PN AAZ 238
54458
3. F 33

FINAL REPORT

Grant No. DPE-5542-G-SS-4026-00

Proposal No. 310177-1

"NEW APPROACHES TO THE CONTROL OF BOVINE BABESIOSIS"

Gerald M. Buening

Department of Veterinary Microbiology

College of Veterinary Medicine

University of Missouri-Columbia

Rec'd in SCI: JAN 22 1983

TABLE OF CONTENTS

	Page
Summary	4
Objective 1 To establish the pathogenicity and genetic stability of clones *in vivo* (the animal)	5
Objective 2 To develop procedures for the preservation of attenuated *B. bovis* by lyophilization	5
Objective 3 To conduct field studies using avirulent clones of *B. bovis* as vaccines	6
Objective 4 To biochemically characterize clones of *Babesia bovis* as to enzymatic and antigenic composition	7
Objective 5 To establish continuous cultures of *B. bigemina in vitro*	8
Objective 6 To develop an ELISA test for *B. bigemina*	9
Objective 7 To isolate clones of *B. bigemina*	10
Objective 8 To establish the pathogenicity and genetic stability of *B. bigemina* clones	11
Objective 9 To conduct field studies using avirulent clones of *B. bovis* and *B. bigemina* as vaccines	12
Achievements	16
Attachments	attached envelope

SUMMARY

This research project resulted in the following achievements:

1. *In vitro* culture systems for *Babesia bovis* and *B. bigemina* were improved.
2. The above culture systems were used in cloning procedures.
3. Clones of *Babesia bovis* and *B. bigemina* were irradiated and evaluated as attenuated vaccine candidates
4. Methods were developed to concentrate *Babesia bovis* and *B. bigemina*-infected erythrocytes from *in vitro* cultures. These concentrated infected erythrocytes were used as immunogens to produce hybridomas and specific monoclonal antibodies were obtained.
5. Procedures were developed for obtaining *Babesia bovis* merozoites free of erythrocyte membranes for biochemical studie
6. Procedures were developed for obtaining *Babesia bigemina* merozoites free of erythrocyte membranes for biochemical studies.
7. Three Mexican national students were involved with the project and two students completed requirements for the Ph.D. degree during the grant period. One Mexican student will complete the requirements for the Ph.D. degree in 1988.
8. Four papers were published in refereed journals. One paper has been submitted and three more are being prepared for publication.
9. Eleven presentations were presented during the grant period and one presentation after the end of the grant.
10. Lyophilization procedures were developed to retain the viability of *Babesia bovis* and *B. bigemina*. However, these procedures need further refinement for consistent results

SPECIFIC OBJECTIVES:

1. To establish the pathogenicity and genetic stability of clones in vivo (the animal)

The following is an abstract from a publication which served as a basis for the present project. The complete publication is Attachment A of this document.

ABSTRACT

A Babesia bovis isolate was cloned by in vitro cultivation. The cloned organism was compared to the original cultured isolate for pathogenicity by animal inoculation. Four yearling heifers were inoculated with cloned material and four with the original culture. The four animals which received the cloned babesia showed comparatively minor hematologic changes and no clinical signs. One animal died in the group that received uncloned babesia and the mean temperature increase and mean reduction in packed cell volume (PCV) was greater in that group. The four animals receiving the cloned material were challenged 100 days following initial inoculation. All of the animals were totally immune with no significant change in temperature or decrease in PCV, whereas control (previously non-inoculated) animals developed significant (P .001) increases in temperature and severe anemia. The cloned organism appears to be a candidate live immunogen for use in endemic areas to induce protection against bovine babesiosis.

Subsequent back passage experiments indicated that the cloned organism was not genetically stable in some animals (unpublished data, Rodriguez, S.D. 1983. Babesia bovis: In vitro Cultivation and Cloning. M.S. Thesis, University of Missouri-Columbia).

It had been suggested that irradiated parasites be tested for avirulence and genetic stability. A number of clones of Babesia bovis have been isolated and several clones have been irradiated. An irradiated clone of Babesia bovis has been tested for pathogenicity by animal inoculation and genetic stability by back passage in animals. The irradiated clone induced a mild reaction and appeared to be less virulent on back passage compared to previously tested clones. One of these clones has been tested for pathogenicity by animal inoculation in a pilot study in Mexico. The clone induced a mild reaction following inoculation by either the subcutaneous, intramuscular or intravenous routes. Back passage of pooled blood (750 ml) from the initial animals into two susceptible animals produced moderate hematological changes but no CNS signs. An additional backpassage to two susceptible animals produced no hematological changes or clinical signs. This irradiated clone was used in field trial #1.

2. To develop procedures for the preservation of attenuated B. bovis by lyophilization.

Live attenuated B. bovis has a limited shelf life of one week at 4° C. The vaccine can be cryopreserved in liquid N_2, but in developing countries this would present distribution problems. Ideally the vaccine should be lyophilized.

The source of the organisms for lyophilization studies were derived from the in vitro culture systems. During the three years of the grant period, sixty different experiments were conducted. Both Babesia bovis and Babesia bigemina were

lyophilized and tested for viability to re-establish growth in vitro. The following is a summary of the procedure which gave the most consistent positive results. This procedure is a modification of the procedure reported for cryopreservation of Babesia bigemina (see Attachment B).

The cultures were initially frozen as reported in Attachment B. The cryoprotectant solution for Babesia bovis was a stock solution of 20% PVP 40 in Puck's saline G containing 20.5 g/L of glucose (PSG+). A similar solution of 20% PVP in Vega y Martinez (VYM) was used for Babesia bigemina. This solution was made according to Vega et al.; VYM solution was composed of: $CaCl_2 \cdot 2\ H_2O$ (16.0 mg), KCl (400.0 mg), KH_2PO_4 (1,415.4 mg), $MgSO_4 \cdot 7\ H_2O$ (154 mg), $Na_2HPO_4 \cdot 7\ H_2O$ (1,450.0 mg), NaCl (7,077.0 mg), and dextrose 20.5 g) in 1 L of double-distilled deionized water containing 0.25 mM adenine and 0.50 mM guanosine. Packed iBRBC or merozoites were mixed with an equal volume of 20% PVP and dispensed in 1- or 2-ml cryotubes. The PVP concentration of the final mixture was 10%. The contents of the vials were frozen at the rate of 20 C/minute in a programmable freezing unit. When the temperature was -80$_o$ C the vials were transferred to a lyophilization chamber.

The lyophilization chamber had been precooled to -50° C. The frozen cultures were placed in the chamber and the vacuum turned on. Cultures were left to stabilize at -50° C for 1 hour. Then the temperature was raised 10° per hour until the chamber temperature was at +10° C. The cultures were then lyophilized over night. Several different types of vials (glass or microfuge) were used. The lyophilized cultures were placed in a refrigerator for at least 1 week at 4° C.

Sample vials were removed from the refrigerator and immediately diluted in distilled water or PSG+ or VYM or complete media. The preparation was centrifuged (4° C) at 1,000 x g for 10 minutes. The resulting pellets were resuspended in fresh culture media containing 10% normal bRBC. The amount of media varied according to whether flasks (25 cm^2) or 24-well plates (16 mm inside diameter) were used to re-establish the cultures. The incubating culture depth and atmosphere varied according to the organism tested. Fresh BRBC were added to the cultures on day 3 after retrieval. Cultures were split 1:1 in complete media containing 10% normal BRBC at three day intervals. Slides were made on days 10, 18, 20, and 28. The criteria for re-establishment of growth was that the cultures could be split twice and greater than 1% parasitemias were observed.

In seven instances Babesia bigemina and Babesia bovis have been re-established in culture. Replicate vials were stored at 4° C and placed in culture at a later date. Results have been encouraging but not always consistent. We still need to define the protocol. At this time optimum conditions for the lyophilization of Babesia bovis and B. bigemina have not been standardized.

3. To conduct field studies using avirulent clones of B. bovis as vaccines.

Initial contacts were made with the veterinary scientists at INIP in Mexico City. Details of the field trials were worked out after an avirulent clone had been confirmed to be genetically stable (see Objective 1). Animals were selected for field trial #1 and conditioned before inoculation. All animals were acquired from a tick-free area (State of Sonora), preferably of the same age (18 months or older). They were tested and selected when shown to be negative for the presence of specific antibodies against Anaplasma marginale, Babesia bovis and Babesia bigemina. The animals were preimmunized against Babesia bigemina and Anaplasma marginale.

Animals were randomly distributed into 4 groups. Three groups were preimmunized against Babesia bigemina and Anaplasma marginale.

I. Control non Babesia bovis-vaccinated (8 animals)
II. Control preimmunized and then vaccinated with 10^8 B. bovis original wild type cultured strain (8 animals)
III. Preimmunized and vaccinated with 10^8 irradiated B. bovis cloned strain (8 animals)
IV. Non-preimmunized and vaccinated with 10^8 irradiated B. bovis cloned strain (8 animals)

The experiment was divided into three phases: Phase 1 was from 0-63 days and consisted of adaptation and preimmunization with Anaplasma marginale and Babesia bigemina. Phase 2 had an observation period of 42 days. During this time the animals were preimmunized/vaccinated with either the wild type or irradiated B. bovis cultures. Animals that had severe reactions were treated. This phase extended until 168 days. Phase 3 consisted of challenging the animals either by infected ticks or needle challenge. Each group was subdivided into two to four animals. One group was challenged by exposure to 2,000 Babesia bovis infected larvae Boophilus ticks. The other group was needle challenged with 10^9 Babesia bovis infected erythrocytes. Following challenge the animals were observed for 42 days.

While in tick-infested area animals were dipped in acaricide bath every 4-7 days. Body temperature data, blood samples (for packed cell volume measurements and blood smears), and serum samples (for serological tests) will be collected throughout the observation periods of the three phases twice a week.

The results of the field trial were as follows: a) Pathogenicity of the irradiated cloned organism: The groups of animals that were vaccinated with an irradiated clone of Babesia bovis experienced no or very mild clinical reactions and did not require treatment. A number of animals in the group preimmunized with the wild type Babesia bovis required treatment. b) Challenge studies: The challenge strain was not as virulent as we had anticipated. The control animals did not experience a severe reaction. Therefore, differences between groups were not as dramatic as we had hoped. The data indicated that the animals vaccinated with the irradiated clone experienced fewer clinical reactions and experienced less weight loss after challenge. One problem encountered during the field trial was that some of the animals preimmunized with Anaplasma marginale experienced recrudescence. This placed additional environmental stress on the trial. Some of these animals died of Anaplasma marginale infection. Our conclusions from the trial were as follows: a) the irradiated strain is safe (nonpathogenic); and b) the irradiated strain will provide at least some protective immunity in endemic areas.

4. To biochemically characterize clones of Babesia bovis as to enzymatic and antigenic composition.

In order to study the biochemical and antigenic composition of Babesia bovis it was necessary to develop procedures to concentrate merozoites and infected bovine erythrocytes. The following is the abstract from a publication which presents the details (see Attachment C).

ABSTRACT

Babesia bovis merozoites, externalized by removal of infected erythrocytes from ordinary culture conditions, were completely separated from red blood cells and stroma by centrifugation in a Percoll gradient. A merozoite band formed at a point corresponding to about 1.087 g/ml specific density. Infected red blood cells were concentrated approximately four-fold to obtain greater than 49.0% parasitemia after centrifugation in Percoll. Most highly enriched fractions positioned between 1.121 and 1.123 g/ml specific density. Full parasite viability was retained.

The procedures for the characterization of isoenzymes in B. bovis infected cells and free parasites were developed. The following is an abstract from a publication which presents the details (Attachment D).

ABSTRACT

Agarose gel electrophoresis was used to identify metabolic enzymes in Babesia bovis and B. bigemina. Glutamate dehydrogenase, lactate dehydrogenase, glucose phosphate isomerase and hexokinase were identified in B. bovis and B. bigemina infected erythrocytes and B. bovis merozoite preparations. Specific electrophoretic mobility was observed for each enzyme. Malate dehydrogenase, glucose 6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase and adenylate kinase were only detected in normal erythrocyte preparations. Inter-species, but not intra-species, variation was noted when comparing electrophoretograms of both species. Kinin-activating activity was not detected in B. bovis-infected erythrocyte or merozoite preparations at pH 4.2 or 7.6.

The isoenzyme patterns of all non-irradiated clones appeared very similar. Results indicate that the hexokinase isoenzyme pattern of two-cobalt irradiated clones are different than non-irradiated clones (unpublished, Rodriguez-Camarillo, S.D. 1985. Babesia bovis Clones: Biochemical Characterization. Ph.D. Dissertation, University of Missouri-Columbia).

Molecular analysis by one and two-dimensional gel electrophoresis of proteins metabolically labeled with ^{35}S-methionine showed that 2 clones derived from the same field strain were similar but not identical to the parent (unpublished, Rodriguez-Camarillo, S.D. 1985. Babesia bovis Clones: Biochemical Characterization. Ph.D. Dissertation, University of Missouri-Columbia).

A number of hybridomas producing specific monoclonal antibodies to Babesia bovis have been obtained. The hybridoma cell lines have been frozen for later characterization.

5. To establish continuous cultures of B. bigemina in vitro.

The procedures for the continuous cultivation of B. bigemina had been developed in our laboratory prior to receiving funding. An abstract of the publication describing this technique follows (see Attachment E).

ABSTRACT

A strain of Babesia bigemina was isolated from an infected calf and propagated in vitro. Culture conditions included washing of infected and normal bovine erythrocytes in a special solution (VYMs), and the use of a 5-10% (v/v)

erythrocyte suspension in medium 199 (with 20-50% fresh normal bovine serum) at a depth of 4 mm in a 5% CO_2, 2% O_2, 93% N_2 atmosphere. The cultured organism, after 36 days in vitro and nine subcultures, was inoculated into a susceptible calf. This calf developed clinical signs of the disease and recovered when treated with 1% trypan blue solution. The strain was also reisolated from the second calf. The original isolate had been maintained in continuous in vitro cultivation for more than 99 days.

We have used this technique to establish cultures from at least six different isolates. The present protocol is as follows: Infected blood is washed in VYMs solution and then resuspended in complete media consisting of 40% normal bovine serum, 60% medium 199 (no L-glutamine). The packed cell volume is adjusted to 10% and the culture vessel is placed in an atmosphere of 5% CO_2, 5% O_2 and 90% N_2. An additional very important factor for successful in vitro cultivation of Babesia bovis and B. bigemina is the source of normal bovine serum and normal bovine erythrocytes. We routinely screen donor animals and select only animals whose serum and cells support satisfactory growth.

6. To develop an ELISA test for B. bigemina.

Preliminary attempts to utilize antigen from in vitro culture in the ELISA test have been done. However, the results were disappointing due to nonspecific reactions. Procedures were developed to concentrate Babesia bigemina infected erythrocytes. The details of the procedures are described in the publication. The following is an abstract from that publication (see Attachment F).

ABSTRACT

Clones of Babesia bigemina-infected erythrocytes were concentrated by several density gradient procedures. The density range of infected erythrocytes containing pairs of parasites was 1.077 to 1.089 g/ml, whereas, the density range of infected erythrocytes containing single parasite was 1.092 to 1.100. Three enzymes, lactate dehydrogenase, glucose-phosphate isomerase and glutamate dehydrogenase, were found associated with infected erythrocytes. The parasite specific enzyme and/or isoenzymes were shown to have different mobility patterns in starch gel electrophoresis from those found in the normal bovine erythrocytes. The enzyme 6-phosphogluconate dehydrogenase was not detected as a parasite specific enzyme in B. bigemina infected erythrocytes.

Procedures to isolate Babesia bigemina merozoites from erythrocyte membranes are being developed. Initially lysing procedures resulted in preparations contaminated with erythrocyte membranes. We have now developed a glycerol lysing procedure and purification methods to obtain viable merozoites free of erythrocyte membranes (Figueroa et al. Purification of the Erythrocytic Stage of Babesia bigemina. Paper in preparation). This will probably be one source of antigen for an ELISA test.

Another approach to obtaining purified B. bigemina antigen for an ELISA test was to obtain monoclonal antibodies and use them in immunosorbent chromatography. A number of hybridomas producing specific monoclonal antibodies have been obtained. These hybridomas have been characterized and the data will be the subject of a paper in preparation for submission. The following is an abstract from that paper.

ABSTRACT

Twenty one mouse hybridomas were isolated that produce monoclonal antibodies (MoAb) which specifically reacted with erythrocytic stages of Babesia bigemina in an indirect immunofluorescence assay on acetone-fixed blood films. Four groups of antibodies were defined on the basis of their staining pattern in IFAT. Four out of eight selected MoAb tested on in vitro cultured B. bigemina were found to have inhibitory activity as analyzed by estimating the percentage of parasitized erythrocytes and incorporation of ^{3}H-hypoxanthine. The four inhibitory MoAb immunoprecipitated ^{35}S-methionine metabolically-labeled parasite proteins. One MoAb from Group II detected a 24 nD protein, whereas another MoAb from the same group precipitated proteins with an approximate weight of 55 kD. MoAb from Group III bound to polypeptides which migrated as 55 kD and 57 kD, these same polypeptides were precipitated by a MoAb from Group IV which also detected protein of 48, 51 and 53 kD.

Another approach that we have investigated is the adaptation of B. bigemina for in vitro cultivation using horse serum instead of cow serum. This adaptation should eliminate nonspecific problems associated with bovine serum. These cultures have been cryopreserved and could be retrieved for antigen production.

7. To isolate clones of B. bigemina.

We now have a total of 6 different isolates of B. bigemina in culture. Some of the isolates have been cloned. Two tertiary clones have been isolated and irradiated and recloned. The cloning procedure is fully described in a publication. The following is the abstract of that publication (see Attachment G).

ABSTRACT

The original Babesia bigemina culture conditions were modified with regard to infected bovine erythrocyte concentration and atmospheric environment. A procedure was designed to obtain a homogeneous parasite population by beginning with a single infected erythrocyte. Calculated dilutions were made in 96-well tissue culture plates to approach one infected erythrocyte per four wells. Growth of parasites in wells was detected between 16 to 28 days after cultures were initiated. Clones were transferred to 24-well tissue culture plates for regular maintenance. Three primary clones were selected for additional recloning. The probability that the parasites detected in one well are the progeny of a single infected erythrocyte approaches 0.99 for tertiary clones.

These clones were biochemically and antigenically characterized. A paper has been submitted describing the procedures and results of these studies. The following is an abstract from that paper (Vega et al. 1988. Incorporation of ^{35}S-methionine by Babesia bigemina Cultivated in vitro. Submitted to Revista Mexicana de Parasitologia).

ABSTRACT

A Babesia bigemina isolate and several in vitro derived clones were metabolically labeled using ^{35}S-methionine. Labeled proteins were separated by SDS-PAGE and clones compared to the original population. Twenty three bands of parasite origin were clearly defined. Six bands of 39,000, 52,500, 79,000, 88,000, 91,000

and 130,000 M.W. showed higher intensity in the film. One polypeptide of 43,000 M.W. was detected in samples from culture supernates. Separation patterns were similar in all samples studied. It was concluded that this similarity might be related to the fact that all clones were derived from the same strain. More studies are required to demonstrate true subpopulation variations in Babesia bigemina.

In addition, monoclonal antibodies are being utilized to study the antigenic composition of the clones and isolates.

8. To establish the pathogenicity and genetic stability of B. bigemina clones.

Several irradiated clones of Babesia bigemina have been obtained. An experiment involving the inoculation of one splenectomized calf at the University of Missouri was completed. Preliminary results indicate that irradiation may attenuate the organism.

Arrangements were made to conduct a study initially at INIP in Mexico City. However, the Department relocated to a research site in Cuernavaca. Because of time constraints, it was decided to investigate pathogenicity, genetic stability and the protective immunity in one experiment. The experiment was designed to investigate the following parameters:

1. The effect of dosage of inoculum (number of Babesia bigemina-infected erythrocytes).

2. The pathogenicity of the inoculum.

3. Tick transmissibility of the various inoculum.

4. Genetic stability of the inoculums.

5. Ability of inoculums to induce protective immunity.

The project is now in progress at Cuernavaca with irradiated clones of B. bigemina. Phase 1 of the trial consists of 20 susceptible animals being divided into 5 groups and each group subdivided into two groups receiving one of the following inoculum.

Group 1: Inoculated intramuscularly with normal red blood cells (subgroup 1 - 1 x 10^8; and subgroup 2 - 1 x 10^9).

Group 2: Inoculated intramuscularly with in vitro culture of Babesia bigemina (subgroup 1 - 1 x 10^8; and subgroup 2 - 1 x 10^9).

Group 3: Inoculated intramuscularly with a virulent field strain of Babesia bigemina (subgroup 1 - 1 x 10^8; and subgroup 2 - 1 x 10^9).

Group 4: Inoculated intramuscularly with in vitro culture of irradiated clone #1 of Babesia bigemina (subgroup 1 - 1 x 10^8; and subgroup 2 - 1 x 10^9).

Group 5: Inoculated intramuscularly with in vitro culture of irradiated clone #2 of Babesia bigemina (subgroup 1 - 1 x 10^8; and subgroup 2 - 1 x 10^9).

Experimental Design: The experimental design for Phase 1 is summarized on the attached Table 1 and Table 2. Phase 2 will consist of evaluation of pathogenicity by back passage of blood to susceptible animals. Ten animals were used in this part of the experiment. Each animal was inoculated with 250 ml of pooled blood from each subgroup. Phase 3 will consist of ten bovine to evaluate whether ticks can transmit the disease. The progeny of the ticks collected from Part 1 will be placed on susceptible animals.

RESULTS

Phase 1: Evaluation of initial pathogenicity: Blood samples were collected and clinical observations made as indicated in Table 2. Group 1 (normal bovine erythrocytes) animals had no clinical reactions; Group 2 (in vitro B. bigemina) animals had a mild clinical reaction. Group 3 (field strain B. bigemina) animals developed severe reactions and one animal died. Group 4 (in vitro B. bigemina irradiated #1) animals had a mild clinical reaction. Group 5 (in vitro B. bigemina irradiated #2) animals had a severe reaction (no deaths). Larvae ticks were allowed to feed on certain animals. These ticks were allowed to drop off then collected and maintained in the laboratory. The progeny of these ticks will be placed on susceptible animals (Phase 3). Blood was collected for back passage into susceptible animals on day 35. The hemolymphs from ticks which fed on the field strain animals contained B. bigemina. No infection was detected in the ticks that fed on the animals receiving in vitro cultures. These animals will be challenged as soon as a sufficient quantity of infected ticks can be obtained.

Phase 2: Evaluation of genetic stability by back passage: A similar protocol for evaluation of pathogenicity as used in Phase 1. Group 1 (normal bovine erythrocytes) animals had no clinical reactions; Group 2 (in vitro B. bigemina) animals had a mild clinical reaction. Group 3 (field strain B. bigemina) animals developed severe reactions. Group 4 (in vitro B. bigemina irradiated #1) animals had a mild clinical reaction. Group 5 (in vitro B. bigemina irradiated #2) animals had a severe reaction. These results would indicate that irradiated clone #1 was genetically stable and relatively nonpathogenic.

Phase 3: Presently in progress.

9. To conduct field studies using avirulent clones of B. bovis and B. bigemina as vaccines.

Due to the time constraints of the project, it was decided to plan a more complete experiment as described under Objective 8. Therefore the cattle which were to be purchased for this objective were reassigned under Objective 8. Veterinary scientists at the University of Missouri and INIP have discussed field trial #2 and it is planned that Mexican scientists will pursue the second field trial at their own expense.

(TABLE 1)

OBJECTIVE 8 - SCHEDULE OF ACTIVITIES

PHASE 1 (20 animals)	DAYS
Temperature	-7, -4, -, 3, 7, 10, 14, 17, 21
Slides	-7, -4, -, 3, 7, 10, 14, 17, 21
Hematocrit	-7, -4, -, 3, 7, 10, 14, 17, 21
CBC	-7, -4, 0, 3, 7, 10, 14, 17, 21
Serum	-7, 0, 7, 14, 21, 28. 35, 42, 49
Infestation with non-infected B. microplus	0, 2, 4, 6, 8
In vitro cultivation of B. bigemina	-7, -4, 0
Inoculation with infected blood	14
Collection of ticks	21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35
Hemolymph from ticks (check for infection)	22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36
Collection of heparinized blood	35

PHASE 2 (10 animals) BACK PASSAGE EXPERIMENT	DAYS
Temperature	28, 31, 35, 38, 42, 45, 49, 52, 56, 59, 63, 66
Slides	28, 31, 35, 38, 42, 45, 49, 52, 56, 59, 63, 66
Hematocrit	28, 31, 35, 38, 42, 45, 49, 52, 56, 59, 63, 66
CBC	28, 31, 35, 38, 42, 45, 49, 52, 56, 59, 63, 66
Serum	28, 35, 42, 49. 56, 63, 70
Inoculation of animals with heparinized blood	35
Collection of blood for cultivation	60

(TABLE 1)

OBJECTIVE 8 - SCHEDULE OF ACTIVITIES (cont'd)

PHASE 3 (10 animals) TICK TRANSMISSIBILITY	DAYS
Temperature	91, 94, 98, 101, 105, 108, 112, 115, 119, 122, 126, 129
Slides	91, 94, 98, 101, 105, 108, 112, 115, 119, 122, 126, 129
Hematocrit	91, 94, 98, 101, 105, 108, 112, 115, 119, 122, 126, 129
CBC	91, 94, 98, 101, 105, 108, 112, 115, 119, 122, 126, 129
Serum	91, 98, 105, 112, 119, 126, 133
Infestation with infected B. microplus	98
Collection of heparinized blood	140

(TABLE 2)

GROUPS / ANIMALS	SUBGROUPS / ANIMALS	INFESTATION DAYS 0, 2, 4, 6 and 8	BLOOD INOCULUM	DOSAGE / ROUTE	DISPOSITION OF TICKS	EFFECT ON FEMALE TICKS	HEPARINIZED BLOOD
GROUP I	A/2	Infestation with 5,000 larvae of *Boophilus microplus* (non-infected), 10 animals (one from each group)	Normal RBC	$1x10^8$/IM	Collection of ticks after natural detachment	Hemolymphs, weight, minimum time for laying eggs, weight of eggs, percent of hatching larvae, estimated production of larvae	Collection of heparinized blood for *in vitro* culture and back passage to other animals
4	B/2			$1x10^9$/IM			
GROUP II	A/2		Culture Strain *B. bigemina*	$1x10^8$/IM			
4	B/2			$1x10^9$/IM			
GROUP III	A/2		Field Strain *B. bigemina*	$1x10^8$/IM			
4	B/2			$1x10^9$/IM			
GROUP IV	A/2		Cultured *B. bigemina* Irradiated #1	$1x10^8$/IM			
4	B/2			$1x10^9$/IM			
GROUP V	A/2		Cultured *B. bigemina* Irradiated #2	$1x10^8$/IM			
4	B/2			$1x10^9$/IM			

ACHIEVEMENTS

PUBLICATIONS:

Publications in refereed journals:

Rodriguez, S.D., G.M. Buening, C.A. Vega and C.A. Carson. 1986. Babesia bovis: Purification and Concentration of Merozoites and Infected Bovine Erythrocytes. Experimental Parasitology 61:236-243.

Vega, C.A., G.M. Buening, S.D. Rodriguez and C.A. Carson. 1986. Cloning of in vitro propagated Babesia bigemina. Veterinary Parasitology 22:223-233.

Rodriguez, S.D., G.M. Buening and C.A. Carson. 1986. Enzymatic Characterization of Babesia bovis. The Journal of Protozoology 33:507-511.

Vega, C.A., G.M. Buening, S.D. Rodriguez and C.A. Carson. 1986. Concentration and enzyme content of in vitro cultured Babesia bigemina Infected Erythrocytes. The Journal of Protozoology 33:514-518.

Publications submitted:

Vega, C.A., G.M. Buening, K. McLaughlin, G.D. Grothaus and C.A. Carson. 1988. Incorporation of ^{35}S-methionine by Babesia bigemina Cultivated in vitro. Submitted to Revista Mexicana de Parasitologia).

Publications in preparation:

Figueroa, J.V., G.M. Buening and T.J. Green. Identification of Common Antigens by Monoclonal Antibodies in Six Isolates of Babesia bovis.

Figueroa. J.V., and G.M. Buening. Inhibition of In vitro Multiplication of Babesia bigemina by Monoclonal Antibodies.

Figueroa, J.V., G.M. Buening and D.A. Kinden. Purification of the Erythrocytic Stage of Babesia bigemina.

Dissertations Completed

S.D. Rodriguez-Camarillo. 1985. Babesia bovis Clones: Biochemical Characterization. Ph.D. Dissertation, University of Missouri-Columbia.

C.A. Vega. 1985. Babesia bigemina: In vitro Cultivation. Ph.D. Dissertation, University of Missouri-Columbia.

Dissertation in Progress:

J.V. Figueroa. Immunological Characterization of Surface Antigens in the Erythrocytic Stage of Babesia bigemina. Ph.D. dissertation in progress.

PRESENTATIONS:

Rodriguez D., G.M. Buening, C.A. Vega and C.A. Carson. Babesia bovis: Separation and concentration of merozoites from in vitro cultures by Percoll gradients. Conf. of Res. Workers in Animal Diseases, Chicago IL. November 12, 1984.

Buening, G.M., C.A. Vega and C.A. Carson. Separation of Babesia bigemina infected erythrocytes. Anaplasma Research Workers Meeting, Pullman WA. September 16, 1985.

Figueroa, J.V. and G.M. Buening. Production of Monoclonal Antibodies Against Babesia bigemina. Anaplasma Research Workers Meeting, Pullman WA. September 17, 1985.

Figueroa, J.V. and G.M. Buening. Production of Monoclonal Antibodies Against Babesia bigemina. Conf. of Res. Workers in Animal Disease, Chicago IL. November 11, 1985.

Buening, G.M. Hemoparasite Research at the University of Missouri. USDA Planning Workshop, Pullman WA. December 4, 1985.

Figueroa, J.V., G.M. Buening and T.J. Green. Antigen Characterization and In vitro Growth Inhibition of Babesia bigemina by Monoclonal Antibodies. Anaplasma Research Workers Meeting, Baton Rouge LA. September 21, 1986.

Figueroa, J.V., G.M. Buening and T.J. Green. Production of Monoclonal Antibodies Against Babesia bigemina. 7th National Congress of Parasitology, Puebla, Mexico. October 16-18, 1986.

Figueroa, J.V., G.M. Buening and T.J. Green. Antigen Characterization and In vitro Growth Inhibition of Babesia bigemina by Monoclonal Antibodies. Conf. of Res. Workers in Animal Diseases Chicago IL. November 17, 1986.

Buening, G.M., P. Myler, A. Barbet and T. McGuire. Development, Specificity and Sensitivity of a DNA Probe to Detect Babesia bigemina Infected Erythrocytes. Conf. of Res. Workers in Animal Diseases, Chicago IL. November 17, 1986.

Buening, G.M. Characterization of a DNA Probe to Detect Babesia bigemina Infection. Seminar at the University of Florida, Department of Infectious Diseases. January 24, 1987.

Figueroa, J.V., G.M. Buening and T.J. Green. Antigens of the Protozoan Parasite Babesia bigemina Detected by Monoclonal Antibodies. American Society for Microbiology, Missouri Branch meeting Osage Beach MO. April 11, 1987.

Presentations Related to the Project After the Closing Date:

J.V. Figueroa. Purification of Babesia bigemina (Erythrocytic-Stage). Veterinary Hemoparasite Research Workers, Los Angeles, CA, December 4-5, 1987.

Graduate students (Mexican nationals) completing a dissertation during this reporting period:

Carlos A. Vega. Babesia bigemina: In vitro Cultivation and Characterization. PhD dissertation, May 1985, University of Missouri, Columbia, MO.

Sergio D. Rogriguez: Babesia bovis clones: Biochemical and Enzymatic Characterization. PhD dissertation, December 1985. University of Missouri. Columbia, MO.

Graduate student (Mexican national) dissertation to be completed December 1988

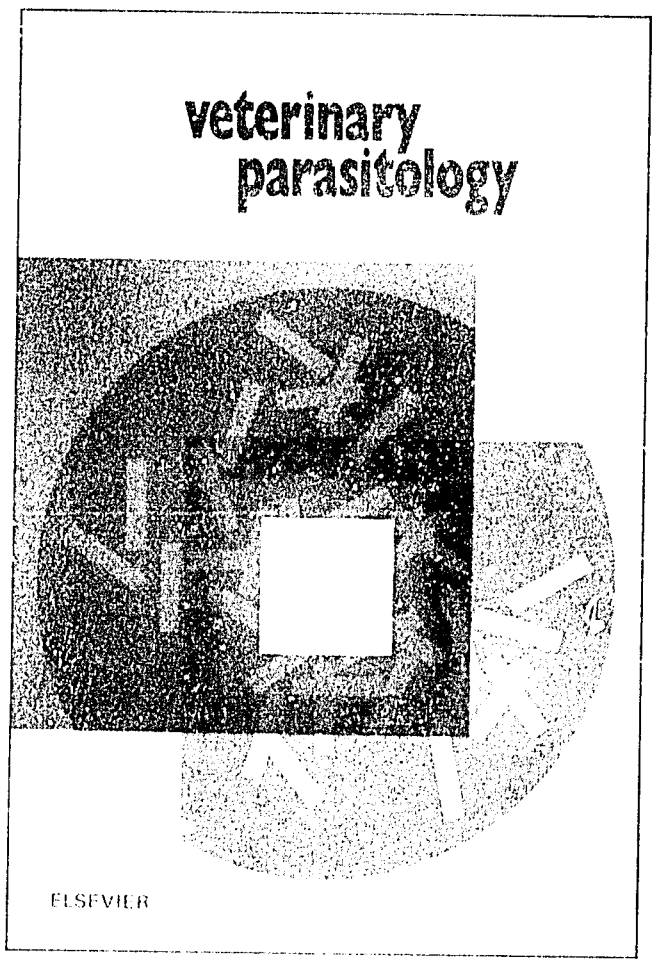
Julio V. Figueroa. Immunological Charcterization of Surface Antigens in the Erythrocytic Stage of Babesia bigemina.

Training of graduate student from other institution:

Steve Hines of the University of Florida at Gainesville spent one week in our laboratory learning cultivation techniques for Babesia bigemina and B. bovis.

ATTACHMENT A

REPRINTED FROM:



ELSEVIER SCIENCE PUBLISHERS, AMSTERDAM

veterinary parasitology

An International Scientific Journal

Editor-in-Chief:
S.M. Gaafar, Department of Veterinary Microbiology, Purdue University, West Lafayette, IN 47907, U.S.A.

Aims and scope.
This journal is concerned with those aspects of helminthology, protozoology and entomology which are of interest to animal health investigators, veterinary practitioners and others with a special interest in parasitology. Papers of the highest quality dealing with all aspects of disease prevention, pathology, treatment, epidemiology and control of parasites in all animals which can be regarded as being useful to man, fall within the scope of the journal. It is intended to give special attention to new aspects of veterinary parasitology, such as problems arising from the intensification of industrialized production systems and mass production, problems of game animals which may be of epizootiological significance in domestic animals, etc. Parasitological studies on laboratory animals fall within the scope of the journal only if they provide a reasonably close model of a disease of domestic animals. Although the journal primarily publishes experimental and clinical research papers, occasionally unique case histories may also be published. Papers on the taxonomy of parasites do not fall within the scope of the journal.

Submission of articles.
Manuscripts should be submitted in triplicate to the Editorial Secretariat, Veterinary Parasitology, P.O. Box 330, 1000 AH Amsterdam, The Netherlands. Authors in N. America should submit their manuscripts directly to Professor Gaafar.

VETERINARY PARASITOLOGY HAS NO PAGE CHARGES
A DETAILED GUIDE TO AUTHORS AND A FREE SAMPLE COPY OF THE JOURNAL ARE AVAILABLE ON REQUEST

Veterinary Parasitology, 22 (1986) 235—242
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

EVALUATION OF A CLONED *BABESIA BOVIS* ORGANISM AS A LIVE IMMUNOGEN

G.M. BUENING[1], K.L. KUTTLER[2] and S.D. RODRIGUEZ[1]

[1] *Department of Veterinary Microbiology, College of Veterinary Medicine, University of Missouri, Columbia, MO 65211 (U.S.A.)*
[2] *U.S. Department of Agriculture, Agricultural Research Service, Hemoparasitic Disease Research Unit, Washington State University, Pullman, WA 99164 (U.S.A.)*

(Accepted for publication 21 November 1985)

ABSTRACT

Buening, G.M., Kuttler, K.L. and Rodriguez, S.D., 1986. Evaluation of a cloned *Babesia bovis* organism as a live immunogen. *Vet. Parasitol.*, 22: 235—242.

A *Babesia bovis* isolate was cloned by in vitro cultivation and compared to the original cultured isolate for pathogenicity by animal inoculation. Four yearling heifers were inoculated with cloned material and 4 with the original culture. The four animals which received the cloned *Babesia* showed comparatively minor hematologic changes and no clinical signs. One animal died in the group that received uncloned *Babesia* and the mean temperature increase and mean reduction in packed cell volume (PCV) was greater in that group. The four animals receiving the cloned material were challenged 100 days following initial inoculation. All of the animals were totally immune with no significant change in temperature or decrease in PCV, whereas control (previously non-inoculated) animals developed significant ($P < 0.001$) increases in temperature and severe anemia. The cloned organism appears to be a candidate live immunogen for use in endemic areas to induce protection against bovine babesiosis.

INTRODUCTION

Major advances in babesiosis research occurred with the development of culture methods for continuous in vitro cultivation of *Babesia bovis*. The early work of Erp (Erp et al., 1980) was modified (Levy and Ristic, 1980) to a microaerophilous stationary phase culture system (MASP). Additional refinements were made by Palmer et al., 1981. Rodriguez et al. (1983) reported a modification of the latter system making it possible to isolate single *B. bovis* parasites in a cloning process, thereby establishing homogeneous parasite populations. The objective of the present study was to compare the pathogenicity and immunogenicity of a fast growing clone to the original culture material.

0304-4017/86/$03.50 © 1986 Elsevier Science Publishers B.V.

MATERIALS AND METHODS

Source of B. bovis *inoculum*

The original culture material was derived from a strain of *B. bovis* established in culture using the technique of Palmer et al. and maintained continuously in vitro for over 2 years. The fast growing clone was obtained by the procedure of Rodriguez et al. (1983) and recloned twice prior to animal inoculation.

Challenge inoculum was collected from a splenectomized calf showing an ascending parasitemia. This calf had been infected by needle inoculation with a frozen, tick-transmitted *B. bovis* stabilate originally isolated in Mexico.

Experimental animals

Eight yearling beef heifers of mixed breeding were used in the experiment. Four animals received the original culture material (heterologous prior to cloning) and four received the cloned material. Cultures were sent from Columbia, MO to Pullman, WA and arrived within 36 h. Each calf was inoculated intravenously with 1×10^9 *B. bovis* organisms, determined by counting the number of infected erythrocytes in the culture material.

Five yearling Holstein steers were used as non-vaccinated controls in the challenge study. These animals and the seven remaining animals previously inoculated with the culture material were challenged intravenously with 1×10^8 *B. bovis* organisms, known to be virulent from previous work.

Serological test

Sera obtained before and after vaccinal and challenge inoculation were tested with the complement fixation (CF) (Mahoney, 1962, 1967) and indirect fluorescent antibody (IFA) tests, using *B. bovis* antigens (Johnson et al., 1973).

Evaluation of pathogenicity and immunogenicity

Blood samples were obtained from all animals prior to inoculation or challenge. In the culture pathogenicity study, blood for packed cell volume (PCV) determinations was collected daily, starting 7 days postinoculation (PI), for 7 days and then twice weekly. In the challenge study blood for PCV determination was collected on Day 1, daily from Day 5 through Day 15 and twice weekly thereafter. Giemsa-stained blood smears were examined to determine parasitemia. Rectal temperatures were recorded before inoculation and challenge, then daily for the first 15 days and then twice weekly.

22

TABLE I

Response of a 2-year-old heifer to infection produced by cloned and non-cloned *B. bovis* cultures

	Pre-infection PCV (%)	Low PCV (%)	Incubation time		Days of parasitemia		Average high CF Titer		Reduction in PCV		Deaths	Group average relative response[c]
			Days	Relative response[a]	Av. No. of days	Relative response	Titer	Relative response	(%)	Relative response		
Culture (n = 4)	42.0 ± 2	19.0 ± 6	3.2 ± 0.5	0.896 (1.12)[b]	3.5 ± 1.7	1.27	1:113	1.015	54 ± 15.0	1.13	1/4	1.13
Clone (n = 4)	40.0 ± 2	24.0 ± 1	4.0 ± 0.8	1.103 (0.91)[b]	2.0 ± 1.0	0.73	1:95	0.978	42 ± 1.0	0.87	0/4	0.87
Significance	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS		$P < 0.05$

*Incubation time: days following exposure required for a sustained temperature of over 40°C to occur after exposure.

[a] Relative response: based on the average of all values being equal to 1.0. Each individual value is then divided by the group average to identify the "relative response" to infection.

[b] Values in parentheses reflect the reciprocals of the relative response, and were used in calculating the "relative response" for each group.

[c] Group-relative response: calculated on 4 parameters: incubation time, days of parasitemia, CF response, and percent reduction in PCV associated with infection. Mortality is not considered.

Presentation of data and analysis

For comparative purposes means and standard deviations were calculated for each data point for all animals of a group. Data was analyzed by F test and by analysis of variances of repeated observations (Gill and Hafs, 1971). In addition, the % change in PCV and rectal temperature based upon pre-inoculation values were calculated. Group-Relative Response was calculated as illustrated in Table I.

RESULTS

All eight cattle inoculated with either the culture or cloned material showed evidence of a *B. bovis* infection as evidenced by an increase in temperature, *B. bovis* parasitemia and by a reduction in PCV (Figs. 1 and 2). One animal receiving the culture material died on Day 18, following the

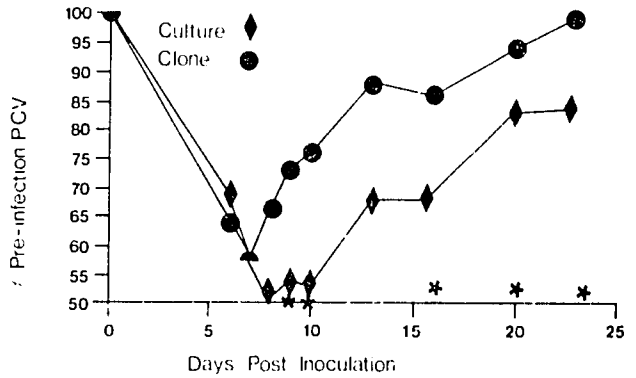


Fig. 1. The percent changes in mean preinfection packed cell volume (PCV) in yearlings inoculated with the original culture (n = 4) and a fast growing clone (n = 4) of *Babesia bovis*. * Point of significant difference $P < 0.05$.

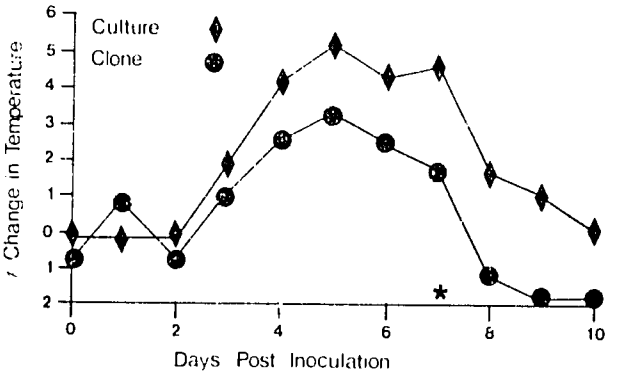


Fig. 2. The percent changes in mean preinfection rectal temperature in yearlings inoculated with the original culture (n = 4) and a fast growing clone (n = 4) of *Babesia bovis*. * Point of significant difference $P < 0.01$.

24

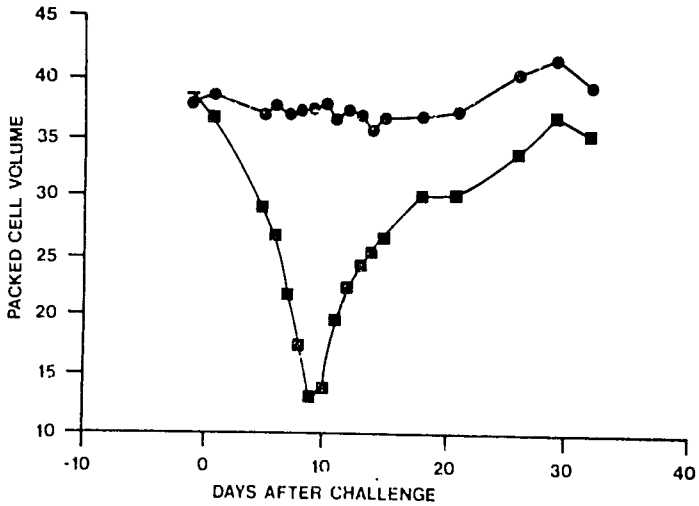


Fig. 3. The changes in mean packed cell volume in control yearlings (■) (n = 5) and clone vaccinated yearlings (●) (n = 4) following challenge with 1 × 10^8 of virulent *Babesia bovis* organisms intravenously.

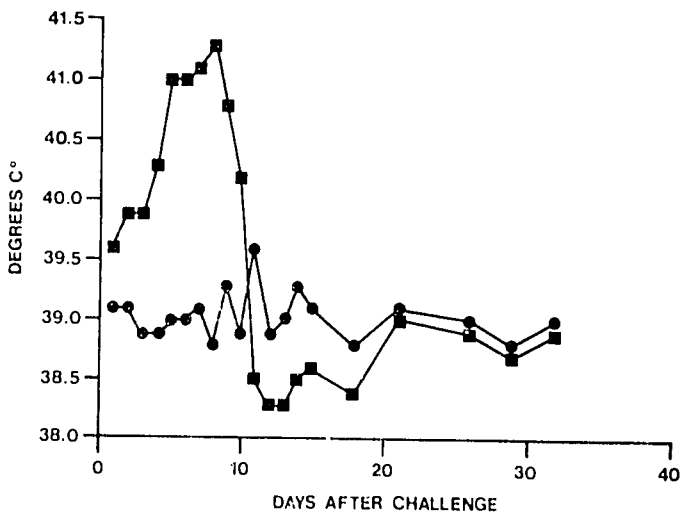


Fig. 4. The changes in mean rectal temperature in control yearlings (■) (n = 5) and clone vaccinated yearlings (●) (n = 4) following challenge with 1 × 10^8 virulent *Babesia bovis* organisms intravenously.

onset of fever, *B. bovis* parasitemia and CNS signs typical of *B. bovis*. Parasitemias were too low to quantitate, but were observed less frequently in cattle receiving the cloned organism. The temperature response and reduction in PCV were less severe in cattle receiving the cloned organism (Figs. 1 and 2).

Each of the parameters measured such as incubation period, days of parasitemia, CF response, and reduction in PCV showed evidence that the

cloned organism was less virulent. This was verified statistically by comparing the composite relative response of animals in each group (Table I).

Based on the onset of fever, arbitrarily established as a sustained temperature of >40°C, animals inoculated with the cloned organism had an incubation time of 4.0 days as compared to 3.2 days for the culture-inoculated group.

In the challenge study control cattle developed significant ($P < 0.001$) increases in temperature followed by severe anemia. Animals receiving the original culture material or the clone were immune as verified by no response to the challenge (Figs. 3 and 4).

Except for the animal which died, all of the animals developed a CF titer of ≥ 1:40 by 24 days PI. Similarly, all animals developed an IFA titer of 1:10, 240 by 77 days PI

DISCUSSION

Continuous cultivation of *B. bovis* in vitro (Erp et al., 1980; Levy and Ristic, 1980; Palmer et al., 1981) has encouraged the development of improved *B. bovis* vaccines. Levy and Ristic (1980) reported that soluble antigens derived from in vitro cultures would reduce mortality on challenge. Additional results were reported by Smith et al. (1981) and Kuttler et al. (1982, 1983), on the inactivated cell culture-derived *B. bovis* vaccine.

However, immunization with live vaccines is the method of choice in all countries where babesiosis is a major disease problem (de Vos, 1980). A highly successful attenuated *B. bovis* vaccine has been used in Australia (Callow, 1977) and South Africa (de Vos, 1980) for many years. All of these previously reported live vaccines have been produced by rapid passage in splenectomized calves. Since donor blood serves as the source of immunogen the danger of incorporation of extraneous pathogens poses a problem as does standardization and quality control. The vaccine has a shelf life of 1 week at 4°C and avirulence is not fully predictable.

In the present approach to obtaining an avirulent population of *B. bovis* for use as a live vaccine an in vitro cloning technique was used to isolate single organisms of modified virulence from a population with various pathogenicities. The differentiation of clones with different growth rates has previously been described (Rodriguez et al., 1983). The results of the present study demonstrate that the chosen clone selected on the basis of rapid growth rate was less pathogenic than the original population.

All of the surviving animals inoculated with cultured *Babesia* developed both CF and IFA antibodies. This is in contrast to animals inoculated with the soluble *B. bovis* antigen, which develop only IFA antibodies (Kuttler et al., 1983). Mahoney (1983) reported that hyperimmune serum from animals infected several times was protective in passive transfer experiments. Since antibodies appear to have a protective role, use of a live organism may be necessary to induce an adequate degree of protection against both homologous and possible variant strains.

In the challenge experiment the cattle which received the cloned organisms were protected equally as well as those which received the uncloned culture. This protective immunity was considerably more effective than that induced by two injections of a cell culture-derived soluble antigen vaccine (Kuttler et al., 1983). When animals vaccinated with the *B. bovis* soluble antigen were challenged an average 50% PCV reduction and average 6 day febrile response were noted. When the animals receiving the live cloned *B. bovis* culture were challenged, no change in temperature or reduction in PCV were observed.

CONCLUSION

The subject cloned organism may be an ideal candidate for use in areas where babesiosis is a disease problem or in animals being transported from free to endemic areas.

ACKNOWLEDGEMENTS

The authors acknowledge the assistance of M.R. Ellersieck, Department of Statistics, University of Missouri. Supported in part by U.S. Department of Agriculture Cooperative Agreement No. 58-9AHZ-2-655.

REFERENCES

Callow, L.L., 1977. Vaccination against bovine babesiosis. Adv. Exp. Med. Biol., 93: 121—149.

de Vos, A.J., 1980. Epidemiology and control of bovine babesiosis in South Africa. Reports and summaries. XI. Int. Cong. Dis. Cattle, 1: 627—641.

Erp, E.E., Smith, R.D., Ristic, M. and Osorno, B.M., 1980. Continuous in vitro cultivation of *Babesia bovis*. Am. J. Vet. Res., 41: 1141—1142.

Gill, J.L. and Hafs, H.D., 1971. Analysis of repeated measurements of animals. J. Anim. Sci., 33: 331--226.

Johnson, L.A.Y., Pearson, R.D. and Leatch, G., 1973. Evaluation of an indirect fluorescent antibody test for detecting *Babesia argentina* infections in cattle. Aust. Vet. J., 49: 373—377.

Kuttler, K.L., Levy, M.G., James, M.A. and Ristic, M., 1982. Efficacy of a nonviable culture-derived *Babesia bovis* vaccine. Am. J. Vet. Res., 43: 281—284.

Kuttler, K.L., Levy, M.G. and Ristic, M., 1983. Cell culture-derived *Babesia bovis* vaccine: sequential challenge exposure of protective immunity during a 6-month post vaccination period. Am. J. Vet. Res., 44: 1456--1459.

Levy, M.G. and Ristic, M., 1980. Continuous cultivation in microaerophilous stationary phase culture. Science, 207: 1218—1220.

Mahoney, D.F., 1962. Bovine babesiosis: diagnosis of infection by a complement-fixation test. Aust. Vet. J., 38: 48—52.

Mahoney, D.F., 1967. Bovine babesiosis: preparation and assessment of complement-fixing antigen. Exp. Parasitol., 20: 232—241.

Mahoney, D.F., 1983. Studies on the protection of cattle against *Babesia bovis* infection. In: J.D. Dunsmore (Editor), 10th Meeting, World Assoc. Adv. Vet. Parasitol., Perth, Aust., pp. 93—104.

21

Palmer, D.A., Buening, G.M. and Carson, C.A., 1981. Cultivation of *Babesia bovis* in vitro. In: R.J. Hidalgo and E.W. Jones (Editors), Proc. 7th Natl. Anaplasmosis Conf., Mississippi State, MS, pp. 207—217.

Rodriguez, S.D., Buening, G.M., Green, T.J. and Carson, C.A., 1983. Cloning of *Babesia bovis* by in vitro cultivation. Infect. Immun., 42: 15—18.

Smith, R.D., James, M.A., Ristic, M., Aikawa, M. and Vega y Murguia, C.A., 1981. Bovine babesiosis: protection of cattle with culture-derived soluble *Babesia bovis* antigen. Science, 212: 335—338.

NOTE TO CONTRIBUTORS

A detailed ***Guide for Authors*** is available upon request and is also printed in the first volume to appear each year. You are kindly asked to consult this guide. Please pay special attention to the following notes:

Types of papers published in the journal

– papers reporting results of original research – review articles – short communications – editorials – book reviews – news and announcements.

Language

The official language of the journal is English.

Preparation of the text

(a) The manuscript should include at the beginning an abstract of not more than 400 words.
(b) It should be typewritten with double spacing and wide margins. Words to be printed in italics should be underlined. SI units should be used throughout.
(c) The title page should include: the title, the name(s) of the author(s), and their affiliation(s).
(d) Submit original plus 2 copies of manuscript (original illustrations or computer printouts plus 2 photocopies – for photographs, 3 prints should be supplied).

References

(a) References in the text should be cited as the name of the author(s), followed by the year of publication.
(b) The reference list should be in alphabetical order and on sheets separate from the text.

Tables

Tables should be compiled on separate sheets. A title should be provided for each table and all tables should be referred to in the text.

Illustrations

(a) Illustrations should be numbered consecutively and referred to in the text. All illustrations (line drawings, computer printouts and photographs) should be submitted separately, unmounted and not folded.
(b) Drawings should be fully annotated, the size of the lettering being appropriate to that of the drawings, but taking into account the possible need for reduction in size (preferably not more than 50%). The page format of the journal should be considered when designing drawings.
(c) Photographs must be of good quality, printed on glossy paper.
(d) Figure captions should be supplied on a separate sheet.

Reprints and page charges

There is no page charge. Fifty reprints of each article published will be supplied free of charge. Additional reprints can be ordered on a reprint order form which is included with the proofs.

All contributions will be carefully refereed for international relevance and quality.

Submission of an article is understood to imply that the article is original and unpublished and is not being considered for publication elsewhere.

ALSO FROM ELSEVIER

AGRICULTURAL ECONOMICS
AGRICULTURE, ECOSYSTEMS AND ENVIRONMENT
AGRICULTURAL WATER MANAGEMENT
ANIMAL FEED SCIENCE AND TECHNOLOGY
ANIMAL REPRODUCTION SCIENCE
APPLIED ANIMAL BEHAVIOUR SCIENCE
AQUACULTURE
AQUATIC BOTANY
COMPUTERS AND ELECTRONICS IN AGRICULTURE
ECOLOGICAL MODELLING
ENERGY IN AGRICULTURE
EXPERIMENTAL AND APPLIED ACAROLOGY
FIELD CROPS RESEARCH
FISHERIES RESEARCH
FOREST ECOLOGY AND MANAGEMENT
LANDSCAPE AND URBAN PLANNING
LIVESTOCK PRODUCTION SCIENCE
PREVENTIVE VETERINARY MEDICINE
RECLAMATION & REVEGETATION RESEARCH
SCIENTIA HORTICULTURAE
SOIL & TILLAGE RESEARCH
VETERINARY IMMUNOLOGY AND IMMUNOPATHOLOGY
VETERINARY MICROBIOLOGY
VETERINARY RESEARCH COMMUNICATIONS

• FULL DETAILS AND A FREE SAMPLE COPY AVAILABLE ON REQUEST •

ELSEVIER SCIENCE PUBLISHERS

P.O. Box 330, 1000 AH Amsterdam, The Netherlands

ATTACHMENT B

Reprinted from the AMERICAN JOURNAL OF VETERINARY RESEARCH, Vol. 46, No. 2, Pages 421-423.
© American Veterinary Medical Association, 1985. All Rights Reserved.

Cryopreservation of *Babesia bigemina* for in vitro cultivation

C. A. Vega, DVM, MS; G. M. Buening, DVM, PhD; S. D. Rodriguez, DVM, MS; C. A. Carson, VMD, PhD; K. McLaughlin

SUMMARY

Babesia bigemina-infected RBC and merozoites were cryopreserved and used to initiate in vitro cultures in normal bovine RBC; the cryoprotectant was a final 10% polyvinylpyrrolidone in Vega y Martinez solution. A cooling rate of 20 C/min until −80 C and then rapid transfer to liquid N_2 storage was satisfactory. Samples for culture initiation were rapidly thawed at 37 C, washed in Vega y Martinez solution and resuspended in complete culture media containing 10% normal bovine RBC. The optimum culture conditions to reestablish cultures were a 24-well plate (16 mm ID), 5 mm in depth, and an atmosphere of 2% to 5% O_2, 5% CO_2, and 93% to 90% N_2.

Babesia bigemina has been cryopreserved for animal inoculation. Barnett[1] reported the use of glycerin as a cryoprotectant, with a cooling rate of 1 to 4 C/min, for storage at −79 C. Dalgliesh[2] studied the infectivity of *B bigemina*-infected blood frozen with glycerin at the rate of 1 C/min. Thawed, infected blood established *B bigemina* infection if inoculated subcutaneously, but not if inoculated IV. Later, Dalgliesh and Mellors[3] reported the use of dimethyl sulphoxide as a cryoprotectant with the best cooling rates being 39 C/min to 212 C/min; they have reported a procedure for the cryopreservation of large volumes of babesial vaccine.[1] The cryoprotectant used was dimethyl sulphoxide, and the cooling rate was approximately 6 to 20 C/min. The successful cryopreservation of *B bovis* for initiation of in vitro cultures was reported by Palmer et al.[5] The cryoprotectant of choice was polyvinylpyrrolidone (PVP) with a cooling rate of 20 C/min.

was to develop procedures to cryopreserve *B bigemina* so that in vitro cultures could be initiated from frozen material.

Materials and Methods

In vitro culture system—An in vitro procedure to propagate *B bigemina* in continuous culture was developed by Vega et al.[6] The original system was modified for the present study. Briefly, the adapted strain of *B bigemina* was maintained in bovine RBC culture consisting of 40% bovine serum + 60% medium 199, 10% PCV of bovine RBC, pH 7.0. The depth of media in the flask was 7 mm and the 37 C incubator had an atmosphere of 5% CO_2 in air. The culture media was replaced daily, and the cultures were subcultured every other day by mixing 1 part of culture with 1 part of media containing 10% PCV of normal bovine RBC. Infected bovine RBC for cryopreservation had a parasitemia between 6 to 10%. Merozoites were obtained from the infected bovine RBC by the procedure of Palmer et al.[4] Briefly, the flask was placed at room temperature in normal atmosphere for 4 to 6 hours. The supernatant was removed, and the merozoites pelleted by centrifuging at 1,000 × g for 10 minutes.

Cryopreservation procedure—The cryoprotectant solution was made according to Palmer et al.[5] Stock solution of 20% PVP 40[a] was made in Puck's saline solution G containing 20 g of glucose (PSG+)/L. A similar solution of 20% PVP in Vega y Martinez (VYM), used in the present study, was made according to Vega et al[6]; VYM solution was composed of $CaCl_2 \cdot 2H_2O$ (16.0 mg), KCl (400.0 mg), KH_2PO_4 (1,415.4 mg), $MgSO_4 \cdot 7H_2O$ (154 mg), $Na_2HPO_4 \cdot 7H_2O$ (1,450.0 mg), NaCl (7,077.0 mg), and dextrose (20.5 g) in 1 L of double-distilled deionized water containing 0.25 m*M* adenine and 0.50 m*M* guanosine. Packed infected RBC or merozoites were mixed with an equal volume of 20% PVP and dispensed in 1- or 2-ml cryotubes.[b] The PVP concentration of the final mixture was 10%. The contents of the vials were frozen at the rate of 20 C/min in a programmable freezing unit (model 801[b]). When the temperature was −80 C the vials were quickly transferred to liquid N_2 storage.

Retrieval from liquid N_2—Sample vials were removed from the liquid N_2 container and rapidly thawed in a 37 C water bath. The thawed samples were immediately diluted into 50 ml of PSG+ or VYM and centrifuged (4 C) at 1,000 × g for 10 minutes. The resulting pellets were resuspended in fresh culture media containing 10% normal bovine RBC. The amount of media varied according to whether flasks[c] (25 cm²) or 24-well plates[d] (16 mm ID) were used. The incubating culture depth and atmosphere varied according to the experiment. Fresh bovine RBC were added to the cultures on day 3 after retrieval; the cultures were subcultured on day 6.

Evaluation of continuous growth—Blood smears were made starting on day 2 after retrieval. The slides were stained with Giemsa's stain and examined. The criteria for growth was based upon parasite morphologic features and on increased numbers of infected bovine RBC. Observations were continued for at least 10 days (except for experiments 1 and 7). Positive growth was reported when the parasitemia was ≥ 1%.

Received for publication Feb 13, 1984.
From the Department of Veterinary Microbiology, College of Veterinary Medicine, University of Missouri, Columbia, MO 65211.
Supported in part by US Department of Agriculture, Agricultural Research Service Cooperative Agreement 58-9AHZ-2-655; Dr. Vega was supported by a fellowship provided by Consejo Nacional de Ciencia y Tecnologia (an agency of the Mexican government) contract No. 09708; Dr. Rodriguez was supported by a grant provided by US Agency for International Development DPE-5542-G-55-3014-00.

[a] Sigma Chemical Co, St Louis, Mo.

[b] Cryo-Med, Mt Clemens, Mich.
[c] Corning Glass Works, Corning, NY.
[d] Costar, Cambridge, Mass.

TABLE 1—The effect of the wash solution on the ability of cryopreserved *B bigemina* merozoites to initiate in vitro cultures

Experiment No.	Days in N_2	Wash solution	Culture atmosphere*	Results†
1	7	Media	5% CO_2 in air	No growth, 5 day observation
2	30	VYM	5.6% O_2, 6.4% CO_2, 88% N_2	Growth, + on day 3
3	35	VYM	5.6% O_2, 6.4% CO_2, 88% N_2	Growth, + on day 2
4	37	PSG‡	5.6% O_2, 6.4% CO_2, 88% N_2	No growth, 10 day observation
5	47	VYM	5% CO_2 in air	Growth, + on day 10

* = 37 C humidified. † = Based upon morphologic features and increased numbers of infected erythrocytes.
‡ = Parasitemia of + 1%.

TABLE 2—The effect of culture atmosphere on the ability of cryopreserved *B bigemina*-infected RBC (experiment 6) to initiate in vitro culture

Incubator	Atmosphere*	Results†
A	2% O_2, 5% CO_2, 93% N_2	Growth, + on day 2
B	5% O_2, 5% CO_2, 90% N_2	Growth, + on day 2
C	5% CO_2 in air	No growth, 11 days observation

* = 37 C humidified. † = Based upon morphologic features and increased No. of infected erythrocytes.
‡ = Parasitemia + 1%.

TABLE 3—The effect of culture atmosphere on the ability of cryopreserved *B bigemina*-infected RBC and merozoites (experiment 7) to initiate in vitro culture

Incubation			Dilutions					
			Infected RBC			Merozoites		
Time	Incubator	Atmosphere*	1:4	1:20	1:100	1:4	1:20	1:100
Before freezing	A	2% O_2, 5% CO_2, 93% N_2	+	+	+	+	+	+
	B	5% O_2, 5% CO_2, 90% N_2	+	+	+	+	+	+
	C	5% CO_2 in air	+	+	Degen	+	+	Degen
	D	10% O_2, 5% CO_2, 85% N_2	+	+	+	+	+	+
After thawing	A	2% O_2, 5% CO_2, 93% N_2	+	+	ND	+	+	ND
	B	5% O_2, 5% CO_2, 90% N_2	+	+	ND	+	+	ND
	C	5% CO_2 in air			ND			ND
	D	10% O_2, 5% CO_2, 85% N_2	+		ND	+		ND

* 37 C humidified.
† = growth based upon morphologic features and increased No. of infected erythrocytes, 5 days after cultures were set, parasitemia + 1%; = no growth; ND = not done; Degen = parasites pyknotic.

Experimental design; experiment 1—Merozoites were stored in N_2 for 7 days, thawed, and washed in complete media. Cultures were set in a flask at a depth of 7 mm in an atmosphere of 5% CO_2 in air.

Experiment 2—Merozoites were stored in N_2 for 30 days, thawed, and washed in VYM. Cultures were set in a flask at a depth of 7 mm in an atmosphere of 5.6% O_2, 6.4% CO_2, and 88% N_2.

Experiment 3—Merozoites were stored in N_2 for 35 days, thawed, and washed in VYM. Cultures were set in a flask at a depth of 7 mm in an atmosphere of 5.6% O_2, 6.4% CO_2, and 88% N_2.

Experiment 4—Merozoites were stored in N_2 for 37 days, thawed, and washed in PSG+. Cultures were set in a flask at a depth of 7 mm in an atmosphere of 5.6% O_2, 6.4% CO_2, and 88% N_2.

Experiment 5—Merozoites were stored in N_2 for 47 days, thawed, and 1 vial was washed in VYM, and a duplicate vial was washed in PSG+. Separate cultures from each wash solution were set in individual flasks at 7 mm depth in an atmosphere of 5% CO_2 in air.

Experiment 6—Infected RBC from an experimentally inoculated calf (parasitemia 6%) were frozen in 10% PVP VYM. The cells were stored for 79 days in N_2, thawed, and washed in VYM. The packed cells were diluted 1:4 in 10% normal bovine RBC and dispensed into 3, 24-well plates (A, B, and C) at 5-mm depth (1 ml/well). Each plate was placed into a different atmosphere: A = 2% O_2, 5% CO_2, and 93% N_2; B = 5% O_2, 5% CO_2, and 90% N_2; C = 5% CO_2 in air (ca, 18-20% O_2).

Experiment 7—Infected bovine RBC and merozoites were obtained from in vitro cultures of *B bigemina* that had been maintained in continuous culture for 90 days. Duplicate samples of each were prepared for freezing in PVP VYM. One sample of merozoites and infected bovine RBC were frozen and stored in liquid N_2 for 7 days. The other sample of merozoites and/or infected bovine RBC was washed in VYM and diluted 1:4, 1:20, and 1:100. Four identical culture sets (A, B, C, and D) were made in separate 24-well plates at 5-mm depth (1 ml/well). Each plate was incubated in the following atmospheres: A = 2% O_2, 5% CO_2, and 93% N_2; B = 5% O_2, 5% CO_2, and 90% N_2; C = 5% CO_2 in air (ca 18-20% O_2); D = 10% O_2, 5% CO_2, and 85% N_2. The cryopreserved merozoites and infected bovine RBC were thawed, and washed in VYM diluted 1:4 and 1:20 in culture media containing 10% normal bovine RBC. Four identical plates were set in the A, B, C, and D atmospheres.

Results

Results of experiments 1 through 5 indicated that the only merozoite samples that could initiate in vitro cultures had been washed in VYM solution (Table 1). In addition, the 5.6% O_2, 6.4% CO_2, and 88% N_2 atmosphere was superior to an atmosphere of 5% CO_2 in air for the initiation of in vitro cultures.

Experiment 6 was a comparison of the effects of culture atmosphere at 5-mm culture depth on the ability of infected bovine RBC to initiate in vitro cultures (Table 2). Cultures were initiated in samples incubated in 2 or 5% O_2, 5% CO_2, and 93% or 90% N_2. The samples incubated in an atmosphere of 5% CO_2 in air did not initiate growth.

In experiment 7, attempts were made to define the effects of culture atmosphere on the ability of cryopreserved *B bigemina* infected RBC or merozoites to initiate in vitro cultures (Table 3). The best atmospheres for infected RBC and merozoites were 2% or 5% O_2, 5% CO_2, and 93% or 90% G_2. The atmosphere of 10% O_2, 5% CO_2, and 85% N_2 supported initiation of growth if a high ratio of infectivity was used. The atmosphere of 5% CO_2 in air did not support the initiation of growth by infected bovine RBC or merozoites at the 5-mm culture depth.

Discussion

The procedure of Palmer et al[5] for the cryopreservation of *B bovis* involved the use of 10% PVP in PSG+. This cryoprotectant solution did not work with *B bigemina* (data not presented). The wash solution used in the retrieval of *B bovis* was PSG+. Again, this solution was not satisfactory for the retrieval of *B bigemina* (Table 1). Vega et al[6] reported the successful continuous propagation of *B bigemina* and used VYM solution to wash the infected bovine RBC and normal RBC used in culture. Results of the present study also indicate that washing in VYM solution is a key factor in the establishment of in vitro cultures from cryopreserved *B bigemina* (Table 1).

The in vitro culture procedure used by Palmer et al[7] for *B bovis* was a modification of microaerophilous stationary phase culture of Levy and Ristic.[8] However, this procedure would not initiate continuous cultures if the parasitemia was < 0.1%. Rodriguez et al[9] developed a cloning procedure for *B bovis* based upon the use of a low O_2 culture atmosphere. Vega et al[6] initially reported the continuous propagation of *B bigemina*, which indicated that low O_2 culture atmosphere is an important factor. This observation was confirmed in the present study. In experiment 7, as the percentage of O_2 in the culture atmosphere increased, the ability of cryopreserved *B bigemina* to initiate in vitro cultures decreased.

The observations with regard to the use of 10% PVP as a cryoprotectant and freezing rate of 20 C/min for *B bigemina* are in agreement with those made by Palmer et al[5] for *B bovis*. These conditions, when combined with the use of VYM solution, provided a reproducible procedure for the cryopreservation of *B bigemina* for the initiation of in vitro cultures.

The reestablishment of in vitro cultures, using frozen *B bigemina* parasites, would eliminate the need for maintaining infected animals and the risk of spread to susceptible animals in the surrounding environment. In addition, this procedure would permit the establishment of seed cultures of *B bigemina* for biochemical and vaccine studies.

References

1. Barnett SF: The preservation of *Babesia bigemina*, *Anaplasma centrale*, and *Anaplasma marginale* by deep freezing. *Vet Rec* 76:4–8, 1964.

2. Dalgliesh RJ: Effects of low temperature preservation and route of inoculation on infectivity of *Babesia bigemina* in blood diluted with glycerin. *Res Vet Sci* 13:540–545, 1972.

3. Dalgliesh RJ, Mellors LT: Survival of the parasitic protozoan *Babesia bigemina* in blood cooled at widely different rates to −196 C. *Int J Parasitol* 4:169–172, 1974.

4. Mellors LT, Dalgliesh RJ, Timms P, et al: Preparation and laboratory testing of a frozen vaccine containing *Babesia bovis*, *Babesia bigemina*, and *Anaplasma centrale*. *Res Vet Sci* 32:194–197, 1982.

5. Palmer DA, Buening GM, Carson CA: Cryopreservation of *Babesia bovis* for in vitro cultivation. *Parasitology* 62:221–231, 1982.

6. Vega CA, Buening GM, Green TJ, et al: In vitro cultivation of *Babesia bigemina*. *Am J Vet Res* 46:416–420, 1985.

7. Palmer DA, Buening GM, Carson CA: Cultivation of *Babesia bovis* in vitro, in Hidalgo RJ, Jones EW (ed): *Proc 7th Natl Anaplasmosis Conf*, Mississippi State, Miss, Mississippi State University Press, 1981, pp 207–217.

8. Levy M, Ristic M: *Babesia bovis*: Continuous cultivation in a microaerophilous stationary phase culture. *Science* 207:1218–1220, 1980.

9. Rodriguez SD, Buening GM, Green TJ, et al: The cloning of *Babesia bovis* by in vitro cultivation. *Infect Immun* 42:15–18, 1983.

ATTACHMENT C

EXPERIMENTAL PARASITOLOGY 61, 236–243 (1986)

Babesia bovis: Purification and Concentration of Merozoites and Infected Bovine Erythrocytes

S. D. RODRIGUEZ, G. M. BUENING, C. A. VEGA, AND C. A. CARSON

Department of Veterinary Microbiology, College of Veterinary Medicine, University of Missouri, Columbia, Missouri 65211, U.S.A.

(Accepted for publication 9 October 1985)

RODRIGUEZ, S. D., BUENING, G. M., VEGA, C. A., AND CARSON, C. A. 1986. *Babesia bovis:* Purification and concentration of merozoites and infected bovine erythrocytes. *Experimental Parasitology* 61, 236–243. *Babesia bovis* merozoites, externalized by removal of infected erythrocytes from ordinary culture conditions, were completely separated from red blood cells and stroma by centrifugation in a Percoll gradient. A merozoite band formed at a point corresponding to about 1.087 g/ml specific density. Infected red blood cells were concentrated approximately fourfold to obtain greater than 49.0% parasitemia after centrifugation in Percoll. Most highly enriched fractions positioned between 1.121 and 1.123 g/ml specific density. Full parasite viability was retained. © 1986 Academic Press, Inc.

INDEX DESCRIPTORS: *Babesia bovis*, Protozoa, parasitic; Merozoites; Cultivation; Isolation; Purification; Centrifugation; Erythrocytes, bovine.

INTRODUCTION

A major problem in the study of the biochemisty and physiology of *Babesia* spp. is the difficulty of separating viable free merozoites, the mature exoerythrocytic stage, from host cells and stroma. Attempts to separate artificially or spontaneously released parasites have been reported. Mahoney (1967) used discontinuous sucrose gradients to separate *Babesia bovis* free forms released by hemolysis with distilled water. Differential centrifugation was used to separate *B. microti* released by continuous-flow sonication and immune hemolysis (Gravely and Kreier 1974; Langreth 1976). Neither technique yielded erythrocytes or stroma-free parasites. Mahoney did not test the viability of the freed parasites but *B. rodhaini* released by a similar lysis method were severely affected (Rickard 1970). Differential centrifugation and Ficoll (a nonionic synthetic polymer of sucrose) density gradient methods were later reported by Gravely *et al.* (1979) to separate spontaneously released *B. bovis* merozoites in short-term *in vitro* cultures (Erp *et al.* 1978). The former technique yielded free merozoites mixed with membrane ghosts, and the latter yielded merozoite fractions mixed with membranes and erythrocytes. Merozoite viability seemed comparable to infected blood when inoculated into susceptible recipients. Greater enriched preparations of infected erythrocytes are also desirable to study the physiology of the intraerythocytic stages. Natural or artificially induced *B. bovis* infections usually lead to a low percentage (<2.0) parasitized erythrocytes but *in vitro* cultivation can produce levels up to [illegible] (Levy and Ristic 1980; Rodriguez *et al.* 1983). Enrichment of infected erythrocytes to 95% parasitized erythrocytes has been achieved by differential lysis of noninfected erythrocytes (Mahoney 1967) and from [illegible] to 80% by affinity chromatography in heparin–Sepharose columns (Goodger *et al.* 1983). Separation of plasmodium infected erythrocytes (Costa *et al.* 1980; Rivas [illegible] *et al.* 1983) and artificially released parasites (Nillni *et al.* 1981) from normal erythrocytes has been achieved using linear gradients of colloidal sols of polyvinylpyrrolidone coated silica beads (Percoll) [illegible] viable free parasites capable of [illegible] and enriched populations of infected erythrocytes. The present report describes methodology for the purification and concentration of *B. bovis* merozoites [illegible] enrichment of infected erythrocytes [illegible] *in vitro* cultures.

MATERIALS AND METHODS

Babesia bovis (an isolate from Texas [illegible] designated TCB), was kindly provided by [illegible] Vega (Hemoparasites Project, Instituto [illegible] Investigaciones Pecuarias, Mexico).

For *in vitro* propagation in culture, no[illegible] erythrocytes were collected into sterile [illegible] containing glass beads and defibrinated by [illegible]. Cells were washed once in cold Puck [illegible] Puck *et al.* 1958), the buffy coat and upper [illegible] layer were removed, and the erythr[illegible] resuspended in Puck's saline G at a 50% [illegible] Red blood cells were passed thro[illegible] powder column (Whatman Co., Engl[illegible] to eliminate leukocytes, following th[illegible] described by Fulton and Grant (1956) and [illegible] Vega (1985). *In vitro* cultures were propa[illegible] described by Levy and Ristic [illegible] modified by Palmer *et al.* (1982) and or by [illegible] technique of Rodriguez *et al.* (198[illegible] The source of merozoites and infected e[illegible] parasites were cultured in 75-cm² (45 ml [illegible] 5% erythrocyte suspension in medium con[illegible] with normal bovine serum, 60% mediu[illegible] salts), and 30 mM TES 2-[2-hydro[illegible] hydroxymethyl) ethyl] aminoethanosu[illegible] Culture suspensions, in volumes of 0.4 ml [illegible], were incubated in atmospheres of [illegible] CO_2, 91% N, or 5% CO_2 in air. Subcul[illegible] every 48 to 72 hr as necessary to [illegible] parasitized erythrocytes.

For density gradients, an aqueous su[illegible] (Pharmacia, Uppsala, Sweden) w[illegible] osmolarity following the manufactur[illegible] One part of Puck's saline G 10x was [illegible] parts of Percoll and used as stock s[illegible] specific density of 1.123 g/ml, calculat[illegible] density) of Puck's saline G (10x) as 1.0[illegible] Percoll as 1.300 g/ml.

To separate merozoites, stationary pha[illegible] with a percentage parasitized erythrocyt[illegible] when most parasites exist as do[illegible] removed from the increased CO_2 atm[illegible] at 22 C (Levy *et al.* 1981). Culture [illegible] collected without disturbing the settl[illegible] and centrifuged at 3000*g* at 4 C for 2

0014-4894/86 $3.00
Copyright © 1986 by Academic Press, Inc.
All rights of reproduction in any form reserved

Best Available Document

34

centration of Merozoites
ythrocytes

. VEGA, AND C. A. CARSON

nary Medicine, University of Min
U.S.A.

tober 1985)

AND CARSON, C. A. 1986. *Babesia*
d infected bovine erythrocytes. *Ex*
merozoites, externalized by re-
nditions, were completely separated
Percoll gradient. A merozoite band
pecific density. Infected red blood
in greater than 49.0% parasitemia
tions positioned between 1.121 and
stained. © 1986 Academic Press, Inc.

ific: Merozoites; Cultivation; Isola-

t al. 1978). The former tech
l free merozoites mixed with
ghosts, and the latter yielded
ractions mixed with membran
ocytes. Merozoite viability se
rable to infected blood when in
nto susceptible recipients. Gre
d preparations of infected ery
re also desirable to study the
of the intraerythocytic stages.
artificially induced *B. bovis*
s usually lead to a low perc
parasitized erythrocytes but *in v*
tion can produce levels up to
and Ristic 1980; Rodriguez *et*
Enrichment of infected erythro
parasitized erythrocytes has
d by differential lysis of nonin
cytes (Mahoney 1967) and fr
by affinity chromatography
epharose columns (Goodger *et*
Separation of plasmodium in
cytes (Tosta *et al.* 1980; Rivad
983) and artificially released
illni *et al.* 1981) from normal er
has been achieved using linear
of colloidal sols of polyvinylpy

[illegible] silica beads (Percoll), yielding [illegible] parasites capable of reinfection [illegible] populations of infected erythrocytes. The present report describes the [illegible] for the purification and con[illegible] of *B. bovis* merozoites and en[illegible] of infected erythrocytes from *in vitro* [illegible]

MATERIALS AND METHODS

[illegible] isolate from Texcoco, Mexico, [illegible] TCB, was kindly provided by Dr. C. A. [illegible] Project, Instituto Nacional de [illegible] Pecuarias, Mexico).

[illegible] propagation in culture, normal bovine [illegible] were collected into sterile flasks con[illegible] beads and defibrinated by gentle agita[illegible] were washed once in cold Puck's saline G [illegible], the buffy coat and uppermost erythrocytes were removed, and the erythrocytes were [illegible] in Puck's saline G at a 50% (v/v) concen[illegible] cells were passed through a cellu[illegible] column (Whatman Co., England, UK) to [illegible] leukocytes, following the procedure [illegible] Fulton and Grant (1956) and modified by [illegible] *In vitro* cultures were propagated by the [illegible] described by Levy and Ristic (1980) and [illegible] Palmer *et al.* (1982) and or by the reduced [illegible] of Rodriguez *et al.* (1983) and used [illegible] merozoites and infected erythrocytes. [illegible] were cultured in 75-cm² (45 ml) flasks as a [illegible] suspension in medium containing 40% [illegible] bovine serum, 60% medium 199 (with [illegible]) and 30 mM TES 2-[2-hydroxyl-1, 1 bis [illegible] ethyl) ethyl] aminoethanosulfonic acid. [illegible] suspensions, in volumes of 0.4 ml/cm² or 0.62 [illegible] were incubated in atmospheres of 2% O_2, 5% [illegible] N_2 or 5% CO_2 in air. Subcultures were [illegible] 48 to 72 hr as necessary to maintain 10 [illegible] parasitized erythrocytes.

[illegible] gradients, an aqueous suspension of [illegible] (Pharmacia, Uppsala, Sweden) was adjusted [illegible] following the manufacturer's instruc[illegible] part of Puck's saline G 10x was mixed with [illegible] Percoll and used as stock solution with [illegible] density of 1.123 g/ml, calculated by using [illegible] Puck's saline G (10x) as 1.059 g/ml and [illegible] Percoll as 1.500 g/ml.

[illegible] merozoites, stationary phase cultures [illegible] parasitized erythrocytes of 10 or [illegible] most parasites exist as double forms, [illegible] from the increased CO_2 atmosphere for [illegible] 22 C (Levy *et al.* 1981). Culture supernates [illegible] without disturbing the settled erythro[illegible] centrifuged at 3000g at 4 C for 20 min. Sediments were washed twice in cold Puck's saline G, resuspended to a final volume of 500 µl, and used immediately or stored at 4 C for no longer than 2 hr. Five-hundred-microliter vol of the sediment was layered over 7.5-ml vol of Percoll prepared at concentrations ranging from 90 to 40% (1.110 to 1.052 g/ml). Tubes (58 mm long) were centrifuged at 26,000g for 30 min in a Sorval (RC2-B) centrifuge with a fixed angle rotor (SS34) at 4 C. Fractions were carefully collected from top to bottom using an angled pasteur pipet and washed three times in cold Puck's saline G. Smears were prepared, Giemsa-stained, and examined microscopically. Density of sample fractions was determined after centrifugation by comparison of the final equilibrium position with calibration tubes of density marker beads (Pharmacia) plotted on the graphic profile of the linear gradient of Percoll.

To concentrate infected erythrocytes, cultures at all stages of growth were used. The erythrocytes were washed three times in cold Puck's saline G at 1000g for 10 min. Packed erythrocytes, in volumes of 500 µl, were mixed with 7.5 ml of stock (100%) Percoll suspension and centrifuged as described above for merozoites. The fractions were collected and washed three times in cold Puck's saline G. The density of the fractions was calculated as described for the merozoites using the profile of a corresponding Percoll gradient.

To quantitate recovered merozoites and infected erythrocytes, merozoite fractions recovered after centrifugation were diluted in either Puck's saline G or 10.61 mM sodium citrate saline solution, and the number of merozoites was determined by counting moving merozoites in a hemacytometer (American Optical, Buffalo, NY, USA) under phase contrast microscopy. Red blood cell fractions were evaluated for enrichment in number of infected erythrocytes by counting at least 1000 erythrocytes in Giemsa-stained preparations and comparing the findings with the initial percentage of infected erythrocytes.

To determine the viability of the parasites, merozoites and enriched fractions of infected erythrocytes were adjusted to obtain various ratios of merozoites/100 uninfected erythrocytes or 2% parasitemia, respectively. Aliquots were delivered into 96-well microtiter plates, cultured under conditions of high oxygen (5% CO_2 in air) and/or reduced oxygen (2% O_2, 5% CO_2, 93% N), and compared to identical numbers of untreated infected erythrocytes from the original culture. Two to 2.5 and 5 hr elapsed, respectively, from the time infected erythrocytes and merozoites from culture supernates were collected until the time the preparations were plated for cultivation. Cultures were monitored for 72 hr, and parasitemias were estimated by counting at least 2000 erythrocytes in Giemsa-stained preparations. Twelve hours after culture initiation, Giemsa-stained smears from free merozoite-initiated cultures were examined by light mi-

croscopy for measurement of invasion rate (Levy and Ristic 1983).

Results

A 65% Percoll concentration was found to be optimal for the separation of *Babesia bovis* merozoites. Merozoites concentrated at a distance of 38 to 40 mm from the meniscus, forming a diffuse band at a specific density of approximately 1.087 g/ml. Figure 1 illustrates both the profile of a 65% Percoll linear gradient and the location of the merozoites. Two additional bands, separate from the merozoites, were observed. The uppermost, located near the top of the gradient (5–7 mm from meniscus), contained mostly red cell membranes and a few merozoites and/or ghosts containing merozoites. The lower band, located 52–55 mm below the meniscus, consisted of both normal erythrocytes and infected erythrocytes. Figure 2 is a photomicrograph of resulting purified merozoites after separation.

A gradient formed with the 100% Percoll stock solution was optimal for enrichment of infected erythrocytes. At lower concentrations, the enriched fractions sedimented toward the bottom of the tube. Figure 3 diagrammatically shows the profile of a [illegible] gradient of 100% Percoll after centrifugation at 26,000*g* for 30 min. Also illustrated is the distribution of infected erythrocytes expressed as a percentage of the [illegible] erythrocytes collected. Red blood cells separated by 100% Percoll formed several distinct bands. The highest concentration of infected erythrocytes appeared in the lower band (1.121 to 1.123 g/ml), while most of the uninfected erythrocytes remained at an intermediate position (1.[illegible] to 1.110 g/ml).

Initial attempts to quantify free merozoites recovered from Percoll, washed and resuspended in Puck's saline G, were severely hampered by clumping and adherence of the merozoites to vessel walls. Such attempts are recorded in Table I (Experiments 1a to 1c). Merozoite clumping precluded accurate evaluation of individual parasites. Merozoites washed in Puck's saline G but resuspended in sodium citrate saline solution for counting resulted in larger counts of free merozoites than those obtained using Puck's saline G throughout (Table I, Experiment 2). Giemsa-stained preparations examined by light microscopy were virtually free of erythrocytes (1 erythrocyte/10^5 merozoites).

To quantitate infected erythrocytes in fractions collected from 100% Percoll gradients, were washed three times in Puck's saline G, and samples were taken for determination of parasitemia. The percentage parasitized erythrocyte values corresponding to original culture, and most enriched fractions from seven experiments are summarized in Table II. The parasitemias in fractions farthest from the meniscus (1.[illegible]) ranged from 49.33 to 65.45% (mean [illegible] ± 5.98 SD), representing 4.50- to 13.4[illegible] enrichment (6.80 mean + 3.30 SD). Fractions located higher in the gradient had a lower percentage parasitized erythrocytes. A band of red cell membranes was located at the top of the gradient, and a fast band of merozoites located beneath it contained

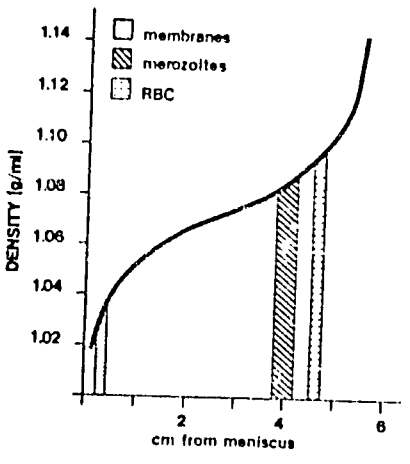


FIG. 1. Density calibration curve and diagrammatic representation of the separation of *Babesia bovis* merozoites in iso-osmotic 65% Percoll after 26,000*g* centrifugation for 30 min.

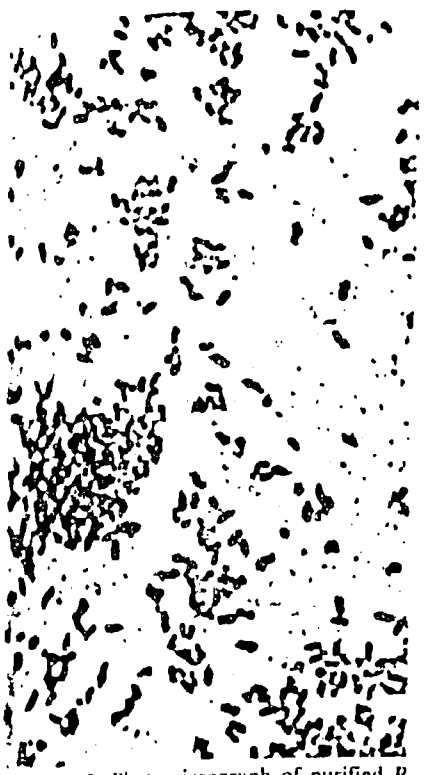


FIG. 2. Photomicrograph of purified *B* [illegible] centrifugation.

[illegible] retrieved without contaminating [illegible] and red cell stroma.

Free merozoites, originally quantif[illegible] Puck's saline G and mixed with n[illegible] erythrocytes, showed 30% viability

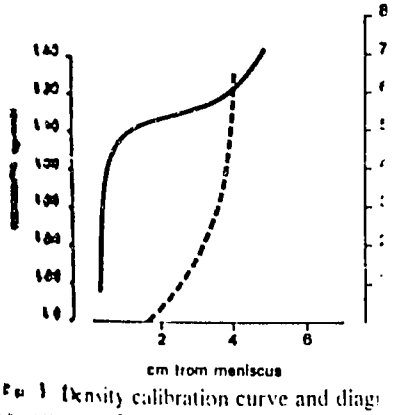


FIG. 3. Density calibration curve and diagrammatic [illegible] of erythrocytes infected with [illegible] *in vitro* culture in 100% iso-osmotic [illegible] centrifugation for 30 min.

iows the profile of a
f Percoll after cent
or 30 min. Also illus
n of infected erythro
percentage of the
ected. Red blood cell
Percoll formed sev
The highest concentra
hrocytes appeared
l to 1.123 g/ml), wh
nfected erythrocytes
ermediate position

s to quantify free
from Percoll, washed
uck's saline G, were
by clumping and
ites to vessel walls
rded in Table I (Exp
Merozoite clumping
evaluation of indiv
tes washed in Puck's
ended in sodium cit
r counting resulted
ee merozoites than
ck's saline G through
ent 2). Giemsa-stai
ined by light micros
e of erythrocytes
rozoites).
ected erythrocytes,
100% Percoll grad
times in Puck's s
re taken for deter
. The percentage
values corresponding
d most enriched
xperiments are sum
e parasitemias in
the meniscus (Fig
o 65.45% (mean
nting 4.50- to 13.47
an + 3.30 SD). B
e gradient had
rasitized erythroc
embranes was loc
lient, and a faint
l beneath it could

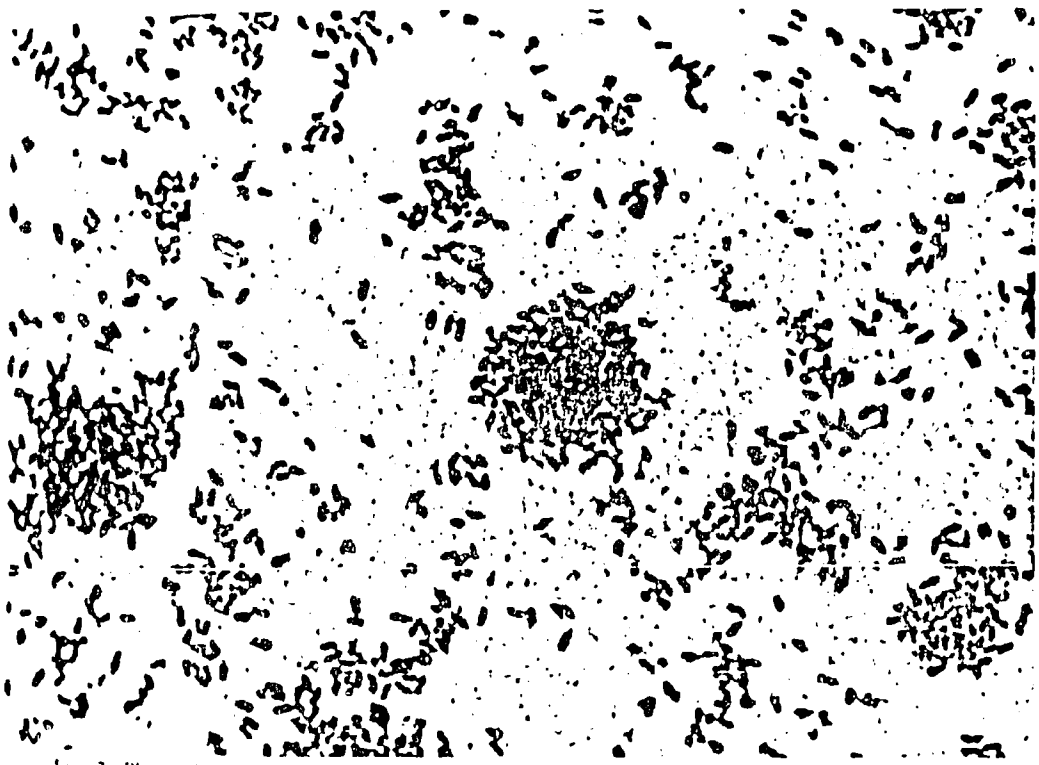


FIG. 2. Photomicrograph of purified *Babesia bovis* merozoites after 65% Percoll linear gradient centrifugation.

retrieved without contaminating membranes and red cell stroma.

Free merozoites, originally quantified in Puck's saline G and mixed with normal erythrocytes, showed 30% viability as estimated by counting the number of infected erythrocytes at 12 hr after culture initiation (Table III, Experiments 1 and 2). At this time, the number of extracellular merozoites was greater than that calculated (6.8 and 3.7 mz/100 normal erythrocytes) at the time of culture initiation, and their quantitation was difficult due to clumping. Cultures started with merozoites counted and resuspended in sodium citrate saline solution prior to mixing with normal erythrocytes showed a much higher viability rate (Table III, Experiments 3 and 4). The reinvasion rate at the reduced O_2 atmosphere was 89.12% (mean of Experiments 3a and 4a) of the originally calculated number of free merozoites compared to 42.69% (mean of Experiments 3 and 4) when the high O_2 atmosphere was used. The number of extracellular merozoites observed at 12 hr after culture initiation was negligible in both cases. At 72 hr after culture initiation, both

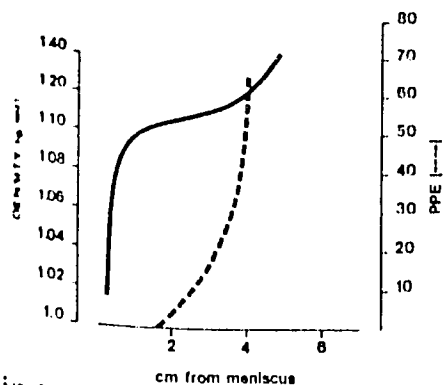


FIG. 3. Density calibration curve and diagrammatic representation of erythrocytes infected with *Babesia bovis* from *in vitro* culture in 100% iso-osmotic Percoll after 26,000*g* centrifugation for 30 min.

TABLE I
Yield of Free *Babesia bovis* Merozoites after Percoll Purification

Experiment No.	mz[a] recovered	
	PSG[b]	SCSS[c]
1 A	4.02×10^7	ND
B	2.81×10^7	ND
C	2.77×10^7	ND
2 A	ND[d]	6.80×10^8
B	2.26×10^7	1.35×10^8
C	9.10×10^7	1.02×10^9
D	6.80×10^7	2.70×10^8

Note. Quantitation of free merozoites was performed by counting moving merozoites in a hemacytometer chamber using phase contrast microscopy.
[a] mz = merozoites.
[b] PSG = Puck's saline G.
[c] SCSS = Sodium citrate–saline solution.
[d] ND = not done.

cultures had lower percentage parasitized erythrocytes than cultures initiated with infected erythrocytes.

Percoll treatment to enrich for infected erythrocytes had no detrimental effect on the capability of infected erythrocytes to initiate cultures, and replication in general seemed comparable to control, non-Percoll treated infected erythrocytes (Table IV). The Percoll method yields leukocyte-free infected erythrocyte preparations [illegible] kocytes have a lower density [illegible] than infected erythrocytes (1.121 [illegible] g/ml).

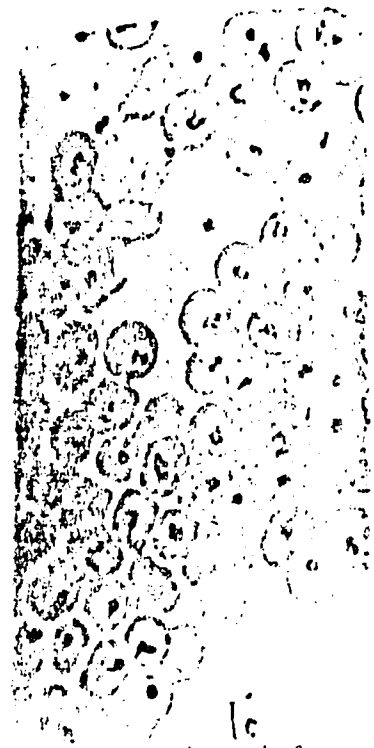
Discussion

Previous reports on separation [illegible] *besia bovis* merozoites from *in* [illegible] tures (Levy *et al.* 1981; Gravely [illegible] resulted in preparations of simil[illegible] the raw material used for Percoll [illegible] used in this report. Spontaneous [illegible] nation of free merozoites obta[illegible] spinner flask cultures (Erp *et al.* 19[illegible] first reported by Gravely and co[illegible] (1979). According to the authors, th[illegible] precluded accurate quantitation of [illegible] forms. They also reported failure to [illegible] rate clumped merozoites by incuba[illegible] acid–citrate–dextrose, Alsever's [illegible] of ethylene–diamine tetraacetic [illegible] agents commonly used to chelate [illegible] Levy *et al.* (1981) reported that [illegible] neous agglutination of free forms [illegible] at physiologic temperatures (37 C) [illegible] this phenomenon could be preven[illegible] duced by handling all materials at [illegible] the present study, spontaneous [illegible] tion was observed at all stages [illegible] preparation despite careful manipu[illegible] 4 C, and this phenomenon seemed [illegible]

TABLE II
Concentration of *Babesia bovis* Infected Bovine Erythrocytes in Percoll Gradients

Experiment No.	Initial culture (PPE)[a]	Band of highest concentration (PPE)	Enrichment	Yield of iRBC[b]
1	9.10	49.33	5.40	[illegible]
2	4.25	57.25	13.47	[illegible]
3	7.57	55.23	7.30	[illegible]
4	9.27	50.68	5.45	[illegible]
5	13.26	59.85	4.51	[illegible]
6	12.70	65.45	5.15	[illegible]
Mean ± SD	NA[c]	56.30 ± 5.98	6.88 ± 3.3	[illegible]

[a] PPE = Percentage parasitized erythrocytes.
[b] Numbers represent the percentage yield of total iRBC mixed with Percoll.
[c] NA = Not applicable.



[illegible] 4. Photomicrograph of concen[illegible] [illegible] gradient centrifugation.

TABLE III
[illegible] of Free *Babesia bovis* Merozoi[illegible]
Percoll Separation

[illegible] No.	mz/100 RBC[b]	PPE 12 hr[c]
[illegible]	6.80	1.97
[illegible]	3.70	1.07
[illegible]	2.30	0.93
[illegible]	0.93	0.81
[illegible]	1.92	0.86
[illegible]	2.37	2.16

[illegible] parasitized erythrocytes.
[illegible] adjusted to initiate cultures w[illegible] [illegible] of merozoites.
[illegible]
[illegible] hr, the cultures were sampled an[illegible] [illegible] examined.
[illegible] 1, 2, 3, and 4 were incubated i[illegible]
[illegible] 4a were incubated in a 2[illegible] atmosphere.

ected erythrocyte preparations si
cytes have a lower density (1.09
n infected erythrocytes (1.121 t
al).

DISCUSSION

revious reports on separation o
ia bovis merozoites from in vitr
es (Levy et al. 1981; Gravely et al
lted in preparations of similar pu
raw material used for Percoll sepa
d in this report. Spontaneous ag
on of free merozoites obtained
ner flask cultures (Erp et al. 1978
reported by Gravely and co-wo
9). According to the authors, this
uded accurate quantitation of the
s. They also reported failure to
clumped merozoites by incubati
citrate–dextrose, Alsever's sol
hylene–diamine tetraacetic a
s commonly used to chelate cal
et al. (1981) reported that spo
agglutination of free forms occ
siologic temperatures (37 C) and
enomenon could be prevented
by handling all materials at 4 C
esent study, spontaneous agglu
as observed at all stages of sa
ition despite careful manipulatio
nd this phenomenon seemed to

throcytes in Percoll Gradients

Enrichment	Yield of iRBC (%)[b]
5.40	34.17
13.47	50.39
7.30	33.99
5.45	17.17
4.51	44.90
5.15	78.74
6.88 ± 3.3	43.2 ± 20.7

Percoll.

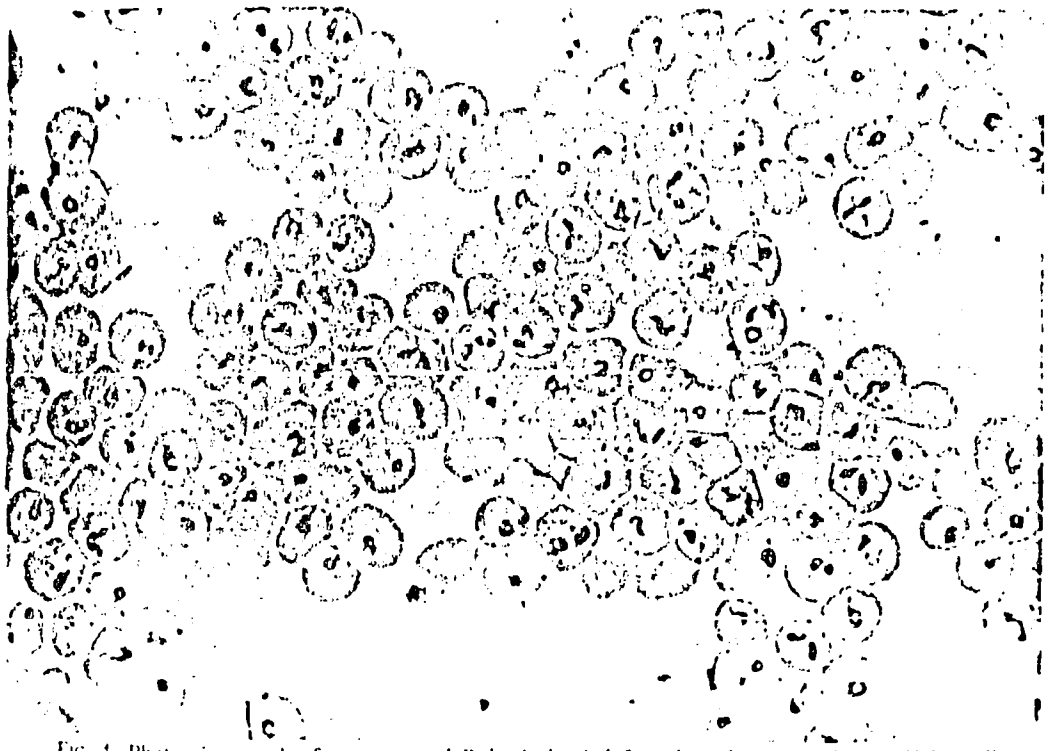


FIG. 4. Photomicrograph of concentrated *Babesia bovis* infected erythrocytes after 100% Percoll linear gradient centrifugation.

TABLE III
Viability of Free *Babesia bovis* Merozoites after Percoll Separation

Experiment No.	m.z. 100 RBC[b]	PPE[a]	
		12 hr[d]	72 hr[d]
1	6.80	1.97	5.35
2	3.70	1.07	1.40
3[c]	2.30	0.93	3.72
3a[e]	0.93	0.81	7.99
4[c]	1.92	0.86	3.99
4a[e]	2.37	2.16	5.63

[a] Percentage parasitized erythrocytes.
[b] Number adjusted to initiate cultures with nRBC for quantitation of merozoites.
[d] PCL 12 hr.
[d] PCL 72 hr: the cultures were sampled and Giemsa-stained preparations examined.
[c] Cultures 1, 2, 3, and 4 were incubated in high oxygen atmosphere.
[e] Cultures 3a and 4a were incubated in a 2% O_2, 5% CO_2, 93% N atmosphere.

accentuated after Percoll separation. The use of a sodium citrate solution (10.61 m*M*) in physiological saline (0.015 m*M* NaCl) reduced agglutination of the free forms, making accurate quantitation of Percoll separated merozoites possible. Superior infectivity of merozoites in sodium citrate saline solution resulted in infected erythrocyte counts 3.9 to 11.2 times greater than those obtained with merozoites in Puck's saline G. This effect of sodium citrate saline solution may simply be related to reduced clumping but since calcium is reportedly important for plasmodial attachment to host cells (Johnson et al. 1980), calcium chelation by the citrate component may warrant further investigation. These results confirm and extend those of Gravely *et al.* (1979) and Levy *et al.* (1981) who assessed the viability of the free forms by animal in-

TABLE IV
Viability of *Babesia bovis* Infected Erythrocytes

		% Parasitized erythrocytes	
		Initial	72 hr
Experiment 1	Control	1.82	14.00
	Percoll enriched[a]	1.97	4.21
Experiment 2	Control	2.12	12.75
	Percoll enriched	2.04	15.34
Experiment 3	Control	2.11	18.09
	Percoll enriched	2.04	19.54
Experiment 4	Control	2.07	18.32
	Percoll enriched	2.01	19.68

[a] Percoll enriched infected erythrocytes were diluted with culture medium, the percentage parasitized erythrocytes adjusted with a 5% PCV nRBC suspension and cultured in a high oxygen atmosphere.

oculation and *in vitro* cultivation, respectively.

Concentration of infected erythrocytes by Percoll density gradients centrifugation was disappointing since 30 to 50% parasitized erythrocytes have been obtained in culture systems (S. D. Rodriguez, unpublished data). The density gradient procedure, however, provided a way whereby infected erythrocytes can be concentrated by a method other than the differential hemolysis of blood from infected animals originally described by Mahoney (1967). Hypotonic lysis of infected erythrocytes to harvest parasites may not only damage the organisms but also yields organisms at all stages of development (Langreth 1976; Gravely and Krier 1974) which often are still attached to red cell membranes (S. D. Rodriguez, unpublished observations). Free *Babesia bovis* merozoites prepared in the manner described in this report can be used for monoclonal antibody preparation and, since they are largely free of contaminants, are useful for biochemical and antigenic characterization.

Acknowledgments

This work was supported in part by the U.S. Department of Agriculture through ARS Grant 58-9AHZ-2-655. Dr. Rodriguez was supported by the U.S. Agency for International Development through Grants DPE-5542-G-SS-3014-00 and DPE-5542-G-SS-4026-[illegible] and by the Instituto Nacional de Investigaciones Pecuarias, Mexico.

References

Erp, E. E., Gravely, S. M., Smith, R. D., Risco, M., Osorno, B. M., and Carson, C. A. 19[illegible] Growth of *Babesia bovis* in bovine erythrocyte cultures. *American Journal Tropical Medicine and Hygiene* 27, 1061–1064.

Fulton, J. D., and Grant, P. T. 1956. The sulfur requirement of the erythrocytic form of *Plasmodium knowlesi*. *Biochemistry Journal* 63, 274–282.

Goodger, B. V., Mahoney, D. F., and Wright, I. G. 1983. *Babesia bovis*: Attachment of infected erythrocytes to heparin-sepharose columns. *Journal of Parasitology* 69, 248–249.

Gravely, S. M., and Kreier, J. P. 1974. *Babesia microti* (Gray strain): Removal from infected hamster erythrocytes by continuous-flow ultrasonication. *Tropenmedizin und Parasitologie* 25, 198–206.

Gravely, S. M., Smith, R. D., Erp, E. E., Cavey, G. J., Aikawa, M., Osorno, B. M., and Risic, M. 1979. Bovine babesiosis: Partial purification and characterization of blood culture-derived *Babesia bovis*. *International Journal of Parasitology* 9, [illegible]–598.

Johnson, J. G., Epstein, N., Shiroishi, T., and Miller, L. H. 1980. Factors affecting the ability of isolated *Plasmodium knowlesi* merozoites to attach to and invade erythrocytes. *Parasitology* 80, [illegible]–550.

Langreth, S. G. 1976. Feeding mechanisms in extracellular *Babesia microti* and *Plasmodium lophurae*. *Journal of Protozoology* 23, 215–223.

Levy, M. G., Erp, E. E., and Rishic, M. 1981. Cultivation of babesia. *In* "Babesiosis" (M. Ristic, [illegible]

J. P. Kreier, eds.), pp. 207–223. [illegible] New York.

Levy, M. G., and Ristic, M. 1980. Continuous cultivation in a microaerophilic phase culture. *Science (Wa[illegible]* 207, 1218–1220.

Levy, M. G., and Ristic, M. 1983. Cu[illegible] *vitro* studies in *Babesia*. *In* "*In vitro* [illegible] Protozoan Parasites" (J. B. Jensen, [illegible] 240. CRC Press, Florida.

Mahoney, D. F. 1967. Bovine babesio[illegible] and assessment of complement fixin[illegible] *Experimental Parasitology* 20, 232–24[illegible]

Nagel, E. A., Londner, M. V., and [illegible] 1981. A simple method for separatio[illegible] erythrocytes from those infected wi[illegible] *berghei* and for isolation of artificiall[illegible] [illegible]sites. *Zeitschrift fuer Parasitenkund[illegible]*

Palmer, D. A., Buening, G. M., and [illegible] 1982. Cryopreservation of *Babesia b[illegible]* cultivation. *Parasitology* 84, 567–57[illegible]

Puck, T. T., Cieciura, S. J., and Robi[illegible] Genetic of somatic mammalian cells.

hrocytes

% Parasitized erythrocytes	
Initial	72 hr
1.82	14.00
1.97	4.21
2.12	12.75
2.04	15.34
2.11	18.09
2.04	19.54
2.07	18.32
2.01	19.68

Jium, the percentage parasitized ery
gh oxygen atmosphere.

S-3014-00 and DPE-5542-G SS-4026-0
stituto Nacional de Investigaciones fo
o.

References

RAVELY, S. M., SMITH, R. D., RISI
D. B. M., AND CARSON, C. A. 19
abesia bovis in bovine erythrocyte
can Journal Tropical Medicine and H
61–1064.

, AND GRANT, P. T. 1956. The sulph
of the erythrocytic form of *Plasmodi*
ochemistry Journal 63, 274–282.

V., MAHONEY, D. F., AND WRIGHT, I. G
a bovis: Attachment of infected ery
heparin-sepharose columns. *Journal o*
69, 248–249.

L., AND KREIER, J. P. 1974. *Babesia*
strain): Removal from infected hamst
by continuous-flow ultrasonicatio
in und Parasitologie 25, 198–206

L., SMITH, R. D., ERP, E. E., CAN
A. M., OSORNO, B. M., AND RISTIC, M
e babesiosis: Partial purification an
tion of blood culture-derived *Babes*
ational Journal of Parasitology 9, 59

., EPSTEIN, N., SHIROISHI, T. A
L. 1980. Factors affecting the ability
nodium knowlesi merozoites to attac
erythrocytes. *Parasitology* 80, 53

. 1976. Feeding mechanisms in ery
ia mitis and *Plasmodium lophurae*
otozoology 23, 215–223.

RP, E. E., AND RISTIC, M. 1981. C
esia. *In* "Babesiosis" (M. Ristic and

Babesia bovis: MEROZOITE PURIFICATION AND VIABILITY 243

J. P. Kreier, eds.), pp. 207–223. Academic Press, New York.

RISTIC, M. G., AND RISTIC, M. 1980. *Babesia bovis*: Continuous cultivation in a microaerophilus stationary phase culture. *Science (Washington, D.C.)* 207, 1218–1220.

RISTIC, M. G., AND RISTIC, M. 1983. Cultivation and *in vivo* studies in *Babesia*. *In* "*In vitro* Cultivation of Protozoan Parasites" (J. B. Jensen, ed.), pp. 222–240. CRC Press, Florida.

MAHONEY, D. F. 1967. Bovine babesiosis: Preparation and assessment of complement fixing antigens. *Experimental Parasitology* 20, 232–241.

SHAW, E. A., LONDNER, M. V., AND SPIRA, D. T. 1981. A simple method for separation of uninfected erythrocytes from those infected with *Plasmodium berghei* and for isolation of artificially released parasites. *Zeitschrift fuer Parasitenkunde* 64, 279–284.

SOMER, D. A., BUENING, G. M., AND CARSON, C. A. 1982. Cryopreservation of *Babesia bovis* for *in vitro* cultivation. *Parasitology* 84, 567–572.

TAO, T. T., CIECHURA, S. J., AND ROBINSON, A. 1958. Genetic of somatic mammalian cells. III. Long term cultivation of euploid cells from human and animal subjects. *Journal of Experimental Medicine* 108, 945–956.

RICKARD, M. D. 1970. Carbohydrate metabolism in *Babesia rodhaini*: Investigations with 14-C-labeled substrates and enzyme assays. *Experimental Parasitology* 28, 512–520.

RIVADENEIRA, E. M., WASSERMAN, M., AND ESPINAL, C. T. 1983. Separation and concentration of *Plasmodium falciparum* by Percoll gradients. *Journal of Protozoology* 30, 367–370.

RODRIGUEZ, S. D., BUENING, G. M., GREEN, T. J., AND CARSON, C. A. 1983. Cloning of *Babesia bovis* by *in vitro* cultivation. *Infection and Immunity* 42, 15–18.

TOSTA, C. E., SEDIGHAH, M., HENDERSON, D. C., AND WEDDERBURN, N. 1980. *Plasmodium yoelii* and *Plasmodium berghei*: Isolation of infected erythrocytes from blood by colloidal silica gradient centrifugation. *Experimental Parasitology* 50, 7–15.

VEGA Y MURGUIA, C. A. 1985. "*Babesia bigemina*: Cultivation and Characterization, pp. 109–140. Ph.D. dissertation, Univ. of Missouri—Columbia.

ATTACHMENT D

22. Muhich, M. L., Neckelmann, N. & Simpson, L. 1985. The divergent region of *Leishmania tarentolae* kinetoplast maxicircle DNA contains a diverse set of repetitive sequences. *Nucl. Acids Res.*, **13**: 3241–3259.

23. Muhich, M. L., Simpson, L. & Simpson, A. M. 1983. Comparison of maxicircle DNAs of *Leishmania tarentolae* and *Trypanosoma brucei*. *Proc. Natl. Acad. Sci. USA*, **80**: 4060–4064.

24. Payne, M., Rothwell, V., Jasmer, D. P., Feagin, J. E. & Stuart, K. 1985. Identification of mitochondrial genes in *Trypanosoma brucei* and homology to cytochrome c oxidase II in two different reading frames. *Mol. Biochem. Parasitol.*, **15**: 159–170.

25. Rigby, P. W., Dieckmann, M., Rhodes, C. & Berg, P. 1977. Labeling deoxyribonucleic acid to high specific activity *in vitro* by nick-translation with DNA polymerase I. *J. Mol. Biol.*, **113**: 237–251.

26. Sánchez, D. O., Frasch, A. C. C., Carrasco, A., González-Cappa, S. M., Isola, E. & Stoppani, A. O. M. 1984. Rapid evolution of kinetoplast DNA minicircle subpopulations in *Trypanosoma cruzi*. *Mol. Biochem. Parasitol.*, **11**: 169–178.

27. Sánchez, D. O., Madrid, R., Engel, J. C. & Frasch, A. C. C. 1984. Rapid identification of *Trypanosoma cruzi* isolates by "dot-spot" hybridization. *FEBS Lett.*, **168**: 139–142.

28. Shlomai, J. & Zadok, A. 1984. Kinetoplast DNA minicircles of trypanosomatids encode for a protein product. *Nucl. Acids Res.*, **12**: 8017–8028.

29. Southern, E. M. 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. *J. Mol. Biol.*, **98**: 503–517.

30. Stuart, K. 1983. Kinetoplast DNA, mitochondrial DNA with a difference. *Mol. Biochem. Parasitol.*, **9**: 93–104.

31. Stuart, K. D. & Gelvin, S. B. 1982. Localization of kinetoplast DNA maxicircle transcripts in bloodstream and procyclic form *Trypanosoma brucei*. *Mol. Cell. Biol.*, **2**: 845–852.

32. Thomas, P. S. 1980. Hybridization of denatured RNA and small DNA fragments to nitrocellulose. *Proc. Natl. Acad. Sci. USA*, **77**: 5201–5205.

Received 4 XI 85; accepted 30 VI 86

J. Protozool., 33(4), 1986, pp. 507–511
© 1986 by the Society of Protozoologists

Enzymatic Characterization of *Babesia bovis*[1]

S. D. RODRIGUEZ,[2] G. M. BUENING, C. A. VEGA, and C. A. CARSON

Department of Veterinary Microbiology, College of Veterinary Medicine, University of Missouri, Columbia, Missouri 65211

ABSTRACT. Agarose gel electrophoresis was used to identify metabolic enzymes in *Babesia bovis* and *B. bigemina*. Glutamate dehydrogenase, lactate dehydrogenase, glucose phosphate isomerase, and hexokinase were identified in *B. bovis*- and *B. bigemina*-infected erythrocytes and *B. bovis* merozoite preparations. A specific electrophoretic mobility was observed for each enzyme. Malate dehydrogenase, glucose 6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase, and adenylate kinase were only detected in normal erythrocyte preparations. Inter-species, but not intra-species, variation was noted when comparing electrophoretograms of both species. Kinin-activating activity was not detected in *B. bovis*-infected erythrocyte or merozoite preparations at pH 4.2 or 7.6.

THE biochemistry and physiology of *Babesia* organisms has generally been documented for murine parasites. Rickard (12–14), studying the carbohydrate metabolism in *B. rodhaini*, found that lactate was the major end product of glucose metabolism and concluded that the overall metabolic pattern was very similar in both the parasite and host red blood cell (RBC). Momen (9) used starch gel electrophoresis to study metabolic differences between *Plasmodium* and *Babesia* in mice and indicated that three enzymes, lactate dehydrogenase (LDH), glucose phosphate isomerase (GPI), and glutamate dehydrogenase (GDH), of eight enzymes tested, were of parasite (*B. rodhaini*) origin. In a later work with several known *Babesia* spp. of rodents including two isolates of *B. microti* from humans (10), inter-species, but not intra-species, variation was demonstrated for GDH and LDH. Intraspecies clone variation (polymorphism) has been reported for GPI, LDH, and 6-phosphogluconate dehydrogenase (6-PGDH) in *P. falciparum* and *P. knowlesi* (3, 4, 18).

Proteolytic activity in *B. bovis* and *B. bigemina* has been documented (22). This esterase was found in particulate extracts prepared by treating infected RBC by sonic disruption or hypotonic shock and was later reported (23) to activate kininogen to kinin. It was suggested that the coagulation cascade was triggered indirectly through kallikrein activation and massive production of cryoprecipitable kininogen, leading to hypotensive shock in *B. bovis*-infected animals. The present study was oriented toward the identification of metabolic and kinin-activating enzymes in both *B. bovis*-infected RBC and spontaneously released merozoites.

MATERIALS AND METHODS

Parasites. a) The KBb strain of *B. bovis* (from Dr. K. L. Kuttler, USDA, Hemoparasitic Research Unit, WSU, Pullman, WA 99164), a Mexican isolate that had been maintained in culture for one year, was passaged through nine splenectomized calves, and returned to in vitro culture for two more years; b) nine tertiary clones (recloned twice) were derived from the KBb strain by the cloning procedure previously described (16) two months after passage in splenectomized calves; c) the Falcon strain of *B. bovis* (from Dr. K. L. Kuttler, USDA, Hemoparasitic Research Unit, WSU, Pullman, WA 99164) was originally isolated at the Falcon Experiment Station of the Texas A&M University from a clinical case, passaged through 13 splenectomized calves, and frozen at −70°C for two years, inoculated into a splenectomized calf, reisolated, and brought to in vitro culture for further work; d) the TCB strain of *B. bovis*, isolated from a clinical case at the municipality of Texcoco, Mexico State, Mexico, was maintained for one year frozen at −70°C, inoculated into a splenectomized calf, and brought to in vitro culture. The TCB strain of *B. bovis*, two *B. bigemina* strains (Mex and CBig), and a tertiary clone derived from CBig were obtained from The

[1] Supported in part by U.S. Department of Agriculture Agricultural Research Service Cooperative Agreement 58-9AHZ-2-655. Dr. Rodriguez was supported by grants provided by U.S. Agency for International Development DPE-5542-G-SS-3014-00 and DPE-5542-G-SS-4026-00. Dr. Vega was supported in part by a fellowship provided by Consejo Nacional de Ciencia y Tecnologia (an agency of the Mexican government) contract No. 09708 and a grant provided by U.S. Agency for International Development DPE 5542-G-SS-4026-00.

[2] Present address: U.S.U.H.S.-Microbiology, 4301 Jones Bridge Rd., Bethesda, MD 20814.

TABLE I. *Metabolic enzymes studied.*

Recommended name	Code number	Systematic name	Abbreviated name
Glutamate dehydrogenase (NADP)	E.C. 1.4.1.4	L-Glutamate: $NAD(P)^+$ oxidoreductase (deaminating)	GDH
Lactate dehydrogenase	E.C. 1.1.1.27	L-Lactate: NAD^+ oxidoreductase	LDH
Phosphogluconate dehydrogenase (decarboxylating)	E.C. 1.1.1.44	6-Phospho-D-gluconate: $NADP^+$ 2-oxidoreductase (decarboxylating)	6PGD
Glucose phosphate isomerase	E.C. 5.3.1.9	D-Glucose-6-phosphate ketolisomerase	GPI
Malate dehydrogenase	E.C. 1.1.1.37	L-Malate: NAD^+ oxidoreductase	MDH
Glucose-6-phosphate dehydrogenase	E.C. 1.1.1.49	D-Glucose-6-phosphate $NADP^+$ 1-oxidoreductase	G6PDH
Hexokinase	E.C. 2.7.1.1	ATP: D-hexose-6-phosphotransferase	HK
Adenylate kinase	E.C. 2.7.4.3	ATP: AMP phosphotransferase	AK

International Hemoprotozoan Project, Instituto Nacional de Investigaciones Pecuarias, Coajimalpa, Mexico D.F.

Purification of merozoites and infected red blood cells (iRBC). Percoll purification of merozoites and iRBC from in vitro culture is detailed elsewhere (17). Infected RBC's in blood collected from a splenectomized calf inoculated with the TCB strain of *B. bovis* were purified by the 100% Percoll procedure (17). *Babesia bigemina* strains and clones were concentrated using a Percoll linear gradient as described elsewhere (21). Cultures were centrifuged at 1000 *g* for 10 min, the packed RBC fraction layered over 68.46% Percoll, and the tubes centrifuged at 30,000 *g* for 6 min. Bands containing 90% iRBC were collected and processed as described for *B. bovis*.

Samples for metabolic enzyme testing. Merozoites and iRBC were adjusted to contain 1×10^6 iRBC or merozoites/μl. Preparations were frozen at −70°C, thawed, and then centrifuged at 10,000 *g* for 10 min in a microfuge (Fisher 235B). Supernatant fluids were aliquoted in 5-μl volumes and stored at −70°C until used. Suspensions of normal red blood cells (nRBC) were prepared in the same manner and adjusted to contain 2×10^6 RBC/μl.

Agarose gel electrophoresis. Gels were cast on chambers consisting of two 100 × 205-mm glass plates with a 1-mm thick "U"-shaped acrylic spacer. One glass plate was covered with a few drops of water and layered with a sheet of GelBond (FMC Corporation, Rockland, ME), hydrophilic side up. The spacer was set between the GelBond-covered plate and a second [illegible] plate. The chamber was held in place with paper clamps. [illegible] percent agarose gels were prepared using low (<0.10) electro[illegible] dosmosis HGT agarose (FMC Corporation) following manu[illegible] facturer's instructions. Agarose was suspended in appropri[illegible] buffer, heated to 100°C with constant gentle stirring, and [illegible] at this temperature for 10 to 15 min so that particles were [illegible] longer visible. The gel solution was allowed to cool to appro[illegible] imately 70°C and poured into prewarmed (70°C) casting cham[illegible] bers with the aid of a pipette, avoiding the formation of bubbles. Gels were cooled to room temperature and later stored at 4[illegible] in a humidity chamber. They were used 24 to 48 h after prep[illegible] aration. The covering glass plate was withdrawn with the spa[illegible] trenches of 4 × 1 mm were cut with a notched trough cutter (Gelman Instruments Co., Ann Arbor, MI), and 3 μl of sample (3×10^6 iRBC or merozoites) delivered into each trench. Trenches were refilled with warm agarose after absorption of the sample to avoid distortion during electrophoresis. The eight metabolic enzymes tested were glutamate dehydrogenase (GDH), lactate dehydrogenase (LDH), phosphogluconate dehydrogenase (6PGD), glucose phosphate isomerase (GPI), malate dehydrogenase (MDH), glucose-6-phosphate dehydrogenase (G6PDH), hexokinase (HK), and adenylate kinase (AK). The eight enzymes studied are listed in Table I. Enzyme activity was detected by the use of MTT-tetrazolium-linked color reactions as described elsewhere (2, 3). Specific reagents for each enzyme were purchased (Sigma Chemical Co., St. Louis, MO). The incubation buffer for GDH, LDH, 6PGD, and GPI was 0.05 M Tris-HCl, pH 8.0, and for MDH, G6PDH, HK, and AK was 0.10 M Tris-HCl (MDH, pH 8.5; G6PDH, pH 7.0; HK and AK, pH 7.4). After reaction, gels were destained, washed twice for 15 min in 10% glacial acetic acid, and dehydrated under two filter paper (Whatman No. 1). Approximately 2 cm of tower paper were placed over the filter and pressure applied with a 2-kg weight. After removing the weight, filter paper and paper towel, gels were dried in a forced-draft oven at 40°C and stored as permanent records.

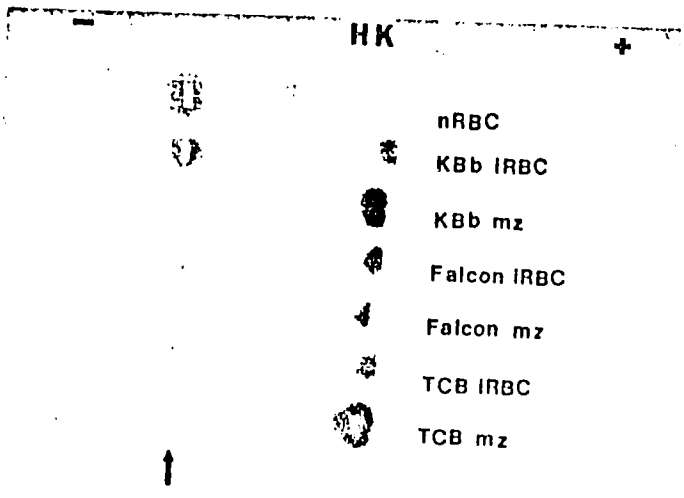


Fig. 1. Agarose gel electrophoresis of hexokinase (HK) of *B. bovis*. Arrow indicates point of origin. mz, merozoites; iRBC, infected RBC; nRBC, normal RBC.

Samples for kinin-activating esterase. Samples for kinin-activating esterase were prepared by hypotonic lysis and [illegible] disruption of parasite material as described (23). Percoll-purified merozoites and iRBC with parasitemias of 65% or higher were lysed with nine volumes of distilled water or sonicated until [illegible] cells remained intact (as assessed by light microscopy). Preparations were centrifuged at 3000 *g* for 15 min, and the sediment was mixed with nine volumes of distilled water. Both supernatant fluid and resuspended sediment were stored at [illegible] until used.

Esterolytic activity. Kinin-activating activity was assayed [illegible] lowing the procedure for measuring the rate of plasmin action on synthetic substrates (15). Fifty-μl samples of *B. bovis* extract [illegible]

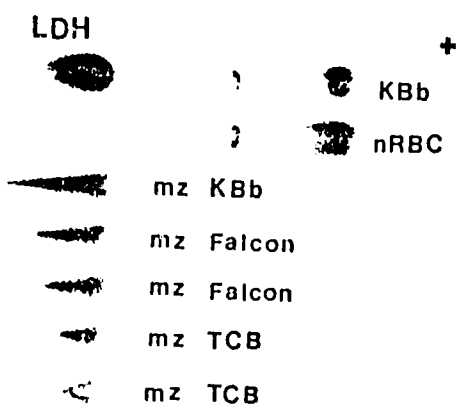


Fig. 2. Agarose gel electrophoresis of lactate dehydrogenase (LDH) of *B. bovis*. Arrow indicates point of origin. mz, merozoites; nRBC, normal RBC; KBb, RBC infected with KBb strain of *B. bovis*.

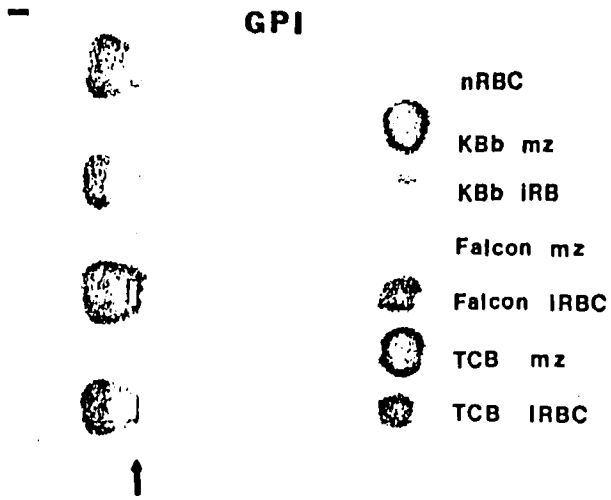


Fig. 3. Agarose gel electrophoresis of glucose phosphate isomerase (GPI) of *B. bovis*. Arrow indicates point of origin. mz, merozoites; iRBC, infected RBC; nRBC, normal RBC.

were delivered into centrifuge tubes (12 × 75) and incubated at 37°C for 60 min in the presence of 50 μl of 100 mM p-toluenesulfonyl-L-arginine methyl ester (TAME), and 100 μl of either 100 mM citrate buffer, pH 4.6, or 100 mM Tris-HCl, pH 8.6. Two hundred μl of 2 M hydroxylamine hydrochloride (stored at 4°C) and 200 μl of 3.5 N NaOH were delivered into each sample, the tubes allowed to stand for 30 min, and 200 μl of 1:1 TCA (one part concentrated HCl diluted with two parts distilled water, plus 6 g of trichloracetic acid dissolved in 100 ml of acid solution) were added. After 30 min at room temperature the tubes were centrifuged at 2000 *g* for 10 min. Five hundred μl of the supernatant fluid were mixed with 2000 μl of 0.1 M ferric chloride in 0.04 N HCl, pH 1.2 ± 0.1, and the color measured within 2 to 3 min of formation. Percent transmittance was compared in a Spectronic 21 (Bausch and Lomb, Rochester, NY) spectrophotometer at 525 nm against blanks prepared by mixing enzyme, substrate (TAME), and corresponding amounts of buffer and reagents. Trypsin (Trypsin 250, Difco), at 1% concentration in 0.01 N HCl, was used as the positive control. Controls for spontaneous TAME hydrolysis were prepared by substituting enzyme for an equivalent amount of appropriate buffer. The rate of disappearance of the ester in tubes containing *B. bovis* extracts minus the rate of the spontaneous hydrolysis control was equal to the rate of ester hydrolysis due to enzyme present in extracts.

Esterase 1 and 3. Presence of Es-1 and Es-3 was determined following the procedure described by Ruddle & Roderick (19), using cellulose acetate electrophoresis. Samples were prepared for kinin-activating esterase assay. One-microliter samples were applied to Titan III Isoflur (Helena Laboratories, CA) cellulose acetate plates previously stabilized in electrophoresis buffer (4 mM Tris, 191 mM glycine, pH 8.5). Plates were electrophoresed at 220 volts (constant voltage) for 30 min. Enzyme activity was revealed by the use of an overlay consisting of 1.0% agarose and 0.06% (w/v) of 4-methylumbelliferyl acetate in 100 mM phosphate buffer, pH 7. Agarose was suspended in 6 ml of buffer, heated to 100°C, and held at this temperature until no particles were visible. Substrate dissolved in 6 ml of buffer was mixed with the hot agarose solution and poured onto the cellulose plate after electrophoresis. Plates were allowed to stand at room temperature for 60 min and then exposed to short (280) and long (350) wave ultraviolet (uv) light.

RESULTS

No variation in the migration pattern of *B. bovis* clones or strains was detected, but clear inter-species variation was observed for parasite-specific enzymes.

Glutamate dehydrogenase. The GDH activity was observed as a faintly stained anodic band in *B. bovis*-infected RBC and merozoite preparations. No activity was detected in nRBC. There was no detectable difference between cloned material and original strains. Evidence of *B. bigemina* activity appeared in a slower mobility range than *B. bovis* (Fig. 4).

Glucose phosphate isomerase. Normal RBC samples showed two cathodic bands of activity, which were close together and stained strongly. *Babesia bovis* samples showed a single anodic band of activity (Figs. 3, 4), which migrated more slowly than the comparable pattern of *B. bigemina*.

Lactate dehydrogenase. Normal RBC presented three anodic bands of fast, intermediate, and slow mobility. The two faster bands were stained strongly while the third was broader, stained weakly, and was less anodic than the others. *Babesia bovis* LDH activity was also anodic in position (Figs. 2, 4), bordering the slow band of nRBC. *Babesia bigemina* activity was faster than *B. bovis* and located between the slow and intermediate nRBC bands.

Hexokinase. Normal RBC showed only one broad anodic band of negligible activity. *Babesia bovis* merozoites or iRBC showed one strongly stained anodic band that moved faster than the nRBC band (Figs. 1, 4). *Babesia bigemina* remained closer to the line of origin.

6-Phosphogluconate dehydrogenase, G6PDH, AK, and MDH. Parasite-specific enzymes could not be detected in any of the *B. bovis* or *B. bigemina* samples tested although activity was observed for each of these enzymes in nRBC extracts.

Kinin-activating esterase. No esterase activity was detected in the samples tested except for the positive (trypsin) control. Trypsin activity was almost absent at pH 4.2, degrading only 0.013 μM substrate/min at 37°C. Activity at pH 8.6, however, could not be distinguished from the spontaneous degradation control, which was total at 15 min. For this reason, a pH 7.6 buffer (100 mM Tris-HCl) was used. Spontaneous hydrolysis at the reduced pH was only 0.017 μM/min of substrate while trypsin action was complete, degrading all substrate by 30 min.

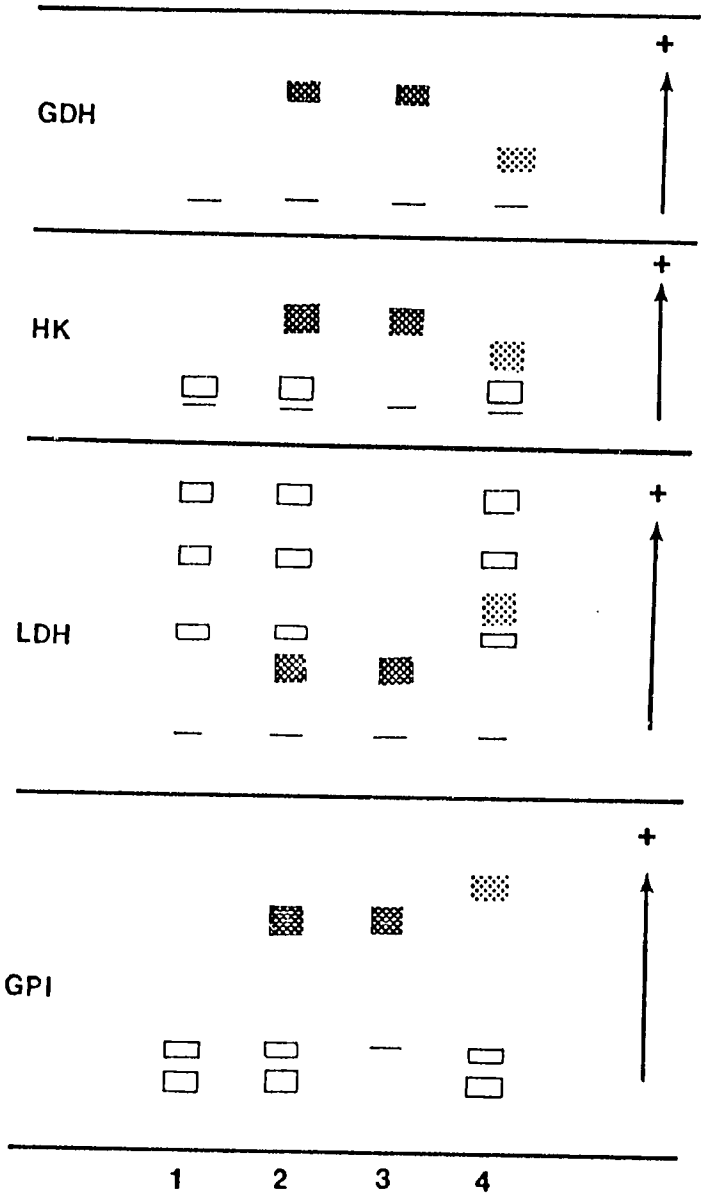


Fig. 4. Schematic representation of the migration pattern of glutamate dehydrogenase (GDH), hexokinase (HK), lactate dehydrogenase (LDH), and glucose phosphate isomerase (GPI). Lanes: 1. normal RBC; 2. *Babesia bovis*-infected RBC; 3. *B. bovis* merozoites; 4. *B. bigemina*-infected RBC.

Esterase 1 and 3. Two bands of activity were developed after uv exposure. One was located close to the origin, and a second band of fast mobility was located close to the anode. These bands of activity were found only in nRBC and iRBC but not in merozoite samples.

DISCUSSION

Results from the present investigation clearly demonstrate that only four metabolic enzymes, LDH, GDH, GPI, and HK, of eight tested are present in *B. bovis* and *B. bigemina*. This is in agreement with observations previously reported (9) for rodent *Babesia* and *Plasmodium* spp. In this study parasite from host GPI, LDH, and GDH, but not G6PDH, MDH, and 6PGDH, were differentiated in *B. rodhaini*. The presence of the same enzymes in *B. bigemina* and the absence of 6PGDH have been documented (22). In these previous works (9, 10), preparations consisting of lysates of iRBC were used. It was noted, however, that failure to detect parasite-derived enzymes was not proof of enzyme absence and could be due either to identical electrophoretic mobility of parasite and host enzymes or the possible presence of enzyme concentrations below the sensitivity of the assay (9). In the present study, use of *B. bovis* merozoite extracts minimized the possibility that host enzyme may mask the parasite activity. In addition, merozoite and iRBC preparations were highly concentrated and staining intensity was usually strong enough to demonstrate the presence of enzymes tested.

The presence of HK, GPI, and LDH is not surprising in view of previous reports (12–14) indicating glycolysis as the main energy production pathway in *Babesia*. Rickard (14) found G6PDH in extracts of *B. rodhaini*-infected RBC but questioned the presence of a functional pentose phosphate shunt since no other enzyme of this pathway could be detected; however, Momen (9) was unable to detect G6PDH. Since the present study ascribed no such activity to merozoites and activity of this enzyme is high in RBC, it is possible that Rickard mistakenly measured host cell activity. In addition it is reasonable to assume that GDH through glutamate deamination could supply, at least in part, NADH or NADPH for other synthetic and degradative purposes. Absence of MDH is in conflict with Rickard's observations that high levels of activity occur in *B. rodhaini* extracts. Momen (9), however, found this enzyme in host RBC preparations and noted that reticulocytes accounted for most of the activity. Rickard (14) had not removed reticulocytes from his preparations and did not include controls for immature erythrocytes. Polymorphism of the parasite-specific enzymes was not observed either in cloned material or strains from different geographic areas. The KBb and Falcon strains were passaged through splenectomized calves (9 and 13 times, respectively) and maintained for at least one month in culture before testing. It could therefore be argued that selection may have occurred, thus eliminating previously existing subpopulations with different electrophoretograms. This phenomenon is known to occur in *Plasmodium* (1, 3, 5, 18) and *Theileria* (7, 8, 11). It seems not to be the case with *B. bovis* since passage of a clinical isolate to a splenectomized calf and further cultivation in vitro for one month showed no changes in serial electrophoretograms. This is in agreement with previously reported observations in *B. microti* strains (10) and *B. bigemina*-cloned materials (20).

Inter-species variation in the electrophoretic migration of parasite-derived enzyme was noted when electrophoretograms of *B. bovis* and *B. bigemina* were compared. Similar observations were reported for *B. hylomysci*, *B. microti*, *B. muratovi*, and *B. rodhaini* (10). Other bovine babesias have not been compared but this system may be useful for taxonomic purposes. Hoare (6), for example, questioned the validity of the taxonomic separation of *B. bovis* and *B. divergens*, which are very similar morphologically.

Kinin-activating esterase was not detected in samples of *B. bovis* clones or strains. This is in contrast with observations reported earlier (23) in which high levels of activity (0.7 enzyme units/ml) were found in extracts prepared in a manner similar to the present study. Further studies (22, 24) showed that vaccine strains, attenuated by rapid serial passages in at least nine splenectomized calves (1), produced no esterase and suggested that this enzyme was related to virulence by virtue of its kinin-activating function through conversion of plasma prekallikrein into the active kallikrein. In the present study, clones tested for this enzyme were derived from one strain (KBb) after passage nine times in splenectomized calves, and another (Falcon) strain after 13 similar passages. Samples from the TCB strain, prepared from infected blood or in vitro culture, had no activity at all. Preliminary work (S. D. Rodriguez, MS Thesis, U. Missouri

(3) indicated that a primary clone derived from the KBb behaved in the same manner as Australian vaccine strains, causing a mild reaction in vaccinates but reverting to full virulence when subinoculated. Thus, it seems possible that the virulence is an inducible or repressible trait. This, however, does not explain the absence of esterase activity in TCB strain samples. A study including samples from other virulent strains seems to be wanted to clarify the problem.

LITERATURE CITED

1. Callow, L. L., Mellors, L. T. & McGregor, W. 1979. Reduction of virulence of *Babesia bovis* due to rapid passage in splenectomized calves. *Int. J. Parasitol.*, 9: 333–338.
2. Carter, R. 1973. Enzyme variation in *Plasmodium berghei* and *Plasmodium vinckei*. *Parasitology*, 66: 297–307.
3. ——— 1978. Studies on enzyme variation in the murine malaria parasites *Plasmodium berghei*, *P. yoelii*, *P. vinckei* and *P. chabaudi* by starch gel electrophoresis. *Parasitology*, 76: 241–267.
4. Carter, R. & Voller, A. 1973. Enzyme typing of malaria parasites. *Trop. Med. J.*, 1: 141–150.
5. Graves, P. M., Carter, R., Keystone, J. S. & Seeley, C. 1984. Drug sensitivity and isoenzyme type in cloned lines of *Plasmodium falciparum*. *Am. J. Trop. Med. Hyg.*, 33: 212–219.
6. Hoare, C. A. 1980. Comparative aspects of human babesiosis. *Proc. R. Soc. Trop. Med. Hyg.*, 74: 143–148.
7. Melrose, T. R. & Brown, C. G. D. 1979. Isoenzyme variation in piroplasms isolated from bovine blood infected with *Theileria annulata* and *T. parva*. *Res. Vet. Sci.*, 27: 379–381.
8. Melrose, T. R. & Sharma, R. D. 1980. Glucose phosphate isomerase isoenzyme patterns in bovine lymphoblastoid cell lines infected with *Theileria annulata* and *T. parva*, with an improved enzyme visualization method using meldola blue. *Res. Vet. Sci.*, 29: 298–304.
9. Momen, H. 1979. Biochemistry of intraerythrocytic parasites. I. Identification of enzymes of parasite origin by starch-gel electrophoresis. *Ann. Trop. Med. Parasitol.*, 73: 109–115.
10. Momen, H., Chance, M. L. & Peters, W. 1979. Biochemistry of intraerythrocytic parasites. III. Biochemical taxonomy of rodent *Babesia*. *Ann. Trop. Med. Parasitol.*, 73: 203–212.
11. Musisi, F. L., Kilgour, V., Brown, C. G. D. & Morzaria, S. P. 1981. Preliminary investigation on isoenzyme variants of lymphoblastoid cell lines infected with *Theileria* species. *Res. Vet. Sci.*, 30: 38–43.
12. Rickard, M. D. 1969. Carbohydrate metabolism in *Babesia rodhaini*: differences in the metabolism of normal and infected rat erythrocytes. *Exp. Parasitol.*, 25: 16–31.
13. ——— 1970. Carbohydrate metabolism in *Babesia rodhaini*: carbohydrate metabolism and infectivity for rats of cells freed from host erythrocytes. *Exp. Parasitol.*, 27: 136–142.
14. ——— 1970. Carbohydrate metabolism in *Babesia rodhaini*: investigations with 14-C-labeled substrate and enzyme assays. *Exp. Parasitol.*, 28: 512–520.
15. Roberts, P. S. 1958. Measurement of the rate of plasmin action on synthetic substrates. *J. Biol. Chem.*, 232: 285–291.
16. Rodriguez, S. D., Buening, G. M., Green, T. J. & Carson, C. A. 1983. Cloning of *Babesia bovis* by in vitro cultivation. *Infect. Immun.*, 42: 15–18.
17. Rodriguez, S. D., Buening, G. M., Vega, C. A. & Carson, C. A. 1986. Purification and concentration of *Babesia bovis* merozoites and infected bovine erythrocytes. *Exp. Parasitol.*, 61: 236–243.
18. Rosario, V. 1981. Cloning of naturally occurring mixed infections of malaria parasites. *Science*, 212: 1037–1038.
19. Ruddle, F. H. & Roderick, T. H. 1966. The genetic control of two types of esterase in inbred strains of the mouse. *Genetics*, 54: 191–202.
20. Vega, C. A., Buening, G. M., Rodriguez, S. D. & Carson, C. A. 1986. Cloning of in vitro-propagated *Babesia bigemina*. *Vet. Parasitol.* (in press)
21. ——— 1986. Concentration and enzyme content of in vitro-cultured *Babesia bigemina*-infected erythrocytes. *J. Protozool.*, 33: 514–518.
22. Wright, I. G. 1975. The probable role of *Babesia argentina* esterase in the in vitro activation of plasma prekallikrein. *Vet. Parasitol.*, 1: 91–96.
23. Wright, I. G. & Goodger, B. V. 1973. Proteolytic enzyme activity in the intra-erythrocytic parasites *Babesia bovis* and *Babesia bigemina*. *Z. Parasitenkd.*, 42: 213–220.
24. Wright, I. G., Goodger, B. V. & Mahoney, D. F. 1981. Virulent and avirulent strains of *Babesia bovis*: the relationship between parasite protease content and pathophysiological effect of the strain. *J. Protozool.*, 28: 118–120.

Received 31 VII 85; accepted 23 VI 86

J. Protozool., 33(4), 1986, pp. 511–513
© 1986 by the Society of Protozoologists

Cultivation of *Phytomonas françai* Associated with Poor Development of Root System of Cassava[1]

MARILENE HENNING VAINSTEIN and ISAAC ROITMAN[2]
Departamento de Biologia Celular, Universidade de Brasília, 70.910 Brasília DF, Brazil

ABSTRACT. In the state of Espirito Santo, Brazil, a cassava disease was recently described, and subsequently a high density of trypanosomatids was revealed in the latex of unhealthy plants. To better characterize this flagellate, *Phytomonas françai*, we tried to grow it axenically. Successful results were obtained using a biphasic medium containing rabbit blood in the solid phase and a defined medium as overlay.

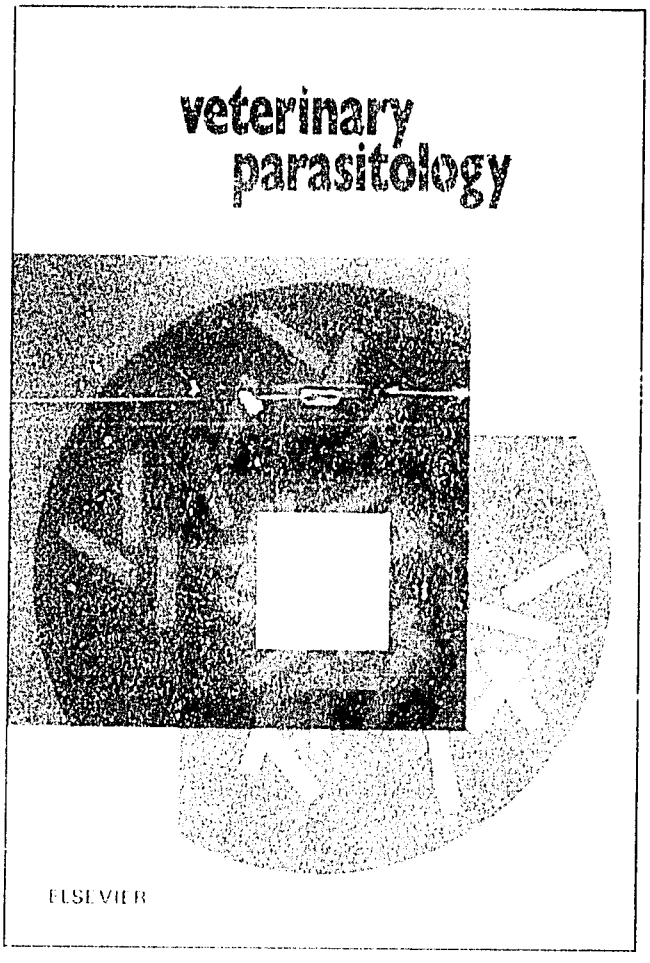
TRYPANOSOMATID flagellates have been described in the laticiferous tubes of several Euphorbiaceae and Asclepiadaceae species after the first report by Lafont in 1909 (12). Donovan (7) suggested the generic name *Phytomonas* for the organisms in latex plants. There are probably six species described in the genus, each from a different family of plants (13).

There are three well-known cases in which *Phytomonas* has proved to be the only microorganism specifically associated with lethal plant diseases: the phloem necrosis of *Coffea liberica* (19), "marchitez" of oil palm (6), and the "hartrot" of coconut palm (17). Recently a species of *Phytomonas françai* was found associated with a disease known as "chochamento das raizes," which means "empty roots," in the cassava (*Manihot esculenta* Crantz); the disease is characterized by a poor root system development and general chlorosis of the aerial part of the plant (11). The present paper gives details of the isolation and cultivation of these flagellates from the cassava laticiferous ducts.

[1] Investigation supported by the National Research Council of Brazil (CNPq). We thank Dr. Philip Marsden for help with the manuscript.
[2] To whom correspondence should be sent.

ATTACHMENT E

REPRINTED FROM:



ELSEVIER SCIENCE PUBLISHERS, AMSTERDAM

veterinary parasitology

An International Scientific Journal

Editor-in-Chief:
S.M. Gaafar, Department of Veterinary Microbiology, Purdue University, West Lafayette, IN 47907, U.S.A.

Aims and scope.
This journal is concerned with those aspects of helminthology, protozoology and entomology which are of interest to animal health investigators, veterinary practitioners and others with a special interest in parasitology. Papers of the highest quality dealing with all aspects of disease prevention, pathology, treatment, epidemiology and control of parasites in all animals which can be regarded as being useful to man, fall within the scope of the journal. It is intended to give special attention to new aspects of veterinary parasitology, such as problems arising from the intensification of industrialized production systems and mass production, problems of game animals which may be of epizootiological significance in domestic animals, etc. Parasitological studies on laboratory animals fall within the scope of the journal only if they provide a reasonably close model of a disease of domestic animals. Although the journal primarily publishes experimental and clinical research papers, occasionally unique case histories may also be published. Papers on the taxonomy of parasites do not fall within the scope of the journal.

Submission of articles.
Manuscripts should be submitted in triplicate to the Editorial Secretariat, Veterinary Parasitology, P.O. Box 330, 1000 AH Amsterdam, The Netherlands. Authors in N. America should submit their manuscripts directly to Professor Gaafar.

VETERINARY PARASITOLOGY HAS NO PAGE CHARGES
A DETAILED GUIDE TO AUTHORS AND A FREE SAMPLE COPY OF THE JOURNAL
ARE AVAILABLE ON REQUEST

Veterinary Parasitology, 22 (1986) 223—233
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CLONING OF IN VITRO PROPAGATED *BABESIA BIGEMINA*

C.A. VEGA[1], G.M. BUENING[2], S.D. RODRIGUEZ[2] and C.A. CARSON[2]

[1] *Depto. Hemoprotozoarios, INIP, Km. 15 1/2 Carr. Mex., Tol. Palo Alto, D.F., C.P. 05110 (Mexico)*
[2] *Department of Veterinary Microbiology, College of Veterinary Medicine, University of Missouri, Columbia, MO 65211 (U.S.A.)*

(Accepted for publication 21 November 1985)

ABSTRACT

Vega, C.A., Buening, G.M., Rodriguez, S.D. and Carson, C.A., 1986. Cloning of in vitro propagated *Babesia bigemina*. *Vet. Parasitol.*, 22: 223–233.

The original *Babesia bigemina* culture conditions were modified with regard to infected bovine erythrocyte concentration and atmospheric environment. A procedure was designed which would yield a homogeneous parasite population, beginning with a single infected erythrocyte. Calculated dilutions were made in 96-well tissue culture plates to approach one infected erythrocyte per four wells. Growth of parasites in wells was detected between 16 and 28 days after cultures were initiated. Clones were transferred to 24-well tissue culture plates for regular maintenance. Three primary clones were selected for additional recloning. The probability that the parasites detected in one well are the progeny of a single infected erythrocyte approaches 0.99 for tertiary clones.

INTRODUCTION

Bovine babesiosis is considered among the most important tick-borne diseases of cattle in tropical and subtropical regions of the world. *Babesia bigemina* and *B. bovis* are recognized as the etiologic agents of the disease in the Americas.

Studies of intra-erythrocytic protozoan parasites have been advanced by the development of procedures that allow their replication under controlled laboratory conditions. Cloning of *Plasmodium falciparum* has employed one of two methods, limiting dilution (Rosario, 1981) and direct microscopic selection (Trager, 1981), utilizing the in vitro culture conditions developed by Trager and Jensen (1976).

Continuous in vitro cultivation of *B. bovis* has been reported by Levy and Ristic (1980) and Erp et al. (1980). Rodriguez et al. (1983) developed a cloning procedure after refining the *B. bovis* culture method. In the latter case a limiting dilution scheme was used and cultures were maintained under a low oxygen atmosphere. Vega et al. (1985a) described a continuous in

0304-4017/86/$03.50 © 1986 Elsevier Science Publishers B.V.

vitro culture procedure for *B. bigemina*, in which a suspension of infected bovine erythrocytes was maintained under low oxygen conditions. In this method, relatively low numbers of infected erythrocytes (0.03% parasitemia) have been used to successfully initiate cultures. The percentage of parasitized erythrocytes (PPE) obtained, however, rarely exceeded 3%. The present investigation was designed to define optimal culture conditions and devise a procedure for cloning *B. bigemina*.

MATERIALS AND METHODS

Babesia bigemina strain

The source of parasites was a Mexican strain provided by Dr. K.L. Kuttler (USDA, Hemoparasitic Disease Unit, Pullman, WA99164). Three different sub-isolates (S, R, K) of the same strain were employed and maintained in continuous culture prior to cloning.

Culture conditions

General culture conditions were basically the same as those described by Vega et al. (1985a). The following experiments were carried out to refine the culture method for the purpose of cloning.

Experiment 1: Determination of optimum red blood cell (RBC) concentration

To establish the limits for the *B. bigemina* culture procedure, five concentrations of red blood cells were compared. A thin smear was prepared from cultures of *B. bigemina*, stained by the Giemsa method and the PPE was determined by counting the number of infected and normal cells in $\geq$ 18 microscopic fields. The PPE was adjusted to 0.1%. Packed erythrocytes were resuspended to a 40% (v/v) concentration by addition of fresh complete medium. Serial 2-fold dilutions with complete fresh medium were made to obtain 40, 20, 10, 5 and 2.5% (v/v) RBC concentrations. Each suspension was then distributed in duplicate into a 24-well plate (Costar, Cambridge, MA 21793) (16-mm ID) in 805-μl volumes with 4 mm depth. The plate was incubated at 37°C in humid 5% CO_2, 2% O_2, 93% N_2 atmosphere. Spent medium was replaced daily with 400 μl fresh medium. Parasite development was monitored by preparing Giemsa-stained thin smears from either of the two culture wells for each different RBC suspension. At 72 h all duplicate cultures were sampled, pooled accordingly and diluted 1:10 with the appropriate nRBC's suspension. The experiment was terminated after 6 days.

Experiment 2: Determination of the effect of 'seed' density

This experiment was designed to assess the possibility of initiating cultures with very low numbers of parasites. Two culture wells containing replicating

B. bigemina parasites in a 10% RBC (v/v) concentration with a PPE of 1.0% were pooled and adjusted to a 5% RBC (v/v) by the addition of fresh medium. Serial 10-fold dilutions were prepared by mixing one volume of the original 5% RBC (v/v) suspension containing 1% infected RBC (iRBC) with 9 volumes of a 5% (v/v) normal RBC (nRBC) suspension in fresh medium. Thus, cultures were initiated with 0.1, 0.01, 0.001, and 0.0001 PPE. Duplicate wells were filled with 805 μl of each dilution in a 24-well tissue culture cluster plate, and incubated at 37°C in a humid 5% CO_2, 2% O_2, 93% N_2 atmosphere. Six hundred μl spent medium per well was exchanged daily. A 2.5% (v/v) nRBC suspension in fresh medium was added on Days 3 and 7 in 600-μl volumes instead of fresh medium exchange. Subcultures were made by 1:2 dilution on Day 5, and 1:4 dilution on Days 9 and 12 after initiation of cultures. Samples of 3—5 μl were obtained from duplicate wells to monitor parasite development through the use of Giemsa-stained thin blood smears. The experiment was terminated after 15 days.

Experiment 3: Determination of optimal fluid level

This experiment was designed to observe the effect of five different fluid levels in four different atmospheres. Several culture plates containing *B. bigemina* iRBC in a 10% v/v concentration were pooled. This suspension was diluted 1:6 with a 10% (v/v) suspension of nRBC in fresh medium to obtain a PPE of 0.05. This suspension was dispensed into four 24-well tissue culture cluster plates in volumes of 603, 804, 1005, 1206 and 1407 μl in duplicate wells for 3, 4, 5, 6 and 7 mm depth, respectively. One plate was incubated at 37°C under each of the following atmospheres: A = 5% CO_2, 2% O_2, 93% N_2; B = 5% CO_2, 5% O_2, 90% N_2; C = 5% CO_2, 10% O_2, 85% N_2 and D = 5% CO_2 in air (approximately 18—20% O_2) in humid chambers. Proportional amounts of medium (75% of volume) were exchanged daily. Each duplicate culture was pooled after medium exchange at 72 h and subcultured by 1:10 dilution, with a 10% (v/v) nRBC suspension in fresh medium. Parasite growth was monitored daily by examination of Giemsa-stained thin blood smears obtained from either duplicate well. The experiment was concluded after 6 days.

Experiment 4: Development of a cloning scheme

Duplicate cultures, which contained a majority of singly-infected erythrocytes, were pooled after medium exchange and mixed with 1.0 ml of VYM solution (Vega et al., 1985a). The initial PPE was determined by counting 18 fields of two thin blood smears by the Giemsa method. A sample of the suspension was used to determine erythrocyte concentration by the Newbauer hemacytometer method. Concentration of erythrocytes was adjusted to contain 10^6 iRBC in 200 μl (based on the average PPE), by the addition of fresh culture medium. Initially a 1:10 dilution was made by transferring 20 μl (10^5 iRBC) of the above suspension into a well (96-well microtiter plate; 9-mm ID) containing 180 μl of fresh medium. Serial 10-fold dilutions were

carried out in single wells in 200 μl volumes up to 10^{-5}. Ten iRBC were theoretically present in the final 200-μl volume. A final 1:10 dilution was made by transferring 20-μl volumes of the previous dilution to 10 different wells, all containing 180 μl of fresh medium. The final 200-μl volume (theoretically containing a single iRBC) was divided into four aliquots of 50 μl each in another 96-well microtiter plate (average 0.25 iRBC/well). These ultimate wells constituted a cloning set. Ten cloning sets were contained in a single plate. The peripheral wells of the plate were filled with sterile distilled H_2O to prevent excessive evaporation. Seeded wells were replenished to a final 160-μl volume (5 mm depth), by addition of a 5% (v/v) nRBC suspension in fresh medium. All plates were incubated at 37°C in a humid atmosphere containing 5% CO_2, 5% O_2, 90% N_2. Fresh culture medium in 100-μl volumes per well was exchanged daily. Addition of 100 μl $well^{-1}$ of a 2.5% (v/v) nRBC suspension in fresh medium was carried out at 48-h intervals after subculture. Subcultures were made every 96 h by a 1:2 dilution with 5% (v/v) nRBC suspension in fresh medium. Detection of parasites was done by preparing thin blood smears for each well at the time of subculture, beginning at 384 h (16 days) after initiation of cloning and every 96 h thereafter up to 768 h (32 days). If the number of parasites found in a smear had a PPE of $\geqslant$ 0.3% the contents of that single positive well were transferred to another well in a 24-well plate. The culture was expanded for continuous cultivation by adding a 5% (v/v) nRBC suspension in fresh medium to a total volume of 1005 μl $well^{-1}$ (5 mm depth) and incubated under the same atmospheric conditions. Clones are designated primary if obtained from the initial cloning procedure. Repeated cloning, or recloning, results in the isolation of secondary and tertiary clones.

It was assumed that the results of the cloning procedure would fit the Poisson distribution for rare events. The model used for the determination of the probability values is the same as the one described by Graves et al. (1984) for *P. falciparum* where the probability that the parasites detected in one well are the progeny of a single iRBC is $\mu e^{-\mu}/(1 - e^{-\mu})$, and the μ value was estimated as the average density of parasites per well per plate using the formula $\mu = 1n$ (1 + total no. positive wells/total no. negative wells).

RESULTS

Experiment 1: Optimum red blood cell concentration

The growth pattern of *B. bigemina* was affected by low and high concentrations of cells (Table I). Suspensions containing 5–10% volumes supported increased replication of the parasite. Total yield of iRBC was largest using a 10% suspension, and all effects were more apparent after subculture at a 1:10 dilution after 72 h in culture.

TABLE I

Effect of red blood cell concentration on the in vitro growth of *Babesia bigemina*

% RBC	Hours in culture						
	0[a] PPE	24	48	72[b]	96	120	144
2.5	0.1	0.183	0.222	0.059	0.000	0.025	0.000
5.0	0.1	0.180	0.652	0.375	0.138	0.233	1.016
10.0	0.1	0.173	0.374	0.487	0.091	0.225	1.064
20.0	0.1	0.170	0.263	0.220	0.044	0.090	0.547
40.0	0.1	0.124	0.064	0.012	0.000	0.020	0.018

[a]Cultures were initiated with 0.1% parasitized erythrocytes (PPE).
[b]Subculture by dilution 1:10.

Experiment 2: effect of 'seed' density

The time required for parasite detection in vitro was extended as decreased numbers of parasites were used to initiate cultures (Table II). Addition of fresh nRBC favored multiplication of the parasite, and extended the time required for subculture. No detrimental effect on parasite growth was observed at the low dilutions (1:2 and 1:4) when the PPE was low prior to subculture. Without adjusting data for the dilution effect, a 100-fold increase in the number of parasites was noted between 6 and 9 days in cultures initiated with PPE $\leqslant$ 0.01.

Experiment 3: determination of optimal fluid level

Fluid levels > 6 mm diminished parasite growth when oxygen tension was $\leqslant$ 10% (Table III). The highest PPE of 3.322 was observed after 144 h in culture with 5 mm fluid level under a 5% O_2 atmosphere. Total parasite yields were optimal when *B. bigemina* cultures were maintained under a humid atmosphere composed of 5% CO_2, 5% O_2 and 90% N_2.

Experiment 4: cloning

More than 20 primary clones were obtained from each of the three subisolates, yielding low probability values (Table IV). The probability that clones were begun from a single iRBC increased after secondary and tertiary procedures (Table IV). Positive wells were detected as early as 16 days and all clones were harvested and expanded between 20 and 28 days after initiation of the cloning procedure. Wells in which parasites were not detected by Day 32 were considered negative and discarded.

Primary and secondary clones maintained in vitro were randomly selected for further cloning. Recloning was not totally predictable, however, since

TABLE II

Effect of the initial seed density (percent parasitized erythrocytes) on continuous in vitro growth of *Babesia bigemina*

Days in culture															
0	1	2	3[a]	4	5	6	7[a]	8	9	10	11	12	13	14	15
0.10[b]	0.068	0.250	0.828	1.317	2.332	0.800	1.155	1.229	Discontinued						
0.01	ND	ND	0.021	0.075	0.312	0.493	1.409	1.626	Discontinued						
0.001	ND	ND	ND	ND	+	+	0.028	0.085	0.385	0.563	1.239	1.528	Discontinued		
0.0001	ND	ND	ND	ND	ND	ND	ND	+	0.011	0.012	0.109	0.353	0.370	1.083	1.345

[a]Replacement 2.5% nRBC Days 3 and 7, subculture by dilution 1:2 on Day 5 and 1:4 on Days 9 and 12.
[b]Seed density of initiating culture in percentage of parasitized erythrocytes (PPE).
+ = Positive but too low to count.
ND = Not determined.

TABLE III

Effect of fluid level under different atmospheric environments on the in vitro growth of *Babesia bigemina*

Depth (mm)	Hours in culture							Atmosphere
	0[a]	24	48	72[b]	96	120	144	
3	0.05	0.207	1.149	2.294	1.232	1.540	2.001	A (2% O_2)
4	0.05	0.217	1.118	2.271	1.139	2.498	2.150	
5	0.05	0.242	1.235	1.938	0.180	0.940	1.844	
6	0.05	0.110	0.897	2.103	0.955	1.379	1.539	
7	0.05	0.236	1.240	1.532	0.613	1.338	0.989	
3	0.05	0.484	1.354	2.535	1.055	3.223	2.409	B (5% O_2)
4	0.05	0.157	1.004	2.377	1.255	2.542	2.280	
5	0.05	0.379	1.259	1.888	0.882	2.294	3.322	
6	0.05	0.336	1.183	1.304	0.787	2.003	2.203	
7	0.05	0.307	1.200	1.743	0.709	1.401	1.926	
3	0.05	0.147	1.682	2.219	0.666	1.718	2.027	C (10% O_2)
4	0.05	0.273	1.116	2.174	0.962	1.364	1.932	
5	0.05	0.194	1.028	1.676	0.601	1.767	1.761	
6	0.05	0.233	1.111	1.713	0.689	2.305	1.803	
7	0.05	0.402	0.857	1.686	0.928	1.785	1.805	
3	0.05	0.022	+	+	+		-	D (20% O_2 approx.)
4	0.05	0.017	+	+	+		-	
5	0.05	0.022	+	+	+		–	
6	0.05	0.075	0.008	+	+	+	-	
7	0.05	0.115	0.023	+	+	+	+	

[a]Initial percentage of parasitized erythrocytes (PPE) = 0.05 at 0 h.
[b]Subculture by dilution 1:10.
+ = Positive but too low to count.
– = Negative.

derived clones initial growth rates in subsequent recloning did not prove to be uniform and several attempts to reclone primary clones derived from the S-sub-isolate were unsuccessful.

DISCUSSION

A sensitive method for in vitro cloning of *B. bigemina* parasites was developed. Contrary to our expectations, a red cell concentration of 2.5% did not support parasite development under in vitro conditions. In contrast, cloning procedures for *P. falciparum* (Rosario, 1981; Trager et al., 1981; Graves et al., 1984) were successful at an initial packed cell volume of 2%. Apparently a strong contact or pressure between host cells was required for *B. bigemina* to penetrate into new cells. The detrimental effect observed with higher concentrations of cells may be explained by the competition for nutrients.

TABLE IV

Derivation of *B. bigemina* clones: identification of sub-isolates, time in culture and associated probability values[a]

K-sub-isolate	R-sub-isolate	S-sub-isolate
52 days in culture primary cloning (x = 0.85 parasites/well)	138 days in culture primary cloning (x = 1.38 parasites/well)	177 days in culture primary cloning (x = 0.79 parasites/well)
III-KC11[b] + 22 primary clones (p_1 = 0.63)	III-RF8 + 29 primary clones (p_1 = 0.[illegible]6)	III-SE2 + 21 more primary clones (p_1 = 0.65)
96 days in culture secondary cloning (x = 0.10 parasites/well)	73 days in culture secondary cloning (x = 0.07 parasites/well)	
III-KC11C10 + 3 more secondary clones (p_2 = 0.94) p_{12} = 0.98[a]	III-RF8F3 + 2 more secondary clones (p_2 = 0.96) p_{12} = 0.97[a]	
58 days in culture tertiary cloning (x = 0.10 parasites/well)	11 days in culture tertiary cloning (x = 0.10 parasites/well)	
III-KC11C10E9 + 3 more tertiary clones (p_3 = 0.94) p_{123} = 0.99[a]	III-RF8F3C3 + 3 more tertiary clones (p_3 = 0.94) p_{123} = 0.99[a]	

aAfter each recloning, the probability that a line arose from a single parasite was obtained by multiplying together the probability that a line arose from more than one parasite in each individual cloning and subtracting from 1.
bClone identification number.

A favorable effect on parasite maintenance was observed when a suspension of fresh normal bovine erythrocytes was added at intervals between subcultures, particularly when parasite numbers are small. This same effect was not observed when the number of parasites was > 1 PPE.

Normal parasite development was improved when cultures were maintained under low oxygen tension, especially at the level of 5% O_2. Vega et al. (1985b) had shown that when cultures were started from cryopreserved samples, optimum growth was obtained using a similar concentration of O_2 in the atmospheric environment. Higher fluid levels were less efficient in sustaining parasite growth compared with lower fluid levels, although the effect is minimized if cultures are maintained in atmospheres with ≤ 10% O_2. When cultures are initiated with low numbers of parasites, high oxygen tensions of 18–20% obtained with a regular CO_2 incubator do not favor growth. Increasing the fluid levels up to 7 mm does not seem to be sufficient to maintain an adequate microaerophilic environment for *B. bigemina*.

Considering the large number of clones derived from the first attempts, also representing low probability values (0.65, 0.46 and 0.63), it is still possible that such populations might not be homogeneous. Therefore recloning two additional times was desirable.

B. bovis clones appeared to have a defined growth rate (Rodriguez et al., 1983). The concept of constant growth rate for *B. bigemina* clones in vitro was not observed. It has been reported that in inoculated animals, *B. bigemina* apparently multiplies faster than other bovine babesia species (Sergent et al., 1927). Callow and Hoyte (1961) and Bishop et al. (1973) were able to separate *B. bigemina* from *B. bovis* by rapid passes in splenectomized calves. In addition, the doubling time in the blood of infected animals has been estimated to be 8.13 h (Wright and Goodger, 1977). In culture, a PPE of 0.3% is a good estimate of parasite development if one considers that clones are initiated with only one iRBC. Also values > 2% are not easy to obtain with the culture conditions described in this report. The PPE of 0.3% represents approximately 3×10^3 iRBC in 10^6 erythrocytes. By simple calculation these 3×10^4 iRBC will convert into 2.4×10^4 iRBC in 24 h and 23 min if the doubling time of 8.13 h is considered. This appears not to be the case in culture; 2.4×10^4 iRBC represents a 2.4% PPE and this is usually not achieved until after 48 h in culture. Therefore, the doubling time in culture must be longer than that observed in inoculated animals. The in vitro culture method still has some limitations. Future studies are required to determine the true doubling time for *B. bigemina* in culture.

It has been known that individual isolates of *Plasmodium falciparum* are heterogeneous (Rosario, 1981; Trager et al., 1981; Graves et al., 1984). Although exactly the same phenomenon has not been described for *B. bigemina*, it has been documented that avirulent strains can be derived after passage in intact calves (Dalgliesh et al., 1981). Apparently, such manipulation allowed the selection of avirulent subpopulations for use as immunizing material (Mellors et al., 1982). It is possible that this and other biochemical and im-

munological properties of the strains or sub-populations of *B. bigemina* could be better differentiated or demonstrated if they are selectively separated by in vitro cloning. These and many other potential applications of the *B. bigemina* cloning procedure have been projected.

ACKNOWLEDGEMENTS

Supported in part by US Department of Agriculture, Agricultural Research Service Cooperative Agreement 58-9AHZ-2-655. Dr. Vega was supported in part by a fellowship provided by Consejo Nacional de Ciencia y Tecnologia (an agency of the Mexican government) contract No. 09708 and a grant provided by US Agency for International Development DPE-5542-G-SS-4026-00. Dr. Rodriguez was supported by grants provided by US Agency for International Development DPE-5542-G-SS-3014-00 and DPE-5542-G-SS-4026-00.

REFERENCES

Bishop, J.P., Adams, L.G., Thompson, K.C. and Corner, D.E., 1973. The isolation, separation and preservation of *Babesia bigemina*. Trop. Anim. Health Prod., 5: 141—145.

Callow, L.L. and Hoyte, H.M.D., 1961. The separation of *Babesia bigemina* from *Babesia argentina* and *Theileria mutans*. Aust. Vet. J., 37: 66—70.

Dalgliesh, R.J., Callow, L.L., Mellors, L.T. and McGregor, W., 1981. Development of a highly infective *Babesia bigemina* vaccine of reduced virulence. Aust. Vet. J., 57: 8—11.

Erp, E.E., Smith, R.D., Ristic, M. and Osorno, M.B., 1980. Continuous in vitro cultivation of *Babesia bovis*. Am. J. Vet. Res., 41: 1141—1142.

Graves, P.M., Carter, R., Keystone, J.S. and Seeley Jr., D.C., 1984. Drug sensitivity and isoenzyme type in cloned lines of *Plasmodium falciparum*. Am. J. Trop. Med. Hyg., 33: 212—219.

Levy, M. and Ristic, M., 1980. *Babesia bovis*: Continuous cultivation in a microaerophilus stationary phase culture. Science, 207: 1218—1220.

Mellors, L.T., Dalgliesh, R.J., Timms, P., Rodwell, B.J. and Callow, L.L., 1982. Preparation and laboratory testing of a frozen vaccine containing *Babesia bovis*, *Babesia bigemina* and *Anaplasma centrale*. Res. Vet. Sci., 32: 194—197.

Rodriguez, S.D., Buening, G.M., Green, T.J. and Carson, C.A., 1983. Cloning of *Babesia bovis* by in vitro cultivation. Infect. Imm., 42: 15—18.

Rosario, V., 1981. Cloning of naturally occurring mixed infections of malaria parasites. Science, 212: 1037—1038.

Sergent, E., Donatien, A., Parrot, L., Lestoquard, F. and Plantureux, E., 1927. Etudes experimentales sur les piroplasmoses bovines d'Alegerir. Ann. Inst. Pasteur, 41: 721—784.

Trager, W. and Jensen, J., 1976. Human malaria parasites in continuous culture. Science, 193: 673—675.

Trager, W., Tershakovec, M., Lyandvert, L., Stanley, H., Lanners, N. and Gubert, E., 1981. Clones of the malaria parasite *Plasmodium falciparum* obtained by microscopic selection: their characterization with regard to knobs, chloroquine sensitivity, and formation of gametocytes. Proc. Natl. Acad. Sci. USA, 78: 6527—6530.

Vega, C.A., Buening, G.M., Green, T.J. and Carson, C.A., 1985a. In vitro cultivation of *Babesia bigemina*. Am. J. Vet. Res., 46: 416–420.

Vega, C.A., Buening, G.M., Rodriguez, S.D., Carson, C.A. and McLaughlin, K., 1985b. Cryopreservation of *Babesia bigemina* for in vitro cultivation. Am. J. Vet. Res., 46: 421–423.

Wright, I.G. and Goodger, B.V., 1977. Acute *Babesia bigemina* infection: Changes in coagulation and kallikrein parameters. Z. Parasitenk., 53: 63–73.

NOTE TO CONTRIBUTORS

A detailed ***Guide for Authors*** is available upon request and is also printed in the first volume to appear each year. You are kindly asked to consult this guide. Please pay special attention to the following notes:

Types of papers published in the journal

– papers reporting results of original research – review articles – short communications – editorials – book reviews – news and announcements.

Language

The official language of the journal is English.

Preparation of the text

(a) The manuscript should include at the beginning an abstract of not more than 400 words.
(b) It should be typewritten with double spacing and wide margins. Words to be printed in italics should be underlined. SI units should be used throughout.
(c) The title page should include: the title, the name(s) of the author(s), and their affiliation(s).
(d) Submit original plus 2 copies of manuscript (original illustrations or computer printouts plus 2 photocopies – for photographs, 3 prints should be supplied).

References

(a) References in the text should be cited as the name of the author(s), followed by the year of publication.
(b) The reference list should be in alphabetical order and on sheets separate from the text.

Tables

Tables should be compiled on separate sheets. A title should be provided for each table and all tables should be referred to in the text.

Illustrations

(a) Illustrations should be numbered consecutively and referred to in the text. All illustrations (line drawings, computer printouts and photographs) should be submitted separately, unmounted and not folded.
(b) Drawings should be fully annotated, the size of the lettering being appropriate to that of the drawings, but taking into account the possible need for reduction in size (preferably not more than 50%). The page format of the journal should be considered when designing drawings.
(c) Photographs must be of good quality, printed on glossy paper.
(d) Figure captions should be supplied on a separate sheet.

Reprints and page charges

There is no page charge. Fifty reprints of each article published will be supplied free of charge.
Additional reprints can be ordered on a reprint order form which is included with the proofs.

All contributions will be carefully refereed for international relevance and quality.

Submission of an article is understood to imply that the article is original and unpublished and is not being considered for publication elsewhere.

ALSO FROM ELSEVIER

AGRICULTURAL ECONOMICS
AGRICULTURE, ECOSYSTEMS AND ENVIRONMENT
AGRICULTURAL WATER MANAGEMENT
ANIMAL FEED SCIENCE AND TECHNOLOGY
ANIMAL REPRODUCTION SCIENCE
APPLIED ANIMAL BEHAVIOUR SCIENCE
AQUACULTURE
AQUATIC BOTANY
COMPUTERS AND ELECTRONICS IN AGRICULTURE
ECOLOGICAL MODELLING
ENERGY IN AGRICULTURE
EXPERIMENTAL AND APPLIED ACAROLOGY
FIELD CROPS RESEARCH
FISHERIES RESEARCH
FOREST ECOLOGY AND MANAGEMENT
LANDSCAPE AND URBAN PLANNING
LIVESTOCK PRODUCTION SCIENCE
PREVENTIVE VETERINARY MEDICINE
RECLAMATION & REVEGETATION RESEARCH
SCIENTIA HORTICULTURAE
SOIL & TILLAGE RESEARCH
VETERINARY IMMUNOLOGY AND IMMUNOPATHOLOGY
VETERINARY MICROBIOLOGY
VETERINARY RESEARCH COMMUNICATIONS

• FULL DETAILS AND A FREE SAMPLE COPY AVAILABLE ON REQUEST •

ELSEVIER SCIENCE PUBLISHERS

P.O. Box 330, 1000 AH Amsterdam, The Netherlands

ATTACHMENT F

J. Protozool., 33(4), 1986, pp. 514–518
© 1986 by the Society of Protozoologists

Concentration and Enzyme Content of In Vitro-Cultured *Babesia bigemina*-Infected Erythrocytes[1]

CARLOS A. VEGA,[2] GERALD M. BUENING, SERGIO D. RODRIGUEZ, and C. ANDREW CARSON

Department of Veterinary Microbiology, College of Veterinary Medicine, University of Missouri, Columbia, Missouri 65211

ABSTRACT. Clones of in vitro-cultured *Babesia bigemina*-infected erythrocytes were concentrated by several density gradient procedures. The density range of infected erythrocytes containing pairs of parasites was 1.077 to 1.089 g/ml, whereas the density range of infected erythrocytes containing single parasites was 1.092 to 1.100 g/ml. Three enzymes—lactate dehydrogenase, glucose-phosphate isomerase, and glutamate dehydrogenase—were found associated with infected erythrocytes. The parasite-specific enzyme and/or isoenzymes were shown to have different mobility patterns in starch gel electrophoresis from those found in the normal bovine erythrocytes. The enzyme 6-phosphogluconate dehydrogenase was not detected as a parasite-specific enzyme in *B. bigemina*-infected erythrocytes.

THE continuous in vitro cultivation method for *Babesia bigemina* was recently described by Vega et al. (23); however, the percentage of parasitized erythrocytes (PPE) seldom exceeds 3 to 6%. Therefore, it was necessary to develop a method to concentrate the infected erythrocytes before attempting an analysis of the enzyme contents of the parasite. Mahoney (14) observed that the concentration of *B. bovis*-infected erythrocytes increased when packed red cells were exposed to hypotonic saline solutions. This concentration method for *B. bovis* has been used for many different purposes by several investigators (10–12, 15, 22, 27, 28). The same hemolytic effect by hypotonic saline solutions was not observed on *B. bigemina*-infected erythrocytes (14, 27).

Pertoff et al. (18) first reported the use of polyvinylpyrrolidone (PVP)-coated colloidal silica particles (Percoll®, Pharmacia Chemical Co., Sweden). Density gradients prepared with Percoll have been used for the separation of different cell populations (8, 20). Percoll density gradients have also been used for the separation of protozoan parasites (6, 19). Aqueous solutions of ditrizoate meglumin (Renografin®, Squibb Lab., Princeton, NJ) have also been utilized in density gradients for the purification of *Rickettsia typhi* (26) and *Anaplasma marginale* (7). Combinations of Percoll-Renografin have been used as density gradient medium for the separation of human erythrocytes into age-dependent fractions (1, 25).

Sherman (21), using infected erythrocytes, detected heterogeneity for lactate dehydrogenase (LDH) in *Plasmodium lophurae* in ducks and *P. berghei* in mice, but could detect no differences for *B. rodhaini*-infected mouse erythrocytes. He concluded that in *B. rodhaini* the concentration of LDH was small and not detectable or that the parasite enzyme had the same mobility as the host cell enzyme. Momen (16), studying the differences in metabolism between *Plasmodium* and *Babesia* in mice, found that three enzymes—lactate dehydrogenase (LDH), glucose-phosphate isomerase (GPI), and glutamate dehydrogenase (GDH)—out of eight enzymes tested could be assumed to be of *B. rodhaini* origin because of different migration patterns in starch gel electrophoresis. Later, Momen et al. (17) studied several known *Babesia* species of rodents and two *Babesia* isolates from humans believed to be of rodent origin. Enzyme variation was demonstrated for all four isolates with LDH, as well as GDH. Intraspecies differences could not be detected. Carter and associates (3–5) were able to identify electrophoretically distinct forms of GPI, LDH, and 6-phosphogluconate dehydrogenase (6-PGD) from different human isolates of *Plasmodium falciparum*. It was also found that individual samples were heterogeneous in that different subpopulations of parasites were present in the blood.

The objectives of the present study were oriented toward 1) the concentration of *B. bigemina*-infected erythrocytes derived from in vitro culture and 2) then utilizing these *B. bigemina*-infected erythrocytes to evaluate parasite enzyme content by means of mobility in starch gel electrophoresis.

MATERIALS AND METHODS

Source of Babesia bigemina. The original Mexican strain was provided by Dr. K. L. Kuttler (USDA, Hemoparasitic Disease Unit, Pullman, WA 99164). Different clones were derived from three different animal subisolates of the same strain by in vitro cultivation (24).

In vitro culture procedure. Normal bovine erythrocytes (nRBC) were collected into sterile flasks with defibrinating glass beads, washed twice with VYM (Vega y Martinez) solution (23), and after removing the buffy coat and uppermost erythrocyte layer, were resuspended at 25% (v/v) concentration in VYM. A modification of the method described by Fulton & Grant (9) utilizing cellulose powder was carried out further to eliminate contaminating leukocytes. The processed erythrocytes were stored at 4°C.

The in vitro culture conditions were similar to those described previously (24). In order to obtain large volumes of *B. bigemina*, infected erythrocyte cultures were grown in 25-cm^2 or 75-cm^2 culture flasks with total volumes of 13.5 or 38.5 ml, attaining a 5-mm fluid depth. Complete culture medium was prepared, and 10 or 30 ml of the culture medium were exchanged daily.

Density gradients. When cultures had reached the optimum percent parasitized erythrocytes (PPE), usually 2 to 8% at 72 h after subculture, supernatant fluids were carefully removed from the flasks, and the sedimented erythrocytes were recovered. Infected erythrocytes were concentrated by one of the following procedures:

1. Percoll-Renografin gradient. An aqueous suspension of Percoll (density 1.130 g/ml) was purchased. Likewise, Renografin-75 (methylglucamine, N,N'-diamino-2,4,6,-triiodobenzoate) was obtained in a 75% aqueous solution (density 1.332 g/ml). A working Renografin solution was prepared as described (1): 15 ml Renografin-75, 22 ml 0.15 M NaCl, and 28 ml distilled H_2O. This solution was checked with a hydrometer and adjusted by the addition of equal volumes of 0.15 M NaCl and distilled H_2O until the density was 1.095 g/ml. The resulting solution was filtered through a 0.45-μm membrane filter and stored at 4°C. The Renografin working solution was mixed with an equal

[1] Supported in part by U.S. Department of Agriculture, Agricultural Research Service Cooperative Agreement 58-9AHZ-2-655. Dr. Vega was supported in part by a fellowship provided by Consejo Nacional de Ciencia y Tecnologia (an agency of the Mexican government) contract No. 09708 and a grant provided by U.S. Agency for International Development DPE-5542-G-SS-4026-00. Dr. Rodriguez was supported by grants provided by the U.S. Agency for International Development DPE-5542-G-SS-3014-00 and DPE-5542-G-SS-4026-00.

[2] Present address: Hemoprotozoan Disease Section, INIP, Palo Alto, D.F., Mexico City, Mexico.

volume of iso-osmotic 95% (v/v) Percoll (95 ml Percoll + 5 ml 3.0 M NaCl).

The density gradient for separation of *B. bigemina*-infected erythrocytes was established as follows. One milliliter of sedimented erythrocytes was mixed with 7.5 ml of Percoll-Renografin in a 14 × 70-mm round bottom polycarbonate tube. The tubes were centrifuged (for exactly 6 min) at 30,000 *g* in an angle rotor (Sorvall Model RC2-B, Rotor No. SS-34) at 4°C, deacceleration without braking. At least four different cell fractions or bands could be recovered by the use of a bent-tip pasteur pipette. Each fraction was washed two to three times with at least 10 volumes of 0.15 M NaCl solution and stored at 4°C until used.

2. Percoll 68.46% iso-osmotic gradient. A 90% (v/v) iso-osmotic Percoll solution (P-iso 90) was prepared by mixing 90 volumes of stock Percoll with 10 volumes of sterile 1.5 M NaCl to attain a density of 1.123 g/ml (manufacturer recommendations). Stock P-iso 90 was adjusted to a density of 1.095 g/ml by mixing 10 volumes of P-iso 90 with 3.146 volumes of 0.15 M NaCl. The resulting solution had a concentration of 68.46% (v/v) Percoll (P-iso 68.46). Packed erythrocytes from infected cultures in volumes of 0.6 ml were mixed with 7.5 ml of P-iso 68.46 in 14 × 70-mm clear polycarbonate tubes. The tubes were centrifuged (for exactly 6 min) (30,000 *g*) at 4°C, deacceleration without braking. A slight modification of the procedure involved adding packed infected erythrocytes with P-iso 68.46 exactly as described except the tubes are centrifuged at 30,000 *g* for 30 min at 4°C. After centrifugation, the fractions were collected and washed as described above. A mixture of density marker beads in a 0.6-ml volume was mixed in a polycarbonate tube with 7.5 ml P-iso 68.46. This tube was centrifuged at the same time as the tube containing the infected erythrocytes to visualize the densities and construct a density curve.

3. Discontinuous density gradient. Concentration of larger volumes of infected erythrocytes was carried out by preparing a discontinuous gradient of Percoll. All iso-osmotic Percoll solutions were made from P-iso 90 (density 1.123 g/ml). Ten volumes of P-iso 90 were mixed with 1.818, 3.928, 11.272, and 29.0 volumes of sterile 0.15 M NaCl solution. The resulting solutions, with densities of 1.105, 1.090, 1.061, and 1.036 g/ml, corresponded to P-iso 76.15, P-iso 64.61, P-iso 42.30, and P-iso 23.07 in percentage (v/v) of Percoll concentration.

Infected erythrocytes were obtained by removal of supernatant fluid from the flasks, and the sedimented cells were resuspended in 10 to 15 ml of VYM solution (23) and transferred to a conical 15-ml sterile polystyrene centrifuge tube. The tube was centrifuged at 750 *g* for 15–20 min at 4°C. Supernatant fluid was carefully removed, and the volume quantified and discarded. Packed erythrocytes were vigorously mixed within the tube with exactly the same volume of P-iso 90. One- to three-milliliter volumes of each of the previously prepared sterile Percoll suspensions were carefully layered on top of the erythrocyte-Percoll mixture, beginning with the highest density of 1.105 g/ml to the last suspension with a density of 1.036 g/ml. Tubes were centrifuged at 750 *g* for 30 to 60 min (average of 45 min) at 4°C. The fractions were collected and processed as described above.

Preparation of samples for enzymatic studies. After elimination of most of the noninfected erythrocytes by any of the aforementioned procedures, cell harvests containing 65% or more of infected erythrocytes were selected. Suspensions of parasitized erythrocytes were adjusted to a concentration of 1×10^9 ml, transferred to cryotubes, and frozen to −79°C for 24–48 h. Vials were quickly thawed at 37°C, resuspended by gentle agitation, and dispensed in 15–20-μl aliquots into previously labeled individual vials and immediately frozen and stored at −79°C. Suspensions of normal bovine erythrocytes maintained under similar culture conditions and separated in an identical manner were included.

Starch gel electrophoresis. Samples for electrophoresis were removed from the freezer and allowed to thaw at room temperature. Lysed erythrocyte material was transferred from the vials to 6 × 6-mm square pieces of Whatman No. 3 paper by dispensing 15 μl with a micropipette. These pieces were placed immediately in the starch gel.

Gels were prepared and used within 24 h according to the procedures described by Carter (2). Gel number and identification were written on the top surface glass, and the gel allowed to solidify overnight at room temperature. The gel was incised all the way through with a scalpel blade. The gel was then carefully separated about 10 mm along the incision. The 6 × 6-mm squares of filter paper containing the lysed erythrocyte material were placed in the edge of the incised gel, which was rejoined. The gel was then placed in an electrophoresis apparatus in a refrigerator. The line of origin (samples) was always 60 mm from the cathodal end of the gel. After electrophoresis, the power supply was turned off and the gel removed for staining as described by Carter (2). Enzyme activity was detected by specific color development as explained by Sherman (21). The appropriate reagents (Sigma Chemical Co., St. Louis, MO) were mixed and poured on the surface of the gel and incubated at 37°C for 30 to 60 min. Results were recorded by photography. The following enzymes were assayed: lactate dehydrogenase (LDH), glucose phosphate isomerase (GPI), 6-phosphogluconate dehydrogenase (6-PGD), and NADP-dependent glutamate dehydrogenase (GDH).

RESULTS

Density gradients. Percoll-Renografin density gradients separated *B. bigemina*-infected erythrocytes from non-infected erythrocytes. Four major fractions or bands were collected and identified by their position from the top to the bottom of the centrifuge tube. A well defined pinkish band at the top of the tube consisted of red cell stroma and parasite-containing ghosts. The next band was a broad zone and contained greater than 90% infected erythrocytes. Most of the infected cells contained pairs of the parasite. The third band was located below the center of the tube and was usually well defined, with a majority of infected erythrocytes containing a single parasite. The fourth band was located close to the bottom of the tube and contained densely packed non-infected erythrocytes with a few erythrocytes containing very immature parasites.

Fractions obtained from the 68.46% (v/v) Percoll centrifuged for 6 min were similar in distribution and composition to those reported for the Percoll-Renografin gradient. Figure 1 is a graphic representation of the fractions obtained by the two methods and presents a density calibration curve. When the time of centrifugation was increased to 30 min, the separation pattern changed. Four major bands were still observed, but the compositions and locations had changed. The uppermost band was composed of non-infected erythrocyte ghosts. The second band contained mostly infected ghosts or pycnotic parasite forms. The third fraction was a broad zone, composed of many (10 to 24) narrow red bands, containing *B. bigemina*-infected erythrocytes in all developmental stages (rings, singles, pairs). The last band, located some distance from the bottom, contained non-infected erythrocytes (Fig. 2).

Density values, the average interphase values between the fractions, for the discontinuous Percoll density gradients were arrived at graphically (Figs. 1, 2). Each value presented is the average of four individual determinations. The calculated average densities for *B. bigemina*-infected erythrocytes containing

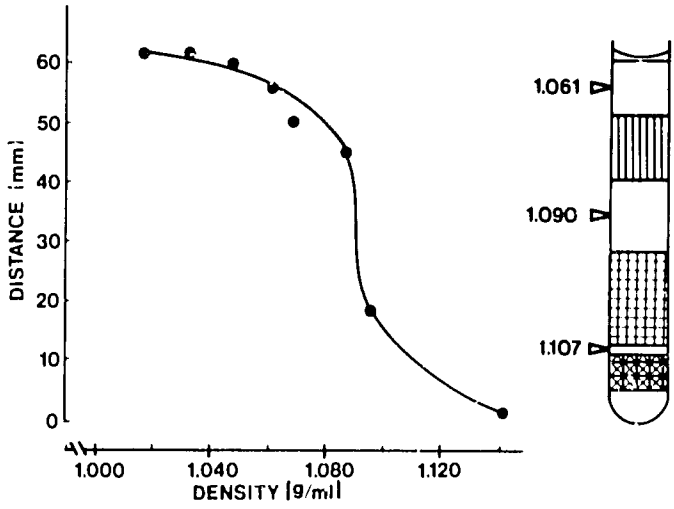


Fig. 1. Density calibration curve and diagrammatic representation of the separation of *Babesia bigemina* culture material in iso-osmotic 68.46% Percoll after centrifugation for 6 min.

pairs of parasites were between 1.076 to 1.089 g/ml and for erythrocytes containing a single parasite between 1.092 to 1.100 g/ml. Figure 3 is a photomicrograph of concentrated *B. bigemina*-infected erythrocytes.

Starch gel electrophoresis. Enzyme activity could be detected for the four enzymes studied by the starch gel electrophoresis method. Five distinct zones of LDH activities were observed when normal bovine serum was used. Three distinct LDH zones were detected in normal bovine erythrocyte extracts and four distinct zones in *B. bigemina*-infected erythrocyte extracts; however, the electrophoretic mobility of one zone of LDH activity in infected extracts was different from that of normal erythrocyte extracts or serum. Figure 4a is a schematic representation of the migration pattern of LDH isoenzymes. No differences were detected between the cloned parasites and the original strain.

The isoenzyme migration pattern of GPI was as follows. Bovine serum and normal erythrocyte extracts had two narrow bands located toward the cathode while *B. bigemina*-infected erythrocyte extracts had the two previously mentioned bands

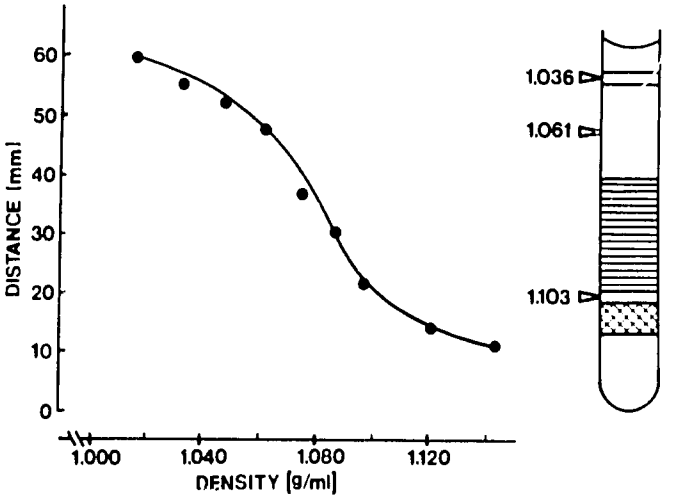


Fig. 2. Density calibration curve and diagrammatic representation of the separation of *Babesia bigemina* culture material in iso-osmotic 68.46% Percoll after centrifugation for 30 min.

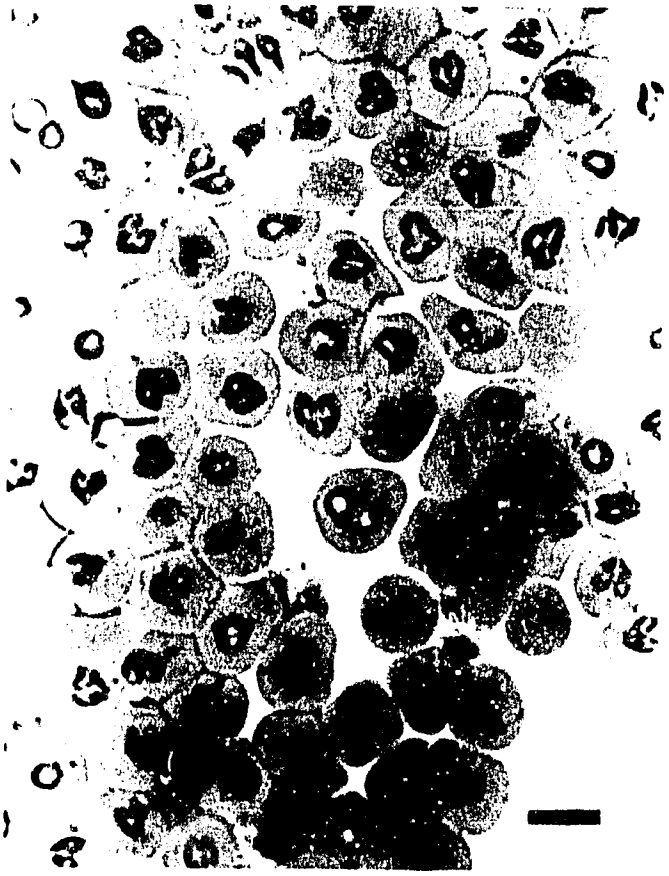


Fig. 3. Photomicrograph of concentrated *Babesia bigemina*-infected erythrocytes. Bar = 1 μm.

plus a fast broader band oriented toward the anode. No differences were detected among the cloned parasites (Fig. 4b).

Only one band of 6-PGD activity was detected in both normal and *B. bigemina*-infected erythrocyte extracts. This band had the same electrophoretic mobility (Fig. 4c). No activity was detected in normal serum.

The activity of GDH was detected only in extracts from *B. bigemina*-infected erythrocytes (Fig. 4d). All of the cloned parasites had enzymes with the same electrophoretic mobility.

DISCUSSION

Babesia bigemina-infected erythrocytes were successfully concentrated at least 20 times by Percoll and Percoll-Renografin density gradients. A report by Wright & Kerr (29), based on the size of infected erythrocytes in splenectomized calves, claimed that *B. bigemina* preferentially invades young erythrocytes. It is known that *B. bigemina*-parasitized erythrocytes appear larger than non-infected erythrocytes, have basophilic inclusions, and tend to accumulate at the edges of thin smears, as do reticulocytes or immature red blood cells. In this study, recorded densities of infected cells were always smaller than those of non-infected erythrocytes.

The density curve for separation of cellular elements in infected *B. bigemina* cultures was based upon determinations made in Percoll gradients because the density beads do not separate in the Percoll-Renografin density medium. Fractions containing infected erythrocytes separated by the Percoll-Renografin procedure showed less non-infected cell contamination than those separated in Percoll alone. The time elapsed after subculture

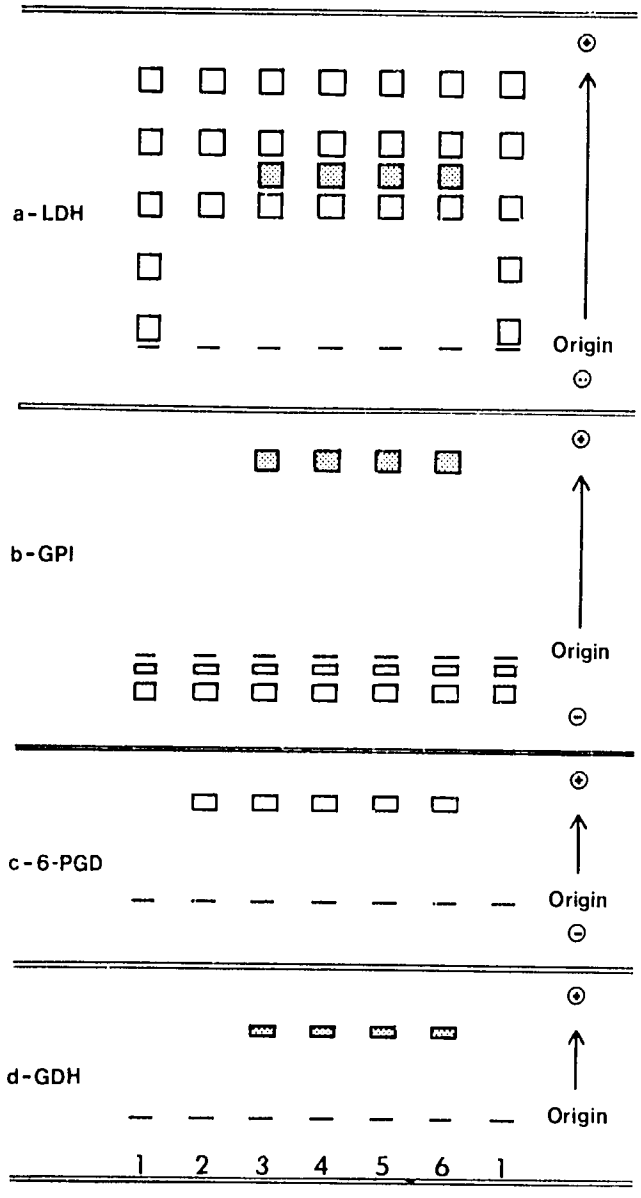


Fig. 4. Schematic representation of the isoenzyme migration patterns from bovine serum and normal and *Babesia bigemina*-infected erythrocytes. Open areas = host origin; shaded areas = parasite origin. Lanes: 1. normal bovine serum; 2. normal bovine erythrocytes; 3. *B. bigemina*—original isolate; 4. Clone III-SE2; 5. Clone III-KC11C10; 6. Clone III-RF8F3C3.

Conditions: LDH—Gel buffer: 65 mM Tris–22.2 mM citrate, pH 6.4; Electrode buffer: 220 mM Tris–78.5 mM citrate, pH 6.2, 70 mA, 100 V, 14 h. GPI—Gel buffer: 10 mM Tris–HCl, pH 8.0; Electrode buffer: 50 mM Tris HCl, pH 8.0, 60 mA, 400 V, 4 h. 6-PGD—Gel buffer: 17.5 mM Tris–6.3 mM citrate, pH 6.2; Electrode buffer: 220 mM tris–78.5 mM citrate, pH 6.0, 75 mA, 190 V, 4 h. GDH—Gel buffer: 5.4 citrate–32 mM Na_2HPO_4, pH 7.0; Electrode buffer: 13 mM citrate–155 mM Na_2HPO_4, pH 7.0, 75 mA, 60 V, 14 h.

also appeared to affect separation. Cultures maintained less than 48 h or more than 72 h after a subculture usually yielded fewer infected erythrocytes. The osmotic pressure may also have an effect on separation patterns in Percoll-Renografin gradients.

Three enzymes—LDH, GPI, and GDH—appear to be of *B. bigemina* origin as visualized in starch gel electrophoresis. Only one band (isoenzyme) for each of the enzymes was found to be different from other bands observed in normal bovine serum or erythrocytes. Momen (16) obtained similar results for *Babesia rodhaini*. Momen et al. (17) also reported similar findings with four species of *Babesia* from rodents, namely *B. hyalomysci*, *B. microti*, *B. muratovi*, and *B. rodhaini*. Momen (16) noted that failure to detect a parasite-derived enzyme was not positive proof that the enzyme was not present. It was possible that it may have the same electrophoretic mobility as the enzyme of the host cell and thus be masked or that the concentration was well below the sensitivity of the assay. In the present study, infected erythrocytes were concentrated. The 15-μl lysate samples contained the equivalent of 1×10^7 parasites. Also the intensity of the reactions was indicative of the presence of the enzymes indicated; however, 6-PGD activity was not detected in any of the electrophoresis systems described in this study or the alternate system mentioned by Carter (2). It is reasonable to assume, in accord with the results presented by Momen et al. (17), that *Babesia* apparently lacks this enzyme.

Polymorphism of the parasite-specific enzymes in the cloned parasites was not observed. Several conclusions could be drawn from this observation. The parasites may not possess isoenzymes for the enzymes studied, for all of the clones studied were from the same strain. An extension of the work would be to evaluate isolates from different geographical areas. The comparative studies by Momen et al. (17) showed that all enzymes of parasite origin had different electrophoretic mobilities for each parasite species. The bovine babesias have not been compared, like the rodent babesias. Hoare (13) for instance, questioned the validity of the taxonomic separation between *B. bovis* and *B. divergens*, which are morphologically very similar. Detailed studies of the biochemical characteristics of each species might clarify this point. Electrophoretic studies of enzyme variants of plasmodia in rodents (2) showed that polymorphism is found in several species.

Glutamate dehydrogenase (NADP-dependent) is present in rodent and bovine *Babesia*. In this study, GDH staining was shown to be parasite specific because mammalian red blood cells lack this enzyme.

The results of this study will contribute to the knowledge base concerning the biochemical characteristics of the bovine parasite, *Babesia bigemina*.

LITERATURE CITED

1. Branch, D. R., Sy-Siok-Hian, A. L., Carlson, M., Maslow, W. C. & Petz, L. D. 1983. Erythrocyte age-fractionation using a Percoll-Renografin density gradient: application to autologous red cell antigen determinations in recently transferred patients. *Am. J. Clin. Pathol.*, **80**: 453–458.

2. Carter, R. 1978. Studies on enzyme variation in the murine malaria parasites *Plasmodium berghei*, *P. yoelii*, *P. vinckei*, and *P. chabaudi* by starch gel electrophoresis. *Parasitology*, **76**: 241–267.

3. Carter, R. & McGregor, I. A. 1973. Enzyme variation in *Plasmodium falciparum* in the Gambia. *Trans. R. Soc. Trop. Med. Hyg.*, **67**: 830–837.

4. Carter, R. & Voller, A. 1973. Enzyme typing of malaria parasites. *Br. Med. J.*, **1**: 149–150.

5. ——— 1975. The distribution of enzyme variation in populations of *Plasmodium falciparum* in Africa. *Trans. R. Soc. Trop. Med. Hyg.*, **69**: 371–376.

6. Castanys, S., Osuna, A., Gamarro, F. & Ruiz-Perez, L. M. 1984. Purification of metacyclic forms of *Trypanosoma cruzi* by Percoll discontinuous gradient centrifugation. *Z. Parasitenkd.*, **70**: 443–449.

7. Davis, W. C., Talmadge, J. E., Parish, S. M., Johnson, M. I. & Vibber, S. D. 1978. Synthesis of DNA and protein by *Anaplasma marginale* in bovine erythrocytes during short-term culture. *Infect. Immun.*, **22**: 597–602.

8. Ellis, W. M., Georgiou, G. M., Robertson, D. M. & Johnson, G. R. 1984. The use of discontinuous Percoll gradients to separate pop-

ulations of cells from human bone marrow and peripheral blood. *J. Immunol. Meth.*, **99**: 9–16.

9. Fulton, J. D. & Grant, P. T. 1956. The sulphur requirements of the erythrocyte form of *Plasmodium knowlesi*. *Biochem. J.*, **63**: 274–282.

10. Goff, W. L., Wagner, G. G., Craig, T. M. & Long, R. F. 1982. The bovine immune response to tick derived *Babesia bovis* infection. Serological studies of isolated immunoglobulins. *Vet. Parasitol.*, **11**: 109–120.

11. Goodger, B. V. 1971. Preparation and preliminary assessment of purified antigens in the passive hemagglutination test for bovine babesiosis. *Aust. Vet. J.*, **47**: 251–256.

12. Goodger, B. V., Commins, M. A., Wright, I. G. & Mirre, G. B. 1984. *Babesia bovis*: vaccination of cattle against heterologous challenge with fractions of lysate from infected erythrocytes. *Z. Parasitenkd.*, **70**: 321–329.

13. Hoare, C. A. 1980. Comparative aspects of human babesiosis. *Trans. R. Soc. Trop. Med. Hyg.*, **74**: 143–148.

14. Mahoney, D. F. 1967. Bovine babesiosis: preparation and assessment of complement fixing antigens. *Exp. Parasitol.*, **20**: 232–241.

15. ——— 1983. Studies on the protection of cattle against *Babesia bovis* infection, *in* Dunsmore, J. D., ed., *Tropical Parasitosis and Parasitic Zoonosis*. Perth, pp. 93–104.

16. Momen, H. 1979. Biochemistry of intraerythrocytic parasites. I. Identification of enzymes of parasite origin by starch gel electrophoresis. *Ann. Trop. Med. Parasitol.*, **73**: 109–115.

17. Momen, H., Chance, M. L. & Peters, W. 1979. Biochemistry of intraerythrocytic parasites III. Biochemical taxonomy of rodent *Babesia*. *Ann. Trop. Med. Parasitol.*, **73**: 203–212.

18. Pertoff, H., Laurent, H., Laas, T. & Kagedal, L. 1978. Density gradients prepared from colloidal silica particles coated by polyvinylpyrrolidone (Percoll). *Ann. Biochem.*, **88**: 271–282.

19. Saul, A., Myler, P., Elliot, T. & Kidson, C. 1982. Purification of mature schizonts of *Plasmodium falciparum* on colloidal silica gradients. *Bull. W.H.O.*, **60**: 755–759.

20. Segal, A. W., Fortunato, A. & Herd, T. 1980. A rapid single centrifugation step method for the separation of erythrocytes, granulocytes and mononuclear cells on continuous density gradients of Percoll. *J. Immunol. Meth.*, **32**: 209–214.

21. Sherman, I. W. 1962. Heterogeneity of lactic dehydrogenase in intraerythrocytic parasites. *Ann. N.Y. Acad. Sci.*, **24**: 944–953.

22. Todorovic, R. A., Gonzalez, E. F. & Adams, L. G. 1973. Bovine babesiosis: sterile immunity to *Babesia bigemina* and *Babesia argentina* infections. *Trop. Anim. Health Prod.*, **5**: 234–245.

23. Vega, C. A., Buening, G. M., Green, T. J. & Carson, C. A. 1985. In vitro cultivation of *Babesia bigemina*. *Am. J. Vet. Res.*, **46**: 416–420.

24. Vega, C. A., Buening, G. M., Rodriguez, S. D. & Carson, C. A. 1986. Cloning of in vitro propagated *Babesia bigemina*. *Vet. Parasitol.* (in press)

25. Vettore, L., De Matteis, M. C. & Zampini, P. 1980. A new density gradient system for the separation of human red blood cells. *Am. J. Hematol.*, **8**: 291–297.

26. Weiss, E., Coolbaugh, J. C. & Williams, J. C. 1975. Separation of viable *Rickettsia typhi* from yolk sac and L cell components by Renografin density gradient centrifugation. *Appl. Microbiol.*, **30**: 456–463.

27. Wright, I. G. 1973. Osmotic fragility of erythrocytes in acute *Babesia argentina* and *Babesia bigemina* infections in splenectomized *Bos taurus* calves. *Res. Vet. Sci.*, **15**: 299–305.

28. Wright, I. G. & Goodger, B. V. 1973. Proteolytic enzyme activity in the intraerythrocytic parasites *B. argentina* and *B. bigemina*. *Z. Parasitenkd.*, **42**: 213–220.

29. Wright, I. G. & Kerr, J. D. 1974. The preferential invasion of young erythrocytes in acute *Babesia bigemina* infections in splenectomized calves. *Z. Parasitenkd.*, **43**: 63–69.

Received 31 VII 85; accepted 23 VI 86

ATTACHMENT 31

Reprinted from the AMERICAN JOURNAL OF VETERINARY RESEARCH, Vol. 46, No. 2, Pages 416-420.
© American Veterinary Medical Association, 1985. All Rights Reserved.

In vitro cultivation of *Babesia bigemina*

C. A. Vega, DVM, MS; G. M. Buening, DVM, PhD; T. J. Green, PhD; C. A. Carson, VMD, PhD

SUMMARY

A strain of *Babesia bigemina* was isolated from an infected calf and propagated in vitro. Culture conditions included washing of infected and normal bovine erythrocytes in a special solution, and the use of a 5% to 10% (v/v) erythrocyte suspension in medium 199 (with 20% to 50% fresh normal bovine serum) at a depth of 4 mm in a 5% CO_2, 2% O_2, 93% N_2 atmosphere. After 36 days in vitro and 9 subcultures, the cultured organism was inoculated into a susceptible calf. This calf developed clinical signs of disease and recovered when treated with 1% trypan blue solution. The strain was also reisolated from the second calf. The original isolate had been maintained in continuous in vitro cultivation for more than 99 days.

Babesiosis, caused by protozoan parasites *Babesia bovis* and *B bigemina*, has impaired bovine livestock production in many tropical and subtropical areas of the world. Long term in vitro cultivation of *B bovis*[1-3] and cloning of *B bovis* has been reported.[4] In vitro cultivation of *B bigemina* has long been a research objective for the production of antigens for serologic test procedures and immunogens for disease prevention. Timms reported[5] that *B bigemina* was successfully maintained in vitro for 96 hours using a method similar to that of Trager and Jensen.[6] The objective of the present work was to develop a procedure by which *B bigemina* parasites can be propagated continuously under in vitro laboratory conditions.

Received for publication Feb 13, 1984.

From the Department of Veterinary Microbiology, College of Veterinary Medicine, University of Missouri, Columbia, MO 65211.

Supported in part by US Department of Agriculture, Agricultural Research Service Cooperative Agreement 58-9AHZ-2-655; Dr. Vega was supported by a fellowship provided by Consejo Nacional de Ciencia y Tecnologia (an agency of the Mexican government) contract No. 09708.

The authors thank Dr. K. L. Kuttler, US Department of Agriculture, Agricultural Research Service, Hemoparasite Research Unit, Washington State University, Pullman, for providing the Mexican isolate of *B bigemina*.

Materials and Methods

Babesia bigemina strain—A frozen stabilate of a Mexican isolate was inoculated into a splenectomized calf and subpassed twice in similarly prepared calves. Blood was collected (parasitemia 2.5%), washed, apportioned into 1.5-ml aliquots, and frozen in liquid nitrogen as described by Palmer et al.[7]

Experimental animals—Two 4- to 5-month-old Hereford-cross calves (A and B) were purchased from a sale barn. Blood samples were examined microscopically, cultured, and found to be free of *Trypanosoma* spp. Serum samples were card test-negative for antibodies to *Anaplasma marginale*. Each calf was splenectomized 2 to 3 weeks before inoculation and maintained in screened isolation stalls with feed, hay, and water ad libitum. One 18-month-old intact Holstein steer, also negative for the aforementioned diseases and maintained at the University of Missouri Veterinary Medicine Research Farm, served as the donor for normal bovine RBC and normal serum throughout the experiment.

Original isolation—Animal inoculum was prepared by quickly thawing five 1.5-ml vials containing *B bigemina*-infected blood and mixing immediately with 22.5 ml of sterile Vega y Martinez (VYM) phosphate-buffered saline solution. The VYM solution was composed of $CaCl_2 \cdot 2H_2O$ (16.0 mg), KCl (400.0 mg), KH_2PO_4 (1,415.4 mg), $MgSO_4 \cdot 7H_2O$ (154 mg), $Na_2HPO_4 \cdot 7H_2O$ (1,450.0 mg), NaCl (7,077.0 mg), and dextrose (20.5 g) in 1 L of double-distilled deionized water containing 0.25 m*M* of adenine and 0.50 m*M* of guanosine. This solution was supplemented with penicillin (100 IU/ml), streptomycin (100 μg/ml), and amphotericin B (0.250 μg/ml[a]) and was filtered. Calf A was inoculated with 15.0 ml of inoculum IV and 15.0 ml subcutaneously on day 0. Ear vein blood samples were collected every 24 hours and rectal temperature was recorded. The PCV was determined by the microhematocrit method. Parasite development was monitored by microscopic observation of Giemsa-stained blood smears prepared daily. Levels of infection were expressed as the percentage of parasitized erythrocytes (PPE). On postinoculation day (PID) 5 when PPE was 0.20 and increasing, 35.0 ml of blood was aseptically collected from the jugular vein into sterile evacuated glass tubes containing heparin (5 to 10 IU/ml of blood) as the anticoagulant. Blood was washed 3 times by centrifugation at 1,000 × g for 15 minutes at 4 C. Supernatant and buffy coat, including one-third of the top RBC layer, were discarded after each washing. *Babesia bigemina*-infected RBC were gently resuspended in VYM solution after each wash.

Preliminary studies had indicated that *B bigemina*-infected blood collected by defibrination with glass beads hemolyzed rapidly and contained fewer infected RBC. The use of heparin as an anticoagulant prevented hemolysis and increased the yield of infected RBC. However, extensive washing was necessary to remove the WBC in heparin-collected blood. The detrimental effect of contaminating WBC in the establishment of parasite cultures was reported by Timms.[5]

Normal venous blood was aseptically collected into a sterile flask while shaking over the contained glass beads. The defibrinated blood was then centrifuged at 1,500 × g for 15 minutes at 4 C. Supernatant serum was recovered, distributed into sterile, disposable 15.0-ml plastic tubes in 5.0-ml aliquots, and immediately placed at −40 C until use. This material was designated as frozen fresh normal bovine serum (FFS). Buffy coat cells were then carefully removed, and the remaining normal RBC were washed twice with VYM solution at 1,000 × g for 15 minutes at 4 C and resuspended

[a] Gibco Laboratories, Grand Island, NY.

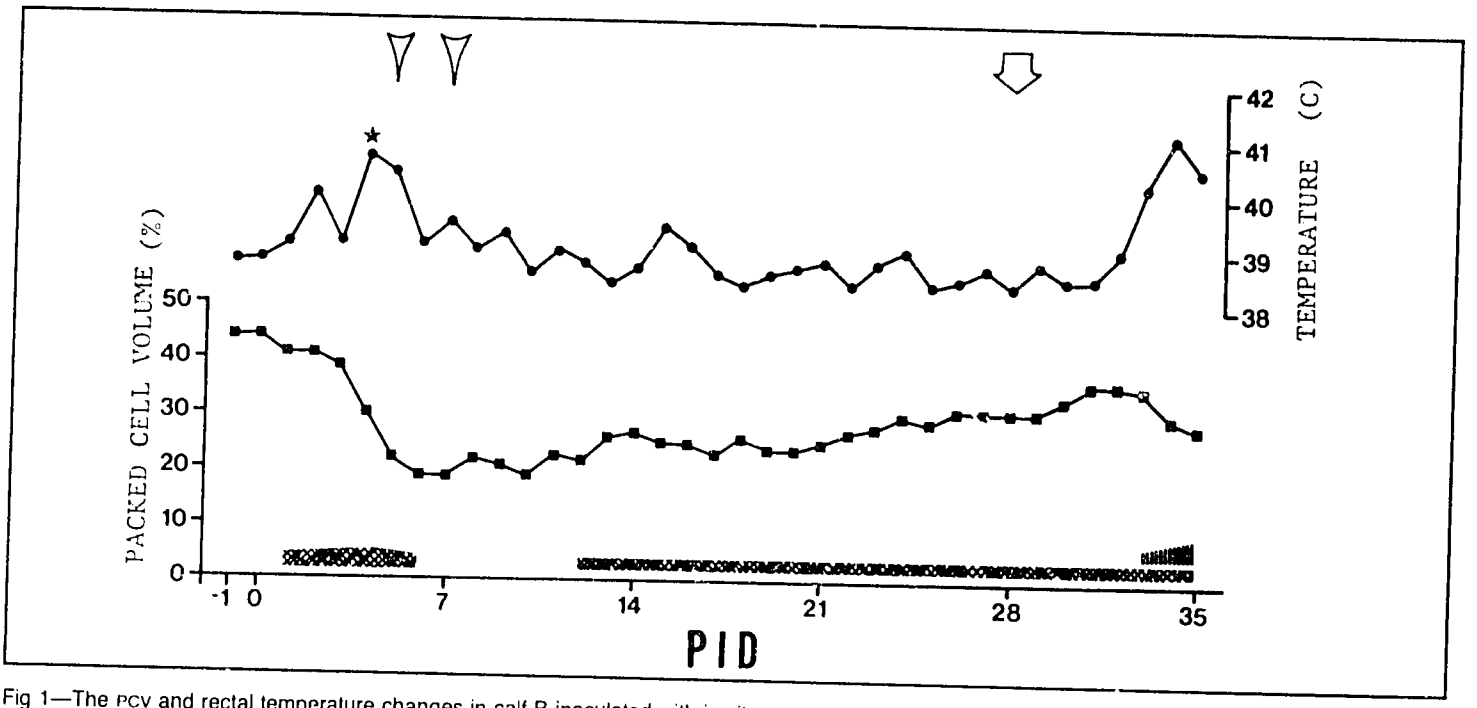


Fig 1—The PCV and rectal temperature changes in calf B inoculated with in vitro-grown *B bigemina* and challenge exposed with in vitro-grown *B bovis*. PID 0: inoculated with 1.5 × 10^8 *B bigemina* organisms produced after 36 days in culture; PID 4: blood sample for reisolation (*); PID 5 and 7: treatment with 1% trypan blue solution (▽); PID 28: challenge exposed with 1.5 × 10^8 *B bovis* organisms grown in vitro (▯); days *B bigemina* were observed in Giemsa-stained blood smears (▪▪▪); days *B bovis* were observed in Giemsa-stained blood smears (▪▪▪▪▪).

TABLE 1—Original isolation: *Babesia bigemina* growth in vitro using six different culture media under different atmospheric environments (I-VI)

Atmosphere	Media	PPE—PID*										
		1	2†	3	4‡	5	6	7	8	9	10	11
I	F-12	0.163	0.013	0.010	0.0[illegible]	0.000	Neg	...	...	...	...	...
	199-HBSS	0.170	0.101	0.107	0.016	0.029	0.043	0.110	0.105	0.035	0.017	0.014
	MEM-EBSS	0.248	0.121	0.148	0.038	0.040	0.017	0.028	0.011	0.112	0.156	0.295
	RPMI 1640	0.090	0.051	0.015	0.061	0.000	0.008	0.009	0.008	0.033	0.016	0.088
	L-15	0.042	0.000	Neg	...	...	...	...	...	...	...	...
	199-EBSS	0.037	0.004	0.000	Neg							
II	F-12	0.200	0.087	0.024	0.029	0.008	0.033	0.030	0.047	0.099	0.098	0.028
	199-HBSS	0.405	0.145	0.069	0.050	0.051	0.033	0.108	0.313	0.616	0.812	1.208
	MEM-EBSS	0.535	0.192	0.163	0.207	0.065	0.045	0.030	0.024	0.178	0.132	0.379
	RPMI 1640	0.121	0.035	0.215	0.070	0.048	0.043	0.025	0.032	0.200	0.177	0.392
	L-15	0.015	0.000	Neg	...	...	...	...	...	...	...	...
	199-EBSS	0.008	0.000	Neg								
III	F-12	0.090	0.016	0.010	0.014	0.000	Neg	...	...	...	...	...
	199-HBSS	0.054	0.009	0.005	0.000	Neg	...	...				
	MEM-EBSS	0.130	0.175	0.100	0.077	0.026	0.000	Neg				
	RPMI 1640	0.162	0.059	0.020	0.024	0.028	0.000	Neg				
	L-15	0.040	0.000	Neg	...	...	...	...				
	199-EBSS	0.014	0.000	Neg	...	...	...	...				
IV	F-12	0.038	0.046	0.012	Neg	...	...	...	...	...	...	...
	199-HBSS	0.010	0.000	Neg	...							
	MEM-EBSS	0.163	0.096	0.019	Neg							
	RPMI 1640	0.104	0.067	0.000	Neg							
	L-15	0.000	Neg	...	...							
	199-EBSS	0.000	Neg	...	...							
V	F-12	0.302	0.152	0.011	Neg	...	...	...	...	...	...	...
	199-HBSS	0.136	0.010	0.000	Neg							
	MEM-EBSS	0.011	0.013	0.000	Neg							
	RPMI 1640	0.102	0.013	0.018	Neg							
	L-15	0.040	0.000	Neg	...							
	199-EBSS	0.000	0.010	0.000	Neg							
VI	F-12	0.028	0.000	Neg	...	...	...	...	...	...	...	...
	199-HBSS	0.151	0.000	Neg								
	MEM-EBSS	0.000	Neg	...								
	RPMI 1640	0.000	Neg	...								
	L-15	0.014	0.000	Neg								
	199-EBSS	0.015	0.000	Neg								

* PID 0: PPE 0.051; 50% BFS in medium; pH 6.85 to 6.95; 5% RBC concentration; 4-mm depth; temperature, 37 ± 0.1 C. † PID 2 and 7: addition of a 2.5% normal RBC suspension. ‡ PID 4 and 9: subcultures 1:2 with a 7.5% normal RBC suspension.
... = no data; Neg = negative.

TABLE 2—Effect of four related media on *B bigemina* growth in vitro

Media	PPE—PID*					
	1	2	3†	4	5	6
199-HBSS	0.192	0.537	1.073	0.114	0.520	1.021
MEM-HBSS	0.335	1.106	0.900	0.271	0.363	0.735
199-EBSS	0.236	0.535	0.747	0.375	1.208	1.331
MEM-EBSS	0.315	0.734	0.762	0.327	0.711	0.902

* PID 0 = 0.178 PPE; culture setting: 40% BFS in medium, pH 6.85 to 6.95; 10% RBC concentration; depth, 4 mm; temperature, 37 ± 0.1 C, and atmosphere II (5% CO_2, 2% O_2, 93% N_2). † PID 3 = Subculture by dilution 1:10.

The data are expressed as the average PPE of duplicate cultures.

TABLE 3—Effect of different serum concentrations on *B bigemina* growth in vitro

Serum (%)	PPE—PID*					
	1	2	3†	4	5	6
50	0.231	1.321	0.478	0.058	0.334	1.188
35	0.336	1.475	0.655	0.134	0.750	1.641
20	0.169	0.980	0.949	0.108	0.774	1.254
5	0.068	0.030	0.020	0.000	0.010	0.007
0	0.008	0.000	0.000	0.000	0.000	0.000

* PID 0 = 0.126 PPE; medium 199-EBSS with $NaHCO_3$, pH 6.85 to 6.95; 10% RBC concentration; 4-mm depth; temperature, 37 ± 0.1 C, and atmosphere II (5% CO_2, 2% O_2, 93% N_2). † PID 3 = Subculture by dilution 1:20.

The data are expressed as the average PPE of duplicate cultures.

TABLE 4—Reisolation: In vitro growth of *B bigemina* from calf B, previously inoculated with in vitro-grown *B bigemina*

	PID							
	0*	1	2†	3	4	5	6	7
PPE	0.736	0.484	0.289	0.755	0.407	0.271	1.105	1.468

* Culture set with 10% RBC in medium 199-EBSS with $NaHCO_3$ with 30% BFS; pH 6.85 to 6.95; depth, 4 mm; temperature, 37 ± 0.1 C; atmosphere II (5% CO_2, 2% O_2, 93% N_2). † Subcultures: 1:4 on PID 2 and 1:10 on PID 4.

in VYM to a final 30% to 50% concentration for storage at 4 C until used (usually within 3 to 4 days).

Culture media were prepared daily by mixing 1 volume of quickly thawed BFS with 1 volume of each of the following commercially available media: Ham's nutrient mixture F-12 (F-12)[b]; medium 199 with Hanks's balanced salt solution (199-HBSS)[c]; minimum essential medium with Earle's balanced salt solution (MEM-EBSS)[c]; RPMI 1640 medium[b]; Leibovitz medium (L-15)[c]; and medium 199 with EBSS (199-EBSS).[b] All media preparations contained $NaHCO_3$ with the exception of L-15 and 199-EBSS. In each case, the pH was adjusted (6.85 to 6.95), using 0.1*N* HCl. Serum-supplemented media were sterilized by passage through a 0.22-µm cellulose filter, and used within 2 hours. Antibiotics were not added to any culture media used in the experiments. Normal or infected RBC suspensions were prepared at 5% concentration (v/v) by mixing 1 part of sedimented erythrocytes, previously centrifuged at 1,000 × g for 10 minutes at 4 C, with 19 parts in each of the serum-supplemented media. One part of a 5% infected RBC suspension was mixed with 3 parts of the 5% (v/v) normal RBC suspension for a final 1:4 dilution of infected/normal RBC suspension with 5% (v/v) concentration for each of the 6 media used. Sterile, disposable 24-well (16 mm ID) tissue-culture cluster plates[d] were used throughout the experiments. Top and bottom (A and D) rows in the plates were filled with 1.5 ml of sterile tap water to prevent excessive evaporation. The final infected normal RBC suspensions with a PPE of 0.05 were distributed in volumes of 805 µl/well (4-mm depth) in duplicate wells. Each plate then contained duplicates of each of the 6 media preparations. Plates were incubated at 37 ± 0.1 C in humidified chambers that had 1 of the following atmospheric conditions: 10% CO_2, 10% H_2, and 80% N_2 (I); 5% CO_2, 2% O_2, and 93% N_2 (II); 6.4% CO_2, 5.6% O_2, and 88% N_2 (III); 5.6% CO_2, 13.6% O_2, and 80.8% N_2 (IV); 5% CO_2 in air (V); and normal air (VI). Spent medium was collected in volumes of 600 µl/well and replaced with freshly prepared 50% serum-supplemented media at 24-hour intervals. Culture samples (3 to 5 µl) were obtained daily at the time of medium exchange and Giemsa-stained blood smears were prepared for microscopic observation. A 2.5% suspension of normal RBC in each medium was added at 48 and 168 hours after initiation of cultures. Cultures, in which the parasites were found, were subcultured on day 4 by diluting 1:2 with a 7.5% (v/v) normal RBC suspension in the corresponding medium and transferred to separate plates under the appropriate atmospheric conditions. A 2nd

[b] KC Biological Inc, Lenexa, Kan.

[c] MA Bioproducts, Walkersville, Md.

[d] Costar, Cambridge, Mass.

TABLE 5—Reaction titer of specific *B bigemina* and *B bovis* serum samples to homologous and heterologous in vitro produced *Babesia* antigens

Sera	Antigens	
	B bigemina	*B bovis*
Negative control	1:20	1:20
B bigemina	1:5,120	1:80
B bovis	1:80	1:10,240

subculture was carried out on day 9 by diluting 1:2 with a 7.5% (v/v) normal RBC suspension. The criteria for parasite growth in culture was a steady increase in the percentage of infected erythrocytes. When parasite development was considered established, a 3rd subculture was performed by a 1:10 dilution with a 10% (v/v) normal RBC suspension in corresponding medium. This *B bigemina* strain was then maintained continuously in vitro.

Media experiment—After original isolation was proven to be successful under 1 of the chosen atmospheric environments, the following closely related media were compared: medium 199-EBSS, 199-HBSS,[c] MEM-EBSS,[c] and MEM-HBSS.[c] These media contained 2.2 g, 1.4 g, 2.2 g, and 0.35 g of $NaHCO_3$/L, respectively. Forty percent concentrations of BFS in each medium were prepared, pH adjusted (6.85 to 6.95), and filtered through a 0.22-µm membrane. A 10% concentration (v/v) of infected RBC and normal RBC, with a PPE of 0.178 was prepared, using each medium tested. Duplicate cultures were set with 805-µl volume/well and incubated at 37 ± 0.1 C under a low oxygen atmosphere (5% CO_2, 2% O_2, 93% N_2). Spent medium (600 µl) was removed from each well and replaced with fresh medium at 24-hour intervals. Subcultures were done at 72 hours by pooling the contents of culture wells with each medium treatment, and diluting 1:10 with normal RBC in fresh medium at a 10% (v/v) concentration. Blood smears were prepared daily and stained by the Giemsa method to observe the parasite and monitor its growth. The experiment was concluded after 6 days of cultivation.

Serum requirement experiment—After 1 medium was selected through the previous study, an experiment was conducted to observe the effect of different serum concentrations. Ten percent (v/v) infected/normal RBC suspensions with a PPE of 0.126 were prepared in medium 199-EBSS with $NaHCO_3$ containing 50%, 35%, 20%, 5%, and 0% serum. Cultures were set in duplicates with 805 µl/well volumes and incubated at 37 ± 0.1 C under atmosphere II (5% CO_2, 2% O_2, 93% N_2). Spent medium was removed from each well and replaced daily. Subcultures were made at 72 hours by pooling

duplicate cultures and diluting 1:20 with a 10% (v/v) normal RBC suspension prepared with the respective serum concentration. Giemsa-stained blood smears were prepared daily, and observed microscopically to evaluate parasite growth. The experiment was terminated after 6 days.

Animal inoculation with material from cell culture—On day 29, after initiation of in vitro cultivation, several culture wells were pooled to obtain a volume of 2.5 ml, mixed with 22.5 ml of a 10% (v/v) normal RBC suspension in medium 199-EBSS with $NaHCO_3$ supplemented with 35% BFS and 10.0 ml transferred to a 15.0-ml plastic flask (25 cm^2 surface) to prepare the inoculum. On day 0, calf B was inoculated IV with 12.5 ml of blood containing 1.5×10^8 *B bigemina* parasites produced after 36 days in vitro. Rectal temperature and PCV were recorded daily. Giemsa-stained blood smears were also prepared and observed each day. Serum samples were collected on PID 0, 7, 28, and 35. After clinical signs of the disease were observed, the animal was treated (IV injection) on PID 5 and 7 with a 1.0% trypan blue sterile solution in normal (0.75% NaCl) saline solution at a dosage of 1.0 ml/kg of body weight.

Reisolation—On PID 4, 40 ml of blood from calf B were aseptically obtained by jugular venipuncture and collected into sterile, evacuated, heparin-containing glass tubes. The infected blood, with a 1.476 PPE, was centrifuged at 1,000 × g for 15 minutes at 4 C and the supernatant, buffy coat, and the upper layer of sedimented erythrocytes were discarded. The original volume was restored using VYM solution. Remaining RBC were subsequently washed twice under the same conditions, removing supernatant and uppermost RBC layers each time. Erythrocytes were mixed 1:2 with fresh normal RBC collected as previously described and resuspended to a final 7.5% (v/v) concentration in medium 199-EBSS with $NaHCO_3$ supplemented with 30% BFS. Cultures were set in duplicate as 805 µl volumes/well and incubated at 37 ± 0.1 C under atmosphere II (5% CO_2, 2% O_2, 93% N_2). Spent medium was replaced at 24-hour intervals. Parasite development was monitored by Giemsa-stained blood smears prepared daily for microscopic observation. Subcultures were made as a 1:4 dilution at 48 and 1:10 dilution at 96 hours using 10% (v/v) normal RBC in freshly prepared serum-supplemented medium.

Challenge exposure—Calf B, which had been given the in vitro cultivated *B bigemina* parasites 28 days earlier, was challenge exposed IV with 1.5×10^8 cultured *B bovis* parasites. Rectal temperature was monitored, and blood samples for PCV and PPE determinations were collected daily.

TABLE 6—Summary of *B bigemina* growth in in vitro erythrocyte cultures from original isolation on day 0 until day 102

Day	Subculture	Dilution (accumulative)	PPE* Initial	PPE* Final
0†	...	2.50×10^{-1}	0.051	0.050
9‡	2	6.25×10^{-2}	0.308	1.208
14§	4	6.25×10^{-4}	0.178	0.747
23‖	7	3.12×10^{-7}	0.126	0.949
26¶	8	1.56×10^{-8}	0.047	1.693
35	11	1.95×10^{-12}	0.031	1.307
53	17	1.95×10^{-18}	0.095	1.942
68	22	1.95×10^{-23}	0.092	1.186
83	27	4.88×10^{-29}	0.103	1.026
99	33	4.88×10^{-35}	0.164	1.393

* Average PPE of duplicate cultures. † PID 0 = original isolation: culture medium 199-HBSS; 50% BFS; 5% RBC concentration; 4-mm depth; 37 ± 0.1 C; and atmosphere II (5% CO_2, 2% O_2, 93% N_2). ‡ PID 11: culture line started by subculture at a 1:10 dilution; 10% RBC concentration. § PID 14: culture medium changed to 199-EBSS; 40% BFS. ‖ PID 23: 20% BFS. ¶ PID 29: culture expanded for animal inoculation.

Indirect fluorescent antibody (IFA) test—Antigens were prepared separately from cultured *B bovis* and *B bigemina*. Infected blood was washed 3 times with sterile saline solution (0.75% NaCl) at 1,000 × g for 15 minutes at 4 C and resuspended to a 50% (v/v) concentration in saline solution with 1.5% bovine albumin. Thin smears made with these suspensions were wrapped in tissue paper and immediately stored at −70 C until use. Serial 2-fold dilutions of positive and negative sera were made with an initial 1:5 dilution in phosphate-buffered saline solution (pH 7.4). The test was carried out as previously described,[8] except that antigens were fixed in acetone for 30 minutes; the slides were incubated at 37 C for 30 minutes.

Results

Original isolation—Babesia bigemina parasites were first identified in Giemsa-stained blood smears from calf A on PID 4. Parasite numbers increased to a maximum PPE of 0.583 on PID 7 and slowly decreased to 0.229 on PID 14, when the calf became moribund and was euthanatized. The PCV decreased from 33% (PID 0) to 18% (PID 14). Temperatures ≥ 40.0 C were recorded on PID 2, 3, 13, and 14. Hemoglobinuria was observed intermittently from PID 7 to 14.

Only 3 media (199-HBSS, MEM-EBSS, and RPMI 1640) of the 6 used in the original isolation procedure supported parasite growth under low oxygen atmospheres I and II. Higher oxygen atmospheres (III, IV, V, and VI) were detrimental to parasite growth (Table 1). Parasites disappeared rapidly in cultures set in media L-15 and 199-EBSS, which lacked $NaHCO_3$. Cultures set in serum supplemented medium 199-HBSS in atmosphere II reached PPE values of > 1.0% before others. By PID 12, cultures set in MEM-EBSS and RPMI 1640 medium in atmosphere II had reached PPE that were indicative of growth (0.723 and 1.108 PPE, respectively). Parasite morphologic features in successful cultures were mostly indistinguishable from forms found in *B bigemina*-infected animals. Subsequent parasite development was continuously maintained by subcultures because the addition of fresh normal RBC enhanced parasite growth. Infected erythrocytes with parasite pairs were proportionally more abundant at 24 hours after subculture, regardless of the initial PPE; ring forms were more numerous at 72 hours. In cultures kept for longer periods without subculture, the PPE decreased with the intracorpuscular forms rounding up and becoming aberrant and degenerative (pyknotic).

Media experiment—All 4 media tested were able to sustain parasite growth under specified conditions (Table 2); however, medium 199-EBSS supported higher parasitemias than did 199-HBSS and it was therefore selected. Sodium bicarbonate was added to all subsequent media preparations.

Serum requirement experiment—Concentrations of fresh serum of 20% or higher seemed to be required to sustain growth. Parasite morphologic features appeared normal in cultures with 5% or more serum (Table 3).

Animal inoculation with material from cell culture—In vitro grown *B*

bigemina retained its infectivity for cattle (Fig 1). Parasites were first noticed in blood smears 24 hours after inoculation of cultured material and PPE peaked at 1.476 on PID 4. On PID 5, calf B became severely ill, its temperature was 40.4 C and the PCV was 22%, which corresponded to a 50% reduction of the original 44% on day 0. Hemolytic crises had occurred overnight on PID 2 through 5, but hemoglobinuria was not observed until PID 4. Treatment with trypan blue solution was effective, because the parasites transiently disappeared from the peripheral blood and PCV values stabilized. Calf B had clinically recovered by day 26 with a PCV of 31%, but remained a positive carrier for the rest of the experiment.

Reisolation—Babesia bigemina was successfully recovered from the peripheral blood of calf B at the time of clinical illness. Parasite cultures were initiated with 0.736 PPE on PID 0 and increased to more than 1.0 PPE by PID 6 (Table 4).

Challenge exposure—Calf B was challenge exposed with *B bovis* after clinical recovery from *B bigemina* infection (Fig 1). Decreases in PCV were not noted. Morphologically distinct *B bovis* infected RBC were detected in Giemsa-stained peripheral blood smears 5 days after challenge exposure. Neither hemolytic crises nor hemoglobinuria were observed. However, calf B had typical clinical signs of CNS damage, such as incoordination and paddling, by day 8 after challenge exposure when calf B died.

The IFA test—Specific positive sera did not cross-react at a dilution > 1:80 when tested against heterologous antigens (Table 5).

Discussion

This report presents evidence of the continuous in vitro cultivation of *B bigemina* (Table 6). The use of VYM solution for the washing and storage of infected and normal RBC was beneficial for the establishment of parasite cultures. De-Verdier et al reported[9] the favorable effect of citrate-phosphate-dextrose supplemented with adenine-guanosine on the stability of human blood stored at 4 C. Such treatment promoted higher levels of nucleotide triphosphates within the RBC and was assumed to provide a reserve of energy-rich compounds. Whether this same effect occurred with respect to the bovine RBC, the parasite, or both is not clear. The present system still may not be optimal, because the PPE rarely becomes > 3.0. One possible explanation may relate to limited availability of key nutrients or critical changes in pH. An illustration of such a critical effect was indicated by the successful parasite reisolation from calf B, as opposed to the original isolation attempt from calf A, using the same culture medium 199-EBSS, with and without $NaHCO_3$, respectively.

The results of this study on the comparison of the effects of various atmospheres on the in vitro cultivation of *B bigemina* emphasized its microaerophilic nature. The best atmosphere was 5% CO_2, 2% O_2, and 93% N_2. Rodriguez et al[4] reported a similar observation when a parasitemia of < 0.1% was used to establish in vitro cultures of *B bovis*, a closely related organism.

The established process does not alter the infectivity of *B bigemina* for the bovine host, as illustrated by the inoculation of calf B. The organism was isolated in pure culture in vitro, inoculated into a susceptible animal, the disease was reproduced, and the organism was reisolated in pure culture. The definitive identity of the parasite as *B bigemina* was substantiated by the results of the challenge study and IFA test results; the IFA observation basically agreed with the data reported by Leeflang and Perie.[10] Immunogenically, the cultured parasite did not elicit protection against challenge exposure with *B bovis*. This result was a confirmation of the absence of cross protection between *B bigemina* and *B bovis*, as reported by Smith et al.[11]

The in vitro procedure of the present study can be used to produce specific antigen for serology and has the potential of contributing to the production of specific immunogens. In addition, in vitro cultivation can be used to study the life cycle, physiologic processes, and biochemistry of the parasite.

References

1. Erp EE, Gravely SM, Smith RD, et al: Growth of *Babesia bovis* in bovine erythrocyte cultures. *Am J Trop Med Hyg* 27:1061–1064, 1978.

2. Levy MG, Ristic M: *Babesia bovis*: Continuous cultivation in a microaerophilous stationary phase culture. *Science* 204:1218–1220, 1980.

3. Palmer DA, Buening GM, Carson CA: Cultivation of *Babesia bovis* in vitro in Hidalgo RJ, Jones EW (ed): *Proc 7th Natl Anaplasmosis Conf*, Mississippi State, Miss, Mississippi State University Press, 1981, pp 207–217.

4. Rodriguez SD, Buening GM, Green TJ, et al: Cloning of *Babesia bovis* by in vitro cultivation. *Infect Immun* 42:15–18, 1983.

5. Timms P: Short term cultivation of *Babesia* species. *Res Vet Sci* 29:102–104, 1980.

6. Trager W, Jensen JB: Human malaria parasites in continuous culture. *Science* 193:673–675, 1976.

7. Palmer DA, Buening GM, Carson CA: Cryopreservation of *Babesia bovis* for in vitro cultivation. *Parasitology* 62:221–231, 1982.

8. Goldman M, Pipano E, Rosenberg AS: Fluorescent antibody tests for *Babesia bigemina* and *B berbera*. *Res Vet Sci* 13:77–81, 1972.

9. De-Verdier CH, Strauss D, Erickson A, et al: Purine metabolism of erythrocytes preserved in adenine, adenine-inosine, and adenine-guanosine supplemented media. *Transfusion* 21:397–404, 1981.

10. Leeflang P, Perie NM: Comparative immunofluorescent studies on 4 *Babesia* species of cattle. *Res Vet Sci* 13:342–346, 1972.

11. Smith RD, Molinar E, Larios F, et al: Bovine babesiosis: Pathogenicity and heterologous species immunity of tick-borne *Babesia bovis* and *B bigemina* infections. *Am J Vet Res* 41:1966–1976, 1980.