FINAL REPORT

Submitted to

US AGENCY FOR INTERNATIONAL DEVELOPMENT; Bureau for Economic Growth, Agriculture and Trade

(USAID CDR PROPOSAL TA-MOU-01-C210-027)

TITLE: "CELL CULTURE DERIVED VACCINE FOR BOVINE ANAPLASMOSIS"

COVERING THE PERIOD FROM
APRIL 2002 TO DECEMBER 2006

SUBMITTED BY:
DR. VARDA SHKAP (Principle Investigator)
KIMRON VETERINARY INSTITUTE, BET DAGAN, PO BOX 12, 50250
ISRAEL
Tel: 972-3-9681758; Fax: 972-3-9681678; Email: shkapv@int.gov.il (KVI)
and

Prof Kathy Kocan & Dr Ed Blouin
Oklahoma State University, Stillwater, USA, Tel: 405-744-7271; Fax: 405-744-5275; Email:
kmk285@okstate.edu (OSU)
And

Dr E. Zweygarth & A.M. Spickett
ARC-Onderstepoort Veterinary Institute, SOUTH AFRICA, and Tel: 27-12-5299-215;
Fax: 27-12-5299-434 (OVI)

Administration official: B Yakobson
Director, Kimron Veterinary Institute
PO Box 12, Bet Dagan 50250
Israel
Tel: 972-3-9681682; Fax: 972-3-9605293
Email: Dir_kimron@int.gov.il

C) Executive Summary

Among tick-borne diseases, bovine anaplasmosis caused by a rickettsial pathogen, *Anaplasma marginale*, is of significant economic importance, as billion losses to livestock production allover the world incur because of the disease. Anaplasmosis is characterized by severe hemolytic anemia, marked weight loss, decreased milk production, abortion and death rate of about 40% among infected cattle. The disease is transmitted by about 20 different tick species, mechanically and by biting flies, which explains its worldwide distribution. Although treatment with tetracycline compounds is effective, it should be administered upon proper diagnosis made on time, however, the incubation period stretches up to 2 months, and mostly when applied late in the infection is not effective. Moreover, it is well acknowledged that control of the vector is neither feasible, nor practical. To control anaplasmosis, in several regions of the world, including Australia, South Africa, Argentina and Israel, a prophylactic immunization is used for decades. The vaccine is comprised of live subspecies organism *A. centrale*, which is on naturally low pathogenicity compared to the field strains of *A. marginale*. The live blood-derived vaccine has serious disadvantages among which risk of spreading silent viral and bacterial pathogens and the use of animals as donors, limited application of such vaccination in most regions of the world.

Propagation of *A. marginale* in tick cells culture has been successfully achieved and encouraged research on development of a potent vaccine against bovine anaplasmosis based on culture-grown of the vaccine less pathogenic *A. centrale*. Tick cell cultures well established in the laboratory of Prof. K. Kocan at the OSU were transferred to the Kimron and the Onderstepoort Veterinary Institutes for initiation of *A. centrale* infection *in vitro*. Culture of Virginia *A. marginale* strain was introduce in all laboratories and served as a control for *in vitro* experiments focused on establishement of A. centrale infected cultures. The *A. marginale* of the Israeli strain was successfully propagated *in vitro*, while numerous attempts in Israel and in S. Africa to grow *A. cenrale* failed. The *A. marginale*- infected cultures retained their ability to infect cattle. Only on a single occasion a culture with transient *A. centrale* infection was obtained, but after only a few subcultures *A. centrale* did not harm the tick cell monolayer, and later disappeared from the cultures. Other the IDE8 and ISE6 lines, other tick cells were obtained from Dr. L.

Bell-Sakyi (Centre for Tropical Veterinary Medicine [CTVM], University of Edinburgh, Scotland, UK), but these failed to support growth of *A. centrale* , as before. The failure to infect tick cell cultures with *A. centrale* led to fundamental questions whether *A. centrale* is tick transmissible rickettsia. The other intriguing question was whether there might be infection-exclusion phenomenon in cattle deliberately inoculated (vaccinated) with the vaccine *A. centrale* strain, and the wild and virulent *A. marginale* strains transmitted by ticks in filed.

The important conclusions that were raised by the research were that *A. centrale* are capable of infecting ticks (*Rhipicephalus* and *Hyalomma* species), but these were not able to transmit the infection to susceptible cattle. Based on the use of primers encoding for various specific genes *A. centrale* presence of *A. centrale* DNA was demonstrated in blood of infected cattle, in acquisition-feeding ticks; the EM detected colonies of *A.centrale*, however no transmission of the disease occurred. We have further shown that vaccination of cattle with *A. centrale* did not prevent infection with *A. marginale*, neither under experimental cross-infection, nor under field conditions. The epidemiological significance of these findings is of importance, as it appears that *A. centrale* vaccination does not provide source for tick infection in field grazing cattle. Aside scientific accomplishments, exposure and experience obtained to apply the most advanced techniques provided to an S. African student, S. Mtshall, will remain of value for the foreseeable future. These include: establishment of tick cells cultures and technology for propagation, handling of the cells, freezing of seeding and working stabilates in liquid nitrogen in both istitutions, OVI and KVI (innovative for both institutions); successful infection of tick cells cultures with *A. marginale* (Israeli and S. African strains) (innovative for both institutions); development of molecular methods for detection of infection in infected bovine blood, ticks and cultured cells (PCR and nPCR assays) (innovative for OVI); extensive training to obtain technical skills was provided to Mr. Sibusiso Mtshali from the Free State University, Qwa Qwa Campus, at the OVI, KVI and OSU on handling tick cell cultures, dissection of infected ticks, preparation of specimens for electron microscopy and PCR methodology, infection of cattle and follow up for clinical and laboratory parameters, publications of joint research in scientific meeting and journals.

D) Research Objectives

The goal of the project was to develop a potent vaccine against bovine anaplasmosis based on culture-derived *A. centrale,* which is at present the blood-derived vaccine strain used for control of the disease in several regions of the world, including S. Africa and Israel. The main specific objectives included:

(1) Infection of susceptible calves with *A. centrale* and *A. marginale* preparation of stabilates to grow *A. centrale* and the local strains of *A. marginale* in tick cell cultures

(2) To establish propagation and continuing maintenance of tick cell cultures

(3) To infect cattle with tick-cell cultures of the Israeli and S. African strains of *A. marginale*

(4) To develop PCR assays specific for detection of *Anaplasma* infection in ticks

(5) To perform tick transmission studies of *A. centrale*, including monitoring of the responses by PCR, and electron microscopy of tick gut and salivary glands

(6) To examine genotype and infection exclusion in experimentally concurrently cross-infected cattle with *A. centrale* and *A. marginale*

E) Methods and Results

Anaplasma strains

The *A. centrale* vaccine stain was obtained from South Africa and brought to Israel in 1952, since used for routine vaccination of cattle. The Israeli tailed and non-tailed *A. marginale* strains (Shkap et al., 2002a,b) were originally isolated from infected cattle graizing in an endemic area. The *A. marginale* Virginia isolate was obtained from Oklahoma State University (de la Fuente et al., 2002a). *A. marginale* and *A. centrale* were maintained by passages in splenectomized calves and kept as frozen stabilates, cryopreserved in DMSO in liquid nitrogen. In South Africa, the original *A. centrale* vaccine strains and local *A. marginale* iosolates were maintained and cryopreserved as above.

Propagation of *A. centrale* and local strain of *A. marginale* in tick-cell cultures

According to published procedures (Munderloh et al., 1996; Blouin and Kocan, 1998; Blouin et al., 1999), blood from calves infected with *A. centrale* or *A. marginale* was collected at a rickettsemia level of about 15%. The erythrocytes were lysed by hypotonic shock, and the suspension was centrifuged to pellet them. Cultures of IDE8 or ISE6 tick-cell were overlaid with 5 ml of resuspended pellet and incubated at 34°C. *A. centrale* cultures were initiated at monthly intervals in the KVI laboratories, and many attempts were made in OVI, but without success; only transiently infected cultures were obtained once, but at subcultivation the cells died out. The Israeli tailed *A. marginale* strain was successfully propagated in IDE8 and ISE6 cultures, which were transferred to S. Africa for further experimental work. Because of the cytotoxicity of DMSO, attempts were made to cryopreserve *A. marginale* cultures by using a method successfully used for *Ehrlichia ruminantium* cultures, whereby DMSO is replaced with a buffer prepared from sucrose, potassium phosphate and glutamate (SPG) (Munderloh et al, 1996; Blouin E. and Kocan, K. 1998; Blouin et al., 1999; Bell-Sakyi, L., 2004). *A. marginale*-infected cultures were harvested and centrifuged (10,000 g, 15 min), the cell pellet was resuspended in SPG and 1-ml aliquots were frozen at –70°C. After 1 week, 1 ml of the stabilate was thawed in a water bath at 37°C and resuspended in 4 ml of complete *Anaplasma* medium. This suspension was added to IDE8-cell culture, which was transferred into an environment at 34°C. The medium was changed after 24 h, and the culture was nicely positive when checked a week later.

Several other tick-cell lines derived from various tick species kindly provided by Dr. L. Bell-Sakyi (Centre for Tropical Veterinary Medicine [CTVM], University of Edinburgh, Scotland, UK) were tested for suitability for infection with *A. centrale*. The tick species and cell line designations, incubation temperatures and culture media used are shown in Table 1. On arrival at OVI, the tick-cell cultures were incubated at the temperature indicated (Table 1). The AVL 13 and IRE 18 lines were the only that remained metabolically active and able to be propagated continuously in Onderstepoort.

Table 1. Data of the tick cell lines obtained from CTVM

Tick species	Cell line	Incubation temp. [°C]	Culture medium
Amblyomma variegatum	AVL 13	32, 37	L-15/L-15B
	AVL 17	32	L-15/L-15B/H-Lac
Boophilus decoloratus	BDE 16	32	L-15
Boophilus microplus	BME 2	32	L-15
	BME 6	32	L-15
Hyalomma anatolicum anatolicum	HAE 9	32	L-15/MEM
	HAE 10	32	L-15/H-Lac
Ixodes ricinus	IRE 18	28	L-15/H-Lac
Rhipicephalus appendiculatus	RAE 1	32	L-15

Growth of *A. marginale* spp in various mammalian cell cultures

In addition to tick cell cultures, mammalian cells were employed for infection with *Anaplasma* spp. Bovine aortic endothelial cells (BA 886), sheep brain endothelial cells (SBE 189), African Green monkey kidney cells (Vero), mouse connective tissue cells (L-929) and Madin Darby bovine kidney cells (MDBK) were propagated, and the tick cells IDE8 infected with *A. marginale* from Israel and *Anaplasma* sp. (Omatjenne) were used as positive control cultures. None of the mammalian cultures supported growth of *Anaplasma in vitro*, except for the tick cells.

Isolation of *Anaplasma* sp. (Omatjenne strain)

A South African *A. marginale*, referred to as the Kaalplaas isolate, was successfully initiated in tick cell culture (Zweygart et al., 2006). This statement has to be revised since the organism propagated *in vitro* was in fact another *Anaplasma* spp., namely *Anaplasma* spp. (Omatjenne). Molecular characterisation revealed that the blood inoculum used to initiate the culture contained both *A. marginale* and *Anaplasma* spp. (Omatjenne), whereas the organisms from established cultures were only *Anaplasma* spp. (Omatjenne). Furthermore, attempts to infect IRE/CTVM18 cell cultures with the *Anaplasma* spp. (Omatjenne) isolate

derived from IDE8 cultures failed, whereas a reference stock of *A. marginale* from Israel infected IRE/CTVM18 tick cell cultures. Whether this is a characteristic true for other *A. marginale* isolates has still to be demonstrated. Therefore attempts are underway to use IRE18 cell cultures to selectively isolate *A. marginale* from the stabilate, which contained *A. marginale* Kaalplaas and *Anaplasma* sp. (Omatjenne). Culture material of *Anaplasma* sp. (Omatjenne) was injected into a splenectomized bovine to demonstrate infectivity of the organisms, however, infection in the animal could not demonstrate.

Infection of susceptible calves with *A. marginale* grown tick cell cultures (Israeli strain)

In Israel, the tailed *A. marginale* isolate, successfully propagated in IDE8 cell culture, was inoculated into two susceptible calves (#647 and #658), both of which showed patent rickettsemia 28 and 21 days after inoculation, respectively. The cultured rickettsia retained their pathogenicity for bovine, as calves developed rickettsemia up to 28% and PCV dropped to 22 %. PCR analyses with specific primers on blood samples performed to determine the expected pattern of reactivity of the tailed Israeli *A. marginale* strain confirmed the stability of *A. marginale* genotypes (Shkap et al., 2002a, b).

Development of PCR assays specific for detection of *A. marginale* and *A. centrale* in infected bovine blood, ticks and cultured tick cells
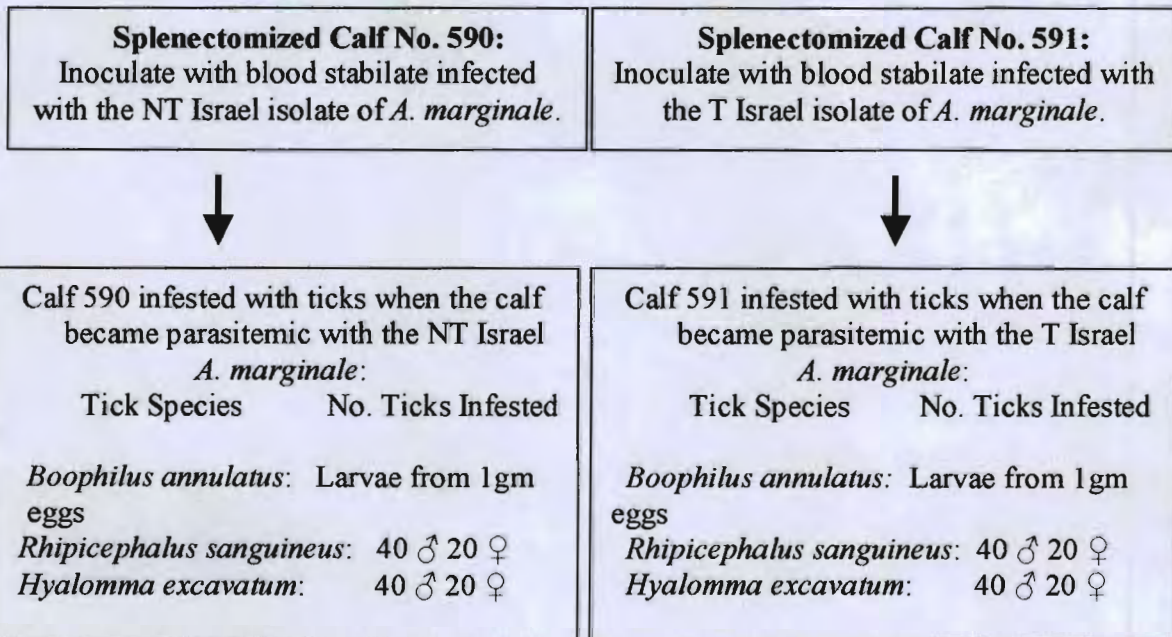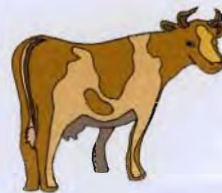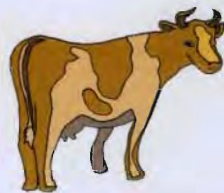
Specific PCR assays to detect and differentiate *Anaplasma marginale* from *A. centrale* DNA in blood of infected cattle have been applied (French et al., 1998; de la Fuente et al., 2001; Kocan et al., 2003). Specific assays to detect *Anaplasma* infection in ticks and cultured cells were developed to detect DNA in 3 tick species samples (gut tissues and salivary glands) including *Boophilus annulatus*, *Rhipicephalus sanguineous* and *Hyalloma excavatum* (Kocan et al., 1981; Kocan 1986; Palmer et al., 2001). 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 (Rodriguex et al., 2005) primers were based on published sequences (GenBank accession number AY132307). Primers based on specific *msp1a*, *msp3* and *msp4*

sequences were used as presented below (Alred et al., 1990; de la Fuente et al., 2001; Molad et al., 2004; 2006).

Transmission Feeding Studies on Susceptible Cattle

Testing for infectivity of three species of ticks (*Boophilus annulatus*, *Rhipicephalus sanguineus* and *Hyalomma excavatum*) for *A. marginale* NT and *A. marginale* T were performed using laboratory-reared ticks. For acquisition feeding of male *Hyalomma*, *Rhipicephalus* and *Boophilus* ticks, splenectomized calves were infected with the Israel NT and T isolates of *Anaplasma marginale (*Samish et al., 1993; Stich, et al., 1998) as follows:

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

| Tick Species   No. Ticks Infested | Tick Species   No. Ticks Infested |

Boophilus annulatus: Larvae from 1gm eggs
Rhipicephalus sanguineus: 40 ♂ 20 ♀
Hyalomma excavatum: 40 ♂ 20 ♀

Boophilus annulatus: Larvae from 1gm eggs
Rhipicephalus sanguineus: 40 ♂ 20 ♀
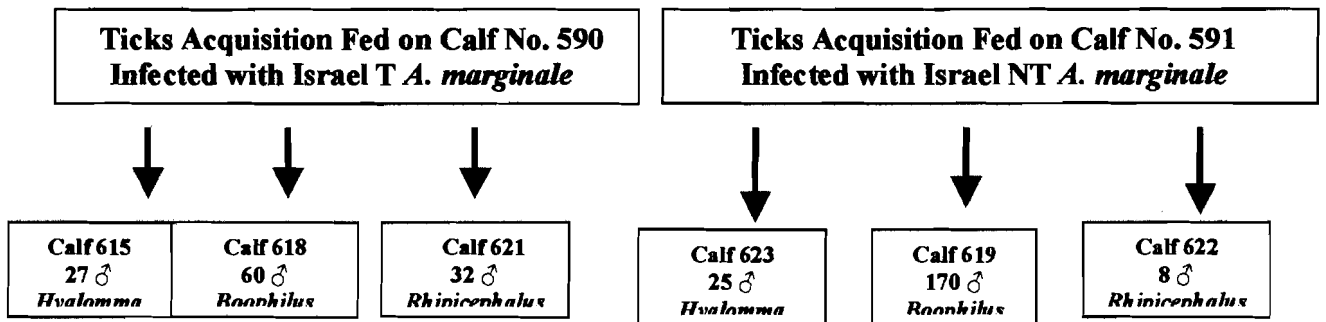*Hyalomma excavatum*: 40 ♂ 20 ♀

All ticks were allowed to feed for 7 days, after which they were removed from the cattle.
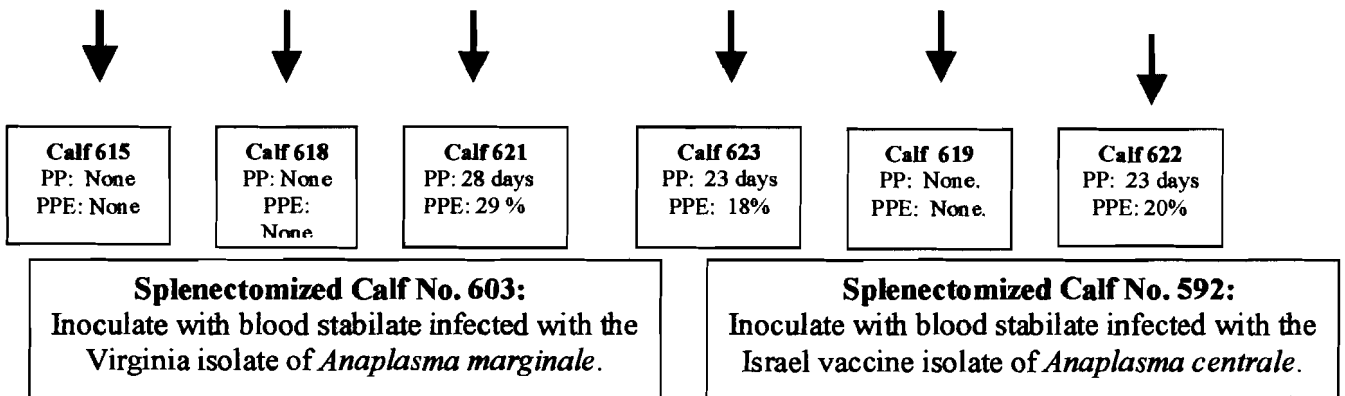
Removal of ticks and Held Period:

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.

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*.

Ticks Acquisition Fed on Calf No. 590 Infected with Israel T *A. marginale*	Ticks Acquisition Fed on Calf No. 591 Infected with Israel NT *A. marginale*

Calf 615 27 ♂ *Hyalomma*	Calf 618 60 ♂ *Boophilus*	Calf 621 32 ♂ *Rhipicephalus*	Calf 623 25 ♂ *Hyalomma*	Calf 619 170 ♂ *Boophilus*	Calf 622 8 ♂ *Rhipicephalus*

All ticks were allowed to feed for 7 days after which they were removed; the cattle were monitored for infection with *A. marginale*.

Transmission Results

Calf 615 PP: None PPE: None	Calf 618 PP: None PPE: None	Calf 621 PP: 28 days PPE: 29 %	Calf 623 PP: 23 days PPE: 18%	Calf 619 PP: None. PPE: None.	Calf 622 PP: 23 days PPE: 20%

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*:

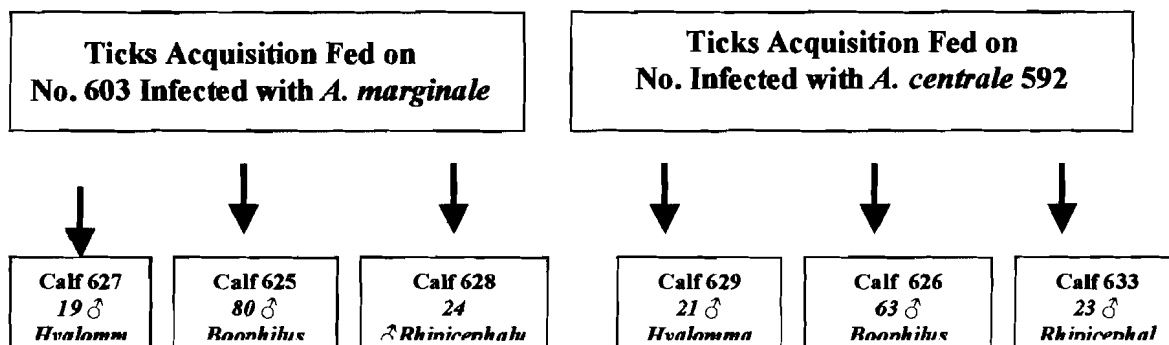| Tick Species	Parasitemia During Tick Feeding
Boophilus annulatus	2.5 to 15.0
Rhipicephalus sanguineus	8.0 to 30.0
Hyalomma excavatum	8.0 to 30.0

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

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.

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.*

Ticks Acquisition Fed on No. 603 Infected with *A. marginale*			Ticks Acquisition Fed on No. Infected with *A. centrale* 592		
Calf 627 19 ♂ Hyalomm	Calf 625 80 ♂ Boophilus	Calf 628 24 ♂ Rhinicephalu	Calf 629 21 ♂ Hyalomma	Calf 626 63 ♂ Boophilus	Calf 633 23 ♂ Rhinicephal

All ticks were allowed to feed for 6 days after which time they were removed.

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.

PCR and microscopy of tick gut and salivary glands for *A. marginale* and *A. centrale* infection in transmission to susceptible cattle

As shown in Figs 1-4, 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. 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.

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 an 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*.

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 transmission feeding (Fig. 4). Analyses for msp1a genotypes were performed according to Allred et al (1990). 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 4th 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.

Tick transmission studies of *Anaplasma* in South Africa and in Israel

Five *Anaplasma*-seronegative by ELISA, splenectomized animals were used for the initiation of transmission experiments. A strain of *R. simus*, the strictly African tick species know as capable of transmitting *A. centrale* was bred to obtain sufficient engorged females to establish a laboratory colony.

Transmission studies of *A. marginale* and *A. centrale* strains by *Boophilus annulatus*, *Hyalomma excavatum* and *Rhipicephalus sanguineus* were performed in Israel with Prof. K. Kocan, Dr. E. Blouin and S. African student Sibusiso Mtshali. The Israeli tailed and *Hyalomma* and *Rhipicephalus* ticks successfully transmitted non-tailed *A. marginale* strains, while there were no signs of clinical anaplasmosis with *A. centrale* infection. However, it must be emphasized that ticks fed on *A. centrale*-infected calves were positive according to PCR assays and to electron microscope examination of the tick gut and, later, of the salivary glands. In fact, the infected ticks were not able to produce an *A. centrale* infection in splenectomized susceptible calves. Experimental transmission of *A. centrale* by ticks was repeated with significantly larger number of ticks.
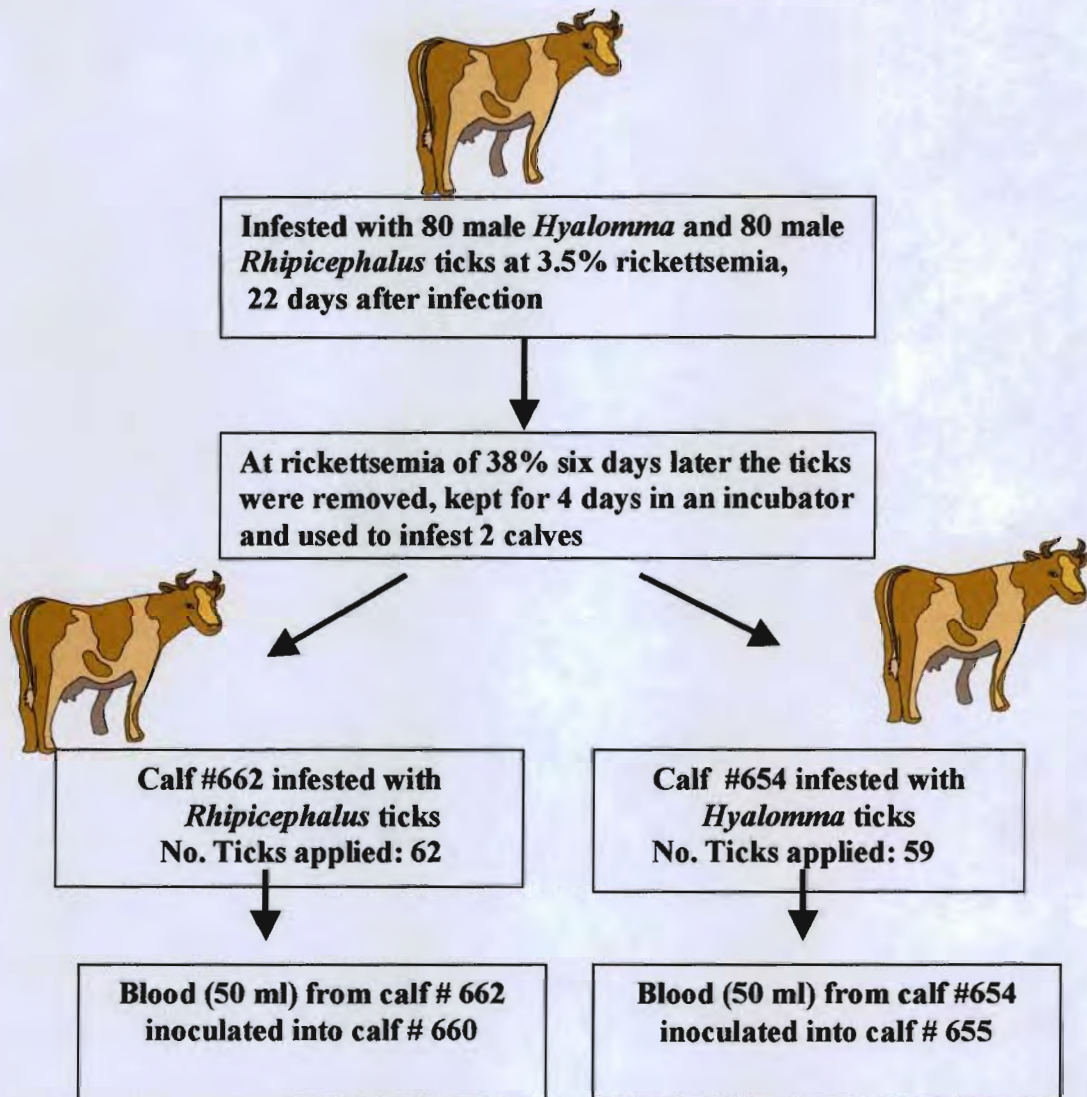
A splenectomized bovine was needle infected with the vaccine strain of *A. centrale* and infested with *Boophilus decoloratus* (body) and *Rhipicephalus simus* (ears)

to attempt acquisition of the infection at high parasitaemia. The *Rhipicephalus simus* adults failed to feed and were dead upon collection. After acquisition feeding, *B. decoloratus* males and females were collected; female and male gut and salivary glands were dissected out for PCR and later EM studies while 120 males were incubated for 5 days post feeding and subsequently placed on a clean splenectomised bovine to attempt transmission of the *A. centrale* organism. Reverse Line Blot hybridization (RLB), microscopic smear examination and antibody ELISA failed to show any sign of subsequent *Anaplasma* infection. PCR of tick gut and salivary glands proved positive for *A. centrale*. It therefore appears that *B. decoloratus* did not successfully transmit the organism in these experiments. EM analysis of gut and salivary glands is pending. During the last 3 months concerted attempts succeeded in establishing a strong *Rhipicephalus simus* colony, which will be used to attempt transmission of *A. centrale*. EM and DNA samples prepared from these experiments will be taken to Oklahoma State for further analysis.

Tick transmission experiments with *A. marginale* in S. Africa

A bovine naturally infected with *Anaplasma* was splenectomized and allowed to reach high parasitaemia (35%). Throughout the course of the infection the bovine displayed clinical signs (supported by microscopic smear examination) typical of *A. marginale* infection. *R. simus* (ears) and *B. decoloratus* (body) were placed on the animal in order to acquisition feed. The *R. simus* did not feed and were dead on collection. *B. decoloratus* were collected but discarded when it became apparent on blood RLB and PCR that the bovine had a mixed infection of *A. marginale* and *Anaplasma* spp (Omatjenne) –see "*Isolation of Anaplasma* sp. (Omatjenne)" above. A second splectomized bovine was infected with a South African isolate of *A. marginale* (Soutpan), which was kept at the OVI's cryobank. *B. decoloratus* (body) were infested to acquisition feed but the animal died of no apparent cause at high parasitaemia resulting in the collection of only 5 males. It was considered wasteful to attempt further transmission to a clean splenectomized bovine with so few ticks, especially as this strain has since been successfully propagated in cell culture.
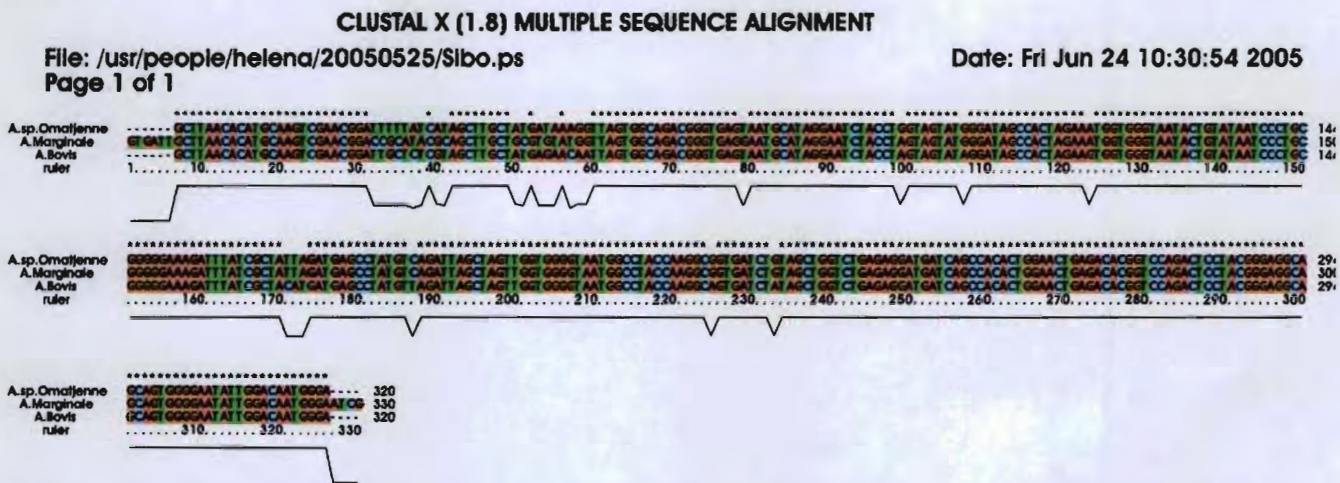
Experimental transmission of *Anaplasma centrale* with higher numbers of *Rhipicephalus* and *Hyalomma* ticks

Infested with 80 male *Hyalomma* and 80 male *Rhipicephalus* ticks at 3.5% rickettsemia, 22 days after infection

At rickettsemia of 38% six days later the ticks were removed, kept for 4 days in an incubator and used to infest 2 calves

Calf #662 infested with *Rhipicephalus* ticks No. Ticks applied: 62

Calf #654 infested with *Hyalomma* ticks No. Ticks applied: 59

Blood (50 ml) from calf # 662 inoculated into calf # 660

Blood (50 ml) from calf #654 inoculated into calf # 655

Inoculation of blood from calves infested with *Rhipicephalus* or *Hyalomma* ticks into susceptible splenectomized cattle did not produce *A. centrale* infection, indicating that transmission did not occur despite infestation of calves with higher numbers of ticks. Application of highly sensitive PCR assays, detecting low rickettsemia, did not result in amplification of specific DNA fragments in the inoculated calves.

PCR, cloning and sequencing

The confusion resulting from the mixed *Anaplasma marginale / Anaplasma* spp (Omatjenne) infection provided an ideal opportunity for training in that Mr. Sibusiso Moses Mtshali (SM) (University of the Free State, QwaQwa Campus), in collaboration with Ms. Steyn (ARC-OVI, Molecular Biology) elucidated organism identity using PCR, cloning and sequencing techniques. A structured sequence for *Anaplasma spp.* (Omatjenne) was constructed and aligned with that of *A. marginale* and *Anaplasma ovis*.

CLUSTAL X (1.8) MULTIPLE SEQUENCE ALIGNMENT

File: /usr/people/helena/20050525/Sibo.ps   Date: Fri Jun 24 10:30:54 2005
Page 1 of 1

Field samples analyzed previously and found positive by IFA for *Anaplasma were tested by* Reverse Line Blot (RLB) hybridization. The 16S gene analysis was performed by PCR, cloning and sequencing. It appears evident that, what was known as *Anaplasma* spp. (Omatjenne) occurred to be widespread in field infections in South Africa.

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

515bp →

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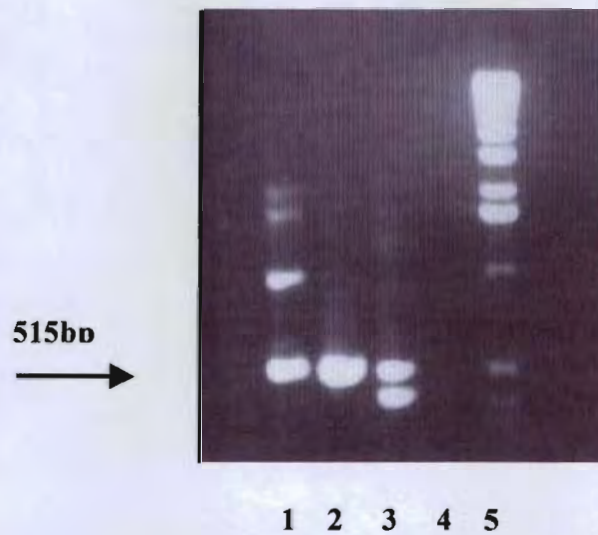
Lane 1 *Boophlus annulatus*; Lane 2 *Rhipicephalus sanguineus*; Lane 3 *Hyalomma excavatum*; Lane 4 negative control without DNA sample, Lane 5 1kb DNA ladder molecular size markers ladder.

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

252bp →

1   2   3   4   5   6 7
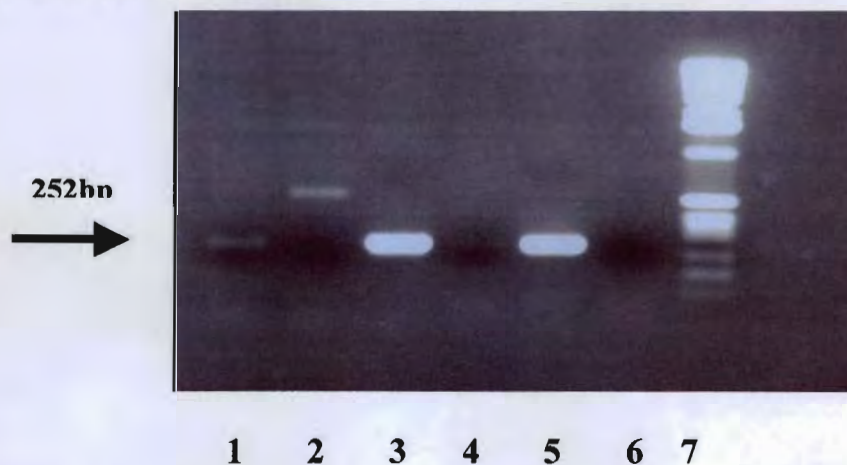
Lane 1 (midgut from *Boophlus annulatus)*; Lane 2 (midgut from *Hyalomma excavatum*); Lane 3 (midgut from *Rhipicephalus sanguineus*); Lane 4 (*Boophilus annulatus* salivary gland); Line

17

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)**
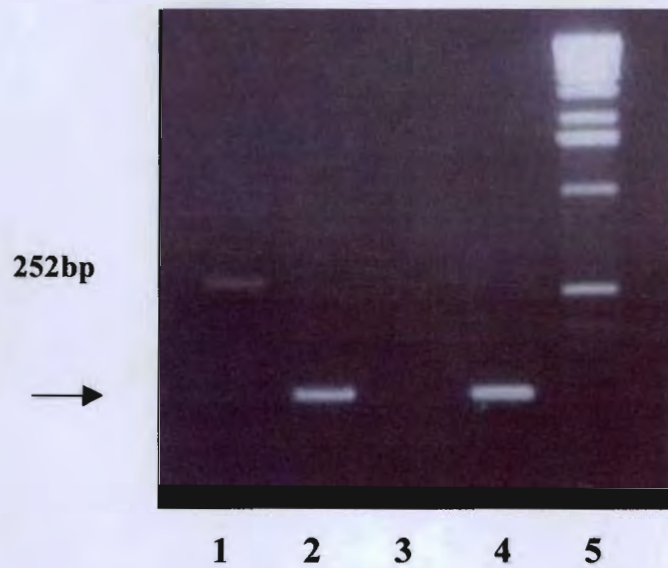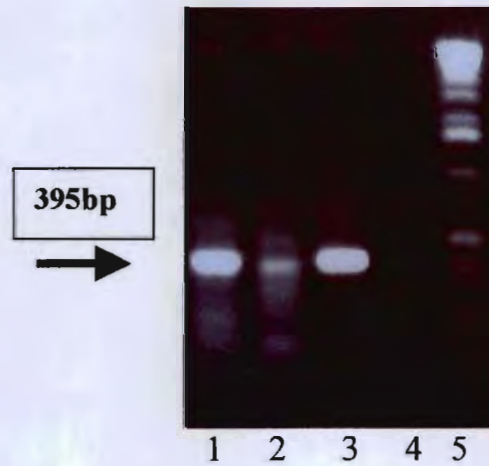

Lane 1, *Hyalomma excavatum;* Lane 2, *Rhipicephalus sanguineus ,* Lane 3, negative control, no DNA template, Lane 4, blood from *A. centrale* infected animal (#592) , Lane 5, 1kb DNA ladder

18

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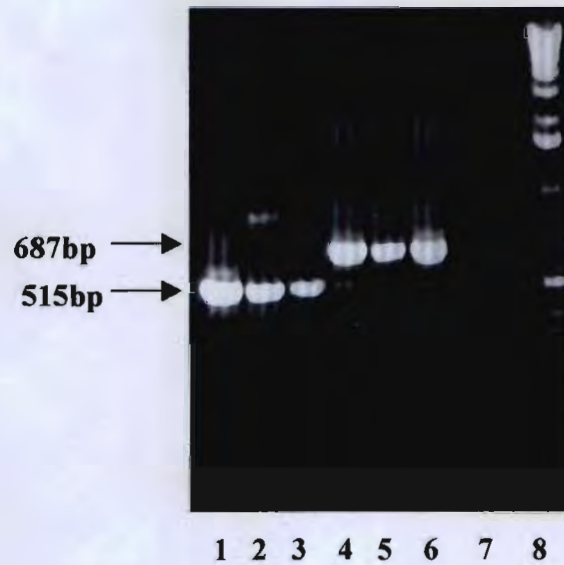
Lane 1, blood from animal #590 infected with AmNT; lane 2, total DNA from AmNT infected *Rhipicephalus sanguineus*; Lane 3, blood from calf #621 infected with AmNT; Lane 4, blood from calf # 591 infected with AmT; Lane 5, total DNA from infected *Rhipicephalus sanguineus* ; Lane 6, blood from calf #622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

19

Fig.5. PCR detection of *A. centrale* with specific *msp4* primers (salivary glands were collected 6 days after transmission-fed on infected calves)

Lane 1, *Rhipicephalus sanguineus,* Lane 2, *Hyalomma excavatum*, Lane 3, blood from *A. centrale* infected animal. Lane 4, negative control, Lane 5, 1kb DNA ladder.

Fig. 6. Electron microscopy of *A. marginale* in guts of transmission-fed *Rhipicephalus sanguineous ticks*
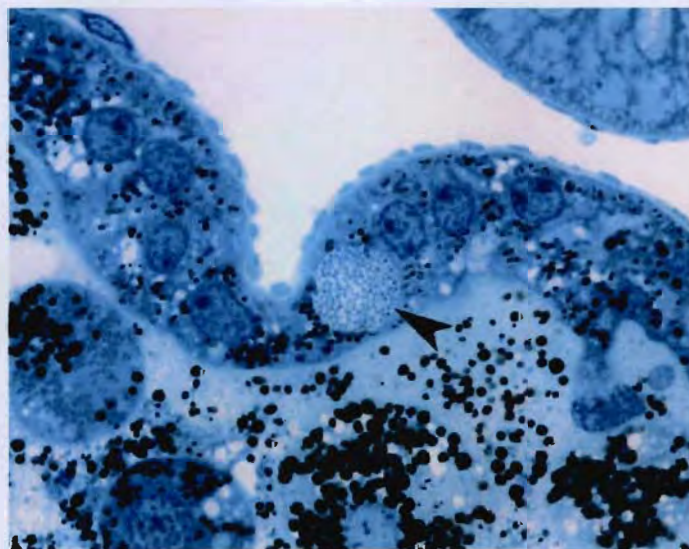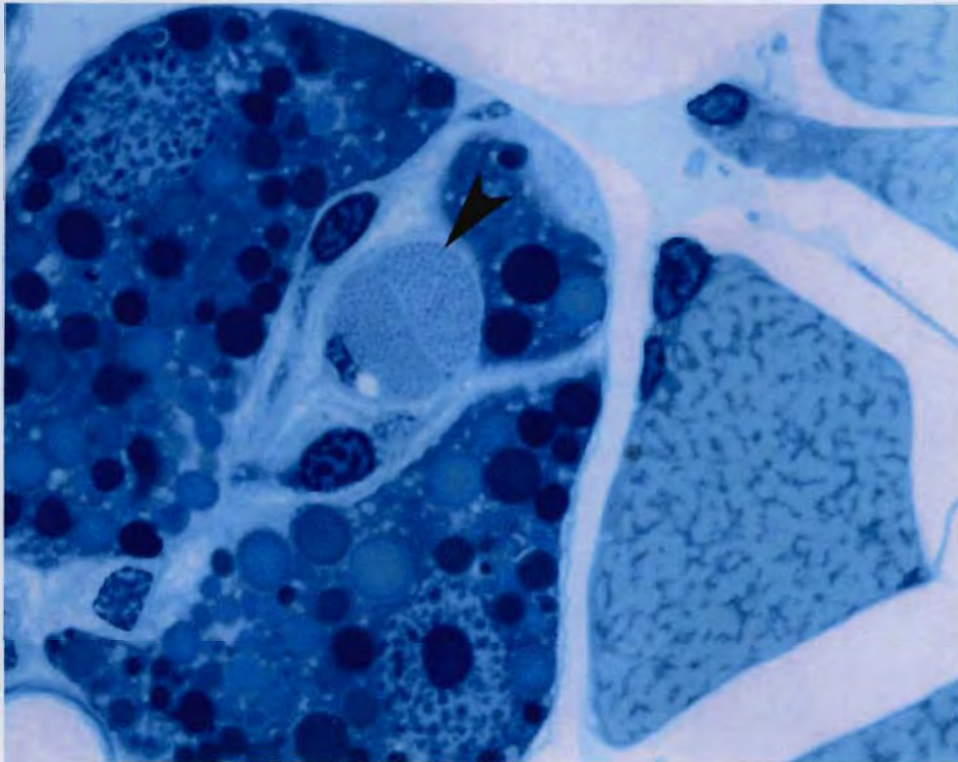
Fig. 7. Electon microscopy of *A. centrale* in salivary glands of transmission-fed
   Rhipicephalus sanguineous

From the tick transmission studies is was clearly demonstrated that *A. marginale*
(Virginia, from the USA, tailed and non-tailed strains from Israel) transmitted efficiently
the infection to susceptible calves. The attempts to transmit *A. centrale*, with small or
larger numbers of ticks, resulted in tick infection, which were incapable of transmitting
the infection to susceptible calves. These results have important epidemiological
significance; it is well possible that *A. centrale* vaccine might not be considered as a
source for field spread of anaplasmosis. Interestingly, it should be emphasized that since
the first isolation of *A. centrale* by Theiler at the beginning of the 20th century, there was
no other isolated of the organisms throughout the world, this fact may support the
suggestion that *A. centrale* is not present in the field, unless cattle were deliberately
inoculated for vaccination.

The genotype and infection-exclusion of *A. centrale* and *A. marginale*

Surface membrane proteins (heterodimer of MSP1a and MSP1b, MSP2, MSP3, MSP4 and MSP5) were described in all examined *A. marginale* strains form various geographical regions. Ortologues of *A. marginale* MSP2, OpAG2 and OpAg3 encoded by the operon-associated gene, were described in *A. centrale*, and high conservation was found in the non-operon linked proteins MSP4 and MSP5 in both rickettsial species. It is well documented for *A. marginale* that the *msp1a*, encoding for the MSP1a protein, is a stable marker used for differentiation of *A. marginale* strains by identification of the variable number of 84 or 87 nucleotide tandem repeats at the 5' end. Although the gene is highly conserved in the rickettsia and in the tick vector of *A. marginale*, while the protein and the encoding gene has not been found in *A. centrale*. Based on the number and sequence of *msp1a* repeats it was found that in naturally, or experimentally infected cattle grazing in an endemic for anaplasmosis area distinct genotypes of *A. marginale* were demonstrated, but a single genotype was detected in an individual animal over a period of two years. These findings led to a suggestion that in cattle persistently infected with a specific genotype, exclusion of another *A. marginale* genotypes takes place. Further it was shown that infection exclusion occurs in both, bovine erythrocytes and tick cells. In the present study, we show presence of concurrent infection with *A. marginale* and *A. centrale* in experimentally or naturally- infected cattle, which were vaccinated earlier with the vaccine strain.

Two splenectomized Friesian-Holstein calves (592 and 603) were infected with *A. centrale* and *A. marginale*, respectively. The calves were seronegative for *Anaplasma* in the IFA test. One month after infection, the two calves were cross-infected. Calf 592 primary infected with *A. centrale* was inoculated iv with 10^5 *A. marginale* infected red blood cells, and that primarily infected with *A. marginale* 603, received a similar number of *A. centrale*-infected cells. Calves were monitored daily for rickettsemia, by examination of Giemsa-stained thin blood smears, and for anemia by determination of packed cells volume (PCV). After the second infection the follow-up lasted for more that 3 months.

Blood samples were collected weekly from the day of the initial infection, and for about 3 months after the cross-infection. For PCR assays blood was washed three times with phosphate-buffered saline (PBS) at pH 7.4 by centrifugation at 1200 x g or 15 min each time. Blood was collected into vacutainer tubes containing sodium citrate or EDTA, blood was washed as described above and kept frozen at -70^0C pending use. The buffy coat was removed, the erythrocytes in the final pellet resuspended in PBS and stored pending use. In addition, blood samples (n=27) were collected from a herd grazing in the northern part of Israel, endemic for anaplasmosis, which was vaccinated a year earlier with *A. centrale* routine vaccine at the waning age of 6-8 months. DNA was extracted from thawed blood by the QIAmp DNA Blood Mini Kit (Qiagen, USA). The purified DNA samples were examined for concentration with the NanoDrop spectrophotometer (USA), resuspended to a DNA concentration of 100 μg ml^{-1}, and stored at -20^0C. Blood samples from a non-infected calf was processed identically and served as negative control. Specific *mspla* primers were applied to genomic DNA to amplify A. marginale, as there were no specific sequences for the gene detected in A. centrale. Primers for msp3 gene were designed for the detection of specific sequences. The primers for msp4 were designed for the specific detection of *Anaplasma* DNA of both species. The details for primers, amplicon size, GenBank accession numbers are given in Table 3. The PCR assays were performed in a final reaction volume of 50 μl: the PCR amplification mix contained 5-10 ng of purified genomic DNA as template, 1x PCR Takara buffer containing 2 mM MgCl$_2$, 4 ng of each primer, 0.2 mM of each deoxynucleotide triphosphate, and 1.25 U of Takara Ex-Taq (Takara Bio Europe SA, France). After preheating at 95^0C for 3 min the cycling conditions were as follows: 35 cycles denaturing at 98^0C for 10s, annealing at 55^0C for 30 sec (for *A. centrale*), or 50 sec (for A. marginale), and extension at 72^0C for 30 sec with final extension at 72^0C for 5 min. Amplicons were analyzed by electrophoresis in 1% agarose gels stained with 0.015% ethidium bromide. For multiplex PCR reaction the conditions were similar, with the exception that the four primers were added into one tube, and the annealing performed at 53° C. PCR amplicons from a single reaction were purified (Purification Kit Qiagen ,USA) and ligated into pGEM-T-EASY with the TA cloning kit (Promega, USA). For reactions with the *mspla* the same conditions were applied, except annealing at 56° C

followed by elongation for 45 seconds, and for the *msp3*, annealing was performed at 57°
C with one-minute elongation.

Table 3. Primers for *msp1a, msp3* and *msp4* genes, GenBank accession numbers, position
and amplicon size obtained following amplification of the genomic DNA from blood
infected with *A. centrale* and *A. marginale*

Rickettsia	Primers	Position	Gene	Amplicon size	Accession number
Anaplasma centrale	Forward CCAGAAGGTGAAGGAGAAGAT Reverse AAGCATTTACAGGAAAGGAA GC	474-495 1347-1369	*msp3*	989 bp	AY586402
	Forward CATGGGGCATGAATCTGTG Reverse AATTGGTTGCAGTGAGCGC	10-28 386-404	*msp4*	395 bp	AY054383
Anaplasma marginale	Forward GCATTACAACGCAACGCTT Reverse ACCTTGGAGCGCATCTCTT	1690-1708 2708-2726	*msp1a*	515 bp 687 bp	M32871
	Forward CATCTCCCATGAGTCACGAAGTG GC Reverse GCTGAACAGGAATCTTGCTCC AAG	86-110 823-846	*msp4*	761 bp	AY786994

Sequences were obtained from the amplified in PCR fragments and further eluted from the agarose
gels. Sequences were analyzed by using the Sequence Analysis Software, GCG (Genetics Computer
Group, University of Wisconsin, USA)

Clinical response of infected calves.

Following initial infection both splenectomized calves developed clinical
anaplasmosis. After the prepatent period of 14 days in both calves (603, infected with *A.
marginale* and 592, infected with *A. centrale*) maximum *A. marginale* and *A. centrale*
rickettsemia reached 25% and 30% on days 27 and 31 after infection, respectively (data
not shown). The minimum PCV of 21% and 15% were recorded in calves 603 and 592,
respectively. Transient fever of > 40° C developed in both calves that lasted 3-4 days. As
shown in Table 4, on the day of the cross-infection, there was 20% *A. marginale* infected

24

red blood cells (iRBC) counted in calf 603, and 18% of *A. centrale* iRBC in calf 592. After the peaks, there was gradual decline in the initial type rickettsemia in both calves, after 7 days no organisms could be detected in blood smears. In the following 19-25 days there was an elevation in the rickettsemia, in the calf 603 maximum of 6% *A. centrale* and 3% *A. marginale* were found on days 52 and 51, respectively. In calf 592 the rickettsemia of *A. centrale* was 7%, and that of *A. marginale* 20% on days 48 and 49. As after initial infection there were no detectable *Anaplasma* until day 73. In the following 10 days in calf 603 peaks of 2.5% of *A. marginale* and 12 % *A. centrale* were recorded.

Detection of *Anaplasma* by PCR assays in the experimentally infected calves. The results of the multiplex PCR based on *msp4* primers and applied to samples from calves #592 and #603 at 86, 88 and 95 days post cross-infection, are presented in Fig.8. There were specific fragments of 395bp and 761bp amplified from *A. centrale* and *A. marginale*, respectively. The sequence analysis of amplified fragments form both calves obtained after elution from the gels, were identical 30 or 60 days after cross-infection (Fig. 9). Application of *msp1a* and *msp3* primers resulted in amplification of specific fragments (Fig. 10) of 989 bp for *A. centrale*, and of 515 bp for *A. marginale* in the same calf at days 44, 46, 60 and 62 after cross-infection. There was no reactivity observed with DNA samples from a non-infected calf, or where no DNA template was added to the reaction mix.

Detection of *Anaplasma* in an endemic herd. As shown in Fig. 11, using the *msp4* primers both *Anaplasma* species were detected in a herd that was routinely vaccinated with *A. centrale* a year and half earlier, and grazing in an endemic area. Both species of *Anaplasma* were detected in most of the animals tested. There were 22 out of 27 calves *A. centrale* positive (81%), and 18 (66%) carried both *A. centrale* and *A. marginale* DNA.

Table 4. Rickettsemia in cross-inoculated calves counted in Giemsa-strained blood smears

Number of calf	Inoculated with:	% iRBC On cross-challenge Ac / Am	Maximum % iRBC Ac	Am
603	*A. marginale / A. centrale*	0 / 20	52	51
592	*A. centrale / A. marginale*	18 / 0	48	49

*Infected red blood cells

Fig. 8. Multiplex PCR assay based on *msp4* primers applied to genomic DNA from blood of calves 592 and 603 infected with *A. centrale* or *A. marginale*, respectively, 86, 88 and 95 days after cross infection with the heterologous species. Lanes 1 and 3, DNA from calf 603, 88 and 95 days after last inoculation; lanes 2 and 4, DNA from calf 592, 86 and 95 days after last inoculation; Nc, no DNA template; M, 1 kb ladder.
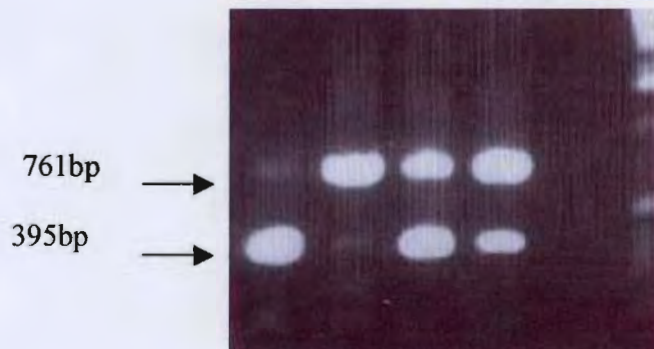

761bp →

395bp →

Fig. 9. Sequence analysis of PCR fragments eluted from gels after amplification with *msp4* specific primers for *A. centrale* (764 bp) and *A. marginale* (761 bp) from calves 592 and 603, 30 and 60 days after heterologous cross-infection. **A, calf 592**, 30 days after cross-infection; **B, calf 603**, 60 days after cross-infection.

Calf 592

A. centrale

```
SPMGHESVSE GSGVMGGSFY VSTAYSPAFP SVTSFDIRES GKETSYVKGY
DKSIATIDVS AHENFSRSSY SFAFSKNLLT SFDGAVGYSL GGARVELEAS
YRRSATLADG QYAKSGAEAL AAVSREAALT ATNYFVVKVD EIINTSVMLN
GCYDVLHTDL PVSPYVCAGI GASFVDISKQ VTAKLAYRGK VGISYQFTPE
ISLVAGGFYH GLFDESYKDI PAHNSVNFPG EAKASIKAHV ADYGFNLGAR
FLFS
```

A. marginale

```
EPSPMSHEVA SEGGVMGGSF YVGAAYSPAF PSVTSFDMRE SSKETSYVRG
YDKSIATIDV SVPANFSKSG YTFAFSKNLI TSFDGAVGYS LGGARVELEA
SYRRFATLAD GQYAKSGAES LAAITRDANI TETNYFVVKI DEITNTSVML
NGCYDVLHTD LPVSPYVCAG IGASFVDISK QVTTKLAYRG KVGISYQFTP
EISLVAGGFY HGLFDESYKD IPAHNSVKFS GEAKASVKAH IADYGFNLGA
RFLFS
```

Calf 603

A. centrale

```
SPMGHESVSE GSGVMGGSFY VSTAYSPAFP SVTSFDIRES GKETSYVKGY
DKSIATIDVS AHENFSRSSY SFAFSKNLLT SFDGAVGYSL GGARVELEAS
YRRFATLADG QYAKSGAEAL AAVSREAALT ATNYFVVKVD EIINTSVMLN
GCYDVLHTDL PVSPYVCAGI GASFVDISKQ VTAKLAYRGK VGISYQFTPE
ISLVAGGFYH GLFDESYKDI PAHNSVNFPG EAKASIKAHV ADYGFNLGAR
FLFS
```
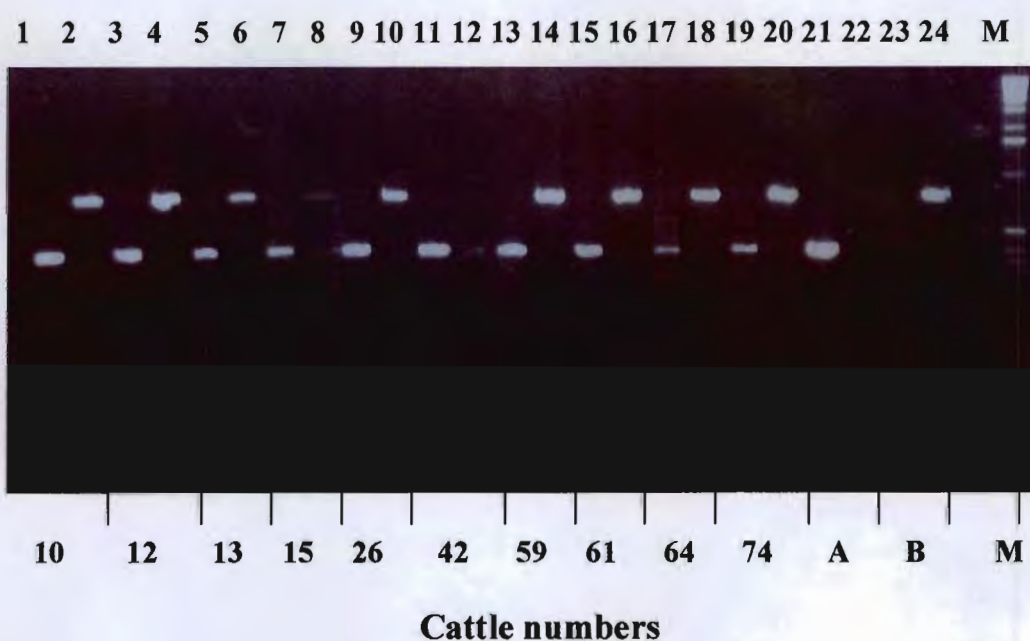
A. marginale

```
EPSPMSHEVASE GGVMGGSFYV GAAYSPAFPS VTSFDMRESS KETSYVRG
YDKSIATIDVKV PANFSKSGYT FAFSKNLITS FDGAVGYSLG GARVELEA
SYRRFATLADGQ YAKSGAESLA AITRDANITE TNYFVVKIDE ITNTTVML
NGCYDVLHTDLP VSPYVCAGIG ASFVDISKQV TTKLAYRGKV GISYQFTP
EISLVAGGFYHG LFDESYKDIP AHNSVKFSGE AKASVKAHIA DYGFNLGA
RFLFS
```

Fig. 10. PCR assays wth *mspla* and *msp3* primers, applied to genomic DNA from calves 529 and 603 infected with *A. centrale* or *A. marginale*, 44, 46, 60 and 62 days after cross-infection with heterologous species. Lanes 1 and 7, calf 529, amplification with *msp3* or *mspla* primers or 62 and 46 days after second inoculation; Lines 3 and 9, calf 603, , amplification with *msp3* or *mspla* primers, 60 and 44 days after second inoculation. Lanes 5 and 6, DNA from non-infected calf with *msp3* or *mspla* primers, respectively. Lane 11, 12, no DNA template. M1 kb ladder

28

Fig. 11. PCR assay using *msp4* primers applied to genomic DNA from calves grazing in an anaplasmosis endemic area and routinely vaccinated with *A. centrale* a year and a half earlier. Odd number lanes (1-19), *A. centrale* amplified fragment of 515 bp, and even number lanes (2-20), *A. marginale* amplified fragment (989 bp form the same animal. Lane 21, *A. centrale* positive control; line 22, 23, no DNA template, lane 24, *A. marginale* positive control, M1 kb ladder.

Cattle numbers

The results presented here clearly showed that cattle could carry infection with *A. marginale* and with the genetically distant *A. centrale* vaccine strain. There was no infection exclusion irrespective of the ricketsstia species involved in the initial infection. While superinfection in *A. marginale* was found to be a relatively rare event, it appeared to be different with concern to the vaccine *A. centrale* strain.

29

F) Impact, Relevance, and Technology Transfer

The project provided a unique opportunity to collaborate on bovine anaplasmosis, one of the world's major diseases in livestock. The aspects on concomitant infection with vaccine and wild virulent strains in cattle grazing in endemic area, and the transmissibility of the vaccine strain by tick vectors were the main subjects studied in close collaboration of the teams in South Africa, Oklahoma State University and the Kimron Veterinary Institute. In South Africa nearly 95% of cattle are found in the area where tock vectors of anaplasmosis are present and, thus the cattle is potentially at risk of contracting the devastating disease. Although the goal of developing a cell culture-derived *A. centrale* vaccine has not been achieved because of the biological feature of the strain not capable of infecting tick or mammalian cells (not known until studied in the present research), the scientists at both institutions (OVI and KVI) gained expertise in vital areas of molecular biology, tick cell culture technology, propagation and maintenance of *A. marginale* infected cell lines, handling of ticks, infection of cattle and ticks, applications of molecular assays for detection of infection in infected cattle and infected ticks, dissecting of ticks, in electron microscopy specimen preparations and examination. Intensive training was provided to the S. African student, S. Mtshali, from the Qwa Qwa University, who worked in the laboratories at the OSU, KIV and at the OVI.

G) Project Activities/Outputs

Over the course of the project, the collaboration, travel and training components consisted of working visits of the participants of the project in the collaborative laboratories. The PI from Israel, Varda Shkap and Mrs. L. Fish, the Co-PI from OSU, Prof. K. Kocan and Dr. E. Blouin visited the OVI, in 2003, where tick cells were first introduced and the technology for cultivation was transferred to OVI and training provided for maintenance of cultures for KVI scientists as well. Dr Erich Zweygarth visited Dr. Lesley Bell's laboratory in Edenborough in 2004 to obtain various tick cell cultures (not available at either f the collaborating groups). Prof. K. Kocan, Dr. E. Blouin and Mr. Sibusiso Mtshali visited KVI in 2004 toconduct transmission experiments; Mr

.Mtshali presented results on the detection of multiple tick-borne pathogen infections in the field at the 20th International Conference of the World Association for the Advancement of Veterinary Parasitology, Christchurch, New Zealand, 16th – 20th October 2005 in a presentation entitled: "Detection of multiple tick-borne pathogens in cattle at Ficksburg and Reitz, eastern Free State, South Africa using reverse line blot hybridization". Dr. E. Zweygarth visited the laboratory in Israel in 2006 in last attempts to cultivate *A. centrale in vitro*; Mrs. Lea Fish paid two weeks visit at the laboratory of Prof. K. Kocan to obtain training in tick dissection methods and electron microscopy preparations. Mr. S. Mtshali visited Prof. Kocan's laboratory in 2006 where he obtained intensive training in molecular biology techniques, electron microscopy and tick cell cultivation.

The following publication were produced from the project:

Zweygarth, E., Josemans, A., Spikett, A., Steyn, H., Bell-Sakyi, L., Shkap, V., Fish, L., Kocan, K., Blouin, E. (2005). *In vitro* cultiavation of a South African isolate of *Anaplasma marginale* in tick cell cultures. *Proceed. STVM, Hanoi, p.135.*

Mtshali, S., Mbati, P., Kocan, K., Shkap, V., Collins, N., Latif, A., Spickett, A. (2005). Detection of multiple tick-borne pathogens in cattle at Ficksburg and Reitz Eastern Free State, South Africa using Reverse Line Blot Hybridization. *Proceedings of the World Association for the Advancement of Veterinary Parasitology, New Zeland, F 4.2, p. 132.*

Zweygarth, E., Josemans, A., Spickett, A., Steyn, H., Putterill, J., Troskie, P., Mtshalli, M., Bell-Sakyi, L., Shkap, V., fish, L., Kocan, K., Blouin, E. (2006). *In vitro* cultivation of a South African isolate of *Anaplasma* sp. in tick cell cultures. *Ondestepoort J. Vet. Res.,* 73: 251-255.

Mtshali, M., De la Fuente, J., Ruybal, P., Kocan, K., Vecente, J., Mbati, P., Shkap, V., Blouin, E., Mohale, N., Moloi, T., Spickett, A., Latif, A. (2007). Prevalence and genetic diversity of *Anaplasma marginale* strains in cattle in South Aftica. *Zoonozes and Public Health*, 54, 23-30.

De la Fuente, J., Lew, a., Lutz, H., Meli, M., Shkap, V., Molad, T., Mangold, A., Almazan, C. Naranjo, V., Gortazar, C., Torina, A., Caracappa, S., Blouin, E., Kocan, K. (2005). Genetic diversity of *Anaplasma* species major surface proteins and implications for anaplasmosis serodiagnosis and vaccine development. *Animal Health Research Reviews*, 6(1), 75-89.

Shkap, V., Kocan, K., Blouin, E., de la Fuente, J., Molad, T., Fish, L., Leibovitz, B., Krigel, Y., Mazuz, M., Fleiderovitch, L. (2007). Concomitant infection and co-exclusion phenomenon in *Anaplasma* spp. infection. Animals Health Research Reviews (submitted for publication)

De la Fuente, J., Ruybal, P., Mtshali, M., Naranjo, V., Shuqing, L., Mangold, A., Rodriguez, S., Jimenez, R., Vicente, J., Moretta, R., Torina, A., Almazan, C., Mbati, P., de Echaide, S., Farber, M., Rosario-Cruz, R., Gortazar, C., Kocan, K. 2007. Analysis of world strains of *Anaplasma marginale* using major surface protein 1a sequences. Vet. Microb., 119, 382-390.

H) Project Productivity:

Anaplasma centrale, the subspecies of *A. marginale* is the strain used for about a century to control bovine anaplasmosis in several regions of the world. The main disadvantage of the vaccine is its bovine blood origin. To ovoid using donor cattle it has been proposed to develop a culture-derived *A. centrale* vaccine, and this based on solid scientific achievements for *in vitro* propagation of the virulent *A. marginale* field strains. However, numerous attempts made in both S. Africa and Israel to grow *A. centrale in vitro*, finally the conclusion for these efforts was that *A. centrale* cannot be propagated *in vitro,* and

therefore the goal of developing anti-anaplasmosis vaccine based on cell cultured *A. centrale* has not been achieved. Despite these difficulties, the "Institutional Strengthening" capacity and training of young scientist has been thoroughly effectively achieved. Experience gained in disease transmission studies, establishing of tick cell culture methodology, molecular biology technology, infection and handling of ticks, specimen preparations form ticks and cattle donors blood for electron microscopy are well established and will contribute to accomplish other goals of research on tick-borne diseases in South Africa.

I) Future work

Tick-borne diseases are considered as main veterinary economic problem, in cattle breeding industry. Future prospects for the control of bovine anaplasmosis include developing of a safe genetically engineered non-viable vaccine. Undoubtedly, research in the post-genomic era will lead to novel genomic-based approaches to the development of recombinant anti-anaplasmosis vaccine, but the knowledge accumulated will inevitable serve the final goals.

I) Literature cited

Allred, D., McGuire, T., Palmer, G., Leib, S., Harkins, T., McElwain, T., Barbet, A. 1990. Molecilar basis from surface antigen size polymorphisms and conservation of a neutralization-sensitive epitope in *Anaplasma marginale*. PNAS, 87, 3220-3224.

Bell-Sakyi, L., 2004. *Ehrlichia ruminantium* grows in cell lines from four Ixodid tick genera. J. Comp. Pathology, 130, 285-293.

Blouin E. and Kocan, K. 1998. Morphology and development of *Anaplasma marginale* (Rickettsiales: Anaplasmataceae) in cultured *Ixodes scapularis* (Acari: Ixodidae) cells. J. Med. Entomol., 35: 788-797.

Blouin , E., Barbet, A., Jooyoung, Y., Kocan, K., Saliki, J. 1999. Establishment and characterization of an Oklahoma isolate of *Anaplasma marginale* in cultured *Ixodes scapularis* cells. Vet. Parasitol., 31, 1705-1714.

De la Fuente, Garcia-Garcia, J., Blouin, E., McEwen, B., Clawson, B, Kocan, K. 2001. Major surface protein 1a effects tick infection and transmission of *Anaplasma marginale*. Int. J. Parasitol., 31, 1705-1714.

De la Fuente, J., Kocan, K., Garcia-Garcia, J., Blouin, E., Saliki, J. Kocan K. 2002a. Infection of tick cells and bovine erythrocytes with one genotype of the intracellular Ehrlichia *Anaplasma marginale* excludes infection with other genotypes. Clin. Diagn. Lab. Immunol., 9, 658-668.

De la Fuente, J., Kocan, K., Garcia-Garcia, J., Blouin, E., Claypool, P., Saliki, J. 2002b. Vaccination of cattle with *Anaplasma marginale* derived from tick cell culture and bovine erythrocytes followed by challenge-exposure by infected ticks. Vet. Microb., 89, 239-251.

French, D., McElwain, T., McGuire, T., Palmer, G. 1998. Expression of *Anaplasma marginale* surface protein 2 during persistent cycling rickettsemia. Inf. Immuni., 66, 1200-1207.

Kocan, K., Hair, J., Ewing, S. Stratton, L. 1981. Transmission of *Anaplasma marginale* Theiler by *Dermacentor andersoni* Stiles and *Dermacentor variabilis* Say. Am. J. Vet. Res., 42, 15-18.

Kocan, K. 1986. Development of *Anaplasma marginale*: coordinated development of a rickettsial organisms and its tick host. In: Sauer, J., Hair, J (eds) Morphology, physiology and behavioral ecology of ticks. Horwood Chichester, pp. 472- 505.

Kocan, K., de la Fuente, J., Gugleilmone, A., Melendez, R. 2003. Antigens and alternatives for control of *Anaplasma marginale* infection in cattle. Clin. Microb. Reviews, 16, 698-712.

Molad, T., Brayton, K., Palmer, G., Michaeli, S., Shkap, V. 2004. Molecular conservation of MSP4 and MSP5 in *Anaplasma centrale* and cattle naturally infected with *A. marginale*. Vet. Microb., 100, 55-64.

Molad, T., Mazuz, M., Fleiderovits, L., Fish, L., savitsky, I., Krigel, Y., Leibovitz, B., Molloy, J., Jongejan, F., shkap, V. 2006. Molecular and serological detection of *A. centrale* and *A. marginale*-infected cattle grazing within endemic area. Vet. Microb., 113, 55-62.

Munderloh, U., Blouin, E., Kocan, K., Ge, N., Edwards, W., Kurtii, T. 1996. Establishment of the tick (Acari: Ixodidae)-borne cattle pathogen *Anaplasma marginale* (Rickettsiales: Anaplasmataceae) in tick cell culture. J. Med. Entomol., 33: 656-664.

Palmer, G., Rurangirwa, F., McElwain, T. 2001. Strain composition of the Ehrlichia *Anaplasma marginale* within persistently infected cattle, a mammalian reservoir for tick transmission. J. Clin. Microb., 39, 2, 631-635.

Potgieter, F., Kocan, K., McNew, R., Ewing, S. 1983. Demonstration of colonies of Anaplasma marginale in the midgut of Rhipicephalus simus. Am. J. Vet. Res., 44, 2256-2261.

Rodriguez, J., Palmer, G., Knowles, D., Brayton, K. 2005. Distinctly different *msp2* pseudogene repertoires in *Aanaplasma marginale* strains that are capable of superinfection. Gene, 361, 127-132.

Samish, M., Pipano, E., Hadani, A. 1993. Inrastadial and interstadial transmission of *Anaplasma marginale* by *Boophilus annulatus* ticks in cattle. Am. J. Vet. Res., 54, 411-414.

Shkap, V., Molad, T., Brayton, K., Brown, W., Palmer, G. 2002a. Expression of major surface protein 2 variants with conserved T-cell epitopes in *Anaplasma centrale* vaccinates. Inf. Immun., 70, 2, 642-648.

Shkap, V., Molad, T., Fish, L., Palmer, G. 2002b. Detection of the *Anaplasma centrale* vaccine strain and specific differentiation from *Anaplasma marginale* in vaccinated and infected cattle. Parasitology Research, 88, 546-552.

Stich R., Kocan, K., Palmer, G., Ewing, S., Hair, J., Barron, S. 1989. Transstadial and attempted transovarial transmission of *Anaplasma marginale* by *Dermacentor variabilis*. Am. J. Vet. Res., 50, 1377-1380.

Zweygart, E., Josemans, A., Spickett, A., Steyn, H., Putterill, J., Troskie, P., Mtshali, M., Bell-Sakyi, L., Shkap, V., Fish, L., Kocan, K., Blouin, E. *In vitro* cultivation of South African isolate of an *Anaplasma* sp. in tick cell culture. Onderstepoort. J. Vet. Res., 2006, 73, 251-255.