BARD

FINAL REPORT

PROJECT NO. US-438-81

**Immunization of Cattle with Antigens Derived from
Continuous *In vitro* Cultures of *Babesia bovis***

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1987

Standard BARD Cover Page for Scientific Reports

Date 6/20/86

BARD
P.O. Box 6
Bet Dagan, ISRAEL

BARD Project No. US 438-81

Title of Research Project:

Immunization of Cattle with Antigens Derived from Continuous in vitro
Cultures of Babesia bovis

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Project's starting date: 5-1-82

Type of Report: 1st Annual 2nd Annual Final x

Signatures:

Principal Investigator

Institution's Authorizing Official

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REPORT OF THE U.S. INVESTIGATOR

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I. ABSTRACT

Five vaccination trials have been conducted under controlled laboratory conditions. The first two experiments were conducted to determine the cross-protective capacity of a vaccine derived from Babesia bovis cultures; the third experiment analyzed the immunoprotective potential of a similar vaccine prepared from B. bigemina cultures; the fourth experiment determined the usefulness of an attenuated vaccine against Anaplasma marginale in the lyophilized state and the fifth trial, terminated in June of this year, studies the efficacy of a bivalent vaccine against bovine babesiosis applied in different doses.

These data have provided the basis for the initiation of vaccine trials under field conditions, necessary for the final evaluation of culture-derived vaccines.

In order to afford maximum protection, vaccination with a polyvalent immunogen against the principal hemotropic agents (B. bovis, B. bigemina, and A. marginale) is recommended in the tropics where susceptible cattle are concurrently infected. It is suggested that mass vaccination of high-risk animals be initiated.

II. ^{The original} OBJECTIVES AND SIGNIFICANCE OF THE RESEARCH

1. To cultivate B. bovis continuously in vitro and to assess infectivity and possible attenuation of virulence of cultured parasites.
2. To prepare live and killed vaccines from cultured parasites free of host material.
3. To evaluate the safety and potency of both types of vaccines for various ages and breeds of cattle.
4. To assess the potential of double vaccination (killed vaccine followed by live vaccine) as a means of avoiding severe reactions while inducing strong immunity in sensitive breeds of cattle.
5. To determine cross-protective immunity between homologous and heterologous isolants of B. bovis using a newly-developed merozoite neutralization test.
6. To assess protective immunity against an individual B. bovis isolant in cattle inoculated with a vaccine containing antigens of multiple isolants.
7. To isolate, purify and determine biochemical properties of merozoite surface coat antigens as a prerequisite for future genetic engineering studies.

Improved control of babesiosis by means of a vaccine that is safe and potent under all conditions

can lead to reducing losses in existing cattle in Israel, and can facilitate the improvement of native cattle by cross-breeding with highly productive imported breeds which are extremely susceptible to babesiosis. The U.S. would be safeguarded from a renewed introduction of bovine babesiosis while foreign buyers would be encouraged to purchase immune cattle from this country.

III. COMPREHENSIVE REPORT

A. Objective 1: Duration of protective immunity to homologous challenge using soluble culture-derived Babesia bovis antigens as immunogens.

While the BARD proposal was under consideration, the study of this objective sponsored by grants from the Rockefeller Foundation of New York and the International Minerals and Chemical Corporation of Terre Haute, IN, was in its initial experimental phase. The study was conducted jointly between the University of Illinois (Levy, Ristic) and the USDA, ARS (Kuttler).

The results of this vaccination and challenge experiment demonstrated that the clinical protection

against homologous challenge in cattle vaccinated with culture-derived soluble exoantigens of B. bovis lasted at least 6 months which was the longest period studied.

The reprint of the paper describing findings of this study and methods and material used is enclosed.

B. Conclusion:

The results of the present study confirmed those of the previous short-term vaccine experiment in that the inactivated cell culture-derived B. bovis vaccine is suitable for induction of protective immunity against bovine babesiosis. It is evident from the results of this experiment, which used greater numbers of susceptible cattle, that the vaccinal immunity, approximately 6 months after vaccination, is sufficient to prevent mortalities. Also, other hematologic and serologic values were significantly in favor of vaccinated animals. The results of these studies are comparable with those of a similar experiment in which animals were challenged by exposure to B. bovis infected Boophilus microplus larvae. Consequently, the duration of protection induced by the vaccine appears sufficiently long to allow for safe introduction of vaccinated cattle into

enzootic areas and provides for the development of long-term protection after natural exposure.

C. Objective 2: Cross-protection between homologous and heterologous isolants using merozoite serum neutralization (MN) test.

The development of a merozoite serum neutralization (MN) test was made possible as a result of the development of MASP culture system for continuous in vitro propagation of Babesia bovis. In the MN test, MASP cultures containing uninfected bovine erythrocytes and test sera in various dilutions (immune and normal) are allowed to preincubate in a CO₂ incubator at 37° C for 1 hour. Following this period, 20 ul of a cell-free merozoite suspension is added to 200 ul of the MASP cultures contained in microtiter plates. These are allowed to incubate for 1-16 hours after which thin films are prepared. The percent parasitemia is determined by examination of the films after Giemsa-staining and the percent infection compared with that of control cultures, containing non-immune serum. The MN titers correlated well with those of the indirect fluorescent antibody test.

Various attempts to correlate the titer of the MN test with actual protection between homologous and heterologous isolants has failed, indicating that the protection is not an expression of antibody activity alone. There is strong evidence that the antibody to soluble exoantigens cooperates with cellular immune elements in the induction of protective immunity. This finding has been confirmed by our Israeli colleagues in their report.

In order to accomplish this objective between homologous and heterologous B. bovis strains, since we were not permitted to work in the U.S. with B. bovis strains other than those of Mexico, we arranged for these studies to be conducted in Maracay, Venezuela in collaboration with scientists at the Center for Veterinary Investigation in that city. Representing our group in this study was Dr. Sonia Montenegro-James, an Assistant Professor in our college. The following are findings of this vaccination trial:

D. Objective of the Trial.

To cross-test protective effect among Babesia bovis isolants from various regions of Latin America using culture-derived soluble MASP antigens.

IV. EXPERIMENTAL DESIGN

Twenty-seven susceptible yearling cross-bred cattle which originated from a high altitude region of Colombia were divided into two groups and each group vaccinated with soluble antigens derived from Venezuelan and Mexican strains, respectively. Equal number of unvaccinated controls were included in each group. Subgroups of cattle from each of the above groups were each challenged three months following the second vaccination with 6×10^8 virulent B. bovis organisms of Colombian, Argentinian, Equadorian, Venezuelan and Mexican origin, respectively. The major parameters used to evaluate cross-protection were the following:

- a. Prepatent period, i.e., time elapsed from challenge exposure to first detectable parasitemia.
- b. Presence, level and duration of parasitemia.
- c. Maximal PCV reduction.
- d. Level and duration of fever.
- e. Presence and concentration of cryofibrinogen in the blood of experimental animals.
- f. Serum antibody levels as determined by the IFA test.

- g. Net gain of body weight 6 weeks following challenge.

A. Grouping and Treatment of Experimental Cattle

1. Vaccinal groups.

- a. Soluble immunogen derived from cultures of the Venezuelan B. bovis strain: 8 animals.
- b. Soluble immunogen derived from the Mexican B. bovis strain: 8 animals.
- c. Unvaccinated controls: 9 animals.

2. Challenge groups.

<u>Group</u>	<u>Challenge Strain</u>	<u>No. Vaccinated Animals & Vaccinal Strain</u>		<u>No. Controls</u>
1	Colombia	2 Venezuelan		2
		2 Mexican		
2	Argentina	2 Venezuelan		2
		2 Mexican		
3	Equador	2 Venezuelan		2
		2 Mexican		
4	Venezuela	2 Venezuelan		2
		2 Mexican		
5	Mexico	2 Venezuelan		1

3. Vaccinal dose and time of administration.

Two 1 ml doses of the antigen-adjuvant (Saponin) mixture administered subcutaneously on day 0 and day 21.

4. Challenge time, dose and route of administration.

Dose: 6×10^8 parasitized erythrocytes.

Route: Intravenous (1 to 5 ml volume).

Time: Three months after the second vaccinal dose.

B. Results.

The results of this experiment revealed:

1. Good homologous protection in cattle vaccinated with Venezuelan strain derived immunogen and challenged with the same strain. This finding confirms previous results in the United States made by Dr. K.L. Kuttler and colleagues using the Mexican isolant of B. bovis. Consequently, the finding ascertains that the technology of using culture-derived soluble antigens as a vaccine is applicable to strains of B. bovis from regions other than Mexico.
2. A good degree of cross-protection was shown when cattle vaccinated with Venezuelan strains were challenged with virulent strains from Ecuador and Argentina, respectively. This finding is significant as it indicates common protective antigens among the strains from various geographical regions. There was

little or no protection in cattle vaccinated with Venezuelan strains and challenged with Colombian and Mexican strains. Similarly, there was a low degree of cross-protection when cattle vaccinated with the Mexican strain were cross-challenged with the above specified heterologous strains.

3. The interesting feature of the above findings is the difference observed in the cross-protective capacity between the Mexican strain and the Venezuelan Equadorian, Colombian, and Argentinian strains. One possible explanation of this difference is the fact that the Mexican border has been closed for the exchange of cattle for the last 30 years due to the control of foot and mouth disease. Moreover, this difference between the Mexican strain and the other strains may explain the failure of our earlier vaccination trials in Colombia.

V. CONCLUSION

1. Based on post-challenge hematologic parameters and weight gain, the protection was demonstrated among Venezuelan, Equadorian and Argentinian strains. Mexican strain protected only against itself.

2. The two scientifically most indicative parameters of protection are considered to be the presence of cryofibrinogen in the blood of challenged animals and the rate of weight gain of these animals post-challenge. These data are graphically presented in figures 1 and 2.

3. Cross-serologic reactivity was demonstrated among all B. bovis strains. The earliest anamnestic response post-challenge was demonstrated with homologous strains.

A. Objective 3: Isolation and purification of cell culture-derived soluble B. bovis exoantigens. The first part of this study has dealt with the development of a test suitable for detection and titration of isolated purified antigens and the second part was concerned with actual isolation methods.

1. Development of a direct Enzyme immunoassay using bovine anti-B. bovis IgG. Bovine anti-B. bovis purified IgG (I.F.A. 1: 163,000) was conjugated with Horseradish Peroxidase. This was accomplished using the one step glutaraldehyde method. The resulting conjugate was tested for working strength.

Fig. 1. Average weight gain at 6 weeks post challenge in animals vaccinated with the antigen derived from Venezuelan B. bovis strain.

Fig. 2. Post challenge cryofibrinogen levels in the blood of cattle vaccinated with the antigen derived from the Venezuelan B. bovis strain.

2. The use of cation exchange chromatography (SP-Trisacryl M) as a preparative step for the partial purification of B. bovis culture-derived antigens. A total of 10 ml (10X concentration) of B. bovis crude supernatant was passed in a continuous NaCl gradient in SP gel. Eluate was collected (80 tubes/2 ml each) and the protein was estimated (samples were tested individually) using the Bradford method. All samples were tested for parasite antigens using direct EIA. Verification of contaminants was carried out by the following methods: EIA using rabbit anti-bovine IgG and immunoelectrophoresis using rabbit anti-bovine albumin. The samples which gave low protein reading and positive reaction to direct EIA were pooled, concentrated, dialyzed and stored.

Figure 3 indicates that direct EIA using bovine anti-B. bovis IgG gave satisfactory results. The optimal conjugate dilution is 1/200. The diluting buffer with 4% polyethylene glycol gave the best results. The use of merozoite lysate as a standard positive reference antigen also was satisfactory.

It appears that parasite antigens may elute at high salt molarities (2.5M). Also at this

Fig. 3. ELUTION PATTERN OF BABESIA BOVIS CRUDE CULTURE SUPERNATANT IN SP-TRYSACRYL M CATION EXCHANGER AND DETECTION OF TARGET PARASITE ANTIGENS USING DIRECT E.I.A.

molarity, contaminants such as IgG elute as well. Current studies indicate that the antigenic fraction may be further purified by specific removal of IgG (DEAE Affi-Gel Blue adsorption).

B. Program for the Second Year.

1. Continue with isolation and purification of protective antigens present in the supernatant medium of MASP culture of B. bovis.
2. Subject purified molecular entities of the above antigens to biophysical and biochemical characterization.
3. Observe immune responses of cattle inoculated with purified antigens.
4. In vitro studies of interaction between soluble B. bovis antigen and bovine monocytes.

C. Training.

Mrs. Leah Fish of the Kimron Veterinary Institute spent one and one-half months on training in our laboratory. The focus of her training was directed towards in vitro cultivation of B. bovis. Mrs. Fish successfully completed her assignment and returned to Israel where she was able to implement acquired knowledge there.

During the second year of the program through the first half of the 3rd year, purification and characterization studies were continued and further immunization studies were conducted.

VI. ISOLATION AND PURIFICATION OF PROTECTIVE ANTIGENS
PRESENT IN THE SUPERNATANT MEDIUM OF MASP CULTURE OF
BABESIA BOVIS

A two-site enzyme immunoassay (EIA) was developed to detect target antigens. This test proved to be specific and reproducible. The specificity was measured by dose-response and cross-reactivity with other antigens. The EIA was able to detect target antigens in samples containing 1 nanogram of total protein. For cross-reactivity studies, a total of 7 antigens were tested and only B. bigemina cross-reacted. The test had a coefficient variation of 9.2%.

Supernatant medium from MASP cultures was subjected to the following preliminary preparative procedures: pooled supernatants were centrifuged at 3,000 x g for 45 minutes and dialyzed by adding 10% dextran sulfate followed by 1M calcium chloride. After centrifugation at 5,450 x g, the supernatant and the precipitate were tested for the presence of target antigens. Results indicated

that the antigenic activity remained in the supernatants. Secondary fractionation of delipidized MASP supernatants was achieved by 40% ammonium sulfate precipitation. The resulting precipitate and supernatants were tested for target antigens using EIA. The antigenic activity was detected in the precipitates.

Further fractionation of antigen-containing ammonium sulfate precipitate was achieved by anion exchange chromatography using DE-52. Two step-wise gradients were used: NaCl and pH. Results indicated that better separation was achieved using pH gradient in 10mM Barbitol-Sodium acetate buffer. Target antigens were detected in the pH 5 eluate. Further purification of target antigens was achieved by using high performance liquid chromatography (HPLC).

VII. BIOPHYSICAL AND BIOCHEMICAL CHARACTERIZATION

Purified target antigens from HPLC were subjected to the following studies: molecular weight determination using vertical sodium dodecyl sulfate (SDS) polyacrylamide (PAGE) electrophoresis, immunoblotting, isoelectric focusing, physical stability using enzyme treatment and thermostability. The antigens were also subjected to amino acid sequencing.

VIII. IMMUNE RESPONSES IN CATTLE INOCULATED WITH BABESIA BOVIS EXOANTIGENS

In last years report we described the results of the vaccination of susceptible cattle with two B. bovis strains when culture-derived antigens from selected, less virulent isolates were used together as components of a soluble B. bovis immunogen.

Thirty-two susceptible yearling cross-bred Holstein cattle from a tick-free zone in Colombia were used in this experiment. Sixteen animals were vaccinated with soluble antigen derived in vitro from high passage Venezuelan, Mexican and Australian strains. Another sixteen animals were retained as non-vaccinated controls. The vaccine was administered subcutaneously in two 3 ml doses on days 0 and 21. The vaccinated and control animals were divided into 4 subgroups (n=4) and challenged one month after the second vaccination with 1×10^9 virulent B. bovis organisms of either the Venezuelan, Mexican, Argentinian or Ecuadorian strains.

A. Collection and Analysis of Data.

Body temperature, PCV and platelet levels, parasitemia (thin blood film), IFA titers and selected erythrophilic proteins were monitored throughout the

experiments. Plasma fibrinogen and cryofibrinogen levels were determined by the radial immunodiffusion test and by cold precipitation, respectively. Homologous and heterologous IFA titers were measured from sera of vaccinated animals. Net gains in body weight post-challenge were also calculated. The statistical significance of the difference between means was established by the student's 't' test.

B. Results.

Homologous antibody levels produced in response to vaccination with the single-strain and multi-strain immunogens are shown in Fig. 4. Pre-challenge IFA titers to the multi-strain B. bovis immunogen were significantly lower with a more rapid diminution to baseline levels than for those of either the single-strain Venezuelan or Mexican immunogens. Maximal post-vaccinal titers corresponded to those induced by the Australian strain (1:1000), followed by those of the Venezuelan (1:766) and Mexican (1:448) strains, respectively.

The degree of protection was also assessed by comparing relevant clinical and pathophysiological parameters with those observed in control animals. Data collected following heterologous challenge are summarized

Figure 4

in Table 1. Results were similar to those obtained in the experiment described in last year's report, as vaccinated animals were protected against heterologous challenge with B. bovis strains from Argentina and Ecuador. Only mild, transient clinical changes were observed among vaccinates, whereas all control cattle manifested severe clinical disease characterized by ataxia, labored respiration, profuse salivation, anorexia and jaundice. Some significant clinical differences were: duration of parasitemia (vaccinated = 2.8 days, controls = 7.3 days after the Argentinian challenge; (p 0.05), duration of anemia (vaccinated = 2.8 days, controls = 11.3 days after the Ecuadorian challenge; p 0.01), duration of elevated fibrinogen (vaccinated of both groups = 4.5 days, controls = 13.3 days and 10.3 days after Argentinian and Ecuadorian challenges, respectively, p 0.05).

A typical IFA response after heterologous challenge is illustrated in Fig. 5. An early anemnestic response, as observed following the Argentinian challenge, was characteristic with antibody titers (1:40, 960 for vaccinated animals and 1:2, 560 for controls) peaking two weeks post-challenge.

Table I. Clinical and pathophysiological responses¹ of vaccinated (multi-strain² B. bovis immunogen) and control animals to challenge exposure with heterologous B. bovis strains.

Type of challenge Measurements	Argentina		Ecuador	
	Vaccinated	Controls	Vaccinated	Controls
Prepatent period (days)	3.3 $\pm$ 0.3	3.3 $\pm$ 0.3	4.5 $\pm$ 0.9	3.7 $\pm$ 0.3
Duration of parasitemia (days)	2.8 $\pm$ 0.8*	7.3 $\pm$ 0.9	1.8 $\pm$ 0.5*	5.0 $\pm$ 0.6
Duration of fever (days)	1.0 $\pm$ 0.**	4.0 $\pm$ 0.7	1.5 $\pm$ 0.3**	4.7 $\pm$ 0.9
Duration of anemia (days)	1.0 $\pm$ 0.4**	9.7 $\pm$ 0.9	2.8 $\pm$ 1.1**	11.3 $\pm$ 3.2
Maximum % PCV reduction	37.5 $\pm$ 0.9**	54.3 $\pm$ 5.5	41.5 $\pm$ 1.5**	54.7 $\pm$ 6.2
Maximum % platelet reduction	75.9 $\pm$ 6.7	83.9 $\pm$ 4.6	72.0 $\pm$ 3.0*	82.6 $\pm$ 6.6
Maximum % fibrinogen increase	67.7 $\pm$ 10.5*	103.5 $\pm$ 16.1	54.2 $\pm$ 10.1	81.4 $\pm$ 13.9
Duration of elevated fibrinogen levels (days)	4.5 $\pm$ 2.1*	13.3 $\pm$ 1.7	4.5 $\pm$ 1.2*	10.3 $\pm$ 1.5
Weight gain (kg) 3 weeks postchallenge	13.5 $\pm$ 2.2*	4.7 $\pm$ 2.9	12.3 $\pm$ 2.8*	3.1 $\pm$ 2.3

1. Group mean + standard error.

2. B. bovis from Venezuela, Mexico, and Australia (high-passage).

* Significantly different from control values, $p < 0.05$.

** Significantly different from control values, $p < 0.01$.

Figure 5

C. In vitro studies of interaction between soluble Babesia bovis antigens and bovine monocytes.

Bovine monocytes from Babesia-free cattle were cultured in vitro. The cell monolayers were treated with MASP culture-derived supernatants. The monolayers were washed free of the supernatants and allowed to develop further in the presence of standard medium. Transfer of supernatant media from monocyte cultures to those of B. bovis revealed the presence of growth inhibition factors. These factors were thermostable, non-dialyzable, degraded by freeze/thawing and their action was concentration dependant. Erythrocytes incubated with supernatant medium from antigen treated monocytes did not support the growth of B. bovis. Scanning electronmicroscopy of treated monocytes showed an increase in size and loss of surface structures.

The results obtained suggests that the mechanism of action of soluble factors could be mediated through the blockage of active transport of essential nutrients into host cells, or by destabilizing the parasitic membrane while in the exoerythrocytic environment. These mechanisms may aid in controlling

parasites early in the course of infection prior to specific humoral-mediated responses and also may play a role in immunity during the post-acute, carrier phase.

IX. STATUS OF ON-GOING FIELD VACCINATION TRIALS IN VENEZUELA
USING BABESIA EXOANTIGENS - YEAR 3

A. Background.

Vaccination trials conducted under field conditions are necessary to provide the final evaluation of the efficacy of exoantigens as immunogens. To that effect, Venezuela represents an ideal epidemiological setting with highly susceptible purebred cattle (imported regularly from the U.S. and Canada), demonstrating an enzootic instability for hemoparasitic infections, and also cross-bred animals with a varying range of enzootic stability for these infections. Since the initiation of the field trials in July 1984, two trials have been completed. As of April 1986, we have 6 ongoing trials in 6 states of Venezuela.

B. Criteria for Selection of Farms.

Dairy farms were selected for the evaluation of the exoantigen vaccine because of the relatively high

quality of animals (most importations consist of dairy cattle). Also, different management practices are found according to the geographic location and size of the farm, offering a wide range of epidemiologic conditions. Finally, the clinical follow-up is conducted at regular intervals.

C. Epidemiological Characteristics of Selected Farms.

Four farms corresponding to field trials No. 1, 2, 3 and 5 are in the category of enzootic instability (susceptible, imported purebred animals, kept in partial confinement) and four farms corresponding to field trials No. 4, 6, 7 and 8 fall into the enzootically stable category (purebred or cross-bred with early exposure to parasites as expressed by prevalent antibody titers).

D. Vaccination Scheme, Dosage and Sampling of Animals.

Days 0 and 21; B. bovis (2 ml-eq.) and B. bigemina (3 ml-eq.) administered subcutaneously with 3 mg saponin adjuvant. On days 0, 21, and monthly thereafter, blood and serum samples are taken for determination of antibody titers, PCV values and PPE (body weights when possible).

E. Summary of Results.

Field trials No. 1.

Location: Zulia State (80% of dairy cattle in Venezuela).

Time period: Initiated July 17, 1984 - terminated January 1985.

Farm identification: Dairy La Guacharaca.

Epidemiological status: Enzootic instability.

Herd size and breed: 2,000 head, consisting of 600 imported pregnant Holstein cows.

Vaccinated animals: 600 cows; data collected from 20 vaccinated cows and 10 non-vaccinated calves

Incidence and prevalence at day 0: negative to hemoparasites.

Mean max. antibody titer after vaccination (day 30 post-II): B. bovis 1:686; B. bigemina 1:905.

Mean antibody titer when taken to the field (day 60 post-II): B. bovis 1:49; B. bigemina 1:80.

Seroconversion after 2 months of natural tick exposure:

<u>Vaccinated</u>	<u>Controls</u>
<u>B. bovis</u> 1:1810	<u>B. bovis</u> 1:335
<u>B. bigemina</u> 1:2152	<u>B. bigemina</u> 1:597

Mean max. decrease in PCV after field exposure:

vaccinated 22.1%; controls 30.6%.

% Babesia-positive animals as determined by blood smears: vaccinated 20%; controls 70%.

Mortality: vaccinated 0/20; controls 1/10.

Trial terminated after 4 months under natural challenge conditions.

Field trial No. 2.

Location: Guarico State

Time period: Initiated June 19, 1985; terminated
March 1986.

Farm identification: Las Palmeras.

Epidemiological status: enzootic instability.

Herd size and breed: 1,500 head, purebred Brahman.

Vaccination animals: 18 calves; control animals: 18 calves - 36 total calves monitored.

Incidence and prevalence at day 0: negative for hemoparasites.

Mean max. antibody titer after vaccination (day 30 post-II): B. bovis 1:190; B. bigemina 1:269.

Mean antibody titer at tick exposure (day 90 post-II):

B. bovis 1:40; B. bigemina 1:40.

Seroconversion after 4 months under natural challenge:

<u>Vaccinated</u>	<u>Controls</u>
<u>B. bovis</u> 1:160	<u>B. bovis</u> 1:67
<u>B. bigemina</u> 1:269	<u>B. bigemina</u> 1:80

Mean max. decrease in PCV after field exposure:

vaccinated 5%; controls 23%.

Average weight gain after 2 months of field exposure:

vaccinated 12.2 kg; controls 4.5 kg.

No parasitemia nor mortality among experimental animals throughout the trial; terminated after 5 months of field exposure.

Field trial No. 3.

Location: Guarico State.

Time period: Initiated July 31, 1985.

Farm identification: FONAIAP Experimental Station
"Calabozo".

Epidemiological status: enzootic instability.

Herd size and breed: 500 calves, Siboney (3/4 Holstein, 1/4 Zebu), one-year old vaccinated animals: 20 heifers; control animals: 4 heifers.

Incidence and prevalence at day 0: negative for hemoparasites.

Mean max. antibody titer after vaccination (day 30

post-II): B. bovis 1:278; B. bigemina 1:403.

Mean antibody titer when taken to field (day 60 post-

II): B. bovis 1:90; B. bigemina 1:160.

Seroconversion after 2 months under field conditions:

<u>Vaccinated</u>	<u>Controls</u>
<u>B. bovis</u> 1:4530	<u>B. bovis</u> 1:127
<u>B. bigemina</u> 1:2903	<u>B. bigemina</u> 1:320

Mean max. decrease in PCV after 2 months in the field:

vaccinated 8%; controls 35%.

Mortality: vaccinated 0/20; controls 1/4 (animal which died showed 50% decrease in PCV and increasing titers against B. bovis).

Mean antibody titer after 6 months of field exposure:

<u>Vaccinated</u>	<u>Controls</u>
<u>B. bovis</u> 1:861	<u>B. bovis</u> 1:202
<u>B. bigemina</u> 1:844	<u>B. bigemina</u> 1:253

Average weight gain after 3 months of field exposure to ticks: vaccinated 50 kg; control 24 kg.

Field trial No. 4.

Location: Miranda State

Time period: November 1, 1985

Farm identification: Dairy Tazon de Cua

Epidemiological status: enzootic instability

Herd size and breed: 300 head, Brown Swiss.

Vaccinated animals: 20 heifers (4-12 months old);

controls: 8 young bulls (4-12 months old).

Incidence and prevalence at day 0: negative to hemoparasites.

Mean antibody titer after vaccination (day 60 post-II); B. bovis 1:113; B. bigemina 1:201.

Outbreak of rabies, three animals died in November 1985.

Field trial No. 5.

Location: Carabobo State

Time period: Initiated January 30, 1986

Farm identification: Dairy La Cuchuela

Epidemiological status: enzootic stability.

Herd size and breed: 120 head, cross-bred Holstein and Brown Swiss.

Vaccinated animals: 100 cows (20 cows followed);

Controls: 20 calves (6-8 months old).

Incidence and prevalence at day 0: 35% positive B. bovis or B. bigemina; 75% positive A. marginale.

Field trial No. 6.

Location: Yaracuy State

Time period: February 27, 1986.

Farm identification: Dairy Rio Tocuyo.

Epidemiological status: enzootic stability.

Herd size and breed: 150 cross-bred Holstein and Brown Swiss.

Vaccinated animals: 100 heifers (20 followed);

Controls: 50 heifers (20 followed).

Incidence and prevalence at day 0: 60% positive to Babesia.

Field trial No. 7.

Location: Zulia State.

Time period: Initiated February 19, 1986.

Farm identification: Dairy La Rosaleda, El Guayabo.

Epidemiological status: enzootic stability.

Herd size and breed: 3,000 head, Holstein.

Vaccinated animals: 120 cows (25 followed);

Controls: 10 cows.

Incidence and prevalence at day 0: 50% Babesia; 40%

A. marginale.

Field trial No. 8.

Location: Portuguesa State

Time period: initiated April 10, 1986.

Farm identification: Dairy Las Majaguas, San Rafael.

Epidemiological status: enzootic stability.

Herd size and breed: 120 head, Holstein.

Vaccinated animals: 80 heifers (20 followed);

controls: 10 heifers.

Incidence and prevalence at day 0: 30% B. bigemina;
10% A. marginale.

F. Description of Collaborative Facilities.

Department of Parasitology, The Kimron Veterinary
Institute:

The Department of Parasitology occupies 1,700 sq. ft. of laboratory space. The laboratory is equipped with the usual items employed in parasitological work, i.e., conventional and fluorescence microscopes, refrigerated conventional and high speed centrifuges, ultrasonic disintegrator, equipment for serology, incubators, etc. Instruments such as ultra-high speed centrifuge, liquid scintillation counter, gamma counter, coulter counter, and others are available as central institutional equipment. Three barn buildings (total 4,500 sq. ft.), two with individual cattle boxes and one that is fly-proof, are used by the department. As a general rule, forty to fifty head of cattle are housed in these barns for vaccine production and research. The

PRUEBAS DE CAMPO CON VACUNA ANTI-BABESIOSIS, INACTIVADA EXPERIMENTAL, OBTENIDA DE CULTIVOS DE B. BIGEMINA Y B. BOVIS (CONVENIO FONAIAP-UNIVERSIDAD DE ILLINOIS)

1. LA GUACHARACA, MACHIKES, EDO. ZULIA
2. LAS PALMERAS, CALABOZO, EDO. GUARICO
3. ESTACION EXPERIMENTAL FONAIAP, CALABOZO
4. HACIENDA TAZON DE CUA, CUA, EDO. MIRANDA
5. LA CUCHUELA, MARIARA, EDO. CARABOBO
6. RIO TOCUYO, YARACAL, EDO. FALCON
7. LA ROSALEDA, EL GUAYABO, EDO. ZULIA
8. LAS MAJAGUAS, SAN RAFAEL, EDO. PORTUGUESA

staff includes 3 veterinarians, 2 PhD parasitologists, 2 MSc, 3 technicians, and 2 animal caretakers. The main activities of the Department are laboratory and field research in parasitic diseases of cattle, parasitological diagnosis (protozoan and helminths), and production of vaccines (A. centrale, B. bigemina, B. bovis, and T. annulata). Field experimentation includes evaluation of experimental vaccination programs and seroepidemiological surveys.

Hemotropic Disease Section, The University of Illinois:

Nine well-equipped microbiology-immunology laboratories are available for these studies. Major equipment includes refrigerated-vacuum centrifuges (4), laminar-flow isolation units (4), column chromatographic (including automated sampler) and immunoelectrophoresis apparatuses, ultraviolet microscopes (2), sonic oscillators (2), variable temperature and gas incubators (6), electron microscope, systems for radioisotopic studies, tissue culture apparatus, histochemical and immunologic staining equipment, ultra-low temperature freezers (4), and cryopreservative apparatus. Seven of the

laboratories are in the Veterinary Research Farm of the University of Illinois situated at the southern outskirts of the campus. Two other laboratories are in the Basic Science Building on the campus of the university. Isolation facilities for cattle and tick vectors are available at the Veterinary Research Farm and the Veterinary Medical Research Building located adjacent to the Basic Sciences Building.

Academic staff of the section includes: 1 full professor, 1 associate professor, 2 assistant professors, 1 post doctoral scientist, 12 graduate students, 2 visiting scientists, 4 technicians, 4 dishwashers and animal caretakers.

G. Expected Contribution (Scientific, Economic).

Development of a safe and potent vaccine from cultured B. bovis parasites may serve as a model for developing vaccines against other species of Babesia in domestic animals as well as against other intraerythrocytic parasites of man and animals.

Corpuscular and soluble antigens free of host cell tissue components can be used for the development of practical and specific serodiagnostic procedures useful for epidemiology and field control studies of

babesiosis. The presently used method of screening potential anti-babesial drugs in animals has been time consuming and costly. The above described culture system provides an inexpensive and rapid tool for that purpose.

A vaccine for babesiosis would find widespread use in Israel and would significantly contribute to the reduction of losses imposed by this disease. Dairy cattle in Israel are not allowed to pasture during the non-lactating period because of the hazard of Babesia infections, among others. The possibility of utilizing pasture land for dairy cattle protected against babesiosis will have a considerable economic impact on milk production. In addition, a completely safe means of vaccinating cattle destined for export could result in a greatly increased demand for the more productive breeds found in the U.S. and Israel. Finally, being non-immune, the U.S. cattle population would be highly vulnerable to reintroduction of babesiosis which would bring about an enormous economic impact on the livestock industry of the country. A vivid example to that affect was a recent sudden entry of Venezuelan equine encephalaomyelitis

from Central American countries and Mexico into the southern portion of the U.S. Resulting consequences might have been long-term and much more devastating had a vaccine not been maintained by, and available from, the U.S. Army. Accordingly, availability of a proven killed vaccine for bovine babesiosis would constitute an insurance and effective deterrent for safeguarding U.S. borders against that disease.

X. PRESENTATIONS AND PUBLICATIONS

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2. Montenegro-James, S., Toro, M., Leon, E., Lopez, R., and M. Ristic. Bovine babesiosis: vaccination with culture-derived exoantigens. 11th Conference of the World Association for the Advancement of Veterinary Parasitology, 5-9 August 1985, Rio de Janeiro, Brazil.
3. Montenegro-James, S., Toro, M., Leon, E., Lopez, R., and M. Ristic. Bovine babesiosis: vaccination with culture-derived exoantigens. 34th Annual Meeting of the American Society of Tropical Medicine and Hygiene. 4-7 November 1985, Miami, Florida.
4. Montenegro-James, S. and M. Ristic. Heterologous strain immunity in bovine babesiosis using a culture-derived soluble Babesia bovis immunogen. Vet. Parasitol. 18:321-337, 1985.
5. Kuttler, K.L., Levy, M.G., and M. Ristic. Cell culture-derived Babesia bovis vaccine: sequential challenge exposure of protective immunity during a 6-months post-vaccinal period. Am. J. Vet. Res. 44: 1456-1459, 1983.

6. Montealegre, F., Levy, M.G., and M. Ristic. Growth inhibition of Babesia bovis in cultures by secretions from bovine mononuclear phagocytes. *Infec. Immun.* 50:523-526, 1985.
7. Montealegre, F., Montenegro-James, S., Kakoma, I., and M. Ristic. Detection of culture-derived Babesia bovis exoantigens using a two-site enzyme immunoassay. Submitted to *Vet. Immunol. and Immunopath.*
8. Levy, M.G., Kakoma, I., Clabaugh, G.C., and M. Ristic. Complement does not facilitate in vivo invasion of bovine erythrocytes by Babesia bovis. *Ann. Trop. Med. Parasit.* (In press).

REPORT OF THE ISRAELI INVESTIGATOR

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Abstract

B. bovis was cultivated in vitro in a microaerophilus stationary system. Non-viable and live vaccines were prepared from culture derived parasites.

Non-viable lyophilized vaccine induced considerable antibody response in calves and caused no adverse effect following inoculation. However following challenge with live parasites the average PCV decrease in vaccinated cattle was about 36% versus about 48% in non-vaccinated control calves. Antibabesial treatment needed to be administered in 5 of 30 (16.6%) vaccinated calves and 15 of 16 (93.7%) control-nonvaccinated-calves.

Doses of live vaccine containing 10^7 B. bovis parasites induced strong protection against virulent blood-derived parasites. The maximum storage period at 4°C was about 7 days, but the parasites were not completely safe for cattle. Inoculation of long-acting oxytetracycline 7 and 14 days post inoculation considerably mitigated the adverse response to the live vaccine.

Another method for safe vaccination of highly sensitive cattle was achieved by a two-step procedure. Heifers were primed with 2 inoculations of non-viable vaccine and 3 weeks later were given live blood derived vaccine. None of the primed cattle required treatment with babesiacides while 4 out of 7 control cattle that received live vaccine only were given diminazene when their PCV dropped to dangerously low levels.

Objectives

1. To cultivate B. bovis continuously in vitro and to assess infectivity and possible attenuation of virulence of cultured parasites.
2. To prepare live and killed vaccines from cultured parasites.
3. To evaluate the safety and potency of both types of vaccines for various ages and breeds of cattle.
4. To assess the potential of double vaccination (killed vaccine followed by live vaccine) as a mean of avoiding severe reactions while inducing strong immunity in sensitive breeds of cattle.
5. To determine immunological cross-reactions between homologous and heterologous isolates of B. bovis using a newly-developed merozoite neutralization test.
6. To assess protective immunity against individual B. bovis isolates in cattle vaccinated with a multiple isolate antigen.
7. To isolate, purify and characterize biochemical properties of merozoite surface antigens as preliminary to future molecular level studies.

Significance of the research

Babesiosis caused by Babesia bovis causes heavy losses to the Israeli cattle industry and is a constant potential threat to cattle in the southern territories of the U.S.

In Israel, beef cattle are reared extensively on pasture all year round and no effective methods are available for control of the babesiosis vector (Boophilus ticks) in the field. Rotation of cattle on pasture in order to allow the ticks to die out is not feasible because of the scarcity of pasture land and the incursions of uncontrolled nomadic herds. Most dairy cattle are kept on zero grazing, but infected ticks may be introduced into dairy sheds by straying beef cattle, by fresh grass from tick infested fields, and by cattle trading.

The extensive borders of Israel with surrounding tick-infested areas make the total eradication of Boophilus ticks in this country a practical impossibility.

In dairy cattle, the growing implications for public health of widespread use of acaricides, and the increasing cost of these chemicals make the frequent application of acaricides undesirable as a sole control measure.

Vaccination against B. bovis has been carried out routinely in Israel for about two decades. The vaccine consists of B. bovis-infected blood drawn from splenectomized cattle during the acute stage of infection with this parasite. The blood derived vaccine has serious drawbacks which can be summarized as follows:

- 1) despite partial attenuation of parasites they can still provoke disease and death in highly susceptible cattle;
- 2) the infected

blood may contain other undetected agents of cattle diseases;
and 3) the vaccine contains an overwhelming quantity of bovine
cellular elements and plasma that may lead to autoimmune compli-
cations.

The results reported here concern trials with non-viable
and live vaccines derived from cell culture parasites, with the
aim of improving the methods of immunization against B. bovis
infection.

Objective 1

To cultivate B. bovis continuously in vitro and to assess infectivity and possible attenuation of virulence of cultured parasites.

The cultivation of B. bovis was performed according to the technique of microaerophilus stationary phase culture described by Levy and Ristic (1980). Cultures were initiated using defibrinated peripheral blood from infected splenectomized calf donors when the parasitemia was between 2-4%. Infected erythrocytes were suspended to a final packed cell volume of 9.1% in a medium consisting of 60% medium M₁₉₉ with Hank's salts and 40% normal bovine serum supplemented with 0.6% Hepes, 100 mcg streptomycin and 100 units/ml penicillin. Serum was obtained from mature Friesian cows. The culture suspension was titrated to pH7 with 0.1N HCL. Portions of the suspension were then placed in 24 well plates in a ratio of 0.62 ml of suspension to 1cm² of culture area. The cultures were incubated at 37°C under a humidified atmosphere of 5% CO₂, 5% O₂ and 90% N₂. Every 24 hours the overlying culture medium was replaced with fresh medium. This medium consisted of 60% M₁₉₉ and 40% serum, supplemented with Hepes and antibiotics and titrated to pH7 as previously described. After 48-72 hours, subcultures were prepared by diluting the culture 4 to 8 times with medium containing freshly collected uninfected bovine erythrocytes. Parasite multiplication in the culture was monitored by counting

500 erythrocytes on Giemsa-stained thin blood films that were prepared from culture wells. The maximal parasitemia observed in these cultures was about 10%. The cultures were maintained during 14 months and 140 subcultures were made during this period.

B. bovis for in vitro cultivation were cryopreserved according to the method described by Palmer et al. When parasitemia in the cultures reached 10%, the infected erythrocytes were pelleted by centrifugation at 200g for 10 min at 4°C. 0.5 ml of packed erythrocytes was mixed with 0.5 ml of cryoprotective solution (10% PVP (W/V) polyvinylpyrrolidone) diluted in PSG + (Puck's Saline G supplemented with glucose 20g/l). The samples were placed in Nunc sterile screw plastic tubes and frozen at a cooling rate of approximately 20°C/min, achieved by immersing vials directly in a -70°C ethanol bath. Samples were then left at -70°C overnight and transferred to liquid nitrogen (-196°C). To re-establish cultures samples were recovered from storage at -196°C by rapid thawing with agitation in a water bath at 37°C. Partly thawed samples were immediately diluted in 50ml of PSG + 4°C and centrifuged at 400g for 10 min. Resulting pellets were resuspended in 1 ml of fresh cultures medium containing 10% uninfected bovine erythrocytes. Aliquotes of 0.2 ml were dispensed in each of 96-well tissue culture plates. The overlying medium was replaced daily with fresh medium and the infection rate in the culture was determined. Usually cultures were ready for subculturing after 5-6 days. The maximal period that cultures were stored at -196°C and then re-established was 9 months.

To assess infectivity of culture-derived B. bovis parasites infected blood was harvested from a 3 - day culture with about a 7% rate of infected r.b.c. The red blood cell suspension was centrifuged and the pellet was resuspended to obtain final concentrations of 10^6 and 10^7 parasitized r.b.c. per 2ml. of suspension. This material was stored at 4°C for 48 h and then inoculated subcutaneously into 3 intact 8-9 month old female calves.

Table 1

Results of inoculation of calves with cultures derived
B. bovis.

Animal No.	No. of organism in inoculum	Max. body Temp. ($^{\circ}\text{C}$)	Min. PCV	Outcome
560	10^6	40	17	* treated and recovered
564	10^6	40	24	recovered spontaneously
566	10^7	40.9	21	recovered spontaneously

* 3.5 mg/kg diminazene

The results presented on Table 1 show that culture derived B. bovis organisms are perfectly infective for cattle. The fact that one animal had to be treated with babesiacide shows that the parasites have retained their virulence.

Objective 2

To prepare killed and live vaccine from cultured parasites.

Babesia bovis was rapidly and sequentially passaged in splenectomized cattle for up to 21 passages. Cultures were initiated with infected blood drawn from the 3rd passage (low passage) and with blood from the 21st passage (high passage).

To prepare non viable vaccine B. bovis organisms from both passages were grown in 150 cm² Corning plastic flasks. The supernatant was harvested 48h. and 72h. after subcultivation and centrifuged at 3000 rpm for 1h. The sediment was discarded and the supernatant was dispensed in 6.7 ml aliquots that were lyophilized and stored at 4°C. Only supernatant from cultures with more than 6% infected r.b.c were used. Before use each lyophilized aliquote was rehydrated with 2ml distilled water containing 1mg/ml Quil A Saponin.^a The rehydrated supernatant was inoculated subcutaneously into experimental animals. Both low and high passage derived supernatant was used in the trials.

Non-lyophilized supernatant was also used for vaccination. Culture supernatant was collected and centrifuged at 1700 g (3000 rpm) and stored at -70°C. Before use the supernatant was thawed and 2ml of 0.1% Saponin solution was added to each dose of 6.7 ml.

To prepare live vaccine the parasites from the 22nd calf passage were used for initiating cultures. The parasites were continuously subcultured for 11 weeks in 24 well culture plates. A 150 cm² plastic culture flask was inoculated from stock cultures to obtain sufficient parasites for preparing live vaccine. Red blood cells from infected cultures were harvested and after centrifugation

^aQuil A Saponin - Superfos AS Denmark.

the pellet was resuspended in culture medium to obtain the concentration of parasites shown in table 13 in a volume of 2ml. (the immunizing dose). The suspension was inoculated subcutaneously in the neck area.

Objective 3

To evaluate the safety and potency of killed and live vaccines derived from in vitro cultures.

A. VACCINATION WITH NON-VIABLE (KILLED) VACCINE

Trial 1

Experimental design

Twenty-four 8-12 month old calves were divided in 5 groups and inoculated as follows:

- Group 1 (5 animals) - Two units of lyophilized high passage antigen 3 weeks apart.
- Group 2 (5 animals) - Two units of lyophilized low passage antigen 3 weeks apart.
- Group 3 (5 animals) - One unit of lyophilized antigen followed 3 weeks later by a second dose of 5 units.
- Group 4 (4 animals) - Two units of antigen (non-lyophilized) stored at -70.
- Group 5 (5 animals) - No inoculation before challenge.

All vaccinated and control animals were challenged with 10^9 B. bovis parasites from a frozen stock 4 weeks after the second inoculation of vaccine. Response to vaccination and challenge was monitored by clinical observations, measurement of body temperature, examination of blood smears and assessment of PCV level. Antibody titer was measured by the indirect fluorescent antibody technique.

Results

No detectable clinical reactions and no difference in behaviour between vaccinated and control animals could be seen during the post-vaccination and prechallenge period.

A considerable level of antibabesial antibody was detected in 16 out of 19 calves on the day of challenge. The remaining 3 animals showed titer of 1:64 (calf 479 - table 2 and calf 607 - table 5) and 1:16 (calf 598 - table 3).

Following challenge with live B. bovis organisms a low level parasitemia was detected in 8 out of 19 (42%) vaccinated calves versus 5 out of 5 (100%) control. None of the 4 calves vaccinated with frozen supernatant antigen showed parasitemia before being challenged with live organisms.

The prechallenge antibody titers, maximum temperature and minimum PCV values are illustrated in tables 3 to 7.

All 5 nonvaccinated control calves showed acute clinical babesiosis accompanied by an important decrease of PCV and treatment had to be administered to avoid serious consequences (table 6).

Table 2

Results of challenge* of calves vaccinated with non-viable culture derived antigen.

Group 1 Antigen: high passage

1st dose : 6.7 ml lyophilized

2nd dose : 6.7 ml lyophilized

No. of calf	Ab. titer on day of challenge	Max. temperature	Min. PCV
478	256***	40.5	21
479**	64	41	17
480	256	41.3	26
481	256	40.7	20
482	256	39.8	21
-			
X		40.7	21

* 10^9 parasites from frozen stock

** treated with one dose of 3.5 mg/kg diminazene

*** reciprocal titers

Table 3

Results of challenge* of calves vaccinated with nonviable culture derived antigen.

Group 2: Antigen: low passage
 1st dose: 6.7 ml lyophilized
 2nd dose: 6.7 ml lyophilized

No. of calf	Ab. titer on day of challenge	Max. temperature	Min. PCV
575**	256 ***	41.0	17.5
582	256	39.8	24
590	256	40.5	21
598	16	40.2	24
605	256	40.6	27
$\bar{x}$		40.4	23

* 10^9 parasites from frozen stock

** treated with one dose of 3.5 mg/kg diminazene

*** reciprocal titers

Table 4

Results of challenge* of calves vaccinated with nonviable culture derived antigen.

Group 3: Antigen: high passage
 1st dose: 6.7 ml lyophilized
 2nd dose: 5x6.7 ml lyophilized

No. of calf	Ab. titer on day of challenge	Max. temperature	Min. PCV
488	1024***	40	21
489	256	40	21
490**	256	40.7	17
491	1024	40.3	22
492	1024	41	23
-			
X		40.4	20.8

* 10^9 parasites from frozen stock

** treated with one dose of 3.5mg/kg diminazene

*** reciprocal titers

Table 5

Results of challenge* of calves vaccinated with non-viable culture derived antigen.

Group 4: Antigen: low passage
 1st dose: 6.7 ml frozen
 2nd dose: 6.7 ml frozen

No. of calf	Ab. titer on day of challenge	Max. temperature	Min. PCV
577	256**	40.5	23
592	256	40.7	28
600	256	39.4	29
607	64	40.7	28
$\bar{x}$		40.3	27

* 10^9 parasites from frozen stock

**reciprocal titers

Table 6

16.

Results of challenge* of non-vaccinated, control calves.

Group 5

No. of calf	Ab. titer on day of challenge	Max. temperature	Min.PCV
927**	Neg	39.9	16
929**	Neg	41.0	13
930**	Neg	40.5	16
931**	Neg	40.4	16
932**	Neg	41.0	18
$\bar{X}$	Neg	40.6	15.8

* 10^9 parasites from frozen stock

** treated with one dose of 3.5mg/kg diminazene at minimal PCV

Table 7

Percentage decrease of PCV after challenge as calculated from the prechallenge values in vaccinated and control calves

Origin of vaccine (passage)	Doses in units of 6.7 ml.	% of decrease PCV	Outcome after challenge
			No. treated No. challenged
High*	1 + 1	42	1/5
Low*	1 + 1	39	1/5
High*	1 + 5	43	1/5
Low**	1 + 1	29	0/4
Control	none	51	5/5

*lyophilized

** frozen

Calves vaccinated with lyophilized antigen (table 2, 3, and 4) showed considerable decrease of the PCV after challenge but only 1 animal in each group required treatment with babesiacide to arrest red blood cell destruction.

Calves vaccinated with antigen stored in frozen state (table 5) had a moderate PCV decrease and all recovered spontaneously.

It appears from the above that vaccination with non-viable antigen engendered a degree of immunity to subsequent infection with live virulent parasites. However, this immunity was only partial since a considerable drop in PCV occurred in the calves and 3 out of 15 animals vaccinated with lyophilized antigen had to be treated with babesiacides (table 7) after challenge with live parasites.

Increasing the quantity of immunizing antigen did not improve the protection against infection with live parasites (Group 3 - table 4 compared with Group 1 - table 2). Antigen harvested from cultures initiated with low passage parasites was not superior to antigen from high passage cultures (Group 2 compared with Group 1).

An unexpected interesting result was the strong protection engendered by frozen supernatant antigen compared to the lyophilized batches. A possible explanation might be that the frozen antigen contained a few live Babesia organisms that were not sedimented and discarded during centrifugation. Such organisms are destroyed during lyophilization but may survive freezing. This phenomenon might have practical applications since no clinical symptoms were observed following vaccination with the frozen

material. It should be noted that this culture was derived from a low passage line that still retained its virulence in ordinary passages in calves.

Trial 2

Experimental design

Fifteen calves were divided in 3 groups (Nos. 6, 7 and 8) of 5 animals each. Group 6 received two units of lyophilized high passage antigen 3 weeks apart. The lyophilized vaccine was rehydrated with 2 ml saponin solution. Simultaneously with the immunizing dose levamisole hydrochloride at a dose of 5mg/kg was inoculated. Group 7 received two inoculations of levamisole hydrochloride 5mg/kg 3 weeks apart, while Group 8 was left as an uninoculated control. All vaccinated and control animals were challenged with 10^9 B. bovis parasites from a frozen stock 48 days after vaccination. Response to vaccination and challenge were monitored as in trial 1.

Results

Vaccination or inoculation of levamisole were not followed by clinical disturbances. On the day they were challenged the vaccinated calves of Group 6 showed relatively high titers in 2 animals (484, 485) and low titers in 3 others (483, 486, 487) (Table 8). Challenge resulted in a considerable decrease in PCV in all animals and 2 of them received diminazene treatment.

Table 8

Results of challenge*of calves vaccinated with non-viable antigen and treated simultaneously with lavamisole hydrochloride.

Group 6

Antigen: high passage

1st dose: 6.7 ml lyophil + 5 mg/kg levamisole

2nd dose: 6.7 ml lyophil + 5 mg/kg levamisole

No. of calf	Ab. titer on day of challenge	Max. temperature	Min. PCV
483**	64 ***	40.8	18
484	1024	40	24
485	256	38.5	20
486**	16	39.2	16
487	64	39	25
-			
X		39.5	20.6

* 10^9 parasites from frozen stock

** treated with one dose of 3.5 mg/kg diminazene

*** reciprocal titers

The animals in the non-vaccinated control group No.8 showed acute clinical babesiosis when challenged and all 5 animals were given a babesiacide (table 9).

Table 9

Results of challenge in susceptible non-vaccinated calves.

Group 8

No. of calf	Ab. titer on day of challenge	Max. temperature	Min. PCV
473*	Neg	41.7	17
474*	Neg	39.7	18
475*	Neg	40.5	19
476*	Neg	39.8	17
477*	Neg	40.5	16
- X	Neg	40.4	17.4

* treated with one dose of 3.5 mg/kg diminazene

A similar reaction to challenge was obtained in group 7 where animals received two inoculations of levamisole only before the challenge (Table 10). Four animals showed a drop of PCV to 18% or below and were treated with diminazene.

Table 10

Results of challenge in animals receiving two inoculations of levamisole 3 weeks apart.

Group 7

No. of calf	Ab. titer on day of challenge	Max. temperature	Min. PCV
493*	Neg	40	17
494*	Neg	40.2	16
495*	Neg	40.2	17
496	Neg	39.3	22
497*	Neg	39.5	18
$\bar{X}$	Neg	39.8	18

*treated with one dose of 3.5 mg/kg diminazene

The results of trial 2 show that a simultaneous inoculation of levamisole hydrochloride and non-viable vaccine does not reinforce immunity to challenge with live B. bovis parasites.

Trial 3

Experimental design

Eleven 9-11 month old female calves were vaccinated with 3 units (6.7 ml each) of lyophilized culture derived B. bovis antigen 3 weeks apart, and 11 other calves of the same age were left non vaccinated. All 22 animals were challenged with live B. bovis parasites 21 days after the second inoculation.

Response to vaccination and challenge was monitored as in trials 1 and 2.

All calves of the vaccinated group (Table 11) responded with a high antibody titer. No parasites were detected after challenge, but a considerable drop in PCV was observed and 2 animals received diminazene.

Table 11

Response of calves vaccinated with culture-derived, lyophilized antigen to challenge with live B. bovis parasites.

Antigen: High passage

1st dose: 3 x 6.7 ml lyophilized

2nd dose: 3 x 6.7 ml " "

No. of calf	Ab. titer on day of challenge	Maximum temperature	PCV		Parasites
			Minimum	Percentage decrease**	
664*	1024	40.6	19	37	Neg.
665*	1024	40.0	19	53	"
666	1024	40.8	20	38	"
667	256	40.5	24	33	"
668	1024	39.6	23	32	"
669	1024	40.5	26	28	"
670	4096	41.0	23	38	"
671	4096	40.0	24	31	"
672	4096	40.0	22	39	"
673	4096	40.5	24	31	"
674	256	40.5	21	38	"
X		40.4	22.3	34	Neg.

*treated with one dose of 3.5 mg/kg diminazene.

** calculated $\frac{\text{Minimum PCV after challenge}}{\text{PCV on day of challenge}}$ as percentage.

Table 12

Response of non vaccinated calves to inoculation with live B. bovis parasites.

No. of calf	Ab. titer on day of challenge	Maximum temperature	PCV		Parasites
			Minimum	Percentage decrease	
675	Neg.	39.5	23	41	Rare
676*	"	40.5	19	44	"
677	"	40.2	18*	49	"
678	"	40.4	20*	47	"
679	"	40.6	19*	49	"
680	"	40.3	19*	46	Neg
681	"	41.5	19*	46	"
682	"	40.1	20*	38	"
683	"	39.6	19*	42	"
684	"	40.5	19*	42	"
685	"	40.0	16*	48	"
- X	Neg	40.3	19.1	45	Rare

* treated with one dose of 3.5 mg/kg diminazene

All but one of the 11 control animals showed parasites in blood films and 10 animals received antibabesial treatment. (table 12).

The average percentage decrease of PCV, calculated from the values on day of challenge and the minimum value following challenge, was lower in the control than in the vaccinated group (45% vs. 34%).

B. VACCINATION WITH LIVE B. BOVIS PARASITES DERIVED FROM
IN VITRO CULTURES

Trial 1:

To assess the response of cattle to live vaccine prepared from in vitro cultured organisms, and to determine the survival period of these organisms when stored at 4°C.

Experimental design

Infected red blood cells were harvested from cultures initiated from parasites that had undergone 22 passages in splenectomized calves. The number of parasites per ml. was calculated from the red blood cell count and the percentage of infected r.b.c. determined by direct count of stained smears. The r.b.c. suspension in culture medium was stored in a conventional refrigerator at a temperature of about 4°C.

Before inoculation the r.b.c. were diluted in fresh culture medium to yield concentrations of 10^6 , 10^7 , 5×10^7 and 10^8 infected r.b.c. per immunizing dose of 2 ml. Forty-three 7 to 10 month old dairy calves were inoculated subcutaneously in the neck area with these dilutions according to the schedule on Table 13.

Response to vaccination was monitored by examination of blood films and measurement of antibabesial antibody level. Some of the animals that did not show parasitemia after vaccination were challenged by inoculation of 10^9 blood derived B. bovis from a frozen stock.

All calves inoculated with 10^6 parasites stored for 2 or 7 days responded by parasitemia, seroconversion or were immune to challenge. Doses containing 10^7 parasites and stored up to 10 days induced seroconversion and immunity although parasitemia was not detectable in all inoculated animals.

When parasites were stored for 11 or 14 days at 4°C , doses containing 10^7 , 5×10^7 or 10^8 parasites did not result in seroconversion nor immunity in all of the inoculated animals. Four of the animals that showed parasitemia following inoculation of the vaccine suffered a decrease of PCV to 18% or below and were treated with babesiacides.

All seroconverted animals were immune to challenge with live organisms and no significant changes in PCV level were noted. Four control animals (not shown on table 13) that were inoculated with the same challenge material required treatment with babesiacides.

It appears that live, culture-derived vaccine against infection with virulent organisms, is not completely safe for susceptible cattle.

Table 13

Response of calves to vaccination with live B. bovis parasites derived from in vitro culture.

Days of storage at 4°C.	Number of parasites per dose	Number of animals inoculated	Number of calves		
			with parasitemia	seroconverted	immune to challenge
2	10^6	5	2	5	5
	10^7	4	1	4	4
7	10^6	3	1	N.D.*	3
	10^7	3	3	3	N.D.
	5×10^7	3	3	3	N.D.
10	10^7	5	2	5	5
11	5×10^7	8	7	7	7
14	10^7	4	0	1	N.D.
	5×10^7	4	2	1	N.D.
	10^8	4	2	2	N.D.

*N.D. = not done

Since live B. bovis parasites derived from in vitro cultures have retained some virulence to cattle a mild palliative drug is needed that will mitigate the clinical response to the vaccines without affecting the development of immunity.

The following trials show that oxytetracycline might be the drug of choice for this purpose.

Trial 2

Laboratory trial

Tetracycline derivatives have been shown to influence the course of Babesia spp. infection in various mammals. Preliminary observation in our laboratory confirmed the therapeutic effect of oxytetracycline in splenectomized calves infected with B. bovis. The following experiments were carried out to evaluate this effect in greater detail.

Experimental design

Experimental animals: Newborn Friesian calves were obtained from a Babesia and Boophilus - free dairy herd kept on zero grazing. The calves were 6-9 weeks old at the beginning of the trial and were splenectomized 4 to 20 days before infection. They were maintained under tick-proof conditions throughout the experiment.

Infection, treatment, and examinations: Six calves were infected with frozen - preserved blood containing 10^9 parasites per dose. From 7 to 11 days later they were inoculated intramuscularly with 20 mg/kg of long-acting oxytetracycline (Table 1). Thin blood films were taken

and body temperatures were measured daily throughout the experiment. Calves surviving the first infection were challenged by infective ticks. Calves (621, 620, 610) treated with oxytetracycline at the onset of parasitemia and 2 or 3 days later showed low parasitemia and recovered. All 3 control non treated calves (587, 592, 605) showed high parasitemia and died from the infection.

Table 1

Results of treating B. bovis infected splenectomized calves with oxytetracycline

No. of calf	Treated with oxytetracycline (days after initial infection*)	Prepatent period (days)	Maximum		Outcome	Outcome following challenge**
			Parasitemia %	Temperature C°		
621	7, 10	7	3	40.7	survived	survived
620	8, 10	7	2	41.1	survived	
610	8, 11	8	2	40.7	survived	survived
587	not treated	7	38	41.3	died	
592	not treated	8	21	40.5	died	
605	not treated	8	36	40.5	died	
616						died

* Initial infection with cryopreserved blood containing 10^9 parasites per dose.

** Challenge with infective ticks.

Two of 3 surviving animals that were challenged with stablate of B. bovis infected ticks survived while an additional control animal (616) died from the challenge dose.

Comments

B. bovis is highly pathogenic for splenectomized calves. The results presented here show that oxytetracycline exerts a powerful anti-babesial activity in these sensitive animals, and when administered at the beginning of patency it inhibited parasite multiplication and prevented the fatal outcome seen in untreated control calves. On the other hand treatment with 2 doses of 20 mg/kg each did not prevent elaboration of immunity to infection with tick derived B. bovis parasites.

Trial 3

Field trial

Dairy farms in Israel maintain their cows at zero grazing and vaccination against babesiosis is usually not recommended under such circumstance. However, in some farms, heifers are sent to pasture before the first calving. In areas infected with Boophilus ticks this type of management exposes the animals to the risk of clinical babesiosis.

In the present experiment the effect of long-acting oxytetracycline administered to intact (not splenectomized) female Friesian calves vaccinated with live anti-B. bovis vaccine was studied.

Experimental design

Vaccination and treatments: Forty 11-13 month old Friesian female calves in a Babesia - free farm were inoculated with 10^9 B. bovis parasites from frozen vaccine. Twenty of the calves received 20 mg/kg of long-acting oxytetracycline (OTC/LA) on days 7 and 15 post-inoculation while the other 20 were left untreated. The

OTC/LA was administered intramuscularly in the neck on the side opposite to that of the vaccine. Animals from both groups that showed a decrease of PCV to 18% or less were treated with one dose of 3.5 mg/kg diminazene.

Examinations: Examinations were performed before inoculation of the vaccine and daily for 28 days after the vaccination. Giemsa stained thin blood films were examined for parasites at a magnification of 1000 X. Films in which no parasites were detected after a 10 min. examination were classified as negative. Conjointly, PCV was evaluated and body temperature was checked. Blood for serological testing was drawn before vaccination and again 2 months later. Antibody titers were measured by the indirect fluorescent antibody technique with antigen prepared from B. bovis parasites of the same passage in cattle that was used for preparing the vaccine.

Results

In both the treated and control groups parasitemia began to appear on day 8 post-vaccination (Table 1). In the week following the first parasitemia (days 8-14) 8 of the OTC/LA treated and 18 of the control calves were patent. In the next 2 weeks (days 15-28) one more treated calf became patent as well as the 2 remaining calves in the control group. The total number of patency days was 56 in the controls versus 18 in the treated animals. In both groups maximal parasitemia remained under 0.5%.

Table 1

Parasitemia following inoculation of live B. bovis vaccine in calves treated with long-acting oxytetracycline (OTC/LA) and in non-treated control calves.

Days after vaccination	Number of calves with parasitemia		Days of parasitemia	
	OTC/LA treated	control	OTC/LA treated	control
0- 7	0	0	0	0
8-14	8	18	9	53
15-28	1	2	9	3
Total	9*	20	18	56

* 11 other treated calves never developed parasitemia.

Average temperatures showed a slight rise in the OTC/LA group on days 8 and 9 after vaccination, but were normal for the remaining period (Fig. 1). In the control group a rise in average temperature was observed during the period 8-12 days post vaccination, after which temperatures faded to normal values. Packed cell volumes of the OTC/LA group showed a decrease from day 9 after vaccination until day 28, but the average minimal value (29%) was considerably higher than that (23%) of the control group. On day 15 (the day of the second treatment) one of the treated animals showed a PCV of 16% and was given diminazene. The PCV values of all remaining OTC/LA treated animals were above 23% during all the experimental period while in the control group 8 animals had PCVs of 16% to 18% and needed to be treated with diminazene.

Antibody levels assessed 62 days after vaccination showed all animals to be positive with titers of 1:64 to 1:1024 (average 1:620) in the OTC/LA group and 1:256 to 1:1024 (average 1:563) in the control group.

Comments

It appears from the above that treatment with OTC/LA on days 7 and 15 post vaccination can considerably mitigate the clinical response to live anti-B. bovis vaccine in sensitive dairy cattle. OTC/LA administered one day before patency was capable of suppressing the parasitemia in treated animals and as a consequence only mild changes in temperature and PCV were observed. In one out of the 20 treated calves the PCV dropped to a critical level on day 15, i.e.

8 days after the first OTC/LA treatment. This suggests that the second OTC/LA treatment should be administered probably 6-7 days after the first.

Although no parasites were detected in 11 of the 20 OTC/LA treated calves it can be assumed on the basis of the positive serologic results that a latent multiplication of B. bovis has occurred in these calves.

Fig. 1. Average body temperature and packed cell volume in calves inoculated with live *B. bovis* vaccine and treated with long acting oxytetracycline (OTC/LA) and in control calves inoculated with vaccine alone. The vertical lines indicate SEM (n=20). Number in circle show the number of calves treated with diminazene.

Objective 4: To assess the potential of double vaccination (non-viable followed by live vaccine) as a means of avoiding severe reactions while inducing strong immunity in sensitive breeds of cattle.

Vaccination with live B. bovis parasites derived from infected calves confers strong long term protection against infections in the field, but involves the risk of severe reactions in some animals. Attenuated parasites, achieved by rapid sequential passages in splenectomized calves have proved to be safe for breeds used mainly for beef production, but in Friesian high grade dairy cattle vaccination is associated with varying degrees of anemia often accompanied by severe clinical manifestations.

Non-viable culture-derived antigen (NCA) of B. bovis was completely safe for cattle but it conferred only a partial protection against challenge with virulent parasites. Under field conditions, where heavy tick challenge may occur, the strongest possible protection is desirable, especially for highly susceptible breeds.

In the present study an effort has been made to provide high grade Friesian cattle with safe, strong protection by the use of live B. bovis vaccine following a priming dose with NCA vaccine.

Experimental design

Preparation of non-viable vaccine from in vitro culture of

B. bovis

B. bovis was rapidly and sequentially passaged through 23 splenectomized calves. The last calf was bled when parasitemia reached 5% and the parasites were grown in a microaerophilus

stationary phase (MASP) culture according to the techniques described by Levy and Ristic. Stock cultures were maintained in flat-bottomed, 24-well microtrays (1.2 ml per well). Supernatants were replaced daily with fresh medium and cultures were transferred every 2-3 days. To obtain NCA for vaccination the parasites were subcultured in 150 cm² plastic flasks which were inoculated with parasites from the maintenance line. Supernatant medium was collected from the flask after 48 and 72 hours of incubation when the average rate of infection of both harvest was 6.5%.

The medium was centrifuged for 30 min at 1200 g and the supernatant was dispensed in doses of 6.2 ml each and lyophilized. For use each dose was rehydrated with 1.5 ml of 0.1% aqueous solution of Quil A saponin.

Experimental animals, vaccination and examinations. Sixteen 10-11 month old Friesian calves negative for antibodies to B. bovis were used in the trial. Eight calves were primed with two subcutaneous doses of NCA vaccine 3 weeks apart. Three weeks after the second inoculation the 8 primed calves and 8 naive control calves were each inoculated with live vaccine containing 5×10^8 organisms drawn from a frozen stock. The vaccine was prepared from the 22nd calf passage of the strain used for initiating the cultures. Starting the day the live vaccine was given and on days 7 to 12 thereafter the calves were

monitored by examining stained blood films and by checking body temperature and packed cell volume. Calves with PCV of 18% or less were treated with 5 mg/kg of diminazene to arrest further red blood cell destruction. Antibody level was assessed by the indirect fluorescent antibody technique using the live vaccine parasites as antigen.

RESULTS

Following administration of live vaccine, no significant difference was found between the primed and control calves in the average prepatent period (10.6 and 9.8 days respectively) and in the average days of patency (2.75 and 2.75) (Table 1). Only rare parasites (1 parasite per 3-4 microscopic field or less) were detected in blood smears from calves of both groups. The average maximum temperature was one degree lower in the primed than in the control calves (39.2°C versus 40.3°C respectively).

The contrast on the response of the two groups to the live vaccine was shown most clearly by the PCV values (Fig. 1). Taking a PCV of 26% as a dividing line, only 3% of the examinations of the primed calves fell below the line compared to 47% of the control calves. Four animals in the control group had PCVs of 18% or below and were treated with diminazene whereas all except one of the primed animals had PCVs of 26% or higher. In one of the primed cattle the PCV dropped to 22% on the 10th post inoculation day but by day 12 it was up to 26%.

There was no correlation between the level of antibody titers induced by the NCA vaccine and the degree of sensitivity to the live vaccine. For example (primed) calf 716 that reached the highest titer

after priming, suffered fever and showed the lowest (22%) PCV value following the inoculation of the live vaccine. Antibody titers measured 3 weeks after the inoculation of the live vaccine was significantly higher in the primed cattle than in the control ones.

Comments

Extensive field experience has shown that Friesian high grade cattle are highly sensitive not only to natural Babesia infection but also to partially attenuated B. bovis parasites. In fact, in the present trial 4 of the 7 one year old female calves in the group that received live vaccine only showed a considerable decrease in PCV and required treatment with babesiacides. On the other hand calves that were primed with non-viable vaccine showed relatively mild decreases in PCV and recovered spontaneously. It is clear then that priming sensitive cattle with non-viable vaccine makes vaccination with live parasites safer.

Trials performed in Australia and long term field experience in Israel show that inoculation of living, blood derived B. bovis parasites confers protection for about 4 years.

It appears from the above that a two step procedure, non-viable vaccine followed by live vaccine, could be a method of choice for vaccinating highly sensitive cattle, combining safety with strong and long term protection.

The role of circulating B. bovis antibody detected by the fluorescent antibody technique is not yet clearly understood. In the present trial primary immunization with NCA induced variable antibody titers

but high antibody levels were not always correlated with clinical response caused by inoculation of live parasites.

It seems therefore that the antibody level detected by the fluorescent antibody technique cannot be used as an indication for the degree of immunity to babesiosis.

Summary

1. Babesia bovis can be grown in vitro for prolonged periods in a relatively simple system. Such cultures provide a reliable and continuous supply of non-viable and live antigens.
2. Vaccination with non-viable antigens is safe for cattle and induces a significant, but partial, protection against challenge with live parasites. For this reason non-viable antigen in itself is not suitable for mass vaccination.
3. Live cultured parasites induce strong immunity in cattle but the post-vaccination reaction may include considerable red blood cell destruction in a portion of vaccinated cattle. Thus, in itself, live cultured parasite antigen also suffers from serious drawbacks as a mass vaccination procedure.
4. Cattle can be vaccinated against B. bovis with a high degree of safety and with strong immunity by a 2 - step procedure in which the animals first receive non-viable culture - produced antigen, followed by live parasites. Cattle vaccinated in this manner show minimal untoward reactions to the vaccine and are solidly resistant to challenge.
5. Long-acting oxytetracycline formulations administered shortly after single vaccination with live parasites protects cattle against red blood cell destruction, without however interfering with the development of immunity. This method, therefore, provides another approach to safe and effective vaccination of cattle against B. bovis infections.