RECOGNITION OF A CANINE RICKETTSIOSIS IN OKLAHOMA AND ITS DIFFERENTIATION FROM BABESIOSIS

by

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DOCTOR OF PHILOSOPHY
May 24, 1964

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ACKNOWLEDGEMENTS

The author expresses appreciation to Doctor Wendell H. Krull for his advice and constructive criticism relevant to experimental design, procedure and organization of data, and for his assistance in editing the manuscript. Members of the committee, viz., Doctor W. H. Krull, Department of Veterinary Parasitology; Doctor W. E. Brock, Department of Veterinary Pathology; Doctor D. D. Dwyer, Department of Agronomy; Doctor D. E. Howell, Department of Entomology; and Doctor A. W. Monlux, Department of Veterinary Pathology, aided with their criticisms and encouragement. Special appreciation is due Doctor W. E. Brock who first observed the leukocytic inclusions described herein and brought them to the attention of the author and who, with Doctor Monlux, permitted use of laboratory space and equipment in the Department of Veterinary Pathology prior to the time that this research was financed sufficiently. The major financial assistance was supplied through Grant Number AI 05508-01, National Institutes of Health, Department of Health, Education, and Welfare. The professional counsel and kind assistance of Doctor R. G. Buckner, Department of Veterinary Medicine and Surgery, is gratefully acknowledged. Professional and/or technical assistance was provided by Drs. E. D. Besch, B. S. Blauch, B. L. Glenn, M. R. Frey, J. Z. Kendrick, R. J. Panciera; Mmes. Linda Carrol, Peggy King, Vicki Mannschreck, Jane Sisk, Robbie Skaggs, Lily Soirez; Messrs. J. H. Brandt, W. C. Russell, and C. E. Ward. Mrs. C. E. Hittle is thanked for the translation into English of important papers originally published in French, and Doctor D. L. Weeks is recognized for his statistical evaluation of a portion of the data.

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I. GENERAL INTRODUCTION

In man's effort to define biological relationships he has encountered a variety of associations between and among organisms; one of these relationships which is often obligatory for one of the partners is called parasitism. The dependent associate, the parasite, is arbitrarily defined as a smaller animal that lives at the expense of a larger animal which is called the host. Like most definitions, this one can be shown to be both insufficiently exclusive and insufficiently comprehensive, and the main difficulty arises in the attempt to define what is meant by expense. Situations are observed in which small organisms live within or upon larger organisms but do not cause any obvious damage. In such cases it is difficult to say whether or not these organisms truly are living at the expense of the host. Consequently it has been necessary to invent other terms such as mutualism, commensalism, symbiosis, and the like as descriptive terms for certain associations. But as one examines the relationships designated by each of these definable terms it becomes obvious that it is not possible to delimit them absolutely, but only arbitrarily. With regard to these designations Caullery (1959) stated:

> Under varied aspects they are only manifestations of the struggle for life, characterized by specialization in the way in which it is exerted, but deprived of all finality or pre-established harmony. Those associations survived which balanced their accounts in a fashion compatible with the existence of perpetuation and the associates; many others must have arisen from time to time but have not lasted through failing to satisfy this necessity.

> Where organisms pass from the normal conditions of existence in free-living forms to those in which they are associates, they undergo very considerable structural changes sometimes of enormous extent, which are perhaps the most striking illustration of the reality of their evolution, and, above all, of the influence of the environment on the organisms; but the capricious diversity of these transformations indicates that the evolutionary changes are principally conditioned by the intrinsic properties of diverse living forms.

One can but admit to the inadequacies of these definitions and justify their usage only on the basis of convenience. A classical definition of parasitism was given by Schwartz (1937):

> ... an ancient but not an honorable partnership between two species of animals, one of the partners, the parasite, getting all that he can from and contributing nothing but grief to the other partner, the host.

Other parasitologists, however, support the point of view that parasitism is neither abnormal nor unusual and that it is simply an extremely common expression of the behavior of living organisms. As an example Elton (1927) stated:

> To imagine that parasites are unique in exploiting the activities and food-products of their hosts is to take a very limited view of natural history. It is common to find parasites referred to as if they were in some way more morally oblique in their habits than other animals, as if they were taking some unfair and mean advantage of their hosts. If we once start working out such "responsibilities" we find that the whole animal kingdom lives on the spare energy of other species or upon plants, while the latter depend upon the radiant energy of the sun. If parasites are to occupy a special place in this scheme we must, to be consistent, accuse cows of petty larceny against grass, and the cactuses of cruelty to the sun.
>
> A parasite's existence is usually an elaborate compromise between extracting sufficient nourishment to maintain and propagate itself, and not impairing too much the vitality, or reducing the numbers of its host, which is providing it with a home and a free ride. In consequence of this compromise, the parasite usually destroys only small portions of its host at a time, portions which can often be replaced fairly quickly by regeneration of the tissues attacked. Or it may exploit the

> energies of its host in more subtle ways, as when it subsists on the food which the host has collected.

Elton's view seems more consistent with the general scheme of interrelationships among organisms in food chains which are basic in our concept of life, and it will be with this guiding philosophy that parasitism is treated in this dissertation.

With refinement in observation man has come to the realization that in cases which are clearly definable as parasitism, the association is not always so simple as a host harboring one parasitic species. The realization has dawned as various host-parasite relationships have been examined (usually with a concern for the well-being of the host as the stimulus for making the examination) that the presence of disease in an animal is usually the result of an interaction of complicated physiological processes of competing organisms, some of which may be parasitic.

Disease has been defined by Hagan and Bruner (1951) as:

> ...an alteration of the state of the body, or of some of its organs, which interrupts or disturbs the proper performance of the bodily functions. Functional disturbance soon is manifested by physical signs which the patient detects by his sensations and which usually can be detected by others.

When the association of living agents in the animal body set up a disturbance of function in any part, infection is said to have occurred. The word infection is derived from the Latin <u>inficere</u> meaning "to put into." An infectious disease then is one caused by the presence in or on an animal body of living organisms of another species which by their presence create a disturbance leading to the development of symptoms.

In the case of some diseases man has passed the descriptive stage in scientific sequence and is now in the experimental stage of the development of knowledge relative to the host-parasite relationship. This is true of relationships in which man or domestic animals are hosts, but not of lower organisms except in a few cases. Unfortunately, even in conditions involving man and his domestic animals, the great majority of diseases are yet incompletely described, and their manifestations in hosts are inadequately understood. The efforts of a great many individuals and an appreciable fragment of the national gross product go into the investigation of these diseases, but as yet the inroads into the problems are meager. The greatest effort naturally goes toward study of relationships in which man is the host, but, in addition to studying diseases of man, it is of course necessary to investigate those in domestic animals for several reasons. In the case of meat animals it is a matter of concern for the quality and quantity of food available to man for consumption. In the case of other animals (non-food) it is sometimes a matter of public health significance, i.e., in conditions in which organisms that are capable of causing disease in man are harbored by such animals. In other cases it is a matter of aesthetic values, humane considerations, or a matter of assuming the responsibility with regard to the health of a work or pet animal.

Modern transportation has complicated studies of infectious diseases. Prior to the period when rapid transportation became commonplace, the spread of diseases of both man and animals was a slow process. Today it is not unlikely that, in spite of efforts to prevent such happenings, organisms which were previously confined to a given geographic area may

unexpectedly appear in a host animal population in a part of the world quite remote from where the parasite was previously isolated. As a result man must be on the alert constantly for new and different manifestations of disease in himself and in his domestic animals. This complication, coupled with the embarrassing fact that diseases which may have been associated with a given population for centuries remain to be recognized and described, makes the problems of the health sciences and allied disciplines quite complex.

It is the purpose of this thesis to describe how such a host-parasite relationship and the resulting disease syndrome was recognized in the United States for the first time and differentiated from a complicating disease entity. Procedures will be discussed which were involved in the final discovery that the animals initially observed to be ill were actually suffering from a disease syndrome caused by at least two organisms present simultaneously.

Babesiosis of the canine caused by Babesia canis (Piana and Galli-Valerio, 1895) has long been recognized as occurring in dog populations and was first reported in the United States in 1934. In March 1962, an Oklahoma dog, suffering from a febrile disease, was proved to be harboring B. canis. In the course of subsequent experiments it was determined that in addition to this organism, another one, apparently a rickettsia or rickettsia-like organism, considered to belong in the genus Ehrlichia Mochkovski, 1937, was found to be associated with B. canis and was later proved to be a complicating factor in the production of disease in the canine host. An attempt will be made to place these parasites and their hosts in proper perspective with special emphasis on the biology of the parasites.

It is the object of the present paper to delineate, insofar as possible, the manifestations of disease caused separately and in combination by these two entities, one previously unrecognized in the United States, and to detail what is known of the host-parasite relationships involved.

In order to understand the hierarchical position of the species involved, it is necessary to relate the organisms to classification.

Whitlock (1960) has emphasized with regard to biological classification that there is really no way to set one thing off from another; a classification is simply one form of description. He stated that:

> Some approximation of biological reality can be reached if one visualizes a net or mesh which extends not only in the three standard dimensions, but also through time. Each individual piece of net represents a species which is closely related to adjoining species and really continuous with them. Measurable values of each species are represented by knots along the strands since these measurements tend to group themselves. The interstices of the mesh are filled by the environment of the species. If, as is often the case with parasitic species, the environment changes with development, the environment can be visualized as oscillating or vibrating along the strands. Since humans cannot visualize four dimensions (most cannot visualize three) we have to get the idea of sampling the meshwork only at our own geologic time. When we do this we find large gaps torn in the mesh. This represents a loss of environment which has removed unadapted species.
>
> Scientists are still finding strands of this mesh and trying to relate them to the known segments. They indicate the uniqueness of their find by designating the species and the relationships of it to the known mesh by classifying the find in genus, family, order, class and phylum. Sometimes the gaps in the mesh are so great that the uniqueness of the find is clear. More often the find is so closely related to known forms that distinctions become arbitrary and trivial. This often calls for a redescription of the characteristics of the group and of the mesh. Inasmuch as the ultimate reality is the individual and the individual has only a transitory existence in time, the opportunities for taxonomic splitting are almost endless because the family, the genus, and the species have no more reality than our network; they are merely conventions which serve to express human ideas.

II. SOME GENERAL CHARACTERISTICS OF THE *BABESIA CANIS* LIFE CYCLE IN THE VERTEBRATE HOST

The class Piroplasmasida to which the genus *Babesia* Starcovici, 1893, belongs is generally defined as including small pyriform, round, ameboid, or rod shaped parasites of vertebrate erythrocytes, sometimes also of leukocytes or histiocytes. The organisms are all parasitic and they are all heteroxenous. Vectors insofar as they are known, are ixodid or argasid ticks. Pigment (hematin) is not formed from the host cell hemoglobin, the organisms possess no spore stages, and the nucleus is vesiculate. These parasites do not possess special locomotor organs, and movement is accomplished by body flexion or by gliding. Reproduction is asexual by binary fission or by schizogony; sexual reproduction is dubious. They are further classified in the order Piroplasmorida, and the family Babesiidae Poche, 1913, the latter including organisms that are relatively large, pyriform, round or oval and occurring in the erythrocytes of vertebrates. Asexual reproduction in the erythrocytes is said to be either by binary fission or by schizogony. The definition at the generic level isolates those in which trophozoites multiply either by binary fission forming pairs or by schizogony forming tetrads in the erythrocytes.

There are opposing views of speciation in the genus, and Levine (1961) has discussed these. Advocates of one view consider breaking it up into several genera or sub-genera, each with a number of species, while exponents of the other view prefer a single genus with a relatively small number of

species each of which may include several strains. The second system seems preferable. The taxonomy of the Piroplasmasida has been discussed by a number of authors and will not be pursued here. It should be indicated, however, that the commonly used generic name Piroplasma Patton, 1895, is not used here because Babesia clearly has priority. The argument that Babesia is a preoccupied name was rejected by the Editorial Board (1953) and the Judicial Commission (1954) of the International Commission of Bacteriological Nomenclature. Hornby (1934) and Dinulescu and Babes (1960) have listed the reasons for rejecting Piroplasma in favor of Babesia. The opinion of a contemporary, Sergent, et al., (1954) that Piroplasma should be used is not accepted herein because no valid reason for rejecting the name Babesia Starcovici, 1893, was presented by these authors.

Neitz (1956) and Levine (1961) listed three Babesia species which occur in the dog: Babesia canis (Piana and Galli-Valerio, 1895), Babesia vogeli Reichenow, 1937, Babesia gibsoni (Patton, 1910). B. canis is said to be pyriform and 4 to 5_u long or ameboid and 2 to 4_u in diameter. B. vogeli is said to be somewhat larger than B. canis, and B. gibsoni is said to be smaller and not possessing the characteristic paired pyriform trophozoites. B. vogeli is considered restricted to Asia and Africa, but Enigk (1944) and Poisson (1953) suggested that this means very little since the species was separated from B. canis on the basis of arthropod host specificity and because dogs that recovered from babesiosis could be infected with this organism. This separation is considered to be inadequate for species recognition. The trophozoites of B. gibsoni are supposedly annular or oval and not more than 1/8 of the diameter of the host erythrocyte. Reported data show, however, that occasionally either large ovoid forms, half the diameter of the host cell, or thin elongate forms reaching almost across the cell may be found.

The life cycle of Babesia canis as described in the dog is essentially the same for all species of the genus parasitizing this host. The trophozoites occur in the erythrocytes in which they multiply either by binary fission or by schizogony. According to Levine (1961), in some species two trophozoites are formed which break out of the erythrocyte and enter new red cells while in others the situation is the same except that four trophozoites are formed. Some authors place the latter in a separate genus Nuttalia, and Ray and Idnani (1943) even proposed a new genus, Pattonella, apparently to accommodate aberrant forms of Babesia gibsoni, but neither of these has been accepted. The formation of more than four trophozoites by schizogony has been described as occurring in erythrocytes, but some workers consider that schizogony is simulated because repeated binary fission or multiple invasion of the host cell may occur.

Method of Reproduction of Babesia canis in Erythrocytes

From the foregoing introduction it is evident that a controversy exists concerning the method of reproduction of B. canis and related species within the erythrocytes of the vertebrate host. This section of this report is concerned with presenting data showing rather conclusively that the method of reproduction of B. canis in the erythrocytes is binary fission.

Materials and Methods

A total of twenty-three dogs was used in this experiment. All except two of them were infected with a strain of B. canis isolated from a dog which was born and raised in the Veterinary Clinic at Oklahoma State University, Stillwater, Oklahoma. The strain was isolated March, 1961, and

the subsequent passages were made until August 1963. The other two dogs were infected with a strain of B. canis acquired from Doctor Ned Rokey, Mesa, Arizona. In all cases the infections in these dogs, which were either intact or splenectomized, were produced by the intravenous transfusion of infectious blood from reservoir animals.

The experimental dogs were housed in quarters which excluded all other animals except arthropods as possible contaminants. Ticks were not observed in the room or on any dog except when they were used experimentally and confined in a certain location until removed. No other arthropods were observed on the dogs during the period of the experiment. A few houseflies (Musca domestica), cockroaches (Blattidae), and spider beetles (Ptinidae) were observed in the room but not on the dogs. None of these insects has piercing mouthparts and they are considered to have represented no threat of contamination as far as the experimental canines were concerned.

Control dogs not exposed to infective blood were kept in cages adjacent to the Babesia-infected dogs throughout most of the experimental period, and the same observations were made on these dogs.

The initiation of the parasitemia and its progress were determined by the daily examination of approximately 100,000 erythrocytes (1,000 oil immersion fields delimited by a grid in which approximately 100 erythrocytes were accommodated) on Romanowsky-stained (Wright's stain, buffer pH 6.8) smears of blood. All examinations were made by the author to reduce sampling errors. The smears were prepared by using the initial drop of blood obtained by puncturing the capillary bed on the ventral surface of the ear.

The evidence for the conclusion that the propagative phase of the life cycle, the erythrocytic stage at least, in the vertebrate host is repeated binary fission is contained in the data in Figures 1-8. Only eight of the

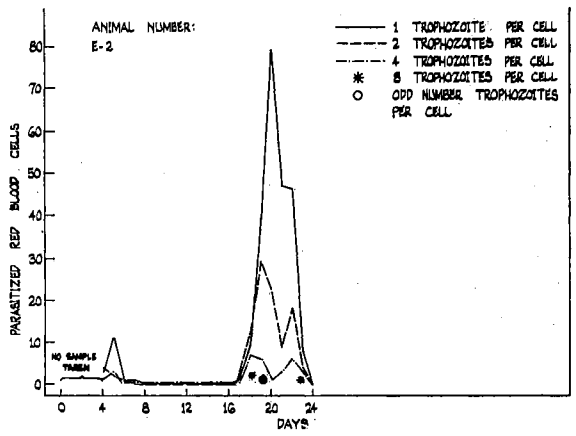

Figure 1. Numbers of Babesia canis trophozoites in each parasitized cell among 100,000 erythrocytes, for E-2.

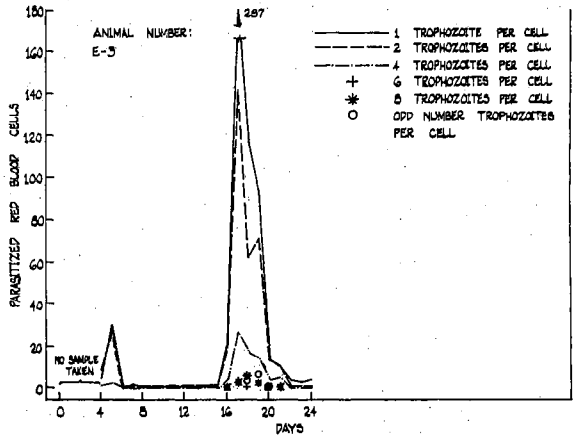

Figure 2. Numbers of Babesia canis trophozoites in each parasitized cell among 100,000 erythrocytes, for E-3.

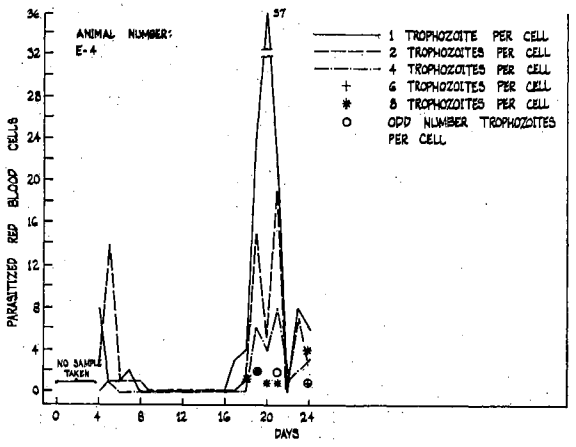

Figure 3. Numbers of Babesia canis trophozoites in each parasitized cell among 100,000 erythrocytes, for E-4.

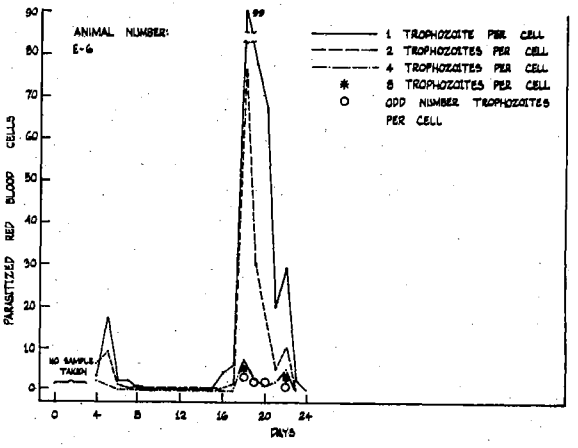

Figure 4. Numbers of Babesia canis trophozoites in each parasitized cell among 100,000 erythrocytes, for E-6.

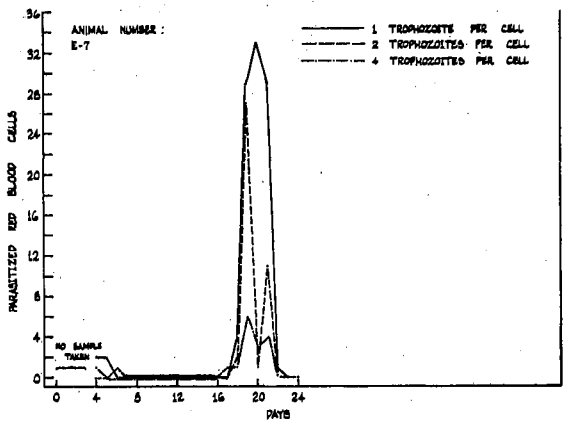

Figure 5. Numbers of Babesia canis trophozoites in each parasitized cell among 100,000 erythrocytes, for E-7.

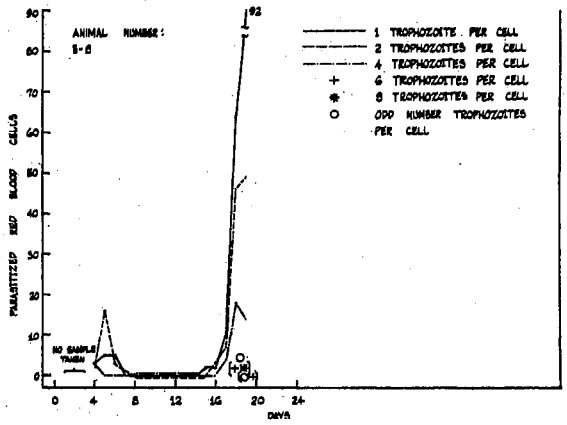

Figure 6. Numbers of Babesia canis trophozoites in each parasitized cell among 100,000 erythrocytes, for E-8.

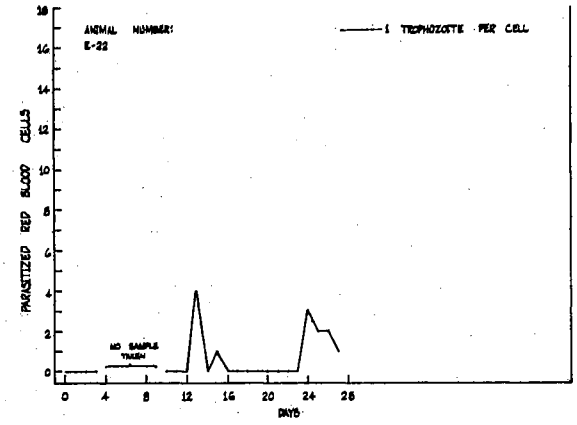

Figure 7. Numbers of Babesia canis trophozoites in each parasitized cell among 100,000 erythrocytes, for E-22.

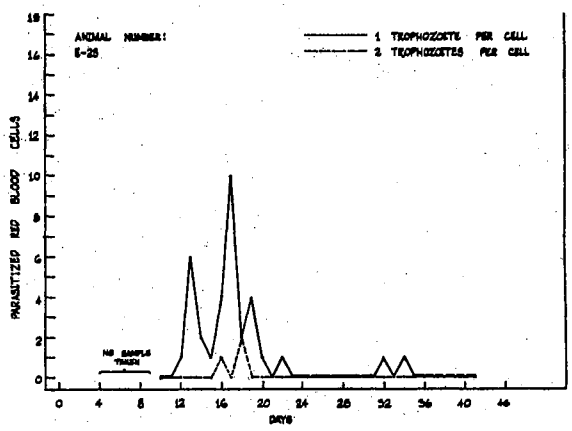

Figure 8. Numbers of Babesia canis trophozoites in each parasitized cell among 100,000 erythrocytes, for E-23.

twenty-three dogs were selected to represent the series. Six of the dogs (E-2, E-3, E-4, E-6, E-7, and E-8) were selected on the basis that they were littermates and represented the largest homogeneous aggregate that could be assembled from among the twenty-three dogs. The other two dogs (E-22 and E-23) were selected on the basis that they were the only ones infected with B. canis in the absence of Ehrlichia, and it was felt that they should be compared with the six littermate dogs in which B. canis and Ehrlichia were concomitantly present.

The protozoan exhibited remarkably consistent life cycle patterns in the six littermate dogs. In general there was an immediate appearance and a sudden rise in numbers of trophozoites in the circulating erythrocytes following injection of infectious blood. This reached a peak in numbers, and then the parasites disappeared from the peripheral blood usually on the third or fourth day after exposure. This marked the beginning of a period in which trophozoites could not be detected. Approximately two weeks after exposure, and thus ten days after the parasites disappeared from the peripheral blood, trophozoites reappeared in the circulating erythrocytes. There was then a rapid increase in the numbers of parasitized cells and in one case (E-8) the dog died at the time the parasites were most numerous. In other cases the numbers declined, sometimes disappearing entirely. The graphs in Figures 1-8 were discontinued at this point because it was neither practicable nor necessary to extent them over longer periods. Data were collected, however, until the other dogs died (E-2, E-3, E-4, E-6, and E-7), were euthanitized (E-22), or lost their active infections (E-23), as judged by absence of trophozoites for an extended period of time. The pattern in dogs (among the fifteen for which graphs are not given) which were bled for long periods was essentially the

same as that just described, i.e., periods of the presence of numerous parasites (marked reproductive activity) interrupted by variable periods of their absence. The length of these subsequent patent periods, which alternated with dormant periods, was not predictable and it was not possible to predict the intensity of the parasitemia which would develop. It was obvious, however, that the same pattern of reproduction occurred in the first, second, and all subsequent relapse periods. This pattern will be discussed.

It is interesting to note that during relapses phagocytosis of erythrocytes and of Babesia canis trophozoites by monocytes sometimes occurred. (Figures 9 and 10) It is assumed that this did not affect the life cycle significantly, but it does indicate at least that the body defenses were active during this time.

The initial reproductive period occurring within three or four days after exposure to infectious blood was never a particularly vigorous one. In parasitized cells harboring more than a single organism, presence of predominantly even numbers of trophozoites (more specifically, exponential progressions of two) indicates that reproduction was by binary fission and not by multiple fission or budding. The reproductive period which occurred following the dormant stage, i.e., about two weeks post exposure, was much more active, and parasitized cells were numerous in the peripheral blood. The preponderance of cells which harbored multiple trophozoites contained either two or exponential multiples of two; two, four, eight, and sixteen parasites were the most common numbers (Figures 11-17). In subsequent relapses in which large numbers of cells were parasitized, the same features were evident.

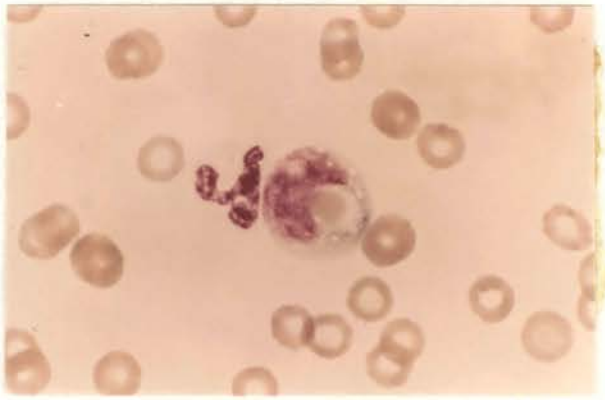

Figure 9. Monocyte containing phagocytized erythrocyte; increased erythrophagocytosis often occurs with extreme parasitemia.

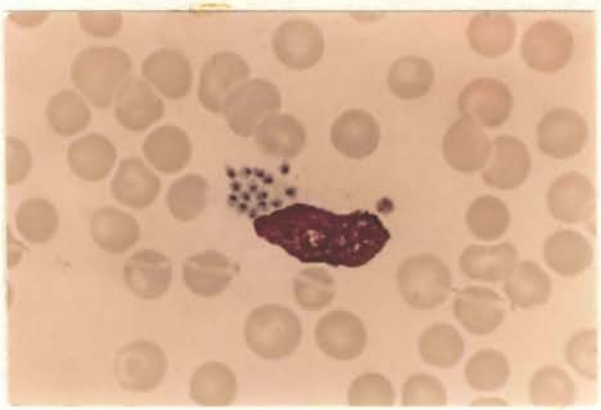

Figure 10. Monocyte containing fifteen phagocytized Babesia canis trophozoites and one intracytoplasmic inclusion typical of those seen in dogs infected with Ehrlichia.

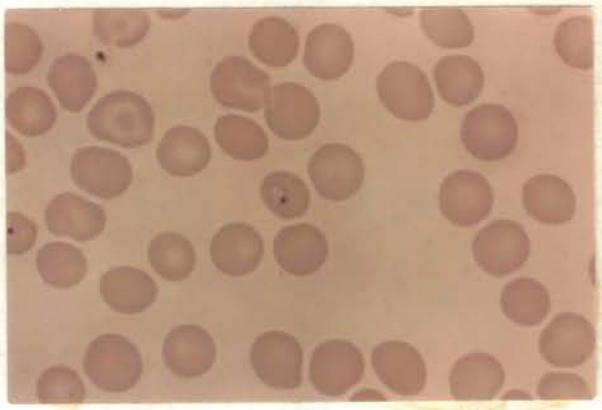

Figure 11. Erythrocyte containing one Babesia canis trophozoite.

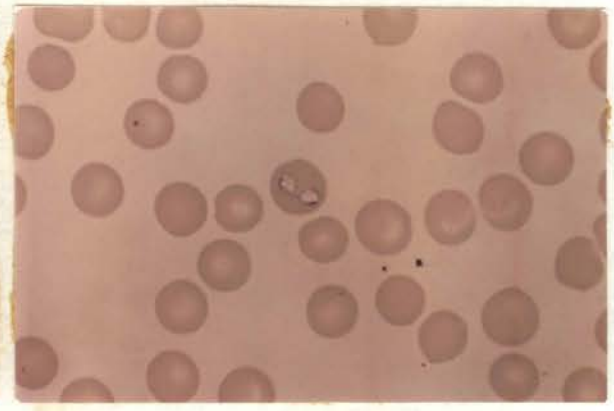

Figure 12. Erythrocyte containing two Babesia canis trophozoites.

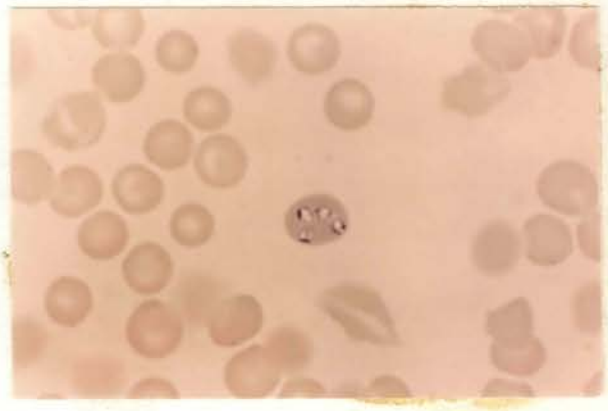

Figure 13. Erythrocyte containing four Babesia canis trophozoites.

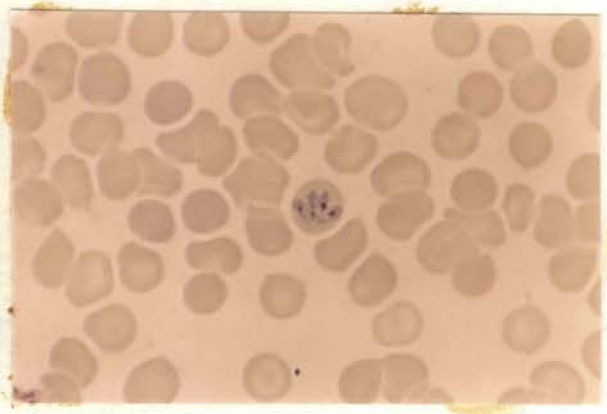

Figure 14. Erythrocyte containing four Babesia canis trophozoites; note the fan-like arrangement of the parasites.

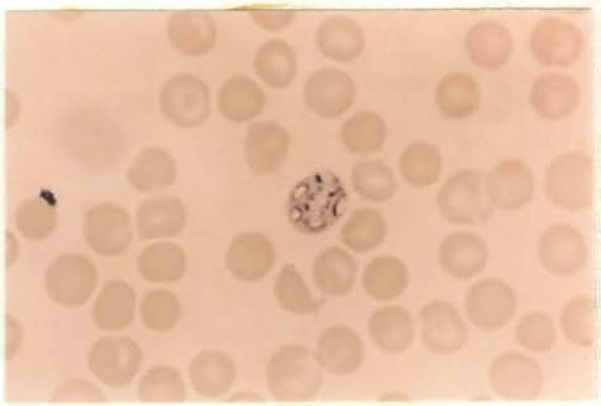

Figure 15. Erythrocyte containing eight Babesia canis trophozoites.

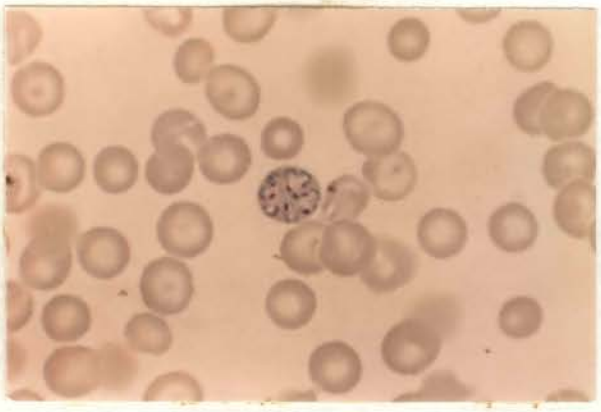

Figure 16. Erythrocyte containing eight Babesia canis trophozoites; note the rosette arrangement of the parasites.

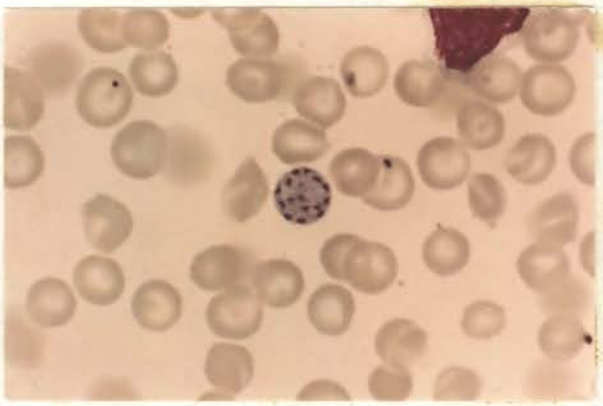

Figure 17. Erythrocyte containing sixteen Babesia canis trophozoites.

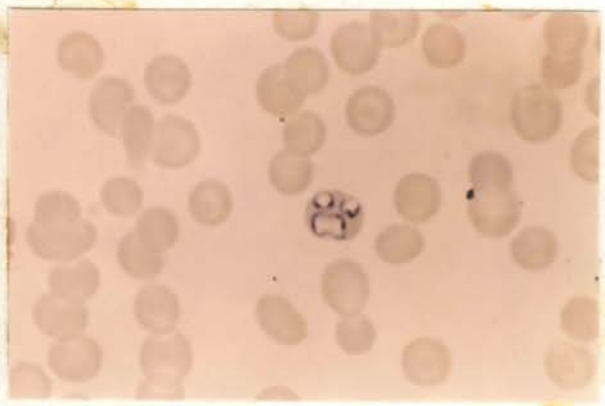

Figure 18. Erythrocyte containing three Babesia canis trophozoites.

In instances in which numbers of trophozoites observed were not exponential multiples of two (e.g., 3, 5, 6, and 7), it was sometimes obvious why the situation existed. Figure 18 depicts one example, viz., a case in which a cell contains three parasites rather than four, and it is obvious on the basis of size and morphology that one parasite is on the verge of completing binary fission. In other instances ruptured cells were observed, and only a single trophozoite had escaped, leaving an odd number inside the ruptured cell which still retained its basic shape.

In comparing the parasite's behavior in the two dogs (E-22 and E-23) which had Babesia in the absence of Ehrlichia, it is seen (Figures 7 and 8) that the same pattern developed, i.e., an initial appearance soon after exposure, followed by a dormant stage, and finally a reappearance (in E-23 only, since E-22 was euthanitized). Thereafter there was no further evidence of infection, and as shown in another section of this dissertation, the dog recovered. The nature of reproduction of parasites within erythrocytes is thus not evident in these two dogs since the numbers of parasitized cells never reached a very high level.

In summary, the data from the twenty-three susceptible dogs exposed to B. canis show that immediately following exposure to whole blood from dogs harboring this protozoan, a transient parasitemia develops, and the increase in numbers appears to be the result of binary fission within erythroctyes. Some cells undoubtedly rupture and release the parasites which then invade other erythrocytes. This lasts for only three or four days, after which the organisms disappear from the peripheral blood, usually for about ten days. A second parasitemia, usually more profound

than the first, develops, beginning about two weeks after exposure, and the pattern is repeated.

It is not possible to state what occurs during the period of absence of parasites. The present study was designed to determine the reproductive cycle in the circulating erythrocytes, and no attempt was made to determine whether an exoerythrocytic reproductive cycle occurs. It is obvious that there is a period when the parasites are not in the peripheral blood, and it seems likely that reproduction of some kind could occur during this time. If such is the case, it remains to be determined where the process occurs. Results of the present study seem to make it unmistakably clear that there is an erythrocytic reproductive phase and that the process is binary fission.

Persistence and Recurrence in Reservoir Animals

In the previous experiments the method used for determining the life cycle of Babesia canis in the dog as related to erythrocytes was discussed. The same methods were employed in this experiment and many of the animals used were the same.

The literature is filled with suggestions, but little proof, that babesiosis is a lifetime infection, and Levine (1961) stated as a fact that cattle infected with Babesia bigemina (Smith and Kilborne, 1893) remain carriers for life. Calzada (1929) indicated that cattle may harbor this parasite indefinitely and remain refractory to reinvasion, i.e., remain in a state of premunization. Stephan and Esquibel (1929) insisted that premunition can be maintained in cattle only by injecting blood from old,

constantly tick-infested cattle. Seifert (1962) lends support to this conclusion with the remark that tick control is the only certain protection against bovine babesiosis. Miessner (1931) reported that dogs which have been infected with B. canis relapse when splenectomized and that the resulting disease is more severe as compared with the original infection and that dogs which survive such relapses eventually become freed of the parasite. Kikuth and Mudrow (1939) indicated that this parasitic species cannot be demonstrated microscopically, or by subinoculation, or by splenectomy in the case of some dogs while in other cases the blood remains contaminated for long periods. One case is cited in which relapse occurred following splenectomy three years after infection. Abramov (1940) gives the following periods of survival for these species: B. bigemina 15 months; B. caballi (Nuttall, 1910), 41 months; B. equi (Laveran, 1901) 5½ years; and B. trautmani (Knuth and du Toit, 1918), 8 months.

Shortt (1935) in considering dogs which survive an attack of Babesia canis stated:

> Those which survive the initial acute attack seem to acquire a degree of immunity or at least tolerance and in these a chronic infection is set up which may persist for a very long time. The condition is more likely to be found in adult dogs and the presumption is that they become infected as puppies and have retained a low grade infection.

It is obvious that there is a lack of unanimity of opinion concerning survival of Babesia in the dog and other mammals. The only constant features of the papers cited is that each in its own way is deficient in the quality and amount of data reported, and the conclusions are presumptuous. It appeared that the differences of opinion which existed were due in part

to the fact that animals from which the data were collected were not kept under circumstances which precluded additional extraneous infection and that they were not bled repeatedly and at short intervals so that examinations could be made on a day to day basis.

In the present experiment nineteen dogs were bled every day from the time of infection until the time of death either naturally or by euthanasia. In the case of four additional dogs which did not expire and were not euthanitized, one (E-01) was bled and the standard number of 100,000 erythrocytes examined repeatedly for twenty-four months. For the first three months post exposure, the dog was bled at frequent but irregular intervals and was then bled every day for twelve consecutive months; this period was followed by a second interval of nine months during which the dog was bled frequently but not daily. Another dog (E-02) was examined similarly every day for over thirteen months. Two additional dogs (E-09 and E-1) were examined every day for four months and seven months respectively after being exposed to Babesia canis. The latter two dogs both had been infected with Ehrlichia prior to the time they were inoculated with blood from a dog having a concomitant infection of B. canis and Ehrlichia; one of these was extraneous (E-1) and occurred about three months prior to the infection with Babesia. The other (E-09) was deliberate and occurred approximately three months before infection with Babesia. (Of course daily observations were made on these two animals for long periods before exposure to Ehrlichia and every day from the time of exposure to Ehrlichia until exposure to Babesia.)

From data given in Tables I-IV it can be seen that Babesia canis persisted in the dog for a maximum period in excess of fourteen months. It

TABLE I

NUMBER OF BABESIA CANIS TROPHOZOITES OBSERVED DAILY AMONG 100,000 ERYTHROCYTES FOR A PERIOD OF FIFTEEN MONTHS (TWELVE CONSECUTIVE) IN ANIMAL NUMBER E-01

4-2-62 ---	3	9-18-62 ---	16	12-17-62---	29	3-17-63 ---	88
4-3-62 ---	5	9-19-62 ---	12	12-18-62---	51	3-18-63 ---	9
4-4-62 ---	*	9-20-62 ---	31	12-19-62---	10	3-19-63 ---	2
4-5-62 ---	12	9-21-62 ---	13	12-20-62---	3	3-20-63 ---	
4-6-62 ---	*	9-22-62 ---	15	12-21-62---	1	3-23-63 ---	> 0
4-7-62 ---	> 0	9-23-62 ---	34	12-22-62---	1	3-24-63 ---	1
4-8-62 ---		9-24-62 ---	38	12-23-62---	3	3-25-63 ---	0
4-9-62 ---	*	9-25-62 ---	43	12-24-62---	3	3-27-63 ---	
4-10-62 ---	> 0	9-26-62 ---	56	12-25-62---	2	3-28-63 ---	8
4-12-62 ---		9-27-62 ---	93	12-26-62---	3	3-29-63 ---	6
4-13-62 ---	> *	9-28-62 ---	169	12-27-62---	1	3-30-63 ---	4
5-9-62 ---		9-29-62 ---	208	12-28-62---	0	3-31-63 ---	38
5-10-62 ---	145	9-30-62 ---	273	12-29-62---	4	4-1-63 ---	33
5-11-62 ---	> *	10-1-62 ---	290	12-30-62---	8	4-2-63 ---	55
7-4-62 ---		10-2-62 ---	139	12-31-62---	33	4-3-63 ---	100
7-5-62 ---	364	10-3-62 ---	114	1-1-63 ---	531	4-4-63 ---	84
7-6-62 ---	123	10-4-62 ---	46	1-2-63 ---	382	4-5-63 ---	28
7-7-62 ---	8	10-5-62 ---	29	1-3-63 ---	530	4-6-63 ---	33
7-8-62 ---	2	10-6-62 ---	36	1-4-63 ---	645	4-7-63 ---	24
7-9-62 ---	3	10-7-62 ---	24	1-5-63 ---	229	4-8-63 ---	5
7-10-62 ---	7	10-8-62 ---	46	1-6-63 ---	12	4-9-63 ---	0
7-11-62 ---	73	10-9-62 ---	66	1-7-63 ---	8	4-10-63 ---	3
7-12-62 ---	298	10-10-62---	88	1-8-63 ---	12	4-11-63 ---	10
7-13-62 ---	545	10-11-62---	121	1-9-63 ---	1	4-12-63 ---	7
7-14-62 ---	550	10-12-62---	102	1-10-63 ---	3	4-13-63 ---	9
7-15-62 ---	163	10-13-62---	66	1-11-63 ---	14	4-14-63 ---	32
7-16-62 ---	75	10-14-62---	+	1-12-63 ---	14	4-15-63 ---	135
7-17-62 ---	39	10-15-62---	146	1-13-63 ---	19	4-16-63 ---	197
7-18-62 ---	17	10-16-62---	9	1-14-63 ---	50	4-17-63 ---	292
7-19-62 ---	11	10-17-62---	0	1-15-63 ---	269	4-18-63 ---	310
7-20-62 ---	7	10-18-62---	13	1-16-63 ---	1713	4-19-63 ---	279
7-21-62 ---	11	10-19-62---	9	1-17-63 ---	526	4-20-63 ---	181
7-22-62 ---	32	10-20-62---	10	1-18-63 ---	336	4-21-63 ---	117
7-23-62 ---	109	10-21-62---	144	1-19-63 ---	316	4-22-63 ---	159
7-24-62 ---	298	10-22-62---	211	1-20-63 ---	268	4-34-63 ---	68
7-25-62 ---	450	10-23-62---	319	1-21-63 ---	24	4-24-63 ---	210
7-26-62 ---	146	10-24-62---	484	1-22-63 ---	6	4-25-63 ---	74
7-27-62 ---	278	10-25-62---	535	1-23-63 ---	3	4-26-63 ---	77
7-28-62 ---	259	10-26-62---	106	1-24-63 ---	6	4-27-63 ---	24
7-29-62 ---	109	10-27-62---	41	1-25-63 ---	159	4-28-63 ---	17
7-30-62 ---	84	10-28-62---	173	1-26-63 ---	24	4-29-63 ---	6
7-31-62 ---	44	10-29-62---	524	1-27-63 ---	10	4-30-63 ---	610
8-1-62 ---	12	10-30-62---	272	1-28-63 ---	554	5-1-63 ---	179
8-2-62 ---	28	10-31-62---	90	1-29-63 ---	214	5-2-63 ---	695
8-3-62 ---	36	11-1-62 ---	76	1-30-63 ---	47	5-3-63 ---	75
8-4-62 ---	39	11-2-62 ---	9	1-31-63 ---	62	5-4-63 ---	24
8-5-62 ---	55	11-3-62 ---	12	2-1-63 ---	52	5-5-63 ---	26
8-6-62 ---	108	11-4-62 ---	5	2-2-63 ---	6	5-6-63 ---	12
8-7-62 ---	185	11-5-62 ---	11	2-3-63 ---	4	5-7-63 ---	542
8-8-62 ---	226	11-6-62 ---	18	2-4-63 ---	4	5-8-63 ---	213
8-9-62 ---	244	11-7-62 ---	17	2-5-63 ---	0	5-9-63 ---	36
8-10-62 ---	102	11-8-62 ---	54	2-6-63 ---	8	5-10-63 ---	81
8-11-62 ---	200	11-9-62 ---	242	2-7-63 ---	22	5-11-63 ---	74
8-12-62 ---	143	11-10-62---	35	2-8-63 ---	37	5-12-63 ---	18
8-13-62 ---	319	11-11-62---	63	2-9-63 ---	28	5-13-63 ---	18
8-14-62 ---	389	11-12-62---	64	2-10-63 ---	28	5-14-63 ---	40
8-15-62 ---	260	11-13-62---	79	2-11-63 ---	216	5-15-63 ---	13
8-16-62 ---	59	11-14-62---	69	2-12-63 ---	37	5-16-63 ---	41
8-17-62 ---	201	11-15-62---	79	2-13-63 ---	20	5-17-63 ---	97
8-18-62 ---	89	11-16-62---	161	2-14-63 ---	4	5-18-63 ---	133
8-19-62 ---	46	11-17-62---	204	2-15-63 ---	15	5-19-63 ---	123
8-20-62 ---	39	11-18-62---	318	2-16-63 ---	2	5-20-63 ---	35
8-21-62 ---	58	11-19-62---	328	2-17-63 ---	4	5-21-63 ---	43
8-22-62 ---	97	11-20-62---	253	2-18-63 ---	1	5-22-63 ---	22
8-23-62 ---	209	11-21-62---	66	2-19-63 ---	0	5-23-63 ---	18
8-24-62 ---	143	11-22-62---	15	2-20-63 ---	1	5-24-63 ---	0
8-25-62 ---	267	11-23-62---	6	2-21-63 ---	24	5-25-63 ---	8
8-26-62 ---	100	11-24-62---	13	2-22-63 ---	8	5-26-63 ---	6
8-27-62 ---	145	11-25-62---	0	2-23-63 ---	4	5-27-63 ---	14
8-28-62 ---	15	11-26-62---	4	2-24-63 ---	6	5-28-63 ---	149
8-29-62 ---	114	11-27-62---	2	2-25-63 ---	18	5-29-63 ---	7
8-30-62 ---	121	11-28-62---	12	2-26-63 ---	26	5-30-63 ---	42
8-31-62 ---	187	11-29-62---	30	2-27-63 ---	23	5-31-63 ---	6
9-1-62 ---	118	11-30-62---	55	2-28-63 ---	105	6-1-63 ---	16
9-2-62 ---	130	12-1-62 ---	117	3-1-63 ---	34	6-2-63 ---	25
9-3-62 ---	44	12-2-62 ---	60	3-2-63 ---	35	6-3-63 ---	68
9-4-62 ---	48	12-3-62 ---	73	3-3-63 ---	8	6-4-63 ---	101
9-5-62 ---	37	12-4-62 ---	43	3-4-63 ---	40	6-5-63 ---	206
9-6-62 ---	167	12-5-62 ---	48	3-5-63 ---		6-6-63 ---	239
9-7-62 ---	183	12-6-62 ---	33	3-6-63 ---	35	6-7-63 ---	141
9-8-62 ---	322	12-7-62 ---	24	3-7-63 ---	13	6-8-63 ---	182
9-9-62 ---	194	12-8-62 ---	81	3-8-63 ---	15	6-9-63 ---	11
9-10-62 ---	227	12-9-62 ---	40	3-9-63 ---	6	6-10-63 ---	2
9-11-62 ---	151	12-10-62---	26	3-10-63 ---	14	6-11-63 ---	> 0
9-12-62 ---	191	12-11-62---	22	3-11-63 ---	63	7-6-63 ---	
9-13-62 ---	246	12-12-62---	6	3-12-63 ---	197		
9-14-62 ---	243	12-13-62---	10	3-13-63 ---	536		
9-15-62 ---	212	12-14-62---	13	3-14-63 ---	557		
9-16-62 ---	73	12-15-62---	13	3-15-63 ---	487		
9-17-62 ---	20	12-16-62---	22	3-16-63 ---	494		

* No sample taken

\+ Trophozoites present but 100,000 erythrocytes not observed due to quality of smear.

TABLE II

NUMBERS OF *BABESIA CANIS* TROPHOZOITES OBSERVED DAILY AMONG 100,000 ERYTHROCYTES FOR A PERIOD OF THIRTEEN CONSECUTIVE MONTHS IN ANIMAL NUMBER E-02

Day	Trophozoites	Day	Trophozoites	Day	Trophozoites	Day	Trophozoites
6-28-62	0	8-22-62	0	10-16-62	1	2-1-63	0
6-29-62	0	8-23-62	1	10-17-62	1	2-2-63	0
6-30-62	0	8-24-62	17	10-18-62	1	2-3-63	0
7-1-62	18	8-25-62	9	10-19-62	1	2-4-63	0
7-2-62	4	8-26-62	14	10-20-62	0	2-5-63	1
7-3-62	1	8-27-62	6	10-21-62	4	2-6-63	0
7-4-62	0	8-28-62	5	10-22-62	27	2-7-63	7
7-5-62	1	8-29-62	2	10-23-62	119	2-8-63	16
7-6-62	0	8-30-62	2	10-24-62	247	2-9-63	10
7-7-62	3	8-31-62	0	10-25-62	1240	2-10-63	14
7-8-62	2	9-1-62	48	10-26-62	1255	2-11-63	66
7-9-62	0	9-2-62	3	10-27-62	386	2-12-63	81
7-10-62	3	9-3-62	10	10-28-62	138	2-13-63	51
7-11-62	0	9-4-62	10	10-29-62	10	2-14-63	30
7-12-62	0	9-5-62	17	10-30-62	0	2-15-63	9
7-13-62	2	9-6-62	5	10-31-62	2	2-16-63	6
7-14-62	1	9-7-62	2	11-1-62	2	2-17-63	> 0
7-15-62	5	9-8-62	0	11-2-62	> 0	7-8-63	
7-16-62	1	9-9-62	0	11-10-62		7-9-63	16
7-17-62	1	9-10-62	0	11-11-62	1	7-10-63	416
7-18-62	125	9-11-62	1	11-12-62	2	7-11-63	213
7-19-62	65	9-12-62	1	11-13-62	0	7-12-63	625
7-20-62	49	9-13-62	33	11-14-62	24	7-13-63	189
7-21-62	5	9-14-62	134	11-15-62	329	7-14-63	123
7-22-62	0	9-15-62	121	11-16-62	894	7-15-63	110
7-23-62	0	9-16-62	178	11-17-62	1110	7-16-63	18
7-24-62	0	9-17-62	249	11-18-62	1088	7-17-63	9
7-25-62	0	9-18-62	120	11-19-62	412	7-18-63	23
7-26-62	0	9-19-62	48	11-20-62	95	7-19-63	4
7-27-62	0	9-20-62	131	11-21-62	0	7-20-63	9
7-28-62	0	9-21-62	596	11-22-62	1	7-21-63	> 0
7-29-62	10	9-22-62	858	11-23-62	6	8-8-63	
7-30-62	2	9-23-62	513	11-24-62	6		
7-31-62	7	9-24-62	378	11-25-62	14		
8-1-62	10	9-25-62	435	11-26-62	42		
8-2-62	19	9-26-62	949	11-27-62	14		
8-3-62	58	9-27-62	124	11-28-62	2		
8-4-62	8	9-28-62	87	11-29-62	> 0		
8-5-62	4	9-29-62	34	12-17-62			
8-6-62	2	9-30-62	23	12-18-62	7		
8-7-62	5	10-1-62	40	12-19-62	4		
8-8-62	2	10-2-62	41	12-20-62	48		
8-9-62	1	10-3-62	61	12-21-62	85		
8-10-62	9	10-4-62	40	12-22-62	120		
8-11-62	7	10-5-62	50	12-23-62	77		
8-12-62	2	10-6-62	16	12-24-62	38		
8-13-62	8	10-7-62	67	12-25-62	22		
8-14-62	18	10-8-62	195	12-26-62	24		
8-15-62	5	10-9-62	545	12-27-62	11		
8-16-62	8	10-10-62	359	12-28-62	11		
8-17-62	7	10-11-62	667	12-29-62	3		
8-18-62	0	10-12-62	208	12-30-62	0		
8-19-62	2	10-13-62	11	12-31-62	> 0		
8-20-62	0	10-14-62	4	1-30-63			
8-21-62	0	10-15-62	1	1-31-63	2		

TABLE III

NUMBERS OF BABESIA CANIS TROPHOZOITES OBSERVED DAILY AMONG 100,000 ERYTHROCYTES FOR A PERIOD OF SEVEN CONSECUTIVE MONTHS IN ANIMAL NUMBER E-1

Day	Trophozoites	Day	Trophozoites	Day	Trophozoites	Day	Trophozoites
11-1-62 ---	> 0	1-9-63 ---	0	2-1-63 ---	4	3-8-63 ---	0
12-18-62---		1-10-63 ---	0	2-2-63 ---	1	3-9-63 ---	0
12-19-62---	1	1-11-63 ---	0	2-3-63 ---	4	3-10-63 ---	2
12-20-62---	96	1-12-63 ---	1	2-4-63 ---	0	3-11-63 ---	> 0
12-21-62---	724	1-13-63 ---	0	2-5-63 ---	0	3-30-63 ---	
12-22-62---	344	1-14-63 ---	0	2-6-63 ---	0	3-31-63 ---	1
12-23-62---	0	1-15-63 ---	7	2-7-63 ---	0	4-1-63 ---	21
12-24-62---	0	1-16-63 ---	0	2-8-63 ---	2	4-2-63 ---	44
12-25-62---	1	1-17-63 ---	3	2-9-63 ---	1	4-3-63 ---	35
12-26-62---	1	1-18-63 ---	0	2-10-63 ---	77	4-4-63 ---	6
12-27-62---	68	1-19-63 ---	0	2-11-63 ---	839	4-5-63 ---	2
12-28-62---	312	1-20-63 ---	0	2-12-63 ---	1127	4-6-63 ---	1
12-29-62---	272	1-21-63 ---	2	2-13-63 ---	19	4-7-63 ---	> 0
12-30-62---	4	1-22-63 ---	5	2-14-63 ---	> 0	5-2-63 ---	
12-31-62---	0	1-23-63 ---	6	2-27-63 ---		5-3-63 ---	5
1-1-63 ---	0	1-24-63 ---	3	2-28-63 ---	2	5-4-63 ---	3
1-2-63 ---	0	1-25-63 ---	7	3-1-63 ---	16	5-5-63 ---	2
1-3-63 ---	0	1-26-63 ---	1	3-2-63 ---	0	5-6-63 ---	7
1-4-63 ---	2	1-27-63 ---	1	3-3-63 ---	0	5-7-63 ---	3
1-5-63 ---	0	1-28-63 ---	0	3-4-63 ---	0	5-8-63 ---	0
1-6-63 ---	0	1-29-63 ---	1	3-5-63 ---	3	5-9-63 ---	0
1-7-63 ---	1	1-30-63 ---	2	3-6-63 ---	2	5-10-63 ---	1
1-8-63 ---	1	1-31-63 ---	0	3-7-63 ---	1	5-11-63 ---	> 0
						6-12-63 ---	

*Euthanitized 6-12-63

TABLE IV

NUMBERS OF BABESIA CANIS TROPHOZOITES OBSERVED DAILY AMONG 100,000 ERYTHROCYTES FOR A PERIOD OF FOUR CONSECUTIVE MONTHS IN ANIMAL NUMBER E-09

Day	Trophozoites	Day	Trophozoites	Day	Trophozoites	Day	Trophozoites
2-8-63 ---	> 0	5-25-63 ---	9	6-23-63 ---	12	7-21-63 ---	0
5-8-63 ---		5-26-63 ---	5	6-24-63 ---	43	7-22-63 ---	6
5-9-63 ---	2	5-27-63 ---	78	6-25-63 ---	2	7-23-63 ---	40
5-10-63 ---	56	5-28-63 ---	50	6-26-63 ---	1	7-24-63 ---	173
5-11-63 ---	22	5-29-63 ---	3	6-27-63 ---	2	7-25-63 ---	19
5-12-63 ---	12	5-30-63 ---	> 0	6-28-63 ---	9	7-26-63 ---	2
5-13-63 ---	4	6-20-63 ---		6-29-63 ---	3	7-27-63 ---	> 0
5-14-63 ---	> 0	6-21-63 ---	5	6-30-63 ---	2	8-4-63 ---	
5-24-63 ---		6-22-63 ---	11	7-1-63 ---	to		

should be mentioned that the dog (E-01) in which the protozoan persisted for the longest period was splenectomized prior to exposure to the parasite. Furthermore, this was the first animal infected with the strain after its discovery in a naturally infected dog. The dog (E-02), in which the organism persisted for seven but not for eight months, was infected with blood taken from E-01 three months after the latter was exposed. E-02 was splenectomized approximately three months after infection and had popliteal lymph nodes removed approximately six months after infection. Approximately five months after the last trophozoite was observed, this dog was challenged with the strain of B. canis acquired from Dr. Ned Rokey. Infection occurred, and trophozoites were present in the circulating erythrocytes for fourteen days after which none was seen; daily examinations were terminated a month after exposure, and frequent samples were examined for the next eight months. In dog E-1 in which the protozoan persisted for four months but not for five, exposure was made from E-01 approximately eight and one-half months after the latter was infected. E-1 had been extraneously infected with Ehrlichia (details given in another section) and had been splenectomized one and one-half months prior to exposure to Babesia. A typical attack of babesiosis occurred, and during some of the subsequent relapses extremely large numbers of parasites, as shown in Table III, were seen in the peripheral blood. Daily examinations were continued for slightly longer than a month after the last trophozoite was seen. The dog (E-09) in which the infection persisted for only about twenty days had recovered from an experimental attack of ehrlichiasis, and was infected from E-01 during the donor's fourteenth month of infection or just prior to the time when the protozoan disappeared from

it. It was suspected that the strain was losing virulence, and thus after a week of negative examinations, E-09 was challenged with massive numbers of trophozoites from E-01, on one of the last days that the donor ever had large numbers of trophozoites in his bloodstream. Nothing happened. About two weeks later splenectomy was performed, and a parasitemia developed, but it lasted less than two weeks. After a negative period of two weeks the dog was challenged with the strain of Babesia acquired from Dr. Ned Rokey (from E-02 which had a patent infection with the strain at that time), but no trophozoites were seen until almost two weeks later when a transient parasitemia developed. The parasites disappeared and were never seen again; daily observations were discontinued a month later, but frequent examinations were made for the next eight months, and no trophozoites were ever observed.

On the basis of these data it is clear that a newly isolated strain of B. canis can persist in the dog for nearly fifteen months. The survival times in these four dogs, one infected immediately with blood from a patent field case and the others infected with blood from this experimental case at three months, eight and one-half months, and fourteen months, suggest that the longer the strain has been reproducing asexually in the dog, (without passage through a tick) the shorter will be the period of survival in susceptible dogs which survive infections. It is not known whether the fact that the dogs were splenectomized (two prior to exposure and two after infections were in progress) affected the length of persistence of the parasites. Neither is it known whether the concomitant presence of Ehrlichia sp. in the dogs affected longevity. In any case, it is proved that under some circumstances dogs may asymptomatically harbor Babesia for several months.

Infections produced experimentally in other dogs, using E-01 as the donor, often terminated fatally; as late as fourteen months after exposure, infections resulting from transfusion of blood from this dog terminated in death. It must be remembered, however, that Ehrlichia sp. was concomitantly present in all these animals and unquestionably contributed to the detrimental effects.

Statistical Evaluation of Methods Used to Detect Babesia canis Infections in Dogs

Confirmatory diagnosis of babesiosis in mammals depends upon detection of trophozoites in the peripheral blood or in bone marrow. Velu (1926) utilized marrow smears from long bones. Girard and Flucher (1937) recommended use of bone marrow smears made from material collected by sternal puncture. Noyan (1954) determined that smears of bone marrow were useful in chronic infections but that blood smears were more useful in diagnosing acute cases. It is now generally agreed that observation of stained smears of blood is the best procedure for diagnosis and that Romanowsky stains are best suited for staining the organisms, but others have been used. Zasukhim (1933-34) reported that certain Piroplasma sp. (Babesia) were Feulgen-negative. Buzanec (1944) described a technique utilizing carbol-fushsin as a stain for the trophozoites after removal of hemoglobin from the erythrocytes by treatment with Ruge's solution (acetic acid and formalin solution). Zottner (1932) described a technique in which blood smears were fixed with a methyl alcohol solution of eosin and stained with a permanganate solution. None of these techniques has been good enough to become widely used, and the great majority of recent workers have stained with either Wright's or

Giemsa's, both of which are classed as Romanowsky stains. Ristic (1964) has utilized a more specialized procedure, viz., the fluorescent-antibody technique, which gives good results but which could not be used under ordinary, routine, diagnostic circumstances.

Utilization of thick blood films has been recommended by Mahoney and Saal (1961) as a method of detecting Babesia sp. in cases in which the organism is not abundant in the peripheral blood. Ono and Isoda (1943) described a procedure for concentrating parasitized cells, and Watkins (1962) recommended the use of a combination technique in which parasites are concentrated and thick smears prepared for staining.

The question arises concerning the type of blood sample which should be used in attempting to diagnose babesiosis. Paine (1934) stresses the importance of using the first drop of blood from a needle prick when making smears for diagnostic purposes. He suggested that the infected erythrocytes are enlarged and tend to become retarded in movement by stasis in the small blood vessels of the skin. Adams (1935) utilized thin blood films taken from the ears of cattle in a study on incidence and distribution of Babesia bigemina and other blood protozoans.

Often additional hematological examinations are to be made on animals which are ill enough to be suspected of having babesiosis, and venous blood is usually utilized for this purpose since a relatively large volume is needed to complete the procedures. It would be useful to know whether a portion of such a sample would be satisfactory for making a smear for observation or whether a separate smear utilizing the first drop of capillary blood must be used.

In the present study experiments were designed to check the comparative efficacy of detecting Babesia canis by (1) using stained smears of the first drop of blood from the ear capillaries and (2) using smears of venous blood drawn from the cephalic vein.

A total of eight dogs was used in this study. Six of the animals were infected by blood transfusion from a reservoir animal, and two were maintained as unexposed controls. The dogs were kept under circumstances which excluded additional exposure to Babesia. The smears of capillary blood were made by using the same procedure as already described. The venous blood was drawn and put into a tube containing EDTA, an anticoagulant. This blood was used for routine hematological studies, and a drop was taken from this quantity and a smear made and stained in the same manner as was the capillary blood.

The examination of approximately 100,000 erythrocytes from capillary blood and an equal number from venous blood per dog per day was carried out using a magnification of 970x beginning on the third day after exposure and continuing for either twenty-one days or until death of the dog. The blood samples from the two sources were drawn at approximately the same time, within two minutes of each other.

A summary of the numbers of Babesia canis trophozoites observed among 100,000 erythrocytes from each of the two sources on each of the twenty-one days is shown in Table V. Only the six principals are included in the table since both control animals had negative counts by both methods throughout the study, and the only significance of the controls in this phase of the study was the indication that no extraneous infections occurred among the experimental subjects.

TABLE V

SUMMARY OF NUMBERS OF BABESIA CANIS TROPHOZOITES AMONG 100,000 ERYTHROCYTES (DAILY) FROM VENOUS BLOOD (V) AND FROM FROM CAPILLARY BLOOD (C)

		Day 1		Day 2		Day 3		Day 4		Day 5		Day 6		Day 7		Day 8		Day 9		Day 10		Day 11		Day 12		Day 13		Day 14		Day 15		Day 16		Day 17		Day 18		Day 19		Day 20		Day 21	
Dog	Trophozoites	V	C	V	C	V	C	V	C	V	C	V	C	V	C	V	C	V	C	V	C	V	C	V	C	V	C	V	C	V	C	V	C	V	C	V	C	V	C	V	C
(E-8)	1/RBC	3	7	5	14	5	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0	2	1	10	3	64		92	91								
	2/RBC	2	3	16	1	3	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3	0	7	1	46		49	5								
	4/RBC	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	4	0	18		14	0		DEAD						
	8/RBC	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2		0	0								
	FREE	0	3	0	1	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	13		0	3								
	TOTAL *	19	16	37	17	11	1	4	8	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0	8	1	40	6	285		255	104								
Dog	1/RBC	2	3	2	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	4	0	29	25	33	52	29	19	1	5	0	0	0	0
(E-7)	2/RBC	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	1	27	4	1	39	11	4	0	1	0	0	0	0
	4/RBC	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1	0	6	2	3	4	4	0	0	0	0	0	0	0	0	0
	8/RBC	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	FREE	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	17	8	12	1	0	0	0	0	0
	TOTAL *	6	5	2	1	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	4	0	12	3	107	46	47	163	75	45	2	7	0	0	0	0
Dog	1/RBC	3	0	17	7	2	3	2	1	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	4	0	6	19	99	108	77	101	67	108	20	10	29	3	2	3	0	1
(E-6)	2/RBC	6	0	9	3	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	3	76	39	30	33	18	69	5	4	10	0	0	0	0	0
	4/RBC	2	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	15	6	5	4	1	12	2	1	5	0	0	0	0	0
	8/RBC	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	5	3	0	1	0	0	0	0	3	0	0	0	0	0
	FREE	2	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	9	6	8	23	3	8	0	10	0	2	1	1	0	0	0	0
	TOTAL *	25	0	39	18	4	3	2	1	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	4	0	17	31	367	263	167	202	110	307	38	24	1014	2	3	0	1	
Dog	1/RBC	8	15	1	17	1	3	2	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3	3	4	19	24	37	37	27	21	34	0	13	8	9	6	2
(E-4)	2/RBC	3	8	14	4	1	0	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	4	15	4	5	3	19	6	1	0	7	0	2	2
	4/RBC	0	0	1	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	6	0	4	0	8	0	1	0	2	0	3	0
	8/RBC	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	2	0	1	0	1	0	0	0	0	0	4	0
	FREE	0	4	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	16	0	15	2	4	3	12	0	1	0	0	0	0
	TOTAL *	14	35	33	48	3	3	4	3	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3	3	16	51	102	60	73	37	10861	6	14	30	9	67	6	
Dog	1/RBC	5	5	30	62	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	9	22	7	287	93	115	78	94	59	14	16	10	2	4	1	3	1	4	12
(E-3)	2/RBC	7	2	27	20	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1	4	17	3	142	32	62	15	71	5	13	15	11	2	0	0	0	0	0	0
	4/RBC	1	0	2	4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	4	0	26	0	17	1	14	0	4	3	5	0	0	0	0	0	0	0
	8/RBC	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	2	0	4	0	2	0	1	0	1	0	0	0	0	0	0	0	0
	FREE	1	1	1	12	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	12	1	5	4	13	6	0	3	0	6	2	0	0	0	0	0	0	0
	TOTAL *	24	10	96	113	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	2	1	2	19	92	14	696	161	367	118	320	72	67	64	62	6	4	1	3	1	4	12	
Dog	1/RBC	3	2	11	1	1	0	1	2	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	9	15	39	46	79	68	47	53	46	26	8	5	0	1
(E-2)	2/RBC	4	0	2	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	12	8	29	3	22	7	9	4	18	5	4	1	0	0
	4/RBC	1	0	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	7	1	6	0	1	0	3	0	6	0	3	0	0	0
	8/RBC	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0	1	0	0	0	0	0	0	1	0	0	0	0
	FREE	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	4	3	0	3	1	3	3	0	3	1	0	1	0	0
	TOTAL *	15	2	27	3	3	0	1	2	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	3	3	81	38	132	55	128	85	80	61	10937	36	8	0	1	

* Deviation from apparent total is due to presence of erythrocyte(s) containing odd numbers of trophozoites.

In general, the data show that a transient parasitemia developed in all the principals, lasted a short time, and was followed by a period of apparent dormancy. Finally the trophozoites reappeared in the bloodstream, and a much more profound parasitemia developed in all the dogs. This condition parallels that of seventeen other dogs similarly affected.

The difference in the numbers of trophozoites observed among 100,000 erythrocytes from each of the two sources is shown in Table VI.

The number of days on which trophozoites were observed in smears made of venous blood and in smears made of capillary blood (Not N) along with the number of days on which no trophozoites were observed by either method (N) is shown in Table VII.

TABLE VII

SUMMARY OF THE DAYS ON WHICH NO TROPHOZOITES WERE OBSERVED BY EITHER METHOD (N) AND DAYS ON WHICH AT LEAST ONE TROPHOZOITE WAS OBSERVED BY EITHER ONE METHOD OR THE OTHER (NOT N)

Dog	Days	N	Not N
E-8	15	7	8
E-7	21	12	9
E-6	21	7	14
E-4	21	8	13
E-3	21	7	14
E-2	21	7	14
	120	48	72

There are inferences which can be made on the basis of the "Not N" data, and these are given in Table VIII; the numerical entry in Table VIII is "days to dormancy", i.e., the days to the time when trophozoites were not observed among the 100,000 erythrocytes examined. Based on data given in Table VIII, the following information is offered:

TABLE VI

DIFFERENCE IN NUMBERS OF TROPHOZOITES OBSERVED AMONG 100,000 ERYTHROCYTES FROM VENOUS BLOOD (V) AND FROM CAPILLARY BLOOD (C)

Dog	Day 1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
E-8	1	1^{+}	1^{+}	0	N	N	N	N	N	N	N	$\overset{x}{1}$	1^{+}	1^{+}	M	1^{+}	D	D	D	D	D
E-7	1	1	$\overset{x}{1}$	N	N	N	N	N	N	N	N	N	N	$\overset{x}{1}$	1^{+}	1^{+}	0^{-}	1^{+}	0	N	N
E-6	1^{+}	1^{+}	T	1	$\overset{x}{0}$	N	N	N	N	N	N	N	$\overset{x}{1}$	0^{-}	1^{+}	0^{-}	0^{-}	1^{+}	1^{+}	0	0
E-4	0^{-}	0^{-}	T	1	$\overset{x}{1}$	N	N	N	N	N	N	N	N	T	0^{-}	1^{+}	1^{+}	1^{+}	0^{-}	1^{+}	1^{+}
E-3	1^{+}	0^{-}	N	$\overset{x}{1}$	N	N	N	N	N	N	1	0^{-}	1^{+}	1^{+}	1^{+}	1^{+}	1	1^{+}	1	1	0^{-}
E-2	1^{+}	1^{+}	1	0	$\overset{x}{0}$	N	N	N	N	N	N	N	$\overset{x}{0}$	T	1^{+}	1^{+}	1^{+}	1^{+}	1	1	$\overset{x}{1}$

Code: T = V - C = 0
1^{+} = 0 < V - C ≤ 5
1^{+} = 5 < V - C
0^{-} = -5 < V - C < 0
0^{-} = -5 ≥ V - C

M = Missing
D = No count due to death of dog

N = Both V and C had no count.
Small x above a number indicates that one method had a count and the other did not.

TABLE VIII

DAYS FROM ONSET OF INFECTION TO BEGINNING OF DORMANT PERIOD (PERIOD WHEN TROPHOZOITES ABSENT FROM PERIPHERAL BLOOD)

	Dog	E-8	E-7	E-6	E-4	E-3	E-2
Method	Vein	4	3	4	5	4	4
	Capillary	4	2	5	4	2	5

TABLE IX

DAYS FROM ONSET OF INFECTION TO SECOND APPEARANCE (END OF DORMANCY) OF TROPHOZOITES

Dog	E-8	E-7	E-6	E-4	E-3	E-2
Vein	12	14	13	14	11	14
Capillary	13	15	14	14	11	13

ESTIMATED MEAN DAYS TO DORMANCY

	Point	Interval (95%)
VEIN	4.00	3.34 to 4.66
CAPILLARY	3.67	2.91 to 4.43

In Table IX, the entries are in each case the number of days which elapsed from the time of first recovery of trophozoites until trophozoites reappeared in the blood smears made from each of the two sources. Based on the data given in Table IX the following information is offered:

ESTIMATED MEAN DAYS TO REAPPEARANCE

	Point	Interval (95%)
VEIN	13.00	11.67 to 14.34
CAPILLARY	13.33	11.89 to 14.77

Using the information from Tables VIII and IX the length of the dormancy period can be estimated.

ESTIMATED DORMANCY PERIOD

	Point	Interval (95%)
VEIN	9.33	8.07 to 10.57
CAPILLARY	9.33	7.17 to 11.49

In summary, the following information seems apparent. From data in Table VI it may be determined that there were seventy-two instances in which trophozoites were recovered either in venous or in capillary blood. In examining these seventy-two cases, (designated "Not N") there were ten in which trophozoites were observed in one type smear but not in the other. In these ten instances, at least one trophozoite was seen in venous blood seven times and in capillary blood three times. This is not strong evidence

that venous blood is more apt than capillary blood to reveal trophozoites; nevertheless in seven out of ten cases (70.0%) trophozoites were detected in venous blood when they were not observed in smears prepared from capillary blood. This information coupled with the discussion which follows supports the conjecture that this result did not arise due to chance fluctuation in sampling.

By eliminating the ten instances just discussed from the total of the seventy-two "Not N" cases, the sixty-one remaining can be assigned to the categories expressed in Table X A. The entries in Table X B are numbers of times these conditions occurred in the data.

TABLE X A

V-C > 0		V-C < 0		V-C=0
V-C > 5	0 < V-C ≤ 5	V-C < -5	-5 ≤ V-C < 0	V-C=0

TABLE X B

ANALYSIS OF "NOT N" CASES IN WHICH TROPHOZOITES WERE DETECTED BY BOTH METHODS

Dog	Vein		Capillary		Ties	Total
E-8	6		1		0	7
	5	1	0	1	0	7
E-7	5		2		0	7
	3	2	1	1	0	7
E-6	6		5		1	12
	5	1	3	2	1	12
E-4	6		4		2	12
	5	1	4	0	2	12
E-3	10		3		0	13
	6	4	3	0	0	13
E-2	9		1		1	11
	6	3	0	1	1	11

Combined for all dogs	Vein		Capillary		Ties	Total
	42		16		4	62
	30	12	11	5	4	62

Several inferences can be made concerning the entries in Table X B. These are made from cases in which trophozoites were observed both in venous and in capillary blood.

Using the lower numbers in Table X B, for each dog, and letting p = estimated probability of five or more trophozoites occurring in venous blood than in capillary (p = V-C ≥ 5 divided by V-C ≥ 5 plus V-C ≤ -5), the results are as follows:

		P	(100p)%
Dog	1	5/5	100%
	2	3/4	75%
	3	5/8	62.5%
	4	5/9	55.5%
	5	6/9	66.7%
	6	6/6	100%
Total		30/41	73.2%

Thus the estimated probability over all dogs in cases in which the venous count was at least five trophozoites higher than the capillary count is .732 or 73.2%. (This is calculated eliminating the three ties.)

If the upper numbers are used, i.e., the estimated probability that the venous count is at least one trophozoite higher than the capillary count, the result is 42/58, i.e., .724 or 72.4%.

Applying a statistical test of the hypothesis that there is no difference in using venous and capillary blood there is evidence to support the rejection of such a hypothesis, i.e., it is very unlikely to observe the above cited values of .724 or .732 when one is sampling from two equally good methods.

Considering all evidence, the results are remarkably consistent. In the ten cases the estimated probability was .700 and in the two concluding analyses, .724 and .732 were observed. This consistent evidence favors the examination of venous blood rather than capillary blood. Indeed, it is not as effective in a few specific cases, but on the average, about 72% of the time, these data show that the venous blood is more apt to reveal Babesia canis trophozoites than is capillary blood taken from the ear.

Using the size sample employed in this study (100,000 erythrocytes), one is likely to find trophozoites if they are present. This is reflected in the similarity between estimated days to dormancy (3.67 by capillary and 4.00 by vein), and estimated dormancy period (9.33 by both methods). If, however, one examined fewer than 100,000 erythrocytes, the probability of observing parasitized cells in the capillary blood would be reduced as compared with venous blood, for the analysis using a difference of five parasitized cells (chosen arbitrarily) as a reference point shows the venous blood to be better in approximately 72% of the cases.

In conclusion, it is obvious from these data that in the dogs used in this study the traditional view that capillary blood should be examined in order to detect B. canis is not supported. In fact the recoveries made in the venous blood are better than those in the capillary blood. Examination of the data from a number of different angles reveals that the same conclusion can be drawn each time, viz., that the venous blood smears will reveal more Babesia canis trophozoites than the capillary blood, when the parasites are present in the peripheral blood.

III. GENERAL CHARACTERISTICS OF *EHRLICHIA CANIS* (DONATIEN AND LESTOQUARD, 1935)

Rickettsia canis was recovered and described by Donatien and Lestoquard (1935) from dogs exposed to tick infestation in Algeria. These same workers (1936) showed that these parasites were distinct and different from *R. conori*, a human parasite which had been reported to infect dogs. The differentiation was based on the fact that *R. canis* was found only in circulating monocytes, and that it caused a severe febrile reaction as well as other clinical symptoms which frequently resulted in death of the animals. This was especially true when concurrent infections such as babesiosis or leishmaniosis existed. *Rickettsia conori* reportedly caused no visible reaction in exposed dogs.

Mochkovski (1937) erected the genus *Ehrlichia* to include rickettsiae which invade monocytes and placed *Rickettsia canis* in the genus but changed the name to *Ehrlichia kurlovi* on the grounds that so-called Kurloff bodies were probably identical with *Rickettsia canis*. This specific epithet has not been accepted, but the genus *Ehrlichia* has persisted. Philip (1953) accepted the name *Ehrlichia* and considered *E. canis* the genotype species.

Rickettsia canis was reported from a dog in Southern Rhodesia by Lawrence (1938), and the parasite was associated with serious disease in dogs originally thought to be suffering from biliary fever (babesiosis). Lawrence accepted the Donatien and Lestoquard designated name of *R. canis*; he either

ignored or was unaware of Mochkovski's publication. Lawrence stated that the probable explanation for R. canis not having been recorded more frequently "... is that private practitioners rarely resort to confirmatory diagnosis in cases of canine biliary fever and that, therefore, this disease is sometimes diagnosed in the rare cases where the cause is a rickettsia."

Neitz and Thomas (1938) reported canine rickettsiosis from the Kruger National Park in South Africa and concluded that the causative agent was R. canis on the basis that "... R. conori develops in the endothelial cells of the blood vessels and the peritoneum, while R. canis parasitizes the monocytes and neutrophiles." These workers stressed the difficulties attendant with making a differential diagnosis of Rickettsia canis because dogs so often had biliary fever (babesiosis) as a complicating disease. Still other diseases, e.g., hepatozoonosis, trypanosomiasis, Weil's disease (spirochaetosis), Stuttgart's disease, leishmaniasis, distemper, dumb form of rabies, verminosis, and malnutrition due to mineral deficiency are listed as possible complicating conditions in naturally occurring disease. Furthermore it was stated that since rickettsiosis is

> ... practically impossible to differentiate clinically, a definite diagnosis can only be made microscopically. It is therefore necessary to be able to distinguish R. canis from other blood protozoa /sic/ and structures such as: 1. Leishmania donovani, 2. Hepatozoon canis, 3. phagocytozed /sic/ P. canis, and Babesia gibsoni, 4. phagocytozed /sic/ bacteria, 5. clusters of blood platelets, and 6. stain deposit and artefacts superimposed on the leukocytes.

Pasquini (1939) reported five cases of canine rickettsiosis. Pigoury and Bernard (1939) reported looking for Rickettsia canis in Beyrouth dogs in

1937 and for a period subsequently and indicated that inclusion bodies were found in the monocytes of the lungs in one stray dog; these bodies were similar in morphology and staining characteristics to R. canis as described by Donatien and Lestoquard. Malbrant (1939) described a disease in dogs in the French Congo in which bodies resembling R. canis were demonstrated in the monocytes of the peripheral blood, lungs, and liver. Thus, R. canis, later called Ehrlichia canis, was reported from various parts of Africa and the Orient within four years after its discovery in Algeria.

Subsequently, Carmichael and Fiennes (1942) described the disease syndrome as it appeared in Uganda and used the term canine typhus to designate the malady. Gillain (1942) reported deaths caused by Rickettsia canis among European breeds of dogs in the Belgian Congo in which R. canis and Babesia canis occurred together causing a complicated syndrome, and Mornet (1942) reported rickettsial infection in European breeds of dogs in French West Africa. Mudaliar (1944) appears to be the first to report R. canis from India; the organisms were found chiefly in circulating monocytes of dogs showing persistent fever and anemia. Malherbe (1947) reported R. canis from the Pretoria district of Africa and pointed out that infections were often clinically obscured by B. canis. Girard and Rousselot (1947) mentioned canine rickettsiosis as being a problem in the Sudan. Malherbe (1948) discussed R. canis on the basis of methods of diagnosis, symptoms, and therapy and indicated that dogs in the Onderstepoort area were often affected. Receveur and Hugaud (1949) reported a fatal case of canine rickettsiosis in a bitch, which confirmed the existence of R. canis in Chad. Thus Ehrlichia canis was reported from dogs in various regions of the Old World, but concomitant infections with B. canis often hindered accurate description of the syndrome produced by the rickettsia. Studies of canine babesiosis were

undoubtedly also complicated by the often unrecognized E. canis. It was not until more than twenty years after the discovery of Ehrlichia in the Old World, that Bool and Sutmöller (1957) recognized this rickettsia in the New World. They published studies involving an agent which they thought to be E. canis isolated from dogs in the Netherlands Antilles. They too reported that Babesia canis was a factor complicating the study of the rickettsiosis just as had been the case in several instances in the Old World. Reports by Cassard (1957) and Raghavachari and Reddy (1958) confirmed that R. canis (E. canis) infections remain a problem in Africa and India, respectively. The latter indicated that the Indian and African strains of the organism were identical. McGaughey, Seneviratna and Mahalingam (1962) reported Ehrlichia canis (R. canis) from Ceylon, and Ewing (1964) suggested that E. canis or a similar species occurs in the United States. In the latter case, it was shown that B. canis could be a concurrent invader in naturally infected dogs.

Malbrant (1945), in the French Congo, isolated and described Rickettsia donatieni from dogs exhibiting conjunctivitis. This organism was reportedly found in epithelial cells of the conjunctiva. No further mention of this organism has been found in the literature, and Philip (in Breed, et al., 1957), failed to mention it as a synonym for Ehrlichia canis or as a valid species (which would undoubtedly require that it be removed from the genus Rickettsia since this is reserved for organisms affecting man.)

Morphology and Life Cycle

Donatien and Lestoquard (1940) described the life cycle of Ehrlichia canis (R. canis) and compared the cycle with that of R. conjunctivae which

causes a severe conjunctivitis in sheep. These workers stated that when the temperature rise is initiated in rickettsiosis of the dog, the first forms of R. canis (E. canis) appear as large dark, homogeneous, circular masses confined to the monocytes, and these were referred to as initial bodies. (These masses are considered to be analogous to the large bodies described for R. conjunctivae which also appear during the first hours of rickettsial conjunctivitis in the sheep.) These initial bodies in both cases apparently undergo fragmentation to pass through a mulberry or morula stage which in turn breaks up to form elementary bodies; the elementary bodies then penetrate monocytes, and each develops into a massive initial body. Thus these workers visualized a cycle in which (1) initial bodies develop in monocytes to become (2) mulberry bodies which in turn dissociate to form (3) elementary bodies. In remarks concerning the conclusion of the cycle the workers stated:

> It is necessary in order to complete the cycle to note the origin of the initial bodies. An initial body can be engendered only by an elementary body which has penetrated into the host cell of the considered rickettsia ... /monocytes in the case of E. canis/.

They admitted that the so-called initial bodies vary in size, but this is accounted for as follows:

> We can assume that the regularly rounded initial bodies of different sizes represent the various stages of the increase in size of the initial body which was irregularly fragmented after having reached its maximum size.

Furthermore these workers cited instances in which at autopsy initial bodies could be found in organ smear preparations but very few mulberry-shaped bodies, and this fact is used to justify

> ... the assumption that the elementary bodies are produced by the mulberry-shaped bodies in the deep organs. These bodies are scattered in the meningeal vessels. The elementary bodies liberated penetrate the monocyte where they become massive initial bodies more and more voluminous. The monocytes thus parasitized are easily observed in the peripheral blood.

In addition these workers stated that the life cycle of Ehrlichia in splenectomy-induced relapses

> ... always develops according to the same cycle: initial bodies first, mulberry-shaped bodies next. The same thing happens in the relapse occurring without apparent cause.

In the present study, involving twenty-one dogs which had both Ehrlichia and Babesia canis and seventeen dogs with Ehrlichia only, the life cycle could not be confirmed as heretofore described, either in initial infections or by splenectomy-induced relapses or relapses occurring without apparent cause. The photographs (Figures 19 and 20) show very clearly the so-called mulberry-shaped bodies which Donatien and Lestoquard and other authors described and reported as occurring in leukocytes. Virtually all previous workers reported that these mulberry-shaped bodies appear in monocytes almost exclusively, but it has been determined in the present work that they are found almost exclusively in lymphocytes, almost never in monocytes, and only rarely as morulae-type aggregates in neutrophils.

As far as so-called initial bodies and elementary bodies are concerned, the observations made in the present study do not substantiate the findings of Donatien and Lestoquard. Although numerous cells containing cytoplasmic granules were observed, the sequential pattern that

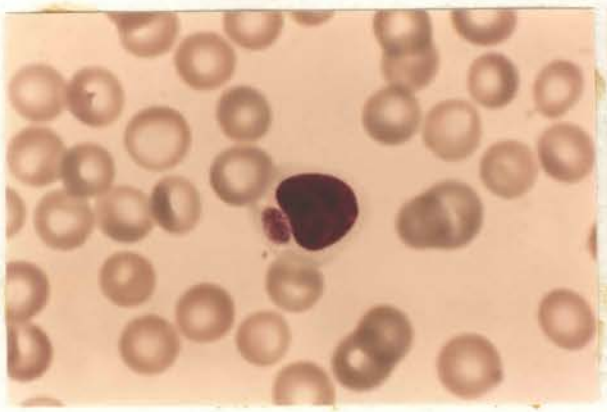

Figure 19. Lymphocyte containing mulberry-shaped inclusion characteristic of Ehrlichia.

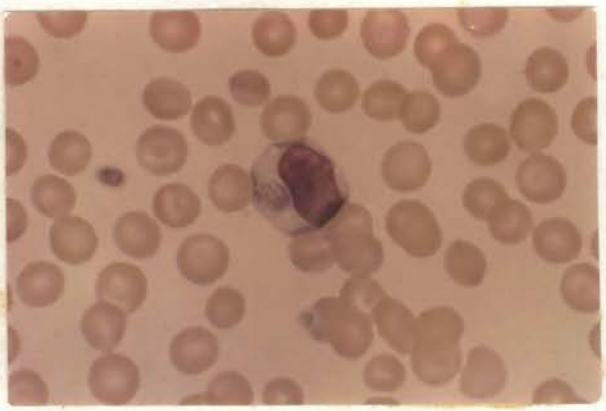

Figure 20. Lymphocyte containing mulberry-shaped inclusion characteristic of Ehrlichia; note granules making up the morula.

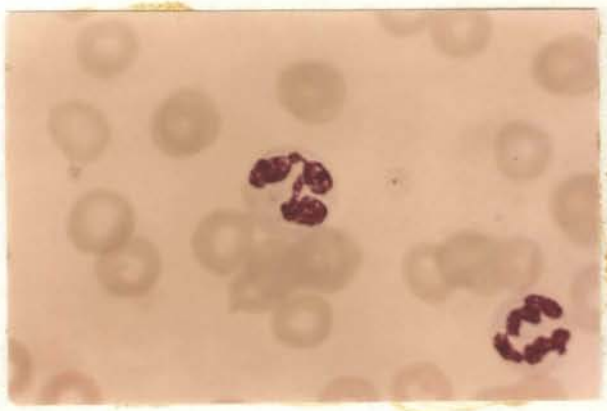

Figure 21. Neutrophil containing small slate-gray intracytoplasmic inclusion.

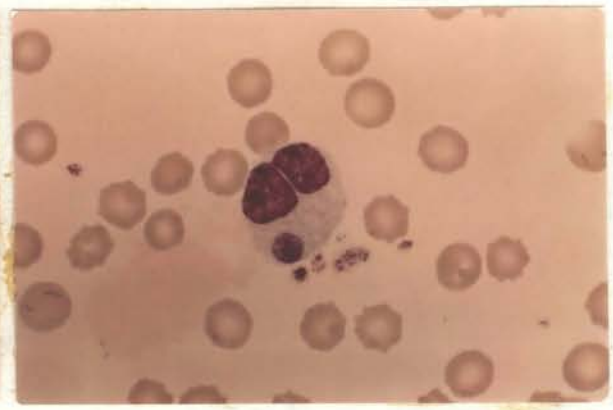

Figure 22. Monocyte containing mulberry-shaped inclusion characteristic of those usually seen in lymphocytes.

they described could not be verified. It seems that Donatien and Lestoquard perhaps were observing rickettsial inclusions in some cases, but it is suggested that they were often observing intracytoplasmic granules which occasionally occur in normal leukocytes of dogs. The small, slate-gray intracytoplasmic granules of neutrophils (Figure 21) observed in this study are of unknown significance. It can be said only, that they were observed consistently in animals in which the mulberry-shaped bodies, which are obviously rickettsiae, were found in lymphocytes; as already indicated mulberry-shaped bodies were found only rarely in neutrophils. From these data the conclusion is obvious that they are seen consistently in animals with rickettsiosis, but there is no way to conclude whether or not they represent rickettsial material. Neither is it possible to determine whether or not they result from rickettsial infections, but their absence from litter-mate control animals is strongly suggestive that they do.

From the data collected it has been determined that cells of the agranulocytic series of dogs experimentally infected with Ehrlichia may harbor intracytoplasmic inclusions of more than one kind. Those in lymphocytes are more apt than those in monocytes to be of the basophilic morula type, i.e., the type which is considered characteristic of Ehrlichia sp. Monocytes were sometimes observed to harbor morulae (Figure 22) but not as commonly as were lymphocytes (Figures 19 and 20), and the inclusions which were most characteristically found in monocytes (Figure 23) were acidophilic and not made up of an aggregate of smaller units. These latter monocytic inclusions possibly could be comparable to the so-called initial bodies mentioned by Donatien and Lestoquard, but there is no evidence that they

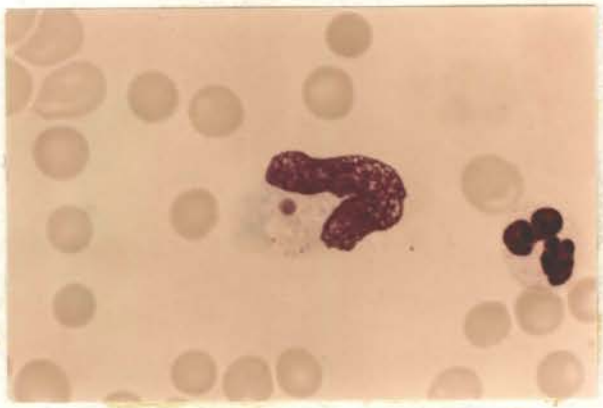

Figure 23. Monocyte containing acidophilic inclusion, perhaps comparable to so-called "initial bodies" described by Donatien and Lestoquard (1940).

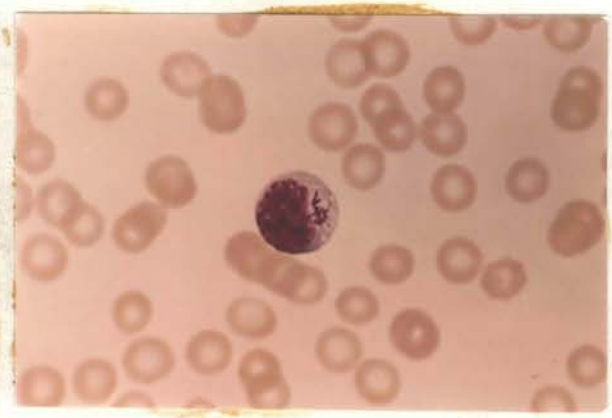

Figure 24. Leukocyte containing mulberry-shaped inclusion which is beginning to dissociate.

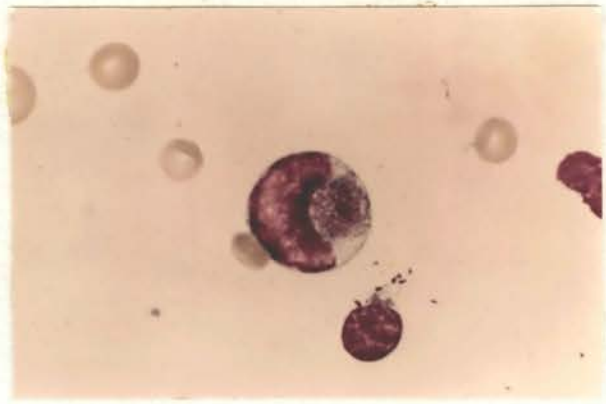

Figure 25. Leukocyte containing mulberry-shaped inclusion which is beginning to dissociate; note large size of aggregate in comparison with Figure 24.

arise from elementary bodies which, according to these workers, are themselves formed by the dissociation of morulae. There is ample evidence that morulae do dissociate within the cytoplasm of the host cell (Figures 24 and 25), but there is no reason to support the so-called life cycle as described by Donatien and Lestoquard. Admittedly their proposed cycle is, in theory, an attractive arrangement, and it does account for three different kinds of leukocytic inclusions observed in Ehrlichia-infected dogs. Nevertheless no sequential arrangement of these inclusions with attendant nominal designations for each seems justified. In order to establish a life cycle for the organism it would be necessary to demonstrate more conclusively that the various inclusions represent a succession of forms. It is not claimed that Donatien and Lestoquard are in error in their conclusions, but it is true that their evidence is not convincing. In fairness to these workers, it must be mentioned that much of the life cycle as they described it was based upon observations made on smears prepared from blood taken from vessels of the pia mater rather than from peripheral blood as in the present study. The rather considerable mass of observations made in the present study show that no orderly and predictable sequential occurrence of inclusions can be determined from peripheral blood smears. Interestingly enough they stated at the conclusion of remarks on the cycle in one dog:

> All the examinations mentioned above were made on smears of peripheral blood. We were able to follow the whole evolutionary cycle of R. canis.

In addition, the granulocytic series of leukocytes is not mentioned by Donatien and Lestoquard as harboring inclusions. In the present study, small slate-gray, intracytoplasmic inclusions were consistently observed in the neutrophils of dogs infected with Ehrlichia, usually before morulae appeared in the agranulocytic series and continuing thereafter for varying periods up to two years. As indicated earlier, these inclusions are of unknown significance, but it cannot be overlooked that they occurred consistently in dogs with ehrlichiosis and were not seen in control dogs. Finally, neutrophils were sometimes observed to harbor the morula-type inclusion characteristically found in lymphocytes, but these were rarely seen other than near the terminus of fatal infections.

Incubation Period and Persistence in the Dog

A discussion of the incubation period of Ehrlichia canis must be based almost completely upon experimental inoculation of the dog because work with tick-inoculated animals has been minimal. Donatien and Lestoquard (1937) stated that the incubation period varies from six to twelve days in natural cases and from three to fifteen days in experimental cases. These workers (1937) however, made such confusing and inconclusive statements as: "... these animals were running a temperature a few days after having been exposed to infection by ticks." and "... /the dog/ shows after a few days a thermic rise..." It is therefore difficult to determine just what the actual range of the incubation period was.

Most of the published reports are concerned with experimental inoculations in which blood from a sick dog was transfused either intravenously or by some other route into a susceptible animal. In these reports the incubation period seems to range from seven to twenty-one days.

Donatien and Lestoquard (1936) indicated that R. canis (E. canis) can persist in an animal "... long after the clinical recovery from an acute natural or experimental attack (at least five months) ...." Previously, Donatien and Lestoquard (1935) claimed that "we were thus able to establish that the virus /sic/ persists long after recovery in the monkey and in the dog." In the same report it was indicated that premunition occurs and that splenectomy causes a relapse and reappearance of the organism in the peripheral blood. Furthermore, it was stated that:

> The seriousness of this disease is brought out in the following figures: Out of 15 dogs affected by the natural disease 14 died; and out of 6 dogs inoculated in the laboratory 4 died. It is therefore difficult to look for the nature of the protection conferred by a first attack.

Finally, however, they concluded "... that an attack of first invasion and the chronic infection following it confer to the recovered animals premunition and not immunity."

In presenting the data in this study the incubation period of Ehrlichia can be judged by the occurrence of a febrile response or by the observation of intracytoplasmic inclusions in leukocytes. It is perhaps presumptuous cite the neutrophilic inclusions described previously (Figure 21) as evidence of ehrlichiosis. However, since these inclusions were consistently found in ill dogs and were never seen in the controls, the author feels justified in using them as an indication of infection. This is done with full awareness that the true nature of these inclusions is unknown. Appearance of lymphocytic inclusions of the morula type (Figures 19, 20 and 22) will be cited as unequivocal evidence of infection. Sixteen dogs (E-09, E-010, E-011, E-9,

E-12, E-15, E-17, E-18, E-24, E-25, E-26, E-27, E-28, E-30, E-33, and E-35) were experimentally infected with Ehrlichia in the absence of Babesia canis and will be discussed. An additional twenty-three dogs which had concomitant infections of B. canis will be discussed in another section.

The pattern of the febrile response which developed in these dogs was remarkably consistent. The day on which the body temperature reached or exceeded 104°F. has been chosen as a point for comparing the dogs. This does not indicate that elevated temperatures below this level are considered insignificant nor does it mean that temperatures did not exceed this figure. It is chosen simply as a figure which was rather consistently reached or exceeded at a time when the dogs were obviously ill. If twelve of the sixteen dogs are considered (omitting E-26, E-27, E-33, and E-35), a temperature of 104°F. or above was reached, on the average, 11.33 days after exposure, with a range of nine (E-25) to fourteen days (E-17). It was considered best to omit the four dogs because of various extraneous complications. E-26 is omitted because it had distemper at the time of exposure to Ehrlichia, and three days post exposure the temperature reached 104°F. but then declined; on the eleventh day the temperature rose to 105.1°F., and it is considered likely that Ehrlichia was involved in this rise. E-27 is omitted because it had an extremely heavy infection of Ancylostoma caninum and was anemic when exposed to Ehrlichia; death occurred on the fifth day after exposure. E-33 is not included because it was a suckling puppy, and daily temperatures were not recorded. E-35 probably could be included except that the dog had a temperature on the second day after exposure, probably due to circumstances attendant to its inoculation, viz., the blood injected accidentally contained

enough succinyl choline to cause respiratory arrest. The dog was revived by applying artificial respiration. After this rise on the second day, the temperature returned to normal, until ten days after exposure when it rose again to 104°F.

The pattern of appearance of intracytoplasmic inclusions in neutrophils in the twelve experimental dogs was not quite as consistent as the febrile response. These inclusions first appeared from eight days post exposure (E-09 and E-010) to sixteen days post exposure (E-17); the average was 12.16 days. Lymphocytes harboring morulae which were unmistakably rickettsial in nature first appeared in the twelve dogs from nine days post exposure (E-30, a young pup) to twenty-seven days post exposure (E-011); the average was 20.08 days.

From these data it can be concluded that dogs, apparently healthy, exposed to Ehrlichia may be expected to show a febrile response with the temperature reaching 104°F. or above, and that on the average this will occur in about eleven days; the range was from nine to fourteen days. Responses in dogs which are infected with distemper and/or with Ancylostoma caninum may deviate from this pattern. Intracytoplasmic inclusions which are characteristically seen in leukocytes of dogs with ehrlichiosis are not as consistent in the time of appearance, but a reasonably consistent pattern was seen.

The sequential occurrence of events in typical, uncomplicated cases of ehrlichiosis is worth noting. On the average, the body temperature becomes elevated to 104°F. or above, 11.33 days after exposure or just prior to the appearance of neutrophilic inclusions 12.16 days post exposure.

Approximately a week later, i.e., about twenty days after exposure, lymphocytic inclusions appear which are definitely identifiable as Ehrlichia.

Persistence of infection in the dog, as judged by infectivity of the blood and by occasional presence of leukocytic inclusions was extended for twenty-four months when B. canis was concomitantly present (for the first several months of infection, only, after which the protozoan disappeared). The longest period of time that a dog with a pure infection of Ehrlichia has been observed is nine months (E-12); it died of an artifically induced Neorickettsia helminthoeca infection. Another dog (E-010) harbored Ehrlichia for seven months before it was euthanitized. Thus it is not possible from these studies to conclude what the maximum period of persistence of Ehrlichia is in the dog. However, it is shown that dogs which have been infected have not become free of the parasite during the period of observation, maximum of twenty-four months. The claim made by Donatien and Lestoquard (1937) and Bool (1959) that premunition develops in infected animals cannot be confirmed nor denied. Attempts were made to produce "super infections" (e.g., E-12 was challenged with blood from E-010 a month after initial exposure), but neither febrile nor other responses occurred.

Serology

Lawrence (1938) reported a study in which serum collected from a dog infected with Rickettsia canis (Ehrlichia canis) was subjected to Weil-Felix agglutination tests and the results compared with tests of serum from four normal animals. Serum from the rickettsia case "... gave a very strong reaction (1: 1,250) with Proteus OX 2 while the other sera produced agglutination only in a dilution of 1: 25 in three cases, and in 1: 50 in the fourth,

using Proteus OX 19, OX 2 and OX K." Malbrant (1939) also reported positive agglutination results of sera from R. canis infected dogs with a strain of Proteus vulgaris. This is strong evidence for believing that the organism is truly a rickettsia and shows a cross agglutination with heterologous antigen. Bool (1959), however, did not get reactions between Proteus OX 2, OX K, or OX 19 and serum collected from each of three dogs, in various stages of infection, viz., three, five, and eight weeks after exposure.

Babudieri (1940) reported results of Weil-Felix tests conducted on the sera of 119 dogs in Italy using Proteus OX 19 and Proteus X K /sic/. Results were positive in a high percentage of cases, particularly with X K strain. Sforza (1947) found that sera from Eritrea dogs which were parasitized by Rhipicephalus sanguineus carried typhus of man. Sforza and Solinas (1947) found 117 dogs from Asmara that had titres of 1: 40 or over to Proteus OX 19, and 105 had titers of 1: 40 or over with Proteus OX 2. They concluded that the dogs were carriers of typhus.

In the present study, sera from dogs in two stages of the disease, i.e., febrile stage and recovered or convalescent stage, were subjected to Weil-Felix reaction and to complement fixation tests using various rickettsial antigens. No significant titers were obtained as shown by data in Tables XI and XII.

The serological findings presented in Table XI were unexpected in view of the positive reports by Lawrence (1938) and Malbrant (1939). It is not possible to determine of course whether the Ehrlichia sp. isolated from Oklahoma dogs is identical with E. canis studied in the Old World. The serological evidence would suggest that they are not identical. The present findings are similar to those of Bool (1959) who was dealing with

TABLE XI

WEIL-FELIX REACTION RESULTS: ANIMALS IN SUCCESSIVE STAGES OF *EHRLICHIA*, *BABESIA*, OR CONCOMITANT INFECTIONS

Dog	History	Antigen	Titer 4	8	16	32	64	128	256
E-12	Exposed to *Ehrlichia* 60 and 90 days prior to date serum collected. "Morulae" observed intermittently in peripheral blood.	*Proteus* OX 2	0	0	0	0	0	0	0
		Proteus OX 19	0	0	0	0	0	0	0
		Proteus OX K	0	0	0	0	0	0	0
E-17	Exposed to *Ehrlichia* 30 days prior to date serum collected.	*Proteus* OX 2	0	0	0	0	0	0	0
		Proteus OX 19	0	0	0	0	0	0	0
		Proteus OX K	0	0	0	0	0	0	0
E-23	Exposed to Arizona strain of *Babesia canis* 30 days prior to date serum collected.	*Proteus* OX 2	0	0	0	0	0	0	0
		Proteus OX 19	0	0	0	0	0	0	0
		Proteus OX K	0	0	0	0	0	0	0
E-01	Exposed to *Ehrlichia* and *Babesia canis* approximately 17 months prior to date serum collected.	*Proteus* OX 2	0	0	0	0	0	0	0
		Proteus OX 19	0	0	0	0	0	0	0
		Proteus OX K	0	0	0	0	0	0	0
E-16	Control animal, never exposed to either *Ehrlichia* or *Babesia canis*.	*Proteus* OX 2	0	0	0	0	0	0	0
		Proteus OX 19	0	0	0	0	0	0	0
		Proteus OX K	0	0	0	0	0	0	0
E-28	Exposed to *Ehrlichia* approximately 65 days prior to date serum collected.	*Proteus* OX 2	0	0	0	0	0	0	0
		Proteus OX 19	0	0	0	0	0	0	0
		Proteus OX K	0	0	0	0	0	0	0
E-30	Exposed to *Ehrlichia* 12 days prior to date serum collected. "Morula stage" in blood stream at time serum collected.	*Proteus* OX 2	0	0	0	0	0	0	0
		Proteus OX 19	0	0	0	0	0	0	0
		Proteus OX K	0	0	0	0	0	0	0

TABLE XII

COMPLEMENT FIXATION TEST RESULTS: SERA OF ANIMALS IN SUCCESSIVE STAGES OF *EHRLICHIA*, *BABESIA*, OR CONCOMITANT INFECTIONS

Dog	History	Antigen	Titer 4	8	16	32	64	128	256
E-28	Exposed to *Ehrlichia* and suffered typical infection. Serum collected 76 days post exposure. Shown to harbor organism (by subinoculation) 14 days prior to date serum collected.	Rocky Mt. Spt. Fever	Not checked	3	0	0	0	0	0
		Typhus Fever		3	0	0	0	0	0
		Q Fever, Phase II		4	2	0	0	0	0
		Q Fever, Phase I		3	0	0	0	0	0
		Colo. Tick Fever		1	0	0	0	0	0
		Psittacosis		0	0	0	0	0	0
		Influenza, Group A		3	0	0	0	0	0
		Influenza, Group B		1	0	0	0	0	0
		RMSF, 2nd Antigen		0	0	0	0	0	0
E-12	Exposed to *Ehrlichia* 60 days and 90 days prior to date serum collected. "Morulae" observed intermittently in peripheral blood.	Rocky Mt. Spt. Fever	+	+	+	+	0	0	0
		Murine Typhus	0	0	0	0	0	0	0
		Q Fever	0	+	+	+	0	0	0
E-17	Exposed to *Ehrlichia* 30 days prior to date serum collected.	Rocky Mt. Spt. Fever	0	0	0	0	0	0	0
		Murine Typhus	0	0	0	0	0	0	0
		Q Fever	0	+	+	0	0	0	0
E-23	Exposed to Arizona strain of *Babesia canis* 30 days prior to date serum collected.	Rocky Mt. Spt. Fever	0	0	0	0	0	0	0
		Murine Typhus	0	0	0	0	0	0	0
		Q Fever	+	0	0	0	0	0	0
E-01	Exposed to *Ehrlichia* and *Babesia canis* 18 months prior to date serum collected.	Rocky Mt. Spt. Fever	+	0	0	0	0	0	0
		Murine Typhus	+	0	0	0	0	0	0
		Q Fever	+	0	0	0	0	0	0
E-16	Control animal, never exposed to either agent.	Rocky Mt. Spt. Fever	+	0	0	0	0	0	0
		Murine Typhus	0	0	0	0	0	0	0
		Q Fever	+	0	0	0	0	0	0

a New World isolate, but as he says his work was "... too limited to attach great value to its negative result."

Data presented in Table XII show that the Ehrlichia sp. isolated here either evokes no immune response or one not detectable by the complement fixation test with the wide variety of antigens used. There were reactions at low dilutions with a few antigens, but as Lackman (1964) has said, this "... is not unusual for dog sera."

Sera were also collected and examined against antigens prepared from the lymph nodes of dogs suffering from so-called Elokomin fluke fever, a disease which has not been described in the literature but which is said to occur in dogs infected with the fluke, Troglotrema salmincola, in the Pacific Northwest. Doctor Keith Farrell of Washington State University conducted these examinations and found no significant reactions.

It appears that currently available antigens are of no value in detecting infections of the organism under study; a logical approach would be to prepare an antigen from this specific agent and determine if predictable reactions could be obtained using sera from infected animals. Bool (1959) apparently attempted to do this for he said: "An effort to prepare an antigen for the C.F. test, from the lung of a dog killed in the acute stage of the disease had no satisfactory result." No details of technique were given, and it is difficult to assess its value.

IV. MANIFESTATIONS OF BABESIOSIS, RICKETTSIOSIS, AND COMBINED INFECTIONS IN THE DOG, INCLUDING HEMATOLOGICAL CRITERIA FOR DIFFERENTIATION

In order to have a realistic approach to the control of disease organisms it is necessary to be able to recognize the manifestations of disease in the host. One of the most baffling problems faced by a practicing veterinarian is arriving at an accurate diagnosis of infectious disease conditions because information which delineates them is often limited or non-existent. Treatment and control mechanisms can not be outlined for infectious diseases without first achieving an appreciation of the biology of the parasitic organism responsible for the detrimental effects in the host. The complications which arise in accomplishing this are multiple, but one of the most important is that a syndrome which is regarded as a specific infectious disease is frequently discovered to be caused by more than one etiological agent. It is necessary then, that specific manifestations of disease be recognized and, insofar as possible, differentiated and identified as to etiological agents involved in causation.

In this dissertation it has been shown already by literature citations that Babesia canis and Ehrlichia canis frequently occur together in the dog. It is obvious from the literature that studies which have been conducted previously on B. canis were frequently complicated by the presence of Ehrlichia or Ehrlichia-like organisms. Shirlaw (1938) described a syndrome which was unmistakably the result of a mixed infection of Babesia and Ehrlichia, and

perhaps the most convincing evidence of this is that in the histopathological description it was reported that granules were observed in well defined clusters in the cytoplasm of polyblasts and reticular cells. These clusters were undoubtedly the so-called mulberry-shaped bodies, i.e., clusters of rickettsiae.

Donatien and Lestoquard (1937), in discussing R. canis (E. canis), stated that they

> ... were amazed for many years by the fact that our statistics of veterinary analysis reveled to the rarity of canine piroplasmosis, in spite of the high number of samples taken on animals suspected of having this disease. From 1926 to 1935 we received 158 blood smears on which Piroplasma canis was found only eighteen times.

They indicated that natural cases of pure rickettsial infections were identified and that "... sixty cases of natural disease /were seen/ in the provinces of Alger and Constantine." In no case did they give details of how they determined when either naturally or experimentally infected dogs had uncomplicated infections, and it is, therefore, difficult to accept all phases of their work. They recognized that dogs frequently had concomitant infections of Piroplasma canis and even admitted that during experimental transmissions of rickettsiosis

> ... we happened to inoculate at the same time R. canis and Piroplasma canis, the donor animal being immune against Piroplasma canis. In this case, the piroplasmosis attack evolves either before the rickettsiosis, or at the same time. Even after stopping, by appropriate therapy, the Piroplasma canis attack, the rickettsiosis always was extremely severe. Inversely, when the acute attack of rickettsiosis evolves on a dog in a state of chronic infection by P. canis, we see, at the end of the acute attack of rickettsiosis, the latent infection of P. canis reappear by temporary suspension of the premunition. Then very severe, sometimes mortal, piroplasmosis attack breaks out.

Obviously these workers were cognizant of complicating concomitant infections, but they never stated how they determined that they actually worked with uncomplicated rickettsiosis. Bool and Sutmöller (1957) discovered Ehrlichia canis in dogs in the Netherlands Antilles and said that

> ... blood examination of dogs on Aruba (near Venezuela) suffering from a serious illness accompanied by fever, anemia, and severe emaciation ... showed that some of these animals were infected not only with one or more blood parasites (Babesia canis, Hepatozoon canis) but also with the above mentioned E. canis.

Furthermore they stated that

> Specimens of Rhipicephalus sanguineus taken from an Aruban dog were used to infect a laboratory dog in Utrecht, after which it was possible to pass the parasite along through six other dogs.

The authors made no mention of how they were able to be certain that the tick did not also transmit Babesia to the experimental dogs in Utrecht and gave no indication of their controls.

It has been emphasized that the rickettsiosis which was recognized in Oklahoma dogs was first found in association with babesiosis. Many of the dogs used in the present study were concomitantly infected with Babesia and the Ehrlichia-like organism before it was recognized that two distinct entities were involved in the disease process. In order to avoid confusion, the details of the separation of the two organisms will be given now, prior to discussion of the respective syndromes produced by these parasites separately and in combination.

The separation was accidental, and it occurred in the following manner. Experimentation on a litter of eight puppies was begun July 13, 1962. The object of the study was to determine the nature of the reproductive process of Babesia canis as reflected in the peripheral blood, and to investigate

the hematological changes occurring in infected dogs. Some pre-exposure bleedings were made for an appropriate period to determine that they were not infected, and the animals were injected with whole blood from a dog known to have Babesia and which we also recognized as harboring the leukocytic inclusions, the nature of which was puzzling at the time. Two of the puppies were retained as uninfected controls.

The six principals died at various intervals and the two controls (E-1 and E-5) were still healthy at the time all the principals had died. One of the controls (E-5) was then infected and used for a periodicity study; following exposure it was bled in the morning and at night in an attempt to determine if there was any fluctuation in the numbers of Babesia in the peripheral blood at different times of the day. The other dog (E-1) was continued as a control for this periodicity study and for subsequent other experimental work done with infected dogs. It was bled on a daily schedule just as were all experimental animals, and no parasites were ever observed until September 21, 1962, (forty-eight days after it ceased to be a control for the original experiment) at which time leukocytic inclusion bodies were found in the peripheral blood. Lymphocytic, monocytic, and neutrophilic inclusions were seen within a period of a few days. This was unexpected inasmuch as the animal had not been exposed to Babesia, and of course at that time the theory still prevailed that the white blood cell inclusions, the lymphocytic morulae at least, represented a schizogonous phase in the B. canis life cycle.

The animal was continued on a day to day bleeding schedule, and the leukocytic inclusion bodies persisted, but no Babesia trophpzoites were

found. This was done until November 2, 1962, at which time it was decided to splenectomize the animal as a method of proving the presence or absence of Babesia. Following the splenectomy bleeding was continued, on a day to day basis, until December 18, 1962. The leukocytic inclusions appeared intermittently, but no Babesia trophozoites were observed. Then the dog was challenged with Babesia with the idea that if immunity had been established by its experience with what was thought to be the schizogonous phase of the parasite, infection probably would not occur. On the other hand, if the dog were totally susceptible, typical babesiosis should develop. It was subjected to infection December 18 by the intravenous injection of 10 cc of whole blood from the same reservoir dog which had been the donor for the six principals in the original experiment that was begun July 13, 1962. Trophozoites were present in the peripheral blood the following day; there was one red blood cell with one parasite among 100,000 erythrocytes observed, the standard sample examined every day. On December 20, there were 55 red blood cells each with one trophozoite, 18 with two trophozoites, one with four trophozoites, and there was one single trophozoite free of any cell. On December 21, there were 412 red blood cells each with one trophozoite, 150 with two, one with four, a peculiar one with three, and five trophozoites free of any cell. On December 22, there were 207 red blood cells each with one parasite, 68 with two, and no others except one that was free of any cell. The parasites disappeared completely for two subsequent days, but on December 25 there was an erythrocyte with one trophozoite, the following day there was another with one trophozoite, and on December 27 a rise in numbers occurred and continued through December 29, 1962. The trophozoites declined, and on December 30 there was only one

infected cell, which contained four trophozoites. A latent or dormant period persisted until January 4, 1963, when two infected red blood cells were observed, each with one parasite.

Following this, there was a rather long period of intermittent appearances of the parasite, 2 to 6 cells being affected on any given day. This pattern changed February 10, when there was a sudden rise in the numbers of affected cells. On the following day there was a profound parasitemia with 245 red blood cells each containing one parasite, 222 containing two, 34 containing four, and a few cells containing some odd numbers of trophozoites. On February 12, an even more profound parasitemia was observed with 314 erythrocytes each containing one trophozoite, 346 containing two, 28 with four, and a few with odd numbers. The following day only eight or nine red blood cells were affected, and on February 14 the parasites had disappeared completely, and no more were observed until February 28, when there was a small number of infected cells, and this situation persisted intermittently until March 10. After March 10 there was another dormant period lasting until March 31, on which day a few parasitized erythrocytes were observed, and from April 1 to 6 a few parasites were seen daily but no profound parasitemias, such as the two sieges which have been described, were observed at this time. There were no parasites from April 7 to May 2, but on May 3 a few trophozoites reappeared in the peripheral blood, a condition which persisted for five consecutive days. Then none was found for two days, but on May 10 one red blood cell with one parasite was seen, which was the last trophozoite ever observed in this animal. Bleeding of this dog continued, and 100,000 erythrocytes were observed as always on a day to day basis, until June 12, 1963, at which time the animal was euthanitized. It

should be stated that a few leukocytic inclusions were observed intermittently throughout the entire period of infection and were seen as late as June 2 and 3, 1963.

On the basis of the details of these observations it is obvious that there were certainly two disease entities. The leukocytic inclusions actually constituted a specific parasitic entity, a rickettsia, probably Ehrlichia sp., and it was shown that they did not constitute a schizogonous phase of the Babesia canis life cycle. The dog obviously had no protection from Babesia by having been host to the other parasite. It now remained to find out something about this organism and its pathogenicity for other dogs, which of necessity required a strain of Ehrlichia not contaminated by B. canis.

On October 19, 1962, 10 cc of whole blood were taken from this same dog (E-1) and injected into a young dog, E-011. Eleven days after exposure, some neutrophilic inclusions, which resembled those seen in dogs suffering from babesiosis (in which these leukocytic inclusions consistently appeared), were seen in the peripheral blood. These persisted but occurred rather intermittently until 27 days after exposure when a perfect morula form was observed in a lymphocyte. The animal was bled on a day to day basis and the standard sample of 100,000 erythrocytes examined from the time of exposure until it died on November 29, 1962. The typical leukocytic inclusions persisted until the time of death, but no trophozoites of Babesia were ever observed. The rickettsia has been passed through dogs a number of times, and studies of these pure infections are related in a subsequent part of this section.

Only two dogs, to be discussed immediately, were infected with Babesia in the absence of Ehrlichia, and this strain of Babesia was acquired from a

different location than the one used in the studies involving dual infections. The discussion of the clinical manifestations of Babesia infections, therefore, had to be based principally on literature, with original data from only two experimentally infected dogs.

Babesia Infections

Two dogs (E-22 and E-23) of a litter of 10 were infected with a strain of Babesia canis isolated in Arizona and supplied to the author by Doctor Ned Rokey. The data in Figures 37, 42, 47, and 52 depict the hematological changes in these dogs from a pre-exposure period until the animals were infected and had recovered clinically. Figures 26 and 27 depict the degree of the parasitemia and body temperature fluctuations in the two dogs. One dog was euthanitized 17 days after exposure so that its tissues could be compared with those of dogs infected for a similar period with Ehrlichia.

It is clear from the data that Babesia was not a particularly serious parasite in these dogs. This does not imply that Babesia alone is never capable of inciting a serious disease, but the clinical picture in these animals was one of a mild infection and was unlike much of what is described in the literature concerning symptoms of Babesia. Even though anemia developed, the dogs recovered through active hemopoesis. The MCV (mean corpuscular volume) and MCH (mean corpuscular hemoglobin) values were calculated, and the results indicated that the anemia, while it persisted, was of the normocytic-normochromic type. Graphs are not included to represent these values since they would not help to clarify the presentation.

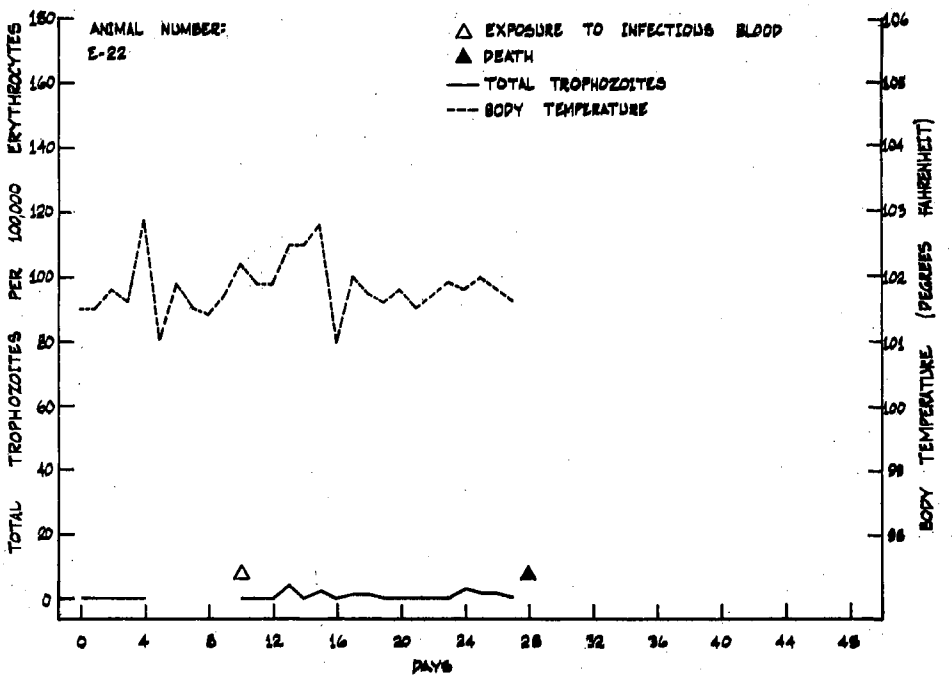

Figure 26. Parasitemia and body temperature fluctuations in E-22, infected with Babesia canis.

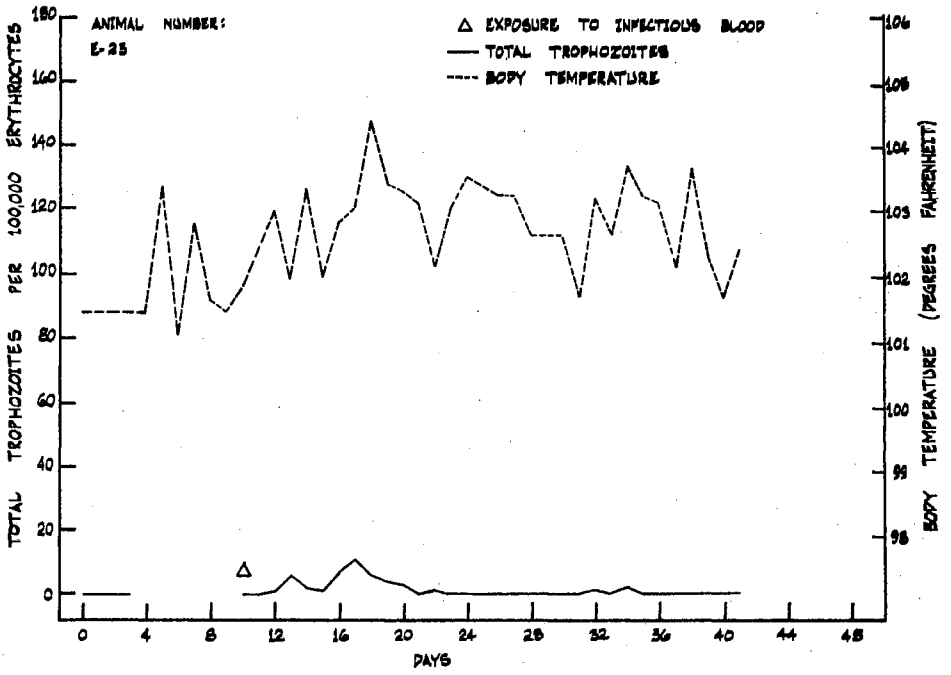

Figure 27. Parasitemia and body temperature fluctuations in E-23, infected with Babesia canis.

A point which must be considered in comparison of these dogs with others as to severity of infection is that this strain of Babesia had been isolated and passed repeatedly from dog to dog without passage through an intermediate host. In the absence of passing through an arachnid where sexual reproduction or some other process of rejuvination presumably takes place, a strain may become altered in virulence. It appears either that the two animals which were infected with Babesia alone harbored a mild strain of the organism or that Babesia alone in some instances is incapable of causing serious disease in dogs. It is not possible to conclude on the basis of present information which, if either, of these is the correct explanation, and there is no reliable information in the literature to supplement these observations.

Concurrent Babesia and Ehrlichia Infections

It has been shown previously in Africa, India and in the Netherlands Antilles (references cited elsewhere) that Babesia and an agent similar to the one which was isolated here often occur together in dogs. Originally it was not recognized that two organisms were present in the dogs involved in this study, and as emphasized earlier, the original aim of the early investigations was to determine whether the inclusions observed in the cytoplasm of leukocytes represented an undescribed schizogonous phase of the life cycle of B. canis. It is the purpose to describe in this section the clinical picture of the disease caused by the concomitant Babesia-Ehrlichia infection as seen in experimentally infected dogs.

The data presented were collected during two intervals, and the experimental dogs were from two litters. Four dogs (E-16, E-19, E-20, E-21)

were part of a large litter, the other members of which were used in the related studies discussed in the immediately preceding section (Babesia Infections) and the section immediately following (Ehrlichia Infections). The two uninfected litter-mate controls (E-16 and E-19) to which E-20 and E-21 are compared in this section are the same controls used for principals in the two sections just mentioned. The experiment involving the other litter, composed of eight dogs, was completed several months earlier and before it was recognized that two disease agents were involved. The experiment included eight dogs (E-1, E-2, E-3, E-4, E-5, E-6, E-7, E-8), two of which were uninfected controls (E-1 and E-5); the other six, as learned later, were exposed to Babesia and Ehrlichia concomitantly.

The methods employed and the nature of the data collected in the two experiments were almost identical, and therefore the results will be discussed as though the experiments were one. The only differences in the two experiments were as follows: (1) the hemoglobin determinations were made by the Spencer Hemoglobinometer in the earlier experiment and by the colorimeter in the second, (2) reticulocyte counts were made only toward the end of the first experiment but were made every day in the second, and (3) the dogs in the first experiment were about three weeks older at the time they were exposed to infectious blood than were those in the second experiment.

Figures 28 through 35 depict the detail of body temperature changes and fluctuations in degree of parasitemia in these dogs, and it is clear that a rather consistent pattern developed. From data in the figures it is shown that a few days after exposure to infectious blood the numbers of Babesia trophozoites rose to a peak and declined rapidly to a point near zero, followed by a period when there were few or no trophozoites observed

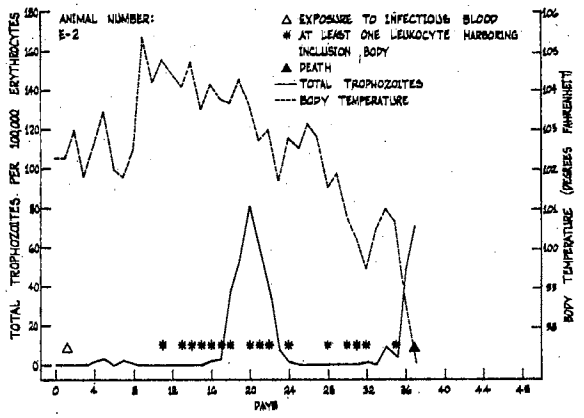

Figure 28. Parasitemia and body temperature fluctuations in E-2, infected with Babesia canis and Ehrlichia sp.

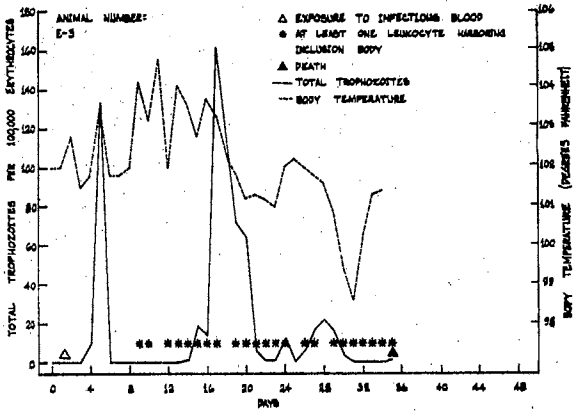

Figure 29. Parasitemia and body temperature fluctuations in E-3, infected with Babesia canis and Ehrlichia sp.

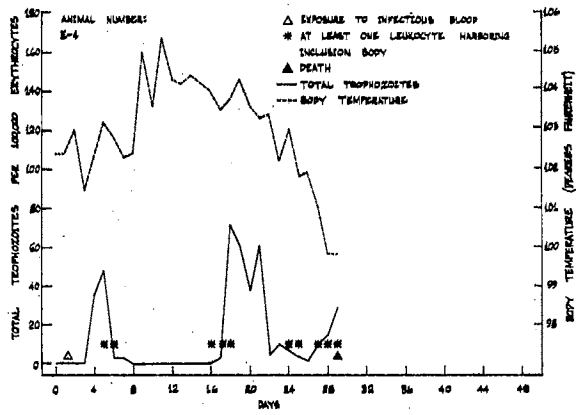

Figure 30. Parasitemia and body temperature fluctuations in E-4, infected with Babesia canis and Ehrlichia sp.

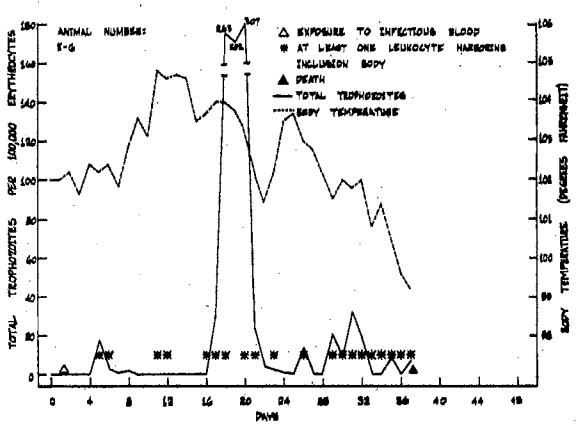

Figure 31. Parasitemia and body temperature fluctuations in E-6, infected with Babesia canis and Ehrlichia sp.

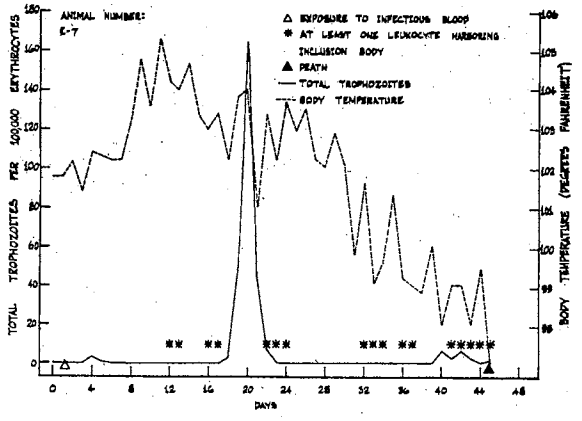

Figure 32. Parasitemia and body temperature fluctuations in E-7, infected with Babesia canis and Ehrlichia sp.

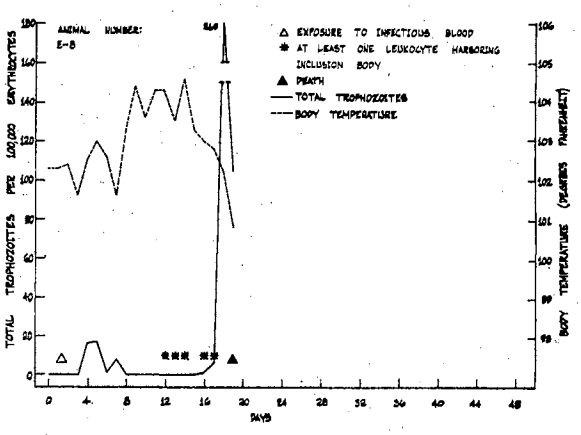

Figure 33. Parasitemia and body temperature fluctuations in E-8, infected with Babesia canis and Ehrlichia sp.

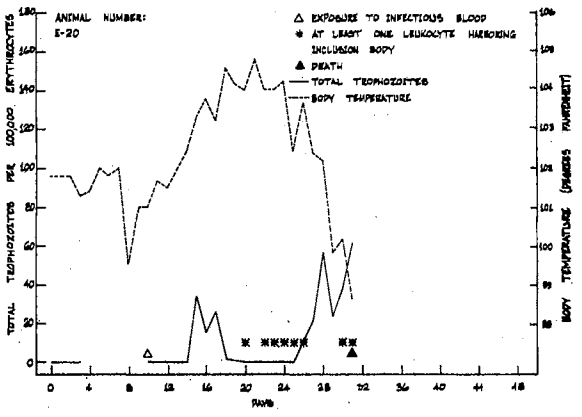

Figure 34. Parasitemia and body temperature fluctuations in E-20, infected with Babesia canis and Ehrlichia sp.

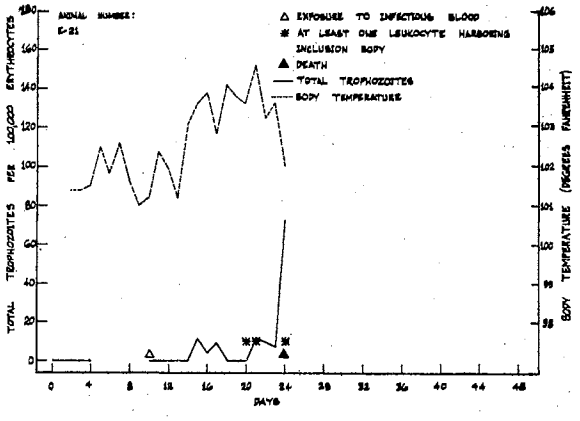

Figure 35. Parasitemia and body temperature fluctuations in E-21, infected with Babesia canis and Ehrlichia sp.

in the peripheral blood for a period of a few days. It was not until approximately two weeks after exposure that the leukocytic inclusions, at least some of which were typical of Ehrlichia, appeared in the peripheral blood for the first time. This was followed by an active reproduction on the part of Babesia canis, and the number of trophozoites in the erythrocytes in the peripheral blood reached a peak approximately two to two-and-one-half weeks post-exposure. The pattern varied by being somewhat erratic after this, but in general, in fatal cases the numbers of Babesia trophozoites rapidly declined and often disappeared before death. The numbers of Ehrlichia-infected leukocytes which could be detected in the peripheral blood varied considerably; sometimes they disappeared entirely, but in other cases appeared in increasing numbers and were quite abundant at the time of death.

The hematological changes exhibited in these dogs are reasonably consistent and can be ascertained from Figures 36, 39, 40, 41, 44, 45, 46, 49, 50, and 51.

When the principals are compared with their uninfected litter-mate controls, the total erythrocytes as well as the hemoglobin and hematocrit values reveal a striking anemia which developed soon after inoculation with infectious blood and persisted and increased in intensity until death of the dogs. The common indices, MCV and MCH, mentioned earlier revealed the anemia to be of the normocytic-normochromic type. The reticulocyte count remained within normal range indicating that the hemopoietic capabilities of the host were impaired. (In E-21 it appears that some hemopoietic response occurred just before death.) This contrasts sharply with the two litter mates infected with Babesia alone, but it resembled the response in the Ehrlichia-infected litter mates rather closely. As indicated

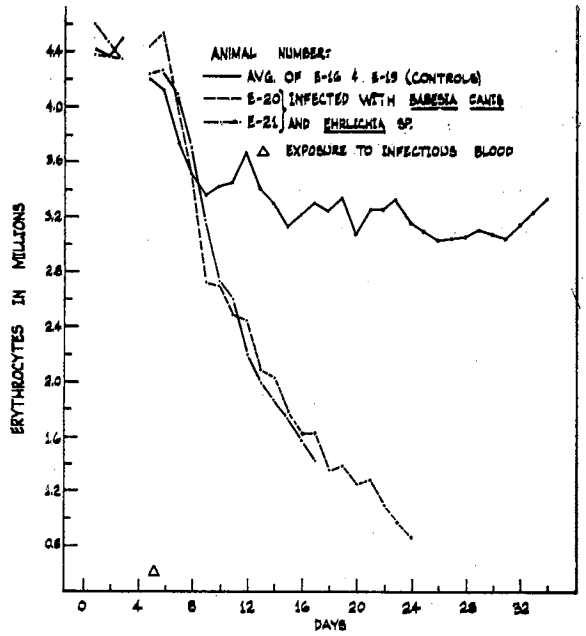

Figure 36. Total erythrocyte counts of E-20 and E-21, compared with the average of two litter-mate controls.

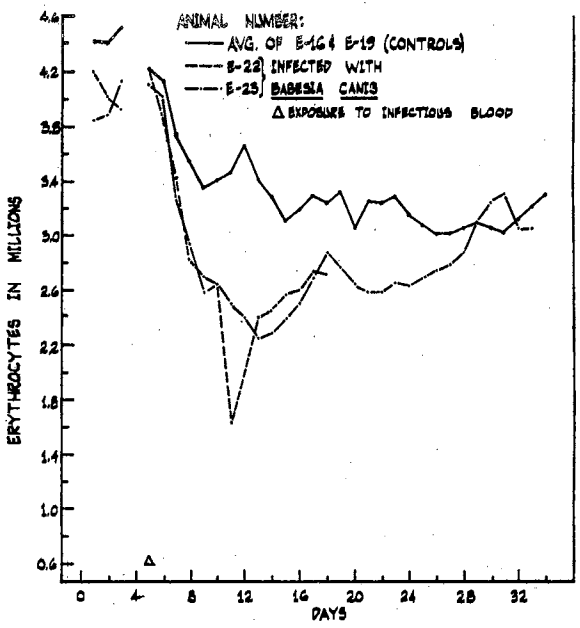

Figure 37. Total erythrocyte counts of E-22 and E-23, compared with the average of two litter-mate controls.

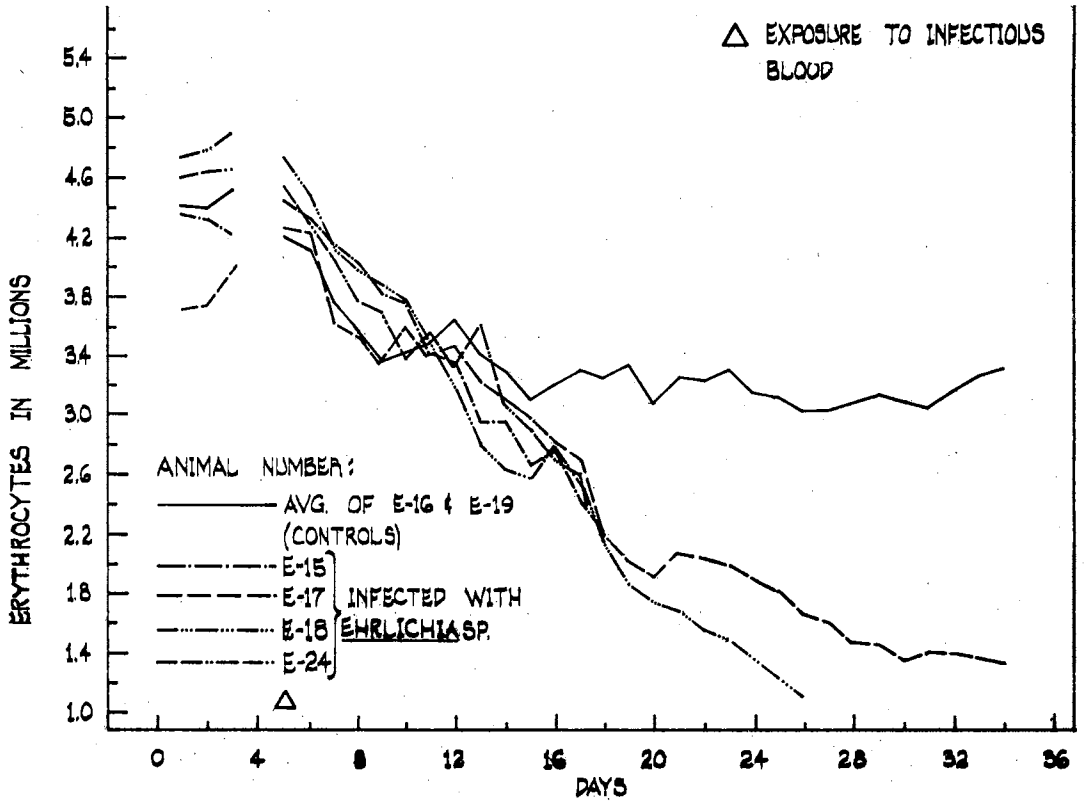

Figure 38. Total erythrocyte counts of E-15, E-17, E-18, and E-24, compared with the average of two litter-mate controls.

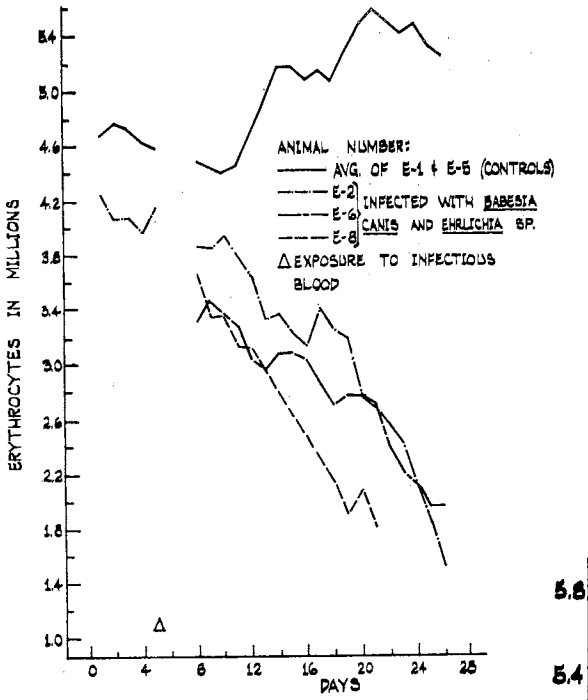

Figure 39. Total erythrocyte counts of E-2, E-6, and E-8, compared with the average of two litter-mate controls.

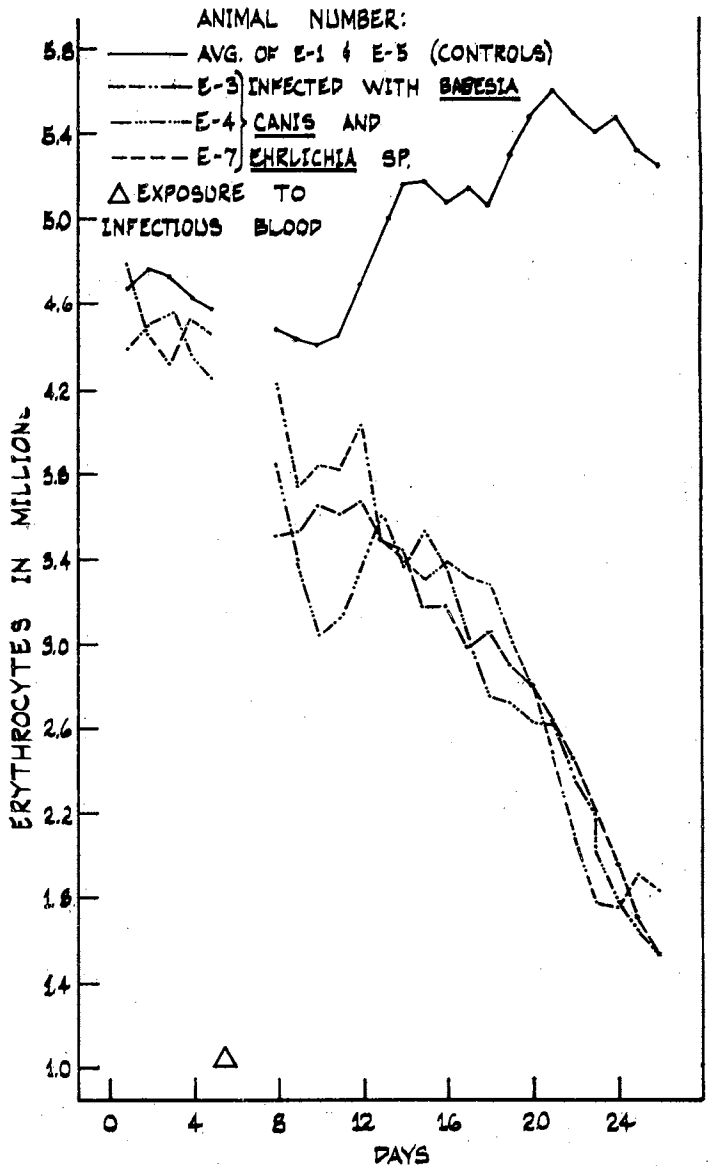

Figure 40. Total erythrocyte counts of E-3, E-4, and E-7, compared with the average of two litter-mate controls.

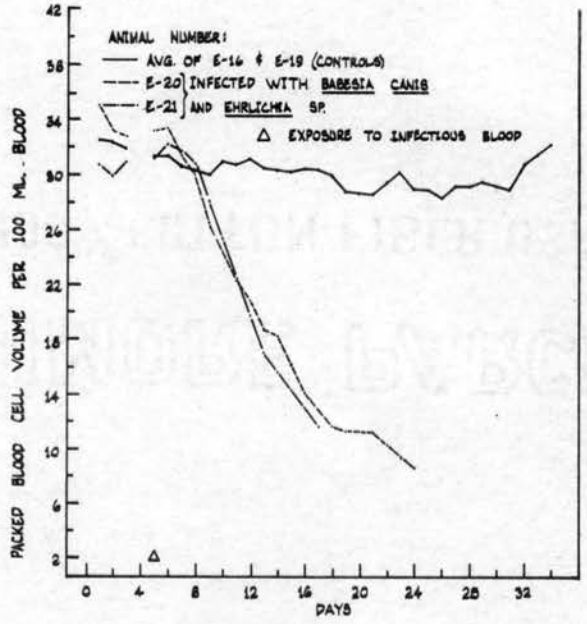

Figure 41. Packed blood cell volume of E-20 and E-21, compared with the average of two litter-mate controls.

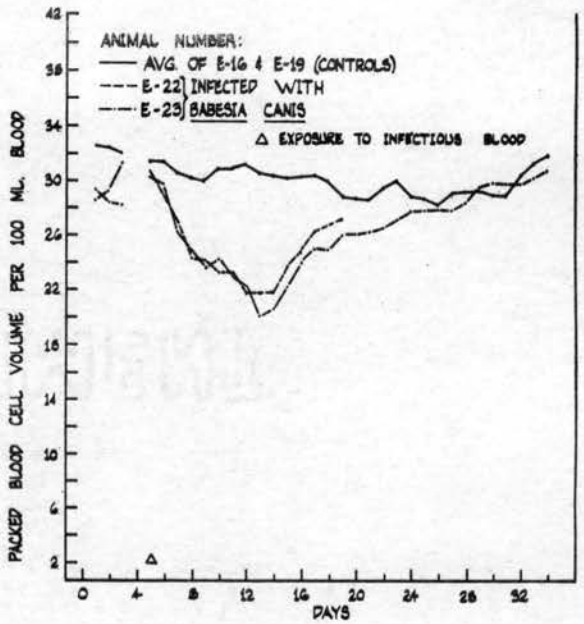

Figure 42. Packed blood cell volume of E-22 and E-23, compared with the average of two litter-mate controls.

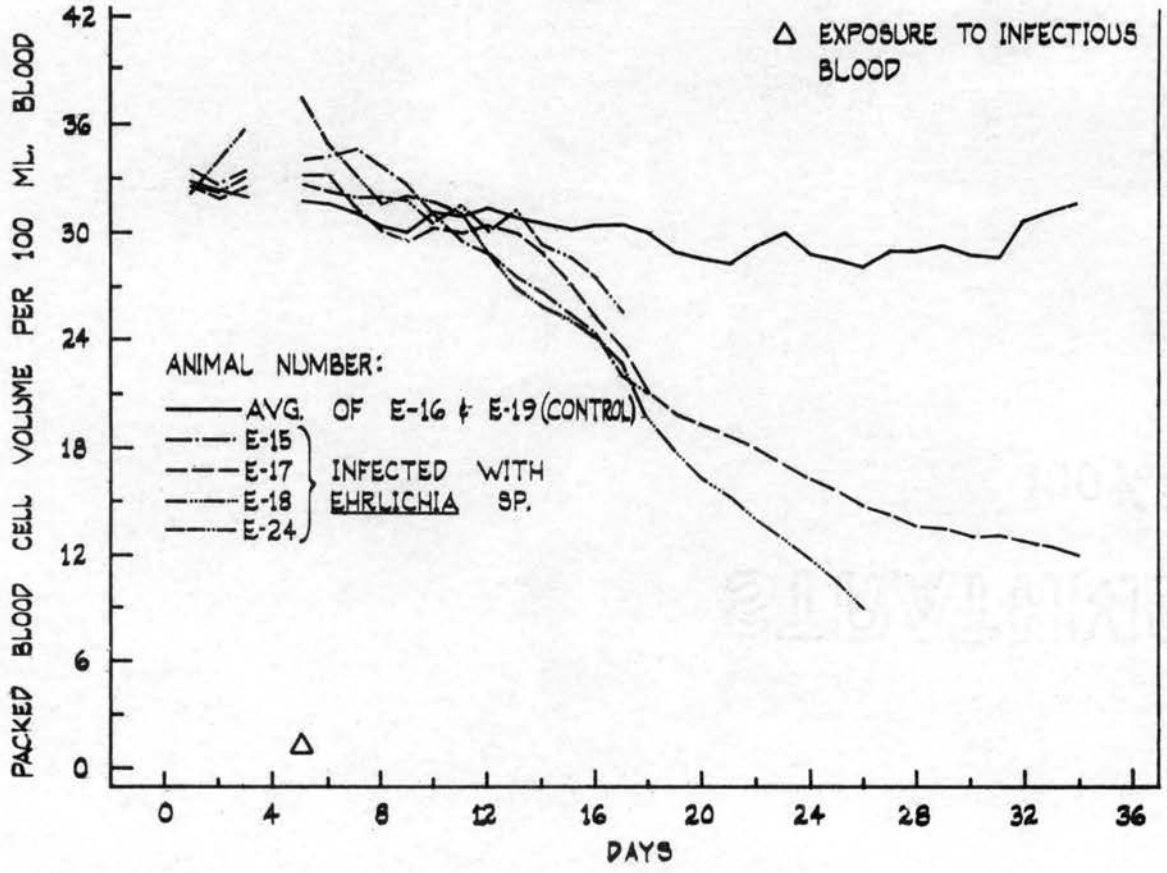

Figure 43. Packed blood cell volume of E-15, E-17, E-18, and E-24, compared with the average of two litter-mate controls.

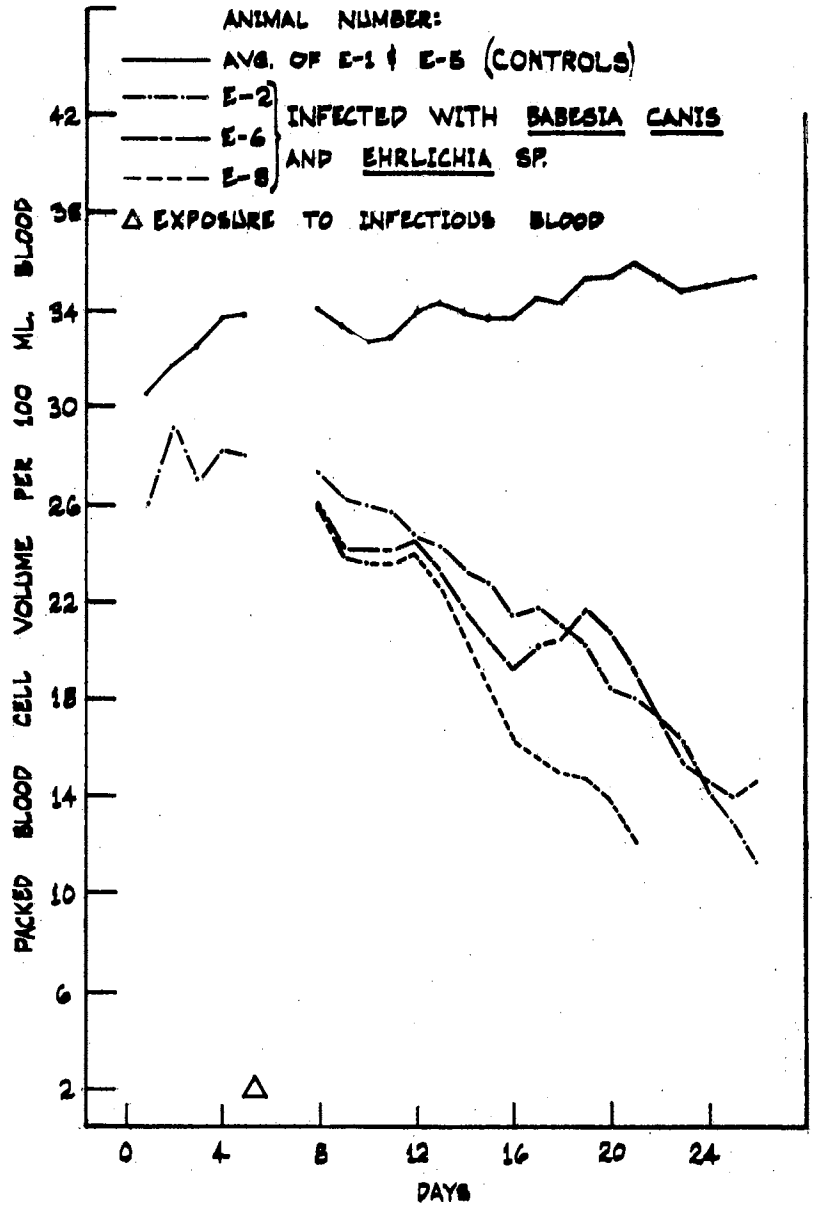

Figure 44. Packed blood cell volume of E-2, E-6, and E-8, compared with the average of two litter-mate controls.

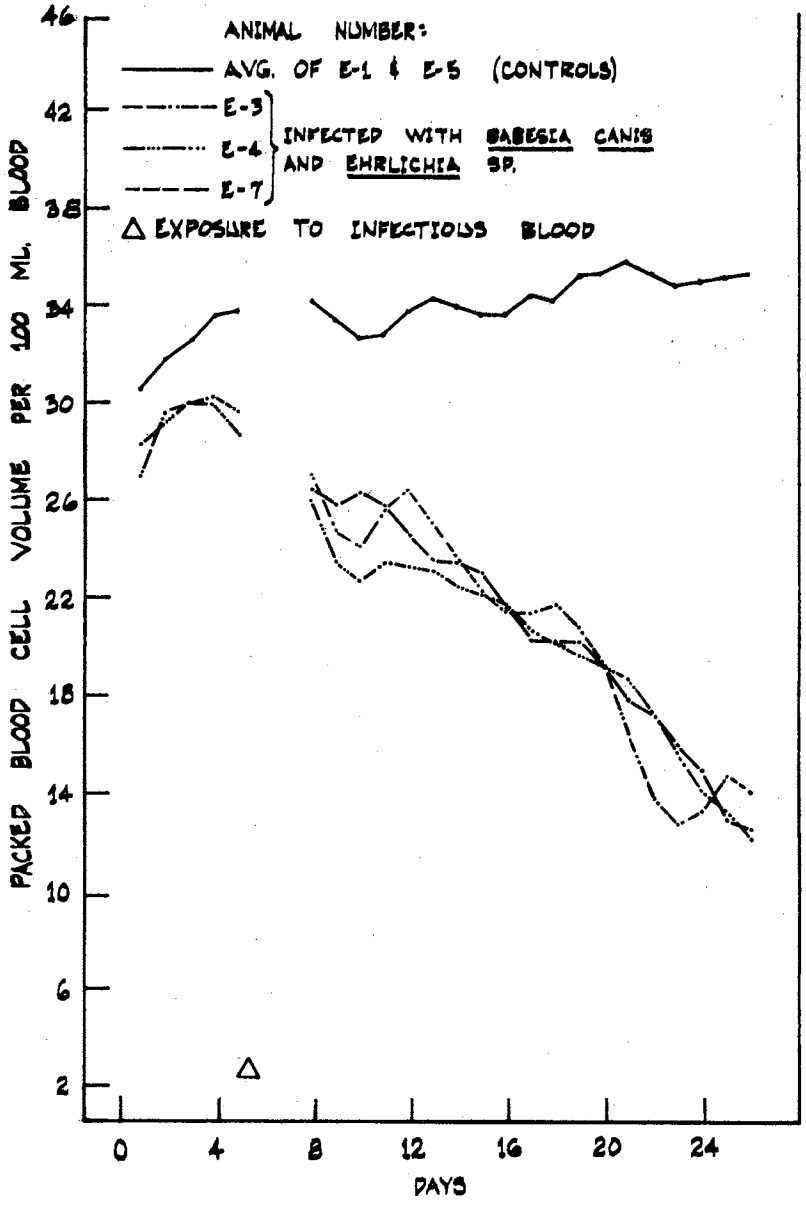

Figure 45. Packed blood cell volume of E-3, E-4, and E-7, compared with the average of two litter-mate controls.

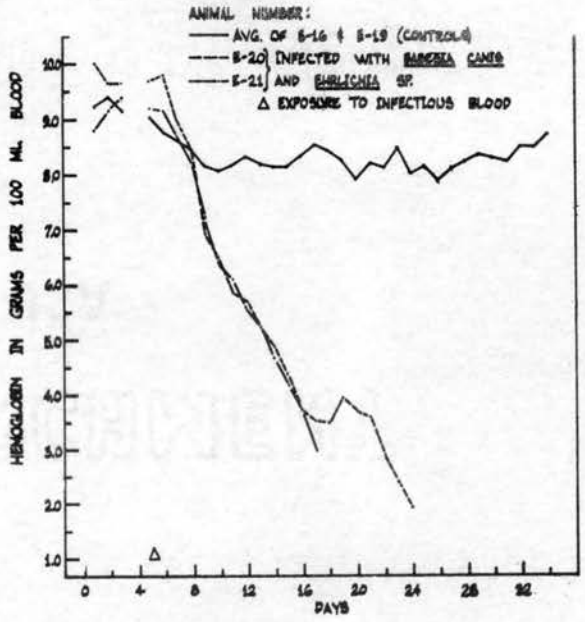

Figure 46. Hemoglobin values of E-20 and E-21, compared with the average of two litter-mate controls.

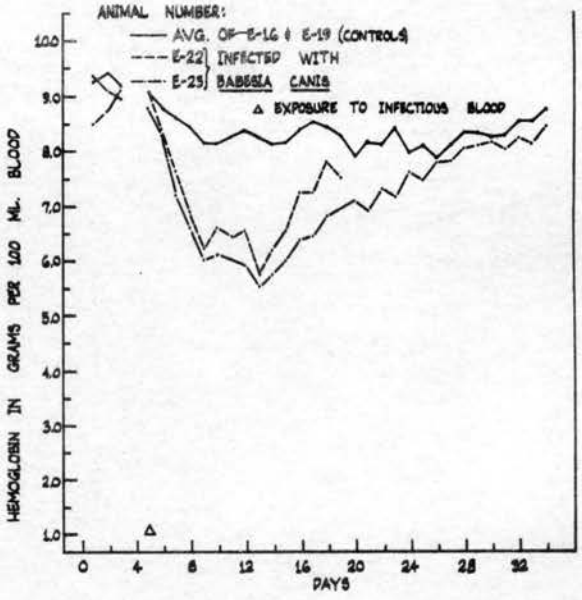

Figure 47. Hemoglobin values of E-22 and E-23, compared with the average of two litter-mate controls.

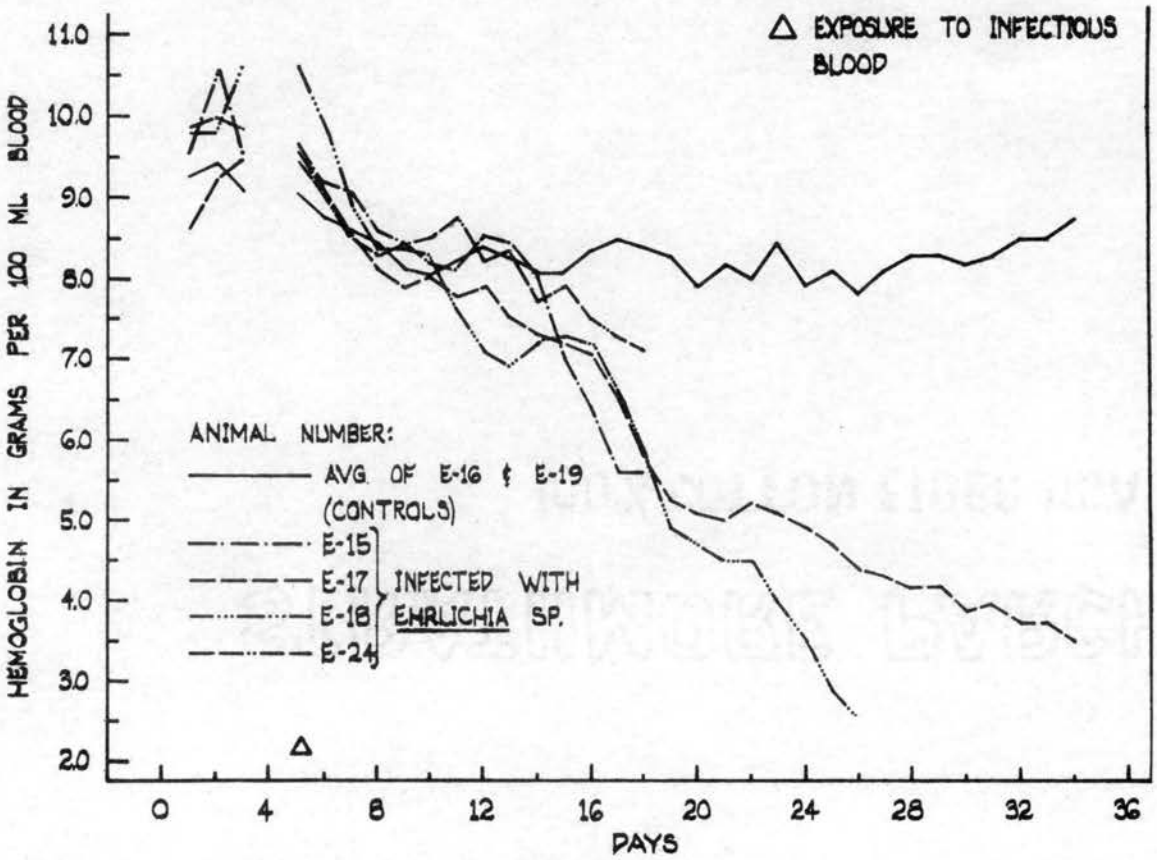

Figure 48. Hemoglobin values of E-15, E-17, E-18, and E-24, compared with the average of two litter-mate controls.

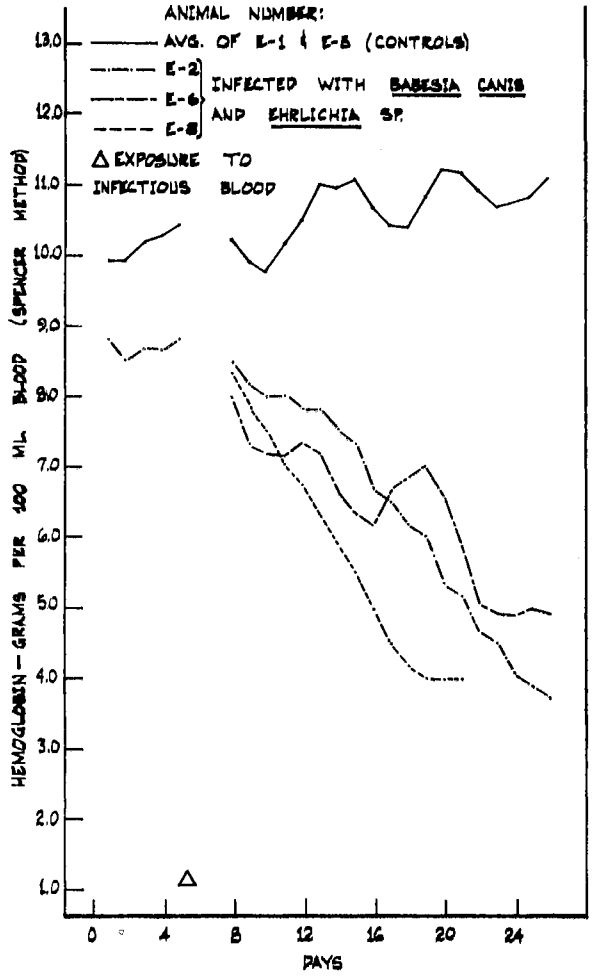

Figure 49. Hemoglobin values of E-2, E-6, and E-8, compared with the average of two litter-mate controls.

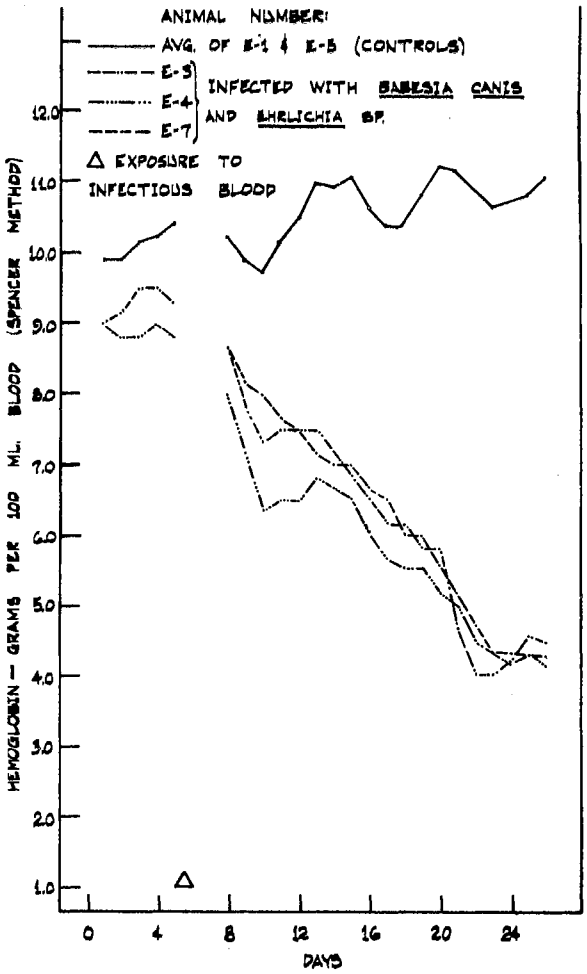

Figure 50. Hemoglobin values of E-3, E-4, and E-7, compared with the average of two litter-mate controls.

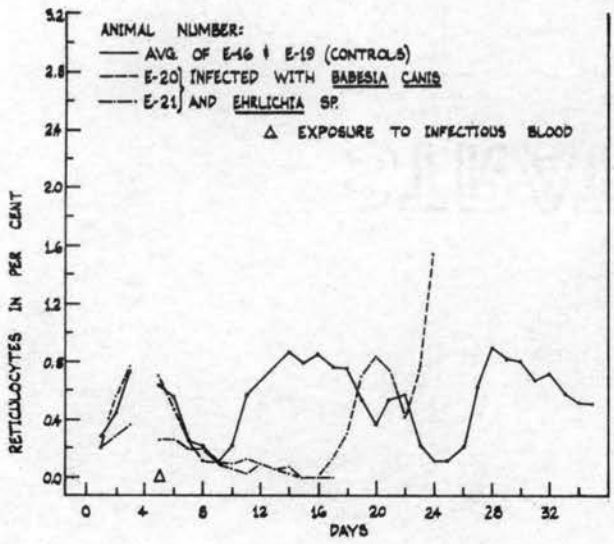

Figure 51. Reticulocyte count of E-20 and E-21, compared with the average of two litter-mate controls.

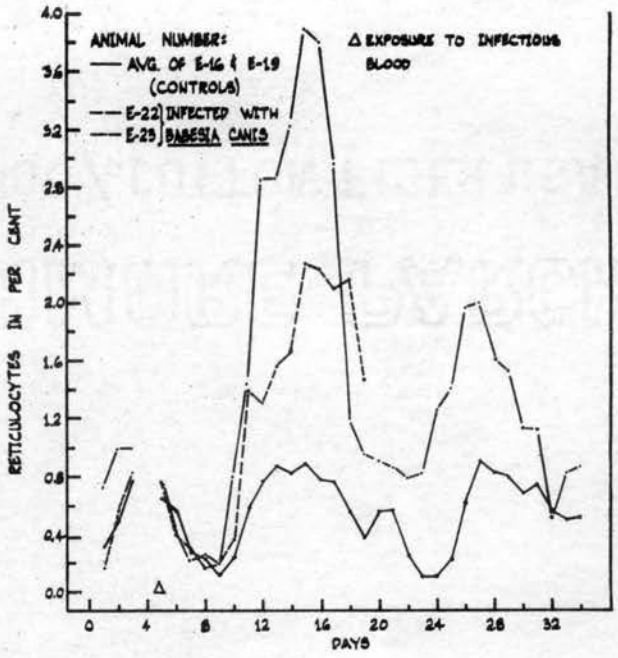

Figure 52. Reticulocyte count of E-22 and E-23, compared with the average of two litter-mate controls.

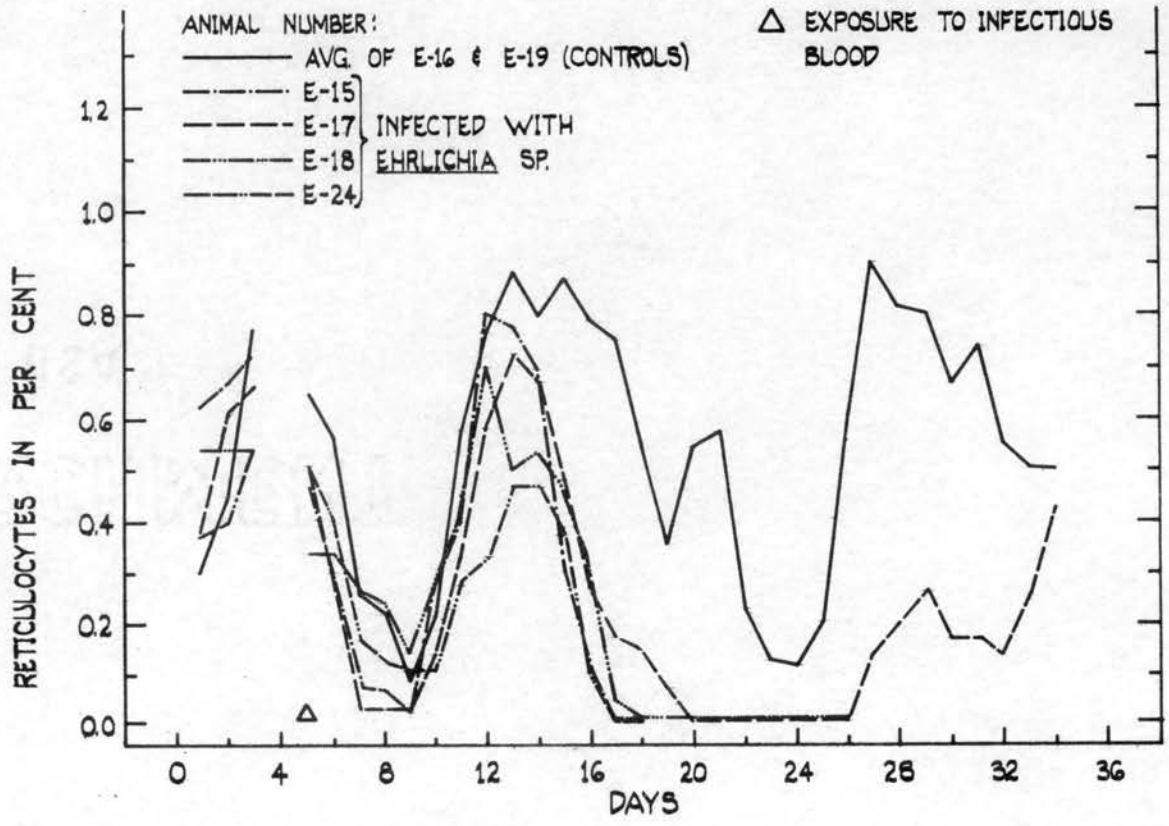

Figure 53. Reticulocyte count of E-15, E-17, E-18, and E-24, compared with the average of two litter-mate controls.

earlier, daily reticulocyte counts were not made throughout the entire observation period on the eight dogs in the first study. Counts were made, however, for nine consecutive days beginning fourteen days after exposure, and these data reveal the same general picture seen in the second experiment, viz., that of an impaired hemopoietic system. There was some evidence of hemopoiesis just prior to death of the dogs, and this parallels the situation in E-21 mentioned above.

From the clinical standpoint there were no pathognomonic signs. In fact the animals appeared, in many cases, to be suffering from a very common disease in dogs, viz., distemper. The most consistent signs were a bilateral, muco-purulent, ocular discharge accompanied by photophobia, and a nasal discharge which was at first serous and later purulent. (None of the dogs being discussed survived the infection, but some older dogs, discussed in an earlier chapter, did survive and in these animals, apparently recovered completely, the nose sometimes remained dry and crusty. See Figure 54.) In a few cases (E-3, E-4, E-6, and E-20) a transient papular dermatitis was observed on the abdomen during the early part of the febrile period. Icterus was occasionally but not consistently observed. Vomiting often followed feeding during the height of the febrile period.

The complete histopathological changes in these animals have not been determined and are not included in this dissertation.

On the basis of the data presented, it is obvious that in young animals suffering from a concomitant infection involving babesiosis and the newly isolated rickettsial infection, a grave illness, often fatal, developed.

Figure 54. Appearance of nose of E-01 after recovery from infection with Babesia canis and Ehrlichia sp. Note dry incrustation.

Death was expected and occurred from nineteen to forty-five days post-exposure, with the average day of death being thirty-three and one-half days post-exposure. Among dogs in the first experiment, the one which died in nineteen days was the smallest of the litter. The two dogs in the second experiment died in fourteen and twenty-one days, respectively, which suggests that very young puppies may be more susceptible than older ones. As related in an earlier chapter, older animals sometimes survive and become carriers for as long as fifteen months in the case of Babesia and for at least two years in the case of Ehrlichia.

Ehrlichia Infections

From the foregoing discussion it is obvious that the rickettsia, probably Ehrlichia sp., was not recognized initially during this study. Indeed a part of the work was completed under the misapprehension that the organism represented an undescribed schizogonous stage in the Babesia canis life cycle. As already indicated, once it was discovered that two agents were involved, and the two were separated, the rickettsia was kept free of Babesia by dog to dog passage using whole blood injected intravenously.

Figures 38, 43, 48, and 53 depict the anemia and other hematological changes which developed in four animals (E-15, E-17, E-18, and E-24) suffering from experimentally induced rickettsiosis, specifically ehrlichiosis. MCV and MCH values were calculated, but these data are not included; suffice it to say that the anemia was of the normocytic-normochromic type. It is clear that a remarkably consistent syndrome developed in animals subjected to this infective agent. The clinical picture was almost identical with that described for the combined Babesia-Ehrlichia infections. That is to

say, there was no clear differentiation between dogs suffering from the rickettsiosis and dogs suffering from combined infections of Ehrlichia and Babesia. In either condition, severe anemia developed, and the dogs were apparently unable to replace functional erythrocytes rapidly enough to keep pace with the need.

The disease produced by the agent which has been isolated from dogs in Oklahoma is quite similar to that described by Donatien and Lestoquard (1935) in Algeria and by Bool and Sutmöller (1957) in the Netherlands Antilles and attributed to a rickettsial agent now known as Ehrlichia canis. The organism under study here is morphologically quite similar to E. canis, and Doctor W. O. Neitz (1964) and Doctor C. B. Philip (1964) concur with the author in considering the agent as belonging to this genus. Neitz examined photographs and stained smears and concluded that "... I feel that I can confirm my original suspicion that the leucocytic inclusion bodies may be Ehrlichia canis." The findings discussed in the section on serology indicate that the affinity of this agent for other rickettsiae is not as close as some reports in the literature would lead one to believe. The reported cross-agglutination of E. canis with Proteus OX 2 was not verified in this study. This may mean that the Oklahoma isolate is serologically but not morphologically nor clinically distinguishable from E. canis of the Old World, but such a conclusion cannot be reached on the basis of presently available data.

V. CONCLUSIONS

The results of studies which have been completed emphasize the fact that a constant vigilance must be kept on the health of domestic animal populations. The isolation of a rickettsial organism, probably Ehrlichia sp., from dogs and the recognition of a distinct disease syndrome caused by it is a potentially important contribution to canine medicine. It is not possible to conclude whether or not the agent has been in the United States for a long period of time or whether the isolation in Oklahoma represents finding an agent soon after its first appearance. The literature suggests, however, that previous American workers have dealt with the disease caused by this organism but have diagnosed it as distemper or possibly some other non-specific condition because it was masked by or confused with other more common and better known disease syndromes.

The discovery emphasizes the fact that our present state of knowledge of infectious diseases in the canine must be considered as being far from satisfactory and complete. It shows that casual observations of ill animals and even detailed laboratory studies may be insufficient to recognize the true nature of infectious disease problems. It was interest in a well-recognized protozoan disease, babesiosis, which led to the discovery of this possibly, previously undescribed agent since the study was originally designed to investigate what was considered to be a phase in the Babesia canis life cycle. The hypothesis that a schizogonous cycle

existed in the Babesia canis life cycle was proved to be erroneous, but in arriving at this conclusion a significant contribution was made.

Detailed studies of the parasitemia which develops in Babesia canis infections both in the presence and in the absence of Ehrlichia were completed. The erythrocytic phase of the reproductive cycle was determined to be binary fission and that neither schizogony nor budding is involved as sometimes reported in the literature. Babesia canis was found to persist in infected dogs for fourteen months, and Ehrlichia was found to persist for at least twenty-four months in the presence of Babesia and nine when present alone. The period for Ehrlichia does not represent an endpoint in the potential duration of this organism. It was proved that venous rather than capillary blood is more apt to reveal Babesia trophozoites. The hematological changes occurring in dogs affected by each of the two agents alone and in combination were found to be similar, viz., a severe anemia of the normocytic-normochromic type develops, but it is more severe in the dual infection. This appears to be due to the fact that there are two detrimental effects involving red blood cells; Babesia affects circulating erythrocytes while Ehrlichia impedes hemopoiesis.

Dogs which survive the acute single or dual infections eventually recover and appear to be healthy; nevertheless their blood remains infectious for other dogs for nearly fifteen months in the case of Babesia and at least twenty-four months in the case of Ehrlichia. Asymptomatic carriers of Babesia and/or Ehrlichia are shown to represent a potentially serious problem as sources of contamination in veterinary hospitals, either from the point of natural transmission to susceptible dogs by ticks

or artificial transmission by blood transfusion. It was shown that Ehrlichia can be transmitted in some other way, but the method(s) was not determined in this study. Data given in this study detailing the fluctuations in numbers of both the rickettsial and the protozoan parasites in the peripheral blood point up the necessity for extensive observation of blood smears in arriving at confirmatory diagnoses.

The reproductive cycle of Ehrlichia canis, as described in the literature, was not confirmed in this study since no sequential pattern was observed in the occurrence of leukocytic inclusions which are thought to be either Ehrlichia or Ehrlichia-engendered.

Serological findings show that existing antigens used for diagnosing rickettsial infections are of no value in detecting infections caused by this Ehrlichia-like organism.

Obviously a great deal of work remains to be done in clearly delineating the symptoms which develop in animals infected with each of these two organisms alone or in combination, and these results show that there is need for more investigation.

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VITA

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