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Studies on *Borrelia coriaceae*, the putative agent of epizootic bovine abortion

Frey, Susan R., M.A.

San Jose State University, 1991

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STUDIES ON *BORRELIA CORIACEAE*, THE PUTATIVE AGENT OF EPIZOOTIC BOVINE ABORTION

A Thesis

Presented to

The Faculty of the
Department of Biological Sciences

San Jose State University

In Partial Fulfillment

of the Requirements for the Degree

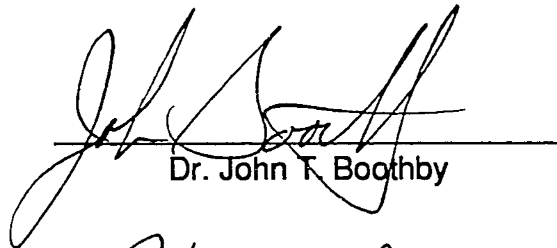
Masters of Arts

By

Susan R. Frey

August, 1991

APPROVED FOR THE DEPARTMENT OF BIOLOGICAL SCIENCES



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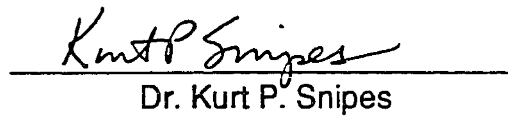

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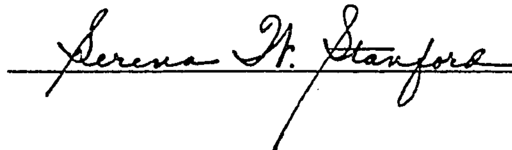

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ABSTRACT

STUDIES ON *BORRELIA CORIACEAE*, THE PUTATIVE AGENT OF EPIZOOTIC BOVINE ABORTION

by Susan R. Frey

Epizootic Bovine Abortion (EBA), a disease of range cattle, causes abortion or birth of weak calves during the third trimester of pregnancy. EBA is transmitted by the tick, *Ornithodoros coriaceus*. A spirochete, *Borrelia coriaceae,* was isolated from *O. coriaceus,* and has been implicated as the etiological agent, though the evidence that *B. coriaceae* causes EBA is circumstantial.

This study describes the development of an enzyme-linked immunosorbent assay (ELISA) to detect antibodies to *B. coriaceae* in cattle. The results indicated that the ELISA could be used as a screening test for EBA with 83% sensitivity and specificity. Two groups of cattle with divergent histories were then evaluated using the screening test.

O. coriaceus were collected and dissected, and 7% of the ticks possessed spirochetes. We found that *O. coriaceus* could be infected with *Borrelia sp.* via a membrane-feeding device.

Our research explored the feasibility of using C3H/He mice in an animal model for EBA. Preliminary results demonstrated the clinical sympton of EBA abortion in 40% (2/5) of the pregnant mice inoculated with *B. coriaceae.*

TABLE OF CONTENTS

Page

ACKNOWLEDGEMENTS iii
LISTS OF TABLES v
LIST OF FIGURES vii

Chapter

I. GENERAL INTRODUCTION 1

II. SEROLOGICAL IMPLICATION OF *BORRELIA CORIACEAE* AS AN AGENT OF EPIZOOTIC BOVINE ABORTION 3

III. *ORNITHODOROS CORIACEUS* KOCH (ACARI: ARGASIDAE) AS AN INVERTEBRATE VECTOR FOR BORRELIOSIS OF CATTLE 21

IV. EXPERIMENTAL INFECTION OF MICE WITH *BORRELIA CORIACEAE* AND *BORRELIA BURGDORFERI* 34

V. GENERAL CONCLUSIONS 53

LIST OF TABLES

Chapter II **Page**

1. Vaccines used for absorbing non-specific IgG antibody in ELISA for the detection of *Borrelia coriaceae.* 19

2. A two-by-two contingency table of the true state of the EBA positive and negative populations and the relevant sensitivity and specificity of ELISA test. 20

Chapter III

1. Results of darkfield observation of *O. coriaceus* in cultures of hemolymph, salivary glands, and tick infected Barbour-Stoenner-Kelly (BSK II)... 33

Chapter IV

1. Proportion of parturitions and teratic pups born to 8-week-old C3H/He mice inoculated with *B. burgdorferi* N40, *B. coriaceae,* and uninoculated medium. 50

2. Proportion of mated and unmated C3H/He mice inoculated at 3 or 8 weeks of age with *B. burgdorferi* N40, *B. burgdorferi* B31, *B. coriaceae,* or medium alone developing arthritis within 35 days. 51

Page

3. Mean EV% of 3-week-old female mice and 8-week-old unmated female mice inoculated with *B. burgdorferi* N40, *B. burgdorferi* B31, *B. coriaceae,* BSK II and RPMI. 52

LIST OF FIGURES

Chapter II | Page

1. *B. coriaceae*-specific reactivity without absorption and after absorption for cows from an EBA enzootic area. 15

2. *B. coriaceae*-specific reactivity without absorption and after absorption for cows in a nonenzootic area for EBA. ... 16

3. *B. coriaceae* specific reactivity without absorption and after absorption of 29 sera samples from cows raised in an EBA enzootic area. 17

4. *B. coriaceae* specific reactivity without absorption and after absorption of 50 sera samples from cows raised in a nonenzootic area for EBA. 18

Chapter III

1. Isolations of the tick, *O. coriaceus,* in California. 29

2. Cattle ranch in Monterey County, California where *O. coriaceae* were collected. 30

3. Carbon dioxide drop-trap placed in one of the 24 locations on study ranch in Monterey County, California. 31

4. Membrane-feeding device, and *O. coriaceus* before (right) and after feeding (left). 32

Chapter IV Page

1. Method used for inoculating mice with *Borrelia*-exposed *O. coriaceus.* 48

2. Rear legs of C3H/He mice inoculated with *B. burgdorferi* (right) and BSK II (left). 49

Chapter I
GENERAL INTRODUCTION

Members of the bacterial genus *Borrelia* are spirochetes transmitted to vertebrates by hematophagous arthropods. They have a specific parasite-vector relationship, but are able to infect and be transmitted by various other arthropods, and have a large range of mammals that can serve as hosts. The majority of the species causes relapsing fever; however, *Borrelia burgdorferi* causes Lyme disease and *Borrelia coriaceae* is the possible agent for Epizootic Bovine Abortion (1). This study concentrated on these two species.

Epizootic Bovine Abortion or Foothill Abortion, is the disease associated with *O. coriaceus* and its putative agent *B. coriaceae*. It is an asymptomatic infection in cattle which results in abortion, characterized by fetal lesions, during the third trimester of pregnancy or birth of weak calves (3). The rate of abortion in pregnant heifers pastured for the first time in EBA enzootic areas can be as high as 85%. In California, the estimated annual calf loss due to EBA is between 5-10% at a cost of up to $15 million (4). In the 1970's, studies by Schmidtmann et al. in which they exposed pregnant heifers to the bite of *O. coriaceus* helped establish that the disease was being transmitted by a tick vector, although, no causative agent was found at that time (8). In the 1980's, a *Borrelia sp.* was isolated from *O. coriaceus*, characterized, and later recognized as a new species *B. coriaceae*, a possible agent of EBA (3, 4, 7). Osebold et al. observed spirochetes in the blood of fetuses with EBA lesions which had similar morphological features as the *Borrelia sp.* isolated earlier (5). Subsequently, Osebold et al. inoculated pregnant heifers with spirochete infected blood from naturally infected EBA fetuses and induced abortion in the heifers (6).

At this time there is still little known about the precise

relationship between *B. coriaceae* and EBA. In this study, we developed ways of dissecting *O. coriaceus* to culture and isolate *B. coriaceae,* and examined the tick's role as a vector for *B. coriaceae* and *B. burgdorferi.* A serological test was also developed for evaluating antibody reactivity in bovine sera to *B. coriaceae*-specific antigens, and the susceptibility of pregnant mice to *B. coriaceae and B. burgdorferi* was studied.

This thesis consists of four chapters: (a) Chapter I is a general introduction; (b) Chapter II describes the development of an enzyme-linked immunosorbent assay (ELISA) to detect antibodies to *B. coriaceae* in cattle and application of this assay studying exposure levels of two cattle populations; (c) Chapter III examines *Ornithodoros coriaceus* as the putative vector for borreliosis; and (d) Chapter IV describes the feasibility of using C3H/He mice as an animal model for EBA.

Chapter II

SEROLOGICAL IMPLICATION OF *BORRELIA CORIACEAE* AS AN AGENT OF EPIZOOTIC BOVINE ABORTION

ABSTRACT

Borrelia coriaceae is considered the putative agent for Epizootic Bovine Abortion (EBA). The following describes the development of an enzyme-linked immunosorbent assay (ELISA) to detect *B. coriaceae* antibodies in cattle and the comparison of EBA positive cow sera to EBA negative cow sera. The sera were absorbed to remove antibodies that could contribute to cross reactivity and then evaluated for specific reactivity to *B. coriaceae.* The results indicated that the ELISA could be used as a screening test for EBA with 83% sensitivity and specificity. Two groups of cattle with divergent histories were evaluated by the screening test. Results indicated that 72% of the cattle from an enzootic region were confirmed *B. coriaceae* reactors as compared to 22% confirmed *B. coriaceae* reactors from a nonenzootic region.

INTRODUCTION

Epizootic Bovine Abortion (EBA) or Foothill Abortion, has become recognized as a major deterrent to maximum calf production in the Western United States (7). It is an asymptomatic infection of cattle which can result in abortion during the 6th to 8th month of pregnancy or in the birth of weak calves (6). The rate of abortion in pregnant heifers pasturing for the first time in EBA enzootic areas can be as high as 85%. In California, estimated annual calf loss, due to EBA, is between 5 to 10 percent at a cost of up to $15 million (7). Osebold et al. inferred that a borrelia-like spirochete could cause

abortion consistent with EBA pathology (12, 13). A new species, *Borrelia coriaceae* has been implicated as the etiological agent and *Ornithodoros coriaceus* as its principal vector (8, 14, 15, 7). Ranchers can do little to prevent EBA except to keep newly integrated heifers from becoming pregnant for at least 4 months, hopefully giving the cattle enough time to be exposed to the bite of the tick and develop immunity against EBA.

In this study we examined the exposure of cattle to *B. coriaceae* in an area of Monterey County, California, thought to be enzootic for EBA. A typical suspected EBA scenario on the Monterey County ranch was experienced in 1987 when 200 heifers were shipped from Pendleton, Oregon, and bred shortly after arrival in California. Seventy-five percent of the pregnant heifers aborted between June and September 1988. The next year these same cattle were bred, but this time only 7% - 8% aborted. Though EBA is thought to be the cause of the cattle abortions in this region, there is little supportive evidence of disease except for the characteristic lesions found on fetuses. No serological, epidemiological or pathological studies have confirmed the diagnosis. The following describes the development of an enzyme-linked immunosorbent assay (ELISA) for the detection of exposure to *B. coriaceae* in cattle, and its use in evaluating specific reactivity in sera from cattle on this ranch.

METHODS AND MATERIALS

Antigen preparation. *B. coriaceae* (ATCC 43381) was supplied by American Type Culture Collection, Rockville, MD, and a low passage, pathogenic strain of *B. burgdorferi*, N40, was supplied by Dr. Stephen Barthold, Section of Comparative Medicine, Yale University, New Haven, CT. Spirochetes were cultured in modified Barbour-Stoenner-Kelly (BSK II) medium at 34°C for 5 to 7 days (1). Following incubation the spirochetes were harvested by

centrifugation (12,000 x g for 30 minutes at 15°C), washed 3 times in phosphate buffered saline (PBS) and the protein concentration determined by the Lowry method (9). Aliquots of antigen (1 mg/ml in PBS) were stored at -20°C.

Serum Samples. Preimmune serum was taken from a 5 month old calf born on the ranch in Monterey County. The calf was then inoculated intramuscularly (IM) and subcutaneously (SC) with 4.0 ml of killed *B. coriaceae* (500 µg/ml) in Freund's Complete Adjuvant (FCA), and 4 weeks later was reinoculated SC with 1.0 ml killed *B.coriaceae* (500 µg/ ml) in FCA. Immune serum was collected from 3 and 7 weeks after the second inoculation. Preimmune serum (N) was used as the *B. coriaceae* negative serum control and immune serum (P) was used as the *B. coriaceae* positive serum control in all assays.

Sera were also collected from 4 groups of cattle. Group one was composed of sera from 10 herefords who were originally part of a group of 34 cows received from Pendleton, Oregon, in December of 1988, bred in April 1989 and which aborted between November, 1989 and January, 1990 (designated EBA positive). Sera from this groups were collected in March, 1990. Group two consisted of 6 sera from Holstein-Friesians raised in a feedlot in a nonenzootic area for EBA (designated EBA negative). Group three was composed of sera from 50 El Monterey heifers received from northern Texas 2 weeks prior to serum collection. Group four consisted of sera from 29 herefords born in Oregon, transferred to the Monterey ranch, raised on the ranch for 3 years, and then returned to Oregon. Sera from this group was collected 11 months after leaving the Monterey ranch.

Inhibitory Antigen Preparations. Immunization histories of all cattle were taken. Preparations used by ranchers to immunize the cattle were identified and used as heterologous antigens to absorb nonspecific reactivity (Table 1). A protein assay was performed on the seven vaccines (9) and equal amounts of each vaccine was combined to form an Inhibitory Antigen Mixture (IAM) at 25 ug/ml in ELISA antibody diluent (PBS containing 0.05% Tween 20)(C. B.

Thomas, Ph. D. dissertation, University of California, Davis, 1983).

Similar preparations were made of *B. burgdorferi* and *B. coriaceae* for use as heterologous and homologous antigens respectively.

Enzyme-linked immunosorbent assay. The ELISA was performed according to the Engvall and Perlmann method with some modifications (5). ELISA plates (Corning Laboratory Sciences Company, Corning, NY) were sensitized overnight at 37°C with 100 µl per well of *B. coriaceae* (20 µg/ml in 0.1 M carbonate, pH 9.6). Plates were washed 3 times with ELISA wash (0.15 M NaCl containing 0.8% Tween 20 [Sigma Chemical Company, St Louis, MO]) and serum samples were added. Each diluted sample to be plated (including the N and P control sera) was prepared in one of 4 ways: 1) unabsorbed serum diluted in ELISA antibody diluent, 2) a mixture of serum and IAM (1:1), 3) a mixture of serum, IAM, and *B. burgdorferi* (2:1:1) or 4) a mixture of serum, IAM, *B. coriaceae,* and *B. burgdorferi* (3:1:1:1). All samples were incubated for 30 minutes on ice then centrifuged (13,000 x g for 10 minutes). A 100 µl volume of each sample (with the final serum dilution in all cases at 1:250 in ELISA antibody diluent) was added to duplicate wells of *B. coriaceae*-sensitized plates and incubated at room temperature for 30 minutes. Plates were washed as before, then incubated for 30 minutes with 100 µl per well of horseradish peroxidase-conjugated rabbit anti-bovine IgG (Sigma Company, St, Louis, MO) diluted 1:4000 in ELISA antibody diluent. Plates were washed 4 times with ELISA wash and incubated at room temperature for 60 minutes with 100 µl per well of ELISA substrate (0.05 M citrate, pH 4.0 containing 2,2'-azino-di{3-ethylbenthi-azoline sulfonic acid} diammonium salt [Sigma Chemical Company, St Louis, MO]; and 1.5 mM hydrogen peroxide). ELISA stop solution (0.005% sodium azide in PBS) was added at 100 µl per well.

Absorbance was measured with a Bio-Tek EL-310 plate reader (Bio-Tek Inc., Burlington, VT) at dual wavelength (A_{405}/A_{450}), data

were transferred to a Macintosh computer with the aid of Datalog (Bio-Tek Inst. Inc, Winooski, VT), and analyzed with Statview 512+ (BrainPower, Inc., Calabasas, CA) and StatWorks (Cricket software, Philadelphia, PA). The mean measurement of duplicate test samples was calculated and used to derive a ratio-transformed expression of relative antibody activity compared with the positive and negative control sera included on the same microtiter plate. A ratio-transformed expression of the A_{405}/A_{450} means, referred to as the ELISA value percent (EV%) (2), was calculated as follows: (Where T, T_n, and T_p were the mean A_{405}/A_{450} values for the test, N and P control sera respectively) $EV\% = 100\ (T - T_n)\ /\ (T_p - T_n)$.

All sera were tested for reactivity against *B. coriaceae* without absorption and those with EV% reactivity of $\geq$ 35% (considered suspect *B. coriaceae* reactors) were submitted to a second ELISA procedure following absorption (using IAM/Bb and IAM/Bb/Bc). An inhibition $\geq$ 25% of the *B. coriaceae* EV% reactivity following absorption was accepted as confirmation of *B. coriaceae* specific reactivity and sera were considered as confirmed *B. coriaceae* reactive.

RESULTS

***B. coriaceae*-specific reactivity in EBA positive and negative populations (groups one and two).** *B. coriaceae*-specific reactivity without absorption and after absorption were compared (Fig. 1, 2). The difference between reactivities without absorption and after absorption represents heterologous reactivity removed with IAM or IAM/*B. burgdorferi.* The difference between reactivities using IAM/*B. burgdorferi*/*B. coriaceae* and IAM/*B. burgdorferi* for absorption represents the amount of specific reactivity remaining after removal of heterologous reactive antibodies.

The mean EV%s for 4 serum treatments for the EBA positive population (Fig. 1) were: Unabsorbed (0.510 ± 0.146), IAM absorbed (0.366 ± 0.158), IAM/*B. burgdorferi* absorbed (0.227 ± 0.166) and IAM/*B.burgdorferi*/*B. coriaceae* absorbed (0.082 ± 0.087). The EBA negative population results were: Unabsorbed (0.094 ± 0.08), IAM absorbed (0.014 ± 0.43), IAM/*B. burgdorferi* (-0.38 ± 0.03), and IAM /*B. burgdorferi*/*B. coriaceae* (-0.068 ± 0.017). This comparison, between the EBA positive and EBA negative populations, established the criteria for developing a screening assay for EBA based on *B. coriaceae* as the causative agent. An arbitrary criterion for classification of a sample as specifically reactive for *B. coriaceae* was based on two conditions: 1) sample EV% ≥35%, and 2) the EV% following the specific inhibition of *B. coriaceae* homologous antibodies ≥25%. Using these criteria to define samples as confirmed *B. coriaceae* reactive, our screening assay was 83% specific and 83% sensitive (Table 2).

***B. coriaceae*-specific reactivity in cattle from an EBA enzootic region (group 3) and cattle from a nonenzootic region (group 4).** Two distinct populations of cattle were evaluated for *B. coriaceae*-specific reactivity. *B. coriaceae* reactivities among sera from 29 cows, raised in a region enzootic for EBA, indicated that 25/29 (86%) were suspect *B. coriaceae* reactors and 21/29 (72%) were confirmed *B. coriaceae* reactors (Fig. 3). *B. coriaceae* reactivities among sera from 50 cows, raised in a nonenzootic area for EBA, indicated that 42/50 (84%) were suspect *B. coriaceae* reactors and 11/50 (22%) were confirmed *B. coriaceae* reactors (Fig. 4).

DISCUSSION

Presently, it is inferred that EBA is caused by *B. coriaceae* because borrelia-like organisms have been observed in aborted fetal

blood, EBA symptoms have been observed following feeding of *O. coriaceus,* and *B. coriaceae* has been isolated from an *O. coriaceus* (12, 15, 7). Diagnosis of EBA is based on clinical symptoms alone, abortion or birth of weak calves, and characteristic fetal lesions (6). There is no direct evidence for the cause of this disease - serological, epidemiological or pathological. Positive diagnosis of EBA could be achieved by isolation of a spirochete from aborted fetuses, but this has not yet been accomplished. Therefore, isolation of the causative agent is not an effective method of diagnosing EBA. A comprehensive diagnosis based on a positive serological test, the cow's history of tick bites, living in or traveling to EBA enzootic areas, and having clinical symptoms suggestive of EBA, could be used.

Indirect fluorescent antibody staining and ELISA have been used to detect antibodies to various bacterial diseases. With its ease of automation and more objective test results, ELISA would be preferred. ELISA diagnosis demonstrates a higher degree of sensitivity and specificity relative to clinical diagnosis of many microbial diseases (10).

In developing an ELISA for *B. coriaceae*-specific reactivity, cross reactivity with heterologous antibodies due to exposure of cattle to other infectious organisms must be taken into account. Routinely, cattle are immunized against various etiological agents as precautionary measures in disease prevention. Vaccines produce specific antibodies which can cross react with antibodies directed against other pathogens in an ELISA. In order to obtain a reliable ELISA for *B. coriaceae*, specificity was established in this study by absorbing the sera with vaccines to which the cattle had been immunized. It was also decided to absorb with *B. burgdorferi,* the causative agent of Lyme disease, since this spirochete is found in many of the same geographical areas as EBA.

An EBA negative population was compared to an EBA positive population and the ELISA profiles were quite distinct. As would be

expected, the EBA positive animals show much stronger reactivity. The change from the negative control serum to the positive control serum demonstrated a dramatic increase. Absorption of the positive control serum with the IAM/Bb/Bc demonstrates the reliability of the test in removing approximately 100% of the *B. coriaceae* homologous antibodies in the immune serum. In developing the criteria for the screening test, the first criterion was reactivity against *B. coriaceae* without absorption with an EV% of $\geq$ 35%. All EBA positive sera, i.e., those sera from cows which lived in an EBA enzootic area and aborted, met the first criterion except for cows #5 and #7 (Fig. 1). Approximately 60% of their unabsorbed EV% were removed with the IAM alone verses 45% - 5% for the rest of the sera. The assumption is that these two animals were probably exposed to some other organism of similar antigenic composition to one of the microbes in the IAM mixture. Both #5 and #7 had very low EV% inhibition for specific *B. coriaceae*. Although included in the EBA positive group by clinical criteria, they were not confirmed *B. coriaceae* reactors by our serological criteria. All EBA negative sera, i.e., those sera from cows raised in a nonenzootic area for EBA, fell below this level except for #8 (Fig. 2). Eighty-one percent of number 8's EV% was removed when *B. burgdorferi* was added, indicating that this animal was probably exposed to this spirochete. It is not known how much ELISA reactivity was due to cross reaction between the two species, therefore we can only say that it had been exposed to *Borrelia* and was not a confirmed *B. coriaceae* reactor by our ELISA. All EBA negative cows fell below the second criterion, in which the EV% inhibition for specific *B. coriaceae* must be $\geq$ 25%.

The results of the established ELISA for the EBA positive and negative populations were placed into a contingency table and the validity of the screening test evaluated (16). The validity of a screening test is judged by its sensitivity and its specificity, and in our case both were 83%. In comparison, one human Lyme disease ELISA was found to have 97% specificity and 92% sensitivity (11).

The screening test was then applied to two additional groups of cattle to test if absorption improved specificity. Of group 3 (from an enzootic area), 25 of the 29 (86%) were suspect *B. coriaceae*-specific reactors, and 21 of the 29 (72%) were confirmed *B. coriaceae* reactors. These results indicate that the majority of the cattle in group 3 had specific exposure to *B. coriaceae* consistent with their long residence in the EBA endemic area. These cattle had been on the Monterey ranch for 3 years, and 21 out of 29 of these cows still had high reactivity to *B. coriaceae* 11 months after being transferred to Oregon.

Group 4 was from a nonenzootic area for EBA. Forty-two out of 50 (84%) were suspect *B. coriaceae* reactors, but only 11 out of 50 (22%) were confirmed *B. coriaceae* reactors. This indicated that many of these cows were exposed to an antigenically related organism, possibly *B. burgdorferi.* A high incidence of Lyme disease have been reported in some areas of Texas (4).

This study has demonstrated that there is strong *B. coriaceae*-specific reactivity in sera from cows that have aborted while living in an EBA enzootic area, compared with sera from cows living in a nonenzootic area for EBA. The ELISA described in this paper might be useful in screening heifers when exposure to *O. coriaceus* is suspected, in evaluating the immune status of individuals or herds, and perhaps to evaluate immune response to EBA vaccine preparations. The next step will be utilization of western blot analysis to enhance studies on antigen specificity by testing sera from EBA enzootic regions against sera from EBA nonenzootic regions.

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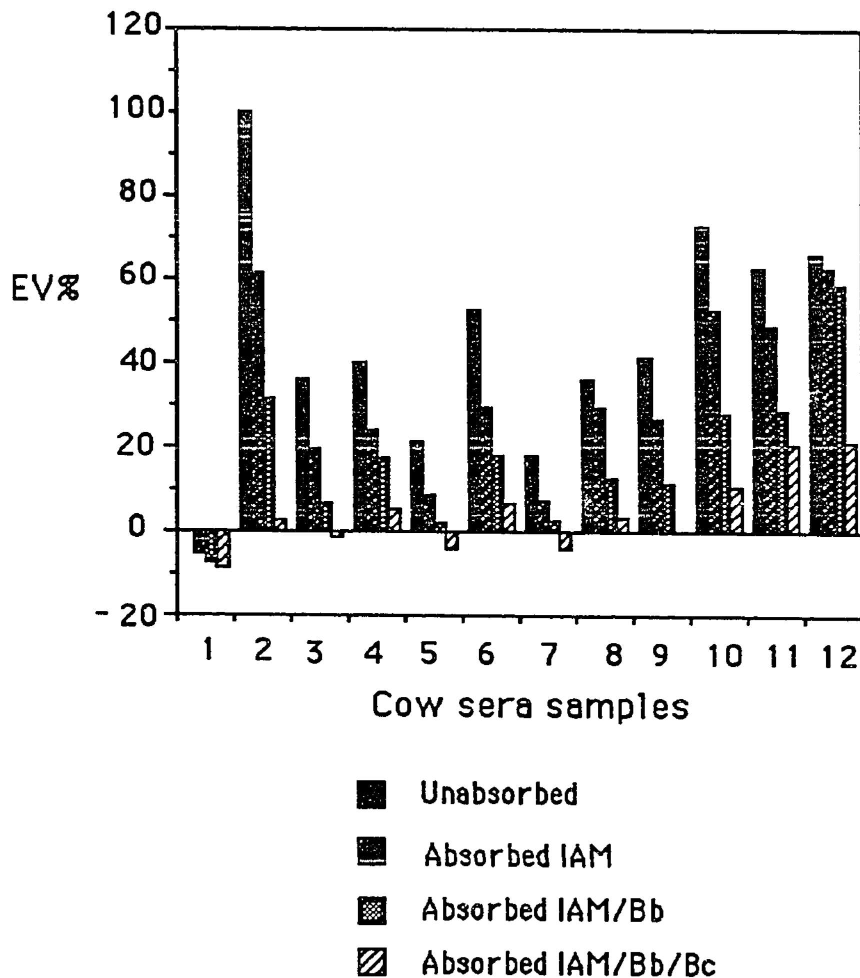

Fig. 1. *B. coriaceae*-specific reactivity without absorption and after absorption for cows from EBA enzootic area. #1 = preimmune (negative control serum), #2 = immune (positive control serum), and #3 - #12 = cows which aborted in an EBA enzootic area. Duplicate tests were performed on each sample.

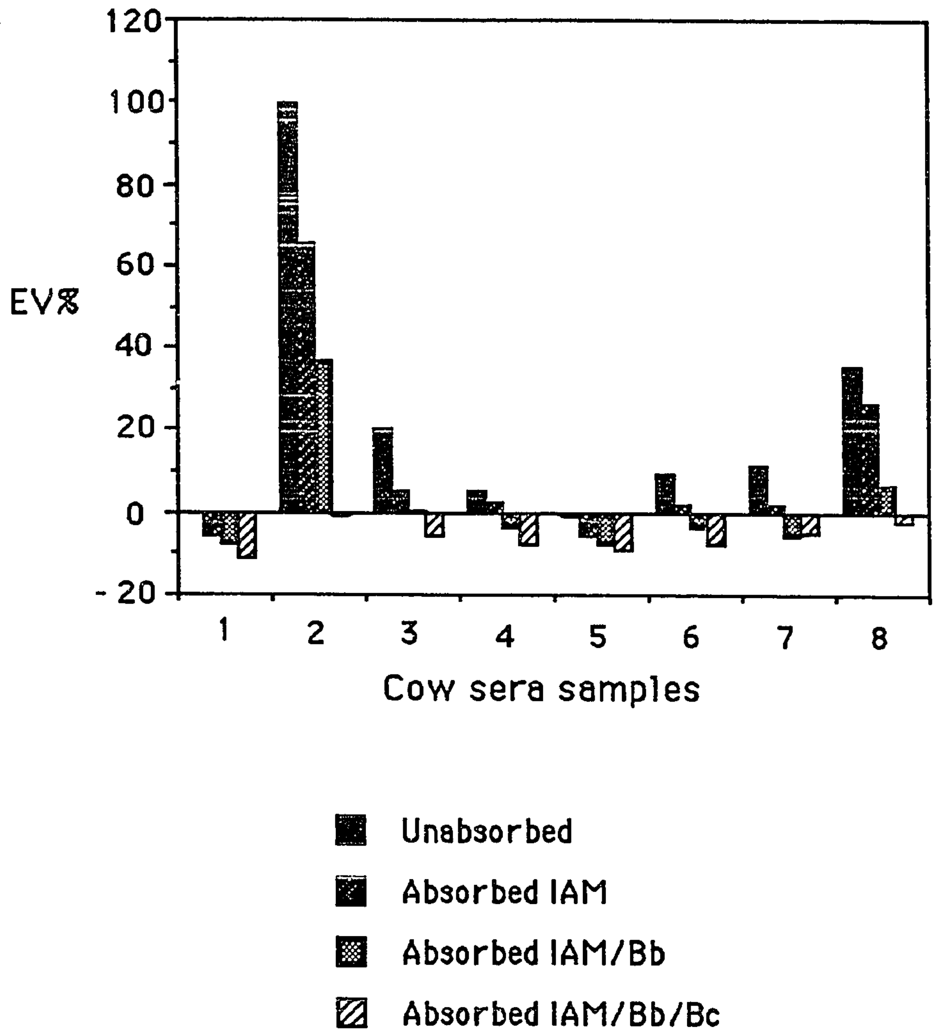

Fig. 2. *B. coriaceae* specific reactivity without absorption and after absorption from cows in a nonenzootic area for EBA. #1 = preimmune (negative control serum), #2 = immune (positive control serum), and #3 - #8 = cows raised in a nonenzootic area for EBA. Duplicate tests were performed on each sample.

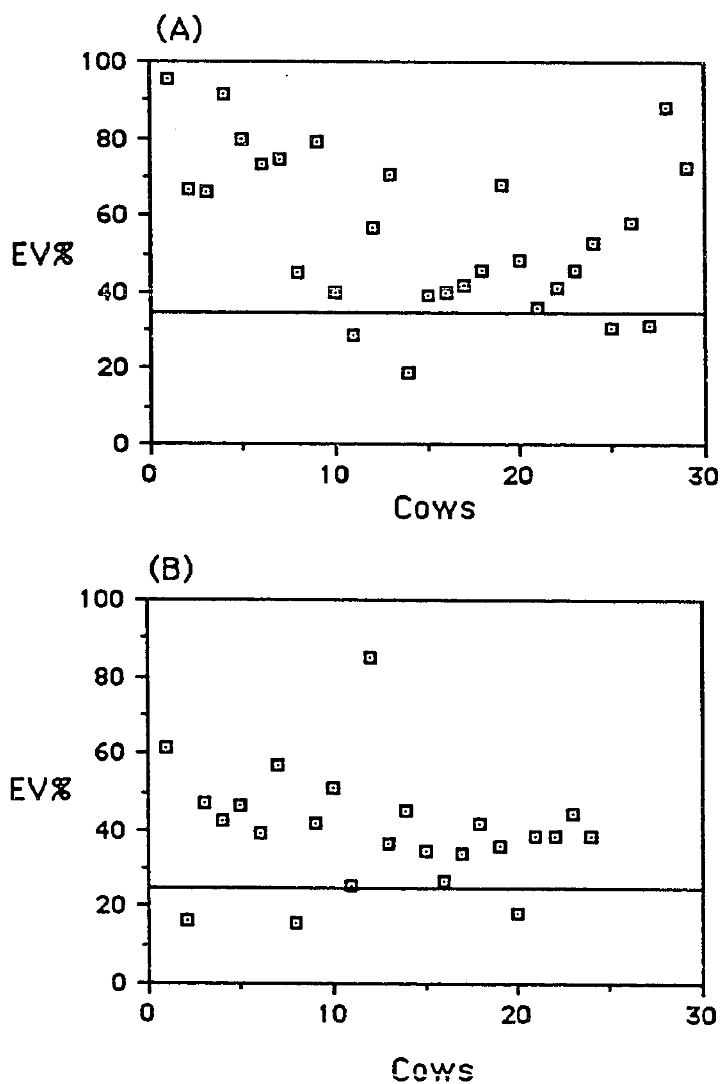

Fig. 3. *B. coriaceae* specific reactivity without absorption (A) and after absorption (B) of 29 serum samples from cows raised in an EBA enzootic area. Fig. 3A shows suspect *B. coriaceae* positive reactors above solid line. Fig. 3B, derived from the percent inhibition of *B. coriaceae* specific reactivity following absorption demonstrates confirmed *B. coriaceae* positive reactors above solid line.

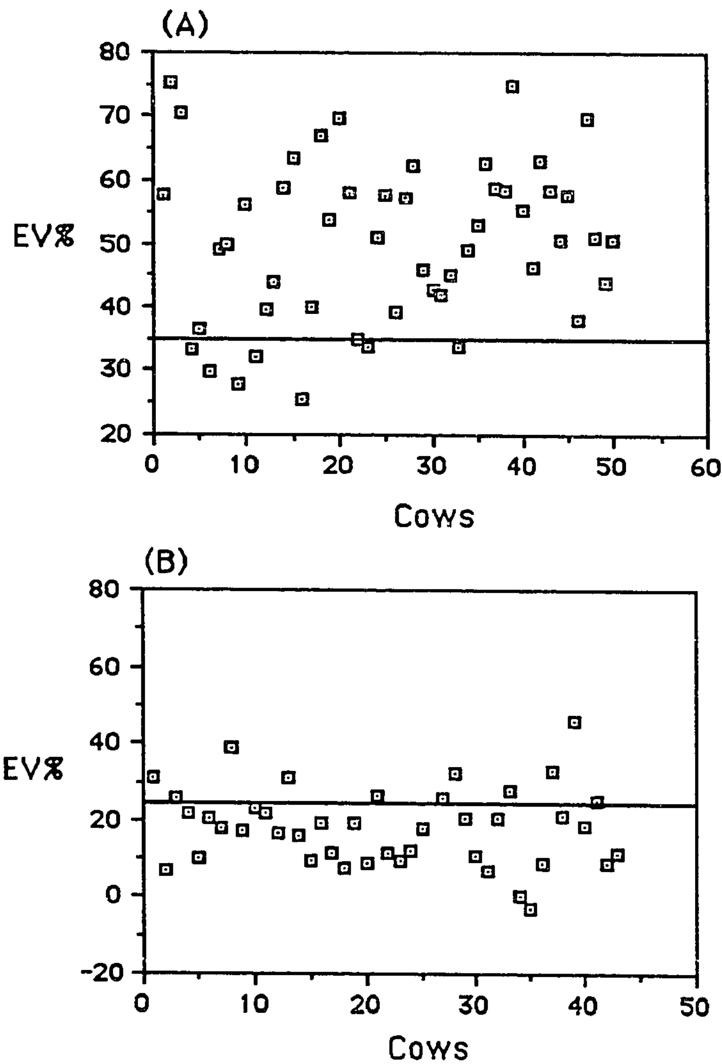

Fig. 4. *B. coriaceae*-specific reactivity without absorption (A) and after absorption (B) of 50 serum samples from cows raised in a nonenzootic area for EBA. Fig 4A indicates suspect *B. coriaceae* positive reactors above solid line. Fig. 4B, derived from the percent inhibition of *B. coriaceae*-specific reactivity following absorption demonstrates confirmed *B. coriaceae* positive reactors above solid line.

TABLE 1. Vaccines used for absorbing non-specific IgG antibody in ELISA for the detection of *Borrelia coriaceae*.

Brand Name	Manufacturer	Organisms Included In Vaccines
Triangle 4	Fort Dodge Lab Fort Dodge,IA	Bovine Rhinotracheitis Bovine Virus Diarrhea Parainfluenza 3 Virus Respiratory Syncytial Virus
Siteguard ML8	Coopers Animal Health Kansas City, KS	*Clostridium chauvoei* *C. septicum* *C. sordellii* *C. novyi* Type B *C. haemolyticum* *C. perfringens* Types B,C,D
Tri Vib 5L	Grand Lab Larchwood, IA	*Campylobacter fetus* *Leptospira canicola* *L. grippotyphosa* *L. icterohaemorrhagiae* *L. hardjo*
Brucella abortus vaccine	Diamond Laboratories, Inc. Des Moines, IA	*Brucella abortus* strain 19
Anaplasmosis vaccine	Fort Dodge Lab Fort Dodge, IA	*A. marginale*
Trichomonas vaccine	Fort Dodge Lab Fort Dodge, IA	*T. foetus*
Somnu Shield	Grand Lab Larchwood, IA	*Haemophilus somnu*

TABLE 2. A two-by-two contingency table of the true state of the EBA positive and negative populations and the relevant sensitivity and specificity ELISA test. (Adapted from N. R. Rose and H. Friedman, 1980, Manual of Clinical Immunology, 2nd ed. American Society for Microbiology, Washington, D. C.)

ELISA Results	EBA Positives[a]	EBA Negatives[b]
Confirmed *B. coriaceae* positive reactors	10	1
Confirmed *B. coriaceae* negative reactors	2	5
Totals =	12	6

Sensitivity = 83%
Specificity = 83%

[a]EBA positive = from enzootic area which aborted
[b]EBA negative = from EBA nonenzootic area

Chapter III

ORNITHODOROS CORIACEUS KOCH (ACARI: ARGASIDAE) AS AN INVERTEBRATE VECTOR FOR BORRELIOSIS OF CATTLE

ABSTRACT

The feeding activity of *Ornithodoros coriaceus* have been linked to Epizootic Bovine Abortion (EBA). In this study, *O. coriaceus* were collected by the use of CO_2 drop-traps on a ranch in Monterey County, California, thought to be enzootic for EBA. Collected ticks were dissected for culture or used for borrelia infection trials. Forty-three ticks were dissected, the hemolymph and salivary glands removed and cultured, and 7% of these ticks possessed spirochetes. Thirty-eight additional ticks were fed through membrane-feeding devices containing *B. coriaceae* or *B. burgdorferi* in BSK II medium, and hemolymph and salivary glands removed and cultured. Twenty-six percent of the samples contained spirochetes. We concluded that borreliae are present in *O. coriaceus* in the wild and that these ticks can be fed with borreliae under laboratory conditions.

INTRODUCTION

Epizootic Bovine Abortion (EBA) is a major cause of abortion and weak calf birth in rangeland cattle in the Western United States (6). The feeding activities of *O. coriaceus*, a tick vector, have been linked to EBA (11). A new species of spirochete, *Borrelia coriaceae*, has been isolated from this tick (7) and spirochetes have been observed in the blood of aborted bovine fetuses with EBA lesions (9).

O. coriaceus was originally collected in Mexico and given the

name parjaroello and is often regionally referred to as the parjaro tick in California. Since 1904 numerous collections of this species have been made in the coastal regions of California and intensive surveys have shown that this tick is widespread in California and other parts of Western United States (2).

O. coriaceus is commonly found in the bedding grounds of deer and cattle underneath foothill chaparral, scrub oak, cottonwood, manzanita, or mahogany brush areas at 185 - 2450 m elevations along the coast from San Diego County north to Humboldt County and to the east of the Sierra Nevada (Fig. 1). Ticks may be collected year round but are found more often from June through September (8). Ticks are attracted to the CO_2 given off by animals and so can be collected in CO_2 drop traps placed in the soil or tree-litter of animal bedding grounds (3, 5). It is not a highly visible tick and it is common for cattlemen to be unaware of its presence on their ranges. Its bite has been implicated in abortion or birth of weak, premature calves from heifers or cows that have grazed on foothill rangeland. It seldom occurs in indigenous cattle that have been sufficiently exposed to the ticks and have developed immunity to EBA. It is postulated that exposure to the ticks during the second trimester will result in abortion during the third trimester (11).

The objectives of this study were to trap *O. coriaceus*, attempt to isolate spirochetes from specific organs by dissection, and to attempt to infect the ticks with *B. coriaceae* and *B. burgdorferi* using a membrane-feeding device.

METHODS AND MATERIALS

Tick collection. Ticks were collected from June to September, 1990, from elevations of 270 - 1130 m at 24 locations on a ranch in Monterey County, California (Fig. 2). Carbon dioxide drop-traps consisted of a plastic photography tray lined with black paper and a

19 L plastic container filled with dry ice placed in the tray. Holes were punched 3 cm from the bottom of the container to allow for the escape of CO_2 to attract ticks. Trays were placed flush with the ground in bedding areas of cattle and deer for 8-10 hours (Fig. 3). Ticks were then collected from the traps and stored at room temperature in glass jars, in a darkened humidified micro-isolator cage (4).

Tick feeding. Ticks were fed through membrane-feeding devices modified from a method of Hokama et al. (4). Five ml of feeding media, sterile Barbour-Stoenner-Kelly (BSK II) broth with rifampicin (50μg/ml, [Sigma Chem. Co., St. Louis, MO]) or BSK II broth containing *B. coriaceae or B. burgdorferi*, was placed in a small petri plate, covered with alcohol-sterilized parafilm (0.12mm thick) secured by a tight-fitting delron ring, and heated to 37°C.

Four to five ticks were then set on top of a layer of guinea pig or mouse hair used as a phagostimulant (4) (Fig. 4). The ticks were contained in the feeding apparatus by a loose-fitted, raised plastic top and allowed to engorge for 30-90 minutes. Ticks were then placed in small, labeled plastic dishes with lids and stored at room temperature in a darkened, humidified containment chamber.

Tick dissection. A special plastic dissecting hood was constructed with two viewing holes for microscopes and a vinyl curtain to minimize air convection. Ticks were soaked in 70% ethanol for several minutes prior to dissection, immobilized on a styrofoam cube by a pin at the dorsal caudal region, wiped dry, and placed under the dissecting scope. Hemolymph was aspirated via a 27 1/2 gauge needle and syringe, and a small amount of BSK II with rifampicin was injected into the caudal region and reaspirated. The hemolymph aspirate was then placed into a tube containing BSK II with rifampicin, and incubated at 34° C for 1-5 days. Salivary glands were excised by dissection of ticks utilizing in incision along the lateral line from posterior to anterior. Salivary glands were

transferred to tubes containing BSK II with rifampicin and incubated as above.

Spirochete detection. Microscope slides prepared from salivary glands, hemolymph, and tick-fed sterile BSK II cultures were observed every day, up to 5 days, after incubation at 34°C. Slides were examined at 400 X magnification under dark phase microscopy.

RESULTS

Of 43 dissected ticks, three (7%) exhibited natural infections. Spirochetes were recovered from cultured hemolymph of 2 ticks and from one culture of BSK II medium on which ticks had fed. We were unable to maintain viable spirochetes, isolated from the ticks, in culture longer then 5 days. Of the 22 ticks experimentally infected with *B. coriaceae* through the membrane-feeding device, five (23%) had spirochetes in the hemolymph. Of the 16 ticks infected with *B. burgdorferi* N40, five (31%) of the ticks were infected. Spirochetes were found in four hemolymph cultures and in one salivary gland culture. In total, for the experimentally infected ticks, 10/38 (26%) were spirochete positive (Table 1).

DISCUSSION

Previous experimentation by Howarth and Hokama established parameters for entrapment and feeding of Argasid ticks; our technique provided an improvement on the membrane-feeding device, i.e. the dish, tight-fitting delron ring securing the parafilm, and the ability to retrieve the medium aseptically after tick feeding. The medium, BSK II, as modified by Barbour (1), was utilized in our

feeding device as a sole food source as well as to inoculate *O. coriaceus* with borreliae. The present study demonstrated that the ticks could be fed on BSK II with borreliae and that the spirochete could be extracted from the hemolymph and salivary glands after infection. Contrary to our findings, previous studies by Howarth and Hokama (5) established that *O. coriaceae* could be infected with arbovirus and pseudorabies virus. The arbovirus could readily pass from the gut into the hemolymph and salivary glands but the larger pseudorabies virus was unable to pass through the gut into the hemolymph.

The results of borreliae ingestion, ticks having consumed spirochete suspensions of BSK II, must be interpreted with caution since spirochetes, morphologically similar to *Borrelia sp.,* appeared in 7% of the sterile BSK II medium after tick feeding. Evidently, borreliae are present in Argasid ticks in the wild and can be introduced into ticks in the laboratory.

Dissection of ticks was performed initially through the technique described by Ribeiro et al. (10); however, it was necessary to alter several components. Since we were unsuccessful in retrieving hemolymph by amputating the tick leg, we extracted hemolymph via a needle inserted in the dorsal caudal area. Though we were able initially visualize the spirochetes microscopically, we were unable to maintain them in culture for prolonged periods. This could have been due to the shock factor of transferring them from tick to medium or the lack of some vital component in BSK II needed to maintain growth.

Of the laboratory infected ticks, spirochetes were microscopically detected in the hemolymph more often than in the salivary glands. Spirochetes may not have been visualized under darkfield microscopy in the salivary glands cultures due to low concentrations of organisms. It was earlier found through the use of direct immunofluorescence that 24% of the hemolymph from naturally infected adult *O. coriaceus* contained spirochetes, and of

that 24%, 71% - 85% had positive salivary gland infections as well (7). Since, by our approximation, an adult tick can consume about 100 µl of cell suspension at 10^6 organisms per ml, the low detection of spirochetes microscopically seen in the hemolymph and the lack of spirochetes seen in the salivary glands might be greatly enhanced with the use of direct immunofluorescence.

In this study we developed procedures for sterile extraction of hemolymph and salivary glands from *O. coriaceus.* These techniques could be used to isolate organisms from *O. coriaceus* which would be an important step in confirming the pathogenic agent for EBA. Inoculation of *O. coriaceus* via a membrane-feeding device would be useful in studying the spirochete's passage though the tick's tissues and transstadial survival. It would also be useful in examining transovarial passage.

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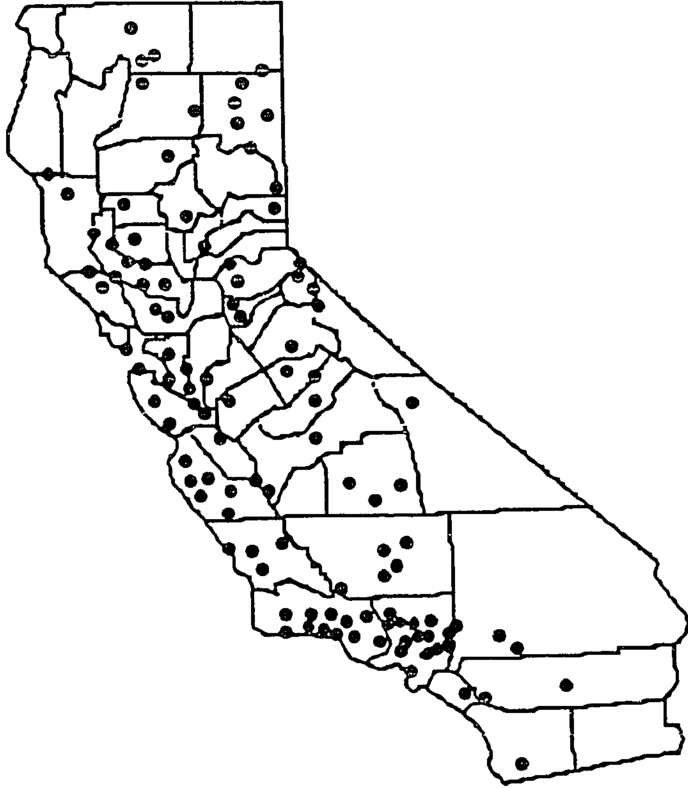

Fig. 1. Isolations of the tick, *O. coriaceus,* in California. (Adapted from D. P. Furman and E. C. Loomis, Bulletin of California Insect Survey, Univ. of California Press, Berkeley, CA, Vol. 25).

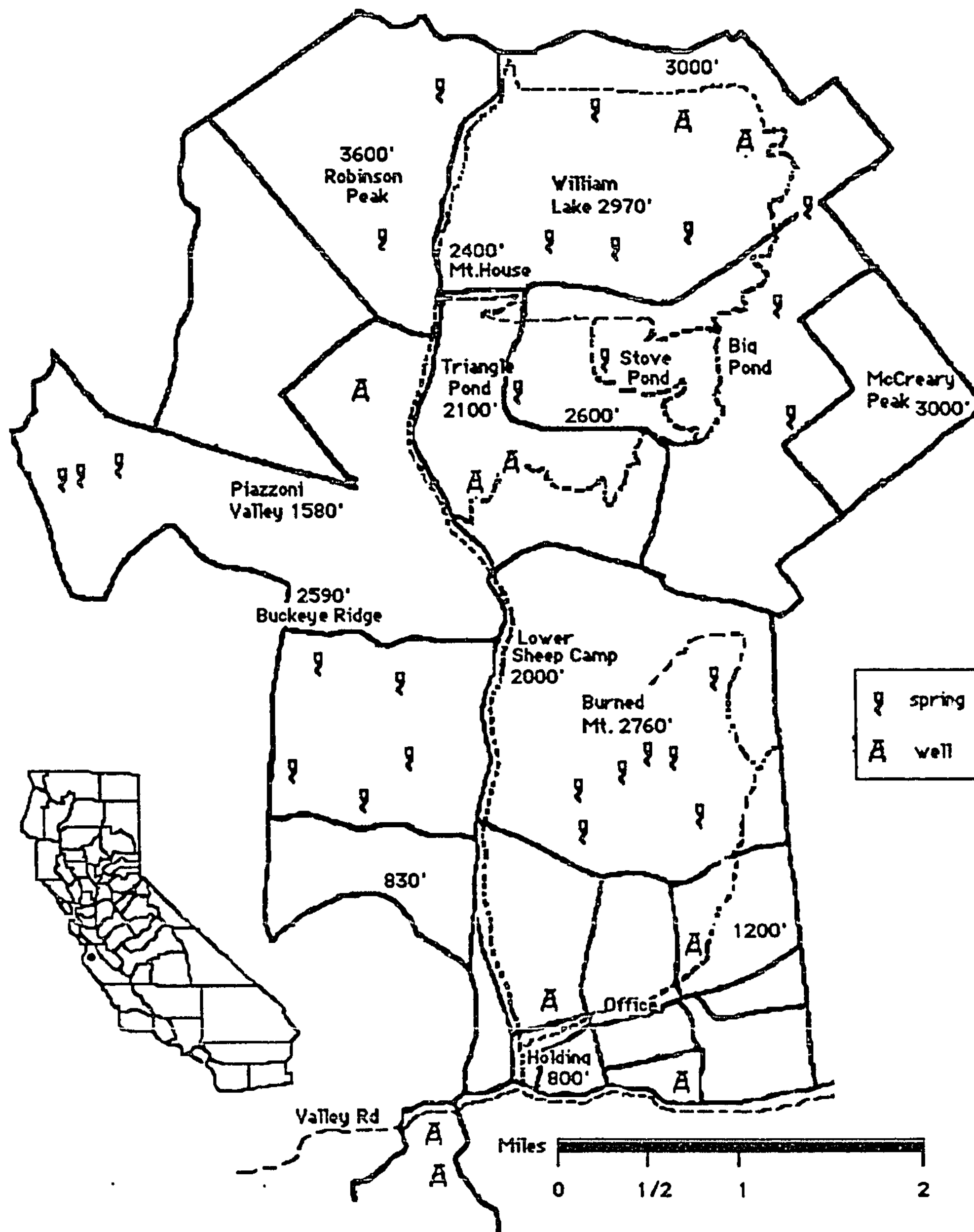

Fig. 2. Cattle ranch in Monterey County where *O. coriaceae* were collected. Dot on California map represents approximate location of ranch.

Fig. 3. Carbon dioxide drop-trap placed in one of the 24 locations on study ranch in Monterey County, California.

Fig. 4. Membrane-feeding device, with BSK II, and *O. coriaceus* before (right) and after feeding (left).

Table 1. Results of darkfield observation of *O. coriaceus* in cultures of hemolymph, salivary glands and tick infected Barbour-Stoenner-Kelly medium (BSK II).

Infection Status of Ticks	# of Ticks Examined	# of Positive Spirochete Cultures Hemolymph	Sal. Gland	BSK II	Total
Non-experimental Infections	43	2 (5%)	0	1 (2%)	3(7%)
Experimental Infections					
B. coriaceae	22	5 (23%)	0	nd	5(23%)
B. burgdorferi	16	5 (31%)	1[a] (6%)	nd	5 (31%)

Exp. Infected total = 26%

[a]Sample from one of the 5 ticks that was hemolymph positive.

Chapter IV
EXPERIMENTAL INFECTION OF MICE WITH *BORRELIA CORIACEAE* AND *BORRELIA BURGDORFERI*

ABSTRACT

The susceptibiiity of laboratory mice to infection with *Borrelia coriaceae* and *Borrelia burgdorferi* was evaluated in mated female C3H/He mice. Female mice were inoculated on day 4 and 10 of mating and observed through gestation for evidence of abortion or teratogenesis in their pups. Forty percent (2/5) of the mice inoculated with *B. coriaceae* and 29% (2/7) of those inoculated with *B. burgdorferi* N40 gave birth to teratic pups. Lyme arthritis developed in 78% (7/9) of the unmated females inoculated with *B. burgdorferi* and only 25% (2/8) of the mated females. Mated and unmated females inoculated with *B. coriaceae* did not exhibit disease symptoms. Unmated mice inoculated with *B. burgdorferi* or *B. coriaceae* uniformly developed ELISA antibody titers twice as high as mated mice. Mated mice which were exposed to *Ornithodoros coriaceus,* which had been previously inoculated with *B. coriaceae* or *B. burgdorferi* via an membraned-feeding device, showed no teratogenesis, no Lyme arthritis and low ELISA antibody titers to both organisms.

INTRODUCTION

Lyme disease has become the most frequently reported tick-borne illness in the United States since it was first reported in 1977 (19). Despite research concerning the clinical manifestation of Lyme disease, the mechanisms of pathogenesis remain poorly understood. A number of attempts have been made to develop an animal model

which could elucidate these mechanisms. Laboratory rabbits and guinea pigs develop skin lesions resembling human erythema migrans after infection with *B.burgdorferi,* but no other clinical manifestations were noted (6, 11). Syrian hamster and white footed mice (*Peromyscus leucopus*) have been infected with Lyme spirochetes but did not develop clinical manifestations of Lyme disease (10, 20). Lyme disease polyarthritis in experimental animals was demonstrated in weanling and 3-week-old inbred Lew/N rats in work by Barthold et al. (3). More recently, Barthold et al. demonstrated polyarthritis in several strains of weanling and 3-week-old inbred mice following laboratory infection. Inbred C3H/He mice were shown to be the most susceptible mice to Lyme borreliosis (4).

Epizootic Bovine Abortion (EBA) is a disease that affects pregnant cattle in the third trimester of gestation. It causes abortion and birth of weak calves (14). The tick vector for EBA was established in 1976 as being *Ornithodoros coriaceus* (18), the pathogenic agent was identified as a spirochete in 1986 (14) and a *Borrelia sp.* was isolated from *O. coriaceus* and later identified as the new species, *B. coriaceae* (12). At this time, there are no reports tying *B. coriaceae* directly to the disease caused in cattle and an animal model has not been developed. An animal model for EBA would be extremely useful in establishing the etiological agent, and also in developing strategies for its control.

With the establishment of clinical manifestations of Lyme borreliosis in mice by Barthold et al. (4), we postulated that C3H/He mice might also serve as a model for EBA. The use of laboratory mice for an animal model is desirable for a number of reasons, including the extensive immunological and genetic information already established for this species. We report here preliminary experiments to establish the feasibility of a C3H/He mouse model for EBA.

METHODS AND MATERIALS

Bacterial strains. *Borrelia burgdorferi* B31 (type strain, ATCC 35210) was provided by Dr. Robert Lane, Department of Entomological Sciences, University of California, Berkeley, CA. A low passage, pathogenic strain of *B. burgdorferi* N40 was supplied by Dr. Stephen Barthold, Section of Comparative Medicine, Yale University, New Haven, CT, and *B. coriaceae* (ATCC 43381) was supplied by American Type Culture Collection, Rockville, MD. Spirochetes were grown in modified Barbour-Stoenner-Kelly (BSK II) medium (1) at 34°C to a concentration of approximately 1 x 10^7 viable organisms/ml for inoculation or ingestion by ticks. Viability was determined by examination under darkfield microscopy.

Experimental Animals. Inbred C3H/HeN (Simonsen Laboratories, Gilroy, CA) were health certified, shipped in filtered crates, housed in micro-isolator cages and provided with food and water ad libitum.

Experimental inoculation of three-week-old mice by injection. Three-week-old C3H/He mice were inoculated intraperitoneally (IP) with one of the following: 1) ≈ 1 x 10^6 *B. burgdorferi* N40 in 0.7 ml of RPMI (Sigma Chem. Co., St. Louis, MO), n = 4 mice; 2) ≈ 1 x 10^6 *B. burgdorferi* N40 in 0.7 ml of BSK II, n = 2 mice; 3) ≈ 1 x 10^6 *B. coriaceae* in 0.7 ml of BSK II, n = 2 mice; 4) ≈ 1 x 10^6 *B. burgdorferi* B31 in 0.7 ml of BSK II, n = 2 mice; and 5) 0.7 ml of BSK II, n = 2 mice. Mice were examined over a 30 day period for arthritis and any other disease symptoms. Blood was collected on day 1 and day 30 post inoculation and small aliquots cultured in 5 ml of BSK II with rifampicin (50 µg/ ml [Sigma Chem. Co., St. Louis, MO]) (9, 21) at 34°C. Immune blood was examined immediately for spirochetes using darkfield microscopy. Other aliquots were allowed to clot, and serum was separated by centrifugation, and frozen at -20°C). Animals were sacrificed with carbon dioxide gas,

dissected, and examined for gross evidence of pathology. Spleen cells and any spirochetes were obtained by injecting BSK II into the spleen, withdrawing the suspension, placing it in 5 ml of BSK II with rifampicin (50 µg/ml) and incubating at 34°C . The cultures were examined by darkfield microscopy for spirochetes after 1, 2 and 3 weeks.

Experimental inoculation of mated mice by injection. Sibling groups of 2 females and 1 male, or 3 females were caged for 10 days and allowed to mate if both sexes present. After 4 days females were inoculated (IP) with one of the following: 1) $\approx 1 \times 10^7$ *B. burgdorferi* N40 in 1.0 ml of BSK II, n = 4 mated females; 2) $\approx 1 \times 10^7$ *B. coriaceae* in 1.0 ml of BSK II, n = 4 mated females; 3) $\approx 1 \times 10^6$ *B. burgdorferi* N40 in 1.0 ml of RPMI, n = 4 mated females; 4) 1.0 ml of RPMI, n = 4 mated females; 5) 1.0 ml of BSK II, n = 4 mated females; 6) $\approx 1 \times 10^6$ *B. burgdorferi* N40 in 1.0 ml of RPMI, n = 4 unmated females; 7) $\approx 1 \times 10^7$ *B. burgdorferi* N40 in 1.0 ml of BSK II, n = 4 unmated females; and 8) $\approx 1 \times 10^6$ *B. coriaceae* in 1.0 ml of RPMI, n = 4 unmated females. All females were reinoculated subcutaneously (SC) with the same inoculum in 0.5 ml on day 10.

Mated animals were examined daily for occurrence of abortion, premature birth, and arthritis. Pups were examined for gross teratogenic effects and symptoms of arthritis. Blood was collected from inoculated mice on days 1 and 35 of the experiment and processed as previously described for 3-week-old mice, and cultured if deemed pertinent.

Experimental inoculation of mice with spirochetes from borrelia fed ticks. *O. coriaceus* were experimentally inoculated by ingestion of viable cultures of *B. burgdorferi* N40, *B. coriaceae* and BSK II via membrane-feeding devices under aseptic conditions (Chapter III). Nine 3-week-old C3H/He mice were anesthetized with 0.15 mls "rodent cocktail" (100 mg/ml ketamine hydrochloride [Ketaset], 20 mg/ml xylazine [Rompun], 10 mg/ml acetapromazine [Acepromazine] and saline) and placed on petri dishes in a darkened

room. An area on the dorsal surface of each mouse was shaved and over that area was placed a delron feeding chamber with gauze filter. Ticks were placed in small beakers in a waterbath at 37°C for 15 min to equilibrate. On top of the filter was placed mouse hair and 5 ticks inoculated with N40, BSK II or *B.coriaceae* (Fig. 1). The ticks were allowed to feed until at least one of the ticks had attached, engorged, and disengaged. Mice were examined over a 30 day period for arthritis and other clinical symptoms. Blood was collected on days 1 and 35. Animals were sacrificed on day 36 with carbon dioxide gas and spleen cells cultured as previously described

Five mated females were exposed to laboratory inoculated ticks, approximately 7 days after gestation. Three of the mice were exposed to ticks inoculated with *B. coriaceae* and two mice were exposed to ticks inoculated with BSK II medium. The same protocol was used for the mated mice as for the 3-week-old mice.

Serology. Specific serological reactivities to *B. coriaceae* and *B. burgdorferi* were determined by ELISA. Microtitration plates (Corning Laboratory Sciences Company, Corning, NY) were sensitized overnight at 37°C with 100 µl per well of *B. burgdorferi* and *B. coriaceae* (20 µg/ml in 0.1 M carbonate pH 9.6). Plates were washed 3 times with ELISA wash (0.15 M NaCl containing 0.8% Tween 20 [Sigma Chem. Co., St. Louis, MO]), antisera were diluted 1:1000 in ELISA antibody diluent (0.95 M Tween 20 in PBS) and were added to the wells in duplicate and incubated for 30 min. at room temperature. Plates were washed as before, and horseradish peroxidase conjugated antibody goat anti-mouse IgG (Antibodies, Inc., Davis, CA) was diluted 1:2000 in ELISA antibody diluent, added to the wells, and incubated for 30 min at room temperature. Plates were washed 4 times with ELISA wash, and incubated at room temperature for 60 min. with 100 µl per well of ELISA substrate (0.05 M citrate, pH 4.0 containing 2,2'-azino-di{3-ethylbenthi-azoline sulfonic acid} diammonium salt [Sigma Chem. Co., St. Louis, MO]; and 1.5 mM hydrogen peroxide). ELISA stop solution (0.005% sodium azide in PBS) was added at 100 µl per well.

Absorbance was measured with a Bio-Tek EL-310 plate reader (Bio-Tek Inc., Burlington, VT) at dual wavelength (A_{405}/A_{450}), data were transferred to a Macintosh computer with the aid of Datalog (Bio-Tek Inst. Inc, Winooski, VT), and analyzed with Statview 512+ (BrainPower, Inc., Calabasas, CA) and StatWorks (Cricket software, Philadelphia, PA). The mean measurement of duplicate test samples was calculated and used to derive a ratio-transformed expression of relative antibody activity compared with the positive and negative control sera included on the same microtiter plate. A ratio-transformed expression of the A_{405}/A_{450} means, referred to as the ELISA value percent (EV%) (5), was calculated as follows: (Where T, T_n, and T_p were the mean A_{405}/A_{450} values for the test, N and P control sera, respectively) $EV\% = 100 \, (T - T_n) / (T_p - T_n)$.

RESULTS

Teratic pups born to mice. Mated mice inoculated with *B. burgdorferi* N40 bore teratic pups in 2/7 (29%) cases (Table 1). Mated mice inoculated with *B. coriaceae* bore teratic pups in 2/5 (40%) cases. Mated mice inoculated with BSK II alone bore teratic pups in 1/4 (25%) of the cases. Mated mice inoculated with RPMI all bore normal pups.

Presence of Arthritis symptoms in mice. Unmated C3H/He mice, both 3-week-old group and the 8-week-old group, were highly susceptible to *B. burgdorferi* N40 and developed swelling in the tibiotarsal joint of the hind legs (Fig. 2, Table 2). Neither mated or unmated C3H/He mice injected with *B. coriaceae* showed any disease symptoms. Mice inoculated with B31, RPMI, or BSK II demonstrated no overt disease symptoms.

Borrelia ELISA Value % (EV%) of mice. The EV% of the

unmated mice inoculated with *B. burgdorferi* N40 was substantially higher than the EV% for mated mice inoculated with *B. burgdorferi* N40 (Table 3). The EV% for mice inoculated with *B. burgdorferi* N40 was much higher than for those inoculated with *B. coriaceae, B. burgdorferi* B31, BSK II or RPMI. The EV% of the unmated mice inoculated with *B. coriaceae* was higher than the EV% for mated mice inoculated with *B. coriaceae*. The EV% for *B. burgdorferi* B31, BSK II and RPMI was low.

Blood and spleen cultures. After 2 weeks of incubation at 34° C, living spirochetes were seen under darkfield microscopy in 2 spleen cultures from 3-week-old mice inoculated with *B. burgdorferi* N40 (1 x 10^6/ml of BSK II). No living spirochetes were observed in any of the blood cultures or in any of the other spleen cultures.

Mated and unmated mice exposed to *O. coriaceus*. There were no arthritic symptoms in mice exposed to the bite of borrelia fed ticks. Blood and spleen cultures microscopically observed, from all mice, were negative. Pups were born normal to mated females exposed to *O. coriaceus* infected with *B. coriaceae*. EV%s for all mice were very low.

DISCUSSION

Presently, the relationship between *B. coriaceae* and EBA is unclear, although *O. coriaceus* is associated with EBA, spirochetes have been observed in tissue from aborted calves after exposure to *O. coriaceus,* blood inoculum from naturally infected EBA fetuses can cause abortion in pregnant heifers, and *B. coriaceae* was isolated from *O. coriaceus* (12, 18, 14, 15). If *B. coriaceae* could be shown to cause serological, pathological, or immunological in vivo changes consistent with EBA, then a more direct link between *B. coriaceae* and EBA could be established.

Experimental models have been developed for a related spirochete pathogen, *B. burgdorferi.* Syrian hamsters and white-footed mice (*P. leucupus*) are easily infected by *B. burgdorferi* and can readily be used as a source for first passage organisms (10, 20). New Zealand white rabbits and guinea pigs develop erythema migrans similar to those observed in humans following infection (6, 11). Lyme arthritis symptoms have been exhibited in hamsters, rats, and mice (3, 4, 10). Antibiotics have been tested in experimentally infected hamsters and immune serum has been used to neutralize infections in rats, hamsters and mice (10, 16, 7, 19). An animal model for *B. coriaceae* could be useful in establishing the role of *B. coriaceae* in infection, fetal teratogenesis, or immunity associated with EBA. As yet, there are no published findings on any animal models for EBA. Our preliminary study demonstrated the possible link between teratogenesis in pregnant mice and *B. coriaceae* infection using pregnant C3H/He mice.

There are many variables in developing an animal model. One variable is choosing a virulent strain of spirochete. It is known for other species, of *Borrelia,* that after numerous in vitro passages virulence is lost (2). Since there has only been one isolation of *B. coriaceae,* that from a tick, it is impossible to compare the original isolates virulence with laboratory passage *B. coriaceae*. We used *B. burgdorferi* N40 for comparison since it is a human pathogen which can be obtained in low passage. Both spirochetes are transmitted by ticks, primary hosts can be deer, and they have antigens in common (2). The difficulty arises in comparing the results of animal inoculations between *B. coriaceae* and *B. burgdorferi* because the clinical symptoms and ELISA reactivity are quite different.

Any conclusions drawn from the results presented here should be regarded as preliminary for several reasons. Conception rates could not be determined with accuracy, hence, the terms "mated" and "unmated" were used. Early abortions or spirochete-prevented

pregnancies were impossible to detect, there are no known chemical pregnancy tests for mice, and physiological tests are unreliable.

We inoculated each mouse twice since it has been determined that cows need to be bitten several times to cause EBA. Barthold et al. used 1×10^7 *B. burgdorferi*/ml of BSK II (4), so this concentration was used initially, though in a replica experiment 1×10^6 spirochetes/ml of RPMI was used. We inoculated the mice with needles containing borreliae, and by feeding borreliae fed *O. coriaceus* on the mice to mimic natural transmission since other factors along with the spirochetes may promote EBA.

We observed our laboratory inoculated ticks feeding on mice, but observable arthritis, positive blood or splenic cultures, and diagnostic antibody EV%s were not obtained. Infection of mice by ticks experimentally inoculated through a membrane-feeding device has not been previously reported. Experiments with *Ixodes dammini* have shown that contact with a host provides stimulation to disseminate *B. burgdorferi* from the guts to the hemocoel and salivary glands for delivery to a new host (17). We postulate this is true for *Ornithodoros* ticks as well, since we found they pass into the hemolymph and salivary glands (Chapter III, Table 1). Since the ticks consumed approximately 1×10^6 spirochetes (100µl of a suspension of 1×10^7 organism/ml), it is unclear why mice did not apparently become infected by the laboratory inoculated ticks. Further studies are needed to establish a model for the natural transmission of spirochete from tick to mouse.

Without considering inoculum size, 40% (2/5) of dams inoculated with *B. coriaceae,* and 29% (2/7) of dams inoculated with *B. burgdorferi* had pups born close to or full-term that were teratic or dead. Any earlier reabsorption of embryos could not be determined. Some animals inoculated with BSK II alone also had teratic pups, the effects due to antigens found in the medium (BSK II) and the effects specifically due to the *Borrelia* inoculum were difficult to delineate. BSK II contains bovine serum albumin and whole rabbit

serum which may adversely affect pregnancy, although adverse effects were not seen by Barthold et al. with nonpregnant mice (4). Garcia-Monco et al. recommended using RPMI 1640, which does not introduce additional antigenic substances, and when used in our study no effect on pregnancy was observed in the control group (8).

The EV% of the unmated mice inoculated with *B. burgdorferi* N40 and *B. coriaceae* was almost twice as high as the EV% of the mated mice (Table 3). In a serosurvey of Lyme disease in which cord blood from a population of pregnant women from an endemic area was compared to cord blood from a population of pregnant women from a nonendemic area no correlation between congenital malformation and the presence of detectable antibody to *B. burgdorferi* could be established (22). Pregnancy may affect the immune response of the mother to such a degree that she fails to respond to various antigens. Adrenocortical hormones increase during pregnancy and these are known to impair the development of sensitivity to many antigens, including tissue grafts. Blocking factors, such as Alfa-fetoprotein (AFP) in murine pregnancies, are directed against antibody formation, especially IgA and IgG and suppress the mixed leukocyte reaction. Because of the suppression of both cellular and humoral immunity during pregnancy, *Borrelia sp.* may more readily attack the uterus, "an immunologically privileged site" (13).

The role of a hyperimmune response in pathogenesis is supported by an inverse correlation between the induction of arthritis and pregnancy in mice. Only 2/8 (25%) of the mated group developed arthritic swelling of the tibiotarsal joints, as compared to 7/9 (78%) in the 8-week-old nonpregnant mice and 2/2 in the 3-week-old mice (Table 2).

Inoculum size may have contributed to teratogenesis. When 1 x 10^7 spirochetes/ml of medium were used, teratogenesis was 1/1 for dams inoculated with *B. coriaceae* and 2/3 for dams inoculated with *B. burgdorferi.* In comparison, when 1 x 10^6 spirochetes/ml of medium were used, the number of teratic pups drops to 1/4 for dams

inoculated with *B. coriaceae* and no teratogenic effects in *B. burgdorferi* inoculated dams, although somewhat confounded by suspending medium. Although there appeared to be some differences in the number of abortions in animals inoculated with borreliae as compared to those that were not, low numbers of animals were used; therefore the experiments need to be repeated with larger populations of mice.

This report summarizes some preliminary data regarding *B. coriaceae* and *B. burgdorferi* infections in mice, and the potential for evaluating *B. coriaceae's* role in EBA. Since some proportion of mated mice did not conceive, the effects of spirochete inoculation on pregnancy observed here may be conservative. A correlation between spirochete inoculation and teratogenic effects in pregnant mice may be useful in the study of EBA.

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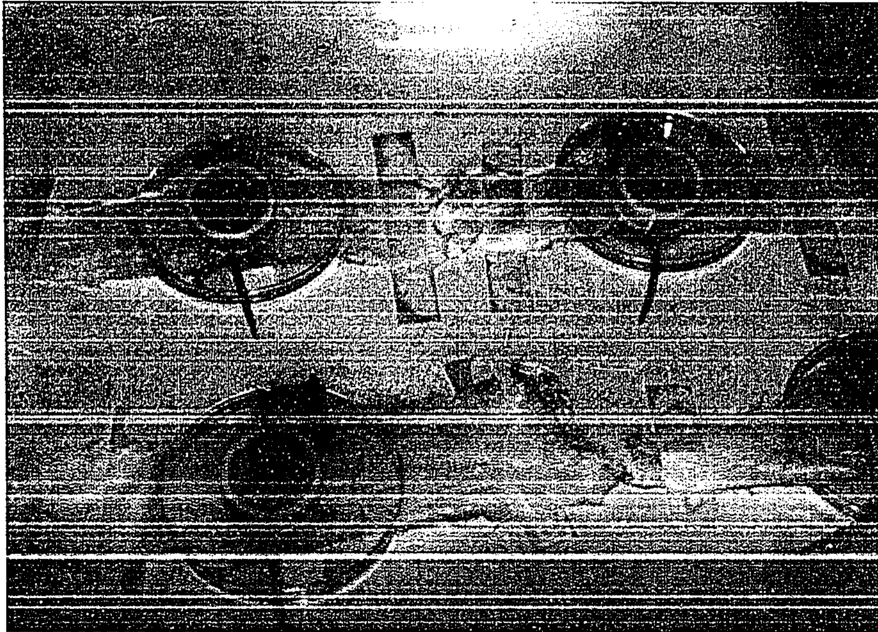

Fig. 1. Method used for inoculating mice with Borrelia-exposed *O. coriaceus.*

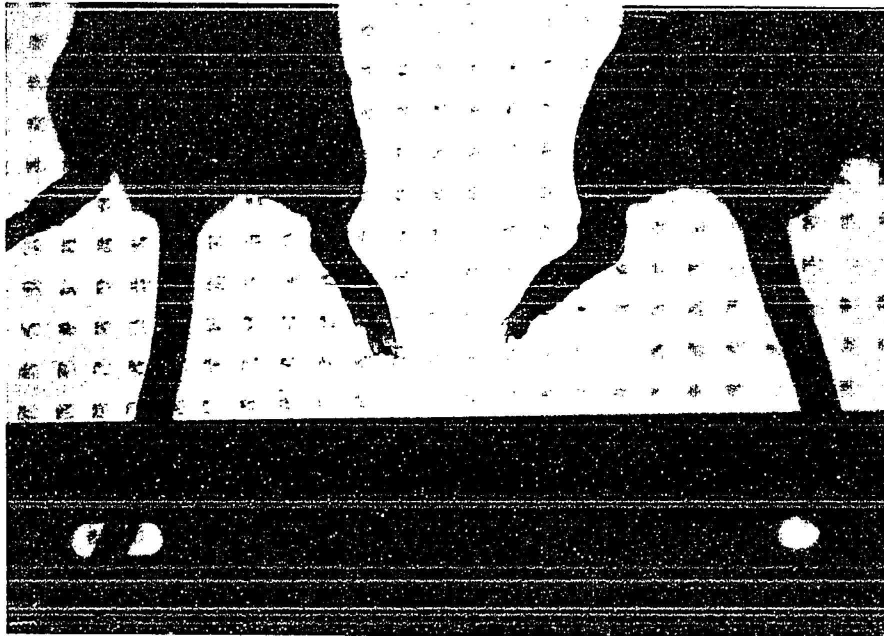

Fig. 2. Rear legs of C3H/He mice inoculated with *B. burgdorferi* (right) or BSK II (left). Note the tibiotarsal swelling in mouse inoculated with *B. burgdorferi.*

Table 1. Proportion of parturitions and teratic pups born to 8-week-old C3H/He mice inoculated with *B. burgdorferi* N40, *B. coriaceae,* and uninoculated medium. Results are combined from two experiments.

Experimental Inoculum	No. of Mice Inoculated	Proportion of Dams with Parturitions	Dams with Teratic Pups
B. burgdorferi N40[a]	4	4/4	0/4
B. burgdorferi N40[b]	4	3/4	2/3
B. coriaceae[a]	4	4/4	1/4
B. coriaceae[b]	4	1/4	1/1
RPMI	4	2/4	0/2
BSK	4	2/4	1/2

[a] 1×10^6 in RPMI
[b] 1×10^7 in BSK

Table 2. Proportion of mated and unmated C3H/He mice inoculated at 3 or 8 weeks of age with *B. burgdorferi* N40, *B. burgdorferi* B31, *B. coriaceae,*or medium alone developing arthritis within 35 days. Data combined from two experiments.

Experimental Inoculum	Mouse Age (Weeks)	Proportion of Mated Females With Arthritis	Proportion of Unmated Females With Arthritis
B. burgdorferi N40[a]	8	1 / 4	2 / 3
B. burgdorferi N40[b]	8	1 / 4	3 / 4
B. burgdorferi N40[c]	3	- - -	2 / 2
B. burgdorferi B31[c]	3	- - -	0 / 2
B. coriaceae[a]	8	0 / 4	0 / 4
B. coriaceae[b]	8	0 / 4	0 / 4
B. coriaceae[c]	3	- - -	0 / 2
RPMI	8	0 / 2	0 / 2
BSK II	8	0 / 4	- - -
BSK II	3	- - -	0 / 2

[a] 1×10^6 in RPMI
[b] 1×10^7 in BSK
[c] 1×10^6 in BSK

Table 3. Mean EV% of 3-week-old female mice and 8-week-old unmated and mated female mice inoculated with *B. burgdorferi* N40, *B.burgdorferi* B31, *B.coriaceae,* BSK II, and RPMI. Sera collected 30 to 35 days post-inoculation. Results are combined from 3 experiments.

Experimental Inoculum	Mouse Age (Weeks)	No. of Mice Inoculated	Mated	No. of Mice Inoculated	Unmated
B. burgdorferi N40[a]	8	4	68.1 ± 23.3	3	118.3 ± 17.1
B. burgdorferi N40[b]	8	4	149 ± 50.8	4	238.1 ± 41.4
B. burgdorferi N40[a]	3		- - - -	4	176.1 ± 29.6
B. burgdorferi N40[c]	3		- - - -	2	180.0 ± 21.0
B. burgdorferi B31[c]	3		- - - -	2	20.8 ± 0.4
B. coriaceae[a]	8	4	8.6 ± 1.0		nd
B. coriaceae[b]	8	4	18.6 ± 10.6		34.5 ± 6.2
B. coriaceae[a]	3		----	2	5.4 ± 0.3
BSK II	8	4	0.06 ± 0.03		nd
RPMI	8	4	0.03 ± 0.001		nd

[a] 1×10^6 in RPMI
[b] 1×10^7 in BSK
[c] 1×10^6 in BSK

Chapter V
GENERAL CONCLUSIONS

Epizootic Bovine Abortion, a disease of range cattle, causes abortion or birth of weak calves during the third trimester of pregnancy (5). It affects pregnant cows or heifers which have had minimum exposure in EBA enzootic areas and is transmitted by the bite of the vector tick, *Ornithodoros coriaceus* (8). Disease symptoms are characterized by fetal lesions (6). A borrelia was isolated from *O. coriaceus* and characterized (4, 7). This new spirochete, *B. coriaceae,* has been implicated as the etiological agent, though isolation of this spirochete, from diseased fetal lesions, has not yet been accomplished. The evidence that *B. coriaceae* causes EBA is still circumstantial.

In an effort to learn more about *B. coriaceae* and its role in EBA, we performed an ELISA serosurvey to test *B. coriaceae*-specific reactivity in cattle from an EBA enzootic area and compared it with *B. coriaceae*-specific reactivity in cattle from a nonenzootic area. From the serosurvey we developed a screening test for *B. coriaceae*-specific reactivity. The specificity and sensitivity of our test was 83% for both parameters. This screening test could be used in developing a vaccine for EBA by identifying major *B. coriaceae* immunogens. There are several factors that make EBA a good candidate for vaccine development: 1) an animal which was experimentally vaccinated with killed, whole cells had no side effects and was able to generate a dramatic change in specific reactivity to *B. coriaceae*, and 2) from the results of the ELISA screening tests performed on the population from an EBA enzootic area, it appears that the antibody production is sustaining. This means the cows could be vaccinated well in advance of becoming pregnant and would have the antibody protection for a considerable length of time. By establishing the criteria for EBA positive sera, an

initial vaccine could be prepared which would elevate a cow's specific antibody level against *B. coriaceae* high enough to protect against this specific antigen. After vaccination, a cow or heifer would be producing its own antibodies against *B. coriaceae* which could protect against EBA during the critical period of pregnancy.

In the second part of this study, we collected *O. coriaceus* from an EBA enzootic area and investigated their role in transmitting spirochetes. We found natural spirochete infections in 7% of the *O. coriaceus* we dissected and spirochetes in 29% of the ticks we inoculated with borreliae through our membrane-feeding device. Because the ticks were not reared in the laboratory, part of the 29% may have been due to natural spirochete infections. If a specific parasite-free colony of *O. coriaceus* was established, this obstacle could be removed. If such a colony was established, many aspects of the relationship between *O. coriaceus* and *B. coriaceae* could be monitored. One application of this work would be to use both wild ticks and experimentally infected ticks as vectors for EBA infection in animal models. If teratogenesis could be established in animals, this would help to establish *B. coriaceae* as the etiological agent for EBA.

Finally, we developed a mouse model using *B. coriaceae* as the causative agent for infecting pregnant mice to emulate the disease symptoms of EBA. We found that teratogenesis occurred in 40% (2/5) of the pregnant mice inoculated with *B. coriaceae,* and that Lyme arthritis developed in 78% (7/9) of the nonpregnant females inoculated with *B. burgdorferi* compared to 25% (2/8) of the pregnant mice. If abortion can be experimentally induced by inoculation of *B. coriaceae*, can abortion be prevented by the use of specific antibodies? Previous research has shown that hamsters and mice can be protected from Lyme arthritis by the use of *B. burgdorferi*-specific antibodies supplied passively (2, 9). Additional studies are needed to determine if these techniques can be applied to the EBA animal model with similar results.

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