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FINAL REPORT
for GRANT no. LAG-G-00-93-00030-00
entitled HEARTWATER RESEARCH PROJECT

submitted to

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SUMMARY OF ACCOMPLISHMENTS

The Project has made a number of significant accomplishments, among the most notable being the following:

- establishment of an internationally recognized heartwater research laboratory in Zimbabwe which is staffed entirely by African nationals
- development of an inactivated vaccine for heartwater which is being commercialized in collaboration with Intervet International
- development of a recombinant DNA vaccine for heartwater
- development of four serological tests for heartwater which have been transferred to SADC laboratories
- completion of detailed epidemiological and economic studies on heartwater in SADC countries
- development of a tick decoy technology as a sustainable and environmentally friendly method of tick control which is being commercialized in collaboration with Intervet International
- training of 13 African scientists to higher degrees
- dissemination of information on heartwater globally via a newsletter and through scientific publications, including recommendations to protect the United States and its livestock industries and wildlife populations from the introduction of heartwater

OVERALL DESCRIPTION OF ACTIVITIES

The UF/USAID/SADC Heartwater Research Project came into existence in 1985, funded by USAID and directed by the University of Florida (UF). Its activities can be divided into four separate phases:

Phase 1 (1985 - 1989)

The first phase was funded by the USAID/Zimbabwe Mission initially as a contract and later as a cooperative agreement. During this phase, a heartwater research laboratory was established in Zimbabwe, located at the government's Veterinary Research Laboratory in Harare, which refined methodology for growth of the causative agent *Cowdria ruminantium* in cell culture and identified the attraction-aggregation-attachment pheromone of the bont tick *Amblyomma hebraeum*, the primary vector of heartwater in southern Africa.

Phase 2 (1989 - 1993)

The second phase was again funded by USAID/Zimbabwe Mission, but this time as a grant to UF. During this phase, the Project expanded its research laboratory in Harare to include biotechnology capabilities, it trained scientists from nine African countries in the new techniques for *C. ruminantium* cell culture and for heartwater diagnosis, it initiated studies on development of diagnostic tests for heartwater, it applied its pheromone discoveries to development of a pheromone-based attractant decoy for tick vector control, and it conducted a registration trial of the decoy technology in Zimbabwe.

Phase 3 (1993 - 1996)

The third phase was funded by the USAID Office of Agriculture in Washington as a grant. During this phase, the Project developed a inactivated culture-derived vaccine for heartwater, it developed DNA probes and PCR assays for *C. ruminantium* diagnosis, it initiated epidemiological and economic studies of heartwater in Zimbabwe, it continued training of Zimbabwean scientists in molecular biology, immunology and epidemiology, and it initiated an information dissemination system through publication of a quarterly newsletter called "The Tick-ler".

Phase 4 (1996 - 2002)

The fourth and final phase of the Project was funded by the USAID Regional Center for Southern Africa (RCSA) as a grant. During this phase, the Project conducted field trials of the inactivated vaccine in four SADC countries (Botswana, South Africa, Zambia and Zimbabwe) followed by a registration trial in South Africa, it developed a DNA vaccine for heartwater, it completed development of serological tests for heartwater, it expanded its epidemiological and economic studies to eight other SADC countries (Angola, Botswana, Malawi, Mozambique, South Africa, Swaziland, Tanzania and Zambia), it conducted a field trial of the tick decoy technology in South Africa, it completed training of Zimbabwean scientists, it continued to disseminate information through "The Tick-ler", and it developed a solid partnership with an international company to commercialize its technologies within the SADC region.

DESCRIPTION OF THE METHODS OF WORK USED

1. Development of Inactivated Vaccine for Heartwater

A number of affected animals may survive primary infection and become fully resistant to re-infection by *C. ruminantium*. This observation initiated the research on development of improved vaccines. The first of these was named the "inactivated vaccine", which became the "first- generation vaccine". The inactivated vaccine for heartwater was developed from cell culture-derived organisms which were harvested, inactivated and included into a vaccine which contained an adjuvant (an immune-modulating chemical which enhances immunity to a vaccine antigen).

The development of this vaccine required five critical steps:

a) demonstration that inactivated organisms in a vaccine with an experimental Freund's adjuvant could protect animals against lethal heartwater challenge;

b) identification of immune responses that are induced in animals that are immune to *C. ruminantium*;

c) inclusion of a strain of *C. ruminantium* in the vaccine that had a wide cross-protective capacity;

d) identification of the optimal adjuvant for the vaccine;

e) registration and commercialization of the vaccine.

One of the first strains of *C. ruminantium* isolated in tissue culture in Zimbabwe was the Crystal Springs strain from the Crystal Springs Farm located in the lowveld region of Zimbabwe. A series of laboratory vaccine trials were conducted with this strain to demonstrate that an inactivated vaccine could protect sheep against a lethal homologous challenge delivered either by needle or by ticks. Initially, the vaccine was formulated with Freund's adjuvant, an experimental but commercially unacceptable adjuvant. The vaccine protected sheep by reducing the severity of infection in the face of lethal challenge. In contrast, the unvaccinated control sheep suffered a more severe disease and exhibited high mortality (up to 60%). Mortality in vaccinated sheep was seldom observed. Subsequently a field trial was conducted with this vaccine whereby vaccinated and unvaccinated sheep were challenged by field ticks on a heartwater-endemic farm in Zimbabwe. The vaccinated sheep were fully protected against a field tick challenge which killed 63% of unvaccinated control sheep. The vaccine did not prevent infection, but prevented death from heartwater challenge by reducing the severity of infection.

The unique feature of the inactivated vaccine was that it could be modified to incorporate new strains of *C. ruminantium* to make it more area, country, or region-specific. However, an inactivated vaccine that has a wide cross-protective capacity would be the ideal choice for use in diverse geographical situations. Hence, a new strain of *C. ruminantium*, the Mbizi strain, also from Zimbabwe, was incorporated into the vaccine in place of Crystal Springs. Initial tests in the laboratory with this strain demonstrated a similar pattern of protection as that observed with Crystal Springs. Thereafter, all trials were conducted using the Mbizi strain.

The inactivated vaccine containing cell-culture-derived Mbizi *C. ruminantium* and Freund's adjuvant reproducibly protected sheep against lethal homologous challenge. The efficacy of the Mbizi inactivated vaccine was then tested in sheep against challenge from heterologous strains of *C. ruminantium* from South Africa, Kenya and Zimbabwe. In that trial, the vaccine protected sheep against challenge from these diverse locations. It became clear that the inactivated vaccine for heartwater could reduce mortality from heartwater challenge and that it was necessary to select adjuvants which were commercially viable and also had a similar level of efficacy in this vaccine as with Freund's adjuvant.

Commercially viable adjuvants were tested in the vaccine to replace the Freund's adjuvant. These were: Alhydrogel, Biocine, Montanide ISA 50, 70, 206, IMS 1113, 1313, 2213 (from Seppic), Quil A, QS-21, Saponin, incomplete Freund's adjuvant, Plazvax, Double oil emulsion and ST 3 (from Merial).

The vaccine containing Montanide ISA 50 was selected since it was the most efficacious and it reproducibly protected vaccinated sheep against lethal challenge. Subsequently, in a field trial, this vaccine also protected sheep against field tick challenge in Zimbabwe. This vaccine containing the ISA 50 adjuvant was then tested in field trials in four countries in southern Africa,

namely Botswana, South Africa, Zambia and Zimbabwe. The species of ruminants used in these field trials were cattle, sheep and goats. Goats were protected by this vaccine against field tick challenge in Botswana, and sheep were protected in Zambia and Zimbabwe. Angora goats were not protected at the field site in South Africa. This indicated that complete cross-protection between the Mbizi strain used in the vaccine did not exist in all situations, and the vaccine would require further improvement by identifying a more efficacious adjuvant in addition to possible inclusion of other strains of *C. ruminantium*. Vaccine efficacy in cattle trials in Zimbabwe yielded the best results, with >80% protection achieved in vaccinated cattle.

The positive results from the testing of the inactivated vaccine has attracted private sector interest, with Intervet International signing an option license agreement with UF. Registration trials are underway currently as the first stage in commercialization of the vaccine.

2. Development of DNA Vaccine for Heartwater

The Project scientists have made significant progress on development of a "second-generation vaccine" against heartwater, utilizing recombinant DNA technology. Several potential vaccine candidate antigens (proteins) of *C. ruminantium* have been identified by studying the immune responses of animal hosts to heartwater infection. Genes representing 35 novel antigens have been cloned, sequenced and characterized, and some of these, based on their cellular location or function, have been further evaluated as DNA vaccines. DNA vaccines represent a novel means of expressing protective antigens of pathogens in vivo and induce humoral and cellular immune responses.

Ten of 35 novel genes of *C. ruminantium* were tested as DNA vaccines in a mouse model. This model is extremely efficient at predicting protective antigens, especially because, in this host, the pathogen induces immune responses which are similar to those detected in cattle and also because the disease pathogenesis is similar. Of the 10 vaccines, the *map1* DNA vaccine has reproducibly shown to protect mice against lethal *C. ruminantium* challenge. A maximum protection of 67% was achieved in the mouse model with the *map1* DNA vaccine. Another five DNA vaccines individually induced lower levels of protection than the *map1* DNA vaccine.

The *map1* DNA vaccine was tested in sheep and in cattle. Evaluation of immune responses following immunization with the DNA vaccines in sheep revealed that the responses were present, albeit of weak intensity which were unlikely to induce the desired levels of protection. However, the *map1* DNA vaccine trials in cattle were encouraging as both humoral and cellular immune responses to the MAP-1 antigen and native *C. ruminantium* antigen lysates were readily detectable.

Further evaluation of regimes of immunization, intervals of administration, combination of vaccines, and identification of new protective genes are required to obtain optimal protection using this methodology. DNA vaccines have been tested for various infection systems affecting both animals and man, and the optimism expressed by the researchers predicts that some day all vaccines are likely to be designed by this protocol.

3. Development of Diagnostic Tests for Heartwater

Ante-mortem detection of *C. ruminantium* infection in ruminant hosts is not easy because of the acute course of infection. Recognizing this fact, the Project has developed and evaluated several diagnostic assays to detect infection or exposure to infection in ruminant hosts. These tests have application in detecting on-going field infections, the spread of heartwater and in understanding the epidemiology of heartwater, providing valuable insight into dynamics of *C. ruminantium* infection in the host and the vectors of the disease. Two types of diagnostic tests have been developed, serological tests which are capable of detecting exposure to infection and DNA-based assays which can detect infection with a high degree of sensitivity and specificity.

The Project has developed four serological tests: the indirect fluorescent antibody test, the immunoblotting assay, the MAP-2 indirect ELISA, and the MAP-1B indirect ELISA. These tests have been used for the detection of antibodies to *C. ruminantium* in laboratory studies and in field surveys of heartwater.

The Project has developed and evaluated two DNA-based assays, the DNA probe assay and the pCS20 polymerase chain reaction (PCR) assay. These assays can detect *C. ruminantium* in ticks and in animals during the clinical and carrier stages of infection. They are highly sensitive and specific and have higher specificity than the serological assays. They have been used to define the infection dynamics of vector ticks and carrier animals, and this information has contributed to a better understanding of the epidemiology of heartwater.

4. Epidemiological and Economic Studies on Heartwater

The Project embarked on a comprehensive study on the epidemiology and economic impact of heartwater in southern Africa. This was an important undertaking as it was necessary to define how the new vaccines under development would perform in the field. As a result of this study, we now have a sound understanding of the factors influencing the distribution of tick vectors, the infection dynamics, and the impacts of the disease in different agro-ecological zones and production systems. We can now predict the technical and economic impacts of control with the new generation of vaccines now emerging. The following key findings were recorded from these studies:

a) Thirty-one million cattle and 28 million small ruminants are at risk to heartwater in the nine SADC countries affected (Angola, Botswana, Malawi, Mozambique, South Africa, Swaziland, Tanzania, Zambia and Zimbabwe).

b) The total annual losses were estimated at US$ 47.6 million, of which 61% were production losses and 39% control costs. Annual total direct losses in Zimbabwe (acaricide costs, milk losses, treatment costs) were estimated at $5.6 million. An inactivated vaccine was predicted to have a benefit:cost ratio of 2.4:1 in the communal sectors and 7.6:1 in the commercial sectors of Zimbabwe.

c) At a SADC-wide level, new vaccines could potentially yield a benefit:cost ratio of 4.4:1, particularly in commercial and emerging market-oriented systems of the region.

d) Intensive use of acaricides will continue to maintain a status of susceptibility to heartwater infection as it will interrupt natural infection and suppress the development of endemic stability.
e) Use of vaccines will allow reduction in acaricide use in many situations and pave the way to transition towards endemic stability, with endemic stability and subsequent natural infection boosting vaccinal immunity.
f) Endemic stability created by vaccination does not affect the productivity of livestock.
g) Both large and small-scale livestock production could be increased significantly with improved and cost-effective heartwater control methods.
h) Six wild ruminant species (African buffalo, eland, giraffe, greater kudu, sable antelope and wildebeest) were shown to be carriers of *C. ruminantium* infection.

5. Development of Tick Decoy Technology

Conventional methods for tick control require frequent applications of toxic pesticides, typically every one to two weeks during the tick season, either by drenching of animals by dipping or spraying or by labor-intensive application as pour-ons. These methods have proven to be unsustainable in Africa, particularly for small farmers, and the first two methods pollute the environment with toxic chemicals. The Project has developed an alternative method of tick control known as the tick decoy technology, and these tick decoys provide a biorational and sustainable approach to targeted vector control for heartwater and other tickborne diseases.

Tick decoys are plastic tags impregnated with an acaricide (a pesticide that kills ticks) and the bont tick pheromone (a mixture of chemicals produced naturally by ticks to attract other ticks for mating). The plastic decoy is attached to the neck of animals with collars, to the ears or to the hairs of the tail switch. After attachment, the pheromone and acaricide emerge slowly from the decoy onto the animal. Bont ticks both on the animal and in the environment are attracted by the pheromone to the area around the decoy where they are killed by the acaricide. The slow continual release of pheromone and acaricide allow one decoy application to control ticks for at least three months.

Tick decoys have been tested in the field in both Zimbabwe and South Africa. The Zimbabwean trial demonstrated excellent control of the bont tick on cattle with decoys containing the acaricides cyfluthrin (98-99% control) or flumethrin (92-97% control). The South African trial showed that the decoys would also control other important tick species including the brown ear tick *Rhipicephalus appendiculatus* which transmits the deadly disease East Coast fever to cattle.

The decoys have many advantages over conventional methods for tick control, including the following:

- they are cheap and easy to produce
- they cause no pollution of the environment with toxic chemicals
- they require no water for application, a major advantage in drought-prone regions

- they are easy to apply, requiring no specific training for application and no mechanical apparatus for operation
- they have a long-lasting effect of at least three months due to their slow-release formulation
- use of decoys greatly reduces labor and infrastructural costs
- use of decoys greatly reduces the need for expensive chemicals, a major advantage particularly in poorer countries with sparse reserves of foreign currency
- they allow for easy identification of treated animals

Commercialization of the tick decoy technology was initially blocked by the major acaricide manufacturers who refused the Project access for their patented technical-grade acaricides because they wanted to maximize sales of their products. However, when these acaricides went off-patent in the late 1990s, the technical-grade materials became available and commercialization of the tick decoy technology was revived. Intervet International is currently negotiating an option agreement with UF to commercialize this technology.

6. Training Activities of the Project

The following 13 African scientists were trained by the Project to higher degrees:

Daniel Mukwedeya	M.Sc. University of Zimbabwe	(Zimbabwean national)
Noel Chigagure	M.Sc. University of Zimbabwe	(Zimbabwean national)
Bigboy Simbi	M.Sc. University of Florida	(Zimbabwean national)
Barbara Byrom	M.Phil. University of Zimbabwe	(Zimbabwean national)
Samuel Masaka	M.Phil. University of Edinburgh	(Zimbabwean national)
Albert Ngakou	M.Sc. University of Zimbabwe	(Cameroonian national)
Julius Mugwagwa	M.Sc. University of Zimbabwe	(Zimbabwean national)
Nigel Bryson	M.V.Sc. University of Pretoria	(South African national)
Owen Kakono	M.Sc. University of Zimbabwe	(Zimbabwean national)
Trevor Peter	Ph.D. University of Florida	(Zimbabwean national)
Shalt Semu	Ph.D. University of Zimbabwe	(Zimbabwean national)
Bigboy Simbi	Ph.D. University of Zimbabwe	(Zimbabwean national)
Aceme Nyika	Ph.D. University of Florida	(Zimbabwean national)

A training workshop in heartwater diagnostics was conducted in 1991/2 in Harare, Zimbabwe, on improved cell-culture techniques and diagnostics. Representatives from the respective SADC national veterinary laboratories participated in this course.

Other training was conducted by special arrangements. Nationals from Kenya, The Netherlands, Mozambique, Madagascar, South Africa, the United States, Zambia, and Zimbabwe were trained in heartwater diagnostics that have been developed by the Project.

7. Dissemination of Information by the Project

The Project used two mechanisms to disseminate information on heartwater globally. The first method was by publication of a newsletter called "The Tick-ler" which was published quarterly from September 1995 to December 1998 and mailed to about 400 individuals in 85 countries. Publication of this newsletter continued while funding for an information specialist was available. The second method was by publication of articles in international journals. The Project has been very productive in this area, publishing 177 full-length papers since 1985, with a further six in press at the time of writing of this report. These 183 publications are listed below:

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3. Burridge, M.J. (1986) Epidemiology of heartwater in the Caribbean. Pages 23-30 in Proceedings of Symposium on Tropical/Subtropical Agriculture Research. Caribbean Basin Advisory Group, Gainesville, Florida.
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5. Norval, R.A.I., Yunker, C.E. & Butler, J.F. (1987) Field sampling of unfed adults of *Amblyomma hebraeum* Koch. Exp. Appl. Acarol. 3: 213-217.
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8. Norval, R.A.I. (1988) The epidemiology of heartwater. Kenya Vet. 12: 9-11.
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15. Norval, R.A.I. (1989) Heartwater disease in Africa and its presence in the Western Hemisphere. USDA Foreign Anim. Dis. Rep. 17: 4-7.
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17. Norval, R.A.I., Butler, J.F. & Yunker, C.E. (1989) Use of carbon dioxide and natural and synthetic aggregation-attachment pheromone of the bont tick *Amblyomma hebraeum* to attract and trap unfed adults in the field. Exp. Appl. Acarol. 7: 171-180.
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In addition to its work in Africa on heartwater, the Project has been active for protecting the United States and its livestock industries and wild ungulate populations from the introduction of heartwater. Some of the ways in which the Project has helped in this task are listed below:

- developed vaccine against heartwater for use in the event that the disease is introduced
- developed PCR assay to screen animals, and in particular wild ungulates, imported from Africa for *C. ruminantium* infection, with subsequent transfer of this diagnostic technology to the U.S. Department of Agriculture
- developed tick decoy technology to help eradicate heartwater tick vectors from the Caribbean
- developed methods for the control and eradication of heartwater tick vectors introduced into the United States on imported reptiles

8. Patenting and Transfer of Technologies

In order to attract the private sector to commercialization of the Project's vaccines and tick decoys, it was essential that these technologies be patented in the United States and certain foreign countries, especially those in the SADC region. Consequently the Project has pursued a number of patent applications and, thus far, the following 8 patents have been issued:

1. "Attractant Decoy for Controlling Bont Ticks" by R.A.I. Norval, D.E. Sonenshine, M.I. Meltzer, M.J. Burridge & C.E. Yunker (U.S. patent no. 5,296,227) issued on 22 March 1994.
2. "Attractant Decoy for Controlling Bont Ticks" by R.A.I. Norval, D.E. Sonenshine, M.I. Meltzer, M.J. Burridge & C.E. Yunker (South Africa patent no. 93/4071) issued on 31 August 1994.
3. "Attractant Decoys for Controlling Bont Ticks and Other Pests" by R.A.I. Norval, D.E. Sonenshine, M.I. Meltzer, M.J. Burridge & C.E. Yunker (Zimbabwe patent no. 74/93) issued on 13 April 1995.
4. "Nucleic Acid Vaccines against Rickettsial Diseases and Methods of Use" by A.F. Barbet, R.R. Ganta, T.C. McGuire, M.J. Burridge, A. Nyika, F.R. Rurangirwa & S.M. Mahan (South Africa patent no. 97/9320) granted on 26 May 1999.
5. "Nucleic Acid Vaccines against Rickettsial Diseases and Methods of Use" by A.F. Barbet, R.R. Ganta, M.J. Burridge & S.M. Mahan (U.S. patent no. 6,025,338) issued on 15 February 2000.
6. "Nucleic Acid Vaccines against Rickettsial Diseases and Methods of Use" by A.F. Barbet, R.R. Ganta, T.C. McGuire, M.J. Burridge, A. Nyika, F.R. Rurangirwa & S.M. Mahan (Zimbabwe patent no. 24/99) issued on 28 April 2000.
7. "Materials and Methods for Prevention or Reduction of the Severity of Heartwater" by S.M. Mahan, A.F. Barbet, M.J. Burridge & G.E. Smith (U.S. patent no. 6,342,230) issued on 29 January 2002 (cell culture deposited in National Bank for Industrial Microorganisms and Cell Cultures in Sofia, Bulgaria, in 1998 as accession no. 3568).
8. "Materials and Methods for Prevention or Reduction of the Severity of Heartwater" by S.M. Mahan, A.F. Barbet, M.J. Burridge & G.E. Smith (Zimbabwe patent no. 57/2000) issued on 1 April 2002.

Patenting of the Project's technologies has led to considerable interest in their commercialization, and Intervet International has signed an option agreement with UF to commercialize the inactivated vaccine for heartwater and is currently negotiating a second option agreement with UF to commercialize the tick decoy technology.

COMMENTS AND RECOMMENDATIONS REGARDING UNFINISHED WORK AND PROGRAM CONTINUATION

1. The Inactivated Vaccine for Heartwater

This first-generation vaccine has been successfully tested in the field to the extent that Intervet International, the largest manufacturer of large-animal vaccines in the world, has signed an option agreement to commercialize the vaccine. Funding needs to be identified to complete commercialization of the inactivated vaccine so that it reaches the marketplace in the SADC region without any undue delay. To this end, a USAID Global Development Alliance (GDA) proposal has been submitted for funding.

2. The DNA Vaccine for Heartwater

This second-generation recombinant vaccine has the promise to be the most cost-effective and sustainable vaccine for heartwater. Funding needs to be identified to complete development of the DNA vaccine. To this end, a proposal is being developed with the United Nations Economic Commission for Africa.

3. The Tick Decoy Technology

This innovative method for tick control has been successfully tested in the field to the extent that Intervet International is negotiating an option agreement to commercialize this technology. Again, funding is needed to complete commercialization of the tick decoy technology so that it reaches the marketplace in the SADC region without any undue delay. To this end, a USAID GDA proposal has been submitted for funding.