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Molecular evaluation of transovarial

transmission of the Uruguayan vaccine strains

of *Babesia bovis* and *Babesia bigemina*

by

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dedicated to my husband, Julio

and children Federica, Josefina and Isabel

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SUMMARY

MOLECULAR EVALUATION OF TRANSOVARIAL TRANSMISSION OF THE URUGUAYAN
VACCINE STRAINS OF *Babesia bovis* AND *Babesia bigemina*

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Babesia bovis and *Babesia bigemina* are both protozoal haemoparasites transmitted to cattle by different hard ticks. *Boophilus microplus* is the only tick vector present in Uruguay. Approximately USD 25 million are lost yearly in Uruguay due to bovine babesiosis. A *B. bovis/B.bigemina*

haemovaccine containing live attenuated organisms has been developed and has been commercially available in Uruguay since 1980.

One of the questions raised since the initiation of vaccine production, was whether there is a possibility that these attenuated organisms could be transmitted by ticks, since a possible reversion to virulence within these ticks could occur.

The work presented in this thesis examines the possibility of transovarial transmission of Uruguayan vaccine strains of *B. bovis/B.bigemina* using PCR tests that had previously been developed in our laboratory. A species-specific PCR was used on DNA extracted from different developmental stages of the vector tick *Boophilus microplus* (viz. engorged ticks, eggs and larvae) which had fed on calves experimentally infected with vaccine strains of *B.bovis/B.bigemina*. All the developmental stages of *B. microplus* gave positive PCR results for the presence of both *B. bovis* and *B. bigemina*, indicating that vaccine strains of *Babesia* could be transmitted via the transovarial route.

A second experiment was carried out to determine whether such organisms could further infect cattle. *B. microplus* larvae, derived from experimentally infected animals, were divided into two groups: one group was derived from cattle during the first 4 weeks of infection (acute phase) and the other from cattle infected after 5 to 13 weeks (chronic phase). PCR was performed on the blood obtained from calves infested with these infected larvae, in order to determine whether *B. bovis* and *B. bigemina* had been transmitted. The PCR was performed in parallel with immunofluorescence antibody tests and Giemsa-stained blood smears. Temperature and haematocrit readings were also determined.

Even though calves infested with larvae from the acute phase of infection neither showed clinical signs nor positive serology, the organisms were still detectable by PCR, indicating that these were still infectious. The absence of seroconversion is unclear but insensitivity of the IFAT or absence of a humoral immune response because of insufficient levels of attenuated organisms may have been responsible. The results obtained differed when using larvae derived from the chronic phase of

cattle infection. In these cases, neither *B. bovis* nor *B. bigemina* could be detected by PCR. The organisms were possibly absent or present in insufficient numbers to infect a new calf.

In conclusion, the results of this investigation indicate that Uruguayan vaccine strains of *B. bovis/B.bigemina* can be retransmitted by their natural vector (*B. microplus*) when the donor calf is still in the acute phase of infection. Further investigations will be directed towards the reproducibility of these tick-transmitted vaccine strains and their possible reversion to pathogenicity.

ABBREVIATIONS

ACD   -anticoagulant citrate dextrose

bp   -base pairs

BVD   -bovine viral diarrhoea

CSIRO   -Commonwealth Scientific and Industrial

                        Research Organization (Australia)

°C   -degrees Celsius

DMSO   -dimethyl sulphoxide

DNA   -deoxiribonucleic acid

DNTP   -deoxynucleoside triphosphates

EDTA   -ethylenediaminetetra-acetic acid

ELISA   -enzyme-linked immunosorbent assay

FAO   - Food and Agriculture Organization

Fig   -figure

FMD   -foot and mouth disease

g   -gravitational force

HCL   -hydrochloric acid

I/V   -intravenous

IBR   -infectious bovine rhinotracheitis

IE   -infected erythrocytes

IFAT   -immunofluorescence antibody test

IFN-γ -interferon – gamma

IgG   - inmunoglobulin G

IgM   -inmunoglobulin M

I.I.C.A.   -Instituto Interamericano de Cooperación
                        para la Agricultura

IU   -international unit

kg	-kilogram
M	-molar
mg	-milligram
MgCl2	-magnesium chloride
MHC	-major histocompatibility complex
ml	-millilitre
mM	-millimolar
NaCl	-sodium chloride
nm	-nanometer
NO	-nitric oxide
PBS	-phosphate buffered saline
PCR	-polymerase chain reaction
PCV	-packed cell volume
r.p.m.	-revolutions per minute
s/c	-subcutaneous
SDS	-sodium dodecyl sulphate
SSC	-sodium chloride and sodium citrate buffer
TAE	-tris acetate EDTA
TNF	-tumour necrosis factor
Tris	-Tris(hydroximethyl)aminomethane
ug	-microgram
µl	-microlitre
µm	-micrometre
UV	-ultraviolet

CONTENTS

LIST OF FIGURES

LIST OF TABLES

TABLE NUMBER AND TITLE   PAGE

CHAPTER ONE

LITERATURE REVIEW

1.1 INTRODUCTION

Babesiosis is a tick borne disease caused by several species of protozoa in the genus *Babesia*, which belong to the phylum Apicomplexa, class Sporozoasida, order Eucoccidionda, suborder Piroplasmorina and family Babesiidae. These intra-erythrocytic and generally host-specific protozoan parasites are capable of infecting all species of domestic animals. The arthropod vectors of *Babesia* are all hard ticks of the family Ixodidae (Mc Cosker, 1981).

VECTORS OF BOVINE BABESIOSIS

AGENT	VECTOR(S)
Babesia bovis	*Boophilus microplus, B. annulatus, B.geigyi, Riphicephalus bursa*
B. bigemina	*B. microplus, B. annulatus, B. decoloratus, R. evertsi*
B. divergens	*Ixodes ricinus, I. persulcatus*
B. major	*Haemaphysalis punctata*
B. beliceri	*Hyalomma anatolicum*
B. occultans	*Hyalomma marginatum*
B. ovata	*Haemaphysalis longicornis*

The only *Babesia spp.* known to infect cattle in Uruguay are *Babesia bovis* and *Babesia bigemina*, and the only vector implicated in their transmission is the one-host tick *Boophilus microplus* (Castro and Trenchi, 1955). The disease caused by these haemoparasites occurs world-wide, but a higher incidence has been observed in tropical and subtropical areas because of the higher prevalence of the natural vector or tick (Callow, 1984).

Uruguay is geographically situated in a marginal area with poor ecological conditions for the development of *Boophilus microplus*. Studies on prevalence and dispersion indicate that the incidence of parasites is higher in the northern parts of the country. Only 2.5 to 3 generations occur yearly, and as a result, most animals are not protected early in life by natural infections with *B. bovis* and *B. bigemina*. This is further demonstrated by the low incidence of

seroconversion found in 6 to 9 month-old calves (Cardozo, *et al.*, 1981). There are therefore great losses when fully susceptible animals are introduced into enzootic areas. The resultant economical burden for Uruguay is substantial, with an annual loss estimated to be about 20-25 million USD. Most outbreaks occur in autumn (February to May), coinciding with the highest level of vector prevalence. The agents of bovine haemoparasitic infections and their incidence in Uruguay are: *B. bovis* (40,2 %), *Anaplasma marginale* (27,3 %), *B. bigemina* (21,3%) and *Babesia spp.* (11,1%) (Solari, 1987).

The acute form of the disease is characterised by fever, anaemia, haemoglobinuria, jaundice and a variable mortality rate. The chronic form is poorly defined clinically but is associated with anaemia and varying degrees of cachexia. The carrier state, however, is asymptomatic.

An epidemiological study of babesiosis in Uruguay over 5 years (1981-1986) showed the incidence of morbidity to be 2.5% and mortality to be 1.6 % and 43 % lethality (percentage of animals dying following the development of clinical symptoms) (Solari, 1987).

1.2 GENERAL CHARACTERISTICS OF *Babesia bovis* AND *Babesia bigemina*

1.2.1 Morphology

Babesia organisms are pleomorphic, assuming round, oval, elongate or amoeboid shapes. They can also appear as pyriform bodies arranged in pairs. Two groups of *Babesia* are distinguished according to the size of the intra-erythrocytic forms. *B. bovis* belongs to the "small species" group which is characterised by pyriform bodies between 1 and 2.5 µm long, while *B. bigemina* belongs to the "large species" group which are 2.5 to 5 µm long (Igarashi *et al.*, 1988).

Organelles found in the anterior region of the merozoite constitute the "apical complex". These include polar granules, micronemes and rhoptries. The polar granule consists of a double membrane beneath the plasma membrane in the anterior region. The rhoptries are electrodense structures with teardrop shapes, and are also located in the anterior region of the cell. The micronemes are osmophilic small structures found around the rhoptries. Rhoptries and micronemes contain proteolytic enzymes which have an important role in the penetration of the merozoite into the erythrocyte. The cytoplasm contains free ribosomes and organelles similar to mitochondria. The nucleus is located centrally in the merozoite and is surrounded by two membranes. The content is homogenous and of low density (Igarashi *et al.*, 1988).

Once the merozoite penetrates the host cell, it multiplies and produces morphologic changes in the erythrocyte. *B. bovis* infected erythrocytes appear spherical in shape with a decreased corpuscular diameter, while those infected with *B. bigemina* appear larger than uninfected cells (Igarashi *et al.*, 1988).

1.2.2 Life cycle of bovine *Babesia spp.*

Babesia bovis and *B. bigemina* have similar developmental patterns in adult *Boophilus microplus* ticks. These ticks are infected during the last 24 hours of their parasitic stage when most blood is imbibed. The infection percentage is directly related to the parasitaemia of the host. The intra-erythrocytic forms ingested by the tick rapidly transform into ray forms called Strahlenkörper (Robert Koch, 1906) before the disintegration of the erythrocyte in the gut of *B.microplus*. The Strahlenkörper usually occur in clusters in the gut lumen. They are irregular in shape, have long spiky projections and appear to undergo five phases of development. It has been demonstrated in the case of *B. bigemina* that ray bodies then divide to form a second generation of ray bodies or gametes. The fusion of two gametes then results in the formation of zygotes which then develop into motile kinetes (Mackenstedt *et al.*, 1995, Ribeiro *et al.*, 1998). These kinetes then differentiate into sporozoites. Sexual stages in *B. bovis* are also suggested to occur (Droleskey *et al.*, 1983). The final phase, which is spherical and motile, then enlarges and possesses a single reduced spike which finally disappears and is thought to invade the basophilic epithelial cells of the tick gut where schizogony or multiple fission occurs with the formation of distinctive, large merozoites (vermicles, sporokinetes) (Friedhoff, 1981 and Droleskey *et al.*, 1983).

Successive cycles of schizogony occur within a variety of cell types and tissues including the oocytes, in *B. bovis* and *B. bigemina*. Transovarial transmission can therefore occur, with further development taking place in the larval stage. In the case of *B. bovis*, a final cycle of schizogony or sporogony takes place within cells in the salivary glands of feeding larvae leading to the formation of small merozoites (sporozoites). These small merozoites are infective for cattle.

Some development takes place in the case of *B. bigemina* in feeding larvae, but schizogony in the salivary glands only occurs in the nymphal and adult stages.

In cattle, *Babesia spp.* develop only in erythrocytes. Each merozoite penetrates the cell membrane with the aid of a specialized apical complex (De Vos and Potgieter, 1995). Once inside, it rapidly undergoes asexual reproduction characterised by the initial formation of uninucleated trophozoites followed by nuclear DNA replication and binary fusion. The resulting two daughter merozoites are set free following erythrocyte rupture and then invade new red blood cells. It has been demonstrated, at least for *B.bigemina*, that finally some merozoites cease multiplication and become spherical gamonts (Mackenstedt *et al.*, 1995).
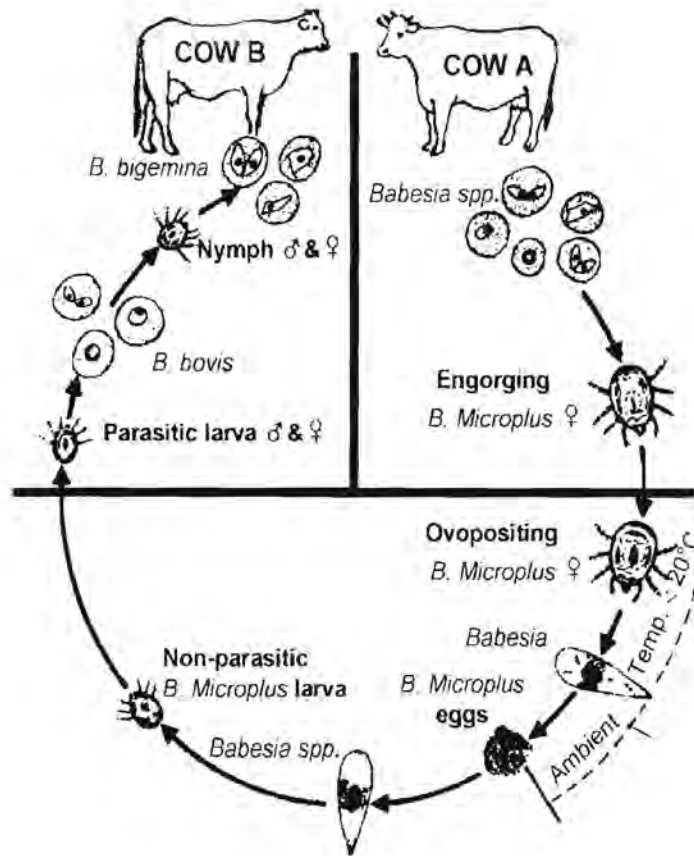
Fig. 1 Life cycle of *Babesia spp.* (Taken from Mahoney and Ross, 1972)

1.2.3 Infection

Babesial infection starts with the invasion of the erythrocyte by a process which consists of:
(1) contact between protozoan and red blood cell; (2) orientation of the protozoan so that rhoptries and granules are in apposition with the cell membrane; (3) membrane fusion; (4) rhoptry release; (5) invagination of the red cell membrane and (6) entry.

The level and duration of parasitemia depends on the species of *Babesia* and on the susceptibility of the host (influenced by age, breed, and level of environmental stress). The incidence of *B. bovis* in infected environments rises from zero at birth to a maximum at one to two years of age and then declines thereafter, while with *B. bigemina* infections the incidence is maximal among calves 6 to 12 months of age (Mahoney, 1979). The parasitaemia levels caused by *B. bovis* in acute-fatal, severe non-fatal and mild cases range from 0.01 to 0.2%, while the acute syndromes caused by *B. bigemina* are associated with levels exceeding 10%. Both *Babesia* species differ with respect to their patterns of distribution in the circulating blood.

4

B. bovis can be readily demonstrated in bovine brain smears, while *B. bigemina* is rarely observed in brain capillaries.

1.2.4 Pathogenesis

The mechanisms by which these organisms cause cellular and tissue injury is mainly due to intravascular hemolysis. This leads to anoxia and secondary inflammatory lesions in various organs, electrolytic changes, complement activation, coagulopathy and the release of pharmacologically active compounds resulting in vascular malfunction and hypotensive shock. *Babesia* virulence - which determines the severity of disease - is not a stable characteristic. Mild and virulent strains of both species have been isolated from the field but are known to undergo rapid change. A major factor in parasite virulence is a history of recent tick transmission. Long residence in a bovine reduces virulence while frequent cyclical transmission in ticks may increase the severity of the ensuing reaction (Callow, 1984).

Parasitic proteases of *B. bovis* cause hydrolysis of fibrinogen resulting in the accumulation of soluble fibrin complexes as well as altered fibrinogen in the circulation (Wright, 1981). This leads to an increased coagulability and viscosity of the blood. Progressive haemolytic anaemia also develops during the course of infection. Fibrinogen and soluble fibrin complexes adhere to infected erythrocyte membranes and contribute to the aggregation of these cells in the vasculature (Goodger *et al.*, 1987). This cytoadherence and the subsequent circulatory stasis result in anoxia with degenerative changes in tissues such as that of the brain, kidneys and skeletal muscles. Parasite proteases also activate macrophages to release pharmacologically-active agents such as histamine and 5-hydroxytryptamine causing vasodilatation, hypotension, increased capillary permeability, oedema and vascular collapse (Wright, 1978). This is due to the predilection of this species for capillaries, but this is not the case with *B. bigemina* infections (Callow, 1977, Coetzer *et al.*, 1995). Massive infiltration of neutrophils into the lung capillaries, with resultant increased vascular permeability and oedema also takes place (Losos, 1986). Similar pathologies are seen with *Plasmodium falciparum* infection, and common underlying mechanisms are probably present. Over-production of interferon-γ (IFN-γ), tumour necrosis factor (TNF-α) and nitric oxide aggravate disease in mice. IFN-γ and TNF-α also enhance expression of adherence molecules on microcapillary endothelial cells, which bind parasite-encoded receptors on the surface of infected erythrocytes. It should be noted that nitric oxide (NO) and several of the inflammatory cytokines are also important for protection (Brown and Palmer 1999).

B. bigemina pathogenesis is almost entirely related to rapid and sometimes massive intravascular haemolysis. The coagulopathy and hypotensive state found with acute *B. bovis* infections are not features of *B. bigemina* infections (Wright, 1981).

1.2.5 Clinical signs

The diseases caused by *B. bovis* and *B. bigemina* are difficult to separate clinically, but *B. bovis* is certainly the more virulent of the two parasites (Callow, 1979). Clinical attacks are essentially acute haemolytical episodes lasting for about one week during which parasites multiply rapidly. Infection with *B. bigemina* is generally mild, primarily resulting in anaemia. *B. bovis* infection results in a high mortality rate in susceptible cattle. It causes a more virulent disease characterised by fever (above 40^{0}C), anaemia, anorexia, weight loss, haemoglobinuria and a hypotensive shock syndrome (Brown and Palmer 1999).

Haemoglobinuria, which often occurs with *B. bovis* infections, appears earlier and more consistently during the course of *B. bigemina* infection. Icterus, muscle trembling and grinding of teeth also develop. Diarrhoea is common and pregnant cattle may abort. Signs reflecting central nervous system involvement such as hyperaesthesia, nystagmus, circling, head pressing, aggression, convulsions and paralysis are often present in *B. bovis* infections. An acute respiratory distress syndrome associated with the development of lung oedema is also seen (Callow, 1977, De Vos and Potgieter, 1995).

1.2.6 Inherent resistance and immunity

There are conflicting reports on the differences in breed resistance to babesiosis.
Brahman and other *Bos indicus* cattle are regarded as more resistant than European breeds *(Bos taurus)* such as the Hereford, the main breed found in Uruguay. This is attributed to differences in the intensity of especially the innate immune response. Resistance is also correlated with resistance to the tick vector (*B.microplus*) (Johnston, 1979 and 1986).

Calves are less susceptible than adults. Calves of immune mothers are protected by specific factors derived from colostrum for the first month or two of life, and older calves, regardless of the status of the dam, develop an enhanced, non-specific immunity which persists for at least 6 months.

Babesia infections are of a long duration indicating survival of the organism in the host despite an immunological response. This is partly due to antigenic variation. The parasites are antigenically unstable during the latent phase of infection, with different variants emerging at regular intervals. This is associated with slight recrudescences of parasitemia during these periods.

Most cattle develop a durable immunity after recovery, although remaining persistently infected (Mahoney *et al.*, 1973). Recurrent relapses, therefore, can still occur. High antibody titres have been detected four years after infection. With *B. bigemina*, a sterile immunity occurs 18 to 21

months after infection. Antibody titres are reduced to undetectable levels, but there is still a strong protection against future challenge (Losos, 1986).

The precise mechanisms of protective immunity against babesiosis have not been defined, but both innate and acquired responses (dependent on both T helper cell and antibody responses) are important for the induction and maintenance of immunity (Brown, 1993a). Innate mechanisms, considered to be of prime importance for resolving acute infection in naïve hosts, involve the activation of phagocytes by cytokines, protozoal DNA and inflammatory mediators. The outcome of an acute infection probably depends on the timing and nature of the inflammatory cytokines produced, leading either to resolution or exacerbation of disease. Evidence suggests that protective immunity against acute infection involves activated macrophages and natural killer cells. Control of parasitaemia during persistent infection, or after infection of immune cattle, involves both cytophilic antibodies and activated macrophages. Since the erythrocyte is devoid of MHC molecules, CD4$^+$ T cells that respond to exogenously presented antigen are an important link between the innate and adaptive immune response (Brown and Palmer 1999).

The infective stages of the organism (sporozoites) invade red blood cells. This invasion apparently involves activation of the alternative complement pathway. Infected erythrocytes incorporate *Babesia* antigens into their membranes. These in turn, induce antibodies that opsonize the red cells and lead to their removal by the mononuclear-phagocytic system. In addition to the humoral response, infected red cells may also be destroyed by an antibody-dependent cell-mediated response. The *Babesia* antigen-opsonizing antibody complex on the surface of infected erythrocytes can be recognised by macrophages which may be important early in infection when the number of infected erythrocytes is small (Tizard, 1996).

Humoral Immune Response: Antibodies develop after infection. Antibody levels increase during the acute phase of infection and decline during the chronic phase. After experimental infection with *B. bovis*, IgM rapidly rises between days 13 and 18, and IgG 21 days post-inoculation. IgM is detectable for 3 months and IgG for several years (James *et al.*, 1981, Goff *et al.*, 1982). IgM is detected 7 days after inoculation with *B. bigemina*, with a peak on day 12 till 22, declining to low levels on day 28. IgG is also detected on day 7, the peak is on day 12 and lasts for 18-20 months (Losos, 1986).

Type I responses involve the production of IFN-gamma induced IgG2 which is the superior opsonizing antibody and also has complement-binding activity (Estes *et al.*, 1994).

It has been shown that certain serologically immunodominant proteins are not protective. Often these proteins contain repetitive epitopes, which can stimulate T cell independent responses of low affinity (IgM) (Wright *et al.*, 1992). In contrast to this, several protective antigens that are relatively minor components of the parasite, have been shown not to induce a dominant antibody response (Wright *et al.*,1992, Brown and Palmer, 1999).

Cellular Immune Response: Specific and non-specific cell-mediated mechanisms involving T-lymphocytes and natural killer (NK) cells appear to be involved in *Babesia* infections. Intracellular parasite death occurs which is not caused by antibodies and which is non-specific. Microorganisms and even microbial extracts exert the same effect on rapidly-dividing babesial organisms. The T cells involved in the immune response against haemoprotozoan parasites, are both helper cells for T-cell dependent antibody production and effector cells acting on intracellular parasites through the elaboration of cytokines (Brown *et al.*, 1993b). Stimulation of IFN-gamma production in $CD4^+$ T cells and type I IFN in macrophages are of importance in the protective response.

The role of the spleen in immunity to babesiosis is well known. The spleen removes infected erythrocytes and splenectomy of latent carriers usually results in *B. bigemina* and *B. bovis* relapses which are sometimes fatal. Cessation of these functions through splenectomy is apparently sufficient to allow the clinical disease to reappear (Tizard, 1996).

1.2.7 Epidemiology.

1.2.7.1 Enzootic stability and instability

Enzootic stability with regard to bovine babesiosis is defined as the condition where there is frequent transmission of the parasites. Infection of all animals occurs during the period when young animals are protected by passively acquired and non-specific factors, i.e. within the first 6 to 9 months of life. Acquired immunity develops without the host becoming obviously sick. Local animals are therefore generally immune to the *Babesia spp* involved and suffer minimally from this disease (Tizard, 1996).

Uruguay is situated between latitudes 30 and 35° South. This constitutes a marginal area for the development of *Boophilus microplus* and, therefore, many animals in the herd fail to become infected for a considerable period after birth. A host-parasite imbalance resulting from infrequent transmission therefore exists, leading to enzootic instability (Nari and Solari, 1990, Solari and Quintana, 1994). Disease is then seen when susceptible animals in a herd encounter infected ticks. This enzootic instability results in significant annual losses in Uruguay estimated to be USD 15 million (Avila, 1999). These direct and indirect losses are due to animal deaths, losses in meat and milk production, and indirect losses due to treatment, technical assistance and restrictions in cattle trade.

1.2.7.2 <u>Epidemiological pattern of *Boophilus microplus* in Uruguay</u>

Boophilus microplus has a one-host life cycle, in which moulting from larva to nymph and nymph to adult takes place on the host. The time spent on the host, from the attachment of the unfed larva until the detachment of the engorged female, is approximately 3 weeks (Nuñez *et al.,*1987). Because of this short life cycle, six generations of ticks can occur yearly, but this is not seen in Uruguay which is situated, as previously mentioned, in a marginal area for the development of *Boophilus microplus*. Only 2.5 to 3 tick generations can develop yearly. The period between December and April is optimal for *B. microplus* development, while the non-parasitic cycle is interrupted due to climatic conditions during the period between May and August . The parasites surviving winter are larvae derived from engorged ticks which had detached during January, February and March, and from eggs from engorged ticks that had detached in April and which then hatched in spring. The highest survival rate of ticks on pasture (7.6 to 8.2 months) is seen with engorged ticks exposed during February, March and April. The non-parasitic cycle starts to shorten from August which then determines the synchronised hatching of eggs in November-December (Cardozo and Franchi, 1994).

1.3 STRATEGIC *B.microplus* CONTROL IN URUGUAY

The present Sanitary Campaign against *B. microplus* in Uruguay has the following objectives:

1) Eradicate ticks in the southern regions of Uruguay (57% of the country) where there is a lower incidence of the parasite, since the ecology there is less suitable for the development of the non-parasitic stages of *Boophilus microplus*.
2) Control the parasite in the north which is an endemic zone with a high incidence of the parasite.
To achieve these objectives the following are being performed:

1) Detection of infected farms.
2) Tick clearance on these farms by the strategic use of acaricides.
3) Safe-guarding these "clean" areas by strict control of incoming animals.

Particular strategy for the use of acaricides in the country.
The use of acaricides in Uruguay is based on knowledge of tick population dynamics. Accordingly, acaricides are used from August to November in order to prevent cattle becoming infected with larvae surviving winter on pasture and which could then complete their cycle and form first generation ticks. A second strategic treatment programme is implemented from February to April, in order to avoid the development of parasites that had escaped the first treatment (Cardozo and Franchi, 1994).

1.4 CONTROL OF BABESIOSIS

Immunisation against babesiosis is widely used throughout the world. A number of laboratories in Australia, South Africa, Argentina, Brazil, Israel, Mexico, Colombia, Cuba and Uruguay produce live attenuated *B. bovis* and *B. bigemina* vaccines. Although progress has been made with recombinant vaccines for babesiosis, no vaccine of this nature is yet available for field use. Attempts to produce non-living *B bovis* vaccines have not succeeded because the immunity produced by these vaccines is unsatisfactory, at least if compared with the immunity elicited by attenuated live *Babesia* vaccines (Timms *et al.*, 1983).

Control methods presently used in Uruguay include the extensive and expensive use of acaricides for strategic tick control based on epidemiological studies, together with the use of a live attenuated haemovaccine produced at DI.LA.VE (Veterinary Laboratory Direction), MGAP (Ministry of Livestock, Agriculture and Fisheries), Montevideo.

1. 4. 1 *B. bovis* and *B. bigemina* haemovaccines

The first law that related to the control of ticks and tick-borne diseases was promulgated in 1940 (National Tick Control Campaign and National Protection Service against Babesiosis). The Uruguayan haemovaccine has been produced since 1980 according to the method described by CSIRO (Australia). This is based on enhancing the development of non-pathogenic organisms within a population (Callow, 1971). The attenuation process is based on the selection of pre-existing parasite subpopulations combined with the ability of them to vary genetically (Gill *et al.*, 1987). These strains are composed mainly of non-pathogenic organisms which do not readily permit the emergence of pathogenic forms (Carson *et al.*, 1990).

1.4.1.1 Isolation of *Babesia bovis*

The isolation methods described below are the ones recommended by Food and Agriculture Organization of the United Nations (F.A.O., 1984).

Isolation of *B. bovis* from ticks.

Engorged *B.microplus* (at least 1000 female ticks) are collected from animals in the field and the larval progeny of these ticks are subsequently used to infect laboratory cattle. The sample should be representative of the herd and should therefore be collected from as many animals as possible. All the pooled larvae should then be placed on one or two susceptible splenectomized calves which have been kept tick-free without the use of acaricides. *B. bovis* is usually transmitted by day 3 after tick attachment and *B. bigemina* after day 8. A separation of these 2 organisms can therefore be achieved either by killing every tick at day 4 of infestation (by saturating the host with an effective acaricide) or by transferring blood to a second splenectomized tick-free animal between day 4 and day 8. Thin blood smears should be

10

transmitted by larval but not by nymphal and adult ticks. *B. bigemina*, however, is transmitted by nymphal and adult ticks but not normally by larvae. As previously explained, only *B. bovis* is transmitted if ticks are removed between day 3 and day 8. By saturating host 1 with an effective acaricide to kill all remaining ticks immediately after the transfer of engorged larvae to host 2, *B. bovis* should be obtained in pure form in host 1.

11

Isolation by passaging.

Tick-infested field-derived cattle under 12 months of age should be used as the source of the strain. The separation of *B. bigemina* from contaminating *B. bovis* and *Anaplasma marginale* can be achieved because it reproduces more rapidly than the other organisms. About 6 susceptible, splenectomized calves should be kept ready to receive in series 0,1-1 ml blood transferred intravenously as soon as *B. bigemina* is found in blood films from the previously inoculated animal. Infected blood should then be collected again from each calf in the series 1-2 days after the passage, and held in the refrigerator should a calf inoculated further down in the series die. If possible, frozen stabilates should be prepared following each passage, particularly when the final animal in the series has developed a significant parasitaemia with *B. bigemina*. When no further material from a calf is required, it may be treated with a babesiacide. Monitoring of blood films from the series for the next 1-3 months should indicate at which stage of passaging contaminating parasites failed to be transmitted.

1.4.2 Preparation of the vaccine at DILAVE (Veterinary Laboratory Direction)

Isolation of *B. bovis*

A 200 ml blood pool from about 20 tick-infested and serologically positive adult cattle from the field, is inoculated into splenectomized calves (3-6 months old). These calves had been previously infested with *B. microplus* (3 times a week) for 15 days in order to coincide with the peak of parasitaemia occurring during the detachment of engorged ticks. The engorged female ticks are then place into an incubator (27°C, 90% relative humidity) to obtain larvae. These larvae are then fed on a susceptible, splenectomized calf and on day 5 an acaricide is then applied. When a parasitaemia appears (approximately day 12), 100 ml blood in anticoagulant citrate dextrose (A.C.D.) is collected. Trypan blue (1%) is added in order to kill *B. bigemina* and then left for one day in the refrigerator before being inoculated into a susceptible calf which is then certified for the presence of *B. bovis* by serology.

Isolation of *B. bigemina*

Isolation of *B. bigemina* is as for *B. bovis,* but no acaricide is applied on day 5 and from day 7 to 18, nymphs and adults are collected and transferred to another splenectomized calf. Blood is collected from day 18 onwards, and blood from the day prior to the detection of parasites by Giemsa stained smears is used to inoculate a splenectomized calf. At each step, some blood is stored as a backup (-196°C) with 50% Alsever solution and 13,5% dimethyl sulphoxide (DMSO).

Attenuation of *B. bovis*

A splenectomized calf is inoculated with the isolated strain and between days 7 to 14 (parasitaemia) it is bled and another splenectomized calf is inoculated. This is done approximately 20 times. Attenuation is therefore achieved using 20 passages during the acute phase in splenectomized 3 month-old calves.

Attenuation of *B. bigemina*

Attenuation for *B. bigemina* is achieved by approximately 5 passages during the chronic phase (3 months) in 5 month-old non-splenectomized calves. After attenuation, Alsever solution (50%) and DMSO (13,5%) are added and the strains are stored at -90°C for 24 hours after which they are kept in liquid nitrogen (-196°C). This is to minimise damage to the parasite.

In order to verify the attenuation of both parasites, about a hundred 3 to 9 month-old susceptible calves are inoculated.

Monthly production

Blood containing the attenuated strains of *Babesia bovis* and *Babesia bigemina* are kept separately in liquid nitrogen (-196°C) in 10 ml tubes. This blood is preserved with Alsever solution (50%) and DMSO (13,5%). Each tube (2 for each Babesia) is rapidly thawed 8 days before the haemovaccine delivery date, at 40°C in warm water and are inoculated into two 1 to 3 month-old (40-45 kg) splenectomized calves which are kept in individual crates. These calves are derived from a herd known to be free of infectious bovine rhinotracheitis (IBR), foot-and-mouth disease (FMD) and bovine viral diarrhoea (BVD) viruses as well as *Brucella*, *Leptospira*, *Babesia spp*, and *Anaplasma*. The presence of *B. bovis* and *B. bigemina* is detected using blood smears after approximately 5 days. On the 8th day, an increase in temperature and a fall in haematocrit is observed, with a parasitemia detected in central blood (usually ranges from 2 to 4 % for *B. bovis* and 3 to 7% for *B. bigemina*). Blood is then collected into sterile bags containing A.C.D. solution, centrifuged at 2000 r.p.m for 15 minutes at 4°C and the buffy coat discarded. The blood concentrate is then resuspended in physiological saline. Packed cell volume (P.C.V.) and parasitaemia are calculated in order to determine infected erythrocytes (I.E.) per ml. The parasite content determines the dilution at which the blood is initially used in order to get a final dose of 10^7 *B. bovis* and 2×10^5 *B. bigemina* infected erythrocytes in 3 ml of blood. Because of the loss of infectivity during storage, the dilution used is decreased each day by a factor of 1.5 and the concentrate is not used for more than 3 days after delivery. A haemodiluent containing glucose, bovine serum and salts are then added with antibacterial agents (2×10^6 I.U. penicillin and 200 µg streptomycin per ml) in a laminar flow hood under sterile conditions. Vaccine is dispensed into sterile plastic bottles of 30 or 80 doses each, packed in ice, and dispatched from the laboratory. The vaccine must be inoculated s/c within 3 days after production and must be kept at 4°C until use.

27 000 doses are produced and sold by DILAVE (Official laboratory) annually. The use of this vaccine, however, has its limitations, including the possibility of disease-transmission, short shelf-life, and the risk of reversion to virulence and subsequent transmission by ticks (Parrodi *et al.*, 1991).

1.5 VACCINATED CATTLE

The strains of *B. bovis* and *B. bigemina* used in the vaccine are of reduced virulence, but are, however, not entirely safe. It is therefore recommended that the use of vaccine be limited to calves in which non-specific immunity will minimise the risk of vaccine reactions.

The most important categories of Uruguayan cattle requiring vaccination are :

1) Susceptible export cattle to be sent to tropical and subtropical countries such as Brazil (one of our most important cattle-trading partners). The severity of challenge faced by such cattle in this new environment is high. Since these cattle are usually pregnant Holstein heifers, abortions have been attributed to vaccination.

2) Cattle for breeding purposes (mainly 2 year old bulls).

3) Protection of susceptible southern Uruguayan cattle introduced into the enzootic northern area.

These requirements lead to the vaccination of adult animals which is far from the recommendations normally prescribed. Varying degrees of surveillance are therefore applied to these vaccinated cattle. Veterinarians are told to anticipate a reaction 7 to 14 days after inoculation. The immunity acquired is life-long.

1.6 AIMS OF THIS INVESTIGATION

The risks associated with the use of this haemovaccine has been a constant concern. One of the questions raised is the possibility of transovarial transmission of the Uruguayan attenuated strain to the vector *Boophilus microplus*. This concern is of importance since should transmission occur, it could possibly lead to a reversion to virulence and subsequent spread of the organism.

The Parasitology Division of DILAVE carried out a study in 1990 to try and determine if transovarial transmission did occur, using xenodiagnosis and detection of *Babesia* by Giemsa-stained smears. Since no *Babesia* organisms were found on the recipient animals, it was assumed that these attenuated organisms were incapable of further development in *B. microplus*. These findings, however, should be regarded as preliminary because of the low sensitivity of the techniques and the limited number of animals used (Solari *et al.*, 1991). We considered using the polymerase chain reaction (PCR) (Saiki *et al.*, 1988), a very sensitive and specific technique, to determine if transovarial transmission of Uruguayan vaccine strains of *B. bovis* / *B. bigemina* does occur.

14

CHAPTER TWO

PCR-BASED DETECTION OF *B. bovis* **AND** *B. bigemina* **VACCINE STRAINS**

PERFORMED ON DIFFERENT STAGES OF *B. microplus* **TICKS**

2.1 INTRODUCTION

The interrelationship between attenuated Babesia strains and their biological vectors, with emphasis on their development within the tick, has been studied in Australia, South Africa, Argentina and Brazil (Stewart, 1978, Dalgliesh *et al.,* 1981a, Mason *et al.,* 1986, Mangold *et al.,* 1993, Mafra *et al.,* 1994). The results of these investigations differed significantly because of different tick and non-tick transmitted strains being used in each country. In Uruguay, studies using xenodiagnosis have not indicated the presence of transovarial transmission (Solari *et al.,* 1991).

In this study, PCR was used on all the different stages of the tick (engorged ticks, eggs and larvae) as well as on calves, in parallel with the evaluation of Giemsa-stained blood smears and clinical data (temperature and haematocrit). Five 1 year-old Hereford calves were used for this purpose, of which three were inoculated with *B. bovis/B. bigemina* vaccine strains, one with *Babesia spp.* field strains, and one was the negative control. Haemoparasite-free *Boophilus microplus* larvae were fed on all the calves, and after these ticks had detached, all subsequent stages (engorged ticks, eggs and larvae) were used for PCR (Fig. 2).

2.2 MATERIALS AND METHODS

2.2.1 Experimental Animals

Five 1 year old intact Hereford calves from a tick-free area were used as tick donors and kept in separate crates throughout the experiment. They were marked and identified as Blue, Red, Orange, Green and White. All tested serologically negative prior to the experiment, for the presence of haemoparasites by Indirect Fluorescent Antibody Test (IFAT), according to the methods of the Inter-American Institute of Agrarian Sciences (I.I.C.A., 1987). Haematocrit and body temperature readings were also recorded.

2.2.2 Strain of *Babesia*-free *Boophilus microplus*

The five calves mentioned were each infected twice weekly (starting on 18/02/96) over three months with 50 mg of *B. microplus* Mozo strain larvae, representing approximately 1000 larvae. This number was based on previous studies showing that this number could effectively transmit Babesia to all test animals (Smith, 1978). Infection was done using closed envelopes containing larvae that were opened onto the back of the animal and which then dispersed by normal migration. The Mozo strain was obtained from naturally-infested animals in a *Babesia*-free farm of Cerro Largo Department (Uruguay) in 1974 and has been maintained at DILAVE by passage on *Babesia*-free calves without any contact with acaricides. This strain has remained sensitive to all acaricides and is used as the reference strain for testing both tick resistance to acaricides and the efficacy of new drugs being considered for use in Uruguay. This strain is also known to be free of haemoparasites since no clinical signs or serology have been detected in infected calves.

2.2.3 *B. bovis* and *B. bigemina* vaccine strains

The *B. bovis* vaccine strain used in this experiment had been attenuated by 27 syringe passages during the acute phase of infection in 3 month old splenectomized calves. Attenuation of *B. bigemina* was achieved after 8 syringe passages during the chronic phase in non-splenectomized 5 months old calves. In short: blood containing the attenuated strains of *B. bovis* and *B. bigemina* that was kept separately in liquid nitrogen (-196°C) in 10 ml tubes, was rapidly thawed (40 °C in a water bath) and inoculated i/v into two 1 month old splenectomized calves. These calves were derived from an IBR, FMD, BVD, *Brucella*, *Babesia spp*, and *Anaplasma*-free herd and were also tested as negative for these pathogens by ELISA except for *Babesia spp*. in which IFAT was used and for *Anaplasma marginale* where Card test was performed. After five days, presence of *Babesia* was detected by Giemsa-stained blood smears in peripheral blood. On the 8th day, an increase in the temperature and a fall in the haematocrit was observed, with a parasitaemia detected in central blood (2 % *B. bovis* and 1.3 % *B. bigemina*). Blood was then collected into sterile bags containing anticoagulant citrate dextrose (A.C.D.) solution, centrifuged at 2000 r.p.m in a bench-top microfuge for 15 minutes at 4°C and then resuspended in physiological saline (0.15 M NaCl). P.C.V. and parasitaemia were determined in order to calculate I.E. per ml. The parasite determined the dilution to be used in order to get a final 3 ml dose containing 10^7 *B. bovis* infected erythrocytes and 2 x 10^5 *B. bigemina* infected erythrocytes.

Twenty days after the first infestation with ticks, three of the experimental calves (Red, Orange and Green) were infected by i/v inoculation of the *B. bovis* (10^7 I.E.) and *B. bigemina* (2 x10^5 I.E) vaccine strains.

2.2.4 *B. bovis* and *B. bigemina* field strains

The Blue calf was infected with *Babesia spp.* field strain which came from an outbreak in Cerro Largo district in 1994. The affected cattle had been diagnosed positive for *B. bovis* and *B. bigemina* by Giemsa-stained bloodsmears and blood from these animals was stored in DMSO at -196^0C. This Blue calf was used as the positive control.

The inoculum for all the animals was on the 8/3/96.

The White calf was not inoculated and was kept as a negative control.
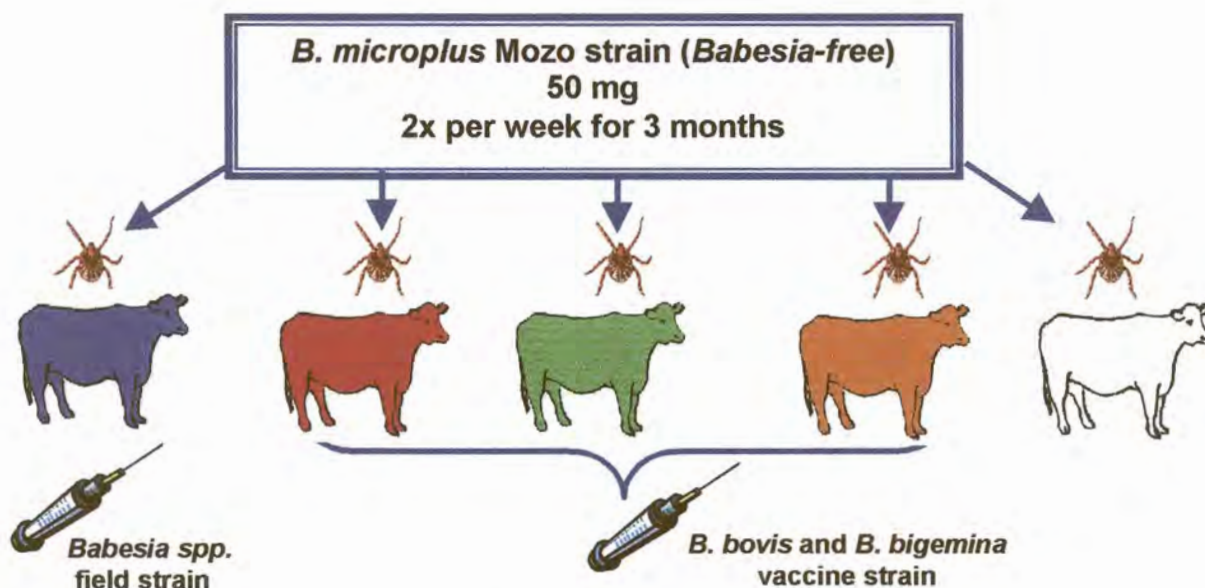
Fig. 2 A schematic representation showing infestation of calves with *B. microplus* and their resultant infection with *B. bovis/B. bigemina*.

2.2.5 Evaluation of infection

The calves were monitored over a three month period using Giemsa-stained blood smears and temperature and haematocrit readings. Blood was also collected for PCR analysis. During the acute phase (from the day of inoculum 08/03/96 till 02/04/96), these procedures were performed three times weekly and during the chronic phase (from one month post-inoculum 02/04/96 till 21/06/96) only once weekly.

A) Giemsa-stained smears

Materials for Giemsa staining

Item	Manufacturer
Giemsa powder	Merck
Glycerol	Merck
Methanol	Fisher Scientific

17

A thin blood smear was dried and then fixed in methanol for 5 minutes. The smear was then stained with Giemsa dye (3g Giemsa powder, 125g glycerol, 100ml water, and 375ml methanol) for 45 minutes, washed in water and air-dried. It was subsequently examined by oil-immersion microscopy and the percentage parasitaemia determined by examining at least three areas for infected and uninfected erythrocytes. This test was done in parallel with PCR.

B) Haematocrit or Packed Cell Volume (PCV)

This value determines the percentage of erythrocytes in the blood. A capillary tube was filled with blood and sealed at one end. It was then centrifuged at 7000 rpm in a bench-top haematocrit centrifuge for 15 minutes and the result read immediately. These values vary according to age, breed, etc. A haematocrit below 20% indicates that therapeutic measures are necessary.

C) PCR analysis

Primer design

Primers sets were designed for *B. bovis* detection (Azambuja *et al.*, 1994), from the BBOMER60 sequence published on the GENBANK (Suarez *et al.*, 1991 and Palmer *et al.*, 1991). This sequence encodes for a 60 kDa merozoite surface protein. The primers used for *B. bigemina* were designed by Figueroa *et al.* (1992), and all were ordered from Gibco BRL.

Primers *B. bovis*	Sequence 5' - 3'
Bov N#1	TCGAACCCTGCCAAGAACAGCG
Bov N#2	CGAGGTCAAGCTACCGAGCAGAACC
Bov # S	TCACCATGTCAGCATAACGACGTGC

Bov N # 1/ Bov N # 2: used for primary PCR

Bov N # 1/ Bov # S: used for secondary PCR (hemi-nested)

Primers *B. bigemina*	Sequence 5' - 3'
Big # IA	CATCTAATTTCTCTCCATACCCCTCC
Big # IB	CCTCGGCTTCAACTCTGATGCCAAAG
Big # IAN	CGCAAGCCCAGCACGCCCCGTGC
Big # IBN	CCGACCTGGATAGGCTGTGTGATG

Big # IA/ Big # IB: used for primary PCR

Big # IAN/ Big # IBN: used for secondary PCR (nested)

Primers B. bovis	Amplicon
Bov N # 1 and Bov N # 2	452 bp
Bov N # 1 and Bov S	423 bp

Primers B. bigemina	Amplicon
Big # IA and Big # IB	278 bp
Big # IAN and Big # IBN	170 bp

Big #IA

5' (+)   TTTTTCATCT AATTTCTCTC CATACCCCTC CAACCACCCC TTCACCGCGT
AAAAAGTAGATTAAAGAGAGGTATGGGGAGGTTGGTGGGGAAGTGGCGCA

Big #IAN

CGACAGACGC CGCAAGCCCA GCACGCCCCG TGCCAATTTT AGTATTTAGC
GCTGTCTGCG GCGTTCGGGT CGTGCGGGGC ACGGTTAAAA TCATAAATCG

TCTTTTAGTA CTTCACTAAG CTTATTAGTG ATGTAATTAT CATACGTTCT
AGAAATCAT GAAGTGATTC GAATAATCACTACATTAATA GTATGCAAGA

AACTGCTTCA TCATCTTTCA CCGCACCAAT GACACCACTC AAAAATCCCA
TTGACGAAGT AGTAGAAAGT GGCGTGGTTACTGTGGTGAGTTTTTAGGGT

TCACCGCATC ACACAGCCTA TCCAGGTCGG AGTACACAAT CCCAGTGCCG
AGTGGCGTAG TGTGTCGGAT AGGTCCAGCC TCATGTGTTA GGGTCACGGC

Big #IBN

TCGTAGCCTT TGGCATCAGA GTTGAAGCCG AGGAATTT 3' (+)
AGCATCGGAA ACCGTAGTCT CAACTTCGGC TCCTTAAA

Big #IB

Fig. 3   Double stranded DNA target sequence of B. bigemina. The primers are
indicated by lines; arrows indicate the direction of polymerase extension.

```
      5'          Bov N#1              3'
      |─────────────────────────────────▶
      |
541 TGCATCGAAC CCTGCCAAGA ACAGCGTAAA ACGCGAATGG TTGCGTTTCA GAAATGGAGC
    ACGTAGCTTG GGACGGTTCT TGTCGCATTT TGCGCTTACC  AACGCAAAGT CTTTACCTCG


601 GAACCATGGT GATTACCACT ACTTCGTCAC TGGTCTGTTG AACAACAATGTTGTGCACGA
    CTTGGTACCA CTAATGGTGA TGAAGCAGTGACCAGACAACTTGTTGTTAC AACACGTGCT


661 GGAAGGAACT ACCGATGTTG AATATCTTGT CAACAAGGTA CTCTATATGGCTACCATGAA
    CCTTCCTTGA TGGCTACAAC TTATAGAACA GTTGTTCCAT GAGATATACC GATGGTACTT


721 CTACAAGACT TATTTGACAG TAAACAGTAT GAACGCCAAG TTCTTCAACA GATTCAGCTT
    GATGTTCTGA ATAAACTGTC ATTTGTCATA CTTGCGGTTC AAGAAGTTGT CTAAGTCGAA


781 CACTACAAAG ATATTCAGTC GTCGTATTAG GCAAACATTG AGTGATATCATCAGGTGGAA
    GTGATGTTTC TATAAGTCAG CAGCATAATC CGTTTGTAAC TCACTATAGT AGTCCACCTT


841 TGTTCCTGAA GATTTTGAAG AAAGGAGCAT CGAACGTATC ACTCAACTTACTAGCAGCTA
    ACAAGGACTT CTAAAACTTC TTTCCTCGTA GCTTGCATAG TGAGTTGAAT GATCGTCGAT


901 CGAAGATTAC ATGTTGACCC AGATTCCAAC TCTTTCCAAG TTTGCACGTC GTTATGCTGA
    GCTTCTAATG TACAACTGGG TCTAAGGTTGAGAAAGGTTCAAACGTGCAG CAATACGACT
                                                 ◀─────────────────
                                           3'        Bov S
961 CATGGTGAAG AAGGTTCTGC TCGGTAGCTT GACCTCGTAC GTTGAAGCTC CTTGGTACAA
    GTACCACTTC TTCCAAGACG AGCCATCGAA CTGGAGCATG CAACTTCGAG GAACCATGTT
    ──────────|─────────────────────────────────────|──
                        Bov N#2
      5'         3'                             5'
```

Fig. 4   Double stranded DNA target sequence from *B. bovis*. The primers are indicated by lines; arrows indicate the direction of polymerase extension.

2.2.6 Vector samples for PCR analysis

Engorged ticks that had detached from the calves, were collected individually every 3 - 5 days. They were washed gently with water and randomly divided into two groups. One group was placed in Petri-dishes in a walk-in incubator (26°C, 90% humidity), and the other group was placed in a freezer (-20°C) for PCR analysis. After the ovipositing ticks had been incubated for approximately 15 days, we collected some eggs for PCR and stored them at -20°C, and the rest were left in the incubator to produce larvae which were then stored at -20°C for PCR analysis (Fig 5).

PCR of eggs and larvae were performed on some dates and not all because of the large amount of samples to be processed.

2.2.7 DNA purification

Materials for DNA purification

Item	Manufacturer
Sodium chloride (NaCl)	Sigma
Ethylenediaminetetracetic acid, disodium salt (EDTA)	Gibco BRL
Tris HCl	Gibco BRL
Triton X-100	Sigma
Proteinase K	Promega
Phenol	J.T. Baker
Chloroform	Merck
Isoamyl alcohol	Sigma
Ethanol	Dorwil
Water bath	Precision Scientific

The samples used for DNA purification were: calf blood collected in EDTA (5mg/ml blood), engorged ticks collected after having detached from calf hosts, eggs and larvae laid and hatched by these ticks in an incubator.

ENGORGED TICKS
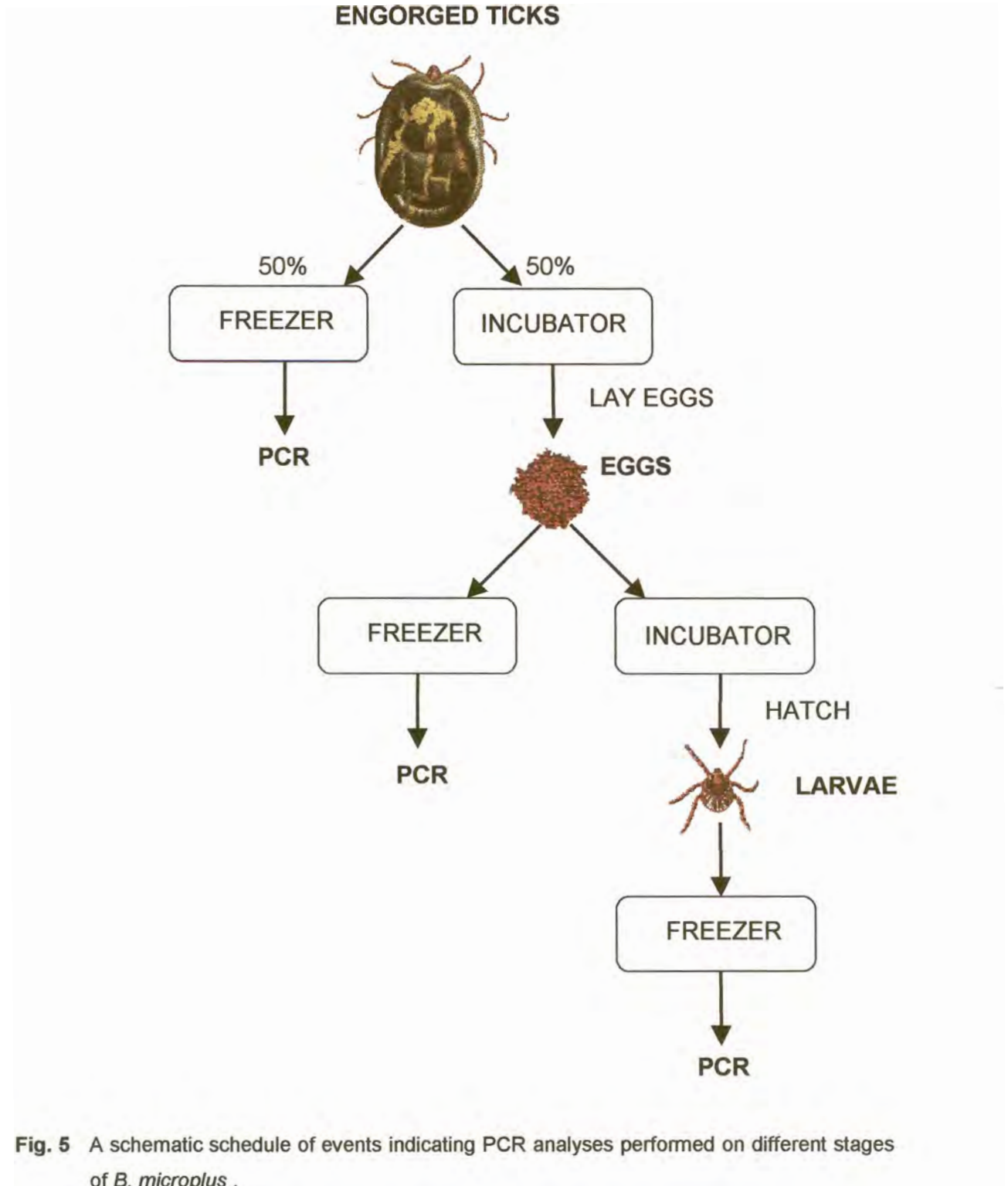
Fig. 5 A schematic schedule of events indicating PCR analyses performed on different stages of *B. microplus* .

DNA purification from blood.

500 µl of blood and 500 µl of Buffer (20mM NaCl, 20mM EDTA, 20mM Tris HCl pH 7.5, and 0.5% Triton X) were mixed using a vortex apparatus and left on ice for 30 minutes. They were then centrifuged at 12000 x g (Eppendorf benchtop centrifuge) for 10 minutes. 500 µl of 1X SSC was added to the pellet, mixed using a vortex apparatus and centrifuged at 12000 x g for 10 minutes. The supernatant was discarded and 500 µl of the Buffer (50mM Tris pH 7.5; 50mM EDTA; 10mM NaCl; and 1% SDS) was added to the pellet with 10 µl Proteinase K (20mg/ml). It was incubated at 56^0C overnight. The following day phenol-chlorophorm-isoamyl alcohol (25:24:1 v/v) was added. The sample was then mixed and centrifuged at 12000 rpm for 15 minutes. The aqueous phase was then transferred into a new Eppendorf tube and 1/3 volume of ammonium acetate (10M) and 2 volumes of cold 100% ethanol were added to precipitate the DNA. The mixture was centrifuged at 12000 rpm for 10 minutes and the DNA was washed with 70% ethanol and air-dried (Sambrook, *et al.*, 1989). The DNA pellet was then re-suspended in 20 to 50 µl Tris-EDTA (TE) pH 8 (depending on the size of the pellet).

DNA purification from ticks

a) Engorged ticks

Three engorged ticks were ruptured and the contents placed into an eppendorf tube. The same extraction buffer (v/v) and the same PCR protocol as used for blood samples were used, but were followed by two phenol-chloroform-isoamyl alcohol extractions.

b) Eggs and larvae

Approximately 50 mg of eggs or larvae were crushed in a glass tissue grinder with 500 µl of Buffer (20mM NaCl, 20 mM EDTA, 20mM Tris pH 7.5, and 0.5% Triton). They were then placed on ice for approximately 30 minutes, centrifuged at 5000 rpm for 5 minutes and the supernatant transferred into a new Eppendorf tube. 500 µl Buffer (50mM Tris pH 7.5; 50mM EDTA; 10mM NaCl and 1% SDS) and 30 µl Proteinase K (20mg/ml) was added. These samples were then incubated at 56 °C overnight and were frozen and thawed four times the following day. After this, the same protocol as for blood was followed but two P:C:I extractions were performed.

DNA concentration and purity was determined on a Pharmacia GeneQuant spectrophotometer. Absorbance readings were carried out at 260 nm and 280 nm, and DNA concentration and purity were determined.

2.2.8 PCR reactions

Materials for PCR

Item	Manufacturer
High quality water	
Buffer (Mg free)	Gibco
dNTP [10mM]	Sigma
$MgCl_2$ [50 mM]	Gibco
Primers [50ng/μl]	Gibco
Taq Polymerase [5U/μl]	Gibco
Thermocycler	Perkin Elmer 2400

The first PCR mix for a final volume of 12.5 μl was prepared as follows:

2.9 μl high quality water

1.25 μl Buffer

0.75 μl $MgCl_2$

1 μl dNTP

0.1 μl Taq Polymerase

2.5 μl Primers

4 μl DNA

For the nested PCR, 0.5 μl of the first PCR amplicon-containing solution was used and the water volume adjusted accordingly.

Cycles

A Perkin Elmer 2400 thermocycler was used.

a (i) The cycles used for the primary B. bovis PCR (outer primers) were:

94°C	40 seconds	
63°C	30 seconds	25 cycles
72°C	40 seconds	

(ii) The cycles used for the secondary B. bovis PCR (hemi-nested primers) were:

94°C	30 seconds	
63°C	30 seconds	25 cycles
72°C	30 seconds	

b (i) The cycles used to amplify *B. bigemina* (outer primers) were:

94°C	40 seconds	
56°C	30 seconds	} 25 cycles
72°C	40 seconds	

(ii) The cycles used to amplify *B. bigemina* (nested primers) were:

94°C	30 seconds	
56°C	30 seconds	} 25 cycles
72°C	30 seconds	

On the conditions previously described, the nested PCR is able to detect a parasitaemia of 0,0005% for *B. bigemina* and of 0,00003% for *B. bovis* (Figs. 6, 7). The dilutions were obtained by mixing blood with known-parasitaemia and making ten fold dilutions with blood from *Babesia*-free calves.

The specificity of the PCR reactions were determined using DNA from *Anaplasma centrale*, *Anaplasma marginale* and bovine DNA. (Figs. 8, 9).

All PCR evaluations were performed in duplicate (including DNA extraction from blood).

Fig. 6 An agarose gel showing results of PCR used to determine PCR sensitivity for detecting presence of *B. bigemina*.

Lanes:
1 123 bp DNA ladder
2 0,5% *B. bigemina* I.E.
3 0,05% *B. bigemina* I.E.
4 0,005% *B. bigemina* I.E.
5 0,0005% *B. bigemina* I.E.
6 0,00005% *B. bigemina* I.E
7 No template DNA (Negative control).

25

Fig. 7 An agarose gel showing results of PCR used to determine PCR sensitivity for detecting presence of *B. bovis.*

Lanes: 1 1kb DNA ladder
       2 3% *B.bovis* I.E
       3 0,3% *B.bovis* I.E
       4 0,03% *B.bovis* I.E
       5 0,003% *B.bovis* I.E

       6 0,0003% *B.bovis* I.E
       7 0,00003% *B.bovis* I.E
       8 0,000003% *B.bovis* I.E
       9 No template DNA (Negative control).
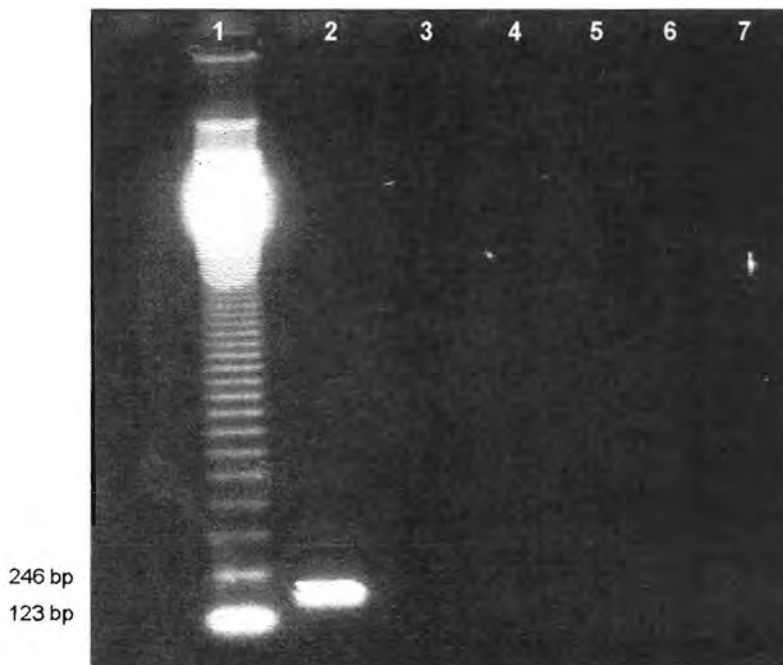
Fig. 8 An agarose gel showing results of PCR used to determine PCR specificity for *B. bigemina.*

Lanes: 1 123 bp DNA ladder
       2 *B. bigemina* DNA
       3 *Anaplasma marginale* DNA
       4 *Anaplasma centrale* DNA

       5 *B. bovis* DNA
       6 Bovine leukocytes DNA
       7 No template DNA (Negative control)

26

2.2.10 Visualisation of PCR products

The amplicons were visualised by UV fluorescence on a Vilber Lourmat UV transilluminator at a wavelength of 254 nm. Photo-documentation was done using a Polaroid DS-34 Direct Screen Instant Camera.

2.3 RESULTS

2.3.1 DNA concentration and purity

The input DNA quality, as determined by the 260nm/280nm ratio, was at all times greater than 1.7 (results not shown).

2.3.2 Analysis of the blood of the *B.bovis* and *B. bigemina* infected calves

The blood samples of the five one year-old calves used in this trial were analysed using Giemsa-stained smears and PCR. Three calves (Red, Green and Orange) were inoculated with the *B. bovis* /*B. bigemina* haemovaccine, one calf (Blue) with a wild type *Babesia spp.* blood inoculum (positive control) and one animal (White) was left uninfected (negative control). Blood was collected at various intervals from all animals over the five month trial period (two months pre-inoculation and three months post-inoculation). The positive control calf (Blue) was treated with 4 ml of 7% diminazine (Berenil) in a 500ml transfusion on day 10 post-inoculation. This was performed when there was a consistent elevation of temperature (above 40^0C) and a PCV count of below 16. On day 12 post-inoculation, treatment was repeated using 5ml diminazine 7% and a 1000 ml transfusion.

2.3.2.1 Detection of *Babesia* organisms by Giemsa staining

Giemsa-stained smears were made using peripheral and central blood. The three vaccine infected animals (Red, Orange and Green calves) were found to be positive for only a short period of time (three days), from approximately day 10 until day 14 post-infection. This window period coincided with an increase in body temperature and a decrease in packed cell volume (P.C.V) (Table 1, 2 and 3).

Table 1 Results from the clinical evaluations and PCR tests done on the *B. bovis* and *B. bigemina* infected Red calf.

RED CALF					
DATE	Temp. ⁰C	P.C.V	Giemsa	P.C.R *B. bovis*	P.C.R *B. bigemina*
22/01/96	38	27	-	-	-
25/01/96	38.30	35	-	-	-
01/02/96	38	38	-	-	-
08/03/96(inoculum)	39	27	-	-	-
11/03/96	40.2	24	-	-	-
12/03/96	38	22	-	+	+
13/03/96	39	23	-	+	+
15/03/96	39.5	22	-	+	+
18/03/96	39	22	-	+	+
19/03/96	39	15	+ tail/+jug	n/d	n/d
20/03/96	38.8	22	+ tail/+jug	+	+
21/03/96	38.5	27	+ tail/+jug	n/d	n/d
23/03/96	38.5	22	-	+	+
26/03/96	38	28	-	+	+
29/03/96	38	30	-	+	+
02/04/96	39	28	-	+	+
10/04/96	37	33	-	+	+
16/04/96	38	31	-	+	+
25/04/96	37.8	34	-	+	+
03/05/96	37	28	-	+	+
08/05/96	38	28	-	+	+
16/05/96	37	25	-	+	+
23/05/96	38	20	-	+	+
29/05/96	37.8	32	-	+	-
05/06/96	38	35	-	+	-
13/06/96	38	27	-	+	-
21/06/96	37	35	-	n/d	n/d
28/06/96	37,5	35	-	n/d	n/d

Temp. = Temperature
Tail = middle coccygeal vein
Jug = jugular vein
n/d = not done
- = negative
+ = positive

29

Table 2   Results from the clinical evaluations and PCR tests done on the *B. bovis* and *B. bigemina* infected Orange calf.

ORANGE CALF					
DATE	Temp. ºC	P.C.V	Giemsa	P.C.R *B. bovis*	P.C.R *B. bigemina*
22/01/96	37.5	37	-	-	-
25/01/96	38	35	-	-	-
01/02/96	38	33	-	-	-
08/03/96(inoculum)	39	34	-	-	-
11/03/96	40.5	30	-	n/d	n/d
12/03/96	40.5	26	-	+	+
13/03/96	39.1	25	-	+	+
15/03/96	39.1	25	-	+	+
18/03/96	38.5	29	-	+	+
19/03/96	39.5	24	+tail	+	+
20/03/96	39	26	+tail /+ jug	+	+
21/03/96	38.5	21	- tail	n/d	n/d
23/03/96	38	28	-	+	+
26/03/96	38	32	-	+	+
29/03/96	38	28	-	+	+
02/04/96	37	33	-	+	+
10/04/96	37	42	-	+	+
16/04/96	37	33	-	+	-
25/04/96	37.8	46	-	+	+
03/05/96	37.5	45	-	+	+
08/05/96	38	35	-	+	+
16/05/96	37.5	33	-	+	+
23/05/96	38	40	-	+	+
29/05/96	38	36	-	+	+
05/06/96	38	40	-	+	+
13/06/96	38	35	-	-	+
21/06/96	37.5	42	-	n/d	n/d
28/06/96	38	42	-	n/d	n/d

Temp. = Temperature
Tail = middle coccygeal vein
Jug = jugular vein
n/d = not done
- = negative
+ = positive

Table 3   Results from the clinical evaluations and PCR tests done on the *B. bovis* and *B. bigemina* infected Green calf.

GREEN CALF					
DATE	Temp. ºC	P.C.V	Giemsa	P.C.R *B. bovis*	P.C.R *B. bigemina*
22/01/96	38	39	-	-	-
25/01/96	38.3	45	-	-	-
01/02/96	38	40	-	-	-
08/03/96(inoculum)	39.2	34	-	-	-
11/03/96	39.2	30	-	-	-
12/03/96	38.4	28	-	+	-
13/03/96	38.3	29	-	+	-
15/03/96	39.2	24	-	+	+
18/03/96	38.8	30	+jug	+	+
19/03/96	39.2	27	+tail	n/d	n/d
20/03/96	39	27	-tail/ - jug	+	+
21/03/96	38.5	26	+tail/ + jug	n/d	n/d
23/03/96	38.3	22	-	+	+
26/03/96	38	25	-	n/d	n/d
29/03/96	38	24	-	+	+
02/04/96	37	28	-	+	+
10/04/96	37.5	35	-	-	-
16/04/96	37	34	-	+	+
25/04/96	38	36	-	n/d	n/d
03/05/96	37	35	-	n/d	+
08/05/96	37	30	-	+	+
16/05/96	38	33	-	+	+
23/05/96	38	32	-	+	n/d
29/05/96	38	35	-	+	-
05/06/96	38	35	-	+	-
13/06/96	38	36	-	+	-
21/06/96	38	38	-	n/d	n/d
28/06/96	38	38	-	n/d	n/d

Temp. = Temperature
Tail = middle coccygeal vein
Jug = jugular vein
n/d = not done
- = negative
+ = positive

The wild-type infected positive control calf (Blue calf) was positive on Giemsa-stained blood smear over the same period of time as the vaccine infected animals (Table 4).

31

Table 4   Results from the clinical evaluations and PCR tests done on the *Babesia spp.* infected Blue calf.

BLUE CALF					
DATE	Temp. °C	P.C.V	Giemsa	P.C.R *B. bovis*	P.C.R *B. bigemina*
22/01/96	38.5	40	-	-	-
25/01/96	38	40	-	-	-
01/02/96	38	36	-	-	-
08/03/96(inoculum)	38.5	45	-	-	-
11/03/96	38.1	41	-	-	-
12/03/96	40	40	-	+	-
13/03/96	39.2	35	-	+	-
15/03/96	40.1	30	-	+	-
18/03/96	41	18	+tail	+	-
19/03/96	40.5	15	+tail	+	-
20/03/96	40	17	-	+	-
21/03/96	37.8	16	+ tail/ - jug	+	-
23/03/96	38	17	-	+	-
26/03/96	37	22	-	+	-
29/03/96	37.8	25	-	+	-
02/04/96	38	29	-	+	-
10/04/96	37.3	33	-	-	-
16/04/96	38.8	35	-	-	-
25/04/96	37.8	44	-	-	-
03/05/96	37.5	38	-	-	-
08/05/96	37.3	28	-	-	-
16/05/96	37.6	31	-	-	-
23/05/96	39	40	-	-	-
29/05/96	38.3	36	-	n/d	n/d
05/06/96	38	37	-	n/d	n/d
13/06/96	37.5	35	-	-	-
21/06/96	38	34	-	n/d	n/d
28/06/96			-	n/d	n/d

Temp. = Temperature
Tail = middle coccygeal vein
Jug = jugular vein
n/d = not done
- = negative
+ = positive

The non-infected calf (White calf) remained negative to both haemoparasites for the whole period of the trial. (Table 5)

Table 5   Results from the clinical evaluations and PCR tests done on the non infected White calf.

WHITE CALF					
DATE	Temp. ⁰C	P.C.V	Giemsa	P.C.R *B. bovis*	P.C.R *B. bigemina*
22/01/96	37	36	-	-	-
25/01/96	37	35	-	-	-
01/02/96	39	29	-	-	-
08/03/96	38	30	-	-	-
11/03/96	38	32	-	-	-
12/03/96	38.5	32	-	-	-
13/03/96	39	31	-	-	-
15/03/96	38.5	34	-	-	-
18/03/96	38	34	-	-	-
19/03/96	39	34	-	-	-
20/03/96	39	36	-	n/d	n/d
21/03/96	38	35	-	n/d	n/d
23/03/96	37	34	-	n/d	n/d
26/03/96	38	36	-	n/d	n/d
29/03/96	37	36	-	-	-
02/04/96	38.5	35	-	n/d	n/d
10/04/96	38	35	-	-	-
16/04/96	38.5	33	-	n/d	n/d
25/04/96	38	34	-	-	-
03/05/96	38.5	34	-	n/d	n/d
08/05/96	38	35	-	n/d	n/d
16/05/96	37	36	-	n/d	n/d
23/05/96	38	35	=	n/d	n/d
29/05/96	38.5	33	-	-	-
05/06/96	38	34	-	n/d	n/d
13/06/96	37	35	-	n/d	n/d
21/06/96	38	36	-	n/d	n/d
28/06/96	39	35	-	n/d	n/d

Temp. = Temperature
Tail = middle coccygeal vein
Jug = jugular vein
n/d = not done
- = negative

B. bovis and *B. bigemina* positive Giemsa-stained blood smears are shown in Figs. 10 and 11.
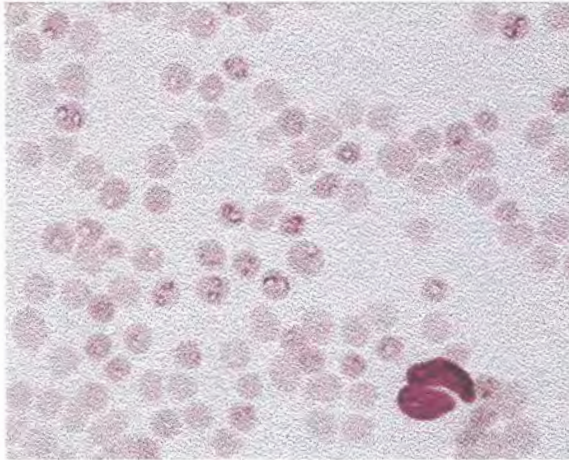

Fig. 10 *B. bovis* in a Giemsa-stained blood smear (400X)
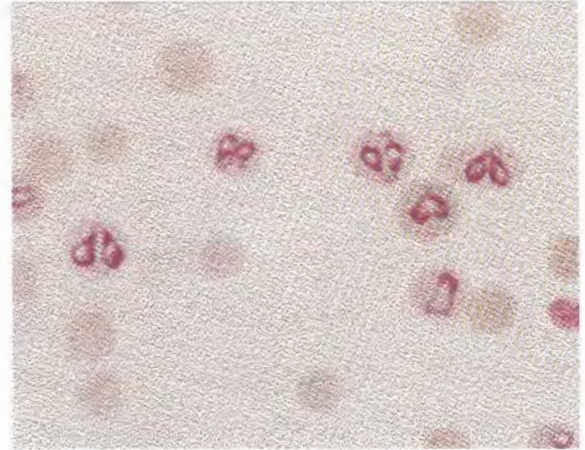
Fig. 11 *B. bigemina* in a Giemsa-stained blood smear (1000X, oil immersion)

2.3.2.2 <u>PCR analysis of blood</u>

PCR analysis was performed from 2 months pre-inoculation until the end of the trial. In general, *Babesia* organisms could be detected in the blood of all the *Babesia* haemovaccine-infected and *Babesia* wild-type infected animals through-out the trial period. A parasitaemia of 0.0005% for *B. bigemina* (Fig. 6) and 0.00003% for *B. bovis* (Fig. 7) was detected using nested PCR with the conditions described. *B bovis* organisms could be detected from day 4 post-inoculation until the end of the trial three months later in the haemovaccine infected calves. (Table 1, 2 and 3). The wild-type infected calf was also PCR positive on day 4 post-inoculation, but was PCR negative one month later (Table 4). Similar results were obtained for the *B bigemina* PCR. Although, *B bigemina* organisms could be detected from day 4 post-inoculation in all the haemovaccine infected animals, only the Orange calf was positive for *B bigemina* using PCR for the whole three month trial period (Table 2). The blood samples from both the Red and Green calves were PCR positive from day 4 post-inoculation but became negative after about 2.5 months, i.e. before the termination of the trial (Table 1 and 3). The positive control (Blue calf) inoculated with blood containing wild-type *Babesia spp* (see Materials and Methods) was only *B. bovis* positive. No *B. bigemina* could be detected by PCR (Table 4). The White calf was negative for both *Babesia spp.* during the whole trial period (Table 5).

2.3.3 <u>PCR analysis of *B.microplus* adult ticks, eggs and larvae infected status</u>

Engorged female *B. microplus* ticks were allowed to feed to completion and were collected when they had detached from their respective host animals. Some were used for PCR and the

rest allowed to lay eggs. These were then divided into two groups: one was used for PCR analysis and the rest placed in an incubator to produce larvae to be also used for PCR analysis. No Giemsa-stained smears were performed on any of the tick stages.

PCR was performed on engorged ticks only during the acute phase of infection (17 days) whereas eggs and larvae were analysed during the whole three-month period. *Babesia bovis* and *B. bigemina* organisms could be detected in engorged ticks from three animals (Red, Orange and Green) from approximately day 4 post inoculation until the last day of analysis (17 days post inoculation) (Table 6, 7 and 8).

Table 6   Results of the PCR tests on the *B. microplus* engorged ticks, eggs and larvae that had fed on the Red calf.

RED CALF			
DATE	Ticks	Eggs	Larvae
22/01/96	n/d	n/d	n/d
25/01/96	n/d	n/d	n/d
01/02/96	n/d	n/d	n/d
08/03/96(inoculum)	n/d	n/d	n/d
11/03/96	- B.big -B.bov.	n/d	n/d
12/03/96	n/d	n/d	n/d
13/03/96	n/d	n/d	n/d
15/03/96	-B. big -B. bovis	+ B.big	n/d
18/03/96	-B. big	n/d	n/d
19/03/96	n/d	n/d	n/d
20/03/96	n/d	n/d	n/d
21/03/96	+B. big + B.bov.	n/d	n/d
23/03/96	+ B.big +B.bov.	+ B.big + B.bov	+ B.big+B.bov
26/03/96	n/d	n/d	n/d
29/03/96	n/d	n/d	n/d
02/04/96	n/d	n/d	n/d
10/04/96	n/d	+ B.big	+B.big
16/04/96	n/d	n/d	n/d
25/04/96	n/d	-B.big + B.bov	n/d
03/05/96	n/d	+ B.big	n/d
08/05/96	n/d	n/d	n/d
16/05/96	n/d	n/d	n/d
23/05/96	n/d	n/d	+ B.big
29/05/96	n/d	n/d	n/d
05/06/96	n/d	+ B.big	n/d
13/06/96	n/d	n/d	n/d
21/06/96	n/d	n/d	n/d
28/06/96	n/d	n/d	n/d

n/d = not done
- = negative
+ = positive

35

Table 7   Results of the PCR tests on the *B. microplus* engorged ticks, eggs and larvae that had fed on the Orange calf.

ORANGE CALF			
DATE	Ticks	Eggs	Larvae
22/01/96	n/d	n/d	n/d
25/01/96	n/d	n/d	n/d
01/02/96	n/d	n/d	n/d
08/03/96(inoculum)	n/d	n/d	n/d
11/03/96	-B. big -B.bov	n/d	n/d
12/03/96	n/d	n/d	n/d
13/03/96	n/d	n/d	n/d
15/03/96	-B. big -B.bov	n/d	n/d
18/03/96	+B. big -B.bov	n/d	n/d
19/03/96	n/d	n/d	n/d
20/03/96	n/d	n/d	n/d
21/03/96	+ B.big +B.bov	- B. big + B. bov	- B.big + B.bov
23/03/96	+B.big +B.bov	+B.big +B.bov	+B.big +B.bov
26/03/96	n/d	n/d	n/d
29/03/96	n/d	n/d	n/d
02/04/96	n/d	n/d	n/d
10/04/96	n/d	n/d	n/d
16/04/96	n/d	n/d	n/d
25/04/96	n/d	+B.big +B.bov	+B.big +B.bov
03/05/96	n/d	n/d	n/d
08/05/96	n/d	+B.big +B.bov	n/d
16/05/96	n/d	+ B.big - B.bov	+B.big + B. bov
23/05/96	n/d	n/d	n/d
29/05/96	n/d	n/d	+B.big – B. bov
05/06/96	n/d	n/d	n/d
13/06/96	n/d	n/d	n/d
21/06/96	n/d	n/d	n/d
28/06/96	n/d	n/d	n/d

n/d = not done
- = negative
+ = positive

36

Table 8   Results of the PCR tests on the *B. microplus* engorged ticks, eggs and larvae that had fed on the Green calf.

GREEN CALF			
DATE	Ticks	Eggs	Larvae
22/01/96	n/d	n/d	n/d
25/01/96	n/d	n/d	n/d
01/02/96	n/d	n/d	n/d
08/03/96(inoculum)	n/d	n/d	n/d
11/03/96	+B. big +B.bovis	n/d	+B.bov
12/03/96	n/d	n/d	n/d
13/03/96	n/d	n/d	n/d
15/03/96	-B. big +B.bovis	+B.big +B.bov	+B.big +B.bov
18/03/96	+B.big +B.bovis	n/d	n/d
19/03/96	n/d	n/d	n/d
20/03/96	n/d	n/d	n/d
21/03/96	+ B. big +B.bovis	+B.big +B.bov	+ B.big +B.bov
23/03/96	+B.big +B.bov	n/d	n/d
26/03/96	n/d	n/d	n/d
29/03/96	n/d	+B.big +B.bov	+B.big +B.bov
02/04/96	n/d	n/d	n/d
10/04/96	n/d	n/d	n/d
16/04/96	n/d	+B.big +B.bov	n/d
25/04/96	n/d	+B.big +B.bov	n/d
03/05/96	n/d	+B.big +B.bov	n/d
08/05/96	n/d	-B.big +B.bov	-B.big +B.bov
16/05/96	n/d	-B.big	n/d
23/05/96	n/d	-B.big -B.bov	-B.big +B.bov
29/05/96	n/d	-B.big	n/d
05/06/96	n/d	n/d	n/d
13/06/96	n/d	-B.big +B.bov	-B.big +B.bov
21/06/96	n/d	n/d	n/d
28/06/96	n/d	n/d	n/d

n/d = not done
- = negative
+ = positive

Only *B. bovis* could be detected in engorged ticks from the Blue calf over the same period that the vaccine strains were detected (Table 9). No *Babesia* organisms were found in the ticks from the White calf (negative control) (Table 10).

Table 9   Results of the PCR tests on the *B. microplus* engorged ticks, eggs and larvae that had fed on the Blue calf.

BLUE CALF			
DATE	Ticks	Eggs	Larvae
22/01/96	n/d	n/d	n/d
25/01/96	n/d	n/d	n/d
01/02/96	n/d	n/d	n/d
08/03/96(inoculum)	n/d	n/d	n/d
11/03/96	-B. big -B. bov	n/d	n/d
12/03/96	n/d	n/d	n/d
13/03/96	n/d	n/d	n/d
15/03/96	-B. big -B. bov	n/d	n/d
18/03/96	-B. big -B.bov	n/d	n/d
19/03/96	n/d	-B. big +B. bov	-B. big +B. bov
20/03/96	n/d	n/d	n/d
21/03/96	n/d	n/d	n/d
23/03/96	+ B. bovis -B.big	+ B.bovis -B.big	+ B.bov, -B.big
26/03/96	- B.big	n/d	-B.big
29/03/96	n/d	-B. big +B. bov	-B. big +B. bov
02/04/96	n/d	n/d	n/d
10/04/96	n/d	n/d	n/d
16/04/96	n/d	n/d	n/d
25/04/96	n/d	-B. big -B. bov	-B. big -B. bov
03/05/96	n/d	n/d	n/d
08/05/96	n/d	n/d	n/d
16/05/96	n/d	n/d	n/d
23/05/96	n/d	-B. big -B. bov	-B. big -B. bov
29/05/96	n/d	n/d	n/d
05/06/96	n/d	n/d	n/d
13/06/96	n/d	n/d	n/d
21/06/96	n/d	n/d	n/d
28/06/96	n/d	n/d	n/d

n/d = not done
- = negative
+ = positive

Table 10   Results of the PCR tests on the *B. microplus* engorged ticks, eggs and larvae that had fed on the White calf.

WHITE CALF			
DATE	Ticks	Eggs	Larvae
22/01/96	n/d	n/d	n/d
25/01/96	n/d	n/d	n/d
01/02/96	n/d	n/d	n/d
08/03/96	n/d	n/d	n/d
11/03/96	n/d	n/d	n/d
12/03/96	- B. bov. - B. big.	- B. bov. - B. big.	- B. bov. - B. big.
13/03/96	n/d	n/d	n/d
15/03/96	n/d	n/d	n/d
18/03/96	- B. bov. - B. big.	- B. bov. - B. big.	- B. bov. - B. big.
19/03/96	n/d	n/d	n/d
20/03/96	n/d	n/d	n/d
21/03/96	n/d	n/d	n/d
23/03/96	n/d	n/d	n/d
26/03/96	n/d	n/d	n/d
29/03/96	n/d	n/d	n/d
02/04/96	n/d	n/d	n/d
10/04/96	n/d	- B. bov. - B. big.	- B. bov. - B. big.
16/04/96	n/d	n/d	n/d
25/04/96	n/d	n/d	n/d
03/05/96	n/d	n/d	n/d
08/05/96	n/d	- B. bov. - B. big.	- B. bov. - B. big.
16/05/96	n/d	n/d	n/d
23/05/96	n/d	n/d	n/d
29/05/96	n/d	- B. bov. - B. big.	- B. bov. - B. big.
05/06/96	n/d	n/d	n/d
13/06/96	n/d	n/d	n/d
21/06/96	n/d	n/d	n/d
28/06/96	n/d	n/d	n/d

n/d = not done
- = negative
+ = positive

In general, the eggs laid from the ticks as well as the larvae derived from the vaccine-infected animal hosts, were also found positive for both *Babesia spp.* (Table 6, 7 and 8). Eggs and larvae from ticks from the Blue calf were PCR positive for *B. bovis* and negative for *B. bigemina* (Table 9). PCR of different tick stages from the negative control (White calf) were all negative (Table 10).

2.4 DISCUSSION

Five 1 year old Hereford calves were used in this experiment to determine whether Uruguayan *Babesia bovis* and *Babesia bigemina* vaccine strains were able to be transovarially transmitted to their natural vector *Boophilus microplus*. All animals were kept in individual crates for 3 months during which engorged ticks detached and were collected daily. Animals were monitored by recording body temperature and haematocrit, by examining Giemsa-stained blood smears and by performing PCR. It was a very costly experiment because of the number of personnel involved and the number of large animals utilised under controlled conditions.

The findings from the Giemsa-stained smears of central and peripheral blood from the tick donors showed, as expected, a very low sensitivity as compared to results from the PCR tests. Differentiation of both Babesia species using Giemsa-stained blood smears could also not be performed in most of the samples tested. All trial animals inoculated with infective blood tested positive for the presence of *B. bovis* (vaccine and field strains) and *B. bigemina* with the exception of the positive control (Blue calf) which didn't show presence of *B.bigemina* during the whole duration of the experiment. The inoculum used possibly only contained *B. bovis* which could not be determined using Giemsa-stained blood smears.

In conclusion, even though *B. bigemina* was eliminated from almost all the animals before *B. bovis*, organisms were still detected for at least 2.5 months after inoculation. This is a much longer period than previously described using Giemsa-stained smears. The positive control became negative one month after inoculation possibly because it had been treated with 7% diminazine 12 days previously. The negative PCR results on some dates interspersed between positive results, could possibly be due to cyclical variations in parasitaemia, with low levels remaining undetected. Parasitaemias in the samples strongly influences the sensitivity and negative predictive value of the PCR-based tests (Calder *et. al.*, 1996).

Engorged ticks that had detached from their experimental hosts, were analysed by PCR and were found positive for both *Babesia* spp. except for those derived from the negative control. *B. bovis* and *B. bigemina* were both found in ticks at different times of infection with the exception of ticks fed on the positive control where no *B. bigemina* was detected. Interestingly, organisms were also found in their progeny (eggs and larvae), indicating that transovarial transmission of not only the wild-type strain, but also of the vaccine strain did in fact occur.

Many articles have been published world-wide on the evaluation of transovarial transmission of different Babesia vaccine strains, mostly with reference to *B. bovis*. The results described differ significantly, depending on the isolate, the number of passages through calves during the attenuation process, etc. The Ka *B. bovis* Australian vaccine was shown to be tick transmissible (Timms *et al.*, 1990). Other vaccine strains appear to have lost their ability to infect *B.microplus*

ticks (Mangold *et al.*, 1993, Mason *et al.*, 1986, O'Sullivan *et al.*, 1966). This is ascribed to a reduction or loss of infectivity in blood-passaged Babesia strains for their tick hosts (O'Sullivan *et al.*, 1966, Dalgliesh *et al.*, 1977, Mason *et al.*, 1986). Similar results with a slow-passaged *B. bigemina* strain were obtained by Dalgliesh *et. al.*, (1981b) which yielded a marked reduction in numbers of ticks infected.

Stewart (1978) studied the differences in the life-cycles of a repeatedly needle-passaged *B. bovis* and an unmodified strain, and came to the conclusion that continuous blood-passaging may result in the selection of parasites incapable of penetrating the gut epithelial cells of the tick.

None of these experiments described, however, used PCR to evaluate the engorged tick and its bovine host.

It can therefore be concluded that the Uruguayan *B. bovis* and *B. bigemina* vaccine strains do have transovarial transmissibility in ticks. Since our initial hypothesis was that these vaccine strains could not be transmitted transovarially, we decided to design a new experiment to investigate whether these transovarially-transmitted organisms were infectious for their bovine hosts.

CHAPTER THREE

EVALUATION OF THE INFECTIVITY OF TICK-TRANSMITTED *B. bovis* **AND** *B. bigemina*

VACCINE STRAINS

3.1 INTRODUCTION

The findings described in the previous chapter indicated that the Uruguayan *B. bovis* and *B. bigemina* vaccine strains can be transmitted transovarially in ticks. Since our initial hypothesis (see Chapter 2) was that this was not the case with vaccine strains, we decided to design a new experiment to investigate whether these transovarially-transmitted organisms were infectious for their bovine hosts.

Additional experiments were thus designed to determine if transovarially-infected larvae were capable of transmitting *Babesia spp* to other calves or whether they had lost this capability. A comparison between the transmissibility of vaccine and field strains by transovarially infected ticks was performed using a number of experimental cattle. The recipient animals were then to be monitored by means of conventional blood smears as well as PCR and IFAT.

The resultant findings and conclusions of this chapter indicate that transovarially transmitted field and vaccine strains of *B. bovis* and *B. bigemina* can also be retransmitted via *Boophilus microplus* to susceptible animals when the donor is in the acute phase, although not during the chronic phase of infection.

Nine animals were utilised for this experiment: three as infected tick donors and six as recipient calves. The six recipients were divided into two groups: one group was infested with larvae from engorged ticks that had detached during the acute phase of infection (first four weeks), and the other group was infected with larvae from engorged ticks that detached during the chronic phase (weeks 5-13).

3.2 MATERIALS AND METHODS

3.2.1 Tick donors

Only three intact 1 year-old Hereford calves were used because of ethical considerations. Two of these, *viz.* Calf 48 and Calf 49, were to be tick donors of the vaccine strains of *B. bovis / B. bigemina* and one *viz.* Calf 50, was to be a tick donor of field strains of *B. bovis* and *B. bigemina.* It was decided to use two animals for the vaccine strains and only one for the field strains since the results from the former were of greater relevance.

No negative control was used due to economic reasons and limited housing facilities.

As in the previous experiment, these calves came from a *Boophilus microplus*-free area and were kept separate in individual crates throughout the duration of the trial.

The Mozo *B. microplus* strain was used as previously described. Briefly, calves were infested with 50 mg larvae (3 times weekly) over a one month period prior to start of the experiment. They were also confirmed negative for both *B. bovis* and *B. bigemina* using PCR. One month later, the three calves were inoculated as follows:

- Calf 49 was inoculated with 200 ml of blood with vaccine strains of *B. bovis* and *B. bigemina* (18% PCV and 2%I.E.).
- Calf 48 was inoculated with 200 ml of blood with vaccine strains of *B. bovis* and *B. bigemina* (43% PCV and 2,4% I.E.).
- Calf 50 was inoculated with 200 ml of a mixture of fresh blood from 20 animals from an outbreak of *B. bovis* and *B. bigemina* in Lavalleja District (Uruguay).

3.2.2 Evaluation of infection of tick donors

We determined the parasitaemia (percentage of infected erythrocytes) by Giemsa-stained blood smears, packed cell volume (PCV), performed species-specific *B. bovis* and *B. bigemina* PCR and indirect fluorescence antibody tests (IFAT) to assess the response of the calves to inoculation.

3.2.2.1 Indirect fluorescence antibody test

This test consists of the detection of immunoglobulins from serum and involves two components: a) the parasite as antigen, and b) fluoroscein-labelled anti-bovine globulin to detect bound antibody.

The antigens for this test were prepared at DILAVE by the i/v inoculation of vaccine strains of *B. bovis* and *B. bigemina* into two different splenectomized 3 month-old calves. Seven days thereafter, blood was collected in PBS (1:9) and centrifuged at 2000 rpm for 15 minutes. This

was repeated three times. The red blood cells were reconstituted in sufficient 4% albumin/PBS so as to obtain a haematocrit of 40%. Thin blood smears were prepared, air-dried and stored at -70ºC. IFAT was performed as described by Instituto Interamericano de Cooperación para la Agricultura (I.I.C.A., 1987) (Figs. 7, 8 and 9). Serum dilutions of 1:60 *(B.bovis)* and 1:30 *(B. bigemina)* were used. The sensitivity and the specificity obtained with this test was 96%.

The other tests were performed as described in the materials and methods section of the previous chapter.
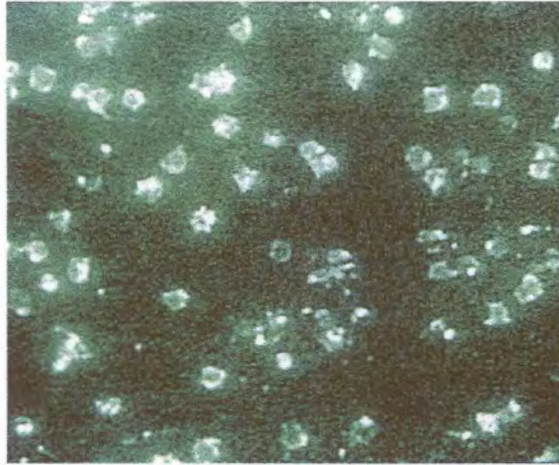
Fig. 12 *B. bovis* positive IFAT (400X)
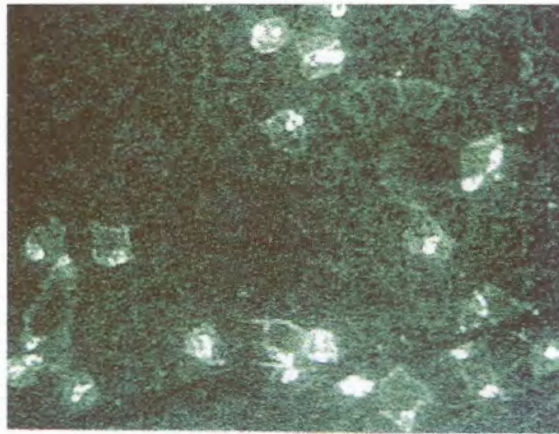
Fig. 13 *B.bigemina* positive IFAT (1000X oil immersion)

Fig. 14 Negative IFAT (400X)

3.2.3 Infestation of calves with larvae from tick donor calves

10 -20 engorged ticks were collected daily from the three animals and were kept separately in a walk-in incubator (27°C, 90% humidity) to allow them to lay eggs, and for the hatching of larvae. Larvae, approximately 20 day-old and derived from ticks that had detached during the first four weeks of the experiment, were fed on three different calves. Calf 70 was infested with ca.150 000 larvae derived from Calf 49; Calf 71 was infested with ca.150000 larvae derived from Calf 48; and Calf 73 was infested with ca. 150000 larvae derived from Calf 50.

The same numbers of larvae derived from ticks that had detached between week 5 and week 13 were fed on three new animals: Calf 64 (larvae from Calf 48); Calf 66 (larvae from Calf 50) and Calf 67 (larvae from Calf 49).
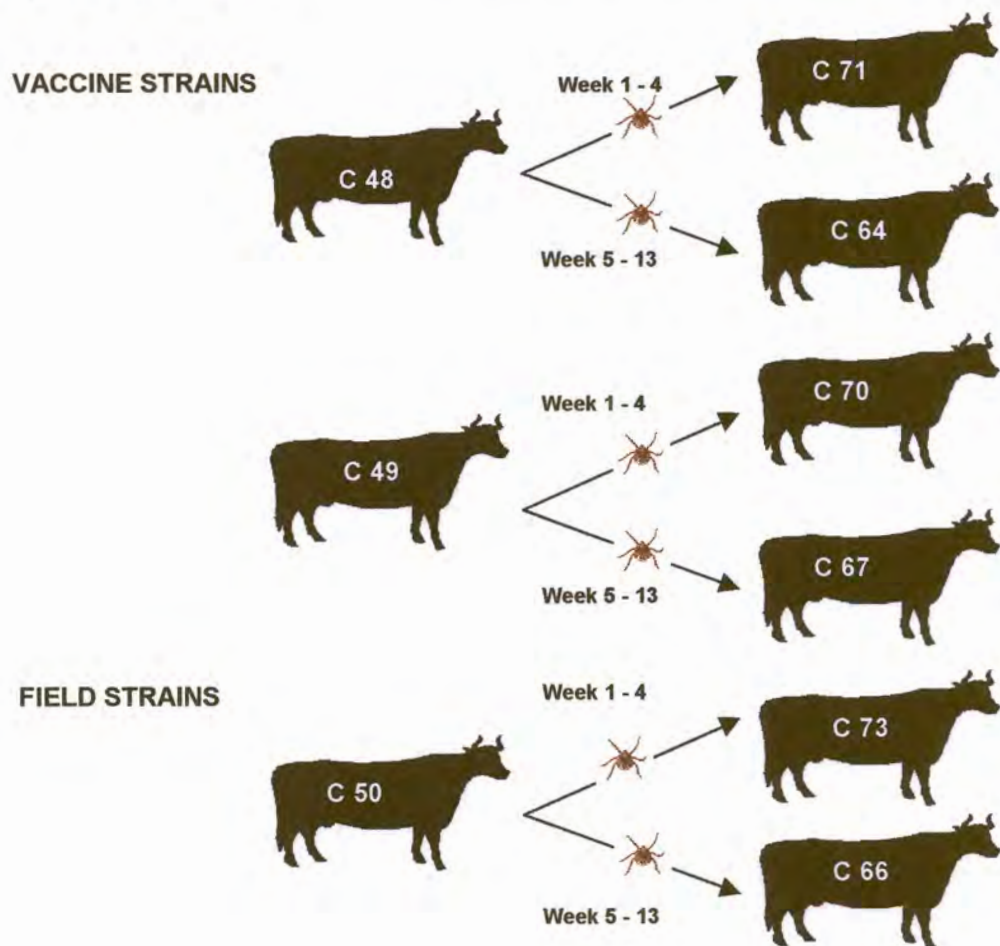
Fig. 15   A schematic time schedule of infestation of recipient calves with larvae derived from the acute and chronic phases of infected donor calves.

3.2.4   Evaluation of tick transmissibility of vaccine and field *Babesia* strains

Blood and serum of each of these calves were periodically collected for testing by PCR and IFAT. Giemsa-stained smears as well as PCV were carried out. All PCR evaluations were repeated at least twice. This involved the DNA extraction from blood as well as the PCR reaction.

3.3 RESULTS

3.3.1 Tick donor calves

Blood of the three calves (48, 49 and 50) that were used as tick donors, were tested using Giemsa-stained blood smears, PCV determinations, IFAT and PCR assays to determine presence of infection.

3.3.1.1 Field strains

Calf 50 was positive on Giemsa-stained blood smears four days after inoculation and remained positive for three days. The PCV was at its lowest level on day 11 (18%). This calf had to receive a blood transfusion with 500 ml blood on days 5, 6 and 7, but was not treated. The PCR assay was positive for the presence of both parasites on days 11 and 21 post inoculation (Table 11).

Table 11   Results obtained from Giemsa- stained blood smears, PCV determinations and PCR tests performed on Calf 50 following inoculation with *B.bovis /B.bigemina* field strains.

Calf 50	Giemsa	PCV	PCR
27/11/98	-	33%	n/d
08/01/99 (inoculum)	-	n/d	n/d
12/01/99	+tail	n/d	n/d
13/01/99	+ tail / + jug	20%	n/d
14/01/99	+ tail / + jug	20%	n/d
19/01/99	-	18%	+B. bov,+B, big
20/01/99	n/d	18%	n/d
26/01/99	n/d	22%	n/d
27/01/99	n/d	27%	n/d
28/01/99	n/d	28%	n/d
29/01/99	n/d	20%	+B. bov,+B, big
01/02/99	n/d	25%	n/d

Tail = middle coccygeal vein
Jug = jugular vein
n/d = not done
- = negative
+ = positive

Because confirmation alone of the presence of *B. bovis* and *B. bigemina* in these donor calves was sought, no further PCR tests were performed. Duration of infection will be evaluated in another trial. IFAT was positive for both parasites on day 142 and 161 post inoculation (Table 12).

Table 12 IFAT results from donor calves 50, 48 and 49.

Calf	Date	Days post-inoculum	*B. bovis*	*B. bigemina*
50	27/12/98	before	-	-
50	27/05/99	142	+	+
50	16/06/99	161	+	+
48	27/12/98	before	-	-
48	27/05/99	142	+	+
48	16/06/99	161	+	+
49	27/12/98	before	-	-
49	09/02/99	31	+	+

Dilution for *B. bovis* (1:60), Dilution for *B. bigemina* (1:30)

- = negative
+ = positive

3.3.1.2 Vaccine strains

Calf 48 was positive on Giemsa-stained smears on day 18 post inoculation and remained positive only for two days. The lowest PCV values were on days 18 and 20 post inoculation (24%). PCR was positive for both parasites on days 18 and 21 post inoculation (Table 13). IFAT was positive 142 and 161 days after inoculation for both parasites (Table 12).

Table 13 Results obtained from Giemsa- stained blood smears, PCV determinations and PCR tests performed on Calf 48 following inoculation with *B.bovis /B.bigemina* vaccine strains.

Calf 48	Giemsa	PCV	PCR
27/11/98	-	n/d	n/d
08/01/99 (inoculum)	-	n/d	n/d
11/01/99	-	30%	n/d
19/01/99	n/d	27%	n/d
20/01/99	-	27%	n/d
26/01/99	+jug	24%	+B. bov,+B. big
27/01/99	+jug / +tail	25%	n/d
28/01)99	-	24%	n/d
29/01/99	-	26%	+B. bov,+B. big
01/02/99	-	26%	n/d

Tail = middle coccygeal vein
Jug = jugular vein
n/d = not done
- = negative
+ = positive

Calf 49 was at all times Giemsa negative and the lowest decrease in its PCV was on day 11 (25%). PCR was positive for both parasites on days 18 and 21 post inoculation (Table 14). IFAT was positive for both parasites on day 31 post inoculation (Table 12). This calf died on day 32 post inoculation because of nutritional problems.

Table 14 Results obtained from Giemsa- stained blood smears, PCV determinations and PCR tests performed on Calf 49 following inoculation with B.bovis /B.bigemina vaccine strains.

Calf 49	Giemsa	PCV	PCR
27/11/98	-	36%	n/d
08/01/99 (inoculum)	-	n/d	n/d
11/01/99	-	n/d	n/d
19/01/99	-	25%	n/d
20/01/99	-	26%	n/d
26/01/99	-	25%	+B. bov,+B. big
27/01/99	-	29%	n/d
28/01/99	-	27%	n/d
29/01/99	-	30%	+B. bov,+B. big
01/02/99	-	30%	n/d

n/d = not done
- = negative
+ = positive

3.3.2 Evaluation of infection in recipient animals

Giemsa-stained blood smears, PCV determinations, species-specific PCR assays and IFAT were performed on each of the 6 recipient calves (C70, C71, C73, C64, C66 and C67). All PCR analyses were repeated at least twice. For each PCR reaction, newly isolated DNA (from starting material) was prepared and an independent PCR was performed.

Table 15 Results obtained from Giemsa-stained blood smears, PCV determinatons and PCR tests performed on Calf 73 infested with larvae obtained during week 1 – 4 from Calf 50.

C 73	Giemsa	PCV	PCR B.bovis	PCR B.big
9/3/99 Infestation with larvae C. 50	-	n/d	n/d	n/d
11/03/99	-	24%	n/d	n/d
12/03/99	-	25%	n/d	n/d
15/03/99	-	30%	-	-
16/03/99	-	27%	n/d	n/d
17/03/99	-	25%	+	n/d
18/03/99	-	20%	+	+
19/03/99	-	21%	+	+
20/03/99	-	20%	n/d	n/d
21/03/99	+	15%	+	+
22/03/99	+	6% (died)	+	+

n/d = not done
- = negative
+ = positive

Table 16   Results obtained from Giemsa-stained blood smears, PCV determinatons and PCR tests performed on Calf 70 infested with larvae obtained during week 1 – 4 from Calf 49.

C 70	Giemsa	PCV	PCR *B.bovis*	PCR *B.big*
09/03/99 Infestation with larvae C. 49	-	n/d	n/d	n/d
11/03/99	-	33%	n/d	n/d
12/03/99	-	38%	n/d	n/d
15/03/99	-	35%	n/d	n/d
16/03/99	-	30%	n/d	n/d
17/03/99	-	33%	+	n/d
18/03/99	-	28%	+	-
19/03/99	-	30%	-	-
20/03/99	-	28%	+	+
21/03/99	-	28%	-	+
22/03/99	-	28%	-	+
23/03/99	-	30%	-	-
26/03/99	-	30%	n/d	n/d
30/03/99	-	n/d	n/d	n/d
05/04/99	-	n/d	-	-

n/d = not done
- = negative
+ = positive

Table 17   Results obtained from Giemsa-stained blood smears, PCV determinatons and PCR tests performed on Calf 71 infested with larvae obtained during week 1 – 4 from Calf 48.

C 71	Giemsa	PCV	PCR *B.bovis*	PCR *B.big.*
09/03/99 Infestation with larvae C 48	-		n/d	n/d
11/03/99	-	29%	n/d	n/d
12/03/99	-	30%	n/d	n/d
15/03/99	-	32%	n/d	n/d
16/03/99	-	30%	n/d	n/d
17/03/99	-	33%	+	+
18/03/99	-	30%	-	-
19/03/99	-	29%	-	+
20/03/99	-	29%	+	n/d
21/03/99	-	29%	+	-
22/03/99	-	30%	-	n/d
05/04/99	-	30%	-	+

n/d = not done
- = negative
+ = positive

49

Table 18   IFAT results of recipient calves 73, 70 and 71 during the acute phase of infection.

Calf	Date	Days post-infestation	*B. bovis*	*B. bigemina*
73	09/03/99	before	-	-
73	22/03/99	13 (died)	+	+
70	09/03/99	before	-	-
70	05/04/99	26	-	-
70	16/06/99	97	-	-
71	09/03/99	before	-	-
71	05/04/99	26	-	-
71	16/06/99	97	-	-

- = negative
+ = positive

3.3.2.1 Acute phase

Calf 70 (infested with the progeny of engorged ticks derived from Calf 49 during the first four week period after vaccine strain inoculation) and Calf 71 (infested with the progeny of engorged ticks from Calf 48 during the first four weeks after vaccine strain inoculation) were both negative on Giemsa-stained smears and didn't show a marked decrease in PCV (Table 16 and 17). IFAT performed on days 26 and 97 after infestation also gave negative results (Table 18). Both, however, were found positive for both *Babesia spp.* using PCR (Table 16 and 17) (Fig. 16 and 17).

Calf 73 (infested with the progeny of engorged ticks derived from Calf 50 during the first 4 weeks after inoculation with field strains) were positive on Giemsa-stained blood smears on day 12 post infestation and the PCV was 6% on day 13. It died on day 13 post infestation even though it had been treated on day 12 with a half dose of Berenil and a 100 ml blood transfusion. PCR was positive for both *Babesia spp.* (Table 15) (Fig. 16 and 17). IFAT results were positive for both *Babesia spp.* on day 12 post inoculation (Table 18).

Lanes:

1 123 bp ladder
2 Calf 73 18/03/99
3 Calf 73 22/03/99
4 Calf 70 22/03/99
5 Calf 71 18/03/99
6 Calf 71 19/03/99
7 Negative control

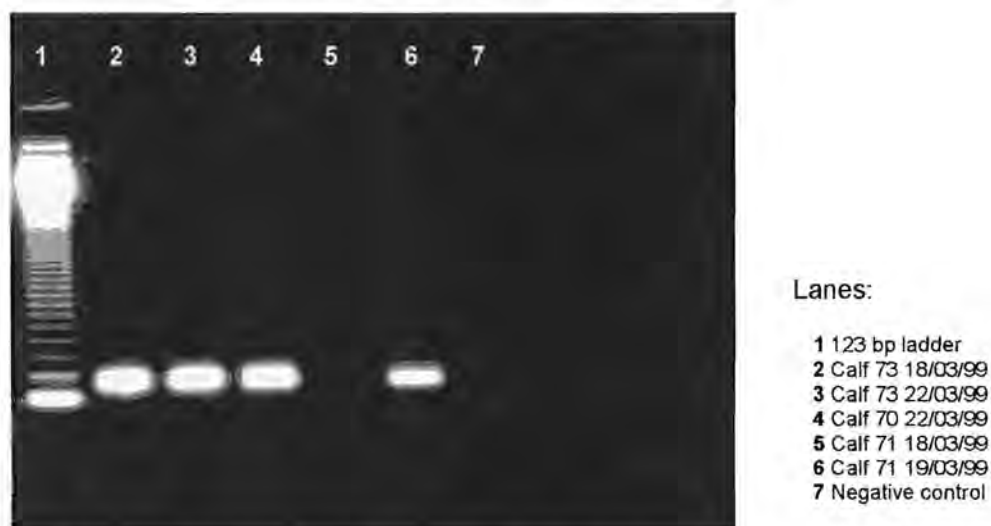**Fig. 16**   An agarose gel showing *B.bigemina* PCR results of samples derived from different dates from calves 73, 70 and 71.

Lanes:

1 123 bp ladder
2 Calf 73 17/03/99
3 Calf 70 17/03/99
4 Calf 70 20/03/99
5 Calf 71 17/03/99
6 Calf 71 21/03/99
7 Negative control

Fig. 17 An agarose gel showing *B.bovis* PCR results of samples derived from different dates from calves 73, 70 and 71.

3.3.2.2 Chronic phase

Calf 64 (infested with the progeny of engorged ticks derived from Calf 48 during weeks 5 to 13 after vaccine strain inoculation) and Calf 67 (infested with the progeny of engorged ticks derived from Calf 49 during weeks 5 to 13 after vaccine strain inoculation) were both negative on Giemsa-stained smears throughout the trial, and didn't show a decline in PCV. No Babesia spp. were detected using PCR (Table 19 and 20). IFAT performed on days 16, 27 and 43 after infestation also gave negative results (Table 21).

Table 19   Results of Giemsa, PCV and PCR tests done on Calf 64 (infested with larvae from week 5 – 13 belonging to Calf 48).

C 64	PCV	Giemsa	PCR *B.bovis*	PCR *B. bigemina*
05/05/99	30%	-	-	-
10/05/99	30%	-	-	-
18/05/99	26%	-	-	-
19/05/99	28%	-	-	-
25/05/99	27%	-	-	-
01/06/99	28%	-	-	-

- = negative

Table 20   Results of Giemsa, PCV and PCR tests done on Calf 67 (infested with larvae from week 5 – 13 belonging to Calf 49).

C 67	PCV	Giemsa	PCR *B.bovis*	PCR *B. bigemina*
05/05/99	30%	-	-	-
10/05/99	33%	-	-	-
18/05/99	32%	-	-	-
19/05/99	32%	-	-	-
26/05/99	29%	-	-	-
01/06/99	28%	-	-	-

- = negative

Table 21   IFAT results of recipient calves 66, 67 and 64 during the chronic phase of infection.

Calf	Date	Days post-infestation	*B. bovis*	*B. bigemina*
66	05/04/99	before	-	-
66	19/05/99	died		
67	05/04/99	before	-	-
67	21/05/99	16	-	-
67	01/06/99	27	-	-
67	16/06/99	43	-	-
64	05/04/99	before	-	-
64	21/05/99	16	-	-
64	01/06/99	27	-	-
64	16/06/99	43	-	-

- = negative

Calf 66 (infested with the progeny of engorged ticks from Calf 50 during weeks 5 to 13 after inoculation with field strains) was positive on Giemsa smears on day 13 post infestation and its PCV had decreased to 16% on the same day. PCR was positive for *B. bovis* and *B. bigemina* on day 13 (Table 22). This calf died of babesiosis on day 14 post infestation and no serum at this stage was available for serology.

Table 22   Results of Giemsa, PCV and PCR tests done on Calf 66 (infested with larvae from week 5 – 13 belonging to Calf 50).

C 66	PCV	Giemsa	PCR *B.bovis*	PCR *B. bigemina*
05/05/99	28%	-	-	-
10/05/99	26%	-	-	-
18/05/99	16%	+	+	+
19/05/99	died			

- = negative
+ = positive

3.4 DISCUSSION

This was a very costly experiment, not only because of the number of personnel involved, but also because of the large number of animals utilised over an extended period . We had already determined that *Babesia* vaccine strains could be found in the different developmental stages of the tick vector. In this additional experiment we attempted to determine if these *Babesia* organisms were also infective. Although recipient calves receiving vaccinal organisms derived from the ticks infected during the acute phase of infection didn't show any clinical signs nor seroconversion, parasite presence could still be detected in blood by PCR. The extremely high sensitivity of PCR allowed for the detection of *Babesia* even though no clinical signs were observed, probably due to the presence of a very low parasitaemia. The negative serological results (IFAT) is unclear but this technique may also have lacked in sensitivity. An ELISA with an appropriate dilution series may have been more effective in this regard. However, it could also be possible that no antibodies were formed because of the low antigenic load, absence of "danger signals" and minimal cell damage (related to the attenuation of the organism), which did not initiate an effective adaptive immune response. An alternative explanation would be that the innate immune response was effective in significantly eliminating organisms before an adaptive immune response could be effectively established and maintained.

Previous articles concerned with the vertical transmission of vaccine strains, have used Giemsa-stained blood smears only. The PCR assays we performed, showed positive reactions (*B. bovis* and *B. bigemina)* for the positive controls as expected. In addition, they also showed seroconversion. When the other two calves were infected with larvae derived from hosts in the chronic phase of infection with attenuated strains, no clinical signs were observed, no seroconversion occurred and no organisms could be detected using PCR either.

The infection of *Boophilus microplus* with *Babesia spp.* depends on, among other things, the phase of infection of the calf on which it feeds. This occurs more readily with ticks fed on infected animals in the acute phase of infection, as compared to those fed on carriers (Smith, 1978). This could have influenced the negative results obtained with the attenuated strains, but was obviously not the only reason since the field strain could still be transmitted. It is known that vaccine strains are a mix of genetically diverse organisms which have been shown to vary in such characteristics as virulence, DNA hybridisation patterns, protein and antigen composition, vector transmission and growth rate *in vitro* (Gill, *et al.,* 1987). It can therefore be assumed that the organisms or parasitic stages that were transmitted were either not present or present in numbers too low to be transmitted to a new animal.

CHAPTER FOUR

CONCLUDING REMARKS

In summary, transovarial transmission of both field and vaccine strains does occur in *Boophilus microplus* tick. They can also be retransmitted to susceptible animals when the donor is in the acute phase but not during the chronic phase of infection.

The number of syringe passages used to obtain a vaccine strain has considerable influence on the tick transmissibility of the organism. Tick transmissibility of *B. bovis* was still present at the 30th passage of the K strain used as a vaccine in Australia as well as in a Brazilian strain after 26 syringe passages. Variability between different strains does therefore exist. The *B. bovis* vaccine strain used in this experiment had been syringe-passaged 27 times, and in the case of the *B. bigemina* strain, 8 times. Tick-transmissibility is therefore still a possibility considering the previously reported findings. Since this capacity appears to have been lost during the chronic phase of infection it could be presumed that transmissible organisms were quite low within this population.

Very little is known about the molecular events associated with changes in *Babesia* virulence and tick-transmissibility. Some authors suggest that the gene product required for vector transmission (an enzyme necessary to penetrate the gut epithelial cells of the tick) is lost in non tick-transmissible strains. The transmissible parasites, however, probably secrete this enzyme in the tick gut thereby allowing them to penetrate the gut epithelium and continue their life-cycle.

The structure of the parasites of tick-transmissible and non-tick transmissible strains in tick gut contents following ingestion also differ. Observations of two strains of *B. bovis*, one infective for ticks (T) and the other no longer infective because of repeated blood passaging in cattle (NT), showed that in tick gut contents, NT strain ticks had morphological forms of Babesia with notable processes measuring up to 81 μm in length in a much higher proportion of parasites than in tick-transmissible strain. The proportion of organisms with obvious processes may therefore be an index of the infectivity of the strain for the tick vector (Stewart, 1978).

The use of a tick-transmissible vaccine in an enzootic area where the tick vector is well established, would probably be effective in promoting enzootic stability by maintaining or increasing the infection rates in the tick population. This, however, would not be the case in Uruguay which is situated in a marginal area for the development of *Boophilus microplus*. On

the contrary, the issuing of a tick-transmissible vaccine could lead to a reversion to pathogenicity, which has been demonstrated after only one tick passage.

Considering the tick transmissibility of vaccine strains during the acute phase of infection, strong recommendations will be given to farmers so as to protect their cattle against ticks following vaccination. Blood transfusions from vaccine recipient animals to new calves will be performed to determine if such Babesia organisms can replicate and revert to pathogenicity. Experiments will also be performed using on-line quantitative PCR to determine the pathogen load and the threshold of detection. Further investigations will be directed towards identifying virulence genes so as to differentiate virulent from avirulent organisms.

It would also be of value to determine the nature of the immunological response and the protection of animals infected with tick-transmitted vaccine strains. More suitable and sensitive methods of characterising the humoral response, and also the cellular immune response (lymphoproliferation assays; gamma-interferon and other cytokine detection assays; flow cytometry) need to be considered. Should such indicators of the adaptive immune response prove negative, then it could be speculated that the innate immune response was effective in eliminating organisms at an early stage. Methods of evaluating the innate immune response could also prove informative (macrophage migration, NO synthesis etc). The nature of attenuation could not only reduce the ease of organisms entering their target cells, it could also mean a reduction in cell damage, a reduction in molecular signatures of pathogen presence or reduced induction of the danger signal believed to be an important prerequisite for the initiation of the innate and adaptive immune responses. In addition, more effective methods to detect presence of parasites in erythrocytes could also be considered (flow cytometry). This would indicate with greater certainty that parasites were in fact entering and replicating in erythrocytes, and that PCR was not just detecting parasite DNA from non-replicating organisms introduced by the large number of infected ticks used. The ability of such organisms to induce a protective immune response, and their transmissibility to other cattle could also be determined.

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