DEVELOPMENT OF NONRADIOACTIVE DNA HYBRIDIZATION ASSAYS FOR DETECTION OF *Anaplasma marginale* IN CATTLE AND TICKS

By

NIE-LIN GE

Doctor of Medicine
Wannan Medical College
Wuhu, P. R. China
1984

Master of Science
Jinan University School of Medicine
Guangzhou, P. R. China
1987

Submitted to the Faculty of the
Graduate College of
Oklahoma State University
in partial fulfillment of
the requirements for
the Degree of
DOCTOR OF PHILOSOPHY
May, 1996

DEVELOPMENT OF NONRADIOACTIVE DNA

HYBRIDIZATION ASSAYS FOR DETECTION

OF *Anaplasma marginale* IN

CATTLE AND TICKS

Thesis Approved:

Katherine M. Kocan

Thesis Advisor

Dean of the Graduate College

PREFACE

This study was undertaken to develop a nonradioactive DNA probe for diagnosis of anaplasmosis in cattle and to use in developmental studies of *Anaplasma marginale* in a tick vector, *Dermacentor andersoni*. The PCR-mediated digoxigenin-labeled DNA probe developed in this study has been successfully used for detection of *A. marginale* in both experimentally- and naturally-infected carrier cattle using slot-blot hybridization assay. The probe proved to be useful in epidemiological investigations and in identification of convalescent carriers of *A. marginale*. This nonradioactive DNA probe has been utilized also for in situ hybridization studies of development of *A. marginale* in paraffin- and LR White-embedded half-tick sections of experimentally-infected male *Dermacentor andersoni*.

The dissertation is divided into six chapters. Chapter I is a literature review on *A. marginale*, the molecular biology of *A. marginale*, DNA probes and hybridization. The chapter ends with a description of the research problem. Chapter II, published in Journal of Veterinary Diagnostic Investigation (1995; 7: 465-472), describes development and utilization of a PCR-mediated digoxigenin-labeled DNA probe for detection of *A. marginale* in experimentally-infected cattle using slot-blot and in situ hybridization. Chapter III, submitted to Journal of Veterinary Diagnostic Investigation in January 1996, describes use of the nonradioactive DNA probe for detection of *A. marginale* infection in naturally-infected carrier cattle and comparison of the slot-blot hybridization assay with

the complement fixation serologic test and microscopic examination. Chapter IV, submitted to The Journal of Histotechnology in February 1996, describes development of a nonradioactive in situ hybridization for detection of *A. marginale* in ticks. Chapter V, submitted to Journal of Medical Entomology in March 1996, describes developmental studies of *A. marginale* in male *D. andersoni* ticks infected as adults using nonradioactive in situ hybridization as compared with microscopy. Chapter VI provides a general summary of the research described herein.

Anaplasmosis has long been a focus of research supported by the College of Veterinary Medicine and the Oklahoma Agricultural Experiment Station. I appreciate the continued support of the Oklahoma Agricultural Experiment Station which provided funds for this research through Projects 1433 & 1669.

I wish to express my great appreciation to all who have contributed to this research in many different ways. First of all, I would like to express my deepest gratitude to my major advisor, Dr. Katherine M. Kocan, for her intelligent advice, constant support and warm-hearted encouragement during the entire period of this research. Her invaluable constructive guidance in experimental design and scientific written skills will never be forgotten. She also provided me with training in electron microscopy techniques. I am so fortunate to be her graduate student. She has cared greatly about my personal and professional development. It would have not been possible for this research to come to fruition without her tireless effort and continued support. Her inspiration and friendship will be missed in the rest of my life.

I would also like to extend my sincerest gratitude to the remaining members of my graduate committee. I am very fortunate to have interacted with this group of wonderful

and nice people. I am indebted to Dr. Edmour Blouin for his creative thought, advice and friendship during my working on this project. His instructive hints to help me understand "Far Side" cartoons are also greatly appreciated. I am indebted to Dr. Sidney Ewing for his excellent editing skills and patience for editing every manuscript. His constructive suggestions and scientific mode of thinking as my scientific editor have been invaluable during this research. I am further indebted to Dr. George Murphy for his intellectual advice regarding the molecular aspects of this project. I have learned a great deal about molecular techniques from Dr. Murphy since I first came to Oklahoma State University in 1991 as a Visiting Scientist. Actually, he is the person who introduced me into the field of molecular biology. I am indebted to Dr. John Sauer for his knowledgeable instruction about insect and tick physiology, which helped me understand host-vector-pathogen interactions. My research project which he directed for the insect physiology class was very productive and provided me with a very positive learning experience.

Special thanks go to Wanda Edwards and Peichun Hu in our laboratory for their excellent technical assistance. I would also like to thank Ms. Jeanenne Duffy at Oklahoma Animal Disease Diagnostic Laboratory for processing many tick samples for paraffin embedding and for teaching me how to cut paraffin-embedded sections.

I appreciate Electron Microscopy Laboratory, Oklahoma State University for allocating me 30 hours of EM laboratory use. I also thank Ms. Ginger Baker, Lab Manager of EM Laboratory, for her instruction of effective use of the STEM.

I am eternally grateful to my mother for the philosophies she instilled in my mind and the love she instilled in my heart. Due to her constant spiritual, emotional support and inspiration, I was able to complete my pre-college education during "Culture Revolution"

in China so that I have had the opportunity to receive higher education after China restored its formal higher education system in 1977 and practiced its open policy in 1978. It was almost impossible for a widow to support her children for education during that period of time in China. Her perseverance, love and dedication have been inspirational throughout my life. I also thank my sisters, Jiuxiang and Meixiang, for relieving me of responsibilities for taking care of my mother, that made possible the continuation of my Ph.D. in the United States. As youngest of all I received constant support and love from them, too.

Finally, with deepest love, I dedicated this dissertation to my mother and my daughter, Zhuanyong Wang and Lu Ge. With this endeavor which involved a large investment of my time and energy, I was unable to take care of them during this time. I am very indebted for their understanding and love.

TABLE OF CONTENTS

Chapter Page

LIST OF TABLES

LIST OF FIGURES

Figure Page

Figure Page

CHAPTER I

INTRODUCTION

Literature Review

Bovine Anaplasmosis

Anaplasmosis is a tick-borne disease of cattle and other ruminants caused by the rickettsia, *Anaplasma marginale* (Ristic 1977). The disease is the most prevalent bovine hemoparasite infection and is enzootic to nearly half the world's livestock production regions (National Research Council 1982). Along with babesiosis, cowdriosis, theileriosis, and trypanosomiasis, anaplasmosis remains a great obstacle to meat, milk and fiber production in tropical, subtropical, and less developed nations (Lawrence et al. 1980; Norval 1983). The annual losses due to anaplasmosis within the United States alone have been estimated at 50,000 to 100,000 head of cattle and $300 million (National Research Council 1982).

Historical Background and Geographical Distribution

Bovine anaplasmosis is endemic in many parts of the world, predominantly in the tropics and subtropics. The disease is common in Africa, the Middle East, Southern Europe, the Far East, Central and South America, and the United States (Soulsby 1982). The disease has been reported from most states in the U. S. A., but is more prevalent in the Gulf states, Lower Plains states, the intermountain Western states and California (Siegmund 1979).

Anaplasma is presently assigned to the Order Rickettsiales, Family Anaplasmataceae, (Weiss & Moulder 1984). *Anaplasma* inclusion bodies can be observed in blood smears stained with Wright's or Giemsa stain as small, round basophilic bodies located near the margins of erythrocytes and range in size from 0.3 to 1.0 μm in diameter. Theiler (1910) first described this organism in erythrocytes of African cattle suffering from acute anemia and named this small punctiform organism on the basis of staining characteristics. The term "anaplasma" indicates an apparent lack of cytoplasm in what was thought to be a protozoan and the term "marginale" represents the peripheral location of the marginal body within erythrocytes. Ultrastructural studies (Ristic 1977) described *Anaplasma* as an initial body that enters the erythrocyte by invagination of the cell membrane, forming an inclusion vacuole. Within this vacuole, the initial body multiplies by binary fission, forming an inclusion body containing 4-8 rickettsiae.

Clinical Manifestations and Diagnosis

The clinical signs of anaplasmosis are related to development of anemia and include depression, inappetence, indolence, and fever (40-41 ^{0}C). The infection may be fatal owing to severe anemia. Organisms appear in erythrocytes several days before the febrile period; the number of parasitized erythrocytes increases as the fever progresses. Lactating dairy cows usually experience rapid fall in milk production, but, in beef cattle, clinical disease is usually not recognized until the affected animal is extremely anemic and weak. Calves undergo mild infections. Increasing severity of disease occurs in adult cattle with development of marked anemia, and mortality may occur in 20-50% of older animals.

Cattle that recover from acute disease remain persistent carriers with low level, often undetectable, parasitemias and serve as reservoirs for mechanical or biological transmission (Swift & Thomas 1983). Convalescent cattle are resistant to clinical disease if they are challenged by the homologous isolate of *A. marginale.*

Diagnosis of acute anaplasmosis is usually based on direct microscopic detection of organisms in Giemsa-stained blood smears (Shkap et al. 1990). Rickettsemias in carriers are often below the limit reliably detectable by examination of stained blood smears (less than 0.1% infected erythrocytes) (Kieser et al. 1990; Eriks et al. 1993) making serologic diagnosis a common alternative method of diagnosis. Serologic tests commonly used include complement fixation (Goff et al. 1990), capillary-tube agglutination (Ristic 1962), card test (Amerault & Roby 1968), latex agglutination (Montenegro-James et al. 1981), indirect fluorescent antibody test (IFAT) (Goff et al. 1990), conventional ELISA (Shkap et al. 1990) and radioimmunoassay (Schunter & Leatch 1988). Lack of sensitivity and specificity have rendered some of these tests impractical for routine use. Montenegro-James et al. (1990) used a dot ELISA with isolated *A. marginale* initial bodies as antigen for rapid detection of antibodies to *Anaplasma* organisms. The sensitivity, specificity and predictive values of the dot ELISA were 93%, 96% and 95%, respectively. Trueblood et al. (1991) developed an antigen capture ELISA with monoclonal antibodies to conserved epitopes on the *A. marginale* MSP-1a surface protein. This assay detected *A. marginale* antigens prior to onset of clinical signs.

A method increasingly used as an alternative to serologic tests, to detect the presence of infectious agents, is nucleic acid hybridization (Goff et al. 1988; 1990; Eriks et al. 1989; 1993; Blouin et al. 1993). A single infected tick salivary gland or 100-1000 infected erythrocytes, which is equivalent to a parasitemia level of 0.00025%, can be detected with a

DNA probe derived from the *msp-1β* gene encoding the MSP-1b subunit of *A. marginale*. Even this method is inadequate to detect infection in all carrier animals (Eriks et al. 1989). A recent application of polymerase chain reaction (PCR), using a thermostable DNA polymerase which can theoretically amplify a single copy of target gene sequence up to 1 X 10^6 fold, may solve this problem. PCR has been developed to detect *A. marginale* from experimentally infected and field-collected ticks and carrier cattle (Stich et al. 1991; 1993 a & b; Figueroa et al. 1993). The technique can detect *A. marginale* from hemolymph, oral secretions and midguts and is especially useful for screening live ticks that can subsequently be used for further study (Stich et al. 1991; 1993 a & b).

Vectors, Reservoirs and Transmission

Approximately 29 species of ticks have been incriminated as vectors of *Anaplasma* (Ewing 1981). Although *A. marginale* can be transmitted mechanically by biting flies and blood-contaminated fomites (Ristic 1977), ticks are the only known biological vectors (Kocan 1986). Biological transmission occurs by transfer of adult ticks from infected to susceptible hosts (intrastadial transmission) or by nymphs or adults infected in a previous stage (interstadial or transstadial transmission) (Kocan et al. 1983; 1992). Transovarial transmission was reported in one of several studies, but has not been confirmed consistently for any tick species (Anthony & Roby 1962; Ewing 1981; Potgieter 1981; Stich et al. 1989). The transmission of *A. marginale* by ticks from persistently-infected carrier cattle may be important in maintaining the organism in enzootic areas (Kocan et al. 1992; Eriks et al. 1993). Although both male and female ticks can transmit *A. marginale* intrastadially, adult males are believed to be of primary importance in this route of biological transmission of *A. marginale* because they are

persistently infected, transfer readily among cattle and maintain high infection rates while off the hosts (Stiller et al. 1989; Kocan et al. 1992).

Mechanical transmission of anaplasmosis may occur during operations of cattle husbandry such as dehorning, castration, vaccination, and blood sampling or by transfer of blood via contaminated mouthparts of biting flies including mosquitoes (Soulsby 1982). Most recently, Baumgartner et al. (1993) reported experimental infection of four calves via ingestion of infected blood.

Cattle are the major domestic ruminant host for *A. marginale* but infections also occur in zebra, water buffalo, bison, various African antelopes, American deer (Southern black-tailed, mule deer), elk and camel (Soulsby 1982). Mule deer have been shown to play an important role in the epizootiology of anaplasmosis in Western U. S. (Ristic 1977). *Anaplasma* may survive in nature in the absence of cattle by transfering among deer. Transmission of *A. marginale* from deer to cattle in California has been demonstrated by several investigators (Ristic 1968).

Development in Tick Vectors

Early studies reported that *A. marginale* was found in the gut contents, excreta, and Malpighian tubules of nymphal and adult ticks by microscopy and by fluorescent antibody studies (Anthony et al. 1964; Friedhoff & Ristic 1966). However, the developmental cycle of *A. marginale* in tick vectors was not described until the early 1980s. Kocan et al. (1980 a & b; 1983; 1984; 1988; 1990) studied development of *A. marginale* in *Dermacentor* ticks from infection of larval or nymphal ticks through transmission to cattle by subsequently-molted adults. Infection of susceptible cattle occurred by inoculation with gut or salivary gland

homogenates and by inoculation of hemolymph from infected adult ticks. Colonies of *A. marginale* were observed by light and electron microscopy (LM and EM) within midgut epithelial cells of adult ticks that were infected as either larvae or nymphs. Initial development occurred in tick midgut epithelial cells, forming three types of colonies, Nymphal type 1 (Ny 1), Nymphal type 2 (Ny2) and transitional nymphal colonies (TsN). Only Ny2 contained organisms infective for cattle. In adult ticks infected as nymphs, an intermediate site of development occurred in gut muscle cells. Five types of colonies contained various forms of *A. marginale* (small electron-dense forms, larger reticulated forms, pleomorphic reticulated forms and small particles). These colony types were present concurrently in gut cells of ticks that were infective for cattle. Colonies in adults exposed to *A. marginale* as nymphs were also confirmed by ferritin- and fluorescein-labeled antibodies and immunoperoxidase techniques (Kocan et al. 1980 a & b; Staats et al. 1982). Furthermore, development of *A. marginale* in salivary glands was confirmed by microscopy and/or DNA hybridization studies of male *D. andersoni* exposed as nymphs or adults (Goff et al. 1988; Kocan et al. 1988; 1989; 1992; 1993). It is presumed that *A. marginale* is transmitted from ticks to cattle via salivary glands where the organism undergoes final development (Kocan et al. 1989).

Immunoprophylaxis

The methods of immunoprophylaxis for anaplasmosis currently used include premunization of cattle with less virulent *A. centrale* or an attenuated *A. marginale* isolate, followed in some cases by tetracycline treatment for control of the initial infection, and vaccination with a killed *A. marginale* vaccine (Palmer 1989). These methods may reduce severe clinical disease. However, cross-protective immunity varies with the *A. marginale*

isolates used in challenge. Also, it has been necessary to collect large quantities of blood from infected animals for preparation of killed vaccines. Neonatal isoerythrolysis may also occur in calves nursing vaccinated dams because some killed vaccines are contaminated with erythrocyte stroma.

Current vaccination strategies are directed toward development of a subunit vaccine using surface-exposed epitopes, that can induce protective immunity. Protein analysis revealed several polypeptides on the erythrocyte-stage of *A. marginale*, with apparent molecular sizes of 105 (MSP-1), 86 (MSP-3), 61, 36 (MSP-2), 31 (MSP-4) and 19 kDa (MSP-5), that have surface-exposed epitopes recognized by neutralizing antibody. These polypeptides were highly conserved among *A. marginale* isolates from Israel, Kenya and the U. S. A. (McGuire et al. 1984; 1991; Palmer et al. 1984; 1986 b; 1987; 1988 a & b; Visser et al. 1992; Oberle et al. 1993). Some of them also share cross-reactive epitopes with tick gut and salivary gland stages recognized by high-titer antibodies from effectively premunized cattle (Palmer et al. 1985; Ge et al. 1993). Immunization of cattle with these surface proteins induced protective immunity against homologous and heterologous *A. marginale* challenge (Palmer et al. 1986 a; 1988 b; 1989). Significant protection against homologous challenge was also reported after vaccination of cattle with outer membranes of the Norton (Zimbabwe) strain of *A. marginale* (Tebele et al. 1991).

Molecular Biology of *A. marginale*

Research on the molecular biology of *A. marginale* has progressed markedly over the past decade. In earlier studies, the reported mol% G+C (guanine and cytosine) content of the *A. marginale* genome varied from approximately 33% to 50% (Ellender & Dimopoullos 1967; Senitzer et al. 1972; Ambrosio & Potgeiter 1987; Bear & Philpott 1987). The 340 kbp of

genome size, as determined by renaturation kinetics (Bear & Philpott 1987) was smaller than the values of 1500-2100 kbp reported for other rickettisae (Frutos et al. 1989). Contamination of anaplasmal DNA by bovine DNA and use of different experimental methods may have caused these inconsistencies. Recently, Alleman et al. (1993) determined that *A. marginale* has a circular 1200-1260 kb genome with a G+C content of 56%, as analyzed by restriction endonuclease cleavage and pulsed-field gel electrophoresis (PFGE).

To date, several genes coding for major surface proteins have been cloned, sequenced and expressed. Barbet et al. (1987) first cloned a gene, from the *A. marginale* Florida isolate encoding MSP-1b, a subunit of a major surface protein complex (MSP-1) that consists of two polypeptides, MSP-1a and MSP-1b with molecular weights of 100 and 105 kDa (formerly called Am 105U and Am 105L). DNA sequence analysis of the *msp-1β* gene revealed that this gene is a member of a polymorphic multigene family (Barbet & Allred 1991). The gene *msp-1α* that codes for the subunit of MSP-1a was shown to be present as a single copy (Allred et al. 1990). Sequence analysis showed that both *msp-1α* and *msp-1β* genes contain domains of tandemly repeated sequences that may be responsible for size variations of the *msp-1α*- and *msp-1β*-encoded polypeptides among *A. marginale* isolates (Barbet et al. 1983; Oberle et al. 1988). Like the *msp-1α* gene, the *msp-5* gene also appears to occur as a single copy in the genome (Visser et al. 1992). The promoters for control of expression of these three genes resemble the *Escherichia coli* consensus sequence (Allred et al. 1990; Visser et al. 1992). Most recently, the gene *msp-4* encoding MSP-4 has been cloned and sequenced (Oberle et al. 1993). When these cloned genes were expressed in *E. coli*, all recombinant proteins were structurally and antigenically homologous to their native major surface proteins (Barbet et al. 1987; Allred et al. 1990; Visser et al. 1992; Oberle et al. 1993). These findings suggest that approaches to

control of anaplasmosis may include production of subunit vaccines through development and application of recombinant DNA techniques.

Nucleic acid probes have been developed for detection of *A. marginale* in bovine erythrocytes and tick tissues. A number of *Anaplasma*-DNA probes have been constructed from *Anaplasma* DNA genomes, as well as by recombinant techniques (Ambrosio et al. 1988; Visser & Ambrosio 1987; Goff et al. 1988). Visser & Ambrosio (1987) isolated four DNA probes by screening an *A. centrale* lambda gt11 DNA library by immunoblotting. 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. Probe AC-2 was further used to detect DNA by Southern blotting from Washington, South-Idaho, Virginia and Florida isolates of *A. marginale*. The hybridization pattern in 3 isolates was identical, the exception being the Washington isolate (Ambrosio et al. 1988). Goff et al. (1988) detected *A. marginale* DNA derived from bovine erythrocytes, midgut and salivary glands of three species of male ticks (*D. andersoni, D. variabilis* and *D. occidentalis*) with a 2 kb DNA fragment isolated from the *msp-1β* gene of the Florida isolate and labeled with ^{32}P. Individual infected salivary glands and 100-1000 infected bovine erythrocytes could be detected with this *A. marginale*-specific DNA probe. Another recombinant DNA probe derived from the *A. marginale* genome of the St. Croix isolate was reported to have similar sensitivity and specificity to the 2 kb DNA fragment (Aboytes-Torres et al. 1989). A 965-bp ^{32}P-labeled DNA probe from within the 2 kb fragment carrying the *msp-1β* gene has been used for monitoring *A. marginale* infection in experimentally-infected ticks and in cell culture (Blouin et al. 1993). It is likely that DNA probes may provide not only an effective diagnostic method but will enhance the

understanding of the genetic relationship among different *A. marginale* isolates and *Anaplasma* species.

The polymerase chain reaction assay has also been used in studies of *A. marginale.* Stich et al. (1991) first used a pair of oligonucleotide primers BAP-2 and AL34S to amplify a 409-bp DNA fragment derived from the *msp-1β* gene of *A. marginale*. The assay was found to be *A. marginale*-specific when tested with 18 species of bacteria and protozoa, and 7 isolates of *A. marginale* from diverse geographic areas of the U. S. A. (Stich et al. 1993 a). This PCR assay has detected *A. marginale* in hemolymph, oral secretions, midguts and salivary glands of infected ticks (Stich et al. 1993 a & b). Most recently, Figueroa et al. (1993) developed a multiplex PCR assay for detection of the hemoparasites *Babesia bigemina, B. bovis* and *A. marginale*, frequently found concurrently in cattle in tropical and subtropical areas. The sensitivity of this multiplex PCR assay was 0.00001%, 0.00001% and 0.0001% parasitemias for *B. bigemina, B. bovis* and *A. marginale*, respectively.

DNA Probe and Hybridization

Hybridization is a reaction whereby two single-stranded nucleic acid molecules recognize one another and bind by means of hydrogen bonding of complementary base pairs (Tecott et al. 1987). DNA/DNA, DNA/RNA and RNA/RNA hybrids all may be formed under appropriate conditions. In situ hybridization (ISH) is a technique that detects DNA or RNA sequences in cytological preparations or sections of tissue (Coghlan et al. 1985). It is based on the method used for studying *in vitro* pairing reactions between labeled RNA or DNA molecules (probe) in solution and complementary nucleic acid (target nucleic acid) either in solution (Marmur & Doty 1961) or bound to membrane (Gillespie & Spiegelman 1965). In the

latter, RNA or purified denatured DNA is bound to nitrocellulose or nylon membranes which are then incubated in appropriate hybridization conditions together with labeled RNA (Gillespie & Spiegeman 1965) or sheared, denatured DNA (Denhardt 1966). Hybrid formation can be detected using autoradiography (for radioactive labels) or an immunologically stained reaction (for nonradioactive labels) after incubation and subsequent washing in buffer (for DNA-DNA hybridization) and an additional RNase treatment (for DNA-RNA hybridization). In ISH, target nucleic acid is maintained in its original location. Therefore, this technique enables precise localization within individual cells containing the specific nucleic acid sequence. The immobility of the target DNA and limitation of probe penetration in tissue sections may influence the properties of the reaction. However, application of molecular hybridization to cytological investigation involves the same principles as aqueous hybridization.

ISH studies were first used to detect amplified DNA targets in cell nuclei (Gall & Pardue 1969; John et al. 1969). Thereafter, the technique rapidly evolved as a suitable method for detecting individual genes on chromosome preparations (Pardue & Dawid 1981), as well as for localizing infectious agents in individual cells (Brahic & Hasse 1978; Brahic et al. 1984). The precise histological localization and great sensitivity of ISH make it possible to detect low copy number mRNA molecules in individual cells (Harper et al. 1986; Shivers et al. 1986) which could not be achieved by a commonly-used method for RNA detection, Northern blotting.

Despite its sensitivity and wide applicability, in situ hybridization has been limited to use in research laboratories due to a variety of problems associated with radioactive probes, including the biohazard of radioactive compounds, limited shelf life, and extensive time required for autoradiography. The commercial availability of non-radioactively labeled

nucleotides (biotin- or digoxigenin (DIG)-nucleotides) has removed the major obstacles of radioactive probes that have hindered general application of ISH. Also, recent developments in molecular biology (genetic recombination, gene amplification or PCR and oligonucleotide synthesis) have resulted in the availability of probes for a rapidly increasing number of target nucleic acids (Morel 1993). PCR has been used to produce a DNA probe by amplifying a specific DNA fragment from a known gene sequence without molecular cloning or by directly labeling a DNA probe by simultaneous incorporation of radioactively or non-radioactively labeled dNTP(s) during DNA amplification (An et al. 1992; Ibrahim et al. 1992; Taveira et al. 1992). This development has greatly simplified procedures for the preparation of probes. More recently, use of PCR to amplify target nucleic acid in tissue sections, followed by ISH (in situ PCR), has made it possible to locate a single-copy gene or single microorganism in tissue sections (Murray 1993). Nuoro et al. (1991) has reported that single-copy HPV type 16 virus could be detected by in situ PCR using biotin or digoxigenin-labeled probes, demonstrating the highest sensitivity of in situ PCR. Instruments for automated colorimetric ISH have been available for clinical virology (Unger & Brigati 1989). Detection of viral messenger nucleic acid, mRNA by ISH has been done at the ultrastructural level with EM (Morel 1993).

Nonradioactive in situ hybridization has been used widely for detection of pathogens from infected tissue sections. Brown et al. (1992; 1993) developed a biotinylated RNA probe to study pathogenesis of foot-and-mouth disease (FMD) in cattle and detected large amounts of viral nucleic acid in multiple epidermal sites only 6 hours after aerosol exposure to FMD virus. Taveira et al. (1992) applied a digoxigenin-labeled probe to detect HIV proviral sequences from peripheral blood mononuclear cell DNA of infected subjects. Nuovo et al. (1991) identified different types of human papillomavirus in formalin-fixed, paraffin-embedded

tissue samples with a biotin-labeled probe. McLaughlin et al. (1992) developed in situ detection of *Plasmodium falciparum* DNA in blood smears. Development of in situ hybridization for detection of microbial genomes within tissues has made it possible to trace the path of organisms through host tissues with greater sensitivity.

ISH has been used to study *A. marginale* in cattle. Krueger et al. (1989) used ISH to determine the presence or absence of *A. marginale* in non-erythrocyte cells from experimentally-infected cattle using a ^{35}S-labeled recombinant DNA probe. Specific radiolabeled signal was found only in association with erythrocytes in tissues of liver and lymph nodes. The lack of resolving power of this radiolabeled probe and abundant non-specific probe binding limited its usefulness in this study (Krueger et al. 1989).

Research Problem

One of the major constraints for effective control of anaplasmosis has been the lack of a reliable diagnostic test. After cattle recover from acute anaplasmosis, convalescent carriers in which *A. marginale* infection is often not detectable by microscopic examination or serologic tests. In addition, the serodiagnostic test currently used most commonly for anaplasmosis, the complement fixation test, lacks sufficient sensitivity to distinguish chronically-infected cattle from uninfected ones. Also, immunized cattle often test serologically positive. Although some sensitive and specific radioactively-labeled nucleic acid probes have proved effective for detection of *A. marginale* in carrier cattle, the biohazard of radioactive compounds, short term of storage and extensive detection time of these probes have limited their use for routine diagnosis. A sensitive nonradioactive nucleic acid probe would provide a more useful diagnostic tool for epidemiological investigations and for identification of carrier cattle.

A nonradioactive nucleic acid probe would also be useful for development of in situ hybridization that allows for the exact localization of the *A. marginale* genome within cells. Use of ISH for identification of *A. marginale* infection in erythrocytes on methanol-fixed blood smears may make it possible to identify infected cattle before onset of clinical disease. Use of the nonradioactive nucleic acid probe for ISH study of development of *A. marginale* in ticks would also enhance our understanding of the complex developmental cycle of *A. marginale* in its tick vectors. In some tick tissues, such as hemolymph and salivary glands of adults infected as nymphs, infection with *A. marginale* was detected by slot-blot hybridization or PCR, but recognizable forms of *A. marginale* were not seen with LM and EM in these tissues. ISH combines sensitivity and specificity with precise histological localization, without disrupting tissue morphology, and can be done on paraffin- and/or LR White-embedded sections of half-ticks. Therefore, many tick tissues could be examined with ISH simultaneously. Tick tissues other than salivary glands and midguts may also be found to be infected with *A. marginale* and contribute to understanding the developmental cycle. In addition, the ISH technique used on half-tick sections may be useful for detection of other tick-transmitted rickettsiae, such as *Ehrlichia* spp. and *Cowdria ruminantium*, as well as tick-transmitted viral pathogens whose life cycles have not been well defined.

The research described herein was focused on development of nonradioactive DNA hybridization for detection of *A. marginale* in cattle and ticks. The specific objectives for this study were: (1) to develop a sensitive nonradioactive DNA probe specific for detection of *A. marginale* in erythrocytes of experimentally-infected cattle using slot-blot and in situ hybridization methods; (2) to test the suitability of the probe for detection of *A. marginale* in the blood of naturally-infected carrier cattle from selected geographic areas in Oklahoma; (3)

to use the nonradioactive DNA probe for ISH detection of *A. marginale* in paraffin- and LR White-embedded half tick sections of experimentally-infected *D. andersoni*; and (4) to use the nonradioactive ISH to study the developmental cycle of *A. marginale* in male *D. andersoni* ticks infected as adults.

References

Aboytes-Torres R, Buening GM, Alvarez A et al.: Recombinant probe construction and application in anaplasmosis research. In: Proc 8th Natl Anaplasmosis Conf, St. Louis, 1989; pp. 305-316.

Alleman AR, Kamper SM, Viseshakul N, Barbet AF: Analysis of the *Anaplasma marginale* genome by pulsed-field electrophoresis. J Gen Microbiol 1993; 139: 2439-2444.

Allred, DR, McGuire TC, Palmer GH et al.: Molecular basis for surface antigen size polymorphisms and conservation of neutralization-sensitive epitope in *Anaplasma marginale*. Proc Natl Acad Sci USA 1990; 87: 3220-3224.

Ambrosio RE, Potgieter FT: The genome of *Anaplasma*: DNA base composition and DNA/DNA hybridization. Onderstepoort J Vet Res 1987; 54: 63-65.

Ambrosio RE, Visser ES, Koekhoven Y et al.: Hybridization of DNA probes to *Anaplasma marginale* isolates from different sources and detection in *Dermacentor andersoni* ticks. Onderstepoort J Vet Res 1988; 55: 227-229.

Amerault TE, Roby TO: A rapid card agglutination test for bovine anaplasmosis. J Am Vet Med Assoc 1968; 153: 1828-1834.

An SF, Franklin D, Fleming KA: Generation of digoxigenin-labeled double-stranded and single-stranded probes using the polymerase chain reaction. Mol Cell Probes 1992; 6: 193-200.

Anthony DW, Roby TO: Anaplasmosis studies with *Dermacentor variabilis* Say and *Dermacentor andersoni* Stiles as experimental vectors. In: Proc 4th Natl Anaplasmosis Res Conf, Reno, Nevada, 1962; pp. 78-81.

Anthony DW, Madden PA, Gates DW: *Anaplasma marginale* Theiler observed in gut and excreta of *Dermacentor andersoni* Stiles (*Dermacentor venustus* Marx). Am J Vet Res 1964; 25: 1464-1471.

Barbet AF, Anderson LW, Palmer GH et al.: Comparison of proteins synthesized by two different isolates of *Anaplasma marginale*. Infect Immun 1983; 40: 1068-1074.

Barbet AF, Palmer GH, Myler PJ et al.: Characterization of an immunoprotective protein complex of *Anaplasma marginale* by cloning and expression of the gene coding for polypeptide Am105L. Infect Immun 1987; 55:2428- 2435.

Barbet AF, Allred D: The *msp-1β* multigene family of *Anaplasma marginale*: Nucleotide sequence analysis of an expressed copy. Infect Immun 1991; 59: 971-976.

Baumgartner W, Stoger J, Marktl W: Demonstration of the oral path of infection with *Anaplasma marginale* in calves. Vet Record 1993; 133: 64-66.

Bear PD, Philpott MS: Isolation and characterization of *Anaplasma marginale* DNA. Current Microbiol 1987; 15: 171-175.

Blouin EF, Kocan KM, Murphy GL, Ge NL: Persistence of tick-derived *Anaplasma marginale* in cultured bovine turbinate and endothelial cells. Rev d'elevage et de Med Vet des Pays Trop 1993: 46: 64-66.

Boehringer Mannheim: Nonradioactive in situ hybridization application manual. Boehringer Mannheim GmbH Miochemica, Germany, 1992a; pp. 1-75.

Boehringer Mannheim: The Genius™ system user's guide for filter hybridization application manual. Boehringer Mannheim GmbH Miochemica, Germany, 1992b; pp. 1-84.

Brahic M, Haase AT: Detection of viral sequences of low reiteration frequency by in situ hybridization. Proc Natl Acad Sci USA 1978; 75: 6125-6129.

Brahic M, Haase At, Cash E: Simultaneous in situ detection of viral RNA and antigens. Proc Natl Acad Sci USA 1984; 81: 5445-5448.

Brown CC, Meyer KF, Olander HJ, House C, Mebus CA: A pathogenesis study of foot and mouth disease in cattle using in situ hybridization. Can J Vet Res 1992; 56: 189-193.

Brown CC, Meyer KF, Grubman MJ: Use of digoxigenin-labeled RNA probe to detect all 24 serotypes of bluetonge virus in cell culture. J Vet Diagn Invest 1993; 5: 159-162.

Coghlan JP, Aldred P, Haralambidis J et al.: Hybridization histochemistry. Anal Biochem 1985; 149: 1-28.

Denhardt RA: A membrane-filter technique for the detection of complementary DNA. Biochem Biophys Res Commun 1966; 23: 641-646.

Ellender RD, Dimopoullos GT: Base composition and thermal denaturation of DNA isolated from *Anaplasma marginale*. Proc Soc Exp Biol Med 1967; 125: 82-85.

Eriks IS, Palmer GH, Goff WL et al.: Review of the development of a DNA probe to detect *Anaplasma marginale* in infected ticks and carrier cattle: a new epidemiological tool. In: Proc 8th Natl Anaplasmosis Conf, St. Louis, 1989; pp. 297-304.

Eriks IS, Stiller D, Palmer GH: Impact of persistent *Anaplasma marginale* rickettsemia on tick infection and transmission. J Clin Microbiol 1993; 31: 2091-2096.

Ewing SA: Transmission of *Anaplasma marginale* by arthropods. In: Proc 7th Natl Anaplasmosis Conf, Mississippi, 1981; pp. 395-493.

Figueroa JV, Chieves LP, Johnson GS, Buening GM: Multiplex polymerase chain reaction based assay for the detection of *Babesia bigemina*, *Babesia bovis* and *Anaplasma marginale* DNA in bovine blood. Vet Parasitol 1993; 50: 69-81.

Friedhoff KT, Ristic M: Anaplasmosis XIX. A preliminary study of *Anaplasma marginale* in *Dermacentor andersoni* Stiles by fluorescent antibody technique. Am J Vet Res 1966; 27: 643-644.

Frutos R, Pages M, Bellis M, Roizes G, Bergoin M: Pulsed-field electrophoresis determination of the genome size of obligate intracellular bacteria belonging to the genera Chlamydia, Rickettsiella and Porochlamydia. J Bacteriolol 1989; 171: 4511-4513.

Gall JG, Pardue M: Formation and detection of RNA-DNA hybrid molecules in cytological preparations. Proc Natl Acad Sci USA 1969; 63: 378-373.

Ge NL, Kocan KM, Blouin EF et al.: Identification of common antigens among erythrocyte stage, tick gut and salivary gland stages of *Anaplasma marginale* using Western immunoblotting methods. The 9th International Veterinary Hemoparasite Disease Conference, Octobr 6-9, 1993; in Merida, Mexico.

Gillespie D, Spiegelman S: A quantitative assay for DNA-RNA hybrids with DNA immobilized on a membrane. J Mol Biol 1965; 12:829-842.

Goff WL, Barbet AF, Stiller D et al.: Detection of *Anaplasma marginale* in tick vectors using a cloned DNA probe. Proc Natl Acad Sci 1988; 88: 919-923.

Goff WL, Stiller D, Roeder RA et al.: Comparison of a DNA, complement-fixation and indirect immunofluorescence tests for diagnosing *Anaplasma marginale* in suspected carrier cattle. Vet Microbiol 1990; 24: 381-390.

Harper ME, Marselle LM, Gallo RC, Wong-Stahl F: Detection of lymphocytes expressing human T-lymphotropic virus type III in lymph nodes and peripheral blood from infected individuals by in situ hybridization. Proc Nalt Acad Sci USA 1986; 83: 772-776.

Ibrahim A, Liesack W, Stackebrandt E: Differentiation between pathogenic *Yersinia enterocolitica* strains by colony hybridization with a PCR-mediated digoxigenin-dUTP-labeled probe. Mol Cell Probes 1992; 6: 163-171.

John HA, Birnstiel ML, Jones KW: RNA-DNA hybrids at the cytological level. Nature 1969; 223: 582-587.

Kieser ST, Eriks IS, Palmer GH: Cyclic rickettsemia during persistent *Anaplasma marginale* infection of cattle. Infect Immun 1990; 58: 1117-1119.

Kocan KM: Development of *Anaplasma marginale* in ixodid ticks: coordinated development of a rickettsial organism and its tick host. In: Sauer JR, Hair JA, eds. Morphology, Physiology and Behavioral Biology of Ticks. Ellis Horwood Ltd, England, 1986; pp. 472-505.

Kocan KM, Hsu KC, Hair JA, Ewing SA et al.: Demonstration of *Anaplasma marginale* Theiler in *Dermacentor variabilis* by ferritin-conjugated antibody technique. Am J Vet Res 1980a; 41: 1977-1981.

Kocan KM, Teel PD, Hair JA et al.: Demonstration of *Anaplasma marginale* Theiler in ticks by tick transmission, animal inoculation and fluorescent antibody studies. Am J Vet Res 1980b; 41: 183-186.

Kocan KM, Oberst RD, Ewing SA et al.: Demonstration of *Anaplasma marginale* in hemolymph of *Dermacentor andersoni* by animal inoculation and by fluorescent-antibody technique. Am J Vet Res 1983; 44: 798-802.

Kocan KM, Yellin TN, Ewing SA, Hair JA, Barron SJ: Morphology of colonies of *Anaplasma marginale* Theiler in *Dermacentor andersoni*. Am J Vet Res 1984; 45: 1434-1440.

Kocan KM, Wickwire KB, Ewing SA et al.: Preliminary studies of the development of *Anaplasma marginale* in salivary glands of adult, feeding *Dermacentor andersoni*. Am J Vet Res 1988; 49: 1010-1013.

Kocan KM, Stiller D, Goff WL et al.: A review of the developmental cycle of *Anaplasma marginale* in *Dermacentor* spp. ticks. In: Proc 8th Natl Anaplasmosis Conf, St. Louis, 1989; pp. 129-148.

Kocan KM, Yellin TN, Claypool PL et al.: Development and infectivity of *Anaplasma marginale* Theiler in *Dermacentor andersoni* Stiles nymphs. Am J Vet Res 1990; 51: 1292-1294.

Kocan KM, Stiller D, Goff WL et al.: Development of *Anaplasma marginale* in male *Dermacentor andersoni* transferred from parasitemic to susceptible cattle. Am J Vet Res 1992; 53: 499-507.

Kocan KM, Goff WL, Stiller D et al.: Development of *Anaplasma marginale* in salivary glands of male *Dermacentor andersoni*. Am J Vet Res 1993; 54: 107-112.

Krueger CM, Buening GM, Kinden DA, Aboytes-Torres R, Turk J: Detection of the *Anaplasma marginale* genome in bovine tissues by in situ DNA hybridization. In: Proc 8th Natl Anaplasmosis Conf, St. Louis, 1989; pp. 83-90.

Lawrence JA, Foggin CM, Norval RAI: The effects of war on the control of diseases of livestock in Rhodesia (Zimbabwe). Vet Res 1980; 107: 82-85.

Marmur J, Doty P: Thermal renaturation of deoxyribonucleic acid. J Mol Biol 1961; 3: 585.

McGuire TC, Palmer GH, Goff WL et al.: Common and isolate-restricted antigens of *Anaplasma marginale* detected with monoclonal antibodies. Infect Immun 1984; 45: 697-700.

McGuire TC, Davis WC, Brassfield AL et al.: Identification of *Anaplasma marginale* long-term carrier cattle by detection of serum antibody to isolated MSP-3. J Clin Microbiol 1991; 29: 788-793.

McLaughlin GL, Montenegro-James S, Vodkin MH et al.: Molecular approaches to malaria and babesiosis. Mem Inst Oswaldo Cruz Rio de Janeiro 1992; 87 (Suppl III): 57-68.

Montenegro-James S, James MA, Levy MG et al.: Utilization of culture-derived soluble antigen in the latex agglutination test for bovine babesiosis and anaplasmosis. Vet Parasitol 1981; 8: 291-297.

Montenegro-James S, Guillen AT, Ma SJ et al.: Use of the dot ELISA with isolated *Anaplasma marginale* initial bodies for serodiagnosis of anaplasmosis in cattle. Am J Vet Res 1990; 51: 1518-1521.

Morel G: Introduction. In: Morel G, ed. Hybridization techniques for electron microscopy. CRC Press, Inc., Boca Raton, Florida, 1993; pp. 1-11.

Murray GI: In situ PCR. J Path 1993; 169: 187-188.

National Research Council: Priorities in biotechnology research for international development---Proceedings of a workshop, directed by the Board on Science and Technique for International Development, Office of International Affairs. National Academy Press, Washington, DC, 1982 ; pp.1-9.

Norval RAI: Arguments against intensive dipping. Zimbabwe Vet Res 1983; 14: 19-21.

Nuovo GJ, MacConnel P, Forde A, Delvenne P: Detection of human papillomavirus DNA in formalin-fixed tissues by in situ hybridization after amplification by polymerase chain reaction. Am J Path 1991; 139: 847-854.

Oberle SM, Palmer GH, Barbet AF et al.: Molecular size variations in an immunoprotective protein complex among isolates of *Anaplasma marginale*. Infect Immun 1988; 56: 1567-1573.

Oberle SM, Palmer GH, Barbet AF: Expression and immune recognition of the conserved MSP4 outer membrane protein of *Anaplasma marginale*. Infect Immun 1993; 61: 5245-5251.

Palmer GH: *Anaplasma* vaccine. In: Wright IG, ed. Veterinary protozoan and hemoparasite vaccines. CRC Press, Inc., Boca Raton, Florida, 1989; pp. 1-29.

Palmer GH, McGuire TC: Immune serum against *Anaplasma marginale* initial bodies neutralizes infectivity for cattle. J Immunol 1984; 133: 1010-1015.

Palmer GH, Kocan MK, Barron SJ et al.: Presence of common antigens including major surface protein epitopes, between the cattle (intraerythrocytic) and tick stages of *Anaplasma marginale*. Infect Immun 1985; 50: 881-886.

Palmer GH, Barbet AF, Davis WC et al.: Immunization with an isolate common surface protein protects cattle against anaplasmosis. Science 1986a; 231: 1299-1301.

Palmer GH, Barbet AF, Kuttler KL et al.: Detection of an *Anaplasma marginale* common surface protein present in all stages of infection. J Clin Microbiol 1986b; 23: 1078-1083.

Palmer GH, Waghela SD, Barbet AF: Characterization of a neutralization sensitive epitope on the Am 105 surface protein of *Anaplasma marginale*. Int J Parasitol 1987; 18: 1279-285.

Palmer GH, Barbet AF, Musoke AT et al.: Recognition of conserved surface protein epitopes on *Anaplasma centrale* and *Anaplasma marginale* isolates from Israel, Kenya and the U. S.. Int J Parasitol 1988a; 18: 33-38.

Palmer GH, Oberle SM, Barbet AF et al.: Immunization of cattle with a 36-kD surface protein induces protection against homologous and heterologous *Anaplasma marginale* challenge. Infect Immun 1988b; 56: 1526-1531.

Palmer GH, Barbet AF, Cantor GH et al.: Immunization of cattle with the MSP-1 surface protein complex induces protection against a structurally variant *Anaplasma marginale* isolate. Infect Immun 1989; 57: 3666-3669.

Pardue ML, Dawid IB: Chromosomal locations of two DNA segments that flank ribosomal insertion-like sequences in *Drosophila*: Flanking sequences are mobile elements. Chromosoma 1981; 83: 29-43.

Potgieter FT: Tick transmission of anaplasmosis in South Africa. In: Whitehead GB, Gibson, eds. Tick Biology and Control. Tick Research Unit, Rhodes University, Grahamston, South Africa, 1981; pp. 53-56.

Ristic M: A capillary-tube agglutination test for anaplasmosis: a preliminary report. J Am Vet Med Assoc 1962; 141: 588-594.

Ristic M: Bovine anaplasmosis. In: Kreier JP, ed. Parasitic Protozoa. Academic Press, Inc., New York, 1977; pp. 235-249.

Ristic M, Sibinovic S, Welfer CJ: An attenuated *Anaplasma marginale* vaccine. In: Proc 72th Annu Meet U. S. livestock Sanit Assoc, 1968; pp. 56-69.

Schunter CA, Leatch G: Radioimmunoassay for *Anaplasma marginale* antibodies. Am J Vet Res 1988; 49: 504-507.

Senitzer D, Dimopoullos GT, Brinkley BR et al.: Deoxyribonucleic acid of *Anaplasma marginale*. J Bacteriol 1972; 109: 434-436.

Shkap V, Bin H, Ungar WH et al.: An ELISA for the detection of antibodies to *Anaplasma centrale* and *Anaplasma marginale*. Vet Microbiol 1990; 25: 45-53.

Shivers BD, Harlan RE, Hejtmancik JF, Conn PM, Pfaff DW: Localization of cells containing LHRH-like mRNA in rat forebrain using in situ hybridization. Endocrinology 1986; 118: 883-885.

Siegmund OH: Anaplasmosis. In: Siegmund OH, Ed. Merck Veterinary Manual. Merck & Co., Inc., New Jersey, 1986; pp. 17-19.

Soulsby ETC: Order: Rickettsiales Gieszkiewicz, 1939. In: Soulsby ETC, ed. Helminths, arthropods and protozoa of domesticated animals. Bailliere Tindall, Philadelphia, 1982; pp. 752-754.

Staats JJ, Kocan KM, Hair JA et al.: Immunocytochemical labeling of *Anaplasma marginale* Theiler in *Dermacentor andersoni* Stiles with peroxidase-antiperoxidase technique. Am J Vet Res 1982; 43: 979-983.

Stich RW, Kocan KM, Palmer GH et al.: Transstadial and attempted transovarial transmission of *Anaplasma marginale* by *Dermacentor variabilis*. Am J Vet Res 1989; 50: 1377-1380.

Stich RW, Bantle JA, Kocan KM et al.: Preliminary development of a polymerase chain reaction assay for *Anaplasma marginale* in ticks. Biotechnol Tech 1991; 5: 269-274.

Stich RW, Bantle JA, Kocan KM, Fakete A: Detection of *Anaplasma marginale* in hemolymph of *Dermacentor andersoni* with the polymerase chain reaction. J Med Entomol 1993a; 30: 781-788.

Stich RW, Sauer JR, Bantle JA, Kocan KM: Detection of *Anaplasma marginale* in secretagogue-induced oral secretions of *Dermacentor andersoni* with polymerase chain reaction. J Med Entomol 1993b; 30: 789-794.

Stiller D, Kocan KM, Edwards W et al.: Detection of colonies of *Anaplasma marginale* in salivary glands of three *Dermacentor* spp. infected as nymphs or adults. Am J Vet Res 1989; 50: 1381-1389.

Swift BL, Thomas GM: Bovine anaplasmosis: elimination of the carrier state with injectable long-acting oxytetracycline. J Am Vet Med Assoc 1983; 183; 63-65.

Taveira NC, Ferrira MOS, Pereira JM: Detection of HIV1 proviral DNA by PCR and hybridization with digoxigenin labeled probes. Mol Cell Probes 1992; 6: 265-270.

Tebele N, McGuire TC, Palmer GH: Induction of protective immunity by using *Anaplasma marginale* initial body membranes. Infect Immun 1991; 59: 3199-3204.

Tecott LH, Eberwine JH, Barchas JD, Valentino KL: Methodological considerations in the utilization of in situ hybridization. In: Valentino KL, Eberwine JH Barchas, eds. In situ hybridization: application to neurobiology. Oxford University Press, New York, 1987; pp. 1-24.

Theiler A: *Anaplasma marginale*. The marginal points in the blood of cattle suffering from a specific disease. In: Theiler R, ed. Report of the Government Veterinary Bacteriologist. Transvaal Department of Agriculture, Transvaal, South Africa, 1910; pp. 1908-1909.

Trueblood ES, McGuire TC, Palmer GH: Detection of *Anaplasma marginale* rickettsemia prior to onset of clinical signs by using an antigen capture ELISA. J Clin Microbiol 1991; 29: 1542-1544.

Unger ER, Brigati DJ: Colorimetric in-situ hybridization in clinical virology: development of automated technology. Current Topics in Microbiol Immun 1989; 143: 21-31.

Venable JH, Coggeshall RA: simplified lead citrate strain for use in electron microscopy. J Cell Biol 1965; 25: 407-408.

Visser ES, Ambrosio RE: DNA probes for the detection of *Anaplasma centrale* and *Anaplasma marginale*. Onderstepoort J Vet Res 1987; 54: 623-627.

Visser ES, McGuire TC, Palmer GH et al.: *Anaplasma marginale msp-5* gene encodes a 19 kD protein conserved in all *Anaplasma* species. Infect Immun 1992; 60: 5139-5144.

Weiss E, Moulder JW: The rickettsias and chlamydias. In: Krieg NR, Holf JG eds. Bergey's manual of systemic bacteriology Vol 1. Williams and wilkins, Baltimore, 1984; pp. 687-739.

CHAPTER II

DETECTION OF *Anaplasma marginale* DNA IN BOVINE ERYTHROCYTES BY SLOT BLOT AND IN SITU HYBRIDIZATION WITH A PCR-MEDIATED DIGOXIGENIN-LABELED DNA PROBE

Abstract

A 409 bp DNA fragment derived from the *msp-1β* gene of *Anaplasma marginale* was amplified and simultaneously labeled with digoxigenin 11-dUTP by a polymerase chain reaction (PCR) assay. The resulting digoxigenin-labeled 409 bp PCR product was used as a probe for slot-blot and in situ hybridization to detect *A. marginale* DNA from experimentally-infected bovine erythrocytes. The hybrid formation was detected with alkaline phosphatase-conjugated anti-digoxigenin antibody and substrates 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium salt. In slot blot hybridizations, the probe detected *A. marginale* DNA from approximately 1,000-10,000 infected erythrocytes in 1.25 ml of whole blood, which is equivalent to a parasitemia level of 0.00001%. The probe proved to be *A. marginale*-specific when tested with 17 species of microorganisms. The applicability of the probe for diagnosis was tested by screening *A. marginale* infections in two experimentally-infected splenectomized cattle before microscopically detectable parasitemias and after acute infection. After inoculation of infected blood, *A. marginale* infections were detected with the probe 14 days prior to detection in stained smears. Microscopically inapparent parasitemias were also detected with the probe for two months after acute disease. When the probe was used for in situ hybridization on methanol-fixed blood smears, probe reaction could be visualized with light microscopy on *A. marginale* inclusions within infected erythrocytes. The probe reaction was not observed on leukocytes and uninfected erythrocytes from infected blood smears, on erythrocytes from uninfected blood samples or on samples infected with *A. ovis*, *Babesia bovis*, or *B. bigemina*. This PCR-mediated nonradioactive DNA probe appears to be a sensitive diagnostic tool for *A. marginale*.

Introduction

Anaplasmosis is a tick-borne disease of cattle and other ruminants caused by the intraerythrocytic rickettsia, *Anaplasma marginale*.[25] The disease is the most prevalent of bovine hemoparasite infections and is enzootic to nearly half the world's livestock production regions.[23] Invasion and multiplication of *A. marginale* in erythrocytes of cattle may result in anemia, weight loss, abortion and sometimes death during acute infections.[26] Cattle recovering from acute disease remain persistent carriers with low to inapparent parasitemias and may serve as reservoirs for transmission of the organism.[32] The annual losses attributed to anaplasmosis within the United States alone were estimated at $300 million.[23]

Diagnosis of acute anaplasmosis is often based on direct microscopic detection of *Anaplasma* inclusions in Giemsa-stained blood smears.[28] Infected erythrocytes in carrier cattle are often not detectable by microscopic examination of stained blood smears (less than 0.1% infected erythrocytes).[18] A variety of serodiagnostic methods have been used to measure *Anaplasma*-specific antibodies, including the complement fixation,[15] capillary-tube agglutination,[24] card test,[3] latex agglutination,[22] indirect fluorescent antibody test (IFAT),[15] conventional ELISA[28] and radioimmunoassay.[27] These tests often lack sensitivity and specificity, or some of them do not distinguish infected from uninfected, vaccinated cattle.

Detection of *A. marginale* using nucleic acid hybridization offers an alternative diagnostic tool. Several radioactive nucleic acid probes have been developed for detection of *A. marginale* from carrier cattle and infected ticks.[12,13,14] *Anaplasma marginale* infection can be detected in individual infected tick salivary glands or in erythrocytes at a level of 100-1000 infected cells using a DNA probe derived from *msp-1β* gene encoding the MSP-1b subunit of

A. marginale.[14] Despite the high sensitivity and specificity of these probes, problems associated with radioactively-labeled probes, such as the biohazard of radioactive compounds, limited shelf life, and extensive time required for autoradiography, have limited their use as diagnostic tools.

Nonradioactively labeled probes have been developed for detection of infectious agents because of their minimal hazard, short detection times, low cost and stability, and because the sensitivity and specificity of these probes approach those of radioactive ones. The recent application of polymerase chain reaction (PCR) for generation of vector-free, digoxigenin-dUTP labeled probes[4,11,17,33] has greatly simplified probe preparation. An easily-labeled, sensitive and specific nonradioactive probe may provide a valuable diagnostic tool for *A. marginale*. Herein we report use of PCR to generate a digoxigenin-11-dUTP labeled DNA probe for detection of *A. marginale* in bovine erythrocytes using slot blot and in situ hybridization.

Materials and Methods

Source of Infected Blood

The Virginia isolate of *A. marginale* (VAM) was used to infect donor calves for this study. This isolate has been successfully used in transmission studies of *A. marginale* involving ticks and cattle at the Anaplasmosis Research Laboratory, Oklahoma State University.[19,20] Two splenectomized calves (No. PA312 & No. PA314), 2 to 6 months of age, tested negative for anaplasmosis by the complement fixation (CF), were inoculated intravenously with blood from a carrier calf and used as a source of infected blood. Calves were monitored for infection by

light microscopic (LM) examination of Giemsa-stained blood smears and by determination of the packed cell volume (PCV).

Preparation of Infected Erythrocytes

For determination of the lowest percentage of infected erythrocytes that the probe could detect, six 10-fold serial dilutions of known numbers of infected erythrocytes (2×10^5 to 2×10^0 infected erythrocytes) were added to a certain amount of uninfected erythrocytes at final concentration of 2×10^{10} erythrocytes per ml. The resulting samples were washed 3 times with phosphate-buffered saline (PBS, 0.137 M NaCl, 10 mM Na_2HPO_4, 3.2 mM KH_2PO_4). For negative controls, blood collected from an uninfected animal was processed in the same manner. Before use, each sample was freeze-thawed 3 times to lyse erythrocytes, after which a 0.5 ml-aliquot equivalent of 1×10^{10} total erythrocytes or 1.25 ml of whole blood, was added to 2 volumes of PBS and centrifuged at 13,000 X g for 30 min. The supernatant containing the released hemoglobin was removed. The resulting pellet was resuspended in 500 µl of PK buffer (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 12.5 mM EDTA, 1% SDS and 0.1 mg/ml proteinase K), incubated at 56 ^{0}C overnight, and extracted with phenol/chloroform/isoamyl alcohol (25:24:1). DNA was precipitated with sodium acetate and absolute ethanol, and resuspended in 300 µl of TE buffer (10 mM Tris-HCl, pH 8.0, 1.0 mM EDTA). Six final samples that contained 1×10^5 - 1×10^0 infected erythrocytes and correspond to levels of parasitemia ranging from 0.001 - 0.00000001% were applied for slot blot hybridizations.

To determine the probe's ability to detect *A. marginale* before occurrence of microscopically detectable infection, blood samples were collected before inoculation and daily after inoculation until a 2-6% parasitemia was detectable microscopically. For detection of *A.*

marginale after acute disease, blood samples were taken from the same two calves twice weekly for two months after *A. marginale* became undetectable with LM in Giemsa-stained blood smears. At each sampling time, five ml of blood from each calf was drawn into sterile EDTA tubes, washed 3 times with PBS, resuspended in PBS to 2.0 X 10^{10} erythrocytes per ml and stored at -70 ^{0}C. Blood samples were collected from an uninfected animal and treated in the same manner to serve as negative controls. Before probing, a 0.5 ml- aliquot of washed erythrocytes representing 1.25 ml of whole blood from each sample was digested and extracted as described above.

Specificity Trials

The DNA probe was tested for its specificity with DNA extracted from bovine thymus and 17 species of microorganisms (Table I). DNA samples were extracted as described above, and DNA concentrations were determined by absorbance at 260 nm. Three, 10-fold serial dilutions from each sample were made from 2 mg of extracted DNA and used for slot blot hybridization.

Generation of a DIG-Labeled DNA Probe Using PCR

Oligonucleotides identical to BAP-2 and AL34S[30,31] were synthesized at the Recombinant DNA/Protein Resource Facility, Oklahoma State University and used as primers. The PCR was done in a 100 µl reaction volume containing 1.25 units of AmpliTaq DNA polymerase,[i] 1 mM of each primer, 1 ng of DNA extracted from *A. marginale*-infected erythrocytes,[6,14] 0.2 mM of dCTP, dATP and dGTP, 0.13 mM of dTTP, 0.07 mM of DIG-11-dUTP,[j] 1.5 mM $MgCl_2$, 10 mM Tris-HCl (pH 8.3) and 50 mM KCl. Amplification was

performed for 35 cycles in a DNA thermal cycler[k] using a denaturing temperature of 95 ^{0}C for 1.5 min, an annealing temperature of 57 ^{0}C for 2 min and an extension temperature of 72 ^{0}C for 3 min. The amplified products were checked on a 1.5% agarose gel. Confirmed DIG-labeled 409-bp DNA fragment was purified by ethanol precipitation. The yield of the DIG-labeled DNA was calculated by the procedure recommended by the manufacturer[8] and used as a probe for slot blot and in situ hybridization.

Slot-Blot Hybridization

DNA samples were treated and bound to nylon membranes[l] using a minifold II slot-blot system[m] according to manufacturer's instructions. The membrane was pre-hybridized for 1 hour at 42 ^{0} C with prehybridization solution (20 ml/100 cm^2 membrane) containing 50% formamide, 5 X SSC, 2% the blocking reagent,[j] 0.1% sarkosyl, 0.02% SDS and 0.1% salmon sperm DNA. The prehybridization solution was removed and 2.5 ml/100 cm^2 membrane of hybridization solution (prehybridization solution + freshly denatured labeled probe at final concentration of 30 ng/ml) was added to the membrane and incubated overnight, after which the hybridization solution was saved at -20 ^{0}C for reuse. The membrane was washed 2 X 5 min with 2 X SSC and 2 X 15 min with 0.1 X SSC at 68 ^{0}C. DIG-labeled probe was detected by specific immunological reaction using 1:5,000 diluted anti-DIG Fab fragment conjugated to alkaline phosphatase and the substrates 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and nitroblue tetrazolium salt (NBT) following the manufacturer's instructions.

In Situ Hybridization

To test the applicability of the probe for detecting *A. marginale* on blood smears using in situ hybridization, blood samples were collected daily from the two infected calves during a period when infection of erythrocytes was apparent by microscopic examination. Blood smears were made on the silanized slides,[n] fixed in 100% methanol for 3 min and air-dried. *A. ovis*-,[a] *Babesia bovis*-[c] and *B. bigimina*-infected blood[a] and uninfected blood were treated in the same manner and used as negative controls. Slides were either used immediately for ISH or stored at -70 ^{0}C for later use. Prior to hybridization, slides were rehydrated in 2 X SSC for 15 min and then prehybridized in prehybridization solution at 42 ^{0}C for 1 hr. To denature the DNA probe and target *Anaplasma* DNA, the DNA probe was heated in boiling water for 10 min and the smears were rinsed in 0.3 N NaOH for 1 min, respectively. A 5 ng DIG-labeled probe in 70 µl of prehybridization solution containing 5% dextran sulfate was then added on each slide, and the slides were covered with coverslips and incubated overnight in a sealed humid pan at 42 ^{0}C. Coverslips were removed the following day and slides were washed in decreasing concentrations of SSC at 48 ^{0}C. To detect DIG-labeled probe, slides were incubated with 1:1,000 diluted anti-DIG conjugated antibody. Color development was achieved by covering slides with chromogenic substrate solution (BCIP/NTP) for 4 hrs. Development was stopped by rinsing in TE buffer. Slides were air-dried, counterstained with eosin, coverslipped in permount and observed with LM. The parasitemias determined by in situ hybridization were compared with those from Giemsa-stained smears made from calf No. PA 312 in 15 successive days, and the data were statistically analyzed using a paired *t*-test.

Results

Digoxigenin DNA Labeling Using PCR

To confirm if digoxigenin-11-dUTP was incorporated during DNA amplification, the electrophoretic mobility of the PCR products amplified with and without digoxigenin were compared using a 1.5% agarose gel electrophoresis (Fig. 1). The labeled 409 bp DNA fragment (Lane 1) migrated more slowly than the unlabeled 409 bp fragment (Lane 3), demonstrating that digoxigenin-11-dUTP was incorporated during the amplification reaction. Approximately, 1-1.5 mg of the purified labeled DNA per 100 µl of reaction mixture was obtained.

Sensitivity and Specificity of the Probe

To determine the sensitivity and specificity of the digoxigenin-labeled probe, the probe was first hybridized to each of 6, 10-fold serial dilutions of DNA extracted from the blood that contained the known numbers of infected erythrocytes, the unlabeled 409 bp DNA fragment and the 2 kb fragment derived from *msp-1β* gene of *A. marginale* (Fig. 2). A very faint band occurred on the 3rd dilution of the blood sample that contained 1,000 infected erythrocytes, corresponding to a parasitemia level of 0.00001%.The probe detected 0.0001 pg of the unlabeled 409 bp DNA fragment and 0.01 pg of the 2 kb fragment derived from the *Anaplasma msp-1β* gene.[14] Color reaction was not observed on the control samples that contained 1 X 10^{10} uninfected erythrocytes. The probe was found to be *A. marginale*-specific and did not hybridize with DNA extracted from bovine thymus and 17 species of microorganisms, including *A. ovis*, *B. bigemina* and *B. bovis* (Fig. 3).

Detection of *A. marginale* during Pre-Acute Infection and after Acute Disease

Anaplasma marginale was detected with the probe in the slot blot hybridizations before the occurrence of microscopically detectable levels of infections (Fig. 4). Probe reactions were seen in samples from the 2 calves beginning at day 10 (calf No. PA 312) and 14 (calf No. PA 314) post-infection. The intensity of the hybridization signals remained constant for several days and then gradually increased. Compared with the infected erythrocyte standards, the number of infected erythrocytes in PA 312 reached to 10^7 per 1.25 ml of blood while parasitemia was first detectable microscopically on day 24 post-infection. Thereafter, the parasitemias, as determined by the DNA probe, were comparable with those determined by LM.

After infections in both calves became inapparent, hybridization signals occurred in most samples collected over the 2-month period. Comparison of test samples with infected erythrocyte standards revealed that the number of infected erythrocytes in the test samples varied from $< 10^3$ to 10^7 per 1.25 ml of blood, which correspond to parasitemias ranging from $< 0.00001\%$ to 0.1% (Fig. 4).

Application of the Digoxigenin-Labeled Probe for In Situ Hybridization

When the probe was used for in situ detection of *A. marginale* in smears of erythrocytes, probe reaction occurred on *A. marginale* inclusions (Fig. 5a). The morphology of the organism with in situ hybridization was comparable to that seen in Giemsa-stained slides. Parasitemias determined by in situ hybridization were slightly lower than those determined by Giemsa stained smears ($0.025>P>0.01$) (Table II). Probe reaction was not observed on

leukocytes nor on uninfected erythrocytes (Fig. 5a), control uninfected erythrocytes (Fig. 5b); *A. ovis*-, *B. bovis*-, and *B. bigemina*-infected blood smears were also negative.

Discussion

The 409 bp DNA fragment derived from the *msp-1β* gene encoding the MSP-1b subunit of *A. marginale*[5] was first amplified by PCR assay from hemolymph, oral secretions, midguts and salivary glands of experimentally infected ticks.[29,30,31] The assay was found to be *A. marginale*-specific when tested with 18 species of bacteria and protozoa, and 7 isolates of *A. marginale* from diverse geographical areas of the U. S. A..[30]

The data presented here have demonstrated the feasibility of using PCR to amplify and simultaneously label this 409 bp DNA fragment with digoxigenin-11-dUTP for development of a probe for detection of *A. marginale*. In this protocol, DIG-11-dUTP was successfully incorporated into the 409 bp DNA fragment during amplification. Greater than 1 mg of the labeled probe per 100 μl reaction mixture was produced in 5 hrs. The labeling reaction was more efficient than random primed DNA labeling, which has been the most efficient method reported previously.[8] As compared with standard PCR reaction conditions,[29,30,31] elongation time was considerably longer since DIG-labeled DNA is synthesized approximately half as efficiently as unlabeled DNA.[11]

Recently, several radioactive nucleic acid probes have been developed for detection of *A. marginale* in bovine erythrocytes and tick tissues. These probes were constructed either from *Anaplasma* DNA genome[2,35] or by recombinant techniques[1,6,12,13,14] that required molecular cloning using vectors, restriction digestion of vector DNA, and recovery or purification of the insert. A sensitive and specific RNA probe was used to detect and quantitate

A. marginale in carrier cattle, and 0.01 ng of *Anaplasma* genomic DNA and 500-1,000 infected erythrocytes were detected.[12,13] The sensitivity and specificity of our PCR-mediated DIG-labeled DNA probe were comparable with those of this RNA probe and other probes reported for detection of *A. marginale*. However, the procedure for preparation of the probe used in this study was simpler.

In situ hybridization combines sensitivity and specificity of traditional nucleic acid hybridization with precise histological localization without disrupting tissue morphology.[7] The technique has been used widely for detection of pathogens in infected tissue sections.[9,10,16,21] In the present study, the DIG-labeled 409 bp DNA probe was used successfully for in situ hybridization to detect *A. marginale* on methanol-fixed blood smears. The probe reaction was specific and was seen only on *A. marginale* inclusions in infected erythrocytes. Nonspecific background staining was not observed. The percentage of infected erythrocytes determined by in situ hybridization was comparable to that determined in Giemsa-stained smears by LM. *Anaplasma marginale* inclusions were easily identified with in situ hybridization. In situ hybridization was especially useful for early detection of *A. marginale* when parasitic inclusions are difficult to differentiate from Heinz bodies, Howell-Jolly bodies and staining artifacts often seen in Giemsa-stained preparations.[34] In addition, the probe is able to detect 1,000 - 10,000 infected erythrocytes in 1.25 ml of whole blood (~1 X 10^{10} erythrocytes), which is equivalent to a parasitemia level of 0.00001%. Microscopically inapparent parasitemias were also detected with the probe for two months after acute disease in two chronically infected, splenectomized cattle. Therefore, this nonradioactive DNA probe may be useful as a diagnostic tool because of its sensitivity in detecting early infections and its potential in identifying intact carrier cattle in the field when parasitemia may be inapparent by LM. Further studies are underway using this

probe for study of *A. marginale* in intact carrier cattle, ticks, and cell culture by slot blot and in situ hybridization.

Acknowledgments

Published by Nie-Lin Ge,[1] Katherine M. Kocan,[1] George L. Murphy[1] & Edmour F. Blouin[1] in *Journal of Veterinary Diagnostic Investigation* 1995; 7: 465-472.

We thank Dr. David Stiller for providing *A. ovis*- and *B. bigemina*-infected fresh blood and Ms. Wanda Edwards & Ms. Suzy Goedeken for technical assistance. We also thank Dr. Corrie Brown for consultation about in situ hybridization technique. Critical review of this manuscript from Dr. Sidney Ewing was greatly appreciated. This study was supported in part by Oklahoma Agricultural Experimental Station project 1433. Published as journal series article J94-017 of the Oklahoma Agricultural Experimental Station, Stillwater.

---

[1] Department of Pathology, College of Veterinary Medicine, Oklahoma State University, Stillwater, Oklahoma 74078.

Sources and Manufacturers

a. Provided by Dr. D. Stiller, Animal Disease Research Unit, USDA-ARS, Moscow, Idaho.

b. Provided by Dr. S. Ewing, Veterinary Parasitology, Oklahoma State University (OSU), Stillwater, OK.

c. Provided by Dr. G. Palmer, Veterinary Microbiology, Washington State University, Pullman, WA.

d. Provided by Dr. A. Kocan, Veterinary Parasitology, OSU, Stillwater, OK.

e. Provided by Dr. J. Bantle, Zoology, OSU, Stillwater, OK.

f. American Type Culture Collection.

g. Provided by Dr. R. Welsh, OADDL, OSU, Stillwater, OK.

h. Provided by Dr. A. Confer, Veterinary Pathology, OSU, Stillwater, OK.

i. Perkin Elmer-Cetus, Norwalk, CT.

j. Boehringer Mannheim Biochemicals, Indianapolis, IN.

k. SingleBlock™ System, Ericomp, Inc., San Diego, CA.

l. Micron Separations Inc. (MSI), Westboro, MA.

m. Schleicher & Schuell Inc., Keene, NH.

n. Fisher Scientific, Pittsburgh, PA.

o. Promega, Madison, WI.

References

1. Aboytes-Torres R, Buening GM, Alvarez A, et al.: 1989, Recombinant probe construction and application in anaplasmosis research. In: Proc 8th Natl Anaplasmosis Conf, pp. 305-316. St. Louis, MO.

2. Ambrosio RE, Visser ES, Koekhoven Y, et al.: 1988, Hybridization of DNA probes to *Anaplasma marginale* isolates from different sources and detection in *Dermacentor andersoni* ticks. Onderstepoort J Vet Res 55: 227-229.

3. Amerault TE, Roby TO: 1968, A rapid card agglutination test for bovine anaplasmosis. J Am Vet Med Assoc 153: 1828-1834.

4. An SF, Franklin D, Fleming KA: 1992, Generation of digoxigenin-labeled double-stranded and single-stranded probes using the polymerase chain reaction. Mol Cell Probes 6: 193-200.

5. Barbet AF, Allred D: 1991, The *msp1-β* multigene family of *Anaplasma marginale*: nucleotide sequence analysis of an expressed copy. Infect Immun 59: 971-976.

6. Blouin EF, Kocan KM, Murphy GL, Ge NL: 1993, Persistence of tick-derived *Anaplasma marginale* in cultured bovine turbinate and endothelial cells. Rev d'elevage et de Med Vet des Pays Trop 46: 64-66.

7. Boehringer Mannheim: 1992a, Nonradioactive in situ hybridization application manual. pp. 1-75. Boehringer Mannheim GmbH Biochemica, Germany.

8. Boehringer Mannheim: 1992b, The Genius™ system user's guide for filter hybridization application manual. pp. 1-84. Boehringer Mannheim GmbH Biochemica, Germany.

9. Brown CC, Meyer KF, Olander HJ, et al.: 1992, A pathogenesis study of foot and mouth disease in cattle using in situ hybridization. Can J Vet Res 56: 189-193.

10. Brown CC, Meyer KF, Grubman MJ: 1993, Use of digoxigenin-labeled RNA probe to detect all 24 serotypes of bluetonge virus in cell culture. J Vet Diagn Invest 5: 159-162.

11. Celeda D, Bettag U, Cremer C: 1992, PCR amplification and simultaneous digoxigenin incorporation of long DNA probes for fluorescence in situ hybridization. Biotechniques 12: 98-102.

12. Eriks IS, Palmer GH, McGuire TC, et al.: 1989, Detection and quantitation of *Anaplasma marginale* in carrier cattle by using a nucleic acid probe. J Clin Microbiol 27: 279-284.

13. Eriks IS, Stiller D, Palmer GH: 1993, Impact of persistent *Anaplasma marginale* rickettsemia on tick infection and transmission. J Clin Microbiol 31: 2091-2096.

14. Goff WL, Barbet AF, Stiller D, et al.: 1988, Detection of *Anaplasma marginale* in tick vectors using a cloned DNA probe. Proc Natl Acad Sci USA 88: 919-923.

15. Goff WL, Stiller D, Roeder RA, et al.: 1990, Comparison of a DNA, complement-fixation and indirect immunofluorescence tests for diagnosing *Anaplasma marginale* in suspected carrier cattle. Vet Microbiol 24: 381-390.

16. Harper ME, Marselle LM, Gallo RC, Wong-Stahl F: 1986, Detection of lymphocytes expressing human T-lymphotropic virus type III in lymph nodes and peripheral blood from infected individuals by in situ hybridization. Proc Nalt Acad Sci USA 83: 772-776.

17. Ibrahim A, Liesack W, Stackebrandt E: 1992, Differentiation between pathogenic *Yersinia enterocolitica* strains by colony hybridization with a PCR-mediated digoxigenin-dUTP-labeled probe. Mol Cell Probes 6: 163-171.

18. Kieser ST, Eriks IS, Palmer GH: 1990, Cyclic rickettsemia during persistent *Anaplasma marginale* infection of cattle. Infect Immun 58: 1117-1119.

19. Kocan KM, Yellin TN, Claypool PL, et al.: 1990, Development and infectivity of *Anaplasma marginale* Theiler in *Dermacentor andersoni* Stiles nymphs. Am J Vet Res 51: 1292-1294.

20. Kocan KM, Stiller D, Goff WL, et al.: 1992, Development of *Anaplasma marginale* in male *Dermacentor andersoni* transferred from parasitemic to susceptible cattle. Am J Vet Res 53: 499-507.

21. Krueger CM, Buening GM, Kinden DA, et al.: 1989, Detection of the *Anaplasma marginale* genome in bovine tissues by in situ DNA hybridization. In: Proc 8th Natl Anaplasmosis Conf, pp. 83-90. St. Louis, MO.

22. Montenegro-James S, MA James, Levy MG, et al.: 1981, Utilization of culture-derived soluble antigen in the latex agglutination test for bovine babesiosis and anaplasmosis. Vet Parasitol 8: 291-297.

23. National Research Council: 1982, Priorities in biotechnology research for international development. In: Proceedings of a Workshop Directed by the Board on Science and Technique for International Development, Office of International Affairs. pp. 1-9. National Academy Press, Washington, DC.

24. Ristic M: 1962, A capillary-tube agglutination test for anaplasmosis: a preliminary report. J Am Vet Med Assoc 141: 588-594.

25. Ristic M: 1977, Bovine anaplasmosis. In: Parasitic Protozoa, ed. Kreier JP, pp. 235-249. Academic Press, Inc., New York.

26. Ristic M, Sibinovic S, Welfer CJ: 1968, An attenuated *Anaplasma marginale* vaccine. In: Proc 72nd Annu Meet U. S. livestock Sanit Assoc, pp. 56-69.

27. Schunter CA, Leatch G: 1988: Radioimmunoassay for *Anaplasma marginale* antibodies. Am J Vet Res 49: 504-507.

28. Shkap V, Bin H, Ungar WH, et al.: 1990, An ELISA for the detection of antibodies to *Anaplasma centrale* and *A. marginale*. Vet Microbiol 25: 45-53.

29. Stich RW, Bantle JA, Kocan KM, et al.: 1991, Preliminary development of a polymerase chain reaction assay for *Anaplasma marginale* in ticks. Biotechnol Tech 5: 269-274.

30. Stich RW, Bantle JA, Kocan KM, Fakete A: 1993a, Detection of *Anaplasma marginale* (Rickettsiales: Anaplasmataceae) in hemolymph of *Dermacentor andersoni* (Acari: Ixodidae) with the polymerase chain reaction. J Med Entomol 30: 781-788.

31. Stich RW, Sauer JR, Bantle JA, Kocan KM: 1993b, Detection of *Anaplasma marginale* (Rickettsiales: Anaplasmataceae) in secretagogue-induced oral secretions of *Dermacentor andersoni* (Acari: Ixodidae) with polymerase chain reaction. J Med Entomol 30: 789-794.

32. Swift BL, Thomas GM: 1983, Bovine anaplasmosis: elimination of the carrier state with injectable long-acting oxytetracycline. J Am Vet Med Assoc 183: 63-65.

33. Taveira NC, Ferrira MOS, Pereira JM: 1992, Detection of HIV1 proviral DNA by PCR and hybridization with digoxigenin-labeled probes. Mol Cell Probes 6: 265-270.

34. Trueblood ES, McGuire TC, Palmer GH: 1991, Detection of *Anaplasma marginale* rickettsemia prior to onset of clinical signs by using an antigen capture ELISA. J Clin Microbiol 29: 1542-1544.

35. Visser ES, Ambrosio RE: 1987, DNA probes for the detection of *Anaplasma centrale* and *Anaplasma marginale*. Onderstepoort J Vet Res 54: 623-627.

Table I. The microorgisms used for specificity trial

Rictettsia	
(1) *Anaplasma ovis*[a]	(2) *Ehrlichia canis*[b]
Protozoa	
(3) *Babesia bigemina*[a]	(4) *Babesia bovis*[c]
(5) *Cytauxzoon felis*[d]	(6) *Theileria cervi*[d]
Spirochete	
(7) *Borrelia burgdorferi*[d]	
Bacteria	
(8) *Brucella abortus*[e]	(9) *Klebsiella pneumoniae*[f]
(10) *Listeria monocytogenes*[g]	(11) *Pasteurella haemolytica*[h]
(12) *P. multocida*[h]	(13) *Pseudomonas aeruginosa*[e]
(14) *Rhizobium meliloti*[e]	(15) *Salmonella typhimurium*[f]
(16) *Serratia marcescens*[f]	(17) *Yersinia enterocolitica*[f]

Table II. Comparison of percentages of infected erythrocytes determined by Giemsa stain and by in situ hybridization during 15 successive days in Calf No. PA 312

Days Postinfection	Giemsa Stain	In Situ Hybridization
27	0.6	0.4
28	0.8	0.5
29	Few/Slide	~0.1
30	1.8	1.3
31	4.0	4.2
32	6.1	7.0
33	12.1	11.1
34	18.4	16.8
35	12.2	11.5
36	16.5	14.7
37	13.0	12.2
38	16.2	14.1
39	10.8	11.1
40	2.0	1.2
41	1.4	0.8
Mean	8.28	7.63

Figure 1. Ethidium bromide-stained 1.5% agarose gel of PCR products amplified from 1 ng DNA extracted from *Anaplasma marginale*-infected erythrocytes.

Lanes: 1) DIG-labeled 409 bp DNA fragment; 3) Unlabeled 409 bp DNA fragment; 2 & 4) Negative controls, no DNA template; 5) pGEM molecular size standards°, arrow: 396 bp.

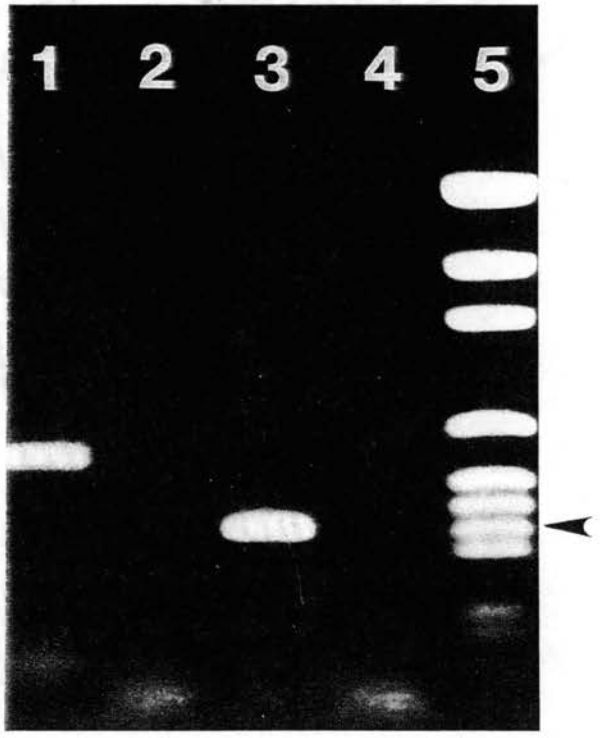

1
2
3
4
5

Figure 2. Sensitivity of the DIG-labeled DNA probe determined by slot blot hybridization.

Slots: 1A-2C, 6, 10-fold serial dilutions of DNA extracted from known numbers ($1X10^5$-$1X10^0$) of infected erythrocytes equivalent to 1.25 ml whole blood (~1 X 10^{10} total erythrocytes), 3A-4C and 5A-6C, 6, 10-fold serial dilutions ($1X10^2$-$1X10^{-3}$ pg) of 2 kb DNA fragment and unlabeled 409 bp DNA fragment, respectively, 7A-7C, DNA extracted from 3, 1.25 ml of control whole blood (~1 X 10^{10} uninfected erythrocytes).

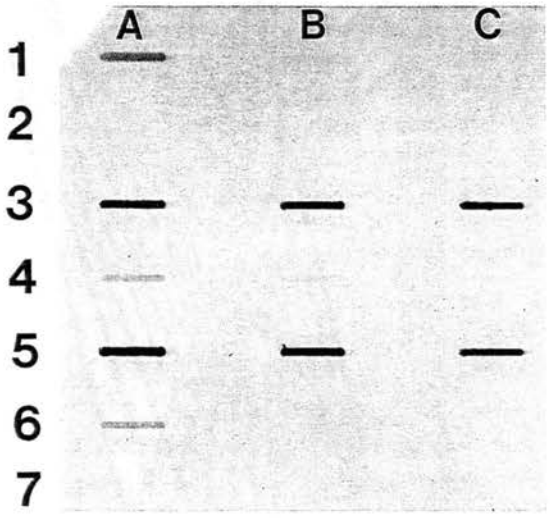

A
B
C
1
2
3
4
5
6
7

Figure 3. Slot blot hybridization of the DIG-labeled DNA probe with DNA extracted from calf thymus and from 15 species of microorganisms.

Slots: 1A-2C and 3A-4C, 6, 10-fold serial dilutions ($1X10^{2}$-$1X10^{-3}$ pg) of 2 kb DNA fragment and unlabeled 409 bp DNA fragment, respectively; 5A-6C and 7A-8C, 6, 10-fold serial dilutions ($1X10^{6}$-$1X10^{1}$) of infected and uninfected erythrocytes, respectively; 9A-24C, 3, 10-fold serial dilutions (2 mg-0.02 mg) of DNAs extracted from calf thymus and from 15 species of microorganisms: 9A-9C, Calf thymus; 10A-10C, *Ehrlichia canis*; 11A-11C, *Babesia bigemina*; 12A-12C, *Cytauxzoon felis*; 13A-13C, *Theileria cervi*; 14A-14C, *Borrelia burgdorferi*; 15A-15C, *Brucella abortus*; 16A-16C, *Klebsiella pneumoniae*; 17A-17C, *Listeria monocytogenes*; 18A-18C, *Pasteurella haemolytica*; 19A-19C, *P. multocida*; 20A-20C, *Pseudomonas aeruginosa*; 21A-21C, *Rhizobium meliloti*; 22A-22C, *Salmonella typhimurium*; 23A-23C, *Serratia marcescens*; 24A-24C, *Yersinia enterocolitica*. (*A. ovis* and *B. bovis* are not shown because they were performed on a different blot).

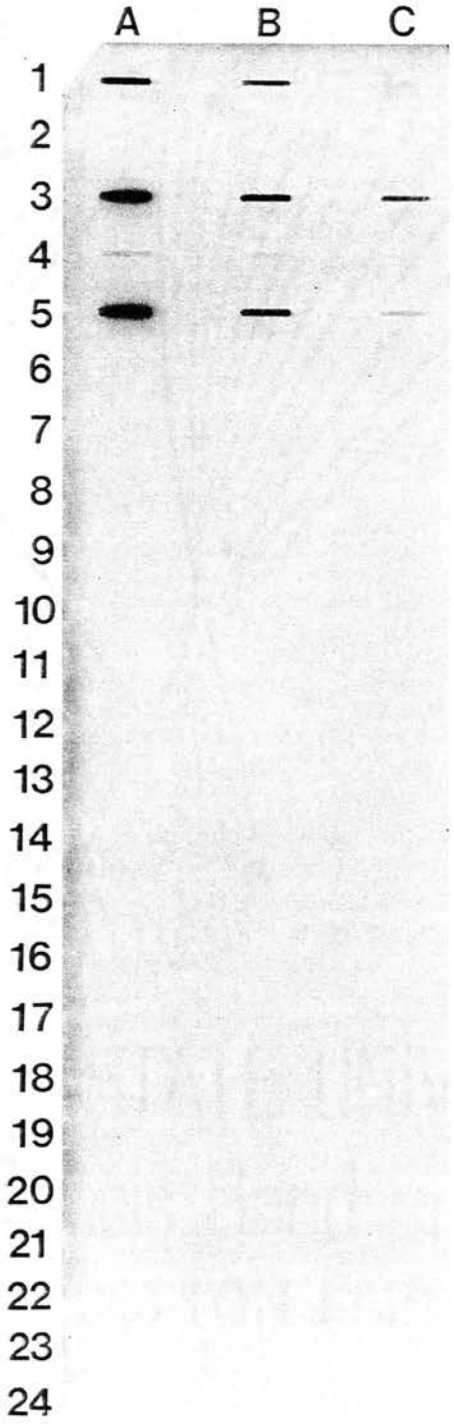
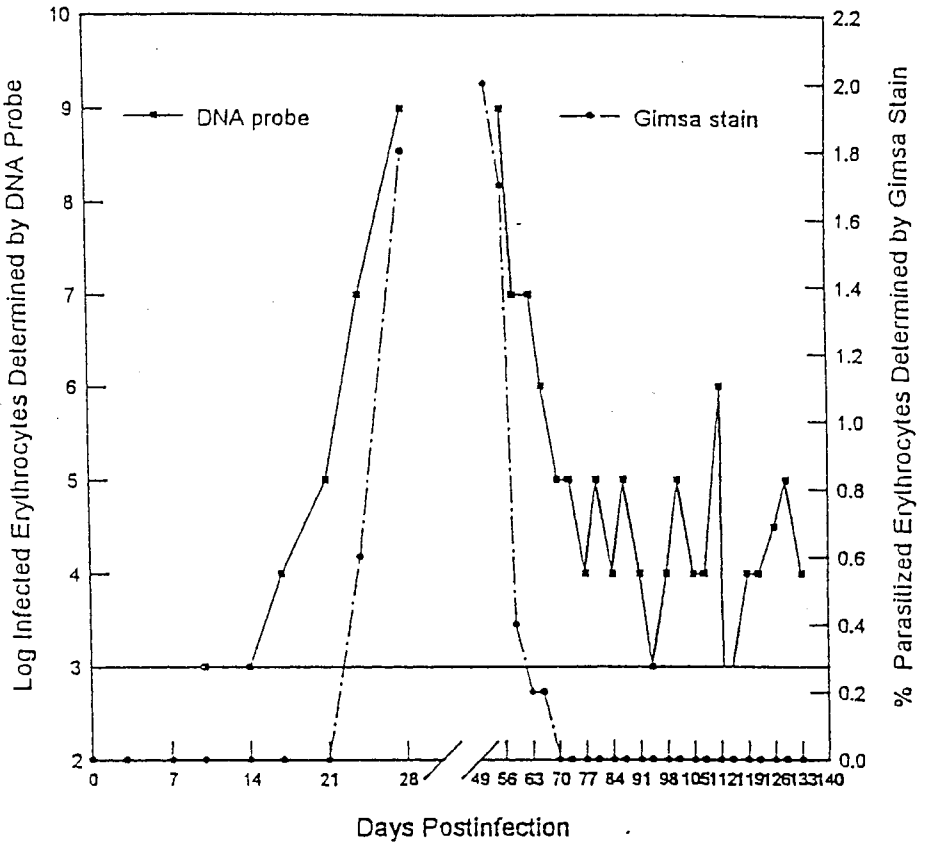

A
B
C
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24

Figure 4. Parasitemia levels determined by the nonradioactive DNA probe and by Giemsa stain during pre-acute infection and after acute disease in two experimentally infected calves.

The number of *A. marginale*-infected erythrocytes per 1.25 ml of blood determined by visual comparison of the intensities of the color hybridization signals obtained from infected blood with those of control standards in slot blot hybridization were expressed as Log_{10}. Levels below 10^3 infected erythrocytes per 1.25 ml of blood could not be detected by using this nonradioactive DNA probe. Parasitemias higher than 2-6% were not tested with this nonradioactive DNA probe.

PA 312


DNA probe
Gimsa stain
Log Infected Erythrocytes Determined by DNA Probe
% Parasilized Erythrocytes Determined by Gimsa Stain
Days Postinfection

PA 314

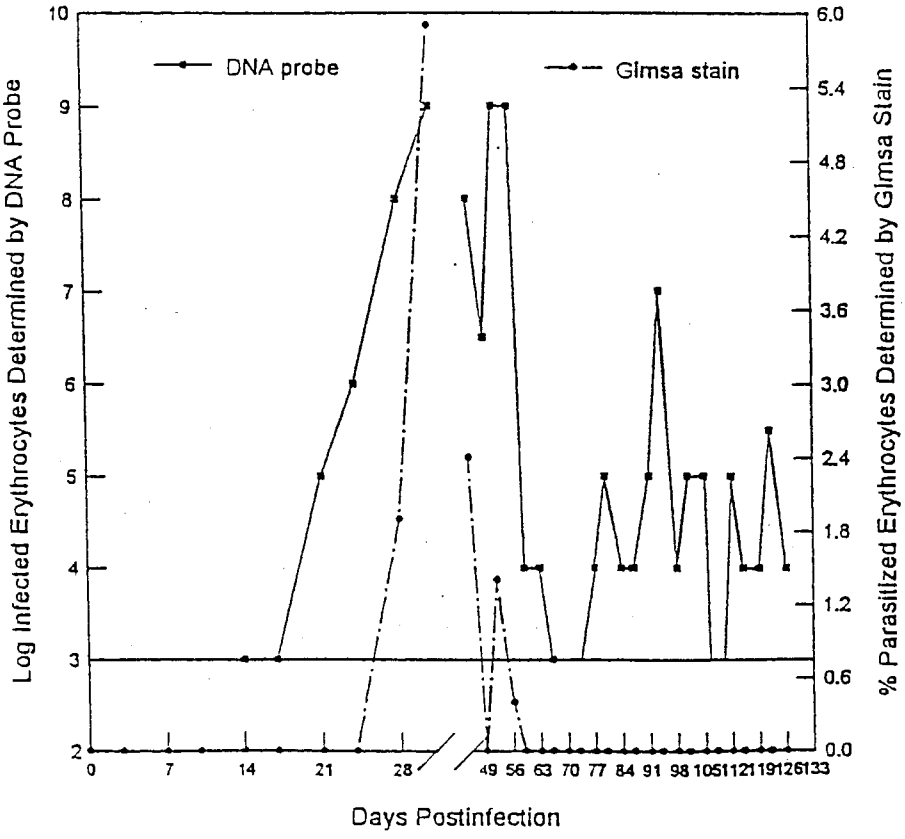
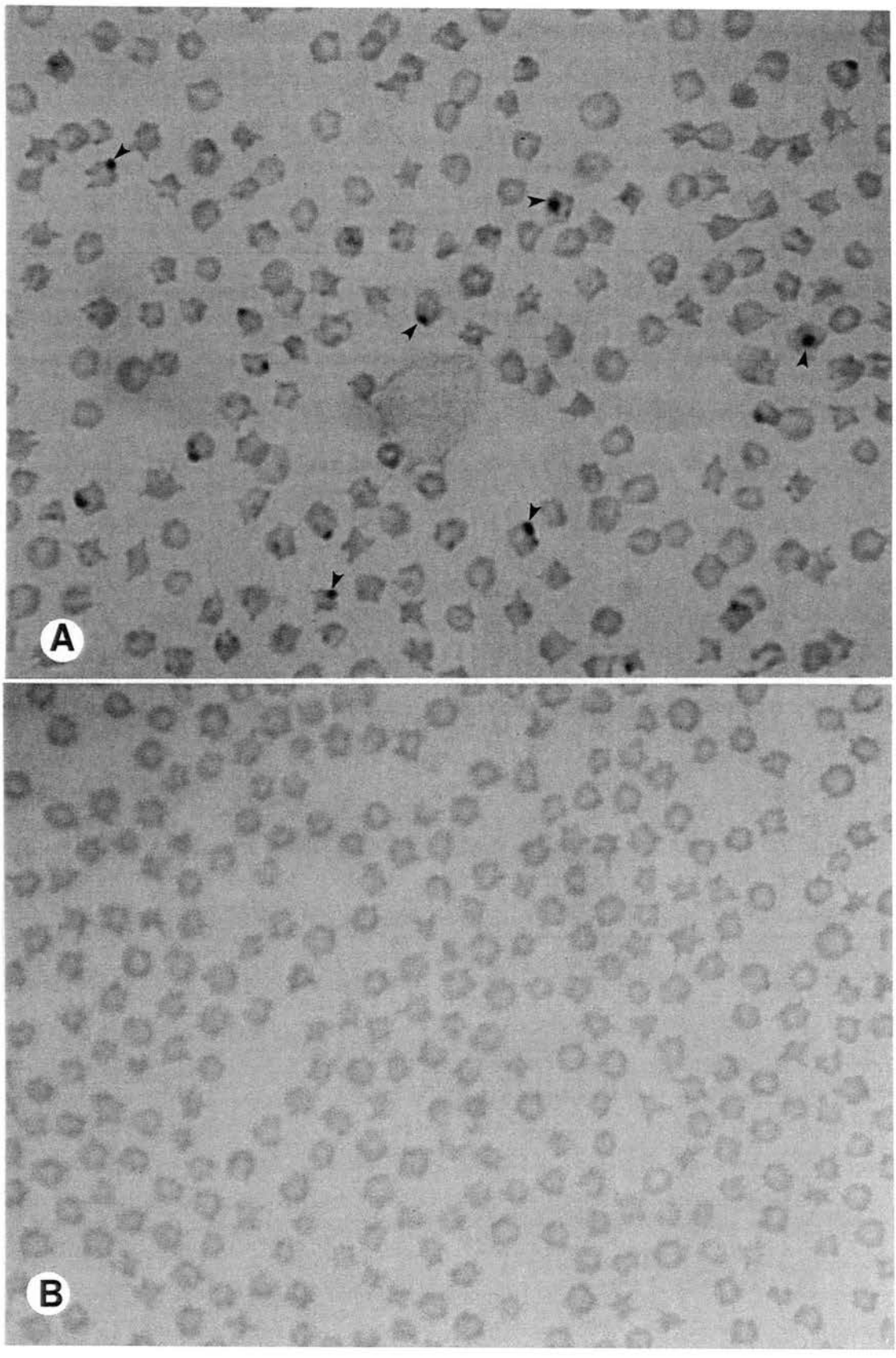

DNA probe
Gimsa stain
Log Infected Erythrocytes Determined by DNA Probe
% Parasilized Erythrocytes Determined by Gimsa Stain
Days Postinfection

Figure 5. In situ hybridization on methanol-fixed blood smears. .

(A) infected blood smear, probe reaction on *A. marginale* inclusions (arrows); (B) uninfected blood smear control.


A
B

CHAPTER III

USE OF A NONRADIOACTIVE DNA PROBE FOR DETECTION OF *Anaplasma marginale* INFECTION IN FIELD CATTLE: COMPARISON WITH COMPLEMENT FIXATION SEROLOGY AND MICROSCOPIC EXAMINATION

Abstract

A sensitive *Anaplasma marginale*-specific 409 bp DNA probe was developed in a previous study for detection of *A. marginale* infection in experimentally-infected cattle which employed slot-blot and in situ hybridization. In order to test the suitability of the probe to detect *A. marginale* in the blood of naturally-infected carrier cattle, slot-blot hybridization was used to determine the infection rate of *A. marginale* in cattle from 3 geographic areas in Oklahoma. For comparison, blood samples from the same cattle were also examined by light microscopy and were tested by the complement fixation (CF) test. For the DNA hybridization assay, the probe was labeled with digoxigenin 11-dUTP by polymerase chain reaction (PCR). DNA was extracted from blood using the QIAamp blood kit, then applied to a nylon membrane and hybridized with the probe. The study herds consisted of 31 beef cows in Harper County, OK, 42 & 70 dairy cows from Payne and Pittsburg Counties, OK, respectively. In the 3 herds, 80.6% (25/31), 92.8% (39/42) and 57.1% (40/70) cows were positive as assessed with the DNA hybridization assay. In contrast, only 25.8% (8/31) and 2.86% (2/70) were CF positive in 2 herds, and no CF positives (0/42) were found in one herd. Uncountable parasitemia (<0.01%) from 29.0% (9/31), 4.8% (2/42) and 11.4% (8/70) samples, respectively was demonstrated by microscopic examination. All CF- and microscopic-positive samples had positive probe reactions in the DNA hybridization assay. Therefore, this PCR-mediated nonradioactive DNA probe may be useful in epidemiological investigations and in identification of carrier cattle. This assay could be adapted for use in diagnostic laboratories because it is sensitive and specific, and is nontoxic, quickly executed, and inexpensive.

Introduction

Anaplasma marginale, an arthropod-borne intraerythrocytic rickettsia, is the causative agent of bovine anaplasmosis.[20] The rickettsial organism invades and multiplies in bovine erythrocytes, causing severe anemia, weight loss, abortion, and sometimes death during acute infection.[20] Cattle surviving acute disease may become permanent carriers and serve as reservoirs.[8,24,26] Currently, the disease is the only major tick-transmitted disease of cattle in the United States.[18] Annual mortality and morbidity in US beef cattle due to anaplasmosis have been estimated at 50,000-100,000 head, with production losses of $300 million.[18]

One of the major constraints for effective control of anaplasmosis has been a lack of accurate epidemiologic information, especially in the detection of *A. marginale* infection in carrier cattle in which parasitemia may be inapparent by microscopic examination.[14] In addition, serodiagnostic tests currently in use[3,19,21,22,23] do not have sufficient sensitivity and/or do not distinguish infected from uninfected, vaccinated cattle.[12,13,16,17] Although some sensitive and specific radioactively labeled nucleic acid probes were developed for detection of *A. marginale* in carrier cattle and/or ticks,[1,2,5,7,11,12,25] biohazard of radioactive compounds, short term of storage and extensive detection time of these probes have limited their use in routine diagnosis.

Recently, we labeled a 409-base pair (bp) DNA probe derived from the *msp-1β* gene[4] of *Anaplasma marginale* with digoxigenin (DIG)-11-dUTP by polymerase chain reaction (PCR).[10] The probe proved to be *A. marginale*-specific when tested with 17 species of microorganisms, including *A. ovis*, *Babesia bovis*, and *B. bigemina*.[10] The probe can detect *A. marginale* DNA from approximately 1,000-10,000 infected erythrocytes in 1.25 ml of whole blood, which is equivalent to a parasitemia level of 0.00001%.[10] Microscopically inapparent

parasitemias were also detected with the probe for 2 months after recovery from acute disease in 2 chronically-infected splenectomized cattle.[10]

In the present study, we report use of this PCR-mediated nonradioactive DNA probe to determine the infection rate of *A. marginale* in cattle from 3 geographic areas in Oklahoma and report on the ability of the probe to detect *A. marginale* in naturally-infected carrier cattle. The sensitivity of the probe is compared with that of complement fixation (CF) test and microscopic examination of stained blood smears.

Materials and Methods

Cattle

Three herds of cattle selected for this study consisted of 31 beef cattle from Harper County in northwestern Oklahoma, 42 and 70 dairy cows from Payne County in north central Oklahoma and Pittsburg County in southeastern Oklahoma. The herd in Harper County had an outbreak of anaplasmosis 5 months prior to this study (S. A. Ewing, unpublished data). There was no history of preventive tetracycline treatment in either dairy herd in Payne or Pittsburg, in which earlier serologic surveys using CF tests and fluorescent antibody (FIAX®) showed numerous seropositive animals.[15,21]

Two 5-ml blood samples from each cattle were collected in EDTA anticoagulant tubes for slot-blot hybridization and microscopic examination, and in non-anticoagulant tubes for CF test, respectively.

Source of Control Blood

Positive control blood samples included 6, 10-fold serial dilutions of *Anaplasma*-infected blood of known parasitemia from an experimentally-infected calf added to uninfected blood at final concentrations of 1,000,000-10 infected erythrocytes/1 X 10^{10} total erythrocytes. Negative controls included blood samples collected from 3 cattle that repeatedly tested negative for anaplasmosis by CF test, the DIG-labeled 409 bp DNA probe,[10] and microscopic examination over a period of 2 months.

Microscopic Examination

Blood smears were prepared from each sample collected in EDTA-anticoagulant tubes and stained with HEMA 3.[a] A positive sample was defined as a blood smear in which at least 5 typical *Anaplasma* inclusion bodies were observed.

CF Test

Serum of each blood sample was separated by centrifugation at 3,000 X g and aliquoted for CF test. The CF test was performed at Oklahoma Animal Diagnostic Laboratory (OADDL) as described previously.[21] Sera with titer of $\geq$ 5 in which none of the sheep erythrocytes were hemolyzed, were defined as positive reactors.

Extraction of DNA from Bovine Blood

QIAamp blood kit[b] was used to isolate DNA from bovine blood. A volume of blood containing 1 X 10^{10} erythrocytes from each animal was washed 2 times with phosphate-buffered saline (PBS; 0.137 M NaCl, 10 mM Na_2HPO_4, 3.2 mM KH_2PO_4) to remove buffy

coat. The erythrocytes were lysed by freeze-thawing and mixing with an equal volume of double-distilled water, after which the hemoglobin was removed by washing pelleted cells (13,000 X g for 3 min) with PBS. The pellet was resuspended in 200 µl PBS, mixed with 25 µl QIAGEN protease[b] and 200 µl Buffer AL[b] by vortexing for 15 sec, and incubated at 70 C for 10 min. After mixing with 210 µl of 100% ethanol, the lysate was applied to a QIAamp spin column, centrifuged at 13,000 X g for 1 min, and washed twice with 500 µl of Buffer AW[b]. DNA was eluted with 200 µl of distilled water preheated to 70 C. Control samples containing an equal volume of erythrocytes as test samples were processed as above.

DNA Hybridization Assay

The procedures used for generation of the DIG-labeled 409 bp DNA probe and slot blot hybridization were followed as described previously.[10] Briefly, a 409 bp DNA fragment derived from the *msp-1β* gene[4] of *A. marginale* was amplified and simultaneously labeled with digoxigenin-11-dUTP[c] by the PCR. The resulting DIG-labeled 409 bp PCR product was purified by ethanol precipitation and used as a probe for slot blot hybridization. For the DNA hybridization assay, DNA samples were first treated and bound to nylon membranes[d] using a slot-blot minifold apparatus[e], then prehybridized without the probe and hybridized with the denatured DIG-labeled 409 bp DNA probe overnight. The membrane was washed with decreasing concentration of standard saline citrate (SSC). The DIG-labeled probe was immunologically detected with anti-DIG Fab fragment conjugated to alkaline phosphatase[6] and the appropriate substrate system. The positive reaction was determined and quantitated by comparison of color intensities of test samples with those of positive controls.

Results

In the 3 herds, microscopic examination detected 9 of the 31 cattle (29.0%) in Harper County, 2 of the 42 cows (4.8%) in Payne County and 8 of the 70 (11.4%) in Pittsburg County as positives for *A. marginale* (Table III). All positive animals had parasitemias of <0.01% except for one from the Harper County herd that had 1.6% parasitemia. With the CF test, only 8 of the 31 (25.8%) samples in Harper, and 2 of the 70 (2.86%) in Pittsburg were identified as reactors, and no reactors were found in Payne.

Using slot blot hybridization, the DNA probe hybridized to 25 of the 31 (80.6%) samples in Harper, 39 of the 42 (92.8%) in Payne and 40 of the 70 (57.1%) in Pittsburg (Table III). In 6, 10-fold serial dilutions of positive controls, a faint band appeared on the 4th dilution that contained 1,000 infected erythrocytes and corresponds to a parasitemia level of 0.00001% (Figure 6). The sensitivity of the probe was confirmed at least as sensitive as described previously.[10] Considerable variations in color intensities of probe reaction indicated various levels of parasitemias among cattle. Compared with the positive controls, the positive animals had parasitemia levels between 0.00001% -0.001%. No probe reactions were observed in negative control samples.

Among a total of 143 cattle examined, all CF- and microscopic-positive samples were correlated with the DNA hybridization assay (Figure 7). Likewise, no negative probe reactions were observed in the CF- and microscopic-positive samples. However, 94 CF-negative and 85 microscopic-negative samples showed positive probe reactions in the DNA hybridization assay. The DNA hybridization proved to be considerably more sensitive in detection of carrier infections than either the CF test or microscopic examination (Figure 8).

Discussion

A number of radioactive nucleic acid probes have been developed for detection of *A. marginale*.[9] An RNA probe was developed to detect and quantitate *A. marginale* in 6 experimentally-infected carrier cattle, and 0.01 ng of *Anaplasma* genomic DNA and as few as 500-1,000 infected erythrocytes were detectable.[7] Parasitemia levels between 0.000025-0.0025% among carrier cattle were detectable with this probe.[7] In another study, a 2 kb DNA probe was used and compared with the CF test and the indirect immunofluorescence (IIF) test for detection of naturally infected carriers in an enzootic region of Washington State.[12] As assessed by the probe, 98.5% of cattle were positive with parasitemia levels between 0.00005 to 0.0005%, 92.3% of samples were seropositive with the IIF, and only 9.2% seropositive as determined by the CF test.[12] A similar pattern was observed in our study; the DIG-labeled DNA probe showed the highest sensitivity (Figure 8) and detected 80.6% (25/31), 92.8% (39/42) and 57.1% (40/70) positive carriers with parasitemia levels between 0.00001-0.001% in 3 selected herds from different geographic regions of Oklahoma. In contrast, only 25.8% (8/21) and 2.86% (2/70) were positive in two locations and no positives (0/42) in the third location as assessed with the CF test. Interestingly, microscopic examination correlated better with the probe than did the CF test, suggesting that it is more sensitive than the CF test (Figure 7). The sensitivity of our nonradioactive DNA probe was comparable to that reported for 2 radioactive ones developed earlier[7,12] and all the probes were much more sensitive than the CF test.

The herd in Harper County had an outbreak of anaplasmosis in January, 1995, in which four cases of acute disease had occurred (S. A. Ewing, unpublished data). Five *Dermacentor albipictus* ticks randomly collected from each of 9 cattle in the herd were tested with the same

DNA probe as used for this study and 23 of 45 ticks were positive. Companion ticks from the same cattle were pooled and transferred to a splenectomized calf; some of these ticks reattached and the animal developed anaplasmosis after a latent period of 22 days. The present study was conducted 5 months after that outbreak. In any case, the CF test revealed a much higher percent positive and higher antibody titer (20 versus 10) in this herd than in the 2 dairy herds in Payne and Pittsburg. Some animals in the herd might still have had high titers of anti-*Anaplasma* antibodies.

According to data collected at OADDL, 4.7-17.6% of 20,155 serum samples from Oklahoma cattle submitted from 1977 to 1991 were positive as assessed by the CF test.[21] There were 11.6 to 30% seropositive samples in Payne and Pittsburg in 1985, 1990 and 1991. However, an epidemiologic survey in 70 counties in Oklahoma using FIAX® microfluorometric immunoassay in 1978 to 1979 showed 55% (1,844/3,367) seropositive for anaplasmosis, in which 43% in Harper, 67% in Payne and 93% in Pittsburg were seropositive.[15] As shown in the present study and the study carried out in Washington State,[12] CF test was much less sensitive than DNA probe in detecting carrier infections. Other workers have also reported that the CF test was unable to identify some proven carriers.[13,17] Those results and our own suggest that the seropositivity for anaplasmosis as assessed by the CF test might be lower than the actual prevalence of *A. marginale* infection in Oklahoma cattle.

We used the QIAamp blood kit for extraction of DNA from bovine erythrocytes in this study. The time required for sample digestion and DNA extraction was significantly shortened; the entire procedure for 60 samples could be completed in 2 consecutive days. In original protocol provided by manufacturer, whole blood was used directly for protease digestion and residual hemoglobin was on the spin column, eluted into purified DNA samples, and stained

the nylon membrane. The stain on the membrane could not be removed and, therefore, was difficult to be identified with the color precipitate of probe reaction. In order to avoid this problem in the present study, we removed the hemoglobin in blood sample before digestion. Using this procedure, a larger volume of whole blood could be processed in a single extraction. This was especially useful for our present study in which DNA extraction was made from larger amounts of whole blood.

In summary, this PCR-mediated nonradioactive DNA probe is highly sensitive and specific for *A. marginale* and provides many advantages over radioactive probes. The probe can be prepared and stored a minimum of one year and used repeatedly, thus significantly reducing the cost of the test. Furthermore, special safety and disposal procedures are not required. The test is quickly executed and the entire procedure can be completed in 2 consecutive days. The results of our study suggest that this nonradioactive DNA probe would be useful in epidemiological investigations and in identification of convalescent carriers of *A. marginale.*

Acknowledgments

Submitted for publication by Nie-Lin Ge,[1] Katherine M. Kocan,[1] S. A. Ewing,[2] Edmour F. Blouin,[1] Wanda L. Edwards,[1] George L. Murphy[1] & Lionel J. Dawson[3] to *Journal of Veterinary Diagnostic Investigation*.

Technical assistance from Ms. Peichun Hu was greatly appreciated. This study was supported in part by Oklahoma Agricultural Experimental Station Projects 1433 and 1669.

[1] Department of Pathology, Colloge of Veterinary Medicine, Oklahoma State University, Stillwater, Oklahoma 74078

[2] Department of Parasitology, Microbiology & Public Health, Colloge of Veterinary Medicine, Oklahoma State University, Stillwater, Oklahoma 74078

[3] Department of Medicine and Surgery, Colloge of Veterinary Medicine, Oklahoma State University, Stillwater, Oklahoma 74078

Sources and Manufacturers

a. Curtin Matheson Scientific, Inc., Houston, TX.

b. QIAGEN Inc., Chatsworth, CA.

c. Boehringer Mannheim Biochemicals, Indianapolis, IN.

d. Micron Separations Inc. (MSI), Westboro, MA.

e. Schleicher & Schuell Inc., Keene, NH.

References

1. Aboytes-Torres R, Buening GM : 1990, Development of a recombinant *Anaplasma marginale* DNA probe. Vet Microbiol 24: 391-408.

2. Ambrosio RE, Visser ES, Koekhoven Y, et al.: 1988, Hybridization of DNA probes to *Anaplasma marginale* isolates from different sources and detection in *Dermacentor andersoni* ticks. Onderstepoort J Vet Res 55: 227-229.

3. Amerault TE, Roby TO: 1968, A rapid card agglutination test for bovine anaplasmosis. J Am Vet Med Assoc 153: 1828-1834.

4. Barbet AF, Allred D: 1991, The *msp1-β* multigene family of *Anaplasma marginale*: nucleotide sequence analysis of an expressed copy. Infect Immun 59: 971-976.

5. Blouin EF, Kocan KM, Murphy GL, Ge NL: 1993, Persistence of tick-derived *Anaplasma marginale* in cultured bovine turbinate and endothelial cells. Rev d'elevage et de Med Vet des Pays Trop 46: 64-66.

6. Boehringer Mannheim: 1992, The Genius™ system user's guide for filter hybridization application manual. pp. 1-84. Boehringer Mannheim GmbH Biochemica, Germany.

7. Eriks IS, Palmer GH, McGuire TC, et al.: 1989, Detection and quantitation of *Anaplasma marginale* in carrier cattle by using a nucleic acid probe. J Clin Microbiol 27: 279-284.

8. Eriks IS, Stiller D, Palmer GH: 1993, Impact of persistent *Anaplasma marginale* rickettsemia on tick infection and transmission. J Clin Microbiol 31: 2091-2096.

9. Figueroa JV, Buening GM: 1995, Nucleic acid probes as a diagnostic method for tick-borne hemoparasites of veterinary importance. Vet Parasitol 75: 75-92.

10. Ge NL, Kocan KM, Murphy GL, Blouin EF: 1995, Detection of *Anaplasma marginale* DNA in bovine erythrocytes by slot-blot and in situ hybridization with a PCR-mediated digoxigenin-labeled DNA probe. J Vet Diagn Invest 7: 465-472.

11. Goff WL, Barbet AF, Stiller D, et al.: 1988, Detection of *Anaplasma marginale* in tick vectors using a cloned DNA probe. Proc Natl Acad Sci USA 88: 919-923.

12. Goff WL, Stiller D, Roeder RA, et al.: 1990, Comparison of a DNA probe, complement-fixation and indirect immunofluorescence tests for diagnosing *Anaplasma marginale* in suspected carrier cattle. Vet Microbiol 24: 381-390.

13. Gonzales EF, Long RF, Todorovic RA: 1978, Compasions of the complement fixation, indirect fluorescent antibody and card agglutination tests for the diagnosis of bovine anaplasmosis. Am J Vet Res 39: 1538-1541.

14. Kieser ST, Eriks IS, Palmer GH: 1990, Cyclic rickettsemia during persistent *Anaplasma marginale* infection of cattle. Infect Immun 58: 1117-1119.

15. Logan TM, Fox JC, Hair JA, Kocan KM, et al.: 1985, The prevalence and distribution of anaplasmosis in Oklahoma. J Oklahoma Vet Med Assoc 37: 96-99.

16. Luther DG, Cox HU, Nelson WO: 1980, Compasions of serotests with calf inoculations for detection of carriers in anaplasmosis-vaccinated cattle. Am J Vet Res 41: 2170-2172.

17. Maas J, Lincoln SD, Coan ME, et al.: 1986, Epidemioloic aspects of bovine anaplasmosis in semiarid range conditions of south central Idaho. Am J Vet Res 47: 528-533.

18. McCallon BR: 1973, Prevalence and economic aspects of anaplasmosis, pp. 1-3. In: Proc 6th Natl Anaplasmosis Conf, Las Vegas, NV.

19. Ristic M: 1962, A capillary-tube agglutination test for anaplasmosis: a preliminary report. J Am Vet Med Assoc 141: 588-594.

20. Ristic M: 1981, Anaplasmosis. In: Diseases of cattle in the tropics, eds. Ristic M, McIntyre I, pp. 327-344. Martinus Nijhoff, Boston, MA.

21. Rodgers SJ, Welsh RD, Stebbins ME: 1994, Seroprevalence of bovine anaplasmosis in Oklahoma from 1977 to 1991. J Vet Diagn Invest 6: 200-206.

22. Schunter CA, Leatch G: 1988, Radioimmunoassay for *Anaplasma marginale* antibodies. Am J Vet Res 49: 504-507.

23. Shkap V, Bin H, Ungar WH, et al.: 1990, An ELISA for the detection of antibodies to *Anaplasma centrale* and *A. marginale*. Vet Microbiol 25: 45-53.

24. Swift BL, Thomas GM: 1983, Bovine anaplasmosis: elimination of the carrier state with injectable long-acting oxytetracycline. J Am Vet Med Assoc 183: 63-65.

25. Visser ES, Ambrosio RE: 1987, DNA probes for the detection of *Anaplasma centrale* and *Anaplasma marginale*. Onderstepoort J Vet Res 54: 623-627.

26. Zaug JL, Stiller D, Coan ME, Lincoln SD: 1986, Transmission of *Anaplasma marginale* Theiler by males of *Dermacentor andersoni* Stiles fed on an Idaho field infected, chronic carrier cow. Am J Vet. Res. 47: 2269-2271.

Table III. Infection rates determined by the DNA hybridization assay, CF test and microscopic examination

Test	Harper County	Payne County	Pittsburg County
DNA hybridization	80.6% (25/31)*	92.8% (39/42)	57.1% (40/70)
CF test	25.8% (8/31)	0.0% (0/42)	2.86% (2/70)
Microscopic examination	29.0% (9/31)	4.8% (2/42)	11.4% (8/70)

*positve samples/total samples examined

Figure 6. Representative slot-blot hybridization results of the DIG-labeled DNA probe with DNA extracted from bovine blood representing 2 herds from Harper and Pittsburg Counties in Oklahoma and controls.

Slots: 1A-2C, 6, 10-fold serial dilutions of known numbers (1×10^6-1×10^1) of infected erythrocytes equivalent to 1.25 ml whole blood (~1×10^{10}); 3A-4C, 3 double 1.25 ml blood samples from 3 cattle repeatedly tested negative for anaplasmosis by CF test, the DIG-labeled 409 bp DNA probe and microscopic examination over a period of 2 months; 5A-24C, 60 blood samples (1.25 ml) collected from herds in Harper (31) and Pittsburg Counties (29).

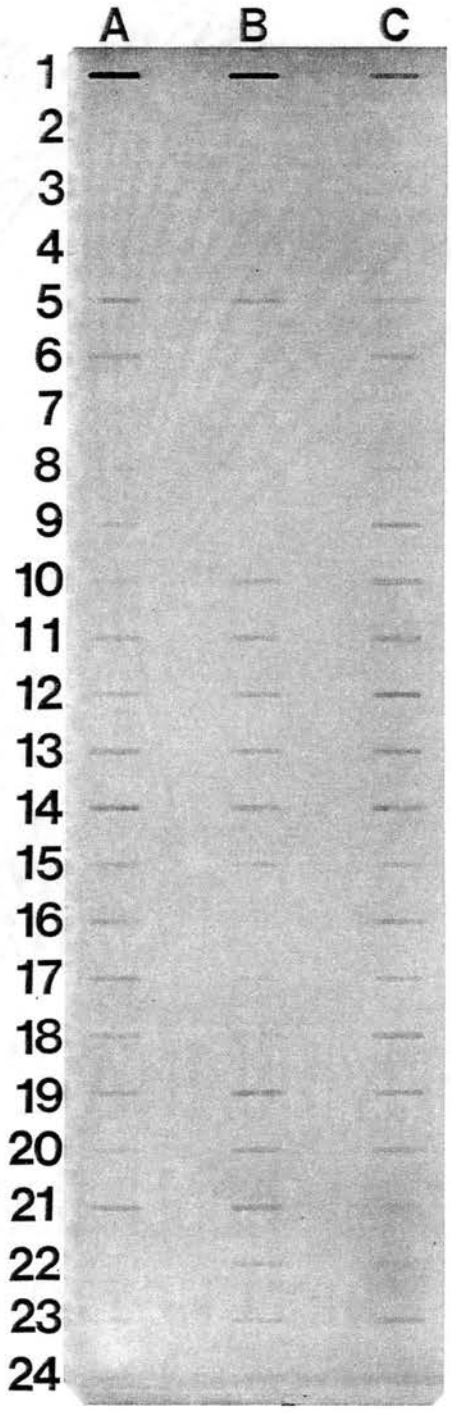
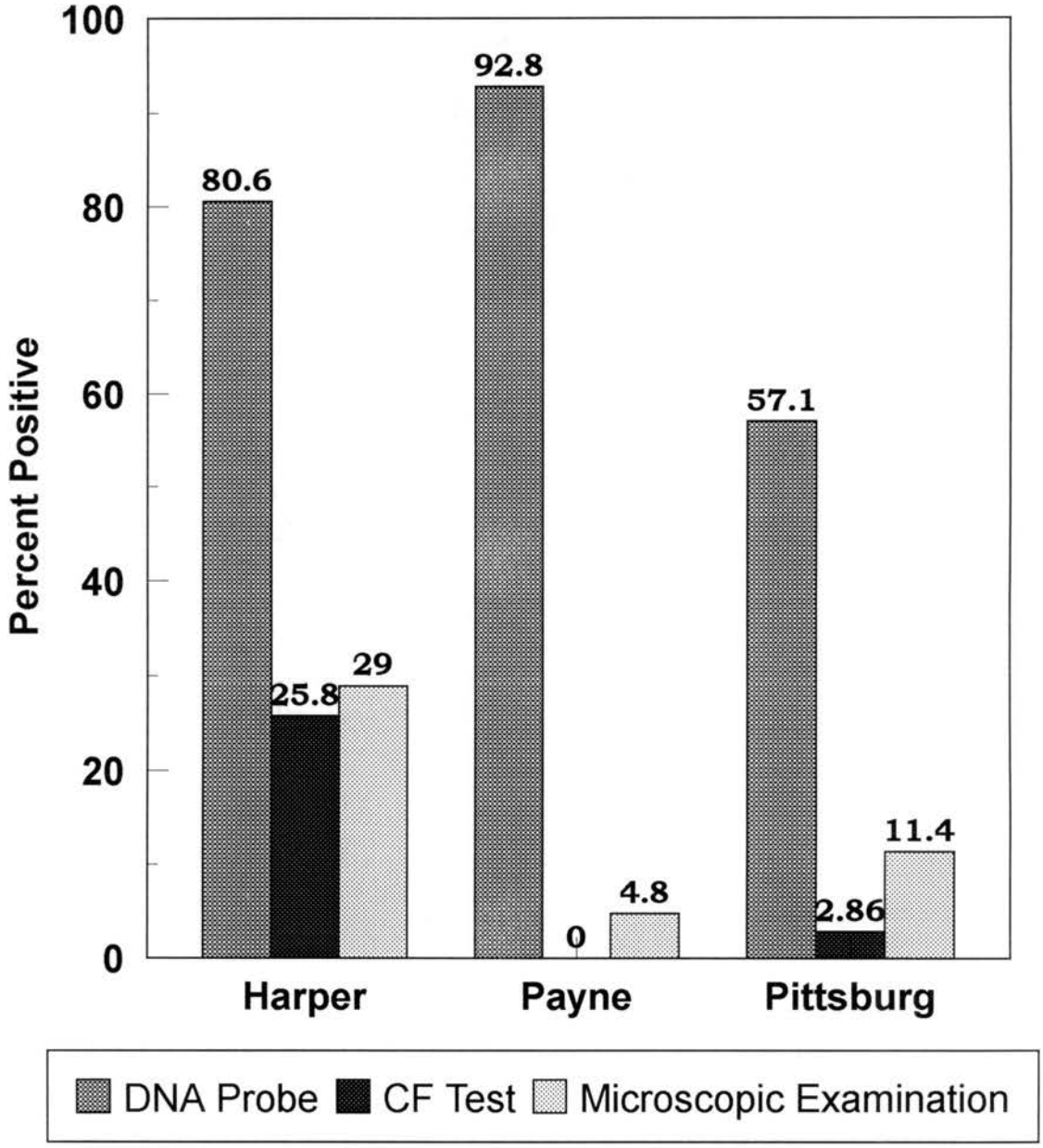

A
B
C
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24

Figure 7. Comparison of efficacy of CF test and microscopic examination with the DIG-labeled DNA probe for detection of *A. marginale* infections in 3 herds from 3 different geographic regions in Oklahoma.

CF TEST		DNA PROBE -	DNA PROBE +
	-	39	94
	+	0	10

MICROSCOPIC EXAMINATION		DNA PROBE -	DNA PROBE +
	-	39	85
	+	0	19

Figure 8. Comparison of *A. marginale* infection rates in 3 herds of cattle from 3 geographic regions in Oklahoma determined by the DNA probe, CF test and microscopic examination.


100
80
60
40
20
0
Percent Positive
80.6
25.8
29
92.8
0
4.8
57.1
2.86
11.4
Harper
Payne
Pittsburg
DNA Probe
CF Test
Microscopic Examination

CHAPTER IV

DEVELOPMENT OF NONRADIOACTIVE IN SITU HYBRIDIZATION FOR DETECTION OF *Anaplasma marginale* IN TICKS

Abstract

In situ hybridization (ISH), which allows for localization of pathogen nucleic acid in tissue sections, was developed to detect *Anaplasma marginale*, a rickettsial pathogen of cattle, in its tick vector. *Dermacentor andersoni* male ticks were experimentally infected with *A. marginale* and one half of each of 20 ticks was embedded in paraffin or LR White for ISH, while the companion halves were embedded in DER resin for light microscopy (LM). Sections were digested with proteinase K and hybridized with a digoxigenin-labeled DNA probe. In both paraffin- and LR White-embedded sections, dark-blue color precipitates of hybridization signals were visualized in both salivary gland and gut cells. *Anaplasma* infections were also confirmed by LM in companion tick halves. Positive hybridization signals and *A. marginale* colonies were not seen in uninfected control tissues. Although ISH detected *A. marginale* in both paraffin- and LR-White embedded sections, LR White was found to be optimum for ISH of *A. marginale* because of improved morphological perservation and the superior resolution of the 1μm sections, allowing for definitive identification of tick tissues that contained parasites as well as host cells that were not infected.

Key Words: *Anaplasma marginale*, *Dermacentor andersoni*, tick, DNA probe, in situ hybridization

Introduction

Anaplasma marginale is an intraerythrocytic rickettsia that causes bovine anaplasmosis (1). Although *A. marginale* can be transmitted mechanically by biting flies and blood-contaminated fomites, ixodid ticks are the only known biological vector (2). Biological transmission occurs by transfer of adult ticks from infected to susceptible cattle (intrastadial transmission), or by nymphs or adults infected in a previous stage (interstadial or transstadial transmission) (3,4). Although both male and female ticks can transmit *A. marginale* intrastadially, adult males are believed to be of primary importance because they are persistently infected, transfer readily among cattle and maintain high infection rates while off the host (2,5).

Much of the developmental cycle of *A. marginale* has been described in *Dermacentor* ticks (2-6). Within male ticks infected as adults, *A. marginale* develops within midgut epithelial, gut muscle and salivary gland cells. In adult ticks infected as nymphs, *A. marginale* was detected in salivary glands with a 2 kb DNA probe derived from the *A. marginale msp-1β* gene (7), but recognizable forms of the parasite were not observed with light and electron microscopy (LM and EM) (8). A technique for detection of the *Anaplasma* genome was needed that would permit localization of this rickettsia within tick cells in order to clarify various aspects of the developmental cycle.

In situ hybridization (ISH) allows for detection of specific nucleic acid sequences in morphologically preserved cells or tissues (9). Development of ISH for detection of microbial genomes within tissues has made it possible to trace the path of organisms through host tissues with great precision (10), and to detect pathogens in infected tissue sections (10-13). In a previous study, a 409 bp DNA fragment derived from the *msp-1β* gene of *A. marginale* was amplified, simultaneously labeled with digoxigenin (DIG)-11-dUTP by PCR, and used as a

probe to detect *A. marginale* from bovine erythrocytes (14). The probe was found to be sensitive and specific for *A. marginale*. In the slot-blot DNA hybridization assay, the probe detected *A. marginale* DNA from approximately 1,000-10,000 infected erythrocytes in 1.25 ml of whole blood, which is equivalent to a parasitemia level of 0.00001%. Microscopically inapparent parasitemias were also detected with the probe in both experimentally- and naturally-infected carrier cattle. The probe was then used for ISH to detect *A. marginale* in bovine erythrocytes on methanol-fixed blood smears.

In this study, we report development of ISH using this nonradioactive DNA probe for detection of *A. marginale* in experimentally-infected ticks. Two embedding media, paraffin and LR White, were compared for their suitability for ISH of *A. marginale* in ticks.

Materials and Methods

Propagation and Infection of Ticks

Male *Dermacentor andersoni* ticks were reared at the Oklahoma State University Centralized Tick Rearing Facility, Department of Entomology (15). Larvae and nymphs, not exposed to *A. marginale,* were fed on rabbits and allowed to molt to the subsequent stage. Adult males were held in a humidity chamber (90-98%, RH) at 25 ^{0}C with a 14-hour photophase period until used for this study.

Infection of male ticks has been optimized in our laboratory as described previously (2,8). Briefly, male *D. andersoni* were placed in an orthopedic stockinette and attached to a donor calf experimentally-infected with *A. marginale* (Virginia isolate) when the ascending parasitemia was between 3 and 5%. After feeding for 7 days, the ticks were removed from the calf, and placed in a humidity chamber for 5 days. The ticks were allowed to feed on a

susceptible calf for 10 days before being dissected for ISH studies. Uninfected male ticks, fed on a susceptible calf in the same manner, served as controls.

Preparation of Tick Tissues for ISH

Two groups of 20 ticks were used for this study, one to test paraffin embedding and the other to test LR White embedding. The ticks in each group were cut in half with a razor blade, separating the right and left sides. One half of each tick was processed for either paraffin or LR White embedding, while the other half of each tick was embedded in DER resin for routine LM. Negative control ticks were collected and processed in a similar manner.

For paraffin embedding, the tick halves were fixed in 4% phosphate-buffered formaldehyde (pH 7.2) for 4 hours, dehydrated and embedded at Histology Laboratory of the Oklahoma Animal Disease Diagnostic Laboratory. Sections (4-μm) were cut and mounted onto Fisher ProbeOn Plus slides (Fisher Scientific, Pittsburgh, PA).

For LR White embedding, tick halves were fixed in 4 % phosphate-buffered paraformaldehyde (pH 7.2) for 4 hours at 4 ^{0}C. After washing twice for 15 min in phosphate-buffered saline (PBS, pH 7.2; 0.137 M NaCl, 10 mM Na_2HPO_4, 3.2 mM KH_2PO_4), the samples were dehydrated in 70% ethanol 2 X 20 min, and infiltrated in LR White (EMS, Ft. Washington, PA) mixed with 70% ethanol at ratios of 1:2, 1:1 and 2:1 for 30 min in each. The tick halves were then infiltrated for 1 hour in 100% LR White, followed by infiltration in fresh LR White overnight. The tick halves were placed in gelatin capsules containing LR White and polymerized at 60 ^{0}C for 24 hrs. Sections were cut at 1 μm thickness using an MT-8000 microtome (Research and Manufacturing Company, Tucson, AZ) and mounted on the Fisher ProbeOn Plus slides.

Probe Labeling and ISH

The procedure used for generation of the DIG-labeled 409 bp DNA probe was followed as described previously (14). A 409 bp DNA fragment derived from the *msp-1β* gene (7) of *A. marginale* was amplified by PCR and simultaneously labeled with digoxigenin-11-dUTP (Boehringer Mannheim Biochemicals, Indianapolis, IN). The resulting DIG-labeled 409 bp product was purified by ethanol precipitation and used as a probe for this study.

For ISH on paraffin-embedded sections, the sections on slides were deparaffinized with xylene and rehydrated with decreasing concentrations of ethanol as described previously (16). To permeate the tissues, sections were digested with proteinase K (25 μg/ml) in 100 mM Tris-HCl, pH 8.0, 50 mM ethylenediaminetetraacetic acid [EDTA] (TE 100, 50) at 42 ^{0}C for 20 min, followed by washing in PBS 2 X 5 min. Slide pairs (1 infection and 1 control) were incubated with prehybridization solution (50% formamide, 5 X standard saline citrate (SSC), 2% blocking reagent (Boehringer Mannheim Biochemicals, Indianapolis, IN), 0.1% sarkosyl, 0.02% SDS, and 0.1% salmon sperm DNA) for 1 hr using a MicroProbe slide incubator (Fisher Scientific, Pittsburgh, PA). After denaturing the target DNA by raising the temperature to 84 ^{0}C for 10 min, 30 ng of the heat-denatured DIG-labeled DNA probe in 150 μl prehybridization solution containing 5% dextran sulfate was introduced into each slide pair and incubated at 42 ^{0}C in a sealed humidity chamber overnight. Slides were washed twice for 5 min with 2 X SSC and twice for 15 min with 0.1% SSC at 50 ^{0}C. Prior to immunological detection of the DIG-labeled DNA probe, the slides were rinsed in 0.3% Triton X-100 for 2 min. Following treatment with 1% blocking reagent, sections were incubated with 1:750 diluted anti-DIG antibody conjugated with alkaline phosphatase (Boehringer Mannheim Biochemicals, Indianapolis, IN). Color development was achieved by covering sections with the chromogenic

substrates, 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and nitroblue tetrazolium salt (NBT) for 4 hr. Development was stopped by rinsing in TE (100, 50) buffer. Slides were air dried, counterstained with eosin, coverslipped in Permount, and observed with LM.

Hybridization conditions used for LR White-embedded sections were similar to those described above. The requirements for achieving the best hybridization signals were less stringent than on paraffin-embedded sections and are summarized in Table IV.

Routine Light Microscopy

Companion tick halves were fixed in cold 2% glutaraldehyde in a 0.25 M sodium cacodylate buffer (pH 7.2) and post-fixed in 2% cacodylate-buffered osmium tetroxide. Subsequently, the fixed tissues were washed three times with 0.02 M cacodylate buffer (pH 7.2), dehydrated through a graded series of ethanol, and infiltrated with DER epoxy resin, using propylene oxide as the intermediate solvent. Sections (1 μm) of tick halves were cut, stained with Mallory's stain for 2 min at 60 ^{0}C, and examined with LM for colonies of *A. marginale*.

Results

Infection of Ticks and Transmission of Anaplasmosis

Male ticks used for this study were from a group of ticks exposed to *A. marginale* on a donor calf that developed a peak parasitemia of 12.0% during tick feeding. After a second feeding on a susceptible calf, the ticks transmitted anaplasmosis with a prepatent period of 26 days and a peak parasitemia of 5.8%. The uninfected calf used for feeding of uninfected (control) ticks, did not develop anaplasmosis and was subsequently proved to be susceptible to

A. marginale by challenge-exposure.

In Situ Hybridization

At least 3 continual sections of each tick half were examined by ISH. Positive hybridization signals were obtained from tick halves embedded with paraffin or LR White (Table V). On paraffin-embedded sections, hybridization signals appeared as dense, diffuse blue-black granularities (Figures 9a-c). Whereas, on LR White-embedded sections, hybridization signals appeared as delicate dot-like blue-black precipitates (Figures 10 a-c). On both paraffin- and LR White-embedded half tick sections, salivary glands were more frequently found to be infected by ISH (Table V). ISH signals were not observed on sections from the uninfected controls.

Microscopy Studies

In companion tick-halves examined by routine LM, colonies of *A. marginale* were present predominantly in salivary glands (Figure 11a) and, with less frequency, in gut tissues (Figure 11b), confirming the findings of ISH studies. A comparison of results obtained from ISH and LM studies is presented in Table V.

Discussion

In this study, ISH detected *A. marginale* DNA in both paraffin- and LR White-embedded sections of experimentally-infected ticks. Specific hybridzation signals were obtained by determining the optimal conditions of tissue fixation, protein digestion, DNA denaturation and hybridization, and DIG-labeled probe detection (Table IV).

Formalin fixed, paraffin embedded tissues were reported to be the best choice for ISH

in a diagnostic setting (17). ISH procedures could be performed on routine histological sections without modification and tissue morphology was adequately preserved (18). Our experiments demonstrated that hybridization conditions were easily adapted from those used for mammalian tissues and optimized for detection of the *A. marginale* genome in paraffin-embedded sections of ixodid ticks. While strong hybridization signals in infected tick tissues were obtained on paraffin-embedded sections, identification of tissue types from which hybridization signals originated was difficult due to the thickness of sections and the digestion of proteins required for unmasking DNA.

Although LR White is a water-soluble resin, it was more difficult for the DNA probe to penetrate tissues than in paraffin-embedded sections. Compared with the hybridization conditions applied on paraffin-embedded sections, less stringency of hybridization and post hybridization washing were used on LR White-embedded sections (Table IV). In addition, the higher concentration of proteinase K (500 μg/ml), 50-500 times that used for LR White-embedded neonatal human skin (19) and EBV-infected cells (20), and 20 times more than used on paraffin-embedded sections, was required in this study for unmasking DNA for ISH on LR White-embedded sections. ISH on LR White sections was not achieved with proteinase K digestion lower than 200 μg/ml. These large amount of proteinase K did not adversely affect identification of tissue types. Denaturation of target DNA on the LR White sections was best obtained with 0.5N NaOH incubation combined with heating at 95 ^{0}C; this combination resulted in good ISH signals and in retention of tissue morphology. Although the intensity of hybridization signals was often weaker than that on paraffin-embedded sections, the tissues where positive hybidization signals were located were easily identified because of the better tissue preservation and higher resolution of the 1 μm sections. Other studies also demonstrated

that LR White-embedded sections resulted in the best combination of morphological preservation and ISH labeling among 4 different plastic embedding media used (19). In addition, ultrathin sections could be cut from the same tissue blocks and used for electron microscopy and ISH studies. LR White/ISH was reported to be considerably easier to perform than the lowicryl procedure which requires a specially equipped laboratory (20). In this study, LR White was found to be the embedding medium of choice for ISH for study of *A. marginale* in ticks.

In the present study, positive hybridization signals of *A. marginale* within colonies were observed in both gut and salivary gland tissues which have been described previously as sites of development of *A. marginale* (3,4). Hemocytes may be an intermediate site of infection of *A. marginale* prior to invasion of salivary gland cells. *Anaplasma marginale* DNA has been detected in hemolymph by PCR assay (21), but recognizable forms of *A. marginale* were not seen with LM or EM extracellularly or within hemocytes in the companion collections. Regulation of gene expression via transcription, mRNA processing, translation or post-translational modification could cause changes in morphology and antigenic composition of *A. marginale* in ticks (22). The genome, however, should remain more stable throughout the rickettsial life cycle. Therefore, all stages, including those previously unrecognized forms of *A. marginale* that may occur in hemolymph or salivary glands of adult ticks infected as nymphs, could be detected with the ISH. The study of the role of hemolymph and salivary glands in the development of *A. marginale* in ticks using ISH methods developed herein is currently underway.

In a previous study, ISH was used for determination of the presence or absence of *A. marginale* in non-erythrocyte cells from experimentally-infected cattle using a ^{35}S labeled

recombinant DNA probe (22). Specific radiolabeled signal was found only in association with erythrocytes in tissues of liver and lymph nodes. However, the lack of resolving power of the radiolabeled probe and abundant non-specific probe binding limited the usefulness of this ISH. DIG labeling and immunological detection system has been found capable of producing high-resolution localization of nucleic acid with minimal background at the LM level (16). In our study, good hybridization signals were obtained on both paraffin- and LR White-embedded sections without background staining. Thus the probe may also be useful for studying of pathogenesis of *A. marginale* in cattle by ISH. In addition, the methodology of ISH on half-tick sections may be adapted for study of other tick-transmitted rickettsiae, such as *Ehrlichia* and *Cowdria*, as well as for tick-borne viral pathogens of veterinary and medical importance of which the life cycles have not been clearly defined.

Acknowledgment

Submitted for publication by Nie-Lin Ge,[1] Katherine M. Kocan,[1] George L. Murphy[1] & Edmour F. Blouin,[1] to *The Journal of Histotechnology*.

The authors like to thank the excellent technical assistance of Ms. Wanda Edwards, Peichun Hu and Jeanenne Duffy. Critical review of this manuscript from Dr. S. A. Ewing was greatly appreciated. This study was supported in part by Oklahoma Agricultural Experimental Station Projects 1433 and 1699.

[1] Department of Pathology, College of Veterinary Medicine, Oklahoma State University, Stillwater, Oklahoma 74078.

References

1. Ristic M: Anaplasmosis. In *Diseases of cattle in the tropics*, Ristic M, McIntyre I (eds). Martinus Nijhoff, Boston, 1981, pp. 327-344

2. Kocan KM, Goff WL, Stiller D et al: Persistence of *Anaplasma marginale* (Rickettsiales: Anaplasmataceae) in male *Dermacentor andersoni* (Acari: Ixodidae) transferred successively from infected to susceptible calves. *J Med Entomol* 29: 657-668, 1992

3. Kocan KM, Ewing SA, Holbert D, Hair JA: Morphologic characteristics of colonies of *Anaplasma marginale* Theiler in midgut epithelial cells of *Dermacentor andersoni* Stiles. *Am J Vet Res* 43: 586-593, 1983

4. Kocan KM, Stiller D, Goff WL et al: Development of *Anaplasma marginale* in male *Dermacentor andersoni* transferred from parasitemic to susceptible cattle. *Am J Vet Res* 53: 499-507, 1992

5. Stiller D, Coan ME, Goff WL et al: The importance and putative role of *Dermacentor* spp. males in anaplasmosis epidemiology: transmission of *Anaplasma marginale* to cattle by ad libitum interhost transfer of *D. andersoni* males under semi-natural conditions (abstract). *Proceedings 8th Natl Vet Hemoparasite Dis Conf* St. Louis, MO, 1989

6. Stiller D, Kocan KM, Edwards W et al: Detection of colonies of *Anaplasma marginale* Theiler in salivary glands of three *Dermacentor* spp. infected as nymphs or adults. *Am J Vet Res* 50: 1381-1386, 1989

7. Barbet AF, Allred D: The *msp-1β* multigene family of *Anaplasma marginale*: Nucleotide sequence analysis of an expressed copy. *Infect Immun* 59: 971-976, 1991

8. Kocan KM, Stiller D, Goff WL et al: Development of *Anaplasma marginale* in salivary glands of male *Dermacentor andersoni*. *Am J Vet Res* 54: 107-112, 1993

9. Coghlan JP, Aldred P, Haralambidis J et al: Hybridization histochemistry. *Anal Biochem* 149: 1-28, 1985

10. Brown CC, Meyer KF, Olander HJ et al: A pathogenesis study of foot and mouth disease in cattle using in situ hybridization. *Can J Vet Res* 56: 189-193, 1992

11. McLaughlin GL, Montenegro-James S, Vodkin MH et al: Molecular approaches to malaria and babesiosis. *Mem Inst Oswaldo Cruz Riode Janeiro* 87: 57-68, 1992

12. Brown CC, Meyer KF, Grubman MJ: Identification of African horse sickness virus in cell culture using a digoxigenin-labeled RNA probe. *J Vet Diagn Invest* 6: 153-155, 1994

13. Nuovo GJ, Margiotta M, Gallery F, Forde A: A comparison of subgenomic and genomic DNA probes for detection and typing of human papillomavirus by in situ hybridization. *J Histotechnol* 18: 105-110, 1995

14. Ge NL, Kocan KM, Murphy GL, Blouin EF: Detection of *Anaplasma marginale* DNA in bovine erythrocytes by slot-blot and in situ hybridization with a PCR-mediated digoxigenin-labeled DNA probe. *J Vet Diagn Invest* 7: 465-472, 1995

15. Patrick CD, Hair JA: Laboratory rearing procedures and equipment for multi-host ticks (Acarina: Ixodidae). *J Med Entomol* 12: 389-390, 1975

16. Fleming KA, Evans M, Riley KC et al: Optimization of non-isotopic in situ hybridization on formalin-fixed paraffin-embedded material using digoxigenin labelled probes and transgenic tissues. *J Pathol* 167: 9-17, 1992

17. Unger ER, Lee DR: In situ hybridization: principles and diagnostic applications in infection. *J Histotechnol* 18: 203-209, 1995

18. Kim SY, Lee JS, Ro JY et al: Interphase cytogenetics in paraffin sections of lung tumors by non-isotopic in situ hybridization. *Am J Pathol* 142: 307-317, 1993

19. Slater M, Mason RS: Immunogold localization of TGFβ 1 protein and mRNA in human skin using a colloidal gold/digoxigenin system. *Histochem* 102: 153-163, 1994

20. Mandry P, Murray AB, Rieke L et al: Postembedding ultrastructural in situ hybridization on ultrathin cryosections and LR White resin sections. *Ultrastruct Pathol* 17: 185-194, 1993

21. Stich RW, Bantle JA, Kocan KM et al: Detection of *Anaplasma marginale* (Rickettsiales: Anaplasmataceae) in hemolymph of *Dermacentor andersoni* (Acari: Ixodidae) with the polymerase chain reaction. *J Med Entomol* 30: 781-788.

22. Krueger CM, Buening GM, Kinden DA et al: Detection of the *Anaplasma marginale* genome in bovine tissues by in situ DNA hybridization. *Proceedings 8th Natl Anaplasmosis Conf* St Louis, 1989, pp. 83-90

Table IV. Comparison of ISH Conditions on Paraffin- and LR White-Embedded Half-Tick Sections

	Paraffin	LR White
Deparaffinization and rehydration	yes	no
PK digestion	25 μg/ml, 20 min	500 μg/ml, 35 min
Target DNA denaturation NaOH	no	15 min
Target DNA denaturation Heat	84 ^{0}C	95 ^{0}C
Hybridization T	42 ^{0}C	39 ^{0}C
Posthybridization-wash T	50 ^{0}C	45 ^{0}C
Anti-DIG Ab	1:750, 60 min	1:100, 90 min
Color development	4 hr	8 hr

Table V. Comparison of *Anaplasma marginale* Infection in Experimentally-Infected Ticks Determined by ISH and LM

Experimental methods	Positives	Positive tissue distribution	
		Salivary gland	Gut
ISH (Paraffin)	20/20	19/20	5/20
LM	20/20	18/20	6/20
ISH (LR White section)	20/20	20/20	6/20
LM	20/20	19/20	6/20

Figure 9. ISH detection of *A. marginale* on paraffin-embedded half-tick sections.

Hybridization signals appeared as dark-blue granularities (arrows): (a) & (b) Salivary glands; (c) Gut. Original magnification: (a) X 100, (b) & (c), X 1,000.

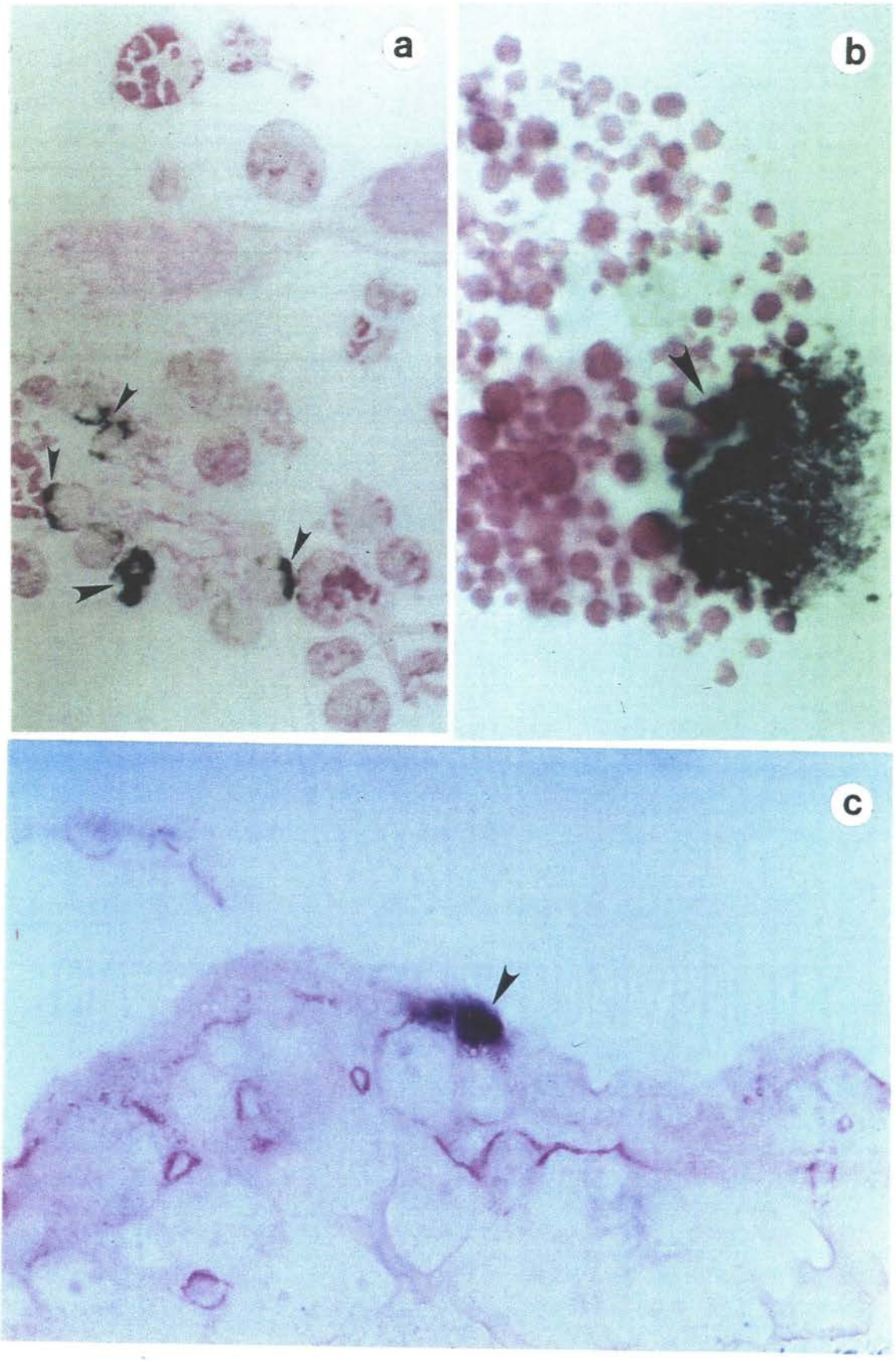

a
b
c

Figure 10. ISH detection of *A. marginale* on LR White-embedded half-tick sections.

Hybridization signals appeared as delicate dot-like dark-blue precipitates (Arrows): (a) & (b) Salivary glands; (c) Gut. Original magnification X 1,000.

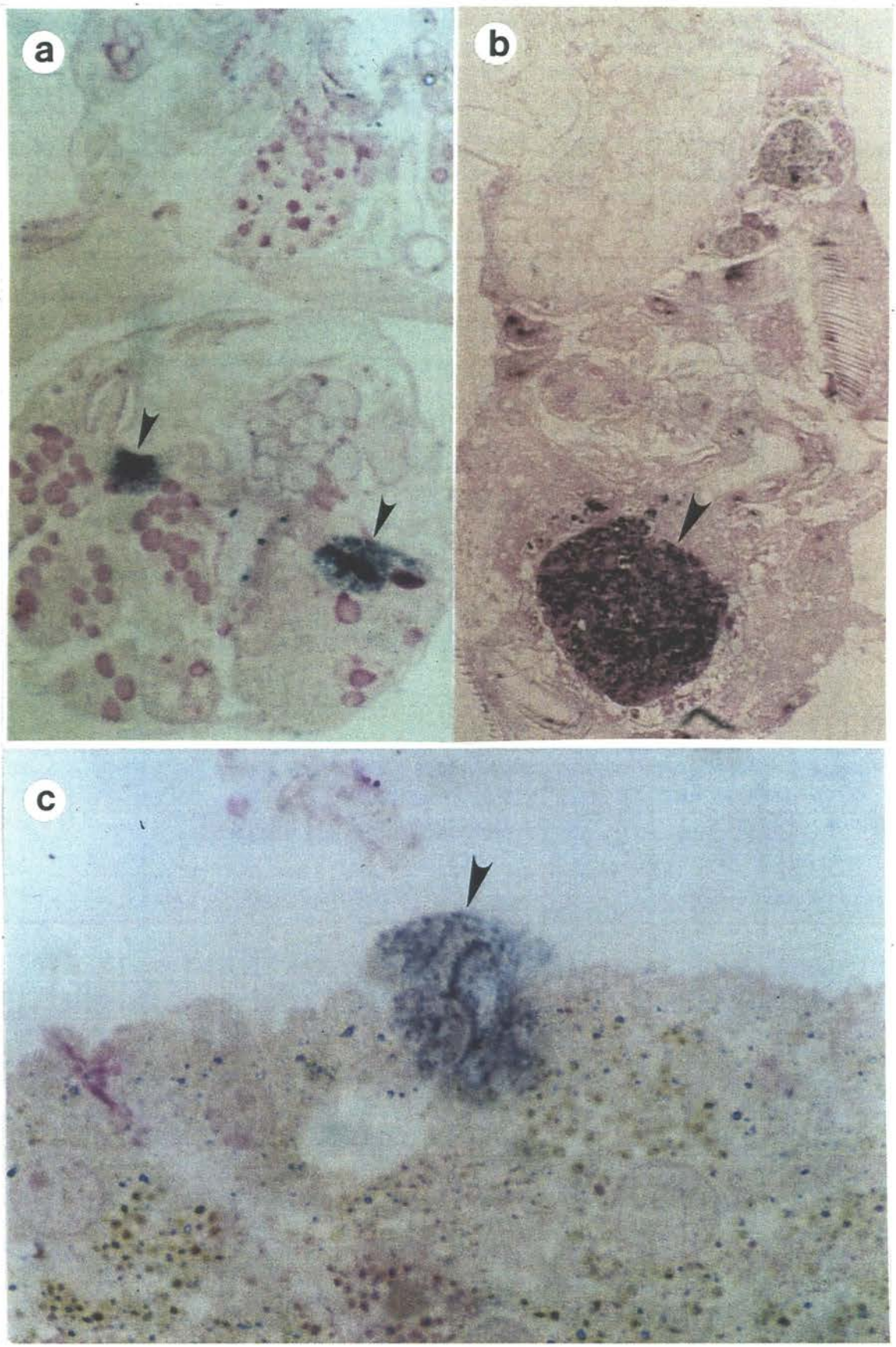
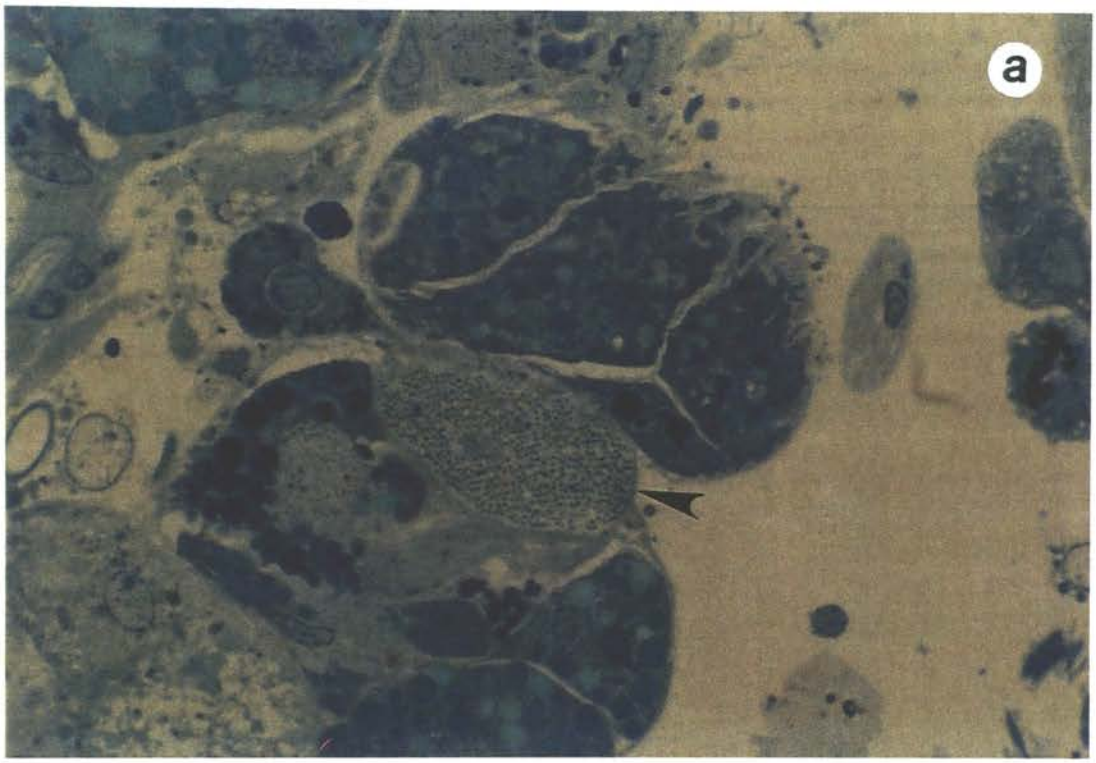
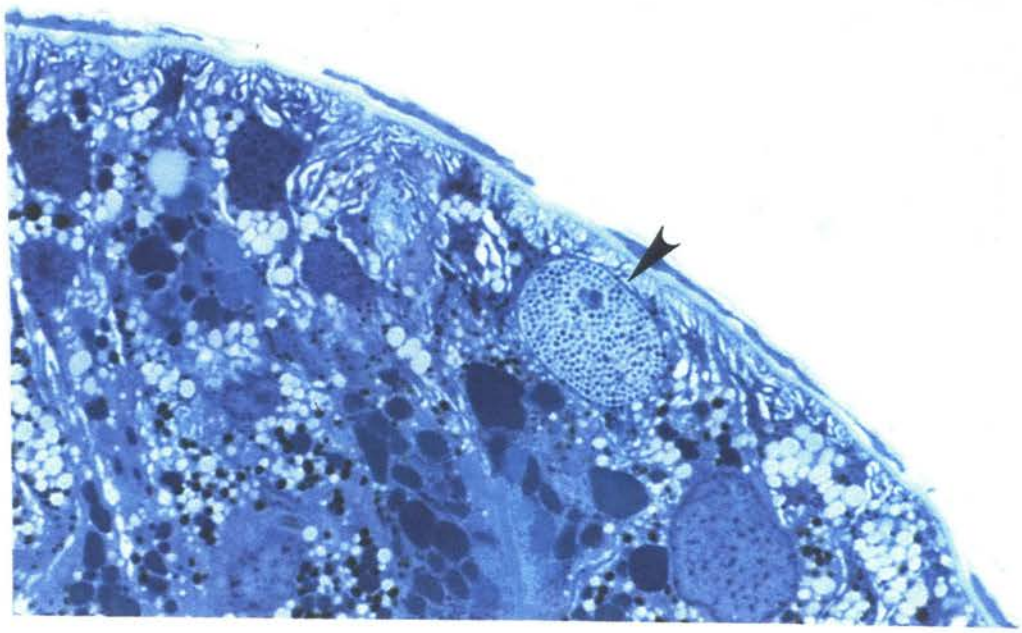

a
b
c

Figure 11. Photomicrographs of colonies (arrows) of *A. marginale* in salivary gland and gut tissues of companion tick-halves.

(a) Salivary gland; (b) gut. Original magnification X 1,000.



b



CHAPTER V

DEVELOPMENTAL STUDIES OF *Anaplasma marginale* (RICKETTSIALES: ANAPLASMATACEAE) IN MALE *Dermacentor andersoni* (ACARI: IXODIDAE) INFECTED AS ADULTS USING NONRADIOACTIVE IN SITU HYBRIDIZATION AND MICROSCOPY

Abstract

The development of *Anaplasma marginale* Theiler was studied in ticks using a nonradioactive in situ hybridization (ISH) method developed in our laboratory. Male *Dermacentor andersoni* Stiles ticks were infected intrastadially by allowing them to feed for 7 days on an infected calf (acquisition feeding). The ticks were then removed and held in a humidity chamber for 5 days before being fed on a second susceptible calf for 10 days (transmission feeding). Two groups of 10 ticks were collected daily during the 22-day experiment. In one group one-half of each tick was processed and embedded in paraffin and in the other group one-half of each tick was embedded in LR White for ISH. The companion tick-halves from each group were fixed and embedded in DER resin for routine light and electron microscopy. As detected by ISH on LR White- and paraffin-embedded sections and by microscopy, initial infection of ticks by *A. marginale* occurred in gut tissues either on the 7th day of acquisition feeding or the 1st day of the holding period, and infection persisted throughout transmission feeding. Two peaks of infection in gut tissues were observed. The first peak occurred on the 4th day of the holding period, and the second peak was observed on the 5th day of transmission feeding. Salivary glands became infected with *A. marginale* on the first day of transmission feeding and remained infected throughout the transmission-feeding period. Peak infection was observed on day 4 of transmission feeding. After the beginning of transmission feeding, *A. marginale* infection was also observed in interstitial, reproductive, skeletal muscle, fat body and Malpighian tubule tissues. While *A. marginale* infection of ticks clearly originates in midgut epithelial cells, many tissues eventually become infected during transmission

feeding, resulting in a generalized infection. The infection of multiple tissues may contribute to the ability of *A. marginale* to persist in intrastadially infected male ticks.

Key Words

Anaplasma marginale, *Dermacentor andersoni*, tick, DNA probe, in situ hybridization

Introduction

Anaplasma marginale Theiler is a tick-borne rickettsia that infects bovine erythrocytes and causes bovine anaplasmosis (Ristic 1981). Although *A. marginale* can be transmitted mechanically by biting flies and blood-contaminated fomites, ixodid ticks are the only known biological vectors (Ewing 1981; Kocan 1986). Biological transmission occurs by transfer of adult ticks from infected to susceptible cattle (intrastadial transmission), or by nymphs or adults infected in a previous stage (interstadial or transstadial transmission) (Kocan 1986; Kocan et al. 1992b; Stiller et al. 1989b). Although both male and female ticks can transmit *A. marginale* intrastadially, males have been shown to be persistently infected, transfer readily among cattle and maintain high infection rates while off the host (Kocan et al. 1992a; Stiller et al. 1989a).

Much of the developmental cycle of *A. marginale* has been described in *Dermacentor* ticks (Kocan 1986; Kocan et al. 1983a; 1984; 1990; 1992a, b). Within male ticks infected as adults, *A. marginale* develops within midgut epithelial, gut muscle and salivary gland cells. This complex developmental cycle appears to be coordinated with the tick feeding cycle (Kocan 1986; Kocan et al. 1992b). Infection originates in midgut epithelial cells during acquisition feeding but infection of tick salivary glands does not occur until the onset of transmission feeding. Transmission of *A. marginale* to cattle appears to be via oral secretions during tick feeding (Kocan 1986; Stich et al. 1993b).

Earlier studies on the development of *A. marginale* in ticks were done on gut and salivary gland tissues that were dissected from ticks and processed individually (Kocan et al. 1992a, b; 1993). Other tick tissues (i.e. Malpighian tubules and muscle) were occasionally dissected with gut and salivary gland tissues and also found to be infected with *A. marginale* (Kocan, unpublished data). We have recently found that bisected ticks could be processed for

microscopy and that sections could be made from the entire half-tick, allowing for examination of all tick tissues. This technique was especially applicable to male ticks because they do not increase in size during feeding and the tick-halves were small enough to fit into embedding molds.

Some tick tissues that we studied previously, such as hemolymph and salivary glands from adults infected as nymphs, were infected with *A. marginale* as determined by slot-blot hybridization or PCR, but recognizable forms of *A. marginale* were not seen with light and electron microscopy (Kocan 1986; Kocan et al. 1983b; 1993). Therefore, we developed is situ hybridization (ISH) for localization of the *A. marginale* genome in tick tissues to facilitate detailed microscopic examination. When ISH was used on half-ticks, many tick tissues could be seen simultaneously, enabling observation of *Anaplasma* development in tissues, other than midgut and salivary glands. ISH has been adapted for detection of *A. marginale* on paraffin- and LR White-embedded half-ticks (Ge et al. 1996). In preliminary ISH studies *A. marginale* inclusions were detected in both salivary gland and gut tissues, and the results correlated well with microscopic observations on companion tick-halves.

In the present study, we describe the use of in situ hybridization coupled with microscopy for detection of *A. marginale* in all tick tissues that may be involved in the developmental cycle of this rickettsia in male *D. andersoni* infected as adults. The distribution of *A. marginale* within tissues of these male ticks was determined and infection rates among the various tissues, as determined by ISH and LM, were compared.

Materials and Methods

Tick Propagation

Dermacentor andersoni ticks used in this study were obtained from a laboratory colony at the USDA-Animal Disease Research Unit, Moscow, Idaho, and were reared for several generations at the Oklahoma State University Medical Entomology Laboratory. Larvae and nymphs, not to be exposed to *A. marginale*, were fed on rabbits and allowed to molt to the adult stage. Adult males were held in a humidity chamber (90-98%, RH) at 25 ^{0}C with a 14-hour photophase period until used for this study.

Exposure of Adult Ticks

Male *D. andersoni* were infected as adults as described previously (Kocan 1992b; 1993). Briefly, male ticks were allowed to feed on a calf (PA 332) inoculated with *A. marginale* (Virginia isolate) for 7 days when the parasitemia reached 3-5% (acquisition feeding). The ticks were removed from the calf, and placed in a humidity chamber for 5 days after which they were fed on a second susceptible calf (PA 337) for 10 days (transmission feeding). Uninfected male ticks were fed on an uninfected calf (PA 357) in the same manner to serve as uninfected controls.

Collection of Ticks and Preparation of Tick-Halves for ISH

Two groups of 10 ticks were collected daily during the 22-day experiment. Each tick from both groups was cut in half longitudinally with a sharp razor blade, separating the left and right halves. One half of each tick was processed for embedding in paraffin (Group 1) or LR White (Group 2). For comparison, the companion half from each tick was processed and

embedded in DER resin for light and electron microscopy (LM & EM). Uninfected ticks (10 per day) that fed on the susceptible, uninfected calf were processed in the same manner to serve as controls.

Tick-halves were embedded in paraffin or LR White as described previously by Ge et al. (1996). For paraffin embedding, half-ticks were fixed in 4% phosphate-buffered formaldehyde and then dehydrated and embedded at Histology Laboratory of Oklahoma Animal Disease Diagnostic Laboratory. Sections (4-μm) were cut and mounted onto Fisher Probe-on Plus slides (Fisher Scientific, Pittsburgh, PA). For LR White embedding, half-ticks were fixed in 4 % phosphate-buffered paraformaldehyde, dehydrated in 70% ethanol, and infiltrated in LR White (EMS, Ft. Washington, PA) mixed with decreasing ratios of 70% ethanol. After 1 hour in pure LR White, the tick-halves were permeated with fresh LR White overnight, then removed, placed into gelatin capsules containing LR White and polymerized at 60 ^{0}C for 24 hrs. Sections (1 μm) were cut using an MT-8000 microtome (Research and Manufacturing Company, Tucson, AZ) and mounted onto the Fisher Probe-on Plus slides.

Probe Labeling and ISH

The DIG-labeled 409 bp DNA probe was made as described previously (Ge et al. 1995). Briefly, a 409 bp DNA fragment derived from the *msp-1β* gene (Barbet & Allred 1991) of *A. marginale* was amplified and simultaneously labeled with digoxigenin (DIG)-11-dUTP by PCR. The resulting DIG-labeled, 409 bp PCR product was purified by ethanol precipitation and used as a probe for the ISH studies.

ISH on paraffin-embedded sections was optimized and performed as described previously by Ge et al. (1996). Sections on slides were deparaffinized with xylene, rehydrated

with decreasing concentrations of ethanol, and made permeable with proteinase K. Slide pairs (1 with infected half-tick section and 1 control) were incubated with prehybridization solution (50% formamide, 5 X standard saline citrate (SSC), 2% blocking reagent (Boehriger Mannheim Biochemicals, Indianapolis, IN), 0.1% sarkosyl, 0.02% SDS, and 0.1% salmon sperm DNA) using a MicroProbe slide incubator (Fisher Scientific, Pittsburgh, PA). The target DNA was denatured by raising the temperature to 84 ^{0}C for 10 min before 30 ng of the heat-denatured DIG-labeled DNA probe was introduced into each slide pair. Slide pairs were then incubated at 42^0 C in a sealed humidity chamber overnight and washed with decreasing concentrations of SSC. The DIG-labeled DNA probe was immunologically detected with anti-DIG antibody conjugated with alkaline phosphatase (Boehriger Mannheim Biochemicals, Indianapolis, IN) and the appropriate chromogenic substrate system. Color development was stopped by rinsing in TE (100 mM Tris-HCl, pH 8.0; 50 mM EDTA) buffer. Slides were air dried, counterstained with eosin, coverslipped in permount, and observed with LM. The procedures for achieving the best hybridization signal for ISH on LR White-embedded sections were performed as described by Ge et al. (1996) and were less stringent than those required for ISH on paraffin-embedded sections.

Light and Electron Microscopy

Companion half-ticks were fixed in cold 2% glutaraldehyde in 0.2M sodium cacodylate buffer and post-fixed in 2% cacodylate-buffered osmium tetroxide (pH 7.2). Subsequently, the fixed tissues were dehydrated in a graded series of ethanol and infiltrated with epoxy resin. Semi-thin sections (1.0-μm) were cut, stained with Mallory's stain (Richardson et al. 1960) and examined with LM for colonies of *A. marginale*. Ultrathin sections (silver-gold reflective) were

cut with a diamond knife (MJO-Diatome Co, Fort Washington, PA), collected on 300-mesh copper grids, and were stained with uranyl acetate and lead citrate. The stained sections were observed and photographed with a JEOL CX 100 transmission electron microscope (JEOL Inc., Boston, MA) operated at 80 kV.

Results

Infection of Ticks and Transmission of Anaplasmosis

The donor calf, PA 332, used for infection of male *D. andersoni* had a peak parasitemia of 51.2% during acquisition feeding. The susceptible calf, PA 337, used for transmission-feeding of the *A. marginale*-exposed ticks developed anaplasmosis with a prepatent period of 28 days and a peak parasitemia of 12.3%. The control calf, PA 357, used for feeding the uninfected ticks did not develop anaplasmosis and was confirmed to be susceptible to *A. marginale* by challenge-exposure after all ticks were removed.

In Situ Hybridization

Positive hybridization signals were observed on the LR White-embedded half-tick sections in midgut tissues from the 7th day of acquisition feeding on the donor calf to last day of transmission feeding on the susceptible calf. The number of ticks with gut cell infection peaked twice during the sampling period. The first peak occurred on the 4th day of the holding period with 6 of 10 ticks infected, and a second peak occurred on the 5th day of transmission feeding with 8 of 10 ticks infected. Salivary gland infection, as determined by in situ hybridization, was first seen on day 1 of transmission feeding and peaked on day 4. Infection of salivary glands persisted throughout the 10-day transmission feeding period. The

number of ticks with infected gut and salivary gland tissues during the 22-day experiment are summarized in Figure 12A. In the paraffin-embedded sections, the number of ticks with infected gut and salivary gland tissues, as determined by ISH, were similar to those observed in LR White-embedded sections (Figure 13A). However, hybridization signals in paraffin-embedded sections appeared first in gut tissues on the 1st day of the holding period rather than on the last day of acquisition feeding.

In addition to *A. marginale* infections detected in gut and salivary gland tissues, several other tissues were found to become infected with *A. marginale* during transmission feeding. In both paraffin- and LR White-embedded sections, positive hybridization signals were observed in skeletal muscle, Malpighian tubule, reproductive, and interstitial tissues (Figure 14A-D & Figure 15A-C). The number of ticks with these tissue infections determined by ISH in LR White sections are shown in Figure 12 and in paraffin sections in Figure 13. Hybridization signals were not observed in sections of the uninfected control tick-halves.

Microscopy Studies

Colonies of *A. marginale* were seen by LM in sections of companion half-ticks embedded in DER resin in gut tissues from day 7 of acquisition feeding to the last day of transmission feeding. Salivary glands contained colonies of *A. marginale* from day 1 through day 10 of transmission feeding. During the entire transmission feeding period in which salivary glands were infected, *A. marginale* colonies were also observed in interstitial, skeletal muscle, Malpighian tubule (Figure 16A, B & D) and reproductive tissues (Figure 17). These findings correlated positively with those obtained by the in situ hybridization studies. In addition, colonies of *A. marginale* were seen in fat body tissues (Figure 16C) from the 5th day of the

holding period to last day of transmission feeding. The colonies observed by LM were confirmed to contain *A. marginale* organisms by EM (Figure 18A-D & Figure 19A-B). Infections of tissues in companion tick-halves determined by LM studies are depicted in Figures 20 & 21. Colonies of *A. marginale* were not seen in sections of uninfected control tick-halves collected during the 22-day experiment.

Discussion

In this study, ISH and LM studies done on male half-ticks enabled simultaneous examination of all tick tissues on a single preparation. *Anaplasma marginale* infections were detected in sections of half-ticks embedded with either paraffin or LR White by ISH, and in companion tick halves embedded in DER resin by LM. The results confirmed previous findings in which tick midgut cells were the first cells observed to become infected near the end of acquisition feeding and remained persistently infected throughout the holding period and during transmission feeding. Salivary glands did not become infected until the ticks fed a second time, thus confirming earlier studies (Kocan et al. 1992b). In addition to gut and salivary glands, long known to be sites of infection, ISH enabled detection of *A. marginale* infection in several other tick tissues including interstitial, reproductive, skeletal muscle and Malpighian tubule. Infection was not observed in these tissues until onset of transmission feeding when salivary gland acini were found to be infected. Most tissues observed to be positive by ISH contained *A. marginale* colonies that were demonstrable by LM and EM. However, peripheral fat body cells which surround tracheal trunks, tracheae, connective tissues or are found as scattered strands below the epidemis (Sonenshine 1991), were difficult to differentiate from other interstitial tissues. This difficulty was experienced in ISH studies on both paraffin- and LR

White-embedded sections because color precipitates of positive hybridization signals were shown only on the *Anaplasma* organisms and, also, because the eosin counterstain did not stain nuclear materials. Therefore, *A. marginale* colonies in fat body cells were more easily seen with routine LM and EM.

We have not determined how *A. marginale* is transferred from midgut epithelial cells to salivary glands. We have hypothesized previously that hemocytes may be the transfer cell because hemolymph has been shown to be infected with *A. marginale* by PCR, animal inoculation and fluorescent antibody studies (Kocan et al.1983b; Stich et al. 1993a) though not by LM and EM. In this study, infection of fat body cells with *A. marginale* was shown to occur one day earlier than the infection of other non-gut tissues. Because fat body cells often occur free in the tick hemocoel (Sonenshine 1991), it seems plausible that these cells may transfer *A. marginale* to various other tissues. In addition, interstitial tissues were found to be strongly positive by ISH (Figure 14D & 15C) and microscopic studies (Figure 16A & 18A) in most ticks during the transmission-feeding period. It may also be possible that organisms released from gut cells invade other tissues directly since many tick tissues are adjacent and/or adhered to the digestive tract.

In this study, male reproductive tissues were found to be heavily infected with *A. marginale* (Figure 14C, 15A, 17 & 18B), possibly reflecting a generalized infection. It would be interesting to determine whether ovaries of female ticks become infected with *A. marginale* during feeding. Further studies may be warranted to determine whether *A. marginale* could be transferred via sperm cells to females during mating.

Male ticks have been shown previously to be persistently infected with *A. marginale* and capable of repeated transmission of the organism to cattle (Kocan et al. 1992b).

Recognition for the first time in this study that many tick tissues were not previously known to be infected may contribute to the maintenance of persistent infections in male ticks.

Acknowledgments

Submitted for publication by Nie-Lin Ge,[1] Katherine M. Kocan,[1] Edmour F. Blouin, [1] George L. Murphy[1] to *Journal of Medical Entomology*.

The authors acknowledge the excellent technical assistance of Ms. Wanda Edwards, Peichen Hu and Jeanenne Duffy. Critical review of this manuscript by Dr. S. A. Ewing was greatly appreciated. This study was supported in part by Oklahoma Agricultural Experimental Station Project 1433 and 1699.

[1] Department of Pathology, College of Veterinary Medicine, Oklahoma State University, Stillwater, Oklahoma 74078-2007

References

Anthony, D. W. 1968. *Dermacentor andersoni* Stiles as a vector of bovine anaplasmosis, pp. 167-171. *In* Proceeding of the 5th National Anaplasmosis Research Conference, Stillwater, Oklahoma.

Barbet, A. F. & D. Allred. 1991. The *msp-1β* multigene family of *Anaplasma marginale*: Nucleotide sequence analysis of an expressed copy. Infect. Immun. 59: 971-976.

Ewing, S. A. 1981. Transmission of *Anaplasma marginale* by arthropods, pp. 395-423. *In* Proceedings of the 7th National Anaplasmosis Conference, Mississippi State University.

Ge, N-L., K. M. Kocan, G. L. Murphy & E. F. Blouin. 1995. Detection of *Anaplasma marginale* DNA in bovine erythrocytes by slot-blot and in situ hybridization with a PCR-mediated digoxigenin-labeled DNA probe. J. Vet. Diagn. Invest. 7: 465-472.

Ge, N-L., K. M. Kocan, G. L. Murphy & E. F. Blouin. 1996. Development of nonradioactive in situ hybridization for detection of *Anaplasma marginale* in ticks. J. Histotechnol. Submitted.

Kocan, K. M. 1986. Development of *Anaplasma marginale* in ixodid ticks: coordinated development of a rickettsial organism and its tick host, pp. 472-505. *In* J. R. Sauer & J. A. Hair [eds.], Morphology, physiology, and behavioral ecology of ticks. Horwood, Chichester, England.

Kocan, K. M., S. A. Ewing, D. Holbert & J. A. Hair. 1983a. Morphologic characteristics of colonies of *Anaplasma marginale* Theiler in midgut epithelial cells of *Dermacentor andersoni* Stiles. Am. J. Vet. Res. 43: 586-593.

Kocan, K. M., R. D. Oberst, S. A. Ewing, J. A. Hair & S. J. Barron. 1983b. Demonstration of *Anaplasma marginale* in hemolymph of *Dermacentor andersoni* by animal inoculation and by fluorescent-antibody technique. Am. J. Vet. Res. 44: 798-801.

Kocan, K. M., T. N. Yellin, S. A. Ewing, J. A. Hair & S. J. Barron. 1984. Morphology of colonies of *Anaplasma marginale* in nymphal *Dermacentor andersoni*. Am. J. Vet. Res. 45: 1434-1440.

Kocan, K. M., R. W. Stich, P. L. Claypool, S. A. Ewing, J. A. Hair & S. J. Barron. 1990. Intermediate site of development of *Anaplasma marginale* in feeding adult *Dermacentor andersoni* ticks that were infected as nymphs. Am. J. Vet. Res. 51: 128-132.

Kocan, K. M., W. L. Goff, D. Stiller, P. L. Claypool, W. Edwards, S. A. Ewing, J. A. Hair & S. J. Barron. 1992a. Persistence of *Anaplasma marginale* (Rickettsiales: Anaplasmataceae) in male *Dermacentor andersoni* (Acari: Ixodidae) transferred successively from infected to susceptible calves. J. Med. Entomol. 29: 657-668.

Kocan, K. M., D. Stiller, W. L. Goff, P. L. Claypool, W. Edwards, S. A. Ewing, T. C. McGuire, J. A. Hair & S. J. Barron. 1992b. Development of *Anaplasma marginale* in male *Dermacentor andersoni* transferred from parasitemic to susceptible cattle. Am. J. Vet. Res. 53: 499-507.

Kocan, K. M., W. L. Goff, D. Stiller, P. L. Claypool, W. Edwards, S. A. Ewing, T. C. McGuire, J. A. Hair & S. J. Barron. 1993. Development of *Anaplasma marginale* in salivary glands of male *Dermacentor andersoni*. Am. J. Vet. Res. 54: 107-112.

Richardson, K. C., L. Jarrett & E. H. Finke. 1960. Embedding in epoxy resins for ultrathin sectioning in electron microscopy. Stain Technol. 35: 313-323.

Ristic, M. 1981. Anaplasmosis, pp. 327-344. *In* M. Ristic & I. McIntyre [eds.], Diseases of cattle in the tropics. Martinus Nijhoff, Boston.

Sonenshire, D. E. 1991. Fat body, pp. 221-231. *In* D. E. Sonenshire [ed], Biology of ticks, volume 1. Oxford University Press, New York.

Stich, R. W., J. A. Bantle, K. M. Kocan & A. Fekete. 1993a. Detection of *Anaplasma marginale* (Rickettsiales: Anaplasmataceae) in hemolymph of *Dermacentor andersoni* (Acari: Ixodidae) with the polymerase chain reaction. J. Med. Entomol. 30: 781-788.

Stich, R. W., J. R. Sauer, J. A. Bantle & K. M. Kocan. 1993b. Detection of *Anaplasma marginale* in secretagogue-induced oral secretions of *Dermacentor andersoni* with polymerase chain reaction. J. Med. Entomol. 30: 789-794.

Stiller, D., M. E. Coan, W. L., L. W. Goff. Johnson & T. C. McGuire. 1989a. The importance and putative role of *Dermacentor* spp. males in anaplasmosis epidemiology: transmission of *Anaplasma marginale* to cattle by ad libitum interhost transfer of *D. andersoni* males under semi-natural conditions, p. 209. *In* Proceedings, 8th National Veterinary Hemoparasite Disease Conference, St. Louis, MO.

Stiller, D., K. M. Kocan, W. Edwards, S. A. Ewing, J. A. Hair & S. J. Barron. 1989b. Detection of colonies of *Anaplasma marginale* Theiler in salivary glands of three *Dermacentor* spp. infected as nymphs or adults. Am. J. Vet. Res. 50: 1381-1386.

Figure 12. Tissue distribution of positive ISH signals on LR White-embedded half-tick sections of male *D. andersoni* infected with *A. marginale* as adults.

(A). Gut; SG, salivary glands; MT, Malpighian tubules; (B) IT, interstitial tissues; RT, reproductive tissues; SM, skeletal muscle.

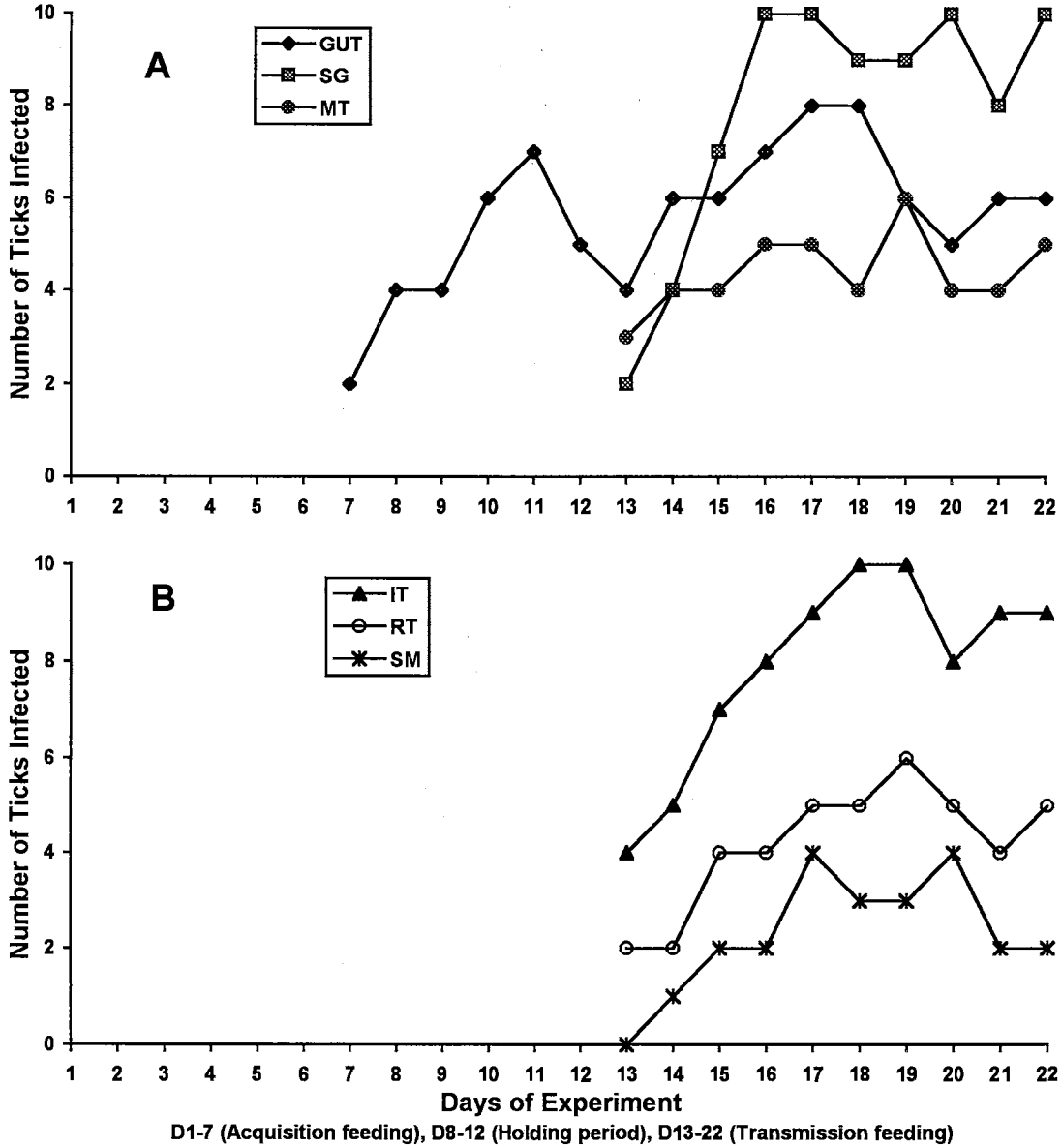

A
GUT
SG
MT
Number of Ticks Infected
B
IT
RT
SM
Number of Ticks Infected
Days of Experiment
D1-7 (Acquisition feeding), D8-12 (Holding period), D13-22 (Transmission feeding)

Figure 13. Tissue distribution of positive ISH signals on paraffin-embedded half-tick sections of male *D. andersoni* exposed to *A. marginale* as adults.

(A). Gut; SG, salivary glands; MT, Malpighian tubules; (B) IT, interstitial tissues; RT, reproductive tissues; SM, skeletal muscle.

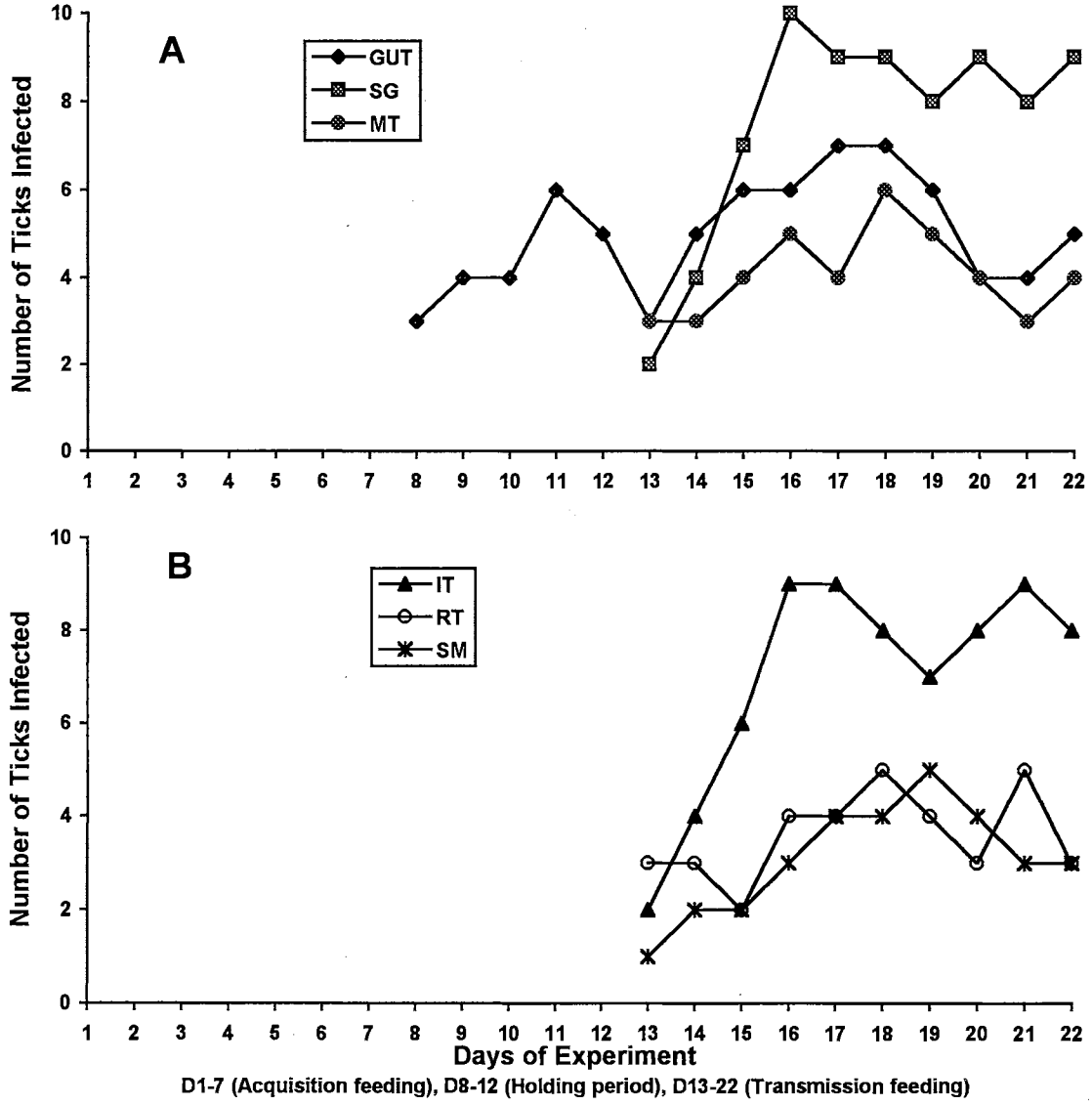

A
GUT
SG
MT
Number of Ticks Infected
B
IT
RT
SM
Number of Ticks Infected
Days of Experiment
D1-7 (Acquisition feeding), D8-12 (Holding period), D13-22 (Transmission feeding)

Figure 14. ISH detection of *A. marginale* on paraffin-embedded half-tick sections of male *D. andersoni* exposed to *A. marginale* as adults.

Positive hybridization signals appeared as dark-blue granularities (arrow head). (A) Skeletal muscle; (B) Malpighian tubules; (C) Reproductive tissues; (D) Interstitial tissues. (X 2,500).

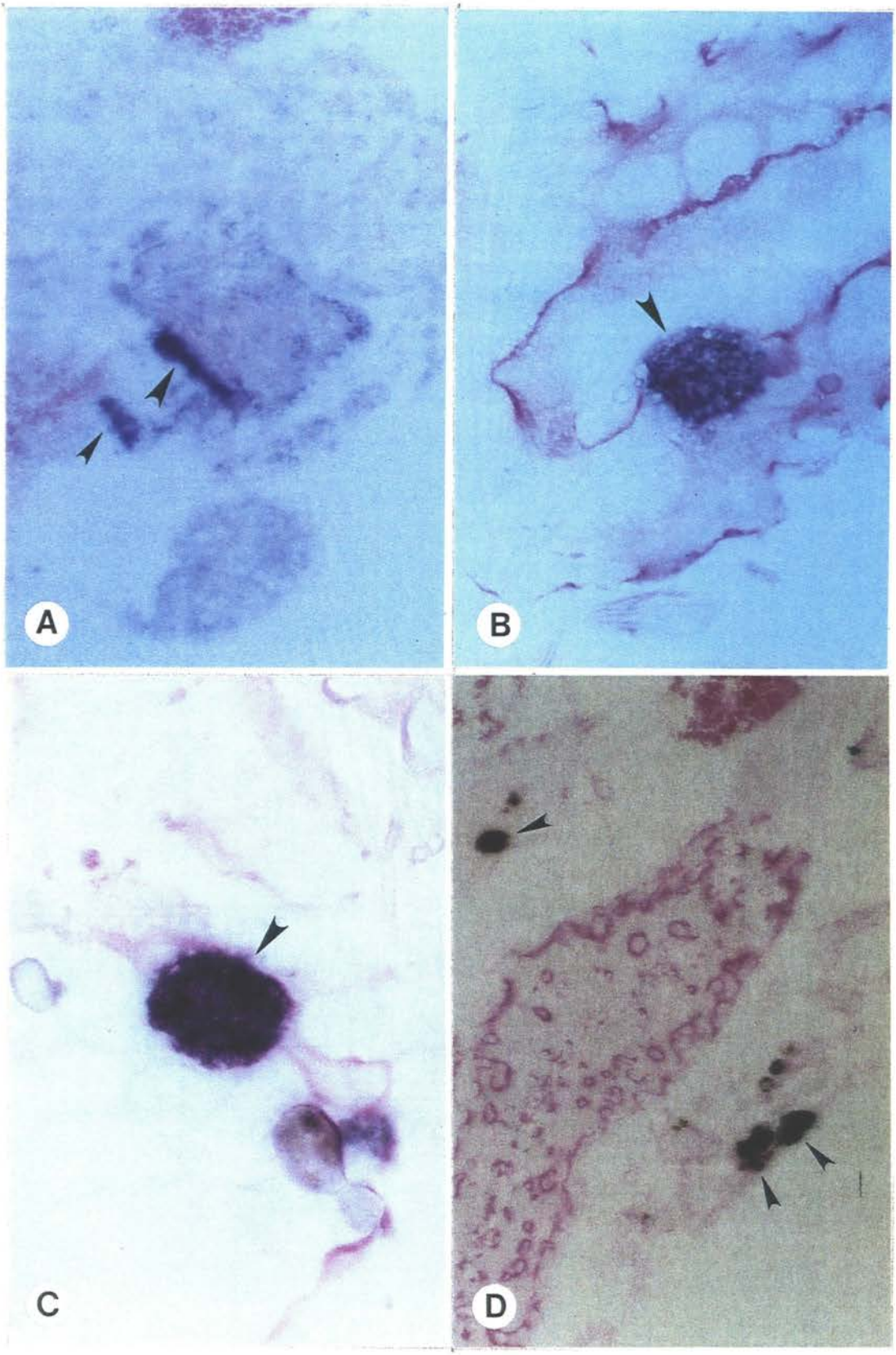

A
B
C
D

Figure 15. ISH detection of *A. marginale* on LR White-embedded half-tick sections of male *D. andersoni* exposed to *A. marginale* as adults.

Positive hybridization signals appeared as delicate dot-like, dark-blue precipitates.

(A) Reproductive tissues; (B) Malpighian tubules; (C) Interstitial tissues. (X2,500).

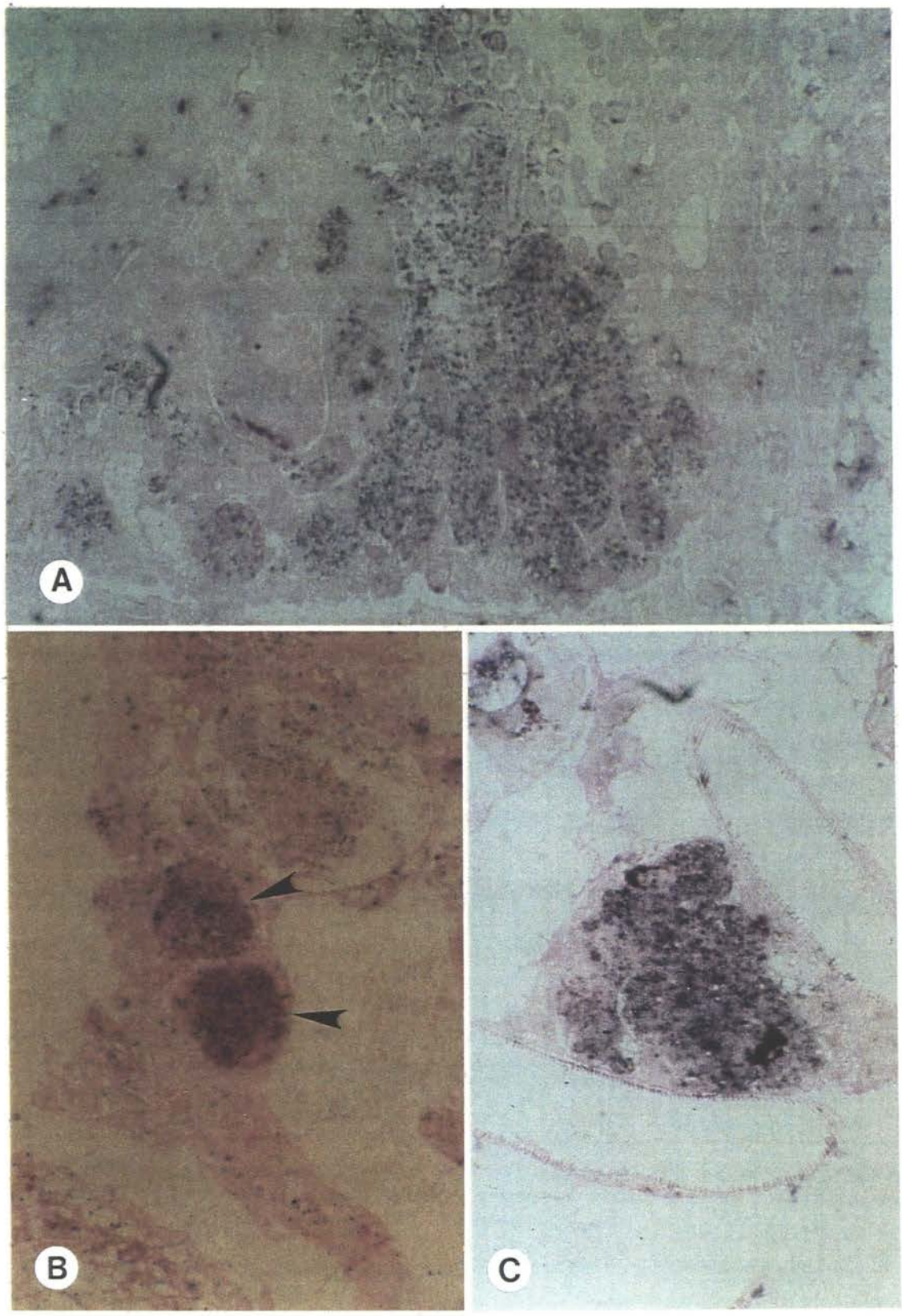

A
B
C

Figure 16. Light photomicrographs of colonies of *A. marginale* (arrows) in tissues of companion tick-halves of *D. andersoni* exposed to *A. marginale* as adults.

(A) Interstitial tissues; (B) Skeletal muscle; (C) Fat body; (D) Malpighian tubules. (X 2,500).

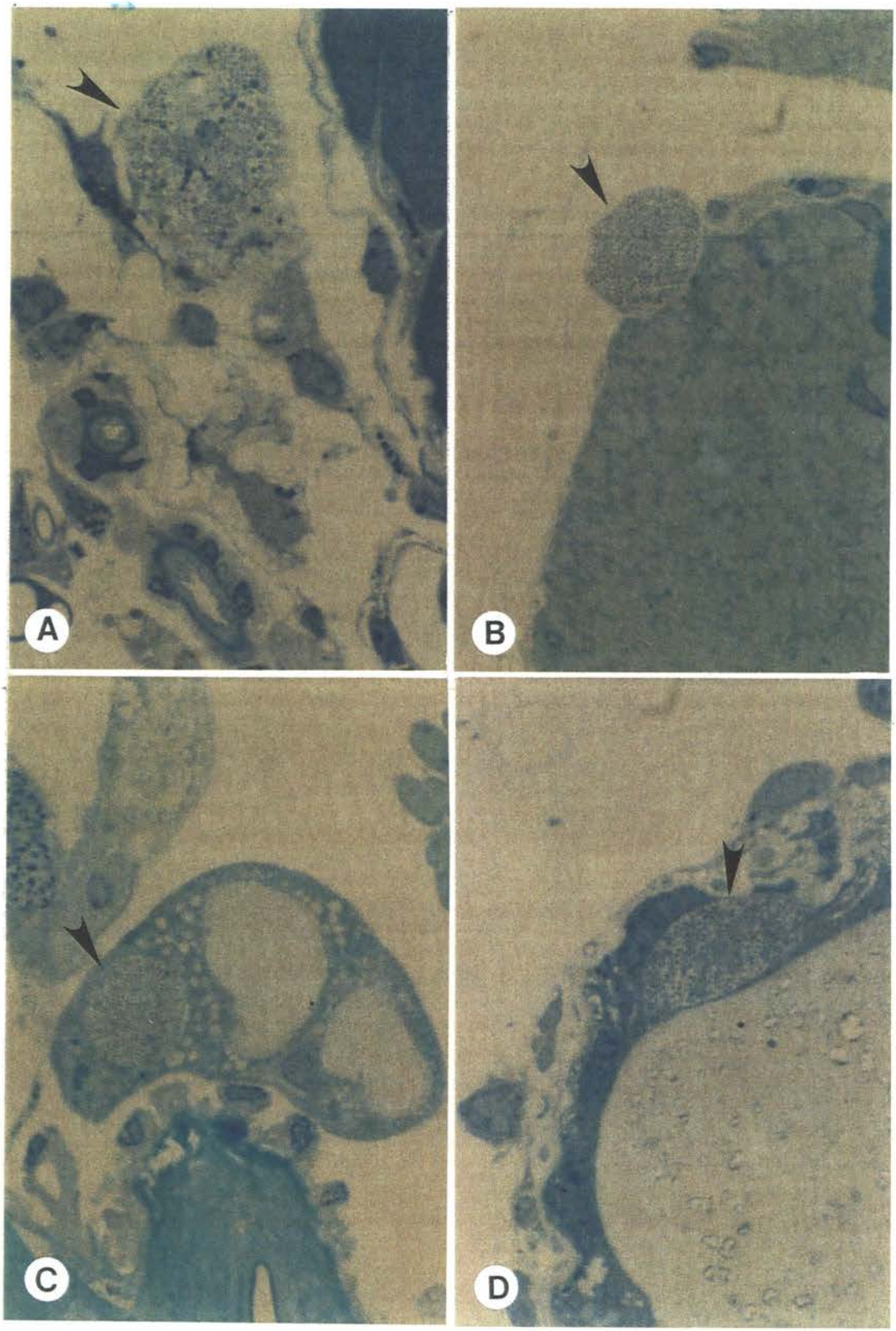

A
B
C
D

Figure 17. Light photomicrograph of colonies of *A. marginale* (arrows) in reproductive tissues of companion tick-halves of *D. andersoni* exposed to *A. marginale* as adults. S: Cross section of spermatocytes (X 2,500).

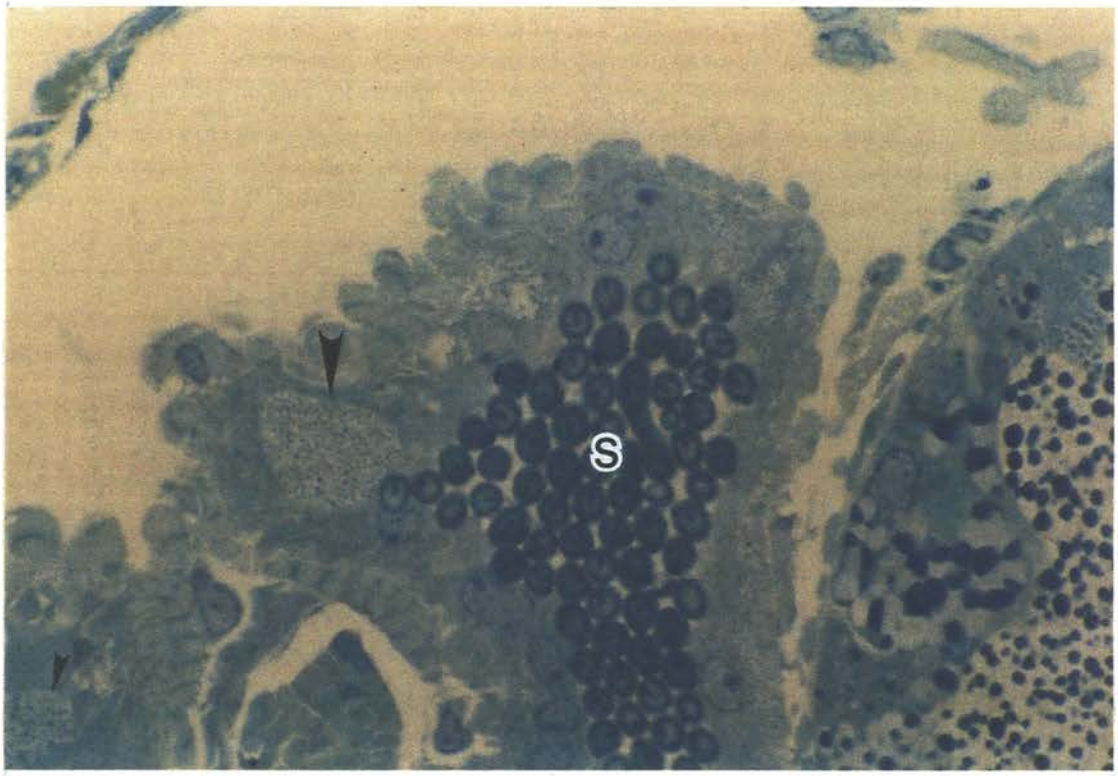

S

Figure 18. Electron photomicrographs of colonies of *A. marginale* in tissues of companion tick-halves of *D. andersoni* exposed to *A. marginale* as adults.

(A) Interstitial tissues (X 29,000); (B) Reproductive tissues (X 36,000); (C) & (D) Skeletal muscle (X 36,000 & X 96,000, respectively).

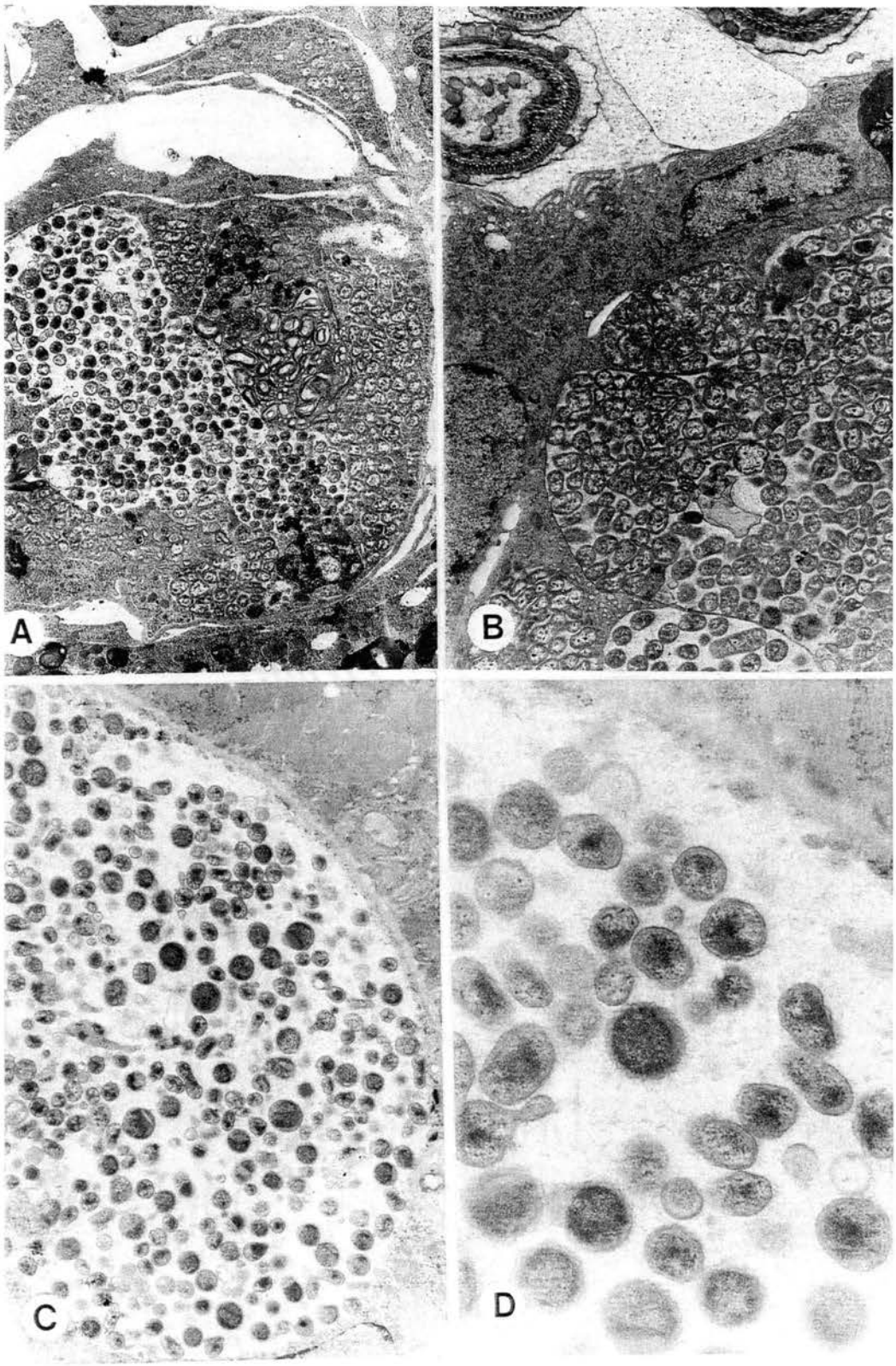

A
B
C
D

Figure 19. Electron photomicrographs of colonies of *A. marginale* in fat body cell and Malpighian tubule of companion tick-halves of *D. andersoni* exposed to *A. marginale* as adults.

(A) Fat body cell (X 29,000); and (B) Malpighian tubule (X 96,000)

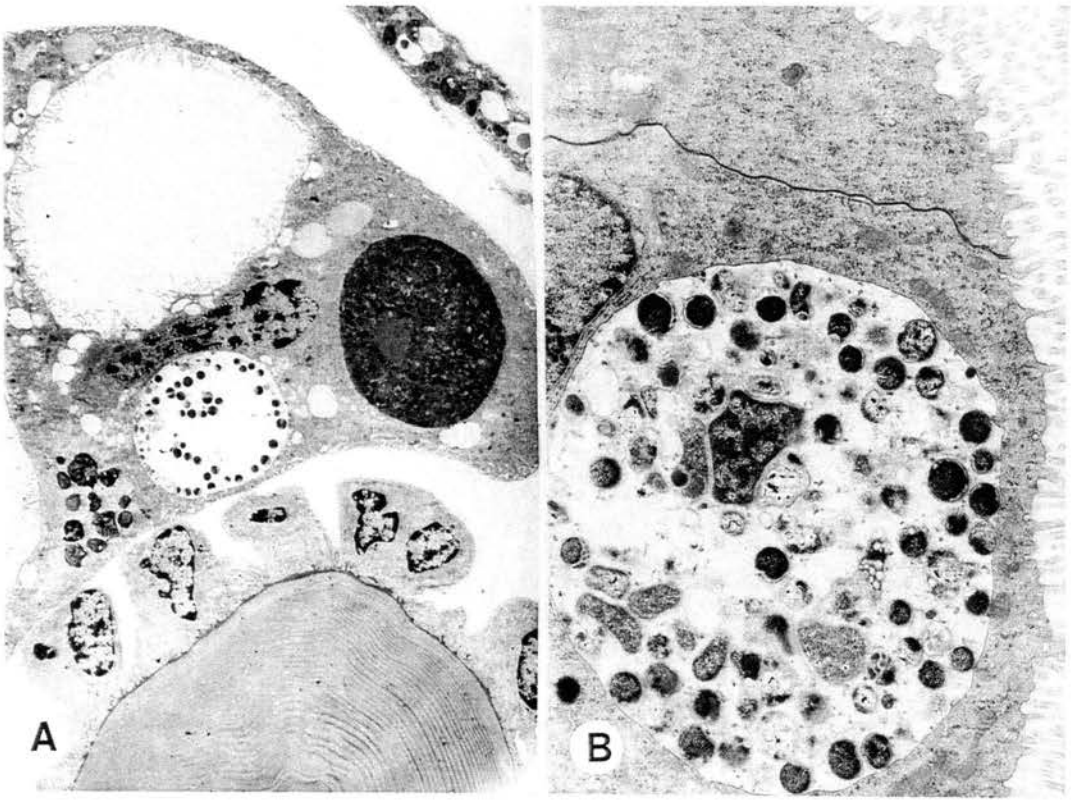
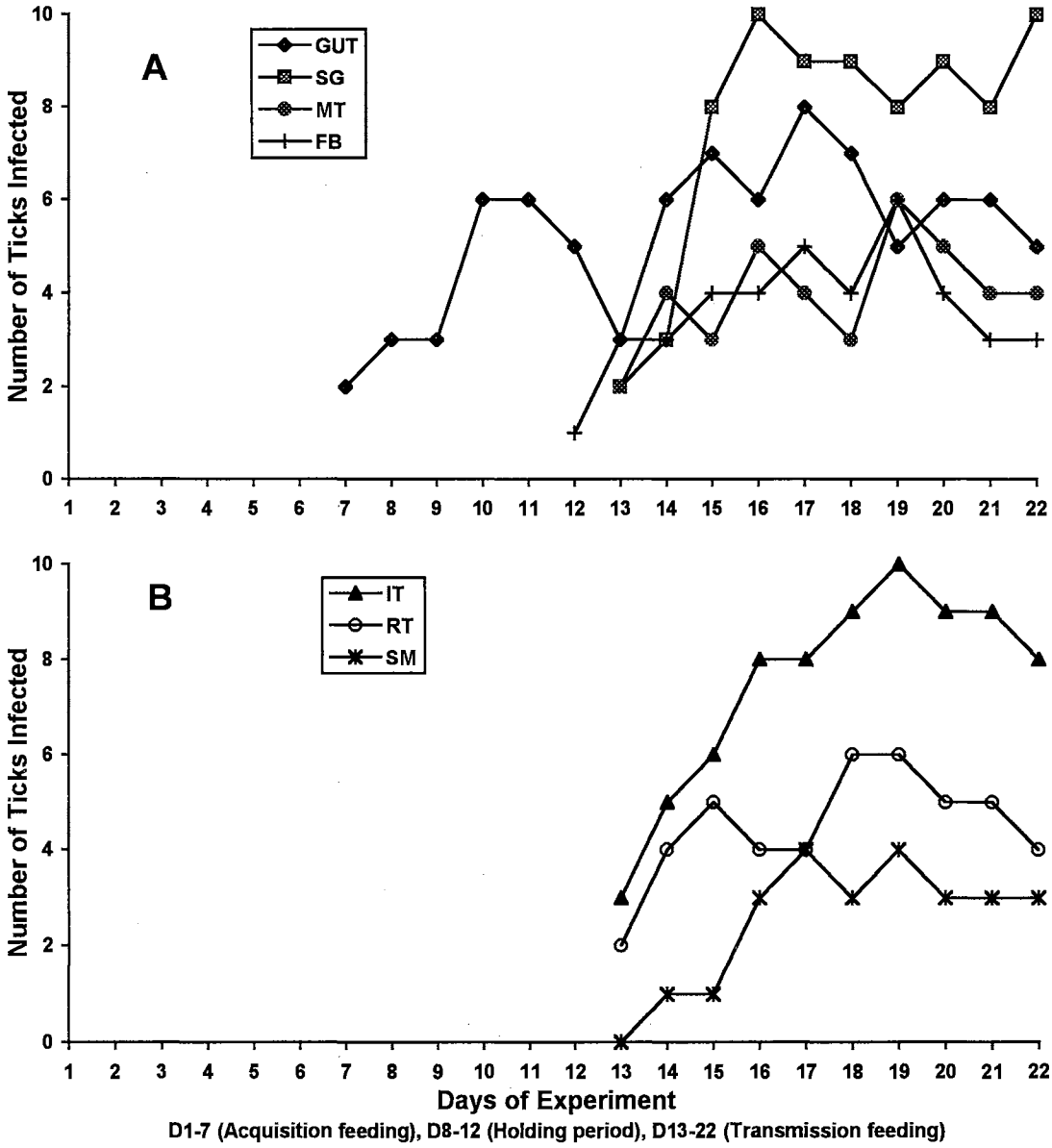

A
B

Figure 20. Distribution of *A. marginale* colonies determined by LM examination of companion tick-halves of LR White-embedded tick-halves of male *D. andersoni* exposed to *A. marginale* as adults.

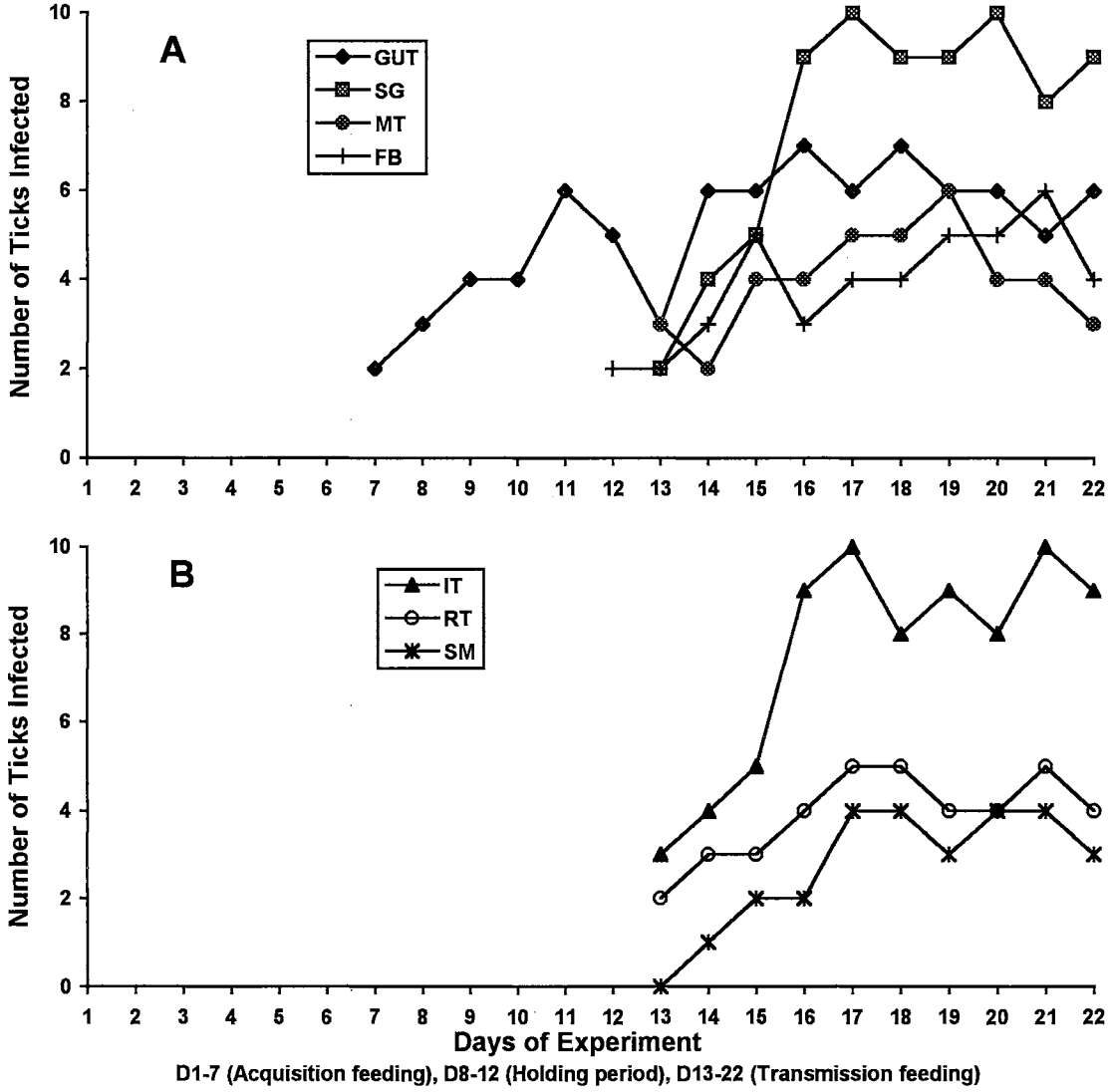
(A). Gut; SG, salivary glands; MT, Malpighian tubules; FB, fat body; (B) IT, interstitial tissues; RT, reproductive tissues; SM, skeletal muscle.



D1-7 (Acquisition feeding), D8-12 (Holding period), D13-22 (Transmission feeding)

Figure 21. Distribution of *A. marginale* colonies determined by LM examination of companion tick-halves of paraffin-embedded half-ticks of male *D. andersoni* exposed to *A. marginale* as adults.

(A). Gut; SG, salivary glands; MT, Malpighian tubules; FB, fat body; (B) IT, interstitial tissues; RT, reproductive tissues; SM, skeletal muscle.



D1-7 (Acquisition feeding), D8-12 (Holding period), D13-22 (Transmission feeding)

CHAPTER VI

SUMMARY

Anaplasmosis is one of the four major tick-borne diseases of cattle and is enzootic in nearly half of the world's livestock production regions. In recent years the disease has become more widely distributed in the United States and Oklahoma than previously known. The widening distribution of anaplasmosis likely has resulted from increased movement of cattle and from reliance upon the complement-fixation test for identification of carrier cattle. The complement-fixation test has been shown to lack senstivity, especially in calves, carrier cattle and in cattle that have been treated with tetracyclines, a procedure which often reduces antibody titers to levels undetectable by this method. Thus, cattle that are falsely regarded as serologically negative may be shipped to non-enzootic areas where they are likely to serve as reservoirs for mechanical transmission by blood-contaminated fomites or biting flies or for biological transmission by ticks. Development of a new diagnostic test would contribute greatly to the control and study of anaplasmosis.

Nonradioactive nucleic acid probes have been used widely in research laboratories for detection of infectious agents and for clinical diagnosis because of their safety, short detection times, low cost and suitability for long-term storage and because of sensitivity similar to that of radioactive probes. Nucleic acid probes detect the genome of the organisms and do not rely on variable antibody response in different stages of the infection. Thus, these probes should be more senstive and useful as diagnostic assays.

The purpose of this study was to develop a nonradioactive DNA probe for diagnosis of anaplasmosis in cattle and to use this probe for developmental studies of *A. marginale* in its tick vector. The study was divided into four parts: (1) development and utilization of a sensitive nonradioactive DNA probe for detection of *A. marginale* in erythrocytes of experimentally-infected cattle during pre-acute disease and after becoming carriers; (2) establishment of the suitability of the nonradioactive probe for detection of *A. marginale* in naturally-infected carrier cattle; (3) development of the nonradioactive ISH for localization of *A. marginale* in ticks; and (4) use of nonradioactive ISH for study of the developmental cycle of *A. marginale* in experimentally-infected ticks.

In this study, a 409-bp DNA fragment derived from the *msp-1β* gene of *A. marginale* was amplified and simultaneously labeled with digoxigenin-11-dUTP by PCR. The resulting DIG-labeled 409 bp PCR product was purified by ethanol precipitation and used as a probe for slot-blot and in situ hybridization in subsequent studies. The probe proved to be *A. marginale*-specific when tested with 17 species of microorganisms, including *A. ovis*, *B. bovis*, and *B. bigemina*. The probe detected *A. marginale* DNA from approximately 1,000-10,000 infected erythrocytes in 1.25 ml of whole blood, which is equivalent to a parasitemia level of 0.00001%. After inoculation of infected blood in 2 splenectomized cattle, *A. marginale* infections were detected with the probe 14 days prior to microscopic detection. Microscopically inapparent parasitemias were also detected with the probe for 2 months after recovery from acute disease. The probe was then used for ISH to detect *A. marginale* in bovine erythrocytes on methanol-fixed blood smears. Positive hybridization signals were visualized with LM on *A. marginale* inclusions within infected erythrocytes.

The suitability of the probe for detection of *A. marginale* in naturally infected cattle was tested in 3 herds from Harper (31), Payne (42) and Pittsburg Counties (70) in Oklahoma, and was compared with complement fixation test and microscopic examination. In the 3 herds tested, 80.6% (25/31), 92.8% (39/42) and 57.1% (40/70) of the cows were positive as assessed with slot-blot hybridization. In contrast, only 25.8% (8/31) and 2.86% (2/70) were positive in 2 herds, and no CF positives (0/42) were found in one herd. With microscopy, uncountable parasitemias (<0.01%) were detected from 29.0% (9/31), 4.8% (2/42) and 11.4% (8/70) samples, respectively. All CF- and microscopic-positive samples had positive probe reactions in the DNA hybridization assay. In this part of study, the QIAamp blood kit was used for DNA extraction from bovine blood. The time required for sample digestion and DNA extraction was significantly shortened. The test was quickly executed and the entire procedure for 60 samples was completed in 2 consecutive days. This probe can be prepared and stored a minimum of one year and used repeatedly, thus reducing considerably the cost of the test. Therefore, the probe was demonstrated to be useful in epidemiological investigations and in identification of cattle that are convalescent carriers of *A. marginale*.

The nonradioactive DNA probe was adapted for in situ hybridization of *A. marginale* in paraffin- and LR White-embedded half-ticks of experimentally-infected *D. andersoni*. In both paraffin- and LR White-embedded sections, dark-blue color precipitates of hybridization signals were visualized in salivary gland and gut cells. The results correlated well with microscopic observations on companion tick-halves. When the hybridization conditions applied on paraffin- and LR White-embedded sections were compared, larger amounts of proteinase K digestion, less stringency of hybridization and post hybridization washes were used on LR White-embedded sections. However, LR White-embedded sections provided superior

morphologic preservation and resolution that allowed for easier identification of most tick tissues.

Finally, nonradioactive ISH was used for developmental studies of *A. marginale* in male *D. andersoni* ticks infected as adults. For this part of study, two groups of 10 ticks were collected daily during 7-day acquisition feeding, a 5-day holding period and 10-day transmission feeding. One half of each tick from each group was embedded in paraffin or LR White, while the companion halves were embedded in DER resin for routine microscopy. As determined by ISH and microscopy studies, initial infection of *A. marginale* in ticks occurred in gut tissues on the 7th day of acquisition feeding or the first day of the holding period, and infection persisted throughout transmission feeding. Salivary glands became infected with *A. marginale* on the first day of transmission feeding and remained infected throughout entire transmission feeding period. The results confirmed those from previous developmental studies on individually-dissected gut and salivary gland tissues. In addition, *A. marginale* infection was also observed in interstitial, reproductive, skeletal muscle, fat body and Malpighian tubule tissues after onset of transmission feeding. *A. marginale* infection originates in gut cells, and many tissues eventually become infected after the onset of transmission feeding, resulting in a generalized infection. The infection of multiple tissues may contribute to the ability of *A. marginale* infection to persist in male ticks.

The nonradioactive DNA probe developed and tested in this study has proved to be a sensitive diagnostic tool for *A. marginale* infection in cattle. When used in ISH for developmental studies of *A. marginale* in its tick vector, the probe allowed for identification of several additional sites of infection and replication that had not been discovered previously. This nonradioactive DNA probe, used either in slot blot hybridizaiton or ISH, will be quite

useful in future research on *A. marginale*. It should be especially useful in development of diagnostic tests, on studies of the development of this rickettsia in ticks, and in study of development of the parasite in continuously-cultured cells.

VITA

Nie-Lin Ge

Candidate for the Degree of

Doctor of Philosophy

Thesis: DEVELOPMENT OF NONRADIOACTIVE DNA HYBRIDIZATION ASSAYS FOR DETECTION OF *Anaplasma marginale* IN CATTLE AND TICKS

Major Field: Veterinary Pathology Specialization: Pathobiology

Minor Field: Microbiology

Biographical:

Personal Data: Born in Qianshan, Anhui Province, China, on August 13, 1962; the son of Liangsong Ge and Zhuanyong Wang.

Education: Graduated from Yezhai High School, Qianshan, Anhui, China, in July 1979; earned Doctor of Medicine degree from Wannan Medical College, Wuhu, China in July, 1984; earned Master of Science degree in Medical Parasitology & Entomology in July, 1987; completed requirements for the Doctor of Philosophy degree at Oklahoma State University in May, 1996.

Professional Experience: Lecturer and Assistant Professor, Department of Medical Parasitology, Jinan University School of Medicine, July, 1987 to April, 1991; Visiting Scientist, Department of Parasitology, Microbiology and Public Health, College of Veterinary Medicine, Oklahoma State University, April, 1991 to April, 1992; Department of Pathology, College of Veterinary Medicine, Oklahoma State University, May, 1992 to July, 1993; Graduate Research Associate, Department of Pathology, College of Veterinary Medicine, Oklahoma State University, August, 1993 to May, 1996.

Professional Memberships: American Society for Microbiology; New York Academy of Sciences; Sigma Xi, The Scientific Research Society; The Society for Tropical Veterinary Medicine.