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"VACCINE AGAINST BOVINE BABESIOSIS IN CENTRAL ASIA"

COVERING THE PERIOD FROM
JANUARY 2003 TO JANUARY 2004

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EXECUTIVE SUMMARY

Developmental: The objective is to isolate and attenuate *Babesia bigemina* and *B. bovis* parasites for the development of an effective live vaccines against bovine babesiosis, a major and economically important disease, which constitutes a significant limiting factor for cattle development in vast tropical and subtropical regions of the world, including Central Asia and Israel.

Scientific: The objective is to isolate and attenuate *Babesia* to develop an effective live anti-babesiosis vaccine based on autochthonous strains of parasites.

Babesiosis is a serious disease of cattle, caused by *Babesia bovis* and *Babesia bigemina*, protozoan parasites transmitted by ticks of the genus *Boophilus*. The vector tick, *Boophilus annulatus*, is entirely a tropical and subtropical ectoparasite. The natural transmission of bovine babesiosis is known to occur during warm seasons under field conditions that include a fair amount of shade and moderate humidity. Central Asian countries and Israel have subtropical climates, and exhibit all the conditions for survival of the vector tick. Babesiosis can be acute and fatal, but upon recovery from acute infection cattle might develop a chronic subclinical infection, so becoming carrier animals that serve as reservoir for infection of ticks, which further transmit the infection when they feed on susceptible cattle. The most effective control of babesiosis is based on prophylactic immunization of cattle with vaccines containing attenuated live parasites, which results in the development of protective immunity against infected ticks. Live vaccines based on attenuated strains are used effectively to control bovine babesiosis in several affected countries, including Israel. Transfer of knowledge for the development of anti-babesiosis live vaccines is one of the major components of the project.

After two years of the project, we are now in the position of having attained one of the important and fundamental goals of the project – isolation of indigenous strains of *B. bigemina* and *B. bovis* from Uzbekistan. We have attenuated *B. Bigemina*, produced an experimental vaccine based on a donor-derived autochthonous strain, and successfully tested it under laboratory and semi-field conditions. The culture-derived *B. bigemina* vaccine is under examination for safety and immunogenicity.
During the early part of the second year, Prof. Rasulov spent 6 weeks at the Kimron Veterinary Institute, in the Division of Parasitology. During this period he, together with two veterinarians, Dr. Y. Krigel and Dr. B. Leibovitz, was actively involved in the attenuation of *B. bigemina* through passages in cattle.

Antigens for serological testing (IFA) of serum samples from immunized cattle were prepared from *BgUz*. These antigens were then transferred to the Tashkent laboratory.

After *B. bigemina* has been established in MASP cultures, they were transferred to Uzbekistan, where Dr. Varda Shkap and Mrs. Lea Fish started growing and maintaining the cultures in the Tashkent laboratory. According to the cooperating Uzbeki scientists, no continuing growth of *B. bigemina* in culture could be achieved; the cultures died out after about three passages. These cultures are successfully maintained in the KVI laboratory, therefore more attempts will be made to continue the MASP cultures, since they should replace bovine donors for vaccine production.

During their visit, Dr Shkap and Mrs Fish collected blood samples from farms in which babesiosis was endemic, for serological screening and for testing with PCR methodology.

During the last half-year, two Uzbeki scientists, Prof. Rasulov and Dr. Shavkat Abdurasulov, a young scientist from the Tashkent laboratory, spent a month in the Division of Parasitology at KVI. During their visit, they were involved in experimental immunization and follow-up, and monitoring the inoculated cattle for reactivity. Dr. Shavkat Abdurasulov was specifically trained in performing splenectomy operations on calves, and serology (IFA testing) for detection of anti- *Babesia* and anti- *Theileria* antibodies. The visiting scientists were involved in both laboratory and semi-field vaccination trials, and they received training in growing *B. bigemina* in MASP cultures, in order to continue the experiments in Tashkent. In each case, training was a necessary preparation for the further work.
SECTION I

A) RESEARCH OBJECTIVES IN YEAR UNDER REVIEW:

During the second year of the program the main activity was focused on:

- Attenuation of the Uzbeki *B. bigemina* strain (designated *BgUz*) by "slow passages" in cattle;
- Cultivation of *BgUz* in MASP culture;
- Preparation of small batches of experimental *BgUz* vaccines containing blood- or culture-derived parasites;
- Testing for safety and immunogenicity of the experimental vaccines for cattle under laboratory conditions. Immunization experiments were conducted in highly susceptible dairy cattle on a tick-free farm.

B) RESEARCH ACCOMPLISHMENTS

Response 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. 1 and in Table 1, 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-babesicidal drugs. However, at a further passage in the splenectomized animal, the calf exhibited acute clinical babesiosis of which the parameters necessitated drug treatment to prevent the death of the animal. At this stage, no further passages were performed. To preserve the blood-derived isolate, infected blood was drawn from calves #576 and #600 and frozen in liquid nitrogen pending use.
B. bigemina parasites 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 1, 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 (#602). At passage 5, as at passage 4, a moderate clinical response was observed: negligible fever, about 30% PCV drop, 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% PCV drop, 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 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 at 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 ten doses (concentrated in a total of 3.6 mls), so when diluted in a cryopreservative, each dose of 2 ml contained 3.6 x 10⁸ parasites. The vaccine was immediately frozen, first in vapor and, after about 1 h, in liquid nitrogen.

The calf from which the vaccine was 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 from 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.

Testing of the experimental vaccine for safety and immunogenicity

The laboratory trial included 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 4x10^8 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 minutes after the blood was thawed.

As shown in Table 2, of the four calves 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 x 10^{11}. Thus at parasitemia of 0.5%, each calf received about 2x10^9 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.
The response of the vaccinated calves to challenge is presented in Table 3. None of the four vaccinates 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, which breeds Friesian cattle 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 3). 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 (#624); four other calves showed *B. bigemina* parasitemia of 0.2-0.5%. None of the vaccinates required babesicidal treatment.

Isolation, propagation and maintenance of *Babesia bigemina* in MASP culture.

After several unsuccessful attempts, we have finally succeeded in growing and maintaining the Uzbeki *B. bigemina* strain in MASP cultures (Fig. 2). *B. bigemina* isolated from an infected calf C585 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 x 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 (1:1; 1:2 and 1:3), at a final volume of 0.8 ml per well. *B. bigemina* were detected in culture after one blind passage, 48 h after seeding. At 72 h parasitemia reached 4% (Fig. 2). The MASP cultures were grown in an incubator in an atmosphere of 2% O₂, 5% CO₂, 93% N₂. For further maintenance, subcultures were performed every 72 h, and when parasitemia reached 8%, frozen stabilates were prepared in DMSO at a final concentration of 15% and kept in liquid nitrogen.
As a further stage of preparation for production of \( B. \textit{bigemina} \) vaccine from MASP culture, an experimental batch of 110 doses was prepared for safety and immunogenicity testing, by inoculation into calves.

The \( B. \textit{bigemina} \) MASP cultures were transferred to the Tashkent laboratory, where no cultures could be initiated during our 2-week visit. After subculturing for only 1 or 2 passages, no multiplication was observed.

**Fig.1. Transmission and attenuation of the Uzbeki \( B. \textit{bigemina} \) strain**
Table 1.
Responses of naive and splenectomized calves inoculated with “slow” passaged
*B. bigemina* originating from tick infection or from blood of a carrier cow

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

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Table 2.
Responses of calves inoculated with the experimental vaccine (laboratory trial)

<table>
<thead>
<tr>
<th>Number of calf</th>
<th>Prepatent period (days)</th>
<th>Clinical and serological response</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Max. Temp (°C)</td>
</tr>
<tr>
<td>607</td>
<td>7</td>
<td>39.8</td>
</tr>
<tr>
<td>608</td>
<td>8</td>
<td>40.1</td>
</tr>
<tr>
<td>612</td>
<td>7</td>
<td>40.3</td>
</tr>
<tr>
<td>614</td>
<td>5</td>
<td>39.5</td>
</tr>
<tr>
<td>Group Mean</td>
<td></td>
<td>6.75</td>
</tr>
</tbody>
</table>

* PCV – Packed cell volume
** PPE – Percent parasitised erythrocytes
*** IFA – Immunofluorescent antibody test three months after immunization
Table 3.
Responses of immunized calves to challenge with Uzbek homologous virulent *B. bigemina* strain

<table>
<thead>
<tr>
<th>Number of calf</th>
<th>Clinical and serological responses to challenge</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Prepatent period</td>
</tr>
<tr>
<td>607</td>
<td>4</td>
</tr>
<tr>
<td>608</td>
<td>4</td>
</tr>
<tr>
<td>612</td>
<td>6</td>
</tr>
<tr>
<td>614</td>
<td>6</td>
</tr>
<tr>
<td>Control</td>
<td></td>
</tr>
<tr>
<td>604</td>
<td>3</td>
</tr>
<tr>
<td>610</td>
<td>3</td>
</tr>
</tbody>
</table>
Table 4
Response of Friesian calves kept on zero grazing to inoculation of the experimental Uzbek *B. bigemina* vaccine (field trial)

<table>
<thead>
<tr>
<th>Number of calf</th>
<th>Clinical and serological responses to challenge</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Prepatent period</td>
</tr>
<tr>
<td>619</td>
<td>6</td>
</tr>
<tr>
<td>620</td>
<td>6</td>
</tr>
<tr>
<td>621</td>
<td>6</td>
</tr>
<tr>
<td>622</td>
<td>6</td>
</tr>
<tr>
<td>623</td>
<td>6</td>
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<tr>
<td>624</td>
<td>6</td>
</tr>
<tr>
<td>625</td>
<td>6</td>
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<tr>
<td>627</td>
<td>6</td>
</tr>
<tr>
<td>628</td>
<td>6</td>
</tr>
<tr>
<td>629</td>
<td>6</td>
</tr>
<tr>
<td>Herd mean</td>
<td>6</td>
</tr>
</tbody>
</table>
C) SCIENTIFIC IMPACT OF COLLABORATION

Participation between collaborating scientists during the last year has included two visits by Prof. Ilhom Rasulov and Dr. Savkat Ablurasulov to the Kimron Veterinary Institute. During the visits, Dr. Ablurasulov, a young veterinarian, received intensive training in serological techniques, parasite DNA extraction from infected blood samples for PCR analyses, use of blood smear examination to differentiate between Babesia-infected cattle and those infected by other tick-borne diseases, and splenectomy of calves. This training is an important component of the program, since it serves to standardize the procedures of the two laboratories. Prof. Rasulov participated in the follow-up of the calves immunized with the experimental donor-derived B. bigemina vaccine, and was part of the team that monitored the outcome of the challenge inoculation. The procedures involved clinical observations, evaluation of parasitological and serological parameters (blood-smear examinations, PCV measurements, and serological testing for antibody levels).

Dr. Shkap and Mrs. L. Fish visited the Tashkent laboratory for 2 weeks, during which the MASP cultures were initiated, but could not be maintained for prolonged cultivation of B. bigemina. During the visit, blood samples from cattle raised in the field were collected for analysis of blood smears, to detect specific Babesia antibodies, in order to determine the seroprevalence of babesiosis in five different regions, and samples were collected for PCR analysis to identify cattle that were carriers for TBDs.

D) DESCRIPTION OF PROJECT IMPACT

The central goal of the project is to isolate indigenous stains of Babesia bovis and B. bigemina from infected cattle in the endemic area in Uzbekistan, and to attenuate the strains, to enable the development of an effective live vaccine.

B. bigemina of an indigenous strain was isolated, attenuated and maintained both in bovine donors and in MASP culture. Further work will be focused on the isolate of the B. bovis Uzbek strain, which is kept frozen to prevent any possible contamination, while both parasites are maintained alive in both laboratories at the same time.
The other major component of the program is training of Uzbeki scientists in all the methodology of diagnosis, isolation, attenuation and immunization against tick-borne diseases.

E) INSTITUTIONAL STRENGTHENING

The need for expertise in the development and production of anti-babesiosis vaccines was the goal of the "institutional strengthening" portion of the project. The goal has been effectively achieved by developing an anti-\textit{B. bigemina} vaccine based on an indigenous Uzbek strain. The Uzbeki scientists collected the local autochthonous specimens from which \textit{B. bigemina} and \textit{B. bovis} parasites could be isolated. The Uzbeki scientists gained expertise in isolation of hemoparasites from either infected vector ticks or from chronically infected carrier cattle; they participated in most steps of the attenuation process, and in monitoring the responses of immunized and challenged cattle.

F) FUTURE WORK

The following are the goals for further work:

- To test the experimental \textit{B. bigemina} vaccine in Uzbekistan, including immunization and challenge follow-up.
- To test the MASP-derived \textit{B. bigemina} vaccine under laboratory and field conditions in Israel and Uzbekistan.
- To attenuate the virulence of the isolated Uzbek strain of \textit{B. bovis}
- To introduce, grow and maintain \textit{B. bovis} in MASP culture.
- To perform cross-immunization and cross-challenge trials between Israeli and Uzbeki strains of \textit{B. bigemina} and \textit{B. bovis}.  

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SECTION II

A) MANAGERIAL ISSUES

The Institute of Zoology (Uzbekistan):

Although the effective date of the proposal was June 2001, the activity actually started in January 2002, after the subcontract had been signed and funding made available. Then there were personnel problems, and it took about a year to activate the scientific part of the program. During the last year equipment was purchased and sent to the Tashkent laboratory, and it is planned to continue to strengthen that facility for further work.

During the last year indispensable equipment was transferred to Uzbekistan, including a pH-meter, a hematocrit centrifuge, a balance, dispensers, and supplies of plastic and glass wear. To improve communication a facsimile instrument was ordered, and when it is delivered the second consignment will be sent to Tashkent. Unfortunately, up to now there has been no facsimile connection. In addition, a refrigerated table-top centrifuge for cell culture work, that had been purchased with funds from the previous CDR program, broke down and cannot be fixed. It is planned to purchase a new refrigerated centrifuge for the Uzbekistan laboratory.

B) BUDGET

A detailed report on the expenses will be submitted separately by the Financial Department of the KVI.

C) SPECIAL CONCERNS

There are no special concerns.

D) COLLABORATION, TRAVEL, TRAINING

Prof. Resulov spent two periods 6 and 4 weeks, respectively, in the Kimron Institute during the last year, and Dr. Shavkat visited the Kimron Institute laboratory for 4 weeks. Both scientists received training, and spent time working with Dr. Y. Krigel and Dr. B. Leibovitz, participating in all the animals experiments, with Mrs. L. Fish, doing cell culture work, and with Mr. I. Savitsky doing serological tests.
Dr. Varda Shkap and Mrs. L. Fish visited the Tashkent laboratory for 2 weeks. During this visit blood samples were collected from farms where babesiosis and theileriosis are endemic. The samples were processed for serological and PCR testing, in cooperation with the Parasitology Laboratory personnel at the KVI. *B. bigemina* cultured in MASP in Israel were transferred to the Tashkent laboratory to initiate *in vitro* cultivation in Uzbekistan.

**E) REQUEST FOR AID ACTIONS IN PROMOTING PROJECT PRODUCTIVITY**

In accordance with the sub-agreement signed between the collaborating institutions, equipment and supplies are bought in Israel and transferred to Uzbekistan. There are difficulties in releasing equipment from the customs. Despite the presentation of a letter from the US Embassy, which states clearly that the equipment is purchased within the framework of cooperative research programs, the problem has still not been solved. Problems are avoided only when a consignment is valued at under $50, which by no means represents the cost of supplies or equipment transferred to Uzbekistan.


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