

THE ISOLATION, SEPARATION AND PRESERVATION OF *BABESIA BIGEMINA*

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SUMMARY

Experiments were performed in Colombia to separate Babesia bigemina from contaminating organisms. Babesia bigemina was passaged serially through five splenectomized calves. The first calf was inoculated with blood carrying several different organisms, and subsequent subinoculations were done soon after blood smears from each calf were found to be positive for B. bigemina. Five blood passages were carried out in 6.5 days. Babesia argentina, B. major and A. marginale were eliminated as contaminants of the B. bigemina isolated after four passages. A stablate of the isolated B. bigemina was established.

INTRODUCTION

The history of the recognition of bovine babesiosis in Colombia and the identification of three species was recounted by Velásquez (1938). The three species, *Babesia bigemina*, *Babesia argentina* and *Babesia major*, may occur together as a mixed infection and the prevalence of infection appears to be related to the occurrence and activity of the tick-vectors at the various altitudes (Todorovic, Adams and Roberts, 1969).

In an attempt to add to our knowledge of the mechanisms involved in acquired resistance to *B. bigemina*, a series of investigations were undertaken. As a starting point, it was necessary to have an isolate of *B. bigemina* free from contaminating organisms. However, *B. bigemina* carriers in Colombia are almost invariably infected with at least one other haemoparasite. *Babesia argentina*, *B. major*, *Anaplasma marginale*, *Trypanosoma vivax*, *Trypanosoma theileri*, *Eperythrozoon wenyoni*, *Eperythrozoon lejanodes* and *Eperythrozoon tuomii* are also parasites of cattle in Colombia in areas where arthropod vectors are present (Kuttler, Adams and Zaraza, 1969; Todorovic *et al.*, 1969; Wells, Betancourth and Page, 1970; Adams, Craig, Platt and Wyss, 1972). Kuttler (1969) isolated *Babesia* spp. and *A. marginale* from a cow from the Llanos Orientales (Eastern Plains) of Colombia. He established a *Babesia* spp. carrier by the simultaneous inoculation of mixed infected blood and alpha-ethoxyethyl-glyoxal dithiosemicarbazone‡ which prevented an *A. marginale* infection.

The organisms originally contaminating our isolate of *B. bigemina* were *B. argentina*, *B. major* and *A. marginale*. This paper describes the isolation, separation and preservation of a *B. bigemina* stablate used at the Laboratorio de Investigaciones Medicas Veterinarias (LIMV), Instituto Colombiano Agropecuario (ICA), Bogotá, Colombia.

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‡Gloxazone. Burroughs-Wellcome and Co., Inc., Tuckahoe, N.Y.

MATERIALS AND METHODS

Three-day-old male Holstein—Friesian calves were obtained from near Facatativá Colombia and were hand-raised on the Tibaitatá ICA experimental station (altitude 2600 m.) in an area free of *Boophilus microplus*.

The isolation of *B. bigemina* was made from a natural case of babesiosis. A four-month-old splenectomized calf was transported by airplane from the ICA-LIMV laboratories in Bogotá to the northern coastal area of Colombia and placed on a tick-infested pasture at the Turipaná ICA experiment station (altitude 24 m) near Montería.

Daily, thin films were made with blood obtained from the jugular vein using 1.3 g/l of disodium ethylenediamine tetra-acetate (EDTA) as an anticoagulant. The films were fixed in absolute methanol and stained with a 1 to 20 solution of Giemsa* for 20 minutes, using phosphate buffered water (pH 7.0-7.1) containing 0.01 per cent alkyl phenoxy polyethoxy ethanol** (APPE) to prepare the stain solution. Stock solutions of M/15 NaH₂PO₄, M/15 NaH₂PO₄, and 10 per cent APPE were prepared and stored in separate glass stoppered bottles. Fresh buffered water containing 0.01 per cent APPE was prepared weekly by using 39 ml of M/15 NaH₂PO₄, 61 ml of M/15 Na₂HPO₄, 1 ml of 10 per cent APPE and 899 ml of distilled water. An electric pH meter*** was used to determine the pH of the freshly prepared buffered water containing APPE. The films were examined microscopically, using a microscope with an oil immersion objective, for 30 minutes in cases when the first appearance of *Babesia* was thought to be imminent. The films were examined about 3 to 6 mm from the end of the film and transversed from one side of the film to the other to give a constant and representative sample. After *B. bigemina* was found in blood smears, 180 ml of blood were withdrawn from the jugular vein of the splenectomized calf, using 1.3 g/l of dipotassium EDTA as an anticoagulant. The blood was transported by airplane to the ICA-LIMV laboratories in Bogotá for the separation of *B. bigemina* from *B. argentina*, *B. major* and *Anaplasma marginale*.

The method of separation of *B. bigemina* from *B. argentina*, *B. major* and *A. marginale* involved rapid passage through five splenectomized calves and was based on that used by Sergeant, Donatien, Parrot, Lestoquard and Planturux (1927) and Callow and Hoyte (1961a). The first calf was inoculated with blood which contained several different organisms, and subsequent subinoculations were done soon after blood smears from each calf were found to be positive for *B. bigemina*, based upon characteristic morphology. Blood for subinoculation was collected from the jugular vein without using an anticoagulant and each passage was carried out immediately by injecting the blood into the jugular vein of the next splenectomized calf.

The drugs used to treat the *Babesia* infections were trypanblue and 4,4'-diamidino-diazoaminobenzene diacetate†. Trypanblue was used for suppressing the *B. bigemina* infection without interfering with the potential *B. argentina* infection.

A stabilate of *B. bigemina* was made using a modification of the method reported by Pipano and Seif (1966). Blood containing 2.5 per cent *B. bigemina* parasitized erythrocytes was collected from the fifth splenectomized calf at the acute stage of the disease by venipuncture using 1.3 g/l disodium EDTA as an anticoagulant. Glycerol at a final concentration of 11.6 per cent was used as a cryoprotective agent. Fifty ml aliquots of the blood-glycerol mixture were dispensed in 60 ml plastic narrow mouth bottles with screw caps‡ and incubated for 30 minutes at 4°C and stored in a dry ice cabinet at -79°C.

*Giemsa Stain. Gradwohl Laboratories, 3513 Lucas Avenue, St. Louis, Mo., 63155.

**Triton X-100, Rohm and Huss Company, Independence Mall West, Philadelphia, Pa. 19105.

***Beckman Expandometric Model 76. Beckman Instruments, Inc., Scientific Instruments Division, Fullerton, California 92634.

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‡Kimble, Owens, Illinois, Toledo, Ohio 43601.

RESULTS

The splenectomized calf placed on a tick-infested pasture at the Turipaná ICA station was found to be heavily infested with *B. microplus* ticks in the larval stage within three days, ticks in the nymphal stage within eight days and ticks in the adult stage within 14 days. After 16 days, the rectal temperature of the calf was 40.4°C and *B. argentina* was found in thin blood smears stained with Giemsa. Two days later, *B. bigemina* was found in blood smears. The results of the rapid passages of *B. bigemina* through five splenectomized calves to separate *B. bigemina* from contaminating organisms are summarised in Table I.

B. bigemina was found in blood smears from the first calf after 36 hours. The first calf, four days after the inoculation, had a *B. argentina* parasitaemia which was treated with ganaseg at 3 mg/kg intramuscularly for five days. *Anaplasma marginale* was present in blood smears from the first calf 44 days after inoculation.

B. bigemina was found in blood smears from the second calf after 30 hours. The second calf, 4 days after the inoculation, had a *B. argentina* parasitaemia which was treated with ganaseg at 3 mg/kg intramuscularly for the following two days, the second calf died from babesiosis one day after the last treatment.

The third calf was positive for *B. bigemina* 24 hours after inoculation with 12 ml of blood from the second calf. The third calf was subsequently treated with an intravenous injection of 20 ml of 1 per cent trypanblue, which suppressed the *B. bigemina* infection. The third calf, 15 days after inoculation with blood, had *B. argentina* and *B. major* which were treated intramuscularly the following day with ganaseg at 3 mg/kg. The third calf died from babesiosis one day after treatment.

The fourth calf was positive for *B. bigemina* 37.5 hours after inoculation with 12 ml of blood from the third calf. The procedure of treating the fourth calf with trypanblue was repeated.

B. bigemina was found in blood smears from the fifth calf after 57.5 hours, and 12 ml of blood was passaged intravenously into an intact calf. A stabilate of *B. bigemina* was made from the fifth calf four days after inoculation when the parasitaemia was 2.5 per cent. The day following freezing, 50 ml of the stabilate was inoculated intravenously into a second intact calf.

The first intact calf was positive for *B. bigemina* two days after inoculation, and the second intact calf was positive five days after inoculation. The intact calves required no treatment with trypanblue. *Babesia argentina*, *B. major* and *A. marginale* were not diagnosed in the two intact calves nor in any smear from the fourth and fifth splenectomized calves for two months after inoculation. *B. argentina* and *B. major* were eliminated as contaminating organisms after four passages. The second and third splenectomized calves died of babesiosis which made it impossible to estimate at which point *A. marginale* failed to be passaged.

TABLE I
The Separation of *B. bigemina* from *B. argentina*, *B. major* and *A. marginale*
by Rapid Passage through 5 Splenectomized Calves

Passage	Inoculum (ml. intravenously)	Prepatent Period (hours)	Parasite	Time of Subinoculation (hours)
1	180 from naturally infected calf	36	<i>B. bigemina</i>	37.5
2	12 from No. 1	30	<i>B. bigemina</i>	31
3	12 from No. 2	24	<i>B. bigemina</i>	24
4	12 from No. 3	37.5	<i>B. bigemina</i>	37.5
5	12 from No. 4	37.5	<i>B. bigemina</i>	—

DISCUSSION

The results of the experiment, designed to obtain *B. bigemina* free from contaminating organisms, show that *B. argentina* and *B. bigemina* were found in blood smears from the splenectomized calf 16 and 18 days, respectively, after the calf had been placed on a tick-infested pasture. Callow and Hoyte (1961*b*) observed the experimental transmission of *B. bigemina* by *Boophilus microplus* and recorded patent infection with *B. bigemina* 11 to 17 days following the placement of larvae on cattle and that transmission did not take place until the ticks became nymphs. Callow and Hoyte (1961*b*) also reported that *B. argentina* could be transmitted by larvae, and therefore, patent infections with *B. argentina* could be detected earlier than patent infections with *B. bigemina* following placement of larvae on cattle. The observations on the natural transmission of *B. bigemina* in the present experiment are in agreement with the observations of the experimental transmission of *B. bigemina* by *B. microplus* recorded by Callow and Hoyte (1961*a*).

The ability of *B. bigemina* to multiply so rapidly that it was found in thin blood smears from 24 to 57.5 hours after calves had been inoculated with relatively small numbers of parasites, made it possible to separate *B. bigemina* from other blood parasites. The prepatent periods for *B. bigemina* observed were in agreement with those prepatent periods reported by Callow and Hoyte (1961*a*).

The lability of newly isolated *Babesia* spp. reported by Irvin and Brocklesby (1969) and Frerichs, Holbrook and Johnson (1969) emphasises the need for the establishment of stabilates. Many factors, such as clonal selection, methods of transmission, type of host animal and antigenic variation are probably involved in the lability of *Babesia* spp. (Phillips, 1969). A stabilate of the *B. bigemina* isolate in this study was established.

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Isolement, séparation et conservation de *babesia bigemina*.

Résumé—Des expériences ont été effectuées en Colombie pour séparer *Babesia bigemina* des organismes infectés. *Babesia bigemina* fut passé en série sur cinq veaux splénectomisés. Le premier veau a été inoculé avec du sang contenant plusieurs organismes différents. Les subinoculations suivantes ne furent faites que lorsque les étalements de sang de chaque veau furent positif avec *B. bigemina*. Les cinq passages furent effectués en 6,5 jours. *Babesia argentina*, *B. major* et *Anaplasma marginale* ont été éliminés, en tant qu'éléments contaminants, après quatre passages. Un stabilat des *B. bigemina* isolés a été effectué.

El aislamiento, separación y preservación de *babesia bigemina*.

Sumario—Se realizaron experimentos en Colombia para separar *Babesia bigemina* de organismos contaminantes. *Babesia bigemina* fue pasada en forma seriada en cinco terneros esplenectomizados. El primer ternero fue inoculado con sangre conteniendo algunos organismos diferentes, y las siguientes subinoculaciones fueron hechas tan pronto como los froticos de sangre de cada ternero fueron encontrados ser positivos con *B. bigemina*. Se llevaron a cabo cinco pases de sangre en 6.5 días. *Babesia argentina*, *B. major*, y *A. marginale* fueron eliminados como contaminantes del aislamiento de *B. bigemina* despues del cuarto pase. Una forma estable del aislamiento de *B. bigemina* ha sido obtenida.