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Thirty-six intact calves were divided into 16 groups of 2 (or 3) and each group inoculated with infective Babesia erthrocytic stabilates. Twenty-eight days later they were homologously and heterologously challenged from the same stabilates, and monitored for an additional 20 days. The antigenic variations were more evident by in vitro than in vivo reactions.

On the basis of the serological results, antigenic variation within an isolate of B. bigemina occurred. Antigenic variation appeared to be influenced by the mode and duration of infection. The hosts' apparent reduced response to homologous challenge and the marked immune response observed with heterologous systems indicated antigenic differences of the B. bigemina and confirmed the serological data under the experimental conditions used in this investigation.

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A COMPARISON OF THE ANTIGENIC PROPERTIES OF ERYTHROCYTIC *BABESIA BIGEMINA* IN ACUTE AND CHRONIC BLOOD BORNE AND TICK BORNE INFECTIONS IN CATTLE

A Dissertation

by

KENNETH CARL THOMPSON

Submitted to the Graduate College of
Texas A&M University in
partial fulfillment of the requirement for the degree of

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A COMPARISON OF THE ANTIGENIC PROPERTIES OF ERYTHROCYTIC *BABESIA BIGEMINA* IN ACUTE AND CHRONIC BLOOD BORNE AND TICK BORNE INFECTIONS IN CATTLE

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ABSTRACT

A Comparison of the Antigenic Properties of Erythrocytic *Babesia bigemina* in Acute and Chronic Blood Borne and Tick Borne Infections in Cattle. (May 1974)

Kenneth Carl Thompson, B.S., Arizona State University; B.S., D.V.M., Texas A&M University

Directed by: Drs. R. J. Hidalgo and R. A. Todorovic

The purpose of this study was to determine possible antigenic differences in the causative organisms of the acute and chronic blood borne and tick borne *Babesia bigemina* infections of cattle. It was anticipated that the results of the research would further aid in the understanding of the mechanism of immunity of babesial infections. In addition, the antigens may possibly be used in the development of new serologic techniques or vaccines.

A *Babesia bigemina* field isolate was purified by 4 rapid serial passages through 5 splenectomized calves within 6½ days, and a stabilate of the isolated *B. bigemina* was established.

The larval progeny of engorged female Boophilus microplus ticks were screened for the presence of Anaplasma marginale or Babesia spp. The noninfected ticks resulting from the screening were maintained primarily on noninfected splenectomized calves. Disease free guinea pigs were also utilized with only moderate success. Various stages of ticks were held without feeding in desiccator jars for periods of 1 to 2 months. Noninfected B. microplus ticks were successfully infected by placing them on calves inoculated intravenously with the B. bigemina stabilate.

With the aid of synthetic corticosteroids, high B. bigemina parasitemias were attained in splenectomized calves subinoculated from intact calves infected by ticks and by artificial inoculation. Babesia erythrocytic antigens and infective blood stabilates were prepared from blood of splenectomized calves with high parasitemias resulting from treatment with synthetic corticosteroids. These splenectomized calves were subinoculated from intact calves with acute (AB) and chronic (CB) blood borne and acute (AT) and chronic (CT) tick

borne infections. Normal bovine (NB) erythrocytic antigen was prepared as a control.

Isolation of B. bigemina antigens was accomplished by erythrocytic lysis and homogenation, differential centrifugation and purification from host materials by means of molecular sieving using Sephadex G-100 and G-200 columns. Antigens were characterized by means of the complement fixation, gel diffusion, agar-gel immunoelectrophoresis and indirect hemagglutination techniques and their antigenic differences observed.

Thirty-six intact calves were divided into 16 groups of 2 (or 3) and each group inoculated with infective Babesia erythrocytic stabilates. Twenty-eight days later they were homologously and heterologously challenged from the same stabilates, and monitored for an additional 20 days. The antigenic variations were more evident by in vitro rather than in vivo reactions.

On the basis of the serological results, antigenic variation within an isolate of B. bigemina occurred. Antigenic variation appeared to be influenced by the mode and duration of infection. The hosts' apparent

reduced response to homologous challenge and the marked immune response observed with heterologous systems indicated antigenic differences of the B. bigemina and confirmed the serological data under the experimental conditions used in this investigation.

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TABLE OF CONTENTS

	Page
ABSTRACT	iii
DEDICATION	vii
ACKNOWLEDGMENTS	viii
LIST OF TABLES	xiv
LIST OF FIGURES	xvi
INTRODUCTION	1
LITERATURE REVIEW	4
General Aspects of Bovine Babesiasis	4
Low Temperature Preservation of *Babesia*	5
Tick Holding and Rearing Methods	9
Infection of *Boophilus microplus* with *Babesia bigemina*	13
Use of Corticosteroids	17
Preparation of *Babesia bigemina* antigens	19
Molecular Sieving	19
Serologic Tests	20
Complement Fixation (CF) Test	20
Gel Diffusion Test	27
Agar-Gel Immunoelectrophoresis Technique	31
Indirect Hemagglutination (IHA) Test	33
Host Response to *Babesia* Infections	36
Antigenic Variation	39

Page

MATERIALS AND METHODS 51

Experimental Animals 51

Calves 51
Guinea Pigs 54
Rabbits 54

Experimental Organism 54

Isolation, Purification and Maintenance of *Babesia bigemina* 54

Experimental Vector 55

Isolation, Purification and Maintenance of *Boophilus microplus* 55
Infection of *Babesia*-free *Boophilus microplus* with *Babesia bigemina* . . . 58

Acute and Chronic Blood Borne and Tick Borne Infections 59

Acute Blood Borne Infection 59
Chronic Blood Borne Infection 63
Acute Tick Borne Infection 63
Chronic Tick Borne Infection 64

Isolation and Preparation of *Babesia bigemina* Antigens 66

Molecular Sieving 66

Preparation of Specific Antiserum 69
Host Response to the Infective *Babesia* Erythrocytic Stabilates 69

Page

Assessment of Reactions 70

Parasitemia 70
Thin and Thick Blood Film 72
Packed Cell Volume (PCV) 75
Temperature 76
Body Weight 76
Complement Fixation (CF) Test 76
Gel Diffusion Test 79
Agar-Gel Immunoelectrophoresis Technique . 83
Indirect Hemagglutination (IHA) Test . . . 85

RESULTS 92

Isolation, Purification and Maintenance of *Babesia bigemina* 92
Isolation, Purification and Maintenance of *Boophilus microplus* Ticks 95
Infection of Hemoprotozoan-Free *Boophilus microplus* with *Babesia bigemina* 97
Monitoring of Acute and Chronic Blood and Tick Borne Infections 97

Acute and Chronic Blood Borne Infections . 97
Acute and Chronic Tick Borne Infections . 101

Isolation and Purification of *Babesia bigemina* Antigens 105
Immune Response of Calves to Various *Babesia bigemina* Stabilates 111
Characterization of the *Babesia bigemina* Antigens 124

Complement Fixation (CF) Test 124
Gel Diffusion Test 124
Agar-Gel Immunoelectrophoresis Technique . 130
Indirect Hemmagglutination (IHA) Test . . 130

Page

DISCUSSION 135

SUMMARY 145

LITERATURE CITED 148

APPENDIX 159

VITA 173

LIST OF TABLES

Table		Page
1.	Utilization of experimental calves	52
2.	Schematic for production of acute and chronic blood borne *Babesia bigemina* stabilates and antigens	61
3.	Schematic for preparation of acute and chronic tick borne *Babesia bigemina* stabilates and antigens	65
4.	Experimental design for primary inoculation and subsequent homologous and heterologous challenge with viable *Babesia bigemina* isolated from acute and chronic blood borne and tick borne infections in cattle	71
5.	The isolation of *Babesia bigemina* from a natural, tick borne, mixed infection also containing *Babesia argentina*, *Babesia major* and *Anaplasma marginale* by rapid serial passage through 5 splenectomized calves . .	94
6.	Summation of host responses by groups to the infective *Babesia* erythrocytic stabilates .	122
7.	Complement fixation titers of 5 crude antigens when tested with 4 positive serums . .	125
8.	Complement fixation titers of 5 crude antigens, standardized to 20 mg/ml protein, when tested with 4 positive and 1 negative serums	127
9.	Indirect hemagglutination titers obtained with 4 antigens and the homologous and heterologous antiserums (each figure represents the reciprocal of an individual dilution)	132

Appendix
Table

Page

I. Concentrate and mineral composition of experimental calf grain ration 160

II. Fecundity of *Boophilus microplus* ticks from calves infected with *Babesia bigemina* 161

III. Hatchability of *Boophilus microplus* eggs of females from *Babesia bigemina* infected calves (Continuation Table II) 162

IV. Standard deviation and means of the packed cell volumes of the calves with blood borne and tick borne *Babesia bigemina* infections 163

V. Standard deviation and means of the rectal temperature of the calves with blood borne and tick borne *Babesia bigemina* infections 165

VI. Standard deviation and means of the complement fixation titers of the calves with blood borne and tick borne *Babesia bigemina* infections 167

VII. Standard deviation and means of the parasitized erythrocytes of the calves with blood borne and tick borne *Babesia bigemina* infections 169

VIII. Standard deviation and means of the body weights of the calves with blood borne and tick borne *Babesia bigemina* infections . . 171

IX. The host response to infective *Babesia* erythrocytic stabilates when measured by means of estimated weekly average weight gains (kg) 172

LIST OF FIGURES

Figure		Page
1.	*Babesia bigemina* parasitized bovine erythrocytes in thin blood film. Giemsa. ×1300 . .	93
2.	Complement fixation titers, percent parasitized erythrocytes, rectal temperatures and percent packed cell volumes for 5 calves inoculated with 1×10^9 *Babesia bigemina* parasitized erythrocytes on day -5. Day 0 indicates that period when 75% or more of the calves showed a parasitemia	99
3.	Complement fixation titers, percent parasitized erythrocytes, rectal temperatures and percent packed cell volumes for 5 calves infected with *Babesia bigemina* by *Boophilus microplus* ticks on day -19. Day 0 indicates that period when 75% or more of the calves showed a parasitemia	102
4.	Thick film of concentrated *Babesia bigemina* used for antigen preparation for the CF microtiter procedure. Giemsa. ×1300 . . .	106
5.	Graph of spectrophotometric absorbance (280 μ) and antigenic activity range (measured by gel diffusion tests) of acute blood, chronic blood, acute tick and chronic tick antigenic fractions collected from a Sephadex G-200 column	107
6.	Graph of spectrophotometric absorbance (280 μ) and antigenic activity range (measured by gel diffusion tests) of acute blood, chronic blood, acute tick and chronic tick antigenic fractions collected from a Sephadex G-200 column	109

Figure

Page

7. Infective *Babesia bigemina* stabilate after thawing, prior to subinoculation into an intact calf. Giemsa. ×1500 112

8. The host response to infective *Babesia* erythrocytic stabilates and challenge with homologous and heterologous stabilates when measured by means of the packed cell volume (%) 113

9. The host response to infective *Babesia* erythrocytic stabilates and challenge with homologous and heterologous stabilates when measured by means of the rectal temperature (°C) 115

10. The host response to infective *Babesia* erythrocytic stabilates and challenge with homologous and heterologous stabilates when measured by means of the complement fixation titer 117

11. The host response to infective *Babesia* erythrocytic stabilates and challenge with homologous and heterologous stabilates when measured by means of the parasitized erythrocytes 119

12. *Babesia bigemina* parasites in a thick bovine blood film. Giemsa. ×1500 123

13. Saphadex antigenic fractions of infected erythrocytes (cells) reacting with specific rabbit antiserum (strongly) in the gel diffusion test. The precipitation line in the center indicated a reaction typical of the 33 antigen fractions tested 129

Figure | Page

14. Agar-gel immunoelectrophoresis of the acute blood (AB), acute tick (AT), and chronic tick (CT) antigens with specific absorbed rabbit antiserum (RAB, RAT, RCB, and RCT). The chronic blood (CB), not shown here, reacted in a reciprocal manner with RAB, RAT, and RCT 131

15. A comparison of the reactions of an indirect hemagglutination tube test (top) and a complement fixation microtiter plate test (bottom) 133

INTRODUCTION

Immunity produced by parasitic protozoa has many common features with that stimulated by other infectious organisms; however, due to the complex life cycles of protozoa, a simple reaction of the host to a single stage of the parasite is not the case. On the contrary, a variety of immunological responses is evoked in the vertebrate host by the various tissue stages of the organism that occur in the invertebrate host as well as by the final products circulating in the blood of the vertebrate host and in some instances, by the continually changing antigenic pattern in vertebrate hosts (Neal et al., 1969).

Clumow (1968, 1973a, 1973b) indicated that antigenic variants possibly occurred in one of more strains of *Babesia argentina* and *Babesia bigemina* arising by a process similar to that demonstrated by the agglutination test in chronic *Plasmodium knowlesi* infections (Brown & Brown, 1965). Evidence that different strains of the

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The citations in this paper follow the style of the *Journal of Parasitology*.

same species of Babesia do exist arose when the movement of cattle in Australia from one locality to another was followed by an outbreak of babesiosis in previously immune cattle (Seddon, 1952). This suggests, by analogy with trypanosomal and malarial infections, that antigenic variation of Babesia does occur after the acute infection.

With respect to Babesia antigens, the majority of the research has been done on blood borne bovine babesiosis and the minority on the tick borne infections. Such experiments usually provide information applicable to the disease as it occurs in the vertebrate, but most of the life cycle of Babesia species occurs in the tick vector (Rick, 1964). Thus, one is probably examining an altered parasite when studying blood borne infections. It is apparent that such experiments should be confirmed with tick borne infections (Brocklesby et al., 1971).

The purpose of the present study was to determine possible antigenic differences in the acute and chronic blood borne and tick borne Babesia bigemina infections of cattle. It is anticipated that this research will

aid in the understanding of the mechanism of immunity to babesial infections. In addition, the antigens may possibly be used in the development of new serologic techniques or vaccines.

LITERATURE REVIEW

General Aspects of Bovine Babesiasis

Babesiasis is a tick transmitted disease of cattle associated with the appearance of the *Babesia* parasite in the host erythrocytes. It is characterized by fever and intravascular hemolysis causing a syndrome of anemia, hemoglobinemia and hemoglobinuria. The disease caused by *Babesia bigemina* occurs in many countries causing considerable economic loss, yet little is known of the host-parasite interactions involved in cattle or in other animal species.

Since the discovery of *Babesia bovis* (Babes, 1888) in Roumania, and *Babesia bigemina* (Smith and Kilborne, 1893) in the United States, attempts have been made to determine their relationship to other Protozoa (Rick, 1968).

The proper taxonomic position of *Babesia* within the phylum Protozoa has been the subject of considerable uncertainty and varies with the authority (Levine, 1973). Levine (1973) classified the *Babesia* under the subphylum

Apicomplexa, class Piroplasmasida, order Piroplasmorida, and the family Babesiidae.

Low Temperature Preservation
of Babesia

Lumsden and Hardy (1965) stated that the term strain, with its connotations of ancestry, descent, race, or stock, is clearly appropriate for living material which is kept available for experimental purposes by being serially passaged in laboratory animals or in culture. Organisms maintained in this way, continuously reproducing, are subject to selection. It may alter in its biological characteristics and become unrepresentative of the populations existing in nature from which it was derived and, therefore, be unsuitable for reference purposes and long term experimentation. However, as Lumsden and Hardy (1965) also observed, the storage of viable parasitic organisms at low temperatures affords a material in which reproduction is arrested and the biological characters are stabilized. Hence, a separate term is now required to convey this implication

of the stabilization of biological characters is preserved material and to distinguish preserved from strain material. The term 'stabilate' was coined and may be defined as a population of an organism preserved in viable condition on an unique occasion. There will be only as many examples of a stabilate as there were individual samples of it laid down on the unique occasion. It will, therefore, be possible to designate each stabilate by a unique code letter or number.

Waddell (1963) reported the survival of B. bigemina in bovine blood with 11.6% (v/v) glycerol for 30 days at -79 C. The blood-glycerol mixture was placed in ampoules that were immersed in a mixture of solid carbon dioxide and alcohol (-79 C) and maintained in a vacuum flask at -79 C.

Barnett (1964) reported bovine blood containing B. bigemina (5 to 20 x 10^8 organisms/ml of blood) with 7% (v/v) glycerol was infective for cattle after storage at -79 C for up to 720 days. Three methods of slow reduction of temperature to -79 C were described and all were successful with no change in virulence or behavior of the parasite resulting from storage. The frozen

blood was usually thawed quickly by immersing and rotating the tube in water at 37 C and the fluid injected without delay. However, a trial was conducted on slow thawing and it was concluded that the parasites will survive slow thawing and remain viable for at least 1 hr after removal from storage in solid carbon dioxide. Stained smears made from the thawed blood showed that most of the erythrocytes were lysed after thawing. The parasites, whether free or in red cells, were somewhat crenated and had dense cytoplasm and usually prominent rounded nuclei. Barnett (1964) reported that it was not possible to assess what proportion of the parasites were viable.

Pipano and Senft (1966) reported that blood containing 27% of the erythrocytes parasitized with B. bigemina was infective for calves after storage at -70 C. Fifty percent glycerol or glucose in phosphate buffer was added to whole blood to a final concentration of 15%. The blood was dispensed in 10 to 50 ml bottles, stoppered with rubber stoppers, and incubated for about 30 min at 4 C. After incubation the blood was stored

in an ultra low temperature cabinet at -70 C. Thawing was accomplished by plunging the bottles into a water bath at 37 C while agitating until the sample was completely thawed. Glycerinated blood containing B. bigemina was infective for calves after storage at -70 C for up to 440 days, and blood containing glucose, for 240 days, the longest period tested. The incubation periods were nearly identical with those observed after the use of fresh infected blood and no change in virulence of the frozen parasites was noted.

Dalgliesh (1969) reported that bovine erythrocytes were freely permeable to glycerol at 37 C but much less so at temperatures around 4 C. Slow equilibration prolonged stress factors imposed by the osmotic effects of extracellular glycerol. The detrimental effect of glycerol for parasitic protozoa may be related to its osmotic activity. Erythrocytes containing glycerol cannot be transferred directly to a glycerol free isotonic medium without swelling and lysis of the cells due to the osmotic effects of intracellular glycerol (Hurn, 1968 as quoted by Dalgliesh, 1972a). Intravenous (iv)

inoculation is equivalent to sudden exposure to an isotonic environment, and Dalgliesh (1972b) showed that this route of inoculation suppressed infectivity of B. bigemina in blood containing glycerol. When parasites were injected subcutaneously (sc), infectivity was not markedly affected, presumably because there was less body fluid available to cause osmotic swelling and rupture of the parasites and the erythrocytes that enveloped them.

Tick Holding and Rearing Methods

Pierce and Pierce (1956) cultivated Boophilus microplus from the larval to the nymphal stage on the air cell membrane of the embryonated chicken egg. Young adults, molted in vitro, also attached to the membrane for short periods.

Riek (1959) showed complete development, including oviposition, of B. microplus on mice and rabbits but not on guinea pigs. Fed females were recovered 23 to 29 and 23 to 31 days after larvae were applied to mice and rabbits, respectively, but were smaller than those which dropped from Zebu-Shorthorn and Shorthorn steers.

The report of rearing Rhipicephalus appendiculatus (Bailey, 1960) on rabbits contains many techniques which readily apply to other Ixodid ticks. For instance, Bailey (1960) discussed the need to ensure that the tick room is free of flysprays or furniture polish containing residual insecticides which may contaminate equipment. Also, tick rooms must not be used to store insecticides nor should the rooms be sprayed or treated against pests.

If a tick incubator is used it must contain shelves which allow free circulation of air throughout the incubator. It should also be equipped with a light source and have some method to maintain the relative humidity inside the incubator.

When using laboratory animals to rear immature stages of ticks, Bailey (1960) noted that proper restraint is necessary for attachment. However, if tape or other materials are used, they must be placed in such a way as not to cause swelling, edema or necrosis of the area which will lead to the death of the ticks. Also, if an ear bag is fitted, it must be removed daily to allow any engorged ticks which have dropped from the

ear to be collected. Prompt removal of these fully fed ticks is important, otherwise the legs of the ticks are liable to become damaged or rubbed off by friction between the ear and the cloth bag. Ticks damaged in this manner can be discarded at this stage as they usually will not lay eggs.

Hoyte (1964) reported limited success in rearing B. microplus to maturity on unrestrained dogs.

The tropical horse tick, Dermacentor (Anocentor) nitens is commonly found on horses in many tropical regions of the Western Hemisphere. It has been considered the probable arthropod vector of equine piroplasmosis. The biologic similarities between D. nitens and B. microplus, which transmits bovine piroplasmosis, are twofold. First, they both are one host ticks; and secondly, they both develop on their preferred hosts in much the same manner. Roby et al. (1964) reported the successful holding of D. nitens in a desiccator jar for 2 months to permit the oviposition of the adults and the hatching of the larvae. The desiccator jar contained about 2 inches of aqueous saturated ammonium

sulfate solution which provided constant relative humidity of 60 to 80% at 23 to 25 C.

Babesia bigemina in ticks grown on non-bovine hosts was reported by Callow (1965). The failure of *B. microplus* to be cleansed of *B. bigemina* while the ticks were growing on non-bovine hosts was demonstrated in experiments in which infected ticks were released on a number of sheep, a goat and a horse. Seven of 10 batches of ticks, collected from 6 non-bovine hosts, retained their *Babesia* infection. Blood subinoculated into cattle from 7 of 9 sheep and from the horse showed that these animals carried *B. bigemina* while the ticks were feeding on them. This suggested that ticks may have become re-infected from the non-bovine hosts, a possibility supported by the finding of *B. bigemina* in blood smears from 2 of the sheep.

Rhipicephalus appendiculatus, an efficient vector of bovine piroplasmosis in Africa, was successfully maintained and reared on mice, rabbits, and calves using tick capsules and ear bags by Irvin and Brocklesby (1970) in Britain. In 1971, Gregson reported a tick

infesting appliance for hamsters which had an added convenience of ease in accessibility to the ticks and a long adhesion period without undue irritation.

Loomis (1971) showed that the common laboratory rabbit was a highly unreliable host for complete development of the cattle tick, B. microplus. Innate resistance in some rabbits and the rapid development of acquired resistance in others did not assure dependability in maintenance of tick cultures. Although the sizes of larvae and nymphs were similar to specimens reared on cattle, the size of fed females was always less than that of bovine engorged specimens.

Mahoney and Mirre (1971) reported the holding of engorged female B. microplus ticks in glass tubes sealed with cotton wool in an incubator at 28 C and a 90 to 95% relative humidity.

Infection of Boophilus microplus
with Babesia bigemina

Callow and Hoyte (1961b) reported 2 methods of infecting B. microplus with B. bigemina. No difficulty

was experienced in infecting ticks with B. bigemina when they were reared on a bovine undergoing a primary infection with B. bigemina induced by an inoculation of infected blood. However, 2 attempts out of 3 to infect ticks from recovered bovine carriers of B. bigemina were unsuccessful. The adult stage of B. microplus was shown to be capable of acquiring infection. One experiment demonstrated that infection of ticks could take place at the end of the adult stage.

Mahoney (1964) reported the success of infecting B. microplus larvae known free of infection with B. bigemina. The larvae were placed on noninfected calves and in 10 to 12 days the calves were injected with blood from an animal with a pure infection of B. bigemina. Female ticks engorging during the ensuing phase of microscopically detectable parasitemia were collected, and their larval progeny successfully used in the tick transmission of B. bigemina.

Hoyte (1965) reportedly placed 2 successive batches of ticks on each of 4 splenectomized susceptible calves. The first batch placed on the calves consisted of about

120,000 noninfected larvae. About 2 weeks later, about 40,000 larvae, which were infected with B. bigemina were released on the calves. This second release was so timed that day 9 of this infestation (when these ticks were starting to inoculate parasites into the calves) coincided with day 22 or 23 of the noninfected ticks (when most of them were completing engorgement). Daily collections were made of the engorged female ticks resulting from the first infestation. Each day's collection was kept separately from the others. The infectivity of the larval progeny of these ticks was tested by placing them on a series of splenectomized susceptible calves. Nine to 12 day (post tick exposure) larval progeny were not found to be infected. The larval progeny of adult ticks collected after day 12 were found to be infected.

Experiments were performed by Callow (1968a) to determine at what phase or phases of parasitic development B. microplus became infected with B. bigemina. Problems due to B. microplus being a one host tick were overcome by artificially transferring ticks from one

host to a second just prior to a molt. In several experiments, a babesiacide was used to eliminate parasites so that the exposure of ticks to infection could be restricted to one phase of their development. Further information was obtained by correlating the presence or absence of patent parasitemia in the bovine with the presence or absence of infection in ticks which fed on it. It was found that ticks were susceptible to infection usually the last day of their parasitic life. Infection at other times, that is, during larval, nymphal, and the major part of the adult stages was rare. Four of 5 attempts to infect larvae and all 5 attempts to infect nymphs were negative. Infection of ticks always resulted when a patent parasitemia coincided with the last day of parasitic life. Ticks were infected in all 13 instances when these conditions existed but in only 7 of 18 instances when the animal was a carrier but was not showing a parasitemia.

Mahoney and Mirre (1971) reportedly placed non-splenectomized calves in tick proof isolation pens and infested them with 20,000 Babesia-free B. microplus larvae. Ten days later the calves were infested again

with larvae infected with B. bigemina. Engorged adult female ticks were collected from the floor of the pens daily, 20 to 25 days after the initial infestation. Babesia were detectable in peripheral blood films of the calves during this period. The infectivity of immature ticks resulting from these adults was tested by infecting noninfected calves with the freshly hatched larvae. In some experiments, an entire batch was used, in others, small replicate samples from a batch were individually placed on at least 10 animals. The calves were tested for evidence of infection by examination of blood smears and by subinoculation of blood 10 to 30 days after infestation. It was concluded that the infection rate in the progeny of the ticks that engorged during the acute phase of infection with B. bigemina was 20 to 44% of the larvae.

Use of Corticosteroids

Callow and Parker (1969) reported an increase in the numbers of Babesia argentina in both brain and thick blood smears from cattle given a cortisone derivative. The cortisone derivative was used to produce nonclinical

relapses of B. argentina in steers carrying this organism. The parasites increased in numbers or appeared for the first time in brain smears after brain biopsies from 12 of 13 treated steers and in the thick blood smears from 10 of these steers which previously were negative.

Frerichs et al. (1969a) reported synthetic corticosteroids had the following effects on horses and burros used in Babesia antigen production for the CF test. The infected horses (or burros) retained their normal clinical appearance and appetite much longer than the controls. Injection of synthetic corticosteroids lowered the body temperature even at the height of the parasitemia. The packed cell volume (PCV) of the infected horses (or burros) usually remained closer to preinoculation levels than the PCV of controls. This effect alone increased yield and potency of the antigen because packed erythrocytes were the beginning material for production of antigen. Since the infected horses (or burros) had greater PCV's along with greater percentage of parasitized erythrocytes, the use of the

drugs greatly increased the number of parasites available for processing into antigen and, consequently, the amounts of antigen recovered.

Young and Cox (1971) found that synthetic corticosteroids had a similar effect on Babesia microti and Babesia rodhaini infections in rodents. When corticosteroids were administered they found an increase in the numbers of parasitized erythrocytes.

Preparation of Babesia bigemina Antigens

A. Molecular Sieving

Sibinovic et al. (1967a, b) reported that antigens in the serums of horses with a mixed Babesia equi and Babesia caballi infection, dogs with Babesia canis, and rats with B. rodhaini were detected by reactions with serums of animals recovered from acute babesiosis. These antigens were freed from the host serum components with the exception of some of the gamma globulins, beta I lipid-poor euglobulins, and beta II globulins by means of molecular sieving techniques and were concentrated by ultracentrifugation.

Mahoney and Goodger (1969) fractionated ammonium sulfate precipitates of lyophilized serum from calves infected with B. argentina by gel-filtration on columns of Sephadex G-200.

Goodger (1971) reported the fractionation of crude bovine babesial extracts by gel filtration on a 37 by 2.5 cm column of Sephadex G-200. The column was eluted with 0.05 M Tris-HCl buffer pH 8.0 at a flow rate of 5 ml/hr. Eluate was collected in 5 ml fractions. Optical density (OD) of each fraction was read at 280 mμ and 413 mμ.

Serologic Tests

A. Complement Fixation (CF) Test

Hirato et al. (1945) reported the application of the CF test in detecting equine babesiosis. The CF antigen was comprised of the erythrocytic stroma from horses acutely infected with B. caballi. The CF antibodies were detected 11 to 15 days after the onset of parasitemia and persisted for at least 100 days.

Mahoney (1962a) reported the application of the CF

test in detecting bovine babesiosis. The results of the tests with serums from cattle given pure infections of either B. bigemina or B. argentina and of tests with serums from noninfected cattle indicated that the antibodies to the Babesia antigens were present only in the serums from the infected calves. These serums reacted at a higher titer with the homologous antigen and antibodies were detected within 7 to 21 days after infection. In the lowest serum dilution of 1:5, slight reactions with serums of noninfected calves were sometimes observed. These were more apparent with B. argentina antigens than with B. bigemina antigens which tended to indicate some impurity of the antigenic material.

Schindler and Dennig (1962) reported the application of the CF test in detecting canine babesiosis. A CF antigen was prepared from erythrocytes of dogs acutely infected with B. canis by means of immunohemolysis or by exposing the erythrocytes to 0.3% saponin. The CF antibody became detectable in 12 to 34 days following infection. No serologic cross-reaction was observed between B. rodhaini and B. canis antigens.

Mahoney (1964) determined weekly CF antibody levels for homologous antigen in calves subjected to infection with either B. argentina or B. bigemina experimentally transmitted by the B. microplus tick. With 11 calves infected with B. argentina, 95 to 100% of serum samples contained antibody at or above the minimum diagnostic level for the ensuing 7 months, but over 5 more months the proportion declined to 50%. In 9 calves infected with B. bigemina the proportion of positive serum was 94 to 100% during the first 4 months, declining thereafter to the low figure of 16% at 12 months. However, in all but 3 of these 20 cattle the continued presence of parasites during periods of 12 to 18 months after infection was proved by the subinoculation of 500 ml of blood into susceptible, splenectomized calves. Thus, in these proven carriers, CF antibody tended to fall below the diagnostic level before infection was finally eliminated. When carriers were superinfected with either species there was either an ensuing rise in homologous antibody level, or a deferment of the expected decline in titer.

Schindler et al. (1966) inoculated dogs with live B. canis and soluble B. canis antigens. Immunity was acquired only after infection with live B. canis. This resistance remained even after disappearance of the CF antibodies. Dogs inoculated with soluble B. canis antigens produced CF antibodies but not protective immunity.

Curnow and Curnow (1967) performed the CF test by the method suggested by Mahoney (1962a) with slight modifications. Erythrocytic suspensions were standardized colometrically and in complement titrations the 50% endpoint (1 unit) was determined in a similar manner. In the test 3.3 such units were used.

In the development of a routine CF test for the diagnosis of bovine babesiosis, it was found by Mahoney (1967a) that the most sensitive CF antigen for B. argentina was a crude suspension of the parasites prepared by distilled water lysis of infected erythrocytes followed by centrifugal concentration. For B. bigemina, a specific, sensitive CF antigen was extracted from the parasite suspension with distilled water. In tests on serums obtained from cattle not exposed to B. bigemina,

1.6% reacted at a 1:5 dilution and 0.2% at a dilution of 1:10. With cattle not exposed to B. argentina, 4.5% reacted at 1:5, 1.4% at 1:10, and 0.5% at 1:20. The nonspecific reactions to B. argentina were prevented by absorption of the serum with a suspension of normal bovine erythrocyte stroma. Cross-absorption tests indicated that the B. argentina and B. bigemina CF reactions each consisted of a major species-specific and a minor group-specific component.

Mahoney (1967b) reported on the immunization of cattle with killed B. argentina. Twenty nonsplenectomized 2-year-old steers were injected with a freeze dried suspension of B. argentina, either sc in Freund's complete adjuvant or iv suspended in distilled water. The steers reacted to the injections by the formation of CF antibodies, and subsequent challenge with the strain of B. argentina used to prepare the parasite suspensions showed that a degree of immunity had been acquired as a result of the inoculations. However, Mahoney (1967c) found no positive correlation between protective capacity and CF titer of serum. Hence, it was concluded

that CF antibodies were unlikely to be concerned with immunological resistance to B. argentina.

Frerichs et al. (1969a) produced antigens for the CF test for B. caballi and B. equi infections. The greatest difficulty in producing B. caballi antigen was the low yield that resulted from low rates of parasitemia. Loss of serologic specificity by B. equi after it was passaged in horses was the greatest problem in producing this antigen.

Frerichs et al. (1969b) reported CF titers of horses infected with B. caballi. Fifteen horses were infected with B. caballi by inoculation with a suspension of packed erythrocytes. Their serologic responses were determined by CF testing. Periodically, erythrocytes from all horses were subinoculated in susceptible ponies to determine duration of the carrier state. The CF titer persisted longer in 14 of the horses than did their capability to transmit the disease and, thus, was a reliable index of exposure to B. caballi. Horses re-exposed to the parasite after losing their CF titer had a typical anamnestic response. The CF test was highly specific for detecting infection with B. caballi.

The CF test used to detect babesial antibodies in the serum of infected cattle was modified by Todorovic et al. (1971) and Todorovic and Adams (1971) from the method described by Mahoney (1967a). One volume of washed infected erythrocytes was resuspended in 10 volumes of veronal buffer and disrupted by sonication. The sonified suspensions were centrifuged for 30 min at 27,000 x g. After centrifugation, the supernatant was collected and the sediment was resuspended in veronal buffer and homogenized with a TenBroeck tissue grinder. The suspension was again centrifuged for 30 min at 27,000 x g and the supernatant collected. The procedure was repeated 3 times. The supernatant fluids were numbered serially in order of collection and were used as the CF antigens. The peak of parasitemia on day 8 post-inoculation coincided with the first day of CF antibody response. A total of 5,420 serum samples of cattle infected with babesiosis were tested. Approximately 95% of these samples were positive whereas about 5% gave discordant reactions.

B. Gel Diffusion Test

Ristic and Sibinovic (1964) obtained an antigen from the lysate of erythrocytes of horses acutely infected with B. caballi by means of precipitation with protamine sulfate. A precipitating antibody was demonstrated in horses with babesiosis by bringing the soluble Babesia antigen in gel into contact with serums of affected horses. Acute and subclinical latent phase serums gave equal precipitation responses. The specificity of the test was shown by the absence of reaction with serums of various other infections, including viral infectious anemia.

Sibinovic et al. (1965) reported an antigen in serums of horses acutely infected with B. caballi. Antibodies against this antigen were demonstrated by the gel diffusion test. Serum from an acutely infected horse was used as the antigen, and serums from convalescing horses were used as antibody. The antigen disappeared from the serum of infected horses approximately 1 month after experimental infection. Antibodies to the soluble Babesia antigen became demonstrable at 3 months after

infection and persisted for at least an additional 7 months.

Mahoney (1966) reported the occurrence of soluble antigens in the serum of cattle heavily infected with B. argentina or B. bigemina. In the preparation of antigens for use in CF tests for the diagnosis of infections with these organisms, heavy parasitemias were produced by serial passage of the organism through splenectomized calves. Plasma from infected calves was lyophilized and afterwards reconstituted to 1/3 of its original volume. These concentrated materials gave 2 precipitin lines in gel diffusion tests with serum obtained from cattle 2 to 4 weeks after recovery from the clinical effects of infection with the homologous organism. As control tests with serum from noninfected calves and with extracts of normal bovine erythrocyte strona were negative, the antigen-antibody reactions were assumed to be associated with babesial infection.

In studies of immunity to babesiosis in cattle, circulating antigens were detected in acute phase serum by gel diffusion tests (Mahoney, 1966) but, because of

weak precipitation shown by the bovine antigen-antibody systems, these reactions were found to be unsuitable for antigenic analysis. Antiserum prepared in rabbits against infected calf serum and then absorbed with normal calf serum contained precipitating antibodies to components apparently absent from the serum of noninfected calves (Mahoney and Goodger, 1969). The first of these was immunologically identical with bovine fibrinogen and its presence in serum was probably the result of intravascular coagulation and subsequent fibrinolysis. The second substance appeared to be a macromolecular complex containing a number of proteins found in the tissues of normal cattle. It was also found in serum of a calf with chemically induced hemolytic anemia and, thus, was unlikely that it contained material derived from the parasites. Antigens of the parasite were notably absent from the serums examined by Mahoney and Goodger (1969). They stated that if soluble products of the parasite were present, they should have been strongly immunogenic for the rabbit which is not the host species. Perhaps, as they further remarked, the soluble products either

did not induce a strong precipitin response or were in such minute quantity that detection was beyond the sensitivity of the gel diffusion method.

Sibinovic et al. (1969) compared the gel diffusion test with the bentonite agglutination and indirect hemagglutination tests for the detection of equine babesiosis. They reported the bentonite agglutination and indirect hemagglutination tests required less antigen and rendered results more rapidly than did the gel diffusion test.

Ristic et al. (1971) found B. canis and Babesia gibsoni to be serologically related on the basis of cross-reactions between erythrocytic and soluble antigens derived from these organisms and serums of dogs recovered from acute infections caused by each of the parasites. By means of a gel diffusion test each Babesia organism possessed at least one species-specific antigen.

Todorovic et al. (1971) used the gel diffusion test to characterize the antigen-antibody reactions of B. bigemina and B. argentina. A cross-reaction was observed

in the gel diffusion system between B. bigemina antigen and antibodies against B. argentina.

C. Agar-Gel Immunoelectrophoresis Technique

Sibinovic et al. (1965, 1967a) reported the serologic characterization of blood serum antigens from horses, dogs and rats with acute babesiosis. Each of the antigens was separated into 2 distinct fractions by density gradient centrifugation. Fraction A, found in the zone containing 25% sucrose, migrated electrophoretically as a gamma globulin and fraction A, found in the zone containing 40% sucrose, migrated as a beta globulin.

Mahoney and Goodger (1969) reported the isolation of 2 antigenic fractions in the serum of calves infected with B. argentina which were absent in the serum of non-infected calves. An antigenic fraction was precipitated from infected calf serum by 30% saturation with ammonium sulfate. Immunoelectrophoretic analysis of this fraction with specific rabbit antiserum showed that the antigen remained near the origin. A second antigen was present in the precipitate formed at 70% saturation of

infected calf serum with ammonium sulfate. Immunoelectrophoresis of this fraction revealed a wide precipitin arc encompassing the alpha, beta, and gamma regions.

Goodger (1973) obtained erythrocytes from a calf heavily infected with B. bigemina and lysed them with 5 volumes of cold distilled water after washing them 3 times with cold saline. The erythrocytes were centrifuged 15 min after the cold distilled water was added and the lysate supernatant collected and fractionated in a column of Sephadex G-100. The eluate was collected and the void volume fraction further separated by agar-gel immunoelectrophoresis. Two ml aliquots were subjected to electrophoresis at 4 C in 10 by 8 by 0.25 inch gel slabs using barbital-acetate buffer, pH 8.6, and a potential of 140 volts for 3 hr. Five fractions were obtained with mobilities corresponding to those of prealbumin, albumin, alpha, beta, and gamma globulins. Disc electrophoresis of B. bigemina antigens obtained by isolation of the material with beta globulin mobility on agar-gel electrophoresis demonstrated 3 faint protein bands. The fastest component migrated about 5 mm into the running gel.

D. Indirect Hemagglutination (IHA) Test

Curnow and Curnow (1967) reported on an indirect hemmagglutination (IHA) test for diagnosis of B. argentina infection in cattle. Formalinized sheep erythrocytes treated with tannic acid were sensitized with an extract of the parasite-erythrocyte stroma mixture used as antigen in the CF test for B. argentina. Bovine erythrocyte stroma antigen prepared from noninfected blood was used to absorb serums before CF testing. By doing this it was possible to remove some of the nonspecific reactions. The sensitivity and specificity of the IHA and CF tests were found to be similar, 70 out of 71 known infected animals being positive to each test.

Sibinovic et al. (1969) prepared a heat stable polysaccharide antigen extracted from Babesia infected erythrocytes of horses. The antigen permitted titration of antibody in the serum of horses affected with babesiosis by means of bentonite agglutination test and IHA test. The IHA test results were free of suspect reactions. In comparison with gel precipitation test, bentonite agglutination and IHA tests required less antigen and rendered results more rapidly.

Goodger (1971) reported on the preparation and preliminary assessment of purified antigens in the IHA test for bovine babesiosis. Two antigens, active in IHA tests for B. argentina infection, were obtained in relatively pure form from erythrocytes infected with the organism. One was obtained by gel filtration of soluble material obtained after sonic disruption of a parasite suspension. The other was prepared from the lysate of infected erythrocytes, first by removing hemoglobin on DEAE cellulose and separating the remaining material on Sephadex gel. Both antigens gave high titers (1:12,000 to 1:25,000) with B. argentina homologous antiserums. There were some low titer (1:100 to 1:200) cross-reactions with acute B. bigemina antiserums, but no reactions were found in preliminary tests of noninfected serum. With B. bigemina, an antigen was purified from sediment following sonic disruption and ultracentrifugation of infected erythrocytes. This antigen gave lower titers (1:1,600) with B. bigemina homologous antiserums than the B. argentina antigens did with homologous antiserums and cross-reacted strongly (1:1,600) with the

latter antiserums. These cross-reactions, however, could be absorbed with B. argentina antigen without affecting the specific B. bigemina reactions.

Ristic et al. (1971) reported on soluble and erythrocytic antigens isolated from blood of dogs infected with B. canis and B. gibsoni. In the dog infected with B. canis, the IHA antibody was detected 6 days after inoculation. Its concentration increased sharply to a titer of 1:640 on day 14 and then gradually subsided to the level of 1:40 at 5 months after the onset of the infection. In the dog infected with B. gibsoni, the rise of the IHA titer was noted approximately 22 days after inoculation, at approximately the same time the first parasites were detected in the peripheral blood films. The titer rose to 1:160 at approximately 4 weeks after inoculation and then gradually subsided to the level of 1:20 at 5 months after inoculation. With cross IHA tests between B. canis and B. gibsoni it was evident that homologous reactions occurred at serum titers which were at least 2 serial dilutions higher than those of heterologous reactions.

Goodger (1973) reported that the sensitivity of the IHA test for B. bigemina antibody was improved by using antigen obtained from the lysate of infected erythrocytes rather than the original antigen prepared from sonically disrupted parasites (Goodger, 1971). The lysate contained a species specific antigen as well as a related antigen associated with B. argentina. High titers (about 1:25,600) were obtained with B. bigemina antiserums. Cross-reaction titers with heterologous B. argentina antiserums were at similar levels, but by comparing the cross-reactions of antigens from both species, it is possible to differentiate between B. bigemina and B. argentina infections without recourse to inhibition techniques. However, inhibition would be needed to distinguish between B. argentina infection and mixed infection.

Host Response to Babesia Infections

Callow (1964) reported all of 9 splenectomized calves carrying B. bigemina at the time of iv challenge with 1×10^9 organisms developed parasitemias. In 2 receiving a homologous challenge, fewer than 1% of the

erythrocytes became infected, whereas 5 of the other 7, which were challenged heterologously, developed parasitemias within the range of 1 to 5%.

Callow (1967) reported on the immunity of steers to B. bigemina. Initial inoculations and subsequent challenges were with heavily parasitized blood collected during acute babesiosis of the donor. The number of organisms in any challenge was not less than 1×10^8 organisms, and when repeated attempts were made to overcome homologous immunity, approximately 1×10^9 organisms of B. bigemina were inoculated. Two strains of B. bigemina were used, the Y strain and the R strain. The Y strain was passaged bi-weekly with blood. The R strain underwent several tick passages during the course of the work, as well as being blood passaged somewhat irregularly. Ten intact steers which had been infected with Y strain B. bigemina 2 to 16 weeks earlier were grouped with respect to time after the primary reaction. One noninfected control steer was added to each group. The groups were challenged. Parasitemias following homologous (Y strain) challenges were shorter and possibly less intense than those following heterologous

(R strain) challenge, even though the control inoculated with Y strain reacted much more acutely than the control inoculated with R strain.

Hall et al. (1968) reported on 2 experiments concerning immunity of calves to B. bigemina infection. Cows were infected with B. bigemina during mid-pregnancy and again in late pregnancy. The cows and their progeny were challenged when the calves were less than 38 days of age. In the first experiment 2 different strains of the organism were used, 1 of them being the same as the strain subsequently used for challenge. In the second experiment only 1 strain was used and a challenge of at least 1000 infecting doses (1×10^8 organisms) of the same strain of organism was given. Comparable groups of animals were used as controls. The calves from cows given the homologous strain were highly resistant and 79% of them did not become infected. Calves from cows given the heterologous strain all became infected and their reactions were almost as severe as those of the fully susceptible control cows and their calves.

Antigenic Variation

Trypanosomes elicit the production of antibodies, yet chronic infections occur, with the periodic recrudescence of parasitemia. This phenomenon has been attributed to the appearance of antigenic variants (populations of trypanosomes which are unaffected by antibody to other variants and which in turn elicit the production of variant specific antibodies). More than 20 such variants were reported from 1 strain (Brown, 1963).

Broom and Brown (1940) reported that passage through a tsetse fly caused a relapse strain to revert to the parent type which had given rise to the initial infection. Gray (1965a, b) investigated this further and also examined the sequence of appearance of variants. During the early stages of a tsetse-transmitted *Trypanosoma brucei* infection, antigens appeared in a predictable order but were more variable in the later stages. The early variants were termed predominant antigens, and 1 antigenic type, called the basic strain antigen,

could always be isolated following cyclical infection. Tsetse flies which ingested the variant forms would transmit trypanosomes with either the basic strain antigen alone or with a mixture of this antigen and the ingested variant form. No antigen of a clone (aggregate of individual variants descended from a single variant) developed twice during an untreated infection (Gray, 1965a) and there was no correlation between drug sensitivity and antigenic variation (Gray, 1966).

The mechanism underlying antigenic variation is still not clear. Some investigators (Watkins, 1964; Seed and Gam, 1966) favor the idea of mutation and selection, perhaps similar to the mechanism suggested by Hawking and Walker (1966) to explain variable drug resistance, while others (Gray, 1965a) believe that adaptation by the trypanosomes best explains some of the observed responses.

Cox (1959) reported that mice with a chronic *Plasmodium berghei* infection were more susceptible to challenge with a relapse strain than with the homologous parent strain, indicating that the 2 strains differed

antigenically. More recently, antigenic variation was demonstrated conclusively in drug induced chronic P. knowlesi infections of rhesus monkeys (Brown and Brown, 1965; Brown et al., 1968). These investigators showed, using a schizont-infected cell agglutination test, that a succession of antigenically distinct populations occurred, each stimulating a specific agglutinin response. Each variant persists for only a few days but variation continues even when the infection is sub-patent (shown by subinoculation). Variants do not appear in a predictable order. Monkeys immune to 1 stabilate showed resistance to homologous challence. There was no cross-reaction when schizonts were tested with serums from monkeys infected with Plasmodium bastianellii or with another strain of P. knowlesi. In their assessment of the significance of antigen variations, Brown et al. (1968) concluded that since the relapses produced only low grade parasitemias in the experimental animals, but fulminating infections after subinoculation into non-immune animals, there must be a generalized immunity which overrode the variant specific response.

Smith and Kilborne (1893) considered that cattle which survived an acute attack with B. bigemina sometimes had a mild relapse after an interval of 3 to 8 weeks. Legg (1928) reported that relapses occurred in cattle subjected to stress. Davies et al. (1958) found 2 episodes of parasitemia in splenectomized calves following the inoculation of blood infected with Babesia divergens. The second parasitemia was lower than the first, but parasites persisted in detectable numbers for several days.

Intact calves, submitted to tick transmitted infection of either B. bigemina or B. argentina, developed a microscopically detectable parasitemia on several occasions over a period of many months. Mahoney (1962b) stated that the initial parasitemias were not high, nevertheless, there were later periods when parasites were sufficiently numerous to be detected again in thick blood films.

Callow (1964) demonstrated that a limited immunity is possessed by calves when they have been previously exposed to the challenging strain, and even then not

100% of these calves possess it. This immunity does not exist when a heterologous challenge is performed.

Barnett (1965), working with Babesia trautmanni, found peaks of parasitemia to occur in 6 to 8 cycles. Subsequent to 3 to 7 such cycles there were 1 to 2 cycles at intervals of 20 to 30 days at which time the parasites became rare and eventually disappeared.

Callow (1967) reported that 21 splenectomized calves subjected to heterologous challenge developed well marked parasitemias with B. bigemina. Nine of these had been highly immune to homologous challenge 2 weeks earlier. Eight of the 21 were carriers at the time of challenge and these showed much lower parasitemias than the spontaneously recovered calves which almost invariably were acutely affected. Challenges of 23 intact steers gave similar results. Six of 12 challenged homologously failed to show detectable parasitemia, whereas all 11 challenged heterologously developed parasitemias.

Mahoney (1967c) reported the presence of protective antibodies to B. argentina was demonstrated by the passive transfer of immunity with serum from carriers of the

organism to highly susceptible splenectomized calves. Serum obtained from donors after 1 infection apparently contained a low concentration of protective antibodies, but higher concentrations were evident in serum from donors after 4 or more repeated infections. Serum from naturally infected donors was less effective in conferring protection than serum from donors infected in the laboratory. Probably only the laboratory infections were homologous for the challenge strain. Mahoney (1967c) stated this anomaly might have been due to antigenic variation among strains of B. argentina.

Callow (1968b) stated homologous strain immunity may have a twofold importance in the study and control of babesiasis. First, in evaluating the production of acquired immunity to natural infection by various vaccination procedures, challenges should be with a heterologous strain. Second, for the long term maintenance of immunity to babesiasis by regular revaccination, either some antigenic variation should be introduced into the vaccine strain, or the strain changed regularly to one capable of causing a mild parasitemia in previously vaccinated cattle.

Curnow (1968) described an in vitro agglutination test of bovine erythrocytes infected with B. argentina. The test was used to investigate the possibility of antigenic variants of 1 or more strains of B. argentina from geographically distinct locations. If basic strain antigens could be identified and strains of the parasite classified, the technique would be valuable in epidemiological studies.

Hall et al. (1968) demonstrated a passive acquired immunity against B. bigemina transmitted from the immune cow to her calf, presumably through the colostrum, but the calf usually remained susceptible to strains of B. bigemina other than those used to immunize the cow.

Neitz (1969) found that vaccine strains of B. bigemina and B. bovis lose their virulence and resistance to chemotherapeutic agents when maintained by serial passage in cattle. This decrease may occur after only 3 passages in intact cattle and after 6 passages in splenectomized cattle.

Phillips (1969a) referred to the isolation of a population of B. rodhaini that was resistant to antiserum

in passive transfer tests which apparently indicated that the organism had the capacity to vary antigenically in order to evade the host's humoral response. Rats which recovered from the acute primary parasitemia of B. rodhaini had protective factors in their serum (Phillips, 1969b). The parasites that occurred during subsequent recrudescences maintained antiserum resistance at least until the second subpassage of the original isolation. Phillips (1969a) further stated that the antiserum resistant population could occur either due to selection and multiplication or by an antigenic variation in response to antiserum. That B. rodhaini definitely undergoes an antigenic variation in a natural chronic infection was not demonstrated (Phillips, 1969a).

Frerichs et al. (1969a) produced antigens for the CF test for B. caballi and B. equi infections. The greatest difficulty in producing B. caballi antigen was the low yield of antigen that was caused by low rates of parasitemia. Specificity of the B. caballi antigen, however, was not a problem. After B. caballi was subjected to extended serial passage from horse to horse

by inoculation the titers of the antigen against the known B. caballi and B. equi reference serums began to approach each other. A passage of B. caballi through its tick vector, D. nitens, caused a return to specificity. With the B. equi antigen the greatest problem in producing satisfactory antigen was its loss of specificity which occurred after the parasite was serially passaged in horses. The Florida isolate, when obtained, was highly specific in that it reacted only with specific B. equi antiserums or cross reacted with B. caballi antiserum only at the lowest antigen dilution tested (1:4). This specificity was lost while the parasite was being maintained in carrier horses; antigen prepared from B. equi recovered from carrier horses reacted at the same titer with both the B. caballi and the B. equi reference antiserums. Attempts to restore specificity by making rapid serial passages through horses, beginning with passage of blood from a carrier horse, were unsuccessful. The New Jersey isolate, when received, also was highly specific in its reactions with known B. equi serums, but this specificity was lost during subsequent

serial passages in horses. Unfortunately, a tick vector was not available for use in these experiments to determine whether the lost specificity could have been regained by passage of the parasites through the vector as was the B. caballi.

Phillips (1971a, b) reported evidence that B. rodhaini can undergo antigenic variation. Populations of B. rodhaini were isolated in 3 different circumstances although in all 3 the variant population eventually emerged in a splenectomized rat which had previously demonstrated acquired immunity to the reference or parent population. Thus, the variant populations probably emerged as a result of the inductive or selective pressures of the host's immune response. It also indicated the role of the spleen in preventing the appearance of acute parasitic recrudescences following the emergence of new variants. Rats immunized with irradiated reference population parasites, although showing no acquired immunity to the variant population in 3 or 4 days immediately after challenge, developed a response to it which was anamnestic in character. This suggested

that variant and reference populations share the same or have similar antigenic groups which are important in the induction of protective immunity to each variant. Five variant populations have been shown to have some antigenic dissimilarity with the parent population, including 2 populations from the same rat. It remains to be shown that the parasite undergoes repeated antigenic variation during the course of a single infection. It is well known that splenectomy of animals carrying subpatent infections of many Babesia parasites is followed by parasitic recrudescence. The experiments of Phillips (1971a, b) indicated that these recrudescences are composed of parasites of a population showing antigenic variation from the infecting population.

Curnow (1973a) found that B. argentina parasites collected at relapses in one calf were antigenically different from one another. When the relapse parasites were transmitted through the tick, B. microplus, they reverted to a common antigenic type which was thought to be strain specific. High agglutination titers were obtained in all homologous tests while heterologous

tests showed insignificant titers in all but one case. This indicated that there was a multiplicity of strain specific antigens present in the parasites found.

Curnow (1973b) reported antigenic differences existing between B. bigemina parasites by means of the agglutination test. Two isolates of B. bigemina were tick transmitted (TT) to 12 calves and antigens were prepared from the TT parasites. Testing of these antigens with antiserums to the TT parasites gave strong reactions in all homologous tests. In heterologous tests 9 of 12 tests showed a reaction but only 3 of these were as strong as in the homologous tests. The test was demonstrated to be capable of detecting antigenic differences between B. bigemina parasites.

MATERIALS AND METHODS

Experimental Animals

A. Calves

A total of 71 four-month-old male and female Holstein-Friesian calves were purchased from two farms in areas known to be free of B. microplus in Cundinamarca, South America. At approximately 5 months of age, after insuring that the calves were free from Anaplasma marginale and Babesia spp. by means of the CF tests, PCV, rectal temperature and blood smears, they were utilized as indicated in Table 1. The calves were given a grain ration and a 1:1 (v/v) mixture of salt and bone meal free choice throughout the experiment (Appendix Table I). Each calf was examined for the presence of internal parasites by fecal examination and treated once with Thibenzole (thiabendazole, Merck, Sharp & Dohme, Bogota, Colombia), and twice at a 15 day interval with Franozan (diethylcarbamazine citrate, Cooper, McDougall & Robertson Ltd., Bogota, Colombia) and Luvorem (isoniazide, Squibb, Bogota, Colombia) before the experiments began.

Table 1. Utilization of experimental calves.

Materials and Methods (Part)	No. of Calves (Splenectomized)	No. of Calves (Intact)
Purification of *Babesia bigemina*	6	2
Isolation of noninfected *Boophilus microplus*	6	0
Infection of *B. microplus* with *B. bigemina*	2	0
Blood borne/tick borne infection	1	10
Antigen production (acute and chronic blood and tick borne infections)	8	0
Host response to erythrocytic *B. bigemina* stabilates	0	36

The calves were vaccinated against foot and mouth disease, virus types A and O and with Tri-bac (*Clostridium chauvoei*, *Clostridium septicum*, *Pasteurella hemolytica* and *Pasteurella multocida* types 1 and 2, Cyanamid de Colombia (Lederle), Bogota, Colombia) before the experiments began.

Splenectomy of calves was carried out under general anesthesia or by combined use of a tranquilizer and a local anesthetic. Using 1 ml/kg of body weight of Equi-Thesin (an aqueous solution of chloral hydrate; 42.50 g/l, pentobarbitol; 9.72 g/l, and magnesium sulfate; 11.26 g/l, Jensen-Salsbury Laboratories, Kansas City, Missouri) or Tranvet (Propiopromazine hydrochloride, Diamond Laboratories, Des Moines, Iowa) and lidocaine locally, the spleen was exposed through a left lateral incision of the paralumbar fossa and removed after ligation of the splenic artery and vein. Bi-weekly blood smears and CF tests were performed for 2 to 3 months after splenectomy to detect natural infections of *Babesia* or *Anaplasma*.

B. Guinea Pigs

Six male and 6 female guinea pigs were obtained from the colony at Laboratorio de Investigaciones Medicas Veterinarias-Instituto Colombiano Agropecuario (LIMV-ICA), Bogota, Colombia. These animals (for use in B. microplus tick experiments) were transported to Turipana-ICA experiment station near Monteria, Colombia, where they were housed in pens constructed on the floor.

C. Rabbits

Twenty New Zealand white rabbits, 3 to 6 months, were purchased from a local supplier and used for the production of specific antiserum for serological tests.

Experimental Organism

A. Isolation, Purification and Maintenance of Babesia bigemina

Babesia bigemina was isolated from the blood of an acutely infected 4-month-old splenectomized calf exposed to field challenge with infected B. microplus ticks in Turipana (Bishop et al., 1973).

Experimental Vector

A. Isolation, Purification and Maintenance of *Boophilus microplus*

Engorged adult female *B. microplus* ticks were collected from adult Hostein-Friesian dairy cattle in Turipana and placed in 2 dram vials (Kimble, Owens-Illinois, Toledo, Ohio) stoppered with cottom plugs. The vials were placed in either a tick incubator (Thelco CO_2 incubator, Precision Scientific Co., Chicago, Illinois) or a desiccator jar (Roby et al., 1964) which had a saturated solution of ammonium sulfate to maintain the relative humidity at 78 to 80%. The temperature of both was maintained at 30 C. One to 3 days after collection, the engorged females began to deposit eggs. The engorged females, which deposited an average of 2,000 eggs/female, were maintained separately so that the progeny of each individual female was known. Approximately 21 days later larvae developed from eggs deposited by the engorged females. One to 2 weeks after the appearance of the first larvae, a period sufficient to allow the

larvae to mature and become active in search of a blood meal, they were placed on the ears (in ear bags) of splenectomized calves known free of A. marginale and Babesia spp. (1 progeny/calf). This permitted the screening of the progeny of each individual engorged female B. microplus for the presence of absence of A. marginale and Babesia spp.

The splenectomized calves were monitored daily by combination thin and thick blood films, PCV, rectal temperatures and weekly CF testing. Any calf found infected with A. marginale or Babesia spp. was treated to eliminate the infection, sprayed to kill the ticks and omitted from further screening trials. The splenectomized calves not showing evidence of A. marginale or Babesia spp. were monitored for 3 months and, after subinoculation of blood (500 ml, iv) to another splenectomized calf, were considered to be noninfected. The progeny of ticks on these calves were said to be free of A. marginale and Babesia spp. The adults of these ticks were again allowed to deposit eggs and the resulting larvae were placed on new splenectomized calves and again monitored to ensure that they were indeed free of A. marginale and Babesia spp.

Disease free guinea pigs were utilized in addition to splenectomized calves for maintenance of B. microplus ticks. The guinea pigs were shaven in the abdominal region, washed free of grease and dirt, and fitted with a band of surgical tape (Elastikon, Porous Adhesive tape, Johnson & Johnson, New Brunswick, N.J.) completely encircling the body. On the abdominal side of the band there was placed the upper 2 cm portion of a 500 ml nalgene plastic bottle (Kimble, Owens-Illinois, Toledo, Ohio) including the screw top. Within the plastic nalgene screw top (5 cm dia.) a circular hole (3 cm dia.) was cut, which was covered by a circular piece of womens nylon hose to prevent the escape of the immature stages of B. microplus. After placement of the band and screw top and allowing 1 day for the guinea pig to become accustomed to the apparatus, the top was unscrewed and 2 to 3-week-old B. microplus larvae (approximately 1000) were placed inside. The band was checked daily to insure that no necrosis occurred, and nymphal B. microplus (ave 100 to 200) were removed 1 to 2 weeks after the placement of the larval stages. The nymphal stages

were then placed on the bodies of splenectomized calves for completion of their life cycle.

Immature stages and adult B. microplus were also kept in desiccator jars with ammonium sulfate in an attempt to see if it were possible to maintain them for long periods without feeding. The temperature was that of the ambient temperature at Turipana (ave 30 C).

B. Infection of Babesia-free Boophilus microplus with Babesia bigemina

Approximately 20,000 larvae (1 gm of eggs = ave of 20,000 larvae) were placed on each of 2 splenectomized calves. These noninfected B. microplus larvae were placed on the bodies of the calves under a feed sack held in place with wire stretched under the neck and tail head. At the time the ticks were undergoing their nymphal molt the calves were inoculated iv with an infection dose (1×10^9 organisms) of purified B. bigemina. The prepatent period of the B. bigemina was 5 to 7 days which coincided with the period of rapid engorgement of the adults (the last 24 hours of adult

engorgement). The procedure of collecting mature ticks was standardized by gathering from the floor of the tick pen those ticks that had completed their final engorgement and detached themselves from the calf during the preceding hours. Collection occurred at 8 A.M. and again at 5 P.M. daily. The infected adults collected from these calves were maintained as previously described and their larvae later used in the tick borne infection of calves.

Acute and Chronic Blood Borne and Tick Borne Infections

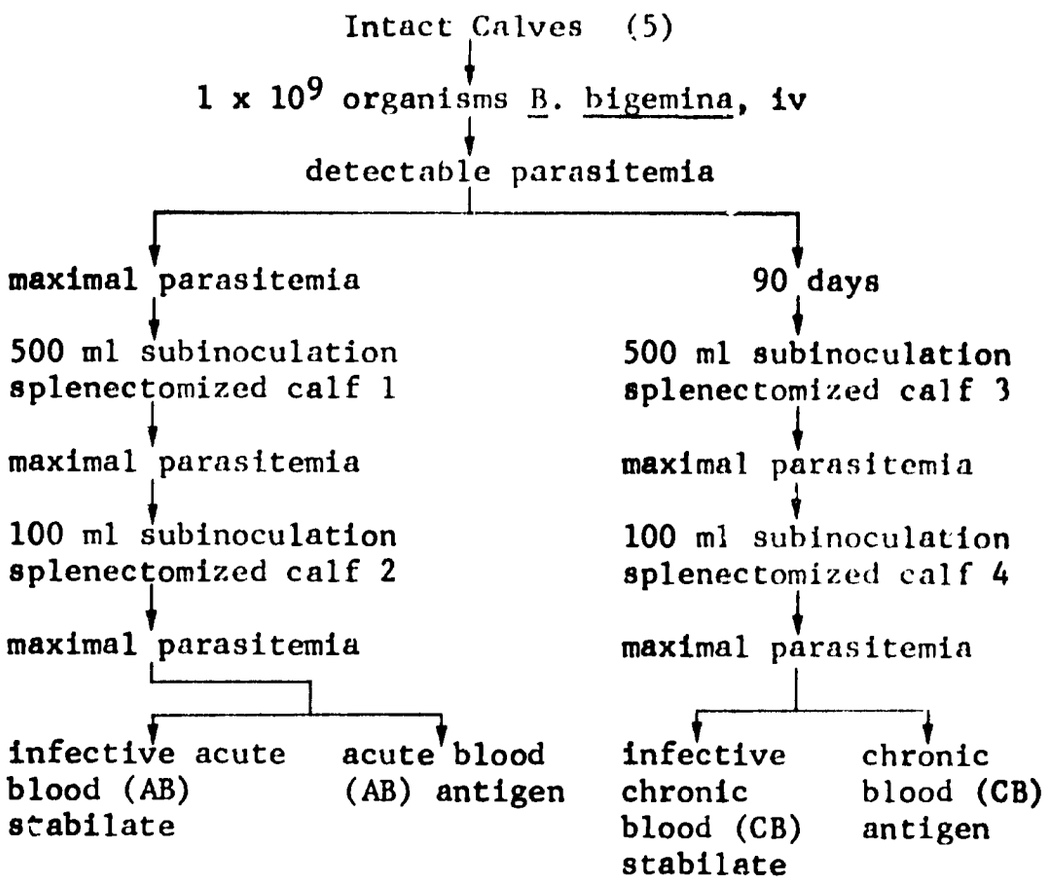
A. Acute Blood Borne Infection

Five intact calves known to be free of *A. marginale* and *Babesia* sp. were inoculated iv with an infection dose of 1×10^9 organisms of purified *B. bigemina*. At the time of maximal parasitemia, 100 ml of blood from each of the 5 calves was pooled and subinoculated iv into a splenectomized calf. At maximal parasitemia, the calf was bled and 100 ml of infected blood was subinoculated into a second splenectomized calf. When the

second splenectomized calf had a peak parasitemia, it was exsanguinated under general anesthesia and 2 l of the blood was mixed with 11.6% sterile glycerol and frozen at -70 C to form an infective blood stabilate. The remainder of the blood was processed to form the acute blood antigen, designated AB antigen (Table 2). All splenectomized calves (sections A to D below) were administered 30 mg/kg of a synthetic corticosteroid, Vetalog (Triamcinolone acetonate, Squibb, Bogota, Colombia) intramuscularly (im) for 3 days prior to and every other day post subinoculation until maximal parasitemia.

The method of Mahoney (1962, 1967) for the preparation of the Babesia erythrocytic antigens was used. Blood was taken by carotid artery cannulation from B. bigemina infected, splenectomized calves with high parasitemias into sterile 4 l Erlenmeyer flasks using EDTA (1.2 g/l, disodium ethylenediaminetetraacetate, J. T. Baker Chemical Co., Phillipsburg, New Jersey). The plasma and buffy coat were removed after centrifugation by aspiration and the erythrocytes were washed

Table 2. Schematic for production of acute and chronic blood borne *Babesia bigemina* stabilates and antigens



twice in sterile physiological saline. The volume of packed erythrocytes was measured and 10 times this volume of 0.35% sodium chloride at 5 C was added. Lysis was allowed to proceed for 1 hr at 5 C. The material was then centrifuged (Sorvall Superspeed, Model RC 2-B, Automatic Refrigerated Centrifuge, Ivan Sorvall Inc., Newtown, Connecticut) at 5,000 x g for 30 min at 5 C. After centrifugation, the sediment was resuspended in sterile physiological saline and was used to prepare the Babesia erythrocytic antigen extract. Distilled water extracts were prepared by mixing 1 volume of erythrocyte-parasite suspension with 2 volumes of distilled water. The preparation was homogenized (TenBroeck Tissue Grinder, Bellco Glass, Inc., Vineland, New Jersey) and then centrifuged at 5,000 x g for 30 min at 5 C. The supernatant fluid was collected and then the above procedure was repeated 5 times.

The supernatant fluids (serially labeled) were tested for antigenic activity by the gel diffusion test (Ristic and Sibinovic, 1964) and the CF test (Mahoney, 1962a) and then frozen rapidly to -79 C in 3 ml amounts

and stored at this temperature until used. Normal bovine erythrocytic antigen, designated NB antigen, was also prepared in a similar manner. The antigens stored by this method retained their antigenicity for at least 6 months (Curnow and Curnow, 1967). On the day of further processing, the antigens were thawed rapidly by immersing the vials in a water bath at 37 C.

B. Chronic Blood Borne Infection

Ninety days after the appearance of a detectable parasitemia the calves (described in section A above) were bled, the blood pooled (in equal volumes) and 500 ml subinoculated iv into a splenectomized calf. A second splenectomized calf was subinoculated iv with 100 ml at the peak of parasitemia of calf 1. When calf 2 had a maximal parasitemia it was exsanguinated and the blood processed as described in section A. An infective blood stabilate was prepared along with a chronic blood antigen, designated CB antigen (Table 2).

C. Acute Tick Borne Infection

Twenty thousand to 40,000 infected B. microplus larvae were allowed to feed on the bodies of 5 intact

Standard U-bottom disposable microtiter plates, 0.025 ml dropping pipettes, 0.025 microdiluters and plas-

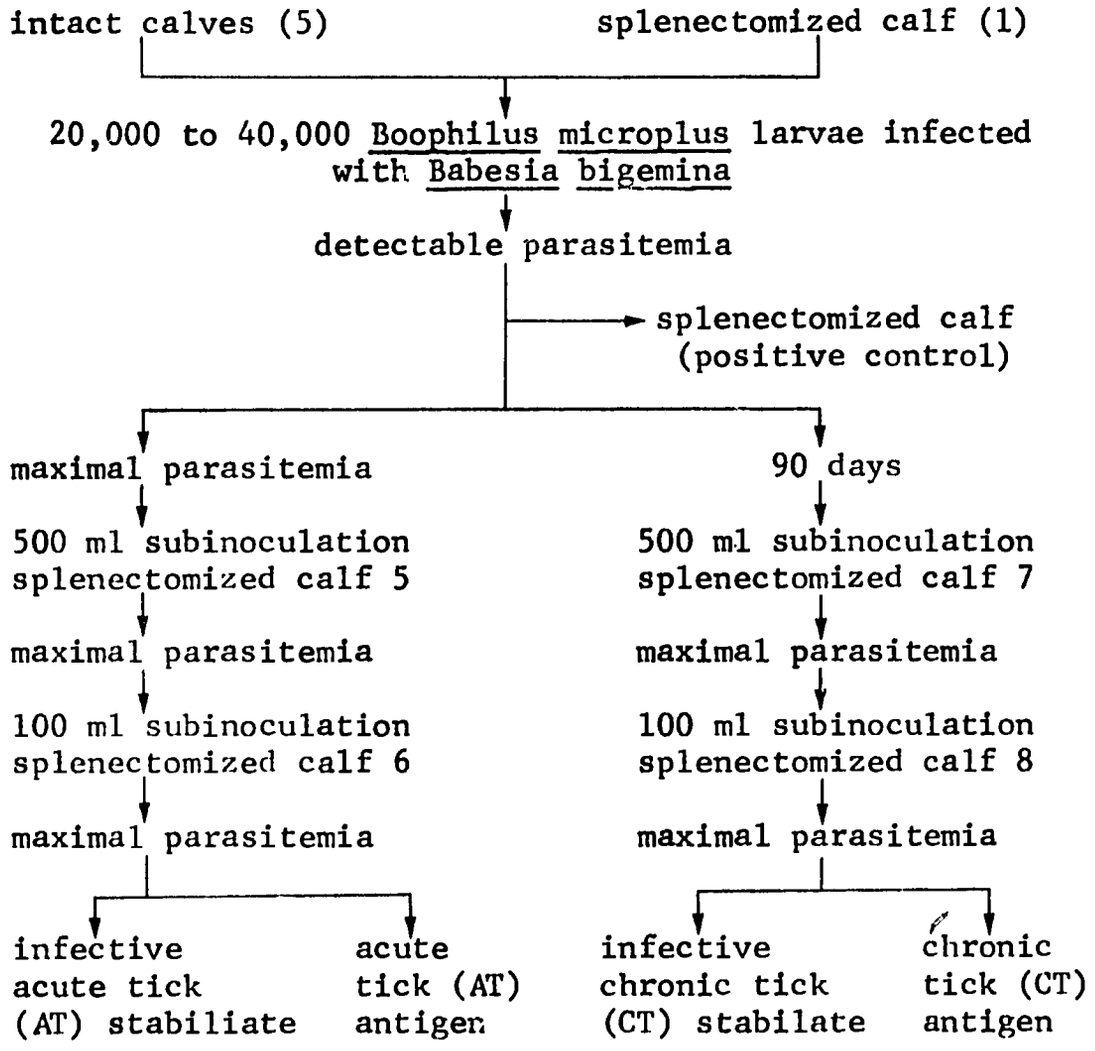
calves and 1 splenectomized calf. At the time of maximal parasitemia, 500 ml of the equally pooled blood from the 5 intact calves was subinoculated iv into a splenectomized calf. At peak parasitemia, the calf was bled and 100 ml of infected blood was subinoculated into a second splenectomized calf, which was managed in a similar manner to that described in section A. An infective blood stabilate was prepared along with an acute tick antigen, designated AT antigen (Table 3).

The 5 intact calves and 1 splenectomized calf were sprayed for 2 consecutive days then at weekly intervals with Pest Spray (Sevin, Salsbury Lab., Kansas City, Missouri) to remove all B. microplus. After the first weekly spraying there was no more evidence of live B. microplus so the calves were placed in clean pens.

D. Chronic Tick Borne Infection

Ninety days after the appearance of a detectable parasitemia the intact calves (section C) were bled, the blood pooled (in equal volumes) and 500 ml subinoculated iv into a splenectomized calf. A second splenectomized calf was subinoculated iv with 100 ml at the peak of

Table 3. Schematic for preparation of acute and chronic tick borne *Babesia bigemina* stabilates and antigens.



parasitemia of calf 1. When calf 2 had a maximal parasitemia it was exsanguinated and the blood processed as described in section A. An infective blood stabilate was prepared along with a chronic tick antigen, designated CT antigen (Table 3).

Isolation and Preparation of *Babesia bigemina* Antigens

A. Molecular Sieving

Purification of *Babesia* antigens by means of molecular sieving (Sibinovic et al., 1967a) was attempted. A 3 to 5 ml sample of *Babesia* erythrocytic antigen mixture was layered on top of a 2.5 by 65 cm Sephadex G-100 (Pharmacia Fine Chemicals, Inc., Piscataway, New Jersey) column (Pyrex Column, Corning Glass Works, Corning, New York) and a 2.5 by 48 cm Sephadex G-200 (Pharmacia Fine Chemicals, Inc., Piscataway, New Jersey) column from which 3 ml samples (Fraction Collector and Collector Activator; Volumetric Control Unit and a Photoelectric Volumetric Dispensing Head, Buchler Instruments, Fort Lee, New Jersey) were collected.

Prior to packing the pyrex glass columns (with fritted glass filters in one end) the gel was allowed to swell in 0.05 M Tris-HCl buffer long enough to reach equilibrium (3 days). The Tris-HCl buffer (pH 8.0) was prepared by first making a stock solution of the ingredients and then mixing the required proportions to yield the proper pH. Thus, to prepare 10 l of 0.05 M Tris-HCl buffer, pH 8.0, 18.15 gm of Tris (Sigma Chemical Co., St. Louis 3, Missouri) was added to 3,000 ml of distilled water and 6 ml of 37% concentrated HCl (11.6 M) was added to 1392 ml of distilled water. Then 2500 ml of Tris solution added to 1340 ml of HCl and made up to 10 l gave a solution with a pH 8.0. Thimersol (sodium ethylmercurithiosalicylate, Grade II, Sigma Chemical Co., St. Louis, Missouri; 1 gm/10 l) was added to the 10 l to prevent bacterial growth. After swelling of the Sephadex, the fine particles were removed by decantation in large beakers, and the gel and all buffers were degassed. The columns were then aligned vertically, filled to 3/4 capacity with buffer and 2 cm of fine sand were placed on top of the fritted glass filters. A

buffer reservoir was attached to the top of each column and the hydrostatic head was kept within 16 to 20 cm. The gel suspension was poured slowly down a glass stirring rod and the gel allowed to settle for a few centimeters with the outlet at the bottom of the columns closed. The outlets were then opened and the columns allowed to slowly pack. After initial packing, Tris-HCl buffer was allowed to flow through the columns for a minimum of 2 days before use and the flow rates were adjusted to approximately 5 ml/hr.

The Babesia erythrocytic antigens (3 to 5 ml) were layered very carefully on the top of the columns and 3 ml fractions were collected. The fractions were tested for antigenic activity and protein content. The gel diffusion test was used to demonstrate the antigenic activity of the column fractions. The amount of protein was measured at 280 mμ using a spectrophotometer (Beckman Spectrophotometer, Model DB, with a hydrogen lamp power supply, Beckman Instruments, Inc., Scientific and Process Instruments Division, Fullerton, California).

Preparation of Specific Antiserum

Three New Zealand white rabbits were utilized for each antigen. Three to 7 mg/ml of erythrocytic antigen plus an equal amount of Freund's complete adjuvant (Bacto Adjuvant, Complete, Freund, Difco Laboratories, Detroit, Michigan) were emulsified for 10 min and inoculated intradermally (1 ml/rabbit) in 6 sites in the axillary and inguinal portion of the legs and the abdominal skin of the rabbits with a 23 gauge needle. After 5 inoculations (day 0, 15 and 22 with adjuvant and day 28 and 38 without adjuvant), the rabbits were bled (10 ml) from the marginal ear vein on day 45 and the serum was tested for the presence of specific antibodies by the gel diffusion test.

Host Response to the Infective *Babesia* Erythrocytic Stabilates

Thirty-six intact calves were divided into 16 groups of 2 (or 3) and each group inoculated with infective *Babesia* erythrocytic stabilates (1×10^9 organisms). Twenty-eight days later they were homologously and

heterologously challenged iv with 1 x 10^{10} organisms from the same stabilates, and monitored for an additional 20 days (Table 4). To measure the response of the calves, the following daily parameters were determined: (1) temperature, (2) PCV, (3) blood smears and (4) CF response. The weights were estimated at weekly intervals.

Assessment of Reactions

A. Parasitemia

The preparation and interpretation of blood smears were performed daily. The blood was taken from the jugular vein using a 12 ml disposable plastic syringe and an 18 gauge needle (Sherwood Medical Industries, Inc., Deland, Florida) with sufficient EDTA to form a final concentration of 1.2 g/l. The blood films were first fixed with absolute methanol and then stained with a 1:20 solution of Giemsa (Giemsa Stain, Gradwohl Laboratories, St. Louis, Missouri) for 20 to 30 min. The Giemsa stain solution was prepared by using phosphate buffered water (pH 7.0 to 7.1) to which was added a 0.01% Triton X-100 (alkyl-phenoxy-polyethoxy-ethanol,

Table 4. Experimental design for primary inoculation and subsequent homologous and heterologous challenge with viable *Babesia bigemina* isolated from acute and chronic blood borne and tick borne infections in cattle.

Stabilate Used for Infection	Number of Calves/Group	Stabilate Used for Challenge (day 28 PI[1])
	2	acute blood borne
acute blood borne	3	chronic blood borne
10 calves	2	acute tick borne
	3	chronic tick borne
	3	acute blood borne
chronic blood borne	2	chronic blood borne
10 calves	2	acute tick borne
	3	chronic tick borne
	2	acute blood borne
acute tick borne	2	chronic blood borne
8 calves	2	acute tick borne
	2	chronic tick borne
	2	acute blood borne
chronic tick borne	2	chronic blood borne
8 calves	2	acute tick borne
	2	chronic tick borne

[1]PI = Post inoculation

J. T. Baker Chemical Co., Phillipsburg, New Jersey). The phosphate buffered water containing Triton X-100 was prepared weekly by adding 39 ml of 0.15 M NaH_2PO_4, 61 ml of 0.15 M Na_2HPO_4, and 1 ml of 10% Triton X-100 to 899 ml of distilled water. An electric pH meter (Beckman Expandomatic SS-2, Model 76, Beckman Instruments, Inc., Scientific and Process Instruments Division, Fullerton, California) was used to determine the pH of the freshly prepared phosphate buffered water.

The blood films were examined for 20 to 30 min each using a Bausch & Lomb microscope (Bausch & Lomb Inc., Rochester, New York) with an oil immersion objective (90 X) by transversing the film from one side to the other approximately 3 to 6 mm from the feathered end.

B. Thin and Thick Blood Film

The examination for the presence of parasitized erythrocytes was facilitated by the use of combination thin and thick blood films (Bishop and Adams, 1973). For preparation of thick blood films a loop of 2.5 mm external diameter was made by twisting 30 gauge wire tightly

around a 14 gauge hypodermic needle and back around itself to form a handle, and then withdrawing the needle. This loop delivered approximately 1.4 mm^3 of blood when it was touched on a clean glass slide. After blood remaining on the wire had been quickly wiped off on a clean cloth, the loop was used to spread the drop evenly over an area of 28 mm^2 which was outlined by a circle 6 mm in diameter marked below the glass slide on a piece of paper. This wet film was approximately 50 mμ thick. Next, the thick film of blood that had been placed 3 to 5 mm from the end of the glass slide was air dried. Final drying was done in an incubator (W. H. Curtin & Company, Chicago Surgical & Electrical Co., Division of Lab-Line Instruments, Inc., Melrose Park, Illinois) at 65 C for 45 min, to prevent the film from washing off during the staining process. After the glass slide with the thick blood film was removed from the incubator and cooled, the thin blood film was prepared over one-half the length of the slide opposite the thick blood film. The thin blood film was fixed with absolute methyl alcohol and the slide was held in a slanted position with

the thin blood film down to prevent alcohol or alcohol fumes from contacting the thick blood film. One to 3 min after the application of absolute methyl alcohol the combination thin and thick blood film was air dried and then stained. The slide was placed in a 1:50 solution of Giemsa stain for 45 min to lyse the nonfixed erythrocytes in the thick film and to stain the thin and thick blood films. Phosphate buffered water containing Triton X-100 was used to prepare the stain solution. After staining, the slide was rinsed briefly in a staining dish filled with the phosphate buffered water. The slide was then placed in the same staining dish with only the thick blood film immersed for an additional 3 to 5 min. The slide was then air dried in the vertical position with the thick blood film down.

An estimate of the number of parasites per cubic millimeter of blood on thick blood films could be made from the number of parasites and leukocytes seen in 20 microscopic fields (using X 10 ocular and X 100 oil objective) and from the number of leukocytes per cubic millimeter of blood. The number of parasites per cubic

millimeter of blood was then converted to the percentage of erythrocytes from the number of erythrocytes per cubic millimeter of blood. Calculations were made according to the following formula:

$$\text{\% infected erythrocytes} = \frac{\dfrac{\underline{B.bigemina}/20 \text{ fields}}{\text{WBC}/20 \text{ fields}} \times \text{WBC}/\text{mm}^{3*}}{\text{RBC}/\text{mm}^{3**}} \times 100$$

*minimal ave WBC (7000 mm^3)
**minimal ave RBC ($5 \times 10^6/mm^3$)

C. Packed Cell Volume (PCV)

The PCV was determined by the micro-hematocrit method using jugular blood in 1.2 g/l EDTA. Plain micro-hematocrit capillary tubes (1.2 by 75 mm, Blu-tip, Sherwood Medical Industries, Inc., St. Louis, Missouri) were filled with jugular blood and the end containing blood was sealed with clay (Seal-Ease, Clay Adams, Parsippany, New Jersey). The tubes were then centrifuged (Adams Autocrit Centrifuge, Clay Adams, Parsippany, New Jersey) at 11,000 x g for 5 min. The PCV's were read directly from the graphic reader of the micro-capillary centrifuge.

D. Temperature

Rectal temperatures were measured on all calves at 9:00 A.M. Individual records were kept and mean values calculated and recorded.

E. Body Weight

The body weight of all calves was estimated by placing a measuring tape (Cinta Metrica para Vacas de Razas Lecheras, Nasco, Fort Atkinson, Wisconsin) around the calf from the point of the shoulders about the ribs and under the brisket. The tape was pulled snugly and the weight in kilograms was read directly from the tape.

F. Complement Fixation (CF) Test

The pre- and post-inoculation calf blood to be tested in the CF microtiter procedure (Mahoney, 1962a; Hidalgo and Dimopoullos, 1967; Todorovic et al., 1971) was collected by jugular puncture, allowed to clot, and the serum recovered after centrifugation (International Centrifuge, Model UV, International Equipment Co., Needham Heights, Massachusetts) at 1,000 x g for 20 min. Serum samples were collected from all experimental calves daily and tested weekly by the CF microtiter procedure.

Standard U-bottom disposable microtiter plates, 0.025 ml dropping pipettes, 0.025 microdiluters and plastic plate sealers (Cook Engineering Co., Medical Research Division, Alexandria, Virginia) were used for the CF microtiter procedure. All reagents in the CF microtiter procedure were diluted with veronal buffer which was prepared and maintained as described in the standard anaplasmosis CF procedure (Anon., 1958).

The hemolytic system was prepared and titrated as previously described (Anon., 1958) and consisted of a 2% suspension of sheep erythrocytes and 2 units/ml of commercially produced hemolysin (Sheep Cell Hemolysin, The Sylvana Co., Millburn, New Jersey). In the preparation of the hemolytic system for the CF microtiter procedure, an equal amount of a 2% sheep erythrocyte suspension was added to hemolysin (2 units/ml) and stored at 5 C for one day before using.

Complement (lyophilized Guinea Pig Complement, Texas Biological Lab., Inc., Fort Worth, Texas) was diluted and titrated according to the scheme described (Anon., 1958).

The prepared antigens were titrated against 3

positive and 1 negative reference serums according to the scheme previously described (Anon, 1958). The positive serums were from cattle that had recovered and were carriers of blood induced infections. A unit of antigen was defined as the highest dilution causing complete fixation of an equal volume of complement (2 units/ml) in the presence of the weakest positive reference serum diluted 1:5.

A 2% sheep erythrocyte suspension and dilutions of antigen, complement and hemolysin each containing 2 units/ml were prepared to conduct the diagnostic CF microtiter procedure. Serum dilutions of inactivated serums (56 C/30 min) from 1:5 to 1:640 were tested. Serum anticomplementary controls on all serums tested were prepared and read according to the previously described scheme (Anon., 1958).

Antigen, complement and hemolytic system controls and at least 1 positive serum of known titer and 1 negative serum were included. After final incubation, the plates were placed for 2 hr at 5 C to allow nonhemolysed cells to form sediments in the bottom of the cups.

Reactions were read by placing the transparent plastic plates on a concave microtiter mirror stand (Cooke Engineering Co., Medical Research Division, Alexandria, Virginia). The titer of the serum was considered to be the highest dilution, having complete fixation.

G. Gel Diffusion Test

The gel diffusion test and the agar-gel immunoelectrophoresis technique (Davis, 1964; Ornstein, 1964; and Todorovic, 1967) were used to further characterize the Babesia erythrocytic antigens.

Nonfrosted microscope slides (Fisher Scientific Co.) were cleaned in a solution of concentrated sulfuric acid. Afterwards, the slides were washed 2 to 4 times in distilled water, then in an ethanol-acetone mixture (v/v) and then dried well with Kimwipes (Kimberly-Clark Corp., Neenah, Wisconsin). The clean slides were placed in Gelman plastic racks which were placed on Gelman leveling tables (Gelman Products, Gelman Instruments Co., Ann Arbor, Michigan) and the tables were leveled. Next, buffer was prepared by placing 1 package of Gelman High Resolution buffer (Gelman Products,

Gelman Instrument Co., Ann Arbor, Michigan) in a sterile flask and bringing the level of distilled water to 1,800 ml. A 1:10,000 solution of Thimersal was added to retard bacterial growth, and then the pH was adjusted to 8.4 to 8.7. The buffer was then filtered using Whatman #1 filter paper (12.5 cm dia, Scientific Products Edison, New Jersey) and placed at 5 C. A 1% agar solution was prepared by placing Ionagar #2 (Consolidated Lab., Inc., Chicago Heights, Illinois; 1 gm/100 ml) in a solution of 25 ml Gelman High Resolution buffer and 75 ml of distilled water. The solution was heated in a flask that was placed in a beaker of boiling water. When the agar went into solution it was pipetted onto the slides that were placed on the leveling table. After 10 to 15 min the plastic trays with the slides were removed from the leveling table and put in a refrigerator to allow the agar to become firm. One hr later the slides were removed from the refrigerator and patterns were cut into the agar-gel (wells 4 mm in diameter, placed 2 mm from the central well or trough) and the wells were filled with antigen and antiserum. Control

patterns employed preinoculation serum of the same calves and preinoculation serum of the rabbits used for the preparation of antiserums against the specific antigens. Serum absorption was accomplished using the following procedure: One drop of normal bovine serum, plasma or normal bovine erythrocytes were added to 5 drops of rabbit antiserum and the mixture incubated first at 37 C for 45 min and then at 5 C for 45 min and subsequently centrifuged at 2,000 x g for 20 min.

After the addition of the antigen and antiserums to the wells, the slides were placed at room temperature in a moist chamber and read in a minimum of 3 to 5 days, after the unreacted protein had been washed out. The plastic trays with slides were placed in a 2% NaCl solution at room temperature and the solution changed about 4 times over a 24 hr period. The slide trays were then rinsed in distilled water for 4 to 6 hr at room temperature, after which they were photographed with an immunodiffusion camera (Cordis Corporation, Miami, Florida) or stained and then photographed.

To dry the slides a piece of filter paper saturated

with distilled water was placed exactly over the gel surface on the slide. As the filter paper dried, it removed water and any salt left from the agar-gel, so that the agar dried evenly. Care was taken to fill all wells and troughs with distilled water and to see that no trapped air bubbles remained, ensuring against retraction or cracking of the agar with distortion of the pattern. When the paper was completely dry it was removed, and the slides were then ready for staining. The protein stain was prepared by dissolving 1 to 2 grams of Buffalo Black (Amido Schwartz 10B, Fisher Scientific Co., Houston, Texas) in 1,500 ml of rinsing solution. It was then filtered and placed in a brown bottle to prevent light from destroying the solution. The rinsing solution (solvent for the stain) was prepared by mixing 45 ml of methyl alcohol, 10 ml of acetic acid, and 45 ml of distilled water. Next, the staining tank was filled with Buffalo Black and the slide racks dipped into it for 5 min. Then, the racks were placed into 5 consecutive rinsing tanks for 10 min in each tank. Afterwards they were removed and allowed to air dry. When the

slides were dry, they were carefully removed from the plastic frames and the slides photographed.

H. Agar-Gel Immunoelectrophoresis Technique

Agar-gel immunoelectrophoresis was performed utilizing a well and trough pattern 2 mm apart. The pattern found most convenient consisted of a central well with 2 troughs on either side. The wells in the agar-gel were cut by using a stainless steel hollow cylinder, 10 mm in length and an outside diameter of 8 mm. The troughs were cut using stainless steel parallel blades 2 mm apart (Gelman Products, Gelman Instrument Co., Ann Arbor, Michigan). The agar-gel was removed from the wells with a Pasteur pipette that had a suction bulb attached to one end. The holes were filled with antigen (about 0.005 ml) using a ½ ml syringe and a 27 gauge needle (or melting point capillary tube drawn to a fine tip). The electrophoresis chamber (Model 51170-1, Gelman Instrument Co., Ann Arbor, Michigan) was then filled with 1 liter of Gelman High Resolution buffer (5 C). After the buffer was equally balanced in the chamber, the slide trays were placed between the two

buffer compartments in such a position that the holes containing the antigen were nearer to the cathode than to the anode. Presoaked lead acetate wicks (Gelman Products, Gelman Instrument Co., Ann Arbor, Michigan) were situated one on each end of the slide, so that each covered about ½ cm of the agar-gel on the slide. The other end of the wicks were allowed to hang into the buffer in the compartments. The chamber was then connected to the power supply (Electrophoresis Power Supply, Model 38201, W. H. Curtin Co., Chicago Surgical & Electrical Co., Division of Lab-Line Instruments, Inc., Melrose Park, Illinois; 3 to 5 ma/tray). The length of time, voltage and amperage depended on the material being separated and was determined empirically for each system. The time found ideal was 2 hr with no more than 250 volts. At the termination of the electrophoretic separation the agar-gel was removed from the trough by using a metal plate (Gelman Products, Gelman Instrument Co., Ann Arbor, Michigan) having a 1 mm wide projection at one end and a 2 mm projection at the other end, and widening out to about 2.54 cm throughout the rest of

the plate. The projection (if the trough was a 1 mm trough, the 1 mm projection was used) was slipped along underneath the agar-gel in the trough and the agar-gel lifted and slid up on the metal plate to facilitate removal. The trough was filled with specific antiserum (about 0.07 ml) and the slide tray was then placed on a level surface at room temperature in a moist chamber to allow the pattern of precipitation to develop. After a minimum of 3 to 5 days, the slides were washed as described in the procedure of the agar-gel precipitation and photographed with the immunodiffusion camera or stained and then photographed.

I. Indirect Hemagglutination (IHA) Test

The indirect hemagglutination test (Stavitsky, 1954; Butler, 1963; Curnow, 1968 and Campbell et al., 1970) aided in further characterizing the Babesia erythrocytic antigens. Sheep blood was aseptically collected in a sterile flask containing an equal volume of sterile Alsever's solution (sodium citrate, 12 gms; sodium chloride, 4.2 gms; dextrose, 20.5 gms; and distilled water to make 1 liter). The blood kept at 5 C,

was allowed to stabilize for at least 3 days before using and thereafter was used for up to 2 weeks. At the beginning of each day 3 ml of sheep red blood cells in Alsevers solution were pipetted into a 20 ml test tube and 10 ml of saline was added. The cells and saline were gently mixed, then centrifuged at 500 x g for 5 min. The supernatant was carefully removed by aspiration and the cells were washed 3 more times. To prepare the cell suspension, 0.5 ml of the packed, washed red blood cells were added to 20 ml of buffered saline (pH 7.2).

Phosphate buffered saline, pH 7.2, was prepared by mixing 100 ml of saline plus 100 ml of buffer consisting of 23.9 ml of 0.15 M KH_2PO_4 and 76.0 of 0.15 M Na_2HPO_4. The pH was checked on a pH meter and adjusted as necessary with either acid or base. Stock solutions of the phosphate buffer components were prepared and stored in brown pyrex bottles in the refrigerator. Potassium acid phosphate (Sorensen Salt, Merck, Sharp & Dohme, Arlington, Texas; 0.15 M KH_2PO_4) was prepared by adding 9.08 gm to enough distilled water to make 1 liter. Disodium phospate (Anhydrous Disodium Phospate, Merck, Sharp &

Dohme, Arlington, Texas) was prepared by adding 9.47 gm to enough distilled water to make 1 liter (0.15 M Na_2HPO_4).

The packed, washed red cells and the buffered saline were gently but thoroughly mixed. After mixing they were standardized by adding 2 ml of the cell suspension to 10 ml of distilled water and the optical density read at 520 mμ in a spectrophotometer. The reading should have been within the range of 0.40 to 0.60. If it was greater or less, it was corrected by the addition of either buffered saline or packed, washed cells. After the cell suspension was standardized, 3 ml was pipetted into a centrifuge tube to which was added 3 ml of dilute tannic acid (Reagent Powder, Matheson, Coleman and Bell, Norwood (Cincinnati), Ohio) solution. A 1:100 dilution of tannic acid was kept at 5 C as a stock solution from which a 1:20,000 dilution was made daily. The cell suspension and tannic acid were gently mixed and placed in a water bath at 37 C for 10 min. After 10 min, the suspension was centrifuged for 5 min at 500 x g. The supernatant was aspirated and the packed

cells were resuspended in 3 ml of buffered saline (pH 7.2). The cells were gently mixed and centrifuged again for 5 min at 500 x g. The supernatant was aspirated and the cells were again resuspended in 3 ml of buffered saline (pH 7.2) and the suspension gently mixed.

To coat the tanned red blood cells with crude antigen (fraction C of AB, CB, AT and CT), 4 ml of buffered saline (pH 6.4) was pipetted into a 12 ml test tube. The buffered saline was prepared by mixing 100 ml of saline plus 100 ml of buffer consisting of 32.2 ml of 0.15 M Na_2HPO_4 and 67.8 ml of 0.15 M KH_2PO_4. The pH was checked on a pH meter and adjusted as necessary with either the acid or the base. To the test tube with buffered saline (pH 6.4), 1 ml of crude antigen solution and 1 ml of tanned red cell suspension were added. The crude antigen solutions were all standardized to contain 0.20 mg of protein/ml as determined spectrophotometrically. Control tanned red blood cells were prepared in a similar manner with 1 ml of saline being added instead of 1 ml of crude antigen suspension. The tubes were mixed gently and allowed to incubate at room temperature

for 10 min. After incubation the tubes were centrifuged for 5 min at 500 x g. The supernatants were aspirated and 2 ml of diluent with normal rabbit serum or gelatin (Bacto "Difco" Certified, Detroit, Michigan) were added. After gently mixing the tubes they were centrifuged for 5 min at 500 x g, and the supernatants were aspirated. The cells were resuspended in 1 ml of diluent-normal rabbit serum (or gelatin) and then used in the test.

Because the coated cells sometimes spontaneously agglutinated in saline alone, saline with normal rabbit serum or gelatin added was used as a diluent. Inactivated, absorbed 1:100 (v/v) dilution or normal rabbit serum in saline or 2 mg/ml (0.2%) gelatin in saline was used. Prior to use both normal serum and antiserums were heated in a 56 C water bath for 30 min and then absorbed with an equal volume of washed sheep red blood cells and then an equal volume of washed normal bovine red blood cells for 15 min each at room temperature.

The antiserums used in the test were pooled bovine antiserums. The AB antiserum was pooled from 4 AB infected calves 28 days post inoculation which had CF

titers of 1:40, 1:80, 1:640, and 1:80. The CB antiserum was pooled from 4 CB infected calves 28 days post inoculation which had CF titers of 1:160, 1:80, 1:160, and 1:80. The AT antiserum was pooled from one AT calf on 4 days (day 27, 28, 29, and 30) post inoculation. This was the only AT calf which showed a detectable parasitemia on thick and thin blood smears. Its CF titers on the days of collection were 1:160, 1:160, 1:160, and 1:80. The CT antiserum was pooled from 4 CT infected calves 28 days post inoculation which had CF titers of 1:40, 1:80, 1:40, and 1:80. The 4 calves in the pools except the AT pool represented those calves with highest CF response of the 8 calves in the respective groups.

Twelve test tubes (13 x 100 mm) were placed in a test tube rack. They were numbered 1 through 12 and then 0.5 ml of diluent was pipetted into tubes 2 through 10 and tube 12. A 0.5 ml of a 1:5 dilution of the pooled bovine antiserum was pipetted into tubes 1, 2, and 11. The contents of tube 2 were mixed and 0.5 ml pipetted from tube 2 into tube 3. These twofold serial dilutions were continued through tube 9, discarding 0.5 ml from

tube 9 after mixing. A 0.05 ml suspension of crude antigen coated tanned cells were added to tubes 1 through 10. A 0.05 ml suspension of control cells were added to tubes 11 and 12. The racks were shaken thoroughly to effect complete suspension of cells and then covered and allowed to stand at room temperature. After 2 hr the patterns formed by the cells at the bottom of each tube were examined. Tube 10 served as a control for the diluent; tube 11, as a control for nonspecific reaction in the antiserum; and tube 12, for nonspecific reaction in the tanned red blood cells. Tubes 10, 11 and 12 should have been negative. Each of the tubes was scored as plus, plus/minus, or minus, in accord with the following definitions. A compact granular agglutination or diffuse film of agglutinated cells covering the bottom of the tube, with edges of the film either folded or somewhat ragged was considered a plus. A narrow ring of cells surrounding a diffuse film of agglutinated cells was a plus/minus. A heavy ring of cells or discrete smooth button of cells in the center of the tube was a minus.

RESULTS

Isolation, Purification and Maintenance of *Babesia bigemina*

Babesia bigemina (Fig. 1) was isolated from a splenectomized calf which was placed on a tick infested pasture at the Turipana ICA station near Monteria, Colombia. The calf was heavily infested with *B. microplus* ticks in the larval stage within 3 days, ticks in the nymphal stage within 8 days, and ticks in the adult stage within 14 days. After 16 days, the body temperature of the calf rose to 40.4 C and *B. argentina* was found in thin blood smears stained with Giemsa. Two days later, *B. bigemina* was found in blood smears. The results of the rapid passages of *B. bigemina* through 5 splenectomized calves to separate it from contaminating organisms are summarized in Table 5. A stabilate of *B. bigemina* was made from the fifth calf, 4 days after inoculation when the parasitemia had reached 2.5%. This stabilate was used in the subsequent experiments to infect *B. microplus* ticks and also to derive the acute

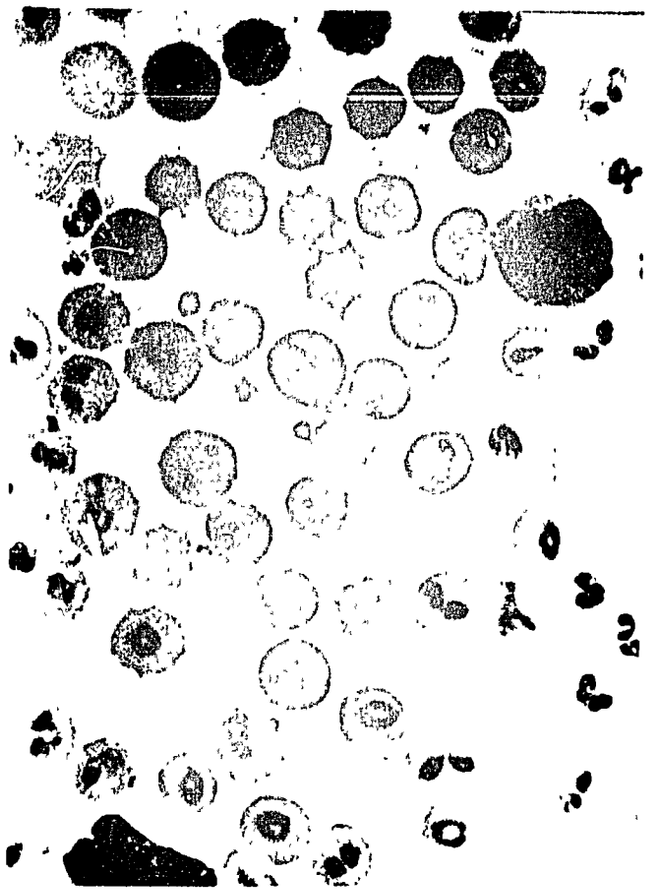


Figure 1. Babesia bigemina parasitized bovine erythrocytes in thin blood film. Giemsa. x1300.

Table 5. The isolation of *Babesia bigemina* from a natural, tick borne, mixed infection also containing *Babesia argentina*, *Babesia major* and *Anaplasma marginale* by rapid serial passage through 5 splenectomized calves.

Passage Level	Inoculum (iv)	Prepatent Period *B. bigemina* (hrs)	Time of Subinoculation (hrs)	Other Organisms Detected
1	180 ml from naturally infected calf	36	37½	*B. argentina* *B. major* *A. marginale*
2	12 ml from No. 1	30	31	*B. argentina* *
3	12 ml from No. 2	24	24	*B. argentina* *B. major* *
4	12 ml from No. 3	37½	37½	0
5	12 ml from No. 4	57½	---	0

*It was not known if *A. marginale* was present in passage level 2 and 3 because these calves died prior to the time that this parasite is normally detected.

blood borne calf infection. This permitted the utilization of a common *B. bigemina* organisms for all experiments.

Isolation, Purification and Maintenance of *Boophilus microplus* Ticks

The complete life cycle of *B. microplus* on the Turipana ICA station was completed in 42 to 52 days. Preoviposition, depending upon temperature and relative humidity, lasted up to 3 days. Deposition of eggs similarly influenced, was completed in 5 to 10 days. Usually within 21 days after preoviposition the eggs hatched into larvae. A small percentage of newly emerged larvae wandered over the host's body for up to 2 days. The larvae fed for 7 days after attachment and then molted into nymphs. Likewise, some nymphs moved about over the body for up to 2 days before feeding. The nymphal period was 7 days after attachment. The nymphal stage then molted to an adult which had an engorgement period of 7 to 10 days.

Fecundity of the females and hatchability of the

eggs are shown in Appendix Tables II and III, respectively.

Disease free guinea pigs were utilized with only moderate success to maintain noninfected B. microplus ticks. Nymphal B. microplus were removed 1 to 2 weeks after the placement of the larval stages. The nymphal stages (10 to 20% recovery) were then placed on the bodies of splenectomized calves for completion of their life cycle. No attempt was made to complete the entire life cycle on the guinea pigs.

Immature stages and adult B. microplus were also kept in desiccator jars, in an attempt to see if it was possible to maintain them for long periods without feeding. The temperature was that of the ambient temperature (30 C) at the Turipana ICA station. Only moderate success was accomplished in that it was possible to keep some of the stages (70 to 80% nymphal recovery) for 1 to 2 months without feeding.

Infection of Hemoprotozoan-Free *Boophilus microplus* with *Babesia bigemina*

Boophilus microplus free from *Anaplasma marginale* and *Babesia* spp. were placed on splenectomized calves which were artificially infected with pure *B. bigemina*. The prepatent period of the *B. bigemina* stabilate coincided with the period of rapid engorgement of the adult *B. microplus*. After engorgement the adults were collected and allowed to complete their developmental cycle. The resulting larval progeny were found to be infected with *B. bigemina* as shown by clinical babesiosis of a splenectomized calf.

Monitoring of Acute and Chronic Blood and Tick Borne Infections

A. Acute and Chronic Blood Borne Infections

Acute blood borne infections of *B. bigemina* were accomplished by inoculating 5 infect calves with an infecting dose of purified *B. bigemina* stabilate. At the time of maximal parasitemia, the 5 were bled

and their blood pooled and subinoculated into a splenectomized calf. Passage through 2 splenectomized calves was required to ensure a parasitemia high enough (7 to 38.4%)for satisfactory antigen production. When the second splenectomized calf reached maximal parasitemia it was exsanguinated and an acute blood (AB) stabilate and antigen were prepared. Ninety days after the appearance of a detectable parasitemia the calves were again bled, their blood pooled and subinoculated into first one and then, at peak parasitemia, another splenectomized calf. A chronic blood (CB) stabilate and antigen were prepared. Figure 2 summarizes the various responses of the 5 calves before and after inoculation with B. bigemina. The first CF antibodies were found on day 6 post inoculation with average titers of 1:5, and the maximum titer of 1:320 occurred on days 13 to 19. After that period, the CF titers persisted with titers between 1:5 and 1:80 for the remainder of the experiment. The first babesia parasites were seen on day 6 post inoculation with a maximum parasitemia (8.44%) between days 8 and 12. After that period the parasitemia

Figure 2. Complement fixation titers, percent parasitized erythrocytes, rectal temperatures and percent packed cell volumes for 5 calves inoculated with 1×10^9 *Babesia bigemina* parasitized erythrocytes on day -5. Day 0 indicates that period when 75% or more of the calves showed a parasitemia.

Group means are represented by horizontal lines and ranges of the means are represented by vertical lines in this figure and in figures which follow.

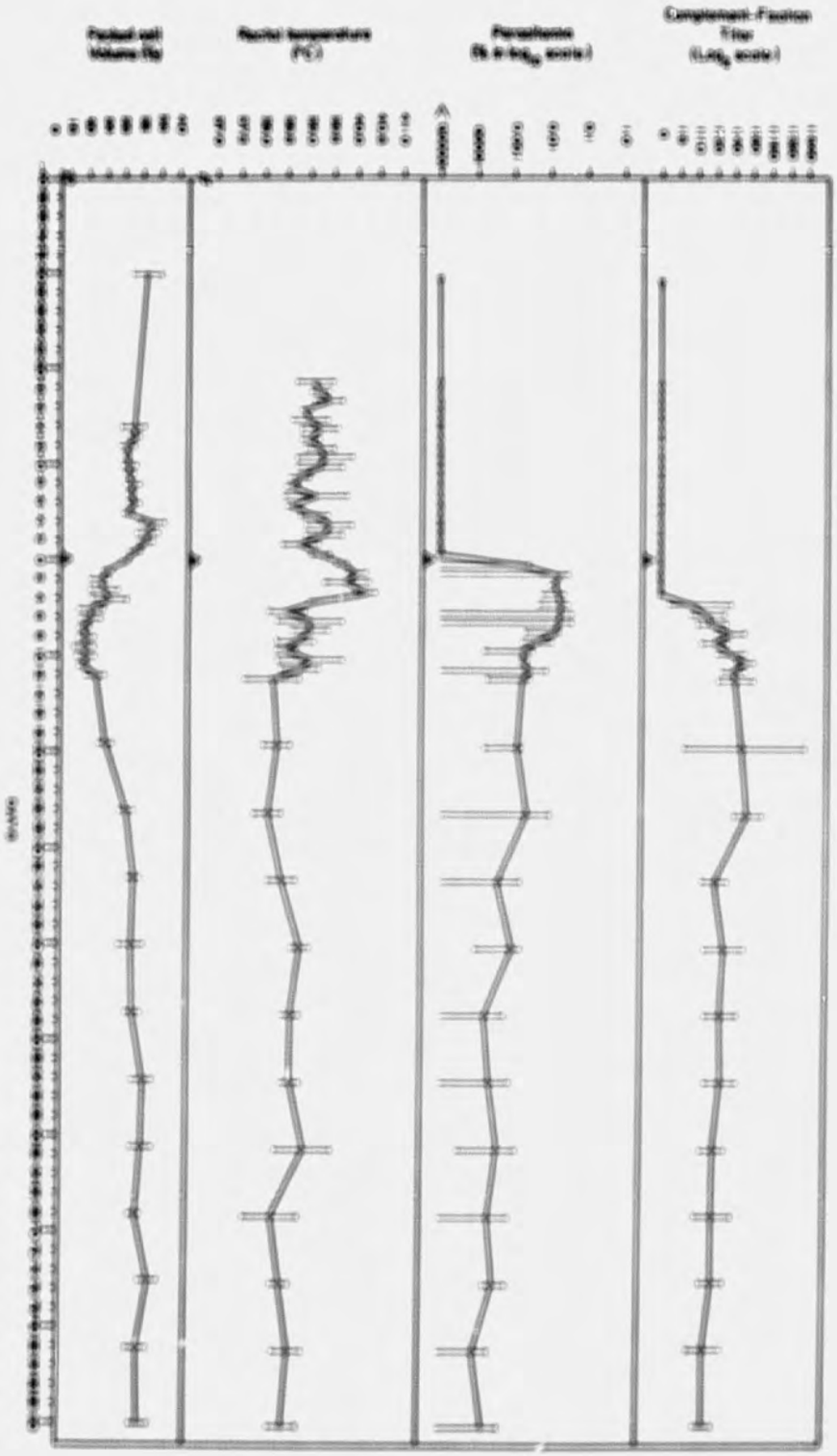
persisted in low levels (<0.00001 to 0.01%) for the duration of the experiment. The maximal rise in temperature (40.4 C) occurred a few days after the maximal parasitemia (day 17). The anemia was evident by low PCV values (ave. 24%) 8 to 17 days after inoculation.

At the time blood was collected for the isolation of a chronic blood (CB) antigen and stabilate the average CF titer was 1:10; the average parasitemia, 0.0001%; the average rectal temperature, 38.3 C and the average PCV, 32%.

B. Acute and Chronic Tick Borne Infections

Five intact calves were infested with B. microplus larvae infected with B. bigemina to initiate a tick borne infection. Blood stabilates and antigens (AT and CT) were prepared identically to the procedure used in the preparation of the blood stabilates and antigens AB and CB. Figure 3 graphically summarizes the responses of these 5 calves which were infected with B. bigemina from B. microplus larvae. The first CF antibody detected was on day 24 after B. microplus exposure at an average

Figure 3. Complement fixation titers, percent parasitized erythrocytes, rectal temperatures and percent packed cell volumes for 5 calves infected with *Babesia bigemina* by *Boophilus microplus* ticks on day -19. Day 0 indicates that period when 75% or more of the calves showed a parasitemia.



titer of 1:10. The maximum CF titer was found on days 31 to 46 post tick exposure (1:640) and decreased thereafter to a titer range of 1:10 to 1:80. The first Babesia parasites were seen on day 19 after tick exposure, with a maximum parasitemia (0.05%) between days 20 and 27. After that period parasitemias persisted in low levels (ave. 0.0001%) for the duration of the experiment. The maximal rise in temperature (40.4 C) occurred on day 22 after tick exposure. The anemia was evident by low PCV values (ave. 20%) on days 24 to 30.

At the time blood was collected for the preparation of a chronic tick (CT) antigen and stabilate the average CF titer was 1:10, the average parasitemia, 0.001%; the average rectal temperature, 38.8 C and the average PCV, 34%.

The mean and standard deviation of the PCV, rectal temperature, CF titers, percent parasitized erythrocytes and weight of the calves with blood and tick borne infections are shown in Appendix Tables IV to VIII, respectively.

The calves with blood borne infections showed a

greater average weight gain, higher average PCV, higher average CF titer and a lower average rectal temperature while the calves infected by ticks exhibited a lower average percent erythrocytes parasitized with B. bigemina.

Isolation and Purification of
Babesia bigemina Antigens

A photograph of concentrated B. bigemina from lysed infected erythrocytes used for antigen preparation for the CF microtiter procedure is shown in Figure 4. This antigen was used to screen all the experimental calves prior to use.

Graphically illustrated in Figure 5 are the 3 ml fractions obtained from the Sephadex G-100 column and their range of antigenic activity. Figure 6 summarizes those samples obtained from the Sephadex G-200 column and their range of antigenic activity. As shown in Figure 5, there does not appear to be any correlation between the level of protein detected at 280 mμ and the antigenic activity of the samples while in Figure 6 this correlation is evident. The AB, CB, and CT antigen

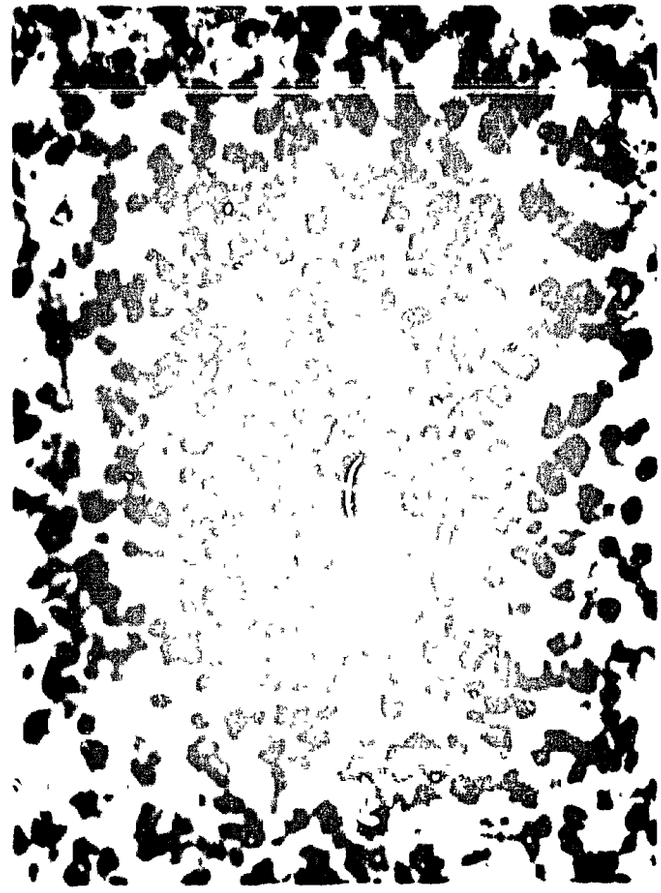


Figure 4. Thick film of concentrated *Babesia bigemina* used for antigen preparation for the CF microtiter procedure. Giemsa. x1300.

Figure 5. Graph of spectrophotometric absorbance (280λ) and antigenic activity range (measured by gel diffusion tests) of acute blood, chronic blood, acute tick and chronic tick antigenic fractions collected from a Sephadex G-100 column.

Vo = Void volume.

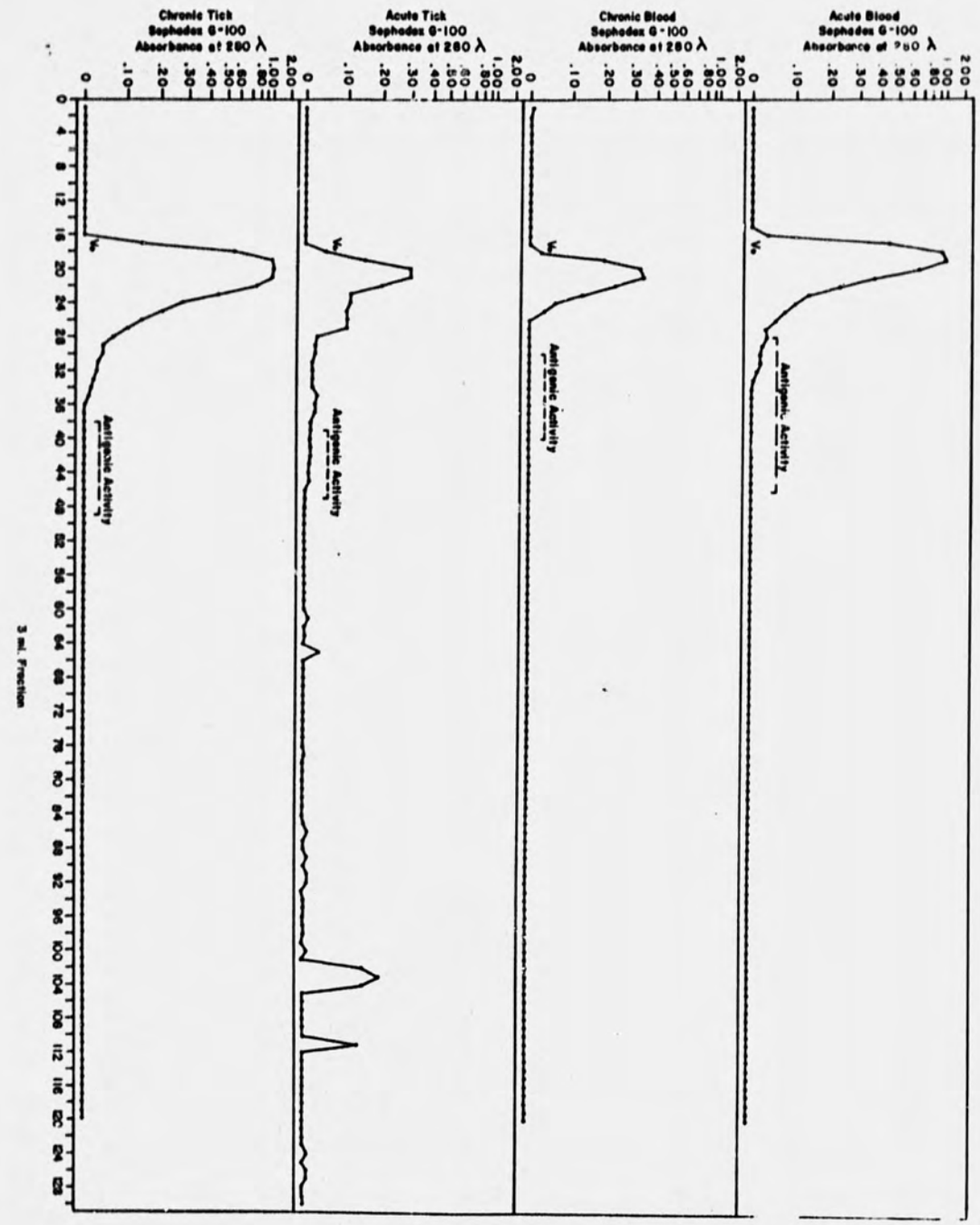

Acute Blood
Sephadex G-100
Absorbance at 280 λ
Antigenic Activity
Vo
Chronic Blood
Sephadex G-100
Absorbance at 280 λ
Antigenic Activity
Vo
Acute Tick
Sephadex G-100
Absorbance at 280 λ
Antigenic Activity
Vo
Chronic Tick
Sephadex G-100
Absorbance at 280 λ
Antigenic Activity
Vo
3 ml. Fraction

Figure 6. Graph of spectrophotometric absorbance (280λ) and antigenic activity range (measured by gel diffusion tests) of acute blood, chronic blood, acute tick and chronic tick antigenic fractions collected from a Sephadex G-200 column.

Vo = Void volume.

fractions did show correlation between antigenicity and protein content. All antigen preparations showed the highest amount of protein eluted with the void volume of the Sephadex G-100 column; however, in the Sephadex G-200 column, protein of AB and CB preparations was not eluted with the void volume. It is interesting to note the range of antigenic activity of most fractions of both the Sephadex G-100 and G-200 columns were similar; the only exception was the AB preparation from the Sephadex G-200 column in which antigenicity started much later and lasted longer than it did from the Sephadex G-100 column (Figures 5 and 6).

Immune Response of Calves to Various
<u>Babesia bigemina</u> Stabilates

A photograph of an infective <u>B. bigemina</u> blood stabilate after thawing, prior to subinoculation into an intact calf is shown in Figure 7.

Figures 8 to 11 graphically illustrate the PCV, rectal temperature, CF titer and parasitized erythrocytes, respectively, of the 16 groups of intact calves

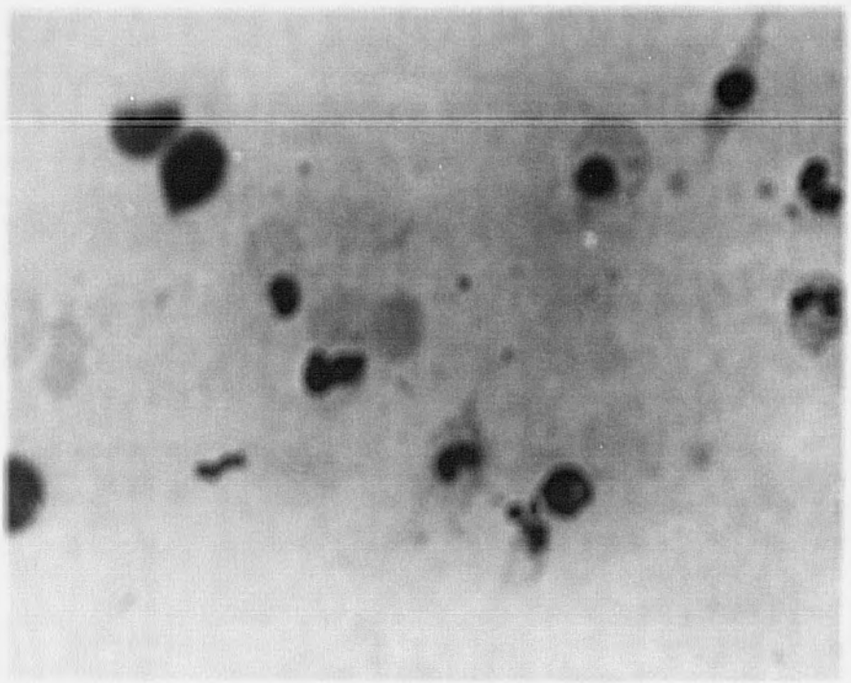


Figure 7. Infective *Babesia bigemina* stabilate after thawing, prior to subinoculation into an intact calf. Giemsa. ×1300.

Figure 8. The host response to infective *Babesia* erythrocytic stabilates and challenge with homologous and heterologous stabilates when measured by means of the packed cell volume (%).

In this figure and the figures which follow, (I) is the inoculation of 1×10^9 *Babesia bigemina* parasitized erythrocytes and (C), the challenging inoculation of 1×10^{10} *Babesia bigemina* parasitized erythrocytes.

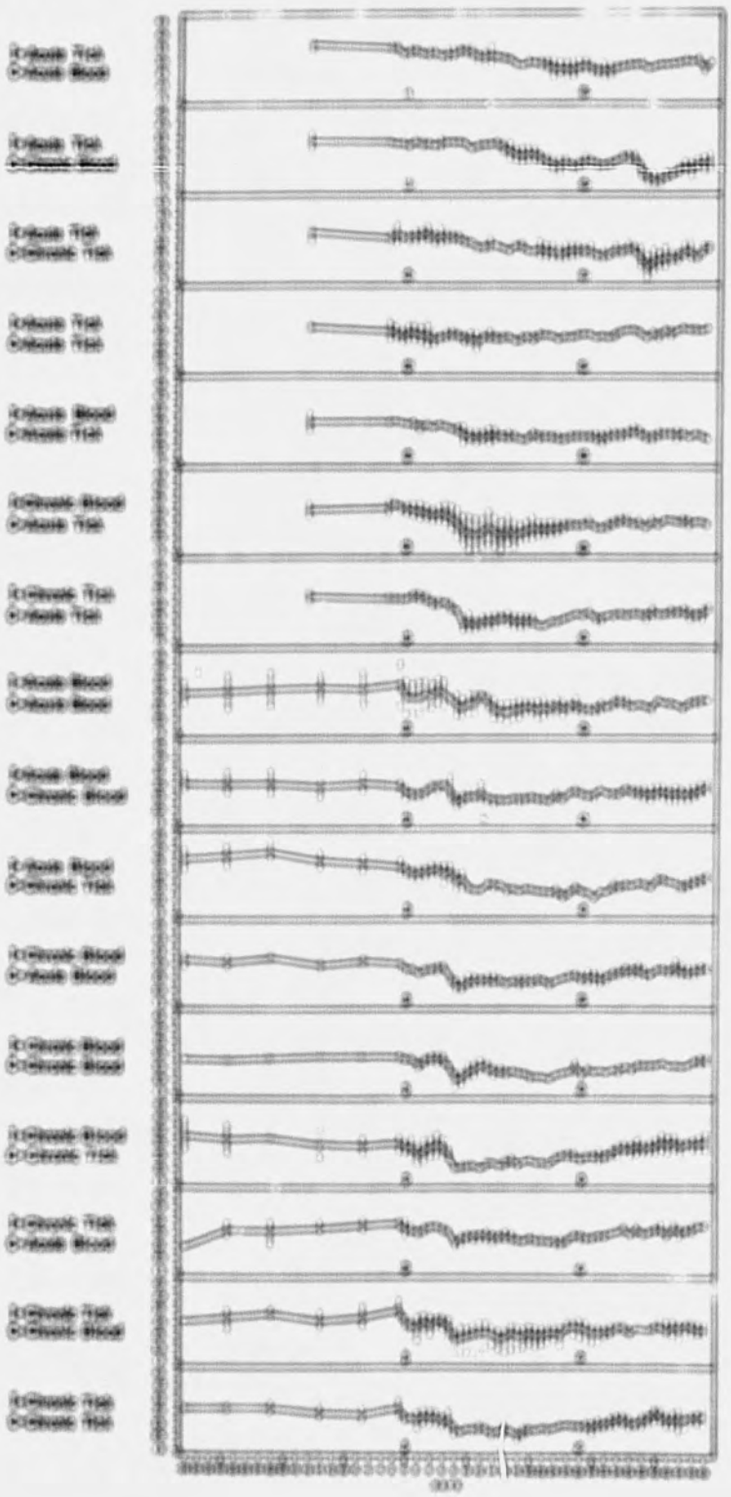


Figure 9. The host response to infective *Babesia* erythrocytic stabilates and challenge with homologous and heterologous stabilates when measured by means of the rectal temperature (C).

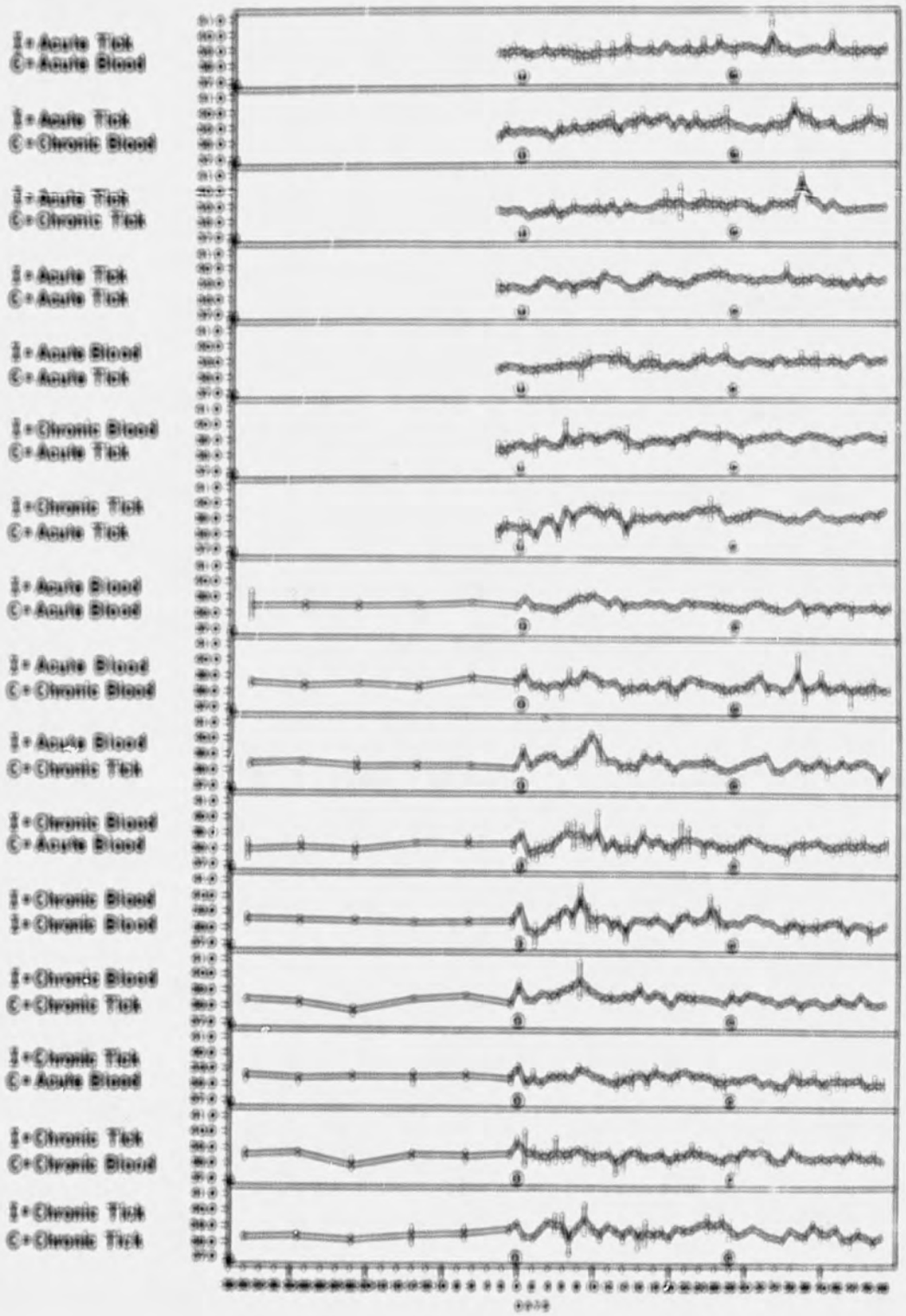

‡ = Acute Tick
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Figure 10. The host response to infective *Babesia* erythrocytic stabilates and challenge with homologous and heterologous stabilates when measured by means of the complement fixation titer.

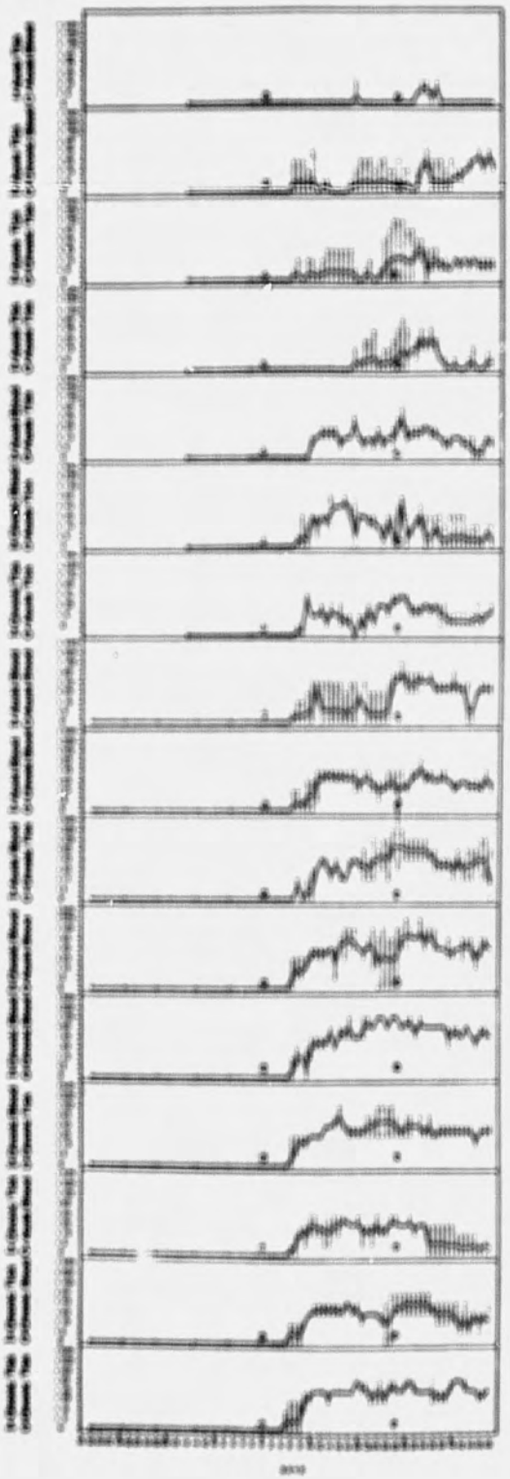
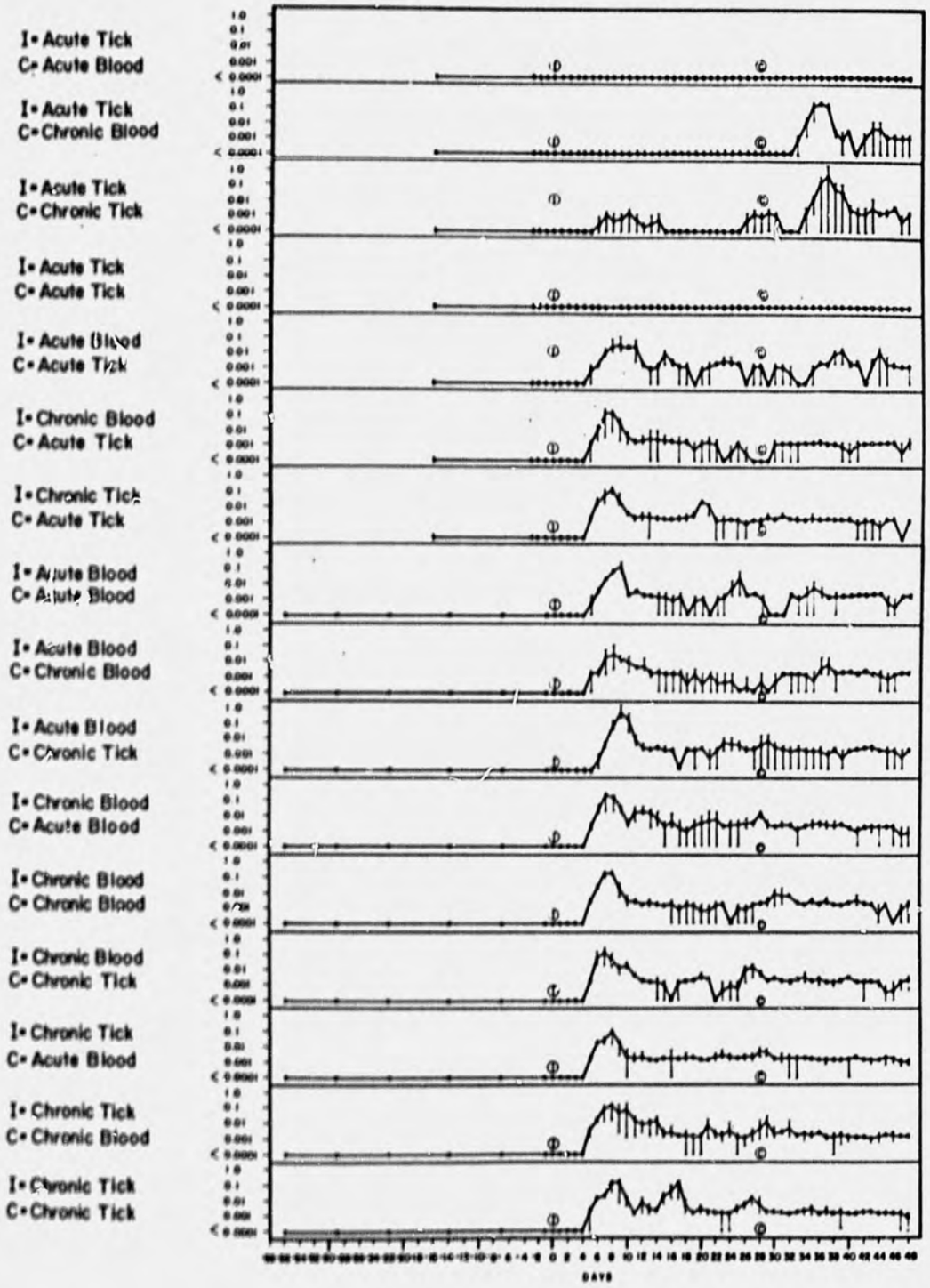


Figure 11. The host response to infective *Babesia* erythrocytic stabilates and challenge with homologous and heterologous stabilates when measured by means of the parasitized erythrocytes.


I = Acute Tick
C = Acute Blood
I = Acute Tick
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DAYS

inoculated with the various stabilates and later challenged with homologous and heterologous stabilates. Appendix Table IX shows the weekly weights of the different groups of calves.

Table 6 is a summation (Figures 8 to 11) of the host responses by groups to the infective *Babesia* erythrocytic stabilates. The lowest average PCV occurred when AT calves were challenged with stabilate CB (17.5%). The highest average rectal temperature was recorded when AT calves were challenged with stabilate CT (40.6 C). The highest average CF titer was observed when CB calves were challenged with stabilate AB (between 1:80 and 1:160). The lowest average parasitemia was recorded when AT calves were challenged with either AT stabilate or AB stabilate (<0.0001%). The highest average parasitemia (0.64%) was found when AT calves were challenged with CT stabilate indicating possible heterogeniety and a difference in the antigens. A photograph of *B. bigemina* in a thick blood film is shown in Figure 12.

Table 6. Summation of host responses by groups to the infective *Babesia* erythrocytic stabilates.

	After Inoculation[1]		After Challenge[2]	
	Minimum	Maximum	Minimum	Maximum
PCV (%)	CB (20)[3]	AT (33)	AT/CB (17.5)	CT/AB (32.7)
Rectal Temperature (C)	CB (37.7)	AB (40.4)	AB/CT (37.6)	AT/CT (40.6)
CF titer	AT (0)	CB (1:160)	AT/AB (0)	CB/AB (1:80 to 1:160)
Parasitemia (%)	AT (0)	AB (0.67)	AT/AB or AT (0)	AT/CT (0.64)

AB = Group receiving Acute Blood stabilate
CB = Group receiving Chronic Blood stabilate
AT = Group receiving Acute Blood stabilate
CT = Group receiving Chronic Tick stabilate

1 Represents response of group within 28 days after receiving 1×10^9 infected erythrocytes.

2 Represents responses of groups within 19 days after challenge with 1×10^{10} infected erythrocytes (initial group/challenge group).

3 Numbers in parenthesis represent average response of group.

Figure 12. Babesia bigemina parasites in a thick bovine blood film. Giemsa. x1300.

Characterization of the *Babesia bigemina* Antigens

A. Complement Fixation (CF) Test

The results of the CF test in which the 5 antigens (NB, AB, CB, AT, and CT) were titrated against 4 positive serums and 1 negative serum are shown in Table 7. Table 8 shows the same 5 antigens which had been standardized at 20 mg/ml of protein titrated against the same 4 positive and 1 negative serums by the CF test. As is shown in Table 8, the AT antigen reacted at a higher titer (trace to 1:20) with all positive serums while the CB antigen showed the lowest titer (trace to 1:10) of all with the exception of the control NB antigen.

B. Gel Diffusion Test

The results of the gel diffusion test indicated that specific precipitation reactions occurred between all the antigens and their corresponding antiserums. These reactions were made visible by the formation of lines of precipitation between the antigen containing wells and the antiserum containing troughs as depicted

Table 7. Complement fixation titers of 5 crude antigens when tested with 4 positive serums.

Antigen (homogenate 3)	Protein (mg/ml)	Antigen Dilution	Positive Serums A	B	C	D
Normal Bovine[1]	4.2	1:5	-	-	-	-
		1:10	-	-	-	-
PCV[2] = 32%		1:15	-	-	-	-
		1:20	-	-	-	-
Bb[3] = 0		1:25	-	-	-	-
		1:30	-	-	-	-
Acute Blood[1]	26	1:5	1/40	1/20	1/40	1/10
		1:10	1/40	1/10	1/40	1/10
PCV = 21%		1:15	1/20	1/5	1/40	1/10
		1:20	1/20	1/5	1/40	1/5
Bb = 38.4%		1:25	1/20	1/5	1/40	-
		1:30	1/20	-	1/20	-
Chronic Blood[1]	17.4	1:5	1/10	1/20	1/20	Tr[4]
		1:10	Tr	1/20	Tr	Tr
PCV = 20%		1:15	Tr	1/20	Tr	Tr
		1:20	Tr	1/20	Tr	-
Bb = 7%		1:25	Tr	1/20	Tr	-
		1:30	Tr	1/20	Tr	-
Acute Tick[1]	14.4	1:5	1/20	1/5	1/40	1/5
		1:10	1/20	-	1/20	-
PCV = 21%		1:15	-	-	-	-
		1:20	-	-	-	-
Bb = 30.8%		1:25	-	-	-	-
		1:30	-	-	-	-

Table 7. (Continued)

Antigen (homogenate 3)	Protein (mg/ml)	Antigen Dilution	Positive Serums A	B	C	D
Chronic Tick[1]	36.8	1:5	1/40	1/20	1/40	Tr
		1:10	1/40	1/20	1/40	Tr
PCV = 12%		1:15	1/20	1/20	1/20	Tr
		1:20	1/20	1/20	1/20	Tr
Bb = 8%		1:25	1/20	1/20	1/10	Tr
		1:30	Tr	1/20	Tr	Tr

1 Negative to all negative serum controls

2 Packed cell volume

3 *Babesia bigemina*

4 Trace

Table 8. Complement fixation titers of 5 crude antigens, all standardized to 20 mg/ml protein, when tested with 4 positive and 1 negative serums.

Antigen	Antigen Dilution	Positive Serums A	B	C	D	Negative Serum
Normal Bovine	1:5	-	-	-	-	-
	1:10	-	-	-	-	-
	1:15	-	-	-	-	-
	1:20	-	-	-	-	-
	1:25	-	-	-	-	-
	1:30	-	-	-	-	-
Acute Blood	1:5	1/5	1/20	1/20	Tr[1]	-
	1:10	Tr	1/20	1/10	Tr	-
	1:15	Tr	1/20	Tr	Tr	-
	1:20	Tr	1/10	Tr	Tr	-
	1:25	Tr	1/10	Tr	Tr	-
	1:30	Tr	1/10	Tr	Tr	-
Chronic Blood	1:5	Tr	1/10	1/5	Tr	-
	1:10	Tr	1/10	1/5	Tr	-
	1:15	Tr	1/10	Tr	Tr	-
	1:20	Tr	1/10	Tr	Tr	-
	1:25	Tr	1/10	Tr	Tr	-
	1:30	Tr	1/10	Tr	Tr	-
Acute Tick	1:5	1/10	1/20	1/20	1/5	-
	1:10	Tr	1/20	1/20	Tr	-
	1:15	Tr	1/20	1/5	Tr	-
	1:20	Tr	1/20	1/5	Tr	-
	1:25	Tr	1/20	Tr	Tr	-
	1:30	Tr	1/20	Tr	Tr	-
Chronic Tick	1:5	1/5	1/20	1/10	Tr	-
	1:10	Tr	1/20	Tr	Tr	-
	1:15	Tr	1/10	Tr	Tr	-
	1:20	Tr	1/10	Tr	Tr	-
	1:25	Tr	1/10	Tr	Tr	-
	1:30	Tr	1/10	Tr	Tr	-

1 Trace

in Figure 13. Figure 13 is a photograph of the Sephadex antigenic fractions of infected erythrocytes reacting with specific rabbit antiserum in the gel diffusion test.

The antigens fractionated by the Sephadex G-100 column (I), the number of fractions tested (II), and the fractions with antigenic activity (III) were as follows:

I	II	III
AB	120	28 to 46
CB	120	30 to 40
AT	130	39 to 47
CT	120	38 to 49

The antigens fractionated by the Sephadex G-200 column (I), the number of fractions tested (II), and the fractions with antigenic activity (III) were as follows:

I	II	III
AB	130	52 to 102
CB	138	27 to 34
AT	100	37 to 51
CT	113	31 to 46

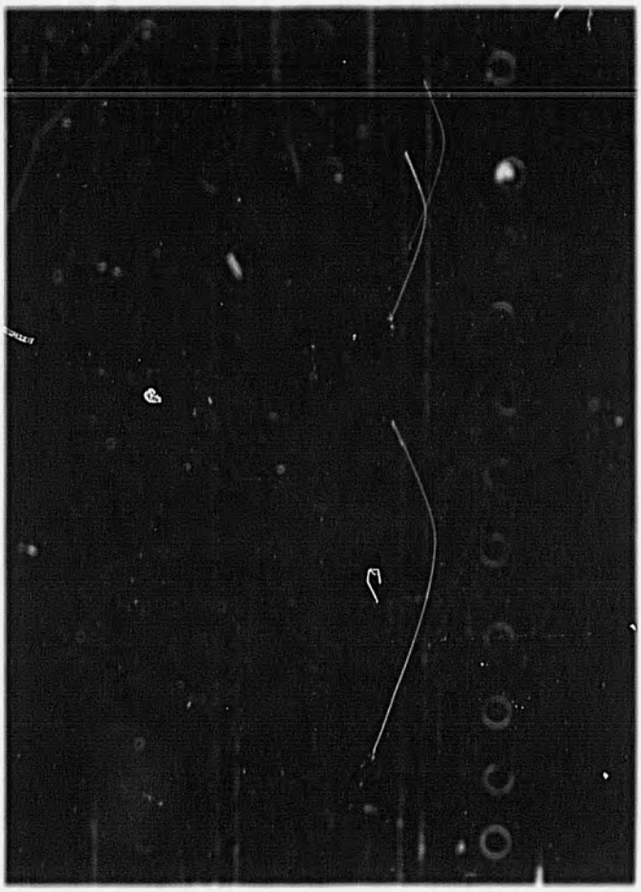


Figure 13. Sephadex antigenic fractions of infected erythrocytes (wells) reacting with specific rabbit antiserum (troughs) in the gel diffusion test. The precipitation line in the center indicated a reaction typical of the 33 antigen fractions tested.

C. Agar-Gel Immunoelectrophoresis Technique

The results of the immunoelectrophoretic reactions of the acute blood (AB), acute tick (AT), and chronic tick (CT) antigens with specific absorbed rabbit antiserum on agar-gel are shown in Figure 14. Antigen AB showed 2 lines of identity with rabbit anti-AT antiserum (RAT) and only 1 line of identity with either rabbit anti-CT antiserum (RCT) or rabbit anti-CB antiserum (RCB). Antigen CT showed 2 lines of identity with RCB, and AT antigen showed 1 line of identity with RCB but no line with RCT. No other antigen-rabbit antiserum combination showed lines of identity. It is interesting to note (Figure 13) antigen AB reacted with RAT in an identical pattern as CT antigen with RCB, and AB antigen reacted with RCT in an identical pattern as AB antigen with RCB.

D. Indirect Hemagglutination (IHA) Test

The results of the IHA test of the 4 antigens reacted with homologous and heterologous antiserums are summarized in Table 9. Figure 15 is a photograph of a

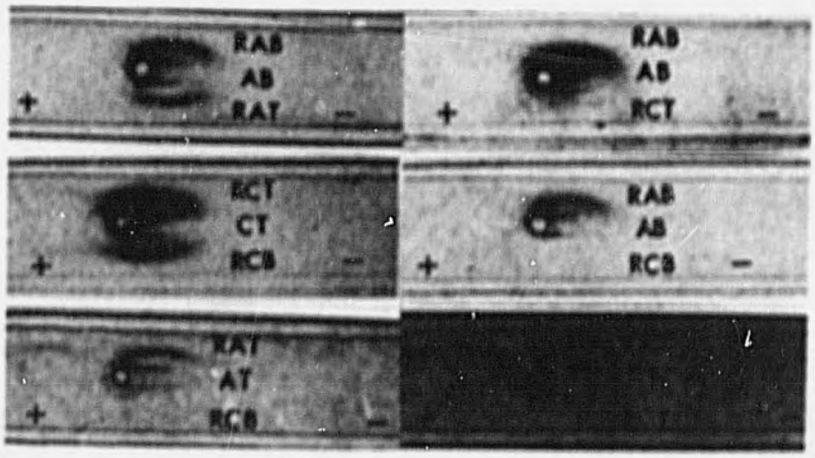


Figure 14. Agar-gel immunoelectrophoresis of the acute blood (AB), acute tick (AT), and chronic tick (CT) antigens with specific absorbed rabbit antiserum (RAB, RAT, RCB, and RCT). The chronic blood (CB), not shown here, reacted in a reciprocal manner with RAB, RAT, and RCT.

Table 9. Indirect hemagglutination titers obtained with 4 antigens and the homologous and heterologous antiserums (each figure represents the reciprocal of an individual dilution).

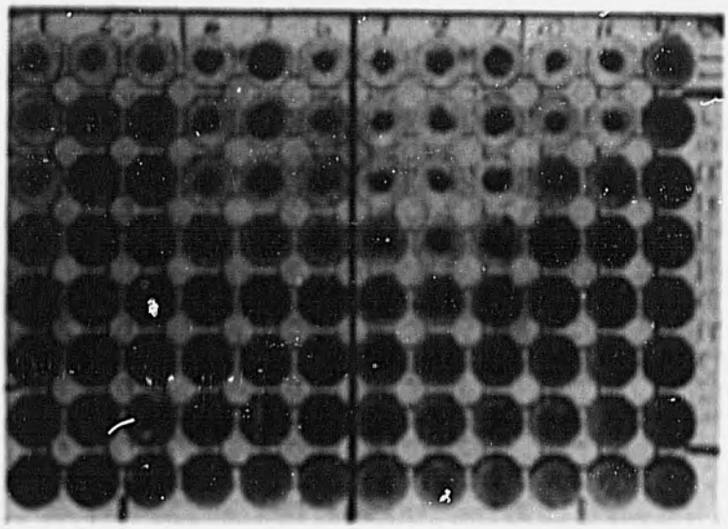
Antiserum to	Antigen			
	AB	CB	AT	CT
AB	640	160	80	20
CB	160	640	80	40
AT	40	80	$\geq$1280	80
CT	80	40	320	160

AB = Acute Blood
CB = Chronic Blood
AT = Acute Tick
CT = Chronic Tick

Indirect Hemagglutination Tube Test

Reactions of the IHA tube test are from left to right; +, +, ±, ±, ±, -, and -.

Complement Fixation Microtiter Plate Test



Results of the CF microtiter plate test are from left to right; 1/20, 1/5, 1/5, 1/20, 1/20, 1/20, 1/20, 1/40, 1/20, 1/10, 1/10, and Trace.

Figure 15. A comparison of the reactions of an indirect hemagglutination tube test (top) and a complement fixation microtiter plate test (bottom).

IHA tube test in comparison with a CF microtiter plate test.

The highest homologous IHA titer was obtained when AT antigen was reacted with AT antiserum ($\geq$1:1280) and the lowest titer when CT antigen was reacted with CT antiserum (1:160). The highest heterologous IHA titer occurred when AT antigen was reacted with CT antiserum (1:320) and the lowest, when CT antigen was reacted with AB antiserum (1:20).

The titer of 1:1280 obtained when AT antigen was reacted with AT antiserum was the highest dilution used in the test procedure.

DISCUSSION

The observations on natural transmission of B. bigemina by B. microplus made during the procedure for separation of B. bigemina from contaminating organisms were consistent with observations made by Callow and Hoyte (1961b) on experimental transmission of this organism by B. microplus. Callow and Hoyte (1961b) observed that the natural transmission prepatent period for B. bigemina had a range of 11 to 17 days which compared favorably with the observed natural transmission prepatent period of 18 days.

The ability of B. bigemina to multiply rapidly enabled it to be found in thin blood smears from 24 to 57½ hr after calves had been subinoculated with relatively small numbers of parasites. This rapid rate of multiplication made it possible to separate it from other blood parasites. The observed prepatent period for the blood borne infection of B. bigemina (26½ to 51 hr) was in agreement with those reported by Callow and Hoyte (1961a).

The procedure designed to obtain B. microplus free of B. bigemina was successful in spite of the fact that

ticks were collected in an enzootic zone of babesiasis. In enzootic areas, parasitized erythrocytes in the indigenous cattle are rarely more than a few/mm^3 (Mahoney, 1969), and even though tick infection rates may not be directly related to the total number of detectable parasites in bovine peripheral blood, infection rates under field conditions are expected to be low. The examination of smears from the hemolymph of oviposition female ticks, eggs, or guts of developing larvae demonstrated the infection rates to be low, with 0.23% being recorded for B. bigemina (Mahoney and Mirre, 1971).

Noninfected B. microplus ticks were maintained on splenectomized calves, disease free guinea pigs and in desiccator jars. Primarily, splenectomized calves were utilized but moderate success was also obtained with guinea pigs. No attempt was made to complete the entire cycle of the B. microplus on guinea pigs. The extremely high ambient temperature in Turipana resulted in an increased irritative dermatitis associated with the containing device which accounted for the decrease in survival of the immature stages of the tick on the guinea

pigs. The technique might be of value in that a less expensive animal could be utilized for maintaining the immature stages of B. microplus. The use of desiccator jars proved to be unnecessary due to the ease of rearing B. microplus on the laboratory bench using ambient temperature and relative humidity.

The results of the infection of B. microplus with B. bigemina agreed with those reported by Callow and Hoyte (1961b), Mahoney (1964), Hoyte (1965), Callow (1968a) and Mahoney and Mirre (1971). The infection of B. microplus with B. bigemina usually occurs only during the last 24 hr of attachment to the host; that is, the final rapid engorgement of the adult tick.

The higher parasitemias observed following the use of synthetic corticosteroids coincided with the results reported by Callow and Parker (1969), Frerichs et al. (1969a) and Young and Cox (1971). Although corticosteroids did increase the number of circulating parasitized erythrocytes, in the present experiment, it had an immunosuppressive response on the host system and the erythrocytes were more fragile. Thus, when preparing

antigens from the infected erythrocytes it was necessary to wash the erythrocytes carefully in order to decrease the amount of free hemoglobin and decrease the amount of anticomplementary activity.

The differences between the calves receiving blood borne and tick borne infections, as shown by Figures 2 and 3, indicated that the cattle tick B. microplus had a severe effect on infested calves. This is in agreement with Riek (1957), Seebeck et al. (1971) and Springell et al. (1971) who reported reductions in the hematocrit and weight gain of tick infested cattle when compared with tick free cattle. A high B. bigemina parasitemia is usually associated with a high rectal temperature, but in this experiment (Figures 2 and 3), the maximum parasitemia of the tick infected calves was 0.05% as compared to 0.44% for the inoculated calves while the maximum rectal temperatures were 40.4 C for both groups of calves. Therefore, it appeared that temperature is not related to the number of parasitized erythrocytes.

The descending order of antigens with respect to percent parasitemia at the time of preparation was AB,

AT, CT, and CB, while the descending order with respect to CF titer was AT, AB, CT, and CB. Although the AT antigen had the highest CF titer when reacted with the positive serums, it was not prepared from blood with the highest percent parasitemia. The AT antigen was prepared from blood with 30.8% parasitemia whereas the AB antigen was prepared from erythrocytes with 38.4% infection. Since the PCV of the blood used to prepare AT and AB antigens were identical, the AT preparation was observed to be more antigenic.

When comparing the 4 antigens by the immunoelectrophoresis technique it was determined that antigen AB was similar to antigen AT and antigens CB and CT were similar. Antigens AB and AT had 2 components that migrated at approximately the same rate and formed similar arcs when reacted with the same antiserums. Antigens CT and CB had 2 components that migrated similarly and formed similar arcs when reacted with the same antiserum. However, components of AB and AT migrated differently than the components of CB and CT. Antigen AT did not form a visible arc with antigen CT.

The indirect hemagglutination test indicated a difference between the 4 B. bigemina antigens. Antigen AB and CB appeared to be similar and antigens AT and CT showed similarity. Antigens AB and CB appeared to be closely related because each had equally high titers when cross reacted with the heterologous antiserums. AT and CT also showed the same association. The similarities observed by the indirect hemagglutination test were different from those of the CF and immunoelectrophoresis tests. These descrepancies are due to the different antibodies being measured by the 3 procedures. Carpenter (1968) reported that the sensitivity of the hemagglutination test was about 1,000 times that of the precipitation test in detecting antibody. Agglutination was 100 times and CF was 50 times as sensitive as the precipitation test. These data compared favorably with those of Curnow and Curnow (1967), Goodger (1971), Ristic et al. (1971) and Goodger (1973) in that the homologous antigen-antiserum reactions showed the highest indirect hemagglutination titers.

The serologic tests reported in the present

experiment confirmed that there are differences between the 4 antigens.

The results of molecular sieving of the antigens were not in agreement with those of Mahoney and Goodger (1969) using B. argentina infected serum and Goodger (1971) using sonicated B. bigemina infected erythrocytes. They reported that the antigenically active portion of their preparation did not enter the pores of the Sephadex G-200 gel and was subsequently eluted in the void volume of the column. In this experiment, the antigenically active portion entered the pores of the gel (Sephadex G-100 and Sephadex G-200, Figures 5 and 6) and was thus eluted after the void volume. The gel filtration indicated that the antigens had molecular weights of 1,000 to 100,000.

The gel diffusion test of the Sephadex fractions indicated that the antigenic fractions of preparation AB and CB had similar molecular weights and that of AT and CT had similar molecular weights but that of the 2 groups differed. This conclusion is made by the fact that the antigenic activity of AB and CB started at

essentially the same fraction. Similarly, the antigenic activity of AT and CT were detected in the same fraction but the latter 2 eluted in much later fractions (Figures 5 and 6). The indication is that fractions AB and CB have a greater molecular weight than do fractions AT and CT.

The determination of the host response to infective Babesia erythrocytic stabilates was difficult to assess due to the variability of infectivity of the AT stabilate. One reason for the lack of infectivity may have been the choice of the route of inoculation. Although Callow and Hoyte (1961b), Mahoney (1964), Callow and Tammemagi (1967), Callow (1968a), and Rogers (1971a, b) all reported success when inoculating Babesia organisms iv, Dalgliesh (1972a, b) showed that this route of inoculation suppressed infectivity of B. bigemina that had been frozen in blood containing glycerol. However, when parasites were injected sc, infectivity was not markedly affected, presumably because there was less body fluid available to cause osmotic swelling and rupture of the parasites and the erythrocytes that enveloped them.

Examination of thin blood smears is a crude method for determining whether or not Babesia is present in circulating blood because the number of erythrocytes that can be examined, relative to the total number in the circulation, is exceedingly small. The thick blood film allowed the examination of approximately 1.4 mm^3 of blood in 25 to 30 min. If this volume of bovine blood contained approximately 5×10^6 erythrocytes and the cells were spread in a single layer as a thin blood film, an observer, examining at the rate of 2×10^4 cells/min, would take over 7 hr to search the film.

In the present experiment, 3 stabilates (AB, CB, and CT) were 100% infective iv; however, stabilate AT was only 12½% infective. In all cattle inoculated with the infective Babesia erythrocytic stabilates no demonstrable reaction occurred with homologous challenge while heterologously the responses were marked. These results confirmed the antigenic differences shown by the serologic tests.

The serological results indicated that antigenic variation within an isolate of B. bigemina occurred.

The host's apparent reduced response to homologous challenge and the marked immune response observed with heterologous systems indicated antigenic differences of the *B. bigemina* and confirmed the serological data under the experimental conditions of this study.

SUMMARY

The purpose of this study was to determine possible antigenic differences in the causative organism of the acute and chronic blood borne and tick borne *Babesia bigemina* infections of cattle. It was anticipated that the results of the research would further aid in the understanding of the mechanism of immunity of babesial infections. In addition, the antigens may possibly be used in the development of new serologic techniques or vaccines.

A *Babesia bigemina* field isolate was purified by 4 rapid serial passages through 5 splenectomized calves within 6½ days, and a stabilate of the isolated *B. bigemina* was established.

The larval progeny of engorged female *Boophilus microplus* ticks were screened for the presense of *Anaplasma marginale* and *Babesia* spp. The noninfected ticks resulting from the screening were maintained primarily on noninfected splenectomized calves. Disease free guinea pigs were also utilized with only moderate success. Various stages

of ticks were held without feeding in desiccator jars for periods of 1 to 2 months. Noninfected B. microplus ticks were successfully infected by placing them on calves inoculated intravenously with the B. bigemina stabilate.

With the aid of synthetic corticosteroids, high B. bigemina parasitemias were attained in splenectomized calves subinoculated from intact calves infected by ticks and by artificial inoculation. Babesia erythrocytic antigens and infective blood stabilates were prepared from blood of splenectomized calves with high parasitemias resulting from treatment with synthetic corticosteroids. These splenectomized calves were subinoculated from intact calves with acute (AB) and chronic (CB) blood borne and acute (AT) and chronic (CT) tick borne infections. Normal bovine (NB) erythrocytic antigen was prepared as a control.

Isolation of B. bigemina antigens was accomplished by erythrocytic lysis and homogenation, differential centrifugation and purification from host materials by means of molecular sieving using Sephadex G-100 and

G-200 columns. Antigen AB and CB were similar, as were AT and CT, when compared by indirect hemagglutination and gel diffusion. Similarities were observed between AB and AT and between CB and CT when these antigens were compared by immunoelectrophoresis and complement fixation.

Thirty-six intact calves were divided into 16 groups of 2 (or 3) and each group inoculated with infective Babesia erythrocytic stabilates. Twenty-eight days later they were homologously and heterologously challenged from the same stabilates, and monitored for an additional 20 days. The antigenic variations were more evident by in vitro rather than in vivo reactions.

On the basis of the serological results, antigenic variation within an isolate of B. bigenina occurred. Antigenic variation appeared to be influenced by the mode and duration of infection. The hosts' apparent reduced response to homologous challenge and the marked immune response observed with heterologous systems indicated antigenic differences of the B. bigemina and confirmed the serological data under the experimental conditions used in this investigation.

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APPENDIX

Appendix Table I. Concentrate and mineral composition of experimental calf grain ration.

Shelled Corn or Sorghum	430 kg
Cottonseed Meal or Soybean Meal	300 kg
Wheat Bran or Rice Bran	230 kg
Iodized Salt	10 kg
Bone Meal	10 kg
Vitamin Premix[1]	10 kg
Mineral Premix[2]	10 kg
	1,000 kg

[1]Vitamin Premix:

Wheat Bran or Rice Bran	10 kg
Roche A-D (Vit. A 750,000 iu/gm and Vit. D 150,000 iu/gm)	8 gm
	10 kg

[2]Mineral Premix:

Copper Sulfate	1.95 kg
Iron Sulfate	7.47 kg
Zinc Oxide	1.24 kg
Magnesium Sulfate	3.09 kg
Cobalt Sulfate	0.20 kg
Potassium Iodide	0.07 kg
Wheat Bran or Rice Bran	85.98 kg
	100.00 kg

Appendix Table II. Fecundity of *Boophilus microplus* ticks from calves infected with *Babesia bigemina*.

Collection (day)	Calf #452 Time 8 AM	Calf #452 Time 5 PM	Calf #452 Calf Parasitemia	Calf #455 Time 8 AM	Calf #455 Time 5 PM	Calf #455 Calf Parasitemia
1	42[1](41/1)[2]	61(61/0)	NPS[3]	10(10/0)	33(32/1)	NPS
2	38(35/3)	25(23/2)	NPS	33(33/0)	77(77/0)	NPS
3	17(16/1)	8(8/0)	NPS	41(41/0)	40(38/2)	NPS
4	11(9/2)	5(5/0)	NPS	16(16/0)	22(22/0)	NPS
5	7(6/1)	8(4/4)	0.2	9(9/0)	13(10/3)	0.05
6	7(6/1)	6(6/0)	3.5	2(0/2)	4(1/3)	0.8
7	5(2/3)	1(1/0)	2.5	1(0/1)	2(2/0)	11.7
8	1(0/1)	0	3.0	2(2/0)	2(2/0)	6.0
9	0	0	3.0	0	2(2/0)	6.0
10	0	0	4.0[4]	1(1/0)	0	6.0[5]
Totals	128(115/13)	114(108/6)		115(112/3)	195(186/9)	
%Eggs	89.84	94.74		97.39	95.38	
%No Eggs	10.16	5.26		2.61	4.62	

1 Total number of engorged females collected.
2 Number of females depositing eggs/number not depositing eggs.
3 No parasite seen in thick blood film.
4 Calf died.
5 Calf was treated.

Appendix Table III. Hatchability of *Boophilus microplus* eggs of females from *Babesia bigemina* infected calves (Continuation Table II).

Collection (day)	Calf #452 Time 8 AM	Calf #452 Time 5 PM	Calf #452 Calf Parasitemia	Calf #455 Time 8 AM	Calf #455 Time 5 PM	Calf #455 Calf Parasitemia
1	41[1](41/0)[2]	61(51/10)	NPS[3]	10(8/2)	32(28/4)	NPS
2	35(29/6)	23(22/1)	NPS	33(26/7)	77(65/12)	NPS
3	16(9/7)	8(6/2)	NPS	41(29/12)	38(24/14)	NPS
4	9(6/3)	5(5/0)	NPS	16(10/6)	22(17/5)	NPS
5	6(4/2)	4(3/1)	0.2	9(8/1)	10(2/8)	0.05
6	6(1/5)	6(4/2)	3.5	0	1(0/1)	0.8
7	2(2/0)	1(0/1)	2.5	0	2(0/2)	11.7
8	0	0	3.0	2(2/0)	2(0/2)	6.0
9	0	0	3.0	0	2(0/2)	6.0
10	0	0	4.0[4]	1(0/1)	0	6.0[5]
Totals	115(92/23)	108(91/17)		112(83/29)	186(136/50)	
%Larvae	80.0	84.26		74.11	73.12	
%No Larvae	20.0	15.74		25.89	26.88	

1 Number of engorged females which deposited eggs.
2 Larvae/no larvae produced by egg batches.
3 No parasite seen in thick blood film.
4 Calf died.
5 Calf was treated.

Appendix Table IV. Standard deviation and means of the packed cell volumes of the calves with blood borne and tick borne *Babesia bigemina* infections.

Day	Blood Borne					Tick Borne				
	N	Ex	Ex^2	SD	Mean	N	Ex	Ex^2	SD	Mean
0	5	154	4748	1.1	30.8	5	140	3922	0.7	28
1	5	146	4270	1.3	29.2	5	120	2884	1.0	24
2	5	128	3334	3.8	25.6	5	113	2567	1.8	22.6
3	5	122	3038	3.9	24.4	5	117	2773	3.0	23.4
4	5	125	3239	5.3	25	5	124	3140	4.0	24.8
5	5	134	3798	7.2	26.8	5	104	2184	2.3	20.8
6	5	122	3002	2.5	24.4	5	103	2139	2.1	20.6
7	5	124	3094	2.2	24.8	5	95	1811	1.2	19
8	5	127	3235	1.5	25.4	5	98	1930	1.5	19.6
9	5	118	2794	1.5	23.6	5	96	1858	1.9	19.2
10	5	126	3222	3.4	25.2	5	99	1969	1.5	19.8
11	5	128	3286	1.5	25.6	5	96	1846	0.8	19.2
12	5	134	3620	2.7	26.8	5	108	2350	2.1	21.6
19	5	148	4400	2.2	29.6	5	119	2839	1.3	23.8
26	5	155	4831	2.5	31	5	141	3987	1.6	28.2
33	5	152	4636	1.9	30.4	5	150	4506	1.2	30
40	5	158	5018	2.5	31.6	5	147	4349	2.6	29.4
47	5	165	5467	2.3	33	5	148	4394	1.8	29.6
54	5	161	5199	1.9	32.2	5	160	5138	2.1	32
61	5	157	4957	2.6	31.4	5	159	5077	2.3	31.8
68	5	172	5930	1.8	34.4	5	153	4687	1.1	30.6

Appendix Table F. (Continued)

	Blood Borne					Tick Borne				
Day	N	Ex	Ex^2	SD	Mean	N	Ex	Ex^2	SD	Mean
75	5	165	5449	1.0	33	5	168	5664	2.2	33.6
82	5	172	5938	2.3	34.4	5	156	4886	2.2	31.2
90	5	169	5731	2.2	33.8	5	159	5071	1.9	31.8

N = Sample size.

Ex = Sum of x (PCV)

Ex^2 = Sum of x^2

SD = Standard deviation

$$\text{Mean} = \frac{Ex}{N}$$

Appendix Table V. Standard deviation and means of the rectal temperature of the calves with blood borne and tick borne *Babesia bigemina* infections.

	Blood Borne					Tick Borne				
Day	N	Ex	Ex^2	SD	Mean	N	Ex	Ex^2	SD	Mean
0	5	194.2	7544.3	0.6	38.8	5	198.6	7888.5	0.1	39.7
1	5	196.9	7754.4	0.3	39.4	5	200.1	8008.2	0.2	40.0
2	5	194.9	7599.8	0.8	39.0	5	198.8	7905.2	0.5	39.8
3	5	193.4	7481.4	0.4	38.7	5	200.8	8064.3	0.2	40.2
4	5	197.2	7778.5	0.5	39.4	5	195.3	7628.5	0.1	39.1
5	5	193.1	7458.3	0.4	38.6	5	192.6	7419.7	0.4	38.5
6	5	194.7	7581.7	0.2	38.9	5	195.0	7606.7	0.7	39.0
7	5	192.7	7427.0	0.3	38.5	5	194.8	7590.1	0.4	39.0
8	5	193.3	7473.3	0.3	38.7	5	193.7	7504.4	0.4	38.7
9	5	193.0	7449.9	0.2	38.6	5	192.3	7396.1	0.2	38.5
10	5	196.5	7723.9	0.6	39.3	5	195.2	7622.5	0.7	39.0
11	5	199.7	7976.9	0.5	39.9	5	194.2	7543.3	0.4	38.8
12	5	192.1	7381.0	0.4	38.4	5	190.9	7290.1	0.6	38.2
19	5	193.3	7473.3	0.3	38.7	5	191.5	7334.8	0.3	38.3
26	5	192.1	7380.7	0.2	38.4	5	190.6	7266.1	0.3	38.1
33	5	193.4	7480.9	0.2	38.7	5	192.1	7380.9	0.3	38.4
40	5	192.4	7403.9	0.3	38.5	5	193.8	7511.9	0.2	38.8
47	5	191.6	7342.2	0.2	38.3	5	193.2	7465.3	0.2	38.6
54	5	193.4	7483.0	0.8	38.7	5	192.9	7442.3	0.2	38.6
61	5	194.2	7543.7	0.5	38.8	5	194.6	7575.4	0.6	38.9

Appendix Table V. (Continued)

	Blood Borne					Tick Borne				
Day	N	Ex	Ex^2	SD	Mean	N	Ex	Ex^2	SD	Mean
68	5	191.6	7342.5	0.3	38.3	5	191.0	7297.5	0.6	38.2
75	5	191.8	7357.8	0.3	38.4	5	191.8	7357.6	0.2	38.4
82	5	191.4	7327.2	0.3	38.3	5	192.9	7442.4	0.3	38.6
90	5	191.7	7350.0	0.2	38.3	5	192.4	7403.8	0.3	38.5

N = Sample size

Ex = Sum of x (Temp)

Ex^2 = Sum of x^2

SD = Standard deviation

$$\text{Mean} = \frac{Ex}{N}$$

Appendix Table VI. Standard deviation and means of the complement fixation titers of the calves with blood borne and tick borne *Babesia bigemina* infections.

Day	Blood Borne						Tick Borne					
	N	Ex	Ex^2	SD		Mean	N	Ex	Ex^2	SD		Mean
				Low	High					Low	High	
0	5	2.3	1.9	1.1	8.5	3.8	5	0	0	0	0	0
1	5	1.3	1.7	0	6.9	1.8	5	0	0	0	0	0
2	5	1.0	1.0	0	4.5	1.6	5	0	0	0	0	0
3	5	3.6	4.7	0	28.2	5.2	5	0	0	0	0	0
4	5	4.3	5.2	1.7	30.2	7.2	5	4.6	5.6	2.2	31.6	8.3
5	5	5.9	7.6	6	38.0	15.1	5	5.9	7.2	8.1	28.2	15.1
6	5	7.1	10.6	12.0	57.5	26.3	5	6.2	7.9	9.8	30.9	17.4
7	5	8.0	13.3	17.0	93.3	39.8	5	7.7	12.1	20.0	61.7	34.7
8	5	7.9	15.9	4.6	316.2	38.0	5	6.5	8.6	12.0	32.4	20.0
9	5	9.8	19.5	51.3	162.2	91.2	5	7.7	12.1	20.0	61.6	34.7
10	5	10.1	20.5	72.4	151.4	104.7	5	8.9	15.9	41.7	871.0	60.3
11	5	10.4	21.7	83.2	173.8	120.2	5	8.0	12.9	24.6	64.6	39.8
12	5	11.6	27.0	144.5	302.0	208.9	5	8.3	14.0	25.7	81.3	45.7
19	5	9.5	18.2	49.0	128.8	79.4	5	8.8	19.4	6.2	549.5	57.5
26	5	8.9	15.9	41.7	87.1	60.3	5	9.5	17.2	38.9	123.0	69.2
33	5	8.3	14.0	25.7	81.3	45.7	5	6.5	8.6	12.3	32.4	20.0
40	5	8.3	14.0	25.7	81.3	45.7	5	7.4	11.4	13.8	66.1	30.2
47	5	9.2	17.0	51.3	93.3	69.2	5	7.1	10.4	14.1	49.0	26.0
54	5	8.0	12.9	24.6	64.6	39.8	5	7.1	10.4	14.1	49.0	26.3

Appendix Table VI. (Continued)

Day	Blood Borne						Tick Borne					
	N	Ex	Ex^2	SD		Mean	N	Ex	Ex^2	SD		Mean
				Low	High					Low	High	
61	5	6.5	8.6	12.3	32.4	20.0	5	6.5	8.6	12.3	32.4	20.0
68	5	6.2	7.8	12.9	23.4	17.4	5	6.5	8.8	10.0	39.8	20.0
75	5	7.4	11.2	16.2	56.2	30.2	5	6.5	8.6	12.3	32.4	20.0
82	5	5.6	6.4	9.1	19.1	13.2	5	5.9	7.3	8.1	28.2	15.1
90	5	5.9	7.4	6.9	33.1	15.1	5	5.9	7.1	10.5	21.9	15.1

N = Sample size

Ex = Sum of x (CF)

Ex^2 = Sum of x^2

SD = Standard deviation

$$\text{Mean} = \frac{Ex}{N}$$

Appendix Table VII. Standard deviation and means of the parasitized erythrocytes of the calves with blood borne and tick borne *Babesia bigemina* infections.

Day	Blood Borne					Tick Borne				
	N	Ex	Ex^2	SD	Mean	N	Ex	Ex^2	SD	Mean
0	5	.0268	.0004	.0083	.0054	5	.0080	.00003	.0022	.0016
1	5	.1641	.0105	.0360	.0328	5	.1128	.0049	.0244	.0226
2	5	.6164	.2071	.1811	.1233	5	.0648	.0009	.0031	.0130
3	5	.3878	.0543	.0781	.0776	5	.1086	.0030	.0141	.0217
4	5	.1650	.0123	.0412	.0330	5	.1342	.0050	.0200	.0268
5	5	.2450	.0416	.0860	.0490	5	.1303	.0076	.0331	.0261
6	5	.2455	.0339	.0741	.0491	5	.1187	.0070	.0331	.0237
7	5	.0393	.0004	.0054	.0079	5	.0671	.0014	.0104	.0134
8	5	.0283	.0003	.0059	.0057	5	.0313	.0003	.0041	.0063
9	5	.0190	.0002	.0056	.0038	5	.0129	.00006	.0024	.0026
10	5	.0054	.000008	.0010	.0011	5	.0142	.00004	.0010	.0028
11	5	.0033	.000003	.0005	.0007	5	.0223	.0002	.0048	.0045
12	5	.0047	.000008	.0010	.0009	5	.0119	.00005	.0021	.0024
19	5	.0268	.0005	.0089	.0054	5	.0066	.00002	.0011	.0013
26	5	.0065	.00003	.0022	.0013	5	.0184	.0003	.0068	.0037
33	5	.0060	.00001	.0009	.0012	5	.0031	.000009	.0014	.0006
40	5	0	0	0	0	5	.0051	.000009	.0009	.0010
47	5	.0060	.00001	.0010	.0012	5	.0011	.000001	.0005	.0002
54	5	.0038	.000005	.0007	.0008	5	.0019	.000002	.0006	.0004
61	5	.0064	.00002	.0018	.0013	5	.0030	.000003	.0006	.0006

Appendix Table VII. (Continued)

	Blood Borne					Tick Borne				
Day	N	Ex	Ex^2	SD	Mean	N	Ex	Ex^2	SD	Mean
68	5	.0007	.0000005	.0003	.0001	5	.0018	.000002	.0006	.0004
75	5	.0020	.000001	.0005	.0004	5	.0027	.000002	.0003	.0005
82	5	.0016	.000001	.0005	.0003	5	.0005	.0000003	.0003	.0001
90	5	.0009	.0000008	.0005	.0002	5	.0013	.000001	.0005	.0003

N = Sample size

Ex = Sum of x (Parasitized erythrocytes)

Ex^2 = Sum of x^2

SD = Standard deviation

$$\text{Mean} = \frac{Ex}{N}$$

Appendix Table VIII. Standard deviation and means of the body weights of the calves with blood borne and tick borne *Babesia bigemina* infections.

	Blood Borne					Tick Borne				
Day	N	Ex	Ex^2	SD	Mean	N	Ex	Ex^2	SD	Mean
2	5	722	104502	7.8	144.4	5	681	93469	13.4	136.2
12						5	673	91207	12.5	134.6
19	5	786	123950	9.9	157.2					
26	5	761	116177	9.4	152.2	5	715	103211	15.5	143
33	5	788	124670	11	157.6	5	702	99692	16.8	140.4
40	5	887	157739	9.8	177.4	5	737	109299	12.9	147.4
47	5	908	165614	13.4	181.6	5	812	132984	16.7	162.4
54	5	914	167322	7.8	182.8	5	860	149002	16.4	172
61	5	900	162694	13.2	180	5	809	132435	19.6	161.8
68	5	989	196689	16.3	197.8	5	822	136078	15.3	164.4
75	5	1007	203695	14.9	201.4	5	915	168299	14.6	183
82	5	1022	210146	17.7	204.4	5	944	179792	19.8	188.8
90	5	1059	225499	17.3	211.8	5	971	190035	19.1	194.2

N = Sample size

Ex = Sum of x (Weights)

Ex^2 = Sum of x^2

SD = Standard deviation

Mean = $\frac{Ex}{N}$

Appendix Table IX. The host response to infective *Babesia* erythrocytic stabilates when measured by means of estimated weekly average weight gains (kg).

	Acute Blood				Chronic Blood			
Day	AB/AB	AB/CB	AB/AT	AB/CT	CB/AB	CB/CB	CB/AT	CB/CT
0	117	119	104	115	122	119	99	114
6	124	122	104	117	138	123	97	108
13	121	121	107	123	132	118	97	107
20	124	127	106	125	141	131	98	110
27	132	129	106	133	144	133	114	119
34	129	137	114	140	147	136	102	119
41	137	137	112	140	154	134	107	119
48	137	140	119	144	160	137	109	123

	Acute Tick				Chronic Tick			
Day	AT/AB	AT/CB	AT/AT	AT/CT	CT/AB	CT/CB	CT/AT	CT/CT
0	104	103	102	106	103	112	104	110
6	100	102	100	102	107	116	106	113
13	105	110	107	102	110	120	109	117
20	105	107	105	104	117	124	112	116
27	107	118	109	108	119	129	110	112
34	108	113	118	110	123	132	118	121
41	115	118	117	115	125	134	120	129
48	116	122	125	116	126	136	125	124

AB = Acute blood, CB = Chronic blood, AT = Acute tick, CT = Chronic tick. Numerator = stabilate used for premunization/denominator = stabilate used for challenge.

VITA

Kenneth Carl Thompson was born on December 17, 1940 in College Station, Texas. He is one of 4 children born to Dr. and Mrs. Lee P. Thompson. He was graduated from Tempe Union High School, Tempe, Arizona in June 1958.

He entered Arizona State University in 1958 and received a B.S. degree in Agriculture in 1962. In 1966 he entered Texas A&M University and in 1968 received a B.S. degree in Veterinary Science, and a D.V.M. degree in 1969.

In September 1969, he accepted a fellowship from the Institute of Tropical Veterinary Medicine, Texas A&M University, supported by the Rockefeller Foundation and the United States Agency for International Development. He is a candidate for the degree of Ph.D. in Veterinary Microbiology.

He was married to Isabel Godoy Bousquet of Vina del Mar, Santiago, Chile on 27 November 1972.

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The typist for this dissertation was Mrs. Dorothy Faver.