DETECTION OF *ANAPLASMA MARGINALE* IN *DERMACENTOR* SPECIES TICKS WITH THE POLYMERASE CHAIN REACTION

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PREFACE

The polymerase chain reaction (PCR) is an effective method of amplifying a specific target fragment of DNA *in vitro*, theoretically detecting a single organism in a tissue sample. Though simple in theory, PCR can be difficult in practice due to the number of parameters that influence the success of each individual system. This research involved application of PCR to the study of ticks that were experimentally- or naturally-exposed to *Anaplasma marginale* (Rickettsiales: Anaplasmataceae), not only to facilitate greater understanding of the association between the parasite and its tick vector, but also in anticipation that these studies may serve as a model for application of PCR to the study of other vector-transmitted organisms.

This dissertation is divided into seven chapters. Chapter I provides a literature review, the research problem and experimental design. Chapter II, published in *Biotechnology Techniques* (Volume 5, pages 269-274, 1991), describes preliminary development and application of a PCR-based assay for *A. marginale* in *Dermacentor andersoni* (Acari: Ixodidae). Chapters III and IV have been submitted to The *Journal of Medical Entomology* and are currently being reviewed. These chapters describe application of the PCR assay to *D. andersoni*

hemolymph as a sensitive, potentially non-lethal test of infection with *A. marginale*, and oral secretions as a test for transmission of *A. marginale* via salivary secretions. Chapter V, which is being prepared for publication, describes application of the PCR assay for *A. marginale* to field-collected ticks for detection of natural infection. Chapter VI is a discussion of various observations during the course of this project, and Chapter VII is a summary of the research.

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This dissertation is dedicated to loved-ones in my life, including my parents, Roger and Patricia Stich, my siblings Steve and Christy, and especially Ms. Lora Lea Reno for her love, support and tolerance of my various moods over the past two years.

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CHAPTER I

INTRODUCTION

Literature Review

Bovine Anaplasmosis

Anaplasma marginale is the causative agent of bovine anaplasmosis. This parasite invades and develops in bovine erythrocytes that are subsequently removed by phagocytic cells of the reticuloendothelial system, resulting in a mild to severe anemia.[1,2,3]

Anaplasma marginale is classified in the family Anaplasmataceae, order Rickettsiales. This family includes those vector-borne, procaryotic parasites that live and multiply only within living cells.[4] *Anaplasma* organisms, as well as *Ehrlichia*, *Cowdria*, and *Coxiella*, belong to a group of rickettsia that are found within membrane-bound inclusions. Some of these organisms also appear to have a specific developmental cycle involving different stages.[5,6] In contrast, *Rickettsia* and *Wolbachia* are found free in the host cell cytoplasm and appear to divide only by binary fission.[5,7]

Anaplasma marginale is distributed world-wide and is found in the tropical and subtropical areas of Africa, Asia, Asia Minor, Australia, the Soviet Union, Indonesia, Taiwan, Philippine Islands, and the Americas.[1,8,9] Areas of the United States where anaplasmosis is enzootic include the southeastern, gulf, lower plains, and western states, with sporadic occurrences in the northern states.[1,10] *Anaplasma marginale* has the greatest enzootic area of any rickettsiae of veterinary medical importance, possibly due to its wide vector range.[7-9] Economic losses due to anaplasmosis in the United States result from weight loss, abortion, testicular degeneration, loss of libido, reduced milk production, and death.[10,11] Annual economic losses to the U.S. cattle industry, including those that result from prevention, treatment, and other veterinary medical services were estimated in 1976 at $100 million.[10] Control of anaplasmosis involves isolation of carriers followed by slaughter or treatment with tetracyclines; anaplasmosis vaccines are available, but they must be administered repeatedly and immunized animals can become carriers.[1,12]

Anaplasmosis is primarily a disease of cattle and affects all breeds. The disease has also been reported in other ruminants including sheep, deer, antelope, buffalo, camel, elk, wildebeest, duiker, and blesbok; some of these wild hosts may have a significant role in the epizootiology of the disease by serving as reservoirs.[1,13-20] *Anaplasma marginale* can be

mechanically transmitted between hosts by blood-contaminated fomites and haematophagous arthropods, but only certain ticks of the family Ixodidae transmit the organism biologically.[21-23]

The characteristic marginal bodies within bovine erythrocytes are inclusions containing one to several initial bodies within a limiting inclusion membrane.[2,22,23] Each initial body is an organism surrounded by a double membrane, with protoplasm resembling that of *Rickettsia* and *Chlamydia* species.[24] The inclusion membrane surrounding the initial bodies has morphology similar to the erythrocytic plasmalemma and is recognized by anti-erythrocyte, but not anti-*A. marginale*, immunogobulin.[24,25]

The morphology of *A. marginale* in bovine erythrocytes is more evident in hemolysed than in intact cells. In some isolates, an inclusion appendage is attached to the inclusion membrane and forms "loop", "dumbbell", or "comet" shapes.[24,25] Ferritin-conjugated antibody studies have shown that the inclusion appendage, as well as the initial bodies, contain parasite antigens. It has been suggested that these may be the particulate antigens responsible for the vertebrate immune response.[26]

A hypothetical life cycle has been proposed for *A. marginale* in the vertebrate host, wherein an intial body enters the erythrocyte by endocytosis, reproduces by binary fission, and is transferred to other erythrocytes.[2] Progression of disease in cattle is correlated with increasing parasitemia and subsequent phagocytosis of infected erythrocytes which results

in anemia.[3,27] A prepatent period of approximately 21-40 days occurs, its length varying with the infective dose. The location of *A. marginale* in its vertebrate host during the prepatent period has not been determined.[3,27] Once the patency is reached the parasitemia rises rapidly, often doubling every 24 hours, until a peak parasitemia is reached in 5-7 days, with the most severe anemia occurring at 8 to 10 days.[3,27] Cattle that recover become carriers; parasitemia may recrudesce if these cattle are immunosuppressed.[3,7,27]

Tentative diagnosis of anaplasmosis involves clinical signs of anemia including accelerated respiration, increased cardiac rate, and pallor and icterus of skin and mucous membranes in the absence of hemoglobinuria. History of the host (age, season, exposure to carriers) is also valuable.[1,12,28] Positive diagnosis requires either demonstration of marginal bodies within the erythrocytes, or a positive complement-fixation (CF) or card serological test.[1,12,28]

Cattle of all ages are susceptible to infection, but those over 2 years old develop clinical symptoms often resulting in death. Younger cattle may have moderate to inapparent symptoms and become carriers.[1,12,28] The mechanism of resistance in younger cattle is not well understood, but it may involve the greater hematopoietic response of younger animals. Splenectomy of calves renders them susceptible to severe anaplasmosis with symptoms similar to those described for adult cattle.[10,28] Immune mechanisms that may be hindered by splenectomy would include

initial body lysis via antibody activation of the complement cascade, phagocytosis of opsonized initial bodies or parasitized erythrocytes, and antibody blockage of the erythrocyte binding site on the initial bodies.[29] All three of these mechanisms have been associated with the bovine immune response to *A. marginale*. Synthesis of bovine complement-fixation (CF) antibodies against *A. marginale* are reported to be exclusively IgM for the first four to five days of synthesis, with IgG synthesis coinciding with patency and reaching a maximum of 25% of the CF titer approximately 30 days post hemolytic crisis.[30] Erythrophagocytosis of parasitized cells has been described, and is believed to be responsible for the absence of hemoglobinuria during clinical anaplasmosis.[3,31]

Transmission of *Anaplasma marginale* by Ticks

Ixodid ticks are the only known biological vectors of anaplasmosis with approximately 20 species incriminated as vectors world-wide.[21,32] Interstadial and intrastadial transmission of *A. marginale* by ticks has been demonstrated repeatedly. Transstadial transmission occurs from nymphs to adults, and from larvae to both nymphs and adults.[21,32-37] The transmission of *A. marginale* by ticks from chronic carrier cattle is considered important in maintaining the organism in enzootic areas.[34,38] Transmission may occur by transfer of adult ticks from infected to susceptible hosts (intrastadial transmission) or by nymphs or adults infected in a previous

stage (interstadial transmission). Intrastadial transmission of *A. marginale* by male and female *Dermacentor andersoni* has been demonstrated, and transfer of both genders between cattle under field conditions has been reported.[39-44] Biological infection of and transmission by adults was confirmed by the presence of colonies in the tick tissues and the inability of the parasite to remain extrinsically within the tick gut lumen for longer than 2 days after feeding on patent cattle.[39,40,45] Interstadial transmission has fallen under scrutiny since the immature stages of several tick vectors do not normally feed on cattle, but there are several symptoms of patent cattle (increased body temperature, odor due to icterus, and increased CO_2 near the ground due to lethargy) that may enhance the attachment of larvae and nymphs.[34,38] Adult *D. andersoni* infected as nymphs have been reported to remain infected with the parasite up to 6 months post-molting.[46] *Dermacentor variabilis* is reported to remain infected up to 12 months, especially when the ticks are stored under winter conditions.[47] Trans-ovarial transmission has been reported in some ixodid tick species, but the phenomenon has not been confirmed consistently in any tick species.[21,32-38,48,49]

Development of *Anaplasma marginale* in Ticks

The developmental cycle of *A. marginale* in ticks has been studied in *D. andersoni* from infection of larvae and nymphs through transmission by the subsequently-molted adults. Sus-

ceptible cattle have been infected by inoculation with gut and salivary gland homogenates from infected adult ticks.[34-48] The process by which *A. marginale* infects the tick midgut is not known.[50,51] Colonies of the parasite were not observed by light (LM) and electron microscopy (EM) within nymphal tissue until 5 days post-repletion, even though the nymphs become infected within as little as 24 hours of feeding on an infected animal.[5,32,50] Two distinct types of anaplasmal colonies, Nymphal type 1 (Ny1) and Nymphal type 2 (Ny2), along with transitional nymphal colonies (TsN) are found in the midgut epithelium as the nymphs molt into adults.[5,50,51] Infection of nymphs is thought to occur concurrent with the rapid digestion of the blood meal after the nymphs are replete from feeding.

Colonies of *A. marginale* have also been demonstrated within the midgut epithelium of *D. andersoni* adults that were infected as larvae, nymphs, and adults by LM and EM.[5,33,34,37-40,50,52-56] Colonies in adults infected as nymphs were confirmed as *A. marginale* by ferritin- and fluorescein-labelled antibody and immunoperoxidase techniques.[36,37,54] Small electron-dense forms, larger reticulated forms, pleomorphic reticulated forms, and small particles are described within the five distinct colony types of adult *D. andersoni* midgut.[5,45,55,56]

Anaplasmal colonies in the gut of unfed adult *D. andersoni* infected as nymphs have often appearred near the epithelial basement membrane.[5] Increasing numbers of colonies were observed within muscle cells on the hemocoel side of the

basement membrane during tick feeding, especially during days 3 through 5. Colony densities sharply decreased on days 7 through 9 of feeding. Colonies were rarely present at 6-9 days of tick feeding. This decrease in the midgut colony density coincides with the 6-7 days of feeding required before the organism can be transmitted from the tick.[5,57] Colonies in muscle cells on the hemocoel side of the basement membrane appear to be an intermediate site of development of *A. marginale* in ticks. Homogenates of salivary glands were infective for calves, and *A. marginale* has been demonstrated in the tick salivary glands of both interstadially and intrastadially infected ticks by EM, and in the hemolymph and salivary glands of interstadially infected ticks by immunofluorescence.[5,37,39,40,42-44,58,59]

Molecular Genetics of *Anaplasma marginale*

Literature on the molecular genetics of *Anaplasma marginale* has increased significantly over the past several years. Several investigators have reported values for the guanine and cytosine (G+C) content of the *A. marginale* genome with varying results.[60-63] The reason for this inconsistency is most likely due to contamination of the anaplasmal DNA by host DNA. Each of these investigations involved purifying the parasite-infected erythrocytes from infected blood by removing the buffy coat. Two of these papers reported further removal of bovine leukocytes by passing the infected blood through a Sigmacell or Whatman CF-11 column.[62,63] Three investigators reported a G+C

content of approximately 50% while one reported a 33% G+C content that is closer to the 29-33% range of other rickettsial organisms.[63] Anaplasmal DNA has been purified to the extent that analysis of restriction fragment legnth polymorphisms (RFLP) is possible.[64] In a similar study the problem of bovine DNA contamination was circumvented by hybridizing the *Eco* RI RFLPs of different isolates to an *Anaplasma*-specific DNA probe.[65]

Several DNA probes have been developed for *A. marginale* and *A. centrale*.[66,67] Several major surface proteins of the *A. marginale* Florida isolate have been isolated with rabbit antisera that is capable of neutralizing virulent initial bodies.[68-71] A 4 kb DNA fragment encoding the AMF 100 (previously named AM105L) surface protein was isolated by screening a pBR322 library with polyclonal antibody against one of these surface proteins (the AM105 complex), indicating that a promoter for this gene was also present.[72] A 2 kb fragment of this AMF 100 gene, from the same pBR322 clone, was used as a probe to detect *A. marginale* within three species of male tick (*D. andersoni, D. variabilis* and *D. occidentalis*) midgut and salivary glands.[67] Oligonucleotide primers (AL52 and AL34S) that are complementary to the reciprocal ends of a fragment of this gene have been developed. Another four DNA probes were isolated by screening an *A. centrale* lambda *gt* 11 DNA library with bovine carrier serum.[66] Three of these probes hybridized to both *A. marginale* and *A. centrale* DNA (AC-2, AC-3 and AC-4)

and one probe (AC-1) hybridized exclusively to *A. centrale* DNA. One of these probes (AC-2) was used to detect *A. marginale* within the midgut of infected *D. andersoni* ticks, but hybridization to salivary glands of the same ticks was not successful.[65]

Polymerase Chain Reaction

The polymerase chain reaction (PCR) is a method of *in vitro* amplification of a specific DNA fragment.[73,74] The procedure originally developed by Mullis involved 20-27 cycles of template DNA denaturation at 95°C, lowering the reaction temperature to allow primer annealing and addition of the *Eschericha coli* Klenow fragment for primer extension at 30°C.[73] Isolation of Taq polymerase from the thermophile *Thermus aquaticus* has allowed development of a superior procedure to that originally described.[74] Taq polymerase is stable at 95°C with optimum activity at 72°C. The use of this enzyme is more economical and can be automated because it does not have to be replenished after each denaturation step. The higher reaction temperature allows for more stringent primer annealing which results in more specific amplification of the target DNA. Furthermore, the more specific PCR results in higher product yield due to reduced competition for Taq polymerase from nonspecificly amplified products. Single copy DNA sequences have been reported to be amplified by a factor of 10^7, and as few as one sequence among 10^5 human cells has been detected.[74]

The extreme sensitivity and specificity of PCR is invaluable for identification of parasites in low numbers. Such methods were reportedly being developed for diagnosis of Chaga's disease and the human leishmaniases.[75] Amplification of DNA from *Brucella* spp, *Chlamydia trachomatis, Rickettsia typhi, Plasmodium falciparum* and *Plasmodium vivax* with PCR has been documented.[76-80] Infected insect vectors of *R. typhi* and *Plasmodium* spp. have been identified with this technique.[78,79] DNA purification has been reported to be the most efficient method of sample preparation,[75] but extremely sensitive techniques have been developed in the absence of DNA extraction.[77,79]

Research Problem

The complex development of *A. marginale* in *Dermacentor* ticks has been studied extensively. Infected ticks were identified by transmission of *A. marginale* to susceptible cattle during tick feeding and by examination of tick tissues by LMand EM. However, demonstration of *A. marginale* in tick tissues by these methods was partially dependent on working with highly infected ticks, and some stages were difficult to differentiate from host cellular inclusions. Furthermore, these techniques were tedious and expensive, owing to the cost of labor, experimental calves and EM. Finally, ticks that had to be killed for these studies were not available for further research. A sensitive and specific method for identification of *A. marginale* within live ticks is needed for laboratory and

field studies. This research involved utilization of a PCR technique for screening live ticks for infection with *A. marginale*. PCR involves specific amplification of target DNA, thus it is specific in distinguishing *A. marginale* from other organisms and sensitive for detection of small numbers of parasites in tick tissue samples. PCR is well suited for epidemiology studies of field-collected ticks in which infections may be low. Furthermore, screening live ticks with PCR would enable further research of known infected ticks. Finally, PCR could be used for identification and study of other organisms in ticks.

Experimental Design

This project was comprised of three objectives: (1) *Objective A* was to develop a PCR test for detection of *A. marginale*. PCR was optimized with DNA extracted from *A. marginale*-infected bovine erythrocytes. Optimum parameters were judged by the appearance of the bands on an ethidium bromide (0.5 ug/ml) stained agarose gel. (2) *Objective B* was to use PCR to detect *A. marginale* in tissues from experimentally infected *D. andersoni*. The optimium method of tick tissue preparation for PCR was determined by comparing freeze/thaw lysis, extended heat denaturation, protein digestion and DNA extraction. Furthermore, PCR was used to attempt to detect *A. marginale* in hemolymph, midgut samples and oral secretions taken from live *D. andersoni* males and females that were exposed

to the parasite during either the nymphal or adult stage. Adult tick hemolymph was screened on each day of feeding to determine whether *A. marginale* can be detected at each phase of biological transmission. (3) *Objective C* was to use PCR to screen field-collected ticks for natural infection with *A. marginale*. Ticks were collected from anaplasmosis-endemic areas of Oklahoma with CO_2 traps, flags, on from vertebrate hosts including cattle. Ticks identified as members of the genus *Dermacentor* were bisected, proteinase-digested and screened for *A. marginale* with PCR.

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CHAPTER II

PRELIMINARY DEVELOPMENT OF A POLYMERASE CHAIN REACTION ASSAY FOR *ANAPLASMA MARGINALE* IN TICKS

Abstract

Polymerase chain reaction (PCR) was applied for detection of *Anaplasma marginale* in tissues of male *Dermacentor andersoni*. Primer sequences were derived from the gene for the MSP1β surface protein of *A. marginale* (Florida isolate). Optimum PCR conditions were used to detect *A. marginale* in individual bisected ticks and salivary glands; associated control tissues were negative.

Introduction

The sensitivity and specificity of the polymerase chain reaction (PCR) is invaluable for identification of arthropod vectors of pathogens (Saiki *et al.* 1988). Such methods have detected *Rickettsia typhi* (Webb *et al.* 1990) and *Borrelia burgdorferi* (Persing *et al.* 1990) in arthropods. DNA purification has been reported to be the most efficient method of sample preparation (de Bruijn 1988), but sensitive techniques have been reported in the absence of DNA extraction (Fekete *et al* 1990; Webb *et al.* 1990). Previously, *Anaplasma marginale* has been detected in cattle and ticks with a DNA probe derived from a gene encoding the β subunit for the Major Surface Protein 1 (MSP1β) of *A. marginale*, Florida isolate (Goff *et al* 1988). Herein we report preliminary development of PCR for identification of ticks infected with the *A. marginale* Virginia isolate. Primers for this assay were derived from the nucleotide sequence of the MSP1β gene (Barbet and Allred, 1991).

Materials and Methods

Infection of Ticks

Dermacentor andersoni were reared at the Oklahoma State University Medical Entomology Laboratory (Patrick and Hair 1975). Male *D. andersoni* were exposed to *Anaplasma marginale* as described previously (Kocan *et al* 1989a; Stiller *et al* 1989a). The exposed males were allowed to feed for 10 days before ticks were bisected or salivary glands removed for PCR. Tissues from unexposed male *D. andersoni*, treated identically, served as controls.

Collection of Tick Tissue

Dissections were performed with aseptic technique by successive rinses of forceps and razor blades in 0.5% (w/v) SDS, 10% (v/v) Clorox Bleach, double distilled H_2O and 95% (v/v) ethanol followed by flame sterilization. Salivary glands from 50 infected males were collected and pooled in 4 ml RPMI 1640 (Gibco). Individual ticks were bisected and salivary glands or bisected ticks were divided and stored in 150 µl RPMI 1640 for PCR or fixed for light microscopy as described previously (Kocan *et al* 1989a; Stiller *et al* 1989a).

PCR Template and Primers

Two 20mer oligonucleotide primers (50 % G+C) flanking opposite strands of a 406 bp fragment of the *A. marginale* Florida isolate MSP1β gene (Barbet 1991) were synthesized. The source of *A. marginale* (Virginia isolate) was bovine blood stabilate prepared by three washes with heparinized RPMI 1640 (buffy coat was removed each time) and stored in liquid nitrogen at 50% packed cell volume in RPMI 1640 and 5% (v/v) DMSO.

DNA was extracted from the blood stabilate for PCR optimization. After erythrocytes were lysed by thawing, hemoglobin was removed by washing pelleted cells (13,000 x g for 1 min) via resuspension once with 2 volumes of TE (10 mM Tris·HCl, pH 8.0, 1 mM EDTA) followed by another two washings and a final suspension with 1 volume of TE. Protein was digested with 100 µg/ml proteinase K and 0.5% (w/v) SDS at 55°C for 5 hr. Digests were heated to 105°C for 10 min and protein was extracted with equal volumes of phenol (once), twice with phenol/chloroform/isoamyl alcohol (25:24:1), and once with chloroform/isoamyl alcohol (24:1). DNA was precipitated with 0.3 M sodium acetate and two volumes of absolute ethanol (-20°C) and resuspended in TE.

Tick Tissue Preparation

Infected salivary glands from 50 male *D. andersoni* infected as adults were pooled and stored in RPMI 1640 at -70°C. Samples were thawed, digested overnight at 37°C in RPMI 1640 with 0.45% (v/v) NP40, 0.45% (v/v) Tween 20 and 100 µg/ml proteinase K, and extracted twice with phenol/chloroform/isoamyl alcohol before DNA was precipitated with 0.3 M sodium acetate and 2 volumes of ethanol (-20°C). Samples equivalent to 1/25 of a salivary gland were collected from the lysate, digest and purified DNA steps, and subjected to optimum PCR amplification with 0.2 µg/µl nuclease- free bovine serum albumin.

PCR of Individual Ticks

For each individual sample, a single salivary gland or tick half was digested with proteinase K. 50 µl of 4X digestion mixture (1.8% NP40, 1.8% Tween 20, 400 µg/ml proteinase K) was added to the 150 µl sample, and incubated overnight at 37°C. Equivalents of 1/160 (2.5 µl) of 5 uninfected and 5 infected bisected ticks, and salivary glands from 10 individual infected ticks, were amplified in 25 µl reactions.

PCR Conditions

Polymerase chain reaction was performed with a Coy Model 60 tempcycler. Master mixes, made with the Perkin-Elmer Cetus Geneamp Reagents, were aliquoted to 50 or 25 µl final reaction

volumes containing 1X PCR buffer (10 mM Tris·HCl, pH 8.3, 50 mM KCl), 0.8 mM dNTP mix and specified amounts of $MgCl_2$, primers and Amplitaq DNA Polymerase. Reactions were overlaid with 35 µl and 20 µl mineral oil, respectively. The reaction profile, except for stated exceptions, consisted of 25 cycles of 94°C for 1.5 min, 60°C for 1.0 min, and 72°C for 1.0 min followed by a final extension at 72°C for 5.0 min.

PCR Optimization

PCR was optimized in 50 µl reaction volumes under conditions previously described. The following reaction parameters were progressively optimized: (1) annealing temperature (37°C, 50°C, 55°C and 60°C with 1.5 mM $MgCl_2$, 0.1 µM primers, 0.025 U/µl Amplitaq and 10 ng/µl template), (2) $MgCl_2$ concentration (0.0-5.0 mM $MgCl_2$ in 0.5 mM increments with 2 ng/µl template), (3) primer concentration (0.00-0.28 µM in 0.04 µM increments with 0.2 ng/µl template), (4) Amplitaq concentration (0.00-0.1 U/µl in 0.01 U/µl increments with 20 pg/µl template), (5) cycle number (25-50 cycles with 5 cycle increments using 2 pg/µl template).

Agarose Gel Electrophoresis

PCR product (20 µl) was added to 5µl of loading buffer (40 % (w/v) sucrose, 89 mM Tris, 89 mM boric acid, 2 mM EDTA) and electrophoresed on a 1 % (w/v) agarose gel with 0.5 µg/ml ethidium bromide in 1 X TBE (89 mM Tris, 89 mM boric acid, 2

mM EDTA) at 60 V for 1-1.5 hr. λ (HindIII) and 123 bp ladder (BRL) were used as molecular weight standards. DNA bands were visualized with ultraviolet light and photographed with a 35 mm camera.

Results and Discussion

Colonies of *Anaplasma marginale* have been described within midgut and salivary gland tissues of *Dermacentor andersoni* males using light microscopy (Kocan *et al* 1989a; Stiller *et al.* 1989a). Male ticks were chosen for these studies because they proved to have highest infection rates of *A. marginale* in salivary glands (Kocan *et al* 1989a). Contamination from residual *A. marginale* in the midgut lumen (from the blood meal) was eliminated by holding the ticks for 9 days; parasites in the blood meal apparently are not infective for cattle after 24 hr, and cannot be detected by DNA probe hybridization after 48 hr (Stiller *et al* 1989b). In addition to histological examination, infection has been detected in ticks by transmission of *A. marginale* to susceptible cattle via tick feeding, inoculation of cattle with tick tissues (Kocan 1986), and DNA probe hybridization (Goff *et al* 1988, Kocan *et al* 1989b). However, due to the extreme sensitivity and specificity of PCR, it would be the appropriate method for detection of *A. marginale* in small samples such as tick hemolymph, midgut biopsy and oral secretions.

In the present study, infected *D. andersoni* transmitted the parasite to a susceptible calf, and colonies of *A. marginale* were observed in individual tick salivary glands with light microscopy. No colonies were observed in uninfected ticks.

Optimum conditions for PCR were determined with *A. marginale* DNA extracted from bovine erythrocytes; they were 60°C annealing temperature, 3.5 mM $MgCl_2$, 0.18 µM total primer concentration and 0.04 U/µl Amplitaq at 45 cycles. These parameters were used for PCR of tick tissues.

To avoid potential PCR inhibitors in tick tissue, several methods of template preparation were tested to determine the best procedure for screening samples. The staining intensity of amplified DNA was but one criterion for determining the best method of tissue preparation; another was to use the fewest steps possible, reducing expense and the potential for contamination and loss of material from small samples. The proteinase K digestion step was chosen on the basis of these criteria and the results shown in Figure I. No amplified product was detected from the freeze-thaw lysate (lane 2), but was present with template from the proteinase K digest (lane 3) and purified DNA (lane 4) steps. Freeze/thaw cell lysates apparently contain PCR inhibitors that are at least partially excluded by protein digestion in the presence of nonionic detergents.

Once an effective sample preparation method was developed, proteinase K digestion was performed on samples from individual infected and uninfected male *D. andersoni*. All exposed ticks tested were PCR-positive for *A. marginale* while the 406 bp band was not seen from uninfected ticks (Figure II). The 406 bp band was not present in uninfected whole *D. andersoni*

controls (lanes 2-5), but was present in the uninfected control combined with 100 pg of *A. marginale* template (lane 6). The *A. marginale* band was present in all reactions of the infected bisected ticks (lanes 8-12) and infected salivary gland preparations (lanes 14-23).

The variable staining intensities of PCR product observed among infected ticks may have resulted from different infection levels in individual ticks, or from well variation in the tempcycler. The number and size of anaplasmal colonies, and organism densities within colonies, have been shown to vary considerably among ticks infected on the same calf (Kocan 1986; Kocan *et al* 1990). In addition, reaction profile variation among tempcycler wells has been reported (Linz 1990). An internal standardization procedure for well variation must be investigated before the number of rickettsiae in ticks can be quantified accurately with PCR.

Additional bands, the brightest of which was approximately 300 bp, were present following PCR of all tick tissues tested. The origin of this DNA is not known. The potential homology between the primers and other symbiotic organisms or tick DNA has been considered. It appears likely that host cell template would result in more consistently staining product among all samples, while posited symbiote template would be present in more variable concentrations, resulting in the different 300 bp band staining intensities observed among samples.

This assay is currently being applied to study of ticks experimentally infected with *A. marginale*. However, further studies are underway to test primer specificity against other tick-borne pathogens preparatory to use with field-collected specimens. Additional studies that include PCR of hemolymph, oral secretions and midguts are being tested on ticks infected as nymphs or adults in order to develop a method of screening live ticks that one wished to save for further study. PCR should be well suited for epizoological studies of field-collected ticks in which infections may be difficult to detect by other methods.

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Footnotes

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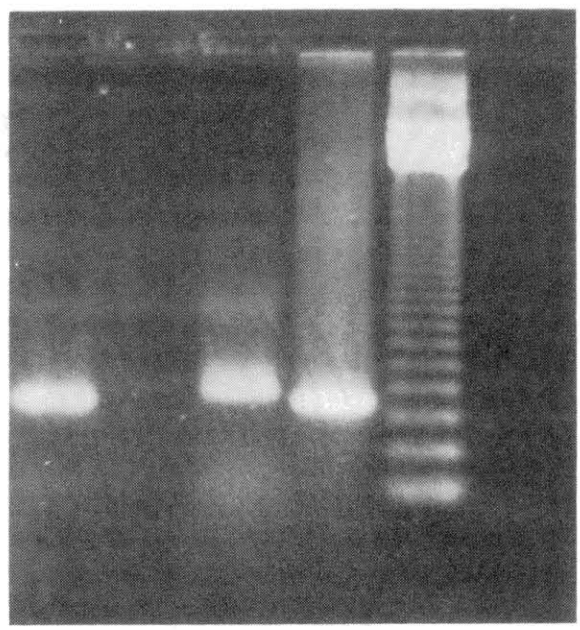
Figure I. Amplification of *Anaplasma marginale* DNA in infected *Dermacentor andersoni* salivary gland pool.

Lanes: (1): 100 pg *A. marginale* DNA extracted from bovine erythrocytes, as positive control, (2): 1/25 salivary glands equivalent freeze/thaw lysate, (3): 1/25 salivary glands equivalent proteinase K digest, (4) 1/25 salivary glands equivalent extracted DNA, (5): 123 bp ladder standard, (6): *A. marginale* primers only, as contamination control.

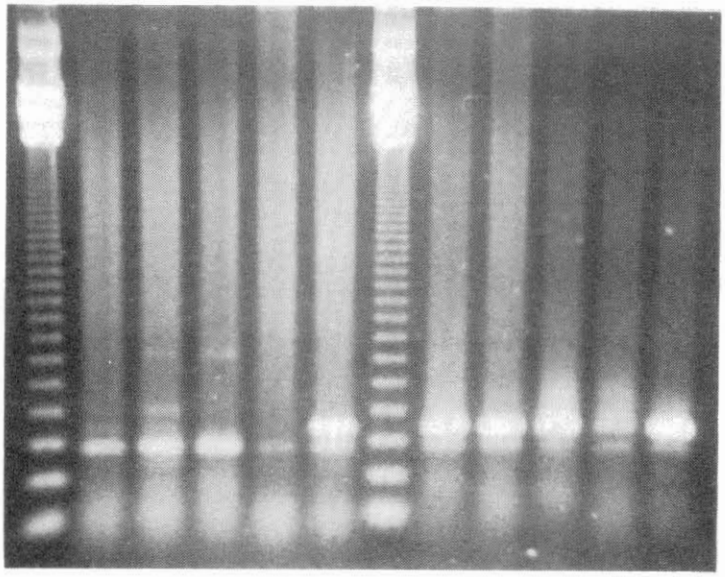


1 2 3 4 5 6

Figure II. Amplification of *Anaplasma marginale* DNA in individual *Dermacentor andersoni* males.

A: Lanes: (1, 7): 123 bp ladder standard, (2-5): 1/160 uninfected entire tick equivalent, as negative controls, (6): 100 pg *A. marginale* DNA from bovine erythrocytes combined with 1/160 uninfected entire tick equivalent, as positive control, (8-12): 1/160 infected entire tick equivalent.

B: Lanes: (13, 26): 123 bp ladder standard, (14-23): 1/160 equivalent of entire infected salivary glands, (24): 1/160 equivalent of entire infected salivary glands lysate pool as negative control, (25): 100 pg *A. marginale* DNA from bovine erythrocytes, as positive control, (27): *A. marginale* primers only, as contamination control.



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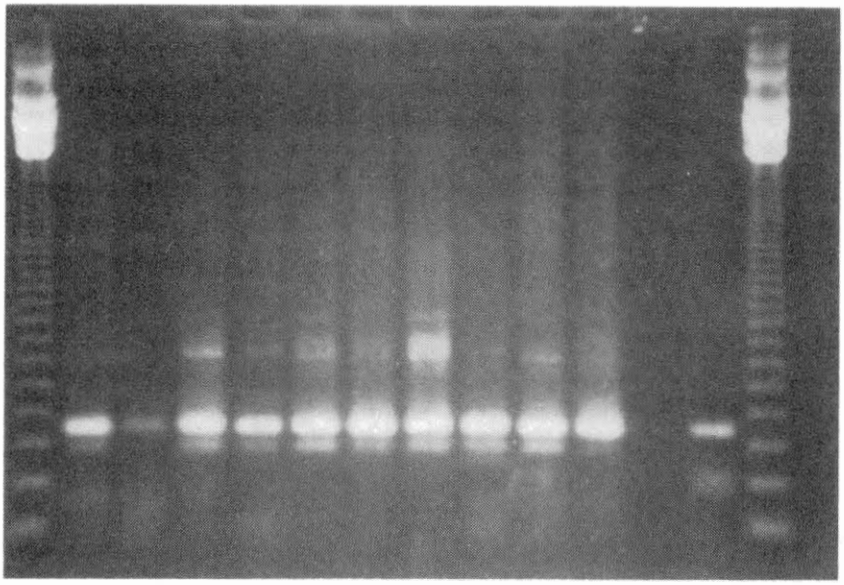

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CHAPTER III

DETECTION OF *ANAPLASMA MARGINALE* (RICKETTSIALES: ANAPLASMATACEAE) IN HEMOLYMPH OF *DERMACENTOR ANDERSONI* (ACARI: IXODIDAE) WITH THE POLYMERASE CHAIN REACTION

Abstract

The polymerase chain reaction (PCR) was used to identify live *Dermacentor andersoni* ticks infected with *Anaplasma marginale*. Hemolymph was collected from severed legs of male and female ticks exposed to *A. marginale* as either nymphs or adults. The optimum method of preparation of hemolymph for PCR was found to be heat treatment. Hemolymph samples were collected and pooled from adult ticks exposed as nymphs prior to feeding and on each of 10 days they fed on a susceptible calf; for male and female ticks exposed as adults, samples were collected on each day that ticks fed on infected calves, while being held between feedings, and during a second feeding when they transmitted the parasite. Hemolymph samples were collected from uninfected ticks at the same times to serve as controls. *Anaplasma* DNA was successfully amplified from hemolymph of infected adult ticks at all collection times, even from ticks that fed on infected calves for only one day. The PCR-based assay of tick hemolymph proved to be a sensitive method for identification of infected ticks, potentially without killing them; it would be well suited for identification of laboratory- or field-infected ticks that could then be used for further studies.

Introduction

Anaplasma marginale is a unique rickettsia that causes anaplasmosis in cattle. The parasite can be transmitted mechanically by blood-contaminated fomites and mouthparts of biting flies, but ixodid ticks are the only known biological vectors. Approximately 20 species of ticks have been incriminated as vectors worldwide (Bram 1975; Ewing 1981; Ristic 1968). Transmission by adult ticks and nymphs, exposed to the parasite in the previous stage of development, has been demonstrated repeatedly (Anthony & Roby 1962; Kocan et al. 1980 a & b; Kocan et al. 1981; Kocan et al. 1982; Kocan 1986; Rees 1934). Recently, transmission has been shown to occur by adult ticks infected as larvae without re-exposure as nymphs (Stich et al. 1989), and by male ticks transferred from infected to susceptible hosts (Potgieter 1979; Stiller et al. 1983; Coan et al. 1987; Zaugg et al. 1986; Stiller et al. 1989 a & b; Kocan et al. 1992 a, b & c).

The developmental cycle of *A. marginale* in ticks is complex; the organism develops in the midgut, gut muscle, salivary gland cells, and hemolymph (Kocan 1986; Kocan et al. 1980 a & b; Kocan et al. 1983; Kocan et al. 1984; Kocan et al. 1990; Oberst et al. 1981). The developmental cycle of *A. marginale* in male ticks transferred from infected to susceptible calves has been reported recently (Kocan et al. 1992 a, b &c).

Infection of ticks with *A. marginale* has been confirmed by feeding ticks on susceptible cattle, light and electron microscopy (Kocan et al. 1986), indirect immunofluorescence (Oberst et al. 1981) and a DNA probe derived from a gene encoding the β subunit for the Major Surface Protein 1 (msp1β) of *A. marginale* (Goff et al. 1988). The success of these tests has depended on high infection levels of *A. marginale* in ticks obtained by experimental infection; these tests may be less effective in the field where infection levels might be minimal within a population. Also, ticks from such studies would be killed and thus not available for further investigation.

The polymerase chain reaction (PCR) has been used for detection of pathogens in arthropod vectors, including *Rickettsia typhi* (Webb et al. 1990), *Borrelia burgdorferi* (Persing et al. 1990) and *A. marginale* in *Dermacentor andersoni* (Stich et al. 1991). The sensitivity of PCR is much greater than other tests; theoretically this assay should be able to detect only a few organisms through amplification of target DNA template of the parasite (Saiki et al. 1988).

The purpose of this study was to adapt PCR for detection of *A. marginale* in ticks by testing hemolymph collected from severed legs. PCR would be ideal for field studies in which a tick population might have minimal infections; once identified as infected by testing of hemolymph, ticks could be used for other studies.

Materials and Methods

Agent

The Virginia isolate of *A. marginale* (VAM) was used to infect donor calves by whole blood transfusion. This isolate has been used successfully in other studies of *A. marginale* involving ticks and cattle in our laboratory (Kocan et al. 1980 a & b; Kocan et al. 1981; Kocan et al. 1982; Kocan et al. 1983; Kocan et al. 1984; Kocan, 1986; Kocan et al. 1990; Kocan et al. 1992 a, b & c; Oberst et al. 1981; Stich et al. 1989; Stich et al. 1991).

Tick Propagation

Dermacentor andersoni nymphs and adult ticks were reared at the Oklahoma State University, Medical Entomology Laboratory (Patrick & Hair 1975). Larvae and nymphs were fed on rabbits and sheep and allowed to molt to the subsequent stage. Nymphs and adult ticks were held in a humidity chamber (90-98% relative humidity) at 25°C with a 14-hour photoperiod, until used for this study.

Experimental Calves

Eight splenectomized calves (2 to 4 months old), determined free of anaplasmosis by the complement-fixation (CF) test, were used for these studies. Two calves were inoculated IV with VAM-infected bovine blood and served as donors for

infection of nymphal or adult ticks. Four susceptible calves were used to feed exposed ticks to test for transmission of anaplasmosis, and two calves were used for feeding uninfected control ticks. All calves were monitored 3 times a week by examination of Wright-stained blood smears and determination of packed cell volume (PCV). Once marginal bodies were detected in blood smears, the calves were monitored daily.

Exposure of Adult Ticks

Dermacentor andersoni males and females (300 pairs) were placed in orthopedic stockinettes attached to the donor calf when the parasitemia reached 3 to 5%. The ticks were allowed to feed for 7 days after which they were removed from the calf and placed in a humidity chamber for 9 days. The ticks were then placed in stockinettes on a susceptible calf and allowed to feed for 10 days.

Exposure of Nymphal Ticks

D. andersoni nymphs (approximately 500) were placed in orthopedic stockinettes attached to the donor calf when the parasitemia reach 3 to 5%. The nymphs were allowed to feed to repletion after which they were removed from the calf and placed in a humidity chamber to molt. Once molted to adult ticks, 150 male/female pairs were allowed to feed on a susceptible calf.

Uninfected Ticks

Uninfected (control) adult ticks (150 males and females) were fed separately on an uninfected (CF negative) calf for 11 days; 35 pairs were removed on feeding day 7 and held 9 days in a humidity chamber.

Collection of Hemolymph

Tick legs were severed with a sterile razor blade and the dribbled hemolymph was collected with finely-drawn sterile Pasteur pipets. Samples were pooled from 10 infected males and females on each day of feeding, and every-other-day of the holding period, and stored at -70°C until tested with PCR for the presence of *A. marginale* DNA. Hemolymph was collected from 5 uninfected (control) male and female ticks on every-other-day of feeding and holding.

DNA Extraction from Infected Bovine Erythrocytes

Blood infected with VAM was processed and used as a control for the PCR tests. The infected blood was washed 5 times with heparinized RPMI 1640 (buffy coat removed with each wash) and stored in liquid nitrogen at 50% packed cell volume in RPMI 1640 and 5% (v/v) DMSO. The erythrocytes were lysed by thawing and mixing with an equal volume of ddH_2O, after which the hemoglobin was removed by washing pelleted cells (13,000 x g for 1 min). The pellet was resuspended once with 2 volumes

of TE (10 mM Tris·HCl, pH 8.0, 1 mM EDTA) followed by two washes with a final suspension in 1 volume of TE. Protein was digested with 100 µg/ml proteinase K and 0.5% (w/v) SDS at 55°C for 5 hr. Digests were heated to 105°C for 10 min and protein was extracted once with equal volumes of phenol, twice with phenol/chloroform/isoamyl alcohol (25:24:1), and once with chloroform/isoamyl alcohol (24:1). DNA was precipitated with 0.3 M sodium acetate and two volumes of absolute ethanol (-20°C) and resuspended in TE.

Preparation of Hemolymph Samples for PCR

Hemolymph pools collected from infected and control adult ticks, that were exposed during the nymphal or adult stage, were thawed and template samples (1.0 µl) were covered with 20 µl mineral oil. The samples were then heated to 105-110°C for 30 min prior to PCR; this was found to be the optimal preparation method. *Anaplasma* DNA extracted from infected bovine erythrocytes served as a positive control, and template-free master mix served as the contamination control.

Polymerase Chain Reaction

Hemolymph samples from feeding and held ticks were assayed with 24'mer and 20'mer oligonucleotide primers (50% G+C), flanking a 409 bp fragment of the *A. marginale* Florida isolate msp1β gene (Barbet 1991), that were synthesized and purified (Synthetic Genetics, LaJolla, CA) with polyacrylamide gel

electrophoresis (PAGE). PCR was performed with Perkin-Elmer Cetus Geneamp Reagents aliquoted from a master mix to 25 µl final reaction volumes containing 1X PCR buffer (10 mM Tris·HCl, pH 8.3, 50 mM KCl), 0.8 mM dNTP mix, 3.5 mM $MgCl_2$, 1.8 µM of each primer, 4% (v/v) heat-denatured fetal bovine serum (Sigma), 0.32 µg/µl BSA and 0.12 U/µl Amplitaq (Perkin Elmer-Cetus). Reaction master mixes were aliquoted to denatured samples that were previously overlaid with 20 µl mineral oil. The reaction profile consisted of 32 cycles of 94°C for 1.0 min, 60°C for 1.0 min, and 72°C for 0.5 min followed by a final extension at 72°C for 3.0 min in a Perkin Elmer-Cetus 9600 Thermocycler.

Agarose Gel Electrophoresis

Amplification products were mixed with 5 µl loading buffer (40 % sucrose, 89 mM Tris, 89 mM boric acid, 2 mM EDTA), and 25 µl loaded and electrophoresed on a 1.5 % agarose gel with 0.5 µg/ml ethidium bromide in 1 X TBE (89 mM Tris, 89 mM boric acid, 2 mM EDTA) at 75-100 V for 1.5 hr. The 123 bp ladder was used as the molecular size standard (Bethesda Research Laboratories). DNA was visualized with ultraviolet light and photographed with a 35 mm camera equipped with a yellow filter.

Results

Infection of Ticks and Transmission of Anaplasmosis

Nymphs were exposed to *A. marginale* on calf PA 135 (Table I); after molting, males transmitted *A. marginale* to PA 151, and females to PA 152 (Table II). Adult ticks were exposed to *A. marginale* on calf PA 80 (Table I); males transmitted the parasite to calf PA 77, and females to calf PA 81 (Table II).

PCR of Tick Hemolymph

A. marginale was detected with PCR in 1.0 µl heat-denatured hemolymph samples collected from unfed males (Fig IIIa) and females (Fig IIIb) exposed to the parasite as nymphs, and on each day that the ticks were tested while feeding on a susceptible calf.

The *A. marginale* target DNA was also amplified in hemolymph from male (Fig IVa) and female (Fig IVb) ticks on each day that the ticks were tested while feeding on an infected calf, while being held between feedings in a humidity chamber, and during transmission feeding on a susceptible calf. Hemolymph collected from control ticks during feeding and holding did not test positive for *A. marginale* DNA (Fig V).

Discussion

Proteinase K digestion was found to be superior to either freeze/thaw lysis alone or to phenol/chloroform extraction of infected tick salivary glands for PCR (Stich *et al.* 1991). However, others have shown that extended incubation of samples at high temperatures is also effective for preparation of tick samples for PCR (Azad *et al.* 1990). Heat denaturation alone was the method of choice for hemolymph template preparation resulting in a reduced number of steps, and minimizing the possibility of contamination from carry over of template DNA.

A. marginale DNA was detected with PCR in this study in all hemolymph samples collected from ticks that had been exposed. Finding *A. marginale* in hemolymph of adult ticks that fed as little as one day on an infected calf, and in unfed adult ticks that were exposed as nymphs, suggests that this test would be useful for field-collected ticks. Strong bands were observed in all samples, except for those collected from females exposed as nymphs; in these, weaker bands occurred as feeding time increased. It is not known whether these fainter bands, seen late in feeding, resulted from smaller numbers of *A. marginale* in the females or from a lower concentration of organisms per sample due to the greater volume of hemolymph collected from females later in feeding.

Further studies are needed to determine the location of *A. marginale* in tick hemolymph. It has been reported that the Florida isolate of *A. marginale*, which does not infect ticks, was not detectable within the tick midgut lumen after a 48 hr incubation period (Stiller 1989b). Thus hemolymph contamination from midgut lumen contents can be disregarded due to detection of the organism in unfed adult ticks exposed as nymphs, and ticks exposed as adults that had been held 9 days. Other possible sources include organisms free in the hemolymph, within hemocytes or from lysed muscle cells that line the visceral midgut (Kocan *et al.* 1990).

Transmission of *A. marginale* appears to be coordinated with development of the tick host (Kocan 1986). Parasite densities progressively increase in midgut epithelium, muscle cells lining the visceral midgut, and salivary glands during tick feeding. Dissemination of tick-borne agents in tick hemolymph is considered a prerequisite to infection of the salivary glands (Ribeiro *et. al.* 1987). Although *A. marginale* had previously been demonstrated in tick hemolymph of feeding ticks using immunofluorescence (Kocan *et. al.* 1983), this is the first demonstration of *A. marginale* in the hemolymph of adult ticks throughout feeding, well before earliest detection in salivary glands (Kocan 1986; Kocan et al. 1992b).

Tick hemolymph volume increased during feeding, especially in females; thus the 1.0 µl template samples were chosen as a conservative estimate of what might be expected from an

individual tick. This assay was repeatable in our hands, but fresh PAGE-purified primers were found superior for production of clear bands with less misprimed amplification products. When degenerated primers were used to assay female ticks infected as nymphs, relatively weak bands and greater non-specific amplification due to mispriming resulted (Fig 1b). However, samples from the same hemolymph pools, assayed with new primers, resulted in clear bands for positive controls and less misprimed background product.

The PCR assay of hemolymph from ticks infected with *A. marginale* proved to be a sensitive method of identifying experimentally-infected ticks, without addition of a DNA probe hybridization step. Experimentally-infected or field-collected ticks could be screened without killing them, enabling one to conduct further experiments. For example, PCR-positive ticks from the field could be fed on susceptible calves to investigate their potential as vectors, perhaps enabling identification of closely adapted tick and rickettsia strains.

The PCR assay of hemolymph may be a useful test for study of other vector-borne pathogens. Use of hemolymph as a source of pathogen template for PCR is a simple, rapid and extremely sensitive method that may circumvent hazards such as carry-over contamination and *Taq* DNA polymerase inhibition by proteinase-resistant proteins, inorganic solvents or denaturing agents.

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Footnotes

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Table I. Infected calves used for tick exposure.

Calf No.	Prepatent Period (Days)	Peak Parasitemia (% Inf RBC)	% Reduced PCV	Ticks Exposure-Fed
PA 80	15	51.2	53.8	300 pr. of males and females
PA 135	5	74.3	68.0	Approximately 500 nymphs

Table II. Susceptible calves used for tick feeding.

Calf No.	Prepatent Period (Days)	Peak Parasitemia (% Inf RBC)	% Reduced PCV	Ticks Transmission-Fed
PA 81	21	63.8	76.7	Females (150) exposed as adults on PA 80
PA 77	21	5.5[a]	47.5	Males (150) exposed as adults on PA 80
PA 152	52	69.3	NA[b]	Females (150) exposed as nymphs
PA 151	28	75.9	62.6	Males (150) exposed as nymphs
PA 89	NA[c]	NA[c]	NA[c]	Uninfected control males (235)
PA 153	7	64.4[d]	NA[b,a]	Uninfected control females (150)

[a]Calf was not splenectomized.

[b]Calf died of anaplasmosis.

[c]Calf was not challenge-exposed.

[d]Data from challenge exposure.

Figure III. PCR assay of hemolymph collected from *Dermacentor andersoni* adults that were exposed as nymphs to the Virginia isolate of *Anaplasma marginale*

a. Males. Lanes: (1-6): 1 µl aliquots of hemolymph pools collected on feeding days 0, 2, 4, 6, 8 and 10, respectively; (7): 123 bp ladder DNA size standard (BRL); (8): Hemolymph collected from feeding day 10 *D. andersoni* males, and spiked with 100 pg *A. marginale* DNA extracted from bovine erythrocytes, as a positive control; (9): Uninfected hemolymph collected from feeding day 10 *D. andersoni* males, as a negative control; (10): 100 pg *A. marginale* DNA extracted from bovine erythrocytes, as a positive control; (11): *A. marginale* primers only, as a contamination control.

b. Females. Lanes: (1-6): 1 µl aliquots of hemolymph pools collected on feeding days 1, 2, 4, 6, 8 and 10, respectively; (7): 123 bp ladder DNA size standard (BRL); (8): Hemolymph collected from feeding day 6 transstadially exposed *D. andersoni* females, and spiked with 100 pg *A. marginale* DNA extracted from bovine erythrocytes, as a positive control; (9): Uninfected hemolymph collected from feeding day 7 *D. andersoni* females, as a negative control; (10): 100 pg *A. marginale* DNA extracted from bovine erythrocytes, as a positive control; (11): *A. marginale* primers only, as a contamination control.

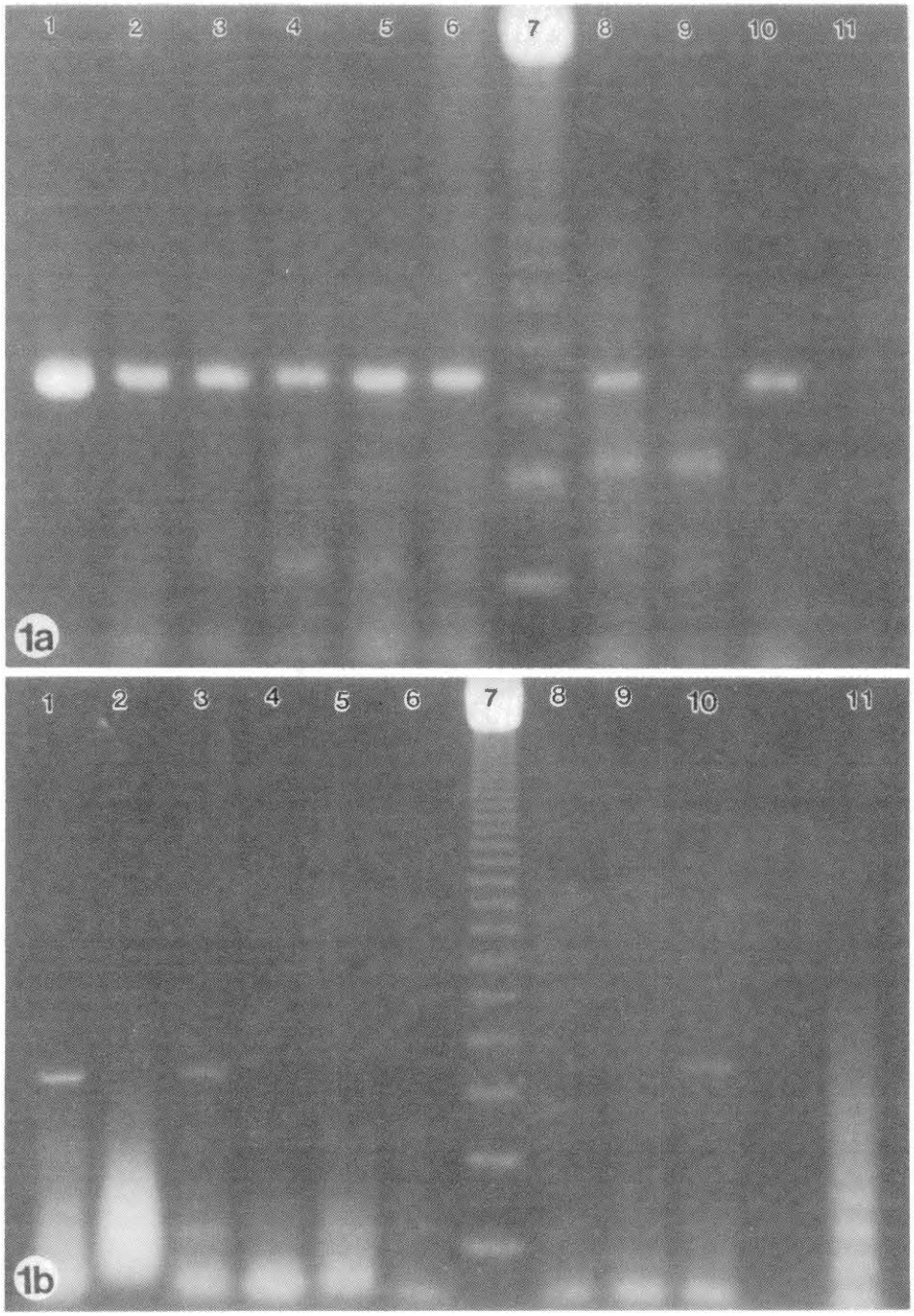
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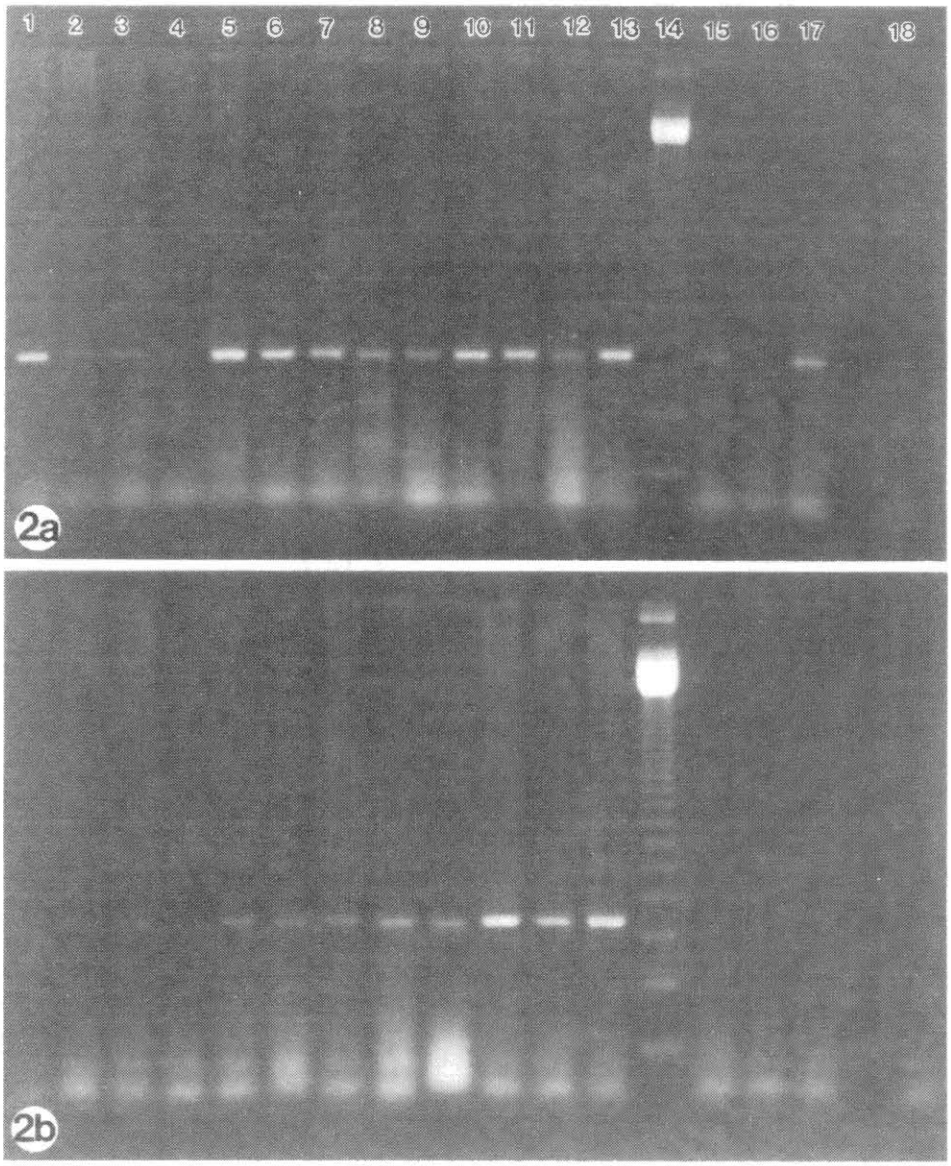
Figure IV. PCR assay of hemolymph collected from *Dermacentor andersoni* that were exposed as adults to the Virginia isolate of *Anaplasma marginale*

a. Males. Lanes: (1-4): Exposure feeding days 1, 3, 5, and 7, respectively; (5-8): Holding days 1, 3, 5 and 9, respectively; (9-13): Transmission feeding days 1, 3, 5, 7 and 9, respectively; (14): 123 bp ladder standard (BRL); (15): Uninfected hemolymph collected from feeding day 6 *D. andersoni* males and spiked with 100 pg *A. marginale* DNA extracted from bovine erythrocytes, as a positive control; (16): Uninfected hemolymph collected from feeding day 6 *D. andersoni* males, as a negative control; (17): 100 pg *A. marginale* DNA extracted from bovine erythrocytes, as a positive control; (18): *A. marginale* primers only, as a contamination control.

b. Females. Lanes: (1-4): Acquisition feeding days 1, 3, 5 and 7, respectively; (5-8): Holding days 1, 3, 7 and 9, respectively; (9-12): Transmission feeding days 1, 3, 5 and 6, respectively; (13): 123 bp ladder DNA size standard (BRL); (15): Hemolymph collected from feeding day 6 *D. andersoni* females exposed as adults, and spiked with 100 pg *A. marginale* DNA extracted from bovine erythrocytes, as a positive control; (16): Uninfected hemolymph collected from feeding day 7 *D. andersoni* females, as a negative control; (17): 100 pg *A. marginale* DNA extracted from bovine erythrocytes, as a positive control; (18): *A. marginale* primers only, as a contamination control.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2a
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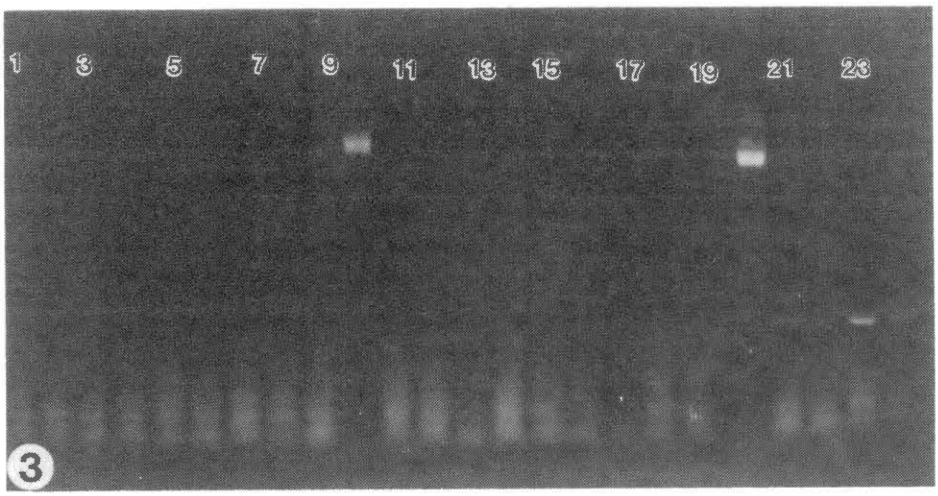
Figure V. PCR assay of hemolymph collected from uninfected male and female *Dermacentor andersoni* during feeding and holding

Lanes: (1-6): Male feeding days 1, 3, 5, 7, 9 and 11, respectively; (7-9): Male holding days 1, 3 and 9, respectively (10): 123 bp ladder standard (BRL); (11-16): Female feeding days 1, 3, 5, 7, 9 and 11, respectively; (17-19): Female holding days 1, 5 and 9, respectively; (20): 123 bp ladder standard (BRL); (21): Hemolymph of intrastadially exposed transmission feeding day 10 males; (22): Hemolymph of feeding day 6 *D. andersoni* females exposed as adults; (23): 100 pg *A. marginale* DNA extracted from bovine erythrocytes, as a positive control; (24): Uninfected hemolymph collected from *D. andersoni* males, feeding day 10, and spiked with 100 pg *A. marginale* DNA extracted from bovine erythrocytes, as a positive control.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CHAPTER IV

DETECTION OF *ANAPLASMA MARGINALE* (RICKETTSIALES: ANAPLASMATACEAE) IN SECRETAGOGUE-INDUCED ORAL SECRETIONS OF *DERMACENTOR ANDERSONI* (ACARI: IXODIDAE) WITH THE POLYMERASE CHAIN REACTION

Abstract

Polymerase chain reaction (PCR) was used to detect *Anaplasma marginale* in secretagogue-induced oral secretions of male and female *Dermacentor andersoni* exposed as nymphs or adults by feeding on infected calves. *Anaplasma* DNA was amplified in oral secretions of female ticks exposed as adults and stimulated to secrete by injection of dopamine. Conversely, *A. marginale* was detected in saliva from prefed female ticks exposed as nymphs only after stimulation with a combination of dopamine, γ-aminobutyric acid, pilocarpine and theophylline. Saliva from ticks exposed as nymphs and stimulated with ergot alkaloids, did not contain *A. marginale* DNA. Saliva collected after 11 d of feeding from dopamine-stimulated male ticks contained *A. marginale* DNA. The results indicate that *A. marginale* is present in tick saliva and suggest that the parasite can be transmitted to cattle via saliva of feeding ixodid ticks. The variable appearance of *A. marginale* in saliva, regardless of the method used to induce salivation, suggests that transmission of *A. marginale* may be affected by the physiological state of the tick.

Introduction

Anaplasma marginale, the etiological agent of bovine anaplasmosis, has been studied extensively in ixodid ticks (Rees 1934; Anthony & Roby 1962; Potgieter 1979; Kocan et al. 1980 a & b; Kocan et al. 1981; Kocan et al. 1982; Stiller et al. 1983; Kocan et al. 1984; Kocan 1986; Zaugg et al. 1986; Coan et al. 1987; Stich et al. 1989; Stiller et al. 1989; Kocan et al. 1990; Kocan et al. 1992 a, b & c). The rickettsia undergoes a complex developmental cycle in its tick vector, and transmission is coordinated with the tick feeding cycle (Kocan 1986). As the tick feeds, anaplasmal colonies develop first in midgut epithelium, followed by proliferation in muscle cells lining the visceral midgut and final development occurs in salivary glands before, presumably, the organism is transmitted to the vertebrate host. The parasite has been found in tick hemolymph, a likely intermediate site that is considered a prerequisite before invading the salivary glands (Kocan 1983; Ribeiro et al. 1987; Stich et al. 1992).

Because *A. marginale* appears in tick salivary glands, which have been shown to be infective by inoculation into cattle, it is likely that transmission occurs via tick salivary secretions during tick feeding (Kocan 1986). Other tick-borne pathogens (*i.e.*, *Theileria parva*, *T. cervi* and *Borrelia burgdorferi*) have been detected in secretagogue-induced oral secretions of partially fed ticks (Purnell et al. 1969; Durham

et al. 1976; Ribeiro et al. 1987). However, *A. marginale* has not previously been demonstrated in secretagogue-induced saliva of partially-fed ticks.

Procedures used in the past to detect *A. marginale* in ticks (*e.g.*, transmission of *A. marginale* to susceptible cattle, *in situ* light and electron microscopy, and DNA probe hybridization) were successful due to heavily infected tick tissues (Kocan et al. 1983; Kocan et al. 1986; Goff et al. 1988). However, even in ticks shown by other methods to harbor large number of parasites, we have been unsuccessful in identifying *A. marginale* in artificially induced saliva from partially-fed ticks. Recently, a polymerase chain reaction (PCR)-based assay for detection of *A. marginale* in *Dermacentor andersoni* has been developed (Stich et al. 1991; Stich et al. 1992). PCR is a sensitive method for extensively amplifying a specific target of template DNA (Saiki et al. 1988), facilitating detection of minimal infections. The purpose of this study was to determine whether *A. marginale* could be detected with PCR in saliva of infected ticks stimulated to secrete saliva by secretogogues (Kaufman 1977; Sauer 1979; Kaufman & Wong 1983; Lindsay 1986; Kaufman 1989).

Materials and Methods

Anaplasma marginale

The Virginia isolate of *A. marginale* (VAM) was used to infect donor calves by whole blood transfusion. This isolate has been used successfully in other studies of *A. marginale* involving ticks and cattle in our laboratory (Kocan et al. 1980 a & b; Kocan et al. 1981; Oberst et al. 1981; Kocan et al. 1982; Kocan et al. 1983; Kocan et al. 1984; Kocan, 1986; Stich et al. 1989; Kocan et al. 1990; Stich et al. 1991; Kocan et al. 1992 a, b & c).

Tick Propagation

Dermacentor andersoni nymphs and adults were reared at the Oklahoma State University, Medical Entomology Laboratory (Patrick & Hair 1975). Larvae and nymphs were fed on rabbits and sheep and allowed to molt to the next stage. Nymphs and adults were held in a humidity chamber (90-98% relative humidity) at 25°C with a 14-hour photoperiod until used for this study.

Experimental Calves

Three splenectomized calves (2 to 4 month old *Bos taurus*), determined free of anaplasmosis by the complement-fixation (CF) test, were used as experimental hosts. Two calves, infected with *A. marginale* by IV inoculation of VAM-infected bovine

blood, served as hosts for exposure of nymphal or adult ticks to *A. marginale*. The third calf served as host to test for tick transmission of *A. marginale* and collection of partially fed ticks for stimulation of salivation. All calves were monitored 3 times a week by examination of Wright-stained blood smears and determination of packed cell volume (PCV). Once *A. marginale* bodies were detected in blood smears, the calves were monitored daily.

Infection of Adult Ticks and Vector Transmission of *A. marginale* to a Susceptible Host

Dermacentor andersoni males and females (150 pairs) were placed in separate orthopedic stockinette cells attached to an infected calf when its parasitemia had reached 3 to 5%. Ticks were allowed to feed on the infected calf for 7 d, and then removed by traction and placed in a humidity chamber (> 90% RH) for 10 d. Male and female ticks were then placed in the same stockinette on a susceptible calf and allowed to feed for 10 d or until replete.

Infection of Nymphal Ticks and Vector Transmission of *A. marginale* to a Susceptible Host

Dermacentor andersoni nymphs (approximately 500) were placed in orthopedic stockinettes attached to an infected calf when parasitemia was between 3 to 5%. Nymphs were allowed to

feed to repletion and placed in a humidity chamber (> 90% RH). After the ticks had molted to adults, 150 male and female pairs were allowed to feed on a susceptible calf.

Collection of Saliva

Ticks were secured in place, dorsal side down, on masking tape and injected through the posterior 1/3 of their ventral scutum; approximately 0.1 µl per mg tick body weight of RPMI 1640 culture medium (Gibco), with indicated concentration of secretagogue, was injected into their hemocoel every 15 min for 1 hr. Ten female ticks exposed to *A. marginale* were removed by traction on each day of feeding on a susceptible host and injected with RPMI and 1 mM dopamine to stimulate salivation. Saliva was collected by capillary action in 50 µl microcapillary tubes placed over the mouthparts of stimulated ticks (females) or drawn-out Pasteur pipets (males), pooled and stored at -70°C until analyzed with PCR for the presence of *A. marginale* DNA. Fifteen prefed females exposed as nymphs were removed daily, and saliva was harvested after stimulating ticks with either dopamine (1mM in RPMI), ergonovine maleate (1 mM in RPMI) or a combination of dopamine, γ-aminobutyric acid, theophylline, ergonovine maleate and pilocarpine (1 mM each in RPMI). Males exposed to *A. marginale* as either nymphs or adults (150 from each group) were removed and injected with approximately 5 µl RPMI containing 1 mM dopamine.

Preparation of *A. marginale* DNA from Infected Bovine Erythrocytes

Anaplasma DNA from VAM-infected blood was processed and used as a control for the polymerase chain reaction (PCR) tests. The infected blood was prepared as described previously (Stich et al. 1991).

Polymerase Chain Reaction

Samples were assayed with 24- and 20-base oligonucleotide primers (50% G+C) flanking a 409 bp fragment of the *A. marginale* Florida isolate msp1β gene (Barbet & Allred 1991). Primers were synthesized and then purified with polyacrylamide gel electrophoresis by Synthetic Genetics, LaJolla, CA. PCR was performed with Perkin-Elmer Cetus Geneamp Reagents aliquoted from a master mix to 25 µl final reaction volumes containing 1X PCR buffer (10 mM Tris·HCl, pH 8.3, 50 mM KCl), 0.8 mM dNTP mix, 3.5 mM $MgCl_2$, 1.8 µM of each primer, 4% (v/v) heat-denatured fetal bovine serum (Sigma), 0.32 µg/µl bovine serum albumin and 0.12 U/µl *Taq* DNA polymerase (Boehringer Mannheim). Reaction master mixes were pipetted onto denatured samples that were previously overlaid with 20 µl mineral oil. The reaction profile consisted of 32 cycles of 94°C for 1.0 min, 60°C for 1.0 min, and 72°C for 0.5 min followed by a final extension at 72°C for 3.0 min in a Perkin Elmer-Cetus 9600 Thermocycler.

PCR Assay of Saliva

Pools of saliva were thawed and 2.5 µl (template samples) covered with 20 µl mineral oil. The samples were then heated to 105-110°C for 30 min prior to assaying for *A. marginale* DNA with PCR. *Anaplasma* DNA extracted from infected bovine erythrocytes served as a positive control, and template-free master mix served as the contamination control.

Agarose Gel Electrophoresis

After the amplification reaction, the 25 µl reaction mixture was added to 5 µl loading buffer (40 % sucrose, 89 mM Tris, 89 mM boric acid, 2 mM EDTA) and electrophoresed on a 1.5 % agarose gel containing 0.5 µg/ml ethidium bromide in 1 X TBE (89 mM Tris, 89 mM boric acid, 2 mM EDTA) at 75-100 V for 1.5 hr. A 123 bp ladder (Bethesda Research Laboratories) was co-electrophoresed in an adjacent lane of the gel to serve as the molecular size standard. DNA was visualized with ultraviolet light and photographed with a 35 mm camera equipped with a yellow filter.

Results

Infection of Ticks

Adult ticks exposed to *A. marginale* either as nymphs or adults transmitted *A. marginale* to a susceptible calf (42 d prepatent period; 48.2% peak parasitemia; 74% reduction PCV).

Collection of Saliva

Salivation was stimulated in females exposed as adults by injection dopamine after 4 and 5 d of feeding. Discolored oral secretions were discarded for fear that it was an indication of gut luminal contamination. Females exposed as nymphs, and removed from the host after feeding for 7, 8 or 10 d, salivated when stimulated with dopamine. Those having fed for 7, 8 and 10 d salivated when stimulated with dopamine, γ-amino butyric acid, theophylline, ergonovine maleate and pilocarpine, and those having fed for 8, 9 and 10 d salivated when injected with ergonovine maleate. Males exposed as either nymphs or adults prior to feeding 11 d, salivated when stimulated with dopamine. For unknown reasons we were unable to stimulate partially-fed infected females to salivate with any secretagogue combinations at other stages of feeding.

PCR of Tick Saliva

Anaplasma marginale DNA was detected with PCR in 2.5 µl heat-denatured saliva obtained from 5 d prefed females that were exposed during the adult stage and stimulated with dopamine to salivate. *Anaplasma marginale* was also detected in saliva collected from female ticks that were exposed as nymphs. *Anaplasma* DNA was detected in saliva of 7, 8 and 10 d prefed female ticks exposed as nymphs, stimulated with dopamine, γ-amino butyric acid, theophylline, ergonovine maleate and pilocarpine. Saliva collected from dopamine-stimulated males, exposed as either nymphs or adults, was PCR-positive for *A. marginale* (Fig VI).

Discussion

Ticks are vectors of a diverse group of hematotropic parasites that may be adapted to several physiological environments within the tick (Friedhoff 1990). Hypothetically, transmission of parasites from a tick to its vertebrate host could occur by salivation, regurgitation, defecation or by host ingestion of infected ticks. The mechanism of transmission may be related to interaction of the parasite with its invertebrate host: regurgitation, defecation and ingestion of the vector may be important for parasites that reside solely in the tick's alimentary canal. Vector ingestion by the host may also be an important method of transmission for parasites restricted to the tick hemocoel. Salivation may be a means for transmission to the host when the tick is infected systemically. The feeding habits and central role of salivary secretions in enabling ticks to remain on a host for relatively long periods of time (Kaufman 1989) strongly suggest that salivation may be an important route of transmission of parasites to vertebrate hosts. The large amount of fluid secreted by the salivary glands, facilitating concentration of the bloodmeal, is well documented (Kaufman 1989). *Anaplasma marginale* has been demonstrated in various ixodid tick tissues including midgut epithelium, muscle cells lining the visceral midgut, hemolymph and salivary glands (Kocan et al. 1980 a & b; Kocan et al. 1981; Kocan et al. 1982; Kocan 1986; Kocan et al. 1992 a & b),

demonstrating that ticks become systemically infected with *A. marginale*. The present results suggest that secretagogue-induced saliva from some infected, partially-fed ticks contains *A. marginale*. Because the secretagogues used to induce salivation in this study have been implicated as physiological effectors of salivary gland secretion *in vitro* and *in vivo* (Kaufman 1989), we believe that one route of transmission of *A. marginale* to cattle is via salivary secretions.

Amplification products in the saliva were greater for male and female *D. andersoni* that acquired *A. marginale* as adults than for those exposed as nymphs. Dopamine was the only agent required to stimulate secretion of *A. marginale* from ticks exposed as adults and males exposed as nymphs. A D-1 dopamine receptor linked to activation of adenylate cyclase has been identified in the salivary glands (Schmidt et al. 1981). Following neural release of dopamine and activation of an adenylate cyclase, an increase in cAMP is an essential link in the mechanism controlling fluid secretion by the salivary glands (Sauer and Essenberg 1984). Conversely, dopamine alone failed to stimulate secretion of the organism by female ticks exposed as nymphs. However, a combination of dopamine, theophylline (inhibitor of cAMP phosphodiesterase), pilocarpine and γ-aminobutyric acid did stimulate a salivary secretion that resulted in relatively small amounts of amplified parasite DNA. Pilocarpine stimulates a cholinergic salivary secretory nerve that in turn stimulates the dopamine receptor in the

secretory cell (Kaufman 1977; Kaufman & Wong, 1983). γ-Aminobutyric acid is believed to potentiate the ability of dopamine to stimulate salivation (Lindsay & Kaufman 1986). Inhibition of PCR by any difference in saliva composition between the two methods of infection is unlikely because of amplification of template spiked into saliva collected from females exposed as nymphs. While ergonovine maleate alone stimulated oral secretion, *A. marginale* was not detected and secretion of the organism was not dependent on the presence or absence of the compound in combination with others. Ergot alkaloids are also agonists of salivary secretion with a separate salivary gland receptor (Kaufman & Wong, 1983).

These observations support previous reports that *D. andersoni* males, exposed to *A. marginale* as adults, had infected salivary glands and readily transmitted the parasite to susceptible cattle (Kocan et al. 1992 a & b). Exposure of ticks to *A. marginale* during the adult stage may provide a physiological "conditioning" that increases salivary gland susceptibility to infection with the rickettsia, resulting in higher densities of parasites within the acini, presumably enhancing transmission in oral secretions.

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Footnotes

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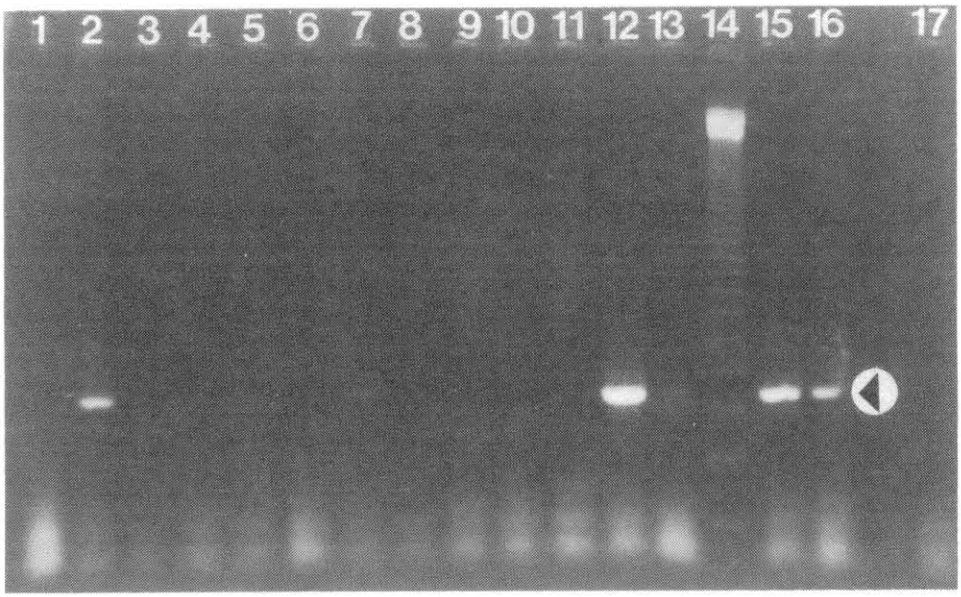
Figure VI. PCR test of saliva collected from prefed male and female *Dermacentor andersoni* that were exposed to *Anaplasma marginale* during the adult or nymphal stage.

Lanes: (1): Females infected as adults, feeding day 4, injected with dopamine; (2): Females infected as adults, feeding day 5, injected with dopamine; (3): Females infected as nymphs, feeding day 5, injected with dopamine; (4): Females infected as nymphs, feeding day 7, injected with dopamine; (5): Females infected as nymphs, feeding day 7, injected with dopamine, γ-amino butyric acid, theophylline, ergonovine maleate and pilocarpine; (6): Females infected as nymphs, feeding day 5, injected with dopamine; (7): Females infected as nymphs, feeding day 8, injected with dopamine, γ-amino butyric acid, theophylline, ergonovine maleate and pilocarpine; (8): Females infected as nymphs, feeding day 8, injected with ergonovine maleate; (9): Females infected as nymphs, feeding day 10, injected with ergonovine maleate; (10): Females infected as nymphs, feeding day 10, injected with ergonovine maleate; (11): Females infected as nymphs, feeding day 10, injected with ergonovine maleate, dopamine, γ-amino butyric acid, theophylline, ergonovine maleate and pilocarpine; (12): Males infected as adults, feeding day 11, injected with dopamine; (13): Males infected as nymphs, feeding day 11, injected with dopamine; (14): 123 bp ladder (BRL) molecular size standard (15): Females infected as nymphs, feeding day 8, injected with

dopamine, γ-aminobutyric acid, theophylline, ergonovine maleate and pilocarpine, spiked with 1 ng *A. marginale* DNA extracted from bovine erythrocytes, as a positive control; (16): 1 ng *A. marginale* DNA extracted from bovine erythrocytes, as a positive control; (17): *A. marginale* primers only, as a contamination control.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CHAPTER V

DETECTION OF *ANAPLASMA MARGINALE* (RICKETTSIALES: ANAPLASMATACEAE) IN FIELD-COLLECTED *DERMACENTOR ALBIPICTUS* (ACARI: IXODIDAE) WITH THE POLYMERASE CHAIN REACTION

Abstract

A polymerase chain reaction (PCR) assay developed for detection of *Anaplasma marginale* in ticks was tested for specificity, and determined to be suitable for seven *A. marginale* isolates found from diverse geographical areas of the USA. Ticks were then collected from anaplasmosis-enzootic areas of Oklahoma and held in a humidity chamber for a minimum of 10 days prior to bisection, proteinase-digestion and screening with PCR for natural-infection with *A. marginale*. Prefed and unattached field-collected *Dermacentor variabilis* adults were PCR-negative for *A. marginale*. Prefed *D. albipictus* adults, removed from cattle on a ranch with a history of anaplasmosis, were PCR-positive for *A. marginale*. The posited involvement of overwintered tick vectors in the transmission of anaplasmosis remains unresolved, but this study suggests infection of *D. albipictus* with *A. marginale* may be involved in the epizootiology of anaplasmosis in Oklahoma.

Introduction

Ixodid ticks are the only known biological vectors of anaplasmosis with approximately 20 species incriminated as vectors world-wide (Ewing 1981; Potgeiter 1981). Transmission may occur by adult ticks transferred from infected to susceptible hosts or by nymphs or adults infected in a previous stage. *Anaplasma marginale* persists transstadially from nymphs to adults, and from larvae to both nymphs and adults (Dikmans 1950; Kocan et al. 1980; Kocan et al. 1981; Ewing 1981; Potgeiter 1981; Stich et al. 1989). Transmission of *A. marginale* by male and female *D. andersoni* exposed as adults has been demonstrated, and transfer of adult ticks between cattle under field conditions has been reported (Kocan et al. 1992 a & b; Stiller 1989a). Biological infection and transmission by adults was confirmed by the presence of colonies in the tick tissues (Kocan et al. 1992 a, b & c). Transovarial transmission of *A. marginale* has been reported in some ixodid tick species, but has not been confirmed consistently in any tick species (Dikmans 1950; Rees 1934; Anthony & Roby 1962; Ewing 1981; Potgeiter 1981; Stich et al. 1989).

Previous studies have involved testing field-collected ticks for infection with *A. marginale* by allowing them to feed on susceptible cattle. Such methods are expensive, owing to the cost of labor and experimental animals, and unreliable due to expected low infection rates in naturally-exposed ticks.

Recently, a polymerase chain reaction (PCR)-based assay has been developed for detection of *A. marginale* in salivary glands, hemolymph and oral secretions of experimentally-infected ticks (Stich et al. 1991; Stich et al. 1992 a & b). The purpose of this study was to assess the feasibility of this assay for testing field-collected ticks for infection with *A. marginale*.

Materials and Methods

PCR Specificity Trials

Specificity of the oligonucleotide primers used (see below) was determined by running the PCR protocol with 100 ng template DNA from 19 different species, including 8 tick-transmitted organisms (Table III). Seven *A. marginale* isolates (Florida, Virginia, Oklahoma, Illinois, Washington-O, S-Idaho and N-Texas) were also tested to determine if a 409 bp band was consistently amplified.

Collection of Ticks and Bovine Blood

Dermacentor variabilis adult ticks were collected from anaplasmosis-enzootic areas between March and April, 1992, using flagging techniques (Bram 1978), CO_2 traps (Garcia 1965) and by removing ticks from canine hosts. Attached *D. variabilis* and *D. albipictus* were removed from cattle on ranches with a history of anaplasmosis; cattle were restrained in a squeeze chute and palpated to detect ticks as described previously (Bram 1978). Blood samples (3-5 ml) were collected in vacutainers containing EDTA, from cows with attached *D. albipictus*. Wright-stained blood smears were made and blood samples were prepared for PCR.

Preparation of Blood for PCR

Blood from *D. albipictus* bovine host samples was centrifuged (1000 x g) in 10-15 hematocrit tubes (approximately 0.5 ml total volume) for 5 min. Buffy coats were separated from from erythrocytes by etching the hematocrit tubes behind the buffy coat, breaking the hematocrit tube, and pushing the erythrocyte fraction into a microfuge tube by pressing the hematocrit sealant with a wire. One volume of RPMI 1640 (Gibco) culture medium (approximately 250 µl) was added to erythrocytes prior to freezing at -20°C. The erythrocytes were lysed by thawing and mixing with an equal volume of ddH_2O, after which the hemoglobin was removed by washing pelleted cells (13,000 x g for 1 min). Washings consisted of pelleting lysed cells (13,000 x g for 1 min) and resuspending the pellet with 1 ml of RPMI (5 washes) with a final suspension in 0.5 ml RPMI. Lysate proteins were digested with 100 µg/ml proteinase K and 0.45% (v/v) Tween 20 and NP40 at 55°C for 5 hr, and stored at -20°C until tested for *A. marginale* DNA by PCR.

Preparation of Ticks for PCR

After collection, ticks were held in a humidity chamber (25°C, >90% RH, 14 hr photoperiod) for a minimum of 10 d, prior to bisection and preparation for PCR as described previously (Stich et al. 1991). Bisections were performed with aseptic technique by successive rinses of forceps and razor blades in 0.5% (w/v) SDS, 10% (v/v) Clorox Bleach, double distilled H_2O and 95% (v/v) ethanol followed by flame sterilization. Bisected

ticks were stored in 150 µl RPMI 1640 (Gibco BRL) at -70°C until prepared for PCR. Each sample was digested with proteinase K. Concentrated (4X) digestion buffer (1.8% NP40, 1.8% Tween 20, 400 µg/ml proteinase K, in RPMI 1640) was aliquoted (50 µl) to 150 µl samples, and incubated 7-13 hr at 55°C prior to testing for the presence of *A. marginale* DNA by PCR.

Polymerase Chain Reaction

Samples were assayed with 24- and 20-base oligonucleotide primers (50% G+C) flanking a 409 bp fragment of the *A. marginale* Florida isolate msp1β gene (Barbet 1991). Primers were synthesized and purified with polyacrylamide gel electrophoresis (Synthetic Genetics, LaJolla, CA). PCR was performed with Perkin-Elmer Cetus Geneamp Reagents aliquoted from a master mix to 25 µl final reaction volumes containing 1X PCR buffer (10 mM Tris·HCl, pH 8.3, 50 mM KCl), 0.8 mM dNTP mix, 3.5 mM $MgCl_2$, 1.8 µM of each primer, 4% (v/v) heat-denatured fetal bovine serum (Sigma), 0.32 µg/µl bovine serum albumin and 0.12 U/µl *Taq* DNA polymerase (Boehringer Mannheim). Reaction master mixes were pipetted onto denatured samples that were previously overlaid with 20 µl mineral oil. The reaction profile consisted of 32 cycles of 94°C for 1.0 min, 60°C for 1.0 min, and 72°C for 0.5 min followed by a final extension at 72°C for 3.0 min in a Perkin Elmer-Cetus 9600 Thermocycler.

Agarose Gel Electrophoresis

After the amplification reaction, the 25 µl reaction mixture was added to 5 µl loading buffer (40 % sucrose, 89 mM Tris, 89 mM boric acid, 2 mM EDTA) and electrophoresed on a 1.5 % agarose gel containing 0.5 µg/ml ethidium bromide in 1 X TBE (89 mM Tris, 89 mM boric acid, 2 mM EDTA) at 75-100 V for 1.5 hr. A 123 bp ladder (Bethesda Research Laboratories) was applied to an adjacent lane of the gel to serve as the molecular size standard. DNA was visualized with ultraviolet light and photographed with a 35 mm camera equipped with a yellow filter.

Results

PCR specificity trials

All *A. marginale* isolates tested produced similar PCR products (409 bp) when amplified with the primers selected from the msp1β sequence of the *A. marginale* Florida isolate (Fig VII). These primers did not result in any amplified DNA bands when tested with DNA other than that of *A. marginale* (Fig VIII), including *A. ovis* and 7 other tick-transmitted organisms (Fig IX).

PCR of field-collected ticks and host blood

Of the 35 *D. variabilis* and 9 *D. albipictus* samples tested, one sample of two *D. albipictus* females (W21) was PCR-positive for *A. marginale* (Fig X). Blood collected from both *D. albipictus* hosts was PCR-positive for *A. marginale*. However, blood from one host (1145) produced an additional, larger band of amplified DNA (Fig X).

Discussion

The PCR assay in this study has been used successfully under experimental conditions (Stich et al. 1991; Stich et al. 1992 a & b). Previous DNA probe studies have shown that *A. marginale* DNA in the tick bloodmeal cannot be detected 48 hr after removing the ticks from an infected host (Stiller et al. 1989a). Thus detection of *A. marginale* in field-collected ticks with PCR, after the minimal 10 day holding period, indicates infection of tick tissues by the parasite. DNA probe hybridization subseqent to target DNA amplification has not been necessary to date, though a hybridization step would facilitate sensitivity for testing field-collected ticks for infections that might be low, and specificity for any unforseen amplification of homologous DNA fragments. Preliminary PCR specificity trials, especially of other tick-vectored organisms, were required to ensure that the assay would only detect isolates of *A. marginale*. Amplification of the same size target DNA fragment resulted from all *A. marginale* isolates tested, and the absence of amplified DNA bands for other organisms tested, including *A. ovis*, suggests that the primers used in this study were species-specific and -universal without the need for a DNA hybridization step.

Despite the results of the specificity trials, an additional, larger band was amplified from one of the *D. albipictus* hosts (1145). The source of this PCR product is not known;

possible sources of template include the bovine host, a heterologous species or an *A. marginale* isolate not verified in the specificity trials. The observation of marginal bodies in peripheral erythrocytes and the demonstrated specificity of this assay suggest that the host may have been infected with an isolate of *A. marginale* in which a portion of the target fragment is deleted in one of the msp1β gene repeats (Barbet & Allred 1991). Additionally, it is noteworthy that only the *D. albipictus* from the host that produced a single band of amplified DNA (W21) were also PCR-positive, indicating that the posited *A. marginale* infection of 1145 may also be an isolate incapable of infecting *D. albipictus*. Other *A. marginale* isolates (*i.e.*, Florida and Illinois) have been demonstrated incapable of infecting *D. andersoni* (Wickwire 1985); a procedure to distinguish between *A. marginale* isolates that are capable or incapable of infecting ixodid species would be useful in epizootiological studies.

The transmission of *A. marginale* by ticks from chronic carrier cattle has been proposed to be important in maintaining the organism in enzootic areas (Kocan et al. 1981; Stich et al. 1989). Adult *D. andersoni* that were infected as nymphs have been reported to remain infected with the parasite up to 6 months post-molting (Kocan et al. 1986). *Dermacentor variabilis* is reported to remain infected up to 12 months, especially when the ticks are stored under winter conditions

(Logan et al. 1987). Attempts to demonstrate *A. marginale* in field-collected overwintered adult *D. andersoni* have been unsuccessful (Maas et al. 1986; Schofield & Saunders 1987).

The role of overwintered tick vectors in the epidemiology of anaplasmosis remains unresolved. Potential transmission of *A. marginale* by ticks infected during a previous stage has been questioned because the immature stages of several 3-host tick vectors do not normally feed on cattle. Howevever, *D. andersoni* males exposed to *A. marginale* by feeding on a naturally infected chronic carrier cow have been shown capable of subsequently transmitting the parasite to susceptible cattle (Zaugg et al. 1986), and it is hypothesized that interhost transfer of adult ticks may initiate anaplasmosis outbreaks (Zaugg et al. 1986; Stiller et al. 1989b; Kocan et al. 1992 a, b & c). Demonstration of naturally-infected *D. albipictus* in this study supports reports of experimental transmission by *D. albipictus* nymphs exposed to *A. marginale* as larvae, and ticks that were exposed to the parasite during the adult stage (Stiller et al. 1980; Stiller & Johnson 1983). Anaplasmosis vectored by interhost transfer of *D. albipictus* may explain transmission of *A. marginale* during the winter months (Wright et al. 1985; Zaugg 1990), suggesting that this species may be a vector of importance in Oklahoma.

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Footnotes

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Table III. Heterologous species tested for specificity of polymerase chain reaction assay for Anaplasma marginale.

Species	Strain	ATTC No.	Source
Candida albicans			H. Vishniac, OSU Botany-Microbiology
Eschericha coli	HB101		S.M. Halling USDA/ARS, Ames, IA
Haemophilus somnus			A. Confer, OSU Veterinary Pathology
Klebsiella pneumoniae		13882	American Type Culture Collection
Listeria monocytogenes	LM1		R. Welsh, OSU, OADDL Field Strain Isolate
Pasteurella multocida	OSU Strain		A. Confer, OSU Veterinary Pathology
Proteus mirabilis		25933	American Type Culture Collection
Serratia marcescens		13880	American Type Culture Collection
Yersinia enterocolitica		23715	American Type Culture Collection
Salmonella typhimurium		29630	American Type Culture Collection
Brucella abortus	S19		APHIS
Babesia odocoiles[a]			A. Kocan, OSU Veterinary Parasitology
Borrelia burgdorferi[a]			A. Kocan, OSU Veterinary Parasitology
Coxiella burnetti[a]			J. Williams, US Army Medical Research Institue
Ehrlichia canis[a]			S. Ewing, OSU Veterinary Parasitology
Francisella tularensis[a]			J. Williams, US Army Medical Research Institue
Rickettsia rickettsii[a]			J. Williams, US Army Medical Research Institue
Theileria cervi[a]			A. Kocan, OSU Veterinary Parasitology
Anaplasma ovis[a]			G. Palmer, WSU Veterinary Microbiology

[a]Tick-associated organisms.

Figure VII. PCR of heterologous *Anaplasma marginale* isolates

Lanes: (1): Primers only, as a contamination control; (2): 123 bp ladder (BRL) molecular size standard; (3): N-Texas isolate; (4): S-Idaho isolate; (5): Washington-O isolate; (6): Illinois Isolate; (7): Oklahoma isolate; (8): Florida isolate; (9): Virginia isolate.

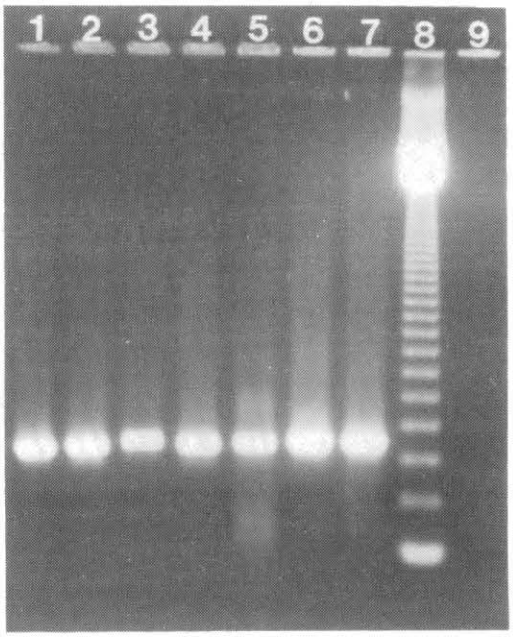
1 2 3 4 5 6 7 8 9

Figure VIII. PCR of heterologous species not associated with ticks

Lanes: (1): *A. marginale* Virginia isolate, as a positive control; (2): 123 bp ladder (BRL) molecular size standard; (3): *C. albicans;* (4): *E. coli;* (5): *H. somnus;* (6): *K. pneumoniae;* (7): *L. monocytogenes;* (8): *P. multicoda;* (9): *S. marcescens;* (10): *Y. enterocolitica;* (11): *S. typhimurium;* (13): *B. abortus*.

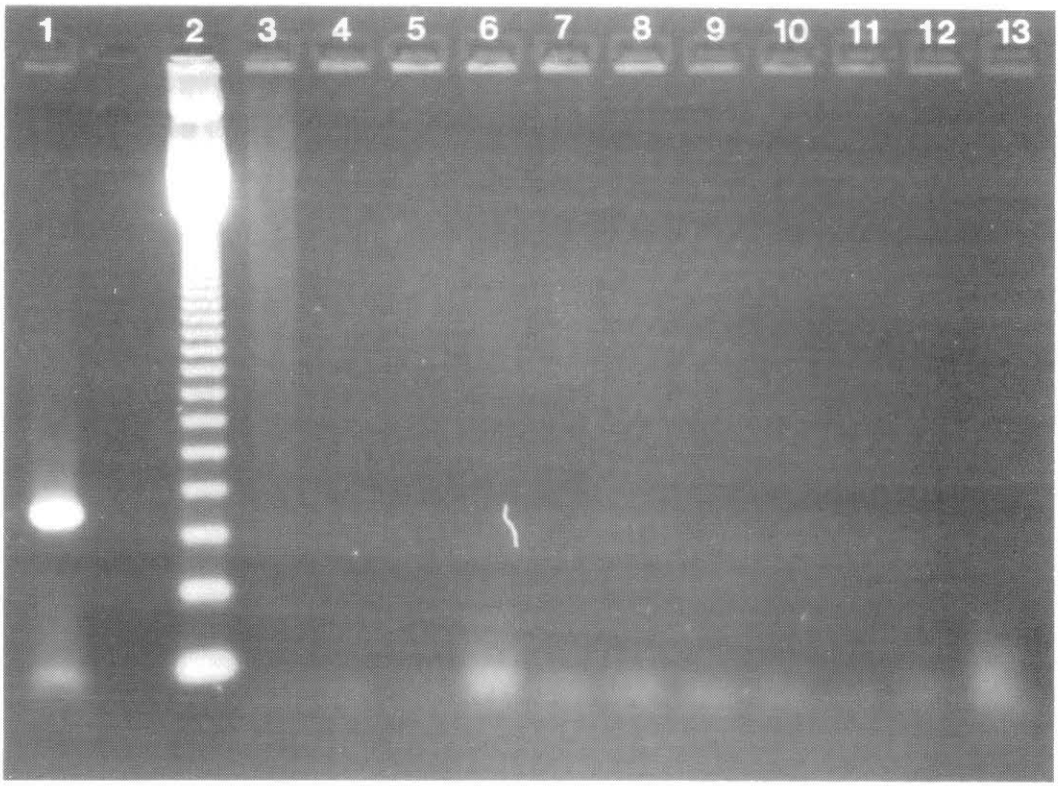
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Figure IX. PCR of heterologous species associated with ticks

Lanes: (1): *B. odocoiles;* (2): *C. burnetti;* (3): *E. canis;* (4): *F. tularensis;* (5): *R. rickettsii;* (6): *T. cervi;* (7): *A. ovis;* (8): 123 bp ladder (BRL) molecular size standard; (9): *A. marginale* N-Texas isolate, as a positive control.

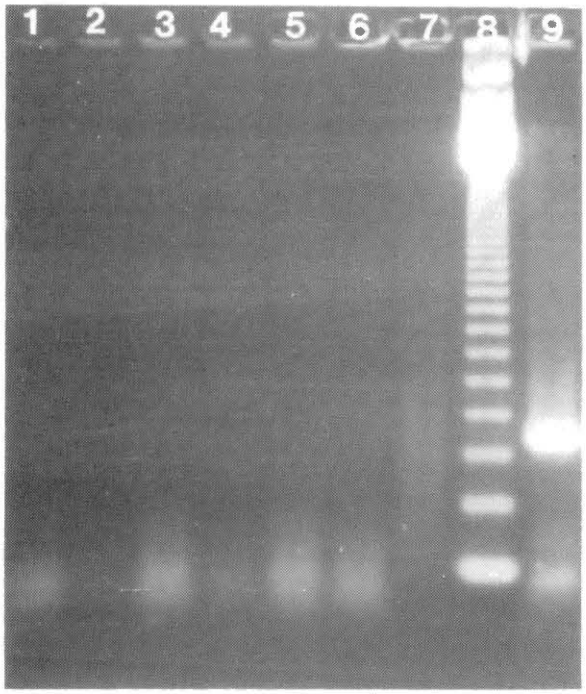
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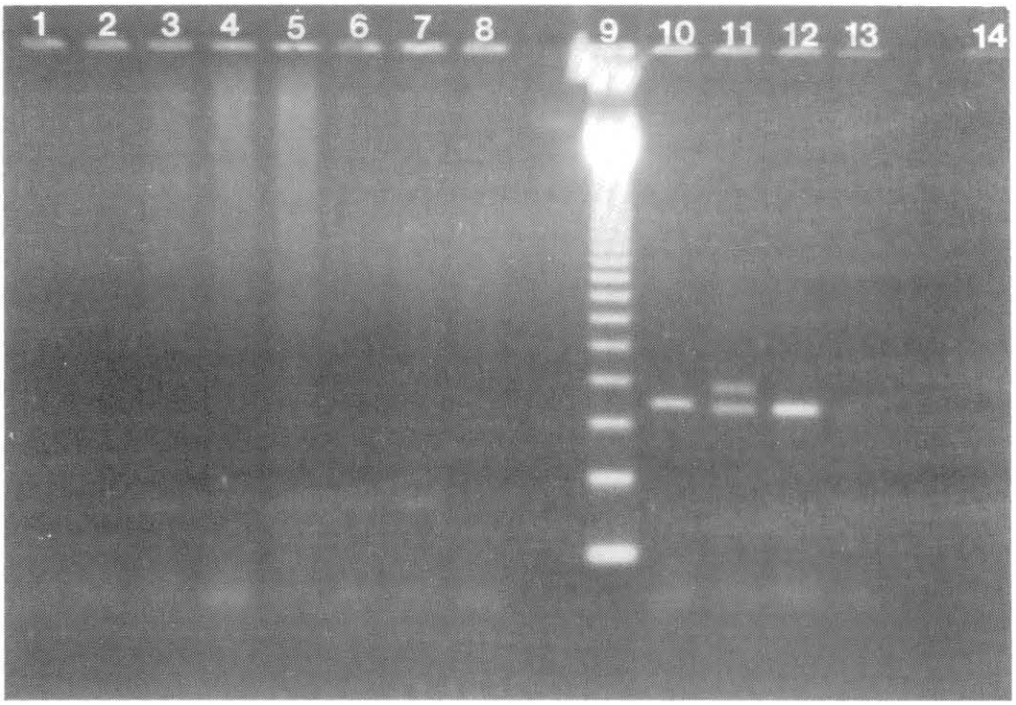
Figure X. PCR of field-collected *Dermacentor albipictus* and bovine host blood

Lanes: (1-2): *D. albipictus* males collected from 1145; (3-8): *D. albipictus* females collected from 1145; (9): 123 bp ladder (BRL) molecular size standard; (10): 1 ng *A. marginale* Virginia isolate, as a positive control; (11): 1145 blood lysate; (12): W21 blood lysate; (13): *D. albipictus* females collected from W21; (14): Primers only, as a contamination control.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CHAPTER VI

DISCUSSION

The purpose of this research was to develop a polymerase chain reaction (PCR) assay for *Anaplasma marginale*, and to test its application for detection of the organism within experimentally- and naturally-infected ticks. The msp1β gene was chosen for this assay for several reasons: (1) The msp1α-β protein complex is well characterized. (2) A 2 kbp fragment of the msp1β gene has been used successfuly as a DNA probe in tick tissues. (3) DNA sequences of both msp1α and msp1β have been reported. (4) The msp1α gene is more variable among isolates of *A. marginale*. (5) The msp1βgene is species-specific for *A. marginale*, while a msp1α homolog is present in *A. centrale*.

Preliminary to application of PCR to experimentally-infected tick tissues, oligonucleotide primers were chosen that flank an appropriately sized DNA fragment of the msp1β gene. Initially, oligonucleotides AL34S and AL52 were provided by Drs. G.H. Palmer and I.S. Eriks of Washington State University, and used to amplify *A. marginale* DNA. However, reactions with these primers proved difficult to repeat, perhaps due to the relatively large size of the two fragments (1100

and 1200 bp) amplified in the *A. marginale* Virginia isolate. The AL52 primer was then replaced with a 20 base, 50% G+C primer (BAP-1) 406 bases downstream of AL34S in the *A. marginale* Florida isolate msp1β sequence. Reaction parameters were optimized for AL34S and BAP-1, resulting in amplification of a single 406 bp band, and adapted to preliminary assay of experimentally-infected tick tissues by PCR.

After preliminary application of PCR for detection of *A. marginale* in ticks, the test was improved with several changes in the protocol, and these adaptations were used for the remaining experiments involving experimentally-infected tick hemolymph and oral secretions, and screening of field-collected ticks for natural infection. First, addition of 4 bases to BAP-1 resulted in less background amplification due to mis-priming; thus the BAP-2 primer (24 bases, 50% G+C) was used for the remaining experiments. Second, addition of heat-denatured fetal bovine serum (FBS) to reaction mixtures greatly reduced nonspecific background amplification and increased sensitivity. The most effective protocol involved incubating the FBS to 105-110°C for 15 min and using the supernatant for the master mix. The amount of FBS used depended on the material assayed; increasing the amount of FBS did not appear to inhibit PCR of pure DNA while amounts greater than 4% (v/v) lowered the sensitivity of the hemolymph test. It is not known what component(s) of FBS was responsible for this effect, but it enabled the use of bovine serum albumin (BSA)

to increase enzyme activity and ultraviolet irradiation to protect against DNA contamination of the master mix. Finally, superior reliability was found in the Perkin Elmer-Cetus 9600 thermocycler, perhaps due to faster ramp times, increased setpoint accuracy and reduced inter-well variation. The best protocol for initiation of reaction profiles was an alteration of the so-called "hot start" method. However, reaction mixtures were not heated prior to addition of *Taq* DNA polymerase, deoxynucleotide triphosphates, or $MgCL_2$; the best results were obtained by completing the master mix on ice, aliquoting to denatured template (on ice) and placing the reactions in a preheated (94°C) thermocycler.

The best method of template preparation for PCR depended on the tick tissue tested. For salivary glands and bisected ticks, proteinase K digestion with nonionic detergents was the most efficient method. However, hemolymph samples were simply overlayed with mineral oil and heated to 105-110°C in a glycerol bath. The latter method was preferred for its simplicity, reduced time required for preparation and reduced opportunity for contamination from template carryover.

The PCR-based assay of tick hemolymph proved to be a sensitive, potentially nonlethal method for identification of infected ticks; it would be well suited for identification of laboratory- or field-infected ticks that could then be used for further research. *Anaplasma marginale* was detected in the hemolymph of experimentally-exposed ticks at all times tested,

including unfed adults that had been exposed to the parasite during the nymphal stage, and previously unexposed adults that had fed on an infected host for as little as one day. These results suggest that hemolymph is a superior tissue for the *A. marginale* PCR assay of ticks and that *A. marginale* is in the hemolymph before the organism develops in the salivary glands. Tick hemolymph may be an intermediate site of development for *A. marginale* prior to infection of the salivary glands. The location of *A. marginale* in tick hemolymph is not known, but possibilities include organisms developing free in the hemolymph, within hemocytes or from lysed muscle cells that line the visceral midgut. Since *A. marginale* is not observed in the salivary glands until after it is found in the hemolymph, which is considered a prerequisite to infection of the salivary glands, further studies are needed to investigate the role of hemolymph in the developmental cycle of *A. marginale* prior to its transmission by the tick host.

Detection of *A. marginale* in secretagogue-induced oral secretions of male and female *D. andersoni* had not been accomplished previously. Detection of the parasite was partially dependent on the sensitivity of the assay, and partially dependent on the secretagogues used, tick gender, method of infection, and feeding time of infected ticks. Male ticks secreted small volumes of saliva, but produced greater amounts of PCR products than female ticks exposed to *A. marginale* during the same developmental stage. This suggests that the

parasite is more concentrated in the saliva of male ticks. *Anaplasma* DNA was amplified in dopamine-stimulated oral secretions of male ticks exposed as either nymphs or adults and female ticks exposed during the adult stage. Conversely, *A. marginale* was detected in saliva from prefed female ticks exposed as nymphs only after stimulation with a combination of dopamine, γ-aminobutyric acid, pilocarpine and theophylline. Saliva from ticks exposed as nymphs and stimulated with ergot alkaloids, did not contain *A. marginale* DNA. Relatively weak bands were observed for PCR-positive saliva collected from male and female ticks exposed as nymphs compared to those exposed during the adult stage, indicating that higher infection rates were achieved with exposure to *A. marginale*. Detection of *A. marginale* in adult stage-exposed females only occurred just prior to detachment of the engorged ticks after transmission feeding for 5 days, suggesting that transmission of the parasite may be coordinated with the rapid feeding stage of female ticks. During rapid feeding, female tick weight increases 10-fold within 24 hr, and copious salivation occurs to as a result of boodmeal concentration. Rapid feeding of female ticks after mating may be an advantage to tick survival due to reduced exposure time of the vertebrate host immune response to tick-related antigens. Transmission of *A. marginale* during the rapid feeding stage may be an advantage for *A. marginale* for similar reasons. Rapid dissemination of large numbers of rickettsiae and immunosuppressive components of

tick saliva could ensure survival of some organisms in the face of a vertebrate host immune response. Male ticks do not undergo rapid feeding, but take in relatively small, intermittent bloodmeals; thus it would be advantageous for *A. marginale* to be secreted during all stages of male feeding. The secretion mechanism of *A. marginale* is probably similar for the salivary glands of both male and female ticks; determination of this mechanism may be facilitated by comparing the role of salivary gland functions during male and female tick feeding.

A prerequisite for application of the PCR assay to field-collected ticks was to determine the specificity of the oligonucleotides used as primers for the *A. marginale* msp1β gene, which was done in several phases. First, amplification of the 409 bp target fragment of the msp1β gene was confirmed by comparing restriction endonuclease-induced fragment lengths to those expected from the sequence of the msp1β gene for the *A. marginale* Florida isolate. Next, it was determined that the assay amplified the same size DNA fragments from *A. marginale* isolates from diverse geographical areas of the USA. Finally, the specificity of the assay for *A. marginale* was demonstrated with 19 different organisms that tested PCR-negative when 100 ng of genomic template was subjected to PCR. Organisms tested in the specificity trials included *Candida albicans, Escherichia coli, Haemophilus somnus, Klebsiella pneumoniae, Listeria monocytogenes, Pasteurella multocida, Proteus mirabilis,*

Serratia marcescens, *Yersinia enterocolitica*, *Salmonella typhimurium*, *Brucella abortus*, and 8 different organisms associated with ticks: *Babesia odocoiles*, *Coxiella burnetti*, *Ehrlichia canis*, *Francisella tularensis*, *Rickettsia rickettsii*, *Theileria cervi*, and *Anaplasma ovis*.

Application of the assay for testing field-collected ticks for natural exposure to *A. marginale* was demonstrated. Unattached *D. variabilis* and feeding *D. albipictus*, and blood from hosts of the latter species, were collected from analplasmosis-enzootic areas of Oklahoma. Initially, the hemolymph test for *A. marginale* was attempted on field-collected ticks, but the methods used to collect hemolymph from *D. andersoni* were not applicable to the smaller *D. albipictus*. Thus the bisected-tick assay was used for field-collected specimens. PCR-positive *D. albipictus* females were identified, from one cow with a history of treatment for clinical anaplasmosis. The blood from this cow was also PCR-positive, and observation of Wright's stained blood smears indicated that it was in a carrier state.

Two bands, one of which appeared to be the appropriate size for the amplified *A. marginale* fragment and the other slightly larger, were produced by PCR of blood from another bovine host from which *D. albipictus* was collected; the ticks collected from this cow were all PCR-negative. Wright's stained blood smears from this cow demonstrated what appeared to be *A. marginale* in the erythrocytes, suggesting that the cow may

have been a carrier of an *A. marginale* isolate that does not infect *D. albipictus*. The msp1β gene is believed to be repeated in the *A. marginale* genome: perhaps the second, larger band amplified in the bovine blood is indicative of an *A. marginale* isolate with an insert in one of the msp1βgene repeats.

This assay for *A. marginale* was successful for all applications tested in the absence of a DNA probe hybridization step. Visualization of the appropriately-sized amplification fragment in an agarose gel proved sensitive and specific enough for each application. However, the addition of a single-stranded oligonucleotide (SSO) hybridization step should be considered for the sake of convenience in field studies where agarose gel electrophoresis may not be practical. Progressive DNA technology has negated the necessity for radioisotope labelling of nucleic acid probes. The sensitivity of chemiluminescent nucleic acid probes approaches that of radioactive probes, and biotinylated nucleic acids allow for colorimetric detection that is no less sensitive than ethidium bromide staining. A "reverse slot blot" test, in which specific oligonucleotide probes are bound to a nylon membrane, has recently been developed commercially (Perkin Elmer-Cetus, Norwalk, CT) for detection of biotinylated multiplex PCR products of *Legionella* spp. A similar method would be useful for screening large numbers of cattle and ticks for infection with *A. marginale*, perhaps eventually being developed to determine if isolates detected in cattle are capable of infecting ticks.

CHAPTER VII

SUMMARY

The polymerase chain reaction (PCR), with primer sequences derived from the gene for the msp1β surface protein of the *A. marginale* Florida isolate, was used for detection of *Anaplasma marginale* in laboratory- and field-infected *Dermacentor* spp. ticks. The study was divided into four parts: (1) Preliminary development involved applying optimum PCR conditions to detection of *A. marginale* in individual tick halves and salivary gland pools. (2) Application of PCR to hemolymph of adult *D. andersoni* that were infected as nymphs or adults. (3) PCR of secretagogue-induced salivary secretions *D. andersoni* males and females that were exposed to the parasite during either the nymphal or adult stage. (4) PCR assay of *D. albipictus* and *D. variabilis* adults collected from anaplasmosis enzootic areas.

First, PCR was used to detect *Anaplasma marginale* in tissues of male *D. andersoni* that were infected as adults. Two 20mer oligonucleotide primers (50 % G+C) flanking opposite strands of a 406 bp fragment of the gene for the MSP1β surface protein of *A. marginale* (Florida isolate) were synthesized. Optimum PCR parameters were initially determined to be 3.5 mM $MgCl_2$,

0.18 µM total primer concentration and 0.04 U/µl Amplitaq with 1X PCR buffer (10 mM Tris·HCl, pH 8.3, 50 mM KCl) at 45 cycles of 94°C for 1.5 min, 60°C for 1.0 min, and 72°C for 1.0 min, followed by a final extension at 72°C for 5.0 min, in a Coy Model 60 Tempcycler. These parameters were used to detect *A. marginale* in individual bisected ticks and salivary glands; associated control tissues were negative.

Second, PCR was used to detect *A. marginale* in the hemolymph of infected *D. andersoni*. Hemolymph samples from feeding and held ticks were assayed with 24mer and 20mer oligonucleotide primers (50% G+C), flanking a 409 bp fragment of the *A. marginale* Florida isolate msp1β gene, optimum conditions for these primers were 0.8 mM dNTP mix, 3.5 mM $MgCl_2$, 1.8 µM of each primer, 4% (v/v) heat-denatured fetal bovine serum (Sigma), 0.32 µg/µl BSA and 0.12 U/µl *Taq* DNA polymerase in 1X PCR buffer. The reaction profile consisted of 32 cycles of 94°C for 1.0 min, 60°C for 1.0 min, and 72°C for 0.5 min followed by a final extension at 72°C for 3.0 min in a Perkin Elmer-Cetus 9600 Thermocycler.

Hemolymph was collected from severed legs of male and female ticks exposed to *A. marginale* as either nymphs or adults. The optimum method of preparation of hemolymph for PCR was found to be heat treatment. Hemolymph samples were collected and pooled from adult ticks exposed as nymphs prior to feeding and on each of 10 days they fed on a susceptible calf; for male and female ticks exposed as adults, samples were collected

on each day that ticks fed on infected calves, while being held between feedings, and during a second feeding when they transmitted the parasite. Hemolymph samples were collected from uninfected ticks at the same times to serve as controls. *Anaplasma* DNA was successfully amplified from hemolymph of infected adult ticks at all collection times, even from ticks that fed on infected calves for only one day. The PCR-based assay of tick hemolymph proved to be a sensitive method for identification of infected ticks, potentially without killing them; it would be well suited for identification of laboratory- or field-infected ticks that could then be used for further studies.

Third, PCR parameters determined for the hemolymph assay were used to detect *A. marginale* in secretagogue-induced oral secretions of male and female *D. andersoni* exposed as nymphs or adults by feeding on infected calves. *Anaplasma* DNA was amplified in oral secretions of female ticks exposed as adults and stimulated to secrete by injection of dopamine. Conversely, *A. marginale* was detected in saliva from prefed female ticks exposed as nymphs only after stimulation with a combination of dopamine, γ-aminobutyric acid, pilocarpine and theophylline. Saliva from ticks exposed as nymphs and stimulated with ergot alkaloids, did not contain *A. marginale* DNA. Saliva collected after 11 d of feeding from dopamine-stimulated male ticks contained *A. marginale* DNA. The results indicate that *A. marginale* is present in tick saliva and suggest that the

parasite can be transmitted to cattle via saliva of feeding ixodid ticks. The variable appearance of *A. marginale* in saliva, regardless of the method used to induce salivation, suggests that transmission of *A. marginale* may be affected by the physiological state of the tick.

Finally, the latter PCR parameters with a 62°C annealing step, was tested for specificity among 19 different genera, and determined to be suitable for seven *A. marginale* isolates found from diverse geographical areas of the USA. Ticks were then collected from cattle and the field in anaplasmosis-enzootic areas of Oklahoma and held in a humidity chamber for a minimum of 10 days prior to bisection, proteinase-digestion and screening with PCR for natural-infection with *A. marginale*. Prefed and unattached field-collected *D. variabilis* adults were PCR-negative for *A. marginale*. Prefed *D. albipictus* adults, removed from cattle on a ranch with a history of anaplasmosis, were PCR-positive for *A. marginale*. The posited involvement of overwintered tick vectors in the transmission of anaplasmosis remains unresolved, but this study suggests infection of *D. albipictus* with *A. marginale* may be involved in the epizoot-iology of anaplasmosis in Oklahoma.

VITA

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