IMMUNOCYTOCHEMICAL LABELING OF *ANAPLASMA MARGINALE* THEILER IN *DERMACENTOR ANDERSONI* STILES WITH PEROXIDASE-ANTIPEROXIDASE TECHNIQUE

By

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CHAPTER I

INTRODUCTION

Bovine Anaplasmosis

Anaplasma marginale, the causative agent of bovine anaplasmosis, causes acute anemia in cattle and a latent infection in black-tailed deer. The disease is associated with the presence of small punctiform marginal bodies in the erythrocytes. These bodies were described originally by Theiler.[1] The scientific name is descriptive morphologically in that *Anaplasma* indicates that the agent lacks cytoplasm and *marginale* refers to the position of the organism at the periphery of the erythrocyte.[2] *A marginale* in the host erythrocyte consists of an inclusion vacuole containing one or more initial bodies, each of which is surrounded by a pellicular membrane.[3] The limiting inclusion membrane has been demonstrated to be of erythrocytic origin.[4]

Anaplasma was originally assigned by taxonomists to an anomolous group in the protozoa. However, further study, especially by electron microscopy, suggested a rickettsia-like morphology and reproductive cycle. According to the 8th edition of *Bergey's Manual of Determinative Bateriology*, *Anaplasma* is currently classified in the Order Rickettsiales, Family Anaplasmataceae.[5]

A developmental cycle of *A marginale* in the bovine erythrocyte has been proposed.[6,7] Ristic suggested that the initial body enters the erythrocyte by evagination of the cytoplasmic membrane. A parasitophorous

vacuole forms the inclusion body in which the initial body multiplies by binary fission. Small, electron-dense particles, which may be a stage in the organism's life cycle, have also been described.[3]

Tick Transmission of Disease-Causing Organisms

Ticks have been shown to be both vectors and reservoirs for a large number of disease-causing organisms including viruses, rickettsiae, bacteria, and protozoans.[8] A number of characteristics make ticks especially efficient disease vectors: (1) they are obligatory blood feeders and, depending on species, may feed a number of times on different hosts; (2) they usually feed for extended periods; (3) both transstadial and transovarial transmission of disease agents is common; (4) they can remain infectious for long periods; and (5) many species are very long lived.

Smith and Kilborne first demonstrated that ticks were involved in transmission of pathogens in their study of *Babesia* in cattle.[9] Other tick-borne cattle disease agents include *Theileria parva* (East Coast fever), *Cowdria ruminantium* (heartwater fever), *Ehrlichia bovis* (ehrlichiosis), and *Anaplasma marginale* (anaplasmosis).[8] *Chlamydia sp* (epizootic bovine abortion) has also been shown to be harbored for long periods by ticks.[10] Most of these microorganisms, although pathogenic for vertebrates, usually cause no detectable harm in the tick vector.[11]

Rickettsiae are believed originally to have been symbiotes of acarines and to have become secondarily pathogenic for vertebrates through the parasitic habits of their arthropod vectors.[12] *Wolbachia*, a nonpathogenic symbiotic rickettsia-like organism in ticks, with no known pathogenicity for vertebrates, may represent an ancestral rickettsial form.[13]

The infection of ticks with disease agents occurs with ingestion of infectious blood from the vertebrate host. The blood flows from the mouth to the digestive diverticula where it is dehydrated and the liquid portion passes to the hemolymph.[11] The erythrocytes are quickly hemolyzed and most of the hemoglobin and proteins pass into the digestive cells.[14] Hemoglobin remaining in the gut is crystallized and eventually egested.

Biological transmission of disease agents in ticks is characterized by growth and/or multiplication of the organisms within various tick organs and cells and subsequent transfer to a susceptible host. _Rickettsia rickettsii_, causative agent of Rocky Mountain spotted fever, is found widespread throughout tick tissues, including sperm, ova, and salivary glands.[15] Transmission of disease agents among tick vectors may be by transstadial (stage to stage) or transovarial (through the egg to successive generations) transmission. Distribution of rickettsiae in ticks is believed to be via the haemocytes.[16] Comparative studies on the development of different species in haemocytes suggest that patterns of rickettsial development are not similar but are species specific.[17]

Ticks are able to infect vertebrates in a variety of ways. The organism may be transmitted in the saliva during the bite[8] or in egested fecal material.[18] Transmission by regurgitation of gut contents occurs with _Cowdria ruminantium_.[13] Rickettsiae excreted by ticks in the coxal fluid may also contaminate the host.[11] Finally, it has been theorized that mechanical transmission of pathogenic organisms can be effected by any blood-feeding arthropod.[19]

Changes in tick metabolism also seem to influence the transmission of disease-causing organisms by inducing changes in the metabolism of the organism transmitted. Induction of infective stages of _Theileria parva_

has resulted from exposure of ticks to elevated temperatures[20] such as would result from feeding on or from close association with a vertebrate host. Rickettsia rickettsii has also been shown to increase in number with increase in tick activity, and as activity decreases, a corresponding decrease in virulence occurs.[21]

Transstadial transmission results when ecdysis occurs and the disease agent is still harbored by the next instar. According to Balashov, not all organs are equally involved in the molting process.[14] Tissues such as midgut and ovaries do not undergo major changes while salivary glands always degenerate.[11] Transstadial transmission of many rickettsial species has been reported.[8,9,22,23,24] Cowdria ruminantium is transstadially transmitted but passage of the microbe through the egg has not been shown.[24] Stage to stage transmission has also been shown in Coxiella burnetii.[22] According to Řeháček,[11] Philip demonstrated transstadial transmission of Ehrlichia canis in Rhipicephalus sanguineus but was unable to achieve similar results with Dermacentor andersoni.[23] D andersoni, however, has been shown to transmit Rickettsia rickettsii through follicular cells to the eggs.[25]

Anaplasma marginale in Ticks

The role of ticks in transmission of A marginale has been under investigation for many years. Several species of ticks have been shown to be capable to biological transmission of anaplasmosis.[26-31] Dermacentor andersoni, D occidentalis, and Ixodes pacificus have remained infective for extended periods in the laboratory.[28] Natural transmission in the field has been shown with D andersoni and D occidentalis.[29-32] Boophilus

annulatus and B microplus have also been incriminated as vectors in tropical and subtropical regions.[33]

Transstadial transmission of A marginale has been shown with selected ixodid ticks.[34,35] Dermacentor andersoni and D variabilis, infected by feeding as nymphs on an infected calf, subsequently tranmsitted the organisms as adults. Inoculation of susceptible animals with gut homogenates from unfed adults, infected as nymphs, also resulted in patent anaplasmosis infections.[34,35] Transovarial transmission has been reported by one researcher;[36] however, work by others has not supported this.[26,34,37]

A marginale is transmitted from vertebrates to ticks during the acquisition of blood meals. Organisms from ingested erythrocytes were present in the gut lumen, prior to digestion, for up to two days after tick detachment.[38] A marginale has been demonstrated by electron microscopy in midgut epithelial cells of replete nymphal and feeding adult ticks, and in unfed incubated and unincubated adult ticks infected as nymphs.[39] The anaplasmal organisms observed in replete nymphal, feeding adult, and unfed adult ticks consisted of individual organisms scattered throughout the cytoplasm of the epithelial cells. Organisms in unfed incubated adult ticks occurred within membrane bound colonies situated primarily along the basement membrane of the midgut epithelial cells.

Studies have shown that colony development occurred as a result of elevated temperatures and/or tick feeding.[34,39] Incubation of infected, unfed adult ticks for 3 days at 37 C has resulted in colony formation.[39,40] Splenectomized calves exposed to gut homogenates from partially fed or from unfed incubated ticks experienced shorter prepatent periods than did those exposed to gut homogenates from unfed, unincubated ticks.[34]

Identification of A marginale in tick organs other than the gut has not been reported. It has been suggested that multiplication by binary fission occurs within the malpighian tubules,[38] but this hypothesis has not been confirmed by other researchers.[41,42] Inoculation of salivary gland homogenate from infected ticks into susceptible cattle has resulted in patent anaplasmosis infection,[35] suggesting the presence of anaplasmal organisms in these organs. However, electron microscopy studies have not confirmed the presence of the organisms in salivary glands.[39]

Immuno-Labeling of A marginale in the Tick

Immunocytochemical techniques were introduced by Coons and Kaplan in their pioneering work with fluorescein-conjugated antibody.[43] This was followed by a myriad of localization techniques in which antibodies were conjugated with numerous marker substances for visualization with both light and electron microscopes. Singer and Schick introduced the use of electron-opaque ferritin molecules, covalently bound to antibody, for use in the electron microscope.[44] Enzyme-labeled antibody was shown by others also to be effective in antigen localization.[45]

A number of labeling methods have been utilized in attempts to identify A marginale in tick tissues. Fluorescent antibody labeling indicated that A marginale remained in gut contents for up to two days post detachment in the case of nymphal ticks that had fed on an infected cow.[38] Identification of the organism was not possible, however, after ticks digested the infected bovine erythrocytes.[41,42] Studies on partially-fed adult ticks that were infected as nymphs have shown positive fluorescence on frozen gut sections.[35] Ferritin-conjugated antibody has also been used to label A marginale in gut homogenates of adult ticks that were infected

as nymphs.[46] More recently, colonies of A marginale have been described in midgut epithelial cells of unfed, incubated adult ticks infected as nymphs.[39] The colonies have been identified with the light microscope by fluorescent antibody labeling technique.[40]

Peroxidase-Antiperoxidase Technique

The unlabeled antibody peroxidase-antiperoxidase (PAP) method was developed by Sternberger and associates.[47] This method differs from conventional histochemical labeling methods in that none of the active antibodies are covalently bound with the marker substance prior to application (Figures 1 and 2). As a result, antibody-antibody interaction is not impaired by chemical alterations due to previous binding. Numerous studies with the PAP technique have shown higher sensitivities and increased binding with unconjugated antibody as opposed to techniques using conjugated antibody.[48-51] Burns found the unlabeled method to be 20 times more sensitive than a similar labeled technique.[48] Titers are often reported that are 100 to 1000 times higher than those obtained by immunofluorescence.[52]

The peroixidase-antiperoxidase complex is especially well suited for electron microscopy. Three peroxidase and two antiperoxidase subunits form a characteristic cyclic structure with an average diameter of 250 Å.[47] These structures are easily discernible with the electron microscope.

Purpose of Study

The colonies of A marginale in the midgut epithelial cells of D andersoni have been well described at the electron and light microscope levels.[39,40] Although fluorescent and ferritin-conjugated antibody

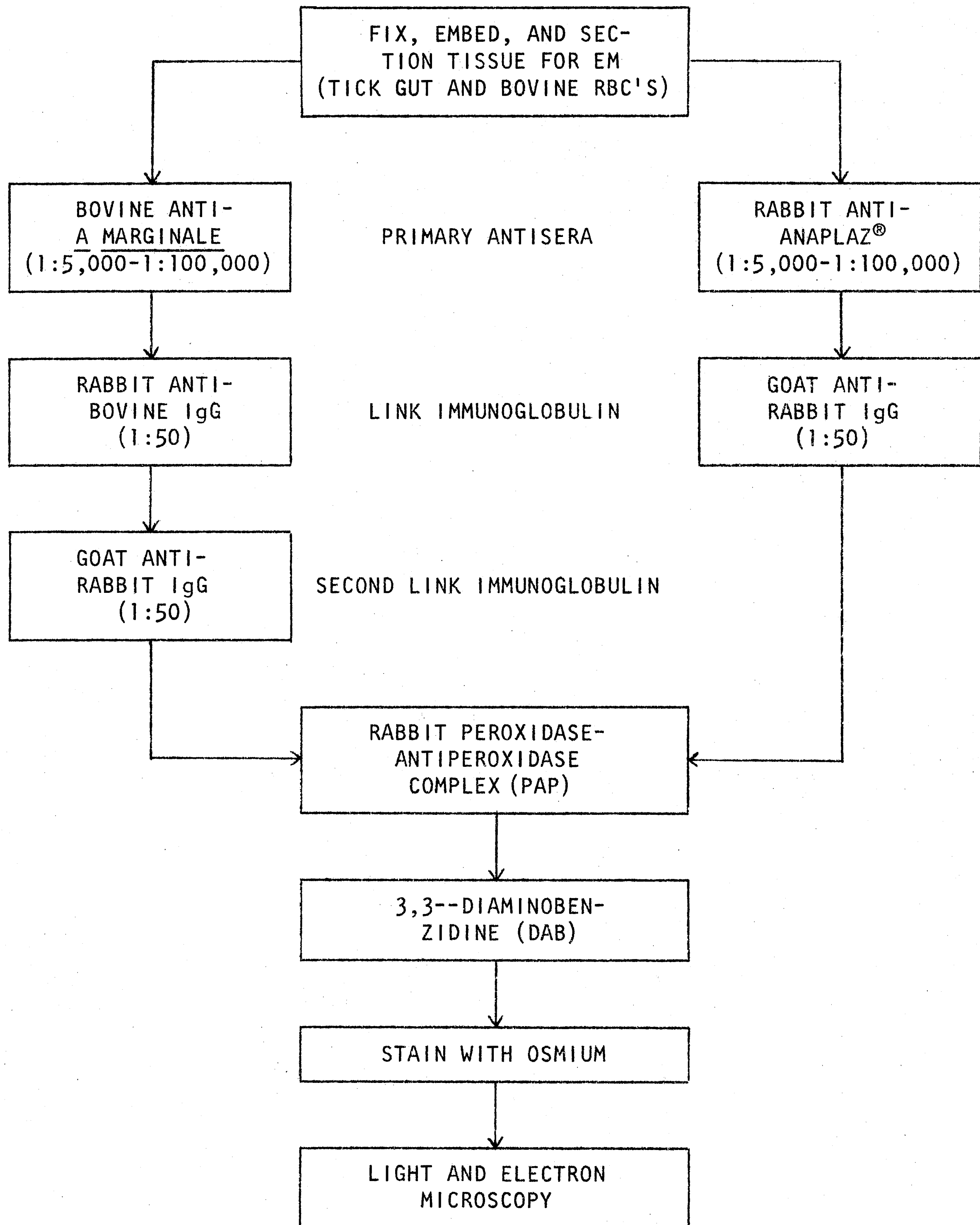
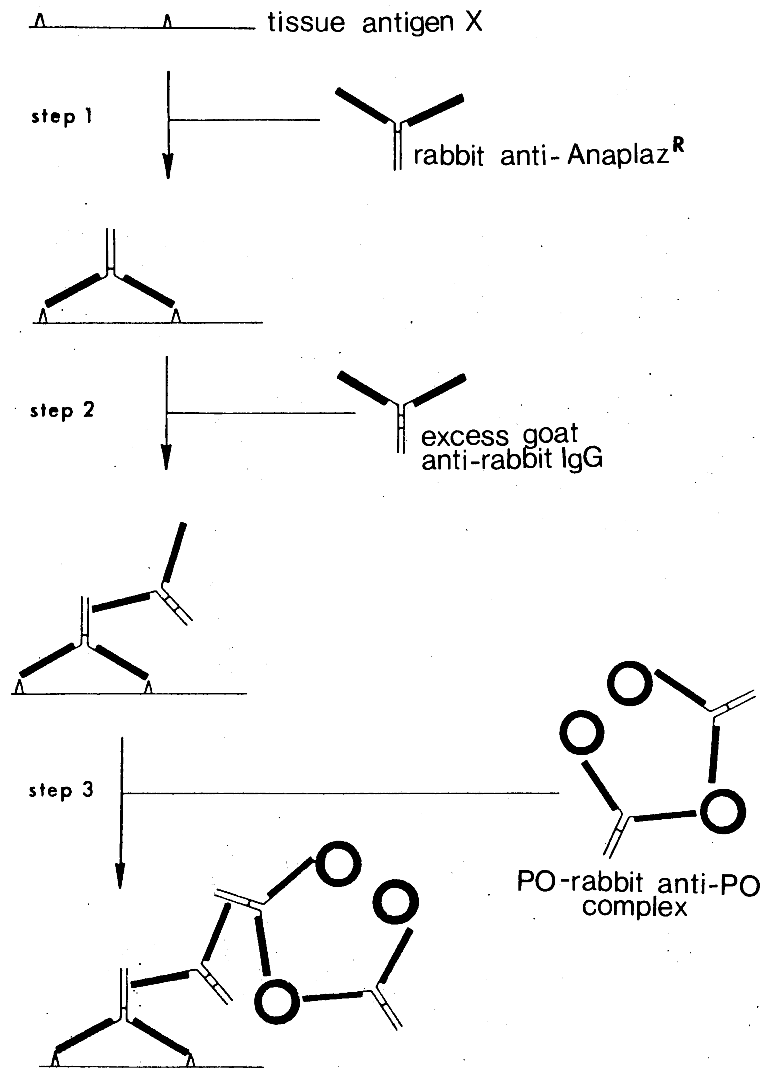


Figure 1. The Experimental Design Used to Identify *Anaplasma marginale* in Tick Midgut by Peroxidase-Antiperoxidase technique



From: Sternberger LA: Immunocytochemistry. New York, New York, John Wiley and Sons, Inc., 1979, p. 109.

Figure 2. Schematic Diagram of Peroxidase-Antiperoxidase Technique Showing Sequential Application of Reactive Immunoglobulins

methods have been used to specifically label organisms within tick midgut cells, neither technique allows positive morphologic identification in conjunction with specific labeling.[40,46] The PAP labeling method was developed to pinpoint antigens in intact tissues. The purpose of the present study was to reconfirm, by specific PAP labeling, that the colonies of organisms described previously in the tick midgut are aggregates of *A marginale*.

CHAPTER II

MATERIALS AND METHODS

Preparation of Primary Antisera

Rabbit Anti-Anaplaz®[a]

Rabbits were injected subcutaneously with 0.5 ml of Anaplaz® vaccine in Freund's complete adjuvant. Two booster injections of 0.5 ml each were given at four-week intervals. Blood was collected from the ear vein seven days after the second booster and allowed to clot. The serum was collected and complement-fixation (CF)[b] tests were conducted to demonstrate the presence of *Anaplasma* antibody. Aliquots of 0.5 ml were frozen at 0 C in BEEM[c] capsules for later use.

Bovine Anti-*Anaplasma marginale*

Bovine serum with a CF titer of 1:40 to *A marginale* was collected from a calf experimentally infected by intravenous injection of 30 ml of carrier blood. The globulin portion of the serum was removed by precipitation with anhydrous sodium sulfate according to the procedures of Stelos[53] and frozen in 0.5 ml aliquots for later use.

Propagation and Infection of Ticks

Dermacentor andersoni were reared and maintained at the Oklahoma State University, Department of Entomology, Tick Laboratory.[54] Larvae

were fed on rabbits to facilitate development of the nymphal stage. Nymphal ticks were then placed on orthopedic stockinettes attached to an Anaplasma-infected cow and allowed to engorge. The stockinettes were attached when the host's parasitemia reached 1 percent; thus feeding coincided with the higher parasitemia. Replete ticks were collected and allowed to molt to the adult stage. Four weeks after molting, the ticks were incubated for $1^1/_2$ days at 37 C to facilitate development of colonies of A marginale.[39] The gut was dissected, fixed for two hours in cold 2 percent glutaraldehyde in 0.27M cacodylate buffer, washed three times in buffer and processed for electron microscopy.

Collection and Fixation of A marginale-Infected Erythrocytes

Twenty-five ml of blood was drawn into a heparinized syringe from the jugular vein of a cow experimentally infected with anaplasmosis. The blood was centrifuged for 20 minutes at 13,925 Xg. The supernatent fluid was removed and replaced with an equal volume of 2 percent glutaraldehyde in 0.27M cacodylate buffer and allowed to fix for two hours. The fixed erythrocytes were spun and the fixative was replaced with an equal volume of cacodylate buffered wash; this step was repeated three times. After the final wash, the supernatent fluid was removed and the erythrocytes were embedded in softened 2 percent agar. Upon hardening, the agar-embedded material was cut into $1mm^3$ blocks and processed for electron microscopy.

Electron Microscopy

The tick gut and agar-embedded erythrocytes were dehydrated by

passing through a graded series of ethanol. Half of the tissue was embedded in Dow Epoxy Resin 732 and the other half was embedded in Araldite 6005. In both cases infiltration was carried out using propylene oxide as the transition fluid. The embedded tissues were cut on an ultramicrotome[d] with a diamond knife[e] and the light gold thin sections were mounted on 200 mesh nickel grids.

Unlabeled Antibody Peroxidase-Antiperoxidase Technique

Grids were floated on drops of aqueous 10 percent hydrogen peroxide for eight minutes, washed immediately by a jet of distilled water and placed in 1:100 normal goat serum (NGS)[f] for three minutes. All dilutions, unless otherwise noted, were made with 0.05M phosphate buffer (pH 7.4) to which was added 2.5 mg/ml crystalline human albumin[g]. Grids were placed in various dilutions (1:5000 to 1:100,000) of primary antisera, rabbit anti-Anaplaz®, in BEEM capsules at 4 C for 48 hours. After exposure to the antisera, grids were jet-washed in 0.5M phosphate buffer and then floated for three minutes on each of the following solutions; NGS, goat anti-rabbit IgG and PAP complex. The blocking reaction with 1:100 NGS was repeated and the grids were placed on drops of goat anti-rabbit IgG[f] diluted 1:50 and were then jet washed. Nonspecific labeling was again inhibited by blocking with 1:100 NGS. The final staining solution of peroxidase-antiperoxidase complex[h] diluted 1:50 was followed by a jet wash.

DAB reaction mixture was prepared just prior to use as follows: 22 mg 3,3--diaminobenzidine[g] (DAB) was added to 175 ml of 0.05M Tris buffer (pH 7.6) to which had been added 1.5 ml of 0.3 percent hydrogen peroxide.

The grids were suspended in a moving solution of DAB for three minutes followed by suspension for three minutes in distilled water. After application of DAB-H_2O_2, the grids were stained on drops of aqueous 2 percent osmium tetroxide for eight minutes.

A modification of the procedure described above was also used in which bovine anti-A marginale serum was the primary antiserum. An additional step involving rabbit and anti-bovine serum was then required (Figure 3).

The peroxidase-antiperoxidase experimental groups and controls were arranged as follows: Experimental groups included: (1) primary antiserum-rabbit anti-Anaplaz®, (2) primary antiserum-bovine anti-A marginale, (3) various primary antiserum dilutions. Controls: 1) 1° antiserum-CF negative bovine serum, (2) 1° antiserum-normal goat serum, (3) 1° antiserum-preinoculation rabbit serum, (4) link antiserum-normal goat serum. Sections of Anaplasma-infected bovine erythrocytes were processed concurrently as an additional method control.

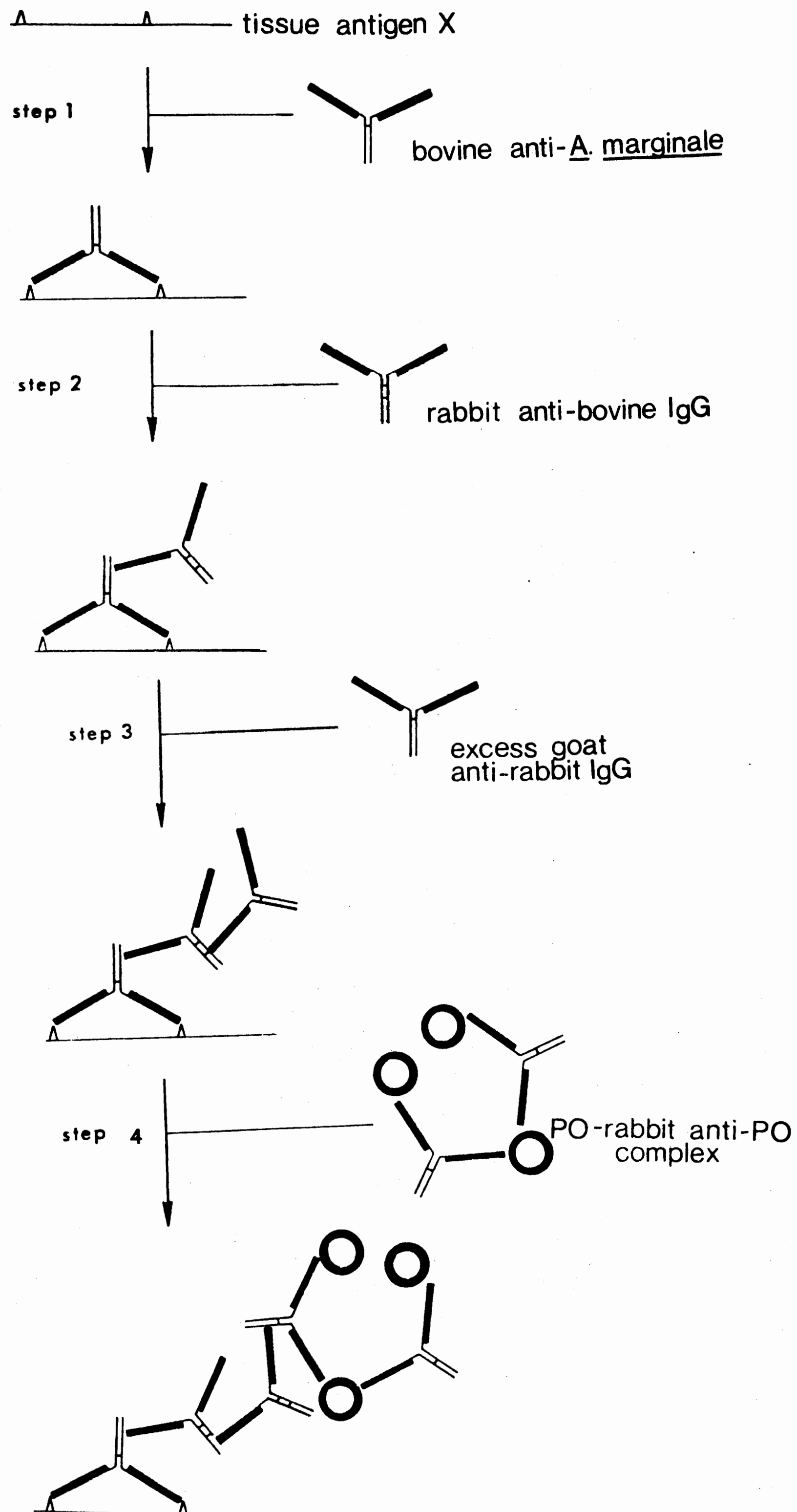


Figure 3. Schematic Diagram of Modified Peroxidase-Antiperoxidase Technique

NOTES

[a]Anaplaz® vaccine, Fort Dodge Laboratories, Fort Dodge, Iowa.

[b]CF titers to A marginale determined by the Oklahoma Animal Disease Diagnostic Lab according to procedure of the National Veterinary Service Laboratory, Ames, Iowa.

[c]Polysciences Inc., Paul Valley Industrial Park, Warrington, PA.

[d]Sorvall MT-2, Sorvall Inc., Newton, Conn.

[e]DuPont Diamond Knife, DuPont Instruments, Wilmington, Del.

[f]Kallestad Labs, Inc., Chaska, Minn.

[g]Sigma Chemical Corp., St. Louis, MO.

[h]Cappell Laboratories, Inc., Cochranville, Penn.

CHAPTER III

RESULTS

Deposition of the ring-like PAP complexes on Anaplasma organisms was obvious on both the tick gut sections and on sections of Anaplasma-infected bovine erythrocytes. The colonies of A marginale organisms in tick tissues were like those described by Kocan et al.[39] and consisted of aggregates of pleomorphic rickettsia-like organisms within a limiting membrane (Figure 4). PAP complexes were deposited primarily over the pleomorphic forms within the colonies (Figures 5, 6, and 7). The density of labeling differed among organisms within a single colony. Intensity of labeling also depended on the dilution of primary antiserum and was most specific at dilutions of 1:20,000 to 1:40,000. Dilutions below this level resulted in excessive nonspecific staining and often the tissue was obliterated. Large electron-dense granules commonly seen in tick gut labeled in Anaplasma-infected ticks but not in uninfected controls. Labeling of A marginale in the tick tissue was most specific when bovine anti-A marginale was used as the primary antiserum. Rabbit anti-Anaplaz® serum resulted in excessive nonspecific staining. Negative controls did not label (Figure 8).

Both of the primary antisera labeled the A marginale initial bodies in inclusions of infected bovine erythrocytes. However, there was more nonspecific staining with the rabbit anti-Anaplaz® serum. Initial bodies in the erythrocytes stained more intensely than did organisms within

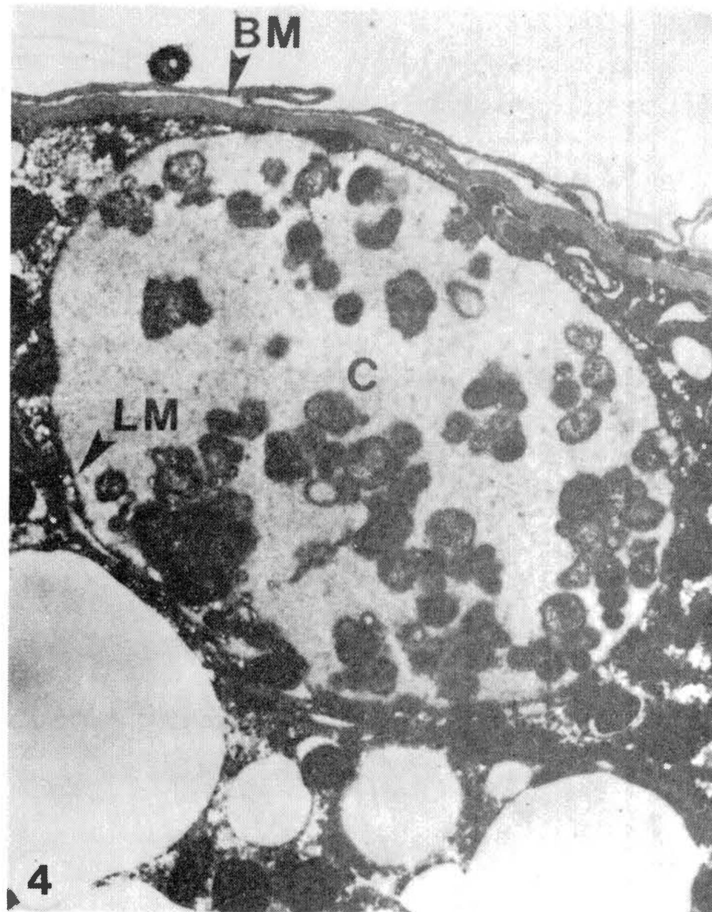


Figure 4. Colony (C) of A marginale Near the Basement Membrane (BM) of a Midgut Epithelial Cell of Dermacentor andersoni (a limiting membrane [LM] is apparent; uranyl acetate and and lead citrate stained; X 13,800)

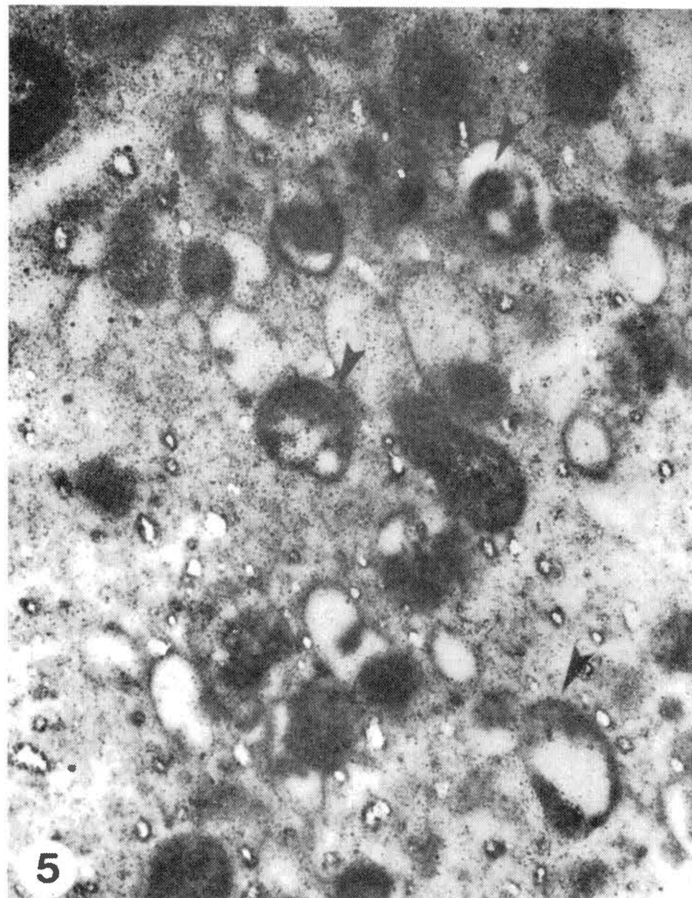


Figure 5. Electron Micrograph of PAP-Labeled A marginale Organisms (Arrows) Within a Colony (individual PAP complexes are apparent; primary antiserum--bovine anti-A marginale diluted 1:20,000; X 23,400)

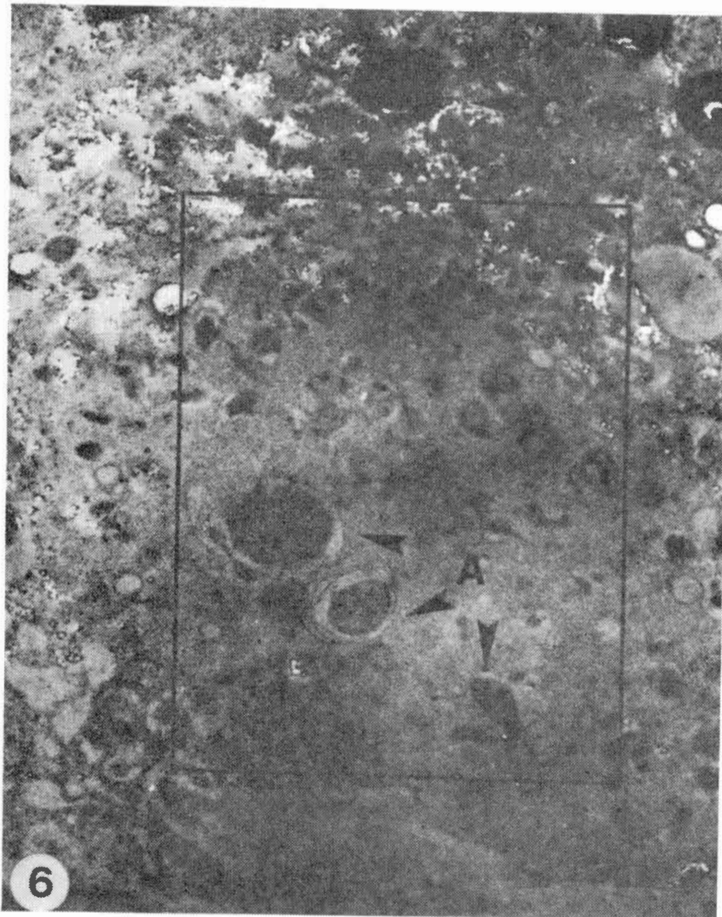


Figure 6. Electron Micrograph of PAP-Labeled A marginale (A) Within a Colony (granules [G] commonly found surrounding colonies also label; bovine anti-A marginale diluted 1:20,000; X 9500)

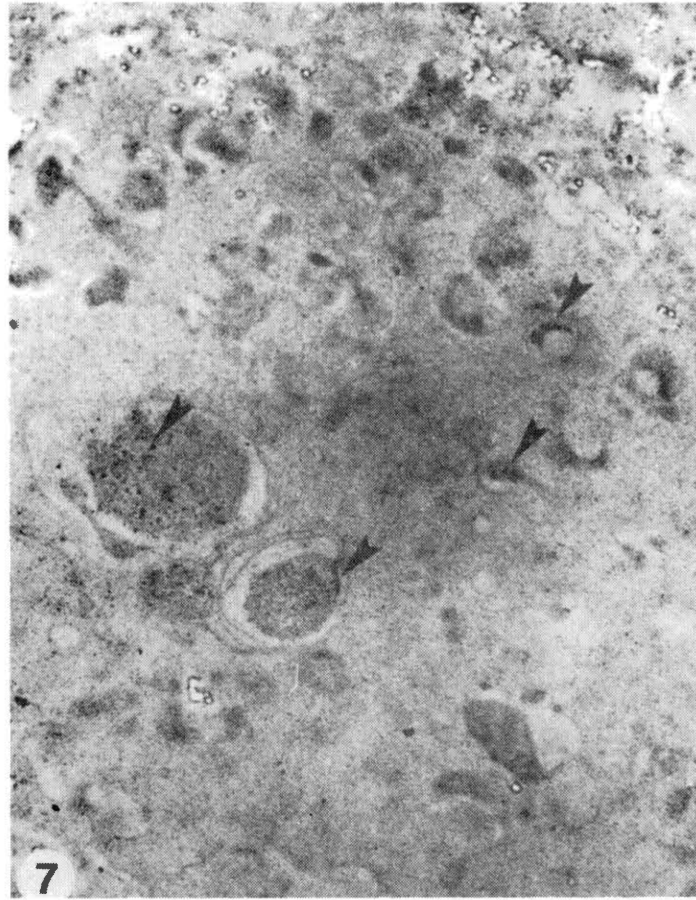


Figure 7. Electron Micrograph of Higher Magnification of 6 (individual PAP complexes [arrows] are apparent on *A marginale* organisms; X 16,500)

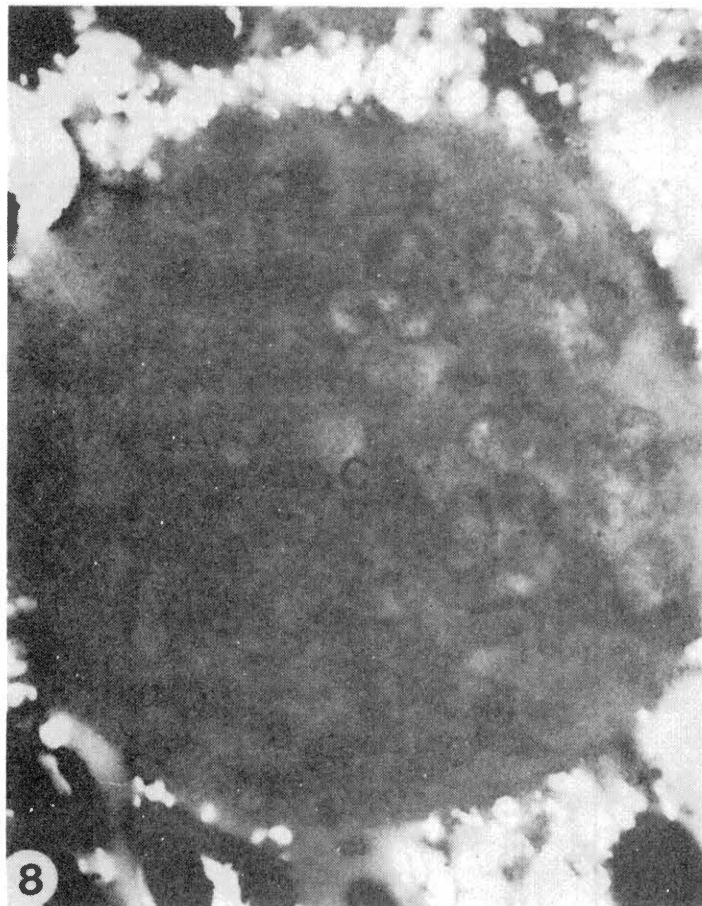


Figure 8. An Electron Micrograph of an *A marginale* Colony (C) (primary antiserum--CF negative bovine serum; no specific labeling of organisms occurred; X 21,300)

colonies in tick midgut tissues. Deposition of PAP complexes occurred primarily on the initial bodies (Figure 9). As with the tick tissue, intensity of labeling differed with varying dilutions of primary antisera, and when negative primary serum was used, no labeling occurred.

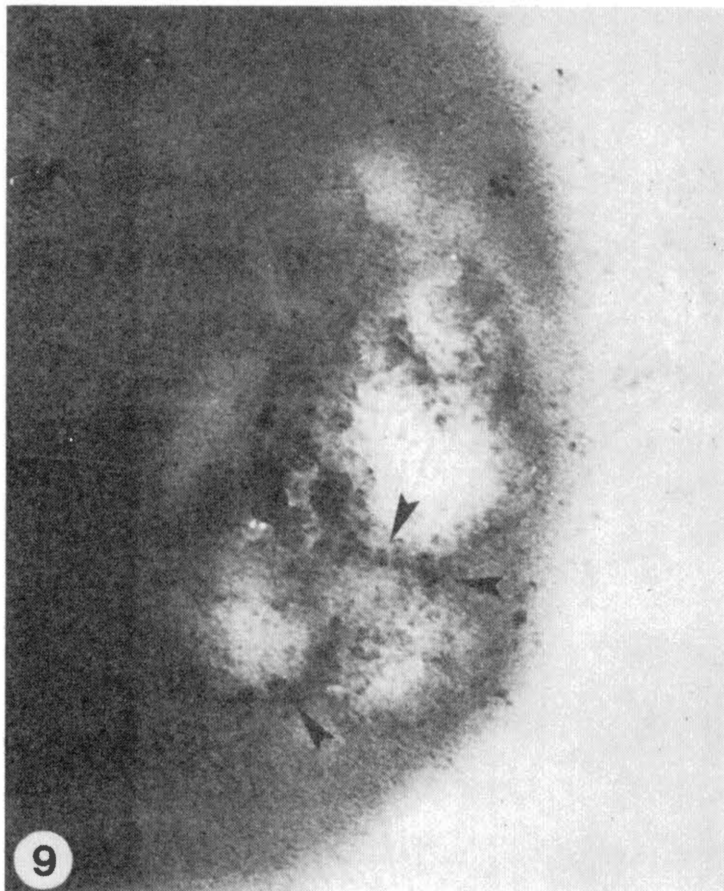


Figure 9. Electron Micrograph of PAP-Labeled A marginale Initial Bodies in Bovine Erythrocyte (deposition of PAP is apparent on pellicular surface of initial bodies [arrows]; primary antiserum--rabbit anti-Anaplaz® diluted 1:30,000; X 110,400)

CHAPTER IV

DISCUSSION

Colonies of *A marginale* in adult *Dermacenter andersoni* were first described using electron microscopy by Kocan et al.[39] The colonies were found in adult, incubated ticks which had been infected as nymphs. They occurred primarily along the basement membrane of midgut epithelial cells and consisted of aggregates of pleomorphic organisms enclosed by a limiting membrane. The present study describes a technique which was used to positively identify structures in intact host cells with the electron microscope. *A marginale* has been identified previously in tick gut by immunolabeling with ferritin-conjugated antibody.[46] However, due to the size of the ferritin-antibody complex, mechanical disruption of the host cell plasmalemma was required before labeling could occur. The PAP method allows labeling of intact structures which have been fixed and sectioned for electron microscopy.

PAP labeling of *A marginale* in the tick appeared as deposits of ring-like complexes distributed primarily over the organisms within the colonies. There was some nonspecific labeling in areas immediately surrounding the colonies, presumably as a result of antigen diffusion due to difficult tissue fixation. Gut from incubated ticks was difficult to fix and infiltrate and therefore, deterioration of sections in the electron beam was not uncommon. It is not clear why the electron-dense granules labeled in infected ticks.

Nonspecific labeling was most obvious when rabbit anti-Anaplaz® antiserum was used. Deposition of PAP complexes occurred indiscriminantly over tick gut tissue and to a lesser degree on the infected erythrocyte sections. It is not certain whether the cross-reactivity occurred with antigens in the tissues or within the resin. The latter is suspected because PAP deposition often occurred on sections of plastic not containing tissue.

Intensity of labeling differed among organisms within a single colony. This was especially apparent on tissues on which a high dilution of primary antiserum was used. In these cases, labeling was apparent over the larger organisms while smaller organisms labeled very little. The difference was less obvious when lower dilutions of antisera were used. The differences in labeling suggest varying antigenicity of organisms, possibly reflecting the presence of multiple stages of development, as suggested by Kocan et al.[55] Such a developmental cycle has been described for Chlamydia sp[56] in which four distinct forms of development can be distinguished; a small, electron-dense elementary body, a large pleomorphic reticulate body, and condensing and dispersing forms which occur between these two stages. Colonial forms also occur in the development of Ehrlichia canis[57] and Cowdria ruminantium[58] in their respective vertebrate hosts.

PAP labeling of A marginale initial bodies in inclusions of infected bovine erythrocytes was also shown. At dilutions of 1:20,000 to 1:40,000 deposition of PAP complexes occurred over the pellicular surface of the initial bodies. At lower dilutions, the chromatin within the initial bodies labeled. Inclusion membranes did not label, a finding that is in agreement with results of previous labeling studies.[4,59] Ristic[7] has reported that A marginale inclusions in bovine erythrocytes contain 2

antigenic components, the initial body and a lipoprotein fraction. Antibody production against these two components in different proprotions may explain the differences in labeling with various dilutions of primary antisera.

The PAP immuno-labeling technique appears to be well suited for identification of microorganisms in intact tissues. The technique can be modified for use at both light[60] and electron microscope levels and it has been shown to be 100 to 1000 times more sensitive than fluorescent antibody techniques.[52] Dilutions of primary antisera are routinely in the range of 1:100,000 to 1:1,000,000. Unlike the ferritin-conjugated antibody, PAP labeling occurs on intact tissues allowing positive morphologic identification of structures. The characteristic ring-like PAP complex is especially well suited for ultrastructural research because it can be identified easily in the electron microscope.

CHAPTER V

SUMMARY

The pathogenicity of *Anaplasma marginale* in the bovine host has long been recognized. Very little is known, however, about the life cycle of the organism in either the bovine host or in ticks that serve as vectors. The structure of *A marginale* in the bovine erythrocyte and its relationship to the host cell has been well described, and knowledge of the organism's morphology in ticks has increased greatly within recent years. Until introduction of the peroxidase-antiperoxidase (PAP) method of immunocytochemistry, however, it was very difficult to correlate information contained in morphological studies with that gained from specific immunolabeling studies. The PAP technique can be a valuable adjunct in specific identification of structures at the cellular level. It may be especially useful in elucidating that portion of the *A marginale* life cycle which is now unknown.

In the present study, colonies of *Anaplasma marginale* in midgut epithelial cells of adult ticks that had been infected as nymphs were specifically labeled using the PAP method. Examination of infected tissue sections by electron microscopy revealed deposition of ring-like PAP complexes over organisms within colonies. The intensity of labeling differed among organisms within a single colony, probably as a result of varying antigenicity among the subunits. Such variation could be due to differences in sequential development of organisms within a given colony. The labeling

observed on organisms in the colonies was similar to that seen on anaplasmal initial bodies in bovine erythrocytes examined concurrently. Control tissue sections prepared with negative antisera did not label.

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Master of Science

Thesis: IMMUNOCYTOCHEMICAL LABELING OF ANAPLASMA MARGINALE THEILER IN DERMACENTOR ANDERSONI STILES WITH PEROXIDASE-ANTIPEROXIDASE TECHNIQUE

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