Molecular Epidemiology of Heartwater (*Ehrlichia ruminantium* Infection) in Domestic Ruminants in the Sudan

By

Mohammed Sayed Mohammed Suliman
BVSc University of Nyala, 1999
MVSc University of Khartoum, 2005

Supervisor

**Dr. Shawgi M. Hassan**
Department of Parasitology
Faculty of Veterinary Medicine
University of Khartoum

Co-Supervisor

**Prof. Abdel Rahim M. El Hussein**
Animal Resources Research Corporation
Ministry of Animal Resources and Fisheries

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Department of Parasitology
Faculty of Veterinary Medicine
University of Khartoum

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Dedication

I dedicate this work with love and respect to my
father, mother, brothers, sisters, my wife, my son Al-fatih
and to all those who have made a positive impact on my life
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ABSTRACT

This study was to investigate molecular epidemiology of heartwater in the Sudan by assessing prevalence of infection in domestic ruminants and vector of the genus *Amblyomma* using the standard PCR and quantitative PCR based on the pCS20 gene as well as to isolate *Ehrlichia ruminantium* stocks in the Sudan. A total of 460 adult *Amblyomma* spp., *A. lepidum* (290), *A. variegatum* (100) and blood samples (170) from cattle (60), sheep (60) and goats (50) were tested with pCS20 PCR. Twenty nine out of 190 (15.3%) and 14 out of 100 (14%) were positive for *E. ruminantium* in *A. lepidum* and *A. variegatum*, respectively. Four out of 60 (6.7%), 2 out of 50 (4%) and 5 out of 60 (8.3%) blood samples from cattle, sheep and goats, respectively were positive for *E. ruminantium*.

A qPCR was applied for detection and quantification of *E. ruminantium* in *Amblyomma* spp. and blood samples. The sensitivity of the qPCR was determined through standard curve generated from the Welgevonden strain. The detection limit was 5 pCS20 copies/µl which was at least 20-times more sensitive than that of standard PCR. Furthermore, the qPCR was compared to the standard pCS20 PCR and nested MAP1 PCR. Out of 50 samples positive with qPCR, 49 (98%) were positive with nested MAP1 PCR and 42 (84%) with standard pCS20 PCR. The specificity of the qPCR was, also, determined using *E. ruminantium* Welgevonden as a reference strain from South Africa, four local *E. ruminantium* stocks (Dinder, Abonama, Nyala and Gdarif) and two other *Rickettsiales* namely *E. canis* and *Anaplasma marginale*. The qPCR specifically detected all *E. ruminantium* stocks and was positive with *E. canis* but negative with *A. marginale*.

The mean copy number of pCS20 was determined. The copy numbers ranged from $7 \times 10^6$ to $7 \times 10^{15}$ in *A. lepidum* with the highest ($7 \times 10^{15}$) copy numbers in Abonama and the lowest ($7 \times 10^6$) in Elhoush. In *A. variegatum*, the copy numbers ranged...
from $1 \times 10^7$ to $4 \times 10^{11}$ with the highest ($4 \times 10^{11}$) copy number in Kass, followed by ($4 \times 10^9$) in Aidelfersan and the lowest ($1 \times 10^7$) was in Nyala. Blood samples from cattle, sheep and goats were tested by qPCR for detection of *E. ruminantium* in Singa, Tamboul and Nyala. Four out of 50 (8%), 3 out of 35 (8.6%) and 5 out of 35 (14.3%) blood samples from cattle, sheep and goats, respectively were positive.

*In vivo* isolation of three *E. ruminantium* isolates (Dinder, Abonama and Nyala) was carried out using Nubian goats reared in *Amblyomma* free zone north of Khartoum. Dinder and Abonama stocks were isolated by inoculating *A. lepidum* homogenates in goats and Nyala stock isolated from *A. variegatum* were inoculated in a goat. Goats developed clinical signs of heartwater by 6 to 12 days. Estimation of copy numbers of *E. ruminantium* infection in *Amblyomma* tick homogenates and blood stabilates of experimentally infected goats during febrile periods were established using qPCR. Nyala stock showed hyperacute form of heartwater. The load of pCS20 copies number in the infective dose was ($5 \times 10^5$) and the animal died on day 9 post infection while the load in Dinder stock was ($1 \times 10^4$) and underwent an acute form and the animal died on day 14. Abonama stock showed a mild form of the disease and the animal survived although the load was the highest ($2 \times 10^5$). The copies number of pCS20 in the blood stabilates of goats inoculated with tick homogenates was, also, estimated during the febrile periods in each stock. The copies number of pCS20 ranged from $2 \times 10^8$ to $1 \times 10^{11}$ copies/µl and the highest ($1 \times 10^{11}$) copies number was in Nyala stock coincided with the highest body temperature ($42.2^\circ C$) followed by ($1 \times 10^{10}$) in Abonama stock and the lowest ($2 \times 10^8$) copies number was reported in Dinder stock.

It conclude that the study defined the spatial distribution and prevalence of *E. ruminantium* in the vector population and domestic ruminant hosts and provided an accurate situation of the potential risk of heartwater that pose livestock in the endemic regions in the Sudan. The qPCR applied for *E. ruminantium* is species specific and sensitive to as few as 5 gene copies/µl. The assay was found to be...
more sensitive than the standard pCS20 PCR assay and nested MAP1 PCR assay. The successful *in vivo* isolation of *E. ruminantium* new stocks (Dinder, Abonama and Nyala) confirmed the high infection rate of ticks and wide distribution of the disease in different localities in the Sudan.

This study has opened the way for a complete investigation of the epidemiology of heartwater in the Sudan. The new technique contributes the knowledge of the diversity of *E. ruminantium* stocks and will facilitate vaccine studies to develop specific vaccines in problem areas.
تقصي وبائية مرض الخدر باستخدام تقارير الأحياء الجزيئية في المجترات المستأنسة في السودان

المستخلص

أجريت هذه الدراسة لتقصي وبائية مرض الخدر في السودان باستخدام تقارير الأحياء الجزيئية لتحديد نسبة انتشار المرض في كل من المجترات المستأنسة والعائل الناقل وهو القدر من جنس أميبيلوما وذلك باستخدام تفاعل البلمرة المسنسل و(pCR) على الجين الدبلوم dodqCR (qPCR) بالاضافة إلى عزل المسبب وهو إريخيا روميناتشيموم.

تم جمع عدد 624عينة لكل من القراد البالغ من جنس أميبيلوما (270) والدم (270) من الأبقار (60)، الضأن (50) والماز (60) وتم فحصه بتقنية PCR للكشف عن الحمض النووي إريخيا روميناتشيموم. وكانت نسبة انتشار المرض 20 من 190 (15.3%) و14 من 100 (14%) في أميبيلوما ق/big و أميبيلوما فاريقاتوم على التوالي. و كانت نسبة انتشار المرض في الدم 4 من 60 (6.7%) و2 من 50 (4%) و5 من 60 (8.3%) في الأبقار، الضأن والماز على التوالي.

تم أيضا استخدام تفاعل qPCR للكشف عن وقياس كمية الحمض النووي لإريخيا روميناتشيموم في الأميبيلوما وعينات الدم. تم تحديد حساسية اختيار qPCR عبر المنحنى القياسي لعمرة وجيلبوندن وكانت بمعدل 61% من جين pCS20 لكل ميكروليلتر والتي تساوي 20 ضعف حساسية اختيار PCR. أيضا تم تمت مقارنة حساسية اختبار qPCR مع كل من اختبار PCR و اختبار تفاعل البلمرة المسنسل الشعبي MAP1 على الجين qPCR. فبينما MAP1 PCR كانت 49 (84%) موجبة باختبار qPCR فقط بينما 42 (84%) فقط nested MAP1 PCR موجبة باختبر qPCR وذلك باستخدام عثرة وجيلبوندن كعترة مرجعية من جنوب أفريقيا وأربعة عثرات محلية (الدود، أوبناما، نيلال والقصاص) واثنين من الريكسما المشابهة لإريخيا روميناتشيموم وهما إريخيا كينس وأنبلازما مارجينال. فكانت نتيجة الاختبار موجبة لكل عثرات إريخيا روميناتشيموم وكذلك لإريخيا كينس وسلانية لفيلة مارجينال.

تم قياس عدد النسخ لإريخيا روميناتشيموم في نوعي القراد أميبيلوما لبيد و أميبيلوما فاريقاتوم. في أميبيلوما لبيدم سجل معدل أعلى معدل (7 x 10^15) في أبوناعمة وأقل معدل (7 x 10^6) في الحوش. في أميبيلوما فاريقاتوم فقد سجل أعلى معدل (4 x 10^6).
تم في هذه الدراسة عزل ثلاث عوائل (الدندر، أبونعامة و نيالا) للأرليخيا روميناشيموب باستخدام الماعز البوني وذل ذلك بدقة أليكس موجات من مسح الوفيات من النوع أميليا لعيد من الدندر وأبونعامة و من النوع أميليا فاريسياتو من نيالا. أظهر الماعز البوني أعراض سريرة متغيرة للإصابة بمضر الخدر وكانت فترة الحضانة 6-12 يوم. تم تقدير كمية إرليخيا روميناشيموب في مسح الوفيات الأميليا وضم حيوانات التجارب المحفوفة بالسنية وذلك خلال فترة الحمى باستخدام qPCR. أظهرت عوائل نيالا حالة مرضية فوق معدل عدد الين في الجرعات المعدية (5 × 10⁴) ونفق الحيوان في اليوم التاسع من العوائل. بينما أظهرت عوائل الدندر حالة مرضية حادة بمعدل عدد الين في الجرعات المعدية (1 × 10⁴) وكان نفق الحيوان في اليوم الـ14 من العوائل. وأظهرت عوائل أبونعامة حالة مرضية خفيفة بمعدل عدد الين في الجرعات المعدية (2 × 10⁴) بالرغم من أنها كانت الأولى إلا أن الحيوان تأثرت. الجدير بالذكر أن معدل عدد الين في ودم الحيوانات المحفزة بمسح الوفيات خلال فترة الحمى في المدى (2 × 10⁴ إلى 1 × 10⁴) حيث سجلت عوائل نيالا أعلى معدل (1 × 10¹¹) وتبين ذلك مع أعلى درجة حرارة الجسم (42.2⁰C). وسجلت أبونعامة (1 × 10¹⁰) بينما سجلت الدندر أدنى معدل (2 × 10⁸).

أظهرت الدراسة مدى واسع لانتشار المرض وكذلك نسبة انتشار المسبب في مجموعة الناقل والمجرات المستأنسة كما أوضحت الوضع الحقيقي للخطورة المتوقعة. امرض الخدر التي تهدد قطعان المجترات في مناطق استيطان المرض في السودان. والتي أستخدمت للكشف عن أرليخيا روميناشيموب وجدت أنها ذات خصوصية نوعية وحساسة إلى ما دون خمس نسخ من الجين لكل ميكرولتر وأن (pCS20 PCR و تناقص (nested MAP1 PCR) هذه التقنية أكثر حساسية من تقنية qPCR للكشف عن ذات الجرثومة. كما أظهرت الدراسة عزل ثلاث عوائل مختلفة في درجة إمراضية للحيوان مما يرهن علي الإصابة العالية في الناقل والانتشار الواسع للمرض في مناطق مختلفة في السودان.

110⁴ في كأس يليه (4 × 10⁹) في عد الفرسان بينما أقل معدل (1 × 10⁷) سجل في نيالا.
INTRODUCTION

Heartwater or cowdriosis is an infectious, non-contagious, tick-borne disease caused by an obligatory intracellular rickettsial pathogen previously known as *Cowdria ruminantium* but recently reclassified as *Ehrlichia ruminantium* (Dumler *et al.*, 2001). The disease is transmitted by the ticks of the genus *Amblyomma* and affects domestic ruminants: cattle, sheep, goats, and several wild ruminants such as buffalo, giraffe, and antelope as well as some wild rodents (Peter *et al.*, 2002). Heartwater is usually an acute disease and may be fatal within days of the onset of clinical signs in naïve susceptible animals. The incubation period of the disease in susceptible animals has an average of less than two weeks (van de Pypekamp and Prozesky, 1987). Adult cattle have a subsequent mortality of up to 82% (Du Plessis and Malan, 1987b) and Merino sheep of up to 95% (Neitz, 1968). Goats and sheep are more susceptible than cattle, and European cattle breeds are generally more susceptible than indigenous African breeds (Uilenberg, 1983).

The endemic area includes the whole of sub-Saharan Africa and, also, the French Antillean islands of Guadeloupe, Antigua and Marie Galante, to which infected *Amblyomma variegatum* ticks were introduced probably in the eighteenth century (Camus *et al.*, 1996). In the sub-Saharan region, the disease is one of the major causes of stock losses, and it has been estimated that more than 150 million animals are at risk in the area (Minjauw and McLeod, 2003). Heartwater is one of the major obstacles to introduction of high producing animals into sub-Saharan Africa, and is of particular importance when susceptible animals are moved from heartwater-free to heartwater-endemic areas (Simpson *et al.*, 1987). The economic impact of the disease is, therefore, difficult to quantify, although estimates made indicated that the losses can be enormous (Mukhebi *et al.*, 1999). Whatever the actual costs may be, it is certain that the economic importance of heartwater in Africa is comparable to that of East Coast fever, trypanosomosis, rinderpest and dermatophilosis (Provost and Bezuidenhout, 1987).
Scanty information on the epidemiology of the disease in most parts of Africa. At present, there is a lack of safe, practical and effective vaccine, and in endemic areas, the control of the disease relies mainly on chemical acaricides to prevent tick transmission and antibiotic treatment of clinical cases. The main problem encountered with the control of the disease is the lack of efficient vaccine in the field. This is thought to be related to the high genetic diversity of strains circulating in a particular area (Allsopp and Allsopp, 2007). However, knowledge on epidemiology of heartwater in the Sudan is very scanty, if any. Two *Amblyomma* species (*A. lepidum* and *A. variegatum*) coexist in the country. In addition, no work has been conducted to elucidate efficiency of these tick species in disease transmission. Few information on infection rates were reported (Muramatsu *et al.*, 2005; Abdel Rahman, 2006).

In order to upgrade the livestock industry and prioritize future research on the development of improved control measures, it is essential to provide an accurate definition of the spatial distribution of the disease risk and determine the prevalence of infection in the vector population and domestic ruminant hosts. However, accurate results completely dependent on the submission of correctly collected and dispatched field samples for an accurate molecular epidemiological study of heartwater. The advent of new molecular techniques, for *E. ruminantium*; specific DNA PCR and the probe pCS20 (Waghela *et al.*, 1991; Mahan *et al.*, 1992; Steyn *et al.*, 2003; van Heerden *et al.*, 2004a) has replaced the older screening techniques and proved to be useful for identification of heartwater in both infected animals and ticks (van Heerden *et al.*, 2004b). Such assays are specific and time consuming (Steyn *et al.*, 2008). Thus, reliable diagnostic assays to obtain better epidemiological information for heartwater in the Sudan are warranted, and could lead to an effective heartwater vaccine development. The development of new diagnostic techniques that included DNA fragments amplified from parasite genomic DNA, revolutionized epidemiological studies.
Since the introduction of sequencing of PCR products, different genes could be accurately identified. This study focused on the use of molecular epidemiology of heartwater, by application of new diagnostic tools, identification and isolation of *E. ruminantium* local stocks in the Sudan.

**Hypothesis:**

Mortality due to heartwater and problems with the disease were observed through planning of the study. Therefore, sufficient samples can be obtained from livestock to elucidate the molecular epidemiology of heartwater using the conventional techniques, coupled with the new molecular techniques, for better definition and control of the disease in endemic areas in the Sudan.

**Aims of the study:**

The aims of the study described in this thesis were:

1- to investigate molecular epidemiology of heartwater in some endemic areas the Sudan by assessing prevalence of infection in the host and vector population based on the conserved pCS20 gene region.

2- to conduct simultaneous detection and quantification of *E. ruminantium* DNA in *Amblyomma* ticks and blood samples collected from the field by quantitative real time PCR based on the pCS20 gene to assess the potential risk of heartwater that pose livestock in the endemic areas of the country.

3- to carry out *in vivo* isolation of *E. ruminantium* and characterize new field isolates in heartwater endemic areas in the Sudan.
CHAPTER ONE

Review of Literature
1.1. The disease

Heartwater (also used to be known as cowdriosis) is an infectious, non-contagious tick-borne disease of sheep, goats, cattle and some wild ruminants (Uilenberg, 1983; Peter et al., 2002). It is caused by *Ehrlichia ruminantium* (formerly *Cowdria ruminantium*) and is transmitted by species of ticks of the genus *Amblyomma* (Uilenberg, 1983; Walker and Olwage, 1987).

The disease occurs in nearly all the Sub-Saharan countries of Africa, in Madagascar and, also, in the Caribbean Islands, threatening the American mainland (Barré et al., 1987; Camus et al., 1996; Burridge, 1998). The course of the disease varies from peracute, acute, sub-acute to mild, depending on age, immune status, breed and virulence of the strain (Uilenberg, 1983). The incubation period of the disease is influenced by the species of animal affected, immune status, breed, age, route of infection, virulence of the organism and amount of infective materials administered (Uilenberg, 1983; Prozesky, 1987b). The disease is characterized by fever, nervous signs, hydropericardium, hydrothorax, lung oedema and high mortality (van Pypekamp and Prozesky, 1987). The name heartwater is derived from the hydropericardium, which is commonly seen with this disease (Uilenberg, 1983; Prozesky, 1987a; Camus et al., 1996). Wild ruminants may play a role as reservoirs, and the wide host-ranges of the pathogen in wildlife is important in the epidemiology and spread of the disease (Mackenzie and Mchardy, 1987; Oberem and Bezuidenhout, 1987; Peter et al., 2002).

The causative agent is Gram negative bacteria, but without typical gram negative bacterial cell wall components such as lipopolysaccharide and peptidoglycan. It is obligatory intracellular bacteria that form micro colonies within membrane-bound cytoplasmic vacuoles, usually in various sizes which infects endothelial cells. Examination of *E. ruminantium* by electron microscopy reveals two distinct
morphologic forms, a larger (0.7-1.9 µm) pleomorphic form with dispersed nucleoid filaments (reticulate) and smaller form (0.4-0.6 µm, dense-cored) with condensed nucleoid filaments (Pienaar, 1970; Camus et al., 1996).

1.2. Historical background

The first description of a disease resembling heartwater was made in South Africa by Trichardt in 1838 upon observation of a fatal nervous disease of sheep following massive tick infestation 3 weeks before appearance of symptoms (Neitz, 1968). Webb in 1877 described enormous losses amongst small ruminants due to heartwater, and believed that the disease was associated with the bont tick A. hebraeum. In addition, Dixon in 1898 was able to transmit heartwater by the intravenous inoculation of infected blood into susceptible animals. It was then concluded that the disease was caused by a living microorganism and was thought to be a virus (Camus et al., 1996). Later, Cowdry (1925a) demonstrated the causative agent of heartwater in tissues of infected animals as well as in infected ticks, thereby, describing for the first time a Rickettsia causing a disease in domestic animals. Initially named Rickettsia ruminantium, the organism was later renamed Cowdria ruminantium in honour of Cowdry redundant (Moshkovski, 1947).

In vitro culture of the organism (Bezuidenhout et al., 1985) facilitated the development of diagnostic tests and the study of immunological responses in hosts to infection (Jongejan et al., 1991a; Martinez et al., 1993a; Totté et al., 1997) and for vaccine protection (Mahan et al., 1994b; 1995; Totté et al., 1997). However, the advent of genomic extraction of DNA allowed the development of PCR-based techniques for identification of the pathogen DNA in ticks and hosts, and to genetically characterize the different isolates from geographically diverse areas (Waghela et al., 1991; Mahan et al., 1992; Peter et al., 1995; Reddy et al., 1996).
1.3. Classification of the agent

Traditional rickettsial taxonomy assigned the causative agent of heartwater (*Cowdria ruminantium*) as the sole member of the genus *Cowdria* in the tribe Ehrlichieae, family Rickettsiaceae, order Rickettsiales (Moshkovski, 1947). The development of serodiagnostic tests revealed a very close antigenic relationship between *C. ruminantium* and the ehrlichial pathogens; *Ehrlichia canis* and *E. chaffeensis* (Jongejan et al., 1993a; Katz et al., 1997). Phylogenetic trees inferred from 16S ribosomal DNA (rDNA) sequences clearly demonstrated that all *Cowdria* genotypes are closely to genogroup comprising *E. canis* and *E. ewingii*, which infect deer in North America and also, pathogenic to human (Walker and Dumler, 1996). Dumler et al. (2001) proposed a new classification of the Rickettsiales based on phylogenetic analysis of the sequences of the 16S rRNA gene and the groESL operon. Accordingly, *Ehrlichia* (*Cowdria*) *ruminantium* was combined together with *E. canis*, *E. chaffeensis*, *E. ewingii* and *E. muris* and all were assigned to the genus *Ehrlichia*. Based on this analysis, *E. ruminantium* is classified as follows:

Phylum: Proteobacteria

Class: Alphaproteobacteria

Order: Rickettsiales

Family: Anaplasmataceae

Genus: *Ehrlichia*

Species: *ruminantium*
1.4. Epidemiology of heartwater

1.4.1. Vertebrate reservoir

*Ehrlichia ruminantium* appears to have evolved in southern Africa (Allsopp *et al.*, 2003) and African wild ruminants are probably the original reservoir of the disease (Neitz, 1967). Domestic ruminants, notably cattle, sheep and goats are most susceptible to heartwater (Camus, *et al.*, 1996). Large number and variety of wild African and non-African ruminants are susceptible to infection with heartwater giving rise to the suspicion that some animals, in heartwater-endemic areas, may serve as reservoirs of the disease (Oberem and Bezuidenhout, 1987). This suspicion was confirmed by the finding that, the African buffaloes (*Syncerus caffer*), which are excellent natural hosts for the vectors are, after recovery from heartwater, intermittently became infective for ticks for many months (Andrew and Norval, 1989a). Peter *et al.* (2002) provided evidence that 12 African ruminants, three non-African ruminants and two rodents can become infected a symptomatically with *E. ruminantium* (Table 1.1). The wide host range of *E. ruminantium* is reflected in the ability to infect *in vitro* endothelial cells from a wide range of species including humans (Totté *et al.*, 1993), African buffalo, warthog, giraffe, greater kudu, eland and sable antelope (Smith *et al.*, 1998). Certain animals such as the crowned Guinea fowl (*Numida meleagris*) and leopard tortoise (*Geochelone pardalis*) can serve as subclinical carriers of *E. ruminantium* and act as a source of organisms for ticks (Oberem and Bezuidenhout, 1987). Although, the precise role of wildlife in the epidemiology of heartwater remains to be fully investigated, they transfer the infection to *Amblyomma* ticks, which feed on these hosts in nature (Peter *et al.*, 2002). The involvement of wildlife in the cycle of heartwater, is a complicating factor for control of the disease and constitutes an important subject for further investigation (Uilenberg, 1983).
Table 1.1. Wild ruminants known to be susceptible to *E. ruminantium* infection, either naturally or after experimental infection (Peter et al., 2002).

<table>
<thead>
<tr>
<th>African ruminant</th>
<th>Scientific name</th>
<th>Non-African ruminant</th>
<th>Scientific name</th>
</tr>
</thead>
<tbody>
<tr>
<td>Common name</td>
<td></td>
<td>Common name</td>
<td></td>
</tr>
<tr>
<td>African buffalo</td>
<td><em>Syncerus caffer</em></td>
<td>Barbary sheep</td>
<td><em>Ammotragus lervia</em></td>
</tr>
<tr>
<td>Black wildebeest</td>
<td><em>Connochaetes gonou</em></td>
<td>Blackbuck</td>
<td><em>Antilope cervicapra</em></td>
</tr>
<tr>
<td>Blesbok</td>
<td><em>Damaliscus dorcas philipps</em></td>
<td>Chital</td>
<td><em>Axis ax</em></td>
</tr>
<tr>
<td>Blue wildebeest</td>
<td><em>Connochaetes taurinus</em></td>
<td>Fallow deer</td>
<td><em>Cervus dama</em></td>
</tr>
<tr>
<td>Bushbuck</td>
<td><em>Tragelaphus scriptus</em></td>
<td>Himalayan tahr</td>
<td><em>Hemitragus jemlahicus</em></td>
</tr>
<tr>
<td>Duiker</td>
<td><em>Cephalophus sp.</em></td>
<td>Mouflon</td>
<td><em>Ovis orientalis</em></td>
</tr>
<tr>
<td>Eland</td>
<td><em>Taurotragus oryx</em></td>
<td>Nilgai</td>
<td><em>Boselaphus tragocamelus</em></td>
</tr>
<tr>
<td>Giraffe</td>
<td><em>Giraffa camelopardalis</em></td>
<td>Timor deer</td>
<td><em>Cervus timorensis</em></td>
</tr>
<tr>
<td>Greater kudu</td>
<td><em>Tragelaphus strepsiceros</em></td>
<td>Water buffalo</td>
<td><em>Bubalus bubalis</em></td>
</tr>
<tr>
<td>Lechwe Kobus</td>
<td><em>leche kafuensis</em></td>
<td>White-tailed deer</td>
<td><em>Odocoileus virginianus</em></td>
</tr>
<tr>
<td>Red hartebeest</td>
<td><em>Alcelaphus buselaphus</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Scimitar-horned oryx</td>
<td><em>Oryx dammah</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sitatunga</td>
<td><em>Tragelaphus spekii</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Springbuck</td>
<td><em>Antidorcas marsupialis</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Steenbok</td>
<td><em>Raphicerus campestris</em></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
1.4.2. Tick vectors of heartwater

*Ehrlichia ruminantium* is transmitted by ticks of the genus *Amblyomma* and the distribution of heartwater in Africa coincides with that of the vector species. Members of this genus are widespread in tropical and sub-tropical zones where they have a wide host range, especially in immature stages (Yunker, 1996; Jongejan and Uilenberg, 2004). The heartwater endemic area includes almost the whole of sub-Saharan Africa, the offshore islands of Madagascar, Mauritius, Reunion, Grande Comore and São Tomé (Provost and Bezuidenhout, 1987; Du Plessis *et al.*, 1989).

Among the 102 *Amblyomma* species ticks presently known, only 10 African species are capable of transmitting the organism that occurs in Africa. Natural and proven experimental vectors of heartwater are summarized in Table 1.2. The most important vectors are *A. variegatum* and *A. hebraeum* in South Africa (Bezuidenhout, 1987) However, *A. pomposum, A. lepidum, A. astrion, A. cohaerens, A. gemma* (Walker and Olwage, 1987) and *A. marmoreum* (Peter *et al.*, 2000) are also, significant vectors. *A. hebraeum* is the main vector of heartwater in southern Africa, while *A. variegatum*, the most widely distributed vector in Africa, has become established in the Caribbean Islands where it transmits the disease on three islands, Guadeloupe, Antigua and Marie Galante (Molia *et al.*, 2008). *A. maculatum* is the only native American vector of *E. ruminantium*, for sheep similar to that of *A. variegatum* (Mahan *et al.*, 2000).

The vectors of heartwater are three-host ticks, and the organism is transmitted transstadially. Both nymphs and adults become infected with *E. ruminantium* after two days of feeding on infected sheep or two to four days of feeding on infected goats (Camus and Barre’, 1992). A single infected nymph can cause a fatal infection in a susceptible animal (Camus *et al.*, 1996) and intrastadial transmission
by a male *A. hebraeum* moving from sick to susceptible animals, also, occurs (Andrew and Norval, 1989b).

1.4.3. Transmission of heartwater

The effectiveness of *Amblyomma* spp. as vectors for heartwater in certain area, depends on their distribution, activity, abundance, and their adaptation to acquire and transmit infection from local wild or domestic carriers of *E. ruminantium* (Uilenberg, 1983). The tick population in an area is heavily influenced by temperature and humidity (Petney et al., 1987). In the drier parts of Africa, this frequently leads to an increased incidence of heartwater after heavy rains when peak numbers of ticks occur. In regions where the climate is temperate and the rainy season is not well defined, occurrence of heartwater is not really seasonal and this is especially true in the Caribbean islands (Camus and Barre´, 1987). Apparently, healthy ruminant hosts, carrying *E. ruminantium* organisms at very low levels, can be infective to ticks for long periods, at least 361 days for cattle (Andrew and Norval, 1989a) and 11 months for goats (Camus, 1992). They pointed out that, the level of the organism were so low, that the carriers only infect ticks intermittently during the 11-months of experiment. This demonstrated the danger that posed by the movement of heartwater carrier animals to non endemic areas (Camus et al., 1996).

Ticks in heartwater endemic areas exhibit surprisingly low infection rates with *E. ruminantium* (Peter et al., 1999). For *A. hebraeum*, the rates were found to be 1–7% in South Africa (Allsopp et al., 1999) and 8.5–11.2% in Zimbabwe (Peter et al., 1999), while it was 1.2–13.3% in *A. variegatum* in Senegal (Gueye et al., 1994). The highest rate reported was 19.1% for *A. variegatum* in Maria Galante (Molia et al., 2008). When *A. variegatum* larvae and nymphs were fed experimentally on *E. ruminantium*-infected sheep, the infection rate was 100% in the ticks at the following stage (Gueye et al., 1994). This suggests that many ticks
in the field feed during the larval or nymphal stages. Several factors may help to explain how relatively small numbers of infected ticks can maintain heartwater infection in a particular area. While infected larvae or nymphs only become infective after molting to the next stage, they then, remain infective for life (Camus and Barre´, 1992). *E. ruminantium*-infective ticks in the field present a highly virulent disease challenge, much more virulent than that presented by elementary bodies contained in an experimental needle challenge with infected blood (Collins *et al*., 2003; Pretorius *et al*., 2008). Nevertheless, vertical transmission of heartwater from dams to their calves has been demonstrated in cattle in Zimbabwe (Deem *et al*., 1996a).

1.4.4. **Tick-host-pathogen interface**

Infected but unfed ticks are not infective until they feed for 38 hr (nymphs) or 75 hr (adults) (Bezuidenhout, 1987) this is so-called ‘grace period’. Ticks undergo long periods of metabolic inactivity while waiting for their next blood meal. It has been speculated that, in the case of *Anaplasma phagocytophilum*, the parasites remain dormant during these periods, and are then activated when feeding begins, possibly in response to temperature changes and/or to chemical signals from host blood (Katavolos *et al*., 1998). In the case of *E. ruminantium*, temperature may not be the trigger, since warming of infected ticks to 37°C without allowing them to feed did not induce infectivity (Bezuidenhout, 1987). *E. ruminantium* organisms delivered by infective tick challenge are much more virulent than elementary bodies delivered as an infected blood needle challenge (Collins *et al*., 2003). This enhanced virulence must, therefore, develop during the grace period, but there is currently no experimental information about the mechanisms involved (Pretorius *et al*., 2008). During the grace period one would expect *E. ruminantium* to be up regulating metabolic pathways and other genes which lead to enhanced survival in the mammalian host (Allsopp, 2010).
Table 1.2. African *Amblyomma* species shown to be experimental and natural vectors of heartwater (Bezuidenhout, 1987).

<table>
<thead>
<tr>
<th>Amblyomma spp.</th>
<th>Reported mode of Transmission</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. hebraeum</td>
<td>I, II, III</td>
<td>Lounsbury (1900)</td>
</tr>
<tr>
<td>A. variegatum</td>
<td>I, II, III</td>
<td>Daubney (1930)</td>
</tr>
<tr>
<td>A. pomposum</td>
<td>I, II</td>
<td>Neitz (1947)</td>
</tr>
<tr>
<td>A. gemma</td>
<td>II</td>
<td>Ngumi <em>et al.</em> (1997)</td>
</tr>
<tr>
<td>A. lepidum</td>
<td>I, II</td>
<td>Karrar (1960); Karrar (1966)</td>
</tr>
<tr>
<td>A. astrion</td>
<td>I, III</td>
<td>Uilenberg and Niewold (1981)</td>
</tr>
<tr>
<td>A. cohaerens</td>
<td>I, III</td>
<td>Uilenberg (1983)</td>
</tr>
<tr>
<td>A. marmoreum</td>
<td>I, III</td>
<td>Bezuidenhout <em>et al.</em> (1987)</td>
</tr>
</tbody>
</table>

I from larval to nymphal stage  
II from nymphal to adult stage  
III from larval through nymphal to adult stage
The genes involved may be those of the pathogen, the tick, or the mammal, and pointers from experiments on other ticks and organisms indicate the sort of changes which may take place (Camus et al., 1996). It is known, for instance, that the saliva of *Ixodes scapularis* and *Rhipicephalus sanguineus* ticks contain molecules that modulate the activity of the mammalian immune system and thereby enhance the survival of transmitted organisms (Ferreira and Silva, 1998; Wikel, 1999). Other mechanisms involve organism surface changes which are activated only during tick feeding (Camus et al., 1996). The mechanisms operating at the tick-host-pathogen interface in the case of *E. ruminantium*, are important potential subjects for future genomic and proteomic-based investigation (Allsopp, 2010).

### 1.4.5. Clinical disease and immunity

Heartwater, in clinically affected animals, is characterized by sudden onset of high fever, that may be accompanied by nervous signs and may followed by death. The disease usually develops within 10 to 30 days after an infective tick bite and the first symptom usually is a sudden rise in body temperature (van de Pypekamp and Prozesky, 1987). The disease occurs in susceptible ruminants following natural transmission by infected *Amblyomma* ticks, artificial transmission by inoculation of infected blood, tissue homogenate, ground-up tick supernatant or infected ruminant endothelial cell culture (Camus et al., 1996). The severity of clinical signs and mortality rate depend on the species, breed and age of the ruminant host, the route of infection (tick transmitted or needle-inoculated), the virulence of the *E. ruminantium* isolate and the size of the inoculums. Mortality rates vary between 5% and 100% (van de Pypekamp and Prozesky, 1987). Newly born and young animals, irrespective of breed and the dam’s immune status have been reported to possess innate resistance to heartwater (Uilenberg, 1983).
The duration of this inverse-age resistance varies amongst species. It is reported to be 9 days in Merino lambs (Camus et al., 1996), 2 weeks in kids (Camus and Barre', 1987a) and 2 to 3 weeks in calves (Uilenberg, 1983). Exotic breeds are more susceptible than indigenous livestock and mortality rate of 50% or greater due to the disease have been reported in sheep and imported cattle in sub-Saharan Africa (Uilenberg, 1983). Generally, indigenous ruminant livestock in heartwater endemic areas are resistant to the disease. However, this is less for sheep and goats (Camus et al., 1996). The course of clinical heartwater in ruminants varies. In the peracute form, death occurs suddenly with little or no prior indication of clinical disease. In the acute form, high fever of rapid onset followed by anorexia, dyspnoea, nervous signs and death within 2-6 days. In the subacute form, the clinical signs are similar to those in the acute form, but less pronounced, and may be followed by death or recovery. In the mild or inapparent form, the only clinical sign is transitory fever, which may not be noticed in the field, followed by recovery and development of immunity. This form is common in neonatal animals, which possess an innate inverse age-related resistance to heartwater. It is also, present in heartwater-endemic areas especially, in disease-resistant indigenous breeds such as West African Dwarf goats and Djallonké sheep (Camus et al., 1996; Allsopp, 2010).

Epidemiological factors such as presence or absence of the vector, of previous occurrence of heartwater in the locality should be considered in making a diagnosis. Immunity to *E. ruminantium* infection, is similar to other intracellular organisms, is considered to be mainly cell-mediated (Totté et al., 1997; 1999). Antibodies produced in response to infection as early as the first day of fever (Viljoen et al., 1987), play little or no role in the protective immune response (Totté et al., 1999). Animals that recovered from heartwater manifest solid immunity against homologous or antigenically related *E. ruminantium* isolate for several years (Camus et al., 1996). Cross-protection between isolates may be
complete, partial or non-existent (Du Plessis et al., 1989) and appears not to correlate with geographic origin. Antigenic diversity between *E. ruminantium* resulting in lack of protection between heterologous strains/stocks is the single most significant obstacle to developing a protective vaccine and consequently constitutes a major constraint to livestock upgrading programmes in sub-Saharan Africa (Allsopp, 2009).

### 1.4.6. Infectivity and pathogenicity

Infectivity to mice was the earliest method used to demonstrate variability between stocks and three different types of pathogenicity were observed; pathogenic genotypes which can kill mice, genotypes that infect mice but are not pathogenic and non-infective genotypes that fail to establish any infection in mice (Table 1. 3). The Welgevonden genotype, for example, is always fatal to mice, whereas the Senegal genotype is only pathogenic when a large infective dose is given. The Ball 3 genotype subclinically infects mice, but it cannot be sub-passaged and the mice do not become long-term carriers. On the other hand, the Gardel genotype does not infect mice at all (Camus, *et al.*, 1996; Allsopp, 2010). The Omatjenne genotype which was first isolated by infecting a mouse with a single *Hyalomma truncatum* tick homogenate, taken off a healthy cow on a farm in Namibia (Du Plessis, 1990). Despite the original isolation of the Omatjenne genotype being made from a *H. truncatum* tick, this species is unlikely to be the primary vector of the organism, since the larvae and nymphs feed exclusively on scrub hares (*Lepus saxatilis*) and rodents, while only the adults feed on cattle (Allsopp *et al.*, 2007).
Table 1.3. Infectivity to mice demonstrates variability among *E. ruminantium* stocks, shows three different types of pathogenicity (Allsopp, 2010).

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Geographical</th>
<th>Biological</th>
<th>Pathogenicity</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Geographical</td>
<td>Biological</td>
</tr>
<tr>
<td>Ball 3</td>
<td>South Africa</td>
<td>Bovine</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Gardel</td>
<td>Guadeloupe</td>
<td><em>A. hebraeum</em></td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Kiswani</td>
<td>Kenya</td>
<td>Bovine</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Mara 87/7</td>
<td>South Africa</td>
<td><em>A. hebraeum</em></td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Omatjenne</td>
<td>Namibia</td>
<td><em>H. truncatum</em></td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Senegal</td>
<td>Senegal</td>
<td>Bovine</td>
<td>+</td>
<td>+/-</td>
</tr>
<tr>
<td>Welgevonden</td>
<td>South Africa</td>
<td><em>A. hebraeum</em></td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

+ = pathogenic; +/- = mildly pathogenic; - = non-pathogenic; 0 = non-infective; ND = not done.
1.4.7. Emergence of novel phenotypes

The development of the pCS20 assay for *E. ruminantium* detection (Mahan *et al*., 1992; van Heerden *et al*., 2004b) has revealed the presence of the organism in a number of atypical or unexplained infections occur after tick bite in both non-ruminants and humans. Canine ehrlichiosis is commonly encountered in South Africa and is normally diagnosed on the basis of clinical symptoms and blood smear examination. Some animals, however, show symptoms suggestive of canine ehrlichiosis but without morulae being seen on blood smears. These are often tested using a PCR assay specific for North American *Ehrlichia canis* (McBride *et al*., 1996; Loftis *et al*., 2006; 2008). However, most of these cases are negative for *E. canis*, but many of them test positive for *E. ruminantium* by the pCS20 assay (Allsopp and Allsopp, 2001). They reported that, in one of these *E. canis*-negative was *E. ruminantium*-positive cases and other *E. ruminantium*-specific gene sequences were obtained. They strongly suggesting that an *E. ruminantium* variant contributed to the animal’s illness.

1.5. Life cycle of *E. ruminantium*

1.5.1. Life cycle in the mammalian host

*Ehrlichia ruminantium* replicates mainly by binary fission of reticulated bodies (Pienaar, 1970; Kocan *et al*., 1987b; Prozesky and Du Plessis, 1987) while the elementary bodies represent the infective stage (Jongejan *et al*., 1991a). Sequential development of the organism has been described in vertebrate (Du Plessis, 1985) and invertebrate host cells (Kocan *et al*., 1987b). In the mammalian host, the organism initially replicates in reticulo-endothelial cells in lymph nodes, and rupture of these cells releases elementary bodies which, then, infect endothelial cells (Du Plessis, 1970). After entry into endothelial cell, by a process resembling phagocytosis, each organism develops within a vacuole to form a colony; a process which eventually leads to rupture of the cell. This disseminates elementary
bodies into the blood stream to continue the infection cycle (Prozesky and Du Plessis, 1987). Several days after infection, rickettsiae were first detected in the cytoplasm of circulating neutrophils (Logan et al., 1987). This neutrophil stage almost coincides with the onset of clinical signs and the invasion of endothelial cells (Du Plessis, 1970). Jongejan et al. (1991a) described the life cycle as a *Chlamydia*-like developmental cycle, which starts with the entry of an infectious stage, an electron dense elementary body, into the intracellular cytoplasmic vacuole of an endothelial cell. Elementary bodies divide by binary fission to produce large colonies of metabolically active reticulate bodies. Infected cells disrupt 5 to 6 days later resulting in the release of numerous elementary bodies in the blood stream of host animals to begin a new cycle of infection.

1.5.2. Life cycle in the tick

*Amblyomma* spp. (three-host ticks) are the known vectors of *E. ruminantium* (Walker and Olwage, 1987). The larvae and nymphs acquire infection by feeding on *E. ruminantium*-infected domestic or wild ruminants. Following the feeding of the larvae, rickettsiae were first seen by light microscopy occasionally in moulting larvae 27 days post detachment, and more frequently in the resultant unfed nymphs up to 101 days post detachment (Cowdry, 1925b). *E. ruminantium* were found in the midgut epithelial cells, and sometimes free in clumps in the gut lumen (Cowdry, 1925b) and also in the salivary gland (Hart et al., 1991; Kocan et al., 1987a). Electron microscopy revealed *E. ruminantium* in the midgut of unfed nymphal or adult *A. hebraeum* and *A. variegatum*, previously fed on rickettsaemic hosts, and up to day 4 following initiation of transmission feeding (Kocan and Bezuidenhout, 1987). In addition, *E. ruminantium* organisms have also been detected in tick haemocytes and Malpighian tubules (Du Plessis, 1985; Kocan et al., 1987b). The presence of the organism in a haemocyte 2 days after transmission feeding (Hart et al., 1991) suggested a possible means of transfer between midgut and salivary gland (Camus et al., 1996). Different morphological forms of the
organism have been demonstrated in the salivary glands of feeding ticks and suggests this the occurrence of a developmental cycle in the tick host. The presence of *E. ruminantium* in the midgut and salivary gland of the tick suggested the colonization of the organism in both organs. This is necessary for the development cycle of the organism and that transmission of the organism by ticks to the vertebrate host occurs by either regurgitation of gut contents and/or through secretion of saliva while feeding (Camus *et al*., 1996). The period required for transmission of the organism to occur after attachment of an infected tick to a susceptible host is estimated between 27 and 38 hours for nymphs and between 51 and 75 hours for adults (Bezuidenhout, 1987). Infected animals serve as source of infection for ticks. The ruminant host, once became infected, may remain a carrier for up to 3.5 years (Andrew and Norval, 1989a) and potentially for the rest of its life, thus, serving as a reservoir of infection for ticks.

1.6. *In vitro* cultivation of *E. ruminantium*

*In vitro* cultivation of *E. ruminantium* has greatly helped research on heartwater. Since *E. ruminantium* is intracellular and pleomorphic, its purifying from the host cell is still problematic (Allsopp, 2010). Organisms have been purified by differential centrifugation (Neitz and Yunker, 1996; Rossouw *et al*., 1990), Percoll gradient purification (Mahan *et al*., 1995) and on immuno-affinity columns (De Villiers *et al*., 1998) were but all these preparations still contain some host cell DNA and proteins. Subsequently, a primary *E. ruminantium*-infected neutrophil culture was maintained *in vitro* for 18 hr to 5 days (Logan *et al*., 1987). It was successfully adapted for production of *E. ruminantium* antigen for heartwater serology (Jongejan *et al*., 1989; Martinez *et al*., 1993a). The *in vitro* cultivation of *E. ruminantium* in calf umbilical cord endothelial cells was first achieved by Bezuidenhout *et al*., 1985a. This created new possibilities, and subsequent propagation of *E. ruminantium* from various organs or anatomical sites of various ruminant or mammalian species. Endothelial cells derived from bovine aorta,
bovine pulmonary artery, foetal bovine heart (Yunker et al., 1988), ovine pulmonary artery (Byrom et al., 1991), bovine saphenous vein (Neitz and Yunker, 1996) and caprine jugular vein were all successfully used. Additionally, endothelial cells derived from brain capillaries of bovine (Martinez et al., 1993b) and human (Totté et al., 1993) supported the in vitro growth of *E. ruminantium*. Continuous *in vitro* propagation of *E. ruminantium* was, also, achieved in endothelial cells obtained from African wild ruminants (Smith et al., 1998) and non-ruminants (Totté et al., 1993). Improvements have subsequently been made by using a chemically defined, serum-free culture medium (Zweygarth et al., 1997). In addition, *E. ruminantium* was shown to grow in monocyte-macrophage cell lines from mice and dogs, and in human leukaemia cell line (HL-60), although with low rates of infection and without persistent infection (Zweygarth, 2006).

Andreason (1974) reported the first successful primary cultures of tick cells initiated from moulting nymphs of *A. hebraeum* and *A. variegatum* that were infected with infective blood from *E. ruminantium* infected sheep. Nine-day-old cultures were injected intravenously into two susceptible sheep, both of which died of heartwater, as diagnosed by the presence of typical *E. ruminantium* organisms in Giemsa-stained brain squash smears. Other attempts to propagate *E. ruminantium* in tick cells failed (Yunker et al., 1988). However, Bell-Sakyi et al. (2000) achieved the first continuous propagation of the Gardel stock of *E. ruminantium* in an *Ixodes scapularis* tick cell line for more than 500 days. Furthermore, Bekker et al. (2002a) infected a *Rhipicephalus appendiculatus* cell line, designated RAN/CTVM3 with the Gardel stock of *E. ruminantium*. Bell-Sakyi (2004a) succeeded to infect continuous tick cell lines derived from *A. variegatum*, *I. scapularis* and *I. ricinus* with *E. ruminantium* derived from bovine endothelial cell cultures. Subsequently this author infected another eight tick cell lines, derived from six different tick species (*A. variegatum*, *Boophilus decoloratus*, *B. microplus*, *I. scapularis*, *I. ricinus* and *R. appendiculatus*), with *E.*
*E. ruminantium* derived from the already established infected tick cell cultures. Five *E. ruminantium* isolates, grew continuously in at least one tick cell line. Three of the isolates were reestablished in bovine endothelial cell cultures following prolonged maintenance in tick cell cultures (Zweygarth, 2006). The above results showed that, growth of *E. ruminantium* is not restricted to cells derived from natural hosts of the organism and could potentially have an expanded host range (Allsopp, 2010). Mass production of *E. ruminantium* elementary bodies using microcarriers as anchors for endothelial cells in stirred tank bioreactors was reported, opening the possibility of up scaling *E. ruminantium* vaccine production (Marcelino et al., 2006).

1.7. Genetic map of *E. ruminantium*

The genome of *E. ruminantium* is circular (De Villiers et al., 2000b) and it is 1.5 megabases in size (Collins et al., 2005). The genome contains 920–957 protein-coding sequences (CDSs) depending on the strain and it shows a low G+C content of 27.5% (Collins et al., 2005; Frutos et al., 2006). This small genome size is an expected trait for an intracellular parasite (Moran and Plague, 2004; Sallstrom and Andersson, 2005). However, it is the largest of all the sequenced Rickettsiales genomes (Frutos et al., 2006). With a coding ratio of 63.7%, *E. ruminantium* is among the bacterial genomes with the smallest coding ratio (Collins et al., 2005). Genetic characterization of *E. ruminantium* has been based on different gene targets, all of which manifest varying degrees of nucleotide sequence polymorphisms among different isolates (Frutos et al., 2007). The 16S rRNA gene was shown to manifest few nucleotide sequence differences in the hyper variable region (the V1 loop) and, therefore, considered most useful and often used for phylogenetic analysis (van Vliet et al., 1992; Rikihsia et al., 1997; Allsopp and Allsopp, 2007). Genetic analyses of 16S rDNA, heat shock protein (groESL) and surface protein genes have reassigned the heartwater agent to the genus *Ehrlichia* (Dumler et al., 2001). In addition, the citrate synthase gene (gltA), which encodes
the first enzyme of the tricarboxylic acid cycle; a key regulator of intracellular ATP production in virtually all living cells (Wiegarg and Remington, 1986), was shown to exhibit higher variation than the 16S rRNA gene. Therefore, analysis of this gene sequence was considered to be the best tools for phylogenetic analysis and identification of *Ehrlichia* species (Inokuma et al., 2001).

The Major Antigenic Protein 1 (MAP1) gene sequences exhibited more polymorphisms (Reddy et al., 1996). It was considered as a useful target to provide information on distribution of *E. ruminantium* genotypes (Allsopp et al., 1999; Martinez et al., 2004). Bekker *et al.* (2002a) reported differential transcription of MAP1 gene paralog among ticks, bovine endothelial cell cultures and tick cell lines. The gene has been confirmed as a multigene family, with 16 tandemly arranged paralogs, all transcribed in *in vitro* infected bovine endothelial cells (van Heerden *et al*., 2004a). The published pCS20 sequence is 1,306 base pairs (bp) long and contains two open reading frames (ORF); ORF1 of 459 bp (nucleotides 717–1175) and ORF2 of 513 bp (nucleotides 205–717) (Waghela *et al*., 1991). The ORF1 is homologous to the cytochrome *c* oxidase assembly protein of various organisms and ORF2 is homologous to the ribonuclease III gene of various organisms. The probe used in the pCS20 assay is the whole 1306 bp insert purified from a plasmid clone of the pCS20 region. It does not detect DNA from other organisms closely related to *Ehrlichia* spp. (Mahan *et al*., 1992; Peter *et al*., 1995). Molecular characterization of *E. ruminantium* using pCS20 sequences also showed the usefulness of the sequences for phylogenetic analysis (van Heerden *et al*., 2004b). Using pCS20 Allsopp and Allsopp (2007) showed no sequence variation among the West African isolates and they indicated that, the level of conservation of the *E. ruminantium* pCS20 region is reliable for differentiation of the organism from other *Ehrlichia* spp. (van Heerden *et al*., 2004b).
1.8. Diagnosis of Heartwater
1.8.1. Serology

The indirect fluorescent antibody (IFA) test (Camus et al., 1996) was the first serological diagnostic assay used for large scale screening of *E. ruminantium* infection. Although, this assay showed to be high sensitive (Du Plessis and Malan, 1987), its specificity was affected by the detection of false-positive results. This is presumably due to closely related *Ehrlichia* spp. in sera from *Amblyomma*-free areas (Du Plessis et al., 1987). With the objective of improving the specificity of heartwater serology, an indirect ELISA based on a recombinant truncated form of the Major Antigenic Protein 1 of *E. ruminantium*, (fragment B, thus named MAP1-B) was developed (van Vliet et al., 1995). This test does not cross-react with the closely related *Ehrlichia* species found in ruminants, like *E. ovina* and *Anaplasma (Ehrlichia) bovis*. Although, it cross-react with *E. canis* (in dogs) and *E. chaffeensis* (in human), two parasites that do not infect ruminants. This cross-reactivity, however, is not expected to hamper the results of heartwater serology. The MAP1-B ELISA (van Vliet et al., 1995) is extensively used by various laboratories (Mahan et al., 1998; Mondry et al., 1998; Mattioli et al., 2000; Semu et al., 2001). The test showed satisfactory results for small ruminants (Mahan et al., 1998; De Waal et al., 2000) with high specificity of 98.9% and 99.4% for caprine and ovine sera, respectively (van Vliet et al., 1995; Mondry et al., 1998). Nevertheless, the performance of MAP1-B ELISA is less satisfactory with cattle exposed to continuous field challenge due to down regulation of MAP1-B antibody responses (Semu et al., 2001) resulting in detection of seropositive lower than expected in heartwater endemic areas (Mahan et al., 1998). Furthermore, a polyclonal competitive ELISA (pc-ELISA) for detection of *E. ruminantium* antibodies was described by Sumption et al. (2003). The assay was comparatively evaluated using field sera from cattle and small ruminants (Bell-Sakyi et al., 2003). Results indicated a better performance for the assay in comparison to MAP1-B ELISA with respect to detection of *E. ruminantium* antibodies in cattle.
In conclusion, serological assays used for *E. ruminantium* unlike PCR-based techniques, where it is only provide information on previous exposure of an animal to infection. In addition they do not differentiate between strains of *E. ruminantium*. Notwithstanding these limitations, serological tools still have important applications for heartwater epidemiology in sub-Saharan Africa, notably their use in disease risk mapping (Awa, 1997; Bell-Sakyi *et al*., 2004; Abdel Rahman, 2006; Faburay *et al*., 2008). In addition, serological tests could be useful, when applied in conjunction with PCR, in determining the true infection status of animals in heartwater endemic regions prior to export to heartwater-free areas.

1.8.2. Molecular detection

Molecular cloning of several *E. ruminantium* genes resulted in the development of improved diagnostic tests for heartwater; the genes, MAP2, encoding the 21 kDa (Mahan *et al*., 1994a) and MAP1 encoding the 32 kDa (van Vliet *et al*., 1994). *E. ruminantium*-proteins have also been cloned, characterized, sequenced and expressed to high levels to produce recombinant analogues, which have found application in subunit ELISA (van Vliet *et al*., 1994). Remarkable progress in the development of PCR-based molecular diagnostics for *E. ruminantium* was associated with the development and use of DNA probes to detect *E. ruminantium* in *Amblyomma* spp. and in animals. The techniques have significant increase in the sensitivity of these assays using PCR (Mahan, 1995). Waghela *et al.* (1991) was the first who described the use of cloned DNA probes to detect *E. ruminantium* in *A. variegatum*. The pCS20, showed high sensitivity and hybridized with eight isolates of *E. ruminantium* tested. It detected *E. ruminantium*-specific DNA from plasma samples of infected sheep before and during the febrile reaction (Mahan *et al*., 1992). Using PCR to amplify the MAP1 gene, *E. ruminantium* could be detected in blood and bone marrow of sheep during the febrile response and up to 4 months later in recovered animals, following treatment (Kock *et al*., 1995). In a comparative evaluation for detection of *E. ruminantium* infection, a DNA probe
based on the pCS20 sequence showed higher sensitivity and specificity compared to probes based on the 16S rDNA and MAP1 gene sequences (Allsopp et al., 1999). The pCS20 PCR assay does not detect DNA of *E. canis* and *E. chaffeensis*, which, serologically and on the basis of the 16S rDNA analysis are closely related to *E. ruminantium* (van Vliet et al., 1992; Peter et al., 2000). Furthermore, a technique, based on the 16S rDNA, for simultaneous detection of a range of ruminant *Ehrlichia* and *Anaplasma* species by Reverse Line Blot (RLB) hybridization detected *E. ruminantium* in experimentally-infected sheep during clinical response, but was not sufficiently sensitive to reliably detect the pathogen in carrier animals confirmed by xenodiagnosis (Bekker et al., 2002b). Similarly, in a serological survey of *E. ruminantium* in cattle in Senegal (Gueye et al., 1994), results was complicated by possible cross-reactions with *Anaplasma (Ehrlichia) bovis* known to occur in Senegal (Gueye et al., 1994). The pCS20 PCR sequences were considered the most sensitive and specific diagnostic test for *E. ruminantium* infection in ruminants and *A. variegatum* (Peter et al., 2000; van Heerden et al., 2004b).

### 1.8.3. Quantitative Real-time PCR

The titration assay based on competitive PCR was first described in 1990 (Gilliland et al., 1990 Leutenegger et al., 1999a). The need for faster, more accurate and more economic systems with a high throughput capacity are required. Three keywords have become important for the development of the next-generation of PCR systems. These are confined in the automation, standardization and miniaturization. The development process was accelerated by combining computer-assisted PCR with laser technology. The laser-guided detection of PCR products, with the help of a so-called TaqMan probe, and the real-time accumulation of fluorescent data points for every PCR cycle virtually replace the need for a time-consuming post-amplification step. Quantitative real time PCR was the selected analytical tool due to its high sensitivity and dynamic range (OIE,
The quantitative real-time TaqMan PCR technique has several advantages over the classical quantitative PCR system. The use of fluorescent dye-labelled probes increases the sensitivity of the system by at least 7 orders of magnitude and gives rise to a linear relationship between copy number and threshold cycle (C\text{T}) values. In addition, the liquid hybridization assay adds further specificity to the system, comparable to hybridization techniques using blotted PCR products. The elimination of post-amplification steps increases reliability and reproducibility of the assay (Leutenegger et al., 1999b). Real-time PCR (qPCR) tests were described for the detection and quantitative determination of *E. ruminantium*. In a first test, a 182 bp fragment from the non-polymorphic MAP1-1 gene was amplified and detection was carried out using the SYBR Green method (Peixoto et al., 2005). DNA from six different isolates was successfully amplified from the blood of experimentally infected sheep. *E. ruminantium* was detected only during the hyperthermia reaction period.

A second SYBR Green-based real-time PCR was described and fully validated for characterization of *E. ruminantium* replication and release kinetics in endothelial cell cultures. Its subsequent use to control the mass production process in bio-reactors was also evaluated (Postigo et al., 2007). A TaqMan probe was designed to recognize the most conserved pCS20 region in 15 different known *E. ruminantium* stocks. The sensitivity of the resulting test was compared to that of a standard pCS20 PCR (Steyn et al., 2008) and the modified pCS20 PCR/32Pprobe technique (van Heerden et al., 2004b). Furthermore, the real-time PCR assay was used to test sheep experimentally infected with *E. ruminantium* (Welgevonden strain) to indicate the presence and number of the organisms in the blood (Steyn et al., 2008).
1.8.4. Loop-mediated isothermal amplification (LAMP) assay

Loop-mediated isothermal amplification (LAMP) assay is a rapid DNA amplification method originally developed by Notomi et al. (2000). It has been applied for the detection of viral (Bista et al., 2007), bacterial (Enosawa et al., 2003), fungal (In?cio et al., 2008), and parasitic agents (Bakheit et al., 2008). Recently the test was been applied to rickettsial agents (Nakao et al., 2011). The method requires a specially designed primer set that recognizes at least six independent regions of the target gene, which increases the specificity as well as the rapidity of the reaction. LAMP results are visualized by turbidity that can be seen by the naked eye (Mori et al., 2001), and optionally by agarose gel electrophoresis or by addition of fluorescent dyes visualized under UV light (Tomita et al., 2008). Since the Bst DNA polymerase used in LAMP allows strand displacement-DNA synthesis, LAMP reactions are performed under isothermal conditions using a simple incubator, such as a water bath or heating block. Furthermore, LAMP reagents are relatively stable for a month, even when stored at 37°C, which is a warmer temperature than recommended by the manufacturer (Thekisoe et al., 2009). With these advantages, LAMP has the potential to be used even in clinical laboratories often poorly equipped, facing problems of constant electricity supply in tropical and sub-tropical countries where heartwater is endemic.

Nakao et al. (2011) developed LAMP assays for the detection of *E. ruminantium* using two sets of LAMP primers designed from the pCS20 and sodB genes. The detection limits for each assay were 10 copies for pCS20 and 5 copies for sodB, which is at least 10 times higher than that of the conventional pCS20 PCR assay. DNA amplification was completed within 60 min. The assays detected 16 different isolates of *E. ruminantium* from geographically distinct countries as well as two attenuated vaccine isolates. No cross-reaction was observed with genetically related Rickettsiales, including zoonotic *Ehrlichia* species from the USA. LAMP
detected more positive samples than conventional PCR but less than real-time PCR, when tested with field samples collected in sub-Saharan countries (Nakao et al., 2011). Due to its simplicity and specificity, LAMP has the potential for use in resource-poor settings and also for active screening of *E. ruminantium* in both heartwater-endemic areas and regions at risk of contracting the disease (Nakao et al., 2011).

1.8.5. Genetic diversity and recombination

The biological variability among strains of *E. ruminantium* is obviously of great practical importance for vaccine development. So, it is surprising that for much of the 20th century it was thought that *E. ruminantium* was a relatively homogeneous organism. Recently, and with the advent of molecular genetic methods for characterization, it has become evident that it is, in fact, an extremely diverse organism (Allsopp and Allsopp, 2001). Even more crucial for diversity is, the discovery that extensive recombination occurs naturally among different genotypes of *E. ruminantium* (Allsopp and Allsopp, 2007). This showed that, the newly generated strains are continuously arising in the field. Recombinational mechanisms are believed to have played an important role in the evolution of prokaryotes (Vetsigian and Goldenfeld, 2005). These mechanisms can be placed in two broad categories: (1) non-homologous recombination or horizontal gene transfer (HGT), which involves the acquisition of a gene or genomic region new to a genome (Ochman et al., 2000); and (2) homologous recombination, which involves the introduction to a genome of a new allelic variant of a gene or genomic region already present in that genome (Hanage et al., 2006; Hughes and French, 2007). Homologous recombination among bacterial genomes was the subject of a number of studies. This is due to its effect on phylogenies reconstructed from a selected set of genes (Lecointre et al., 1998). Intracellular bacteria being unable to regain the lost sequences from other bacterial species through horizontal gene transfer thus suffer a loss of genes whose products must
be obtained from the host (Collins et al., 2005). These deletions could result in the creation of new genotypes of *E. ruminantium* with possible phenotypic consequences. For example, the MAP1-2 in the Gardel stock of *E. ruminantium* is shown to bear a deletion of 48 bp, which accounts for 80% of the size difference between the map1 clusters of the Gardel stock and the Welgevonden stock (Frutos et al., 2006). MAP1-2 was shown to recombine with map1-3 in a sub-strain, Gardel-CTVM, of the parent Gardel stock (Bekker et al., 2005). It indicated that recombination can occur in *E. ruminantium*, thus influence the phenotypic characteristics of the bacteria (Bekker et al., 2005). These intrachromosomal recombination events could probably explain the wide genetic diversity observed amongst *E. ruminantium* stocks in the field (Allsopp and Allsopp, 2007).

Extensive recombination was shown to have occurred among Southern and East African stocks, but not with West African stocks. The only evidence of recombination among the West African stocks was between Pokoase and Senegal. The Kumm 1, a Southern African stock, showed recombination with all the West African stocks (Allsopp and Allsopp, 2007). Since homologous recombination results in both synonymous and non-synonymous changes, these recombination events could potentially have phenotypic effects on the recipient genotype (Hughes and French, 2007). It is postulated that the most likely time for recombination to occur is in the tick vector after ingestion of a blood meal from animal carrying a mixture of *E. ruminantium* genotypes and before the establishment of the organism in gut epithelial cells (Allsopp, 2010).
1.9. Heartwater in the Sudan

1.9.1. Background

The Sudan has an official livestock population estimate of over 140 million head (ARSC, 2009) (Table 1.4). The livestock production areas of Sudan are ethnically, geographically, and biologically diverse. However, livestock owners throughout the extensive pastoral lands in the east, central, west and south face common problems, including displacement owing to civil unrest and agricultural mechanized schemes and development. Livestock production systems have changed over time owing to internal and external influences. Large development schemes, desertification, drought, war, population growth, and other factors have affected pastoral systems of livelihood.

The different production systems present are pastoral nomads, transhumance, and sedentary farmers possess up to 90% of the animal wealth; therefore they are responsible for the major source of meat for domestic and export markets (Mufarrih, 1991). Cattle have great social and economic importance in the Sudan. There are twenty types of cattle but the main groups of cattle are Zebu including Kenana, Butana, Baggara, Murle, Nuba Mountain and Nilotic (Ageeb and Hayes, 2000). In addition, Holstein-Friesian cattle from Europe were imported for milk production and breeding (Ahmed and El Amin, 1997; Ageeb and Hayes, 2000).

Sheep are the top Sudan’s livestock export. There are four main types of Sudanese sheep (Desert, Nilotic and Arid Upland) and seventeen eco-types (El-Hag, 2001). Sudan Desert sheep comprise more than 65% of the total sheep and nearly 100% of sheep exports (Mufarrih, 1991; El-Hag et al., 2001). Goats are an important source of milk and meat. There are 11 eco-types of goats in the country. The most common being Sudan Desert and Nubian goats. The Nubian goat is the only specialized milk eco-types (Kamal et al., 2005). Three exotic breeds of goats (Saanen, Toggenburge, and Anglo-Nubian) were imported to the Sudan in 1976 to
improve milk production of local types (Kamal et al., 2005). There are five types of camels in the Sudan. Camels are generally found in the desert and semi-desert regions between latitudes 12° and 16° N. Most of the camels are located in the Darfur, Kordofan, and Eastern Sudan (ARSC, 2009). Disease control in the Sudan is challenging owing to the sheer size of the country, the ease of border crossings with neighbouring countries, the size of the livestock populations, and the difficulty of reaching migratory populations. The Sudanese tick fauna comprises 68 tick species. Many of which have veterinary importance. The distribution map of ticks and tick borne diseases has changed in the country, due to the extensive animal movement, deforestation, desertification and establishment of large mechanized schemes (Osman, 1978; Jongejan et al., 1987).

In the Sudan, heartwater was first reported in sheep and goats in Eastern Sudan (Kassala province) (Karrar, 1960), White Nile (Kosti) (Karrar, 1966), in goats at Um Banein (Jongejan et al., 1984) and in cattle in South Darfur (Abdel Wahab et al., 1998). A. lepidum was considered to be the chief vector of heartwater in Eastern Sudan (Karrar, 1968) and in Central Sudan (Jongejan et al., 1984; Mohammed and Hassan, 2007). Moreover, A. variegatum was incriminated to transmit the disease in Western and Southern regions of the country (Abdel Wahab et al., 1998). Fawi et al. (1977) studied the infectivity in goats and showed that E. ruminantium was associated with the red blood cells. Shommein and Abdel Rahim (1977) studied the clinical and histopathological changes and they suggested that the lymph nodes and the spleen might be possible sites for replication of the organism. Abdel Rahim and Shommein (1978) studied haematological changes in experimentally infected goats. Serological surveys using an indirect MAP1-B ELISA were carried out in sheep, goats and cattle (Abdel Rahman, 2006). Prevalence of the antibodies was 69% in sheep, 75.2% in goats and 38.7% in cattle (Abdel Rahman, 2006). E. ruminantium was isolated in experimental Nubian goats and seven E. ruminantium stocks were collected from New Halfa, Gadarif,
Tamboul, Singa, Rabak, Abu Karshola, and Pibore (Abdel Rahman, 2006). Molecular techniques using PCR, Reverse Line Blot (RLB) were attempted in animals, infected cultures and *Amblyomma* spp. ticks by Abdel Rahman (2006). The MAP1 nucleotide sequence of Gadarif strain (GenBank accession no. AB218278) was found to be closely related to those of Senegal and Pokoase strains from West Africa and to Canine and Kümm1 strains from southern Africa. Gadarif clustered with these 4 strains and with 6 other strains, including Kiswani from eastern Africa and Antigua from the Caribbean Islands. In contrast, the nucleotide sequence of Gadarif showed 84.8% similarity with that of Um Banein, which was known as the only strain of *E. ruminantium* in the Sudan (Muramatsu *et al.*, 2005).

Table 1.4. The total livestock population reported in the old Sudan (ARSC, 2009).

<table>
<thead>
<tr>
<th>Livestock</th>
<th>Number (million)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cattle (<em>Bos indicus</em>)</td>
<td>41.6</td>
</tr>
<tr>
<td>Sheep (<em>Ovis aries</em>)</td>
<td>51.5</td>
</tr>
<tr>
<td>Goats (<em>Capra hircus</em>)</td>
<td>43.3</td>
</tr>
<tr>
<td>Camels (<em>Camelus dromedarius</em>)</td>
<td>4.5</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>140.9</strong></td>
</tr>
</tbody>
</table>
1.9.2. Economic importance of heartwater

Heartwater disease is one of the most important obstacle facing animals upgrading in Africa or replacing local stock (Uilenberg, 1982). It is a major disease when local animals are, moving from heartwater-free to heartwater-endemic areas and it remains a threat in endemic areas especially among small stock (Bezuidenhout and Bigalke, 1987). It is a threat in areas such as the American mainland where potential vectors are present but do not harbour the disease (Uilenberg, 1982). It is also, a threat to countries where the vectors may be introduced and become established (Barré. et al., 1987). The effect of dipping and environmental changes influence endemic stability, which is often difficult or impossible to manipulate. Moreover, in susceptible animals, heartwater can cause mortalities ranging from 20% to 90% depending on virulence of the strain, immune status, age, breed and species involved (Mahan et al., 2001).

Therefore, the disease remains of major importance until an effective and safe vaccine becomes available (Bezuidenhout and Bigalke, 1987). Reliable economic loss figures in the Sudan and East Africa generally are unavailable, the prevalence of heartwater in the endemic regions of South Africa is under-reported because a definitive diagnosis (brain smear) is not usually done in the field. Furthermore, immunization by infection and treatment can result in the disease cost being partially suppressed, so it is hard to quantify the resistance of different animals or endemic stability. Measurement of the economic impact is difficult to quantify as it should include not only losses due to animal mortalities, but should also include purchase of stock remedies for tick control and immunization and treatment of naturally exposed livestock. However, an annual national loss due to cowdriosis in Zimbabwe alone is estimated at US$5.6 million (Chamboko et al., 1999; Mukhebi et al., 1999).
1.10. Control of heartwater

1.10.1. Treatment and chemical acaricides application

Treatment of heartwater with sulfonamides or antibiotics was found to be effective at the onset of clinical symptoms and showed ineffectiveness when defined nervous symptoms occurred (Camus et al., 1996). In general, antibiotics of the tetracycline group are effective chemotherapeutic agents for successful treatment of heartwater. However, their use is constrained by the acute nature of the disease, which does not always allow timely intervention to prevent a fatal outcome (Zweygarth, 2006). Tick control with chemical acaricides, although expensive, has been in use for more than a century and it was very effective in the past. Today, however, chemical acaricide resistance is widespread and the environmental pollution caused by the chemicals is generally deplored (Schröder, 1987; Jongejan and Uilenberg, 2004; Allsopp, 2009). Intensive tick control measures may, under certain conditions, succeed in preventing outbreaks of heartwater, even in endemic areas (Bezuidenhout and Bigalke, 1987). The disease can be successfully controlled if all the animals on the farm can be regularly dipped in chemical acaricides throughout the year (Pentney and Horak, 1987). The establishment of endemic stability, using strategic tick control is a state of equilibrium between an infectious agent, its vector and host animal population. (Bezuidenhout, and Bigalke, 1987; Norval et al., 1995).

1.10.2. Inactivated vaccines

Inactivated heartwater vaccines consist of organisms derived from tissue culture which have been rendered non-viable by chemical treatment is available. The first successful application of such material was in goats using the Gardel isolate. Fifty to 80% of the animals were protected against a homologous needle challenge which killed 100% of the negative controls (Martinez et al., 1994). The next report used the Crystal Springs isolate in sheep. In this case, 50–100% of the animals
were protected against a homologous needle challenge that killed 60% of the control animals (Mahan et al., 1995). These early successes were unfortunately not repeated when trials were conducted in a field situation, where natural tick challenge with genotypes having differing immunogenicities would have occurred (Allsopp, 2009). Several reports indicate that under these circumstances the vaccine reduces mortality levels, but protection levels have been disappointing (Mahan et al., 2001; Faburay et al., 2007b). A summary of all field trials conducted by one research group over a period of years showed that, overall, mortality levels of 71% in naïve animals could be reduced to 36% by vaccination (Mahan et al., 2003). The high cost of batch culture systems for *E. ruminantium* is another obstacle facing the development of large scale use in the field (Esteves et al., 2004). Later, however, there were notable improvements in mass production of the organism which could make commercial scale preparation feasible (Marcelino et al., 2006). However, its limited protection against tick challenge in the field, necessitate urgent improvements is required before inactivated heartwater vaccines could become a commercially viable proposition (Allsopp, 2009; 2010).

1.10.3. Attenuated vaccines

The Senegal isolate of *E. ruminantium* was the first isolate to be attenuated in culture. It conferred 100% protection on animals subjected to a homologous needle challenge (Jongejan, 1991). As with the inactivated vaccine, the results were far less satisfactory when field trials were conducted. About 70% mortality in the controls being reduced to 43% in the vaccinated animals (Gueye et al., 1994). The Welgevonden isolate provided cross-protection with a range of other isolates against a needle challenge (Collins et al., 2003). This could make the Welgevonden isolate a good attenuated candidate vaccine. Unfortunately it did not get attenuate when grown in culture through hundreds of passages over several years (Gueye et al., 1994; Zweygarth et al., 1997). However, the Welgevonden isolate was attenuated when cultured in a canine macrophage-monocyte cell line,
after which it was re-adapted to grow in bovine endothelial cells (Zweygarth et al., 2005). When the attenuated organisms were used to infect sheep or goats there were no adverse symptoms, except for a brief rise in body temperature. Thereafter, the animals were subsequently found to be fully protected against a lethal needle challenge with the homologous isolates (Zweygarth et al., 2005). This attenuated vaccine has not yet been tested in the field against natural tick challenge. However, it could provide a cheap and effective vaccine for use in endemic heartwater areas (Allsopp, 2009).

1.10.4. Recombinant vaccines

The first attempts to develop a recombinant vaccine involved immunization of mice with a plasmid clone expressing the MAP1 gene of *E. ruminantium* against a lethal homologous challenge at levels ranging from 23–88% (Nyika et al., 1998). In another experiments, the naked DNA induced immunity was boosted with MAP1 protein and as a result the protection levels increased from 13 to 27% without boosting to a range of 53 to 67% (Nyika et al., 2002). Denatured MAP1 protein confer no protection in ruminants (van Kleef et al., 1993). Therefore, the MAP1 gene might not be the best choice for recombinant vaccine experiments. The completion of the genome sequence of *E. ruminantium* showed that there were 888 genes that can be tested as vaccine candidates (Collins et al., 2005). Nevertheless, there are no reliable strategies to identify the genes that code for antigens stimulate the protective T-cell response (Esteves et al., 2004). One attempt to overcome this difficulty involved selection of clones from *E. ruminantium* expression libraries on the basis of their expression products. It was firstly recognized by anti-*E. ruminantium* antibodies, and secondly that they stimulated proliferation of peripheral blood mononuclear cells (PBMC) from cattle immunized against *E. ruminantium* by infection and treatment (Barbet et al., 2001). Lysates of recombinant bacterial cultures expressing the selected genes were, then, used to immunize mice. About 58–89% survival was observed with
some pools of recombinants (Allsopp, 2009). The levels of protection found, similar to those obtained by the MAP1 gene. *E. ruminantium* immunization trials performed in mice which gave unpredictably variable results. Genes conferred immunity in such trials were often not protective in ruminants (Louw *et al*., 2002; Collins *et al*., 2003). Vaccination trials conducted in sheep, however, showed to be reproducible. A cocktail of four *E. ruminantium* genes cloned in a DNA vaccine vector consistently stimulated 100% protection in sheep against a virulent needle challenge with both homologous and heterologous *E. ruminantium*-infected blood (Collins *et al*., 2003; Pretorius *et al*., 2007). In trials against a natural tick challenge in the field this vaccine was poorly protective, even when used in a prime–boost format with recombinant *E. ruminantium* proteins (Pretorius *et al*., 2008). Pretorius *et al*. (2010) studied the ability of Erum2510 to provide protection against heartwater. It was tested in sheep using two different immunization strategies namely homologous DNA immunization and aDNAprime/rprotein boost strategy. The DNA prime/rprotein boost immunization strategy with Erum2510 induced complete protection in sheep against a virulent needle challenge. Therefore, this open reading frame (ORF) may be useful component of a vaccine against heartwater. To date none of these vaccines have showed complete protection under natural field conditions.
CHAPTER TWO

Prevalence of *Ehrlichia ruminantium* in *Amblyomma* spp. and Blood of Domestic Ruminants by Conventional PCR Based on pCS20 Gene Region in the Sudan
2.1. Introduction

Heartwater or cowdriosis, is a rickettsial disease cause high mortalities in sheep, goats, and cattle and is of major economic importance to livestock production and development in sub-Saharan Africa and several Caribbean Islands (Uilenberg, 1983). The causative agent is an obligate intracellular gram-negative bacterium, *Ehrlichia ruminantium* (formerly *Cowdria ruminantium*) (Dumler et al., 2001). Ticks are of great significance in the epidemiology of tick-borne diseases (Norval et al., 1991). The disease is only transmitted by ticks of the genus *Amblyomma* and its distribution coincides with that of its vector species (Walker and Olwage, 1987).

The specific diagnosis of heartwater is based on the observation of colonies of *E. ruminantium* in capillary endothelial cells of the brain of clinically animals suspected, during the acute disease. It is not possible to detect pre-symptomatic and carrier animals (Camus et al., 1996). Several serological tests have been extensively employed for epidemiological studies. Although, they do not discriminate among different *Ehrlichia* species because of antigenic similarity (Palmer, 1989; Dreher et al., 2005). Molecular methods, with a high degree of sensitivity and specificity, have been developed to identify *E. ruminantium* DNA (Waghela et al., 1991; Steyn et al., 2003; van Heerden et al., 2004b). PCR techniques are highly sensitive and specific they could improve the understanding of the disease epidemiology. The techniques have the ability to detect low levels of *E. ruminantium* infection in *Amblyomma* ticks (Peter et al., 2000). Estimations of the prevalence of infection rates in *Amblyomma* spp. are important because they reflect the true prevalence of *E. ruminantium* in the field population. A wide variety of different methods were used for determining the infection rates of *E. ruminantium* in *Amblyomma* ticks in Africa and Caribbean Islands. Generally, in the Sudan the real or potential economic losses due to the disease are not known.
because the incidence/prevalence is not well studied or documented. In this study, therefore, the conventional pCS20PCR gene was applied to determine the prevalence and spatial distribution of *E. ruminantium* infection rates in *A. lepidum*, *A. variegatum* and blood samples from selected endemic localities in the Sudan.

2. 2. Materials and Methods

2. 2. 1. Study area

This study was conducted in heartwater endemic areas in four States in the Sudan. These included Sennar State (Singa, Dinder and Abonama), Gezira State (Tamboul and Elhoush), North Kordofan State (Elnihoud and Um Rawaba) and South Darfur (Nyala, Kass and Aidelfersan) (Figure 2.1).

2.2.2. Tick collection

Tick collection was conducted in the wet seasons (August, September and October) during the period 2007 to 2009. Partially engorged adult (males and females) *A. lepidum* (190 samples) were collected from cattle and sheep throughout the suspected heartwater endemic areas of the Sudan as shown in Figure 2.1. *A. variegatum* (100 samples) were collected from cattle in South Darfur State only (Table 2.1). The collected ticks were preserved in tubes containing 70% ethyl alcohol. They were examined morphologically in the laboratory according to Walker et al. (2003).

2. 2.3. Whole blood

Whole blood samples (170) also were randomly collected in the wet seasons (August, September and October) during the period 2007-2009 from apparently healthy cattle, sheep and goats from the above mentioned localities (Figure 2.1; Table 2.1). Approximately 1-2 ml of blood was collected from the jugular vein into vials with ethylene diaminetetraacetic acid (EDTA) using plain vacutainers and needles. The collected blood was kept in Eppendorf tubes and stored at -20°C until used.
Figure 2.1. Localities (●) at which blood and *Amblyomma* spp. ticks were collected in the endemic regions of the Sudan.

Source: Remote Sensing Authority (RSA) University of Khartoum, Sudan.
Table 2.1. *Amblyomma* spp. ticks and blood samples collected in different localities in the Sudan during the wet seasons of 2007 to 2009

<table>
<thead>
<tr>
<th>State</th>
<th>locality</th>
<th>A. <em>lepidum</em></th>
<th>A. <em>variegatum</em></th>
<th>Blood</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Cattle</td>
<td>Sheep</td>
</tr>
<tr>
<td>Sennar</td>
<td>Singa</td>
<td>40</td>
<td>-</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>Abonama</td>
<td>20</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Dinder</td>
<td>40</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>100</td>
<td>0</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td>Gezira</td>
<td>Tamboul</td>
<td>-</td>
<td>-</td>
<td>10</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>Elhoush</td>
<td>20</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>20</td>
<td>0</td>
<td>10</td>
<td>20</td>
</tr>
<tr>
<td>North Kordofan</td>
<td>Um Rawaba</td>
<td>10</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Elnihoud</td>
<td>20</td>
<td>-</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>30</td>
<td>0</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>South Darfur</td>
<td>Nyala</td>
<td>20</td>
<td>40</td>
<td>20</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Aidelfersan</td>
<td>20</td>
<td>20</td>
<td>-</td>
<td>-</td>
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<tr>
<td></td>
<td>Kass</td>
<td>-</td>
<td>40</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>40</td>
<td>100</td>
<td>20</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>190</td>
<td>100</td>
<td>60</td>
<td>50</td>
</tr>
</tbody>
</table>

- = Not taken

43
2. 2.4. DNA extraction
2.2.4.1. Genomic DNA purification from ticks

DNA was extracted according to the manufacture's instruction (Promega, Madison, USA) using Wizard® SV Genomic DNA Purification System. Prior to extraction, ticks were washed with 70% ethanol followed by twice wash with distilled water and blotted dry on filter paper. Each tick was, then, separately cut into small particles using a sterile blade in a sterile Petri dish and placed into a 1.5 ml microcentrifuge tube. It was then digested with 275 µl Digestion Solution Master Mix (Proteinase k) (Promega, Madison, USA). The mixture was incubated overnight at 55°C heat block and centrifuged at 2000×g to pellet any undigested tissues. Supernatant was transferred to a new 1.5 ml microcentrifuge tube and a volume 250 µl of Wizard® SV Lysis Buffer were added to each sample and vortexed. The entire lysate sample was transferred to a Wizard® SV Minicolumn assembly and centrifuged at 13,000×g for 3 minutes to bind the genomic DNA to the Wizard® Minicolumn. Thereafter, the Wizard® SV Minicolumn was removed from the Minicolumn assembly and the liquid in the Collection Tube was discarded. Finally the Wizard® SV Minicolumn was placed into the Collection Tube and 650 µl of Wizard® SV Wash Solution was added and centrifuged at 13,000×g for 1 minute. The flow through was discarded. This step was repeated three times for a total of four washes of the Wizard® SV Minicolumn. After the last wash, the Wizard® SV Minicolumn was replaced into the empty Collection Tube and centrifuged at 13,000×g for 2 minutes to dry the binding matrix. The Wizard® SV Minicolumn was placed in a new labeled 1.5 ml microcentrifuge tube and a volume 200 µl of room temperature Nuclease-Free Water was added to the Wizard® SV Minicolumn and incubated for 2 minutes at room temperature. It was, then, centrifuged at 13,000×g for 1 minute for elution. The purified genomic DNA was stored at -20°C until used.
2.2.4.2. Genomic DNA purification from whole blood

DNA was extracted according to the manufacture’s instruction (Promega, Madison, USA) using Wizard® SV Genomic DNA Purification System. A volume of 200 µl whole blood was placed into a sterile 1.5 ml microcentrifuge tube, followed by 40 µl Proteinase K (20 mg/ml). The mixture was incubated at room temperature (25°C) for 10 minutes. The tube was occasionally inverted. A Whole Blood Lysis Buffer (Wizard® SV Lysis Buffer + 1% Triton® X-100 [Promega, Madison, USA]) was prepared and 400 µl was added to the Proteinase K-treated whole blood sample. The mixture was vortexed and incubated at room temperature (25°C) for 10 minutes with occasionally vortexing. It was then transferred to Wizard® SV Minicolumn assembly. Thereafter, extraction was carried out as previously described in the Genomic DNA Purification from ticks. DNA concentrations and purity were determined by NanoDrop® Spectrophotometer (A260 and A280). The samples were then stored at -20°C until further processing.

2.2.5. Polymerase chain reaction (PCR) amplification

The PCR was performed according to van Heerden et al. (2004b) using the CowF as forward primer and CowR as reverse primer to amplify a 226-bp fragment of the conserved pCS20 region of *E. ruminantium* DNA. The sequences of the two primers as follows:: CowF 5'-CAA AAC TAG TAG AAA TTG CA A-3' CowR 5'-TGC ATC TTG TGG TGG TAC-3'. A total reaction volume of 25 µl containing 5 µl of 5X GoTaq green buffer [100 mM Tris–HCl (pH 9), 500 mM KCl, 1% Triton X-100 (Promega, Madison, WI, USA)], 200 µM of each of the four deoxynucleotide triphosphates (dNTP), 0.675 U GoTaq DNA polymerase (Promega, Madison, WI, USA), and 0.5 µM of each primer (TIB-Molbiol, Berlin, Germany). Distilled water and *E. ruminantium* DNA (Welgevonden reference strain, GenBank accession no. X74250) were used in each test as negative and positive controls, respectively. The reaction conditions on a GeneAmp PCR
System 9700 (Perkin-Elmer Applied Biosystems) were: initial denaturation, 6 min at 94°C; 35 cycles of 30 sec at 94°C, 45 sec at 55°C and 30 sec at 72°C; final extension, 10 min at 72°C; and then hold at 4°C.

2.2.6. Gel analysis of PCR products

The PCR products were separated by electrophoresis on 1.5% agarose gel (FMC BioProducts, Rockland, ME, USA) in 1X TBE loading buffer [0.089 M Tris-hydroxymethyl, 0.089 M boric acid and 0.002 M EDTA (pH 8.0)] buffer, using voltage gradients of 70 V for 90 minutes. Results were visualized after staining the gels in 0.5–1 µg/µl ethidium bromide. PCR product was mixed and the equivalent of about 10 µl was loaded in each gel lane, in the loading buffer. The DNA was visualized under UV illumination. The images were captured using Kodak gel imaging system.

2.3. Results

2.3.1. Detection of E. ruminantium in A. lepidum

A total of 190 samples of A. lepidum adults were initially tested by pCS20 PCR. Twenty nine out of 190 (15.3%) A. lepidum were positive for E. ruminantium (Table 2.2 and Figure 2.2). The highest (25%) infection rate was found at Abonama and the lowest (10%) was detected at Elnihoud, Um Rawaba and Aidelfersan (Table 2.2).

2.3.2. Detection of E. ruminantium in A. variegatum

A total of 100 samples A. variegatum adults were tested with pCS20 PCR. Fourteen out of 100 (14%) were positive for E. ruminantium (Table 2.3 and Figure 2.3). High prevalence of E. ruminantium was detected in A. variegatum samples
collected from Aidelfersan (20%) infection followed by Kass (17.5%) and the lowest (7.5%) rate was reported in Nyala (Table 2.3).

2.3.3. Prevalence of *E. ruminantium* in cattle, sheep and goats

A total of 170 blood samples from cattle, sheep and goats were subjected to pCS20 PCR. Eleven out of 170 (6%) were positive for *E. ruminantium* (Table 2.4). Four out of 60 (6.7%) cattle blood were positive for *E. ruminantium* (Figure 2.4). The highest prevalence (10%) of *E. ruminantium* in cattle was reported in Tamboul and Elnihoud followed by (5%) in Singa and Nyala. Only two out of 50 (4%) sheep blood samples were positive for *E. ruminantium*. The highest prevalence (5%) of *E. ruminantium* in sheep was reported in Tamboul and Singa and the lost prevalence (0%) was reported in Elnihoud (Table 2.4; Figure 2.5). On the other hand, five out of 60 (8.3%) blood samples from goats were positive for *E. ruminantium*. The highest prevalence (15%) was reported in Nyala followed by (5%) in Singa and Tamboul (Table 2.4; Figure 2.5).
Table 2.2. Infection rate of *E. ruminantium* in *A. lepidum* using pCS20 PCR in different localities in the Sudan during the wet seasons of 2007 to 2009

<table>
<thead>
<tr>
<th>Locality</th>
<th>Source of ticks</th>
<th>No. tested</th>
<th>No. +ve</th>
<th>Infection rate%</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Sennar State</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Singa</td>
<td>Cattle</td>
<td>40</td>
<td>8</td>
<td>20</td>
</tr>
<tr>
<td>Dinder</td>
<td>Cattle</td>
<td>40</td>
<td>5</td>
<td>12.5</td>
</tr>
<tr>
<td>Abonama</td>
<td>Cattle</td>
<td>20</td>
<td>5</td>
<td>25</td>
</tr>
<tr>
<td><strong>Gezira State</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Elhoush</td>
<td>Cattle</td>
<td>20</td>
<td>3</td>
<td>15</td>
</tr>
<tr>
<td><strong>South Darfur State</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nyala</td>
<td>Cattle</td>
<td>20</td>
<td>3</td>
<td>15</td>
</tr>
<tr>
<td>Aidelfersan</td>
<td>Cattle</td>
<td>20</td>
<td>2</td>
<td>10</td>
</tr>
<tr>
<td><strong>North Kordofan State</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Elnihoud</td>
<td>Cattle</td>
<td>20</td>
<td>2</td>
<td>10</td>
</tr>
<tr>
<td>Um Rawaba</td>
<td>Sheep</td>
<td>10</td>
<td>1</td>
<td>10</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td></td>
<td>190</td>
<td>29</td>
<td>15.3</td>
</tr>
</tbody>
</table>
Table 2.3. Infection rate of *E. ruminantium* in *A. variegatum* using pCS20 PCR in South Darfur State during the wet season in 2007

<table>
<thead>
<tr>
<th>Locality</th>
<th>Source of ticks</th>
<th>No. tested</th>
<th>No. +ve</th>
<th>Infection rate%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nyalá</td>
<td>Cattle</td>
<td>40</td>
<td>3</td>
<td>7.5</td>
</tr>
<tr>
<td>Aidelfersan</td>
<td>Cattle</td>
<td>20</td>
<td>4</td>
<td>20</td>
</tr>
<tr>
<td>Kass</td>
<td>Cattle</td>
<td>40</td>
<td>7</td>
<td>17.5</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td></td>
<td><strong>100</strong></td>
<td><strong>14</strong></td>
<td><strong>14</strong></td>
</tr>
</tbody>
</table>

Table 2.4. Prevalence of *E. ruminantium* in blood samples from domestic ruminants using pCS20 PCR in different localities in the Sudan during the wet seasons in 2007 to 2009 [No. tested (No. +ve, %)]

<table>
<thead>
<tr>
<th>Locality</th>
<th>Cattle</th>
<th>Sheep</th>
<th>Goats</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Sennar State</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Singa</td>
<td>20 (1, 5)</td>
<td>20 (1, 5)</td>
<td>20 (1, 5)</td>
<td>60 (3, 5)</td>
</tr>
<tr>
<td><strong>Gezira State</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tamboul</td>
<td>10 (1, 10)</td>
<td>20 (1, 5)</td>
<td>20 (1, 5)</td>
<td>50 (3, 6)</td>
</tr>
<tr>
<td><strong>South Darfur State</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nyalá</td>
<td>20 (1, 5)</td>
<td>Nt</td>
<td>20 (3, 15)</td>
<td>40 (4, 10)</td>
</tr>
<tr>
<td><strong>North Kordofan State</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Elnihoud</td>
<td>10 (1, 10)</td>
<td>10 (0, 0)</td>
<td>Nt</td>
<td>20 (1, 5)</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>60 (4, 6.7)</td>
<td>50 (2, 4)</td>
<td>60 (5, 8.3)</td>
<td>170 (11, 6.5)</td>
</tr>
</tbody>
</table>

Nt: not tested
Figure 2.2. Amplification of *E. ruminantium* genomic DNA detected in *Amblyomma lepidum* using the Wizard® SV Genomic DNA Purification System. Lane (M) 100 bp molecular marker. Lane (-) PCR negative control. Lane (+) positive control lanes 1, 2, 3, 4, 5, 6, 7 and 8: PCR products obtained from *A. lepidum* collected from Singa, Dinder, Abonama, Elhoush, Nyala, Aidelfersan, Elnihoud and Um Rawaba, respectively, showing single specific band at approximately 226 bp.
Figure 2.3. Amplification of *E. ruminantium* genomic DNA detected in *Amblyomma variegatum* feeding on cattle using the Wizard® SV Genomic DNA Purification System. Lane (M) 100 bp molecular marker. Lane (-) PCR negative control. Lane (+) positive control. lanes 1, 2 and 3: PCR products obtained from *A. variegatum* collected from Nyala, Aidelfersan and Kass, respectively, showing single specific band at approximately 226 bp.
Figure 2.4. Amplification of *E. ruminantium* genomic DNA detected in blood of cattle using the Wizard® SV Genomic DNA Purification System. Lane (M) 100 bp molecular marker. Lane (-) PCR negative control. Lane (+) positive control. Lanes 1, 2, 3 and 4: PCR products obtained from Cattle blood collected from Singa, Tamboul, Nyala and Elnihoud, respectively, showing single specific band at approximately 226 bp.
Figure 2.5. Amplification of *E. ruminantium* genomic DNA detected in blood of sheep and goats using the Wizard® SV Genomic DNA Purification System. Lane (M) 100 bp molecular marker. Lane (-) PCR negative control. Lane (+) positive control. (A) lanes 1, 2, and 3: PCR products obtained from goats’ blood collected from Singa, Tamboul and Nyala respectively. (B) lanes 1 and 2: PCR products obtained from sheep blood collected from Singa and Tamboul respectively, showing single specific band at approximately 226 bp.
2.4. Discussion

Accurate estimates of tick infection prevalence are essential in the development and validation of heartwater disease transmission dynamics models (Deem et al., 1996b). These models are useful to evaluate the effectiveness of different disease control strategies, such as vaccination and chemical acaricide treatment. It assists in the formulation of cost-effective control programmes (O’ Callaghan et al., 1998). The use of polymerase chain reaction for epidemiological surveys and clinical diagnosis offers an ideal combination of sensitivity and specificity, while being relatively high cost. Previous reports demonstrated that, PCR assays could detect the pathogen in ticks and peripheral blood of clinically healthy animals in heartwater endemic areas (Peter et al., 1995). This indicated that a DNA-based technique is useful even for the diagnosis of latent infection.

This study presents the pCS20 PCR of *E. ruminantium* prevalence in adult *A. lepidum* and *A. variegatum*; the major vectors of heartwater in the Sudan, as well as the prevalence of *E. ruminantium* in the blood samples of cattle, sheep and goats collected from the field in different localities in the Sudan. The overall infection rate of *A. lepidum* was 15.3%. This indicated that nearly one in seven adult *A. lepidum*, collected was infected with *E. ruminantium*. This infection rate was high than those (1.9% and 1.8%) previously reported by Muramatsu et al (2005) and Abdel Rahman (2006) respectively. This infection rate was higher than that reported in *A. hebraeum* in South Africa (5.4%) (Du Plessis, 1981); Zimbabwe (1.7%) (Peter et al., 1999). On the other hand, the results were more in line with those recorded in *A. hebraeum* in Zimbabwe by Norval, et al. (1990) (10-40%) and Peter et al. (1999) (11.5%). The high prevalence (25%) reported in Abonama, was by far the highest value in the study, and a large sample size would have given a more accurate result. Nevertheless, one in four ticks in Abonama was infected with *E. ruminantium*.
The overall prevalence of *E. ruminantium* in *A. variegatum* collected in South Darfur State (Western Sudan) was 14%. The high prevalence (20%) reported in Aidelfersan, Nevertheless, one in five ticks in Aidelfersan was infected with *E. ruminantium*. The overall prevalence estimates for *A. variegatum* (14%), fall within the wide range found in heartwater endemic regions in Senegal (11%) (Gueye *et al*., 1994) and are higher than those determined previously in the South Sudan (8.2%) (Muramatsu *et al*., 2005) and Caribbean Islands (2%) (Camus and Barré, 1987b).

The exact reasons for this higher infection rates are difficult to explain, however, it is clear that there are a wide range of results from even within one country. Du Plessis and Mahan (1987) recorded prevalence values up to 30%, while Peter *et al.* (1999) only found 1.7% of ticks infected in South Africa. The consequences of the high infection rates in *A. lepidum* and *A. variegatum* in different localities in the Sudan have important repercussion for the endemic stability of heartwater on the communal grazing. This high infection rate in ticks is not surprising because, as mentioned earlier, *Amblyomma* spp. is a three host-tick and it is known that heartwater infection could be transmitted transstadially, thus larvae and nymphae that feed on an infected animal will, also, be positive for heartwater in their adult stage (Camus *et al*., 1996). Thus, the chances of a tick to become infected with heartwater are high.

The prevalence of *E. ruminantium* detected in cattle (6.7%), sheep (4%) and goats (8.3%) in different localities in the Sudan was higher than that observed in South Africa (3.9%) by Steyn *et al.* (2008) who reported prevalences ranging from 0.2 to 3.9% but lower than the 43.3% and 45.5% reported by Jongejan *et al.* (1988) in Zambia. The prevalences recorded in this study are however, lower than the sero-prevalence reported in the Sudan in cattle (38.7%), sheep (69%) and goats
(75.2%) (Abdel Rahman et al., 2006), and, also, lower than those reported in Cameroon (61%) (Awa, 1997), Kenya (61%) (Maloo et al., 2001), Tanzania (50.3%) (Swai et al., 2005) and Ghana (70%) (Bell-Sakyi et al., 2003). In this study the PCR assay demonstrates an effective tool for determination *E. ruminantium* infection prevalence and pCS20 PCR was found a powerful tool for the epidemiological study of heartwater and as a rapid and sensitive diagnostic technique to detect infected animals in the disease-endemic areas.
CHAPTER THREE

Detection and Quantification of *Ehrlichia ruminantium* DNA in *Amblyomma* spp. Ticks and Blood samples by Plexor® qPCR System based on pCS20 gene in the Sudan
3.1. Introduction

Diagnosis of heartwater is based on the observation of *E. ruminantium* colonies in capillary endothelial cells of the brain in clinically suspected animals, during the acute disease. However, it is not possible to detect the disease pre-symptomatic and carrier animals (Camus *et al*., 1996). Molecular techniques, with a high degree of sensitivity and specificity, have been developed to identify *E. ruminantium* DNA (Waghela *et al*., 1991; van Heerden *et al*., 2004b). However, the conventional PCR assays do not permit an accurate estimation of the initial concentration of target DNA, as they only provide semi-quantitative results (Steyn *et al*., 2003). The recent advent of the real-time quantitative PCR technique has proven useful in various applications, including pathogenic detection (Francino *et al*., 2006), gene expression and regulation (Follo *et al*., 2006), and allelic discrimination (Best *et al*., 2005).

Real-time PCR assays have been developed for quantification of *E. ruminantium* MAP1 gene in order to monitor cell culture vaccine production (Peixoto *et al*., 2005). Similarly, Postigo *et al*. (2007) quantified *E. ruminantium* DNA in ticks and in infected endothelial cell cultures using the MAP1-1 gene. These methods, based on detection of amplified DNA, are known to be polymorphic when use SYBR Green intercalating dye. A TaqMan-based real-time PCR assay was designed to recognize the most conserved pCS20 region (Steyn *et al*., 2008). The assay provides best sensitivity with a detection limit of one gene copy per reaction, which is 100 times higher than that of conventional pCS20 PCR (van Heerden *et al*., 2004b). However, this assay was reported to cross-react with both *E. chaffeensis* and *E. canis* (Steyn *et al*., 2008).

The Plexor® qPCR system is a novel real-time PCR system for the quantification of specific sequences within a DNA sample. It is designed to work with pairs of
PCR primers where one of the primers contains a fluorescent label adjacent to an iso-dC residue at the 5´ terminus. The other primer is unlabeled. The key to the Plexor® technology is the quenching of a fluorescent reporter due to the site-specific incorporation of dabcyl (4-(dimethylamino) azobenzene-4'-carboxylic acid)-iso-dGTP. As a result, the fluorescent signal from a Plexor® System reaction decreases as PCR product accumulates (Sherrill et al., 2004).

The basis for this assay is the use of two isomers of the bases cytidine and guanine, iso-C and iso-G (Figure 3.1) (Johnson et al., 2004). The iso-bases will only base pair with the complementary iso-base and DNA polymerases will only add an iso-base when the cognate complementary iso-base is present in the existing sequence. One primer is synthesized with a fluorescently labeled iso-C on the 5´ end. The PCR master mix contains free iso-dGTP coupled to a dark quencher dye (DABSYL) (Sherrill et al., 2004). As each amplification cycle progresses, the fluorescent signal from the free fluorescently tagged iso-C primers is progressively quenched as the labeled primers are incorporated into the growing amplified PCR products (Figure 3.2). The quenching is accomplished following the synthesis of the complementary strand bearing the iso-G-dark dye.

The present study described the application of Plexor® qPCR System assay for the quantitative detection of *E. ruminantium* and to evaluate the diagnostic sensitivity and specificity of the assay using a DNA samples of *Amblyomma* spp. and blood of sheep, goats and cattle.
Figure 3.1. Structures of the natural guanidine and cytosine bases showing hydrogen bonding compared with the hydrogen bonded iso-base structures. The iso-bases will only base pair with themselves and not the natural dG and dC residues nor dA, dT or dU. They are recognized as dNTPs and incorporated by DNA polymerase along with natural DNA bases into newly synthesized DNA (Johnson et al., 2004).

Figure 3.2. Schematic diagram of the Plexor® system real-time PCR process illustrating quenching of the fluorescent signal by dabcyl during product accumulation. One primer is labeled at the 5’ end with an iso-dC and a fluorescent reporter dye. The primer binds to the complementary template and is extended. In the next cycle, a complementary strand is made during the PCR, using a second, unlabeled primer. An iso-dG-Dabcyl base, present in the PCR master mix along with the four natural dNTPs, is incorporated as a complement to the iso-dC in the new strand, quenches the signal from the reporter dye (Sherrill et al., 2004).
3.2. Materials and Methods

3.2.1. Tick Samples

Adult male and female *A. lepidum* and *A. variegatum* ticks (130 samples) (an average of one to two ticks per animal) feeding on cattle were collected from heartwater endemic areas including Singa, Dinder, Abonama, Elhoush, Elnihoud, Um Rawaba, Nyala, Aidelfersan and Kass (Figure 2.1; Table 3.1).

3.2.2. Blood Samples

Whole blood (120 samples) was collected from cattle, sheep and goats in heartwater endemic areas (Singa, Tamboul and Nyala) in sterile vacutainer tubes containing EDTA as anticoagulant and stored at -20°C until used (Table 3.1). and blood stabilates of Nubian goats experimentally infected with *E. ruminantium* stocks (Abonama, Nyala and Dinder).

3.2.3. Positive controls

The Welgevonden strain was used as positive reference sample (Du Plessis, 1985) and samples used as specificity controls; *Anaplasma marginale* and *E. canis* (known to cause cross-reactions) were kindly provided by Prof. A. A. Latif, Onderstepoort Veterinary Institute, South Africa. Furthermore, *E. ruminantium* local stock (Gadarif) was cultured *in vitro* as described previously by Abdel Rahman (2006) also used as reference sample.

3.2.4. DNA Extraction

DNA was extracted from the blood and ticks using Wizard® SV Genomic DNA Purification System (Promega, Madison, USA). Extraction was carried out according to the manufacturer’s instruction and performed as described in chapter two (2.2.4.).

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3.2.5. Primer preparation

In order to apply the Plexor® qPCR System real-time PCR assay for detection and quantification of \textit{E. ruminantium} DNA, the CowF forward primer contains an iso-dC residue at the 5’ terminus and CowR reverse unlabeled primer were used to amplify a 226 bp fragment of the conserved pCS20 region (van Heerden \textit{et al.} 2004b) (Table 3.2). Primer stocks (25X) were prepared using the MOPS/EDTA Buffer (1mM MOPS, 0.1mM EDTA) [pH 7.5] to maintain primer integrity.

\textbf{Table 3.1.} \textit{Amblyomma} spp. ticks and blood samples collected from different localities in the Sudan during the wet seasons of 2007 to 2009

<table>
<thead>
<tr>
<th>State</th>
<th>Locality</th>
<th>\textit{A. lepidum}</th>
<th>\textit{A. variegatum}</th>
<th>Blood</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>\textit{Cattle}</td>
<td>\textit{Sheep}</td>
<td>\textit{Goats}</td>
<td></td>
</tr>
<tr>
<td>Sennar</td>
<td>Singa</td>
<td>20</td>
<td>-</td>
<td>20</td>
<td>15</td>
</tr>
<tr>
<td></td>
<td>Abonama</td>
<td>20</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Dinder</td>
<td>20</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>60</td>
<td>0</td>
<td>20</td>
<td>15</td>
</tr>
<tr>
<td>Gezira</td>
<td>Tamboul</td>
<td>-</td>
<td>-</td>
<td>10</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>Elhoush</td>
<td>5</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>5</td>
<td>0</td>
<td>10</td>
<td>20</td>
</tr>
<tr>
<td>North Kordofan</td>
<td>Um Rawaba</td>
<td>5</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Elnihoud</td>
<td>10</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Total</td>
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<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>South Darfur</td>
<td>Nyala</td>
<td>10</td>
<td>10</td>
<td>20</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Aidelfersan</td>
<td>-</td>
<td>10</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Kass</td>
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<td>20</td>
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<td>-</td>
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<tr>
<td>Total</td>
<td></td>
<td>10</td>
<td>40</td>
<td>20</td>
<td>0</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td></td>
<td>90</td>
<td>40</td>
<td>50</td>
<td>35</td>
</tr>
</tbody>
</table>

- = Not taken
3.2.6. Standard curves construction

The South African Welgevonden reference strain GenBank accession number (X74250) was used for generation of standard curve from which the efficiencies of the qPCR assays were determined. A ten fold dilution series \(10^0\) to \(10^9\) of \textit{E. ruminantium} stock was prepared. Regression analysis, standard curve slopes [Threshold cycle \((C_T)\) versus log DNA concentration], and amplification efficiencies \((E)\) were calculated from given slopes in the Swift Spectrum 48 software according to the equation: \(E = 10^{-\frac{1}{\text{slope}}} - 1\) (Rasmussen, 2001). The accepted amplification efficiency range is 0.9–1.1.

3.2.7. Internal control

The Plexor® qPCR control included a positive control reaction (internal control, 5X, contained a pair of primers and a synthetic template) was used to verify the consistency of reagents and instrumentation as well as the presence of amplification inhibitors in the DNA samples. One of the primers has an iso-dC residue adjacent to a 5´ fluorescein (FAM) label (Promega, Madison, USA).

3.2.8. pCS20 real-time PCR assay

Real-time PCR for simultaneous detection and quantification of \textit{E. ruminantium} DNA was performed on a Swift Spectrum 48 (Esco globule) with Plexor® qPCR system (Promega, Widson). The CowF forward primer contains an iso-dC residue at the 5´ terminus and CowR reverse unlabeled primer (Table 3.2) were added with dNTP mix contains dabcyl-iso-dGTP to induce quenching of the fluorescent dye on the complementary strand during amplification. A volume of 25 µl reaction mixture was made containing 12.5 µl of Plexor® qPCR System, each primer at a concentration of 0.5 µM and 5 µl of DNA template at the appropriate dilution. All reactions included a pure water as negative control and \textit{E. ruminantium} Welgevonden strain DNA as a positive control were included in the run. The
thermal cycling consisted of activation at 95°C for 10 min, 40 cycles of
denaturation at 95°C for 30 sec and annealing-extension at 60°C for 1 min. The
clamp of fluorescent signal was registered during the extension step of reaction.
The data were analysed with the “FitPoints method” software as described by
Rasmussen et al. (1998). The growth of PCR product was proportional to the
exponential increase in fluorescence (\(R_n\)). The software showed the amplification
curve resulting from a plot of \(R_n\) versus cycle number. \(C_T\) for each analysed
sample was regarded as the cycle number at which the amplification curve crossed
the threshold. This is automatically selected from the average of the \(R_n\) of the
samples.

3.2.9. Evaluation of sensitivity and dynamic range

Data from the above standard curve were used to determine the real-time assays
detection limit. The detection limit describes the lowest amount of a template that
can be detected under optimal conditions. It was defined as the highest dilution
factor at which all samples were positive in the specific real-time assay. This was
done by entering amount of DNA (ng) from respective serial dilutions of \(E.
ruminantium\) (Welgevonden strain) and length of pCS20 template (226 bp) in
calculator formula using the following calculations (Staroscik, 2004).

\[
\text{number of copies} = \frac{\text{amount} \times 6.022 \times 10^{23}}{\text{length} \times 1 \times 10^9 \times 650}
\]

\[
\text{number} = \frac{\text{ng} \times \text{number/mole}}{\text{bp} \times \text{ng/g} \times \text{g/mole of bp}}
\]

This calculation is based on the assumption that the average weight of a base pair
(bp) is 650 Daltons. This means that one mole of a bp weighs 650 g and that the
molecular weight of any double stranded DNA template can be estimated by taking the product of its length (in bp) and 650.

The inverse of the molecular weight is the number of moles of template present in one gram of material. Using Avogadro's number, 6.022x10^{23} molecules/mole, the number of molecules of the template per gram can be calculated:

\[ \text{mol/g} \times \text{molecules/mol} = \text{molecules/g} \]

Finally, the number of molecules or number of copies of template in the sample can be estimated by multiplying by 1*10^9 to convert to ng and then multiplying by the amount of template (in ng)

### 3.2.10. Evaluation of specificity

The test was conducted to exclude cross-reactivities between *E. ruminantium* and other TBDs pathogens. These include the closely related *A. marginale* and *E. canis*. DNA of positive blood samples of *A. marginale* and *E. canis* were included in the run as well as sterile water as negative control.
**Table 3.2.** Oligonucleotide primers for *E. ruminantium* used for pCS20 real-time PCR, conventional pCS20 PCR and MAP1 nested PCR assays

<table>
<thead>
<tr>
<th>Code</th>
<th>Sequence 5-3</th>
<th>Annealing Tm</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>CowF</td>
<td>CAAAACCTAGTAGAAAATGGCAA</td>
<td>58</td>
<td>van Heerden <em>et al.</em> (2004b)</td>
</tr>
<tr>
<td>CowR</td>
<td>TGCATCTTGTGGTGTTAC</td>
<td>58</td>
<td>van Heerden <em>et al.</em> (2004b)</td>
</tr>
<tr>
<td>Outer primers</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Map1NT</td>
<td>CTCGTAAGAAGTTGGTTAAC</td>
<td>50</td>
<td>Marteniz <em>et al.</em> (2006)</td>
</tr>
<tr>
<td>MapCT1</td>
<td>TTAATAACAAAAACCTTCTCC</td>
<td>50</td>
<td>Marteniz <em>et al.</em> (2006)</td>
</tr>
<tr>
<td>Inner primers</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Map1LP</td>
<td>CTTGGTGTTGCTTTTTTCTGA</td>
<td>55</td>
<td>Marteniz <em>et al.</em> (2006)</td>
</tr>
<tr>
<td>Map1CT2</td>
<td>CCTTCCTCCAATTTCTATACC</td>
<td>55</td>
<td>Marteniz <em>et al.</em> (2006)</td>
</tr>
</tbody>
</table>
3.2.11. Calculation of *E. ruminantium* pCS20 copies in field samples

The number of pCS20 copies/µl was determined in each sample. The standard curve generated from the Welgevonden reference strain of known concentration (128 ng/µl) was used to measure the concentration of DNA. Typically the standard curve was plot of the threshold cycle, C\_T versus log DNA concentration. A linear regression analysis of the standard plot was used to calculate the amount of DNA in samples of unknown concentration. The C\_T was manually determined shifting the line to the steepest part of amplification curves. The following formula was applied to convert the values determined using the standard curve to the pCS20 copies/ml of the sample (Matiaszek, 2007).

\[ x = 10^{(b - y)/\text{slope } a} \]

Where a and b are some numbers defined in a logarithmic curve, \( x \) is the copy number per reaction and \( y \) is the C\_T number.

A volume of 5 µl DNA was added to the qPCR master mix for each reaction. The total copy numbers per sample was based on the 200 µl elution volume in ticks and 100 µl elution volume in blood samples.

\[ \text{Result (copies/ml)} = \frac{\text{Result (copies/µl)} \times \text{Elution volume (µl)}}{\text{Sample volume (ml)}} \]
3.2.12. Gel-based PCR

3.2.12.1. pCS20 PCR assay

A conventional pCS20 PCR assay, based on ethidium-bromide staining, was performed for detection of *E. ruminantium* DNA in *Amblyomma* spp. and blood samples as described by van Heerden *et al.* (2004). The reaction volume was 25 µl. CowF and CowR primers (Table 3.2) with 5X GoTaq green buffer [100 mM Tris–HCl (pH 9), 500 mM KCl, 1% Triton X-100 (Promega, Madison, WI, USA)] were used. The assay was performed as described in chapter two (2.2.5).

3.2.12.2. MAP1 Nested PCR assay

MAP1 Nested PCR amplifications were performed as described in Martinez *et al.* (2006) in iycler thermal cycler (BioRad) using primers Map1NT, MapCT1, Map1LP and MapCT2 (Table 3.2). The reaction volume was 25 µl contained 5 µl of 5X GoTaq green buffer [100 mM Tris–HCl (pH 9), 500 mM KCl, 1% Triton X-100 (Promega, Madison, WI, USA)], 200 µM of dNTPs, 0.675 U GoTaq DNA polymerase (Promega, Madison, WI, USA), and 0.4 µM of each primer (TIB-Molbiol, Berlin, Germany). Distilled water and *E. ruminantium* DNA (Welgevonden reference strain) were used in each test as negative and positive controls, respectively. The first round PCR program consisted of an initial denaturation phase of 3 min at 94°C followed by 35 cycles of 45 sec denaturation at 94°C, 45 sec annealing phase at 50°C and 45 sec elongation phase at 72°C, followed by a final extension phase of 10 min at 72°C. The machine was hold at 4°C. The second phase was done using 1 µl of PCR product from the first phase as template. Amplification reactions (25 µl) contained 200 µM of dNTPs, 0.675 U GoTaq DNA polymerase and 0.4 µM of each primer. Reaction conditions were as follows: initial denaturation 1 min at 94°C, followed by 35 cycles of 50 sec denaturation at 95 8C; 50 sec annealing at 55°C; 50 sec elongation at 72°C, with a
final 10 min extension at 72°C. Then hold at 4°C. PCR products were visualized after electrophoresis on 1.5 % agarose gels.

3.2.13. Statistical analysis

The procedure of statistical analysis system (SAS) package was used to perform analysis of frequencies (Chi-square test). Correlation analysis was carried out to relate prevalence of *E. ruminantium* in *A. lepidum* and *A. variegatum* as well as sensitivity among pCS20 PCR, nested MAP1 PCR and pCS20 real-time PCR.
3. 3. Results

3.3.1. Standard curve

The standard curve for the serial dilutions (10-fold) of *E. ruminantium* Welgevonden strain was carried out using the software available in Swift Spectrum 48, to optimize the efficiency of Plexor® qPCR system. The calibration curve indicated a linear correlation (R² > 0.98) and the mean slopes (-3.12) with an amplification efficiency (E = 1.089) and these indicate a very efficient PCR (Figures 3.3; 3.4).

3.3.2. Sensitivity and dynamic range of the pCS20 qPCR assay

In order to estimate the sensitivity of the Plexor® qPCR system, FAM fluorescence signals were detected by real-time PCR at an a range of Cₜ values from 18 for the undiluted sample (10⁴) up to 38 for the highest dilution (10⁸). The Cₜ for each analysed sample was regarded as the cycle number at which the amplification curve crossed the threshold which is usually automatically selected from the average of the ?Rₙ of the samples. Lower Cₜ values corresponded to a greater amount of initial template and a negative result was considered to have a Cₜ value of 39 or more cycles (Figures 3.2; 3.3). The qPCR could detected pCS20 DNA at a dilution of 10⁻⁸ (6 pCS20 copies/μl) (Table 3.3).

3.3.3. Specificity of the pCS20 real-time PCR assay

The specificity of the Plexor® qPCR System was determined in triplicate using *E. ruminantium* Welgevonden as reference strain, a total of 4 local *E. ruminantium* stocks (Dinder, Abonama, Nyala and Gadarif), and two other *Rickettsiales* namely *E. canis* and *A. marginale*. The pCS20 real-time PCR assay specifically detected all *E. ruminantium* stocks (Dinder, Abonama, Nyala and Gadarif) and was positive with *E. canis* but negative with *A. marginale* DNA (Table 3.4).
Figure 3.3. The main graph containing the corresponding amplification plots of ten fold serial dilution of the *E. ruminantium* Welgevonden strain (128 ng/µl). Fluorescent profiles of the PCR products plotted against cycle numbers. Numbers (1 to 7) at curves-DNA Log concentrations (10^7, 10^6, 10^5, 10^4, 10^3, 10^2 and 10^1, respectively) at which the curves were obtained.
Figure 3.4. Regression analysis and standard curve generated from the amplification plot on ten fold serial dilutions of Welgevonden strain with linear correlation ($R^2 > 0.98$) and mean slopes (-3.12). The Y-axis represents the Log concentration of the PCR products. The X-axis represents the detectable signal (C_T-threshold cycles) of the Welgevonden DNA.
Table 3.3. Sensitivity and detection limit of pCS20 real time PCR (Plexor® qPCR System) using serially diluted Welgevonden strain

<table>
<thead>
<tr>
<th>Dilution</th>
<th>Concentration (ng/µl)</th>
<th>Copy number (copies/ µl)</th>
<th>qPCR</th>
</tr>
</thead>
<tbody>
<tr>
<td>$10^{-1}$</td>
<td>0.0128</td>
<td>$5 \times 10^7$</td>
<td>+</td>
</tr>
<tr>
<td>$10^{-2}$</td>
<td>0.00128</td>
<td>$5 \times 10^6$</td>
<td>+</td>
</tr>
<tr>
<td>$10^{-3}$</td>
<td>0.000128</td>
<td>$5 \times 10^5$</td>
<td>+</td>
</tr>
<tr>
<td>$10^{-4}$</td>
<td>0.0000128</td>
<td>$5 \times 10^4$</td>
<td>+</td>
</tr>
<tr>
<td>$10^{-5}$</td>
<td>0.00000128</td>
<td>$5 \times 10^3$</td>
<td>+</td>
</tr>
<tr>
<td>$10^{-6}$</td>
<td>0.000000128</td>
<td>$5 \times 10^2$</td>
<td>+</td>
</tr>
<tr>
<td>$10^{-7}$</td>
<td>0.0000000128</td>
<td>$5 \times 10^1$</td>
<td>+</td>
</tr>
<tr>
<td>$10^{-8}$</td>
<td>0.00000000128</td>
<td>$5 \times 10^0$</td>
<td>+</td>
</tr>
</tbody>
</table>
3.3.4. Internal control detection

The Plexor® qPCR control (internal control) was detected in all examined samples, with the range of threshold (C_T) values of 33 ± 3. Therefore, significant DNA losses did not occur during nucleic acid extraction, as well as no DNA polymerase inhibition was observed during real-time PCR amplification.

3.3.5. Determination of *Amblyomma* spp. infection rates by real-time PCR

Out of 90 *A. lepidum* collected from cattle and sheep in seven localities in the Sudan, 32 (35%) were positive for *E. ruminantium* with the pCS20 qPCR. The highest prevalence (55%) was reported in Abonama followed by (40%) in Singa and Elhoush, (30%) in Dinder and the lowest prevalence (20%) was reported in Elnihoud, Um Rawaba and Nyala (Table 3.5). Of 40 *A. variegatum* collected from cattle in different localities in South Darfur western Sudan, 18 (45%) were positive with the pCS20 qPCR. The highest prevalence (50%) was reported in Aidelfersan followed by (45%) in Kass and the lowest prevalence (40%) was reported in Nyala. (Table 3.6).

Standard curve generated from serial dilutions of the Welgevonden strain allowed the determination of *E. ruminantium* pCS20 copy numbers in *A. lepidum* and *A. variegatum* collected from cattle and sheep in localities mentioned. The mean quantities (copy number) of pCS20 was ranged from 7 x 10⁶ to 7 x 10¹⁵ in *A. lepidum* and the average copy number was highest (7 x 10¹⁵) in Abonama and the lowest (7 x 10⁶) average copy number was reported in Elhoush (Table 3.5). On the other hand, the average copy number of pCS20 in *A. variegatum* was ranged from 1 x 10⁷ to 4 x 10¹¹ with the greatest (4 x 10¹¹) copy number in Kass, moderate (4 x 10⁹) in Aidelfersan and the lowest (1 x 10⁷) average copy number was reported in Nyala (Table 3.6).
Table 3.4. Specificity of the two pCS20 assays using genomic DNA of known *E. ruminantium* stocks and two closely related bacteria

<table>
<thead>
<tr>
<th>Samples/ location</th>
<th>pCS20 PCR</th>
<th>pCS20 real-time PCR</th>
<th>Copy number (copies/ µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Welgevonden</td>
<td>+</td>
<td>+</td>
<td>3 x 10^{10}</td>
</tr>
<tr>
<td>Dinder</td>
<td>+</td>
<td>+</td>
<td>2 x 10^{8}</td>
</tr>
<tr>
<td>Abonama</td>
<td>+</td>
<td>+</td>
<td>1 x 10^{10}</td>
</tr>
<tr>
<td>Nyala</td>
<td>+</td>
<td>+</td>
<td>1 x 10^{11}</td>
</tr>
<tr>
<td>Gadarif</td>
<td>+</td>
<td>+</td>
<td>1 x 10^{6}</td>
</tr>
<tr>
<td><em>Ehrlichia canis</em></td>
<td>+</td>
<td>+</td>
<td>1 x 10^{10}</td>
</tr>
<tr>
<td><em>Anaplasma marginale</em></td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

+ = Positive 
- = Negative
Table 3.5. Infection rate and average copies number of *E. ruminantium* in *A. lepidum* using qPCR in seven localities in the Sudan in the wet seasons of 2007-2009

<table>
<thead>
<tr>
<th>Locality</th>
<th>No. tested</th>
<th>No. +ve (%)</th>
<th>Average copy number (copies/µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Singa</td>
<td>20</td>
<td>8 (40)</td>
<td>$4 \times 10^{10}$</td>
</tr>
<tr>
<td>Dinder</td>
<td>20</td>
<td>6 (30)</td>
<td>$2 \times 10^9$</td>
</tr>
<tr>
<td>Abonama</td>
<td>20</td>
<td>11 (55)</td>
<td>$7 \times 10^{15}$</td>
</tr>
<tr>
<td>Elhoush</td>
<td>5</td>
<td>2 (40)</td>
<td>$7 \times 10^6$</td>
</tr>
<tr>
<td>Nyala</td>
<td>10</td>
<td>2 (20)</td>
<td>$6 \times 10^9$</td>
</tr>
<tr>
<td>Elnihoud</td>
<td>10</td>
<td>2 (20)</td>
<td>$4 \times 10^8$</td>
</tr>
<tr>
<td>Um Rawaba</td>
<td>5</td>
<td>1 (20)</td>
<td>$3 \times 10^7$</td>
</tr>
<tr>
<td>Total</td>
<td>90</td>
<td>32 (35.6)</td>
<td>$1 \times 10^{15}$</td>
</tr>
</tbody>
</table>

Table 3.6. Infection rate and average copies number of *E. ruminantium* in *A. variegatum* using qPCR in South Darfur State during the wet season of 2007

<table>
<thead>
<tr>
<th>Locality</th>
<th>No. tested</th>
<th>No. +ve (%)</th>
<th>Average copy number (copies/µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nyala</td>
<td>10</td>
<td>4 (40)</td>
<td>$1 \times 10^7$</td>
</tr>
<tr>
<td>Aidelfersan</td>
<td>10</td>
<td>5 (50)</td>
<td>$4 \times 10^9$</td>
</tr>
<tr>
<td>Kass</td>
<td>20</td>
<td>9 (45)</td>
<td>$4 \times 10^{11}$</td>
</tr>
<tr>
<td>Total</td>
<td>40</td>
<td>18 (45)</td>
<td>$1 \times 10^{11}$</td>
</tr>
</tbody>
</table>
3.3.6. Analysis of blood samples by real-time PCR

A total of 120 blood samples from cattle, sheep and goats were tested by real-time PCR for detection of *E. ruminantium* DNA in Singa, Tamboul and Nyala (Table 3.7). Twelve out of 120 (10%) were positive for *E. ruminantium*. Four out of 50 (8%) blood samples from cattle were positive for *E. ruminantium*. The highest prevalence (10%) of *E. ruminantium* in cattle was reported in Nyala and Tamboul followed by (5%) in Singa. Moreover, three out of 35 (8.6%) blood samples collected from sheep were positive for *E. ruminantium*. The highest prevalence (10%) of *E. ruminantium* in sheep was in Tamboul and the lowest prevalence (6.7%) was in Singa. Five out of 35 (14.3%) blood samples from goats were positive for *E. ruminantium*. The highest prevalence (30%) of *E. ruminantium* in goats was in Nyala moderate (10%) in Singa and the lowest prevalence (6.7%) was reported in Tamboul (Table 3.7).

3.3.7. Comparison of real-time PCR with conventional PCR

The ability of the qPCR assay to detect *E. ruminantium* in field samples was determined (Table 3.8). Of 50 field samples (blood from cattle, sheep and goats, *A. lepidum* and *A. variegatum*) tested positive with pCS20 qPCR. Of these 49 (98%) were positive with MAP1 nested PCR and 42 (84%) were positive by conventional pCS20 PCR. The pCS20 qPCR detected 100% positive samples than the conventional PCR (*P*=0.001). Twelve blood samples that tested positive with the qPCR, also tested positive (100%) with the MAP1 nested PCR compared to 10 positive samples (83%) with the conventional PCR. Of the 22 *A. lepidum* a 100% concordance was demonstrated by qPCR and MAP1 nested PCR and 86.4% concordance (19/22) between these techniques and the conventional PCR. Of the 16 *A. variegatum* that tested positive with the qPCR, only 15 (93.7%) tested positive by MAP1 nested PCR and 13 (86.7%) by conventional pCS20 PCR.
Table 3.7. Prevalence of *E. ruminantium* in blood samples from domestic ruminants using qPCR in Singa, Tamboul and Nyala localities in the Sudan in the wet seasons of 2007-2009 [No. tested (No. +ve, %)]

<table>
<thead>
<tr>
<th>Locality</th>
<th>Cattle</th>
<th>Sheep</th>
<th>Goats</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Sennar State</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>-Singa</td>
<td>20 (1, 5%)</td>
<td>15 (1, 6.7%)</td>
<td>10 (1, 10%)</td>
<td>45 (3, 6.7%)</td>
</tr>
<tr>
<td><strong>Gezira State</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>-Tamboul</td>
<td>10 (1, 10%)</td>
<td>20 (2, 10%)</td>
<td>15 (1, 6.7%)</td>
<td>45 (4, 8.9%)</td>
</tr>
<tr>
<td><strong>South Darfur State</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>-Nyala</td>
<td>20 (2, 10%)</td>
<td>Nt</td>
<td>10 (3, 30%)</td>
<td>30 (5, 16.7%)</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>50 (4, 8%)</td>
<td>35 (3, 8.6%)</td>
<td>35 (5, 14.3%)</td>
<td>120 (12, 10%)</td>
</tr>
</tbody>
</table>

Nt: not tested
### Table 3.8. Comparison among pCS20 real-time PCR, conventional pCS20 PCR and nested MAP1 PCR for the detection of *E. ruminantium* DNA in field samples

<table>
<thead>
<tr>
<th>NO.</th>
<th>Locality</th>
<th>Samples</th>
<th>pCS20 PCR</th>
<th>Nested MAP1 PCR</th>
<th>pCS20 real-time PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Singa</td>
<td>Cattle blood</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>2</td>
<td></td>
<td>Sheep blood</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>3</td>
<td></td>
<td>Goat blood</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>4</td>
<td></td>
<td><em>A. lepidum</em></td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>5</td>
<td></td>
<td><em>A. lepidum</em></td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>6</td>
<td></td>
<td><em>A. lepidum</em></td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>7</td>
<td></td>
<td><em>A. lepidum</em></td>
<td>–</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>8</td>
<td>Abonama</td>
<td><em>A. lepidum</em></td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>9</td>
<td></td>
<td><em>A. lepidum</em></td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>10</td>
<td></td>
<td><em>A. lepidum</em></td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>11</td>
<td></td>
<td><em>A. lepidum</em></td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>12</td>
<td></td>
<td><em>A. lepidum</em></td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>13</td>
<td>Dinder</td>
<td><em>A. lepidum</em></td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>14</td>
<td></td>
<td><em>A. lepidum</em></td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>15</td>
<td></td>
<td><em>A. lepidum</em></td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>16</td>
<td></td>
<td><em>A. lepidum</em></td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>17</td>
<td></td>
<td><em>A. lepidum</em></td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>18</td>
<td>Elhoush</td>
<td><em>A. lepidum</em></td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>19</td>
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<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>20</td>
<td>Tamboul</td>
<td>Cattle blood</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>21</td>
<td></td>
<td>Sheep blood</td>
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<td>+</td>
<td>+</td>
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<tr>
<td>22</td>
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<td>Sheep blood</td>
<td>–</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>23</td>
<td></td>
<td>Goat blood</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>24</td>
<td>Elnihoud</td>
<td><em>A. lepidum</em></td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>25</td>
<td></td>
<td><em>A. lepidum</em></td>
<td>–</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>26</td>
<td>Um Rawaba</td>
<td><em>A. lepidum</em></td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>27</td>
<td></td>
<td><em>A. lepidum</em></td>
<td>–</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>28</td>
<td>Nyalu</td>
<td>Cattle blood</td>
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<td>+</td>
<td>+</td>
</tr>
<tr>
<td>29</td>
<td></td>
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<td>32</td>
<td></td>
<td>Goat blood</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>33</td>
<td></td>
<td><em>A. lepidum</em></td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>34</td>
<td></td>
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</tr>
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<tr>
<td>36</td>
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<tr>
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<td>49</td>
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</tr>
<tr>
<td>50</td>
<td></td>
<td><em>A. variegatum</em></td>
<td>–</td>
<td>–</td>
<td>–</td>
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</table>

Total +ve: 42, 49, 50

(+) = Positive  
(–) = Negative
3.4. Discussion

The Plexor® qPCR System assay based on a sequence in the pCS20 region was used for the detection and quantification of *E. ruminantium* genome in *A. lepidum*, *A. variegatum* and blood samples of cattle, sheep and goats collected from the field at different localities in the Sudan. The sensitivity of the Plexor® qPCR system, was determined through standard curve generated from the Welgevonden strain. The analytical analysis indicated that the dynamics of quantitative analysis ranged among cycles 18 and 38 (from $10^1$ to $10^8$ Log concentration). The detection limit of the Plexor® qPCR system was 5 pCS20 copies/µl which is 20-times more sensitive than that of the conventional pCS20 PCR (Peter *et al*., 1995; Steyn *et al*., 2008). However, slightly it is less sensitive than the pCS20 real-time PCR TaqMan probe assay, that has high detection limit of one pCS20 copy/µl (Steyn *et al*., 2008).

Moreover, sensitivity of the Plexor® qPCR system was compared with two other tests, namely a standard pCS20 PCR (van Heerden *et al*., 2004b) and a MAP1 nested PCR (Marteniz *et al*., 2006). More samples tested positive using the Plexor® qPCR assay than the other two assays, probably as a result of its higher sensitivity. Of the 50 samples tested positive using the pCS20 qPCR collected from endemic areas in the Sudan, 49 were positive with the nested MAP1 PCR, while only 42 tested positive with the standard pCS20 PCR under optimum conditions. Similarly, in South Africa, Steyn *et al*. (2008) tested 179 tick samples, of these only 79 were positive using the pCS20 real-time PCR TaqMan probe assay. Using the same genomic DNA from these positive qPCR samples, only 66 tested positive with the pCS20 PCR/32P-probe and 54 with the standard pCS20. Therefore, this showed that the qPCR could detect lower concentrations of the pCS20 DNA. These results indicated that at the present time the qPCR assay could be more suitable for epidemiological studies and as a diagnostic test.
Likewise, cross-reactivity in the pCS20 real-time PCR TaqMan probe assay, were reported in *E. ruminantium* and both *E. chaffeensis* and *E. canis* (Steyn *et al.*, 2008). It is worth mentioning that, a close phylogenetic relationship existed among *A. marginale*, *E. canis* and *E. ruminantium* (Dumler *et al.*, 2001; van Heerden *et al.*, 2004b). This may explain why the PCR assays cross react. However, *E. canis* only infect the dogs (Allsopp *et al.*, 1997), this DNA should not pose a problem for qPCR detection of heartwater.

In this study, pCS20 qPCR was used to quantify the copy numbers of *E. ruminantium* in both *A. lepidum* and *A. variegatum* naturally infected and collected from cattle in different localities in the Sudan. As previously reported, there is one copy of pCS20 per genome equivalent of *E. ruminantium* (Collins *et al.*, 2005). It can be assumed that each copy of pCS20, measured by the qPCR assay, is equivalent to one genome copy of *E. ruminantium*. This was supported by Steyn *et al.* (2008) who and pCS20 TaqMan® probe assay indicated that approximately 10^8 - 10^9 *E. ruminantium* organisms per ml blood were present in infected sheep with Welgevonden blood stabilate. The result was also, correlated with the amount of *E. ruminantium* copies found in cell culture (10^9 copies/ml) when using MAP1 real-time PCR (Peixoto *et al.*, 2005). Furthermore, Postigo *et al.* (2007) reported on the quantification of *E. ruminantium* in the midgut and salivary glands in ticks (10^6 copies/tick) used qPCR and MAP1-1 multigene family.

The low level of positive blood samples (10%) may be ascribed to the fact that, blood was collected from healthy animals that did not show any symptoms of disease. However, Steyn *et al.* (2008) reported that TaqMan probe qPCR could only detect *E. ruminantium* organisms in the blood of infected animals during the febrile stage of infection. This could be linked to the life cycle of the parasite in the ruminant host. After an infected tick bite, the initial replication of the organism
takes place in reticulo-endothelial cells within the lymph nodes (Allsopp et al., 2005). Furthermore, it has been postulated that the febrile stage coincides with the release of organisms from the lymph to the blood stream (Du Plessis, 1970). In contrast, the qPCR field positive samples from healthy animals, therefore, can be indication of state. It has been known for many years that animals can have a "premunity" (in other words, heartwater organisms remain in the animal and stimulate ongoing immunity, with no symptoms being shown) that results in endemic stability of heartwater (Camus et al., 1996; Allsopp et al., 2005). Domestic ruminants are known to occasionally harbour E. ruminantium without any clinical signs and to serve as reservoirs of the disease after recovery (Faburay et al., 2007b). Nakao et al. (2011) showed that, out of 150 bovine, 35 goats, and 19 sheep blood samples from a heartwater-endemic tested, only two sheep blood samples were positive by conventional PCR, real-time PCR, and LAMP. It is also known that cattle is less susceptible to the disease than sheep and goats (Allsopp et al., 2005) and different breeds of cattle are more resistant to the disease than others. Another factor to consider is reported infections of immune animals, if animals are infected with heartwater organism multiple times at an early stage by tick transmission they became immune (Martinez et al., 1999).

In conclusion, the Plexor® qPCR system based on the pCS20 region was more sensitive than the standard pCS20 PCR and MAP1 nested PCR when applied to tick DNA. Blood samples of infected animals could be diagnosed before they succumb the disease and can thus, be treated appropriately. Furthermore, DNA in the samples could be quantified for diagnostic and epidemiological studies. qPCR was found to be rapid and accurate for identification and quantification of E. ruminantium.
CHAPTER FOUR

*In vivo* Isolation of *Ehrlichia ruminantium* Local Stocks using Nubian Goats (*Capra hircus*) and Estimation of Infective dose by Real-time PCR
4.1. Introduction

*Ehrlichia ruminantium* is a tick-borne intracellular *Rickettsia* causing a disease of ruminants called heartwater (Cowdry, 1925a, b). The disease is characterized by high fever, pulmonary distress and nervous symptoms, and is frequently fatal in naïve animals (van de Pypekamp and Prozesky, 1987; Camus *et al*., 1996). Heartwater is transmitted by several members of the tick genus *Amblyomma* and occurs commonly throughout most of sub-Saharan Africa and in the eastern Caribbean Islands (Walker and Olwage, 1987).

*In vivo* and *in vitro* isolation of *E. ruminantium* is required for propagation of the organism for serological and biological purposes (OIE, 2010). *In vivo* isolation is used to assess presence of heartwater in a herd, a region or a country. *E. ruminantium* is isolated by inoculating blood or tick homogenate into a susceptible animal (Ngumi *et al*., 1997). This done by slowly inoculation of blood from individual animals, or pooled blood intravenously at a dose of 10-100 ml into a susceptible sheep or goat. However, the method will rarely detect infection in carrier/recovered animals (Camus *et al*., 1996). Alternatively, tick homogenate inoculation into susceptible animals can assesse the presence of the disease (Birnie *et al*., 1985). This method is more sensitive than inoculation blood from of suspect animals (especially if blood is from recovered animals) (OIE, 2010). However, the tick infection rate in the field is variable and sometimes is as low as 1% (Peter *et al*., 1995).

Methods to quantify *E. ruminantium* include: (i) cell counting using microscope chambers either by conventional phase contrast microscopy, which has a significant associated error due to the *E. ruminantium* size (0.3–2.8 µm), or by using fluorescent stains (Camus *et al*., 1996). Nevertheless, several problems were found in the quantification of *E. ruminantium* using this method mainly due to the presence of nuclei from endothelial cells, *E. ruminantium* aggregation phenomena
and the rapid decrease observed in fluorescence for both strains of *E. ruminantium* tested (Mutunga *et al.*, 1998); (ii) LD<sub>50</sub> method (Brayton *et al.*, 2003) or plaque assay (TCLD<sub>50</sub>) (Totte´ *et al.*, 1993) for quantification of *E. ruminantium* infection: an important feature to ensure the consistency of *E. ruminantium* batches of challenge material (LD<sub>50</sub>) or to calculate *E. ruminantium* inoculum size for continuous *in vitro* propagation (TCLD<sub>50</sub>), for dosage establishment. (iii) Total protein content: although simple and inexpensive this method has interferences due to the presence of cell debris, often overestimating the bacterial protein content of the samples (Totté *et al.*, 2006).

Quantitative real time PCR was developed to quantify *E. ruminantium* using the SYBR Green I dye based on MAP1-1 gene (Postigo *et al.*, 2007) and MAP1 (Peixoto *et al.*, 2005). The pCS20 real-time PCR TaqMan probe assay was provide higher sensitivity than that of conventional PCR (Steyn *et al.*, 2008). This study aimed to isolate, characterize and quantify *E. ruminantium* field isolates in heartwater endemic areas in the Sudan.

4.2. Materials and Methods

4.2.1. Tick homogenates preparation

Adults males and females of partially engorged *A. lepidum* were collected from cattle at Dinder and Abonama while *A. variegatum* ticks were collected from Nyala in September 2009 (Figure 2.1). The ticks were kept alive and identified according to Hoogstraal (1956). They were cleaned and thoroughly washed by cool Phosphate Buffered Saline (PBS). According to Birnie *et al.* (1985) method, a hundred of these ticks (80 males and 20 females) were ground up using sterile pestle and mortar after adding 50 ml of cool PBS (pH 7.2) as diluents (0.5 ml per tick). The tick homogenate was transferred to a clean sterile glass cylinder and
allowed to stand at room temperature for 10 minutes and the supernatant immediately inoculated intravenously into each naïve goat.

4.2.2. Experimental animals and blood stabilates preparation

Six-month-old Nubian goats were obtained from Alhalfaya town (16° 0′ 32° 6′) a heartwater-free region (Khartoum North) (Abdel Rahman, 2006). Three goats, that tested *E. ruminantium* negative with pCS20 PCR assay, were used for *E. ruminantium* isolation. A volume of 5 ml tick supernatant was immediately inoculated intravenously into these three naïve goats. The inoculated animals were kept in tick proof sheds and rectal temperatures were taken daily. At rise of goats' temperatures to 41°C, blood in EDTA was taken from the jugular veins and Dimethyl Sulphoxide (DMSO) (1:10) was slowly added to the blood in Petri-dish over ice that continuously stirring. The blood was divided into 2 ml aliquots in cryo-vials and immediately kept in liquid nitrogen till used.

4.2.3. Postmortem examination

Postmortem examination was performed on the dead animals and confirmation of heartwater was made by preparation of brain-crushed smears for detection of *E. ruminantium* colonies (Purchase, 1945). A small fragment of the grey matter (approximately the size of a match head) was placed on a microscope slide, crushed paste consistency by another slide while maintaining pressure. The slides were drawn over each other length wise to produce a single layer of cells. They were then air-dried, immediately fixed in methanol and stained with Giemsa's stain. The slides were examined under a microscope at a low magnification (×10 objective) to identify the colonies of *E. ruminantium*. 

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4.2.4. DNA extraction and PCR analysis

DNA was extracted from tick homogenates and the blood of the inoculated goats using Wizard® SV Genomic DNA Purification System (Promega, Madison, USA). Extraction was carried out according to the manufacturer’s instruction. The pCS20 PCR and nested MAP1 PCR were carried out as previously described in chapters two and three.

4.2.5. Estimation of the infective dose by real-time PCR

Estimation of *E. ruminantium* infective dose in ticks homogenates were carried out on a Swift Spectrum 48 (ESCO globule) using Plexor® qPCR System (Promega, Widson). The CowF forward primer contains an iso-dC residue at the 5´ terminus and CowR unlabeled reverse primer were used to amplify a 226 bp fragment of the conserved pCS20 region (van Heerden *et al.* 2004b). The pCS20 copy numbers of *E. ruminantium* in tick homogenates and blood stabilates of infected goats were calculated. The total copy numbers per samples were based on the 200 µl in tick samples and 100 µl in blood samples according to elution volume as previously described in chapter three (3.2.9.).
4.3. Results

4.3.1. Local stocks isolation

Three stocks of *E. ruminantium* were obtained from Dinder, Abonama and Nyala (Table 4.1; 4.2). The Nubian goats inoculated with *Amblyomma* spp. homogenates collected from cattle in the three mentioned endemic areas developed clinical signs of heartwater by 6 to 12 days after inoculation. During this period, body temperatures and clinical signs of the goats were daily recorded. Goats were considered in hyperthermia when their rectal temperature was equal to or greater than 40°C.

4.3.2. Heartwater confirmation

The clinical signs included elevated temperature up to 41°C, an increase in respiration tempo, loss of appetite, diarrhoea, nervous symptoms (constant movement of the lower jaw and tongue, muscular twitching and squinting), frothy fluid in nostrils and finally death. Post mortem signs included an enlarged spleen and visible haemorrhages on the lung and heart. Brain oedema and effusion into body cavities of transparent light yellow fluid, were also seen, particularly, in the heart and lungs. Recovery occurred in one goat that was inoculated with tick homogenates from Abonama area.

4.3.2.1. Microscopical detection

*Ehrlichia ruminantium* blue-purple colonies were found in the cytoplasm of brain endothelial cells stained with Giemsa’s. The clusters of granules showed different forms of *E. ruminantium* (Figure 4.1).
Figure 4.1. Goats; brain crushed smears stained with Giemsa showing dark blue colonies (arrowhead) of *E. ruminantium* in cytoplasm of endothelial cells (A) Nyala isolate (B) Dinder isolate. (X100).
4.3.2.2. Molecular detection

_Ehrlichia ruminantium_ DNA extracted from blood stabilates collected during the febrile period of the Nubian goats, experimentally infected with tick homogenates were subjected to pCS20 PCR and MAP1 nested PCR. All samples tested produced a specific DNA amplicons of the pCS20 (226 bp) and the MAP1 (800 bp) (Figure 4.2).

4.3.3. Estimation of the infective dose material by real-time PCR

The pCS20 copies numbers were calculated in 5 ml challenge materials of both _A. lepidum_ and _A. variegatum_ homogenates collected from Dinder, Abonama and Nyala using the standard curve generated of serial dilution of the Welgevonden strain (Figure 4.3). Five ml dose of each stock homogenate was inoculated into Nubian goats. The copies number of pCS20 ranged from 1 x 10^4 to 2 x 10^5/ml in _A. lepidum_. The greatest (2 x 10^5) copies number was in Abonama stock, followed by (5 x 10^4) in Nyala stock and the lowest (1 x 10^4) copies number was in Dinder stock (Table 4.1).

Goat inoculated with 5 x 10^4 copies/ml of Nyala stock died on day 9, while that inoculated with 1 x 10^4 copies/ml of Dinder stock died on day 14 but the goat inoculated with 2 x 10^5 copies/ml of Abonama stock survived (Table 4.2). The copies number generated by pCS20 in the blood stabilates of goats inoculated with tick homogenates was also estimated during the febrile periods for each stock. The copies number of pCS20 ranged from 2 x 10^8 to 1 x 10^{11} copies/µl. The greatest (1 x 10^{11}) copies number was reported in Abonama stock, followed by (1 x 10^{10}) in Nyala stock and the lowest (2x10^8) copies number was reported in Dinder stock (Table 4.2).
Figure 4.2. Amplification of *E. ruminantium* genomic DNA detected in blood of goats experimentally inoculated with *Amblyomma* spp. homogenates. Lane (M) 100 bp molecular marker; Lane (-) PCR negative control; Lane (+) positive control. (C) lanes 1, 2 and 3: pCS20 PCR products obtained from blood stabilates of Dinder, Abonama and Nyala stocks respectively, showing single specific band at approximately 226 bp. (D) lanes 1, 2 and 3: MAP1 nested PCR products obtained from Dinder, Abonama and Nyala stocks respectively, showing single specific band at approximately 800 bp.
Figure 4.3. Flourescent profiles of blood stabilates of *E. ruminantium* local stocks. The small letters at curves were: (a) The genomic Welgevonden DNA (positive control), (b) Abonama stock (c) Nyala stock (d) Dinder stock (e) Gadarif stock and (f) internal control.
Table 4.1. Copies number of *E. ruminantium* DNA in infective dose (5 ml) of tick homogenates used to infect Nubian goats

<table>
<thead>
<tr>
<th>Origin</th>
<th>Source of infection</th>
<th>Incubation period (Days)</th>
<th>Copy number (copies/ml)</th>
</tr>
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<tr>
<td>Dinder</td>
<td><em>A. lepidum</em></td>
<td>9</td>
<td>1 x 10⁴</td>
</tr>
<tr>
<td>Nyala</td>
<td><em>A. variegatum</em></td>
<td>6</td>
<td>5 x 10⁴</td>
</tr>
<tr>
<td>Abonama</td>
<td><em>A. lepidum</em></td>
<td>12</td>
<td>2 x 10⁵</td>
</tr>
</tbody>
</table>

Table 4.2. Copies number of *E. ruminantium* DNA in blood stabilates during febrile period of Nubian goats experimentally infected with tick homogenates

<table>
<thead>
<tr>
<th>Stock origin</th>
<th>Days of blood stabilates preparation</th>
<th>Maximum temperature (°C)</th>
<th>Days to death</th>
<th>Copy number (copies/µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dinder</td>
<td>11-14</td>
<td>41.9</td>
<td>Death/14</td>
<td>2 x 10⁹</td>
</tr>
<tr>
<td>Nyala</td>
<td>7-9</td>
<td>42.2</td>
<td>Death/9</td>
<td>1 x 10¹¹</td>
</tr>
<tr>
<td>Abonama</td>
<td>12-15</td>
<td>40.5</td>
<td>Survived</td>
<td>1 x 10¹⁰</td>
</tr>
</tbody>
</table>
4.4. Discussion

*In vivo* isolation of *E. ruminantium* was carried out using Nubian goats purchased from Alhalfaya area Khartoum North, an area proved to be heartwater free zone (Abdel Rahman, 2006). Dinder stock and Abonama stock were isolated by inoculation of *A. lepidum* homogenates in naïve goats and Nyala stock was isolated by inoculation of *A. variegatum* homogenate. Likewise, Abdel Rahman (2006) isolated seven stocks of *E. ruminantium* in Nubian goats from different localities in the Sudan including: New Halfa, Gadarif, Tamboul, Singa, Abukarshola, Rabak, and Pibore from the South Sudan. Similarly, Ilemobade (1977) and Ilemobade and Blontkamp (1978) isolated the organism in Nigeria using the same method in West African Dwarf (WAD) goats. Sheep and cattle were, also, used for isolation of Um Banein strain by Jongejan *et al.* (1984).

The Nubian goats inoculated with tick homogenates developed clinical signs of heartwater by 6 to 12 days in agreement with previous studies. Abdel Rahim and Shommein (1984) reported that the mortality rate due to heartwater in Eastern Sudan were 17% and 11% among sheep and goats, respectively. The incubation period was 6-12 days under laboratory conditions but 14-28 days in the field. Abdel Rahman (2006) reported incubation periods that ranged from 9 to 18 days in Nubian goats inoculated with blood stabilates from experimentally infected animals with tick homogenates from various regions of Sudan.

The Plexor® qPCR System was used to estimate the intensity of *E. ruminantium* infection in the tick homogenates used to infect Nubian goats. However, this the first study on quantification of *E. ruminantium* in infective dose. Real-time PCR, shown to be more sensitive method for quantification of *E. ruminantium* than counting fluorescence stained organisms. Counting is difficult due to the presence of endothelial cell nuclei and aggregation of the parasites (Peixoto *et al.*, 2005).
Nevertheless, several problems were found in the quantification of *E. ruminantium* using this method mainly due the rapid decrease observed in fluorescence for *E. ruminantium* tested (Mutunga *et al*., 1998). Quantification using total protein content, although simple and inexpensive, this method has interferences due to the presence of cell debris, often overestimating the bacterial protein content of the samples (Totté *et al*., 2006). Likewise, Zanetti *et al*. (2008) used qPCR to characterize the growth of *Rickettsia amblyommii* in tissues of naturally infected *Amblyomma americanum*.

The varying degrees of susceptibility and classification into hyperacute, acute and mild form as observed in this study was also reported by some workers in other breeds of goats (Uilenberg, 1983; Losos, 1986 and Okaiyeto *et al*., 2009). However, Abonama stock showed mild form of the disease and the animal survived although the load of pCS20 copies number in the infective dose was the highest ($2 \times 10^5$) and the copies number of pCS20 during the febrile period (12 – 15) was high ($1 \times 10^{11}$). There is great variation in the pathogenicity of various stocks of *E. ruminantium* fore different animals (mice, sheep, goats and cattle) (Camus, *et al*., 1996). Mortality in susceptible sheep varied from 30% using Salem stock to 99% using Schalekamp stock while Kumm and Mara 88/9 stocks were virtually non-pathogenic for cattle (Camus, *et al*., 1996). Such differences are apparently independent from geographical remoteness or proximity (Allsopp, 2010). For instance, Du Plessis *et al*. (1992) found that three isolates in one area of South Africa differed considerably in pathogenicity to mice, sheep and cattle and in cross-immunity tested in sheep. The host species is important in demonstrating antigenic differences; these appear to be more pronounced in goats than in sheep (Jongejan *et al*., 1991). In addition, Ilemobade (1977) suggested that goats are not preferential hosts of adult *Amblyomma* ticks which prefer cattle for feeding. This may further reduce their chances of developing immunity. However, the new *E. ruminantium* stocks showed different heartwater forms and this thought to be due...
to a wide diversity of *E. ruminantium* stocks that simultaneously circulates in different localities of endemic areas in the Sudan.

The successful *in vivo* isolation of *E. ruminantium* new stocks (Dinder, Abonama and Nyala) confirmed the high infection rate of ticks and wide distribution of the disease in different localities in the Sudan. However, the isolated *E. ruminantium* stocks showed different heartwater forms and this is thought to be due to a wide diversity of *E. ruminantium* stocks that simultaneously circulates in different regions of endemic areas in the Sudan.
CHAPTER FIVE

General Discussion and Conclusions
Tick-borne diseases continue to be a hindrance to the development of the livestock industry in many countries. Sensitive, specific and cost-effective diagnostic methods are important component in the study and control of these diseases. Heartwater is regarded as one of the most important infectious tick borne disease of domestic livestock and wildlife in Sub-Saharan Africa according to World Organization of Animal Health (OIE, 2010). It is caused by the obligate intracellular rickettsial agent *Ehrlichia ruminantium* and is transmitted to livestock and wild ruminants by *Amblyomma* tick species.

Since the advent of biotechnology and genomics, molecular epidemiology has evolved. Traditional epidemiology coupled with the new molecular techniques will lead to better definition and control of animal diseases. The molecular epidemiology of heartwater was not previously well understood, because the disease could only be diagnosed after death in crushed-brain endothelial cells of smears. However, the new molecular techniques can be applied for diagnosis of heartwater in live animals, ticks and cell cultures, as well as for identification of different genotypes by sequencing.

In the Sudan, however, prior to the study of Adel Rahman (2006) who used conventional and molecular techniques for country wide survey of *E. ruminantium*, information on the presence and distribution of the disease was fragmentary and based on clinical observations of affected animals, limited isolation trials and definitive post mortem diagnosis (Karrar, 1960; Fawi *et al.* 1977; Shommein and Abdel Rahim, 1977; Abdel Rahim and Shommein, 1978). The absence of systematic studies and lack of diagnostic capacities in field laboratories upcountry resulted in lack of adequate records on the impact of the disease in the endemic areas. Few epidemiologic data exist on infection rates of *Amblyomma* spp. with *E. ruminantium*. The pCS20 PCR reported to be more sensitive to detect *E. ruminantium* infection in ticks and blood samples was used to assess the level and distribution of heartwater-risk in the Sudan in a
countrywide as well as point seroprevalence survey involving local cattle, sheep and goats (Abdel Rahman, 2006). Another PCR assay for the MAP1 gene was, also, used to differentiate strains of *E. ruminantium* (Muramatsu *et al.*, 2005). In order to control heartwater in susceptible livestock in the Sudan, it is important to understand the epidemiology of the disease, particularly the prevalence and distribution of infection in the target population. The current study described a systematic epidemiological investigation into heartwater in some endemic areas in the Sudan. Each of the aims of the study is listed below and the findings summarized. All the aims were achieved.

The study applied the pCS20 PCR to determine *E. ruminantium* infection rate in *A. lepidum* and *A. variegatum*. The overall infection rate of *E. ruminantium* in *A. lepidum* was 15.3% and 14% in *A. variegatum* were quite high. This infection rate was higher than many of the previous surveys for *E. ruminantium* prevalence in the Sudan of 1.9% (Muramatsu *et al*, 2005) and 1.8% (Abdel Rahman, 2006). Furthermore, this infection rate was higher than many of surveys for *E. ruminantium* prevalence in *A. hebraeum* in South Africa (5.4%) (Du Plessis, 1981); Zimbabwe (1.7%) (Peter *et al.*, 1999). On the other hand, the results were in line with some of the higher values recorded in *A. hebraeum* in Zimbabwe (10-40%) (Norval, *et al.*, 1990) and (11.5%) (Peter *et al.*, 1999). However, there are a wide range of results from even within one country. The consequences of the high infection rates in *A. lepidum* and *A. variegatum* in different localities in the Sudan have important repercussions for the endemic stability of heartwater in the communal grazing. However, this high infection rate in ticks is not surprising because, *Amblyomma* spp. have a three stage life cycle and it is known that heartwater infection could be transmitted transstadially, larvae and nymphae that feed on an infected animal will be infective for heartwater in their adult stage (Camus *et al.*, 1996). Thus, the chances of a tick to become infected with heartwater are high. In contrast, the prevalence of *E. ruminantium* detected in
blood samples from cattle (6.7%), sheep (4%) and goats (8.3%) was lower than prevalence in ticks but higher than that observed in South Africa (3.9%) by Steyn et al. (2008), who reported prevalences ranging from 0.2 to 3.9%.

Accurate estimates of tick infection prevalence are also essential in the development and validation of models for heartwater disease transmission dynamics (Deem et al., 1996b). These models can be used to evaluate the effectiveness of different disease control strategies such as vaccination and chemical acaricides treatment, and assist in the formulation of cost-effective control programmes (O’Callagham et al., 1998). PCR assays have high sensitivity and specificity for amplification of E. ruminantium DNA. Previous reports demonstrated that PCR assays could detect the pathogen in ticks and in the peripheral blood of clinically healthy animals in heartwater endemic areas (Peter et al., 1995) and are useful even for the diagnosis of latent infection. The current study demonstrated that the PCR assay is an effective tool for determining the prevalence of E. ruminantium infection in the vertebrate and invertebrate hosts.

A pCS20 quantitative real-time PCR (Plexor® qPCR System) was successfully applied as a diagnostic assay for simultaneous detection and quantification E. ruminantium in Amblyomma spp. and blood sample. The sensitivity of the Plexor® qPCR system, was determined through standard curve generated from the Welgevonden strain (South Africa). The detection limit of the Plexor® qPCR system was 5 pCS20 copies/µl which was at least 20-times more sensitive than that of conventional pCS20 PCR. These finding were more or less similar to that obtaining by Peter et al. (1995) Steyn et al. (2008) but slightly less sensitive than the pCS20 real-time PCR TaqMan probe assay, with a reported detection limit of one pCS20 copy/µl (Steyn et al., 2008). The pCS20 qPCR was compared with the conventional pCS20 PCR and nested MAP1 PCR. It was found more sensitive than the two assays. The specificity of the Plexor® qPCR System was, also, determined. The assay specifically detected all E. ruminantium local stocks of
Dinder, Abonama, Nyala and Gadarif. It was positive with *E. canis* but negative with *A. marginale* DNA similar to pCS20 real-time PCR TaqMan probe assay (Steyn *et al.*, 2008). Since *E. canis* only infect the dogs, this DNA should not pose a problem for qPCR detection of heartwater. To further investigate the risk situation of heartwater in the country, it is necessary to estimate *E. ruminantium* infection rates in the vector tick population. The study has applied the pCS20 qPCR to detect and quantify the copies number of *E. ruminantium* in *A. lepidum* and *A. variegatum* collected from cattle in different localities in the Sudan.

This study showed that the Plexor® qPCR system based on the pCS20 region was more sensitive than the standard pCS20PCR and MAP1nested PCR when applied to tick DNA. The qPCR offers an ideal combination of sensitivity and specificity while being relatively high throughput. It was rapid and accurate method for identification and quantification.

*In vivo* isolation of *E. ruminantium* was carried out in Nubian goats from *Amblyomma* free zone of north of Khartoum. Dinder stock and Abonama stock were isolated by inoculating *A. lepidum* homogenates into naïve goats, however, Nyala stock was isolated from *A. variegatum* homogenate. Similarly, Abdel Rahman (2006) isolated seven stocks of *E. ruminantium* in Nubian goats from different localities in the Sudan that included New Halfa, Gadarif, Tamboul, Singa, Rabak, Abukarshola and Pibore stocks. Sheep and cattle were, also, used for isolation of Um Banein strain (Singa-Sudan) by Jongejan *et al.* (1984).

In this study, estimation of the intensity of *E. ruminantium* infection in *Amblyomma* tick homogenates and blood stabilates of experimentally infected goats was achieved by the Plexor® qPCR system. Nyala stock showed hyperacute form of heartwater. The load of pCS20 copies number in the infective dose material was \(5 \times 10^4/ml\) and the animal died on day 9 post infection while
Dinder stock on the other hand, showed an acute form of the disease and the animal died on day 14. The load of pCS20 copies number in the Dinder infective dose of Dinder was the lowest (1 x 10^4/ml). Abonama stock, however, showed a mild form of the disease, the animal survived and the load of pCS20 copies number in the infective dose was the highest (2 x 10^5/ml). The quantification of organisms in samples is important to determine the infectivity of in vitro cell cultures and infective blood that will be used as a challenge material.

The varying degrees of susceptibility and classification into hyperacute and acute as observed in goats in this study was, also, reported by some workers in other breeds of goats (Uilenberg, 1983; Losos, 1986 and Okaiyeto et al., 2009). The successful in vivo isolation of E. ruminantium new stocks (Dinder, Abonama and Nyal) confirmed the wide distribution of the disease in different localities in the Sudan. However, the new E. ruminantium stocks showed different heartwater forms and this thought to be due to a wide diversity of E. ruminantium stocks that simultaneously circulates in different localities of endemic areas in the Sudan.

The current study investigated the epidemiology of heartwater using the methodology of conventional epidemiology coupled with the advanced molecular techniques. The study defined the spatial distribution and prevalence of E. ruminantium in the vector population and domestic ruminant hosts and provided an accurate situation of the potential risk of heartwater that pose livestock in the endemic regions in the Sudan. This study has also paved the way for a complete investigation of the epidemiology of heartwater in the Sudan.
Recommendations

The epidemiological data and isolation of \textit{E. ruminantium} and their analysis presented in this study have raised major areas of focus for research that are relevant and important for issues which may form the basis for further studies to control heartwater in the Sudan:

i- to conduct large-scales Serological surveys complemented by molecular epidemiology of heartwater in the hosts and vectors population to assess the potential risk of the disease throughout the Sudan. These surveys will result in the identification and mapping of risk areas of heartwater thus facilitating the harmonization of heartwater disease control policies in the country.

ii- It is also recommended to carry out \textit{in vivo} and \textit{in vitro} isolation of \textit{E. ruminantium} new stocks from different endemic areas in the Sudan and to carry out cross-protection studies to identify best vaccine candidates.

iii- It is further recommended to elucidate genetic diversity of \textit{E. ruminantium} in the Sudan using phylogenetic analysis among \textit{E. ruminantium} stock sequences to determine where recombination takes place, whether it is in the vector or in the host.

iv- Further research should be aimed at developing a heartwater vaccine.

v- Indigenous small ruminants, sheep and goats, in comparison to local cattle, are generally more susceptible to heartwater (Yunker, 1996), and therefore should be given particular attention in heartwater epidemiology. In addition studying the epidemiology of heartwater in camels and wild animals should be undertaken.
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