STUDIES ON TWO *EHRLICHIA* SPECIES

(RICKETTSIALES: EHRLICHIEAE)

By

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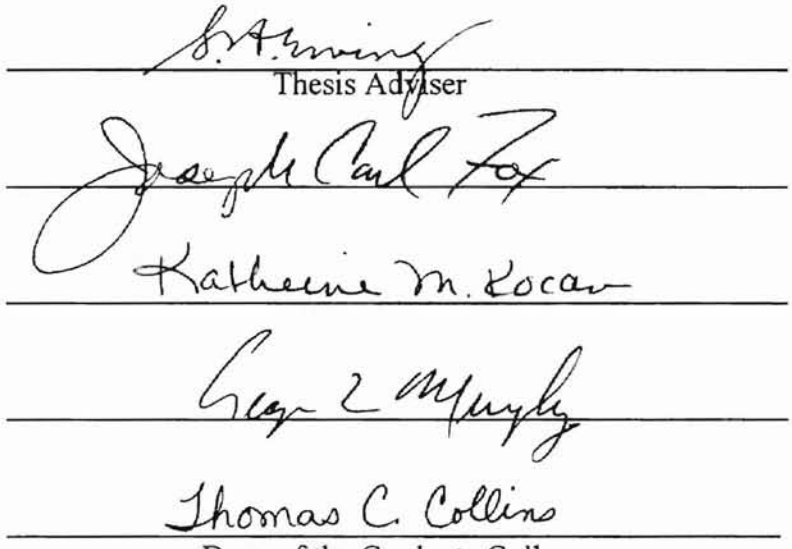
STUDIES ON TWO *EHRLICHIA* SPECIES

(RICKETTSIALES: EHRLICHIEAE)

Thesis Approved:



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CHAPTER I

Failed transmission of *Ehrlichia canis* by *Rhipicephalus sanguineus* after passage in tissue culture

Summary:

A recent isolate of *Ehrlichia canis* (Ebony) and another (Oklahoma) which had been passaged in tissue culture (DH82 cells) for an undetermined number of generations before being reintroduced into dogs were studied for transmissibility by the brown dog tick, *Rhipicephalus sanguineus*. Ticks were acquisition fed on acutely infected dogs as larvae or nymphs and, after molting, were transmission fed as nymphs and adults on seronegative dogs. Dogs were monitored daily for clinical signs and by blood smear evaluation and weekly by serologic and hematologic parameters. The Ebony isolate was successfully transmitted to dogs by nymphs and by adults while all ticks that were exposed to the tissue-cultured isolate failed to transmit it. Sequencing of 16S rDNA showed 99.9% similarity between the two agents; one nucleotide present in the Ebony isolate was different from the Oklahoma isolate.

Introduction:

Canine agranulocytic ehrlichiosis, caused by *Ehrlichia canis,* is a tick-borne disease first discovered in Africa.[1] It has been reported in many parts of the world including the United States of America.[2-5] The role of the brown dog tick *(Rhipicephalus sanguineus)* as a vector was first demonstrated by Donatien and Lestoquard,[1] and later the vector potential of this ixodid was studied experimentally by Groves et al.[6] and Lewis et al. [7] This ixodid is generally acknowledged to transmit the agent transstadially under natural conditions, although we have found no reports of more recent experimental transmission studies except those from our laboratory.[a,b] One unconfirmed report that suggested transovarial transmission was based upon intravenous inoculation of larval tick tissues into a monkey.[8] *Dermacentor variabilis* has been shown experimentally to transmit *E. canis* transstadially.[a] In the present study we attempted transmission of two isolates (designated Oklahoma and Ebony) of *E. canis*; the Oklahoma isolate had been passaged in tissue culture[9] and the other (Ebony) had been isolated recently from a naturally infected dog from southwestern Oklahoma. The Oklahoma isolate had been successfully transmitted by ticks prior to passage in tissue culture.[b]

Material and Methods.

Animals: Eleven Walker Hound pups and one adult Walker Hound (#2100) raised by the Laboratory Animal Resources Unit (LAR) of Oklahoma State University and one mixed-breed pup were utilized in this experiment. The Walker Hound pups numbered 2207, 2208, 2271, 2272, 2442, 2444, 2445, 2446, 2448, 2449, 2478 and the mixed breed pup (#2399) were found seronegative for *E. canis* infection before being used for the experiments. The animals were housed in conventional laboratory animal facilities and were kept in isolation while being exposed to ticks. Pups that served as uninfected controls, or which did not acquire infection as principals in one phase of the study, were used again in subsequent phases as principals or controls. All dogs were maintained in accordance with standards of humane laboratory practice under the care of LAR.

Infective agents: The two isolates of *E. canis* used in this study were both originally isolated in Oklahoma. The Oklahoma isolate was initially recovered from a naturally infected dog in the early 1980s and had been passaged in tissue culture (DH82 cells) for an undetermined number of times at the Centers for Disease Control and Prevention, Atlanta, GA, before being reintroduced into dogs by intravenous inoculation of infected DH82 cells. The Ebony isolate was recovered from a naturally infected dog from southwestern Oklahoma in December, 1994; it was passed by intravenous inoculation of infected whole blood to dog #2100 that was then maintained as a reservoir host. Dog #2399 was infected by intravenous inoculation of 9 ml of infected blood from the reservoir host.

Ticks: Laboratory-reared *R. sanguineus* were obtained as pathogen-free larvae and nymphs

from the Centralized Tick Rearing Facility of the Oklahoma Agricultural Experiment Station. Transmission was attempted with nymphs and adults that acquisition-fed as larvae and with adults that acquisition-fed as nymphs.

Tick feeding: The following method was used for both acquisition- and transmission-feeding of ticks. Initially each dog was placed in an enclosed ventilated wooden box for 16-22 h to facilitate tick attachment. Cartons containing ticks were placed under stockinettes that were snugly fitted to the body of the dogs. Once attachment was accomplished, the stockinette was removed and the tick-infested dogs were maintained in separate metabolism cages positioned over water-filled pans. As engorged ticks left the host and fell into the refuse pan or into the water moat, they were collected at 6-8 h intervals (nymphs and larvae) or twice daily (adults). Engorged ticks were kept at ambient room temperature of about 22 C in a humidity chamber maintained at 90% relative humidity (RH) and a photoperiod of 14:10 h (Light:Dark) to molt; after molting they were held in the humidity chamber until transmission feeding was attempted.

Evaluation of dogs: Dogs exposed by inoculation of either infected tissue culture material or infected whole blood and those subjected to transmission feeding by ticks and all control dogs were monitored daily by physical examination and microscopic examination of peripheral blood smears. We monitored the dogs for 35 days (*E. canis* infection typically has an incubation period of 14-21 days) or until infection was confirmed by observation of morulae in peripheral blood smears. Hematologic and serologic evaluations were performed weekly. Complete blood count and thrombocyte count were

included in the hematologic studies. Although clinical observation and serologic monitoring were done, definitive diagnosis of infection was based upon demonstration of typical morulae of *E. canis*[10,11] in peripheral blood films. The prepatent period of infections was calculated from the day of tick exposure (or from the day of intravenous inoculation of blood or tissue culture material) to the first observation of morulae in circulating leukocytes. We did not attempt culturing of organisms from any of the ticks and dogs.

Serology : An objectively quantitative fluorescent immunoassay (FIAX) was used for serology.[12] The FIAX serology has been reported to be rapid and economical and it provides a precise estimate of IgG levels.[12-14] Antibody levels for the samples were expressed as FIAX values and these values are rankings and not expressions of titer. The positive control was a serum sample that consistently provided high fluorescent readings and the negative control, low fluorescent readings. The positive control was arbitrarily assigned a value of 300 and the negative a value of 25. FIAX values for all other sera were determined from a regression analysis constructed from FIAX fluorescent readings and standard values for the high and low control sera. A FIAX 100™ fluorometer[c] was used to measure the fluorescence values for all samples. FIAX values for all pre-inoculation sera were averaged and the standard deviation (SD) was calculated. The upper value for negative sera was determined (using the SD) with a 95% confidence interval. Based on this a FIAX value of 58 or higher was considered as seropositive.

Characterization of 16S rDNA of Ebony Isolate: DNA was extracted from the blood of dog #2100 which had been infected with the Ebony isolate of *E. canis* as previously

described.[15] The 5' end of the 16S rDNA gene was amplified using a nested Polymerase Chain Reaction (PCR) as described previously.[15] The template DNA was sequenced using an automated sequencer[d] and a PRISM™ Ready reaction DyeDeoxy™ Terminator Cycle Sequencing Kit[d] according to the recommendations of the manufacturer.

The sequence was analyzed using GCG version 8.0 DNA analysis software[e]. Other *E. canis* sequences used for comparison are *E. canis* (Oklahoma), GenBank accession number M3221; *E. canis* (Florida), GenBank accession number M73226; and *E.canis* (Israel), GenBank accession number U26740.

Experimental Design: The Oklahoma isolate was used in phases I & II (Fig 1 and 2) and the Ebony isolate was used in phase III (Fig 3). Once morulae were detected in the peripheral blood, dogs infected by intravenous inoculation of whole blood from carrier donors or of infected DH82 culture material were exposed to ticks for acquisition-feeding (Fig 1-3). Transmission feeding was attempted on littermate pups with nymphs and adults acquisition-fed as larvae or with adults acquisition-fed as nymphs. Aside from the principals, appropriate controls were used in phases II and III, but no controls were used in phase I.

Results

Phase I

Oklahoma isolate

Dog # 2208, infected by intravenous inoculation of tissue-cultured Oklahoma isolate in DH82 cells, developed ehrlichiosis with morulae in mononuclear cells 19 days post exposure. Adult ticks that acquisition-fed as nymphs on this dog did not transmit the infection when transmission-fed on dog #2271 (Fig 1). The body temperature was within the normal range and there were no signs of illness; there were no hematologic abnormalities and the dog remained seronegative.

Dog #2207 inoculated intravenously with infected whole blood from dog #2208 developed infection (Fig 1); the dog had morulae in the peripheral blood 20 days post exposure. Other evidence of ehrlichiosis included moderate fever, thrombocytopenia, mild normocytic normochromic anemia, and seroconversion (data not shown).

Phase II

Oklahoma isolate

Dog #2271, to whom infection was not successfully transmitted by ticks in phase I, was exposed to Oklahoma isolate through intravenous inoculation of infected whole blood from dog #2207 that was infected by whole blood inoculation from #2208 in phase I. Dog #2271 developed ehrlichiosis with morulae observed in the peripheral blood 21 days post exposure, at which time ticks were acquisition-fed, larvae for 3-4 days and nymphs for 5-7 days (Fig 2). When these larval ticks were transmission-fed as nymphs, on dog #s2444 and 2449, neither dog developed ehrlichiosis (Fig 2). Peripheral blood smear evaluation for 35

consecutive days revealed no morulae; the dogs remained clinically normal with body temperature within the normal range. Hematologic values, determined weekly, remained within normal range and the dogs did not seroconvert. Control dogs #2448 (exposed to uninfected nymphs) and #2446 (environmental control) did not show any evidence of ehrlichiosis and were seronegative throughout the experiment (data not shown) and they were used later as principals in this phase (#2448) or in phase III (#2446).

Adult ticks that acquisition-fed as nymphs on dog #2271 did not transmit the infection when transmission-fed on dog #2448 (Fig 2). The dog was monitored daily for 35 days by blood smear evaluation and weekly by hematologic and serologic techniques; no evidence of ehrlichial infection was seen. Adults that acquisition-fed as larvae also failed to transmit the infection when transmission-fed on dog #2449 (Fig 2). A control (dog #2478) exposed to uninfected adult ticks did not show evidence of infection (Fig 2); no environmental control animal was maintained in this part of phase II.

Phase III

Ebony isolate

Dog #2399 exposed to Ebony isolate by blood transfusion from #2100 developed ehrlichiosis with a rather short incubation period; morulae were found in the peripheral blood 13 days post exposure. Larval ticks were acquisition fed on this dog for 4 days (Fig 3). When these ticks were transmission fed as nymphs on two dogs, one developed ehrlichiosis (Fig 3). Morulae were observed in the peripheral blood of #2442 28 days post-exposure but the companion principal (#2445) did not develop infection. Clinical response to infection in #2442 included high fever (Fig 4), serous nasal discharge, and ocular

discharge that led to matted eyes. Hematologic studies revealed severe thrombocytopenia, mild leukopenia and normocytic normochromic anemia (Table 1). The dog became seropositive by 7th week (Fig 6). The control dogs, #2448 (on which uninfected nymphs fed) and #2446, (environmental control) remained clinically normal throughout the experiment and did not seroconvert (Fig 6).

Adult ticks that acquisition-fed as nymphs on dog #2399 transmitted the infection to dog #2446 (Fig 3). The prepatent period was 20 days and the dog had high fever (Fig 5), severe thrombocytopenia, mild leukopenia and normocytic normochromic anemia (Table 1). The dog seroconverted by the 4th week after exposure (Fig 6). Ticks that acquisition-fed as larvae (on #2399) and transmitted infection to dog #2442 (but not to #2445) as nymphs also transmitted infection to dog #2444 when fed as adults (Fig 3); morulae were observed in the peripheral blood 21 days post exposure. The dog showed clinical evidence of infection with high fever (Fig 5), serous nasal discharge, and ocular discharge. Hematological evaluation revealed thrombocytopenia, leukopenia and normocytic normochromic anemia (Table 1). The dog seroconverted by the 4th week post exposure (Fig 6). The control (dog #2478) on which uninfected ticks fed remained sero-negative throughout the experiment; no environmental control animal was used in this part of phase III.

Sequencing of 16S rDNA:

The sequence of 723 nucleotides (NTs) at the 5' end of the Ebony isolate 16S rDNA was determined. Comparison of these NTs in the Ebony sequence to the region of the Oklahoma isolate shows that they differ by only one nucleotide (A-- G at position 199 in the Oklahoma

sequence). The other *E. canis* isolate from the United States, *E. canis* (Florida) is identical to the Oklahoma isolate in this region of the 16S rDNA. The Ebony isolate is identical to the only other *E. canis* isolate (Israel) for which sequence data are available.

Discussion

The role of *R sanguineus* in transstadial transmission of *E. canis* has been conclusively documented experimentally.[6,7] Substantial evidence exists to suggest that transmission under natural conditions also involves this tick species.[6,8] Another canine ehrlichial agent, *E. ewingii* which causes canine granulocytic ehrlichiosis, was successfully transmitted experimentally with *Amblyomma americanum* but not by *Dermacentor variabilis*[16] and *R. sanguineus*[b]. In the present study, successful transmission of the Ebony isolate confirms the vector potential of brown dog ticks for *E. canis*. Except for one instance both nymphs and adults that were experimentally infected as larvae transmitted the agent after molting (Fig 2). We are unable to explain the cause in the failed instance (dog #2445) . It is especially puzzling because dog #2445 is a littermate of its companion principal (#2442), and in a later study proved susceptible to infection (data not shown).

Like Groves et al.[6] we have shown that larvae, after acquisition-feeding on a carrier dog, can transmit the infection as nymphs and again as adults without re-exposure. Unfortunately nymphs from companion principals (dog #2442 and #2445) were pooled for molting and feeding as adults (Fig 3). Therefore, we do not know on which dog(s) the adult ticks that transmitted *E. canis* to #2444 fed as nymphs.

Before the Oklahoma isolate was passaged in DH82 cells it was transmissible by tick.[b] Even though it was easily reintroduced into dogs following several generations in DH82 cells, all the ticks exposed to this agent after reintroduction into dogs failed to transmit it when transmission-fed on susceptible dogs (Fig 1 and 2). We speculate that during repeated passage in DH82 cells *in vitro*, the agent lost infectivity for ticks, but we have no substantive

basis to explain this apparent change in infectivity. Not only did the isolate maintain infectivity for dogs (Fig 1 and 2), it also maintained its pathogenicity (data not shown).

The prepatent period of Ebony infection was approximately a week longer with nymphal (28 days) transmission than with adult tick (21 days) vectors. The reason for this difference is unknown, but may be due to lower infective dose from nymphs. Groves et al.[6] also found that infections developed more slowly when nymphs (rather than adult ticks) transmitted *E. canis* under experimental conditions. These workers defined the prepatent period as the time from day of exposure to ticks to the first rise in body temperature, and it was 19.2 and 14 days for transmission by nymphs and adults, respectively. Our results are similar but the periods are slightly longer. Because we used the appearance of morulae in the peripheral blood as our indicator of prepatent period, the differences are accentuated. The infected dogs in our study usually had an increase in body temperature 2-3 days prior to the observation of morulae in peripheral blood smears (Fig 4 and 5), and morulae were usually observed after bouts of elevated body temperature. The acute phase of infection was characterized in some dogs by undulating body temperature. A pattern of about every 24 h is suggested by data displayed in Fig 4 and 5, but since body temperature was recorded only once/day we can only speculate about the actual period of undulation. All dogs that became infected--irrespective of whether transmission was via nymphal or adult tick feeding--developed severe thrombocytopenia of <25,000/*u*l (Table 1). Of all parameters measured, thrombocytopenia and pyrexia proved to be the most consistent indicators of acute ehrlichial infection. Appearance of morulae in peripheral blood smears was the only criterion accepted as unequivocal evidence of infection. Mild leukopenia and normocytic

normochromic anemia are also seen with regularity. These findings are consistent with earlier studies on the clinicopathological changes associated with canine ehrlichiosis.[2,6,10]

Serologic evaluation of dogs using the FIAX system to detect IgG shows that dogs exposed to *E. canis* via tick bite took longer to seroconvert than did dogs infected by inoculation with infected whole blood (Fig 6). (The apparently aberrent FIAX value for dog #2444 on day 14 post exposure was repeated and is inexplicable; it may represent a sampling error.) This finding is not surprising and is consistent with the longer prepatent period observed in dogs exposed via tick feeding. We do not know how soon after tick attachment the organism is transmitted, and we cannot quantitate infective dose. Furthermore, we did not quantitate infective dose when exposure was by injection of whole blood. Variation in prepatent period and in the time of seroconversion could be due to difference in infective dose. Alternatively, host response could also be influenced by immunosuppressive agents present in tick saliva. It has been demonstrated that tick salivary gland-derived material can modulate host cytokine, antibody and cell mediated host responses.[17]

The 16S rDNA is the most accessible feature by which previously uncharacterized bacteria can be identified.[18-20] The 16S rDNA sequences of more than 20 *Ehrlichia* (or *Ehrlichia*-like) species are currently available. The sequence analysis of the Ebony 16S rDNA identifies this organism as a member of the *E. canis* species. Although less than 750 NTs of sequence were determined, the location of these NTs is very significant for *Ehrlichia* identification. A very small portion of this region (corresponding to NTs 32 to 91 in the *Escherichia coli* rDNA sequence) has been especially interesting in the identification of *Ehrlichia* species.[21] Almost all of the variation known within the 16S rDNA of *Ehrlichia*

species occurs within these 60 NTs. The Ebony isolate sequence is identical to one of the *E. canis* isolates (Israel) in this region and differs by only one NT from the other two *E. canis* isolates (Oklahoma and Florida) for which sequence is available. It should be noted perhaps that both the Oklahoma and Florida isolates were sequenced only after having been passaged in cell culture for many generations. Unfortunately there is no pre-passage material of either isolate available for study, and we have no way of knowing whether culturing affected the nucleotide configuration. By the same token, we do not know whether the change in a nucleotide had any impact on transmissibility by ticks. We know from our own work that the Oklahoma isolate was transmissible by ticks before passage in DH82 cells and that the Florida isolate was studied in ticks before continuous cell-line culture was developed[22]. We know of no recent work involving tick transmission of the Florida isolate.

It has recently been demonstrated that different isolates of the same species of the tribe Ehrlicheae may differ slightly in NT sequence. Specifically, Dumler et al.[23] isolated a strain of *E. chaffeensis* (91HE17), the cause of human (predominantly agranulocytic) ehrlichiosis, which lacked an epitope in its 16S rDNA identified in the *E. chaffeensis* Arkansas strain. Results of our sequencing of Ebony isolate 16S rDNA provides further support for the usefulness of NT sequence data in characterization of ehrlichial agents.

Foot notes

a. E.M. Johnson, Experimental transmission of *Ehrlichia* spp., Ph.D thesis, Oklahoma State University, Stillwater, 1989.

b. Ewing, S.A., Unpublished data.

c. Bio-Whittaker Inc., Walkerville, MD.

d. Model 373A; Perkin-Elmer/Applied Biosystems Division (PE/ABD), Foster City, CA.

e. Madison, WI.

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Table 1. Hematologic values of dogs infected with Ebony isolate of *Ehrlichia canis* by feeding ticks (*Rhipicephalus sanguineus*) and control dogs (mean values), n=3

Dog #	Indices	Time following exposure to ticks Day 0	Day 7	Day 14	Day 21	Day 28	Day 35
2442*	WBC x1000	12.7	7.1	8.8	4.3	6.2	4.9
	HCT %	30	31.1	34.0	26.8	26.3	24.5
	MCV	66	65	64.9	63.3	64	64.4
	MCHC	34.6	34.7	33.8	35.4	33.5	33.5
	PLT x1000	585	492	465	55	31	25
2444**	WBC x1000	8.6	8.3	7.0	5.4	3.8	3.5
	HCT%	43.5	42.9	40	33.9	31.0	25
	MCV	64.0	64.7	64.3	62.5	62.0	63.9
	MCHC	35.0	34.8	34.3	35.7	35.5	34.0
	PLT x1000	520	494	375	103	32	26
2446**	WBC x1000	9.2	10.0	7.7	4.3	5.4	ND
	HCT%	40.1	38.0	39.2	36.1	32.8	ND
	MCV	64.9	65.9	65.5	64.4	64.8	ND
	MCHC	35.1	35.3	35.7	35.5	35.4	ND
	PLT x1000	503	390	250	58	19	ND
control dogs	WBC x1000	12.5	10.0	12.0	11.0	10.0	12.0
	HCT%	33.0	35.0	37.5	38.2	42	39.5
	MCV	65.7	65.0	65.3	65.4	65.7	64.7
	MCHC	35.4	35.1	34.3	35.0	35.1	35.1
	PLT x1000	500	521	481	523	460	477

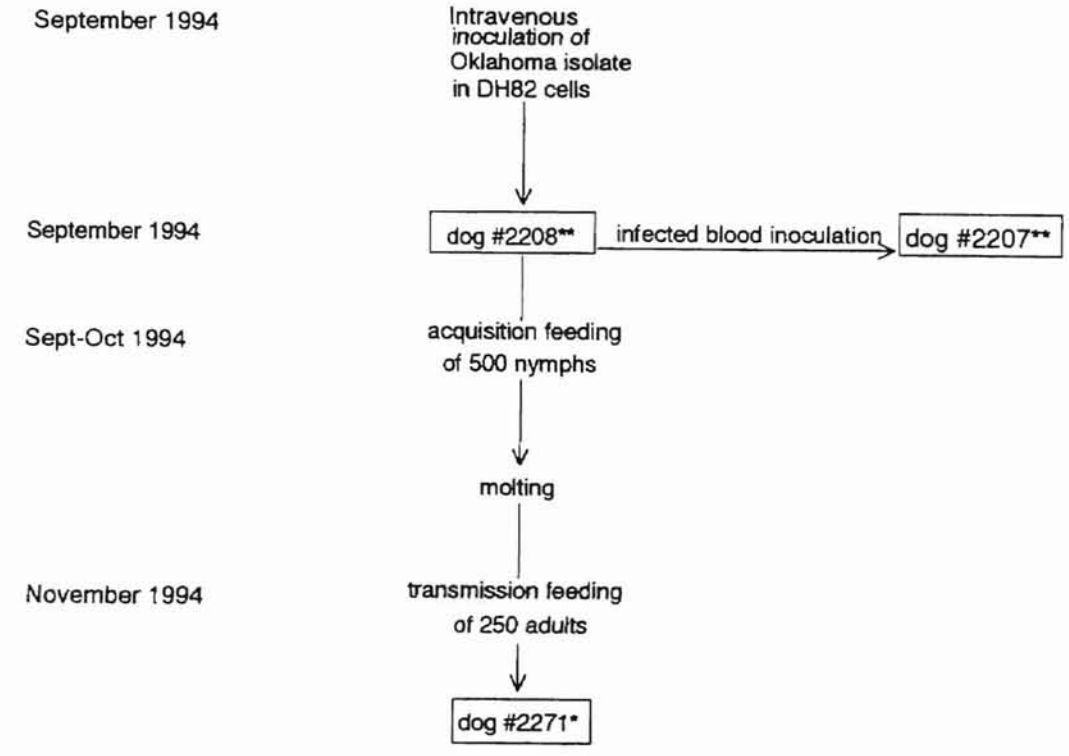
* infected by nymphal tick bite
** infected by adult tick bite

ND- Not Determined; WBC-White Blood Cells; HCT-Hematocrit (Packed Cell Volume) value; MCV-Mean Corpuscular Volume; MCHC-Mean Corpuscular Hemoglobin Concentration; PLT-Platelets

Fig 1. Diagramatic representation of experimental design and results of phase I of the experiment in which dogs were exposed to Oklahoma isolate of *Ehrlichia canis* by inoculation of blood or DH82 cells or by *R. sanguineus* bite.

*Dogs clinically normal during this phase

**Dogs that showed evidence of ehrlichial infection



(dog #2271 was monitored for 35 days)

Fig 2. Diagramatic representation of experimental design and results of phase II. Ticks (*R. sanguineus*) and dogs exposed to Oklahoma isolate of *Ehrlichia canis* in this phase of the experiment.

*Dogs clinically normal throughout this phase

**Dogs that showed evidence of ehrlichial infection

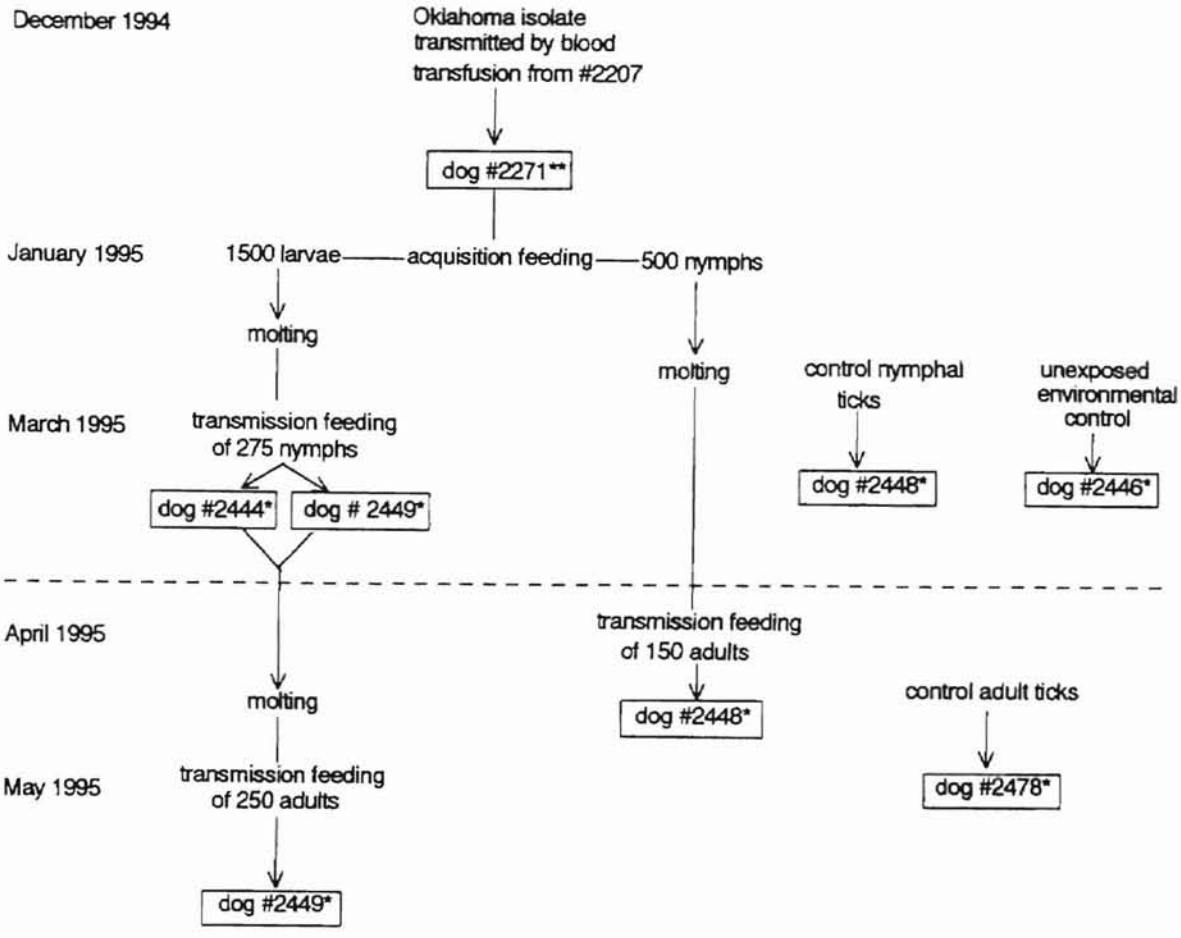

December 1994
Oklahoma isolate transmitted by blood transfusion from #2207
dog #2271**
January 1995
1500 larvae
acquisition feeding
500 nymphs
molting
molting
control nymphal ticks
unexposed environmental control
March 1995
transmission feeding of 275 nymphs
dog #2444*
dog # 2449*
dog #2448*
dog #2446*
April 1995
transmission feeding of 150 adults
molting
dog #2448*
control adult ticks
May 1995
transmission feeding of 250 adults
dog #2478*
dog #2449*

Fig 3. Diagramatic representation of experimental design and results of exposure of dogs to Ebony isolate of *Ehrlichia canis* by blood inoculation and by transstadial tick *(R. sanguineus)* transfer.

*Dogs clinically normal throughout the experiment

**Dogs that showed evidence of ehrlichial infection

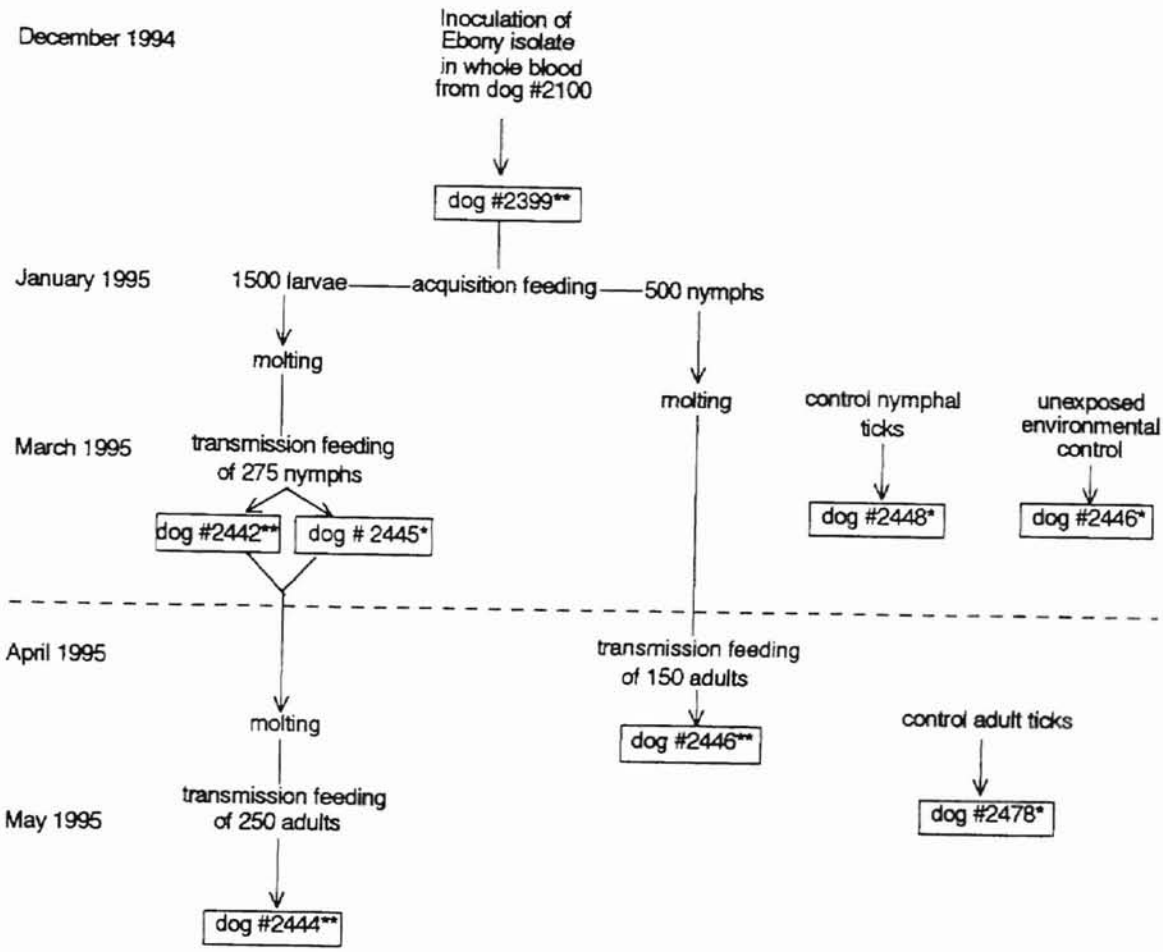


Fig 4. Body temperature response of dog (#2442) infected with Ebony isolate of *Ehrlichia canis* by bite of nymphal *R. sanguineus* that were acquisition-fed as larvae; and those of two control dogs, one exposed to uninfected nymphs (#2448) and an environmental control(#2446). Arrow from morulae indicates first day on which parasites (morulae) were observed in peripheral blood.

Fig 4. Body temperature response of dog (#2442) infected with Ebony isolate of *Ehrlichia canis* by bite of nymphal *R. sanguineus* that were acquisition-fed as larvae; and those of two control dogs, one exposed to uninfected nymphs (#2448) and an environmental control(#2446). Arrow from morulae indicates first day on which parasites (morulae) were observed in peripheral blood.

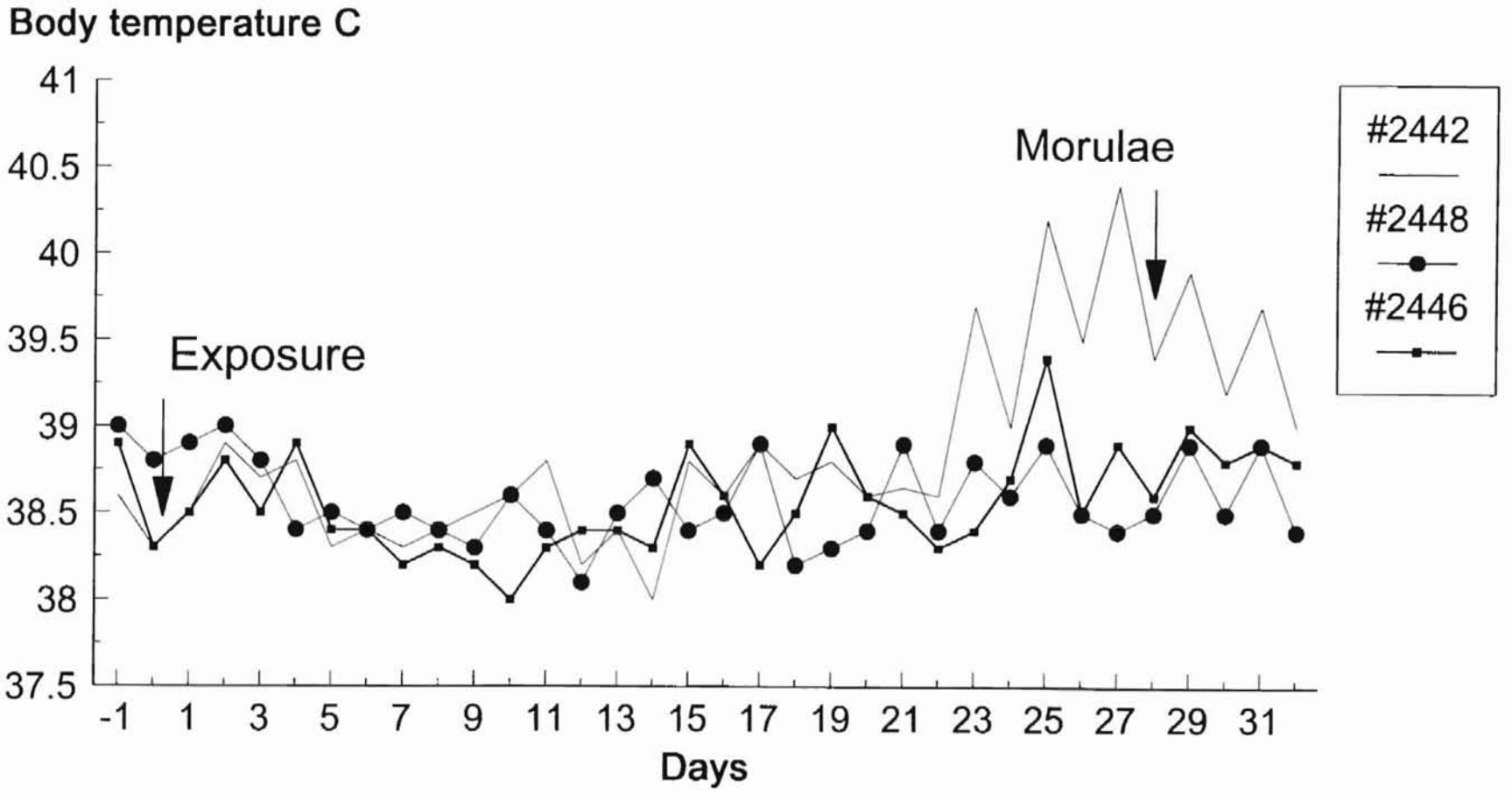
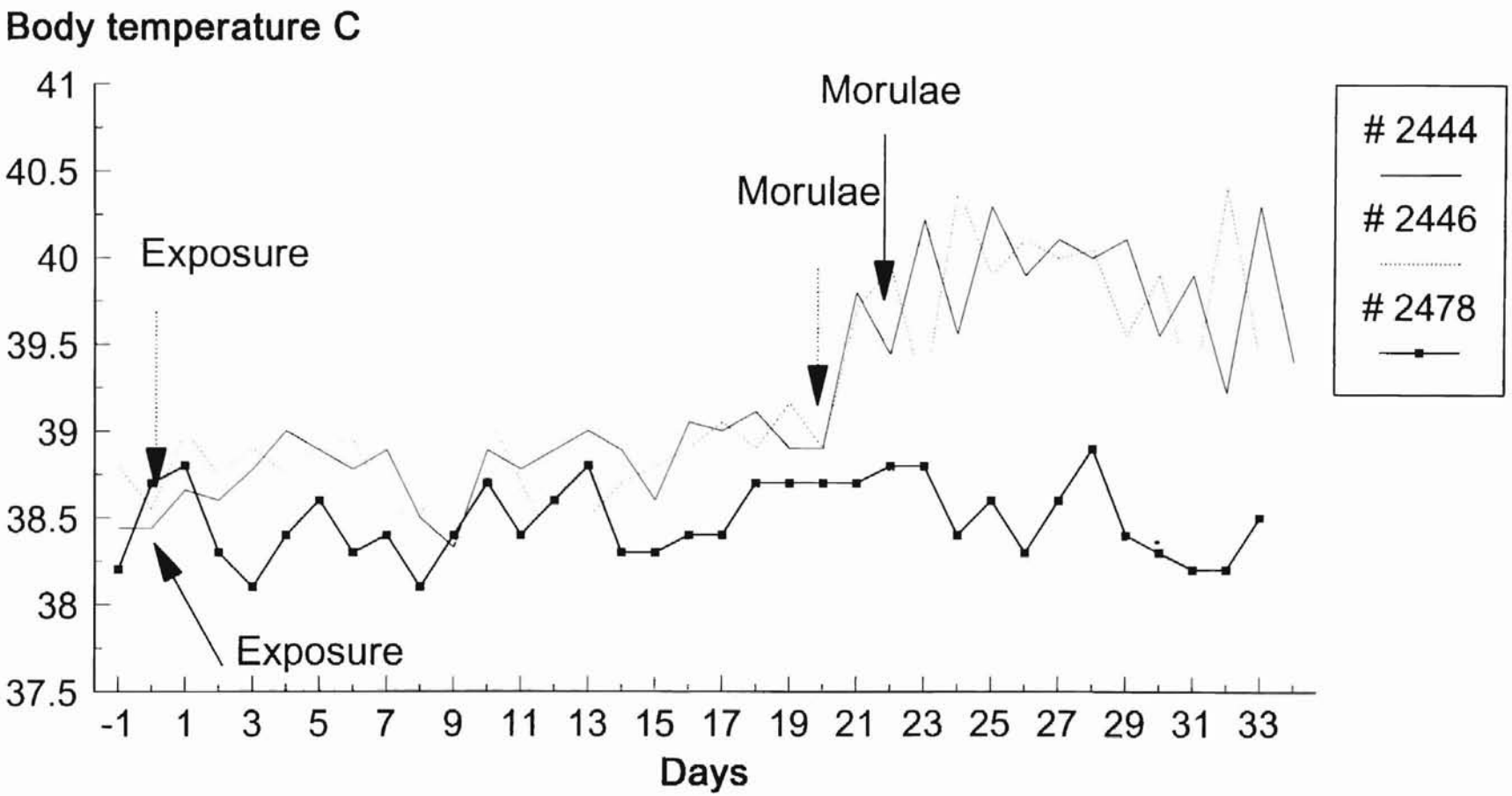

Body temperature C
41
40.5
40
39.5
39
38.5
38
37.5
Exposure
Morulae
#2442
#2448
#2446
-1 1 3 5 7 9 11 13 15 17 19 21 23 25 27 29 31
Days

Fig 5. Body temperature response of two dogs (#s 2444 and 2446) exposed to Ebony isolate of *Ehrlichia canis* infection by the bite of adult *R. sanguineus* and that of #2478 exposed to uninfected adult ticks. Arrow from morulae indicates first day on which morulae were observed in peripheral blood.


Body temperature C
41
40.5
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38.5
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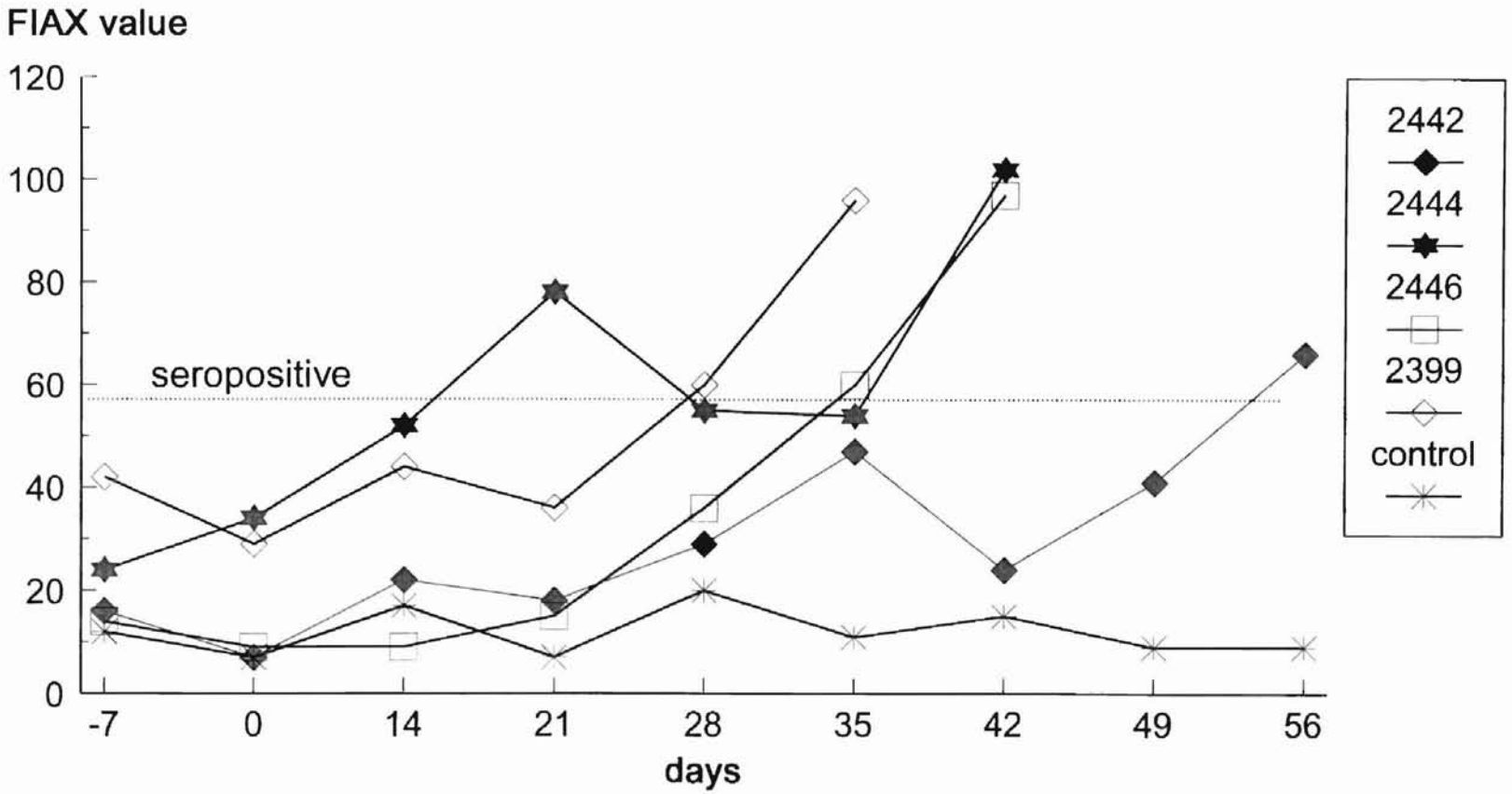
37.5
Exposure
Exposure
Morulae
Morulae
-1 1 3 5 7 9 11 13 15 17 19 21 23 25 27 29 31 33
Days
2444
2446
2478

Fig 6. FIAX value reflecting weekly serum IgG levels of four dogs infected with Ebony isolate of *Ehrlichia canis*. One dog (#2399) was exposed by whole blood inoculation and three by tick (*R. sanguineus*) feeding; dog #s 2444 and 2446 were exposed to adult ticks and #2442 to nymphal ticks. Control represents the average value of three dogs, one environmental and two uninfected tick-exposed controls.

Serum IgG levels



CHAPTER II

Characterization of a new isolate of *Ehrlichia platys* (Order Rickettsiales) using electron microscopy and polymerase chain reaction

Abstract

A mixed-breed pup obtained in north central Oklahoma by the Laboratory Animal Resources Unit of Oklahoma State University presented with platelet inclusions. The dog developed severe thrombocytopenia (<10,000/*u*l) following the appearance of inclusions. Blood films were monitored daily and when about 75% of platelets had inclusions, samples were collected in EDTA and processed for electron microscopical (EM) studies and polymerase chain reaction (PCR). EM studies on glutaraldehyde-fixed buffy coat revealed rickettsia-like inclusions in numerous platelets. Serologic examination, using *Ehrlichia platys* antigen, showed high titer suggestive of *E. platys* infection. PCR primers derived from a highly variable region of the 16S rRNA gene sequence of *E. platys* were used to specifically amplify that region of the parasite's DNA. Sequencing of the PCR product obtained by use of general *Ehrlichia* primers showed one nucleotide difference from the published sequence for *E. platys* which suggests possible strain variation of this intracellular parasite. Our results indicate that PCR may be a useful tool in the diagnosis of *E. platys* infection and that, like some other *Ehrlichia* spp., *E. platys* isolates may vary slightly.

Key words: *Ehrlichia platys*; dog; polymerase chain reaction; electron microscopy; 16S rRNA gene sequence.

1. Introduction

Infectious cyclic thrombocytopenia (ICT) of dogs was first described in 1978 (Harvey et al., 1978) and its etiology was ascribed to a new species which selectively infects dog platelets. Electron microscopical studies confirmed that the platelet inclusions within membrane-lined vacuoles were rickettsiae, and the name *Ehrlichia platys* was proposed (Harvey et al., 1978). In stained platelets of peripheral blood smears the parasite appears as clusters, called morulae, or as individual organisms called elementary bodies. The agent causes cyclic thrombocytopenia which recurs about every 2-3 weeks for variable periods. ICT is a mild, usually asymptomatic disease, but may prove fatal when infected dogs hemorrhage after accidents or during surgery.

Diagnosis of ICT by demonstration of organisms in blood smears is often not reliable because of i) the cyclic nature of parasitemia, ii) low parasitemia during thrombocytopenic phases, and iii) the tendency for few platelets to be infected in secondary and subsequent bouts of parasitemia. Serological studies using indirect fluorescent antibody (IFA) technique with *E. platys*-infected platelets as antigen is very useful in diagnosis (French and Harvey 1983; Baker et al., 1987), but recovered animals may remain seropositive for long periods, making it difficult to differentiate actively infected from convalescent animals.

Polymerase chain reaction (PCR) has been used widely to characterize different ehrlichial organisms (Anderson et al., 1992; Dumler et al., 1995; Dawson et al., 1996). Studies of the 16S rRNA gene sequences of different isolates of *E. chaffeensis* and *E. canis* suggested possible strain variation within both species (Dumler et al., 1995; Mathew et al., 1995). Variations in one or more nucleotides have been reported among different isolates for both

these species.

Development of a PCR-based diagnostic technique to detect *E. platys* infections may be important for the accurate and prompt diagnosis of ICT. It has been demonstrated that agranulocytic canine ehrlichiosis caused by *E. canis* can be diagnosed earlier with PCR than by other currently available methods (Iqbal et al., 1994; Ewing et. al., 1995). A PCR-based technique may also help to differentiate *E. platys* from other putative agents that infect platelets, such as one that we recently reported from dogs (Mathew et al., 1995).

The present study was undertaken when a mixed-breed pup presented with platelet inclusions. The dog developed severe thrombocytopenia shortly after large numbers of platelet inclusions were observed. Serologic and electron microscopical studies suggested that the inclusions were *E. platys*. As a means to confirm our suspicion, we investigated the potential of *E. platys*-specific primers as a diagnostic tool. We amplified a 359 base pair (bp) segment in the *E. platys* 16S rRNA gene by using PCR primers from a highly variable region in that gene sequence. Further characterization was achieved by sequencing the amplified PCR products.

2. Materials and Methods

2.1. The agent

The isolate of *E. platys* used in this study was obtained from a spontaneously-occurring case of ICT observed in a mixed-breed pup (#2431) obtained by the Laboratory Animal Resources Unit (LAR) of Oklahoma State University (OSU) from a dog shelter in Payne County, Oklahoma, in February, 1995. We have no information about how the dog acquired the infection.

2.2. Monitoring the infection

The dog was monitored by physical examination and blood smear evaluation on a daily basis for 15 days. Hematological evaluations (CBC and platelet count) were performed every-other-day for 8 days and serologic examinations were done on a weekly basis for 5 weeks. Blood samples were collected in EDTA during peak parasitemia and processed for EM and PCR.

2.3. Serology

Serum samples were diluted 1:20 with phosphate-buffered saline (pH 7.2) and antibodies to *E. platys* were measured by an indirect fluorescent antibody technique (IFA) as previously described (French and Harvey 1983) using infected platelet suspension in PBS as antigen. Once samples were found positive (>1:40), a titer was determined by two-fold serial dilutions until specific fluoresence was no longer observed. All serum samples were also tested for evidence of *E. canis* infection by the FIAX system as described by Gorton et al. (1992) and Mathew et al. (1995).

2.4. Electron Microscopy

Blood samples collected in EDTA were spun down using microhematocrit tubes to separate buffy coat which was then processed for EM as described by Kocan et al. (1978). Briefly, buffy coats were fixed immediately in 2% glutaraldehyde in 0.2M sodium cacodylate buffer overnight, and post fixed in 2% buffered osmium tetroxide for 1 hr. The samples were then dehydrated through ascending grades of ethanol (70%, 80%, 90% and absolute) and infiltrated and embedded in epoxy resin using propylene oxide as intermediate solvent.

Thick sections were made, stained with Mallory's stain for 2 minutes at 60 C, and examined for organisms in platelets using light microscopy. Ultrathin (silver reflective) sections were cut using a Sorval MT-7000 ultramicrotome (RMC, Tucson, Arizona, USA) and Dupont diamond knife (Dupont, Irving, Texas, USA), collected on 300-mesh grids, stained with uranyl acetate and lead citrate. Specimens were observed and photographed using a JEOL 100CX electron microscope (JEOL, Peabody, Massachusetts, USA) at 80KV.

2.5. Polymerase chain reaction

2.5.1. Extraction of DNA

DNA was extracted from whole blood samples in EDTA collected from the infected dog with the QIAamp blood kit (QIAgen Inc. Chatsworth, California, USA) following the procedures of the manufacturer. We used DNA extracted from an uninfected dog and from an *E. canis*-infected dog as negative controls. DNA extracted from *E. platys*-infected platelets supplied by Dr. R. E. Corstvet of Louisiana State University served as positive control.

2.5.2. Oligonucleotide primers and PCR

Oligonucleotides used as primers in PCR were purchased from the OSU Recombinant DNA/Protein Core Facility. The 16S r RNA gene sequences, deposited with the GenBank DNA sequence data bank (GenBank accession number M82801), were aligned using MacVector DNA sequence analysis software (Eastman Kodak Company, Rochester, New York, USA). Primers EPLAT5 (5'-TTTGTCGTAGCTTGCTATGAT-3') and EPLAT3 (5'-CTTCTGTGGGTACCGTC-3') were used for the amplification of *E. platys* DNA and were selected from highly variable regions near the 5' end of the 16S rRNA gene sequence (Weisburg et al., 1989; Anderson et al., 1991). Primers defined a segment of 359 bp upon amplification.

PCR was performed using the GeneAmp kit (Perkin Elmer-Cetus, Norwalk, Connecticut, USA). Reactions were performed in a volume of 50*u*l, and contained 10*u*l of DNA extracted from whole blood, primers (0.5*u*M each), $MgCl_2$ (2mM), nucleotides (100*u*M) and Taq polymerase (1.25U). For amplification, an initial denaturation step at 94^{0}C for 3 minutes was followed by 3 cycles of 94^{0}C for 1 min, 55^{0}C for 2 min, and 72^{0}C for 1.5 min, and 37 cycles of 92^{0}C for 1 min, 55^{0}C for 2 min, and 72^0 C for 1.5min. PCR products were separated by electrophoresis through 1.5% agarose and visualized with ethidium bromide and ultraviolet (UV) illumination.

2.6. DNA Sequencing

DNA extracted from *E. platys*-infected whole blood was amplified using oligonucleotide primers ECC (5'-AGAACGAACGCTGGCGGCAAGC-3') and ECB (5'-CGTATTACCGCGGCTGCTGGCA-3') (Anderson et al., 1991). For amplification, 30

cycles at 92^{0}C for 1 min, 60^{0}C for 2 min, and 72^{0}C for 2 min, were used following initial denaturation at 94^{0}C for 4 min. The PCR products (approximately 450bp long) were sequenced at the OSU Recombinant DNA/Protein Core Facility on an ABS 373 DNA sequencer (Perken Elmer/Applied Biosystems Division, Foster City, California, USA).

3. Results

3.1. Evaluation of the dog

The dog appeared clinically normal by physical examination. There was no pyrexia, and feeding habits were normal. Basophilic inclusions resembling *E. platys* (Fig. 1) were observed in platelets on the first 4 of 15 days of continuous blood smear evaluation. Some platelets had more than one inclusion, and about 75% of the platelets were infected on the day of peak parasitemia. The number of infected platelets decreased rapidly following the peak parasitemia, and no infected platelets were detected in the peripheral blood smears from the 5th day to 15th day when monitoring by this method was discontinued.

Hematological evaluation of the dog revealed severe thrombocytopenia (<10,000/*u*l) immediately after the peak parasitemia (Fig 2), and the platelet count started to increase by the 4th day following peak parasitemia. All other hematological values (RBC, WBC, HCT, MCV, MCHC, MCH) were within normal range throughout the obsevation period.

3.2. Serology

Samples were serologically positive for *E. platys* from the 3rd week. Titers ranged from 1: 20 to 1: 640 during the 5-week observation period with peak titer at the 4th week (Fig 3). Samples were sero-negative for evidence of *E. canis* infection.

3.3. Electron microscopy

Electron microscopical studies on buffy coat preparations revealed rickettsia-like inclusions in the platelets. The inclusions were morphologically indistinguishable from structures reported to be *E. platys* (Harvey et al. 1978). Many platelets had aggregates of organisms (called morulae) within membrane-lined vacuoles; most parasitized platelets had

individual organisms (Figs 4 and 5) but a few platelets contained multiple morulae (Fig 6).

3.4. PCR studies

The DNA from the *E. platys*-infected platelets (positive control) and from dog #2431 yielded 359 bp product (Fig 7) upon amplification. Neither of the two negative control samples (uninfected and *E. canis*-infected dog blood) yielded a product with primers EPLAT5 and EPLAT3.

3.5. DNA sequencing

The oligonucleotide primers ECC and ECB used to amplify DNA extracted from dog #2431 yielded a product of approximately 450 bp. Sequencing of this PCR-derived product revealed one nucleotide difference from the published *E. platys* sequence obtained from the GenBank. Specifically there was an insertion of a nucleotide (an 'A' at position 249). We repeated PCR and sequencing five times to ensure accurate results and the difference was seen consistently.

4. Discussion

When a dog (#2431) presented with inclusions in platelets and was shown to be developing thrombocytopenia, we were stimulated to determine whether the animal was infected with *Ehrlichia platys*. This interest was based in part upon recent experience with appearance of unidentified inclusions in platelets of dogs that failed to show serologic evidence of *E. platys* infection (Mathew et al., 1995). Spontaneously occurring *E. platys* infections have been reported earlier from Oklahoma (Johnson and Morton, 1987).

In order to determine with certainty whether the dog was infected with *E. platys,* we used various methods including blood smear evaluation by light microscopy, hematology, serology, EM and PCR to characterize these inclusions. Severe thrombocytopenia of <10,000/*u*l was the only abnormal hematologic finding recognized during the observation period. Results of blood smear evaluations and other hematologic examinations were consistent with acute *E. platys* infection as reported earlier (Harvey et al., 1978; Johnson and Morton, 1987; Baker et al., 1987).

Serologic studies using *E. platys* antigen showed positive titers (Fig 3), further suggesting *E. platys* infection. We have no explanation for the decline in IFA titer at the 5th week; the finding may represent a sampling error. If the finding is valid, it contrasts rather markedly with our experience with *E. canis* infections in which convalescent animals remain seropositive for long periods, many months or even years.

EM studies confirmed rickettsia-like inclusions in platelets (Figs 4, 5 and 6). Since the inclusions appeared within membrane-lined vacuoles and their ultrastructure was morphologically indistinguishable from dog platelet inclusions identified as *E. platys* by

Harvey et al. (1978), we tentatively concluded that we were dealing with that organism. To provide additional evidence we explored the effectiveness of PCR to diagnose *E. platys* infection. We used oligonucleotide primers specific for the species, selected from a highly variable region in the 16S rRNA gene. To our knowledge use of PCR as a means to diagnose *E. platys* infection has not been reported previously.

We were able to recover a PCR product from a positive control and from dog #2431, but not from either of two negative controls, viz., an *E. canis*-infected dog and an uninfected dog. We cannot assess the sensitivity of the method by comparison with other previously reported methods for detecting *E. platys*; but studies with *E. canis* infections (Iqbal et al., 1994; Ewing et al., 1995) suggest that PCR-based techniques are efficient in early and accurate detection of that organism in dogs. PCR has also proved useful previously in differentiating closely related *Ehrlichia* species isolated from human beings and other hosts (Anderson et al., 1992). The present study illustrates that PCR can be used to confirm preliminary diagnostic impressions in *E. platys* infections.

The 16S r RNA gene sequence analysis is a widely used method for characterization of pathogenic bacteria, especially those in the genus *Ehrlichia.* We used primers ECC and ECB (Anderson et al., 1991) to amplify a segment of 16S rRNA gene which includes the *E. platys*-specific primer region. Amplification of the DNA extracted from dog #2431 using these primers confirmed the presence of DNA from a member of the genus *Ehrlichia.* We sequenced the PCR product which was approximately 450bp long and compared the results with that published for *E. platys.* While sequence analysis was compatible with the published *E. platys* values at the primer region there was one nucleotide difference ,viz.

insertion of an 'A' at position 249. All other species of the genus *Ehrlichia* for which the sequences are available have an 'A' at this position. Difference in the sequence suggests at least minimal variation among isolates of *E. platys*. This is not surprising, perhaps, given results obtained in studies of four isolates of *E. canis* (Israel, Florida, Oklahoma, and Ebony) which showed that some differed by one nucleotide in this region of their 16S rRNA gene sequence (Anderson et al., 1991; Mathew et al., 1995; Dawson, personal communication). Likewise, a new isolate of *E. chaffeensis* (91HE17) from Texas differed from the original Fort Chaffee, Arkansas, isolate of that species by two nucleotides in the 16S rRNA gene; the Texas isolate lacked an epitope present in the original isolate (Dumler et al., 1995). Though variations in pathogenicity, vertebrate host range, and vector associations are very important in characterization of *Ehrlichia* spp., it appears that analysis of the 16S rRNA gene sequence may be valuable for detecting subtle differences at the molecular level.

Acknowledgement

Prepared for submission by J. S. Mathew, S. A. Ewing, G. L. Murphy, K. M. Kocan, R. E. Corstvet and J. C. Fox, to *Veterinary Parasitology*, March, 1996.

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Weisburg, W. G., Obson, M. E., Samuel, J. E., Dasch, G. A., Mallavia, A. P., Baca, O., Madelco, L., Sechrest, J. E., Weiss, E., and Woese, C. R. 1989. Phylogenetic diversity of rickettsiae. J. Bact., 171: 4202-4206.

Fig 1. Basophilic inclusions in dog platelets observed by light microscopy; x2500 (Diff-Quik stain)

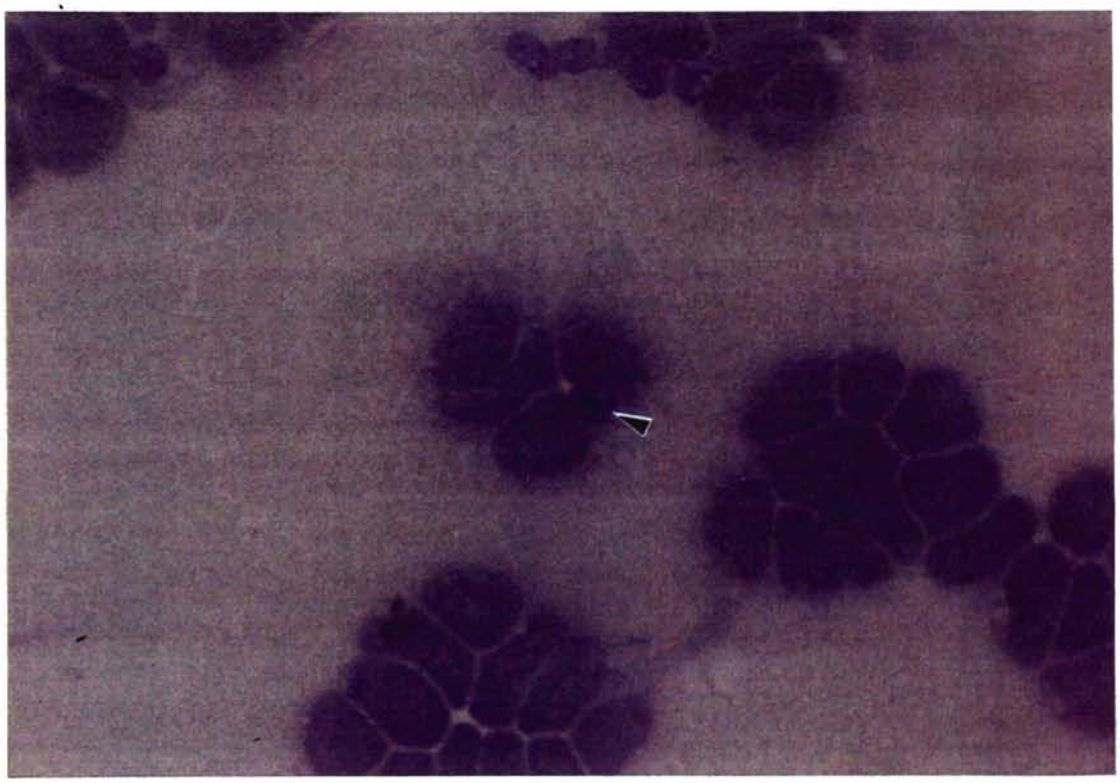


Fig 2. Thrombocyte count of dog #2431 during parasitemic phase of *Ehrlichia platys* infection. Arrow indicates the day of peak parasitemia.

Thrombocyte count

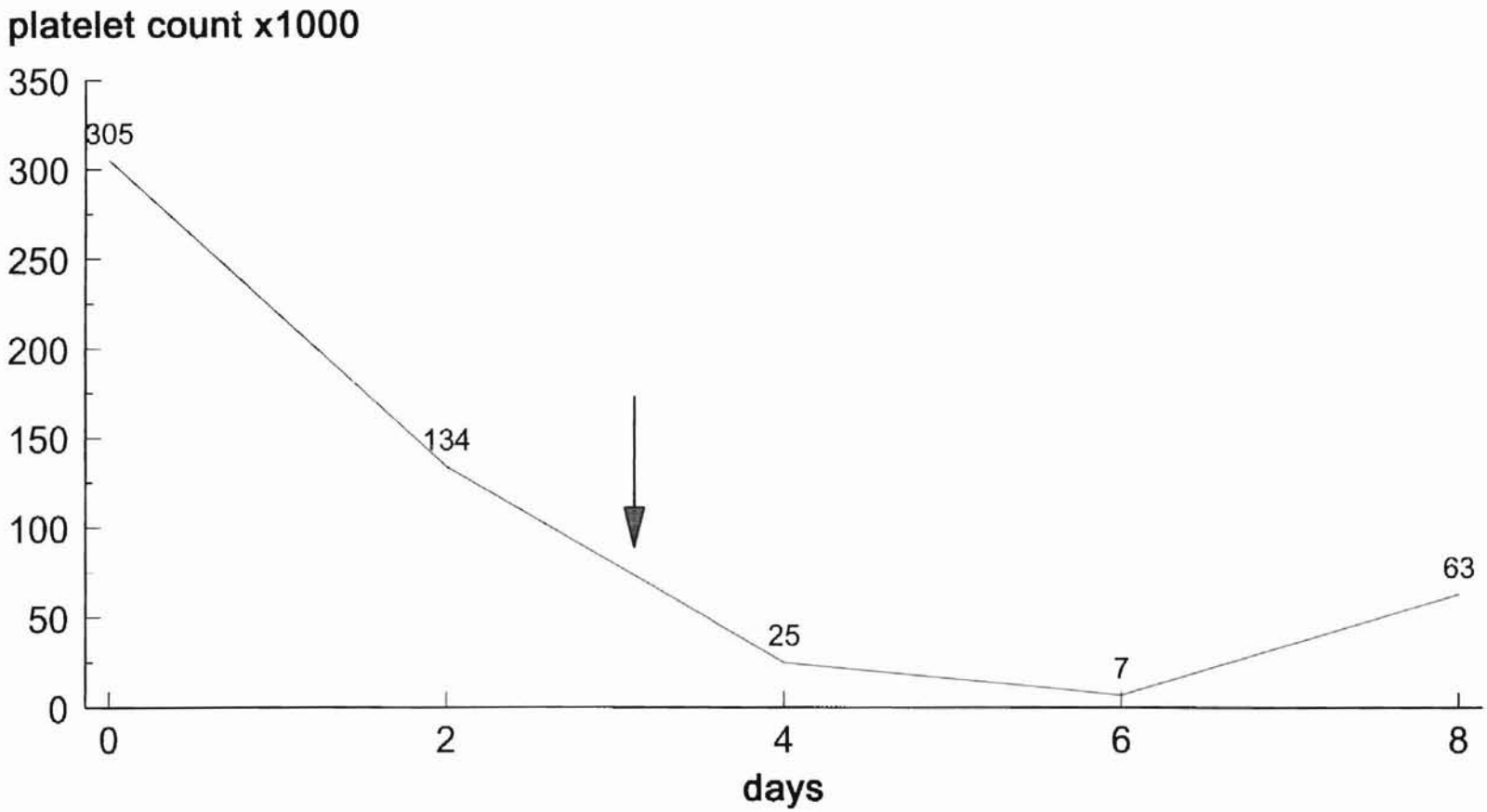


Fig 3. The IFA titer of dog #2431 performed using *Ehrlichia platys* antigen.

Serology

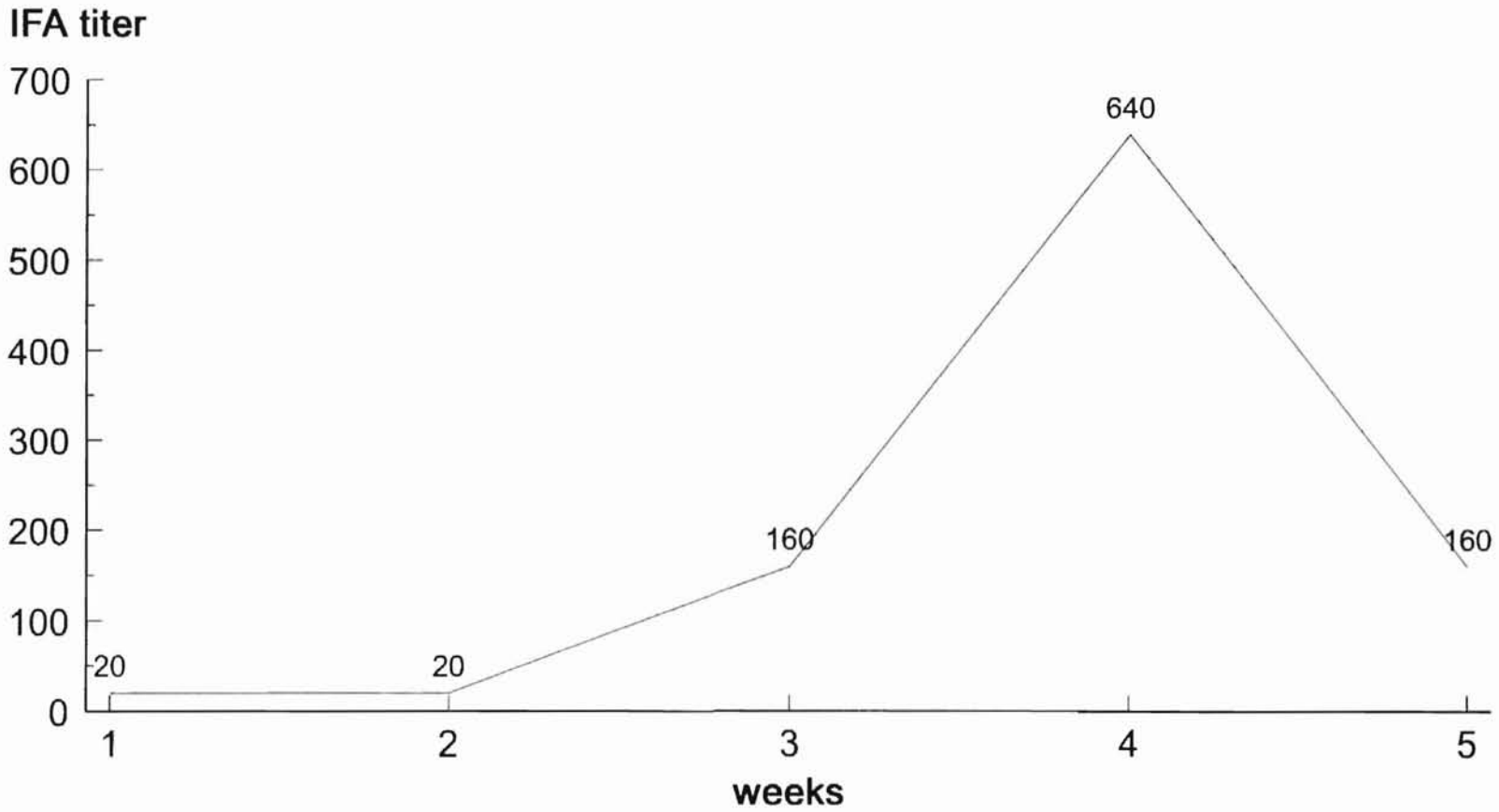

IFA titer
700
600
500
400
300
200
100
0
20
20
160
640
160
1
2
3
4
5
weeks

Fig 4. Electron micrograph of a single organism in a dog platelet. Arrows indicate the double membrane around the organism which is characteristic of species in the Order Rickettsiales. (Bar= 0.5*um*)

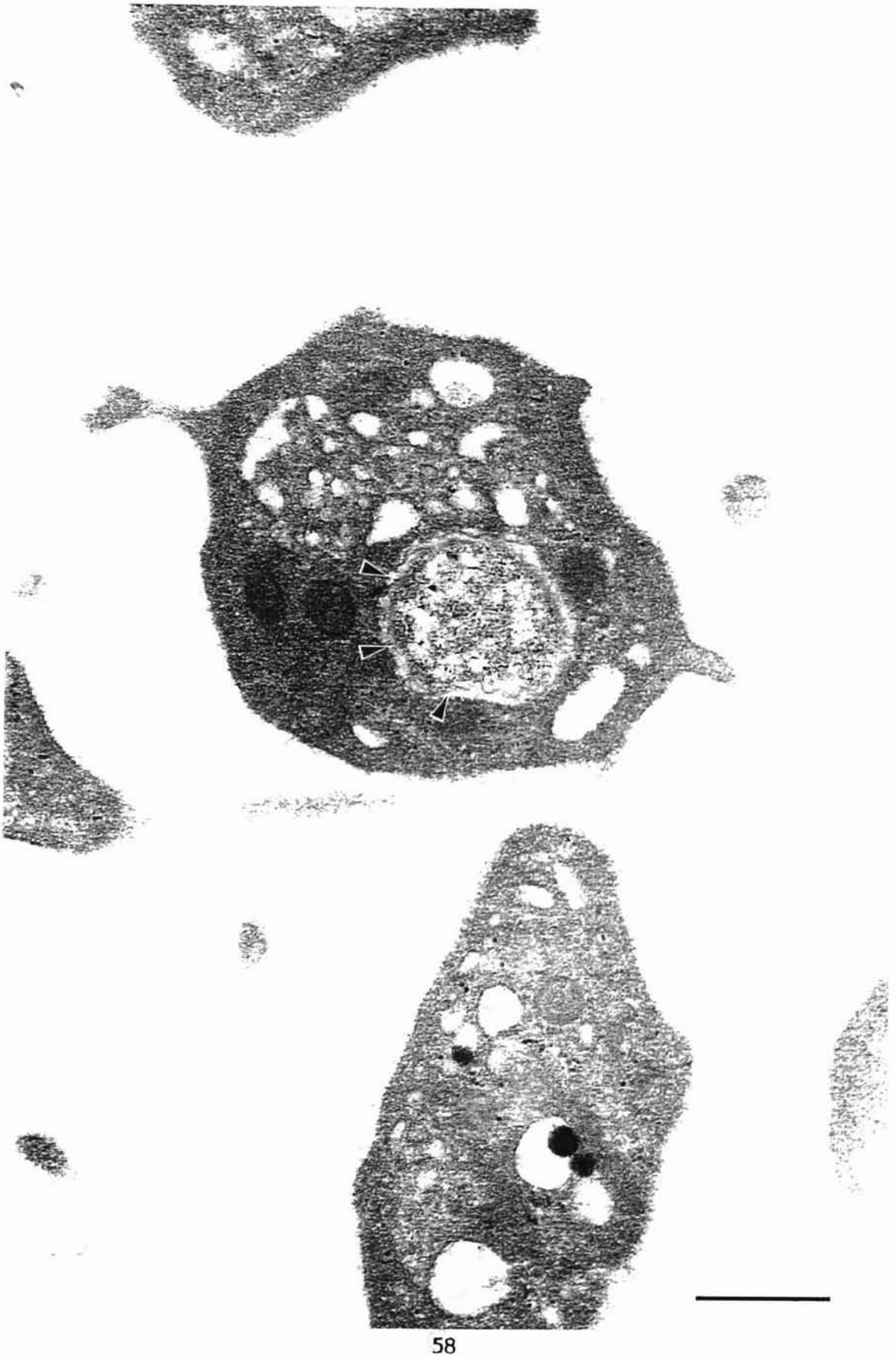


Fig 5. Electron micrograph of three individual *Ehrlichia platys* organisms (arrows) in a dog platelet (Bar=0.5*um*).

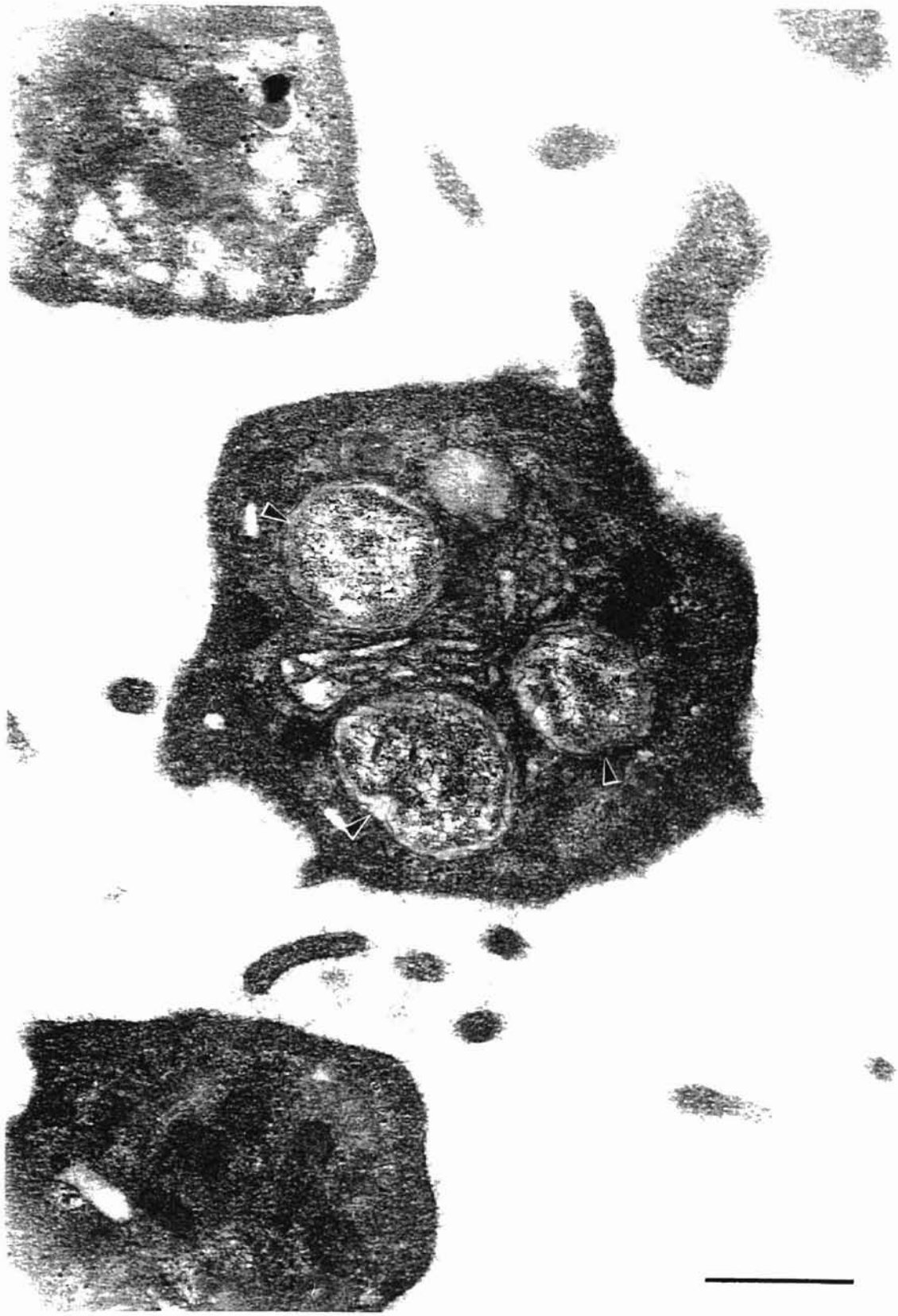


Fig 6. Electron micrograph depicting multiple (three) aggregates (called morulae) of *Ehrlichia platys* organisms in a dog platelet (Bar=0.5*um*).

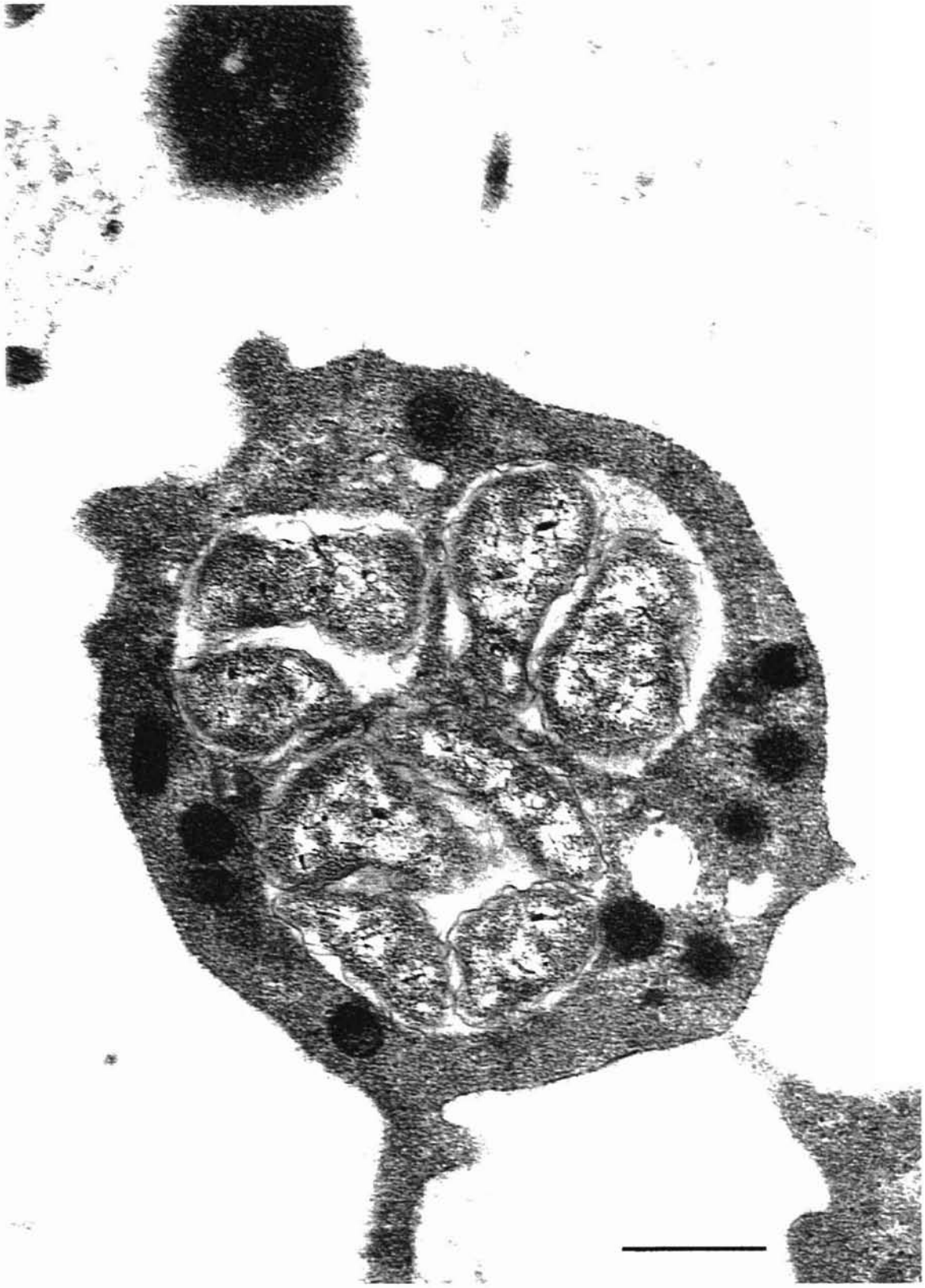
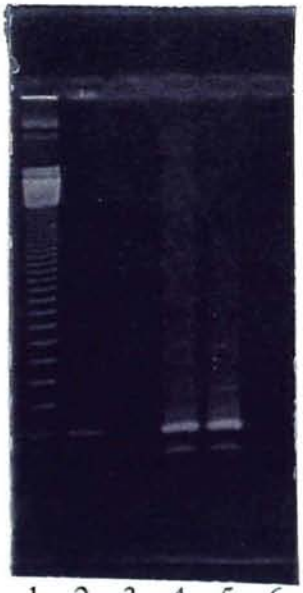


Fig 7. Agarose-gel visualization of PCR products obtained using *Ehrlichia platys* specific primers.

Lanes: (1) 123 bp ladder standard, (2) DNA extracted from *E. platys*-infected platelets as positive control, (3) DNA extracted from an uninfected dog, (4, 5) DNA extracted from dog #2431, and (6) DNA extracted from dog infected with *E. canis*


1 2 3 4 5 6

VITA

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