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 passed 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
stabilate of the isolated B. bigemina was established.

INTRODUCTION

The history of the recognition of bovine babesiosis in Colombia and the indenti-
fication 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

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 wenyonii, Epery-
 throzoosangenodes and Eperythrozoon tuominii are also parasites of cattle in Colombia
in areas where arthropod vectors are present (Kuttler, Adams and Zaraza, 1969;
Todorovic et al., 1969; Wells, Betancourt and Page, 1970; Adams, Craig, Platt
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 dihydroximicarbazonet, which prevented an A. marginale infection.

The organisms originally contaminating our isolate of B. bigemina were B. argen-
tina, B. major and A. marginale. This paper describes the isolation, separation and
preservation of a B. bigemina stabilitate used at the Laboratorio de Investigaciones
Medicas Veterinarias (LIMV), Instituto Colombiano Agropecuario (ICA), Bogotá,
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MATERIALS AND METHODS

Three-day-old male Holstein-Friesian calves were obtained from near Facatativá, Colombia and were hand-raised on the Tibaita 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 Na₂HPO₄, 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 Sergent, Donatien, Parrot, Lestoquard and Planturux (1927) and Callow and Hoyte (1961). 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'-diamidinodiazooaminobenzenc diaceturate†. Trypanblue was used for suppressing the *B. bigemina* infection without interfering with the potential *B. argentina* infection.

A stabulate 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.

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**Giemsa Stain. Gradwohl Laboratories, 3513 Lucas Avenue, St. Louis, Mo., 63135.**  
**Triton X-100, Rohm and Haas Company, Independence Mall West, Philadelphia, Pa. 19105.**  
**Beckman Expandumeter Model 76. Beckman Instruments, Inc., Scientific Instruments Division, Fullerton, California 92634.**  
*Ganoysq. Squibb, Cati, Columbia.  
†Kimbie, Owens, Illinois, Toledo, Ohio 43601.
ISOLATION OF B. BIGEMINA

RESULTS

The splenectomized calf placed on a tick-infested pasture at the Turipani 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 1.

B. bigemina was found in blood smears from the first calf after 36 hours. The first calf, four days after the inoculation, had B. argentina parasitaemia which was treated with gansev 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 gansev 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 gansev 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 stablate 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 stablate 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 1

<table>
<thead>
<tr>
<th>Passage</th>
<th>Inoculum (ml. intravenously)</th>
<th>Preparative Period (hours)</th>
<th>Parasite</th>
<th>Time of Subinoculation (hours)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>180 from naturally infected calf</td>
<td>36</td>
<td>B. bigemina</td>
<td>37.5</td>
</tr>
<tr>
<td>2</td>
<td>12 from No. 1</td>
<td>30</td>
<td>B. bigemina</td>
<td>31</td>
</tr>
<tr>
<td>3</td>
<td>12 from No. 2</td>
<td>24</td>
<td>B. bigemina</td>
<td>24</td>
</tr>
<tr>
<td>4</td>
<td>12 from No. 3</td>
<td>37.5</td>
<td>B. bigemina</td>
<td>37.5</td>
</tr>
<tr>
<td>5</td>
<td>12 from No. 4</td>
<td>37.5</td>
<td>B. bigemina</td>
<td>—</td>
</tr>
</tbody>
</table>
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 (1961b) 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 (1961b) 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 (1961a).

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 (1961a).

The lability of newly isolated *Babesia* spp. reported by Irvin and Brocklesby (1969) and Freirichs, 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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REFERENCES


ISOULATION OF B. BIGEMIA


Isolamento, separação e conservação de babesia bigemina.


El aislamiento, separación y preservación de bheeia bigemina.

Súmario—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 frotis 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 después del cuarto pase. Una forma estable del aislamiento de B. bigemina ha sido obtenida.