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Molecular Detection of *Ehrlichia ruminantium* in Ruminants in Al Gezirah State, Sudan

By

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DEDICATION

To
my parents,
sisters,
and brothers
with love

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Abstract

The objective of this study was to determine the existence of heartwater disease in ruminants at the central parts of Al Gezirah State (central Sudan). This was accomplished by using PCR technique to detect the DNA of the causative agent, *Ehrlichia ruminantium*, in blood collected as spots on filter paper. The samples were collected from three locations in the State: Tambool, El Hasaheissa and Madani. A total of 170 samples were collected, 100 from sheep, 40 from goats, 20 from cattle and 10 from camels. The DNA was extracted by using the phenol-chloroform method. Using the specific primers (HH1F and HH2R) for amplification of the target sequence (980 bp fragment) of pCS20 gene of *E. ruminantium*, only three animals were positive and were sheep. Two of them were from Tambool area and were males of Dobasee breed and the third was from El Hasahiessa area and it was a female of cross breed. This positive result represents only 1.8 % of the total number of animals investigated and 3.0 % of the total number of sheep. The two positive cases from Tambool counted for 4.4 % of number of sheep form this area and the one positive case from El Hasahiessa counted for 2.9 % of sheep from this locality. It was concluded that the heartwater disease exists in the central parts of Al Gezirah State.

المستخلص

الغرض من هذه الدراسة هو التحقق من وجود مرض القلب المائي (الخدر) في المجترات في الأجزاء الوسطى من ولاية الجزيرة (وسط السودان). تم انجاز ذلك باستخدام تقنية التفاعل البولمريزي المتسلسل لاكتشاف الدنا (الحمض النووي منقوص الاوكسجين) لمسبب المرض، *ارليخيا المجترات* من الدم المأخوذ كبقع علي ورق الترشيح. جمعت 170 عينة دم من ثلاث مناطق بالولاية: تمبول، الحصاحيصا و ود مدني: 100 من الضان و40 من الماعز و20 من البقر و 10 من الإبل. استخدمت طريقة الفينول-الكلوروفورم لاستخلاص الحمض النووي. باستخدام بادئات خاصة (HH1F و HH2R) لتضخيم التسلسل المستهدف (شدفة بحجم 980 زوج قاعدي) من الجين pCS20 *لارليخيا المجترات*، كانت فقط ثلاثة حيوانات موجبة وكانت من الضأن، اثنتان منها من منطقة تمبول وهي ذكور من السلالة الدباسية و الثالثة من منطقة الحصاحيصا وهي أنثى من سلالة هجين. مثلت هذه النتيجة الموجبة 1.8 % فقط من العدد الكلي للحيوانات التي فحصت و 3.0 % من العدد الكلي للضأن. بلغت نسبة الإصابة في الضأن بمنطقة تمبول 4.4 % وبمنطقة الحصاحيصا 2.9 %. خلصت هذه الدراسة إلى أن مرض الخدر موجود بوسط ولاية الجزيرة.

INTRODUCTION

Tick-borne diseases and their vectors are most important constraint to livestock development in Africa (Norval *et al*., 1983). Heartwater (cowdriosis) is the major rickettsial infection of ruminants and the second most important tick-borne disease after East Coast Fever in Africa (Mahan *et al*., 1995).

Heartwater disease is an infectious, non contagious and fatal disease of domestic and wild ruminants (Peter *et al*., 2002). The disease is caused by an obligate intracellular bacterium, *Ehrlichia ruminantium*, previously *Cowdria ruminantium* (Dumler *et al*., 2001). Vascular endothelial cells and to a lesser extent leukocytes are primarily infected (Logan *et al*., 1987). It is transmitted by ticks of the genus *Amblyomma* (Uilenberg, 1983). The distribution of heartwater coincided with the distribution of the vector and the disease is endemic in the sub-Saharan Africa and Caribbean island (Jongejan and Uilenberg, 2004). Cowdriosis has a significant economic importance, particularly where livestock improvement projects are established (Camus *et al*., 1996).

Economic impact of the disease falls into two categories: disease related mortality and non lethal losses (Burridge *et al*., 2000). Cowdriosis mortality rates ranging from 20 to 90% in susceptible ruminants (Mahan *et al*., 1998b). A study in Zimbabwe evaluated the estimated total annual losses due to heartwater, US $ 5.6 million annually (Mukhebi *et al*., 1999).

In the Sudan, the disease is known as *Khadar* and it was first reported in sheep and goats in eastern Sudan associated with presence of the tick A. *lepidum* (Karrar, 1960). In western Sudan, A. *variegatum* was incriminated to transmit the disease (Abdel Wahab *et al.*, 1998). The disease is endemic in eastern, central, western and southern Sudan and no *Amblymma* ticks were identified in northern Sudan (Abdel Rahman,

2005). Serologically, prevalence of *E. ruminantium* antibodies was 58.9% in sheep in eastern Sudan (Abdel Rahman *et al*., 2003) and 76.6% in sheep in Blue Nile State (Sayed, 2004). A prevalence of 69% in sheep, 75.2% in goats and 38.7% in cattle was reported in Sudan (Abdel Rahman, 2005).

Depending on the age, immune status, individual or breed susceptibility of the animal and virulence of the isolate, the course of heartwater disease ranges from mild to peracute (Alexander, 1931; Van de Pyepkam and Prozesky, 1987). Presumptive diagnosis of heartwater in living animals is usually based on clinical signs, epidemiological observations and presence of *Amblyoma* vector (Prozesky, 1987a). However clinical signs are not pathognomonic (Camus and Barre*,* 1987; Van Viliet *et al.,* 1995). In dead animals, tentative diagnosis could be made by presence of transudations in the pericardium and thorax at postmortem, but definitive diagnosis requires the demonstration of *E. ruminantium* colonies in crushed smears (Peter *et al*., 2002). The frequent lack of characteristic clinical signs, and sometimes a total absence of lesions, makes the diagnosis of the disease particularly difficult (Camus *et al*., 1996).

Detection and identification of *E. ruminantium* in live animals is obviously difficult by conventional methods such as direct blood smear examination, indirect immunofluorescence, and isolation of the organism in cell culture or using histochemical stain techniques (Abdel Rahman, 2005). Therefore, simple, sensitive and specific discriminating techniques are required at species or strain level. Successfully, Van Viliet *et al*. (1995) developed a sensitive recombinant major antigen protein (MAP1) of *E. ruminantium* for indirect antibody ELISA. However, molecular diagnosis is presently the most characterized and reliable approach for *E. ruminantium* in carrier animals or ticks which are

carrying low levels of infection and thus is highly useful for field and laboratory epidemiological investigations of heartwater (Allsop *et al*.,2001). It included DNA hybridization (Mahan *et al*., 1992), polymerase chain reaction (PCR) (Peter *et al*., 1995) and reverse line blot (RLB) (Bekker *et al.,* 2002).

The aim of this study was to collect blood samples on filter paper, as a simple method of blood collection from ruminants at Al Gezirah State and to extract the DNA for detection of *E. ruminantium* using PCR technique.

CHAPTER ONE
Literature Review

1.1 Heartwater

1.1.1 Definition

Heartwater (cowdriosis) is an acute, febrile infectious and often fatal tick-borne disease of domestic and wild ruminants, caused by the rickettsia, *Ehrlichia ruminantium* and transmitted by ticks of the genus *Amblyomma* (Uilenberg, 1983; Semu *et al*., 2001).

The disease was observed for the first time in 1837 in South Africa (Provost and Bezuidenhout, 1987). It is characterized by a sudden high fever often exceeds 41˚C. Death is common within a week after the appearance of clinical signs, which include acute gastroenteritis, hydro-pericardium, hydrothorax, and respiratory distress.

1.1.2. Epidemiology

1.1.2.1. Distribution of heartwater

Heartwater occurs only where the vector ticks of the genus *Amblyomma* are present (Provost and Bezuidenhout, 1987). The disease is endemic in the sub-Saharan Africa and Caribbean islands (Uilenberg, 1983). In Sudan, heartwater is endemic in eastern, central, western and southern parts of the country, while no vector tick of the genus *Amblyomma* was identified in northern Sudan (Abdel Rahman, 2005). The disease was confirmed in sheep and goats in the White Nile province (Karrar, 1968), in goats at Um Banien (Jogejan *et al*., 1984) and in cattle in South Darfur (Abdel Wahab *et al*., 1998).

1.1.2.2 Host

All the domestic representatives of the family Bovidae are susceptible to clinical disease, namely sheep, goats, cattle and Asian buffalo (Mammericky, 1961). Nevertheless, susceptibility varies within animal breeds and for example, *Bos taurus* is more susceptible than *Bos indicus* (zebu cattle) (Uilenberg, 1983). Several wild animal species have been implicated as host of *E. ruminantium* (Oberem and Bezuidenhout, 1987; Peter *et al*., 2002). Infection has been proven in 12 African wild ruminants, three non African wild ruminants and two African rodents.

1.1.2.3 Transmission and vectors of heartwater

Heartwater is transmitted by ticks of the genus *Amblyomma*. The hardiness and exceptional longevity of *Amblyomma* species make them an excellent reservoir of heartwater. The organism can persist in the tick host for 15 months (Ilemobade and Blotkamp, 1976). Depending on the season and locality, infection rates in ticks vary from 0-44.9% in males, 20-36.1% in females and 0-13.4% in nymphs (Du Plessis, 1982; Norval *et al*., 1990). There are 13 *Amblyomma* species known to transmit *E. ruminantium* (Uilenberg, 1982; 1983; Jongejan, 1992). Ten species are African *Amblyomma* ticks: *A. variegatum, A. hebraem, A. pomposum, A. tholloni, A. gemma, A. lepidum, A. sparsum, A. astrion, A. cohaerence* and *A. marmoreum* (Uilenberg, 1983) and three American species of *Amblyomma*; *A. cajennense, A. maculatum* (Uilenberg, 1982; 1983) and *A. dissimile* (Jongejan, 1992).

E. ruminantium is transmitted transtadially but not to next generation (transovarially) of ticks (Bezuidenhout, 1987a). *Amblyomma* spp. are three-host tick. Larvae and nymphs become infected when feed on domestic and wild ruminants at time when *E. ruminantium* is circulating in the blood of the host. After replicating in the host, *Amblyomma*

nymphs transmit the organism to susceptible host (Jongejan and Uilenberg, 1994). The minimum period required for transmission of the parasite after tick attachment is 27-38 hours in nymphs and 21-75 hours in adults (Bezuidenhout, 1987a). The incubation period in naturally infected cattle is from 9 to 29 days (average 18) and that in sheep and goats is 7 to 35 days (average 14) (Uilenberg, 1983). Incubation period is shorter (4 days) when infected culture is used (Bezuidenhout *et al*., 1985). Incubation period depends on route of infection, infecting dose, species of affected animal and its susceptibility, and virulence of isolate (Van de Pypekamp and Prozesky, 1987).

In Sudan, *A. lepidum* was reported from central and eastern Sudan (Karrar *et al*., 1963) and *A. variegatum* was incriminated to transmit the disease in western part of the country (Table 1) (Musa *et al*., 1996)

Table 1: *Amblyomma* spp. identified from different geographical locations in the Sudan.

Location	***Amblyomma* spp. identified**	**Geographical location**
1- Kassala (E/S)	*A. lepidum*	15˚13˙N 35˚51˙E
2- Halfa Eljadida (E/S)	*A. lepidum*	15˚19˙N 35˚ 35˙ E
3- Gadarif (E/S)	*A. lepidum*	14˚02˙N 35 ˚22˙ E
4- El Showak (E/S)	*A. lepidum*	14˚37˙N 35 ˚45 ˙ E
5- Tambool (C/S)	*A. lepidum*	14˚52˙N 33˚31 ˙ E
6- Singa (C/S)	*A. lepidum*	13˚09˙N 33˚55 ˙E
7- Abu Naana (C/S)	*A.lepidum*	12˚48˙N 33˚59 ˙E
8- Dindir (C/S)	*A. lepidum*	13˚21˙N 24˚28 ˙E
9- Um Banien (C/S)	*A. lepidum*	13˚16˙N 33 ˚55 ˙E
10- Rabak (C/S)	*A. lepidum*	13˚10˙N 32˚44 ˙E

Table 1: (Contd.)

11- Kadugli (S/K)	*A. variegatum* *A. lepidum*	11˚16˙N 29˚12˙E
12- Dallang (S/K)	*A. lepidum*	12˚07˙N 29˚51 ˙E
13- Abu Karshol (S/K)	*A. variegatum* *A. lepidum*	12˚08˙N 30˚47 ˙E
14- Nyala (W/S)	*A. variegatum*	12˚08˙N 24˚46 ˙E
15- Tulus (W/S	*A. variegatum*	10˚58˙N 23 ˚58 ˙E
16- Buram (W/S)	*A. lepidum*	10˚52˙N 24 ˚50 ˙E
17- Pibore (S/S)	*A. lepidum*	7˚ 35 ˙N 33˚14 ˙E
18- Juba (S/S)	*A. variegatum*	5˚56˙N 31˚ 31˙E
19- Chukudum (S/S)	*A. lepidum*	4˚ 21˙N 32˚ 12˙E
20- Bentio (S/S)	*A. variegatum*	10˚14˙N 22˚12 ˙E

Source: Abdel Rahman (2005)

Key: E/S = Eastern Sudan, C/S = Central Sudan, S/K = Southern Kordofan, W/S = Western Sudan, S/S = Southern Sudan

1.1.2.4 Immunology

Interferon gamma (IFN-γ) is considered as a key factor in protection against heartwater of ruminants. A better definition of the molecular masses of IFN-γ inducing proteins of the Gardel strain of *E. ruminantium* was obtained by the use of continuous flow electrophoresis (CFE) and sensitized polyclonal lymphocytes. Out of 15 *E. ruminantium* CFE fractions tested within the 14–39 kDa region, eight were commonly reacted to by all goats. Interestingly, half of these fractions fall within the 23–29 kDa region, which was shown previously to contain polymorphic B-cell epitopes (Esteves *et al.*, 2004). Thus, the region also contains T-cell epitopes potentially involved in protection. Also, several proteins were found to be more immunogenic than the serologically

immunodominant MAP1 protein. *E. ruminantium* induced IFN-γ secretion was observed in 24h stimulated blood. Flow cytometric analysis of stimulated peripheral blood mononuclear cells (PBMCs) collected after each infection inoculation indicated that immune CD4 and CD8 T cells contribute to the same extent to the production of IFN-γ. This was confirmed by blocking the secretion of IFN-γ with anti-classes I and II major histocompatibility complex antibodies (Esteves *et al*., 2004). Blocking experiments also suggests that CD8 needs the help of CD4 T cells in order to produce IFN-γ. Thus, underling the key role of CD4 T cells in the production of IFN-γ by immune goat PBMC. It also describes, for the first time in ruminants, *E. ruminantium*-specific CD8 effector T cells. Since CD4 and CD8 T cells collectively contribute to the production of IFN-γ in most vaccinated animals, these responses are associated with protection (Esteves *et al*., 2004). Antibodies starts to appear from 12 to 24 days post infection and remain detectable between 100 to 200 days in sheep, 100 days in goats and for shorter period (50 to 100 days) in cattle infected with *E. ruminantium* (Van Vileit *et al*., 1995).

1.1.3 *Ehrlichia ruminantium*

Ehrlichia ruminantium (formerly *Cowdria ruminantium*) is an obligate intracellular bacterium (rickettsia) that primarily infects vascular endothelial cells, leukocytes and also occurs in the blood (Dumler *et al*., 2001). It is transmitted by ticks of the genus *Amblyomma* (Uilenberg, 1983). The American scientist Cowdry was the first who fully described the pathogenic agent as *Rickettsia ruminantium* in host and tick vector (Cowdry, 1925). *E. ruminantium* was first successfully cultivated *in vitro* in calf endothelial cell line in 1985 (Bezuidenhout *et al*., 1985). Until 2005, 56 strains of *E. ruminantium* have been identified worldwide

(Abdel Rahman, 2005). In Sudan there are two strains, Um Banien strain (Jongejan *et al*., 1984) and Gadarif strain (Muramatsu *et al.,* 2005).

The nucleotide sequences of *E. ruminantium* from *A. variegatum* ticks from Juba and *A. lepidum* ticks from Gadarif using same gene were identical (Muramatsu *et al.,* 2005) (gene bank accession no. 218277) and they were similar to those of Walgenvendon, Vosloo and Ball 3 strains from South Africa and Caribbean islands (similarity = 99.64%), but different from that of Western Africa based on the 855 *map*1 gene. The nucleotide sequence obtained from *A. lepdium* ticks from Gadarif State was closely related to those of Senegal and Pokoase strains from Western Africa and to the South Africa canine and Kumml strains (similarity = 90.53%-97.93%) and lesser relationship was showed (84.8%) with that of Um Banien strain (Muramatsu *et al.,* 2005).

1.1.3.1 Morphology

Ehrlichia ruminantium is pleomorphic with small coccoid forms and large ring, horseshoe shape and bacillary shape and a diameter that varies from 0.2 to 2.5 μm (Pinaar, 1970; Prozesky 1987a). The ultra structural morphology of *E. ruminantium* consisting of a wall, made up of 2 membranes, and internal structure consisting of electron-dens and electron-pale areas (Pinaar, 1970). *E. ruminantium* is Gram negative and stains purplish–blue with Giemsa, the organism replicates mainly by binary fission and is non motile (Prozesky, 1987b; Camus *et al*., 1996). Most often, *E. ruminantium* occurs in colonies varying in number (from one to several thousands) in the cytoplasm of endothelial cells of mammalian host (Camus *et al*., 1996).

1.1.3.2 Taxonomy

The organism was traditionally classified as the sole member in the genus *Cowdria* in tribe *Ehrlichieae*, family *Rickettsiaceae*, order *Rickettsiales*, which were previously placed in taxa based upon morphological, ecological, epidemiological and clinical characteristics (Weiss and Moulder, 1984). Phylogenetic tree inferred from 16S rRNA sequences clearly demonstrated the existence of three major taxa (genogroup I, II, III) within the *Ehrlichia.* All *Cowdria* genotypes are closely related within the genogroup that include *Ehrilichia canis* and *E. ewingii* (Allsopp *et al*., 1996; Walker and Dumler, 1996). According to the biological, molecular and antigenic characteristics, tribes of family Rickettsiacae were eliminated. Memders of the tribes *Ehrlichieae* and *Wolbachieae* were transferred to the family anaplasmataeceae and the genus *Ehrlichia* was amended to include the new species, *Ehrlichia ruminantium.* Now the classification of *E. ruminantium* is: Order: *Rickettsiales*, family: *Anaplasmataeceae*, Genus: *Ehrlichia* and species: *Ehrlichia ruminantium* (Dumler *et al*., 2001).

1.1.3.3 Pathogenecity

Various strains (isolates and stocks) of *E. ruminantium* that vary in their virulence have been isolated (Jongejan, 1990). Heartwater disease development usually depends on the virulence of the isolate and species, breed, degree of natural resistance, immune status and age of the animal (Alexander, 1931; van de Pypekamp and Prozesky, 1987).

1.2.3.4 Life cycle of *Ehrlichia ruminantium*

Initial replication of the organism takes place in the intestinal epithelium of ticks and that the salivary gland eventually become parasitized. Feeding of an infected tick on susceptible vertebrate host

results in transfer of the organism into the blood stream of the host (Kocan and Bezuidenhout, 1987). The organism initially replicates in reticulo–endothelial cells and macrophages in regional lymph nodes and then released into the efferent lymph stream and eventually into the blood stream where endothelial cells are parasitized. Demonstrated developmental stages resemble those of the chlamydial species. Extracellular elementary body (EB) adheres to, and invades an endothelial cell, it then transformed into a metabolically active intracellular stage, the reticulate body (RB) (Jongejan *et al*., 1991). Chromosomal DNA replicates mainly by binary fission and possibly by endosporulation (Pinaar, 1970). After 5-6 days of infection, large number of elementary bodies are released due to rupture of endothelial cells to initiate a new infectious cycle.

1.1.4 Pathogenesis

The pathogenesis is still poorly understood, but there are some hypotheses that have been proposed. After infection of the host with *E. ruminantium*, initial replication of organism in reticulo-endothelial cells and macrophages in the regional lymph nodes, the organism disseminates via the blood stream invading endothelial cells of blood vessels of various organs where its multiplication occurs. Cerebrocortical capillaries are particularly invaded (Du Plessiss, 1970; Hirsh *et al*., 2004).

Increased vascular permeability allowing the seepage of plasma protein which results in transudation through serous membranes with a result of oedema and effusion into body cavities which causes the drastic fall in blood volume before death (Brown and Showronek, 1990; Prozesky, 1987). Oedema of the brain is responsible for the nervous signs, while hydropericardium contributes to cardiac dysfunction during the terminal stages of the disease and progressive pulmonary oedema and

hydrothorax result in asphyxiation (Owen *et al*., 1973; Camus *et al*., 1996). The pathogenesis of vascular permeability remains speculative as the intracytoplasmic development of the organism seems have little detectable cytopathic effect upon the endothelial cells (Pinaar, 1970). There is also no apparent correlation between the number of parasitized cells in the pulmonary blood vessels and severity of pulmonary oedema. It has also been proposed that an endotoxin and increased cerebrospinal fluid pressure play a role in the development of lung oedema (van Amsted *et al*., 1987).

1.1.5 Diagnosis

Diagnosis of heartwater depends on clinical signs, postmortem change, laboratory microscopic examination, isolation of organism, serological tests and molecular diagnosis (Alexander, 1931; Camus *et al*., 1996; Waghela *et al*., 1991).

1.1.5.1 Clinical signs

The incubation period is 1-3 weeks after transmission in tick saliva. Depending on the susceptibility of individual animals, age, immune status and the virulence of the infecting organism, the resulting disease may be peracute, acute, subacute, mild or inapparent (Alexander, 1931; van de Pypekamp and Prozesky, 1987).

Peracute cases show only high fever (over 40˚C), prostration and death with terminal convulsions in 1-2 days. Acute cases are more common, of about 6 days with sudden febrile reaction which is accompanied by inappetence, listlessness and rapid breathing, and followed by the classical nervous syndrome that is characteristic of heartwater, including chewing movement, twitching of the eyelids, recumbancy, diarrhoea and convulsions. Subcute cases are less severe but

may terminate in death in two weeks or the animal gradually recover. Mild form is subclinical, seen in indigenous animals and wild ruminants with high natural or induced resistance (Mahan *et al*., 1998a; van de Pypekamp and Prozesky, 1987).

Differential clinical diagnosis should be made with bovine cerebral babesiosis, theileriosis, anaplasmosis, botulism, anthrax, rabies, small ruminant PPR, malignant ovine theileriosis, haemonchosis, meningitis, encephalitis, plant poisoning and pesticides (chlorinated hydrocarbons and organic phosphate) poisoning (Van de Pypekamp and Prozesky, 1987).

1.1.5.2 Post-mortem examination

In almost all cases of heartwater, hydropericardium and hydrothorax are predominant pathological changes (Neitz *et al.*, 1986). The pericardial and thoracic fluids are yellowish in colour and sometimes slightly blood tinged. The fluid, clots few seconds after opening the thorax or the pericardium, and become jelly-like in appearance. Other gross lesions include splenic tumor, edema of lung and bronchi. The liver engorges and gall bladder distends with bile. Lymph nodes show swelling and are hyperaemic and the heart muscles show small petechial haemorrhages (Shommein and Abdel Rahim, 1977).

1.1.5.3 Isolation

1.1.5.3.1 *In vitro* isolation

In-vitro isolation of *E. ruminantium* is necessary for diagnosis, typing of strains, epidemiological, and immunological studies and vaccine production. Numerous attempts have been made to cultivate the organism *in-vitro*. Jongejan *et al*. (1989) reported one of these attempts and the organism was persistent for a period of 13 days in primary kidney

culture of goats. Chicken embryo (Haig, 1952) and primary cell culture from *A. hebraem* (Alexander, 1946) remain infective only for 9 days after inoculation. However, all these trials were of limited success or failed (Uilenberg, 1983). *E. ruminantium* can be isolated from the blood of a reacting animal by cultivation on endothelial cells from umbilical cord, aorta, or the pulmonary artery of different ruminant species (cattle, sheep and goats) (Bezuidenhout *et al*., 1985; Bezuidenhout, 1987b). Other endothelial cells have been described for routine culture of the microorganism, such as brain capillaries, circulating endothelial cells, as well as cultivating *E. ruminantium* in neutrophils (Logan *et al*., 1987). *E. ruminantium* has also been propagated for over 500 days in *Ixodes* tick cell line (Bell-Sakyi *et al*., 2000).

Ehrlichia ruminantium can be propagated in baby hamster kidney (BHK) cells, Chinese hamster ovary (CHO-K1) cells and Madin Darby bovine kidney (MDBK) cells (Zweygarth and Josemans, 2003). Endothelial cell lines of African mammal species were also successfully infected by *E. ruminantium* (Smith *et al*., 1998). The organism from plasma or blood of clinically febrile animals is routinely isolated in these endothelial cultures (OIE, 2002).

Bacterial growth was monitored by microscopic examination of Giemsa-stained cytocentrifuged smears. When *E. ruminantium*-infected cultures showed about 90% of cytolysis due to infection, the remaining adherent cells were scraped from the bottom of the culture flasks and the cultures centrifuged and cry preserved (Jongejan *et al*., 1991).

1.1.5.3.2 *In vivo* isolation

This method is suitable to assess the presence of heartwater in a herd, a region or a country or to isolate a strain of *E. ruminantium* by inoculating blood or tick supernatant into susceptible animal (OIE, 2002).

The efficacy of material used as source of E. *ruminantium* depends on the heartwater isolate, immune status of the donor animal, bacteria concentration in infective material, time of collection of infected material after infection and species of susceptible animal (Prozesky, 1987b; Camus *et al*., 1996).

Different stocks of the organism behave differently in laboratory animal; some are neither infective nor pathogenic, others are able to survive for over a year without being pathogenic (Camus *et al*., 1996).

1.1.5.4 Serological tests

Serological tests that have been developed for cowdriosis are based on detection of antibodies by immunofluorescence or by indirect enzyme-linked immunosorbent assay (iELISA) with *in vitro*-cultured *C. ruminantium* organisms. However, cross-reactivity between *C. ruminantium* antigens and antibodies to several *Ehrlichia* spp. has been observed (Jongejan, 1991a). In addition, a competitive ELISA (cELISA) and an immunoblotting technique have been developed (Jongejan and Bekker, 1999). These tests are based on an immunodominant 32-kDa surface protein of *E. ruminantium* designated as major antigenic protein, MAP1 formerly known as Cr32 (Jongejan, 1991b). This protein is conserved within all tested *E. ruminantium* isolates originating from different geographic regions, although its size varies when it is tested under certain electrophoretic conditions. However, this gene is conserved amongst all *E. ruminantium* isolates tested. The gene for the immunodominant 32 kDa protein has been cloned recently. Analysis shows that this protein varies structurally between different *E. ruminantium* isolates.

Several immunogenic proteins of *E. ruminantium* have also been identified and monoclonal antibodies have been developed to some of

them. The gene for the 21 kDa *E. ruminantium* protein has been cloned, characterized, sequenced and expressed to high levels to produce a recombinant analogue.

Recombinant protein analogues will have application in vaccine studies and subunit enzyme linked immunosorbent assay (ELISA) developments. A 32 kDa protein specific competitive ELISA is available and a 21 kDa protein specific direct and cELISA was being developed (Jongejan *et al*., 1991). At present monoclonal antibodies are available for the 21 and 32 kDa proteins.

1.1.5.4.1 Serologic Detection of *E. ruminantium* antigen from tissues

First serological test for *E. ruminantium* diagnosis was capillary tube flocculation using acetone extracted brain material of infected goats and cattle (Ilemobade and Blotkamp, 1976). The short period of antibody detection limited the practical value of the test.

Complement fixation test utilized sucrose acetone extraction of infected calf brain and blood was also used (Du Plessis, 1982; Musisi and Hussein, 1985). The sensitivity and specificity of capillary tube flocculation and complement fixation are low (van Viliet *et al*., 1995). A dot immunobinding assay (DIA) is a rapid test and utilizes detergent extracted antigen of organisms placed on nitrocellulose strips which were then treated with primary antibody conjugate (van Viliet *et al*., 1995). Infected neutrophils as source of antigen were reported (Logan *et al*., 1986). Detection of false positive case due to cross-reaction with *Ehrlichia* species was reported (OIE, 2002).

Infected peritoneal mouse macrophage as a source of *E. ruminantium* antigen for the indirect fluorescent antibody test was used. The test was based on kömm strain of south Africa (Du Plessis, 1981). Its difficulty is in producing large quantities of infected macrophages (Jongejan, 1991a).

1.1.5.4.2 Enzyme-linked immunosorbent assay (ELISA)

ELISA assay based on recombinant major antigenic protein (MAP1) has been developed (Jongejan, 1990; van Viliet *et al*., 1995). These include a competitive enzyme-linked immunosorben assay (cELISA) using MAP1 gene, cloned in the baculovirus, and monoclonal antibodies (Mabs) (Jongejan, 1990). The other one is an indirect ELISA using specific immunogenic region on the *E. ruminantium* (MAP-1B). Two immunogenic regions on the MAP1 protein of *E. ruminantium* were identified; namely MAP-1A and MAP-1B. MAP-1A was found reactive with antisera *E. ruminantium*, *E. ovina*, *E. bovis* and *E. plagocytophila*, while MAP-1B was reactive with *E. ruminantium*, *E. canis* and *E. chaffeensis*. The last two species don't infect ruminants (van Viliet *et al*., 1995).

A polyclonal competitive ELISA assay (PC-ELISA) was developed for detection of antibodies to *E. ruminantium* by using a soluble extract of endothelial cell culture-derived *E. ruminantium* as the antigen and biotion–labeled polyclonal goat immunoglobulins as the competitor (Sumption *et al*., 2003). The test format is simple, and the test is economical to perform and has a level of sensitivity for detection of low titer positive bovine sera that may prove to be of value in epidemiological studies on heartwater (Bell-Sakyi *et al*., 2000).

Overall specificity from 11 heartwater free Islands was 98.5% and 99.4% for the Map1-cELISA and MAP-1B ELISA respectively, but still a few false positive sera were detected (Mahan *et al*., 1998a; OIE, 2002). Despite the fact that MAP-1B ELISA showed improved specificity than other serological tests, it demonstrated low seropositivity in cattle in Zimbabwe (Mahan *et al*., 1998b), which may indicate down regulation of antibodies production against *E. ruminantium* antigen by these animals (Semu *et al*., 2001).

1.1.5.5 Molecular diagnosis

A new generation of assays which are based on identification of DNA sequence specific for the genome of *E. ruminantium* and characterization of the molecular level, using recombinant DNA and hybridization techniques has been introduced (Ambrosio *et al*., 1987; Wikins and Ambrosio, 1989).

1.1.5.5.1 Polymerase chain reaction (PCR)

The application of PCR using specific primers proved to be a reliable approach to the detection and identification of pathogenic *rickettsiae* within infected vector and animal host (Waghela *et al*.,1991). With the advent of the polymerase chain reaction, small numbers of the organism can be positively amplified (Figueroa and Buening, 1995).

Waghela *et al*. (1991) cloned two genes of *E. ruminantium*, namely; pCS20 and PCR9. pCS20 obtained from Crystal Springs strain (Zimbabwe) that was collected from *A. variegatum* which had 1,306 bp insert and PCR9 from Kiswami strain (Kenya) that had 754-bp insert. Three target genes are available now and have been used for detection of *E. ruminantium* by PCR and hybridization assays. These genes are; pCS20 gene, the major antigenic protein (map1) gene and the 16S ribosomal RNA gene (Waghela *et al*., 1991; Allsopp *et al*., 1997). The PCR greatly amplified the targeted 279 bp fragment of pCS20 using primers AB/128 and AB/129 (Peter *et al*., 1995). This gene was selected as a diagnostic probe to identify clinically sick animals and *E. ruminantium* in experimentally infected *A. hebraeum* and *A.variegatum* ticks with different strains (Mahan *et al*., 1992). Multigene families encoding for outer membrane proteins have been identified in the genus *Ehrlichia* and designated P28 (OMP-1) in *E. chaffeensis*, P30 in *E. canis*

and the major antigenic protein 1 (MAP1) family in *E. ruminantium* (Ohashi *et al*., 1998a, Ohashi *et al*., 1998b). Recently, the entire *map1* cluster of the Welgevonden isolate of *E. ruminantium* was characterised and shown to contain 16 genes located upstream of a *secA* gene (van Heerden *et al*., 2004). All the 16 genes were transcriptionally active when *E. ruminantium* was grown in bovine endothelial cells, whereas between 4 and 11 paralogs were found to be transcribed in *E. ruminantium*-infected tick cell lines (Bekker *et al*., 2005; van Heerden *et al*., 2004) and transcripts of only two out of three paralogs were detected in unfed infected adult *A. variegatum* ticks (Bekker *et al*., 2002). *E. ruminantium* DNA was detected by MAP1-PCR in blood and bone marrow samples of clinically healthy carrier wildlife ruminants free ranging in Zimbabwe (Kock *et al*., 1995). The 16S ribosomal RNA gene was amplified by PCR and subsequently cloned from several isolates of *E. ruminantium*. It has been used to elucidate the phylogenetic relationship of other rickettsials (Allsopp *et al*., 1997).

DNA probe hybridization has been identified for the diagnosis of *E. ruminantium* in ticks and animals and the technique is usually used to confirm PCR findings. The assay was utilized to determine the prevalence of *E. ruminantium* in nymphs and adults of *A. hebraeum* from two heartwater – endemic areas in Zimbabwe (Peter *et al*., 1999).

A cloned nucleic acid probe (DNA probe), pCS20 that recognizes all strains of *E. ruminantium* was developed by Waghela *et al*. (1991) from the DNA of the crystal spring heartwater strain from Zimbabwe. The probe detected *E. ruminantium* in DNA preparation from plasma and *in vitro* culture from infected animal before and during the febrile stages as well as from *Amblymma* ticks (Mahan *et al*., 1992; Yunker *et al*., 1993; OIE, 2002).

1.1.5.5.2 A quantitative real-time PCR assay for *E. ruminantium*

A pCS20 quantitative real-time PCR TaqMan probe assay to detect *E. ruminantium* in livestock blood and ticks from the field was developed (Steyn *et al*., 2008). The assay is based on the conserved pCS20 gene region of *E. ruminantium* that contains two overlapping genes, rnc and ctaG. The genome of *E. ruminantium* contains multiple tandem repeats of actively variable copy number. The pCS20 quantitative real-time PCR TaqMan probe was compared to the currently used pCS20 PCR and PCR/^{32}P-probe test with regards to sensitivity, specificity and the ability to detect DNA in field samples and in blood from experimentally infected sheep (Peixoto *et al*., 2005). It showed that the pCS20 quantitative real-time PCR TaqMan probe was the most sensitive assay detecting seven copies of DNA/ml of cell culture. All three assays, however, cross react with *E. canis* and *E. chaffeensis* (Allsopp and Allsopp, 2001; Peixoto *et al*., 2005). The pCS20 real-time PCR detected significantly more positive field samples. Both the PCR and pCS20 real-time PCR could only detect *E. ruminantium* parasites in the blood of experimentally infected sheep during the febrile reaction. The PCR/^{32}P-probe assay, however, detected the parasite DNA 1 day before and during the febrile reaction. Thus, because this new quantitative pCS20 real-time PCR TaqMan probe assay was the most sensitive and can be performed within 2 h it is an effective assay for epidemiological surveillance and monitoring of infected animals (Peixoto *et al*., 2005).

1.1.5.6.3 Reverse line blot (RLB)

Reverse line blot combines hybridization and PCR that can differentiate between *Theileria* and *Babesia* on the basis of their differences in the 18S rRNA gene sequences. This has encouraged Bekker *et al*. (2002) to detect and differentiate *Anaplasma* and *Ehrlichia* spp. on the basis of the 16S rRNA gene differences. The essence of the technique is hybridization of

PCR product to species–specific probes immobilized on nitrocellulose membrane.

1.1.6 Prevention and control of heartwater

1.1.6.1 Tick control

Intensive tick control measures may, under certain conditions succeed in preventing outbreaks of heartwater, even in endemic areas, where the disease normally occurs and tick vector perminantely established (Bezuidenhout and Bigalke, 1987). Intensive dipping programmes may be applied, however, high frequency dipping may carry a high risk as far as the development of tick resistance to the dipping compound is concerned (Schröder, 1987). Genetic resistance to ticks with-breed variation could potentially be used for breed resistance.

Development of the combined use of acaricides and pheromones is believed to have a potential role in controlling ticks in a more selective manner and using less acaricide (De Castro, 1997). Alternatively, control measures adopted are immunization and or maintenance of endemic stability through strategic acaricides application. This approach implies that animal become naturally infected by ticks or immunized and this immunity is maintained by continuous tick challenge (Norval *et al*., 1995).

1.1.6.2 Vaccination

An animal that recovers from heartwater is protected against subsequent homologous challenge (Camus *et al*., 1996). A conclusive evidence that immunity to heartwater is T-cells mediated and that circulating antibodies play a minor role in immunity has been established (Du Plessis *et al*., 1991). Vaccination strategies are based on the initiation of infections with blood or culture derived organisms followed

by treatment with tetracycline (Jongejan, 1991b; Mahan *et al*., 1998a). The commercial vaccine available consists of the blood of sheep infected with live virulent *E. ruminantium* organism (Ball3 - strain) (Bezuidenhout, 1981). It produces high titer of *E. ruminantium* specific anibodies. The attenuated Senegal isolate confers a strong homologous protection in sheep and goats, but limited heterologous protection against geographically diverse isolates (Jongejan, 1992; Mahan *et al*., 1998a).

On contrary, a chemically inactivated *E. ruminantium* infected endothelial culture induces heterologous protection in sheep (Mahan *et al*., 1998a). A recombinant vaccine for *E. ruminantium* in mice made antibodies to MAP-1, but its capacity to produce heterologous protection remains to be tested (van Villiers, 2001).

1.1.6.3.Treatment

The most effective treatment of heartwater is the administration of tetracycline antibiotics (van Amstel and Oberem., 1987). Treatment is effective when given early and repeated administrations are preferable to single treatment (Ilemobade and Blotkamp, 1976). In goats, short-acting tetracyclines are administered at a dosage rate of 3 mg/kg body weight on 10, 20, 30, 45 and 60 days after introduction into an endemic area. Similarly, injection of a long acting tetracycline formulation (10-20 mg/kg body weight) given after introduction of cattle into endemic area on days 7, 14 and 21, or even on only two occasions (days 7 and 14) are sufficient to protect them from contracting heartwater, while at the same time allowing them to develop a natural immunity (Purnell, 1987). Animals treated in this manner will develop immunity to challenge with *E. ruminantium* (Oberem and Bezuidenhout, 1987; Norval *et al*., 1995). This finding forms the basis of the infection and treatment regimen that

used in South Africa. A successful therapy depends on timing, dose rate, formulation and route of administration (van Amstel and Oberem, 1987).

CHAPTER TWO
Materials and Methods

2.1 The study area

This study was conducted in Al Gazirah State (central Sudan) at three locations; Madani, El Hasahiesa and Tambool.

2.2 Samples

A total of 170 blood samples (as spots on filter paper) were collected in summer season from sheep, goats, cattle and camels from both sexes and all ages. Hundred samples were from sheep, 40 from goats, 20 from cattle and 10 from camels. According to localities, 70 samples were from Tambool, 52 from El Hasahiessa and 48 from Madani. Distribution of samples according to sex, age and breed are shown in Tables 2 to 5.

2.2.1 Collection method

A blood spot was collected from jugular vein by a sterile needle and spotted on filter paper making a circle of about 2 cm in diameter, triplicate spots were made from each animal. Spots were allowed to air dry and filter papers were individually sealed off in polyethylene bags and labeled to indicate locality, sample No., animal spp. and date of collection prior to storing at room temperature until being used. Age of animal, sex and breed were recorded in a separate sheet according to sample number.

Table 2. Distribution of ovine blood samples according to location, gender, age and breed

Location	Gender		Age			Breed		
	Male	**Female**	**6 months - < 1 year**	**1- 4 years**	**> 4 years**	**Cross breed**	**Hamaree breed**	**Dobasee breed**
Tambool	29	16	8	28	9	7	0	38
El Hasahiessa	25	10	7	22	6	7	3	25
Madani	15	5	3	13	4	9	4	7
Total	69	31	**18**	**63**	**19**	**23**	**7**	**70**

Table 3. Distribution of caprine blood samples according to location, gender, age and breed

Location	Gender		Age			Breed	
	Male	**Female**	**6 months -<1 year**	**1- 4 years**	**> 4 years**	**Nubian**	**Cross breed**
Tambool	5	10	4	7	4	15	0
El Hasahiessa	2	8	2	6	2	9	1
Madani	4	11	3	7	5	10	5
Total	**11**	**29**	**9**	**20**	**11**	**34**	**6**

Table 4. Distribution of bovine blood samples according to location, gender, age and breed

Location	Gender		Age			Breed	
	Male	**Female**	**6 months - <1 year**	**1-4 years**	**> 4 years**	**Botana**	**Cross breed**
Tambool	_	_	_	_	_	_	_
Hasahiessa	1	6	2	5	2	3	2
Madani	3	10	3	5	3	12	3
Total	**4**	**16**	**5**	**10**	**5**	**15**	**5**

Table 5. Distribution of cameline blood samples according to location, gender and age

Location	Gender		Age	
	Male	**Female**	**1 - 4 years**	**> 4 years**
Tambool	2	8	3	7

2.3 DNA extraction

The DNA from all blood samples was extracted by the phenol chloroform method (Herrmann and Frischauf, 1987) with some modification made by the Central Laboratory of Ministry of Science and Technology (unpublished data).

2.3.1 Procedure

The filter paper with blood spots was put on a dry clean rapper pad cleaned with 70% ethanol. Blood spots were punched out by a special tool for producing 4 circles of 3 mm diameter for each spot and the tool was sterilized by 70% ethanol after each puncturing. The punctured-out material containing blood was put into 1.5 ml eppendorf tube. A volume of 500 µl STE-buffer (Sodium Tri base boric EDTA) and 10 µl proteinase K (10 mg/ml) and 20 µl sodium dodecyl sulphate (SDS) 10% were added to the punctured-out blood spots. The mixture was then incubated over night at 56˚C in a water bath. The tubes were centrifuged at 9000 *g* for 5 min and all of the lysate (supernatant) was transferred into a phase lock gel tube. A volume of 500 µl phenol-chloroform–isoamylethanol (25:24:1 ratio) was added and the tube was centrifuged for 5 min at 9000 *g*. The supernatant was transferred into a new 1.5 ml eppendorf tube and volumes of 45 µl 3M sodium-acetate solution and 1 ml 99% ethanol (ice-cold) were added and the tube was let to stand for 15 min. The mixture was centrifuged at 9000 *g* for 10 min to precipitate the DNA. The supernatant was discarded and the DNA was washed by adding 200 µl 70% ethanol and then centrifuged at 9000 *g* for 10 min. This washing step was repeated once again. The liquid phase was discarded and the tube was left opened to dry the DNA. Then 30 µl distilled water were added to the DNA and the tube was stored at 20˚C. All steps of aseptic nature were conducted in an air laminar flow (BIOAIR instruments, Siziano, Italy).

2.4 Primers

The modified pCS20 oligonucleotides HHIF (5`-CCC TAT GAT ACA GAA GGT AAC CTC GC-3`) and HH2R (5`-GAT AAG GAG ATA ACG TTT GTT TGG-3`) were used. They were produced by 1sBASE Company, Malaysia. These primers were designed to amplify the 980 bp fragment of the pCS20 gene. The primers were delivered lyophilized. To prepare the primers for use, a volume of 143 µl of distilled water was added to the HH1F lyophilized oligonucleotide primer to make the working primer solution with 100 µM, and 328 µl of distilled water added for HH2R primer to make the working solution with a concentration of 100 µM (Table 6).

Table 6. Data sheet of primers used in this study

Primer	Sequence	Len.	Scale	Tm (C)	GC (%)	MW	OD	µ g	pmol.	Make 100 µM
HH1F	(5`-CCC TAT GAT ACA GAA GGT AAC CTC GC-3`)	26	50 nmole	66.2	50	7924	3.6	112.9	14252	Add 143 µl of d H_2O
HH2R	(5`-GAT AAG GAG ATA ACG TTT GTT TGG-3`)	24	50 nmole	59.4	37.5	7487	8.1	245.9	32847	Add 328 µl of d H_2O

Source: 1sBASE Company (Malaysia)

Len.= Number of nucleotides, **TM**= Annealing Temperature, **MW**= Molecular Weight, **OD**= Optical Density, **pmol.**= Picomole

2.5 Polymerase Chain Reaction

The PCR method of Peter *et al*. (1995) was adopted using the HH1F and HH2R primers).

Master mix. A master mix of reagents of 25 µl was used, which contained 200 µM of mix of dNTPs (dATP, dCTP, dGTP, dTTP), volume of dNTP in reaction 0.5 µl, 2.5 U Tag polymerase, 0.5 µl, 0.125 µl of each of oligonucleotide primer HHIF (5`-CCC TAT GAT ACA GAA GGT AAC CTC GC-3`) and HH2R (5`-GAT AAG GAG ATA ACG TTT GTT TGG-3`), 2.5 µl 10x PCR buffer, 2.5 µl $MgCl_2$ and 5 µl template DNA and 13.75 µl distilled water. The mixture (usually for 8 or 16 samples in 1.5 µl eppendorf tube) was vortexed thoroughly and then distributed as 25 µl volume into 0.5 ml PCR tubes.

PCR amplification. The PCR reaction was carried out in Primus Thermal cycler (PeQ Lab, Biotchnoloie, GmbH, Germany). PCR tubes were loaded into the machine, which was set at 94˚C for 10 minutes for initial denaturation followed by 35 cycles. Each cycle consisted of denaturation at 94˚C for 30 seconds, annealing at 62˚C for 45 seconds, and exraction at 72˚C for 30 seconds. After amplification the thermocycler was set at 72˚C for 6 min as extension period for the newly formed DNA and then held at 4˚C.

2.6.1 Gel electrophoresis

Gel prepration. 1.5gm of agarose (Sigma, UK) was dissolved and boiled in 100 ml TBE buffer (0.089 M Tris-hydroxymethyl, 0.089 M boric acid and 0.002 M EDTA). The mixture was left to cool to 65˚C and the gel was stained by 0.4 µg ethidium bromide (10 mg/ml). The solution was thoroughly mixed and poured on gel tray and left for 20 min to solidify.

PCR products were analyzed on the agarose gel (1.5%) and 10 µl PCR product mixed with 3 µl of 6X loading dye were loaded and run in electrophoresis apparatus for 1 hour at 90 V. The gel was visualized by UV light transilluminator (BIO-RAD Laboratories, Italy) and photographed by Polaroid camera. Appropriate controls including DNA molecular marker (1sBASE Company, Malaysia), positive control DNA of *E .ruminantium* Tambool (isolated by Abdel Rahman, 2005) and non–infected Nubian goat blood as negative control were included in each run.

CHAPTER THREE

Results

3.1 PCR Results

Out of 170 DNA extracts from blood of 100 sheep, 40 goats, 20 cattle and 10 camels only three samples (1.8 %) of sheep blood were positive by PCR assay using the modified primers (HH1F and HH2R) for detection of pCS20 gene of *E. ruminantium*. The size of the bands of the PCR products of these three sample were exactly matching the size of 980 bp amplified fragment of the target gene (Figs. 1 and 2).

Two of the three positive sheep were from Tambool area (males of Dobasee breed) and the third was from El Hasahiessa area (female of a cross breed). The two positive cases from Tambool accounted for 4.4 % of number of sheep (45) from this area and the one positive case form El Hasahiessa accounted for 2.9 % of number of sheep (35) from this locality (Tables 7 and 8).

3.2 Epidemiological observations

During the period of samples collection, it was observed that animals of Tambool area were most infested by ticks, followed by El Hasahiessa area and the least infested was Madani area. Amongst the ticks observed there was *Amblyomma lepidum*.

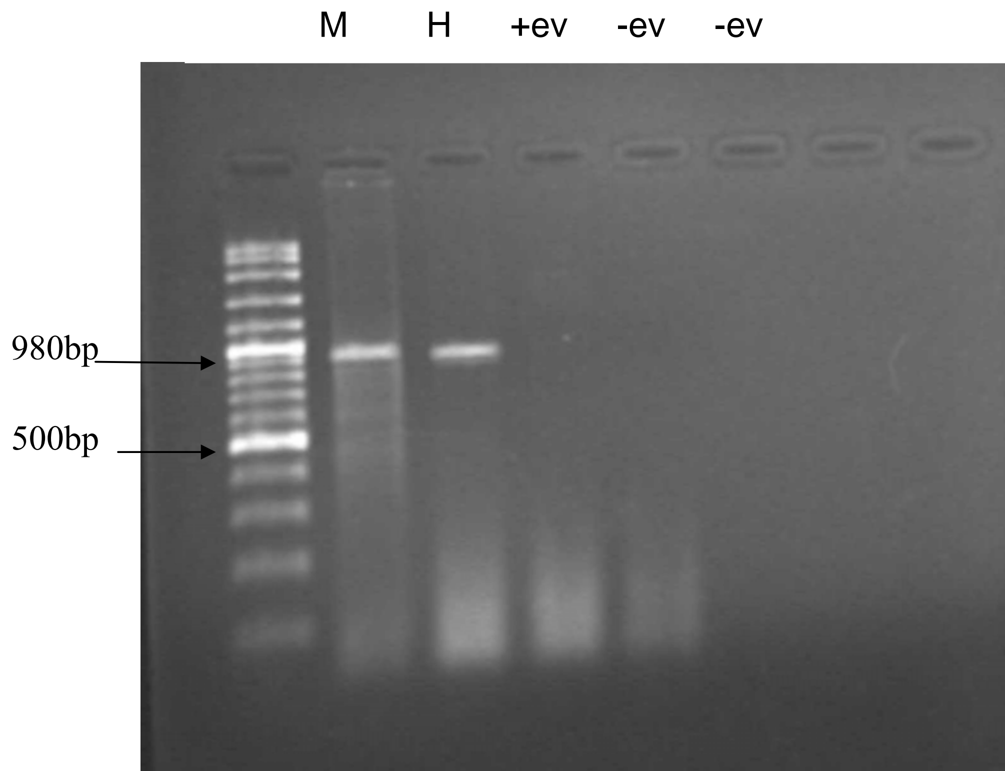

Fig.1. PCR detection of *E. ruminantium* from blood of a sheep from El Hasahiessa area.

Lane M, molecular marker (1500bp); lane H, a positive result for *E. ruminantium* DNA from a sheep naturally infected; lane +ev, positive control; lane –ev, negative control

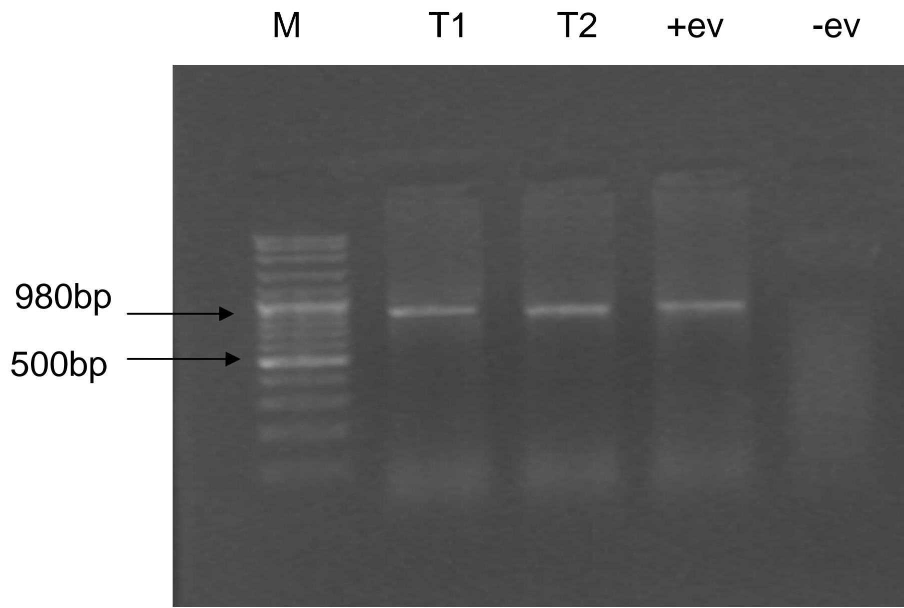

Fig.2. PCR detection of *E. ruminantium* from two naturally infected sheep from Tambool area.

Lane M, molecular marker 1500bp; lane T1, a positive result for *E. ruminantium* DNA from one sheep; lane T2, a positive result for *E. ruminantium* DNA from the a second sheep; lane +ev, positive control; and lane –ev, negative control.

Table 7. Prevalence of *E. ruminantium* in ruminants from three locations in Al Gazirah State using PCR technique

Location	**No. of animals tested**	**No. of positive animals**	**Percentage of positive cases**
Tambool	70	2	2.9
El Hasahiessa	52	1	1.9
Madani	48	0	0.0
Total	170	3	1.8

Table 8. Distribution of *E. ruminantium*-positive sheep from Al Gazirah State according to location, gender, age and breed

Location	**Gender**		**Age**			**Breed**		**% from total no. of sheep at specific location**
	Male	Female	6 months – <1 year	1-4 years	4-7 years	Cross	Dobasee	
Tambool	2	0	0	2	0	0	2	4.4%
El Hasaheissa	0	1	0	1	0	1	0	2.9%

CHAPTER FOUR

Discussion

Diagnosis of active infection of hearwater in live animals was not possible in the near past due to lack of simple and accurate diagnostic test and it relied on post-mortem findings. The PCR assay is of high sensitivity and specificity for detection of *E. ruminantium* in blood of infected animals at the febrile stage of infection, in carrier animals and in ticks (Peter *et al*., 1995; Allsopp and Allsopp, 2001; van Heerden *et al*., 2004). In this study PCR was used for detection of *E. ruminantium* in blood of ruminants from Al Gazirah State. Out of 170 DNA extracts, only three (1.8%) were positive. The set of primers (HH1F and HH2R) used in this assay was targeting a fragment of 980 bp in the pCS20 gene of *E. ruminantium*. These primers are designed in a more conserved region than the primes (AB128 and AB129) previously used by Allsopp and Allsopp (2001). These primers (HH1F and HH2R) were succefully used for amplification of visible PCR products of all *E. ruminantium* strains (van Heerden *et al*., 2004). DNA-based diagnostic assays targeting three DNA regions were developed for the amplification and detection of *E. ruminantium*, but pCS20 DNA region was found of most superior specificity in PCR assays (Allsopp *et al*., 1999). The sequence of pCS20 from all different virulent heartwater-producing isolates has been shown to be phylogenetically closely related (Van Heerden *et al*., 2004). So, due to these superior characteristics of the primers used and the highly conserved and specific targeted gene, the obtained prevalence (1.8%) of heartwater is most probably true. This prevalence rate is not unexpected and is comparable with the previous PCR results, but, however, far below the results obtained by serology. Serologically, prevalence of *E. ruminantium* antibodies was 58.9% in sheep in eastern Sudan (Abdel Rahman *et al*., 2003) and 76.6% in sheep in Blue Nile State (Sayed,

2004). A prevalence of 69% in sheep, 75.2% in goats and 38.7% in cattle was reported in northern Sudan (Abdel Rahman, 2005). However, seroprevalence is usually partly due to antibodies from a previous infection or cross-reactivity. The low prevalence of heartwater infection found in this study is in line with the previous studies which used the PCR to detect *E. ruminantium* pCS20 DNA in ticks. In *A. lepidum* ticks, a prevalence of 1.9 % was found from Gedarif State and 8.2% in *A. variegatum* from Juba (Muramatsu *et al.,* 2005). Also, a prevalence of 1.8 % was reported in *A. lepidum* (Abdel Rahman, 2005). No previous study was found done in the same area of this study. The prevalence of the infection using pCS20-PCR in some other African countries was higher than the one found here. In Zimbabwe, prevalences of *E. ruminantium* in *A. hebraeum* was found to range between 3.16% and 12.45% and in South Africa the prevalence ranged between 4.7% and 11.3 in wildlife reserves (Bryson *et al*., 2002). This study is different from the previous study in that the blood samples were taken as spots on filter paper, while in the previous studies; jugular blood in tubes was used. Using filter paper was found easier, simple.

The three positive animals in this study were sheep, two of them were from Tambool area and were males of Dobasee breed and the third was from El Hasahiessa area and was a female of a cross breed. Their age ranged between 1 to 4 years. Many reasons might be involved to interpret this result. From the epidemiological observation in the areas investigated in this study and from previous reports, the vector in this area is *A. lepidum* (Abdel Rahman, 2005). Cattle are rare hosts of *A. lepidum* nymphae but sheep and goats are important hosts (Hoogstraal, 1956; Karrar *et al*., 1963). Local breeds of domestic ruminants may become resistant to cowdriosis if exposure has been observed elsewhere (Uilenberg, 1990). It was observed that animals of Madani and El

Hasahiessa areas receive more care from their owners in terms of tick control and use of oxytetracycline for different pathological conditions compared with animals from Tambool area. Oxytetracycline is the most effective treatment of heartwater (van Amstel and Oberem, 1987). Also, it was observed that sheep of Tambool area were the most heavily infested with ticks, followed by sheep of El Hasahiessa. This is in accordance with the finding that the three positive animals were sheep and two of them were from Tambool. Two of the three positive sheep from Tambool area were clinically infected. No other animal was found clinically infected. So, the PCR method used was able to detect the organism in blood of the two infected animals as well as a suspected carrier or latent infection case. This is in line with the report of Allsopp and Allsopp (2001), who said that pCS20-PCR diagnostics would allow detection of *E. ruminantium* in both infected and carrier animals. The PCR assay has the potentiality to detect low level of infection (70 to 200 organisms) (Peter *et al*., 1995).

4.2 Conclusions

- Three sheep were found positive by Using PCR for detection heartwater in the central parts of Al Gezirah State .

- The usage of pCS20-PCR was found sensitive and detected clinically infected as well as a suspected latent infection case.

- The usage of filter paper to collect blood for DNA extraction was found efficient, in that it was cheap.

4.3 Recommendations

- The isolation of the organism from different parts of the Sudan, genotyping of the isolates and production of a vaccine to prevent the disease is highly recommended.

- This study recommends the usage of the PCR method for diagnosis of heartwater in a large number of animals from different parts of the Sudan.

- The susceptibility of camels and cattle to heartwater disease needs more elucidation.

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