PN-ADI-347

# FINAL REPORT

**Submitted to the**

**U.S. AGENCY FOR INTERNATIONAL DEVELOPMENT;**
**Bureau for Economic Growth, Agriculture and Trade**

## "VACCINE AGAINST BOVINE BABESIOSIS IN CENTRAL ASIA"

**(USAID CDR PROPOSAL TA-MOU-00-CA20-013)**

**COVERING THE PERIOD FROM**

**JANUARY 2002 TO DECEMBER 2006**

**SUBMITTED BY:**

**DR. VARDA SHKAP (Principal Investigator)**
**(KIMRON VETERINARY INSTITUTE, BET DAGAN, P.O.BOX 12, 50250, ISRAEL**
**Tel: 972-3-9681758; FAX 972-3-9681678; Email: shkapv@int.gov.il ( KVI)**
And
DR. I. RASULOV (Co-Principal Investigator)
**(INSTITUTE OF ZOOLOGY, NIAZOV STR. 1, TASHKENT, UZBEKISTAN,**
**Tel: 998-71-146-07-18; Fax: 998-71-120-67-91, Email: i.rasilov.rambler.ru**

**Administration Official: (B. Yakobson)**
**Director, Kimron Veterinary Institute**
**P.O. Box 12, Bet Dagan 50250**
**Israel**
**Tel: 972-3-9681682**
**FAX: 972-3-96052-93**
**Email:Dir_kimron@int.gov.il**

**Administration Official: D. Azimov**
**Director, Institute of Zoology**
**1, Niazov str., Tashkent,**
**Uzbekistan**
**Tel: 998- 71-146-07-18**
**Fax: 998-71-120-67-91**

| | |
|---|---|
| **B) TABLE OF CONTENTS** | **2** |
| **C) EXECUTIVE SUMMARY** | **3-4** |
| **D) RESEARCH OBJECTIVES** | **4** |
| **E) METHODS AND RESULTS** | **5-31** |
| **F) IMPACT, RELEVANCE AND TECHNOLOGY TRANSFER** | **32** |
| **G) PROJECT ACTIVITIES/OUTPUTS** | **32-33** |
| **H) PROJECT PRODUCTIVITY\FUTURE WORK** | **33-34** |
| **I) LITERATURE CITED** | **34-35** |
| | |
| **Abstract presented at the STVM Symposium** | **36** |
| **Manuscripts accepted for publication** | **37-53** |

## EXECUTIVE SUMMARY

The main objective of the present project was to develop safe and potent live attenuated blood- or culture-derived vaccines based on the *Babesia bigemina* and *B. bovis* strains originating from Uzbekistan.

*B. bigemina* and *B. bovis* were isolated from engorged *Boophilus annulatus* ticks collected from pasture-raised sick or carrier cattle, in areas where babesiosis is endemic (Sir-Daria, Ushkent). Calves were infected either by infestation with infected ticks or by transfer of infected blood obtained from the tick-infected animals into splenectomized calves. MASP cultures were initiated from the infected calves for attenuation and further development of culture-derived vaccines for each parasite species.

Loss of virulence of *B. bigemina* has been achieved after four "slow passages" *in vivo*; virulence reduction was evidenced by the response of calves inoculated with an experimental live frozen vaccine produced from the following passage. The vaccine was safe and protective against homologous virulent challenge under laboratory conditions. The culture-derived experimental anti-*B. bigemina* vaccine was produced from cultures initiated after three passages of the parasites *in vivo* followed by 22 passages *in vitro.* The cultured parasites did not elicit any clinical sign, and the inoculated calves seroconverted following vaccination, and were protected against the virulent homologous challenge. Both calf- and culture-derived vaccines were safe for cattle grazing in an endemic area in Uzbekistan. Despite the high polymorphism of *B. bigemina*, as reported from various geographical regions, the Central Asian strain was attenuated similarly to those that form the basis of the existing live *B. bigemina* vaccines in other parts of the world.

The Uzbek *B. bovis* strain exhibited reduced virulence after nine passages. Calves inoculated with the experimental blood-derived vaccine prepared from passage 15 did not develop clinical signs of babesiosis, and were protected against virulent homologous and heterologous challenge. *In vitro*, *B. bovis* could be cultured for a limited number of passages (up to 10), but the parasitemia in these cultures did not reach adequate levels for preparation of an experimental vaccine.

The trivalent vaccine consisting of attenuated schizonts of *Theileria annulata* (a vaccine developed under our previous CDR grant TA_MOU-94-CA-14-040), *B. bigemina*, and *B. bovis* (attenuated under the present grant), was safe for

immunization use under either laboratory (tested in Israel) or field conditions (tested in Uzbekistan). There was a high degree of immunity against challenge with virulent live parasites. Challenge experiments proved that the trivalent vaccine was immunogenic: no signs of theileriosis or babesiosis were elicited, whereas only non-immunized control animals that received the virulent parasites required drug treatment. Similarly to South Africa, Israel, Argentina and Australia, the trivalent vaccine based on Uzbek autochthonous strains can be introduced for routine vaccination of cattle.

It is important to note that, in addition to the specific achievements in the development of effective anti-babesiosis vaccines, the Uzbek scientists were exposed to and learned the technology for vaccine production from the very beginning, including parasite isolation, attenuation, animal experimentation, understanding and evaluation of responses on the basis of either clinical or laboratory parameters. These skills will remain of value in research and practical applications for the foreseeable future. The Uzbek scientists obtained extensive training and actively participated in all the experimental laboratory and fieldwork, and it was very important that they were able to participate in the International Meeting on Tropical Diseases held in Hanoi, Vietnam, where they gained direct contact with the international community involved in research into tick-borne diseases, and were able to present our cooperative work.

## D) RESEARCH OBJECTIVES

The main overall aim of the project was to develop safe and potent vaccines against *B. bigemina* and *B. bovis* babesiosis; vaccines based on the autochthonous strains from Uzbekistan. As cattle breeding is one of the most important activities in Central Asian countries, and animal protein forms the major component of the diet of the human population, great benefits will be gained through improving cattle health and protect cattle by using these vaccines against high morbidity and mortality the use of these vaccines initiallyin Uzbekistan and subsequently in the surrounding countries, in which babesiosis is endemic. Frozen blood- and culture-derived vaccines were developed against *B. bigemina* infection, but only blood-derived vaccine was produced for use against *B. bovis* infection. All the vaccines were safe and potent under laboratory and field conditions in Uzbekistan. The application of a trivalent combination of *B. bigemina, B. bovis* and *Theileria*

*annulata* vaccines in an endemically infected field in Uzbekistan demonstrated an additional benefit to the cattle industry in this country; farmers engaged in small-scale husbandry, lacking the infrastructure for anti-tick control, such as dipping facilities, could protect their cattle against major tick-borne diseases by simultaneous prophylaxis provided by administration of the trivalent vaccine.

## E) METHODS AND RESULTS

### Isolation of *Babesia* from ticks and blood of carrier cattle

In Uzbekistan, engorged *Boophilus annulatus* adult ticks were collected from pasture-raised sick or carrier cattle in babesiosis-endemic areas (Sir-Daria, Ushkent). Numerous ticks were kept at room temperature pending transfer to Israel. Upon arrival the adult ticks were incubated at 28° C and 85% humidity for 3 days, after which the ticks started to lay eggs. The resulting eggs were kept at 14° C, 85% humidity pending use. In addition to the collected adult ticks, larval stages obtained after oviposition in Uzbekistan were transferred to the KVI laboratory. Unfed larvae obtained from several collections of hatched eggs were pooled and were applied for feeding on three 2-month-old spleen-intact calves that were seronegative for *Babesia* (Fig. 1, calves 565, 566 and 567). All the calves were examined for increased body temperature, microscopic examinations of peripheral blood smears were done to detect infection, and packed cell volume (PCV) was determined daily. *B. bovis* is transmitted by the larval stage, and *B. bigemina* by the nymph stage. Approximately 10 days after infestation of cattle with larvae, nymphs had developed, and were further transmission-fed to allow transmission of *B. bigemina* infection. After the nymphs had fed for about a week, all the calves were sprayed with an acaricide to prevent the spread in Israel of exotic ticks. Blood from calves that had been monitored for the presence of *Babesia* parasites was drawn into tubes containing anticoagulant (EDTA), and was either frozen with 15% DMSO, or transferred to other animals for transmission of the infection, or for identification of parasite DNA by PCR. Serum samples were collected for detection of antibodies specific to either *B. bigemina* or *B. bovis*.

Blood collected from a carrier and from a clinically apparent case of babesiosis in Uzbekistan (Fig. 1, cows 253 and 254) was inoculated into splenectomized seronegative calves (559 and 560, respectively). DNA was prepared to test for the

presence of parasites and for their identification by using specific primers for PCR (Fig. 2 A, B). The calves were monitored for infection as described above.

A total of 18 calves were used for the isolation of either *B. bigemina* or *B. bovis* from ticks or from infected blood. Fig. 1 summarizes the experiments performed up to the present, on susceptible animals infected by tick transmission feeding or by inoculation of blood from chronically infected cows in Uzbekistan. Infection of calves was followed by passages in intact or splenectomized calves, for maintenance and further attenuation of virulence. *B. bovis* was isolated from calf 565, which was infested with one of the pooled larvae collections. At least three rapid passages (transfer of blood at the first appearance of parasites in peripheral blood) were performed in splenectomized calves (577, 578 and 551). At the height of parasitemia blood was frozen at -70° C pending use.

*B. bigemina* was isolated from ticks (on calves 567 and 559) and from the blood of a chronically sick cow (253), respectively. At least four slow passages were performed in intact calves (563, 570, 576 and 585). After recovery from infection the calves were splenectomized, and then, following the recrudescence of parasitemia, their blood was cyopreserved or further inoculated into a spleen-intact calf for slow passages.

As shown in Table 1, all five calves infected either by the *Boophilus* larval stage or by blood transfusion developed high fever characteristic of *Babesia* infection. Although in four out of six calves the PCV was below 20%, compared with 25 and 26% in the other two animals, all six had to be treated with babesicides (Berenil – diminazene aceturate, or Lomidine, pentamidine) to prevent further destruction of red blood cells, and thus to prevent the death of the animals. Calf 566, which had been infected via ticks, became infected with both *B. bigemina* and *B. bovis* and, therefore, was not used for further experiments. It appears that treatment with Benenil destroyed *B. bigemina*, which is considered to be more sensitive to the drug than *B. bovis*, so that at blood transfusion only *B. bovis* infection was established. Three passages were performed, but the strain was virulent and needed further procedures for attenuation. The infected blood from animals with high parasitemia was frozen with DMSO at –70 ° C to serve as a stabilate for the Uzbek strain of *B. bovis*.

In Tables 2 and 3 are summarized the transmission/ feeding experiments and the isolation of *B. bigemina* from blood collected from chronically infected cow no. 253 from Uzbekistan. The *B. bigemina* strain was isolated from tick infection and

from blood. To maintain the infection, the first transfusions were performed in splenectomized animals, but for attenuation, passages were done in intact calves, to produce chronic infection, and were followed by splenectomy, with further passages to be performed at the recrudescence of infection. At high parasitemia blood was cryopreserved and will serve as a stabilate for the Uzbek strain of *B. bigemina.*

**Isolation of *Babesia* parasites in MASP culture**

Cultures were initiated from animals infected with *B. bigemina* or *B. bovis,* according to Levy and Ristic (1980). Blood was drawn under sterile conditionsfrom calves that showed parasitemia in peripheral blood smears. Infected blood at 0.5 to 2% parasitemia was collected into vessels with glass beads and was defibrinated, and the erythrocytes were suspended to a final packed cell volume (PCV) of 9% in medium comprising 60% RPMI 1640 and 40% normal bovine serum, supplemented with 15 mM HEPES buffer, pH 7.0, and 100 IU penicillin G and 100 μg streptomycin sulfate per milliliter. Cultures were incubated in flat-bottomed micro-trays at 37° C under a mixture of gases comprising 5% $CO_2$, 2% $O_2$ and 93 % humidified air. Overlaying medium was removed and replaced with fresh medium daily. Subcultures were made initially when parasites could be detected in blood smears prepared from the culture wells, and then every 2-3 days, by diluting the culture to 0.5% parasitemia with a 10% suspension of uninfected erythrocytes in culture medium.

**PCR assays**

To test for the presence of specific *Babesia* parasites by PCR (Fig. 2A, B), DNA was extracted from infected erythrocytes by applying the Purogene kit (Gentra Systems Inc., Minneapolis, USA) to the blood samples that were taken from chronically infected cows in Uzbekistan and brought to Israel. Blood samples from an uninfected calf and from experimentally infected calves were processed similarly and used as negative and positive controls. The PCR assays were based on primers published in GenBank, as well as those we have designed for the present study (for *B. bigemina* - GenBank accession number S45366, Figueroa et al., 1992; for *B. bovis* - GenBank accession number M138218, Suarez et al., 1991).

**Responses of cattle to blood- or tick-derived *B. bigemina* during the attenuation process**

Blood infected with two parallel strains that had been obtained, respectively, from the blood of a naturally chronically infected cow and from infected ticks collected in Uzbekistan, was passaged in spleen-intact calves to attenuate *B. bigemina* further. As shown in Fig. 3 and in Table 4, passages 2 and 3 of the blood-derived strain were performed in calves 576 (naive, spleen-intact calf) and 600 (splenectomized calf). *B. bigemina* appeared to be virulent at this stage, although the calves exhibited relatively low parasitemia (6%) and only a 15% decrease in PCV, and recovered without treatment with anti-*Babesia* drugs. However, as a result of a further passage in the splenectomized animal, the calf exhibited acute clinical babesiosis, with parameters that necessitated drug treatment to prevent the death of the animal. At this stage, no further passages were performed. *B. bigemina* parasites (Fig. 3) originating from infected ticks were subjected to "slow passages", as described by Dalgliesh et al. (1981): the parasites used came from a relapsed parasitemia case, after a naive and chronically infected calf had been subjected to splenectomy. As shown in Table 4, the passages were performed in three naive calves (575, 585 and 597). At "slow" passage 4 (calf 597), a slightly elevated fever (normal body temperature in bovines is 39.5° C) and a moderate PCV drop were recorded, and parasitemia was below 0.01%. Blood from calf 597 was subinoculated into a naive calf, no. 602. At passage 5, as at passage 4, a moderate clinical response was observed: negligible fever, about 30% drop in PCV, and minimal – below 0.01% – parasitemia. At this stage of attenuation (from passage 5), an experimental vaccine was prepared. As expected, inoculation of a splenectomized calf with this material resulted in elevated fever (maximum 41.0° C), a 20% drop in PCV, and parasitemia up to 23%. These data were considered to indicate suitability for vaccine production, and blood was drawn and processed, as described below, for production of a frozen experimental *B. bigemina* vaccine.

**Experimental *B. bigemina* vaccine production**

The frozen *B. bigemina* vaccine was prepared from calf 595 at maximum parasitemia of 23%. Infected blood was collected by jugular cannulation into anticoagulant 4% sodium citrate solution, and cooled to 4° C. The number of parasites per milliliter of infected blood was calculated from the percentage of

infected erythrocytes, as determined from Giemsa-stained smears, and the total red blood cell count. Blood was diluted with PBS containing cold DMSO, which was added slowly on a magnetic stirrer. The final concentration of DMSO in the mixture was 15%. Vials were loaded with 10 doses (concentrated in a total of 3.6 ml, so that, when diluted in a cryopreservative, each 2-ml dose contained $3.6 \times 10^8$ parasites. The vaccine was immediately frozen, first in nitrogenvapor and then, after about 1 h, in liquid nitrogen.

The calf from which the vaccine had been produced was tested for viral and bacterial contaminants, including bovine leucosis, infectious bovine rhinotracheitis, mucosal disease, ephemeral fever, bluetongue, leptospira, and salmonella. Blood in the final vaccine product was tested similarly.

**Measurements of responses to vaccination**

Clinical condition was monitored for parasitemia, as counted in Giemsa-stained blood smears, and percentage PCV volume and body temperature were measured daily from day 5 after vaccination until day 14, when no parasites, no fever, and normal PCV were recorded. Blood for serological testing was collected before vaccination and weekly after vaccination. Levels of specific antibodies were recorded by the indirect fluorescent antibody (IFA) test according to Goldman et al. (1972).

**Testing of the experimental vaccine for safety and immunogenicity**

The laboratory trial (Table 5) used four intact 6-month-old Friesian calves (607, 608, 612 and 614) that were kept in closed barns under tick-free conditions and inoculated with a dose of $4 \times 10^8$ frozen parasites. Before inoculation the frozen vial was thawed in a water bath at 40° C, and the contents were transferred immediately into the diluent containing PBS and DMSO, to obtain a final DMSO concentration of 15%. Each animal was inoculated subcutaneously within 15 min after the blood was thawed. As shown in Table 5, of the four calves that were inoculated, two responded with elevated fever. Calf 608 developed fever of 40.1° C, its minimum PCV was 20% and its peripheral blood parasitemia was 0.7%. The PCV depression was calculated as the difference between the pre-infection level and the minimum level recorded. An additional calf (612) developed fever (40.3° C), but its minimum PCV was 25%, and only a few parasites could be detected in smears

from its peripheral blood. Following vaccine inoculation, calves 607 and 614 exhibited no severe clinical or parasitological responses.

To test for immunogenicity the four immunized calves (607, 608, 612 and 614) were challenged by inoculating them with 50 ml of blood from a calf infected with a virulent Uzbek *B. bigemina* strain at 0.5% parasitemia (homologous challenge). The average total number of red blood cells inoculated was $3.88 \times 10^{11}$. Thus, at parasitemia of 0.5%, each calf received about $2 \times 10^{9}$ infected erythrocytes. The challenge inoculation was performed 4 months after the initial vaccination. Two additional calves (604 and 610) that had not been vaccinated served as controls for the challenge inoculation.

**Challenge trial for potency of the *B. bigemina* vaccine**

The responses of the vaccinated calves to challenge are presented in Table 6. None of the four vaccinated animals developed fever, their PCV depressions ranged from 14.3 to 24.2%, and none required specific drug treatment. In contrast, the two control calves (604 and 610) responded with severe clinical babesiosis. Their body temperature reached 41.4° C, their PCV depressions were 62.9 and 61.7%, and their minimum PCV was 13%. Both calves received diminazene aceturate (Berenil) to prevent death. The experimental *B. bigemina* vaccine was tested for safety in a tick-free and babesiosis-free dairy farm in which Friesian cattle are bred under zero-grazing conditions, which ensures that they never encounter tick vectors; such cattle are considered to be highly susceptible to *Babesia* infection.

Ten calves aged 5-6 months were vaccinated with the frozen experimental vaccine, and were monitored for clinical and serological responses (Table 7). After 6 days of the prepatent period, 5 out of 10 calves developed fever, with a maximum of 40.3° C. The group average body temperature was 39.9° C. The group mean PCV was 23.4%, with a minimum of 20%. Maximum parasitemia of 1% was observed in only one animal (calf 624); four other calves showed *B. bigemina* parasitemia of 0.2-0.5%. None of the vaccinated animals required babesicidal treatment.

**Propagation and maintenance of *Babesia bigemina* in MASP culture**

*B. bigemina* isolated from an infected calf, no. 585, at 0.2% parasitemia was introduced into MASP culture. Aliquots of 5 ml of blood were collected into tubes with EDTA. The blood was washed three times with VYM solution by centrifugation

at 1200 × *g*. The final pellet of infected erythrocytes was dispersed into 24-well culture plates after dilution with growth medium (MEM 199, 50% bovine serum, or 20% serum in HL-1 medium at 1:1, 1:2 and 1:3), at a final volume of 0.8 ml per well. *B. bigemina* (Fig. 5) parasites were detected in culture after one blind passage, 48 h after seeding. At 72 h parasitemia reached 4%. The MASP cultures were grown in an incubator in an atmosphere of 2% $O_2$, 5% $CO_2$, 93% $N_2$. For further maintenance, subcultures were performed every 72 h (Figs 6 and 7), and when parasitemia reached 8%, frozen stabilates were prepared in DMSO at a final concentration of 15%, and kept in liquid nitrogen (Fig. 8)

As shown in Table 8, doses of *B. bigemina* culture-derived frozen vaccine containing $1 \times 10^9$ infected erythrocytes, prepared with DMSO as cryoprotectant, were tested for safety in two calves (632 and 634). The immunized calves showed mild parasitemia, none developed fever, and their minimum PCV was in the normal range (Table 8); these calves developed IFA titers up to 1:64. Five months after immunization the calves were challenged with the virulent homologous Uzbek strain (blood from calf 600 at passage 3, before *B. bigemina* became attenuated). Two additional non-immunized calves were given the challenge inoculation, to serve as challenge controls.

As a further stage of preparation for production of *B. bigemina* vaccine from MASP culture, an experimental batch of 110 doses was tested for safety and immunogenicity by inoculation into susceptible calves, whose clinical and parasitological responses were then monitored.

The blood-derived *B. bigemina* vaccine was transferred to Uzbekistan (Fig. 8), where its safety was tested under field conditions. Forty calves, aged 2 to 5 months, on a farm near Tashkent, in the babesiosis-endemic area, were immunized with the frozen *B. bigemina* vaccine. During the 16-day follow up the only clinical response recorded was slight fever (up to 40° C) in a few animals. Serum samples collected a month later and examined for specific antibodies all showed seroconversion (data not shown).

**Preparation and testing of *B. bovis* vaccine**

The Uzbek *B. bovis* strain was passaged *in vivo* through up to 16 "rapid" passages, to obtain attenuated parasites (Fig. 4). The responses of splenectomized calves during attenuation are presented in Tables 9 and 10. An experimental frozen vaccine was prepared for safety and virulence testing of the calf-derived *B. bovis* in Israel and in

Uzbekistan, under laboratory and field conditions A total of 600 doses, each containing $3,56 \times 10^8$ infected erythrocytes were frozen with cryoprotectant 15% DMSO, at the final concentration (Fig. 9). In light of publications in the scientific literature and experience gained in vaccine development in Australia and in Israel, it is well known that some strains are less immunogenic than others (Callow and Dalgliesh, 1980; DeVos, 1989). There is evidence that multiple passaging of a strain may result in loss, not only of virulence, but of immunogenicity as well. It is well documented that after about 23-28 passages most *B. bovis* strains remain protective; however, it has been found recently that at lower passages (15-20), the protection imparted by the vaccinates was superior to that imparted at higher passages of the strain (Bock and deVos, 2001). Therefore, in the present work, it was decided to produce an experimental vaccine from the calf-derived passage 15.

**Monitoring of responses to immunization with trivalent anti-*B. bigemina*, anti-*B. bovis* and anti- *Theileria annulata* vaccines under laboratory conditions.**

Six intact calves aged 5-7 months were inoculated with attenuated, frozen live vaccines against *B. bigemina*, *B. bovis* and *Theileria annulata*. All the vaccines were produced from the Uzbek isolates. The anti-*Babesia* vaccines were calf-derived and contained, respectively, $3.8 \times 10^6$ and $1.78 \times 10^6$ live *B. bovis* and *B. bigemina* parasites per dose before freezing. The anti-*Theileria* vaccine was produced from monolayers of culture-derived schizont-infected cells; each dose contained $5 \times 10^6$ infected cells. All the inoculated calves were monitored for clinical signs: elevation of fever, PCV drop, and presence of parasites in smears of the peripheral blood.

As shown in Table 11, all the calves developed fever above the normal level of 39.5° C at various days post-vaccination, but the elevated temperatures persisted for a maximum of 2 days, after which they returned to normal. The PCV depression was dramatic in calf 701 (50%), and its absolute value was 18% for 2 days; however, in light of the clinical condition of the animal, no drug treatment was required. Table 12 summarizes the parasitemia and serological responses in vaccinated calves.

Countable parasitemia was recorded in all calves except calf 693 – no parasites were found in that animal's peripheral blood smears. In an examination based on the biology of the parasite only a few *B. bovis* individuals could be found in the circulating blood during 1 or 2 days, although at a roughly similar level of *B. bigemina* parasitemia, those parasites could be detected. The presence of the parasites

persisted for about 1 week post vaccination. All the calves, including calf 693 – the one without visible parasitemia – developed significant antibody titers to all the parasites in the trivalent vaccine. This indicates that infection took place; the live organisms in the vaccines did multiply and elicited a humoral immune response (Table 12).

The potency of the trivalent vaccine was tested by challenge inoculation of the previously immunized calves with virulent parasites (Table 13), and for each parasite component control calves were added to test the virulence of the inocula. All the control calves developed parasitemia, which ranged from 1 to 4% with *B. bigemina* infection, from 1 to 20% in response to *Theileria annulata*, and reached only 1% parasitemia in *B. bovis*-infected animals, as expected. The PCV depression in response to *B. bovis* was around 20%, except for one calf with 47.8%; 4 out of 6 calves responded to *T. annulata* with about 49% PCV depression. Out of 6 calves infected with *T. annulata,* three required treatment. One calf required treatment to prevent death from *B. bigemina* infection. Following challenge with virulent parasites it was evident that pre-immunized calves developed solid immunity, despite elevated fever and moderate PCV drop, and none showed parasitemia or required drug treatment. On the other hand, the control non-immunized calves showed signs of infection, and some suffered severe clinical diseases, which required treatment to ensure recovery.

In a field trial performed in Uzbekistan, 38 calves aged from 4.5 to 9 months were immunized with the trivalent vaccine containing three pathogens, *B. bigemina, B. bovis* and *Theileria annulata.* As shown in Table 14, none on these calves developed fever, their PCV values remained in the normal range, *B. bigemina* and *T. annulata* parasites (and, as expected, not *B. bovis*) appeared in blood smears from about one-third of the vaccinated animals, and in all the calves specific antibodies were detected a month after vaccination. In light of the field trial, it can be concluded that simultaneous immunization against three pathogens is safe for grazing cattle in Uzbekistan when it is applied at the weaning age.

**Fig. 1. Schematic presentation of isolation of *Babesia* parasites**

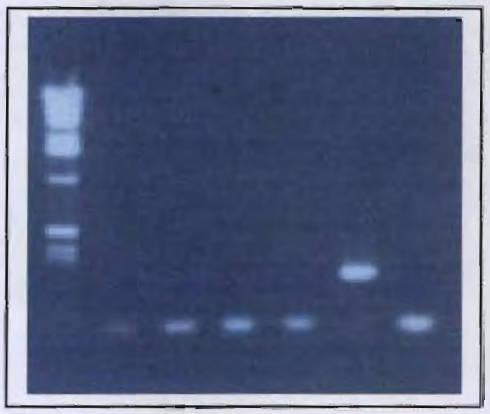
BEST AVAILABLE COPY

**Figs. 2 A and B**

Results of PCR for presence of *Babesia bovis* and *B. bigemina* in blood samples from chronically infected cow in Uzbekistan.

**A. *B.bovis* primers**

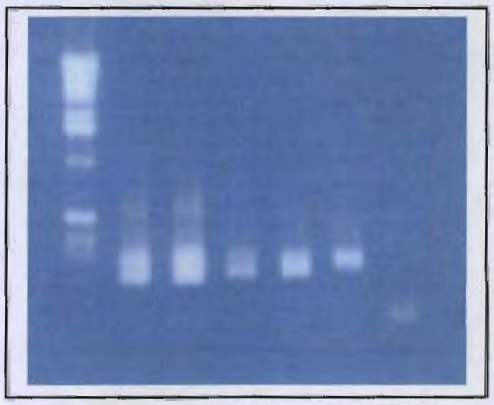
1. **Marker**
2. **Blood DNA from cow 253**
3. **Duplicate of 253**
4. **Blood DNA from cow 254**
5. **Duplicate of 254**
6. **Positive control**
7. **Negative control**



1 2 3 4 5 6 7

**B. *B.bigemina* primers**

1. **Marker**
2. **Blood DNA from cow 253**
3. **Duplicate OF 253**
4. **Blood DNA from cow 254**
5. **Duplicate of 254**
6. **Positive control**
7. **Negative control**



1 2 3 4 5 6 7



BEST AVAILABLE COPY

**Table 1. Isolation of *B.bovis* from calves infested with the *Boophlus* ticks collected in Uzbekistan by transmission / feeding**

| Number of calf | Inoculated or infested | Max fever (° C) | Min PCV | Established infection | Splenectomy/ Treatment | Outcome |
|---|---|---|---|---|---|---|
| 565 | *Boophilus* larvae | 40.2 | 25 | *B.bovis* <0.01% | +/ Berenil | *B.bovis* |
| 566 | *Boophilus* larvae | 40.3 | 18 | *B.bovis* <0.001% *B.bigemina* 3% | +/ Lomidine | Mixed infection |
| 571 | Blood from 565 | 40.8 | 26 | *B.bovis* <0.01% | - | *B.bovis* |
| 577 | Blood from 571 | 41.0 | 14 | *B.bovis* 14% | +/ Berenil | Survived, passage #1 |
| 578 | Blood from 577 | 41.1 | 16 | *B.bovis* 6% | +/ Berenil | Survived, passage #2 |
| 551 | Blood from 578 | 40.8 | 16 | *B.bovis* 5% | +/ Berenil | Survived, passage #3 |

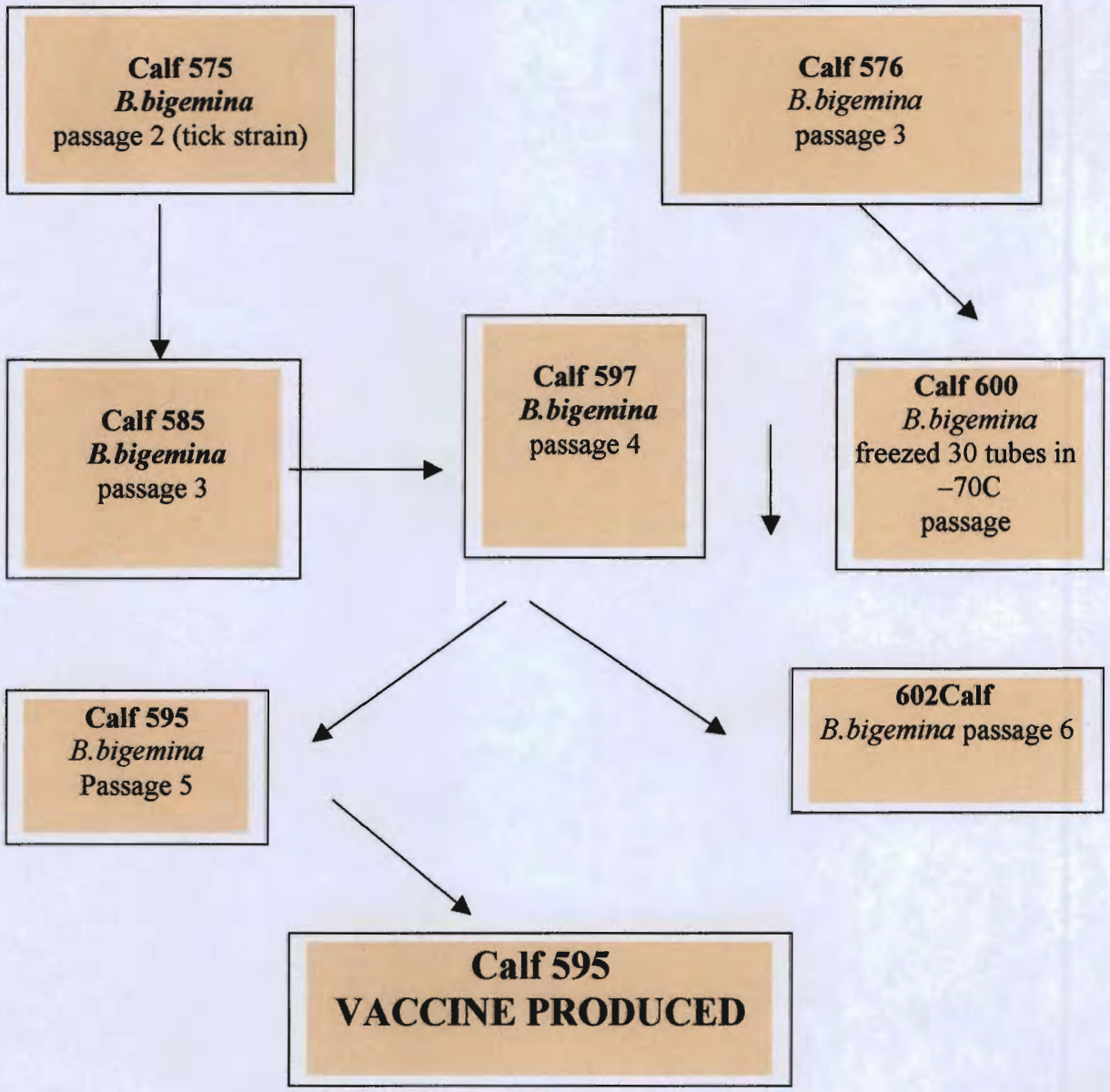
**Table 2. Isolation of *B.bigemina* from calves infected with blood from chronically infected cows in Uzbekistan**

| Number of calf | Infected with blood | Max fever (° C) | Min PCV | PPE (%) | Treatment | Passage number | Splenectomy at stage |
|---|---|---|---|---|---|---|---|
| 559 | Cow 253 40ml sc | 40.6 | 12 | 6 | _ | | + acute infection |
| 560 | Cow 254 50ml sc | 41.3 | 33 | 0 | + | | + acute infection |
| 563 | Calf 559 | 41.0 | 17 | 28 | + | 1 | + acute infection |
| 570 | Calf 563 | 40.2 | 18 | 0.5 | + | 2 | + chronic infection |
| 576 | Calf 570 | 40.0 | 15 | 6 | _ | 3 | + chronic infection |

**Table 3. Isolation of *B.bigemina* from infected *Boophilus* ticks collected in Uzbekistan by transmission / feeding on calves**

| Number of calf | Infected with | Max fever (° C) | Min PCV | Infection / Max PPE | Splenectomy at stage | Outcome / passage number |
|---|---|---|---|---|---|---|
| 567 | Tick nymphs | 40.1 | 19 | 1% | - | |
| 569 | Blood from 567 | 40.5 | 21 | 4% | - | Survived 1 |
| 575 | Blood from 569 | 40.1 | 13 | 8% | + chronic | Survived 2 |
| 568 | Blood from 575 | 40.3 | 16 | 3% | + chronic | Survived, 3 |
| 585 | Blood from 575 | Beginning of reaction | | | | 4 |

**Fig. 3. Attenuation of the Uzbeki *B. bigemina* strain**






BEST AVAILABLE COPY

**Table 4. Responses of naive and splenectomized calves inoculated with "slow" passaged *B. bigemina* originating from tick infection or from blood of a carrier cow**

| Number of calf | Inoculated with: | Number of passage and origin | Prepatent period (days) | Max fever (°C) | Min PCV (%) | Max. PPE (%) | Treatment | Calves With/Wo spleen |
|---|---|---|---|---|---|---|---|---|
| 576 | Blood from calf 570 | 2 Blood strain | 3 | 40.8 | 15 | 6 | no | naive |
| 600 | Blood from 576 | 3 Blood strain | 8 | 40.8 | 34 | 10 | Berenil | splenectomy |
| 575 | blood from calf 569 | 2 Tick strain | 4 | 40.1 | 13 | 15 | no | naive |
| 585 | blood from 575 | 3 Tick strain | 6 | 39.6 | 28 | 0.5 | no | naive |
| 597 | blood from 585 | 4 Tick strain | 5 | 39.6 | 28 | > 0.01 | no | naive |
| 602 | blood from 597 | **5** Tick strain | 4 | 39.6 | 32 | > 0.01 | no | naive |
| 595 | blood from 597 | **5** Tick strain | 3 | 41.0 | 20 | 23 | Berenil | splenectomy |

**Table 5. Responses of calves inoculated with the experimental vaccine (laboratory trial)**

| Number of calf | Prepatent period (days) | Clinical and serological response | | | | | |
|---|---|---|---|---|---|---|---|
| | | Max. Temp C | Min PCV* (%) | PCV depression (%) | Percent PPE** | Treatment | Antibody IFA titer ($log_4$)** |
| 607 | 7 | 39.8 | 28 | 15.1 | <0.01 | no | 3 |
| 608 | 8 | 40.1 | 20 | 39.4 | 0.7 | no | 3 |
| 612 | 7 | 40.3 | 25 | 26.5 | <0.01 | no | 3 |
| 614 | 5 | 39.5 | 26 | 23.5 | <0.01 | no | 2 |
| Group Mean | 6.75 | 39.9 | 24.7 | 27.2 | 0.01 | | 2.75 |

* PCV – Packed cell volume

** PPE – Percent parasitised erythrocytes

*** IFA – Immunofluorescent antibody test three months after immunization

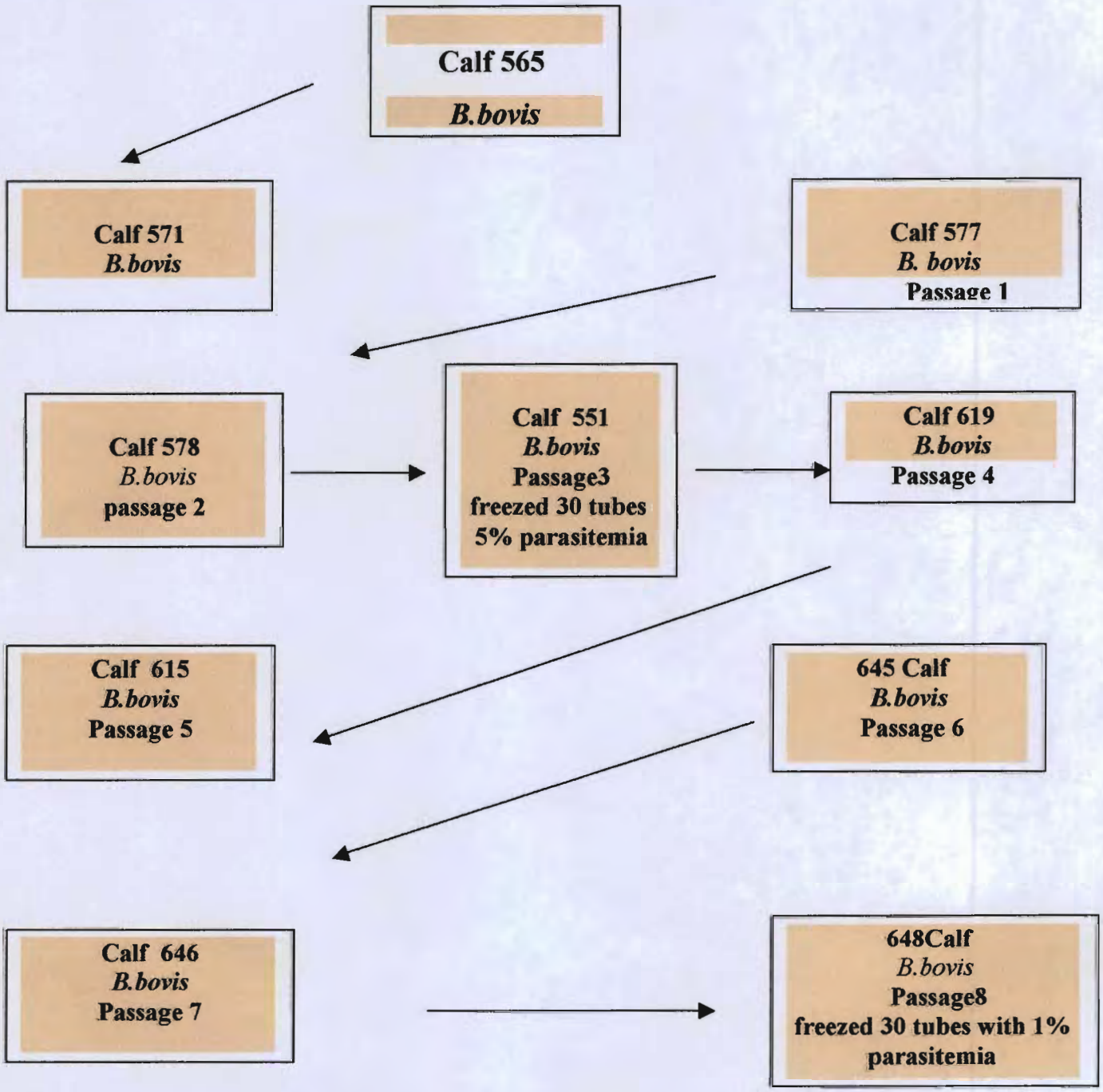
**Table 6. Responses of immunized calves to challenge with Uzbek homologous virulent *B. bigemina* strain**

| Number of calf | Clinical and serological responses to challenge | | | | | | |
|---|---|---|---|---|---|---|---|
| | Prepatent period | Max. Temp (°C) | Min PCV (%) | PCV Depression (%) | PPE (%) | Treatment | Antibody titer in IFA ($log_4$) |
| 607 | 4 | 39.0 | 30 | 16.7 | <0.01 | Nr* | 4 |
| 608 | 4 | 39.5 | 30 | 14.3 | <0.01 | Nr* | 4 |
| 612 | 6 | 39.4 | 25 | 24.2 | <0.01 | Nr* | 3 |
| 614 | 6 | 39.1 | 30 | 14.4 | <0.01 | Nr* | 4 |
| Control | | | | | | | |
| 604 | 3 | 41.4 | 13 | 62.9 | 10 | Berenil | Not determined |
| 610 | 3 | 41.4 | 12 | 61.7 | 10 | Berenil | Not determined |

**Table 7. Response of Friesian calves kept on zero grazing to inoculation of the experimental Uzbek *B. bigemina* vaccine (field trial)**

| Number of calf | Clinical and serological responses to challenge | | | | | | |
|---|---|---|---|---|---|---|---|
| | Prepatent period | Max. Temp C)ؤ( | Min PCV (%) | PCV depression (%) | PPE (%) | Treatment | Antibody titer in IFA ($log_4$) |
| 619 | 6 | 40.1 | 24 | 8.0 | 0.5 | Na* | 4 |
| 620 | 6 | 40.0 | 26 | 9.1 | <0.01 | Na* | 3 |
| 621 | 6 | 39.4 | 24 | 8.4 | 0.3 | Na* | 4 |
| 622 | 6 | 39.7 | 24 | 9.0 | 0.2 | Na* | 4 |
| 623 | 6 | 39.8 | 22 | 7.7 | 0.5 | Na* | 3 |
| 624 | 6 | 40.5 | 20 | 7.0 | 1.0 | Na* | 4 |
| 625 | 6 | 39.5 | 20 | 7.4 | 0.5 | Na* | 4 |
| 627 | 6 | 40.0 | 22 | 6.8 | <0.01 | Na* | 4 |
| 628 | 6 | 40.3 | 24 | 9.8 | 0.2 | Na* | 3 |
| 629 | 6 | 39.6 | 27 | 9.2 | <0.01 | Na* | 3 |
| Mean | 6 | 39.9 | 26 | 9.16 | 0.3 | | 3.6 |

**Fig. 4. Attenuation of the Uzbeki *B. bovis* strain**






BEST AVAILABLE COPY

**Table 8. Response of calves immunized with the culture-derived *B.bigemina* vaccine of the Uzbeki strain**

| Number of calf | Prepatent period (days) | Max. Temperature °C | Minimal PCV (%) | Maximum parasitemia % | Treatment |
|---|---|---|---|---|---|
| 632 | 7 | 39.6 | 31 | 0.4 | No |
| 634 | 7 | 39.5 | 29 | 0.01 | No |

**Table 9. Responses of splenectomized calves during established infection and "rapid" passages of *B. bovis* (Uzbeki strain)**

| Number of calf | Infected with: | Splenectomized (S) or Naive (N) | Number of passage | Prepatent period (days) | Max fever (°C) | Min PCV (%) | Max. PPE (%) | Treatment applied |
|---|---|---|---|---|---|---|---|---|
| 565 | Tick larvae | N | Establishing infection | 8 | 40.2 | 28 | 0.5 | no |
| 571 | Blood from 565 | N | Establishing infection | 5 | 40.3 | 26 | 0.01 | no |
| 577 | Blood from 571 | S | **1** | 3 | 41.0 | 14 | 4 | + |
| 578 | Blood from 577 | S | **2** | 3 | 41.1 | 17 | 4 | + |
| 551 | Blood from 578 | S | **3** | 1 | 41.3 | 14 | 5 | + |
| 619 | Blood from 551 | S | **4** | 8 | 42.2 | 23 | 3 | + |
| 615 | Blood from 619 | S | **5** | 2 | 41.2 | 32 | 0.01 | + |
| 645 | Blood from 615 | S | **6** | 2 | 41.2 | 18 | 3 | + |
| 646 | Blood from 645 | S | **7** | 2 | 41.3 | 22 | 4 | + |
| 648 | Blood from 646 | S | **8** | 3 | 41.2 | 31 | 4.5 | + |

**Table 10. Responses of splenectomized calves during established infection and "rapid" passages of *B. bovis* (Uzbek strain)**

| Number of calf | Number of passage | Blood inoculated | Prepatent period (days) | Max fever (°C) | Min PCV (%) | Max. PPE (%) | Treatment applied |
|---|---|---|---|---|---|---|---|
| 654 | 9 | Frozen stabilate | 11 | 40.5 | 33 | 0.01 | none |
| 653 | 10 | 50 ml from 654 | 1 | 40.5 | 32 | 0.01 | none |
| 656 | 11 | 60 ml from 653 | 4 | 40.7 | 41 | 0.01 | none |
| 655 | 12 | 60 ml from 656 | 2 | 38.6 | 34 | 0.01 | none |
| 659 | 13 | 60 ml from 655 | 3 | 40.1 | 41 | 0.01 | none |
| 660 | 14 | 60 ml from 659 | 3 | 40.9 | 23 | 0.01 | none |
| 662 | 15 | 200 ml from 660 | 1 | 41.1 | 18 | 0.01 | none |
| Experimental vaccine prepared | | | | | | | |
| 661 | 16 | 200ml from 662 | 1 | 41.3 | 19 | 13 | Berenil |

**Table 11. Monitoring of fever and packed cell volume (PCV) in calves vaccinated with the trivalent vaccine containing *B.bigemina, B.bovis and T. annulata***

| Number of calf Vaccinated | Maximum fever °C | Fever on day / Lasting for days | Minimum PCV value | PCV depression (%) |
|---|---|---|---|---|
| 691 | 39.8 | 12/1 | 24 | 30.3 |
| 693 | 40.0 | 7/1 | 26 | 21.2 |
| 694 | 39.4 | 13/1 | 22 | 33.3 |
| 699 | 40.3 | 12/2 | 24 | 35.1 |
| 700 | 40.5 | 5/1 | 24 | 32.3 |
| 701 | 41.0 | 12/2 | 18 | 50.0 |
| **Mean** | **40.1** | **8.3 / 1.3** | **23** | **33.7** |

**Table 12. Percentage parasitemia (PPE) recorded following vaccination, and antibody level in the IFA test expressed in log $_4$ (four-fold serum dilution) to *Babesia bovis, B. bigemina* and *Theileria annulated***

| Number of vaccinated calf | Maximum PPE* (%) during (days) | | IFA** titer (log $_4$) to: | | |
|---|---|---|---|---|---|
| | *B. bovis* | *B. bigemina* | *B. bovis* | *B. bigemina* | *T. annulata* |
| 691 | <1 (2) | 0.2 (7) | 5 | 4 | 4 |
| 693 | Neg | <1 (6) | 4 | 3 | 4 |
| 694 | <1 (2) | 0.2 (5) | 5 | 5 | 5 |
| 699 | <1 (1) | <1 (6) | 5 | 5 | 3 |
| 700 | <1 (1) | <1 (6) | 5 | 4 | 4 |
| 701 | <1 (2) | <1 (6) | 5 | 5 | 3 |

**Table 13. Response of non-imminized calves served as control to challenge inoculation with virulent parasites (laboratory trial)**

| Control of challenge | No calves infected | Response | | | |
|---|---|---|---|---|---|
| | | Max. Fever | Decrease PCV (%) | PPE (%) | Drug-treated |
| ***B.bigemina*** | 2 | 39.2; 41.4 | 22.0; 35.4 | 1.0; 4.0 | 1 |
| **Mean** | | **40.3** | **28.7** | **2.5** | |
| *B. bovis* | 6 | 40.1; 40.5; 40.3; 39.4; 40.4; 40.2 | 21.0; 21.0; 27.0; 28.0; 47.8; 22.2 | 1,0; 1,0; 1,0; 1,0; 1,0; 1,0; | None |
| **Mean** | | **40.1** | **27.8** | **1.0** | |
| *Theileria annulata* | 6 | 39.1; 41.2; 39.2; 40.7; 41.0; 40.8 | 18.6; 19.0; 44.4; 11.4; 44.4; 48.7 | 1.0; 20; 1; 1; 20; 14 | 3 |
| **Mean** | | **40.3** | **31.0** | **9. 5** | |

**Table 14. Response of calves immunized with the trivalent vaccine under field conditions in Uzbekistan (n=38)**

| Clinical and serological response | |
|---|---|
| Fever recorded in number of calves | 0 |
| Mean PCV (%) on days<br>0, 9, 13, 16 | <br>34.0; 32.3; 30.5; 35.1 |
| Percent of calves showing parasitemia<br>*B.bigemina*<br>*Theileria annulata*<br>*B.bovis* | <br>31.5<br>26.3<br>0 |
| Percent seropositives before immunizaion<br>to : *B.bigemina*<br>*Theileria annulata*<br>*B.bovis* | <br>24.2<br>27.2<br>15.1 |
| Percent seroconverted after immunization<br>*B.bigemina*<br>*Theileria annulata*<br>*B.bovis* | <br>100<br>100<br>100 |

**Fig. 5. *Babesia bigemina* in cell culture (MASP), Giemsa stained smear**

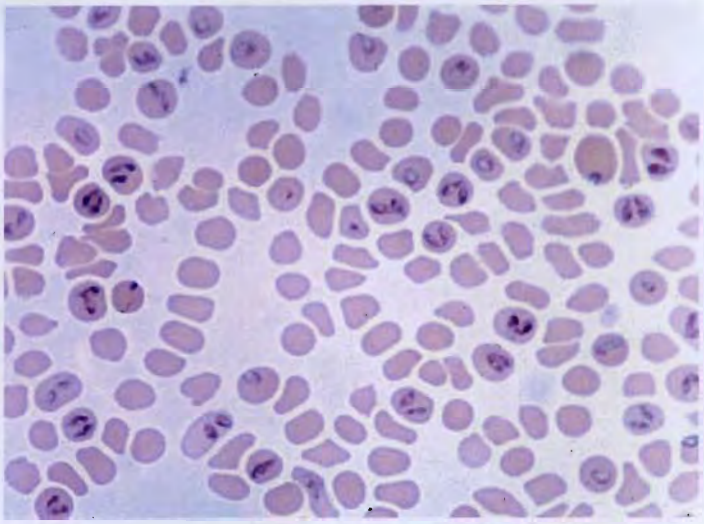
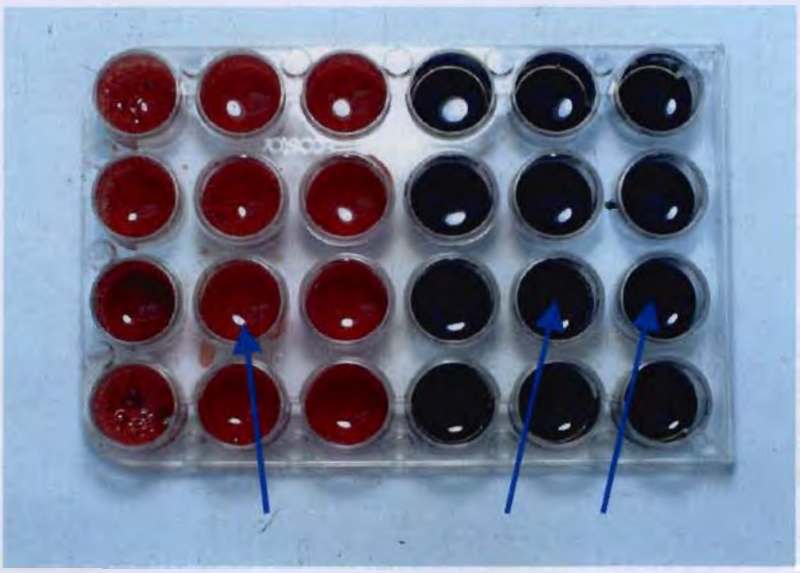


**Fig. 6. Growth of *Babesia* in 24 well culture plates**



**Negative culture** **Positive culture**



BEST AVAILABLE COPY

**Fig. 7. Propagation of Babesia MASP cultures in large culture flasks.**

**Fig. 8 . Frozen vaccines and diluents for *B. bigemina* and *B. bovis***

***B. bovis*** ***B. bigemina***



BEST AVAILABLE COPY

## F) IMPACT, RELEVANCE AND TECHNOLOGY TRANSFER

The project provided a unique opportunity to collaborate on bovine babesiosis, the major world's diseases of significant importance to livestock production, and Uzbekistan in particular. Prophylactic immunization is the most effective means to control tick-borne diseases. Research and development provided by the present grant t led to production of anti-*Babesia* vaccines, which can be used for mass vaccination of cattle within Uzbekistan, and further expanded to surrounding countries. The knowledge and technology transferred to the Uzbekistan scientists laid the basis for future research on tick-borne diseases highly endemic in Central Asia. The Uzbek scientists gained expertise in vital areas of handling infected ticks, step by step methodology for isolation of *Babesia* parasites form infected ticks or bovine hosts, red blood cell technology for in vitro cultivation of hemoparasites, splenectomy and attenuation processes either by "slow" or "fast" passages of infection in susceptible cattle, serological assay for monitoring antibody responses following vaccination. Intensive training was provided to a young scientist, Shavkat Abdirasulov, who revealed a considerable capability in applying laboratory techniques.
The project had a beneficial effect in improving the infrastructure of the laboratory, an autoclave, fluorescent microscope, refrigerated tabletop centrifuge, pH-meter, liquid nitrogen contained for vaccine storage and for field work, computer and FAX for better communication, vacuum pump, hematocrit centrifuge, balance, and this in addition to the equipment that was purchased in the previously funded CDR (TA-MOU-94-CA-14-040).

## G) Project Activities/Outputs

Over the course of the project, the collaboration, travel and training components consisted of working visits of the participants of the project in the collaborative laboratories. The PI from Israel, Varda Shkap and Mrs. L. Fish, visited the laboratory in Tashkent and performed field trials to test safety of the blood- and culture-derived anti-babesiosis vaccines. The Uzbeki counterparts, Prof. Ilhom Rasulov, Dr. Pack Den Sun (who was the Uzbeki PI initially, but left the Institute of Zoology later), Dr.

Shavkat Abdurasulov paid visits to the Bet Dagan laboratory in Israel for intensive training and performance of trials in cattle during attenuation, laboratory examination of vaccines for safety and potency. In Israel the Uzbek scientists were trained for *in vitro* cultivation of blood parasites, serology, and by themselves performed splenectomy. In 2004 Uzbek and Israle scientists attended the Symposium of the society of Tropical Medicine held in Vietnam, where a poster was presented on the development of anti-*B. bigemina* vaccine. The Abstract will be published in the Annals of New York Academy of Sciences. Following this presentation a manuscript has been accepted for publication in Veterinary Parasitology (peer- reviewed journal). In April 2007 our cooperative research will be presented at the Asian component of the Integrated Consortium on Tick and Tick-borne Diseases (ICTTD-3) to be held in Borstel, Germany.

**H) PROJECT PRODUCTIVITY**

The following are the publications:

1. Shkap, V., Rasulov, I., Abdurasulov, S., Fish, L., Leibovitz, B., Krigel, Y., Molad, T., Mazuz, L. M., Savitsky, I. (2005). *Babesia bigemina*: development of a live vaccine from an attenuated Central Asian isolate.
*Proceed. STVM, Hanoi, p.141 and N. Y Academy of Sciences (in press).*

2. Varda Shkap, I. Rasulov, L. Fish, S. Abdurasulov, B. Leibovitz, Y. Krigel, T. Molad, M. Mazuz and Savitsky I. (2007)
*Babesia bigemina*: development of live calf- and culture-derived vaccines from an attenuated Central Asian autochthonous isolate.
*(Vet Parasitology, in press)*,

The publications are attached to this report.

**FUTURE WORK**

Tick-borne diseases, babesiosis and theileriosis specifically, are considered as main economic problem in the cattle industry in Central Asia. It is expected that the anti-*Babesia* vaccines will be produced and applied in the field to reduce the economic losses in livestock. The same diseases affect cattle in neighbor to Uzbekistan countries, therefore it is also expected that the anti-tick-borne vaccines developed for

Uzbekistan will lay the basis for further developments in the countried of the Cetral Asia region, this considerably reduce the morbidity and the mortality rate in herds in the whole area. The infrastructure built by the support of USAID is adequate to perform cooperative research with the institutions in Uzbekistan primarily, and to expand to Kazakhstan. The Israeli team will provide any assistance to promote practical application of the vaccines developed. In addition to the practical aspects, the knowledge about antigenic and genetic characteristics of strains present in the regions is extremely scarce. Unfortunately, there are difficulties to obtain indispensable equipment and reagents produced abroad, therefore it is impossible to predict the possibilities and the amount of doses that can be produced after the project is finished.

## I) LITERATURE CITED

Bock, R., and DeVos, A. 2001. Immunity following use of Australian tick fever vaccine: a review of the evidence. Aust. Vet. J., 79, 12, 832-839.

DeVos, A., 1978. Immunogenicity and pathogenicity of three South African strains of *Babesia bovis* in *Bos indicus* cattle. Onderstepoort J. Vet. Res., 45, 119-124.

Callow,L., Dalgliesh, R. 1980. The development of effective, safe vaccination against babesiosis and anaplasmosis in Australia. In: Johnston, L., Cooper, M (Ed)., Tick and Tick Borne Diseases. Proceed., 56th Annual Conference of the Australian Veterinary Association, Townsville, pp. 4-8.

Dalgliesh, R.J., et al., Development of a highly infective *Babesia bigemina* vaccine of reduced virulence. Australian Veterinary Journal, 1981. 57(1): p. 8-11.

Figueroa, A., Chievers, L., Johnson, G., Buening, G., 1992. Detection of *Babesia bigemina*-infected carriers by polymerase chain reaction amplification. J. Clin. Microbiol. 30, 2576-2582.

Goldman, M., Pipano, E., Rosenberg, A., 1972. Fluorescent antibody test to *Babesia bigemina* and *B. berbera*. Res. Vet. Sci. 3, 77-81.

Lery, M and Ristic, M. 1980. *Babeisia bovis*: continues cultivation in a microaerophilous stationary phase culture. Science 207, 1218-1220.

Suarez, C., McElwain, T., Stephens, E., Mishra, V., Palmer G. 1991. Sequence conservation among merozoite apical complex proteins of *Babesia bovis* and *Babesia bigemina* and other apicomplexa. Mol. Biochem., Parasitol., 49, 329-332.

**Abstract of the Poster presented at the STVM Symposium**

## *Babesia bigemina*: Development of a Live Vaccine from an Attenuated Central Asian Isolate

**Shkap Varda**[1], I. Rasulov[2], S. Abdurasulov[2], L. Fish[1], B. Leibovitz[1], Y. Krigel[1], T. Molad, M. Mazuz, I. Savitsky[1]
[1]Kimron Veterinary Institute, Bet Dagan Israel
[2]Institute of Zoology, Tashkent, Uzbekistan

Babesiosis, caused by *Babesia bigemina*, is recognized as one of the most important cattle diseases in Central Asian countries. Splenectomized calves were infested with larvae hatched from adult *Boophilus annulatus* ticks harvested from a cow with clinical babesiosis, from an enzootic area in Uzbekistan. PCR assays with specific primers applied to the ticks confirmed *Babesia bigemina* infection. Calf- and culture-derived live vaccines based on an Uzbeki *B. bigemina* field strain were developed from attenuated parasites obtained by "slow" passages in splenectomized calves, or from *in vitro* subcultured parasites initiated after partial attenuation in calves. Based on clinical and parasitological parameters in calves inoculated during the attenuation process, an experimental calf-derived vaccine was produced after five passages in animals or from culture-derived vaccine after eight passages in culture. Frozen calf- and culture-derived vaccines were both tested for safety under laboratory and field conditions in Uzbekistan and in Israel. Complete protection was attained upon challenge of immunized calves with a homologous virulent strain. It can be concluded that, similarly to *B. bigemina* strains from other geographical areas, autochthonous Central Asia strains can be attenuated *in vivo* or by *in vitro* cultivation, for use as a safe and efficacious vaccine.

*Babesia bigemina*: attenuation of an Uzbek isolate for immunization of cattle with live calf- or culture-derived parasites

Varda Shkap[2*], I. Rasulov[1], S. Abdurasulov[1], L. Fish[2], B. Leibovitz[2], Y. Krigel[2], T. Molad[2], M. L. Mazuz[2], I. Savitsky[2].

[1]Institute of Zoology, 1, Niazov St., Tashkent, Uzbekistan
[2]Kimron Veterinary Institute, P.O. Box 12, Bet Dagan, 50250, Israel

*Author to whom correspondence should be sent:
Division of Parasitology, Kimron Veterinary Institute, P.O. Box 12, Bet Dagan 50250, Israel; Tel: 972-3-9681758; Fax: 972-3-9681678; Email: shkapv@int.gov.il

**Abstract**

The virulence of an Uzbek isolate of *Babesia bigemina,* obtained from infected *Boophilus annulatus* ticks from an endemic area in Uzbekistan, was attenuated for immunization of cattle with autochthonous calf- or culture-derived parasites in Uzbekistan. After four "slow passages" *in vivo* the virulence was reduced, as evidenced by the response of calves inoculated with an experimental live frozen vaccine produced from the following passage. The vaccine was safe and protective against homologous virulent challenge under laboratory conditions. The culture-derived experimental vaccine was produced from cultures initiated after three passages *in vivo* followed by 22 passages *in vitro.* The cultured parasites did not elicit any clinical sign, but inoculated calves seroconverted following vaccination and were protected against the virulent homologous challenge. Both calf- and culture-derived vaccines were safe for cattle grazing in an endemic area in Uzbekistan. Despite the high polymorphism of *B. bigemina*, as reported from various geographical regions, the Central Asian strain was attenuated similarly to those that form the basis of the existing live *B. bigemina* vaccines in other parts of the world.

*Key words*: *Babesia bigemina*, autochthonous, calf-derived, culture-derived, vaccine, Central Asia

## 1. Introduction

*Babesia bigemina*, which causes bovine babesiosis, is an intraerythrocytic protozoan parasite that, in the Central Asia and Mediterranean region, is transmitted by a one-host tick, *Boophilus annulatus*. The disease is one of the most important cattle diseases in Central Asia, where it poses a major threat to the livestock industry. It is well established that protection against babesiosis can be achieved by vaccination of cattle, which thus prevents losses caused by the disease. The vaccines that are presently employed in Australia, South Africa, several countries of South America, and Israel (DeVos et al., 1979; Jorgensen et al., 1989; 1992; Echaide et al., 1993; Mangold et al., 1996; Callow et al., 1997; Shkap et al., 2005) consist of bovine blood containing attenuated parasites. However, by no means all of these attenuated parasites are completely avirulent. It is generally known that, because of the wide diversity of *B. bigemina* in different geographical regions, it would be preferable to use autochthonous strains to obtain a highly potent vaccine. In addition, introduction of new exotic strains, or new genetic/antigenic variants into a geographical area might present a serious risk for the severity of the disease status in the field. The calf-derived vaccines were developed by "slow" passages *in vivo* in intact calves (Dalgliesh et al., 1981), they were shown to be highly effective, but have the potential of transmitting latent pathogens if testing is insufficiently stringent (DeVos and Jorgensen, 1996). Following the introduction of the *in vitro* culture technology for *Babesia bigemina* (Vega et al., 1985), culture-derived vaccines, which present minimal risk of the inadvertent spread of pathogens, were adopted, and showed immunogenicity comparable with that of calf-derived vaccines (Jorgensen et al., 1989; Echaide et al., 1993; Shkap and Pipano, 2000). In countries where there is no possibility of maintaining cattle under highly stringent conditions, vaccines derived from an *in vitro* culture might be a suitable alternative source for parasites to be used for immunization.

In the present study a *B. bigemina* strain originating from Uzbekistan was attenuated after five "slow passages" *in vivo*. Experimental frozen calf- and culture-derived vaccines, prepared from attenuated autochthonous parasites were found safe for vaccination field cattle in Uzbekistan. Although no field challenge with virulent parasites was feasible, both, calf- and culture-derived vaccines were shown to provide protection against virulent homologous challenge under laboratory conditions.

## 2. Materials and methods

## 2.1. *Cattle and parasites*

A total of 23 calves were used in the study for isolation, attenuation of the Uzbek *B. bigemina* isolate, and for laboratory trials, in Israel, of experimental calf- or donor-derived vaccines. An additional 30 calves were immunized with the same vaccines on a farm in Uzbekistan. The 23 calves were Friesians; they were acquired from a dairy herd maintained on a zero-grazing regime, and were further raised under tick-free conditions at the Kimron Veterinary Institute. The 5- to 6-month-old calves, which had been shown to be serologically negative by the indirect fluorescent antibody (IFA) test for *B. bigemina*, were used for "slow" passages of *B. bigemina* according to Dalgliesh et al. (1981). The Uzbek isolate was obtained by infestation of three naïve calves with larvae hatched from adult *Boophilus annulatus* ticks that had been harvested from a cow with clinical *B. bigemina* babesiosis in Uzbekistan. Only one out of the three calves developed parasitemia. At 1% parasitemia, 15 ml of blood was drawn and inoculated subcutaneously into a naïve calf. Six weeks after recovery the calf was splenectomized, and when *B. bigemina* reappeared 5-8 ml of its blood were inoculated subcutaneously into another naïve calf. Five "slow" passages were performed in eight calves (Table 1). After the 5$^{th}$ passage 50 ml of blood were inoculated into a splenectomized calf to produce recrudescent parasitemia.

## *2.2. Preparation of vaccines*

Blood was drawn into a 0.4% sodium citrate solution and mixed with phosphate buffered saline (PBS) and dimethyl sulfoxide (DMSO) to a final concentration of 15% (Shkap et al., 2005). Each dose of the frozen calf-derived vaccine contained $2 \times 10^8$ infected erythrocytes in a volume of 2 ml. Microaerophilous stationary phase cultures (MASP) of *B. bigemina* were initiated from a calf at the third *in vivo* passage, when parasitemia was 0.2% according to Vega et al. (1985). The subcultures were performed every 72 h and after 22 *in vitro* passages, with the cultured parasites at 10% parasitemia in the Giemsa-stained smears, their pathogenicity was determined by inoculating them into two naïve calves. As no clinical babesiosis was elicited following inoculation (data not shown) a culture-derived experimental vaccine was prepared from parasites propagated in large (225 cm$^2$) cell culture flasks (Costar, USA). Each dose contained $4 \times 10^8$ infected erythrocytes in a volume of 2 ml that was frozen with DMSO at a final concentration

of 15%. Both calf- and culture-derived vaccines were kept in liquid nitrogen pending use. The vaccines based on the Uzbek *B. bigemina* isolate were tested for bacterial and viral contaminants according to the requirements of the Unit for Testing of Vaccines at the Veterinary Services, in a procedure similar to that used for testing the conventional vaccines produced by the Kimron Veterinary Institute (Pipano et al., 1986). Before inoculation the vaccines were thawed at 40 °C and immediately transferred into PBS containing 15% DMSO for inoculation.

### *2.3. Vaccination and challenge*

A total of eight Friesian bull calves, aged 5-6 months, were used in a laboratory trial. Four of them were inoculated subcutaneously with the thawed calf-derived vaccine, and the remaining four with culture-derived parasites. The follow-up of the calves included daily recording of body temperature, examination of blood smears and packed cell volume (PCV), for 12 days post immunization. PCV depression was calculated according to DeVos (1979). To test the potency of the vaccines, the calves were challenged, 6 months after immunization, with $2 \times 10^8$ homologous virulent parasites obtained from a calf infected with the second *in vivo* passage. Three non-immunized calves received the challenge inocula and served as control. Animals exhibiting fever above 40° C for more than 2 days, PCV below 20% and parasitemia exceeding 2% were treated with 3.5 mg/kg of diminazene aceturate (Berenil, Hoechst, Germany).

To test their safety for use in the locally bred cattle, the same vaccines were inoculated into twenty-four 6- 8-month-old calves in Uzbekistan, and the animals were then monitored for responses for one month. Six additional calves that had not been immunized served as controls. Challenge with virulent parasites was not feasible under field conditions.

### *2.4. Serology*

Antibodies specific to *B. bigemina* were assessed in the calves, before and after immunization, by the indirect immunofluorescence antibody test (IFA), with blood slide antigen prepared from the Uzbek isolate according to Goldman et al. (1972).

#### *2.5. Nested polymerase chain reaction (nPCR)*

To ensure freedom of the *B. bigemina* Uzbek strain from *B. bovis*, a PCR reaction with previously described primers (Shkap et al., 2005) was used. The primers for *B.*

*bigemia* were based on published sequences (GenBank accession number S45366, Figueroa et al., 1992). Primers for *B. bovis* were also based on published sequences (GenBank accession number M138218, Suarez et al., 1991). The PCR amplified products were subjected to electrophoresis on 1.5% agarose gel and visualized by ethidium bromide staining.

## 3. Results

Nested PCR analysis of blood samples obtained from Uzbekistan revealed a fragment of the expected size of 170 base pairs (Fig. 1, lines 3 and 4). Amplification was not obtained when the same *B. bigemina* specific primers were used to test DNA extracted from erythrocytes infected with *B. bovis* (Fig. 1, lane 5). No amplification was obtained when the *B. bovis*-specific primers were applied to DNA isolated from cattle infected with the Uzbek *B. bigemina* isolate (not shown), indicating that the source of the parasites used to initiate attenuation was not contaminated with *B. bovis*. The purity of the infection was also confirmed by propagation of *B. bigemina* in MASP culture (Vega et al., 1985; Echaide et al., 1993): after serial passages *in vitro*, no parasites other than *B. bigemina* were detected in the blood smears made from the culture. As shown in Table 1, the four calves in which the first two passages were performed responded with elevated fever up to 41.0°C, compared with the normal body temperature of 39.5°C. The minimum PCV ranged from 12 to 20%, and parasitemia, although relatively low at the 1st passage (4 and 6%), was significantly higher at next passage (15 and 28%). These calves recovered without drug treatment.

Following inoculation of parasites from passage 3, one calf developed fever of 40.2°C and relatively low PCV (21%), whereas the second one showed slightly elevated body temperature (39.7°C) and minimum PCV of 28%. The blood smears from both calves showed low parasite counts of 0.1 and 0.2%. In the calves that received parasites from passages 4 and 5 no fever was recorded, PCV values were normal, and parasitemia was less than 0.01%. It appears that after four "slow" passages the Uzbek isolate became attenuated. To ensure safety, an experimental vaccine was prepared from the 5th passage 23% recrudescent parasitemia in a splenectomized calf, with packed cell volume of 35%.

Following immunization (Table 2) with calf-derived parasites there was slightly elevated group mean fever of 39.9 °C, the mean PCV depression 27.2%, the mean

parasitemia of <0.01%, and none of the vaccinated animals required drug treatment. Three weeks after vaccination all the animals developed specific *B.bigemina* IFA antibody titers. Following immunization with cultured parasites none of the calves developed a clinical response, but all seroconverted. Upon challenge, 6 months after immunization (Table 3) all the vaccinated calves were protected against the virulent challenge. None of the calves immunized with calf-derived parasites developed fever, there was low PCV depression (17.7%) and low parasitemia (< 0.01%). Specific IFA antibodies were detected 3 weeks after immunization with titer of 3.5 ($\log_4$). The three control animals given the virulent challenge inocula responded with high fever (mean of 40.9°C), 54% PCV depression, and 17.5 % parasitemia; two out of three control non-immunized calves received drug treatment to prevent death.

As shown in Table 4, the locally bred cattle in Uzbekistan responded to the calf-derived vaccine with slightly elevated mean group fever (39.9°C), with group minimum PCV of 26%, and 0.3% parasitemia. Fourteen calves that received cultured *B. bigemina* did not develop fever; their PCV was in the normal range and negligible parasitemia was recorded (<0.01%). As expected there was no fever and no parasites were detected during a month of observations. Specific antibody titers developed only in the vaccinated calves, and ranged from 2.75 to 3.6 ($\log_4$) dilutions of serum samples. Antibody titers observed in the control group at 1:4 and 1:16 dilutions are considered non-specific (Shkap et al., 2005).

## 4. Discussion

Safe and potent calf- or culture-derived frozen anti-*B. bigemina* vaccines, based on an autochthonous Uzbek isolate were developed by "slow" passages in calves, according to Dalgliesh et al. (1981), or by subculturing in MASP cultures. Despite the high polymorphism of *B. bigemina* parasites that has been reported from several geographically distinct regions (McElwain et al., 1992; Palmer and McElwain, 1995; Fisher et al., 2001), the Uzbek strain was attenuated similarly to the parasite strains from which vaccines used in Israel, Australia and Argentina are derived (Echaide et al., 1993; Bock and DeVos, 2001; Shkap et al., 2005). It seems that the initial state of virulence determines the attenuation process: for the Australian *B. bigemina* vaccine, reduced virulence was achieved after a series of seven "slow" passages in calves (Dalgliesh, 1981), whereas with the Israeli (Pipano, 1995) and the Uzbek isolate it

was obtained after four passages. Relatively mild responses were recorded in the immunized calves; there was only transient fever of short duration, insignificant PCV reduction, no high parasitemia developed, and none of the inoculated calves required drug treatment. Although effective and satisfactory, the calf-derived vaccines are difficult to produce, because of the highly stringent conditions required to maintain the vaccine donor calves (DeVos, 1979; Jorgensen et al., 1992; DeWaal, 1996). The calf-derived vaccine has serious drawbacks, including the potential for contamination with silent pathogens that might be present in the blood of an infected donor (Rogers et al., 1988; De Waal and Combrick, 2006) and limitations of use for animals older than 8-9 months. The donor animals require thorough examination for possible contaminants for either calf-, or culture-derived vaccine production. Although the *Babesia* cultures require constant supply of fresh bovine erythrocytes and serum, these are obtained from one or two spleen-intact cows, whereas for each batch of calf-derived vaccine a number of splenectomized calves need to be maintained. The culture-derived vaccines might have the advantage of a better quality assurance, while exhibiting immunogenicity comparable with that of the calf-derived vaccine (Jorgensen et al., 1989; Mangold et al., 1996). On the other hand, production of frozen vaccines enables thorough post - production testing for contaminants, irrespective of the vaccine source, donor calves or cultures. The preparation of culture-derived vaccines requires specific blood culture technology and laboratory skills, but in countries where calf-derived vaccine production is not feasible, the cultured parasites present an alternative. According to the OIE standards for vaccines, culture-derived vaccines are of high cost production, and possible antigenic drift during long-term maintenance in culture, make mass culture of *Babesia* impractical in most laboratories (Bock et al., 2004b). However, in Argentina 300,000 doses of culture-derived vaccines against *B. bigemina* and *B. bovis* are produced annually, and successfully applied in the field (Mangold et al., 1996; Echaide, 2005, personal communication). In Israel, the use of culture-derived vaccine has been suggested for vaccination of sensitive older cattle, by priming them with avirulent cultured parasites followed by immunization with the conventional donor-derived vaccine (Shkap et al., 2005). As shown in the present study, vaccine, whether produced from a donor calf or by propagation in culture, offered complete protection from clinical babesiosis upon challenge with virulent homologous parasites, therefore, both were found to be equally safe and effective for use in Uzbekistan. They offer the added advantage of

feasibility of production of frozen vaccines such as are routinely used in South Africa, Australia, Argentina and Israel (Echaide et al., 1993; DeWaal, 1996; Bock and DeVos, 2001; Shkap et al., 2005). Despite the high polymorphism of the *B. bigemina* merozoite antigens (Brown and Palmer, 1999; Fisher et al., 2001; Precigout et al., 2006), the G strain attenuated in Australia has been shown to be protective in other parts of the world including South Africa, Zimbabwe, and South-East Asian and Central American countries (Bock et al., 2004a). However, except for S. Africa, the performance of the G strain vaccine for routine field vaccination in other countries is not documented. Whether the anti-*B. bigemina* vaccine based on the Uzbek isolate might be used for protection of cattle in the neighboring countries needs to be examined and considered yet.

**Acknowledgments**

This work was supported by the AID CDR grant TA-MOU-CA-021-013.

## References


Bock, R., De Vos, A., 2001. Immunity following use of Australian tick fever vaccine: a review of the evidence. Aust. Vet. J. 79, 832-839.

Bock, R., Jackson, L., DeVos, A., Jorgensen, W. 2004a. Babesiosis of cattle. Parasitology, 129, S247-269.

Bock, R, Jorgensen, W.K., Molloy, J.B. 2004b. Bovine babesiosis, In: Manual of standards for diagnostic tests and vaccines for terrestrial animals. Office International des Épizooties. http://www.oie.int/eng/normes/mmanual/A_00059.htm, Paris, pp. 507-518.

Brown, W., Palmer, G. 1999. Designing blood-stage vaccines against *Babesia bovis* and *B. bigemina*. Parasitol. Today 15, 275-281.

Callow, L., Dalgliesh, R., DeVos, A., 1997. Development of effective living vaccines against bovine babesiosis – the longest field trial? Int. J. Parasitol. 27, 747-767.

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

**DeVos, A. 1979. Epidemiology and control of bovine babesiosis in South Africa. In: Soulsy, E. (Ed.), Immune Responses in Parasitic Infections: Immunology, Immunopathology, and Immunoprophylaxis. III. Protozoa. CRC Press, Boca Raton, FL., pp. 183-222.**

DeVos, A., Jorgensen., W., 1996. Bovine babesiosis. In: Manual of Standards for Diagnostic Tests and Vaccines. Office International des Epizootie, 3rd Edit. Paris. pp. 333-338.

DeWaal, D., 1996. Vaccination against babesiosis. Acta Parasit. Turcica. 20, 487-499.

De Waal, D., Combrink, M., 2006. Live vaccines against bovine babesiosis. Vet. Parasitol., 138, 88-96.

Echaide, I., DeEchaide, S., Guglielmone, A., 1993. Live and soluble antigens for cattle protection to *Babesia bigemina*. Vet. Parasitol. 51, 35-40.

Echaide, I., 2005. Personal communication.

Figueroa, A., Chievers, L., Johnson, G., Buening, G., 1992. Detection of *Babesia bigemina*-infected carriers by polymerase chain reaction amplification. J. Clin. Microbiol. 30, 2576-2582.

Fisher, T., McElwain, T., Palmer, G., 2001. Molecular basis for variable expression of merozoite surface antigen gp45 among American isolates of *Babesia bigemina*. Infect. Immun. 69, 3782-3790.

Goldman, M., Pipano, E., Rosenberg, A., 1972. Fluorescent antibody test to *Babesia bigemina* and *B. berbera*. Res. Vet. Sci. 3, 77-81.

Jorgensen, W., De Vos, A., Dalgliesh, R., 1989. Comparison of immunogenicity and virulence between *Babesia bigemina* parasites from continuous culture and from splenectomized calf. Aust. Vet. J., 66, 371-372.

Jorgensen, W., Waldon, S., McGrath, J., Roman, R., DeVos, A., Williams, K., 1992. Growth of *Babesia bigemina* parasites in suspension cultures for vaccine production. Parasitol. Res. 78, 423-426.

Mangold, A., Vanzini, V., Echaide, I., DeEchaide, S., Volponi, M., Guglielmone, A., 1996. Viability after thawing and dilution of simultaneously cryopreserved vaccinal *Babesia bovis* and *Babesia bigemina* strains cultured *in vitro*. Vet. Parasitol. 61, 345-348.

McElwain, T., Misra, V., Stephens, E., 1992. Development of an antigenically defined vaccine against *Babesia bigemina*. In: T.T. Dolan, (Ed), Recent Developments in the Control of Anaplasmosis, Babesiosis and Cowdriosis, Proc. Workshop, ILRAD, Nairobi, Kenya, May 1991, pp. 105-112.

Palmer, G., McElwain, T., 1995. Molecular basis for vaccine development against anaplasmosis and babesiosis. Vet Parasitol. 57, 233-253.

Pipano, E., Krigel., Y., Frank, M., Marcovics, A., Mayer, E. 1986. Frozen *Anaplasma centrale* vaccine against anaplasmosis in cattle. Br. Vet. J., 142, 553-556.

Pipano, E. 1995. Live vaccines against hemoparasitic diseases in livestock. Vet. Parasitol., 213-231.

Precigout, C., Schetters, E. Gorenflot, A. 2006. Genetic basis for GPI-anchor merozoite surface antigen polymorphism of *Babesia* and resulting antigenic diversity. Vet. Parasitol. 138, 33-49.

Rogers, R., Dimmock, C., DeVos, A., Rodwell, B., 1988. Bovine leukosis virus contamination of a vaccine produced *in vivo* against bovine babesiosis and anaplasmosis. Aust. Vet. J. 65, 285-287.

Shkap, V., Pipano, E., 2000. Culture-derived parasites in vaccination of cattle against tick-borne diseases. In: J. House, K. Kocan, P. Gibbs (Eds), Tropical Veterinary Diseases: Control and Prevention in the Context of the New World Order. Ann. N.Y. Acad. Sci. 916, 154-171.

Shkap, V., Leibovitz, B., Krigel, Y., Hammerschlag, J., Marcovitcs, A., Fish, L., Molad, T., Savitsky, I., Mazuz, M., 2005. Vaccination of older *Bos taurus* bulls against bovine babesiosis. Vet. Parasitol. 129, 235-242.

Suarez, C., McElwain, T., Stephens, E., Mishra, V., Palmer G. 1991. Sequence conservation among merozoite apical complex proteins of *Babesia bovis* and *Babesia bigemina* and other apicomplexa. Mol. Biochem., Parasitol., 49, 329-332.

Vega, C., Buening, G., Green, T., Carson, C., 1985. *In vitro* cultivation of *Babesia bigemina*. Am. J. Vet. Res. 46, 416-420.

**Table 1**

Clinical response of naïve calves during attenuation of the Uzbek isolate of *B. bigemina* by "slow" passages

| Calf number | Passage Number | Prepatent period (days) | Max fever (°C) | Min PCV* (%) | Max PPE** |
|---|---|---|---|---|---|
| 559 | 1 | 4 | 40.6 | 12 | 6 |
| 569 | 1 | 4 | 40.1 | 20 | 4 |
| 563 | 2 | 4 | 41.0 | 17 | 28 |
| 575 | 2 | 4 | 40.1 | 13 | 15 |
| 570 | 3 | 5 | 40.2 | 21 | 0.1 |
| 585 | 3 | 6 | 39.7 | 28 | 0.2 |
| 597 | 4 | 5 | 39.3 | 28 | < 0.001 |
| 602 | 5 | 4 | 39.4 | 32 | < 0001 |

Minimum percent packed cell volume *

** Maximum percent parasitized erythrocytes

**Table 2**

Response of calves to immunization with calf- or culture-derived *B. bigemina* (n = 4 per group; mean values in a laboratory trials)

| **Response parameters** | **Type of vaccine inoculated:** | |
|---|---|---|
| | Calf-derived | Culture-derived |
| Prepatent period (days) | 6.7 | 8.0 |
| Max. body temp (°C) | 39.9 | 39.3 |
| PCV* depression (%) | 27.2 | 14.0 |
| Max parasitemia (%) | <0.01 | <0.01 |
| Drug treatment | 0/4 | 0/4 |
| IFA** antibodies ($\log_4$) | 3.0 | 3.25 |

Packed cell volume *
Immunofluorescent antibody assay **

**Table 3**

Response to virulent challenge of calves immunized with calf- or culture-derived *B. bigemina* (mean values in a laboratory trials)

| Calves immunized with parasites: | Response to challenge | | | | |
|---|---|---|---|---|---|
| | **Prepatent period (days)** | **Max body temp (°C)** | **PCV* depression (%)** | **Maximum PPE** (%)** | **Drug Treatment** |
| Calf-derived (n=4) | 6.5 | 39.2 | 17.7 | <0.01 | 0 |
| Culture-derived (n=4) | 8.0 | 38.6 | 23.0 | <0.01 | 0 |
| Non-immunized (n=3) | 4.0 | 40.9 | 54.0 | 7.5 | 2 |

* Packed cell volume

Table 4

**Response of Friesian calves to immunization with calf- or culture-derived Uzbek *B. bigemina* vaccine in a field trial (group mean values)**

| Calves immunized with parasites | Response to immunization | | | | |
|---|---|---|---|---|---|
| | **Prepatent period (days)** | **Max. body temp (°C)** | *** PCV depression (%)** | **Max. **PPE (%)** | **IFA titer ($log^4$)** |
| **Calf-derived (n=10)** | **8** | **39.9** | **18.7** | **0.3** | **3.6** |
| **Culture-derived (n=14)** | **9** | **38.5** | **20.8** | **<0.01** | **2.75** |
| **Non-immunized (n=6)** | **0** | **38.7** | **21.2** | **0** | **<0.1** |

* Packed cell volume

** Percent parasitized erythrocytes

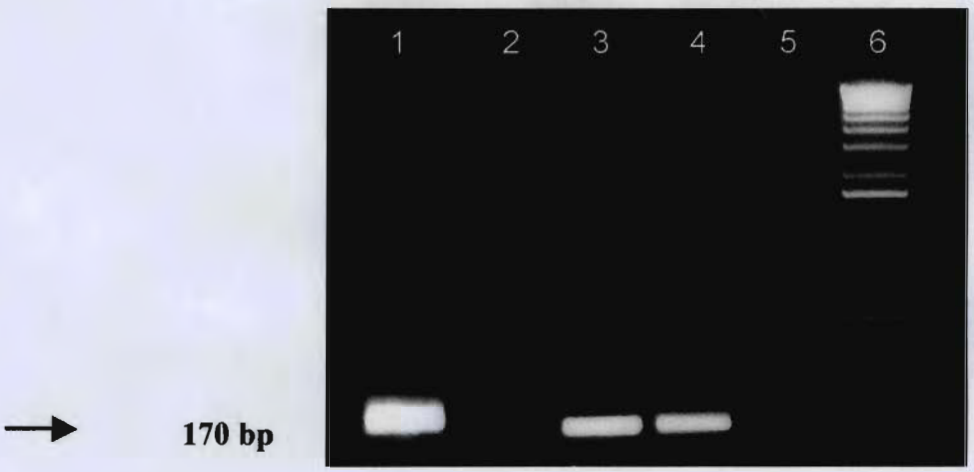



**Figure** 1

Nested PCR analysis for *B. bigemina* in blood of infected cattle.

Lane 1, positive control for *B. bigemina*; lane 2, no DNA template;

lanes 3 and 4, DNA from blood of cattle infected with the Uzbek isolate;

lane 5, DNA of *B. bovis*; lane 6, 1-Kbp marker



BEST AVAILABLE COPY