Heartwater

Potential Worldwide Threats for Livestock

Gap Analysis Workshop Report

October 2018

The vision of the Heartwater Gap Analysis Workshop was to provide a foundation for controlling transboundary tick-borne pathogens of livestock, such as *Ehrlichia ruminantium*. Multidisciplinary scientists from several continents including Africa, Europe, South and North America came together and brought their expertise in immunology, entomology, parasitology, pathology, microbiology and molecular biology. The primary goals of this workshop were: 1) to facilitate research collaborations, 2) to conduct strategic research, and 3) to develop next generation strategies of controlling pathogen spread.

The purpose of this report is to 1) provide current scientific knowledge of heartwater, 2) identify potential threats to livestock worldwide, 3) identify research needs and priorities, 4) offer an in-depth analysis of available countermeasures to contain and mitigate threats, and 5) deliver specific recommendations for research and countermeasure development.

HEARTWATER ANALYSIS WORKSHOP

This gap analysis report is a collaborative effort of international scientists with expertise in tick and tick-borne diseases of livestock. This report intends to show how to control and mitigate the impact of a heartwater outbreak in new geographical areas, and also support global control and eradication initiatives in heartwater-endemic regions including sub-Saharan Africa and the Caribbean.

The gap analysis, was conducted both by presented research updates reported from 12 research institutes representing 10 different countries across the world, coupled with scientific literature reviews. Using this information, areas of heartwater research were prioritized.

To cite this report:
Heartwater Disease: Potential Worldwide Threats for Livestock. Gap Analysis Workshop Report. 2018. Agricultural Research Service, Washington, D.C. (https://go.usa.gov/xVszZ).

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GROUP PICTURE

Heartwater Gap Analysis Workshop
Guadeloupe, October 9-11, 2018

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EXECUTIVE SUMMARY

The Heartwater Gap Analysis Workshop met in Gosier, Guadaloupe, October 9-11, 2018 with support of the USDA-ARS, USDA-APHIS, CIRAD and CaribVET. The workshop group evaluated available countermeasures, identified and assessed strengths and weaknesses, and ranked existing methods to counteract against heartwater. Three groups were divided to assess current scientific knowledge and the available countermeasures to effectively control and mitigate the impact of a heartwater outbreak in new geographical areas, as well as, to support global control and eradication initiatives in heartwater-endemic countries. Each group used a decision model to assess potential countermeasures to control disease outbreaks caused by *Ehrlichia ruminantium*, including diagnostics and experimental vaccines. The groups used decision modeling as a means of classifying the efficacy of vaccines, diagnostics or tick control methods for preventing heartwater outbreaks, which led to understanding the gaps in our knowledge, research needs and priorities. The decision model was a simple tool that allowed focusing on critical criteria and ranking the current interventions relative to each other.

Scientific Information
Experts provided reviews of the current state of research and knowledge of *E. ruminantium* and tick vector biology, including immunology, vaccine development, epidemiology, tick control methods, bacteriology, pathology and laboratory diagnostics.

Countermeasure Assessment
The Heartwater Workshop assessed both commercial products and products known to be in the "pipeline." The assessment led to a prioritized list of segments where vaccines, diagnostics and tick control methods would have the greatest impact for preventing heartwater outbreaks. The decision model criteria, and their respective weight, were selected by each working group. We used numerical ranks for each available product. These countermeasures must significantly improve the ability to control and eradicate a heartwater outbreak. The goal of the countermeasure assessment was to develop a decision model and criteria to identify gaps in our knowlege regarding heartwater disease.

Research Needs
Research needs and priorities were identified to address knowledge gaps and, importantly, to advance the development of control strategies in order to mitigate a heartwater outbreak. Research priorities focused on 1) understanding bacterial infection in animal populations, 2) bacteria pathogenesis, transmission and epidemiology, and 3) development of improved countermeasures such as diagnostics, vaccines and tick control to yield significant improvements in mitigating heartwater outbreaks.

The gap analysis developed a comprehensive list of research needs and priorities.

Vaccine group

- Understanding strain diversity by sequencing strains from different regions. The sequences will allow comparative genomics to better understand phenotypic characteristics, virulence determinants in cattle vs sheep vs goats, identify putative vaccine candidates/conservation of vaccine candidates, and discover diagnostic markers

- Explore the dynamics of host-pathogen-vector interactions
- Compare different *E. ruminantium* strains from wildlife and livestock to determine pathogen virulence
- Identify mechanisms of immune evasion by *E. ruminantium*, and the correlates of protection
- Analyze vaccine delivery methods, including subcutenous, intramuscular and intradermal, that provides optimal protection and is accessible to the end user
- Identify optimal delivery system such as nanospheres or viral particles
- Evaluate adjuvants to enhance immune protection
- Develop tick challenge systems
- Develop cell-free systems for growing *E. ruminantium* to facilitate vaccine development and research such as targeted mutagenesis

Diagnostic group

- Understand global protein expression and antibody responses to better identify *E. ruminantium* carrier animals
- Early detection of infection for outbreaks in disease-free countries
- Characterize kinetics and features of early responses (i.e. acute phase markers, IgM)
- Validate and/or develop ELlSAs for wild ungulates
- Characterization of strains from wildlife for epidemiology studies
- Develop ELISAs or other technologies to detect multiple strains of *E. ruminantium*
- Determine how genetic diversity relates to epidemiology and strain tracking
- Determine how tick infection levels correlate to the risk of tick transmission
- Disease modeling and risk assessment are necessary to prioritize surveillance efforts

Vector control group

- Understand potential tick vectors for *E. ruminantium* transmission other than *Amblyomma variegatum*
- Determine tick efficiency in acquring bacteria during persistent infection
- Develop better acaricide formulations for tick control
- Develop an integrated control strategy that includes vaccines against ticks, acaricides and pasture rotation
- Vaccines that confer cross-protection against other *E. ruminantium* vectors
- An integrated control strategy that exhibits transmission-blocking affects
- Understand the tick microbiome and how that affects *E. ruminantium* transmission

GLOSSARY

ANSES: Agence nationale de sécurité sanitaire

APHIS: Animal and Plant Health Inspection Service

ARS: Agricultural Research Service

CaribVET: The Caribbean Animal Health Network

CENSA: Centro Nacional de Sanidad Agropecuaria

CIRAD: Centre de coopération internationale en recherche agronomique pour le développement

DIVA: Differentiating between infected and vaccinated animals

ELISA: Enzyme-linked immunosorbent assay

E. ruminantium: *Ehrlichia ruminantium*

FRET: fluorescent resonance energy transfer

Ig: Immunoglobulin

LAMP: Loop mediated isothermal amplification

nPCR: nested PCR

OIE: World Organization for Animal Health

ORF: Open reading frame

OVI: Onderstepoort Veterinary Institute

PBMC: Peripheral blood mononuclear cells

PCR: Polymerase Chain Reaction

pDNA: Plasmid DNA

PME: Panola Mountain Ehrlichia

qPCR: quantitative PCR

rRT-PCR: Real-time reverse transcription-polymerase chain reaction

US: United States of America

USDA: United States Departament of Agriculture

INTRODUCTION

The family Anaplasmataceae includes several tick-transmitted pathogens belonging to the genera *Ehrlichia* and *Anaplasma* (1). *Ehrlichia ruminantium* is the causative agent responsible for an important ruminant disease (heartwater) throughout sub-Saharan Africa and parts of the Caribbean (1, 2). Heartwater is characterized by fever, neurological signs, hydropericardium, hydrothorax, ascites, edema of the lungs, and high mortality rates (3). *E. ruminantium* multiplies in vascular endothelium throughout the body and in the lymph node reticuloendothelial cells. The name "heartwater" is derived from the hydropericardium, which is commonly observed with this disease. *E. ruminantium* is transmitted by ticks of the genus *Amblyomma*; the major vectors, originating from sub-Saharan Africa, are *A. variegatum* and *A. hebraeum* (4). *A. variegatum* is well established in several Caribbean islands (5) and consequently, its presence in the Caribbean poses a continuous threat of the spread of heartwater to livestock in North, Central, and South America (5, 6). The cattle egret (*Bubulcus ibis*), also a native of Africa (7), is established in the Western Hemisphere, including in the Caribbean and the USA (8, 9). This bird is commonly associated with cattle in pastures (8-10) and can serve as a host for *A. variegatum* ticks (11). Data on the migratory pattern of this bird suggest that it is the second most important potential vehicle for disseminating *E. ruminantium*-infected ticks to the American mainland (12). Further, two North American *Amblyomma* species, *A. maculatum* and *A. cajennense*, can serve as vectors for *E. ruminantium* (13, 14), though *A. cajennense* was a poor vector. Introduction of heartwater disease into a non-endemic area, such as to the USA, can result in 80-90% mortality rates in domestic and wild ruminants (14, 15). Although *E. ruminantium* persists in both vertebrate and tick hosts for long periods of time, little is known about the molecular basis for its pathogenesis and the pathogen evasion mechanisms supporting the persistence.

Heartwater is a non-contagious, infectious tick-borne disease of domestic and wild ruminants caused by *Ehrlichia ruminantium*. This disease is one of the most devastating tick-borne diseases of livestock, with mortality rates up to 80% in naive domestic animals, including bovine, ovine and caprine (16). This pathogen is an obligate intracellular bacterium and the disease severity in domestic ruminants depends upon the species and breed of ruminant affected as well as the strain of *E. ruminantium*. Animals that survive serve as reservoirs for tick transmission (17). Pathogen transmission occurs when tick larval or nymphal stages feed initially on an infected animal and then, following interhost transfer, feed on a susceptible animal (18). Following acquisition feeding and ingestion of the blood meal, *E. ruminantium* infects midgut epithelial cells and undergoes development with subsequent migration to and invasion of the salivary glands (19, 20). For transmission, infectious organisms are secreted in tick saliva (2).

The distribution of heartwarter is dependent on commingling of competent vectors and infected animals. Though surveillance and reporting efforts are not consistent, heartwater is likely a major cause of livestock loss in sub-Saharan Africa and the Caribbean (16). The risk of disseminating *E. ruminantium* to new areas exists due to the presence of potential tick vectors and susceptible animals that could become carriers. Should an outbreak occur in areas free of heartwater, the livestock industry's ability to produce food and fiber would be significantly reduced.

A few disease control strategies, including acaricides and live vaccines, have been used with limited success in countries where heartwater is endemic. The most common strategy of controlling tick-borne diseases is by preventing ticks transmission of *E. ruminantium* through the use of acaricides. However, the long-term and wide spread acaricide use has resulted in acaricide-resistant tick populations, which underscores the need for new methods to control ticks and tick-borne pathogens. Live vaccines, based on infection and treatment, are used in some countries however, they are blood-based and may possess inherent risks of disseminating other blood-borne pathogens including bacteria, viruses and hemoprotozoan parasites. In addition, *E. ruminantium* live vaccines confer limited protection due to strain diversity. Vaccine strains establish infection in naïve animals and vaccinated animals may serve as reservoirs for tick transmission. Within the tick vectors, *E. ruminantium* could mutate and revert to a virulent strain.

The main concerns for the spreading of heartwater is the inability to detect infected, but clinically normal animals, the potential for chemical acaricide resistant tick populations capable of transmitting *E. ruminantium*, climate and land usage changes that favor expansion and survival of ticks in heartwater-free areas, and the lack of an effective recombinant or killed vaccine that confers robust immune protection. The lack of tools to control and/or limit transmission of tick-borne *E. ruminantium* may render the livestock industry vulnerable to the consequences of heartwater if *E. ruminantium* is introduced into areas that have competent tick vectors.

DEFINITION OF THE THREAT

Economic Impact

No recent studies are available measuring the economic impact of heartwater in endemic areas or the estimated cost of an introduction into *E. ruminantium*-free areas. One older study demonstrated that countries endemic for heartwater have economic losses of approximately US$ 30 million annually (21). In another study, the annual losses was over US$ 5.5 million annually. That study included the cost of acaricide use, losses in milk production and antibiotic treatment (22). Introduction of heartwater into disease-free countries has important economic consequences for the livestock industries. It is estimated that up to 90% of immunologically naïve animals exposed to the pathogen would die as a consequence of *E. ruminantium* infection (16). Importantly, the bacterium is spread by tick vectors and, therefore, containment of heartwater requires robust vector control. If heartwater is introduced into disease-free countries important control measurement need to be implemented, including restricting animal movement to prevent the spread of *E. ruminantium* to other farms, controlling tick populations using acaricide treatment, and identifing and euthanizing all infected animals to eliminate potential carriers. These control strategies would predominantly affect small ruminant and cattle production.

Epidemiology and vectors

Introduction of *E. ruminantium*- infected *A. variegatum*, which is an aggressive tick and the primary vector of *E. ruminantium* in the Caribbean, arguably serves as the most likely route of *E.*

ruminantium introduction into the US. Establishment of *E. ruminantium* in mainland America relies on the lack of early detection and adequate vector populations and mammalian hosts to sustain transmission, both of which are theoretically available. Currently, *Amblyomma maculatum*, which was shown experimentally to be an efficient vector of *E. ruminantium* is well established in the US (14, 23). Additionally, *A. cajennense*, also present in limited areas of the US, can serve as a poorly efficient vector. Wildlife, including white tailed deer, can serve as reservoirs for *E. ruminantium*, thus presenting greater risk of long term establishment of a sylvatic disease cyle. Currently, suitable habitat for *A. variegatum* in mainland America can be found in Florida and the extreme south of the US. Environmental conditions coupled with global warming and the effect on tick populations together with the lack of rigorous and recent risk asessment addresseing this problem presents a major gap in ability to prioritize resources required for disease surveillance.

Surveillance

Surveillance is critical for early detection and rapid containment of a disease outbreak and thus is a critical countermeasure. In the US, with some exceptions, detection of transboundary diseases, including heartwater, relies on our generally robust passive surveillance system. A major component of this surveillance system is accredited, private veterinarians who serve as the eyes and ears of our national Veterinary Services and our network of diagnostic laboratories.

There are three primary limitations in our surveillance system to detect *E. ruminantium* rapidly following an introduction. First, there is a shortage of veterinarians that serve agricultural animals in rural areas, thus creating a weakness in our passible surveillance system. Second, the clinical presentation and size of the initial outbreak, and thus likelihood of rapid detection, is difficult to predict and is dependent on the species of ruminant affected and the rate of transmission, which is in turn dictated by the number of transmission competent ticks in the environment. Because ruminant herds in the US are naïve to *E. ruminantium*, unexpected death or severe disease and death is likely. There are some possible exceptions as cattle in general suffer less severe disease than sheep and goats, though whether this holds true for naïve U.S populations is unknown. Importantly, animals previously exposed to Panola Mountain Ehrlichiosis, may have some degree of immunity, thus reducing clinical disease and masking a disease outbreak. While the clinical disease is most likely to be dramatic, the number of animals affected may be small and the rate of spread slow if the number of competent tick vectors is low, thus decreasing the chances for early detection.

Third, robust surveillance system for the detection of tick introductions, with the exception of *Rhipicephalus microplus* in the US, are generally poorly developed and difficult to implement. Introduction of *E. ruminantium* would likely involve the concurrent introduction of *Amblyomma variegatum.* Early detection of this tick could prevent the introduction of *E. ruminantium*. Should this tick arrive on wildlife or illegally transported animals that circumvent a port of entry, rapid detection is unlikely.

Biosecurity

Implementing biosecurity measures on the farm is one of the most important countermeasures to prevent and protect the livestock industry operations, but specific measures need also to be included and integrated in an eradication campaign to prevent further transmission via tick

vectors. Restricting animal movement, controlling tick population, and identifying and euthanizing all infected animals are critical to preventing the dissemination of the disease to other areas free of *E. ruminantium*.

Vaccines

A vaccine is one of the most effective means to prevent and control the devastating effects of a disease. The only commercial vaccine available for heartwater is a live sheep blood vaccine with numerous inadequacies. Because it is a live vaccine, it cannot be used in non-endemic areas for fear of establishing *E. ruminantium* in those disease-free areas. In particular, this vaccine cannot be used where tick vectors are present but not *E. ruminantium*. Therefore, an effective alternative vaccine is needed to control the disease in endemic areas and its spread into new territories. During the recovery phase from a disease outbreak, a marked vaccine to differentiate infected from vaccinated animals (DIVA) may also be needed.

Diagnosis

The application of diagnostic tools is somewhat different in terms of early detection and diagnosis and outbreak control and pathogen elimination. Early detection in the face of an introduction, would rely on passive surveillance, and thus, would be dependent on the scale of the initial outbreak and the scope of the initial diagnostic efforts. As *E. ruminantium* would not necessarily be suspected, it could be missed in an initial diagnostic workup because clinical signs can be non-specific and gross and histologic lesions can be variable and subtle, particularly in cattle. Additionally, in the absence of heartwater as a differential diagnosis, the pathogen may only be visualized in the brain during routine histologic examination. Unfortunately, this organ is often not collected during field necropsies. A strong index of suspicion is required to conduct a brain smear, one of the best means for diagnosing heartwater in the field. This is unlikely to be done in the US.

Following the initial detection of *E. ruminantium*, the inadequacies of the available diagnostic tests may present challenges for control and eradication efforts. First, based on the biology of the pathogen, no reliable diagnostic tests exist to detect infected animals after exposure but prior to the onset of clinical disease because pathogen levels remain low in peripheral blood, which is the sample of choice. This period is variable and lasts from days to weeks. Though PCR is a robust means of diagnosing clinically affected animals. Second, in the face of establishment of the pathogen, a major limitation in the existing diagnostic tools is the ability to detect individual, inapparent carrier animals because pathogen levels are variable and often low and antibody levels wane through time.

In the context of *E. ruminantium* endemic areas, there are no diagnostic tests that that can be reliably applied to individual animals to determine their infection status. This greatly limits herd-level and regional control and eradication efforts and limits potential for animal export.

GAP ANALYSIS

The following section summarizes what we know about *E. ruminantium*, heartwater and highlights gaps in our knowledge.

Pathology and Pathogenesis

Heartwater, caused by *E. ruminantium*, is a disease of ruminants. In naïve populations, mortality rates can be up to 100%, however disease severity is dependent on the species and breed of animal, with goats and sheep being more susceptible than cattle. European breeds of ruminants are typically more severely affected than African breeds (24). The pathogen is transmitted by *Amblyomma* ticks. Following tick feeding, the incubation period is generally 2 to 3 weeks. The spectrum of disease can vary from peracute with fever, seizures and rapid progression to death to subacute characterized by prolonged fever, coughing and mild incoordination and recovery. However, acute disease is most typical. Clinical signs include fever, respiratory signs, such as cough and increased respiratory rate progressing to dyspnea. Profuse or hemorrhage diarrhea develops in some animals, though this is not consistent. Typically, neurologic signs such as chewing, circling, abnormal gait and posture, muscle tremors with terminal seizures, lateral recumbency, paddling, hyperesthesia and nystagmus (25). Importantly, animals that are treated early in disease or survive infection, generally become clinically inapparent, long term carriers of the pathogen and thus serve as a reservoir for ongoing transmission.

The disease is named for the remarkable hydropericardium that is more commonly seen in sheep and goats as compared to cattle. Additional common lesions include pulmonary and mediastinal edema, hydrothorax, ascites and perirenal edema. Endocardial petechiae are common and petechiae may be present in other organs, particularly the abomasum and kidneys. Cerebral congestion and edema may be present, but are not consistent findings. In some cases, lesions are absent (25).

E. ruminantium are obligate intracellular bacteria that reside in endothelial cells. Although edema is the predominant lesion, vasculitis is variable and tends to be a minor histologic finding. Perivascular malacia in white matter is evidence of vascular damage. Overall, the cause of the edema is poorly understood. Organisms are most readily identified in the cerebral endothelium. Thus, it is likely that immune dysregulation and alteration of the permeability of the vascular endothelium results in cerebral edema. Because few organisms are present in the pulmonary or cardiovascular endothelium, the cause of the hydropericardium and pulmonary edema is controversial (26). Some postulate that the pulmonary edema is due to immune dysregulation due to host response to the pathogen, while a second hypothesis is that the pulmonary edema is primarily neurogenic. Acute onset of pulmonary edema can occur following a significant central nervous system insult, particularly seizures.

While we have some understanding of the outcome of infection, the early stages of infection, prior to the development of fever are poorly understood. Based on early studies, the initial replication takes place in the reticulo-endothelial cells of the lymph nodes draining the site of

infection, with subsequent infection of endothelial cells (27). The pathogen is thought to enter endothelial cells by a process resembling phagocytosis, and forms a large colony within the vacuole, eventually causing the cell to burst, disseminating elementary bodies into the blood stream (20). Histologic, evidence of cell lysis or necrosis is absent, suggesting small numbers of cells may rupture at any given time.

E. ruminantium colonies can also be identified in monocytes of infected animals and in neutrophils in culture. The biological significance in the development of immunity and disease remain largely unknown. Ultimately, capillary endothelial cells serve as the primary host cell in the vertebrate host (16), with organism being most numerous in the brain, but also present, in alveolar endothelium, renal endothelium and the endothelium of large vessels.

Gaps and Research needs

1. Identification of the host cells responsible for the systemic spread of the pathogen will inform vaccine development and may provide opportunities to develop improved diagnostics designed to detect early infection.

2. An improved understanding of the pathophysiology of disease, particularly the development of edema, is useful the understanding how a vaccine should bias immune response toward protective immunity.

Bacteriology and Comparative Genomics

Heartwater is caused by a small (0.2-1 µm), Gram-negative obligate, intracellular bacterium belonging to the Order Rickettsiales. For most of the 20th century the bacterium was known as *Cowdria ruminantium*, but when a number of organisms in the order were re-classified based on molecular evidence in 2001, it was renamed *Ehrlichia ruminantium* (1). Figure 1 shows a phylogenetic tree of representative species in the order to show the positioning of *E. ruminantium*. Genetic diversity is one remarkable and biologically significant feature of pathogens in the family Anaplasmataceae, including *E. ruminantium*. A number of *E. ruminantium* strains have been "captured" and studied in varying detail, but mostly suggesting that there is relatively little cross protection when experimental vaccine studies are done. Therefore, strain diversity is of great interest.

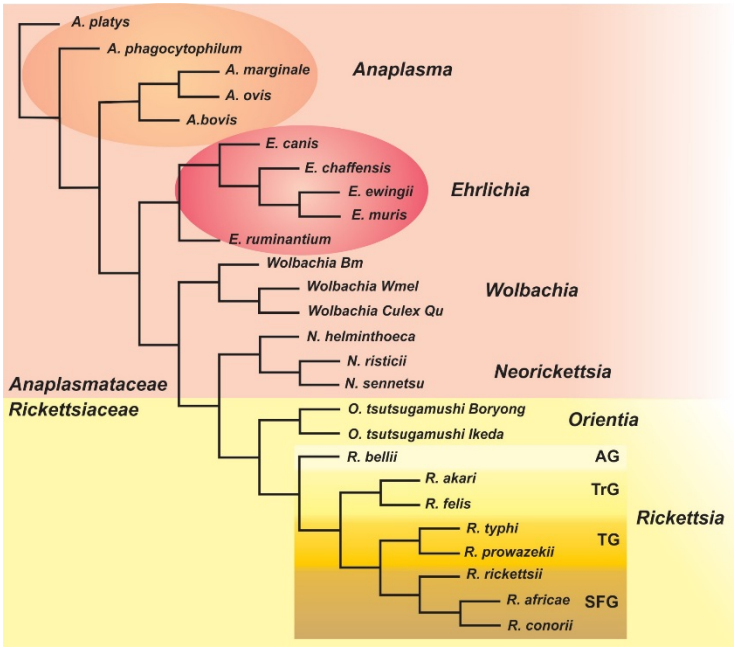


Figure 1: Phylogenetic tree of selected species in the order Rickettsiales. The tree was constructed using the 16S rRNA gene sequence. The families Anaplasmataceae and Rickettsiaceae are shown on different colored backgrounds. SFG = Spotted Fever Group; TG = Typhus Group; TrG = Transitional Group; AG = Ancestral Group.

Much of the early work assessing the diversity between strains focused on analysis of the *map1* gene, which encodes an immunodominant surface protein. The gene for *map1* was first cloned in 1994 from the Senegal isolate, and further analysis from additional isolates showed that there was three variable regions when comparing between isolates (Figure 2) (28, 29). The size of the *map1* gene, 854 bp, is similar to the *msp4* gene of *Anaplasma marginale* and P28/P30 genes of other *Ehrlichia* species. Indeed, these proteins are thought to cross react on Western blot analysis (30). However, although sera could cross react with the total protein, the distinct differences in the sequences between isolates indicated there were likely to be separate introductions of ER into the Caribbean (29).

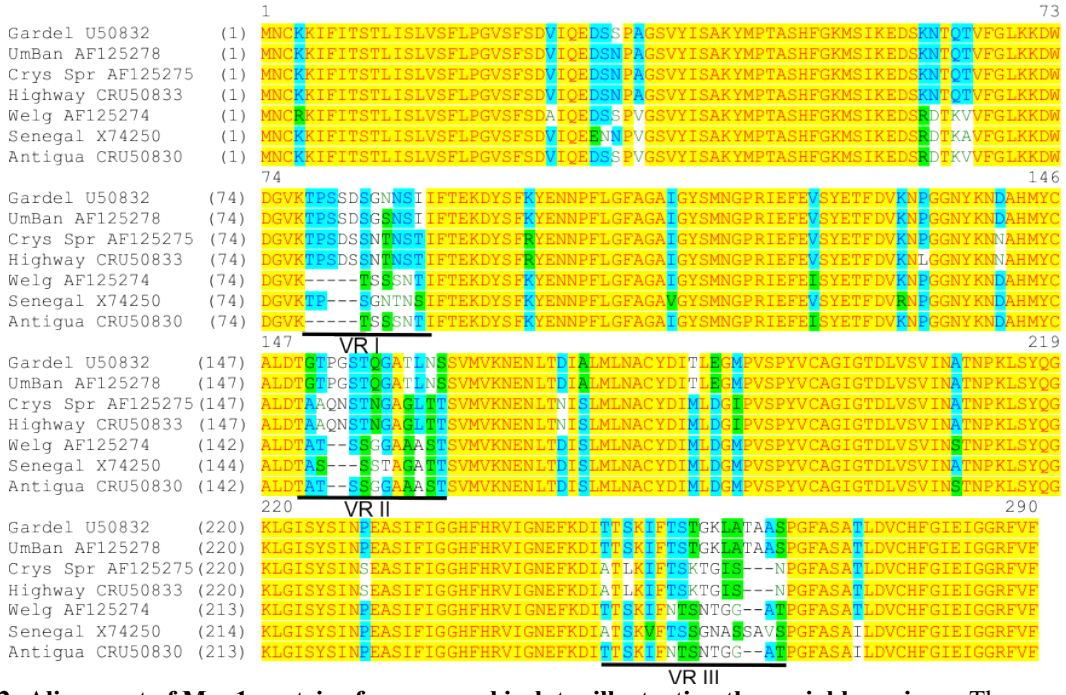


Figure 2: Alignment of Map1 proteins from several isolates illustrating the variable regions. The sequences are shown with their strain name or abbreviation and Genbank accession number. Identical amino acids have red text on a yellow background, blue indicates that the majority of the sequences are the same, while green background indicates conservative substitutions and green text indicates a weakly similar amino acid. Variable region I, II and III are underlined. Gardel and Antigua are from the Caribbean, Um Banein (UmBan) is from Sudan, Crystal Springs (Crys Spr) and Highway are from Zimbabwe, Welgevonden (Welg) is from South Africa, and Senegal is from Senegal.

The Allsopps did an analysis of eight "core function" genes from 12 strains (Table 1) and showed that the genes were a "mix and match" between strains, and their explanation for this was that there was extensive recombination between strains and suggested that recombination could take place while the organisms are extracellular in the tick (31). While *E. ruminantium* has a Type IV Secretion System (T4SS), it has never been demonstrated to transfer DNA, and these organisms are not known to have plasmids, making this idea harder to visualize.

Table 1. Color coded sequence matches of eight genes from 12 strains

	Sequence match of gene or region						
	16S	pCS20[a]	*gltA*	*groEL*	*ftsZ*	*sodB*	*nuoB*
Welgevonden	[b]						
Mara 87/7							
Ball 3							
Blaauwkrans							
Umbanein							
Kiswani							
Kumm2			NA				
Kumm1							
Senegal							
Sankat							
Pokoase							
Gardel							

[a]pCS20 is a DNA segment that corresponds to two genes, *rnc* and *cta*G.
[b]Color represents a sequence type, ie blue is the Welgevonden sequence, and when the same sequence is found in another strain it is identified with the color of the first strain tested. The *sodB* gene of Ball3 has the same sequence as Welgevonden. Adapted from Allsopp and Allsopp, 2007 (31).

Following from this analysis, a multi-locus sequence typing (MLST) assay was developed using the *gltA*, *groEL*, *lepA*, *lipA*, *lipB*, *secY*, *sodB* and *sucA* genes (32). The initial study employing this analysis suggested that some strains were in a state of genomic stasis, while others were rife with polymorphism. Further studies employing this technique indicate that recombination plays a role in the diversification of *E. ruminantium* (33, 34). As with the earlier study by the Allsopps, the phylogeny of these genes does not clearly segregate with their geographic origin. The CIRAD group suggests that the isolates are clustered into two main groups, a West African group and a worldwide group represented by West, East, and Southern Africa, Indian Ocean, and Caribbean strains (34). These authors suggest that both ancient and recent cattle movement has shaped *E. ruminantium* diversity by facilitating recombination between strains to generate the different subgroups shown in Figure 3.

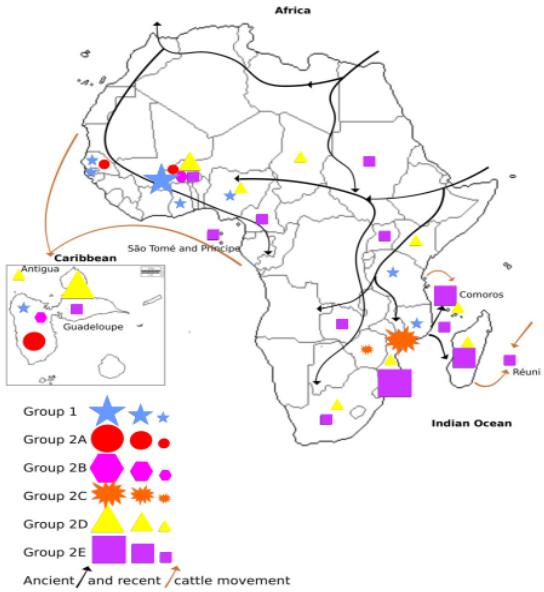


Figure 3: Distribution of *E. ruminantium* genetic groups and subgroups 1, 2A, 2B, 2C, 2D, and 2E in each sampled country within Africa, Caribbean, and Indian Ocean Islands. Groups are coded by symbols according to the legend. Symbol size corresponds to sampling size defined by the following sample threshold: >15 samples (big symbol), < 15 samples (medium symbol), and < 5 samples (small symbol). Recent (< 400 years ago; brown arrows) and ancient (>400 years ago; black arrows) movement of cattle is represented in the map. Group 1 = blue; Group 2 = hot colors. Adapted from Cangi et al., 2016.

The first strain to be sequenced was the Welgevonden strain from South Africa, revealing that the genome was 1.5 Mb is length, and encodes 920 genes (3). The genome has a low G+C content which is known to make cloning fragments unstable in *E. coli* (35). The genome contains numerous repetitive regions, with 126 short, simple repeats of 2-5 base pairs (homopolymeric tracts) and

158 long tandem repeats ranging from 6 to several hundred base pairs. The numbers of repeats are actively variable – that is, different clones contain different numbers of a particular repeat. With sequencing of a second strain, Gardel, comparative analyses revealed that gene order (synteny) was highly conserved between strains, with two small inversions corresponding to two or three coding sequences (Figure 4). Further, the gene content is highly conserved. When comparing between Gardel and Welgevonden only six coding sequences are detected that are substantially different between the two strains. Other differences are detected, but these correspond to either annotation differences, or in frame stop codons or frameshifts, which could be due to sequencing errors, while others appear to be truncated pseudogene copies of an otherwise intact full length gene elsewhere in the genome (36).

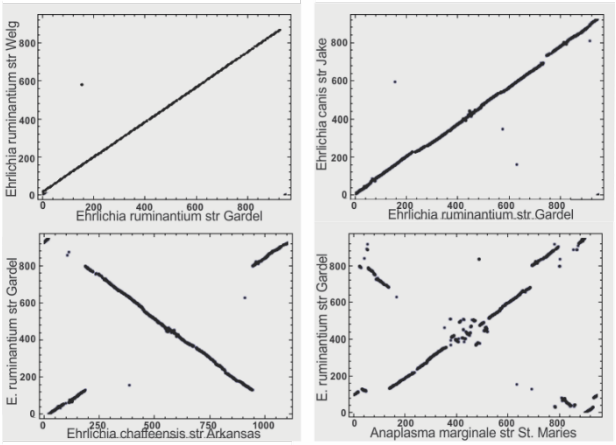


Figure 4. Whole genome alignments of *E. ruminantium* and other genomes. Strains Gardel and Welgevonden exhibit almost perfect syteny. There is significant synteny between *E. ruminantium* and other Ehrhichial genomes, with *E. chaffenesis* exhibiting an inversion around the origin of replication. There is much higher gene rearrangement when comparing with *A. marginale*.

More recently, draft stage genome sequences from the Crystal Springs strain from Zimbabwe, the Kerr Seringe strain from The Gambia, and the Sankat 430 strain from Ghana were reported (37). Interestingly, altogether, there are 10 genome sequences available for *E. ruminantium* representing 9 different strains, as the Welgevonden strain has been sequenced by two different groups (Table 3). While there are a number of sequences available, two points are readily discernable: 1) Little in-depth analysis has been done at the genomic level and 2) The strains that have been sequenced have been maintained in the laboratory for a long time and are not likely representative of *E. ruminantium* circulating in the field today. The oldest strains have been isolated since 1981 (Welgevonden and Senegal), while the "youngest" sequenced strain, Ker Seringe, was isolated in 2001 (Table 3).

Table 3: Available genomes for *E. ruminantium* at Genbank

Strain name	Country of origin	submitter	year[a]	year[b]	Accession #	size	# of contigs
Welgevonden	South Africa	UP	1981	2005	CR767821	1.52	1
Welgevonden	South Africa	CIRAD	1981	2006	CR925678	1.51	1
Gardel	Caribbean	CIRAD	1982	2006	CR925677	1.50	1
Crystal Springs	Zimbabwe	Hokkaido	1990	2016	BDDK00000000	1.48	34
Ker Seringe	The Gambia	Hokkaido	2001	2016	BDDL00000000	1.45	118
Sankat 430	Ghana	Hokkaido	1996	2016	BDDN00000000	1.46	183
Senegal (vir)	Senegal	CIRAD	1981	2017	MQUJ00000000	1.46	8
Senegal (p63)	Senegal	CIRAD	-	2017	MRDC00000000	1.46	8
Palm River	Zimbabwe	µFORGE	1989	2016	LUFS00000000	1.49	368
Pokoase	Ghana	Hokkaido	1996	2016	BDDM00000000	1.47	390

[a]year isolated from the field
[b]year sequence deposited or published

A number of isolates have been reported, spanning the breadth of sub-Saharan Africa and the Caribbean. A partial list of known strains is reported in Table 2. The blanks in the table show how salient information is often not recorded, or easy to find about strains; even more difficult to track down is an assessment of what hosts these strains are known to infect and how virulent they are in each host.

Table 2. Isolates of *E. ruminantium*

Country	Strain	Source	Year Isolated	Ref	Country	Strain	Source	Year Isolated	Ref
South	Mara 87/7			(38)	Zimbabwe	Crystal Springs		1990	(39)
Africa	Ludlow	tick		(38)		Palm River			(40)
	Morgenswag 1	goat		(38)		Mbizi			(39)
	Morgenswag 2	goat		(38)		Beatrice	Ah	1997	(41)
	Nonile	sheep		(38)		Finale	Ah	1997	(41)
	Welgevonden	Ah[a]		(42)		Mubayira	Ah	1997	(41)
	Ball 3	bovine		(43)		Rusape	Ah	1997	(41)
	Skukuza	Ah	1997	(44)		Hunyani	Ah	1997	(41)
	Kumm 1			(38)		Lemco T3			(40)
	Kumm 2			(38)		Highway			(40)
	Kwayanga			(45)		Plumtree		1992	(46)
	Vosloo	Ah		(47)		Kwekwe	Ah	1997	(41)
	Blaaukrans			(38)					
	Zeerust		1979	(48)	Kenya	Kiswani		1985	(38)
	Pretoria North	Dog		(49)		Asembo Bay	Av[b]		(50)
						Marigat	Av		(50)
Burkina Faso	Burkina Faso		1997	(38)		Isiolo	Ag/Al[c]		(50)
	Lamba 479	tick		(51)					
	Sara 450T	tick		(51)	Uganda	A004; A006	Av	2008-9	(33)
	Sara 371T	tick		(51)		D002	Av	2008-9	(33)
	Bek448FC2	tick		(51)		P003; P006	Av	2008-9	(33)
	Ban455Cer	brain		(51)		T009	Av	2008-9	(33)
	Sara 292F1	tick		(51)		S001; S013	Av	2008-9	(33)
	Lamba 465T	tick		(51)					
	Bank 421Cer	tick		(51)	Ghana	Sankat 430		1996	(52)
	Bek 313 Cer	brain		(51)		Pokoase 417		1996	(52)
	Ban 181T	tick		(51)					
	Sara 409 Cer	brain		(51)	Caribbean	Antigua	Av		(53)
						Gardel		1982	(54)
Zambia	Lutale		1986	(55)					
Mozambique	Umpala			(38)	Nigeria	Nigeria D225	bovine		(56)
Mali	Mali			(38)		Ifé Nigeria		1983	(33)
Senegal	Senegal		1981	(38)					
Tanzania	Tanga	Av	1997	(41)	Sudan	Um Banein	sheep	1981	(57)
Cameroun	Cameroun			(38)	Gambia	Kerr Seringe		2001	(58)
Sao Tom é	Sao Tom é		1981	(33)					

[a]Ah = *Amblyomma haebraeum*
[b]Av = *A. variegatum*
[c]Ag/Al = *A. gemma*/*A. lepidum*

Gaps and Research needs

1. *E. ruminatium* strain diversity needs to be more fully addressed. Genome sequences of current field strains should be obtained and comparative analysis done not only with other *E. ruminantium* strains, but also with other *Ehrlichia* species.
2. Host range is not well known, it is often assumed that the field isolates will infect cattle, sheep and goats (and mice), but this has not been examined experimentally. For example, are

there isolates with a predilection for small ruminants over large ruminants or vice versa? Can comparative genomics address this gap? Is *E. ruminantium* undergoing a host range shift as has been suggested?

3. Comparative virulence of strains has been studied to some degree (59, 60). While this is a difficult trait to dissect because dose can play a role, comparative genomics may again shed some light on this phenotype when the right strains are analyzed.

Plainly put, this boils down to acquisition of genome data from a variety of sources: ticks, bovine, ovine and caprine hosts from a variety of geographically diverse regions. Complete genome sequences will facilitate comparative analyses and it is recommended that researchers use long sequence read technology such as PacBio in the near term to obtain these sequences in a cost-effective manner. Whole genome comparative analyses will shed light how much of the genome is undergoing recombination, whether there is a stable core set of genes that can be targeted for vaccine development and robust diagnostic assays.

Immunology

Detailed understanding of the host response is valuable in devising effective interventions, including the development of robust diagnostics and treatment and control strategies. Our understanding of the host immune response to *E. ruminantium*, including the components that provide protection in contrast to those that lead to immune dysregulation and disease or death, is somewhat limited. Additionally, much of the work, particularly the most relevant work carried out in ruminants, was done over two decades ago.

The immune response against *E. ruminantium* has been assessed in a number of experimental models including infection or protective immunization of cattle, sheep, and goats (61-63). Protective immunization typically consists of the infect and treat method of immuniztion, whereby animals are infected with *E. ruminantium* and treated with oxytetracycline to prevent severe disease and death. This method relies on the fact that animals that recover from infection develop robust, long-lasting immunity to challenge with a homologous strain, though not heterologous strains. Alternatively, immunization with inactivated organisms can also resulted in protective immunity and has provided insight into the protective immune response to *E. ruminantium* (62, 64, 65).

Mice have been used as an experimental model in passive transfer experiments and immunization and challenge studies. It is important that findings in mice are confirmed in ruminants as mice are not natural hosts for *E. ruminantium*. The response of cultured cerebral bovine microvascular endothelial cells to *E. ruminantium* infection serve as relevant models because these cells likely play a central role in pathogenesis of disease and initiation of the immune response (66, 67). Finally, the response of peripheral blood mononuclear cells from protectively imunized animals provides further insight into the role of T cells in protective immunity and the antigens they recognize (68, 69). Overall, these studies indicate that animals develop cell-mediated immunity involving CD4+, CD8+, γδ T-cells and B-cells, and Th1 cytokines including variable levels of cytokines including IL-2, IL-1b, IL-6, IL-8, IFN-γ, INF-α and TNF-β, TNF- α (68).

Host cytokine response against *E. ruminantium*

In general interferons have an important role in the immune protection against intracellular bateria in the order Rickettsiales. The foundational studies involving characterization of the cytokine response to *E. ruminantium* were conducted in bovine microvessel endothelial cells. Infection of these cells resulted in the production of IL-1b, IL-6 and IL-8 mRNA in the presence and absence of IFN-γ, though the the effect was enhanced by the presence of IFN- γ (66). IFN-α and IFN-γ are of particular importance and play a major role in the protection against *E. ruminantium*. Both are produced in response to infection with *E. ruminantium* or vaccination with killed organisms (61, 64). Survival to challenge has correlated with levels of IFN-α and IFN-γ (61, 64), though these findings are not consistent in the case of IFN-γ. In fact some vaccinated goats survived lethal challenge without a measurable IFN-γ response in plasma (70).

In vitro, both cytokines have an inhibitory effect on *E. ruminantium* (61). Though, even at high concentrations of IFN-α, *E. ruminantium* growth was not completely abrogated (61), while IFN-γ completely blocked the replication of *E. ruminantium* in bovine endothelial cells (71-73). The dose dependent decrease in *E. ruminantium* viability in bovine respiratory endothelial cells in the face of treatment with IFN- γ correlated with an increase in nitric oxide, suggesting production of nitric oxide may contribute to the anti-*E. ruminantium* effects of IFN- γ, though these findings are not consistent (73, 74).

Recently a more holistic approach was taken to characterize the innate and adaptive immune response to *E. ruminantium* infection in sheep PBMC during tick infection and tick challenge (75); (personal communication; unpublished results). Cytokine rRT-PCR (IFN-γ and TNF-α) and immune transcriptome sequencing was performed with PBMC at different time points during infection and challenge. Cytokine profiling indicated that the cytokine levels peaked around 12-13 days after infection and challenge.

Functional analysis of up-regulated genes indicated that the most pathways were upregulated during the febrile response and 6 days after challenge. String analysis showing the network analysis of protein-protein interactions in up-regulated genes indicated a significant increase in the number of protein interactions on day 16 after infection. The top five innate immune response pathways included Toll-like receptor signaling, NOD-like receptor signaling, chemokine signaling, cytosolic DNA sensing and cytokine receptor interaction. The number of genes in these pathways increased significantly during the febrile response and day six after challenge. A number of genes were identified that were unique or shared between these two time points. Several adaptive immune response pathways were also identified. Thus, innate and adaptive immune response pathways to *E. ruminantium* infection in sheep PBMC during natural infection and challenge were identified that can assist in vaccine development studies. This will help us to design vaccines which elicit much more focused and effective immune responses.

Cell mediated immunity against *E. ruminantium*

Antigen presentation is one of the key initiating events in development of an adaptive immune response. Endothelial cells and monocytes, the primary host cells for *E. ruminantium*, potentially have a major role in antigen presentation. In general, IFN-γ upregulates major histocompatibility complex (MHC) I and MHC II expression on a variety of cell types, including endothelial cells. Importanly, *E. ruminantium* infection inhibits the expression of MHC I and

MHC II molecules in endothelial cells, though endothelial cells do maintain the capacity to present antigen (68), at least *in vitro*.

CD4+, CD8+ and γδ T cells from protectively immunized animals proliferate in the presence of E. *ruminantium* infected endothelial cells and monocytes (65). These cells are all sources of IFN-γ, while CD4+ T-cells are required in helping CD8+ T-cell activation (67, 76, 77). The role of CD8+ T cells in anti-*E. ruminantium* immunity remains to be determined. In one study, a rise in CD8+ T cells occurred late after challenge of immunized animals and after non-immunized animals succumbed suggesting mechanisms other than CD8+ T cell killing were responsible for the protective immune response (78). However, in mice, both CD8+ and CD4+ T-cells play a role in protection as judged from adoptive transfer experiments in wild type mice (79). Importantly mice lacking CD4+ T cells were able to resist challenge following immunization via infection and treatment (80). Thus the current effort in vaccine development is focused on producing both CD4+ and CD8+ T cell response (81, 82).

Antibody response to *E. ruminantium*

The predominant, measurable antibody response targeting the major immunodominant antigens in the MAP1 family in both cattle and sheep does not correlate with recovery or progression to severe disease (67, 83, 84). In general terms, antibodies are important effectors of the adaptive immune response, including opsonization, complement mediated killing and cell mediated cytotoxicity and thus cannot be completely dismissed as relevent effectors against *E. ruminantium*. Additionally, antibodies relevant for protective immunity may be subdominant and thus difficult to measure.

Impact of field challenge

The outcome of immunization and challenge experiments is dependend on the route of challenge. For example, there was 100% protection against needle challenge using DNA vector vaccine and prime boost strategy. In contrast, there was 20% protection against a natural tick challenge. There are a number of variables that could account for this difference including challenge dose, which cannot be controlled by tick challenge, strain and the potential for immuno-suppressive effects of tick feeding. The innate immune response is delayed in response to either tick feeding or direct effects of the pathogen (75). Overall these findings highlight the necessity for testing vaccines via tick challenge in a controlled environment, prior to field testing (85, 86).

Gaps and Research needs:

Despite a greater understanding of immunity exerted by vertebrate hosts against *E. ruminantium*, several knowledge gaps exist which warrant the renewed research efforts on this important disease. A greater strain variation exists in *E. ruminantium* isolates recovered from across the Sub-Saharan Africa and parts of the Caribbean. Pathogenicity of *E. ruminantium* isolates is also consequently highly variable; as some isolates cause more severe disease in ruminants while others may cause more moderate disease. Likewise, host response and pathogenesis may differ considerably for infection acquisition by a natural route of tick transmission compared to needle inoculation experiments. Furthermore, host responses and pathogenesis vary greatly depending on the host species acquiring the infection and host-specific differences for ruminants originating from diverse geographic regions. Ruminants in non-endemic regions are likely to develop significantly higher morbidity and mortality to *E. ruminantium* infections compared to those

originating from sub-Saharan Africa. Considering these challenges, investigations on heartwater should also be focused on the following areas:

1. Compare host response outcomes for tick-transmitted infections with *E. ruminantium* isolates representing diverse geographic locations causing a severe and moderate disease.
2. Compare host responses between needle infection experiments and tick-transmitted infections to define how tick feeding alters the course of host response.
3. Define host response variations in small and large ruminants originating from endemic regions and non-endemic regions to tick transmission of *E. ruminantium*.
4. Define immunological correlates critical for the protective host response and having the ability to confer protection against diverse *E. ruminantium* isolates and for small and large ruminants originating from diverse geographic locations.
5. Assess indigenous *Amblyomma* species of ticks from North America as vectors for transmitting *E. ruminantium* and in causing heartwater.
6. Pursue the research in developing innovative vaccine strategies, such as to assess live attenuated vaccines as described for *E. chaffeensis* (87-89). Similarly, efforts should continue in evaluating inactivated whole cell-derived antigen vaccines and subunit vaccines having the ability to confer protection to genetically diverse strains of *E. ruminantium* (90-92).

Vaccines

The only commercial vaccine available for heartwater is a live sheep blood vaccine containing the Ball3 strain (93). This is injected intravenously into ruminants. Rectal temperatures must be monitored daily and antibiotic treatment administered soon after a febrile response develops. The vaccine must be preserved at -70°C limiting its use in rural areas. Furthermore, the Ball3 strain does not protect against all field strains. Although the highly virulent Welgevonden strain is known to protect against most strains (86) it causes death very shortly after a rapid temperature rise. In contrast the Ball3 strain produces a febrile reaction several days before any other clinical signs appear which makes it easy to treat in time, making it the vaccine strain choice.

Attenuated vaccines

The first *E. ruminantium* organism to be cell culture attenuated was the Senegal strain (94). This vaccine conferred 100% protection to homologous needle challenge in sheep, and limited protection against heterologous natural tick challenge (95, 96). A cell culture attenuated Welgevonden strain resulted in 100% protection in sheep, goats and cattle from a homologous and heterologous needle challenge (63, 97). It can provide full protection for approximately six months and is currently being developed for commercial distribution in southern Africa.

Inactivated vaccines

The first inactivated vaccine (Gardel strain) protected 50-80% of goats against a homologous needle challenge (62). Similarly, the Crystal Springs strain inactivated vaccine protected 50-100% of sheep against a homologous needle challenge (98). However, two doses of vaccine are needed and protection against heterologous strains is low. Importantly the inactivated heartwater vaccine can be stored at ambient temperature and several strains can be included.

Recombinant subunit vaccine development
A recombinant vaccine is cheaper to manufacture, can be stored at ambient temperature, and is easier to transport and administer. Several promising recombinant vaccines have been tested.

The major antigenic protein 1 (*map*1) gene was the first DNA vaccine tested that protected 23-88% of mice against a needle challenge (99). Priming the mice with the *map*1 DNA vaccine followed by boosting with the recombinant MAP1 protein increased protection from 13-27% to 53-67% (100).

When *E. ruminantium* genomic mini-libraries were cloned into a *Salmonella* vaccine delivery system and used to immunize outbred mice, no protection was obtained with needle challenge (101). However, a low level of protection (14%) was induced when individual clones were tested.

An *E. ruminantium* genomic expression library was screened with sera from heartwater-immune sheep and positive clones were selected. These open reading frames (ORFs) were cloned into an expression vector and recombinant proteins generated (102). Those that reacted positively with heartwater-immune antisera and stimulated proliferation of PBMC from cattle immunized by an infection and treatment method were used to immunize mice (103). Two pools induced 58-89% protection and five of these individual DNA constructs induced cell-mediated immune responses and partial protection in mice.

The first heartwater DNA vaccine that was developed at ARC-OVI was with four ORFs called 1H12. They were identified from an ER cosmid library and when tested in mice they induced variable protection (104, 105). Three of these four ORFs (Erum2550, Erum2580 and Erum2590) are predicted to be genes belonging to an ABC transporter system while Erum2540 is an exported protein (3). They were tested in sheep as a vaccine using four different immunization strategies: 1) plasmid DNA (pDNA) cocktail (86, 106), 2) pDNA with individual genes (106), 3) pDNA cocktail prime followed by recombinant protein cocktail boost (85), and 4) a pDNA cocktail prime followed by recombinant lumpy skin disease virus (rLSDV) vectored cocktail boost (85). Strategy 1) reproducibly gave 100% protection against needle challenge with five different strains (Welgevonden, Blaauwkrans, Ball3, Gardel, Kwanyanga, and Mara 87/7). All four strategies were able to fully protect sheep against Welgevonden strain needle challenge, except the LSDV boost strategy where only 4/5 animals survived. However, natural tick challenge experiments resulted in 0-20% protection after immunization using strategy 1) (85, 86).

Subsequently, the *E. ruminantium* polymorphic gene 1 (*cpg1*, Erum2510), was evaluated at the ARC-OVI as a DNA and DNA prime/recombinant protein boost vaccine in sheep. DNA immunization alone gave variable and limited protection whereas the DNA prime/recombinant protein boost gave full protection against Welgevonden strain needle challenge (107, 108).

Reverse vaccinology (RV) using bioinformatics algorithms was used to search the Welgevonden genome sequence and select possible vaccine candidate genes based on selected criteria (surface-associated proteins; secreted proteins; transporters; environmental stress adaptation proteins; proteins containing tandem, tetratricopeptide or ankyrin repeats; adhesins; proteases; iron-

binding proteins; methyltransferases; GTPases; and homologs of proteins identified as vaccine candidates in other pathogens) (109). The initial identification of 419 genes was reduced to 272 by eliminating patented genes, genes tested previously, and genes with more than four predicted transmembrane helices. These genes were expressed in *E. coli* and the recombinant proteins were screened for their ability to induce cellular immune responses by PBMC from heartwater-immune cattle and sheep using several immunological techniques (109-111). Five low molecular weight proteins were identified that induced cellular immunity and these were tested as a cocktail pDNA vaccine in sheep and 20% protection was obtained against Welgevonden strain needle challenge (110).

It appears that a recombinant vaccine should be able to induce innate and adaptive immunity (CD4+, CD8+ T cells, γδ T cells and B cells). To achieve this it will require the identification of antigens/peptides/epitopes that induce this immunity and to determine the cell types producing appropriate cytokines.

In order to further improve the DNA vaccine it must induce a broad spectrum immune response that will protect animals against a wide range of *E. ruminantium* strains and must thus include several protein antigens. The current vaccine candidates encode proteins of 100-300 amino acids. However only a small region (<20 amino acids) induces protective immunity and is called an epitope. Using only epitopes in a vaccine will allow discarding of a large amount of unnecessary, harmful or inhibitory sequences as well as efficient packaging of multiple epitopes.

Scientists at the ARC-OVI have designed and synthesised 16 mer overlapping peptides and identified and characterized individual peptides that induce: IFN-γ by CD4+ and CD8+ memory T cells, Th1 cytokines (IL-1α, TNF-α, iNOS, GM-CSF) and/or CD8+ cytotoxic activity (82); (personal communication; unpublished). A 12-peptide DNA vaccine was constructed in a mammalian vector designed for dual expression of eight CD4+ and four CD8+ specific peptides. This multi-peptide DNA with adjuvant vaccine protected 60% sheep (with different MHC II DRB1 alleles) against laboratory tick challenge. Once again it was noted that the presence of antigen specific IFN-γ and CD4+ and CD8+ memory T cells was not an indication that an animal will be protected after challenge.

Targeting the early immune response

We have determined that different *E. ruminantium* proteins are present in the ruminant host, tick host and at the tick bite site (early antigens) (112); (personal communication). Including early antigen peptides in a vaccine will ensure that *E. ruminantium* is detected and eliminated soon after being introduced via the tick before it has a chance to establish an infection. In addition, including B-cell peptides will ensure that the extracellular stage is also detected by the immune system resulting in a more effective protective mechanism. Furthermore, epitopes presented by B cell MHC II to Th2 cells ensures effective memory B cell/antibody response.

Transcriptome analysis of *E. ruminantium* in the ruminant host at the *E. ruminantium* infected tick bite site, in the *E. ruminantium* infected tick vector salivary glands and *E. ruminantium* infected ruminant and tick cell cultures was performed (112), (personal communication). A number of *E. ruminantium* genes were identified that are unique to each sample or shared between the datasets. Bovine cell culture *E. ruminantium* elementary body or sheep skin biopsy

E. ruminantium unique genes can be targets to block infection of mammalian host cells or to induce early and B cell immune responses. Bovine cell culture reticulate body unique genes can be targets for cytotoxic immune responses. Tick cell culture *E. ruminantium* elementary body or *E. ruminantium* in tick salivary gland unique genes can be targets to block infection of the tick host cells.

Characterization of the immune response induced by selected recombinant *E. ruminantium* early proteins when incubated with immune sheep PBMC identified proteins that induce significant IFN-γ and/or IL-4. They were selected for immune transcriptome sequencing analysis where significantly up and down regulated genes were analyzed with the KEGG pathway mapping tool. Several up and down regulated genes were identified in KEGG immune pathways. The immune transcriptome findings indicate that ErumC induced down-regulation of cellular and humoral immune pathways and up-regulated genes of the innate immune pathways. This may indicate that this protein inhibits the immune response when *E. ruminantium* enters the ruminant host, thus allowing it to infect the host before a protective immune response in induced. Thus, ErumC is a vaccine candidate antigen that possibly contains peptides that will induce an early innate and adaptive memory immune response to *E. ruminantium*. Including these peptides in the current multi-peptide DNA vaccine may improve its efficacy to protect sheep against a virulent tick challenge.

Acknowledge the research team from ARC-OVI (some members have left): Ms. Erika Faber, Dr. Jeanette Heath, Dr. Junita Liebenberg, Dr. Tshifhiwa Nefefe, Ms. Abigail Ngoepe, Dr. Alri Pretorius, Ms. Lauren Son, Mr. Rae Smith, Dr. Helena Steyn, Dr. Topsy Thema, Dr. Mabotse Tjale, Mr. Ndavhe Tshikhudo, Dr. Ivy Tshilwane and Dr. Mirinda van Kleef. We would like to thank the following: University of Pretoria, University of Minnesota as the source of the tick cells (IDE8) and Dr Lesley Bell-Sakyi of the Tick Cell Biobank at The Pirbright Institute for providing the tick cells and funders (ARC, ECSP, THRIP, DoA, NRF).

Gaps and Research needs

Vaccine development problems that were identified that need solving:

1) Strain diversity
    a. Sequence strains from different regions
    b. Comparative genomics to:
        i. Better understand phenotypic characteristics
        ii. Understand virulence determinants in cattle vs sheep vs goats
        iii. Identify putative vaccine candidates/conservation of vaccine candidates in pipeline
        iv. Look for diagnostic markers
2) Host-pathogen-vector interactions
3) Immunity (Immune profile has been characterized):
    a. Identify mechanism of immune evasion by ER
    b. Identify the correlates of protection
    c. Analyze delivery: SC, IM, ID,
        i. That will provide optimal protection (ie, will ID provide faster immunity against tick bite)
        ii. Be accessible to end user

iii. Identify optimal delivery system, ie nanospheres vs viral etc
iv. Adjuvants
v. Develop tick challenge system (It is available in South Africa)
vi. Develop cell free system for growing ER to facilitate vaccines, and research
    1. Such as targeted mutagenesis

Diagnosis

Diagnosis in mammals

The spectrum of clinical disease can vary from unexpected death to mild symptoms that include fever, cough and incoordination. Neurologic disease, including chewing movement, circling, high stepping, rigidity progressing to recumbency, paddling, opisthotonos and death are the most distinct clinical manifestations. As such, differential diagnoses include bovine cerebral babesiosis, rabies, theileriosis, anaplasmosis, anything that causes unexpected death in small ruminants, rabies and other causes of neurologic disease. In short, a definitive diagnosis based on the clinical signs, post mortem and histologic lesions cannot be made.

Post mortem lesions are also variable, but can include marked hydropericardium, hydrothorax and pulmonary edema. Such lesions, in combination with neurologic disease are somewhat distinct for heartwater.

Brain smears

Diagnosis of heartwater post mortem can be done by direct visualization of the pathogen in Giemsa-stained, impression smears of brain or intima of blood vessels. The advantages of this technique are that it is specific, low cost, requires only basic laboratory equipment and does not require extensive training. The disadvantages are that this procedure is not a routine part of a post mortem diagnostic work up in *ER* free areas and would only be done if heartwater is suspected. Additionally, in the context of brain smears, sample acquisition is inconvenient and the sensitivity is low at 76%. In rabies endemic areas, including much of the Americas, this technique presents serious biosafety concerns.

Isolation using *in vitro* culture

Numerous cell lines, including endothelial cells from umbilical cord, aorta and pulmonary artery from cattle, sheep or goats support the growth of *E. ruminantium*. However, in terms of rapid diagnosis, detection of growth generally requires 2 weeks (113). Thus, culture has little utility apart from use for strain typing.

Polymerase Chain Reaction (PCR)

PCR can serve as both a post-mortem and ante-mortem test. Various PCR based tests have been developed using different platforms, gene targets and sample types. In animals that succumb to heartwater, validation to varying degrees has been done in fresh, frozen and ethanol-fixed lung, brain and blood. Detection from the buffy coat isolated from peripheral blood using PCR detection is most reliable in animals one to two days prior to onset and during fever. In animals that are treated or survive infection, *E. ruminantium* is not consistently detected in individual animals through time.

One study has been done tracking the PCR detection through time using nested PCR (nPCR) (83). In this study, all animals were PCR positive at least one time between weeks 4 to 6 following either field exposure or tick transmission in a laboratory setting (Fig. 1). In the field setting, all animals were positive at week seven. In the laboratory setting, all animals were positive at week four. Through time, the number of positive animals at any given time following infection decreased.

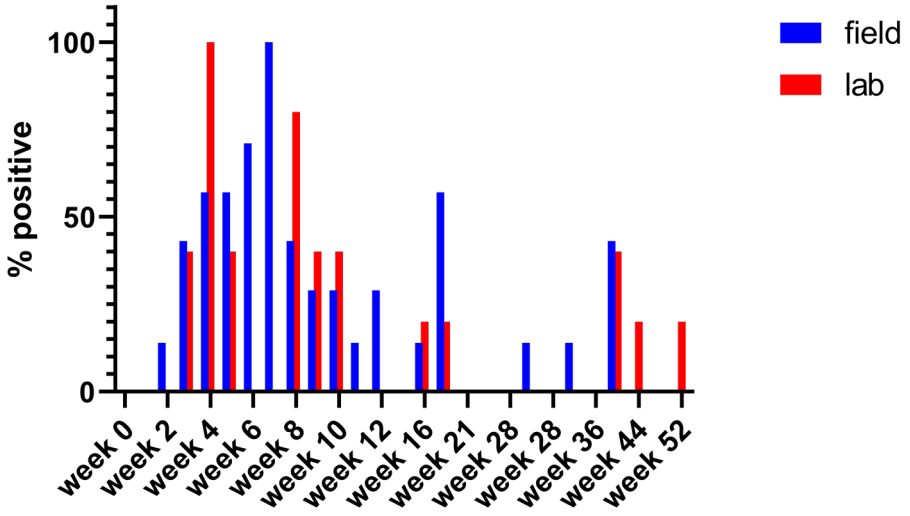


Fig. 5. Percent PCR positive cattle through time, using AB129/AB129 primers. Animals were either infected under laboratory conditions using *A. habraeum* ticks fed on *E. ruminantium* inoculated sheep or infected under field conditions.

PCR

The primary gene target is pCS20, originally identified as a 1,306 base pair sequence derived from Crystal Springs strain of *ER* (GenBank X58242.1) (114, 115). pCS20 has two open reading frames, a ribonuclease III and a cytochrome C oxidase assembly protein. All PCR targets are in the ribonuclease III open reading frame. MAP 1 has also been used a target for diagnostic PCR, however variability in this gene among strains precludes its use.

The primers AB128 and AB129, which amplify a 279 base pair region (Table 1) of the ribonuclease III open reading frame, were initially demonstrated to have high specificity for *E. ruminantium* by the absence of amplification of bovine, *A. marginale*, *B. bigemina*, *T. brucei brucei* or *E. coli* DNA in a non-nested PCR (116). The detection limits of this reaction were 10 to 100 organisms, while dot blotting and hybridization increased the detection limits to 1 to 10 organisms (116).

Nested PCR (nPCR)

Based on this primer pair, there are two nPCR protocols targeting the same region of pCS20 using different combinations of external and internal primers (83, 117). One uses U24 and L24 as the external primers and AB128 and AB129 as the internal primers (83). A second uses AB128 and AB130 as the external primers and AB128 and AB129 as the internal primers (117).

Post mortem blood, brain and lungs samples either preserved frozen or in 70% ethanol were tested. The analytic sensitivity for this latter assay was 6 organisms per reaction.

Finally, these primers (AB128', AB129', AB130') were modified with degenerate nucleotides to account for SNPs in the target region and allow for detection of a broader range of *ER* strains (118). This nPCR and has been routinely used at the OIE Reference Laboratory for diagnostic purposes and can detect 15 copies per reaction, but was recently shown to cross react with Panola Mountain Ehrlichia (PME) (119).

Nested PCR presents serious challenges for diagnostic labs in terms of rapid turn around and requires dedicated, separate space for gel running to avoid the potential for amplicon contamination. Thus, real-time PCR, which in general has similar analytic sensitivity is currently the preferred platform.

Table 4. Primers used in PCR-based assays.

Assay name	Primer name	Primer sequence (5' to 3')
nested PCR	U24	TTTCCCTATGATACAGAAGGTAAC
	L24	AAAGCAAGGATTGTGATCTGGACC
	AB128	ACTAGTAGAAATTGCACAATCTAT
	AB128'	ACTAGTAGAAATTGCACAATCYAT
	AB129	TGATAACTTGGTGCGGGAAATCCTT
	AB129'	TGATAACTTGGWGCRRGDARTCCTT
	AB130	ACTAGCAGCTTTCTGTTCAGCTAG
	AB130'	RCTDGCWGCTTTYTGTTCAGCTAK
real time PCR	Sol1F SG/TqM	ACAAAT CTGGYCCAGATCAC
	Sol1R SG/TqM	CAGCTTTCTGTTCAGCTAGT
	Sol1 TqM probe	FAM-ATCAATTCACATGAAACATTACATGCAA CTGG- BHQ1
	CowF	CAAAACTAGTAGAAATTGCACA
	CowR	TGCATCTTGTGGTGGTAC
	CowTqMm probe	**FAM** -TCCTCCATCAAGATATATAGCACCTATTA-**TAM**
	groEL290F	GTTATTGAGGAAGTATCTAAGG
	groEL390R	TTAAAGCTTCTAATACTGCCTC
	groeL-PMEpr	**HEX**-TACACCTTCCCTAACACAAATAATATCTGCAC-**BHQ1**
	groEL-HWpr	**HEX**-TACACCTTCCCTAACACAAATAATATCTGCAC-**BHQ1**
LAMP pCS20	F3	CTTGATGGAGGATTAAAAGCA
	B3	GTAATGTTTCATGTGAATTGATCC
	FIP	TGTGCCCATTCTTGTAAGATAGTTT-TTTCTATTCTGGAAAAATTCTGC
	BIP	TAAAGGATTTCCTGCACCAAGTT-ACTTCTACAGTAAAACAAGGATTG
	LF	TGCATCTTGTGGTGGTACTTTCA
	LB	AATAAACAAATCTGGCCCAGATCA
LAMP sodB	F3	GCCCCATATTTGAGTGCTAA
	B3	CGTAACAACACCATTCTTTGT
	FIP	ACAGAAATCAGTCCCTGCAACA-TGTTGAATTATCACTATGGAAAGC

	BIP	ACCTGCGGTTATTAAAGCTACACA-TATGATTCCATACTTGACCAGC
	LF	AAGCATTTACATAACCTTGATGAT
	LB	ATAGTGATTTAGCAACTAGATCAA
FRET-qPCR	F	GAGGATTTTATCTTTGTATTGTAGCTAAC
	R	TGTAAGGTCCAGCCGAACTGACT
	probe	ACGCGAAAAACCTTACCACTTTTTGAC-**6-FAM**
16S rDNA-tick	16SF	CTGCTCAATGATTTTTTAAATTGCTGTGG
	16sR2	TCTTAGGGTCTTCTTGTCDTTAATTTT

Real-time PCR

Sol1, which also targets pCS20, was designed to replace nPCR in the OIE reference lab. This assay was development using SYBR Green and TaqMan probes as SYBR Green is a less expensive than TaqMan probes and thus could be more readily adopted in areas with limited resources. The analytical sensitivity was three copies per reaction for both chemistries using the Gardel isolate grown in culture. Additionally, 16 strains from 8 geographically dispersed African countries and Guadeloupe and 10 isolates from S. Africa were all successfully detected by the pCS20 Sol1 taqman as well as the gold standard nested PCR.

There was no detection of 10 related pathogens from the genera *Anaplasma*, *Babesia*, *Erhlichia*, including PME, and *Rickettsia*. Primers and probe were checked against *E. chaffeensis* using BLAST and the probe did not align with the *E. chaffeensis* pCS20 gene region and only 13 of 20 nucleotides, in the middle of one primer had identity with the gene, thus no positive results are expected from *E. chaffeensis* (119). Though not rigorously evaluated in clinical cases, pCS20 Sol1 TqM was able to detect *E. ruminantium* in blood samples from three experimentally infected goats during hyperthermia and is likely to perform similarly to the nested PCR on clinical samples.

Another real-time, taqman probe-based PCR (Cow) was developed to amplify a 227 base pair region internal to the initial AB128/AB129 primer set (120). This assay could detect 14 copies per reaction and was able to detect a panel of 15 *ER* strains. However, it cross reacts with *E. chaffeensis* and *E. canis*, but not *T. parva*, *A. centrale* or *A. marginale*. This cross-reactivity precludes its use in the USA and Caribbean. In comparison with Sol1, it suffered from low amplification efficiency for reasons which could not be determined (119).

With the discovery of PME in the US, diagnostic tests that can differentiate between PME and *E. ruminantium* are necessary (121). Using groEL as the target gene, primers were designed to amplify both PME and *ER*, while two Taqman probes were designed to differentiate between the two species and excluding other *Ehrlichia* spp. in a single reaction. The analytic sensitivity was 10 copies of DNA and all 23 geographically diverse strains of *ER* and PME from ticks was detected. The *E. ruminantium* samples were negative for PME and vice versa, suggesting high specificity, though the detection threshold to identify a Ct cut-off to differentiate between positive and negative results was not calculated.

Finally, a fluorescent resonance energy transfer (FRET)-qPCR was designed to detect five major *Ehrlichia* spp by targeting the 16srRNA gene based on differences in melt curves (122). The detection limit was 5 copies per gene. In samples with roughly equal copies of *E. ruminantium*,

E. chaffeensis, *Ehrlichia* sp Bov2010 and PME, all were detected. No additional determination of sensitivity in the context of mixed infections or mixed samples was done. Little work done specifically on the performance of this test for the detection of *E. ruminantium*.

Reverse line blot (RLB)
The V1 region of 16s ribosomal RNA gene was amplified and hybridized to a membrane with eight species-specific oligonucleotide probes and one covalently-linked probe designed to detect *Anaplasma* spp and *Ehrlichia* spp. in order to have one test to diagnosis *Ehrlichia* spp and *Anaplasma* spp that infect domestic ruminants (123). The performance of this test was promising based on known positive and negative samples, including those with protozoal pathogens. Based on spiking experiments, mixed pathogen infections could be readily differentiated.

The RLB was able to detect many strains of *E. ruminantium* though not all animals were positive by RLB during acute infection and none of the animals were positive following antibiotic treatment. However, neither the analytic nor diagnostic sensitivity or specificity were determined.

Loop Mediated Isothermal Amplification (LAMP)
Lamp assays using both pCS20 and SodB have also been developed (124). The analytical sensitivity was 5 and 10 copies of SodB and pCS20, respectively as determined by using serial dilutions of plasmids containing the target region of the gene. Both assays detected 16 different, geographically diverse *E. ruminantium* isolates and there was no cross reaction with *E. chaffeensis*, *E. ewingii* and PME. These assays successfully detected *E. ruminantium* in two samples or sheep blood from a heartwater endemic area, demonstrating potential utility of these assays to detect persistently infected animals.

As real-time PCR become the gold standard for pathogen detection in diagnostics, the importance of LAMP and other low input diagnostic tools should not be discounted as such tools assist individual veterinarians and veterinary services to improve diagnostic capacity, particularly in underserved areas, to obtain definitive diagnoses and build more robust surveillance systems throughout the world.

Serology
Similar to other infection diseases, the development of the initial IgG response generally requires 3 to 4 weeks, though can be up to 6 weeks and is generally co-incident with fever (83). Thus, serology based on detection of IgG cannot be reliably used to identify infected animals prior to the development of fever.

A widely acknowledged limitation of ELISAs or other antibody-based tests as a diagnostic tool to detect individual animals persistently infected with *E. ruminantium* is the marked decay in antibody through time. Total IgG levels as measured by end point titer using whole cell antigen and IFAT were below detectable limits in 4 of 5 experimentally infected animals by 30 to 45 weeks post infection (83, 125). In contrast, using western blots, IgG against total *E. ruminantium* antigen was detected in all four animals tested at 1:100 titers at 45 weeks post infection (83), though detection in one animal was weak. Interestingly, antibody titers decay even in the presence of repeat exposure to the pathogen.

Enzyme-Linked Immunosorbent Assays (ELISA)
There are two ELISAs based on the immunodominant MAP1 protein neither of which is commercially available. The MAP1-cELISA is based on the *map1* gene expressed in baculovirus and monoclonal antibodies raised against MAP1 (126). Depending on the context, serologic specificity and sensitivity are problematic. The second test, an indirect ELISA targeting MAP-1B, has been more widely used (83, 113, 127). MAP-1B includes amino acids 47-152 of the *map1* gene and encompasses two variable regions that are interspersed among highly conserved regions (29). Though MAP-1B has variable regions, it is recognized by antibody raised against at least nine isolates from geographically separate regions (127).

In the initial validation, anti-MAP-1B antibody was detectable for up to 50 days post infection in cattle and up to 75 to 80 days in sheep and goats. Though animal numbers were low, antibody levels tended to be highest in sheep and goats as compared to cattle (127), suggesting important species differences. This suspected species difference was further highlighted by testing the performance of the MAP 1B ELISA in cattle and goats from endemically stable regions of Zimbabwe (128). These regions had heavy tick burdens and infection pressure, minimal tick control and an expected prevalence of nearly 100%. In goats, MAP-1B specific antibodies were detected in 67 to 100% of goats depending on the area. Though at one site, the MAP1B antibody was detected in 43% of goats. In contrast, seroprevalence in cattle was more variable from 24 to 61%, depending on the study site. These findings suggest antibody decay is more pronounced in cattle, though the possibility of cross reaction with related organism in goat serum could not be excluded. The use of MAP-1B ELISA to accurately determine true prevalence of *E. ruminantium* infected animals is problematic.

In a large, later study done only in cattle, anti-Map 1B IgG was detectable between 3 to 4 weeks and peaked between 4- and 9-weeks post infection (83). The decline in antibody levels to below detectable limits was generally between 14-33 weeks, though there was a great deal of variably among animals. For example, in two field infected animals, antibody did persist for 52 weeks. The general decline in antibody occurs even though animals remained persistently infected as determined by xenodiagnosis (83).

It is currently well recognized that the false positive results are problematic with anti-MAP 1 ELISAs because MAP is conserved among other *Ehrlichia* spp. In initial studies, cELISA and MAP 1B ELISA performed similarly with >98% specificity in animals in heartwater free islands of the Lesser Antilles that have the tick vector (126). A lack of specificity becomes problematic in animals exposed to or infected with other *Ehrlichia* spp. species. For example, anti-*E. canis* and anti-*E. chaffeensis* antibodies reacted with MAP1b during initial validation (127). Of additional relevance to the Americas is that antibody from PME infected goats reacted with *E. ruminantium* MAP 1B, thus this target antigen cannot be used for serodiagnosis in the US (121). Serum from animals in *E. ruminantium* affected areas of the Caribbean reacted with PME further complicating the identification of *E. ruminantium* carriers.

In a different study, also testing serum from cattle, sheep and goats on seven different islands of the lesser Antilles free of *E. ruminantium*, the overall specificity of the MAP-1B test was high, though there were important differences between islands, with up to 4% false positive in sheep

on Monserrat and Dominica, and 3 and 4% in cattle and goats, respectively on St. Kitts, suggesting that the diagnostic performance varies based on location and likely the presence of other circulating *Ehrlichia* spp. (129).

Diagnosis in ticks

Detection of *E. ruminantium* in *Amblyomma* spp.

Xenodiagnostics, inoculating small ruminants or mice with tick organ homogenates and monitoring for clinical disease or seroconversion, can be used to identify the presence *E. ruminantium* in a particular geographic region and estimate the tick infection prevalence (130). This technique is expensive, slow and unreliable in mice, thus using small ruminants is preferred. Additionally, this use of animals raises ethical concerns at a time when reducing the use of animals in research is a high priority.

Given these limitations, coupled with the limitations of PCR and serology in detecting persistently infected animals, much effort has been dedicated to the validation of PCR to detect *E. ruminantium* in *A. variegatum* and *A. hebraeum*, the primary tick vectors. This approach is promising because it is likely that *E. ruminantium* replicates in tick organs, which in turn improves detection, allowing for the possibility of using ticks to detect persistently infected animals. Additionally, it may be possible to accurately estimate herd prevalence or provide data for the establishment of *E. ruminantium* free herds (113). While many of the technical challenges of this approach have been met, the work to validate this approach for a specific purpose has yet to be done.

Detection of infected ticks

PCR

Using AB128 and AB129 primers, the specificity as determined by using laboratory, reared, uninfected nymphal and adult *A. habraem* was 98% (130). There was no amplification of *E. chafeensis* DNA in spiked samples. The limits of detection were not strictly calculated, though 97-88% of ticks spiked with 10^7 to 10^4 organisms were PCR positive, while 28% of samples were considered positive with 10^3 to 10^2 spiked organisms.

In field samples, *E. ruminantium* was detected in *A. hebraeum* from 13 sites in Botswana, S. Africa, Swaziland and Zimbabwe and in *A. variegatum* from two sites in Zambia and two sites in Zimbabwe. There was no gold standard for detection of *E. ruminantium* -infected ticks, as a surrogate, groups of PCR positive ticks from 17 different locations and feeding trials were fed on animals. In all but 4 cases, transmission occurred. In one case, *A. variegatum* failed to attach for the transmission feed (130).

Real time PCR

pCS20 taqman assay using CowF and CowR primers was validated in part via at inter assay comparison using cultured ER, blood from experimentally infected animals and field samples including ticks from heartwater endemic areas (120). The highest number of positive ticks was detected using the taqman PCR than a direct PCR, though no other validation was done using tick tissues. Most recently, the pCS20 Sol1 qPCR was largely validated using tick tissues.

Concurrently, a tick 16s rDNA real time PCR using SYBR Green was developed in order to evaluate the quality of tick DNA extraction and verify the absence of PCR inhibitors. Additionally, automated and manual DNA extraction performed similarly thus allowing for processing of large numbers of ticks, which could be necessary for surveillance efforts (119).

The limit of detection was six organisms for both nested PCR, the former OIE reference laboratory standard and Sol1 qPCR as determined by spiking unfed, naïve tick lysates with serial dilutions of *E. ruminantium* from cell culture. A gold standard for identifying true positive ticks does not exist, however in an inter test comparison, the kappa value when comparing nPCR and manual DNA extraction with pCS20 Sol1 qPCR and automated extraction with 49%, demonstrating moderate agreement between the tests (119).

LAMP

The performance of LAMP was compared to that of pCS20Sol1 qPCR using DNA extracts from *A. variegatum* spiked with ER (124). The limits of detection for both LAMP assay was > 10 copies. *E. ruminantium* was detected in 12 of 140 ticks, while a real time pCS20 PCR detected *E. ruminantium* in only one additional tick as compared to LAMP.

RLB

An RLB designed as a single diagnostic test to detect *Anaplasma* spp and *Ehrlichia* spp. was tested using adult *A. variegatum* fed as nymphs on either rabbits or *E. ruminantium* infected sheep during clinical infection (123). RLB was positive in ticks feed on 4 of 5 infected sheep. Negative control ticks were consistently negative. The percent of RLB positive ticks varied from 15-70%. Interpretation of these results is confounded by a lack of knowledge concerning the expected tick infection rate from these ticks or a gold standard method to detect *E. ruminantium* in the ticks. However, detection in ticks did correlate with transmission, though the sample size was low.

Gaps and Research needs

The primary gap is our inability to reliably detect individual, persistently infected, clinically normal *E. ruminantium* -infected domestic and wild animals. In cattle, sheep and goats, this significantly limits our ability to prevent and control disease spread within and between herds and prevents the development of export markets in heartwater endemic regions. In wildlife, this limits our ability to identify wildlife reservoirs and fully understand the epidemiology heartwater at the interface between wildlife and domestic animals. There are two primary reasons of this limitation. First, the antibody response to MAP-1, the immunodominant antigen of *E. ruminantium*, is inconsistent and wanes through time. Second, organisms reside primarily in endothelial cells and cannot be consistently detected using PCR during persistent infection.

It is possible that detection of *E. ruminantium* in ticks collected or purposefully fed on animals could be used to enhance our diagnostic capacity in the detection of individual, infected animals. A number of knowledge gaps remain in our understanding of the efficiency of tick acquisition relative to the pathogen load in the animal. Addressing this knowledge gap will pave the way for the validation of a diagnostic test fit for this purpose.

The second most significant limitation in diagnostic tools is the ability to detect early infection, prior to the development of clinical disease. This is relevant in the context of introduction of the pathogen into an *E. ruminantium* free country or territory as well as the ability to institute early and effective treatment in exposed animals. The primary reason for this limitation is the long incubation period in which levels of *E. ruminantium* are low in peripheral blood, which is the sample of choice for testing. Additionally, there is little understanding of the early immune responses and the mechanisms by which ER disseminates throughout the body after infection by tick feeding.

Currently, diagnosis of clinically affected or dead animals relies on PCR from blood or a Giemsa –stained impression stain of a large vessel or brain smear. In resource poor areas, PCR may not be available and brain smears are not possible ante-mortem. Brain smears require training to recognize the pathogen, lack sensitivity, and present significant health risks to diagnosticians in rabies endemic areas. This is major limitation in the passive surveillance efforts, particularly in ER-free areas. Since many *E. ruminantium* free countries and regions at risk for introduction of ER are resource poor, a low input test to diagnose heartwater would be valuable.

A fourth limitation is a lack of standardized method for genotyping *E. ruminantium*. This will improve our understanding of the strain structure of *E. ruminantium* in wildlife reservoirs as compared to domestic animals and improve our understanding of the epidemiology of heartwater, including the ability to trace strains and identify sources of pathogen introduction. Genotyping will also help link particular genetic signatures to phenotypic traits, particularly virulence and host susceptibility.

Epidemiology and Vectors

Pathogen distribution

Because *E. ruminantium* is transmitted by ticks in the genus *Amblyomma*, the distribution of *E. ruminantium* is dictated by the distribution of *Amblyomma* spp, particularly *A. variegatum* and *A. habraem,* which is the primary vector in southern Africa. Consequently, *E. ruminantium* is present in most of sub-Saharan Africa and associated islands of east and west Africa. *E. ruminantium* and a primary tick vector *A. variegatum* were introduced into the Lesser Antilles from Senegal during the slave trade in the 18th or 19th century. The pathogen and tick became well established on Guadeloupe and two neighboring islands, Marie Galante and Antigua. In the 1940's, *A. variegatum* was introduced to Martinique via animals imported from Guadeloupe (131). In the 1960s, the cattle egret (*Bubulcus ibis*) became established in the region. This bird closely co-habitats with cattle and can serve as a host for larval and nymphal *A. variegatum*. Co-incident with the introduction of the cattle egret, *A. variegatum* spread to 14 Caribbean islands from Barbados in the south to Puerto Rico in the north between 1967 and 1988 (131). Much of the dissemination of the tick could not be accounted for by movement of domestic animals, thus primarily implicating the cattle egret, though movement of ruminants and dogs did play a role (131). Today, despite an intensive eradication effort, the tick remains widespread in the lesser Antilles, though it was eradicated from the Grenadines and Puerto Rico (132, 133). Interestingly for over 150 years, *E. ruminantium* has remained confined to Guadeloupe, Marie Galante and Antigua. Additionally, A. *variegatum*, despite the migratory patterns of the cattle egret, has not been introduced to the mainland Americas.

***Amblyomma* ticks**

Both introduction of *A. variegatum*, and introduction of *E. ruminantium* via importation of infected ticks are threats to livestock agriculture in mainland America. In addition, *A. variegatum*, a large and aggressive tick, reduce animal growth and productivity even in the absence of pathogens (134). *Amblyomma* spp are tropical to subtropical ticks and are typically found in environments with continuously high relative humidity. Generally, *Amblyomma* spp. require annual rainfall between 25-280 cm/year (9.8 to 110 inches/year). Suitable habitat for *A. variegatum* in mainland America can be found in Florida and the extreme south of the US, throughout central America, the northeast coast of S. American and extending south and west to the Parana basin of Brazil, Paraguay, northern Uruguay, northern Argentina. This is a single prediction based on a paper published in 1985 using computer systems for matching climates in ecology (135). More recent modeling published in 2007, suggests suitable habitat for *A. variegatum* exists in the peninsula of Florida, central America, the Caribbean, large areas of Colombia and Venezuela, and parts of Brazil (136). The number of ticks and density of hosts required to establish a population in a new area is unknown.

Risk of *A. variegatum* and *E. ruminantium* spread

Though the ecological niche for *A. variegatum* exists in mainland America, the true risk of introduction of *A. variegatum* and specifically, *E. ruminantium* infected *A. variegatum* via the cattle egret remains unknown. One paper from 1992 that describes the tick infestation rates of the cattle egret (18). In this study, there was 1.7 larvae and 0.05 nymphs per bird from 80 examined birds. *E. ruminantium* is maintained through each tick molt (transtadial transmission), but is not maintained from one generation to the next (transovarial transmission). Consequently, only nymphs or adult ticks, exposed to *E. ruminantium* in a previous lifestage serve as the primary means of transmission of *E. ruminantium*. Thus, the authors estimated there was a potential for 0.05 infected ticks per bird, which is overall low. However, generally the number of ticks per host can vary dramatically temporally and spatially and there is a complete absence of data regarding *A. variegatum* burdens on cattle egrets, particularly from the islands endemic for *E. ruminantium*.

When estimating risk, another important factor is the tick infection rate, particularly in ticks that feed on inapparent, carrier animals. Initially, these estimates were made by collecting ticks from ruminants, homogenizing individual ticks and inoculating the homogenates into susceptible goats or mice. More recently, tick infection rates have been estimated using nested PCR. In the late 1980 or early 1990s, the tick infection rate on the two islands of Guadeloupe, Basse Terre and Grande Terre were 1.2% and 0.6%, respectively (137). The tick infection rate on Marie-Galante and Antigua was 2.5% and 0.2%, respectively (137). More recently, in 2003 the tick infection rate was 36.7% in Guadeloupe (138). In Marie Galante, the tick infection rate was similar at 35.6% and dropped to 19.1% in 2005 (118, 138). Similar to the previous study, the tick infection rate in Antigua remained relatively low at 5.8% (138). Of note, is the marked increase in tick infection rates between the early 1990s and early 2000s. It is possible that tick infection rates have increased during that time; however, that remains unknown due to the different methods used to determine the tick infection rates. Another major knowledge gap that exists in terms of accurately determining the risk of spread of *E. ruminantium* is how the infection rate and/or level in ticks as determined by PCR correlates to risk of transmission of *E. ruminantium* to a new host.

Competent tick vector in mainland Americas

Following acute infection, ruminants that survive become long-term inapparent carriers. Thus, a second route of introduction to mainland America is via infected ruminants. However, transmission and ultimately establishment of the pathogen would require the presence of a competent tick vector. Of the at least 50 species of *Amblyomma* ticks that reside in the Americas, the host preference is not well described in the Anglophone literature. However, only six, excluding *A. variegatum*, are known to favor feeding on ruminants in at least two lifestages, a requirement for an efficient *E. ruminantium* vector. These *Amblyomma* spp include *A. neumanni*, *A. cajennense*, *A. maculatum*, *A. americanum* and *A. parvum* (18).

In the early to mid-1980s *A. maculatum* (Texas), *A. cajennense* (Mexico), *A. imitator* (Mexico), *A. americanum* (Texas) and *A. neumanni* (Argentina) were tested for their ability to transmit *E. ruminantium* via transtadial transmission (18). In all experiments, ticks were exposed as larvae to *E. ruminantium* infected animals. Ticks were allowed to molt to nymphs and adults and transmission to naïve ruminants was tested in these two later life-stages. *A. maculatum* proved be the most efficient vector with successful transmission of several strains of *E. ruminantium* by nymphal and adult ticks. In the case of *A. cajennense*, transmission was successful with only nymphal ticks in one of 8 experiments testing various strains of *E. ruminantium*. Thus, it is considered a poor vector for *E. ruminantium* (14). None of the other species tested proved capable of transmitting *E. ruminantium*.

A. maculatum, the Gulf Coast tick, was confined to the coastal areas of the Gulf of Mexico and Caribbean Sea. However, its range in North America has undergone remarkable expansion in the last several decades and now includes the mid-Atlantic states as far north as Maryland and Delaware and the Midwest, into northern Kansas and west to the Oklahoma panhandle (23, 139). Reports of its distribution in Central and South America are unavailable in the Anglophone literature.

Tick control

Tick control is one of the mainstays of the control of heartwater. Currently acaricides are the primary method used to control *Amblyomma* ticks. Acaricide resistance has been rapidly developing in some tick species, particularly *Rhipicephalus microplus*; while little to no resistance has been identified in *A. variegatum* tick populations to date (134). However, little current data is available regarding acaricide resistance among populations of *Amblyomma* spp. tick. In one study conducted in Veracruz, Mexico, there was medium to high resistance among *A. cajennense* to organophosphates and some degree of resistance against amitraz. In this study, nearly all ticks remained susceptible to synthetic pyrethroids and fipronil (140). In general, acaricides are only partially effective in control of pathogen transmission of tick-borne diseases due to the necessity of obtaining nearly complete removal of all ticks from all animals for extended time periods. Acaricides are expensive and often have negative environmental effects.

Anti-tick vaccines are a potential alternative or addition to acaricides. Currently, a single anti-tick vaccine based on the midgut protein BM86 has been developed. This vaccine is effective against some, though not all, populations of *R. microplus*. Vaccines targeting other ticks have yet to be developed.

Gaps and Research needs

Modeling of various aspects of *E. ruminantium* transmission is a high priority research need as this will serve as an important tool to assess the risk of introduction of *E. ruminantium* into pathogen-free areas given various conditions, thus informing surveillance strategies. Modeling is also useful in the design of disease control and eradication efforts to reduce the burden of heartwater in endemic areas.

There are several significant knowledge gaps concerning vector competence. First, the relationship between the level of *E. ruminantium* in the tick midgut and salivary glands and the risk of tick transmission is unknown. Second the influence of the composition of the tick microbiome, including the virome, on pathogen transmission is unknown. Third, the competence of many tick species in the Americas for *E. ruminantium* is unknown.

1. Develop and validate a model to assess the risk of *A. variegatum* introduction and establishment in *A. variegatum*-free areas in order to inform surveillance efforts.
2. Develop a model to assess the risk of introduction and establishment of *E. ruminantium* in *E. ruminantium*-free areas.
3. Assess acaricide resistance of known *E. ruminantium* vectors.
4. Develop a vaccine against *E. ruminantium* vectors.
5. Investigate the role of wild ruminants in the maintenance of *E. ruminantium* vector populations in the Americas.
6. Identify additional competent vectors in the transmission of *E. ruminantium* in the Americas.
7. Test alternative control methods and their cost in developing an integrated approach for *E. ruminantium* vector control.
8. Evaluate the economic losses associated with *E. ruminantium* vectors in endemic areas.

COUNTER MEASURES ASSESSMENT

ASSUMPTIONS

The following captures assumptions made by heartwater working groups to assess potential countermeasures to enhance our ability to contain and eradicate an outbreak of heartwater.

Situation
Countermeasures assessed for worst case scenario.

Target Population
Countermeasures assessed for target farm production segments in priority order:

1. Goats
2. Sheep
3. Dairy cattle
4. Beef cattle
5. Cervids

Scope of Outbreak
Countermeasures assessed for multiple outbreaks occurring simultaneously in a new naïve geographical area.

Vaccine Administration
No effective vaccine available for use in the US., therefore the only control strategy would be based in the early detection of infected animals and their elimination.

DECISION MODEL

The Heartwater Working Group used the quantitative Kemper-Trego (KT) decision model to assess available vaccines and diagnostics, including experimental products. Instructions for using the model were provided prior to the workshop (see Appendix I). Criteria and weights in the model were modified by the working groups for the purpose of assessing available countermeasures as well as experimental heartwater vaccines and available diagnostics and available acaricides for ruminants (See Appendices II, III and IV).

Criteria
The working groups selected critical criteria and weights to allow a quantitative comparison of the impact of the selected interventions, as follows:

Vaccines

Critical Criteria	Weight
Prevent clinical disease	8
Prevent transmission	10
One dose	6
Speed of scale-up	4
Safety	8
High yield manufacturing process	6
Cost of goods	8
Duration of immunity	6
Cross-protection	10
Storage	6
DIVA	2
Withdrawal	2

Diagnostics

Critical Criteria	Weight
Sensitivity	10
Specificity	8
Validation to purpose	10
Speed of scale-up	6
Throughput	8
Need for a confirmation	5
Handling and caution to avoid cross contamination (molecular only)	8
Rapid result (less than one day)	8
Detection of different strains (molecular only)	4
Implementation of the assay (commercial or currently used)	8
Easy to perform (including sample preservation)	8
Cost	4

Acaricides for controlling *Amblyomma* spp in domestic ruminants

Critical Criteria	Weight
Efficacy	10
Formulation/application method	10
Residual activity on host	8
Frequency of Application	8
Market Availability	8
Toxicity for non-target species	6
Safety for humans	6
Cost of Acaricide	6
Withdrawal	6
Toxicity for target host	2

Product profile

To ensure a consistent and meaningful assessment, the desired product profile was identified for each countermeasure:

Desired Vaccine Profile

1. Highly efficacious: prevents transmission; efficacy in all ruminants of any age, including maternal antibody override; one-year duration of immunity
2. Safe in all age ruminants; no reversion to virulence for attenuated live vaccines
3. Only one dose required
4. Rapid speed of production and scale-up, can deliver finished product quickly, and manufacturing method yields high number of doses
5. Expiration date of 24 months or greater
6. Manufacturer has effective storage and distribution capability
7. Quick onset of protection, 7-days or less
8. DIVA compatible: Can effectively and reliably differentiate infected from vaccinated animals
9. Short withdrawal period for food consumption
10. Reasonable cost of goods, cost of administration, cost of storage

Desired Diagnostic Test Profile

1. Detect all heartwater genotypes
2. Direct tests (pathogen detection) for early detection
3. Indirect tests (antibody detection) for post-control monitoring/detection non-clinical cases
4. Rapid test (less than one day) - early detection
5. >95% specificity
6. >95% sensitivity
9. Validation to purpose
10. Easy to perform/easily train personnel
11. Scalable
12. Reasonable cost

There are currently no commercial or experimental vaccines available against the recognized vectors of *E. ruminantium*, though this is the preferred intervention.

Desired Anti-tick Vaccine Profile

1. Minimize the use of other means of control
2. Safe for target host
3. Produce long-lasting immunity
4. Single dose application
5. Easy to produce and maintain (no cold-chain)
6. Have a long shelf life
7. Have no side effects
8. Cross-protection against all *Ehrlichia ruminantium* vectors
9. Exhibit transmission-blocking effects

Values

The values assigned for each of the interventions reflect the collective best judgment of Heartwater Working Group members (see Appendices II, III and IV).

Vaccines

The Heartwater Gap Analysis Working Group noted that current research into a suitable vaccine for heartwater is limited to only a few research groups worldwide.

Summary

Vaccination against heartwater may be an option. Progress has been made towards the development of an attenuated Welgevonded vaccine and a rationally designed multi-peptide vaccine that shows promise.

Assessment of Experimental Vaccines

The Heartwater Working Group discussed the characteristics of the different available experimental vaccines. Following is a summary of the group's opinion for each of them.

The commercial and experimental vaccines were rated according to several critical criteria and according to the resulting scores they ranked as follows:
Heterologous prime boost DNA vaccine = Multi-peptide DNA Vaccine > Live attenuated > Inactivated/Adjuvanted > Infection/Treatment

In the NVS heartwater Decision Model for Ruminant Vaccines the **heterologous prime boost DNA vaccine and the multi-peptide DNA Vaccine** both achieved the highest and equal values. Their ability to prevent clinical disease when vaccinated sheep are needle challenged, received the highest rank of 10 because it is important that vaccinated animals do not require antibiotic treatment because of the rise in antibiotic resistance. Additional advantages of a DNA vaccine is that it is easy to scale up resulting in high yields, is safe, can be stored at 4°C and is DIVA compliant. However, these vaccines were tested using three doses which is not acceptable, particularly in rural areas in Africa. It is not known if these vaccines can protect using only one dose, therefore it was ranked lowest at 2.

Live attenuated vaccines particularly the attenuated Welgevonden strain is known to prevent clinical disease, has a wide range of protection, can be scaled up speedily to high yields, does not require a withdrawal period because antibiotics are not administered, and only requires one dose led to favorable ranking of between 6-10. The disadvantages of this vaccine is that it may result in reversion to virulence and transmission causing a safety risk, it requires ultra-cold chain for storage and the possibility of developing a DIVA test is low therefor these were rated 2. However, if *E. ruminantium* was attenuated by gene manipulation the latter may be possible making this a more favorable vaccine.

Inactivated vaccines have the advantage that several strains can be included to increase its protective range and it can be stored at 4°C making it suitable for use in rural areas. The disadvantages of this vaccine is that it may result in clinical disease and transmission if not adequately inactivated causing a safety risk, two doses are required and it is not DIVA compliant.

Infection/Treatment vaccine. The only commercial vaccine available for heartwater is a live sheep blood vaccine containing the Ball3 strain. This is injected intravenously into animals where after rectal temperatures must be monitored daily, and antibiotic treatment must be administered during the febrile response. If treatment is not administered at the correct time the animal may die or not develop an effective protective immunity. The vaccine must be preserved on dry ice or in liquid nitrogen. Cannot be used in non-endemic areas. The Ball 3 strain was chosen as the vaccine strain because it produces an early temperature rise several days before any other serious clinical signs appear. This makes it relatively easy to treat timeously. Although the highly virulent Welgevonden strain is known to protect against most strains it is not suitable as a live vaccine because it can cause death very shortly after a rapid temperature rise. Unfortunately, the Ball3 vaccine does not protect against all the strains which circulate in the field. The many drawbacks of this vaccine make it unsuitable to be used in rural areas of Africa.

Based in this assessment the Heartwater Working Group decided that the most promising experimental vaccines are based on the use of rationally designed DNA vaccines.

	Rank - comment				
Critical Criteria	**Live blood Infection/Treatment (Ball 3)**	**Inactivated/Adjuvant**	**Live attenuated**	**Heterologous prime boost DNA vaccine**	**Multipeptide DNA Vaccine**
Prevent clinical disease	10 – this should be 2 as the vaccine does lead to HW	6 – possible	10 - possible	10 - no	10 - no
Prevent transmission	2 - yes	2 - possible	4 - possible	4 - no	4 - no
One dose	8 - yes	2 - no	8 - yes	2 - no	2 - no
Speed of Scaleup	8 - moderate	4 – fast if strain is in culture otherwise very slow	8 – fast is strain is in culture otherwise very slow	8 - fast	8 - fast
Safety	2 – transmission of other blood pathogens is possible	6 - safe	4 - safe	8 - safe	8 - safe
High manufacturing yield	6 - yes	6 - yes	8 - yes	8 - yes	8 - yes
Cost of goods	8 - high	6 - moderate	8 - moderate	6 - moderate	6 - moderate
Duration of immunity	6 – 12 months. Protective immunity for life if kept on heartwater infected veld	4 - unknown. Protective immunity for life if kept on heartwater infected veld	6 - unknown. Protective immunity for life if kept on heartwater infected veld	4 - unknown	4 - unknown
Cross-protection	2 – not to all field strains	2 – not to all field strains but possible because many strains can be added to the vaccine	6 - not to all field strains	6 - unknown	6 - unknown
Storage	2 - < -70°C	10 - < 4°C	2 - < -70°C	8 - <4°C	8 - <4°C
DIVA	2 - no	2 - no	2 - no	8 - yes	8 - yes
Withdrawal	6 – 28 days	6 - no	8 - no	6 - no	6 - no

See Appendix II for Assessment of Experimental Vaccines.

Diagnostics

Diagnostic tests were evaluated in the context of different epidemiological situations relevant for *E. ruminantium*. These situations, which demand different performance from a diagnostic test, include *E. ruminantium*-free areas with clinical suspicion after outbreak, endemic area, outbreak area, follow-up testing following an outbreak. Serologic tests and molecular – based tests were evaluated separately.

Summary

ELISA to detect an antibody response to *E. ruminantium* are only suitable for detecting infection in animals following seroconversion, typically 2 to 3 weeks post infection. The limitations of this testing modality are due to the decay of antibodies through time in persistently infected, clinically normal animals and a lack of specificity in areas in which there is circulation of multiple *Ehrlichia* spp.

Despite these limitations, in all situations the indirect ELISA is expected to perform better than the cELISA due to lower cost and the fact it is in current use. No ELISAs are commercially available.

In terms of molecular tests, qPCR Sol1 generally outperforms the other PCR based tests. The test is in current use by the OIE reference lab and has been well validated. The multiplex PME/ER is also expected to perform well based on the working group's assessment, though the validation performed to date is less extensive.

See Appendix III for Assessment of Laboratory Tests.

Acaridides

Existing acaricides were evaluated in the context of controlling *Amblyomma* spp. ticks in domestic ruminants. Organophosphates, macrocyclic lactones, pyrethroids, formamidines and botanicals were considered. The overall scoring was as follows: pyrethroids > macrocyclic lactones > formamidines > organophosphates > botanicals.

In general, the pyrethroids due to low toxicity for non-target species, low cost and short withdrawal time. In contrast, the macrocyclic lactones have longer residual activity on the host thus require reduced frequency of application. While considered safe for humans, the macrocyclic lactones were more toxic to non-target species than the pyrethroids, tend to be more expensive and have a longer withdrawal time.

Importantly all had similar and high efficacy and ease of application, with the exception of botanicals, which tend to have low efficacy.

See Appendix IV for Assessment of Acaricides.

RECOMMENDATIONS

The heartwater gap analysis working group recommends the implementation of the following research priorities to advance our ability to prevent or rapidly detect and control a heartwater outbreak in a free area as well as improve control and eradication efforts in endemic areas.

Epidemiology and Vectors
Rigorous and quantitative risk assessment to identify the regions of mainland America most vulnerable to introduction of infected *A. variegatum* vectors or infected animals is required to prioritize and bolster surveillance efforts. This should include evaluation of habitats suitable for likely vectors of *E. ruminantium* as well as predictive maps of how vector distribution is likely to change through time. Understanding wildlife as reservoirs for maintaining *E. ruminantum* circulating through tick vectors will be useful for pathogen eradication efforts.

Pathology
Identifying the relevant host cells during early infection and understanding the pathophysiology in the development of cerebral and pulmonary edema will inform vaccine development.

Immunology
Immune transcriptome sequencing must be further investigated to identify correlates of protection to facilitate vaccine development.

Vaccines
The DNA vaccine development strategy is most promising and should be developed further to improve its effectiveness. This vaccine is also the most suitable for use in non-endemic areas where the vector is present but not the disease.

Diagnostics
High-performance diagnostic tests are needed to: 1) detect infected animals prior to the development of clinical diseases; 2) identify clinically normal, reservoir animals. The first would aid in pathogen eradication effort in the face of an introduction to a free area. The second is needed as a tool for herd and region-level pathogen eradication in endemic areas and to mitigate the risk of spread of *E. ruminantium* through animal movement.

Vector control
Develop an anti-tick vaccine targeting *Amblyomma* spp ticks as an alternative or adjunct method of tick control and thus disease prevention.

PREPAREDNESS

The primary countermeasures available for controlling an introduction of *E. ruminantium* into a free area such as the US. include acaricides for control of competent vectors and PCR to detect clinically affected animals. There are no commercially available vaccines and available diagnostic tests can only reliably detect clinically affected animals.

CONCLUSIONS

Heartwater is one of the deadest diseases of livestock, but despite this, it is markedly understudied. This pathogen is transmitted by *Amblyomma spp* ticks and is endemic in much of sub-Saharan Africa and a limited area of the Caribbean. Given the proximity to the Caribbean and the presence of potential tick vectors in mainland of America, the potential exists for this pathogen to spread to mainland America. Tools to control *E. ruminantium* in such circumstances, including vaccines and diagnostic tests to identify infected, pre-clinical animals and inapparent carries are lacking. Additionally, methods to control vectors, apart from acaricides, which are expensive and often only partially effective at controlling pathogen spread are lacking. Enhanced research effort and capacity is required to address these deficits.

APPENDIX I: Countermeasures Working Group Instructions

Decision Model

We will use a decision model to assess potential countermeasures to stockpile. These countermeasures must significantly improve our ability to control and eradicate an outbreak of heartwater in an heartwater-free country such as the US. The decision model is a simple tool that will allow us to focus on critical criteria for veterinary stockpiles, and rank the available interventions relative to each other. The decision model is available as a Microsoft Excel spread sheet, which has been prepared to quantitatively assess the rankings we assign to a set of selected criteria that will lead to the selection of the highest cumulative option. We can use as many criteria as we want but the objective is to get down to the ones that will make or break success. The criteria for each intervention will be selected by the Heartwater Working Groups (vaccines, diagnostics, and acaricides) but a preliminary set has been identified to expedite the process. You are encouraged to review the criteria prior to coming to the meeting and be prepared to modify the criteria as needed with the working group. The following provides an example of criteria and assumptions for assessing vaccines.

Criteria

If a vaccine is going to be used as an emergency outbreak control tool for heartwater, then we need to know: 1) is it efficacious (does it effectively eliminate shedding or just reduce shed by a known log scale); 2) does it work rapidly with one dose (probably do not have time for a second dose); 3) whether it is available today from the perspective of having a reliable & rapid manufacturing process (need to know it can be up & running rapidly and will yield a predictable amount of vaccine; 4) can we get the product to the outbreak site rapidly & safely; 5) once at the site, can we get it into the target population rapidly; 6) type of administration- mass or injected, people and equipment to do the job become important); and 7) are diagnostics available to monitor success and or DIVA compliant. While cost is important, the cost of the vaccine in an outbreak will be small in comparison to the other costs. In addition, how fast the product can be made is important because that will have a big impact on how big a stockpile will be needed. Accordingly, you will see from the Excel sheets that have been prepared for vaccines that the following critical criteria and assignment of weights for each criterion are proposed.

Weight	Critical Criteria
10	Efficacy
6	Safety
8	One dose
6	Speed of Scale up
2	Shelf life
2	Distribution/storage
10	Quick Onset of Immunity
8	DIVA Compatible
2	Withdrawal
2	Cost to Implement

APPENDIX II: Assessment of Experimental Vaccines

Weight	Critical Criteria	Infection/Treatment (Ball 3)	Inactivated/Adjuvanted	Live Attenuated	Heterologous Prime Boost DNA vaccine	Multipeptide DNA Vaccine
8	Prevent clinical disease	10	6	10	10	6
10	Prevent transmission	2	2	4	4	4
6	One dose	8	2	8	2	2
4	Speed of Scaleup	8	4	8	8	8
8	Safety	2	6	4	8	8
6	High yield manufacturing process	6	6	8	8	8
8	Cost of goods	8	6	8	6	6
6	Duration of immunity	6	4	6	4	4
10	Cross-protection	2	2	6	6	6
6	Storage	2	8	2	8	8
2	DIVA	2	2	2	8	8
2	Withdrawal	6	6	8	6	6

Critical Criteria	Infection/Treatment	Inactivated/Adjuvanted	Live Attenuated	Heterologous prime boost DNA vaccine	Multipeptide DNA Vaccine
Prevent clinical disease	80	48	80	80	48
Prevent transmission	20	20	40	40	40
One dose	48	12	48	12	12
Speed of Scaleup	32	16	32	32	32
Safety	16	48	32	64	64
High yield manufacturing process	36	36	48	48	48
Cost of goods	64	48	64	48	48
Duration of immunity	36	24	36	24	24
Cross-protection	20	20	60	60	60
Storage	12	48	12	48	48
DIVA	4	4	4	16	16
Withdrawal	12	12	16	12	12
Value	380	336	472	484	452

APPENDIX III: Assessment of Laboratory Diagnostics

Evaluation of Laboratory Diagnostics Based on Antibody Detection

Weight	Critical Criteria	HW free area		Endemic area		Outbreak area		Follow-up of outbreak	
		Competitive ELISA**	Indirect ELISA	Competitive ELISA**	Indirect ELISA	Competitive ELISA**	Indirect ELISA	Competitive ELISA**	Indirect ELISA
10	Sensitivity	8	7	8	7	8	7	8	7
8	Specificity	2	3	2	3	2	3	2	3
10	Validation to purpose	7	7	7	7	6	5	7	7
6	Speed of Scaleup	3	5	3	5	3	5	3	5
8	Throughput	8	8	8	8	8	8	8	8
5	Need for confirmation	3	4	8	9	3	4	3	4
8	Handling[1. 2.]								
8	Rapid Result (less than one day)	8	8	8	8	8	8	8	8
4	Detection of different strains[2.]								
8	Implementation[3.]	2	5	2	5	2	5	2	5
8	Easy to perform[4.]	8	8	8	8	8	8	8	8
4	Cost	6	9	6	9	6	9	6	9
	Sensitivity	80	70	80	70	80	70	80	70
	Specificity	16	24	16	24	16	24	16	24
	Validation to purpose	70	70	70	70	60	50	70	70
	Speed of Scaleup	18	30	18	30	18	30	18	30
	Throughput	64	64	64	64	64	64	64	64
	Need for confirmation	15	20	40	45	15	20	15	20
	Handling[1. 2.]	0	0	0	0	0	0	0	0
	Rapid Result (less than one day)	64	64	64	64	64	64	64	64
	Detection of different strains[2.]	0	0	0	0	0	0	0	0
	Implementation[3.]	16	40	4	25	4	25	4	25
	Easy to perform[4.]	64	64	64	64	64	64	64	64
	Cost	24	36	24	36	24	36	24	36
	Value	431	482	444	492	409	447	419	467

Evaluation of Laboratory Diagnostics Based on Pathogen Detection

Weight	Critical Criteria	Endemic area				Outbreak area				Follow-up of outbreak			
		nested pCS20	qPCR Sol1	qPCR Cow	Dual Plex PME/ER	nested pCS20	qPCR Sol1	qPCR Cow	Dual Plex PME/ER	nested pCS20	qPCR Sol1	qPCR Cow	Dual Plex PME/ER
10	Sensitivity	8	9	7	8	8	9	7	8	8	9	7	8
8	Specificity	8	9	6	9	8	9	6	9	8	9	6	9
10	Validation to purpose	7	8	8	5	5	10	5	8	5	10	5	8
6	Speed of Scaleup	8	8	8	8	8	8	8	8	8	8	8	8
8	Throughput	4	10	10	10	4	10	10	10	4	10	10	10
5	Need for confirmation	8	10	9	10	5	7	5	7	7	10	5	7
8	Handling[1. 2.]	1	9	9	9	1	9	9	9	1	9	9	9
8	Rapid Result (less than one day)	4	10	10	10	4	10	10	10	4	10	10	10
4	Detection of different strains[2.]	9	9	7	8	9	9	7	8	9	9	7	8
8	Implementation[3.]	7	9	9	9	7	9	9	9	7	9	9	9
8	Easy to perform[4.]	9	9	9	9	9	9	9	9	9	9	9	9
4	Cost	5	4	4	3	5	4	4	3	5	4	4	3
	Sensitivity	80	90	70	80	80	90	70	80	80	90	70	80
	Specificity	64	72	48	72	64	72	48	72	64	72	48	72
	Validation to purpose	70	80	80	50	50	100	50	80	50	100	50	80
	Speed of Scaleup	48	48	48	48	48	48	48	48	48	48	48	48
	Throughput	32	80	80	80	32	80	80	80	32	80	80	80
	Need for confirmation	64	80	72	80	40	56	40	56	56	80	40	56
	Handling[1. 2.]	8	72	72	72	8	72	72	72	8	72	72	72
	Rapid Result (less than one day)	32	80	80	80	32	80	80	80	32	80	80	80
	Detection of different strains[2]	72	72	56	64	72	72	56	64	72	72	56	64
	Implementation[3]	56	72	72	72	56	72	72	72	56	72	72	72
	Easy to perform[4]	72	72	72	72	72	72	72	72	72	72	72	72
	Cost	40	32	32	24	40	32	32	24	40	32	32	24
	Value	638	850	782	794	594	846	720	800	610	870	720	800

[1.] Ease of handling in laboratory, including potential for cross contamination of samples.
[2.] Relevant only to molecular diagnostics.
[3.] Commercially available or in current use.
[4.] Ease of performance, including sample preservation.

APPENDIX IV: Evaluation of Acaricides

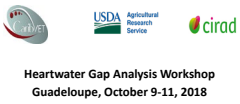
Weight	Critical Criteria	Organophosphates	Macrocyclic Lactones	Pyrethroids	Formamidines	Botanicals
10	Efficacy	10	10	10	10	2
10	Formulation/application method	10	10	10	10	10
8	Residual activity on host	6	8	6	6	2
8	Frequency of Application	6	8	6	6	2
8	Market Availability	6	8	8	8	4
6	Toxicity for non-target species	4	4	6	2	8
6	Safety for humans	2	8	4	6	8
6	Cost of Acaricide	8	6	8	8	6
6	Withdrawal	4	2	8	8	8
2	Toxicity for target host	8	8	8	8	8

Critical Criteria	Organophosphates	Macrocyclic Lactones	Pyrethroids	Formamidines	Botanicals
Efficacy	100	100	100	100	20
Formulation/application method	100	100	100	100	100
Residual activity on host	48	64	48	48	16
Frequency of Application	48	64	48	48	16
Market Availability	48	64	64	64	32
Toxicity for non-target species	24	24	36	12	48
Safety for humans	12	48	24	36	48
Cost of Acaricide	48	36	48	48	36
Withdrawal	24	12	48	48	48
Toxicity for target host	16	16	16	16	16
	468	528	532	520	380

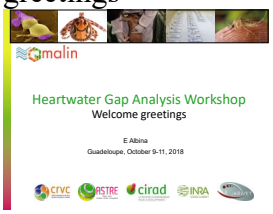
APPENDIX V: Contributors

POWERPOINT PRESENTATIONS
Click on slides below to open presentations

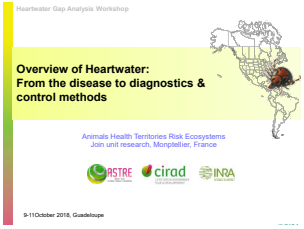
Cyril Gay, USDA-ARS: Heartwater Gap Analysis Workshop



Emmanuel Albina, CIRAD Guadaloupe: Heartwater Gap Analysis Workshop: Welcome greetings



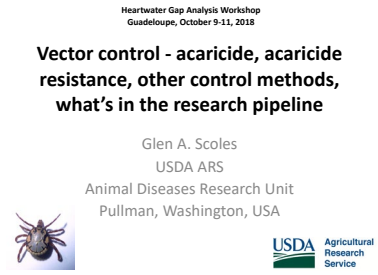
Nathalie Vachiéry, CIRAD France: Overview of Heartwater: From the disease to diagnostics and control methods



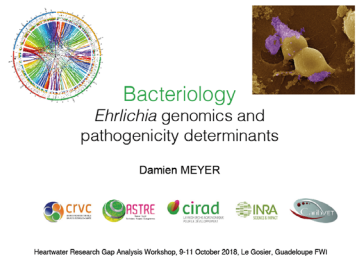
Laure Bournez, ANSES: Hearwater epidemiology

Juan Mosqueda, Universidad de Queretaro: Acarology - vector competence and transmission

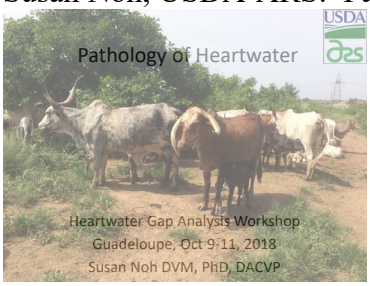
Glen A. Scoles, USDA ARS: Vector control - acaricide, acaricide resistance, other control methods, what's in the research pipeline



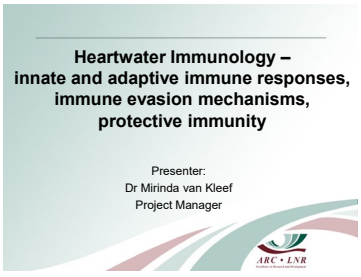
Damien Meyer, CIRAD Guadaloupe: Bacteriology, *Ehrlichia* genomics and pathogenicity determinants



Susan Noh, USDA-ARS: Pathology of Heartwater



Mirinda van Kleef, OVI: Heartwater Immunology-innate and adaptive immune responses, immune evasion mechanisms, protective immunity



Kelly A. Brayton, Washington State University: Vaccinology

Valérie Pinarello, CIRAD Guadaloupe: Laboratory Diagnosis: Available tests and new generation approaches

Jennifer Pradel, CIRAD Guadaloupe: The Caribbean Animal Health Network, Integrated platform of research and surveillance in the Caribbean

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