

## FINAL REPORT

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### VACCINE AGAINST *Theileria annulata* in CENTRAL ASIA

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### III Executive Summary

The project aimed to develop a safe and potent vaccine against *Theileria annulata* infection a bovine disease of considerable economic importance in Central Asia. *T. annulata* parasites isolated from vector ticks in Uzbekistan were attenuated by long-term growth and passages in cell culture. The degree of attenuation was assessed by inoculating susceptible calves and by evaluating the level of proteolytic enzyme activity of the parasites. Complete loss of virulence of the schizonts occurred after about 100 passages in culture. Correlation between the level of proteolytic activity and the virulence of the parasites was observed. This is of important potential value as a means of simplifying the testing of vaccines in the future. Experimental vaccine prepared in the Uzbekistani laboratory was tested for safety and effectiveness in laboratory and field cattle, starting with groups of 10 animals and progressively augmenting the number up to a total of about 3,500 cattle. There were no undue reactions to of the vaccine. In a farm where cattle were immunized during a theileriosis outbreak the disease was arrested among the immunized cattle, while new cases continued to occur among the non-immunized animals. An application for a patent on the attenuated vaccine strain of *T. annulata* was submitted in Uzbekistan and a permit for routine use of the vaccine in that country was requested from the relevant authorities by the Uzbekistani collaborators. The immunogenic relationship between Uzbekistani and Israeli isolates of *T. annulata* was studied in a cross-immunization laboratory trial. There was a high degree of immunity against the homologous isolates and a lesser degree against the heterologous isolates. Although this difference was not great, it seems preferable at the present time to prepare vaccine from autochthonous isolates in order to achieve the strongest immunity. Aside from the specific achievements of the project in developing an effective vaccine, the project has had a general felicitous effect that is worth noting. The Central Asian scientific staff was exposed to and learned to use, some of the most advanced techniques of

research and development in the field of vaccine preparation against tick-borne parasitic diseases. These skills will remain of value for all the foreseeable future.

#### **IV Research Objective**

Cattle breeding is one of the most important activities in the rural economies of Central Asia and animal protein forms a major component in the diet of the human population.

Theileriosis transmitted by ticks is responsible for a high mortality rate among young and adult cattle. The main overall aim of the program was to develop a safe and potent vaccine against theileriosis infection, that could be used initially in Uzbekistan and then in the surrounding area.

Culture-derived antitheilerial vaccine was first developed in Israel (Pipano 1977), subsequently Mediterranean and some Asian countries reached various stages in the development or application of this system (Brown 1990).

The present program was initiated in a large theileriosis-infected area covering several national entities. Available literature did not include any data about the immunological relationship of *T. annulata* isolates from this area with theilerial parasites from other geographically remote areas. This subject is important because it affects the degree to which local vaccination program can be relied upon in the face of the possible incursions of foreign parasitic strains from other areas.

#### **V Methods and Results**

*T. annulata* is a protozoan parasite with a complex life cycle, of which three developmental stages are infective for cattle. The sporozoites that develop in the salivary glands of the vector ticks transform into intracellular schizonts when they are inoculated into cattle. The schizonts represent the pathogenic stage of *T. annulata*, and are responsible for most of the pathological

lesions and clinical manifestations. The schizonts yield merozoites, which penetrate the red blood cells and represent the erythrocytic stage of *T. annulata* (known as erythrocytic merozoites or piroplasms). The erythrocytic stages are capable only of infecting ticks.

## 1 Isolation of *T. annulata* stock in Uzbekistan

Engorged *Hyalomma anatolicum* nymphs were collected from pasture-raised cattle in south-east Uzbekistan. The nymphs were allowed to molt in the laboratory and the female and male ticks which issued were kept for 2 months at 22°C and 85% relative humidity (RH). The adult ticks were then incubated at 37°C and RH for 4 days. The live ticks were separated from the dead, rinsed and then triturated by mortar and pestle in a small amount of 42% bovine fetal serum in Eagles Minimum Essential Medium (Samish et al. 1983). The coarse tick debris was sedimented by centrifugation for 3 min at 400 g. A 2-month-old calf was inoculated subcutaneously in the neck area with 1.8 ml of supernatant suspension containing the equivalent of eight ground ticks. When a body temperature increase was observed and schizonts were detected in smears from liver biopsy material, the calf was used as a source of *T. annulata* schizont-infected cells.

## 2 Cultivation and cloning of schizont-infected cells

Cultures of schizonts were initiated by the three methods described by Pipano et al. (1989), which involve buffy coat cells, mononuclear cells separated by Ficoll gradient and cells obtained by liver needle biopsy. Primary cultures were initiated by incubating  $5 \times 10^5$  cells/ml in Leibovitz L-15 medium supplemented with 20% newborn calf serum. Established monolayer cultures were removed by rinsing with 0.025% EDTA in phosphate buffered saline. The dispersed cells were suspended in complete culture medium to obtain a concentration of  $2 \times 10^5$  cells/ml and subcultured.

in fresh vessels 25 cm<sup>2</sup> tissue culture flasks. Primary cultures and subcultures were incubated at 37°C.

Cells obtained from the second passage were cloned by the limiting dilution technique (Rodrigues et al 1983). Parallel cells from a virulent Israeli strain were cloned in the Bet Dagan laboratory.

### 3 Testing for virulence

The virulence of the culture-derived schizonts from every 10 to 20 passages was assessed by subcutaneous inoculation of  $2 \times 10^6$  schizont-infected cells into two or four 3-5 month-old calves. The calves were acquired from a theileriosis-free dairy farm in which the cattle were raised on zero grazing. After purchase the calves were kept under tick-free conditions and examined for a period of at least one month before inoculation. No anti-theilerial antibody (for technique see IV 6) or parasites were detected in these animals during the pre-experimental period (Dargouth et al , 1996). The response of the calves was monitored by measurement of body temperature and by examination of Giemsa stained thin blood films and smears made from liver needle biopsy material. The results of the attenuation experiment are summarized in Table 1.

After an initial growing period of 2 weeks a uniform monolayer of round bright cells was observed. Giemsa-stained preparations revealed schizont-infected mononuclear cells. The cells grew vigorously in each subsequent passage until the end of the trial.

Four calves inoculated with schizonts from passages 1 and 20 developed fever, schizonts were detected in the liver smears of all four animals, and erythrocytic parasites in their blood. One animal inoculated with schizonts of the first passage was treated with Buparvaquone.

**Table 1**  
**Response of calves inoculated with schizonts of *Theileria annulata* from different passages in cell culture (Uzbekistan isolate)**

No of passage in culture	Number of calves				Average	
	Inoculated	Schizonts in liver	Erythrocytic merozoites	Fever	Days over 39.5°C	Antibody titer*
1	2	2	2	2	3.8	3.5
20	2	2	2	2	2.5	4.5
40	2	0	2	1	2.0	4.0
50	4	2	4	2	1.5	3.5
60	2	0	1	0	0.0	3.5
71	2	0	1	0	0.0	3.0
80	2	0	1	1	1.0	3.0
102	2	0	1	0	0.0	3.0
112	2	0	0	0	0.0	3.0

\*Antibody titer is expressed as a log<sub>4</sub> where 1/4=1, 1/16=2 etc

(Pitman-Moore, USA) to prevent eventual death. The other three animals recovered spontaneously. One of the two calves inoculated with schizonts from passage 40 suffered fever for several days, schizonts could not be detected in liver smears but erythrocytic parasites appeared in both animals.

Two of the four calves inoculated with schizonts from passage 50 developed fever and exhibited schizonts in liver smears and erythrocytic merozoites in blood films, while the two remaining animals showed only a few erythrocytic merozoites. No schizonts were detected in the calves inoculated with schizonts from passages 60, 71, 80 and 102, and only one of the two calves inoculated with schizonts from these passages showed a few erythrocytic parasites, 4 to 8 weeks after inoculation. One animal inoculated with schizonts from passage 80 showed a rise in temperature during one day. Neither of the animals inoculated with schizonts from passage 112 developed fever or showed parasites in liver smears or blood films. Most calves inoculated with schizonts grown through up to 60 passages *in vitro* showed a considerable swelling of the superficial lymph nodes 10 to 15 days post-inoculation. All calves developed anti-theilerial antibodies following the inoculation of the schizont-infected cells.

**Table 2**  
**Response of calves to inoculation with *in vitro* cloned or passaged *T. annulata* schizonts**  
**Uzbekistani isolate**

Source of Parasites	Maximum (%)		Body temperature	
	Sch	Em	Max	Days over 39.5°C
Passage 1	2	8	41.3	8 t
Clone 3F	1	<1	39.8	1
Clone 3F	0	<1	38.8	0
Clone 6s	0	0	39.2	0
Passage 15	1	<1	40.4	3
Passage 15	3	<1	40.8	2

0=no parasite found

Sch=schizont in liver smears

Em =erythrocytic merozoites in blood films

C=treated with Buparvaquone

\*=passaged in culture during the period required for cloning

t=treated with Buparvaquone

**Table 3**  
**Response of calves to inoculation with *in vitro* cloned or passaged *T. annulata* schizonts**  
**Israeli isolate**

Source of Parasites	Maximum (%)		Body temperature	
	Sch	Em	Max/	Days over 39.5°C
Passage 1 Ln	20	3	41.5	7 t
Passage 1 Ln	1	6	40.7	4
Passage 1 Lv	1	1	40.5	4
Passage 1 Lv	1	<1	40.5	2
Clone C <sub>1</sub> Ln	7	4	40	4
Clone C <sub>3</sub> Ln	39	11	40.7	5 td
Clone C <sub>6</sub> Ln	13	7	40.5	4
Clone C <sub>5</sub> Lv	0	0	39.2	0
Clone C <sub>5</sub> Lv	3	1	40.8	4
Clone C <sub>6</sub> Lv	11	19	40.8	7 t
Passage 12 Ln	11	12	40.7	4 t
Passage 12 Ln	<1	<1	40.3	3
Passage 37 Ln	0	<1	40.5	3
Passage 37 Ln	0	<1	39.2	0

0=no parasites found

Sch=schizonts in liver smears

Em=erythrocytic merozoites in blood films

Ln=culture or clone derived from lymph node cells

Lv=culture or clone derived from liver cells

t=treated with Buparvaquone

td=died despite treatment

In the cloning experiment the schizont-infected cells from the first passage, from which the cells were derived for cloning, induced moderate parasitemia accompanied by severe clinical manifestations and fever which continued for 8 days (Table 2) The calf was treated with an antitheilerial drug to prevent eventual death

The inoculation of three clones derived from the first passage in culture into three calves caused no clinical manifestations in any of them, but a low-level parasitemia was observed in two of them Schizont-infected cells that were passaged in culture during the period required for cloning (15 passages) showed moderate parasitemia and fever during two or three days The results obtained from cloning an Israeli isolate in the Bet Dagan laboratory were significantly different from those obtained with the Uzbekistani isolate Two cell lines were obtained from an infected calf One line was derived from cells withdrawn with a syringe from a hypertrophied prescapular lymph node (ln-line) and the other from cells obtained by liver needle biopsy (lv-line)

Schizonts from the first passage in culture from both lines caused clinically expressed theileriosis following inoculation into calves All three clones derived from the ln line as well as two clones derived from the lv cell line induced severe disease in the inoculated calves A third clone from the derived lv line caused no clinical manifestation and no parasitemia Cells passaged in culture during the cloning period also retained considerable pathogenicity However, when these cell lines were grown in culture during 25 supplementary passages (a total of 37 passages), they induced a mild response in calves

All calves used in the cloning experiment were serologically negative (titers of 1/4 to 1/16) before the inoculation and exhibited significant levels (1/256 to 1/1024) 4-5 weeks after inoculation

In the process of attenuation of *T. annulata* schizonts by growth and passages in culture three stages are observed (Pipano, 1989) 1) During the early period of cultivation the schizonts

induce clinical theileriosis in most inoculated cattle and may cause death in some of them 2) Further cultivation yields less virulent schizonts, and their inoculation into cattle results in milder clinical manifestations and a lower level of parasitemia. Some animals may exhibit clinical manifestations while others remain symptomless. Response to a higher passage may be more severe than to lower passages 3) At a certain stage of cultivation, which differs among the various isolates, the inoculated schizonts no longer induce clinical manifestations and no schizonts can be detected in smears from liver biopsies. By this stage, erythrocytic merozoites are rarely seen, and subsequently they are no longer detected in the blood films from inoculated calves.

All three stages of attenuation were observed with the Uzbeki isolate: severe theileriosis up to passage 20, variable response up to passage 50, and no pathogenicity after passage 60. The variable response of the calves to inoculation with schizonts from passage 50 can be explained by the varying sensitivity, among cattle of the same breed, to disease agents including *T. annulata*. This variability in response necessitates the use of completely attenuated schizonts for preparation of vaccine, which must be safe even for the most sensitive animals. The finding of a few erythrocytic merozoites in calves inoculated with schizonts grown for 60 or more passages in culture suggests that there was a microscopically undetectable level of schizonts in these animals. The average antibody level in calves inoculated with virulent schizonts was slightly higher than that in calves which received attenuated schizonts.

It seems from the present cloning experiment that low pathogenic parasite populations can be obtained by this technique. All the clones derived from the Uzbeki isolate were considerably less virulent than those passaged in culture during the period required for cloning of parent schizont-infected cells. Contrarily, only one of the six Israeli clones was avirulent while the remaining five provoked clinical manifestation of theileriosis.

It appears from this study that virulent as well as low-virulence or avirulent clones may result from cloning a virulent *T. annulata*-infected cell line. These results differ from those obtained in cloning *Babesia bovis*, where most of the clones are low-virulent for cattle (Buening et al 1986, Pipano et al 1991). The cloning approach can considerably shorten the period required for obtaining avirulent parasites to be used for vaccine production. However, a cloned *in vitro* schizont-infected cell line is not necessarily genetically homogenous, since more than one sporozoite may infect a bovine cell (Irvin and Boarer 1980). On the other hand, the relatively homogenous population of a clone might be less immunogenic than the heterogeneous parasite population of a cell line. In this context, attenuation of cell lines by long-term cultivation has also been associated with phenotypic and genotypic alterations of the parasites as well as with clonal selection during the cultivation process (Sutherland et al 1996, Somerville et al, 1998).

#### 4 Proteolytic enzyme activity of cultured schizonts

Cells infected with schizonts of the Uzbekistan (Uz) or the Israeli (Is) isolates were grown and subcultured as described (above). Three-day-old cultures at various passages (from 10 to 81 of the Uz isolate, and from 18 to 197 of the Is isolate) were harvested and washed three times with cold PBS by centrifugation at 1500 x g for 10 min at 4°C. The cell pellet was resuspended in an equal volume of cold sterile lysis buffer comprised of 0.25% sucrose and 0.5% Nonidet P-40 at final concentration. After 30 min incubation on ice the lysate was centrifuged at 14,000 x g for 5 min. The supernatant collected, dispensed into small aliquots and immediately frozen in liquid nitrogen. The concentration of proteins in the preparations was examined according to Bradford (1976). Proteins were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) according to Laemmli (1970) on a Protean II vertical slab gel unit (Bio-Rad, Richmond, CA) on 10% running gel to which 0.2% gelatin at final

concentration was added as a substrate, and 3% stacking gel (without gelatin). Samples of cell lysates were adjusted before application onto the gel to 5 µg per lane with a sample buffer containing 2% SDS, 10% glycerol, 80 mM Tris-HCl and 0.005% bromphenol blue (pH 6.8). Samples of lymphocytes obtained from healthy calves were processed in parallel and served as controls. The samples to be loaded to the gel were not boiled in order to preserve the proteolytic activity. Electrophoresis was carried out at a constant current of 30 mA in Tris-glycine buffer on ice for 1 h. Molecular weights were approximated by comparison with protein standards run on an SDS-PAGE without gelatin. After electrophoresis the gel was washed in double-distilled water and then incubated twice in large volumes of 2.5% Triton X-100 for 30 min to remove SDS. After rinsing in distilled water the gel was immersed overnight in incubation buffer (50mM Tris/HCl pH 8.0, 5mM CaCl<sub>2</sub>) at 37°C with agitation. To visualize the enzyme activity the gel was stained with 0.5% Coomassie blue R-250 dissolved in 45% methanol and 10% acetic acid, for a few hours at room temperature. Destaining was then performed in 25% methanol and 10% acetic acid, until clear bands of proteolytic hydrolysis of the substrate-embedded gel were apparent.

The electrophoretic separation of schizont-infected cells of the Uzbek1 isolate at passage 10 revealed a profound proteolytic activity in the gelatine substrate gel (Fig 1). Four distinct bands were detected at the lowest passage examined (Fig 1, lane A), and two bands at passage 23 (lane B). A gradual reduction in the protease activity was observed in lysates of cells of higher passages. The longer were the cells had been subcultured, i.e., the higher the passage number, the lower were the observed levels of protease activity. In the preparation from passage 43 (Fig 1, lane C), one clear band was observed. Two preparations of lysates were examined at passage 60, and a very faint band could be seen in lane D, while no reactivity was observed in lane E. There was no reactivity with non-infected lymphocytes (Fig 1, lane F). A similar pattern of gradual reduction of enzyme activity was observed with

the Israeli (Is) isolate of *Theileria*-infected cells. As displayed in Fig 2, one strong band indicating intensive proteolytic activity was detected in passages 18, 32 and 50 (Fig 2, lanes A-D). A weak band showing a reduction in the magnitude of reactivity was detected in higher passages – 149 and 181 (Fig 2, lanes E, F). No reactivity was observed in preparations from passages 197 (Fig 1, lane G) and higher (not shown, (Fig 3)). No reactivity was observed in non-infected cells (Fig 2, lane H).

In order to characterize the proteolytic enzymes, several different inhibitors were added to the incubation buffer during final incubation. Tosyl (tested at concentrations of 0.01, 0.05, 0.1 and 0.2 mM), E-64 (tested at concentrations of 1, 2 and 5 mM), Iodoacetamide (5, 10 and 50 mM), EDTA (0.5, 1.0 and 5 mM), PMSF (1-2 mM), Antipain 5-100 µg/ml, TLCK (1-2 mM), TCPK (1-2 mM), Leupeptine 5-100 µg/ml). There was dose-dependent inhibition with Tosyl (Fig 3), which was potent at concentrations of 0.1 and 0.2 mM, but not at 0.05 mM and lower. None of the other inhibitors tested at various concentrations was effective (Figs 4 and 5) except EDTA which effectively inhibited enzyme activity from 5 mM down to the lowest concentration tested 0.5 mM (Figs 6 and 7).

From the results obtained it appears that there is a substantial difference in proteolytic enzyme activity at various levels of schizont-infected cell subculture. We tested two isolates (Uzbekistani and Israeli) and, based on the proteolytic enzyme activity, traces of enzyme were found in the Uz isolate in passage 60, beyond which the activity vanished completely. From the results obtained with the inhibitors, it appears that the enzymes detected were metalloproteases which use  $Zn^{2+}$  molecules for their activity. These results are in agreement with those obtained by Balys et al (1992), in studies with *Theileria annulata* from India and Turkey. Similar proteolytic enzyme activities have been described in other protozoan parasites, such as *Leishmania* (Bordier et al, 1987) and helminths (Hotez et al, 1990, Marco and Nieto, 1991).

Inoculation into calves (Table 1) of schizont-infected cells subcultured from the Uzbeki isolate for up to 60 *in vitro* passages resulted in the appearance of clinical signs of theileriosis, while no detectable signs of the disease could be seen when cells of higher passage level were introduced into cattle. Thus, a degree of correlation between the enzyme activity *in vitro* and the virulence of schizont-infected cells *in vivo* was found. On the other hand, cells of the Israeli isolate showed a weak enzyme activity in schizonts from higher passages (up to 181), while no reactivity was observed at still higher passages. Inoculation onto two calves of passage 43 cells resulted in detection of schizonts and merozoites in one animal, but no signs of theileriosis in the other (Table 4). Infected cells at passage 200 did not produce clinical reactions.

**Table 4**  
**Virulence to cattle and proteolytic enzyme activities in Uzbekistani and Israeli *T. annulata* isolates from various culture passages**

No of passage	Response to inoculation				Enzyme activity
	Sch	Em	Max temp	Days over 39.5	
Uzbekistani Isolate					
10	Nd	Nd	Nd	Nd	4 bands
20	Yes	Yes	40.4	3	2 bands
20	Yes		40.8	3	
40	0	Yes	40.8	4	1 band
40	0	Yes	39.1	0	
60	no	Yes	39.4	0	1 band
60	no	No	38.1	1	
71	no	Yes	39.2	0	no bands
71	no	No	39.4	0	
81	no	No	39.9	1	no bands
Israeli isolate					
43	Yes	Yes	40.2	3	1 band
43	No	Yes	39.3	1	
200	No	No	39.1	0	no bands
200	no	No	38.5	0	

Sch=Schizonts in liver

Em =Erythrocytic merozoites

Fig 1 Gelatin- substrate SDS-PAGE gel of *Theileria annulata* - infected lymphocytes Uzbeki isolate, preparations obtained from passages A-10, B-23, C-43, D-60, E-60 (another preparation), F-non-infected lymphocytes

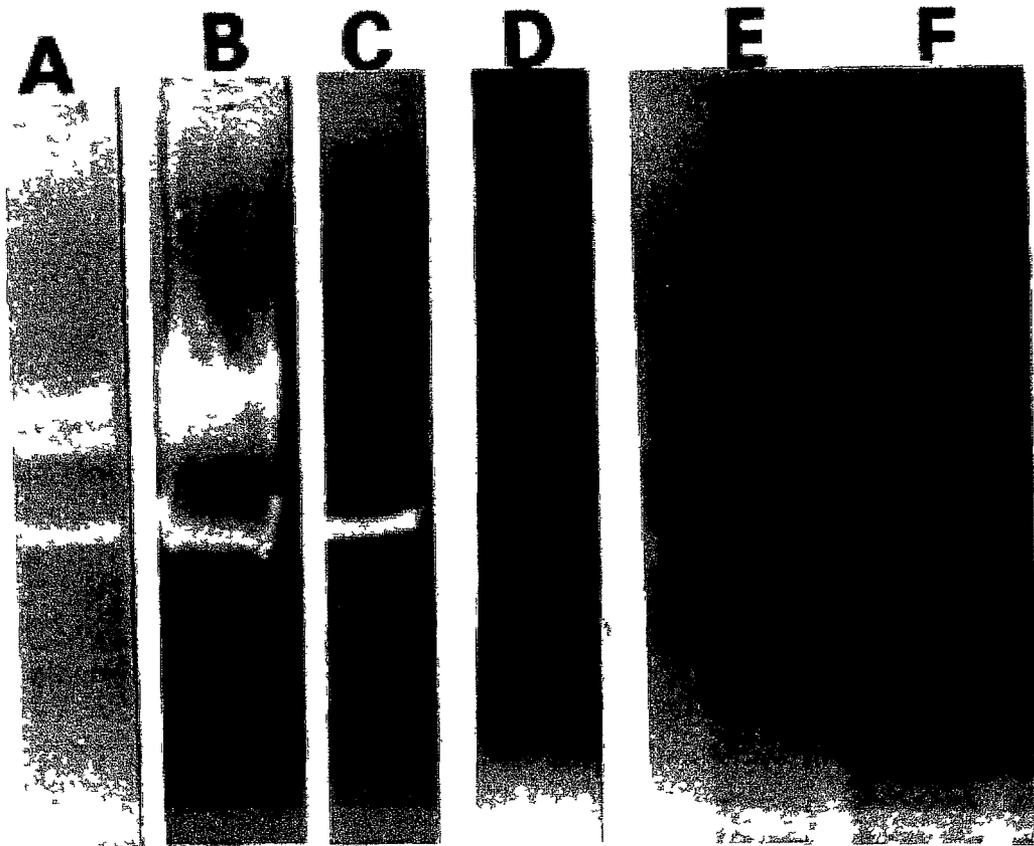


Fig 2 Gelatin-substrate SDS-PAGE of *Theileria annulata* - infected lymphocytes Israeli isolate, preparations obtained from passages A-18, B, C from passage 32 (two different preparations), D -50, E - 149, F -181, G -197, H - non-infected lymphocytes

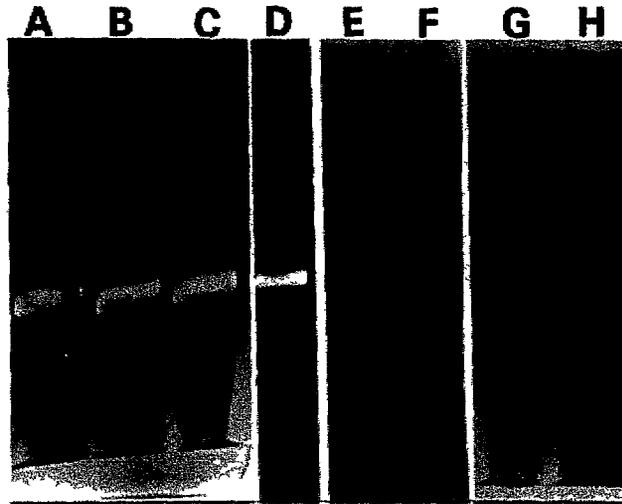


Fig 3 Gelatin-substrate SDS-PAGE gel of *Theileria annulata* - infected lymphocytes Uzbeki isolate, passage 43 Lanes A, B C, and D - Tosyl inhibitor added to the incubation buffer at concentrations of 0.01, 0.05, 0.1 and 0.2 mM, respectively



Fig 4 Gelatin-substrate SDS-PAGE gel of *Theileria annulata* - infected lymphocytes Uzbeki isolate, passage 20 Lanes A, B and C – E-64 inhibitor in the incubation buffer at concentrations of 1, 2, and 5 mM, respectively

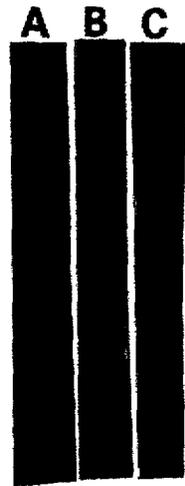


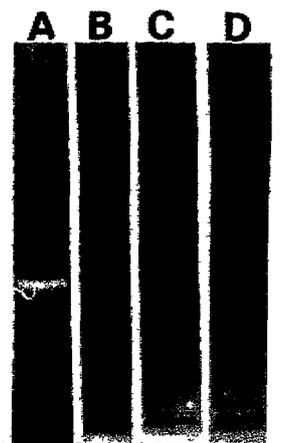
Fig 5 Gelatin-substrate SDS-PAGE gel of *Theileria annulata* - infected lymphocytes Uzbeki isolate, passage 32 Lanes A, B and C – Iodoacetamide in the incubation buffer at concentrations of 5, 10 and 50 mM, respectively



Fig 6 Gelatin-substrate SDS-PAGE gel of *Theileria annulata* - infected lymphocytes Lanes A – preparation from Uzbeki isolate from passage 23 without inhibitor, B, C, D, and E - EDTA inhibitor added at concentrations of 0.5, 1.0, and 5 mM, respectively



Fig 7 Gelatin-substrate SDS-PAGE gel of *Theileria annulata* - infected lymphocytes Lanes A – preparation from Israeli isolate, passage 149 without inhibitor, B, C, D, and E - EDTA inhibitor added at concentrations of 0.5, 1.0, and 5 mM, respectively



## 5 Preparation of experimental vaccine

Schizont-infected cells from passage 112 were propagated to obtain about  $1.3 \times 10^8$  cells in a single culture of passage 116. Dimethyl sulfoxide (DMSO) and culture medium were added to obtain a concentration of  $5 \times 10^6$  schizont-infected cells in 1.8 ml. Aliquots of 1.8 ml were dispensed in cryotubes and cryopreserved in liquid nitrogen as the stock of seed cells to be used for the preparation of batches of experimental vaccine.

To prepare and test the first batch of vaccine a cryotube was retrieved from storage and after thawing and rinsing the cells from the DMSO, fresh medium was added and the cells were incubated at  $37^\circ\text{C}$ . Several passages were made in order to obtain the  $1 \times 10^9$  schizont-infected cells required for the preparation of about 200 doses of vaccine.

A roller culture system was set up in the Tashkent laboratory, based on a roller apparatus and equipment acquired with the project funds. This facility allowed the production of a quantity of vaccine sufficient for the experimental vaccination of cattle. The cells grown in roller bottles were dispersed by rinsing and incubation with EDTA and then concentrated by centrifugation. Aliquots of 1 ml containing  $5 \times 10^7$  cells comprising 10 doses of a concentrated vaccine ( $5 \times 10^6$  viable schizont-infected cells per dose) were dispensed in cryovials. The first batch of experimental vaccine prepared from the seed-cell stock consisted of about 200 doses. Two supplementary batches of vaccine were prepared after the testing of the first batch. The first batch of vaccine was tested for safety in the laboratory (Table 5): four calves were each inoculated with a dose of  $5 \times 10^6$  schizont-infected cells. Only one calf showed a mild rise in temperature for one day, no schizonts were seen in smears from liver puncture material obtained on day 14 post-inoculation, nor were parasites seen in blood during the prechallenge period. Slight to moderate swellings of the prescapular lymph nodes draining the site of inoculation of the vaccine were observed. All calves became seroconverted as determined by the indirect fluorescent antibody technique.

The four immunized calves and three non-immunized control calves were challenged by inoculation of a frozen (thawed) stabulate of ground *Theileria* infected ticks. Each animal received the equivalent of eight ground ticks in the neck area (Table 6). All immunized calves showed rare schizonts (below 1%) in the smears made from liver biopsy as well as few parasites in the blood films. However, transient fever lasting 2-3 days occurred, and erratic values were measured during the period of pyrexia. No visible changes were observed in the behavior of the animals. All control non-immunized calves suffered clinical theileriosis accompanied by anorexia and recumbency, and a relatively high parasitemia was seen in the liver and blood smears. The control calves were treated with Buparvaquone, but one calf died in spite of the treatment. The test for safety proved that vaccine produced from seed cells originating from passage 120 or higher were safe for young animals, therefore, such vaccines could be used in further experimental immunization of field cattle.

Obviously, the method of propagation of the seed cells in order to obtain the quantities needed for vaccine production involves further passaging of the cells. Present experience shows that once schizonts have become attenuated, no reversal to virulence occurs following further passages in culture (Pipano, 1989). On the other hand, it is not yet clear whether the immunogenicity of the schizonts is altered during prolonged cultivation. For this reason it is recommended that when the seed cells have been passaged 20-25 times during the production process, further production should be started anew from another tube of cryopreserved seed cells.

The efficacy of the vaccine was tested against a heavy dose of infective material.

Paradoxically, inoculation of ground infected ticks triggers a considerably more severe infection than infestation with live ticks which produce sporozoites in the salivary glands (Pipano, 1994). The immunized cattle were not completely protected against the tick infection but the short-term fever and the low infection rate caused no obvious damage to the

vaccinated animal. Such results have been reported also by other investigators who tested a cultured antitheilerial vaccine (Pipano 1989a, Shirong 1997)

The control non-immunized calves showed no signs of spontaneous recovery. The rate of parasitemia would have increased and fever would have continued if treatment with an antitheilerial drug had not been administered. In fact, the maximum values appearing in Table 5 are those measured on the day of treatment. At that stage of the disease the control cattle were treated since a clear difference between the responses of the immunized and control animals was observed.

The reasons for the incomplete protection induced by schizont vaccine appears to be related to the antigenic difference between the main developmental stages of *T. annulata* (Pipano 1974) and the genotypic and phenotypic alteration of the schizonts following prolonged cultivation (Sutherland et al 1996). Nevertheless, the schizont vaccine has proved to be highly efficient in protecting cattle against theileriosis under field conditions (Brown 1990).

**Table 5**  
**Response of calves to experimental culture-derived *Theileria annulata* vaccine – laboratory trial**

No. of calf	Sch %	Em %	Maximum temperature	Fever over 39.5 (days)	Swelling of ln
T21	0	0	39.8	1	Moderate
T22	0	0	39.4	0	Slight
T23	0	0	39.5	0	Slight
T24	0	0	39.1	0	Slight

Sch=Schizonts in liver

EM=Erythrocytic merozoites

ln=lymph nodes

0=no parasites or fever detected

**Table 6**

**Response of calves immunized with culture-derived *T annulata* vaccine to challenge infection with ground, *Theileria*-infected ticks Each animal received an equivalent of eight ticks**

No of calf	Sch %	Em %	Maximum temperature	Fever over 39.5 (days)	Swelling of l n
Immunized					
T21	Rare	Rare	41.0	3	Moderate
T22	Rare	2	40.8	3	Considerable
T23	<1	rare	40.4	3	Considerable
T24	<1	<1	39.9	2	Moderate
Non-immunized control					
T25	8	20	41.2	6 (t)	Considerable
T26	9	8	41.5	7 (t)	Considerable
T27	16	24	41.6	8 (td)	Considerable

Sch=schizonts in liver

Em =Erythrocytic merozoites

ln =lymph nodes

(t)=treated with buparvaquone

(td)=died despite treatment

**Table 7**

**Response of calves to inoculation with experimental *T annulata* vaccine (field trial)**

No of animals		Maximum average temperature °C	Range	Size of prescapular lymph node	Antibody* titer
10	Inoculated with experimental vaccine	39.2	38.6-39.6	Slightly swollen	3.5
10	Inoculated with diluent (for frozen vaccine)	39.0	38.3-39.3	No detectable changes	1.2

\*Antibody titers are presented as log<sub>4</sub> where 1.4=1, 1.16=2 etc

**Table 8**

**Milk yield in dairy cows before and after immunization with culture-derived antitheilerial vaccine**

Group	Number of animals	MILK YIELD (liters)			
		For 15 days before immunization	Cow per day (average)	For 30 days after immunization	Cow per day (average)
1	24	3,204	8.9	6,614	9.2
2	19	3,135	11.0	6,360	11.1
3	21	3,087	9.8	5,885	9.3
4	35	5,617	10.7	11,445	10.9
Total	99	15,032	10.1	30,629	10.2

**Table 9**  
**Numbers of pastured cattle with acute theileriosis induced by natural tick-transmitted infection since the day of vaccination**

	Number of cattle		
		Suffered acute theileriosis	
		Days post vaccination	
		1-12	13-90
Immunized with anti-theilerial vaccine	247	7	0
Non-immunized	259	6	28

Based on the results of the laboratory trial a field trial was performed in a state farm (Krasni vodopad) belonging to the Animal Research Institute of Uzbekistan. Twenty 6-7-month-old Friesian calves were divided at random in two groups of 10 animals each (Table 6). All animals were theileriosis free as determined by the serological test and blood film examination. The calves in the first group of 10 were each inoculated in the neck area with a dose of anti-theilerial vaccine containing  $5 \times 10^6$  schizont-infected cells. The vaccine was transported to the farm in a field liquid nitrogen container. The vial containing 10 doses of concentrated vaccine was thawed on the spot and the vaccine was transferred to a bottle containing diluent for the vaccine. Each animal received 2 ml of diluted vaccine. The second group of 10 calves received 2 ml of diluent only.

Body temperature was checked every two days for 3 weeks. Palpation of the prescapular lymph node draining the site of inoculation was done from day 8 after inoculation until the end of the experimental period. Blood for serological tests was drawn before and 30 days after inoculation. The results summarized in Table 6 show that there was no significant difference in the body temperature of the animals in both groups. A slight swelling in the lymph nodes was found in the cattle inoculated with the vaccine. All immunized animals became seroconverted.

A possible influence on the milk yield was tested in four groups comprising a total of 99 milking cows (Table 7). The milk yield was measured during 15 days before immunization.

and 30 days post-immunization. The results showed no significant difference between the preimmunization and post-immunization period. The safety of the antitheierial vaccine for adult cattle has been proved previously (Pipano 1989a), but the present trial was one of the requirements for licensing the vaccine produced from an autochthonous *Theileria* strain in Uzbekistan.

The efficacy in protection against natural disease was tested in the Yangiabad farm belonging to the Railroad Administration of Uzbekistan. A total of 890 cattle of all ages are bred on this farm. The male calves are sent to pasture during the day and return to the barns for the night (except for the winter period of 3-4 months). An outbreak of theileriosis was signaled in June 1998. A total of 506 calves (grown for beef) aged from 6 months to about 3 years, that did not show disease symptoms on the day of vaccination were included in the trial (Table 8).

Two hundred and forty seven calves were immunized with the antitheierial vaccine and 259 other calves of approximately the same age were left as controls. When sick cattle were seen on the farm, investigators from the Tashkent laboratory checked these animals for specific clinical symptoms, and examined blood films for parasitemia.

During 12 days postvaccination, acute theileriosis was diagnosed in seven immunized and six non-immunized cattle. Twenty-eight more cases of acute theileriosis were detected in the non-vaccinated cattle during the following 80 days of observation and none in the vaccinated ones. In this trial antitheierial vaccination prevented clinical theileriosis from day 12-post vaccination. It follows that vaccination can be used not only as a long-term prophylactic measure but also as a means of intervention during a theileriosis outbreak, with the aim of considerably decreasing the losses caused by the disease.

Some of the immunized cattle might have been silently infected by *T. annulata* beforehand, in which case, not all the cattle included in the trial would have been sensitive to theileriosis. On the other hand, it is likely that cattle which had recovered from previous infection

occurred in both groups, and there is clear evidence that the vaccine prevented the appearance of new cases of theileriosis after day 12 post-vaccination, while 28 new cases were diagnosed in the non-vaccinated group of cattle

The field trials described here do not have the basic value of laboratory experiments performed under controlled conditions. However, they supplied an obvious indication concerning the safety and efficacy of the vaccine.

About 2 500 supplementary doses of vaccine were administered to cattle on several farms without a planned follow-up by the staff of the Tashkent laboratory. The general information collected from these farms did not contradict the results reported above.

#### 6 Immunogenic relationship between Uzbekistani and Israeli isolates

Controversial findings concerning the immunogenic difference of *T. annulata* isolates from geographically separated areas exist in the literature. The reported responses of cattle recovered from theilerial infection, to challenge with heterologous isolates range from acute disease to no clinical response.

In order to test the immunogenic relationship between the Uzbekistani and Israeli isolates, frozen stabilates from macerated ticks infected with sporozoites of the above isolate were each inoculated into a susceptible calf. Blood drawn from these calves during the acute stage of the theilerial infection was subinoculated into four susceptible calves for each isolate (Table 10). Ninety-two days after the primary infection, the calves were reinoculated with blood from the homologous or the heterologous isolate. Finally, calves that had received one or two inoculations with homologous isolate were challenged with homologous or heterologous stabilate (Table 11) containing sporozoites (used in the beginning of the trial). Susceptible control calves were added in the reinoculation and challenge infections.

All calves inoculated with blood infected with Israeli or Uzbeki isolate (Table 10) developed fever accompanied by schizonts in liver and parasites in blood films (the response to the primary inoculation is not shown in the table), but all recovered from the infection. Reinoculation of the recovered calves with blood from homologous or heterologous isolate caused no fever (except for one calf with 39.6°C) and no schizonts were seen in smears made from liver puncture material on day 14 after the infection. The rare erythrocytic merozoites occurring in the blood films were seen after the recovery from the primary infection. All susceptible control calves (three for each isolate) showed fever and parasites in liver and blood preparations.

Table 11 shows the results of challenge with sporozoites of calves that had previously been inoculated with blood from a single isolate. Schizonts were detected only in calves that received sporozoites from heterologous isolate. Average temperature above 39.5°C occurred for 1.6 to 2.5 days. Low levels of erythrocytic parasitemias, similar to those observed before the challenge were detected in blood films. The non-immunized calves suffered severe theileriosis and recovered following treatment with buparvaquone.

It appears from the above results that an appreciable cross-immunity existed between the Israeli and Uzbeki isolates. The immunity against the homologous tick-derived sporozoites was stronger than that against the sporozoites from the heterologous isolate. However, as mentioned elsewhere, attenuation of schizonts may accentuate immunogenic strain differences that exist among natural isolates (Pipano 1994).

According to literature data, most field isolates conferred a high degree of reciprocal immunity (Sergent et al. 1945, Gill et al. 1980, Pipano 1974). A previous investigation carried out by the Uzbekistani participant in this program (Rasulov 1977) showed that most autochthonous isolates from Central Asia protect against each other. It has been suggested that in nature a constant mixing and crossing of genetically differing parasite populations

occurs (Irvin and Boarer 1980), therefore, most isolates are likely to consist of mixtures of "strains" A noticeable immunogenic difference was reported between Israeli and Algerian isolates (Sergent et al 1945, Adler and Ellenbogen 1935, 1936) out of 25 calves recovered from an Algerian isolate and challenged by an Israeli isolate five died four suffered severe theileriosis and 16 showed a more feeble reaction Virulence of the parasite stock may play a role in immunization challenge experiments according to Sergent et al (1945) virulent parasites engender a stronger immunity than those which cause a mild infection In this context six out of 10 calves immunized with a field isolate of naturally low virulence induced partial immunity (six out of 10 exhibited clinical theileriosis, but none died) when challenged with a more virulent laboratory stock (Pipano et al 1974)

Laboratory *in vitro* techniques have been used in attempts to distinguish among different *T annulata* parasite populations isoenzyme electrophoresis revealed differences among schizonts from Turkish, Iranian and Indian isolates as well as among six isolates from Sudan (Melrose et al 1980, 1984) A series of monoclonal antibodies, reacting with intracellular macroschizonts, has been used to examine the level of antigenic diversity between and within stocks of *T annulata* The binding of the antibodies varied when tested against different stocks some monoclonal antibodies failed to react against a number of stocks and others recognized the macroschizonts of all stocks but revealed difference among stocks in their degree of antibody reactivity In addition to the antigenic variability between stocks variations have also been observed within the stocks, and this variability segregated when the stocks were cloned, indicating that the original stock consisted of several types of parasites (Shiels et al 1986) Based on up-to-date experience from anti-*T annulata* vaccination campaigns it appears that the diversity in field parasite populations detected by laboratory techniques is not necessarily related to immunogenic differences and, therefore, has a limited impact on immunization against theileriosis

**Table 10**  
**Reciprocal immunity conferred by virulent blood-derived schizonts from Uzbekistani and Israeli isolates of *T. annulata***

Number of calf	Inoculated with	Reinoculated with schizonts				
		Isolate	Response			
			Sch%	E m %	Max F	D F
312	Virulent schizonts Israeli isolate	Israeli	0	<1	39.2	0
318			0	<1	39.4	0
319		Uzbekistan	0	<1	39.0	0
321			0	<1	39.2	0
322	Virulent schizonts Uzbekistani isolate	Israeli	0	<1	39.4	0
323			0	<1	39.4	0
324		Uzbekistan	0	<1	39.6	1
326			0	<1	39.2	0
327	Non immunized	Israeli	10	21	41.3	6
328			1.5	5	41.2	4
340			<1	<1	39.8	1
329	Non immunized	Uzbeki	5	24	42.0	8
330			12	15	41.5	6
341			3.5	7	40.8	3

Sch=schizonts in liver, E m=Erythrocytic merozoites

Max F = Maximum temperature, D F =Days with temperature over 39.5°C

0=no parasites or fever detected

Specific anti-theileria antibody in cattle used for immunization-challenge trials were assessed by the indirect fluorescent antibody technique (Pipano and Cahana 1969) using culture-derived schizonts as antigen. The antibody response to the schizont and sporozoite inoculations with Uzbekistani and Israeli isolates are summarized in Table 12. All animals had titers of 1:4 (log<sub>1</sub>) before the beginning of the experiment (not shown in the tables). Two months after the first inoculation with schizonts considerable antibody levels were measured in all calves. A similar average level of antibody was found after the second inoculation. A more significant rise was found following the inoculation of sporozoites. Except for one calf (318) the antibody level after the sporozoite challenge was higher (one or two fourfold dilutions) than the level after the first schizont inoculation. Although antibodies against *T. annulata* may not play a major role in the immunity against theileriosis the presence or rise

of antibodies indicates that an immune response against the parasites has occurred in the vaccinated animal (Preston et al 1997)

**Table 11**  
**Response of calves immunized with virulent blood-derived schizonts from Uzbekistan and Israeli isolates of *T. annulata* to challenge infection with homologous and heterologous sporozoites derived from macerated ticks**

Number of calves	Immunized with schizonts	Challenge with sporozoites				
		Isolate	Response (average values)			
			Sch %	E m %	Max F	D F
3	Israeli	Israeli	0	<1	40	1.6
3	Uzbekistan	Israeli	<1	<1	40	2.0
2	Uzbekistan	Uzbekistan	0	<1	39.8	2.0
2	Israeli	Uzbekistan	<1	<1	40.1	2.5
2	Non-immunized	Israeli	24.5	20.5	41.3	7.5t*
2	Non-immunized	Uzbekistan	21.0	29	41.4	6.5t

Sch=schizonts in liver

E m =Erythrocytic merozoites

Max F =Maximum temperature

D F =Days with temperature over 39.5°C

0=no parasites detected

t\*=treated with buparvaquone

**Table 12**  
**Antibody levels in calves inoculated with Uzbekistan or Israeli isolates of *T. annulata* and challenged with homologous or heterologous sporozoites**

No. of calf	Inoculated with schizonts				Challenge with Sporozoites	
	First inoculation		Second inoculation		Isolate	Ab titers*
	Isolate	Ab titers*	Isolate	Ab titers*		
312	Israeli	4	Israeli	4	Israeli	6
318		5		5	Uzbekistan	5
319		5	Uzbekistan	5	Israeli	6
321		4		5	Uzbekistan	6
322	Uzbekistan	4	Israeli	4	Israeli	6
323		4		5	Uzbekistan	5
324		4	Uzbekistan	5	Israeli	5
326		5		5	Uzbekistan	6
Average		4.4		4.7		5.6

Antibody titer log<sub>4</sub> where 1.4=1, 1.16=2 etc

## VI. Impact, Relevance and Technology Transfer

Since theileriosis is considered to be one of the economically most important diseases in Uzbekistan it is expected that prophylactic immunization with the antitheilerial vaccine will have a considerable beneficial effect on the cattle industry in this country. The vaccine will probably be effective also for vaccinating of cattle in the countries surrounding Uzbekistan. The decrease in losses caused by theileriosis in high-grade cattle will encourage the breeding of improved stock. Small farmers' units that do not possess the infrastructure for application of anti-tick control measures like dipping etc. could protect their animals by a single administration of vaccine. Immunity induced by the vaccine would then be reinforced by natural infection in the field without losses of production in the vaccinated animals.

Despite the research on theileriosis that was conducted in the past by the Soviet scientists from the All-Union Research Institute in Moscow, *in vitro* cultivation and immunological studies of *T. annulata* were not performed in Uzbekistan before the present project. The Uzbekistani research personnel are showing interest and support in economically beneficial subjects of research since the latter may attract the interest of local institutions concerned with economic development. Among the staff involved in the joint program a veterinarian Dr. Nina Pak Den Sun revealed a considerable capability in applying laboratory techniques related to the development of the antitheilerial vaccine. It appears that she will be one of the leading scientists in future research on tick-borne diseases aimed at developing effective methods for their control.

The modified theilerial strain, TaV-219 will be used in the future for production of antitheilerial vaccine, initially for application within Uzbekistan. Successful results of mass vaccination will probably expand the use of the vaccine in the surrounding countries.

The project has had a beneficial effect on the infrastructure of the Tashkent laboratory. Several items of nonexpendable equipment – among the most important, a roller apparatus

incubator refrigerated centrifuge, inverted microscope, laminar flow sterile hood, liquid nitrogen containers – were acquired with the support of the project. All non-expendable and expendable items were acquired by the Bet Dagan laboratory and sent to Uzbekistan. Although this arrangement added an extra cost for transport, it was justified by guaranteeing that only equipment adequate for the research was acquired. With this equipment several tens of thousands of doses of vaccine can be prepared, but such quantities are still insufficient for vaccinating of most of the newborn cattle in Uzbekistan. Furthermore, the Tashkent laboratory has an insufficient infrastructure of supporting services such as supply of deionized (distilled) water, sterilization of glassware, etc. Also, diversion of this equipment to production of vaccine by this laboratory would have a negative impact on its involvement in research activities. However, at present, adequate support for research into tick-borne diseases can be expected only from international sources.

The management staff of the large farms have great interest in using the antitick vaccine, and requests for production of several hundred to several thousands of doses for "special" use are received by the laboratory. Although it is not yet clear who will produce the vaccine in the future, it seems that more attention to these issues will be given by the relevant authorities after having received the official approval of the vaccine for mass application.

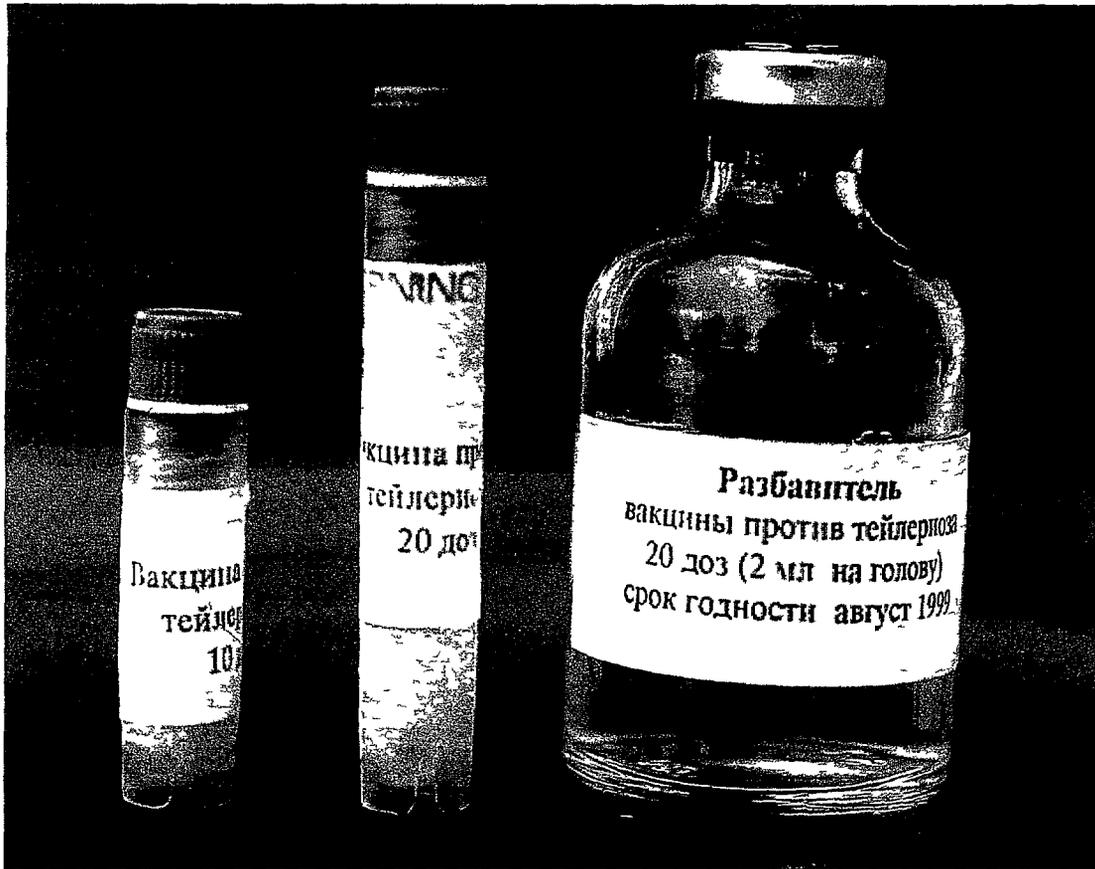
## **VII. Project Activities/Outputs**

- 1 The Israeli investigators, E. Pipano and V. Shkap, paid four visits to the Tashkent laboratory each time for three weeks.
- 2 The Uzbekistani counterparts Prof. Rasulov and Prof. Azimov paid four visits to the Bet Dagan laboratory in Israel each time for 2-4 weeks.
- 3 A request for a license for use of anti-tick vaccine in Uzbekistan was submitted to the relevant authorities (see copy of application attached).

- 4 A request for a patent on the modified theilerial strain TaV-219 was submitted in Uzbekistan (see copy of application attached)
- 5 Results related to attenuation of the virulence of Uzbekistani *T. annulata* isolates were presented in Punjab, India in the International Veterinary Immunology Symposium No 8-13, 1998 Papers in preparation will be released after the decision of the Committee for Patents in Uzbekistan

## **IX. Future Work**

Tick-borne diseases are considered to be the main veterinary and economic problem in the cattle breeding industry in Uzbekistan. It is expected that the anti-theilerial vaccine will considerably decrease the incidence of theileriosis among the immunized anti-theilerial herds. As a result another tick-borne disease – bovine babesiosis caused by two *Babesia* species (*B. bovis* and *B. bigemina*) - will become an important cause of cattle morbidity and mortality. Further research on tick-borne diseases in Uzbekistan should target bovine babesiosis with the main efforts directed at the development a culture-derived vaccine against the two *Babesia* species occurring there. Most of the existing infrastructure in the Tashkent laboratory is adequate for this research objective. The continuing cooperation of the Uzbekistani and the Israeli teams will also support the practical application of the anti-theilerial vaccine resulting from the present project.



Experimental anti-theilerial vaccine and diluent

Application for a patent in Uzbekistan  
for the *Theileria annulata* TaU219 strain

Форма ИИ -1-98

(22) Дата поступления	Входящий №	(21) № госрегистрации
Приоритет	(51) МПК	
<p><b>ЗАЯВЛЕНИЕ</b> о выдаче патента предварительного патента на изобретение (необязательно зачеркнуть)</p> <p>Нижесоподписавшийся (еся)</p> <p>(71) Заявитель(и) <b>Пиано Евгений Шкап Варда Расулов Ильхом Хасанович</b></p> <p>Представляю указанные ниже документы, прошу(просят) выдать патент предварительный патент (необязательно зачеркнуть) на имя</p> <p><b>Пиано Евгений Шкап Варда Расулов Ильхом Хасанович</b></p> <p>(указывается полное имя или наименование и местожительство или местонахождение заявителя и лица на чье имя испрашивается охраняемый документ. Данные о местожительстве авторов-заявителей принимаются в графе с кодом 97)</p>		<p>В Государственное патентное ведомство Республики Узбекистан 700047 г Ташкент ул Луйтепа, 2а</p> <p>Код организации, предприятия по ОКПО (если он ус ановлен) Код страны по стандарту ВОИС ST 3</p>
<input type="checkbox"/> Прошу (просим) установить приоритет изобретения по дате <ul style="list-style-type: none"> <li><input type="checkbox"/> подачи первой(ых) заявки(ок) в стране-участнице Парижской конвенции</li> <li><input type="checkbox"/> поступления более ранней заявки в Патентное ведомство</li> <li><input type="checkbox"/> поступления по кдественной заявки в Патентное ведомство</li> <li><input type="checkbox"/> поступления дополнительных материалов к более ранней заявке</li> </ul> <p>(Заполняется только при испрашивании приоритета более ранней чем дата поступления заявки в Патентное ведомство)</p>		
(31) № первой более ранней тойдественной заявки	(32) Дата испрашиваемого приоритета	(33) Код страны подачи по ST 3 (при испрашивании конвенционного приоритета)
1. —		
2.		
3.		
<p>(54) Название изобретения</p> <p>На русском языке <b>Штамм <i>Theileria annulata</i> TAU - 219 пригодный для изготовления вакцин</b></p> <p>На узбекском языке <b>Вакцина тайерлаш учун яратилган <i>Theileria annulata</i> TAU - 219 штамми</b></p> <p>Шифр проблемы (ткмы) ГКНТ и т а _____</p>		
(74) Патентный поверенный (полное имя, регистрационный номер местонахождение)		
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(98) Адрес для переписки (полный почтовый адрес имя или наименование адресата) г Ташкент, 700179, ул. Нозимаханум, пр 1, д. 40		
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(72) Автор(ы) (фамилия, имя отчество должность ученая степень и место работы)	(97) Полный домашний адрес (область, район город улица, дом для иностранцев код страны по стандарту ВОИС ST.3)	Подпись(и) автора(ов) дата
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# Application for a licence for the production and use of anti-theilerial vaccine in Uzbekistan

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## ШТАММ THEILERIA ANNULATA TAU-219, ПРИГОДНЫЙ ДЛЯ ИЗГОТОВЛЕНИЯ ВАКЦИНЫ

6 A 61 K 39/00

Изобретение относится к области ветеринарии и паразитологии и может найти применение в производстве вакцинных препаратов для профилактики тейлериоза крупного рогатого скота

Тейлериоз является наиболее опасным и тяжелым заболеванием региона жаркого климата центральной Азии и особенно в Узбекистане. Ущерб, наносимый тейлериозом, велик и выражается в падеже больных животных, потере всех видов продуктивности. Возникает большая опасность заболеваемости и смертности для вноса, завозимого для племенных целей скота.

В зонах неблагополучных по этой болезни практически болеет весь крупный рогатый скот. Чтобы предотвратить ущерб и сохранить животных от болезни необходимо проводить регулярные обработки их ядохимикатами. Широкое применение ядохимикатов обусловлено загрязнением окружающей среды ядохимикатами.

Выделение остатков ядохимикатов с продуктами является препятствием для обработки животных.

И только разработка метода аттенуации *Theileria Annulata* в культуре тканей, не нарушая экологического равновесия окружающей среды и продуктов животных, может обеспечить надежную профилактику животных от тейлериоза.

Испытание жидкой культуральной противотейлериозной вакцины ВИЭВ при иммунизации молодняка 6-18 месячного возраста подкожно в дозе 1 мл позволило предохранить крупный рогатый скот от тейлериоза. Степанова Н И, Заблоцкий В Т и другие.

- Ж. «Ветеринария» №3, 1987, стр 3-8

Недостатком штамма, из которого приготовлена вакцина, является то, что он не гарантирован полной аттенуацией и не исключается возможность его реверсии в исходное вирулентное состояние.

Наиболее близким к заявляемому штамму является штамм *Th Annulata* включающий культивирование возбудителя тейлериоза, изучение его биологии, использование культуральной биомассы для приготовления средств профилактики и диагностикумов. Для выделения возбудителя животных убивали на разных этапах развития болезни (инкубационный период, острый период болезни и во время паразитоносительства). Приготовленные различными способами культуральные антигены испытывались в качестве диагностикума для выявления больных тейлериозом животных и паразитоносителей. З П. Мутузкина «Культивирование и морфологическая характеристика инвазированных *Th annulata* клеток. Антигенные свойства паразита в РДСК // - Бюлл ВИЭВЮ1977, вып 31, стр 13-18

Недостатком штамма, полученным в различных географических зонах Советского Союза является и то, что штаммы выделены из клещей не адаптированных к местному среднеазиатскому региону, занимали иную экологическую нишу.

Задача изобретения - получение штамма *Th annulata*, пригодного для изготовления вакцины.

Поставленная задача решается штаммом *Th annulata* TAU-219, пригодным для получения вакцины.

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Штамм *Th annulata* TAU-219 выделен из клещей среднеазиатского региона в 1996 году (Расулов И.Х., Пилано Е., Шап В.) и относится к семейству Theileriidae, роду *Theileria* виду *Theileria annulata*

Штамм имеет индекс TAU-219 и хранится в Лаборатории паразитологии Института зоологии АН Республики Узбекистан под №219

#### ПРИЧИННО – СЛЕДСТВЕННАЯ СВЯЗЬ

Штамм *Th annulata* TAU-219 выделен в условиях Узбекистана из местных видов клещей рода *Hyalomma* вид *Hyalomma anatolicum*

Благодаря, использования этого штамма, включающего в себя все биологические основы возбудителя тейлероза может найти применение при разработке новых профилактических средств от тейлериоза крупного рогатого скота

Штамм *Th annulata* TAU-219 имеет следующие биологические свойства

Морфологические свойства - изучены на суспензионных инвазированных тейлериям лимфоидных клетках, при этом суспензию клеток высевают в пластиковых колбах для тканевых культур, которые извлекают в разные сроки культивирования, фиксируют и окрашивают по Романовскому - Гимза

Авторы впервые изучили параллельно с этой микроскопией фиксированных мазков и модификацию метода, а именно – жизнеспособность лимфоидных клеток изучали микроскопически в нативной культуре, что позволило иметь истинную картину наблюдения за живыми клетками тейлерий

При исследовании мазков –отпечатков из органов больного тейлериозом животного выявлено значительное изменение соотношения клеточных элементов - усиление гиперпластических процессов в лимфоидных органах и увеличение содержания крупных клеток с обширной цитоплазмой и с ядром четкой структуры. В цитоплазме многих пролиферирующих клеток находились гранатные тела *Th annulata* TAU-219

На представленном ниже Рисунке 1 отчетливо видны клетки культуры *Th annulata* TAU-219 слева - А – фигура - клетки лимфоцитов/лейкоцитов и В – фигура - шизонты в культуре клеток.



« Interrupted Transmission »

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В процессе длительного культивирования в большом количестве наблюдаются формы, двухядерные, многоядерные и гигантские клетки. Отличался и фагоцитоз – при длительном культивировании крупные многоядерные клетки фагоцитируют клетки меньших размеров.

Деление клепок и гранатных тел *Th annulata* TAU-219 происходит одновременно, проходя стадии клеточного деления в дочерних клетках остаются одинаковые гранатные тела.

### КУЛЬТУРАЛЬНЫЕ СВОЙСТВА

С момента прикрепления клещей к коже животного возникают изменения в коже, затем в регионарных лимфатических узлах. Появляются очаговые пролифераты в печени, селезёнке, почках с последующей реакцией всех внутренних органов. В это время формируются гранулематозные образования, а стенки сосудов подвергаются набуханию, затем некрозу. Происходит инвазирование эритроцитов тейлериями. При культивировании гранатных тел *in vivo* установлен активация и пролиферация недифференцированных клеток и по нахождению в них гранатных тел практически во всех органах животного можно судить о его заражении.

Саму культуру *Th annulata* TAU-219 можно получить из селезёнки и лимфатических узлов, взятых от больных тейлериезом телят, которые были покусаны клещами *H. anatolicum* или *H. detritum*.

### ПАТОГЕННЫЕ СВОЙСТВА *Theileria annulata* TAU-219

Штамм сохраняет свои инвазионные свойства при температуре минус 70 и 79 °C, культура, "гранатных тел" тейлерий в замороженном состоянии (минус 196 °C) способна сохранять инвазионные свойства паразита.

Для получения перевиваемых суспензионных лимфоидных культур, инвазированных тейлериями, исходный материал (органы лимфатической системы) необходимо брать от животных в период острого течения болезни.

Размножение гранатных тел суспензионных лимфоидных культур происходит синхронно с митотическим делением поражённой клетки. Специфичность антигенных свойств культуральных тейлерий не изменяется в процессе их длительного пассирования.

### СТАБИЛЬНОСТЬ БИОЛОГИЧЕСКИХ СВОЙСТВ *Th annulata* TAU-219 В ПРОЦЕССЕ ДЛИТЕЛЬНОГО ХРАНЕНИЯ

Инвазионный материал сохраняемый при температуре минус 196 °C в течение трех лет сохранял жизнеспособность в размороженном состоянии, что определялось методом субвитаального окрашивания раствором трипановой сини. Около 85% лимфоидных были жизнеспособны и сохраняли инвазионные свойства.

Изобретение иллюстрируется примерами по использованию  
Штамма *Th annulata* TAU-219

#### Пример № 1

Штамм *Th annulata* TAU-219 выделяется из клещей *H. anatolicum* и *H. detritum* среднеазиатского региона. Штамм культивируют в лимфоидной ткани, а затем пассируют в среде, состоящей из среды Лейбовича с добавлением 20% сыворотки телёнка пятидневного возраста. Субкультивирование проводили через каждые 4-5 дней, до концентрации клеток 3-5 миллионов в одном миллилитре с пересевом в новые колбы.



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## РЕФЕРАТ

- Использование - паразитология, ветеринария
- Задача - получение нового штамма *Theileria annulata* TAU-219 ,  
пригодного для получения (изготовления) вакцины
- Сущность - предлагается новый *Theileria annulata* TAU-219 в качестве  
основы для получения вакцинных препаратов

### ФОРМУЛА ИЗОБРЕТЕНИЯ

Штамм *Theilera annulata* TAU-219 пригодный для изготовления вакцины

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