VIABILITY OF A METHOD FOR THE ISOLATION OF *BABESIA BOVIS* AND *BABESIA BIGEMINA* TO CREATE A STRAIN BANK FROM FIVE PHYSIOGRAPHICAL REGIONS OF BRAZIL..

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SUMMARY:Pure isolates of *Babesia bovis* and *Babesia bigemina* were obtained from *Boophilus microplus* ticks, from the five brazilian physiographic regions. Engorged female ticks from these regions were placed in Petri dishes and incubated at 28°C and 80% of humidity. After oviposition (day 7 - 14), eggs were weighted distributed in adapted disposable syringes or metal screen tubes and incubated at the same temperature and humidity. Splenectomized Nelore calves (*Bos indicus)* were infected with *B. microplus* larvae or engorged larvae. The animals were evaluated for parasitaemia, packed cell volume and once a week serologically for antibodies against *Babesia* (indirect fluorescent antibody). *B. bovis* was transmitted only by the larvae, while *B.bigemina* was transmitted during the nymph and adult stages. When parasitaemia was detected by stainned thin slide smears, infected blood samples were titrated and stored in liquid nitrogen.

KEY WORDS: *Babesia*, isolation, transmission, tick, *Boophilus microplus.*

INTRODUCTION

Babesiosis has been considered one of the limiting factors for the increase of productivity of the cattle industry in countries with tropical and subtropical climates. In Brazil the tick *Boophilus microplus* is the principal vector of *Babesia bovis* and *Babesia bigemina*. The tick larvae transmit *B. bovis* (MAHONEY & MIRRE, 1979), and the nymphs and adults transmit *B. bigemina* (CALLOW & HOYTE, 1961b). Isolation of these agents based on the transmition properties by the *B. microplus* tick has been studied by several authors (CALLOW & HOYTE, 1961a; CALLOW, 1977; KESSLER & BELLATO, 1981; GUGLIELMONI *et alii*, 1981; DALGLIESH & STEWART, 1983; BAI *et alii*, 1987; KESSLER *et alii*, 1987). Genetic and antigenic diversities have been demonstrated in the organisms of the *Babesia* genus, in isolates from different geographical regions of the world (COWMAN *et alii*, 1984; PALMER *et alii*, 1991; DALRYMPLE, 1992). These variations between populations of *Babesia* appear to have a significant effect on the duration of efficacy of the attenuated vaccine (BOCK *et alii*, 1992; DALRYMPLE, 1992), as well as in the elaboration of the subunit vaccine, extensively studied in recent years (TIMMS & BARRY, 1988; REDUCKER *et alii*, 1989; HINES *et alii*, 1991; WRIGHT *et alii*, 1992). The objective of this work was to create a bank of isolates of *B. bovis* and *B. bigemina*, from the different physiographical regions, aiming at a better understanding of the Brazilian isolates for the improvement and development of new diagnostic and immunization techniques.

MATERIALS AND METHODS

The work was carried out at the "Centro Nacional de Pesquisa de Gado de Corte (CNPGC)" - National Beef Cattle Research Center , "Empresa Brasileira de Pesquisa Agropecuária" (EMBRAPA) - Brazilian Agricultural Research Company, in Campo Grande, Mato Grosso do Sul. Initially engorged female ticks were obtained from the five physiographical regions of Brazil: North, Northeast, Central-West, Southeast and South. The method described by KESSLER *et alii* (1987) was used for the isolation of *B. bovis* and *B. bigemina*. The engorged females were placed in Petri dishes and incubated at 28°C, with 80% relative humidity. Eggs from the 7th through the 14th day of oviposition were weighed, distributed in 0,5g batches (equivalent to 10,000 larvae) in adapted disposable syringes or in metalic mesh tubes and incubated in the same conditions of temperature and humidity. For the isolation of the samples, four to six months old Nelore calves, born and raised tick-free were used. The animals were placed in individual pens, in an isolation area, and monitored before and after splenectomy by rectal temperature (T), hematocrit (Ht), by direct examination of blood smears stained by May-Grünwald/Giemsa, clinical examination and serological testing by indirect fluorescent antibody technique (IFAT).

Ten or twenty thousand larvae were placed in a capsule of cotton fabric (musseline), attached to the back of one calf (Fig. 1). On the fifth day, the metalarvae were removed from this calf

(Fig. 2) and transferred to another capsule that was attached to the back of another calf. After removal of the metalarvae the first calf was sprayed with an acaricide to eliminate remaining ticks. Some isolates were subinoculated intravenously. The animals were examined daily as described above. Serological testing was carried out weekly by the IFAT. Parasitized blood was collected, titrated, stabilized by the addition of 10% of glycerol, and preserved by freezing in liquid nitrogen in 1 ml aliquots. The same procedure was applied for each isolated sample.

Fig. 1. Calf with capsule attached to the back, being infested with *Boophilus microplus* larvae.

Fig.2. Collection of metalarvae, after removal of the capsule, to be transferred to another calf.

RESULTS

Tables 1 and 2 present the values of the prepatent period (PPP), percentage of parasitemia (PP), rectal temperature (T) and hematocrit observed during the isolation of the samples.

Table 1. Prepatent period (PPP), percentage parasitemia at freezing (PPF), maximum parasitemia (PPM), maximum rectal temperature (T) and reduction in hematocrit, of splenectomized calves, infested with *Boophilus microplus* larvae, during the process of isolation of *Babesia bovis* from the five physiographical regions of Brazil.

Region	PPP (days)	PPF (%)	PPM* (%)	T* (ºC)	Ht red.* (%)	Obs.
North	11	0.6	0.6	40.8	30.3	died
Northeast	15	0.5	-	-	-	medicated
Southeast	10	0.1	-	-	-	medicated
Mid-West	13	0.08	-	-	-	medicated
South (Bagé)	14	0.4	-	-	-	medicated
South (Rosário)	25	**	0.01	40.4	33.3	no medication

*Maximum values obtained were considered only for calves that recovered from infection without medication.
**Subinoculation was done in another calf.

Table 2. Prepatent period (PPP), percentage parasitemia at freezing (PPF), maximum parasitemia (PPM), maximum rectal temperature (T) and reduction in hematocrit, of splenectomized calves, infested with *Boophilus microplus* larvae, during the process of isolation of *Babesia bigemina* from the five physiographical regions of Brazil.

Region	PPP (days)	PPF (%)	PPM* (%)	T* (ºC)	Ht red.* (%)	Obs.
North	13	0.8	-	-	-	medicated
Northeast	16	4.8	4.8	41.2	43.2	no medication
Southeast	10	0.1	-	-	-	medicated
Mid-West	11	19.5	19.5	40.3	84.0	no medication
South (Bagé)	13	0.18	0.18	-	34.2	no medication

*Maximum values obtained were considered only for calves that recovered from infection without medication.

North isolate (Rondônia): in the first calf, infested with 10,000 larvae, the presence of *B. bovis* was detected 11 days after infestation. On this date, a subinoculation (20 ml of blood) was carried out with another calf. A sample of the isolated was frozen in liquid nitrogen at the 13th day, when parasitemia was 0.5%. During infection by *B. bovis* the calf had a maximum PP of 0.6%, maximum T of 40.8% and 30.3% reduction in Ht, as well as anorexia and motor incoordination, and died on the 18th day post-infestation. Brain smears showed capillaries filled with *B. bovis*-parasitized erythrocytes. The subinoculated calf had a PPP of 9 days and a parasitemia of 0.3% when samples were frozen. The calf infested with metalarvae removed from the first one did not show parasitemia for an observation period of 30 days. In an attempt to isolate *B. bigemina*, the process was repeated with a new batch of engorged females from the same origin, again unsuccessfully. A third attempt was carried out the same way as the other two, when the presence of *B. bigemina* was detected 13 days after infestation with metalarvae. On the 14th day, when the PP was 0.8%, the isolate was frozen. After subinoculation in another calf a second sample, with a 1.3% parasitemia, was also frozen.

Northeast isolate (Bahia): the first attempt of isolation of *B. bovis* and *B. bigemina* was frustrating. None of the calves infested with larvae or metalarvae showed parasitemias detectable in thin smears. In the second attempt, the first calf

was infested with 20,000 larvae of *B. microplus* from the same origin. Fifteen days after infestation, samples with 0.5% parasitemia of *B. bovis* were frozen. On the 16th day post-infestation with metalarvae, the calf showed patent infection by *B. bigemina* and on the 17th day maximum parasitemia reached 4.8%, then samples were frozen. The maximum T was 41.2% and reduction in Ht was 43.2%. The animal recovered normal conditions without specific medication.

Southeast isolate (São Paulo): the first calf had a PPP of 10 days after infestation with 20,000 larvae and 0.1% parasitemia by *B. bovis* when samples were frozen. The second calf infested with metalarvae showed patent infection with *B. bigemina*, with 0.1% parasitemia and 31.0% Ht, 10 days after infestation when blood for freezing was collected. During the observation period, this animal had hemoglobinuria, and parasitemia reached 3.6% when it was medicated with a babesicide, recovering normal conditions.

Central-West isolate (Campo Grande): the first calf was infested with 10,000 larvae of *B. microplus*. The presence of *B. bovis* was detected 13 days after infestation. Samples were frozen when parasitemia was at 0.08%. The calf was medicated with a babesicide. The second calf infested with metalarvae showed patent infection by *B. bigemina* 11 days post-infestation. Samples were frozen when the calf had a 19.5% parasitemia. This calf recovered from infection without medication.

South isolate (Bagé): 14 days after infestation with 20,000 larvae, samples of *B. bovis* were isolated, with a parasitemia of 0.4%. Thirteen days after infestation with metalarvae, the presence of *B. bigemina* was detected. Samples were frozen with 0.18% parasitemia. Maximum PP was 0.9% and the reduction in Ht was 34.2%. The animal recovered without specific treatment.

South isolate (Rosário do Sul): the presence of *B. bovis* was observed 25 days after infestation with 20,000 tick larvae when a second calf was subinoculated with 20 ml of blood. Samples of *B. bovis* of the subinoculated calf were frozen when parasitemia was 0.2%. The calf that was infested with metalarvae did not show parasitemia, and *B. bigemina* was not isolated from this tick sample.

DISCUSSION

The prepatent period varied for *B. bovis*, from 10 to 25 days and for *B. bigemina*, from 10 to 16 days post-infestation with larvae and metalarvae, respectively. Considering six days for larvae developing to nymphs, the pre-patent period for *B. bigemina* may be projected as 16-22 days. These periods were higher than 8 to 16 days, normally encountered in natural infestations (MAHONEY, 1977). The isolation of pure samples of *B. bovis* and *B. bigemina* was confirmed by IFAT testing. The rates of success in the isolation were 85.7% for *B. bovis* (6 isolates in 7 attempts) and 55.5% for *B. bigemina* (5 isolates in 9 attempts). This lower success rate in the isolation of *B. bigemina* might be attributed to the survival of a smaller number of nymphs in relation to the number of larvae used in the initial infestation and to the higher resistance of Nelore cattle (McCOSKER, 1981; GOMES *et alii*, 1989) used in this work, despite that the calves had been previously splenectomized.

The isolation method proved very practical, since the localized infestation allows for an easy recovery of a significant number of metalarvae that will go through ecdysis on the calf, in an environment restricted by the capsule, diminishing the losses caused by falling. On the other hand, the method is more economical than that described by the authors who used the transference of adults, males and females (GUGLIELMONE *et alii*, 1981), only males (DALGLIESH & STEWART, 1983; BAI *et alii*, 1987), or successive blood passages containing a mixed infection (CALLOW & HOYTE, 1961b; BISHOP *et alii*, 1973), for the isolation of *B. bigemina*, because it makes possible the isolation of *B. bovis* and *B. bigemina* using only two calves. However, in this work, *Theileria* spp., *Eperythrozoon* spp. or *Borrelia* spp. were not found in thin smears of blood from the splenectomized calves, probably because in the microregion cattle are not naturally infected by those microorganisms, what facilitated much the process of isolation.

ACKNOWLEDGMENTS

The authors thank the valuable help from Dr. Maria Ângela Ornelas, Dr. Arlete Del Porto and Dr. Francelino Goulart, for sending the engorged females.

SUMÁRIO

Isolaram-se amostras puras de *Babesia bovis* e *Babesia bigemina* de carrapatos *Boophilus microplus* provenientes das cinco regiões fisiográficas do Brasil. Teleóginas procedentes das respectivas regiões foram colocadas em placa de Petri e incubadas a 28°C com umidade de 80%. Após a postura (7° ao 14°dia) os ovos foram pesados e distribuídos em seringas descartáveis adaptadas ou tubos de tela metálica e incubados nas mesmas condições de temperatura e umidade. Bezerros da raça Nelore (*Bos indicus*), esplenectomizados, foram infestados com larvas ou metalarvas de *B.microplus*. Diariamente avaliaram-se os animais observando-se a parasitemia, hematócrito e, semanalmente, realizou-se a sorologia pelo teste de imunofluorescência indireta. A fase larval transmitiu somente a *B. Bovis*. A *B. bigemina* foi transmitida durante a fase de ninfa e adulto. Quando a

parasitemia tornou-se patente amostras de sangue parasitado foram colhidas, tituladas e congeladas em nitrogênio líquido.

PALAVRAS-CHAVE: *Babesia*, isolamento, transmissão, carrapato, *Boophilus microplus*.

REFERENCES

BAI, Q.; YIN, S.X.; CHEN, Z.H. ; LIU, G.Y. & ZHOU, J.Y. (1987). Studies on isolation and preservation of a single species of haematocytozoon in bovines: isolation of a pure strain of *Babesia bigemina*. *Chinese Journal of Veterinary Science and Technology*. 9:25-27.

BISHOP, J.P.; ADAMS, L.G.; THOMPSON, K.C. & CORRIER, D.E. (1973). The isolation, separation and preservation of *Babesia bigemina*. *Tropical Animal Health and Production*. 5: 141-145.

BOCK, R.E.; de VOS, A.J.; KINGSTON, T.J.; SHIELS, I.A. & DALGLIESH, R.J.; (1992). Investigations of breakdowns in protections provided by living *Babesia bovis* vaccine. *Veterinary Parasitology* ,43:45-56.

CALLOW, L.L.& HOYTE, H.M.D. (1961a). The separation of *Babesia bigemina* from *Babesia argentina* and *Theileria mutans*. *Australian Veterinary Journal*. 37:66-70.

CALLOW, L.L. & HOYTE, H.M.D. (1961b). Transmission experiments using *Babesia bigemina, Theileria mutans*, *Borrelia* spp. andthe cattle tick *Boophilus microplus*. *Australian Veterinary Journal*; 37:381-90.

CALLOW, L.L. (1977). Vaccination against bovine babesiosis. In:. MILLER, H.L.; PINO, J.A.; McKELVEY JR, (Editors), *Immunity to Blood Parasites of Animals and Man*. Plenum Press, New York NY, pp121-149.

COWMAN, A.F. ; TMMS, P. & KEMP, D.J. (1984). DNA polymorphism and sub populations in *Babesia bovis*. *Molecular and Biochemichal Parasitology*: 46: 45-52.

DALGLIESH, R.J.& STEWART, N.P. (1983). The use of tick transmission by *Boophilus microplus* to isolate pure strains of *Babesia bovis Babesia, bigemina* and *Anaplasma marginale* from cattle with mixed infections. *Veterinary Parasitology*, 13: 317-23.

DALRYMPLE, B.P. (1992). Diversity and selection in *B. bovis* and their impact on vaccine use. *Parasitology Today*, 8: 21-23.

GOMES, A.; HONER, M.R.; SCHENK, M.A.M. & CURVO, J.B.E. (1989). Populations of the cattle tick (*Boophilus microplus*) on purebred Nellore, Ibage and Nellore x european crossbreds in the brazilian savanna, *Tropical Animal Health and Production*, 21(1):20-24.

GUGLIELMONE, A.; BERMUDEZ, A.; HADANI, A.; MANGOLD, A.; VANZINI, V.; LUCIANI, C. de ; RIOS, L.G. ; GALLATTO, C. (1981). Aislamiento de una cepa de *Babesia bigemina* por la parasitación de un ternero con *Boophilus microplus* adultos. *Gaceta Veterinária*, 43:341-7.

HINES, S.A.; PALMER, G.H.; JAMES, D.P.; McGUIRE, T.C. & McLWAIN, T.E.(1991). Neutralization-sensitive merozoite surface antigens of *Babesia bovis* encoded members of a polimorfic gene family. *Molecular and Biochemical Parasitology*, 46: 45-52.

KESSLER, R.H. & BELLATO, V. (1981). Measure of the protective immune response to *Babesia bovis* culture derived antigens. isolation of *Babesia bovis* and *Babesia bigemina* strain from Rio grande do Sul, Brazil. s.l., s.ed., 7p. Trabalho apresentado no "II Research Coordination Meeting Coodinated research programmee on the Use of Isotope Techniques in Research and Control of Ticks and Tick Borne Diseases", Nairobi, Quênia.

KESSLER, R.H.; MADRUGA, C.R.; DE JESÚS, E.F. & SEMPREBON, D.V. (1987a). Isolamento de cepas puras de *Babesia bovis, Babesia bigemina* e *Anaplasma marginale* em área enzóotica. *Pesquisa Agropecuária Brasileira.*, Brasilia 22 (7): 747-752.

MAHONEY, D.F. (1977) Babesia of domestic animals. In: KREIER, J.P., ed. *Parasitic protozoa*. New York, Academic Press, v. 4, p. 1-52.

MAHONEY, D.F.& MIRRE, G.B. (1979). A note on the transmission of *Babesia bovis* (syn. *B. argentina*) by the one-host tick, *Boophilus microplus*. *Research Veterinary Science*, 26: 253-4.

McCOSKER, P.J. (1981). The global importance of babesiosis. In: RISTIC, M.; KREIER, J.P. (ed), *Babesiosis*, Academic Press, New York. p. 1-24.

PALMER, G.H.; MCELWAIN, T.F.; PERRYMAN, D.P.; DAVIS, W.C.; REDUCKER, D.R.; JASMER, D.P.; SHKAP, V. PIPANO, E.; GOFF, W.L. & McGUIRe, T.C. (1991). Strain variation of *Babesia bovis* merozoite. Surface exposed epitopes. *Infection and Immunity* 59: 3340-42.

REDUCKER, D.W.; JASMER, D.P.; GOFF, W.L.; PERRYMAN, L.E.; DAVISW.C. & McGUIRE, T.C. (1989). A recombinant surface protein of *Babesia bovis* elicits bovine antibodie that react with live merozoites. *Molecular and Biochemical Parasitology*, 35:239-248.

TIMMS, P.; BARRY, D.N. (1988). Failure of a recombinant *Babesia bovis* antigen to protect cattle agains heterologus strain challenge. *Research Veterinary Science*, 45:267-269.

WRIGT, I.G.; CASU, R.; COMMINS, M.A.; DALRYMPLE, B.P.; GALE, K.R.; GOODGER, B.V.; RIDDLES, P.W.; WALTISBUHL, D.J.; ABETZ, A.; BERRIE, D.A.; BOWLES, Y.; DIMMOCK, C.; HAYES, T.; KALNINS, H.; LEATH, G.; McCRAE, R.; MONTAGUE, P.E.; NISBET, I.T.; PARRODI, F.; PETERS, J.M.; SCHEIWE, P.C.; SMITH, W.; RODERAMAINS & K.; WHITE, M.A. (1992). The development of a recombinant *Babesia* vaccine. *Veterinary parasitology*. 44:3-13.

(Received 2 December 1997, Accepted 28 April 1998)