to live for up to 4 years without a blood-meal (Furman & Loomis 1984). Larvae feed on hosts for approximately 9 days, nymphs approximately 8-100 minutes, and adults 5-50 minutes (Furman & Loomis 1984). For males, egg to sexual maturity takes approximately 159 days and for females about 343 days. The bite is described to be venomous to humans, monkeys, rabbits, and mice with a small purple ring surrounding a bright red spot at the point of detachment (Herms, 1916). Previous research has indicated a strong correlation between cattle and deer populations and the occurrence of the tick, as cattle and deer are the most common hosts for *O. coriaceus* (Loomis et al., 1974). However, this tick has been known to feed on almost any warm-blooded animal including human as an incidental host (Furman & Loomis 1984).

Ornithodoros coriaceus ticks have been identified in the same geographic locations that have cases of EBA. The presence of *Ornithodoros coriaceus* in the same places as the abortions strongly indicates this disease is endemic (Hall et al., 2002). The bacterial agent of EBA has been recognized as an intracellular bacterium. It is in the δ -proteobacteria class, of the *Myxococcales* order (King et al., 2005), and previous research suggests that tick salivary glands are the anatomical location within the tick most likely to harbor the aoEBA. The salivary glands and bodies of 25 nymphs, 25 females, and 25 males were tested for the presence of aoEBA. The results found 1 nymph, two males, and three female salivary glands positive for aoEBA with no detection of aoEBA in the bodies. One additional male body was positive for aoEBA but not the corresponding salivary gland (Chen et al., 2007).

Infected ticks carry relatively low numbers of the pathogen, and ticks range from 5-20% infected in varying geographical locations (Chen et al., 2007). In Nevada infection has been identified in all areas where aborted cattle fetuses tested positive for EBA; most sites were in northern and northwestern Nevada (Hall 2002). Sites tested positive for the presence of aoEBA in *Ornithodoros coriaceus* include but are not limited to: the Alturas area, California; Susanville area, California; Lakeview area, Oregon; Wellington area, Nevada; Reno area, Nevada (Chen et al., 2006); Alakali Lake, Paradise Valley, Gund Ranch, Virginia Range, Wellington Hills, Hat creek, Klamath falls, and Hallelujah Junction (Teglas et al., 2011).

Transstadial transmission is the transmission of a pathogen from one developmental stage to the next within an arthropod vector. Transovarial transmission is the transmission of the pathogen to a succeeding generation through the ovaries and eggs of the pathogen's respective vector species (Macaluso et al., 2002). Transovarial transmission has been studied more closely in conjunction with ticks that transmit *Borrelia* spirochetes and ticks that transmit bacteria in the genus *Rickettsiae*. According to a study done in 1987, transovarial transmission of *Borrelia burgdorferi* has been known to occur in *Ixodes dammini* (renamed *scapularis*) at a high variable rate. Seven of the eighteen infected female ticks passed spirochetes transovarially, and 1.9% of the larvae contained spirochetes (Magnarelli et al., 1987). The study ultimately concluded that transovarial passage of the spirochetes was of little epidemiological importance. The larvae were more likely to acquire the bacteria by feeding on infected rodents than actually having the bacteria passed onto them from their infected mothers. However, the question not answered by this study is whether the transovarial infected larvae can transmit the bacteria to their vertebrate hosts.

Technology has progressed since the study done in 1987. There are now more accurate ways to extract DNA, analyze, and understand the presence of specific bacteria in the DNA through PCR and QPCR. Newer technology has led to greater understanding of pathogen transmission properties within the tick *Ixodes scapulari* (the tick that vectors *Borrelia burgdorferi*). This has led to new conversations on whether the bacteria in the genus *Borrelia*, *Borrelia burgdorferi*

and *Borrelia miyamotoi* are actually transovarially transmitted by *Ixodes scapularis*. This particular study by Rollend et al., 2012 looking into transovarial transmission with new technology counters the argument of the epidemiological importance of transovarial transmission. They argue that, "The presence of infected larvae in the environment would lengthen the transmission season well beyond the currently recognized nymphal activity period...and larval (*Ixodes scapularis*) do feed upon people, and their extremely small size would make them difficult to detect" (pg 46). The fact that the transmission season could be lengthened and that larvae can feed on people furthers the importance of determining whether transovarial transmission does indeed occur.

After Rollend et al., 2012 analyzed previous studies on this subject, there was much concern regarding methodology. Older methods of determining transovarial transmission of *B. burgdorferi* were done using the older technology of detecting spirochetes through staining with Direct or Indirect Fluorescent Antibody methods. However, when *B. burgdorferi* was analyzed using the newer technology of DNA extraction and PCR, transovarial transmission was not observed. After Rollend et al., 2012 ran their own study infecting and breeding ticks, and then testing them using DNA extraction and PCR, the results provided strong evidence that transovarial transmission of *B. burgdorferi* does not occur. However the study did show that transovarial transmission of *Borrelia miyamotoi*, a more recently described species of bacteria in the Relapsing Fever Group, does occur. *Borrelia miyamotoi* is probably

responsible for earlier reports of transovarial transmission that were originally credited to *B. burgdorferi* (Rollend et al., 2012).

Transovarial and transstadial transmission of *Rickettsiae africae* (the causative agent of Africa tick bite fever) in the hard tick *Amblyomma variegatum*, has been determined. *Amblyomma variegatum*'s main hosts are large ruminants and wildlife, and *Amblyomma variegatum* is the most common vector of *R. africae*. Larvae and nymphs are known to parasitize birds and rodents. These ticks are also known to bite humans. After Socolovschi et al., 2009 finished collecting, feeding, breeding, and testing there was a 93.4% filial infection rate of *R. africae* in *A. variegatum* of which 57/61 larvae of the third generation tested positive. The pools of eggs collected from infected females also tested positive in 20/20 egg masses. This study effectively shows transstadial and transovarial transmission of *R. africae* in the tick's lifecycle. These results demonstrate that *A. variegatum* not only acts as a vector of the disease but also as a reservoir. The high transovarial rate of 100%, and the high transstadial rate of 93.4%, explains the high population of infected ticks in nature (Socolovschi et al., 2009).

The primary objective of our study was to determine whether the aoEBA could be maintained within the tick vector *O. coriaceus*; either between life stages (transstadial transmission) or passed along to their progeny via infected eggs (transovarial transmission). Understanding the route of pathogen transmission within the tick vector will greatly contribute to the understanding of how the aoEBA

is maintained within the environment. It can potentially provide useful information as to the eradication of this disease through the control of the tick vector itself.

Methodology: *1. Collection of O. coriaceus:* Ticks were collected near the Nevada/California border north of Reno, NV during two different collection periods (5/16/2012 and 9/16/2012). Ticks were collected by depositing pieces of dry ice (approximately 12-13 centimeters in diameter) along a modified transect in areas with evidence of occupation by deer or other small mammals, as this can serve as an indication of the presence of this tick. A small amount of earth was removed to create a shallow depression for the dry ice to sit within and left for 10-20 minutes. Upon returning to the dry ice, any ticks that were drawn to the carbon dioxide were collected, put into collection tubes, and transported back to the laboratory at the University of Nevada Reno. Ticks were then sorted by sex and life stage (nymphs, adult females and adult males) for use in the remainder of the study. In order to determine the overall prevalence of aoEBA in ticks from our collection area, 73 ticks were sacrificed and their genomic DNA extracted using the DNeasy extraction kit according to the manufacturer's instructions. They were then tested by qPCR specific for the detection of the aoEBA as described by Teglas et al. (2006).

2. Experimental infection of collected ticks: Eighty-four nymphs and 29 females were experimentally infected with an inoculum containing the aoEBA at a concentration of approximately 50 aoEBA infected cells per ul of material. Each cell contained variable amounts of aoEBA bacteria making it difficult to determine the exact number of bacteria, so the inoculum was diluted to deliver a standardized dose of infected cells. Specifically, ticks were experimentally infected with a preparation made of a single cell suspension of

SCID mice splenic cells infected with the aoEBA mixed with sterile glycerin and DMSO. Four hundred and fifty microliters of inoculum was mixed with 550 microliters of sterile 1X PBS. Cell counts were performed, and the number of infected cells were determined within the inoculum. Sterile PBS was added to this solution in order to dilute the number of bacterially infected cells to approximately 50 infected cells per microliter. Two microliters of this diluted inoculum was then injected into each tick's coelomic cavity through the dorso-caudal aspect of the tick (about 2/3 of the way towards the rear) using a 30-gauge hypodermic needle and a Hamilton repeating micropipetor. A total of twenty-nine females and 84 nymphs were injected in this manner.

3. *Maintenance and feeding of ticks:* The ticks were then maintained in an insect growth chamber at 24 degrees Celsius, 70% humidity, and a 12-hour light cycle. They were maintained in this environment through the study in order for them to complete their lifecycle. In order for ticks to gain the energy required to molt and reproduce, experimental ticks were fed on commercially purchased defibrinated rabbit blood using an artificial feeding membrane created out of silicon, mesh fabric, and animal fur based on previous success with the same feeding membrane described in (Chen et al., 2007).

4. *Testing:* Nymphal *O. coriaceus* were harvested after molting to the next nymphal instar. They were marked with wax or nail polish so when they molted their exoskeleton no longer contained a mark, molted nymphs could be differentiated from others that had yet to molt. Larval ticks were collected via aspiration after they had hatched from eggs lain by infected females. The larvae were counted out and batched. Each batch had approximately 125 larvae per batch, which was .1 ml in a 1.5 ml ependorf tube. All testing was done through DNA extraction on each nymph and batch of larvae;

detection of DNA of the agent of EBA was done through qPCR as described by Teglas (2006). Results of these experiments were compared to the estimated prevalence of the aoEBA initially found in the study population. Data were maintained in Excel and a student T test was conducted to compare the prevalence of aoEBA infection between the wild caught and experimentally infected groups of ticks.

Results: Of the ticks collected and tested from our collection site, one nymph out of 16 was naturally infected with EBA (6.25%), and one female out of 49 was naturally infected (2.04%)

Table 1: Number of ticks tested for aoEBA that were found in the wild, and then 1 month, and 4 months after artificial infection. Percent positive for aoEBA in parentheses.

Life Stage	Wild ticks	1 month (artificially infected)	4 month (artificially infected)
Nymphs	1/16 (6.25%)	3/46 (6.52%)	0/20 (0%)
Females	1/49 (2.04%)	0/6 (0%)	0/10 (0%)

Of the experimentally infected ticks, including those that died within the first month of inoculation, three nymphs out of 46 were positive (6.52%), and zero out of six females were positive. Of the experimentally infected ticks, and of those that died within in four months of inoculation, zero nymphs and 1 female was positive (Table 2). When the prevalence of aoEBA infection in the wild population of infected ticks is compared to experimentally infected ticks the P value was non-significant at 0.84, indicating no statistical significant differences exist in the number of ticks naturally infected verses those experimentally infected. Of the 15 females that bred, one was positive (6.67%), and zero of the larvae produced by these females were positive (Table 2).

Table 2: Number of larvae, males, and females tested for aoEBA after breeding. Percent positive indicated in parentheses.

Life Stage	#Positive/#Tested
Larvae	0/1750 (0%)
Male	0/24 (0%)
Female	1/15 (6.67%)

Discussion of Results: The results of this study can be interpreted in several ways: one, there is no evidence of transovarial transmission that occurred especially because one female tested positive for aoEBA, and none of her offspring were positive for pathogen. Two, there was no evidence of transstadial transmission as there was no statistically significant difference in the infected wild ticks and the experimentally infected ticks although other studies with large sample sizes have indicated that this method of transmission may occur (Chen et al., 2007). Three, there was a problem with physically inoculating the tick. The females and nymphs that bred and molted may not have originally been infected with the aoEBA, therefore, had no way of passing aoEBA onto their offspring or into the next life stage. When the ticks were inoculated via syringe, the inoculum frequently spilled out when the syringe was removed. A tick's exoskeleton is made of chitin the same hard non-pliable substance found in the shells of crabs and lobsters, so when the exoskeleton is punctured the hole remains open, and has no way of resealing itself to keep the injected fluid in. The exoskeleton may have led to many of the ticks never becoming infected with aoEBA in the first place.

In order to remedy this problem, other methods of infecting *O. coriaceus* ticks need to be tested. Laboratory experiments using Severe Combined Immuno Deficient (SCID) mice could be used to infect ticks instead of injecting the inoculum with a syringe. Successful passage of the bacteria into mice with severe combined immunodeficiency has been proven. These mice can be used to grow aoEBA, and the aoEBA can then be harvested and administered to susceptible pregnant heifers (Blanchard et al., 2010). It would be recommended for future work with aoEBA and the infecting the tick vector to, infect a SCID mouse with a known amount of aoEBA, allow the ticks to feed on the mouse, and then allow the ticks to breed and reproduce. This would insure infection of the ticks. Since these ticks readily feed on an artificial membrane, a system using a circulating pool of infected blood may potentially be utilized to experimentally infect ticks. This method could also minimize the use of animals in experimentation. Regardless of the method used, a technique that ensures ticks can become infected with aoEBA, in a manner that more closely resembles infection in nature would allow for the question of how these ticks transmit this pathogen to be fully answered.

Conclusion: *Ornithodoros coriaceus* were collected from an area where the prevalence of the disease was well documented. A subset of these ticks was tested for aoEBA through DNA extraction and QPCR to determine the wild population infection. The rest of the ticks were experimentally infected, fed, and allowed to reproduce. Then through DNA extraction and QPCR ticks were tested for the prevalence of aoEBA. It does not appear that the ticks have a transstadial or transovarial transmission.

Any information regarding the maintenance of the pathogen in the environment will lead to a better understanding of the disease. The more we know about the reservoir of the disease and the pathogen transmission, the better we will be able to combat and protect against infection in cattle herds. This study does not advance the current knowledge of how aoEBA is maintained in the environment, but it does provide a clear direction for future researchers to take the next step in furthering this research.

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