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"VACCINE AGAINST BOVINE BABESIOSIS IN CENTRAL ASIA"

COVERING THE PERIOD FROM

JANUARY 2002 TO DECEMBER 2002

SUBMITTED BY:

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EXECUTIVE SUMMARY

Developmental: To generate data, which will lead to an effective live vaccines against bovine babesiosis, one of the major and economically important diseases caused by haemoparasites, *Babesia bigemina* and *B. bovis*.

Scientific: To isolate and attenuate autochtonous strains of *Babesia* to develop an effective live anti-babesiois vaccine.

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 that dies out when introduced into the cold northern regions. The natural transmission of bovine babesiosis is known to occur during warm seasons under field conditions with a fair amount of shade and moderate humidity. Central Asia countries and Israel have subtropical climate and all the conditions for survival of the vector tick. Babesiosis can be acute and fatal, but upon recovery from acute infection cattle might develop chronic subclinical infection. Thus the carrier animals serve as reservoir for continuous infection of ticks, which further transmit the infection when fed on susceptible cattle. The most effective control of babesiosis is based on prophylactic immunization of cattle with vaccines containing attenuated live parasites followed by development of protective immunity against infected ticks.

During the first year of the program we were greatly encouraged when we managed to identify and isolate *B. bigemina* and *B. bovis* from both, infected ticks and blood from chronically infected cattle transferred to Israel from Uzbekistan. There were about 1000 adult ticks collected in the endemic for babesiosis areas. After oviposition the adult ticks produced thousands of larvae. To determine their infectivity transmission/feeding experiments were conducted, which led to infection of cattle with *B.bigemina* or *B. bovis*. The isolated parasites are maintained by continuous infection of cattle, and attempts were made to introduce the isolated parasites into MASP cell culture. Parasites of both species were cryopreserved with 15% DMSO to serve as a source for further work. After isolation and several passages in cattle, *B.bovis* are kept cryopreserved to prevent any possible contamination

between the species, and at present the work is focused mainly on aspects of attenuation virulence and cell cultivation of *B. bigemina.*

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.

The first six months of the year (when the project has been activated) are characterized by low temperatures, i.e., climatic conditions when there was no tick activity, therefore we palnned to collect infected ticks during the late summer or autumn period. Dr. Nina Pak Den Sun spent 3 weeks in the Division of Parasitology at the KVI to strengthen the knowledge and capabilities in identification, growing the vector ticks *Boophilus* under laboratory conditions. During her visit she monitored the developmental stages (larvae, nymph and adults, female feeding and drop for oviposition). Dr. Pak Den Sun was specifically trained in performing splenectomy operations on calves. The anti-*Babesia* vaccines are routinely produced in Israel from splenectomized calves inoculated with live attenuated *Babesia* parasites. The process of attenuation involves rapid passages in splenectomized calves for *B. bovis* and slow passages for *B. bigemina*. In each case, training was a necessary preparation for further work with splenectomized cattle, for isolation, attenuation, and virulence testing of the parasites.

During the last six month of the year, Prof. Rasulov imported into the Division of Parasitology *Boophilus* ticks collected from calves grazing in endemic for babesiosis areas. Blood was collected into vessels containing anti-coagulants from chronically infected with *Babesia* cattle (although no parasite species was determined when blood was collected). During spent six weeks of Dr. Rasulov's visit *Boophilus* ticks were applied to calves for transmission/feeding experiments, blood from infected animals was inoculated into splenectomized cattle, all that in attempt to isolate *Babesia* parasites originating from Uzbekistan.

SECTION I

A) RESEARCH OBJECTIVES IN YEAR UNDER REVIEW:

- ☒ To identify and collect *Babesia*-infected ticks from cattle with clinical signs of babesiosis or from carrier animals
- ☒ To isolate the *Babesia* parasites by passage in cattle or by cell culture (MASP) for anti-babesiosis vaccine to be developed from local Uzbek strains

B) RESEARCH ACCOMPLISHMENTS

Isolation of Boophilus from ticks and blood of carrier cattle

In Uzbekistan, engorged *Boophilus annulatus* ticks were collected from pasture-raised sick or carrier cattle in endemic for babesiosis areas (Sir-Daria, Ushkent). Numerous adult ticks were kept at room temperature until transferred to Israel. Upon arrival the adult ticks were incubated at 28 ° C at 85% humidity for 3 days, then started the egg hatch. The eggs were kept at 14° C at 85% humidity until use. In addition to the adult tick collection, larval stages obtained after oviposition in Uzbekistan were transferred to the KVI laboratory as well. Unfed larvae obtained from several collections of hatched eggs were pooled and applied for feeding on three 2-months-old spleen-intact calves seronegative for *Babesia* (#565,566 and 567). All calved were examined for rise of body temperature, microscopic examinations of peripheral blood smears were made to detect infection, and packed cell volume (PCV) were determined daily. *B. bovis* is transmitted by the larval stage, and *B. bigemina* by the nymph stage. Approximately 10 days after infestation of larvae on cattle nymphs were developed and further fed to allow transmission of *B. bigemina.* After about a week of feeding as nymphs all calves were sprayed with Amitraz to prevent spreading of exotic for Israel ticks. Blood from calves monitored for presence of *Babesia* parasites was drawn into tubes containing anticoagulant (EDTA), and was either frozen with 15% DMSO, or transferred to another animals for infection

transmission, or for identification of parasite DNA by PCR. Serum samples were collected for detection of specific antibodies to either *B. bigemina* or *B. bovis*.

Blood collected from carrier or clinically apparent cases of babesiosis in Uzbekistan (cow #253 and $254) was inoculated into splenectomized seronegative calves (#559 and #560, respectively). DNA was prepared to test presence of parasites and for their identification by using specific primers for PCR. 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 date on susceptible animals infected tick transmission feeding or 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 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 until use.

B.bigemina was isolated from ticks (calf 567 and 559) and from blood 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 calves were splenectomized, and then at recrudescence of parasitemia blood was cyopreserved, or further inoculated into an spleen-intact calf for slow passages.

Isolation of Babesia parasites in MASP culture.

Blood was drawn sterile from calves showing parasitemia in peripheral blood smears. Infected blood at 0.5% to 2% parasitemia collected into vessels with glass beads was defibrinated, and the erythrocytes suspended to a final packed cell volume (PCV) of 9% in medium comprised of 60% RPMI 1640, 40% normal bovine serum supplemented with 15mM HEPES buffer, pH 7.0, and 100 IU penicillin G and 100 µg streptomycin sulfate per ml. Cultures were incubated in flat-bottomed micro-trays at 37 ° C in an mixture of gases 5% CO_2, 2% O_2 and 93 % humidified air. Overlaying medium was removed and replaced with fresh medium daily. Subcultures were made when parasites could be detected in blood smears prepared form the culture wells, and

then every 2-3 days by diluting culture to 0.5% parasitemia with 10% suspension of uninfected erythrocytes in culture medium.

Cultures were initiated from animals infected with *B. bigemina* or *B. bovis*.

As shown in Table 1, all five calves infected either by Boophilus larvae stage or by blood transfusion developed high fever characteristic for Babesia infection. Although in 4 out of 6 calves the PCV was below 20% while in other animals 25 and 26%, all six had to be trated with babesicides (Berenil – diminazene aceturate, or Lomidine – pentamidine) to prevent further destruction of red blood cells, and thus prevent death of the animals. Calf #566 infected with 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 are considered as more sensitive to the drug, and thus at blood transfusion only *B.bovis* infection was established. There were three passages performed, the strain is virulent and need further procedures for attenuation. The infected blood from animals with high parasitemia was frozen with DMSO at –70 ° C to serve a stabilate for *B.bovis* Uzbek strain.

In Table2 and 3 are summarized transmission/ feeding experiments and isolation of *B.bigemina* from blood collected from chronically infected cow #253 in 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 followed by splenectomy and further passages to be performed at the recrudescence of infection. At high parasitemia blood was cryopreserved and will serve as stabilate for *B.bigemina* Uzbek strain.

Attempts were made to grow and propagate the isolated parasites of both species in MASP cultures (Table 4). At present there are no established cultures of either species obtained. In 2 experiments parasites survived for 7 days and parasitemia reached 4% and 5%, however they did not multiplied beyond 2 passages.

To test for presence of specific Babesia parasites by PCR (Fig. A and B), DNA was extracted from infected erythrocytes using Purogene (Gentra Systems Inc., Minneapolis, USA) of the blood samples from chronically infected cows in Uzbekistan that were brought to Israel. Blood from an uninfected calf and from experimentally infected calves were processed similarly and used as a negative and positive controls.

UZBEKISTAN

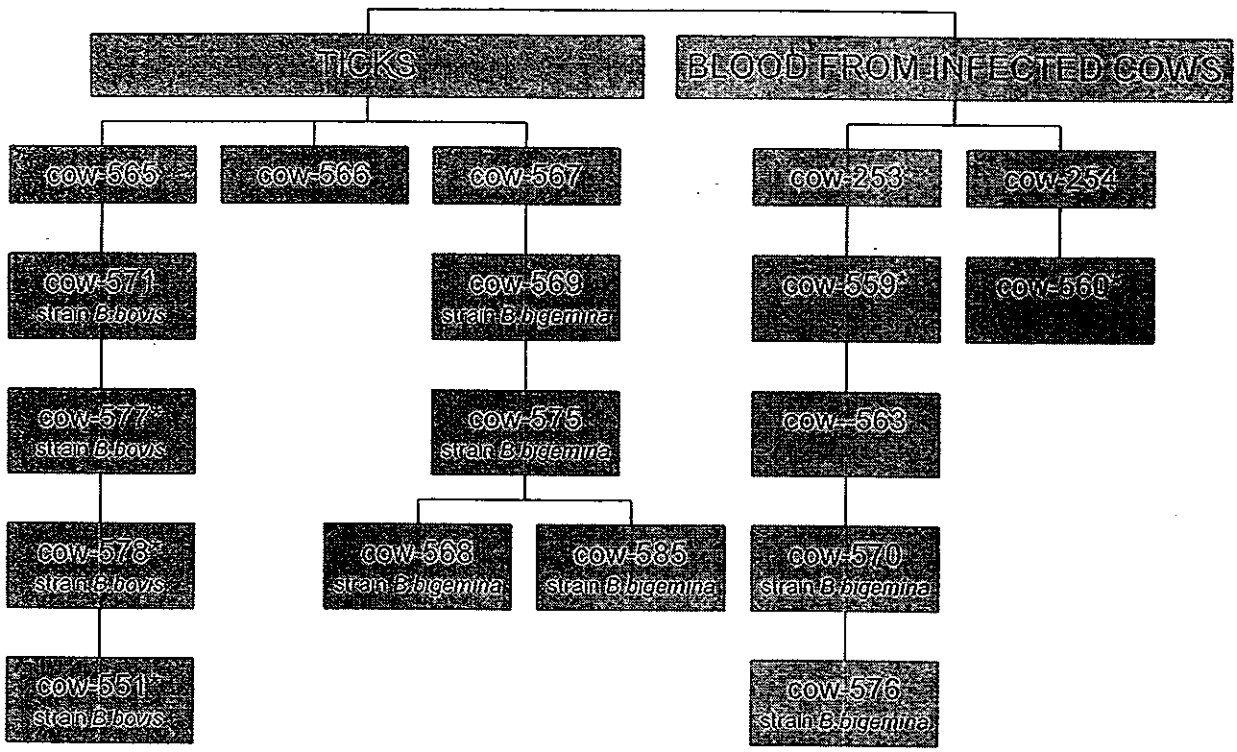

TICKS
BLOOD FROM INFECTED COWS
cow-565
cow-566
cow-567
cow-253
cow-254
cow-571
strain B.bovis
cow-569
strain B.bigemina
cow-559
cow-560
cow-577
strain B.bovis
cow-575
strain B.bigemina
cow-563
cow-578
strain B.bovis
cow-568
strain B.bigemina
cow-585
strain B.bigemina
cow-570
strain B.bigemina
cow-551
strain B.bovis
cow-576
strain B.bigemina

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

Table 4. Attempts to isolate *Babesia* parasites *in vitro*

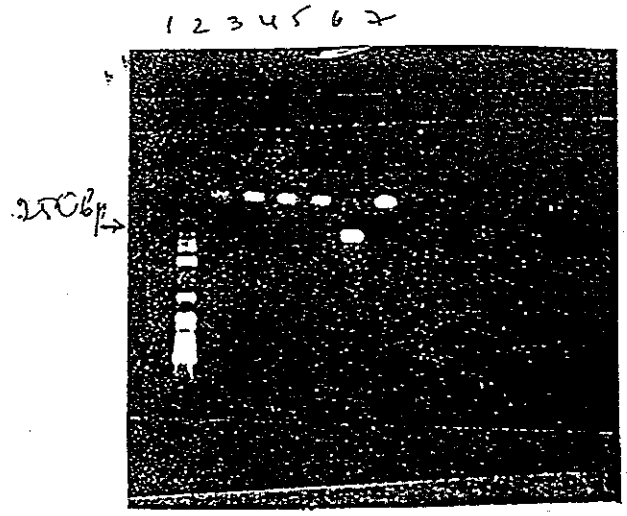
Calf number	Parasite	Number of cultures initiated	Parasitemia	Culture established
571	*B.bovis*	2	<0.001	No
577	*B.bovis*	4	<0.001; <0.001; 4%; 5%	No
578	*B.bovis*	1	<0.001	No
575	*B.bigemina*	2	0	No
576	*B.bigemina*	2	0	No

Figs. A and B

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

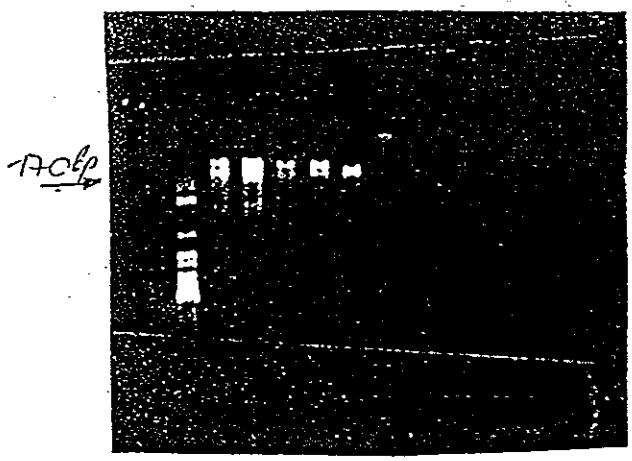
A. *B.bovis* primers

1. blood DNA from cow 253
2. duplicate
3. blood DNA from cow 254
4. duplicate
5. positive control
6. negative control



B. *B.bigemina* primers

1. blood DNA from cow 253
2. duplicate
3. blood DNA from cow 254
4. duplicate
5. positive control
6. negative control



C) SCIENTIFIC IMPACT OF COLLABORATION

Participation between collaboration scientists in the past year consisted of two visits at the KVI laboratory of Dr. Nina Pak Den Sun and Prof. Ilhom Rasulov. The primary stages of the program necessitated training in the techniques of maintaining ticks (either infected or non-infected), identification of the developmental stages in ticks, differentiation of *Babesia*-infected cattle from other TBDs by blood smear examination. Identification and differentiation of hemoparasites of the tick-borne diseases group (TBD) – *Babesia bovis, B. bigemina, Theileria annulata* and the rickettsia of red blood cells – *Anaplasma,* was another goal to start the project, this in order to standardize the procedures between the two participating laboratories. During the visit Dr. Pak Den Sun performed four splenectomy operations on calves. The anti-*Babesia* vaccines are routinely produced in Israel from splenectomized calves inoculated with live attenuated *Babesia* parasites. The process of attenuation involves rapid passages in splenectomized calves for *B. bovis* and slow passages followed by splenectimy after recovery for *B. bigemina*. In each case, training was a necessary preparation for further work with splenectomized cattle, for isolation, attenuation, and virulence testing of the parasites.

Prof. Rasulov collected tick and blood specimens, which were transferred these to Israel. During his visit the imported tick were identified, and used for transmission-feeding experiments. Blood from chronically infected cows was injected into calves, which were monitored for *Babesia* infection. Prof. Rasulov participated in all experimental work, which included tick rearing, monitoring of infested and infected calves.

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 for the development of an effective live vaccine.

E) INSTITUTIONAL STRENGTHENING

The need for expertise in development and production of anti-babesiosis vaccines was the goal of the "institutional strengthening" portion of the project. This first step towards this goal has been effectively achieved. The Uzbeki scientists collected the local autochthonous specimens from which *Babesia* parasites could be isolated. They gained the expertise in isolation of hemoparasites from either infected vector ticks, or from chronically infected carrier cattle.

F) FUTURE WORK

To achieve the scientific goal of the project requires attenuation of virulence of the B.bovis and B. bigemina isolates. This is associated with the biological nature of the isolates, which cannot be predicted at that early stage. Both species of parasites will be modified by *in vivo* and *in vitro* methodology. The achievements of the last year constitute solid progress towards this goal.

SECTION II

A) MANAGERIAL ISSUES

The Institute if Zoology (Uzbekistan):

Although the effective date of the proposal was June 2001, the actual activity started in January 2002 after the subcontract had been signed and funding made available. In order to start the project a protion of equipment was purchased in Israel and transferred to Tashkent laboratory. The parcel included light/fluorescence microscope and supplies of pipettes, tissue culture flasks, glass slides, supplies of chemicals for preparations of various solutions and culture medium.

We have purchased indispensable equipment, including pH-meter, hematocric centrifuge, balance, dispensers, and supplies of plastic and glass wear.
To improve communication FAX was ordered, and when obtained the second parcel is going to be sent to Tashkent.

To ease the procedure of releasing the parcel from the customs, it was agreed with the USA/AID in Tel Aviv that we are going to ask for help of the USA/AID Authorities in Tashkent.

B) BUDGET

To activate the project an advance of $20 000 and $15, 000 was obtained for Uzbekistan and Israeli laboratories, respectively. Of the $ 20 000 allocated for Uzbekistan the total expenses up to date were about $ 17 000, and in Israel the expenses were about $13 000. In Israel, about $7, 500 were spent to purchase materials and indispensable equipment. Three-month salary ($3,665) was paid for animals experimentation. The Uzbekistan budget was spent to purchase equipment, including fluorescent microscope, pH-meter, balance, FAX, pipette dispensers , plastic wear, chemicals. The other major portion (about $5000) was spent on International travel of 2 scientists from Tashkent to Israel, where they worked for about 9 weeks total. There was $500 transferred to cover salary. A sum of $475 were transferred to cover the expenses of local field travel, which resulted in collection of infected ticks. Detailed report on the expenses are submitted separately by the Department of Finances of the KVI.

C) SPECIAL CONCERN

There is no special concern.

D) COLLABORATION, TRAVEL, TRAINING

Dr. Pak Den Sun spent 3 weeks with the personnel of the KVI laboratory, Dr. Y. Krigel, Dr. B. Leibovitz. Dr. Pak Den Sun studied the examination of TBD parasites by light microscopy and the technique of calf splenectomy, she was trained in tick-rearing techniques with Dr. M. Samish. The KVI laboratory has a good and long-time experience with cultivation of *Babesia bigemina* and *B. bovis.* Mrs. Lea Fish, who is the expert in *in vitro* cultivation of these parasites, has introduced Dr. Pak Den Sun to the cultivation methodology, using the Israeli *Babesia* isolates.
Prof. Rasulov spent 6 weeks at the KVI working on aspects of isolation of *Babesia* parasites from infected ticks by transmission/feeding on intact calve. Attenuation procedures, whether "slow" for *B. bigemina* or "rapid" passages for B. bovis, in intact or splenectomized cattle, respectively started during his visit in Israel. During the spring two scientists from KVI are planning to visit the laboratory in Uzbekistan with the intention of establishing the culture system with either Israeli or Uzbek strains of *Babesia* parasites. The collaboration, travel and training component of the project are

mainly constituted in the provision for training and establishment of methodology towards developing of an effective live anti-hemoprotozoa vaccines.

E) REQUEST FOR AID ACTIONS IN PROMOTING PROJECT PRODUCTIVITY

Upon singed subagreement between the collaborating institutions, equipment and supplies are bought in Israel and transferred to Uzbekistan. There were difficulties in receiving the equipment sent from Israel to Tashkent. The parcel was kept for a long time by the custom services. We would like to ask for assistance with further deliveries.

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