BABESIA CANIS: A CHARACTERIZATION OF FIVE FIELD STOCKS IN SOUTHERN AFRICA

THESIS

Submitted in fulfilment of the requirements
for the degree

MASTER OF SCIENCE (ZOOLOGY)

in the Faculty of Science, Rhodes University,
Grahamstown, South Africa.

by

GARTH WILLIAM VAN HEERDEN

January 1994

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ACKNOWLEDGEMENTS

I am most grateful to my supervisor, Professor Bruce Fivaz, for the help and guidance he has given me over the past three years while I was doing this research. I would also like to thank the rest of the staff of the Tick Research Unit at Rhodes University, Sally Terry, Edward Williams, Charles and Elaine Williams, for their assistance in the laboratory and in maintaining the dogs.

My gratitude also goes to Buzz Bowker for the use of his veterinary clinic when splenectomising dogs, to Professor Don Henry at the Microbiology Department for the use of his Western blotting apparatus, to the Regional Veterinary Laboratory, Grahamstown, for the use of their Coulter counter and also to Neil Cannon at the Electron Microscopy Unit at Rhodes University for the developing of photographs.

I am grateful for funding from the Rhodes University Graduate Assistance Bursary and the Foundation for Research and Development Masters Bursary.

I must also thank Martin Hill for proof reading my final draft.

DEDICATION

I dedicate this thesis
to my mother, Gwen,
who has always supported
me in everything I did.

ABSTRACT

This study attempted to characterise 5 field stocks of *B. canis*, collected from various geographic areas of southern Africa in terms of pathogenicity, immune response, cross resistance and antigenic composition. This was done by means of artificial infection of two to four year old Beagle dogs. Serological tests included indirect fluorescent antibody tests, polyacrylamide gel electrophoresis, Western blotting of *B. canis* antigen and immune serum transfers. The ZIM1, GHT1, QTN1 and DBN1 stocks were found to be virulent and the JHB1 stock, which might be a cardiac form of babesiosis, was found to be highly virulent. Immune responses of dogs to primary challenges were poor. A second challenge or relapse was important in the development of a resistant immunity. Heterologous challenges showed that dogs which were immune to the Zim1, QTN1 and DBN1 stocks were partially immune to the JHB1 stock. The dog that was immune to the GHT1 stock was the only one that was totally resistant to the JHB1 stock. Western blots revealed interstock antigenic variation between the JHB1 and the other four stocks. These differences were, however, not great enough to separate the stocks taxonomically.

CHAPTER ONE

INTRODUCTION

Canine babesiosis or biliary fever is caused by a protozoan parasite in the genus *Babesia* and is transmitted by several species of ixodid ticks (Taboada & Merchant, 1991). All stages of the tick have been shown to transmit infection but the adult female is the most important for parasite transmission (Friedhoff, 1988). The dog is the most important companion animal host for *Babesia* parasites and the disease appears to be increasing world wide (Taboada & Merchant, 1991). In most cases the species found in wild canids appear to be innocuous to their hosts but domestic dogs may succumb to the parasite resulting in high mortality (Shortt, 1973).

There are two species of *Babesia* which are known to parasitize the dog (Friedhoff, 1988). These include *Babesia canis* which measures 2.4 µm X 5.0 µm; it is piriform and is usually paired within the erythrocytes. The range of this species covers most of southern Europe, Africa, Asia, and North, Central, and South America (Table 1). Its vector ticks include *Rhipicephalus sanguineus*, *Dermacentor reticulatus*, and *Haemaphysalis leachi* (Breitschwerdt, 1988). The second species, *Babesia gibsoni* is found primarily in North Africa and the Far East (Table 1). The parasite is a small (1.0 µm X 3.2 µm), pleomorphic organism usually found singly within

erythrocytes. The vector ticks of *B. gibsoni* include *Haemaphysalis bispinosa* and *Rhipicephalus sanguineus*.

Table 1. The vectors and world distribution of the genus *Babesia* (Taboada & Merchant, 1991).

Species	Tick Vectors	Geographic Distribution
Babesia canis vogeli *Babesia canis canis* *Babesia canis rossi*	*Rhipicephalus sanguineus* *Dermacentor reticulatus* *Haemaphysalis leachi*	Africa, Asia, Australia, Europe, Central, South and North America
Babesia gibsoni	*Rhipicephalus sanguineus* *haemaphysalis bispinosa*	Far East, Asia United States, Southern Europe

B. canis appears to cause a wide range of symptoms of varying severity and this has led to a reappraisal of *B canis* especially in terms of the occurrence of strains within the species. Uilenberg *et al.* (1989) proposed a trinomial nomenclature system for *B. canis* (Table 1). Based on pathogenicity and vector range, *B. canis* can be divided into *B. canis vogeli* which occurs in tropical and subtropical regions of most continents and is transmitted by the brown dog tick, *Rhipicephalus sanguineus*. It is the least pathogenic of the three subspecies and is the one found in the United States. *B. canis canis* is the name proposed for the subspecies occurring in Europe and parts of Asia. It is intermediate in pathogenicity and is transmitted by *Dermacentor reticulatus*. The third subspecies, *B. canis rossi*, a highly pathogenic species transmitted by *Haemaphysalis leachi*, is found in southern Africa (Uilenberg *et al.*, 1989).

1.1 The life cycle in the arthropod and mammalian host

Ticks infected by infected dogs can transmit the parasite through several generations, and still remain infective when fed on a susceptible dog. Following ingestion of infected blood by the adult, the parasites which survive the massive destruction in the gut, leave the red cells, penetrate the walls of the tick diverticula to enter the coelomic cavity. They then make their way in the haemolymph to the ovaries and invade the ova. Multiplication occurs in the ova and these parasites are the source of transovarian transmission when the ova hatch into larvae. It seems likely that the parasites may be contained in certain cells in the haemocoele and remain dormant until the larvae feed (Ewing, 1965; Shortt, 1973; Young & Morzaria, 1986).

The parasites are extra-cellular in unfed nymphs and may occur in large concentrations in the haemolymph. The salivary glands are not invaded. A very small proportion of the parasites ingested survive to undergo further development following feeding on an infected dog. By the second or third day the parasites have become intracellular. At any time after 10 or 11 days after detachment of the tick and after a series of multiplications, the parasites migrate by chemotaxis towards and invade muscle tissue (Shortt, 1973).

When the adult tick containing the parasites begin to feed the parasites are activated and migrate towards the salivary glands. They then enter the salivary ducts for inoculation

into the tissue of the dog (Shortt, 1973). Once in the host, *Babesia* spp. only parasitize erythrocytes. They attach to the erythrocyte membrane and are subsequently engulfed by endocytosis (Igarashi *et al.*, 1988). Complement plays a role in recognition of red cells and attachment by the parasite (Taboada & Merchant, 1991). Once in the erythrocyte, the red cell membrane that surrounds the parasite disintegrates, and all subsequent stages are in direct contact with the host cell cytoplasm. *B canis* are now termed merizoites and multiply within the erythrocytes by binary fission.

1.2 Signs and symptoms of Babesiosis in dogs

Hyperacute and chronic infections may be seen with *B. canis rossi* in South Africa while *B. gibsoni* and the more virulent strains of *B. canis canis* and *B. canis vogeli* usually result in acute infections (Taboada & Merchant, 1991). When the parasites first appear in the circulating blood the dog seems perfectly well and is normally lively. This condition may continue for a few days while the parasites proliferate. The first clinical symptoms are a slight listlessness, lethargy and/or variable appetite. The body temperature is often raised and may go as high as 42°C (Shortt 1973). The dog may lose condition very rapidly even in the early stages. The mucous membranes of the mouth and eyes become pale due to anaemia because of a massive destruction of red cells which may result in haemoglobinuria. Death often results from complications as a result of anaemia and/or hypotensive shock.

B. canis vogeli and *B. canis canis* usually result in mild infections (Taboada & Merchant, 1991). In such cases the signs and symptoms described above are less severe, although pyrexia and anaemia may be marked. Recovery is slow and the anaemia may persist for several months. In many dogs there is an apparent complete return to health although a very mild parasitemia may persist for 14 months or more (Shortt, 1973; Young & Morzaria, 1986). If treatment becomes necessary, the parasitemia may clear within 48 hours after treatment (Stewart, 1983). Relapses may be associated with the development of parasitemia despite treatment (Stewart, 1983) and may sometimes be more severe than the initial reaction (Uilenberg *et al.*, 1989). In other relapsing cases, clinical symptoms may be very mild and may only be noticed at an advanced stage of the disease when liver and kidney damage have developed due to tissue anoxia (Stewart, 1983).

1.3 Pathogenesis

The pathogenicity of *Babesia* organisms is determined primarily by the species and strain involved (Wright, 1979; Wright & Goodger, 1988; Zwart & Brocklesby, 1979; Uilenberg *et al.*, 1989). In general, small babesias (1.0 µm x 3.0 µm) are highly pathogenic and more virulent than the larger babesias (2.5 µm x 5.0 µm)(Taboada & Merchant, 1991). Two broad syndromes can be defined clinically; one characterized by hypotensive shock and the other by haemolytic anaemia.

A hypotensive shock syndrome, which causes hypoxia and extensive tissue damage, is produced by the most pathogenic species. Vascular stasis from sludging of parasitized cells within the capillary beds also contributes to many of the signs (Taboada & Merchant, 1991). The cause of the shock syndrome is multifactorial and is not completely understood. Some babesial organisms contain a protease with direct hypotensive properties (Wright, 1979). This protease may also activate kallikrein or the alternate complement cascade, contributing to hypotension. The release of biogenic amines may be triggered by the initial release of kinins, which independently and synergistically produce further vasodilation and hypotensive shock. *B. canis rossi* and *B. bovis* are the two parasites species associated with this syndrome (Taboada & Merchant, 1991).

Vascular stasis and sludging is a prominent finding during severe babesiosis resulting in lowered packed cell volume (PCV). Factors involved in the development of vascular stasis include the parasite itself, alterations in the erythrocytic and endothelial membranes, and changes in the physical properties of plasma (Wright & Goodger, 1988). Increased lipid peroxidation in response to the infection increase red cell rigidity, affecting passage through the capillary bed. Infected erythrocytes develop stellate protrusions from their cell membranes that can attach to the capillary endothelium (Igarashi *et al.*, 1988). The most severe sludging occurs in the central nervous system and in muscles. Virulent strains

of *Babesias* are also known to cause disseminated intravascular coagulation (DIC) as a result of the procoagulant activity of babesial organisms (Moore & Williams, 1979). This factor can markedly influence the pathogenesis of the disease.

Haemolytic anaemia is caused by invasion of erythrocytes by parasites resulting in hemolysis and subsequent anaemia. Direct parasitic damage is most important in the pathogenesis of hemolysis but immunologic destruction is known to play a role (Taboada & Merchant, 1991). The resultant anaemia contributes to tissue hypoxia resulting in lactic acidosis (Button, 1979). Pyrexia in *Babesia* infections probably occurs secondary to the release of pyrogens during erythrolysis and parasite/erythrocyte interaction with the macrophage-monocyte system (Breitschwerdt, 1984; MacWilliams, 1987). Haemolysis, pyrexia and anaemia are caused by *B. canis canis* and *B. canis vogeli* (Taboada & Merchant, 1991).

In assessing the initiation and progress of an infection the following should be taken into account:

(i) The age of the infected dog. As a general rule the younger the dog the more susceptible it is to babesiosis (Shortt, 1973; Taboada & Merchant, 1991).

(ii) Whether the dog is splenectomised. A dog splenectomised before infection is more susceptible and usually acquires a more severe infection (Shortt, 1973).

(iii) The general physical condition of the dog. A poorly nourished dog is usually more susceptible than a well fed one (Shortt, 1973).

(iv) *Erhlichia canis* has been shown to predispose dogs to babesiosis. (Van Heerden *et al.*, 1983).

(v) Route of infection by virulent blood. The most certain early infections are those produced by intravenous injection of parasitized blood (Shortt, 1973).

(vi) Volume of inoculum. The result depends less on the volume of inoculum or on the route employed than on the virulence of the strain or the individual susceptibility of the dog (Wright, 1979; Wright & Goodger, 1988; Zwart & Brocklesby, 1979).

(vii) The degree of parasitemia in the inoculum. The higher the number of organisms, the sooner an overt blood parasitemia appears (Shortt, 1973).

(viii) The particular strain of *B. canis* used. The virulence of strains vary considerably both from different areas and individually in the some area and this is probably the most important governing factor in the severity of the infections produced (Wright, 1979; Wright & Goodger, 1988; Zwart & Brocklesby, 1979).

(ix) The final result of the infection - death or chronicity. This depends on the strain or species of parasite and is also related to the individual susceptibility of the dog (Shortt, 1973).

1.4 Pathology

Pathological changes are due to the effects of anaemia and hypotensive shock. (Basson & Pienaar, 1965; Shortt, 1973). A massive invasion of parasitized red cells may block the liver sinuses and so interfere with the nutrition of the liver cells leading to their destruction. The lung is more heavily parasitized than other organs due to the larger proportion of circulating blood in that organ. The capillaries of the septal airspaces may become heavily loaded with normal and parasitized red cells leading to laboured breathing (Basson & Pienaar, 1965; Shortt, 1973). The spleen may become enormously enlarged, congested and dark in colour (Basson & Pienaar, 1965; Shortt, 1973). There are no marked changes in the small intestine but the parasites are present in considerable numbers in the capillaries of the intestinal villi. These do not appear to be numerous enough to cause any haematological changes sufficient to have any marked effect on digestion (Shortt, 1973).

1.5 Immunology of babesiosis

Immunity to *Babesia* infection depends on both the innate resistance of the host and an active immune response directed against babesial antigens (James, 1988). Both cell-mediated and humoral processes play a role in immunity to babesial organisms. Cell-mediated immunity probably is of greater importance against *B. canis* infections (Carson & Phillips, 1981; James, 1988). Natural killer cells and T-effector lymphocytes may be important mediators of functional splenic

activity (James, 1988). The macrophage-monocyte system is critical to the host response during active infection and the spleen and liver are responsible for phagocytosis of free and intra-erythrocytic and damaged red blood cells.

The spleen is an important site of anti babesial antibody production. Both IgG and IgM are involved in the humoral response. When bound to babesial antigens, IgG and IgM fix complement and serve as opsonizing agents, increasing phagocytosis by the macrophage-monocyte system (James, 1988). The anaemia seen in babesia-infected dogs is usually not proportional to the parasitemia. Very anaemic animals may have few organisms, and at other times highly parasitized animals may not be anaemic. Both the haemolytic anaemia and thrombocytopenia probably have an immune-mediated component. The immune response may contribute to the development of clinical disease (Taboada & Merchant, 1991).

Immunosuppression has been demonstrated in dogs with experimentally induced *B. gibsoni* infections (Kawamura, 1985; Kawamura *et al.*, 1987). The immunosuppression seen in naturally occurring babesiosis may predispose affected animals to other infectious processes.

While there appears to be a good understanding of the immunology of *B. canis* infection it is clear that resistance to infection is highly variable within the dog population and

between individuals within any breed (Martinod *et al.*, 1986). In contrast with the situation in cattle the dog appears to develop a relatively weak resistance to *B. canis* (Bigalke, 1976). In southern Africa it is commonly observed that some dogs acquire infection once only and never appear to acquire a further infection while others do acquire further infections - or are they relapses? Similarly an apparently immune dog which is moved to a different endemic area may become infected again. Is that infection due to a relapse of a different strain?

1.6 Project aims

Serologic and cross-immunity studies reported by Uilenberg *et al.* (1989) have revealed antigenic differences among different stocks of *B. canis*. It is on this basis as well as differences in pathogenicity and transmitting vectors, that the trinomial nomenclature system was proposed which split up *B. canis* into *B. c. vogeli*, *B. c. canis* and *B. c. rossi*.

Uilenberg's classification is tentative and his conditions need to be confirmed using a more homogenous experimental dog population which was not the case in his study (Uilenberg *et al.*, 1989). Nevertheless there is a need to study more carefully the existence and nature of various stocks of *B. canis* which may appear in southern Africa especially in the view of various clinical manifestations of the disease in the field situation.

The aim of this project is to attempt to characterise 5 field strains of *B. canis*, collected in various geographic areas of southern Africa, in terms of their pathogenicity, immune response, cross resistance and antigenic components.

CHAPTER TWO

MATERIALS AND METHODS

2.1 Experimental animals

Experimental animals comprised purebred Beagle dogs purchased from the Animal Resource Centre, University of Durban Westville. Both male and female dogs were used, ranging in age from 2 to 4 years old. The animals had been maintained under tick-free conditions since birth. Freedom from *B. canis* was confirmed serologically and by regular direct examination of blood smears. Experimental animals also included cross bred puppies obtained at 6 weeks of age from a breeder in Grahamstown.

All animals were maintained in concrete floored kennels under tick free conditions at the Tick Research Unit (T.R.U.) experimental farm and routinely screened for evidence of *Babesia* infection using serology and direct observation of blood smears. Additionally the Beagles were bled at 2-weekly intervals from the cephalic vein and the sera stored at -20°C until needed for further use. All the crossbred puppies were splenectomised on arrival at the T.R.U. according to standard surgical procedures. Animals were smeared daily for evidence of *Babesia* infection and were used approximately 1 month after splenectomy.

2.2 Stocks of *Babesia canis*

The stocks of *B. canis* used were all isolated from confirmed cases of clinical babesiosis presented to veterinary

practitioners in the field. In all cases 2ml venous blood was collected into heparin tubes, submitted to the T.R.U. on ice and the blood was stored in a 20% solution DMSO/PBS (Dimethyl Sulphoxide/Phosphate buffered saline, pH 7.2) - the DMSO/PBS pH 7.2 solution was slowly added to the blood (1:1) in a test tube and mixed gently for 5 min). The mixture was divided into 1ml aliquots and this stabilate was stored in the air phase of liquid nitrogen for not longer than 12 months before it was used.

The *B. canis* stocks included the following:

Zimbabwe (ZIM1) - The stock was collected from a dog in Harare which displayed anorexia, listlessness, haemoglobinuria and progressive anaemia. The infected blood was preserved in heparin and transported to Grahamstown in liquid nitrogen.

Grahamstown (GHT1) - This stock was collected from a mildly infected dog. Symptoms included anaemia and lethargy.

Queenstown (QTN1) - This stock was collected from a dog showing progressive anaemia and which had relapsed following treatment for babesiosis 1 month previously. Relapsing canine babesiosis had been occurring commonly in the practice concerned.

Durban (DBN1) - This stock was collected from a dog showing signs of severe vomiting, pyrexia and diarrhoea. Vomition was the most consistent finding in terms of canine babesiosis in this practice (Dr. Perchman, *pers. comm.*) and

therefore it was considered worthwhile to collect this particular stock of *B. canis*.

Johannesburg (JHB1) - This stock was collected from a puppy showing nervous symptoms followed by sudden death. The clinical symptoms and post mortem were consistent with cerebral babesiosis. This type of clinical babesiosis was commonly encountered in the practice concerned and accounted for a high mortality in that area.

2.3 Artificial infection of dogs with stocks of *B. canis*.

Six Beagle dogs were each infected with one of the *B. canis* stocks respectively according to the following protocol:
Vials of deep frozen *B. canis* infected blood were thawed out by immersion in water at 37°C. The thawed blood was then injected intravenously into a splenectomised cross bred puppy. The puppy was monitored for evidence of babesiosis using direct blood smear examination. Parasitemia was determined by estimating the mean percentage of parasites in 20 fields of vision on a smear of venous blood. The mean percentage of the total red cell count (RCC) yielded the approximate number of parasites per millilitre. Red cell counts were carried out using a Coulter counter at the Regional Veterinary Laboratory, Grahamstown. Approximately 10^7parasites were injected intravenously into each of the respective Beagle dogs. The recipient was monitored for infection in terms of clinical symptoms, body temperature, blood smear and packed cell volume (PCV). Dogs were treated with diminizene aceturate (Berenil®) at 3mg/kg when PCV, clinical

symptoms and parasitemia suggested that the infection was life threatening. A 2ml sample of infected venous blood was collected from the Beagle dogs before treatment for use in homologous challenges. The blood was stored in liquid nitrogen as described earlier. Beagles recovering from their first artificial infection were monitored closely for the evidence of relapses following treatment. Sera were collected regularly for serology as described earlier.

2.4 Homologous and heterologous challenges

Six weeks after the primary infection and treatment, the dogs were given an homologous challenge, 1ml aliquot of the appropriate *B. canis* stabilate, to boost immunity. The dogs were closely monitored as discussed earlier and if treatment was needed again, dogs were given further homologous challenges at six weekly intervals (Table 2) until homologous immunity was established. After all the dogs had proven to have homologous immunity, a heterologous challenge was given not sooner than 6 weeks later to test for cross protection. Reports from veterinary practitioners suggested that the Johannesburg stock was the more virulent stock and was therefore chosen as the stock for the heterologous challenges. Each dog received an intravenous injection of 0.5ml of stabilate which contained the Johannesburg stock on 12 November 1991.

2.5 Preparation of *B. canis* antigen using saponin

The cross-bred puppies which provided the infective *B. canis* blood were euthanased when the parasitemia reached at least 50%

in a peripheral blood smear. At euthanasia, 500ml of blood was collected by cardiac puncture into a vacuumised blood collecting set containing citrate anticoagulant. The blood was then allowed to stand over night at 4°C. 100ml of pooled blood, collected from each of the naive Beagles, was put through the same process to serve as control.

The plasma was drawn off the parasite rich blood by centrifuging at 3500 rpm for 30 minutes in a Sorvall GSA refridgerated centrifuge at 4°C and the supernatant and buffy coat was discarded. The remaining red cells were resuspended in more than 3 volumes of PBS (pH 7.2), shaken and centrifuged as before. This step was repeated twice. The cells were finally resuspended in more than three volumes of TEN buffer (20mM Tris, 10mM EDTA, 100mM NaCl, pH 8.0). The suspended cells were centrifuged as before and the supernatant discarded. The cells were warmed to 37°C before an equal volume of saponin (Sigma Chemical Co., St. Louis, USA) (1mg/ml prewarmed to 37°C) was added. The mixture was mixed gently for 10 seconds and four volumes of TEN buffer was then added and mixed gently at room temperature. This mixture was centrifuged at 3500 rpm for 20 minutes. The cloudy supernatant was retained and centrifuged at 10 000 rpm for 30 minutes at 4°C in a Sorvall HB4 swingout rotor. This left three layers in the tube - from the bottom; a small hard pellet (WBC nuclei), a soft pellet (which contained the parasites) and the supernatant. The supernatant was discarded and the soft pellet was resuspended in more than six volumes of TEN buffer and transferred to another centrifuge tube leaving the

small hard pellet to be discarded. The mixture was centrifuged again at the same speed. This step was repeated until a white pellet was obtained. A drop of this pellet was then smeared onto a microscope slide, stained with RapiDiff stain (Clinical Science Diagnostics) and viewed under a microscope to confirm the presence of parasites. The parasite rich fraction was divided into 1 ml aliquots and stored at -20°C until needed (Dr. W K Jorgensen - Tick Fever Research Centre, Wacol, Australia, *pers. comm.*).

2.6 Serological tests

2.6.1 Indirect fluorescent antibody test

Preparation of antigen slides

A splenectomised dog was inoculated intravenously with the ZIM1 stock and exsanguinated after a 15% parasitemia was reached on the fourth day (Levy *et al.*, 1987). 200ml blood was washed 4 times in phosphate-buffered saline (PBS; pH 7.2). The cells were then resuspended to yield a final PCV of 50% in PBS pH 7.2 to which was added 1.75% bovine serum albumin (BSA, Sigma Chemical Co., St. Louis, USA). Thin blood smears were prepared on glass slides, air dried, wrapped in tin foil and frozen at -20°C until required.

Immediately prior to use, antigen slides were warmed to room temperature in a desiccator jar to prevent lysis by moisture condensation on the cold slides, fixed for 10 minutes in acetone at room temperature, and dried. Each blood smear was divided up into 15 squares by drawing lines on the back of the slide with

an indelible pen. Pooled negative serum from *B. canis* susceptible dogs was used as a negative control. Positive and negative sera were titrated to establish a screening dilution. Test blood samples were firstly diluted 1:10 (in PBS pH 7.2 containing 0.5% BSA) in Bijou bottles before they were subjected to a two fold serial dilution in a round bottom microtitre plate.

Five microliters of each serum dilution together with the positive and negative controls were applied to the centre of the squares and incubated in a humidified chamber at 37°C for 30 minutes. Each slide was washed for 1 minute with PBS pH 7.2 and the excess PBS was shaken off lightly. Drops of rabbit anti-dog immunoglobulin G (whole molecule) florescein isothiocyanate-conjugate (Sigma Chemical Co., St. Louis, USA) diluted 1:80 in PBS/BSA(0.5%), were applied to the centre of each square, incubated, washed and dried as before. 10% Buffered glycerol (pH 7.2) was applied to each slide, a cover slip was applied and slides were examined at 400X magnification, using a Zeiss epifluorescence microscope. Fluorescence was graded 1 - 5. Titres which yielded a 4 - 5 fluorescence were regarded as positive.

2.6.2 Changes in host serum immunoglobulin levels following infection by *B. canis*

A commercial immunoglobulin serial radial immunodiffusion (SRID) Kit (Veterinary Medical Research and Development, Inc.) was obtained to quantitatively determine how the level of immunoglobulin (Ig) alter with the progression of canine babesiosis infection. In this system antiserum specific for

canine Ig is incorporated into agarose gel and the test antigen diffuses into the gel containing the antibody forming a ring of precipitation that is proportional to the concentration of antigen. A linear relationship exists between the diameter of the ring and the concentration of the antigen when plotted on semi-log graph paper. Test sera were taken from the same samples collected for the IFAT.

Reference standards were determined by pipetting 3µl of supplied antigen into the first wells while the remaining wells were each filled with 3µl test sera. The gel was left at room temperature for 18 hours, the diameter of the rings were determined and read off against the standard curve. In case of very high Ig values the serum was diluted two-fold in PBS (pH 7.2) before testing.

2.6.3 Passive immune serum transfer

One of the Beagles, Jason, was found to be resistant to the Zimbabwe stock following 2 homologous challenges. The dog also manifested a high antibody titre to *B. canis* as confirmed by the indirect florescent antibody test (IFAT). Jason was bled weekly over a period of three months to obtain at least 100ml of immune serum. The venous blood collected, was allowed to clot and the serum was drawn off by centrifugation and stored at -20°C until needed. Antibody levels of individual batches were confirmed using IFAT. Non immune control serum was acquired by exsanguinating a *B. canis* susceptible dog by cardiac puncture.

The donor dog was confirmed to be susceptible to *B. canis* using the IFAT.

The blood was collected into a vacuumised collecting container and the serum was drawn off as before. The batches of immune serum were pooled and these together with the non-immune serum were incubated at 56°C for one hour to eliminate complement immediately prior to inoculation of recipients. Five out of 10 *B. canis* susceptible cross-bred dogs were each slowly inoculated intravenously with 10ml of immune serum while the remaining five received the same volume of non-immune serum. All the dogs were then immediately challenged subcutaneously with 0.5ml of *B. canis* infected blood containing approximately 10^6 parasites of the ZIM1 stock. Dogs were monitored daily for evidence of pyrexia, parasitemia, PCV and clinical symptoms. 2ml Venous blood was collected once a week from all recipients and serum stored at -20°C for determination of antibody titres.

2.6.4 Polyacrylamide gel electrophoresis

Crude *B. canis* antigen was resolved by SDS polyacrylamide gel electrophoresis (PAGE) (Laemmli, 1970) to determine differences in parasite proteins between the respective experimental stocks used. A UVP Gel Analyzing computer software package was use to analyze the gel.

The first lane of the gel was loaded with Sigma molecular weight marker proteins and the next five lanes with 30µg of crude *B. canis* protein from each of the respective stocks. The last lane was loaded with control antigen (uninfected host

erythrocytes). A standard polyacrylamide gel (stacking gel 4%, resolving gel 10%) was run at 60 Volts for ±12 hours. The gel was removed from the electrophoresis tank and stained with Coomassie brilliant blue to identify the resolved protein. A slab gel drier was then used to vacuum dry the gel onto filter paper. This was done for three hours using heating for the first hour.

2.6.5 Western blotting

The sera used for western blotting were collected from the Beagle dogs during the period after their homologous challenge up to before their heterologous challenges. Sera collected from the different Beagles were screened against the respective *B. canis* stock e.g. blood collected from Pompies (infected with the GHT1 stock) was screened against GHT1 antigen.

The first lane of the gel was loaded with Sigma molecular weight marker proteins and the next five lanes with crude *B. canis* antigen of the five respective stocks. A standard polyacrylamide gel (stacking gel 4%, resolving gel 10%) was run at 60 Volts for ±12 hours. The gel was removed from the electrophoresis tank placed in a basin of transfer buffer (25mM Tris, 190mM Glycine, 20% methanol - prechilled) and left to equilibrate for 30 minutes in the buffer.

Proteins were transferred to nitrocellulose paper (Hoeffer Scientific) in a Bio-Rad transblotting tank. Fibre pads, Whatman No. 3M filter paper and nitrocellulose strips were pre-soaked in

transfer buffer. The equilibrated gel was placed on the filter paper and the pre-wet nitrocellulose strips were placed over the different lanes of the gel. This was covered with a second piece of filter paper and fibre pad. The gel holder was closed and placed in the transblotting tank with the gel on the anode and nitrocellulose on the cathode side. The transblotting tank was filled up with transfer buffer and placed on a magnetic stirrer. Electroblotting was allowed to continue at 0.15A for at least 20 hours at room temperature.

After transblotting, the nitrocellulose was removed form the holder, incubated in 0.5% gelatine in TPBS (PBS pH 7.2, containing 0.5% Tween 20) at 37°C for 1 hour and rinsed 3 x 10min in TPBS. The nitrocellulose was then incubated in appropriate homologous serum primary antibody solution diluted 1:75 in TPBS for 3 hours, washed as before and incubated in rabbit anti-dog IgG (whole molecule) peroxidase conjugate (Sigma Chemical Co., St. Louis, USA) diluted 1:2000 in TPBS for 3 hours. Again the nitrocellulose was washed 3 times. A colour solution was then made up as follows: 60mg HRP colour developing reagent (Sigma Chemical Co.) was added to 20ml ice cold methanol in one bottle and 60µl ice cold hydrogen peroxide was added to 100ml TPBS in a second bottle. The contents of the two bottle were mixed, poured over the nitrocellulose and left to develop for 20 minutes. The blots were air dried and photographed within the hour.

2.6.6 Indirect microhemagglutination test

Crude *B. canis* antigen from the ZIM1 stock prepared by saponin treatment as discussed earlier was washed three times in PBS (pH 7.2). It was then sonicated with a MSE sonicator for 3 minutes. 10ml of fresh goat blood was collected in an equal volume of Alsevers solution (0.05% dextrose, 0.42% sodium chloride, 0.8% sodium citrate, 0.055% citric acid, pH 6.1). The blood was then washed three times with PBS and the goat erythrocytes (GE) were resuspended to 20% in PBS containing 10% formalin. This mixture was incubated at 37°C for 1 hour. After washing three times with PBS the formalin-fixed red cells were resuspended to 10% in PBS containing 0.5% formalin, and stored at 4°C until ready for use. Activation and sensitization of formalin fixed GE were processed following the method described by Fujita *et al.* (1991).

This method was used because of the relatively small amount antigen required. After washing 3 times with PBS, 1 volume of this suspension was mixed with an equal volume of PBS containing 0.02% tannic acid. After incubation for 30 minutes at 37°C, the GE were washed four times with PBS. For sensitization of the cells with *B. canis* antigen, 1 volume of the activated cells was resuspended in 2 volumes of PBS again, and 1 volume from the suspension was mixed with an equal volume of antigen solution containing 0.75mg protein/ml. The mixture was incubated at 37°C for 2 hours, agitating every 20 minutes. After incubation, the sensitized cells were washed four times in PBS and suspended to 1% in 0.075M PBS containing 0.25% arabic gum, 1% normal goat

serum, 0.01% Tween 80, and 0.1% sodium azide, and stored at 4°C until ready for use.

The test was performed as follows: Test and negative control sera were diluted out two fold in PBS diluent into a round bottom Cooke microtitre plate starting with neat serum and ending with a PBS control so that each well contained 20µl of the serum/PBS mixture. 5µl of sensitized cells were pipetted into each well, the plate was agitated and left for 12 hours at room temperature before the titres were read.

CHAPTER THREE

RESULTS

3.1 Artificial infection of dogs with stocks of *B. canis*. Primary and homologous challenges.

Results of the primary, homologous and heterologous challenges of Beagles with respective *B. canis* stocks are summarized in Table 2.

3.1.1 Zimbabwe stock (ZIM1)

The first male Beagle, Jason, developed a detectable parasitemia 3 days after a primary challenge with $\pm 10^7$ *B. canis* parasites (Fig. 1). Jason's temperature on day 3 was raised above 40°C and by the 4th day the parasitemia increased rapidly to 17 parasites per field. The dog remained lively with a PCV of 42% but there was evidence of a severe haemoglobinuria. Prior to treatment with 45mg berenil on day 3, 3ml of Jason's infected blood was collected into heparin for the homologous challenge. The dog's PCV decreased to 35% on the following day (4) but returned to 42% after 48 hours. Jason's recovery was uneventful and rapid.

Six weeks after the primary challenge with the ZIM1 stock, Jason, together with a naive splenectomised dog, which served as control, was challenged with 0.5ml of the homologous stock. Parasites appeared in the peripheral blood smear of the control and Jason 3 and 7 days respectively after inoculation. Jason's

Table 2. Results of cross-immunity tests in Beagle dogs. Dates of initial, homologous and heterologous challenges are in 1991, except where otherwise indicated. P = Parasitemia, F = Fever, D = Disease symptoms and R = Treatment.

Stock (Dog)	Date of 1st infection & results	R	Relapse	R	Date of homologous challenge & results	R	Relapse	R	Date of heterologous challenge & results
Zimbabwe (Jason)	16/4 PFD	19/4	-	-	28/5 PFD 10/8 PFD 12/11 PFD	6/6	18/6	-	6/3/92 FP
Grahamstown (Pompies)	20/5 PFD	23/5	-	-	01/7 PF	-	-	-	6/3/92 -
Queenstown (Ali)	13/7 PFD	19/7	4/8	-	(Died 30/9)				
Queenstown (Phoebe)	7/10 PFD	16/10	-	-	12/11 PF	-	-	-	6/3/92 P
Durban (Katryn)	9/9 PFD	13/9	3/10	12/10	12/11 PF	-	-	-	6/3/92 P
Johannesburg (Blondie)	25/5 PFD	(Died 29/5)							
Johannesburg (Ghost)	14/9 PFD	16/9	-	-	18/10 PFD 12/11 PFD	26/10 22/11	(Died 28/12)		

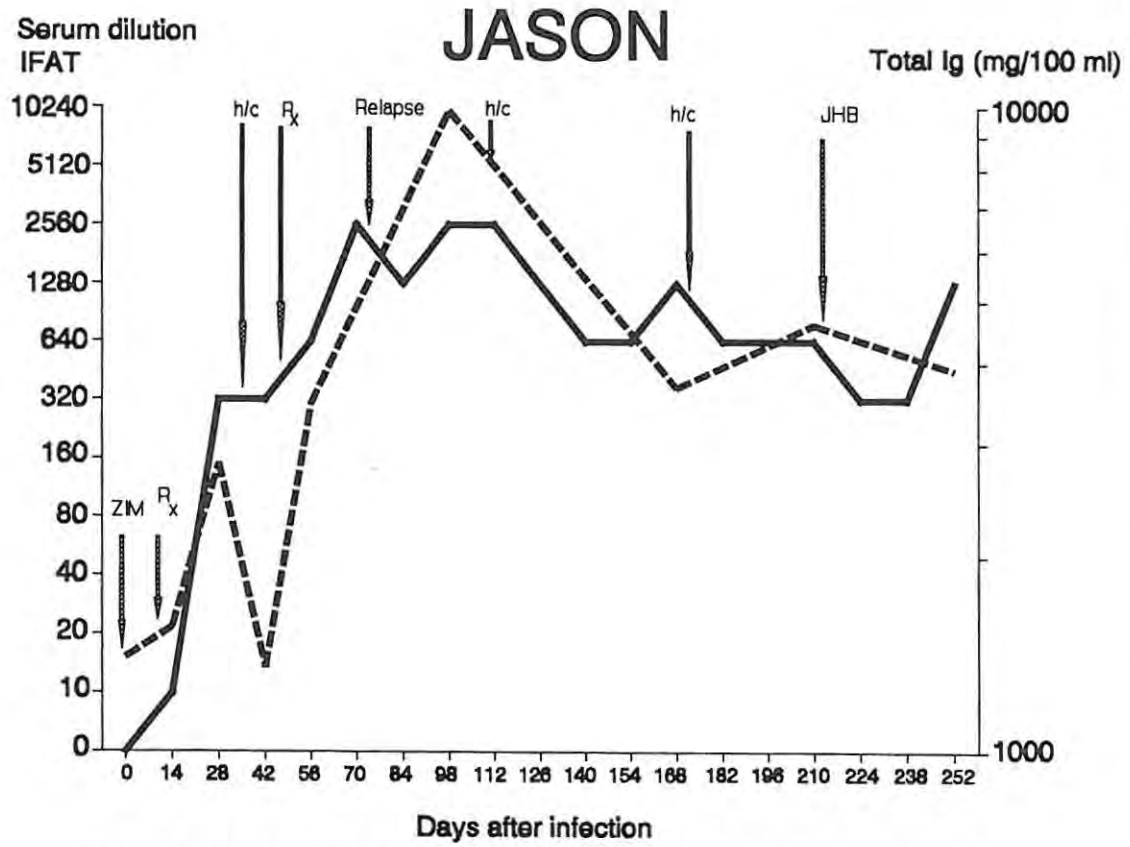


Fig. 1 Profile of antibody titres (IFAT ———) and Ig levels (– – – –) in Jason following homologous and heterologous challenge with the ZIM1 stock.

Legend to figures 1 to 5 .

R_x - Treatment

h/c - homologous challenge

JHB - heterologous challenge with the JHB1 stock

------ - Total serum Ig concentration

——— - Antibody titre (IFAT)

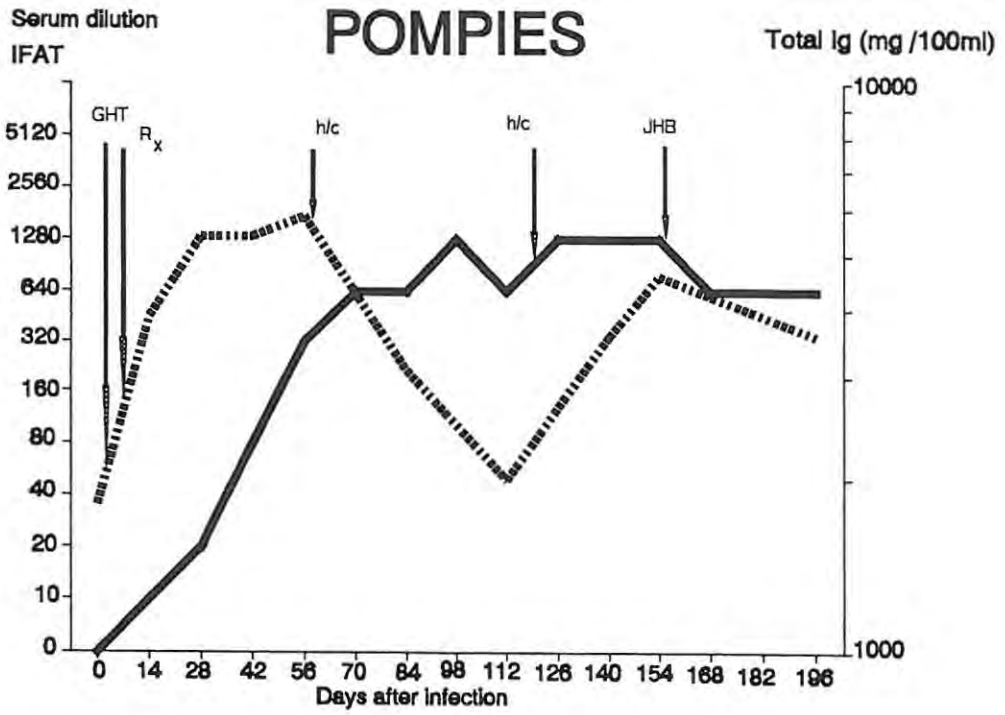


Fig. 2. Profile of antibody titres (IFAT ———) and Ig levels (– – –) in Pompies following homologous and heterologous challenge with the GHT1 stock.

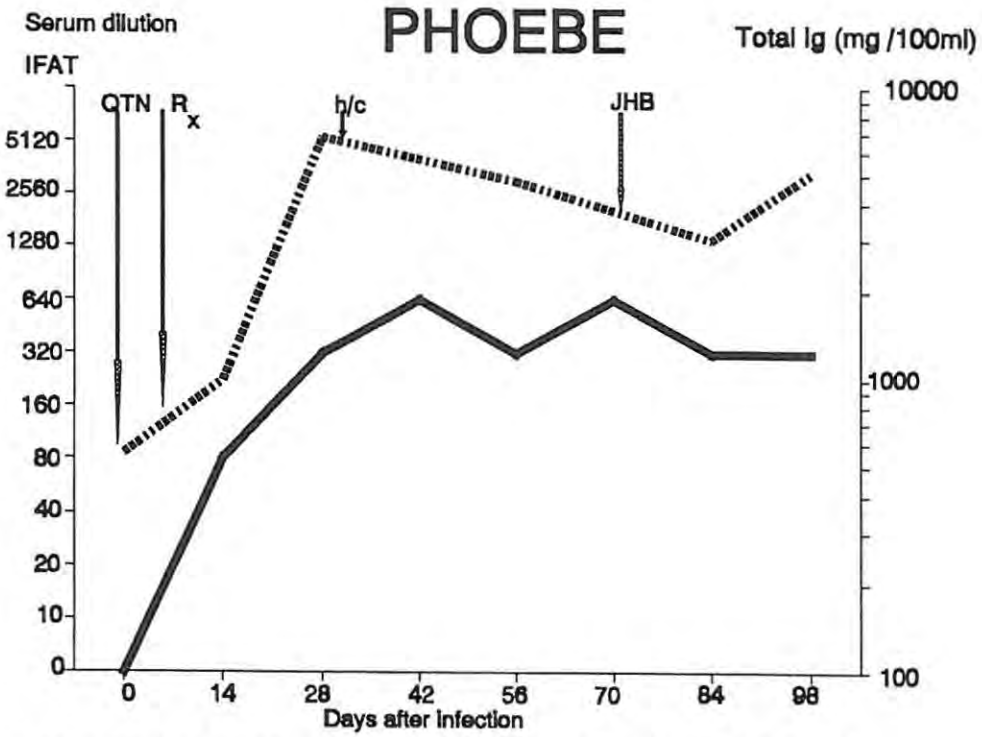


Fig. 3. Profile of antibody titres (IFAT ———) and Ig levels (– – –) in Phoebe following homologous and heterologous challenge with the QTN1 stock.

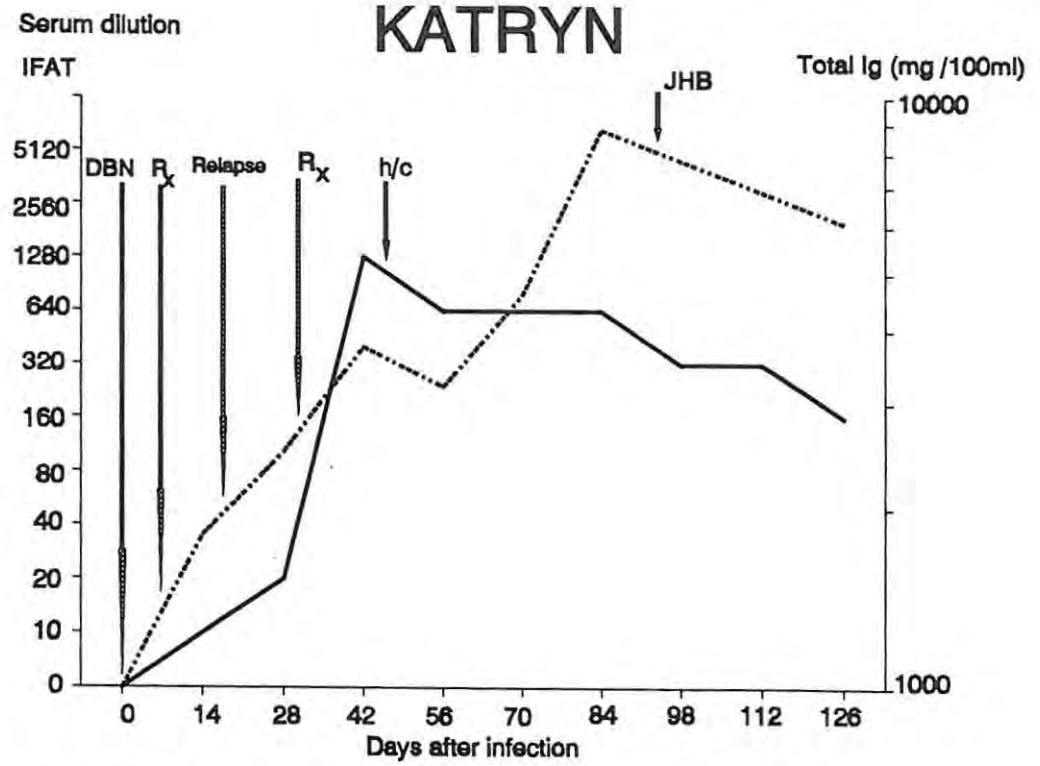


Fig. 4. Profile of antibody titres (IFAT ——) and Ig levels (– – –) in Katryn following homologous and heterologus challenge with the DBN1 stock.

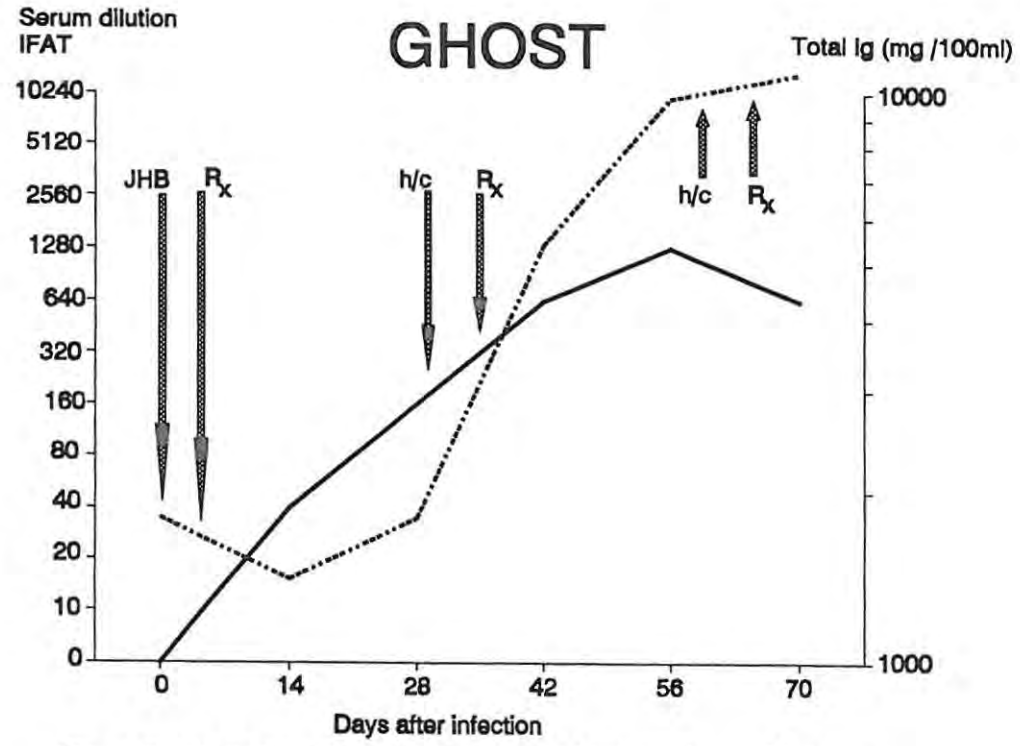


Fig. 5. Profile of antibody tires (IFAT ——) and Ig levels (– – –) in Ghost following homologous and heterologous challenge with the JHB1 stock.

parasitemia increased from less than (<) 1 parasite per field on day 7 to 8 parasites per field on day 9 and the PCV had declined to 25%. He manifested a haemoglobinuria, inappatence and slight weight loss. Jason was again treated with 36mg Berenil on day 9. However, 12 days after this treatment, he had a relapse, as evident from a peripheral blood smear. He developed a fever but showed no clinical symptoms and remained lively and ate well. He was not treated for the relapse although the PCV had declined from 45% to 35.7%. The parasitemia remained low (<4 parasites per field) for another 3 weeks before the infection cleared. Prior to his heterologous challenge Jason received 2 more homologous challenges at 6 weekly intervals but failed to develop parasitemia or obvious clinical symptoms after each challenge dose.

3.1.2 Grahamstown stock (GHT1)

A male Beagle, Pompies, was inoculated with 1ml of stabilate containing $\pm 10^7$ *B. canis* parasites and showed a parasitemia of <1 parasites per field as well as a temperature of 40.1°C, 2 days later (Fig. 2). Parasitemia increased to 4 parasites per field 4 days after inoculation. At this stage Pompies was very listless and anorexic. He had a deep yellow urine and became progressively anaemic. The dog's PCV had dropped to 22% when he was treated with 39mg Berenil on day 4 after inoculation. His recovery was rapid and uneventful.

Six weeks after the primary challenge, Pompies as well as a splenectomised control were each inoculated with 0.5ml of

infected homologous blood. Pompies and the splenectomised dog manifested positive peripheral blood smears 4 days later. Pompies developed a fever but showed no clinical symptoms and remained lively. The parasitemia remained low (<2 parasites in 10 fields) until day 7 after which no *B. canis* were found in the smears. His PCV came down to 33.9% from 42% on day 8 and he remained untreated. The splenectomised dog died on day 8. Six weeks before his heterologous challenge, Pompies received a further homologous challenge to which he was totally resistant i.e. no parasites were encountered in peripheral blood smears and he remained clinically normal.

3.1.3 Queenstown stock (QTN1)

Ali, a bitch, was inoculated with 0.5ml of stabilate containing $\pm 10^7$ *B. canis* parasites of the Queenstown (QTN1) stock and parasitemia was recorded 4 days later. On the seventh day, the parasitemia increased to 17 parasites per field. The symptoms were less severe than in the splenectomised dog and no vomiting was noticed. The dog had a PCV of 40% when it treated on the same day (7). 16 days later the dog relapsed without showing clinical symptoms. Peripheral smears were still positive for *B. canis* for 2 months when she unfortunately died as result of a dog fight.

The trial was repeated on a 7 months old, Beagle bitch, Phoebe, who was inoculated with the same dose of the original QTN1 stock as Ali (Fig. 3). She developed a parasitemia 7 days after inoculation. The symptoms were less severe than for Ali and was

restricted to listlessness and loss of appetite. The parasitemia remained low (<3 parasites per field) but the dog became progressively anaemic until she was treated when the PCV reached 22% on day 10. This dog was given a homologous challenge 4 weeks later to which she was partially resistant i.e. a parasitemia was detected 5 days after inoculation. Other than a fever of 39.8°C no obvious clinical symptoms were noticed and she was not treated. The parasitemia remained less than 2 parasites per field for 2 more weeks, following which no further parasites were detected in peripheral smears. No treatment was needed and she was therefore not given a second homologous challenge.

3.1.4 Durban stock (DBN1)

A female Beagle, Katryn, was inoculated with the Durban (DBN1) stock and developed a parasitemia 2 days later (Fig. 4). Clinical symptoms included listlessness and by day 4 anaemia and a parasitemia was 10 parasites per field was recorded. Katryn's PCV had come down to 32% to from 38% when she was treated with 33mg Berenil on day 4. 20 days after treatment Katryn suffered a relapse. The parasitemia remained low (<1 parasite per field) and she became listless and anaemic. She was treated a second time with 30mg Berenil when her PCV reached 10% on day 10. She recovered uneventfully despite her low PCV. Katryn was given a homologous challenge by intravenous injection of infected blood containing 10^7 parasites 4 weeks later to which she was totally resistant.

3.1.5 Johannesburg stock (JHB1)

The Beagle, Blondie, a bitch, was inoculated with 1.5ml of infected blood containing a Johannesburg stock of *B. canis* and 3 days later the parasitemia was ±1 parasite per field. The dog still seemed perfectly well, she was lively and had a good appetite. Early on day 4 the parasitemia was determined at 5.2 per field. At this stage the dog still showed no obvious symptoms of babesiosis. Late afternoon, the same day, the dog appeared dyspnoeic and manifested a haemorrhagic vaginal discharge. After a blood smear was taken, the parasitemia was estimated at 38 parasites per field. The dog died the same afternoon. A histopathological examination (conducted by Prof. M. Collett of the University of Pretoria, Dept. of Pathology, Faculty of Veterinary Science) revealed a severe lung oedema with scattered *Babesia* parasitized red cells in the lung capillaries. The heart vessels also showed massive sludging by *Babesia* parasitised red cells. The capillaries of the spleen and stomach also contained many parasitised red cells.

Eight weeks later a second Beagle bitch, Ghost (Fig. 5) was infected with the same Johannesburg stock by an intravenous injection of $\pm 10^7$ parasites. Again after 2 days incubation an overt parasitemia was observed. The dog had a fever but no clinical symptoms was evident at this early stage. Although she had a PCV of 47%, Ghost was treated immediately for fear of losing another dog to this particular stock. 6 weeks later Ghost received her homologous challenge and was positive 6 days later. She had a fever but the parasitemia remained <1 per field. By

day 8 the bitch was showing clinical symptoms i.e. vaginal haemorrhage, haemoglobinuria and loss of appetite. She became progressively anaemic (PCV 13%) and was treated with 39mg Berenil. 6 weeks later, Ghost was given a second homologous challenge to which she was susceptible. This time symptoms were less severe and were restricted to listlessness and progressive anaemia. She was treated on day 9 when her PCV dropped to 12%. Ghost only became partially resistant after her third homologous challenge. She developed a parasitemia of not more than 2 parasites per field, a fever of 39.5°C and became inappatent but did not require treatment.

3.2 Heterologous challenges.

Following heterologous challenge with the Johannesburg (JHB1) stock, Jason (ZIM1) and Phoebe (QTN1) had positive blood smears on day 4 and Katryn developed a parasitemia on day 8 (Table 3). At this stage Jason had a temperature of 39.8°C and a PCV of 45.2% and Phoebe had a fever of 40.2°C and a PCV of 37.5%. In both cases the parasitemia remained <1 parasites per field.

Except for the febrile reaction and low parasitemia no clinical symptoms were observed in these two dogs. Like the other dogs, they remained typically lively and ate well. This febrile reaction declined by day 8 when the body temperatures of both dogs declined below 39°C. Jason had a PCV of 40.8% and his parasitemia was detectable on blood smears until day 12 while Phoebe had a PCV of 17.5% and her parasitemia persisted until day 11.

On day 8 Katryn (DBN1) showed a positive smear with a parasitemia of 5 in 10 fields and a body temperature of 38.9°C. On day 12 the PCV's of all the dogs were determined as follows: Jason 49.8%, Pompies 36%, Katryn 25% and Phoebe 23%. Although Phoebe (QTN1) had no fever at this stage, she began to show signs of listlessness, loss of appetite and started vomiting. These symptoms gradually cleared after 1 week without appropriate treatment. By day 14 all the dogs had negative smears. None of the dogs required any treatment. Pompies (GHT1) was the only dog that was totally resistant to the heterologous (JHB1) stock.

Table 3. Results of the heterologous challenge on 4 Beagle dogs. **(+)** - positive blood smear, **(p/f)** - parasites per field

	Temp. (°C)	Parasitemia	PCV (%)	Symptoms
Jason	39.7	+, day 4, <1 p/f	40.8	None
Pompies	-	-	46.7	None
Phoebe	39.5	+, day 4, <1 p/f	17.5	aneamia, lethargy, anorexia.
Katryn	38.9	+, day 8, <1 p/f	19.4	anaemia, lethargy

3.3 Serology

3.3.1 IFAT assays

The IFA test was found to be sensitive and relatively easy to interpret despite variations in fluorescence on certain days. In dubious cases the tests were repeated in order to confirm end-point fluorescence. Typical positive and negative fluorescence are shown in figures 6 and 7 respectively. Titrations of negative and positive serum samples indicated the sera could be

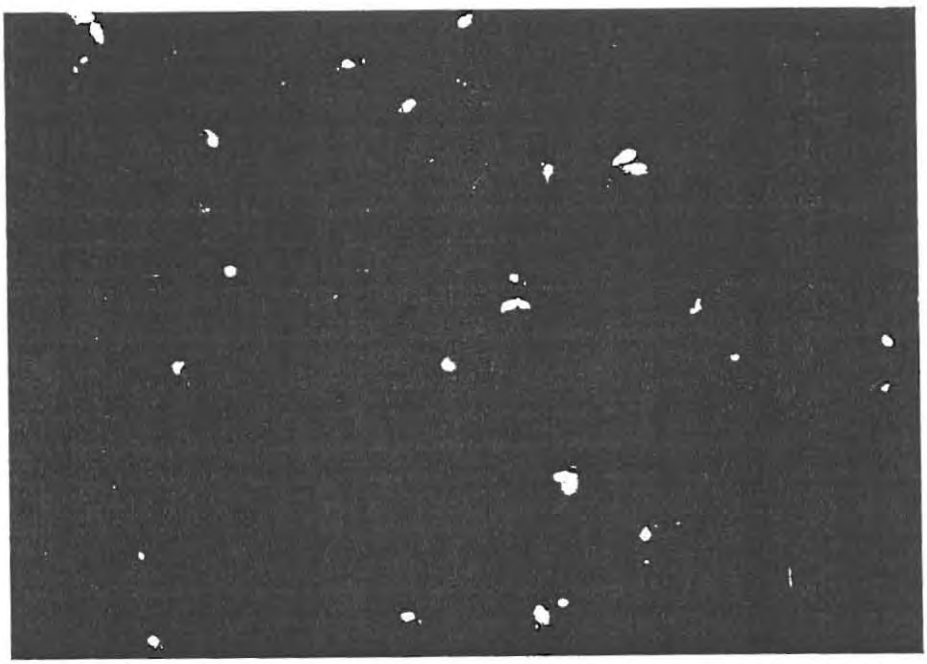


Fig. 6. Typical example of positive fluorescence obtained with rabbit anti-dog immunoglobulin G (whole molecule) florescein isothiocyanate-conjugate diluted 1:80 in PBS/BSA (0.5%). 1000X mag.

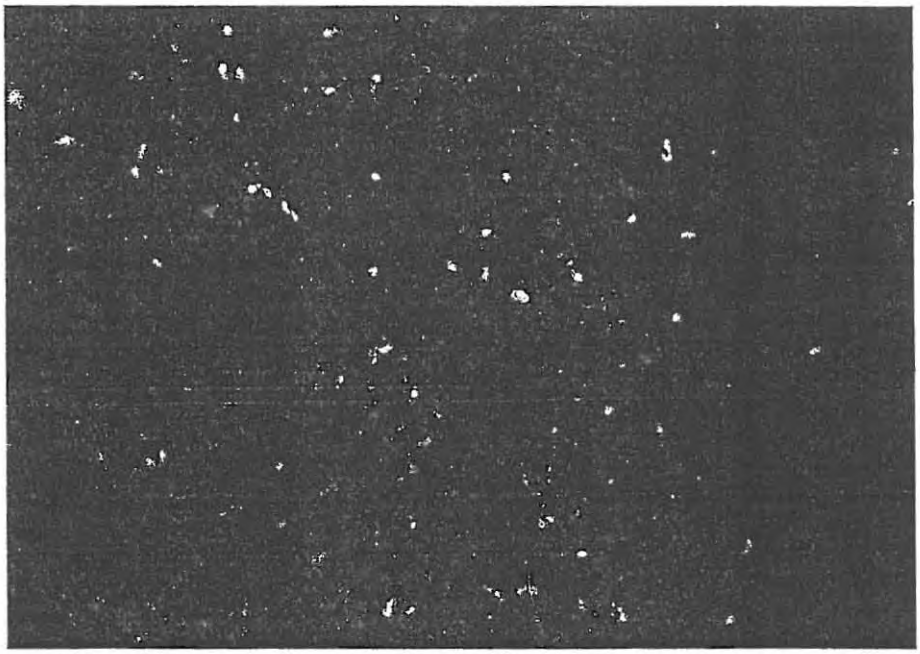


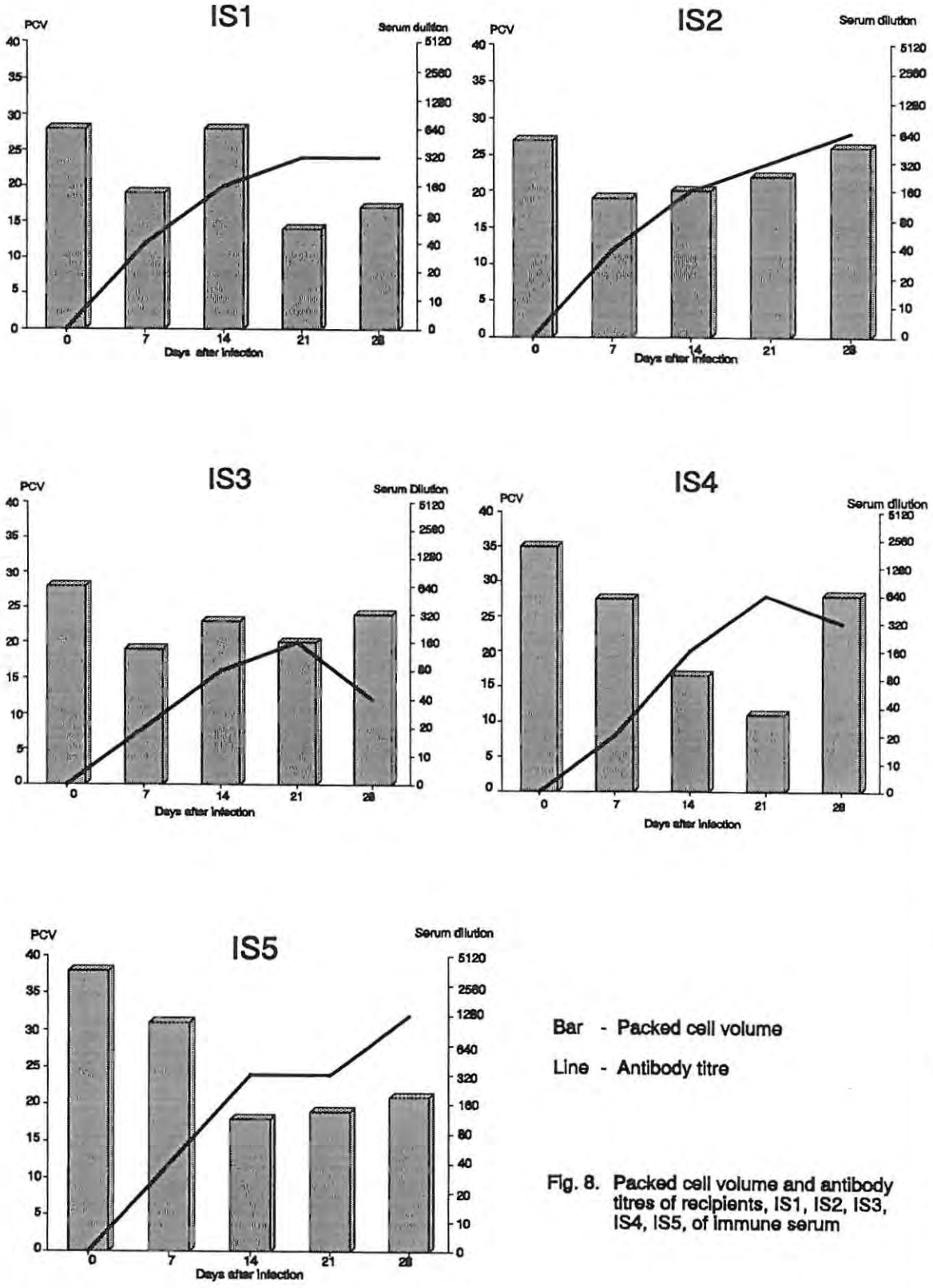
Fig. 7. Typical example of negative fluorescence abtained with rabbit anti-dog immunoglobulin G (whole molecule) florescein isothiocyanate-conjugate diluted 1:80 in PBS/BSA (0.5%). 1000X mag.

screened at 1/20 to avoid nonspecific fluorescence. All dogs tested negative for *Babesia* according to the IFA test and had a total serum Ig concentration of between 560 and 1800 mg Ig/100ml before they received their primary challenge.

Changes in antibody (IFAT) titres (IgG) and total serum Ig levels are shown in figures 1 - 5. The first antibody titres to *Babesia canis* were detected after the first bi-monthly sample was taken. Upon their homologous challenge the dogs had IFAT titres of 1:1280 and above except for Jason (1:320), Ghost (1:320) and Pompies (1:80). Total serum Ig concentrations were above 3750mg Ig/100ml except for Jason (1350 mg Ig/100ml) and Ghost (1800 mg Ig/100ml). Heterologous challenges did not bring about any dramatic changes in IFAT titres nor in total serum Ig concentrations.

3.3.2 Passive immune serum transfer

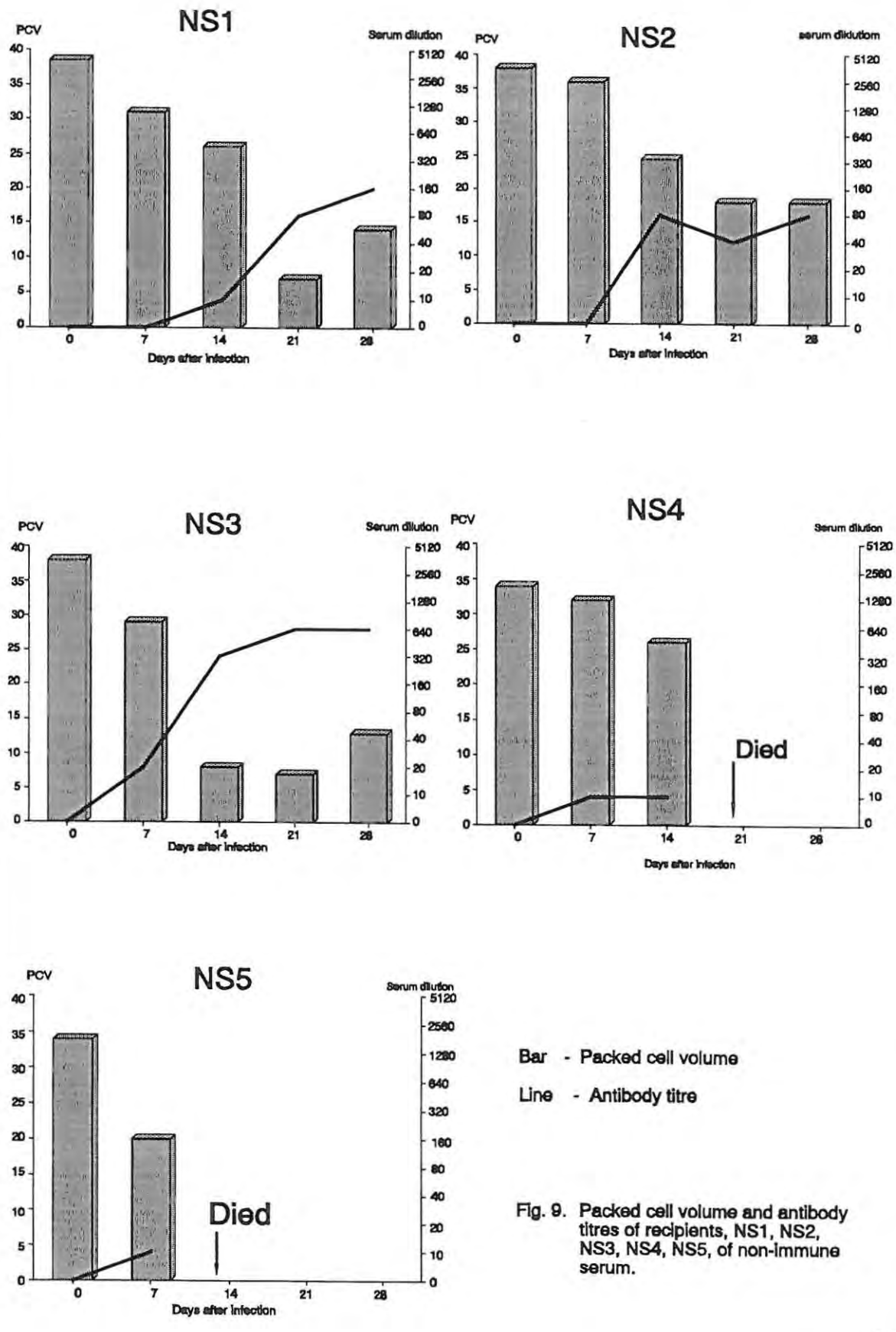
Figures 8 & 9 and table 4 summarize the results of the serum transfers. All dogs had positive smears and a fever on day 4 after inoculation (Table 4). Although all dogs demonstrated disease symptoms, recipients of non-immune serum (NS dogs) showed more severe symptoms (vomiting, anorexia and death) than recipients of immune serum (IS dogs) (loss of appetite and listlessness). All the dogs that received immune serum survived without treatment whereas 3 of the five dogs that received non-immune serum died. The parasitemia remained very low for IS dogs (less than 1 parasite per field) except for the cross bred, IS3, which had a parasitemia of ±8 parasites per field from days 7 to



Bar - Packed cell volume

Line - Antibody titre

Fig. 8. Packed cell volume and antibody titres of recipients, IS1, IS2, IS3, IS4, IS5, of immune serum



Bar - Packed cell volume

Line - Antibody titre

Fig. 9. Packed cell volume and antibody titres of recipients, NS1, NS2, NS3, NS4, NS5, of non-immune serum.

9 while NS dogs had significantly higher parasitemias (±5 parasites per field). PCV's of the IS dogs remained above 25% except for that of two cross bred German Shepherd dogs that went as low as 11.5% and 14% respectively (average PCV for IS dogs = 23.2 ± 6.3). PCV's of the NS dogs were also lower and at one stage were all below 10% except for the Beagle (average PCV for NS dogs = 17.8 ± 13.8).

On day 7 after inoculation antibody titres of IS dogs ranged from 1:20 to 1:40 and 3 weeks later Ab titres ranged from 1:160 to 1:640. On day 7 the NS dogs had no detectable Ab levels and at 3 weeks had titres ranging from 1:20 to 1:40 except for the one Beagle which had a titre of 1:320. By day 11 all the IS dogs had normal body temperatures; they were all eating well and showed no obvious clinical symptoms. The NS dogs still had body temperatures above 40°C and still showed signs of listlessness and anorexia.

Table 4. Results of immune serum transfers. IS dogs 1 & 2 were beagles, 3, 4 & 5 were cross breds. NS dogs 1 was a Beagle and 2,3,4 & 5 were cross breds.

	First Para-sitemia	PCV (%)	Temp. (°C)	Symptoms	Antibody titre (4 weeks)
IS Dogs					
1	day 4	20.7	40.0	anaemia,	1:640
2	day 4	22.7	39.8	listlessness,	1:640
3	day 4	29.8	39.6	anorexia	1:1280
4	day 4	14.0	39.8		1:640
5	day 4	11.5	39.6		1:640
NS Dogs					
1	day 4	16.1	39.8	vomiting, anorexia	1:640
2	day 4	13.4	39.6	haemoglobinuria,	1:320
3	day 4	16.3	39.9	weight loss,	died
4	day 4	6.2	40.0	anaemia,	died
5	day 4	7.0	39.9	listlessness	1:40

3.3.3 Polyacrylamide gel electrophoresis (PAGE)

Staining with coomassie brilliant blue revealed numerous protein bands on the 5 lanes containing a crude extract of whole *B. canis* merizoites and the lane containing uninfected erythrocyte control antigen. The major protein bands present only in the experimental lanes included proteins of molecular weight 85 kDa, 69 kDa, 54 kDa and a number of bands of molecular weights ranging between 46 kDa and 36 kDa.

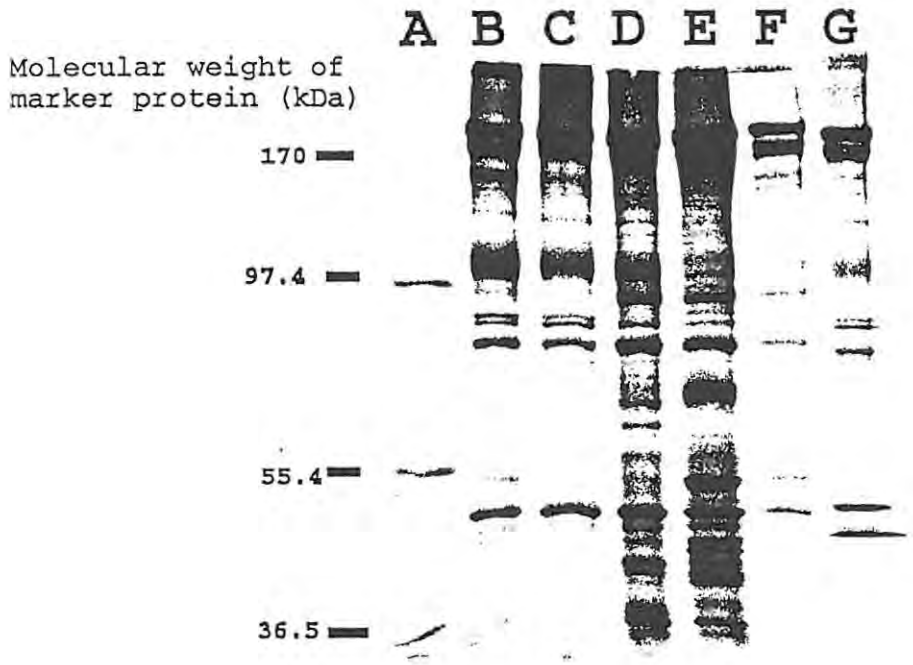
Proteins which showed differences between the stocks included:

1) 54 kDa, shared only by lanes 1 (ZIM1), 4 (DBN1) and 5 (JHB1);

2) 85 kDa, shared only by lanes 1 (ZIM1), 2 (GHT1) and 4 (DBN1);

3) 42 kDa, shared only by lanes 3 (QTN1), 4 (DBN1) and 5(JHB1).

Proteins that were unique to specific lanes included 62 kDa, unique to lane 3, and 69 kDa unique to lane 4.

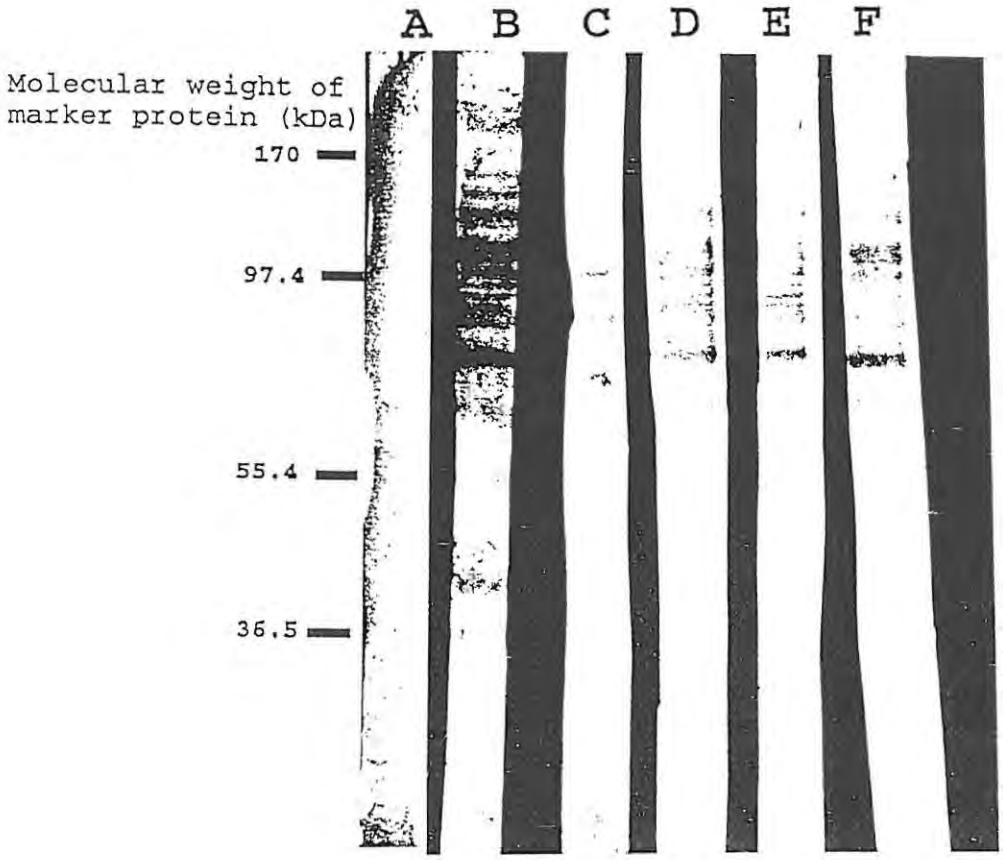


Lane: A - Molecular weight marker protein
B - Zim1 antigen
C - GHT1 antigen
D - QTN1 antigen
E - DBN1 antigen
F - JHB1 antigen
G - Uninfected eryhtrocytes

Fig. 10. Polyacrylamide gel electrophoresis (PAGE) of protein from various southern African *Babesia* stocks.

3.3.4 Western blots

All the post challenge sera used were serologically positive for *B. canis* as determined by the indirect fluorescent antibody test. These sera, taken from the Beagle infected with the respective stocks, reacted with numerous protein bands on the corresponding immunoblots. Some reactivity against proteins present in uninfected erythrocytes was detected at 25 kDa. The major bands in the merozoite extract recognized by the antiserum had approximate molecular weights of 220 kDa, 145 kDa, 124 kDa, 85 kDa, 63 kDa and 43 kDa. Lane C (JHB) was the lane that differed most from the other 4 experimental lanes. It reacted with a smaller number of bands and more specifically showed little reactivity at 124 kDa and 85 kDa, two protein bands that showed a high degree of reactivity on immunoblots containing the other four stocks. Serum collected from dogs that were serologically negative to *B. canis* (IFAT determined) did not react with any *B. canis* protein on immunoblots.



Lane: A - Uninfected erythrocytes
B - Zim1 antigen
C - JHB1 antigen
D - GHT1 antigen
E - QTN1 antigen
F - DBN1 antigen

Fig. 11. Serologic reactivity of *Babesia canis*-immune serum with various southern African stocks. Western blots were done with serum that was immune to the respective stocks. Serum used in lanes B to F was pooled for use in lane A.

CHAPTER FOUR

DISCUSSION

The methods used in this study provided a satisfactory way of characterising the five stocks of *B. canis* collected from different geographic areas in southern Africa.

Uilenberg *et al.* (1989) characterised a number of *B. canis* stocks originating from France, North Africa and South Africa in terms of vector specificity, cross immunity tests and indirect fluorescent antibody tests (IFAT). They distinguished between three groups of parasites, *B. canis canis*, *B. canis vogeli* and *B. canis rossi* having *Dermacentor*, *Rhipicephalus* and *Haemaphysalis* ticks as their vectors respectively. This study is different in that the methods used did not involve vector specificity but rather pathogenicity, cross resistance, antigenic composition and the immune response of Beagle dogs.

All the stocks used in this study proved to be potentially fatal if left untreated. Of the five stocks collected, four, namely ZIM1, GHT1, QTN1 and DBN1, were found to be virulent. Symptoms caused by these stocks were very similar in nature and severity. The first clinical symptom was usually a fever followed by listlessness and anorexia. Massive destruction of red cells commonly led to haemoglobinuria and in all these cases severe symptoms usually resulted from complications as a result of anaemia such as tissue hypoxia resulting in lactic acidosis and

pyrexia. All cases were characterized by relatively low rates of infections which caused progressive anaemia with PCV's as low as 10% being recorded with the primary challenges. Farwell *et al.* (1982) found that nearly 85% of dogs with babesiosis had positive results of Coombs' test (test for auto immune haemolytic anaemia). The anaemia was, therefore, most probably caused by immunologic destruction due to the immune response rather than a direct effect of parasitemia.

The 5th stock (JHB1) differed from the four stocks described above. It was highly virulent and could almost be said to be asymptomatic. It was only in the last stages that severe symptoms became apparent. The incubation period was comparable to that of the other stocks but differed in that it had a very low concentration of parasitized red cells in venous and arterial blood and showed a high affinity for capillaries. This increased attraction of parasites for the capillaries is also characteristic of virulent *B. bovis* strains (Callow *et al.*, 1979). In this case capillaries of the lung were heavily packed with parasites and the dog more than likely died of cardiac failure and anoxia due to the lung oedema. Histopathological results indicated massive numbers of sludged cells in cardiac vessels which may suggest a cardiac form of babesiosis.

Taboada and Merchent (1991) distinguish between two syndromes of babesiosis. One, characterised by haemolytic anaemia, is displayed by the least pathogenic species. The second is a hypotensive shock syndrome which causes hypoxia and extensive

tissue damage and is caused by the most pathogenic species. The symptoms displayed by the various stocks suggest that the first four stocks, i.e. ZIM1, GHT1, QTN1 and DBN1 stocks, could be classified under the first syndrome of haemolytic anaemia while the JHB1 stock is possibly an example of the hypotensive shock syndrome.

Beagles are among dog breeds which are known to have a strong innate resistance to babesiosis (Martinod *et al*, 1986). Adult dogs are also known to cope better with babesiosis than younger dogs (Shortt, 1973). In spite of this, all the dogs in this experiment showed an initial weak immune response to *B. canis* infection as shown by the slow increase in IFAT titres after their primary challenges. In most cases it was only after a homologous challenge or a relapse that dogs were able to build up a resistant immunity to overcome subsequent *B. canis* challenges which coincided with peak IFAT titres. It is only after a minimum of 2 challenges that antibody levels raised to levels that would provide a measure of resistance.

Three out of eight Beagles suffered relapse infection between 12 and 20 days after treatment with Berenil. Two of these, Ali (QTN1) and Katryn (DBN), occurred after treatment of a primary challenge while the third dog, Jason (ZIM1) only suffered a relapse after he received treatment for his first homologous challenge. These dogs never showed any symptoms of clinical babesiosis and blood smears showed a very low percentage of infected red cells. Parasitemia remained low (<1 per field), but

it was after Katryn (DBN) PCV had dropped below 10% that symptoms of listlessness and anorexia became apparent and the dog had to be treated. The dogs which overcame the relapse infections were subsequently totally resistant to challenges with *B. canis*.

These results agree with that of Stewart (1983), who, comparing the efficiency of three different drugs for the treatment of *B. canis*, found that dogs which relapsed after an initial treatment were able to withstand the challenge with homologous parasites whereas dogs which did not suffer relapses developed severe babesiosis following a homologous challenge. Stewart (1983) suggested that dogs develop a poor immunity against *B. canis* and it needs to be continually stimulated to be maintained. It is likely that under natural conditions dogs relapse to *B. canis* following treatment but do not show clinical disease and symptoms remain unnoticed by the owner. In this way relapse infections may play an important role in continually stimulating the dogs immune system to maintain a resistant immunity.

The dog (Ghost) infected with the JHB1 stock was the only one that failed to develop an immunity to the this stock even after her third homologous challenge. Serologically this bitch showed a very slow immunological response and this might be explained by the fact that she was treated as soon as the first parasitemia appeared. This may have impeded stimulation of the immune system. Another reason might be explained by the observations of Callow *et al.* (1979) who concluded that, because *B. bovis*, which like the JHB1 stock has a tendency to sludge into

capillaries, is less prone to immune effector mechanisms.

All the Beagles had relatively high IFAT titres at the time of their heterologous challenge. This seems to be the reason that although some of the dogs showed a parasitemia, none of them developed life threatening symptoms. Uilenberg *et al.* (1989) working with isolates from Tunisia, Egypt, France and South Africa found that IFAT antibody titres to a heterologous stock where several dilutions lower than those for homologous stocks. They used this in support of their argument that these stocks should be separated at the subspecies level. In the present study, serum taken from dogs which were immune to the respective stocks, reacted very satisfactory to the QTN1 antigen used for IFAT's. This may indicate a high degree of homogeneity between southern African stocks. However, this would have to be checked using heterologous and homologous antigens.

The *B. canis* antigens detected by the PAGE and Western blots corresponded well with the major antigens identified by Brown *et al.* (1991) for *B. bovis*. Although some variation was detected, PAGE, generally implied a high degree of similarity between all the stocks used. The PAGE and Western blots showed proteins of approximately similar molecular weights. Antigenic differences detected by Western blots, seems to be between the JHB1 stock and the four other stocks (ZIM1, GHT1, QTN1 and DBN1). Serum from dogs infected with the latter four stocks reacted strongly with proteins with approximate molecular weights of 124 and 85 kDa whereas serum taken from the JHB1 dog showed no reactivity with

these protein bands. Although these antigenic differences do exist between stocks, the fact that all dogs survived the heterologous challenge suggest that these differences are not sufficient enough to impede cross resistance between the southern African stocks. This high degree of antigenic similarity coupled with the knowledge that the southern African parasites are highly immunogenic (Uilenberg *et al.*, 1989) makes the development of a vaccine a real possibility.

In their trinomial system, Uilenberg *et al.* (1989) classify the southern African stocks of *Babesia* as *B. canis rossi* which would be transmitted by *Haemaphysalis leachi*. The ZIM1, GHT1, QTN1 and DBN1 stocks may have been different to the JHB1 stock in terms of virulence but cross resistance studies, immunoblots and IFAT results generally show a high degree of similarity between all stocks. All the stocks used in this experiment could, therfore, probably be classified under *B. canis rossi* and may only be variation of this subspecies.

Difference in virulence between the ZIM1, GHT1, QTN1 and DBN stocks and the JHB1 stock may be explained by the findings of Wright (1981) who reported that virulent strains of *B. bovis* contained high levels of protease, while stocks rendered avirulent by rapid passage or by irradiation contained insignificant levels of the enzyme. They suggest that the virulence of *B. bovis* is dependent on the ability of the parasite to induce kinin formation and hypotensive shock - an ability dependent on the level of the protease it contains. The ZIM1,

GHT1, QTN1 and DBN1 stocks may therefore contain only a small amount of these substances as opposed to the highly virulent JHB1 stock. The effect of various vectors also represents a very likely cause for differences in virulence of different stocks of *Babesia* but this still needs to be investigated.

Brown *et al.* (1991) found that *B. bovis* protein was not mitogenic for T cells. Adoptive transfer experiments in mice showed that only B lymphocyte enriched spleen cell subpopulation were effective in protecting mice against primary infections with *B. microti*, whereas T cell-enriched subpopulation were best at transferring immunity to a second reinfection (James, 1988). This suggested that primed helper T cells regulated B cell expression of the protective antibody response. The development of an anamnestic humoral response is dependent on the proliferation of memory B cells (James, 1988) indicating that humoral immunity was important in the resistance of mice to *B. microti*.

Passive transfer of immune serum into susceptible animals has been shown to confer partial protection against *Babesia* infections (Zwart & Brockelsby, 1979). These findings could be confirmed during this study. All the dogs that received immune serum (IS dogs) survived and only 40% of the dogs that received non-immune serum (NS dogs) survived following infection with *B. canis*. Although this study only considered one stock (ZIM1), Mohoney *et al.* (1979) suggest that this adopted immunity is predominantly species specific. IS dogs also showed less severe

symptoms, lower parasitemias and maintained higher PCV's than recipients of non-immune serum. Meeusen *et al.* (1984) similarly reported that animals which received immune serum developed parasitemias at a much slower rate and reached a lower peak parasitemia (25%) as compared to that of untreated mice (48%). Recipients of immune serum in the present study were also able to respond immunologically much quicker to infection and consequently recovered sooner than did NS dogs as assessed by antibody titres.

Interstrain antigenic differences within the *B. bovis* species have been well documented (Callow, 1968; Curnow, 1968). Montenegro-James *et al* (1985), after studying the cross protection between *B. bovis* isolates from various regions in Latin America, found that an isolate from one particular area failed to impart protection against isolates form other areas. Uilenberg *et al.* (1989), working on *B. canis* has also been able to show a lack of protection by the French, Egyptian and Tunisian stocks against the South African stock. Recovery from the South African stock did however provide protection against French, Egyptian and Tunisian stocks. The South African stocks therefore seem to have definite immunogenic properties. Of the various southern African stocks used in this study, the ZIM1, QTN1 and DBN1 stocks were able to induce partial protection (following homologous challenge) against the highly virulent JHB1 stock while the GHT1 stock provided total protection against the JHB1 stock.

The majority of effective immunization strategies in cattle are based on the use of living organisms as vaccine (Callow *et al.*, 1979; De Vos & Jorgensen, 1991). The purpose of using these vaccines is to give the animal a mild form of the disease which then imparts lasting protection against subsequent natural infections. In preparing vaccines for cattle, *B. bigemina* and *B. bovis* parasites with low virulence can be obtained by passaging the strain in a number of, either splenectomised or intact, calves depending on the strain used. This procedure exerts selective pressure on parasite populations which result in survival of predominantly avirulent organisms.

During this experiment it was also possible to attenuate the ZIM1 stock. A three month old mixed breed German Shepherd was infected with the ZIM1 stock after the stock had been passaged through two splenectomised dogs. Symptoms were very mild and involved mainly loss of appetite, listlessness and progressive anaemia. No treatment was required and this dog also did not receive any homologous challenge. He was given the highly virulent JHB1 stock as a heterologous challenge to which he was partially resistant. Given that these southern African stocks can be attenuated and that the South African stocks have already proven to be highly immunogenic (Uilenberg *et al*, 1989) the possibility of developing a vaccine is likely. Of all the stocks used in this experiment, one representing the more mild haemolytic anaemia syndrome would be an appropriate choice i.e. the ZIM1, GHT1, QTN1 or DBN1 stock. The JHB1 stock has already proven to have limited immunogenicity and because of its highly

virulent and asymptomatic nature would probably not be a wise choice. Of course whether the attenuated *B. canis* remains the same after passage through ticks would need to be investigated; it may revert to the virulent form after passage.

CONCLUSION

It is evident from this study that within *B. canis rossi* (Uilenberg *et al.*, 1989) at least two variations exist. The ZIM1, GHT1, QTN1 and DBN1 stocks conforming to a haemolytic anaemia syndrome and the JHB stock to the hypotensive shock syndrome. It would now seem that although different southern African stocks display different symptoms, as observed in this study, they have a high degree of cross resistance, evident from the fact that all the dogs which received a heterologous challenge survived without treatment. All dogs, except those which suffered relapse infections after treatment to primary infections, required treatment after homologous challenges. These results, in agreement with Stewart (1983) suggest that dogs develop a poor immune response to primary infections of *B. canis* and it is only after a second infection or relapse that a resistant immunity develops. This observation is confirmed by relatively low IFAT titres after primary challenges followed by a sharp rise after a relapse or second challenge. Separation of protein bands on immunoblots suggest a high degree of similarity between the ZIM1, GHT1, QTN1 and DBN stocks. It also revealed interstock antigenic variation between these four virulent stocks and the highly virulent JHB1 stock.

The antigen used in the micro hemagglutination tests was frozen before use. One possible reason that no meaningful results could be obtained from these tests might be because proteins were disrupted by thawing and therefore antigens were lost while washing this material.

The preservation method used during the present study worked well and with the artificial infection produced a 100% infection rate. However, some workers are of the opinion that artificial infection of dogs (needle passage) with *B. canis* causes a change in parasite antigens and thus also affects the virulence (De Waal, *pers comm.*). Infecting with approximately 10^7 parasites may be too high and combined with the use of an intravenous route might overwhelm the immune system. Giving consideration to these facts a more natural infection might be obtained by means of subcutaneous injection of fewer parasites or even better by placing infected ticks on the dogs. This would, however, be very time consuming and expensive in terms of maintaining the dogs.

Further study might include attempts to attenuate a highly immunogenic stock by syringe passage through splenectomised dogs in the way it is done with cattle (De Vos *et al.*, 1991) with the aim of producing a vaccine. Skin test to determine the specific nature of the immune response involved and DNA fingerprinting are further methods which can be utilised to characterise these stocks. In drawing conclusions it should also be taken into account that the particular breed (Beagle) used has been

documented as "more resistant ... to canine babesiosis" (Martinod *et al*, 1986). Further studies should possibly include other breeds that are "less resistant to canine babesiosis" e.g. Spaniel, Cocker, Griffon, Yorkshire terrier, Dobberman and Pekingese (Martinod *et al*, 1986).

REFERENCES

Basson, P.A. & Pienaar, J.G. 1965. Canine babesiosis: A report on the pathology of three cases with special reference to the "cerebral" form. *J S Afr vet med Ass* **36**: 333-341.

Bigalke, R.D. 1976. Relapses and immunity in canine babesiosis. *J S Afr vet med Ass* **47**: 281-287.

Breitschwerdt, E.B. 1984. Babesiosis. In Green C.E. (ed). Clinical Microbiology and Infectious Diseases of the Dog and the Cat. Philadelphia, WB Saunders, pp 796-805.

Brown, W.C., Logan, K.S., Wagner, G.G. & Tetzlaff, C.L. 1991. Cell-mediated immune responses to *Babesia bovis* merizoite antigens in cattle following infection with tick-derieved or cultured parasites. *Infection and Immunity* **59**: 2418-2426.

Button, C. 1979. Metabolic and electrolyte disturbances in acute canine babesiosis. *J Am Vet Med Assoc* **175**: 475-479.

Callow, L.L. 1968. A note on homologous strain immunity in *Babesia argentina* infections. *Aust. Vet. J.* **44**: 268.

Callow, L.L., Mellors, L.T. & Mcgregor, W. 1979. Reduction in virulence of *Babesia bovis* due to rapid passage in splenectomized cattle. *Int J Parasitol* **9**: 333-338.

Carson, C.A. & Phillips, R.S. 1981. Immunologic response of a vertebrate host to babesia. In Ristic, M. & Kreier, J.P. (eds). Babesiosis. New York, Academic, pp 411 - 444.

Curnow, J.A. 1968. Studies on antigenis changes and strain differences in *Babesia argentina* infections. *Aust Vet J* **49**: 279.

De Vos, A.J., Bessenger, R. & Fourie, C.G. 1991. Virulence and heterologous strain immuntiy of South African and Australian *Babesia bovis* with reduced pathogenicity. *Onderstepoort J vet Res* **49**: 133-136.

De Vos, A.J. & Jorgensen, W.K. 1991. Protection of cattle against babesiosis in tropical and subtropical countries with a live, frozen vaccine. From the Tick Fever Research Centre, Department of Primary Industries, Wacol, Australia.

Ewing, S.A. 1965. Method of Reproduction of *Babesia canis* in erythrocytes. *Am J Vet Res* **26**: 727-733.

Farwell, G.E., Le Grand, E.K. & Cobb, C.C. 1982. Clinical observations on *Babesia gibsoni* and *Babesia canis* infections in dogs. *J Am Vet Med Assoc* **180**: 507-511.

Friedhoff, K.T. 1988. Transmission of babesiosis. In Ristic, M (ed): Babesiosis of Domestic Animals and Man. Boca Raton, FL, CRC Press, pp 23 - 52.

Fujita, H., Sato, T., Ohara, Y & Homma. 1991. An indirect microhemagglutination test for Lyme disease. *Microbiol Immunol* **35**: 589 - 592.

Igarashi, I., Aikawa, M. & Kreier, J.P. 1988. Host cell-parasite interaction in babesiosis. In Ristic, M (ed): Babesiosis of Domestic Animals and Man. Boca Raton, FL, CRC Press, pp 53 - 69.

James, M.A. 1988. Immunology of Babesiosis. In Ristic, M (ed): Babesiosis of Domestic Animals and Man. Boca Raton, FL, CRC Press, pp 119 - 130.

Kawamura, M. 1985. Mitogen induced transformation of lymphocytes in canine babesiosis with special reference to the effect of splenectomy on it. *Jpn J Vet Res* **33**: 77.

Kawamura, M., Maede, Y. & Namioka, S. 1987. Mitogenic responsibilities of lymphocytes in canine babesiosis and the effects of splenectomy on it. *Jpn J Vet Res* **35**: 1 - 10.

Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**: 680 - 685.

Levy, M.G., Breitschwerdt, E.B. & Moncol, D.J. 1987. Antibody activity to *Babesia canis* in dogs in North Carolina. *Am J Vet Res* **48**: 339 - 341.

MacWilliams P.S. 1987. Erythrocytic rickettsia and protozoa of the dog and cat. *Vet Clin North Am Small Anim Pract* **17**: 1443-1461.

Mahoney, D.F., Kerry, D.J., Goodger, B.V. & Wright, I.G. 1979. The immune response of cattle to *Babesia bovis (B. argentina)*. Studies on the nature and specificity of protection. *Int J Parasitol* **9**: 297.

Martinod, S., Laurent, N. & Moreau, Y. 1986. Resistance and immunity of dogs against *Babesia canis* in an endemic area. *Vet Parasitol* **19**: 245-254.

Meeusen, E., Lloyd, S. & Soulsby, E.J.L. 1984. *Babesia microti* in mice. Adoptive transfer immunity with serum and cells. *Aust J Exp Biol Med Sci* **62**: 551.

Montenegro-James, S., Toro Bentinez, M., Leon, E., Lopez, R. & Ristic, M. 1985. Heterologous strain immunity in bovine babesiosis using a culture-derived soluble *Babesia bovis* immunogen. *Vet Parasitol* **18**: 321.

Moore, D.J. & Williams, M.C. 1979. Disseminated intravascular coaggulation: a complication of *Babesia canis* infection in dogs. *J S Afr Vet Ass* **50**: 265 - 275.

Shortt, H.E. 1973. *Babesia canis*: The life cycle and laboratory maintenance in its artropod and mammalian hosts. *Int J for Parasitol* **3**: 119 - 148.

Stewart, C.G. 1983. A comparison of the efficacy of isometamidium, amicarbalide and diminizene against *Babesia canis* in dogs and the effect on subsequent immunity. *J S Afr Vet Ass* **54**: 47 - 51.

Taboada, J. & Merchant, S.R. 1991. Babesiosis of companion animals and man. *Vet Clin North Am Small Anim Pract* **21**: 103 - 123.

Uilenberg, G., Franssen, F.F.J., Perie', N.M. & Spanjer A.A.M. 1989. Three groups of *Babesia canis* distinguished and a proposal for nomenclature. *The Veterinary Quarterly* **11**: 33 - 40.

Van Heerden, J., Reyers, F. & Stewart, C.G. 1983. Treatment and thrombocyte levels in experimentally induced canine ehrlichiosis and canine babesiosis. *Onderstepoort J vet Res* **50**: 267 - 270.

Wright, I.G. 1979. The kallikrein-kinin system and its role in the hypotensive shock syndrome of animals infected with the haemoprotozoan parasite, *Babesia*, *Plasmoium*, and *Trypanosoma*. *Gen Pharmacol* **10**: 319-325.

Wright, I.G. 1981. Biochemical charateristics of *Babesia* and physiochemical reactions in the host. In Ristic, M. & Kreier, J.P. (eds): Babesiosis. New York, Academic, pp 171 - 205.

Wright, I.G. & Goodger, B.V. 1988. Pathogenesis of babesiosis. In Ristic, M. (ed). Bebesiosis of domestic animals and man. Boca Raton, FL, CRC Press, pp 99-118.

Young, A.S. & Morzaria, S.P. 1986. Biology of *Babesia*. *Parasitology Today* **2**: 211-219.

Zwart, D. & Brocklesby, D.W. 1979. Babesiosis: non-specific resistance, immunological factors and pathogenesis. In Lumsden, W.H.R., Muller, R. & Baker, J.R. (eds). Advances in Parasitology, Academic Press, London, pp 49 - 113.