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# ANNUAL PROGRESS REPORT (1)

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TITLE: "CELL CULTURE-DERIVED VACCINE FOR BOVINE ANAPLASMOSIS"

COVERING THE PERIOD FROM

JANUARY 2003 TO JANUARY 2004

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## EXECUTIVE SUMMARY

***Developmental***: To major goal of the project is to develop an effective live culture-derived vaccine against bovine anaplasmosis, one of the major and economically important diseases, which constitute a significant limiting factor for cattle development in vast tropical and subtropical regions of the world, including South Africa, the USA and Israel.

***Scientific:*** An effective vaccination against anaplasmosis is based on immunization with live cattle-derived *A. centrale*, which differs from *A. marginale* morphologically, antigenically and genetically. The main objective of the research is to establish tick cell cultures for continuous propagation of *A. centrale*, this in order to provide a basis for the development of a cell culture derived vaccine against bovine anaplasmosis in order to replace the existing blood-derived vaccine.

## SECTION I

### A) RESEARCH OBJECTIVES IN YEAR UNDER REVIEW:

The major objectives of the first year were as follows:

(1) To transfer the tick cell culture technology to OVI and KVI.

(2) To establish *A. marginale* and infections in cattle and prepare infected blood stabilates at both OVI and KVI.

(3) Perform tick studies to confirm infection of ticks by transmission of *A. marginale* isolates from the U.S. and Israel and *A. centrale* to susceptible cattle and by PCR, and microscopy of tick gut and salivary glands.

(4) To establish *Anaplasma marginale* and *A. centrale* infections in the tick cell cultures and to prepare cell culture stocks for storage in liquid nitrogen.

(5) To develop PCR assays specific for detection of *A. marginale* and *A. centrale* in infected bovine blood, ticks and cultured tick cells.

(6) Provide training for Mr. Sibusiso Mtshali, Graduate Student from the University of the Free State, Qwa Qwa Campus.

## B) RESEARCH ACCOMPLISHMENTS

**Description of the Work Activities:**

1. **Transfer the tick cell culture to OVI and KVI.**

   The IDE8 tick cell line was transferred to OVI and KVI in January 2003. Training was provided by Dr. E. Blouin of Oklahoma State University at both OVI and KVI on the preparation of the complex cell culture medium, propagation and handling of the cells, techniques for freezing the cells and preparing cell stocks for storage in liquid nitrogen and preparation of cell smears for examination by light microscopy. A second tick cell line, ISE6, was transferred to and established at KVI in January 2004.

   Uninfected IDE8 cells were successfully adapted to laboratory conditions at OVI and KVI. The cells are maintained at 32°C in L-15B medium. The cells are now subcultured at a ratio of 1:3 and 1:5. During continious subcultivation confluent monolayers were frozen in liquid nitrogen or in a -80°C freezer using a 5% DMSO freezing medium.

2. **To establish *A. marginale* and *A. centrale* infections in cattle and to prepare infected blood stabilates at both OVI and KVI.**

   Cattle were splenectomized at KVI and infected with either the Virginia isolate of *A. marginale* or the Israel strain of *A. centrale*. Blood was collected aseptically and used to prepare stabilates to (1) serve as parasite stocks and (2) to use as inoculum for tick cell cultures. In addition, splenectomized calves were also inoculated with a tailed *A. marginale* isolate or a non-tailed Israel isolate, both of which were isolated recently from the field.

3. **Establish *A. marginale* and *A. centrale* infections in the tick cell cultures.**

IDE8 cells infected with the Virginia isolate of *A. marginale* were established at KVI. The establishment of cultures with *A. marginale* was necessary to use as controls for PCR studies. Examination of infected cultures also provide infected cell culture material for comparison with cultured cells that were exposed to *A. centrale*. At present, attempts to infect tick cells lines *with A. centrale* were not successful in both OVI and KVI laboratories.

*A. marginale* of Israeli tailed strain (IDE8-AmIT) has been successfully propagated and maintained in the IDE8 cells. Frozen stocks of the infected cells were prepared and kept until use in liquid nitrogen. Similarly, ISE6 cells, another tick cell culture line, was infected with the AmIT strain. Comparatively slow growth of the *A. marginale* was observed with the new line, a few subcultures were performed in which the organisms were detected in the Cytospin Giemsa-stained preparations the infected monolayer. The IDE8-AmIT cell line was transferred to South Africa to serve a control for initiation of S. African *A. marginale* cultured cells line.

4. **Develop PCR assays specific for detection of *A. marginale* and *A. centrale* in infected bovine blood, ticks and cultured tick cells.**

Specific PCR assays to detect and differentiate *Anaplasma marginale* from *A centrale* DNA in blood of infected cattle have been developed. During the last year specific assays to detect *Anaplasma* infection in ticks and cultured cells were applied to detect DNA in 3 tick species samples (gut tissues and salivary glands) including *Boophilus annulatus*, *Rhipicephalus sanguineous* and *Hyalloma excavatum*. The ticks were obtained from cattle after acquisition and transmission feeding experiments. The primers used for *A. marginale* PCR assays were based

on published sequences of *msp1a* flanking the conserved 5' repeat region. To specifically amplify *A. centrale msp2* cistron primers were based on published sequences (GenBank accession number AY132307).

As shown in Fig 1, amplification of specific *A. marginale* DNA was obtained from *Rhipicephalus* and *Hyalloma* tick gut tissues collected after acquisition feeding on *A. marginale* (Virginia strain– infected calf).

Following transmission feeding, *A. centrale* DNA was amplified from *Rhipicephalus* and *Hyalloma* salivary gland tissues (Fig. 2). The amplified fragments were of the expected molecular size, and were similar to the band observed with specific DNA from infected blood, which were run in parallel in agarose gels.

5. **Perform tick studies of *A. marginale* and *A. centrale* to confirm infection of ticks by transmission of *A. marginale* and *A. centrale* to susceptible cattle and by PCR and microscopy of tick gut and salivary glands.**

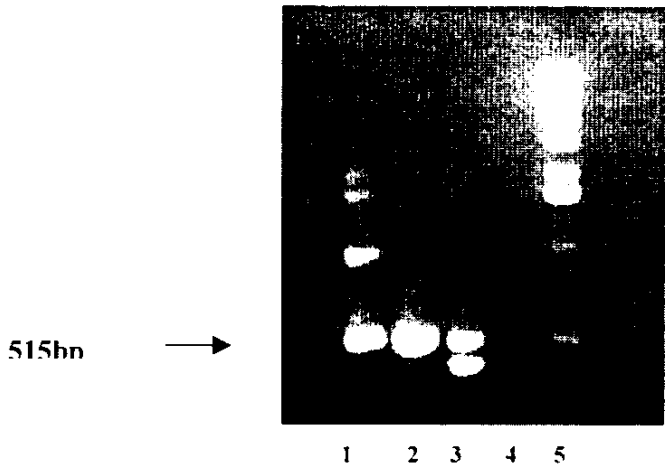
Experiments were conducted to determine whether *A. centrale* was infective for ticks. In additional isolates of *A. marginale* (U. S. Virginia isolate, Israel tailed (T) and non-tailed (NT) were included in these studies to serve as positive controls, thus confirming that the three tick species were susceptible to *Anaplasma* spp. infection. The question of whether *A. centrale* is infective for ticks is fundamental to our attempts to infect tick cells cultures. Previous research has shown that infection of ticks and cell cultures is dependent on the ability of the *A. marginale* major surface protein (MSP) 1a to adhere to tick cells. Isolates of *A. marginale*, which have proved to be non-infective for ticks, have also had a MSP1a that was not adhesive for tick cells. If the results these tick transmission studies demonstrate that *A. centrale* is not infective for and transmissible by ticks, it will be unlikely that *A. centrale* can be propagated in cell culture. If *A. centrale* is transmitted by at least one of these three tick species, then our efforts to incorporate various means of infection of the tick cell cultures will be warranted and eventual success is probable. At present MSP1a has not been identified on *A. centrale*.

As shown in Fig. 4, PCR amplifications performed on DNA samples obtained from calves infected with Israeli strains of *A. marginale*, tailed and non-tailed (AmIT and AmINT), from total DNA extraction from ticks that were fed on the infected calves, and from calf blood after transmoission feeding . Invariance of the *msp1a* genotype of repeat region amplicons was observed during 1) acute calf infection, 2) acquisition-fed ticks and in 3) blood of calves infected by ticks transmission.

Cattle experimentally infected with *A. marginale* or *A. centrale* were infested with three species of male ticks: (1) *Boophilus annulatus*, (2) *Rhipicephalus sanguineus* and (3) *Hyalomma excavatum*. After the ticks were allowed to feed and acquire infection for 6 days, they were removed and held in a humidity chamber. After 4 days of being held each species of ticks from each of the two cows was allowed to transmission feed on a susceptible splenectomized cow in order to test for the ability of the ticks to transmit *A. marginale* or *A. centrale*. Tick gut tissues were collected on the 4$^{th}$ day after the ticks were removed from the acquisition feeding cattle and salivary glands were collected from ticks as the end of transmission feeding on susceptible cattle. These tissues were fixed for both PCR and light and electron microscopy studies.

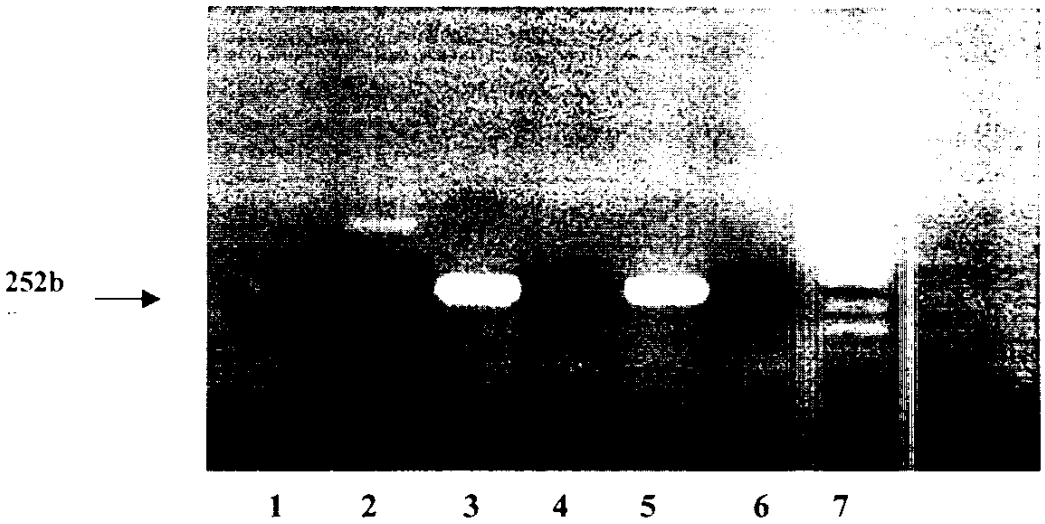
The experimental design in Figure 5 compares tick transmission of the Israel tailed (AmIT) and nontailed *A. marginale* (AmINT) isolates in the three tick species, while Figure 6 is the experimental design for comparison of the tick transmission of the Virginia isolate of *A. marginale and A. centrale.*

**Figure 1. DNA from gut tissues from three tick species fed on *A. marginale* (Virginia strain) infected calf (#603)**



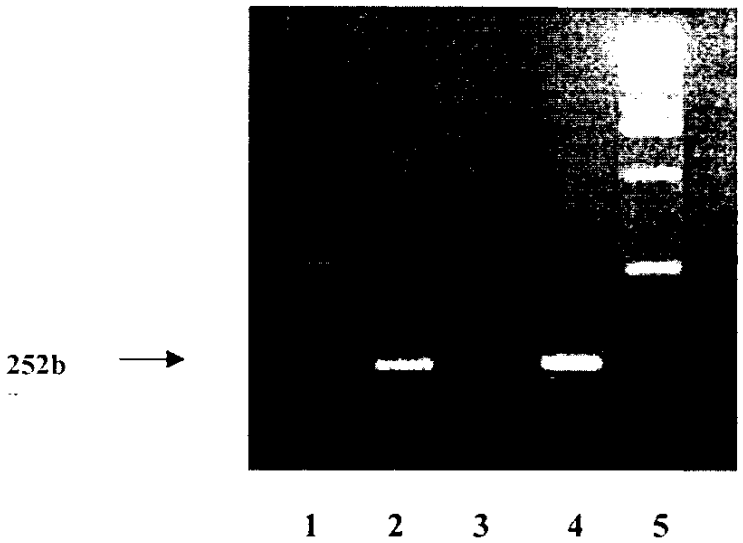

**Lane 1 (*Boophlus annulatus*; Lane 2 (*Rhipicephalus sanguineus*); Lane 3 (*Hyalomma excavatum*); Lane 4 (negative control without DNA sample), Lane 5 (1kb DNA ladder molecular size markers ladder)**

**Figure 2. DNA from gut tissues from three tick species fed on *A. centrale* infected calf (#592)**



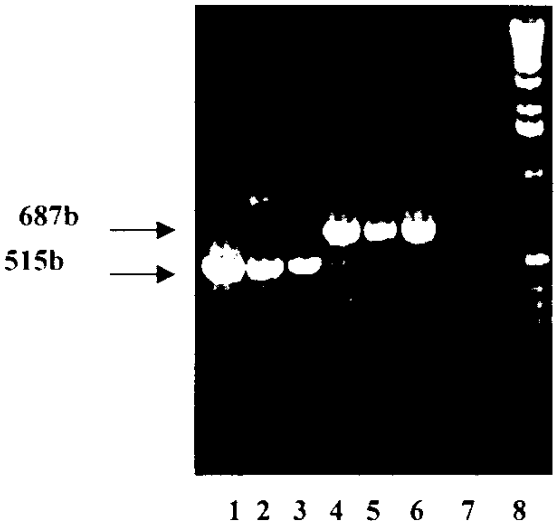

**Lane 1 (midgut from *Boophlus annulatus)*; Lane 2 (midgut from *Hyalomma excavatum*); Lane 3 (midgut from *Rhipicephalus sanguineus*); Lane 4 (*Boophilus annulatus* salivary gland); Line 5 (*A. centrale* DNA from blood sample); Lane 6 (no DNA template); Lane 7 (1 kb ladder molecular size marker)**

**Figure 3. PCR amplified DNA *(msp2* operon) from salivary glands from two tick species transmission-fed on a calf infected with *A. centrale* (calf # 592)**




**Lane 1, *Hyalomma excavatum;* Lane 2, *Rhipicephalus sanguineus* , Lane 3, negative control, no DNA template, Lane 4, blood from *A. centrale* infected animal (#592) , Lane 5, 1kb DNA ladder**

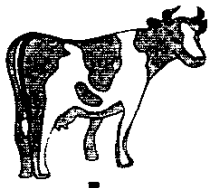
**Fig. 4. PCR analysis of genomic DNA of *A.marginale msp1a* in ticks after acquisition-feeding for 3 days on calf (# 590) and transmission feeding on calf # 621, infected with the Israeli *A. marginale* NT strain (AmINT); after acquisition-feeding on calf #591 and transmission feeding on calf # 622 infected with AmIT strain), compared to blood-derived DNA from the respective calves during acute rickettsemia after transmission by infected ticks.**

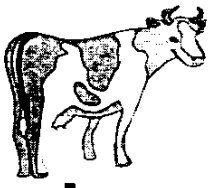



**Lane 1, blood from animal #590 infected with AmNT; lane 2, total DNA from AmNT infected *Rhipicephalus sanguineus;* Lane 3, blood from calf #621 infected with AmNT; Lane 4, blood from calf # 591 infected with AmT; Lane 5, total DNA from infected *Rhipicephalus sanguineus* ; Lane 6, blood from calf #622 infected with AmT; Lane 7, negative control ( no DNA template); Lane 8, molecular size marker, 1kb ladder .**

**Figure 5: The experimental design for testing infectivity of three species of ticks (*Boophilus annulatus*, *Rhipicephalus sanguineus* and *Hyalomma excavatum*) for *A. marginale* NT and *A. marginale* T from Israel.**

**PART I: Acquisition feeding of male *Hyalomma*, *Rhipicephalus* and *Boophilus* ticks on splenectomized calves infected with the Israel NT and T isolates of *Anaplasma marginale*.**





**Splenectomized Calf No. 590:**

Inoculate with blood stabilate infected with the NT Israel isolate of *A. marginale*.

**Splenectomized Calf No. 591:**

Inoculate with blood stabilate infected with the T Israel isolate of *A. marginale*.

**Calf 590 infested with ticks when the calf became parasitemic with the NT Israel *A. marginale*:**

| Tick Species | No. Ticks Infested |
|---|---|
| *Boophilus annulatus*: | Larvae from 1gm eggs |
| *Rhipicephalus sanguineus*: | 40 ♂ 20 ♀ |
| *Hyalomma excavatum*: | 40 ♂ 20 ♀ |

**Calf 591 infested with ticks when the calf became parasitemic with the T Israel *A. marginale*:**

| Tick Species | No. Ticks Infested |
|---|---|
| *Boophilus annulatus*: | Larvae from 1gm eggs |
| *Rhipicephalus sanguineus*: | 40 ♂ 20 ♀ |
| *Hyalomma excavatum*: | 40 ♂ 20 ♀ |

**All ticks were allowed to feed for 7 days, after which they were removed from the cattle.**

## Part II: Removal of ticks and Held Period:

All ticks were removed and held in a humidity chamber for 4 days, after which each species of ticks was allowed to transmission feed on a susceptible calf.

## PART III: Transmission Feeding Studies on Susceptible Cattle

Transmission feeding of male *Hyalomma*, *Rhipicephalus* and *Boophilus* ticks that were allowed to acquisition feed on calf No. 590 infected with the NT Israel isolate of *A. marginale* or calf No. 591 infected with the T Israel isolate of *A. marginale.*

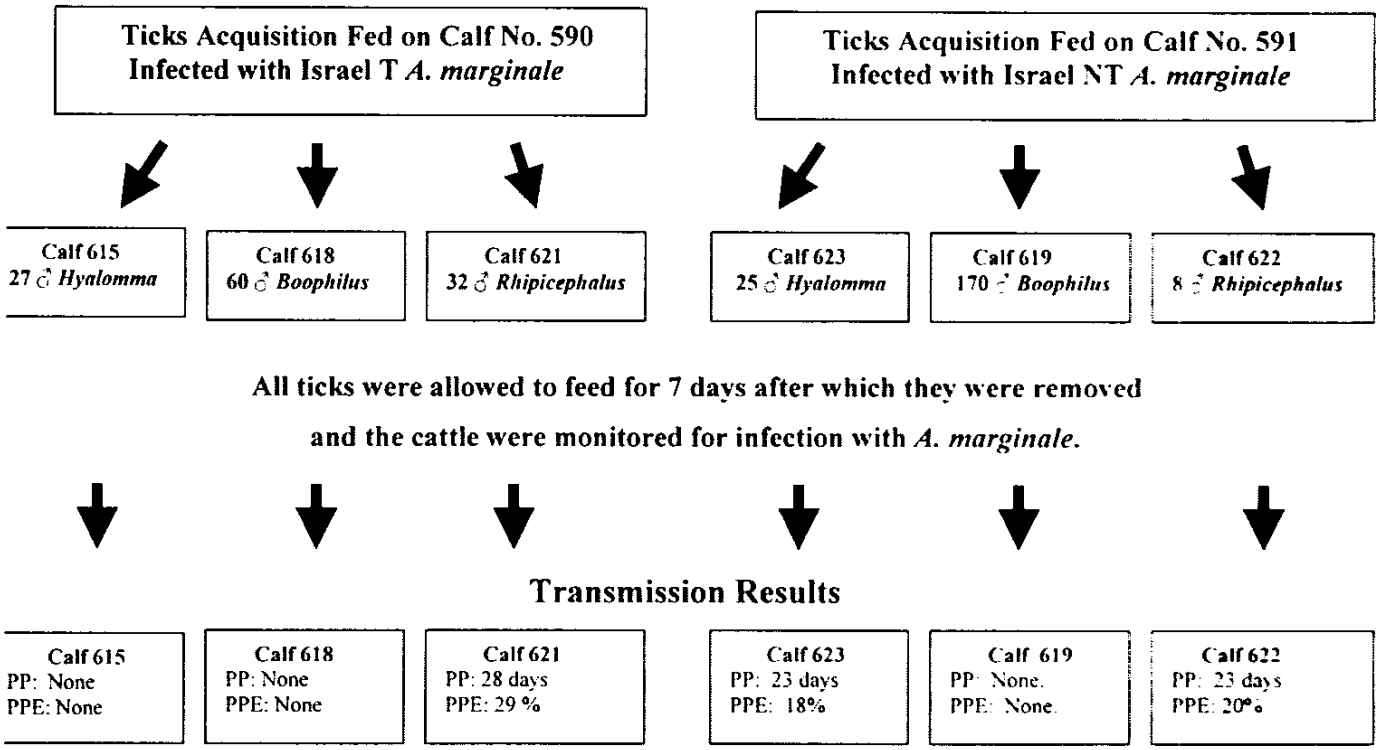
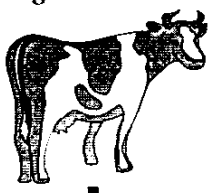
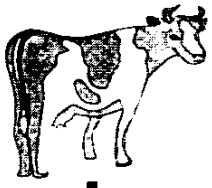


**Figure 6: The experimental design for testing infectivity of three species of ticks (*Boophilus annulatus*, *Rhipicephalus sanguineus* and *Hyalomma excavatum*) for *A. marginale* and *A. centrale*.**

**PART I: Acquisition feeding of male *Hyalomma*, *Rhipicephalus* and *Boophilus* ticks on splenectomized calves infected with the Virginia isolate of *Anaplasma marginale* or the Israel vaccine train of *Anaplasma centrale*.**





**Splenectomized Calf No. 603:**

Inoculate with blood stabilate infected with the Virginia isolate of *Anaplasma marginale*.

**Splenectomized Calf No. 592:**

Inoculate with blood stabilate infected with the Israel vaccine isolate of *Anaplasma centrale*.

Calf 603 infested with ticks when the calf became parasitemic with *A. marginale*:

| Tick Species | Parasitemia During Tick Feeding |
|---|---|
| *Boophilus annulatus* | 1.5 to 28.0 |
| *Rhipicephalus sanguineus* | 8.0 to 23.0 |
| *Hyalomma excavatum* | 8.0 to 23.0 |

Calf 592 infested with ticks when the calf became parasitemic with *A. centrale*:

| Tick Species | Parasitemia During Tick Feeding |
|---|---|
| *Boophilus annulatus* | 2.5 to 15.0 |
| *Rhipicephalus sanguineus* | 8.0 to 30.0 |
| *Hyalomma excavatum* | 8.0 to 30.0 |

**All ticks were allowed to feed for 6 days, after which they were removed from the cattle.**

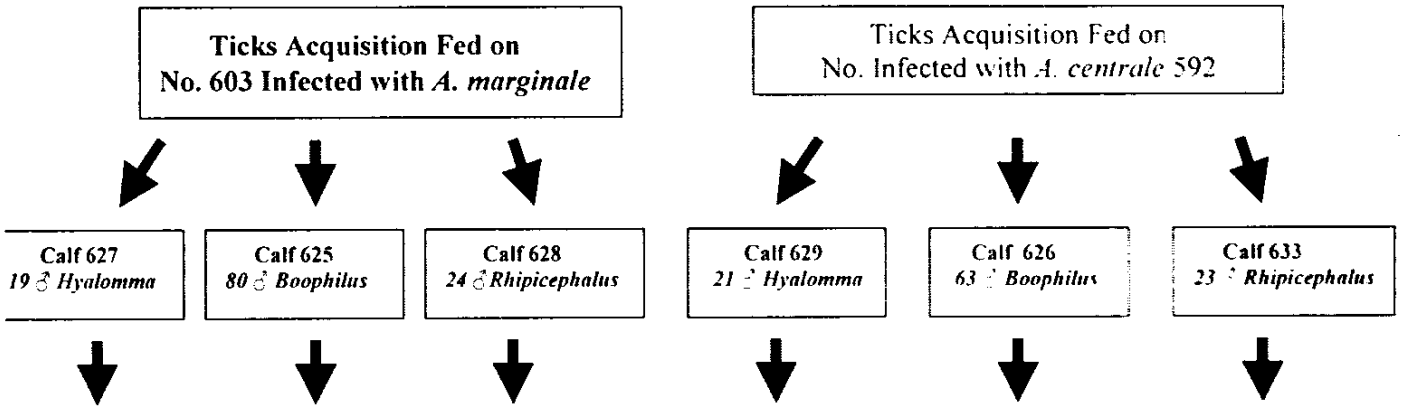
## Part II: Removal of ticks and Held Period:

All ticks were removed and held in a humidity chamber for 4 days, after which they were allowed to transmission feed. For *R. sanguineus* and *H. excatavum*, 10 ticks for each tick species were dissected for collection of gut tissues. The guts from ½ tick were pooled in 500 ml RNALater and the other ½ tick was fixed in glutaraldehyde fixative for LM and

EM studies. The numbers of *Boophilus* ticks were too low and therefore not available for microscopy studies.

## PART III: Transmission Feeding Studies on Susceptible Cattle

Transmission feeding of male *Hyalomma*, *Rhipicephalus* and *Boophilus* ticks that were allowed to acquisition feed on calf No. 603 infected with the Virginia isolate of *Anaplasma marginale* or calf No. 592 infected with the Israel vaccine strain of ***Anaplasma centrale.***




1. All ticks were allowed to feed for 6 days after which time they were removed.
2. Ticks were dissected and the salivary glands collected from ½ of the tick and pooled in RNALater, while the other half of the tick was fixed in glutaraldehyde for LM and EM studies.

*Boophilus* were removed from cow 625 and cow 626 on Friday, February 20:

**0** ♂ ticks were removed from Cow No. 625.

**4** ♂ ticks were removed from cow No. 626, the salivary gland dissected and placed in RNALater for PCR studies.

*Hyalomma* and *Rhipicephalus* ticks acquisition fed on cow No. 603 were removed from cow No.s 627 and 628, respectively, on Sunday, February 22, 2004

**16** ♂ *Hyalomma* ticks were removed for cow No. 627.

½ of each tick was fixed for LM and EM and the salivary gland from the other half was pooled for PCR (2 tubes of 8 salivary glands each).

**24** ♂ *Rhipicephalus* ticks were removed from cow No. 628.

½ half of each tick was fixed for LM and EM and the salivary gland from the other half was pooled for PCR (2 tubes of 12 salivary glands each).

*Hyalomma* and *Rhipicephalus* ticks acquisition fed on cow No. 592 (*A. centrale*) were removed from cow No.s 629 and 633, respectively, on Tuesday, February 24, 2004:

_**16**_ live *Hyalomma* ticks were removed for cow No. 629.

_**18**__ live *Rhipicephalus* ticks were removed from cow No. 633.

**6. Provide training for Sibusiso Mtshali, Graduate Student from the University of the Free State, Qwa Qwa Campus.**

A Ph.D. graduate student, Mr. Sibusiso Moses Mtshali, Qwa Qwa University, was identified and interviewed to fill the trainee position on the project. Mr. Mtshali was found to be well suited for this training position and he was enthusiastic about conducting research on tick-borne diseases of cattle. His graduate advisor, Dr. Peter Mbati, was present at the initial meeting at OVI., and became acquainted with the objectives of the CDR project.

Mr. Mtshali spent 6 weeks at KVI, January 25 to March 5. During this time he was provided training in PCR, tick transmission studies, tick dissection, and fixation of tick tissues for light and electron microscopy and serologic tests. He was provided

opportunity to meet with Drs. Shkap, Blouin and Kocan and had free access to interaction with KVI personnel. Reprints of key journal articles on anaplasmosis were provided to him and he was encouraged to read and discuss them.

## C) SCIENTIFIC IMPACT OF COLLABORATION

Collaborative activities involving scientists in the last year included visits by Prof. Kathy Kocan and Dr. E. Blouin of OSU to KVI, and by Dr. Varda Shkap and Mrs. L. Fish of KVI to the Parasitology Laboratory at the OVI, S. Africa. During the visits tick cells (Tick cell line IDE8) and cultivation methodology were transferred betweenOVI and KVI scientists. Plans for further work in S. Africa and for the introduction of a young South African scientist into the project were discussed with Dr. E. Zweygarth, Dr. A. Spicket and Dr. F. Potgieter. During recent months Dr. Eric Zwegyrth visited Dr. Lesley Bell's laboratory in Edinburgh, UK, where he received various tick cell lines in order to introduce *A. centrale* into these cells, since, up to now it could not be grown in IDE8 cells. Prof. Kocan, Dr. E. Blouin and Mr. Sibosiso Moses Mtshali from Qwa Qwa University visited the KVI for about a month, during the performance of the tick transmission studies reported here.

## D) DESCRIPTION OF PROJECT IMPACT

The central goal of the project is to develop a live and effective *centrale* culture-derived vaccine against bovine anaplasmosis. Tick cell cultures were shown to support propagation of the *A. marginale* pathogen. In addition, it is well documented in the scientific literature that *A. marginale* can be transmitted by about 20 different species. However, information concerning tick transmission of *A. centrale*, the strain used in the vaccine against *A. marginale* is limited, and *in vitro* cultivation of *A. centrale* has not been reported hitherto. The findings of the experimental work described in this report include new knowledge concerning the biology and transmission of *A. centrale*.

which will be used in the development of a novel culture-derived vaccine against bovine anaplasmosis.

## E) INSTITUTIONAL STRENGTHENING

With activation of the project the OVI and KVI laboratories gained the expertise to grow and maintain non-infected and *A. marginale*-infected tick cell lines. New cell lines, which are held by only a few laboratories in the world have been introduced in the OVI. Animal experimentation and tick transmission experiments were performed while a young scientist; Mr. Sibosiso Moses Mtshali from Qwa Qwa University visited the KVI. Ticks were dissected to enable infection of the guts and salivary glands to be examined by electron microscopy and PCR, and the "know how" were transferred.

## F) FUTURE WORK

The following are the goals for further work:

- ☒ To collect ticks in regions endemic for anaplasmosis in S. Africa, in order to perform tick acquisition and transmission studies with *A. centrale* and S. African *A. marginale* strains.
- ☒ To grow and maintain at least four different tick cells lines and to infect them with *A. centrale* and *A. marginale* (S. African and Israeli strains) in both OVI and KVI laboratories.
- ☒ To perform EM and PCR examination of ticks collected in Israel during the transmission studies.
- ☒ To continue the follow-up examinations of the calves that develop anaplasmosis in the course of the transmission studies: light microscopy of blood smears, PCR and serology will be used.

# SECTION II

## A) MANAGERIAL ISSUES

There are no special managerial issues

## B) BUDGET

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

## C) SPECIAL CONCERN

There are no special concerns.

## D) COLLABORATION, TRAVEL, TRAINING

The project was initiated at a meeting held from January 20 to February 14, 2003, with the participation of the P.I., Dr. Shkap, and co-P.I.s Drs. Kocan and Blouin. The meeting took place at Onderstepoort Veterinary Institute (OVI), where Dr. Erich Zweygarth and Mr. Arthur Spickett and staff will perform the research. The objectives and timetable were reviewed and a detailed plan was developed for the first year of the project. During this visit, the cells and cell culture technology were transferred to OVI. Cell cultures were delivered by hand, and several subsequent shipments were sent during this time to ensure the availability of adequate amounts of cells and medium to provide for production of initial cell stocks.

A student was selected and interviewed to fill the trainee position on the project, and was found to be well suited for this training position. Mr. Sibusiso Moses Mtshali, of Qwa Qwa University, will receive training through this project. His advisor, Dr. Peter Mbati, also joined the meeting in order to become acquainted with the objectives of the project.

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

F) There is no special request.