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

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ABSTRACT

This study was carried out to investigate molecular epidemiology of heartwater in the Sudan by assessing prevalence of infection in domestic ruminants and the vector tick of the genus *Amblyomma* using the standard PCR technique based on the pCS20 gene. Four hundred and sixty samples were collected from different regions in the Sudan. They included 190 *Amblyomma lepidum* and 100 *Amblyomma variegatum* ticks, and 50, 60 and 50 blood samples from cattle, sheep and goats respectively. The samples were tested with pCS20 PCR. The infection rate of *E. ruminantium* was 15.3% (29/190) and 14% (14/100) in *A. lepidum* and *A. variegatum*, respectively. The prevalence of *E. ruminantium* in the blood samples was 6.7% (4/60), 4% (2/50) and 8.3% (5/60) from cattle, sheep and goats, respectively. It defined the spatial distribution and prevalence of *E. ruminantium* in the vector population and domestic ruminants and provided an accurate assessment of the potential risk of heartwater to livestock in some endemic regions in the Sudan. This study has proved the way for a national program for the investigation of the epidemiology of heartwater in the Sudan.

INTRODUCTION

Heartwater or cowdriosis, is a rickettsial disease causing high mortalities in sheep, goats, and cattle and is of major economic importance to livestock production and development in sub-Saharan Africa and on several Caribbean Islands (Uilenberg, 1983). The causative agent is an obligate intracellular gram-negative bacterium, *Ehrlichia ruminantium* (formerly *Cowdria ruminantium*) (Dumler *et al*., 2001). 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 detection of colonies of *E. ruminantium* in capillary endothelial cells of the brain in animals clinically suspected, during the acute disease, whereas it is not useful 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 (Dreher *et al*., 2005). The control of heartwater depends, to a large extent, on the availability of accurate data on the epidemiology of the infection in the vector and host. 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*., 2004). PCR-based tests are highly sensitive and specific and could improve the understanding of the epidemiology of cowdriosis by their ability to detect low levels of *E. ruminantium* infection in *Amblyomma* ticks (Peter *et al*., 2000).

Ticks are of great significance in the epidemiology of tick-borne diseases and planning of appropriate tick control measures is crucial in preventing tick borne diseases (Norval *et al*., 1991). 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 (Camus and Barré, 1987). In the Sudan generally, the real or potential economic losses due to the disease are not known because the incidence/prevalence is not well studied or documented. This urged the current investigation employing PCR based on pCS20 gene to determine the prevalence and spatial distribution of *E. ruminantium* infection rates in the respective *A. lepidum*, *A. variegatum* ticks and blood samples from cattle, sheep and goats selected endemic areas in the Sudan.

MATERIALS AND METHODS

Study area:

This study was conducted in heartwater endemic areas of four states in the Sudan. *E. ruminantium* occurs only where the *Amblyomma* spp. prevail. The study area 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 1). A total of 460 samples were collected (blood, *A.*

lepidum and *A. variegatum*) from cattle, sheep and goats (Table 1).

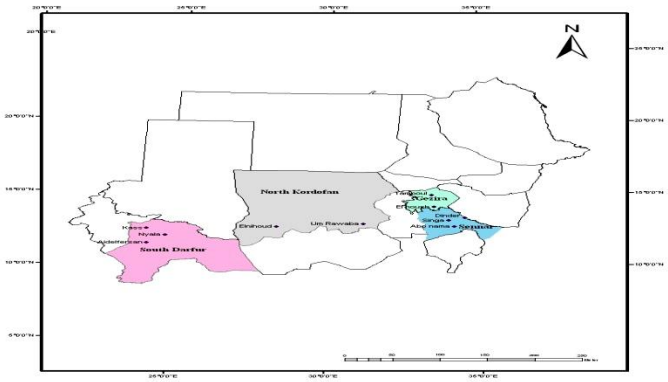

Figure 1: Localities (♦) from where blood and *Amblyomma* spp. ticks were collected in the endemic regions of the Sudan.

Source: Remote Sensing Authority (RSA) University of Khartoum, Sudan.

Tick collection:

Tick collection was conducted in the wet seasons (August, September and October) during 2007 to 2009. Partially engorged adults (males and females) of 190 *A. lepidum* were collected from cattle, sheep and goats in the above mentioned localities (Figure 1), while 100 *A. variegatum* were collected from cattle in South Darfur State (Table 1). The ticks were preserved in tubes containing 70% ethyl alcohol and were morphologically examined using the method described by Walker *et al.*, (2003).

Table 1: *Amblyomma* spp. ticks and blood samples collected in some localities in the Sudan during the wet seasons of 2007 to 2009

State	locality	*A. lepidum*	*A. variegatum*	Blood			Total
				Cattle	Sheep	Goats	
Sennar	Singa	40	-	20	20	20	100
	Abonama	20	-	-	-	-	20
	Dinder	40	-	-	-	-	40
Total		100	0	20	20	20	160
Gezira	Tamboul	-	-	10	20	20	50
	Elhoush	20	-	-	-	-	20
Total		20	0	10	20	20	70
North Kordofan	Um rawaba	10	-	-	-	-	10
	Elnihoud	20	-	10	10	-	40
Total		30	0	10	10	0	50
South Darfur	Nyala	20	40	20	-	20	100
	Aidelfersan	20	20	-	-	-	40
	Kass	-	40	-	-	-	40
Total		40	100	20	0	20	180
Total		190	100	60	50	60	460

Whole blood from the field:

Whole blood (170) were randomly collected in the wet seasons (August, September and October) during 2007-2009 from apparently healthy cattle, sheep and goats from the same localities (Figure 1; Table 1). Approximately 1-2 ml of blood was collected from the jugular vein into vials with ethylene diaminetetra-acetic acid (EDTA) using plain vacutainers and needles. The collected blood samples were kept in Eppendorf tubes and stored at -20ºC until used.

DNA extraction

Genomic DNA purification from ticks

DNA was extracted using Wizard® SV Genomic DNA Purification System (Promega, Madison, USA). Prior to extraction, ticks were washed in 70% ethanol followed by twice rinse with distilled water and blotted dry on filter paper. Each tick was, then, separately cut in small pieces using a sterile blade in a sterile Petri dish and placed into a 1.5 ml microcentrifuge tubes. Ticks were then digested in 275 µl of the prepared Digestion Solution Master Mix (Proteinase k) to each sample tube (Promega, Madison, USA) and, the mixture was then incubated overnight at 55°C (heat block) and centrifuged at 2000×g to pellets. The supernatant was then transferred to a new 1.5 ml microcentrifuge tube and 250 µl of Wizard® SV Lysis Buffer were added to each sample and vortexed. The entire lysate sample was transferred from the 1.5 ml microcentrifuge tube to a Wizard® SV Minicolumn assembly and centrifuged at 13,000×g for 3 minutes to bind the genomic DNA to the Wizard® Minicolumn. The Wizard® SV Minicolumn was then removed from the Minicolumn assembly and the liquid in the Collection Tube was discarded. The Wizard® SV Minicolumn was replaced into the Collection Tube and 650 µl of Wizard® SV Wash Solution was added to each Wizard® SV Minicolumn assembly and centrifuged at 13,000×g for 1 minute, and 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 250 µl at room temperature Nuclease-Free Water was added to the Wizard® SV Minicolumn and incubated for 2 minutes at room temperature and, then, centrifuged at 13,000×g for 1 minute for elution. The purified genomic DNA was stored at -20°C until used.

Genomic DNA purification from whole blood

DNA was extracted using Wizard® SV Genomic DNA Purification System (Promega, Madison, USA). 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) and 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. The mixture was transferred to Wizard® SV Minicolumn assembly. Thereafter, extraction was carried out as previously described to genomic DNA Purification from ticks. DNA concentrations and purity were determined by NanoDrop® Spectrophotometer (A_{260} and A_{280}). The samples were, then, stored at -20°C until further processing.

Polymerase chain reaction (PCR) amplification

The PCR was performed according to van Heerden *et al.* (2004) using the Cow F as forward primer and CowR as reverse primer to amplify a 226-bp fragment of the conserved pCS20 region of *E. ruminantium* DNA, and had the following sequences: 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 hold at 4°C.

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 [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 60-90 minutes. Results were visualized after staining the gels in 0.5–1 µg/µL ethidium bromide. The equivalent of about 10 µL PCR product was loaded in each gel lane, after mixing it in loading buffer. The DNA was visualized under UV illumination. The images were captured using Kodak gel imaging system.

RESULTS

Detection of E. ruminantium in A. lepidum

A total of 190 *A. lepidum* adults were initially tested with pCS20 PCR. Twenty nine out of 190 (15.3%) of *A. lepidum* were positive for *E. ruminantium* (Table 2 and Figure 2). The highest (25%) infection rate was at Abonama and the lowest (10%) was at Elnihoud, Um Rawaba and Aidelfersan (Table 2).

Table 2: Infection rate of *E. ruminantium* in *A. lepidum* using pCS20 PCR in some localities in the Sudan during the wet seasons of 2007 to 2009

Locality	Source of ticks	No. tested	No. +ve	Infection rate%
Singa	Cattle	40	8	20
Dinder	Cattle	40	5	12.5
Abonama	Cattle	20	5	25
Elhoush	Cattle	20	3	15
Nyala	Cattle	20	3	15
Aidelfersan	Cattle	20	2	10
Elnihoud	Cattle	20	2	10
Um Rawaba	Sheep	10	1	10
Total		190	29	15.3

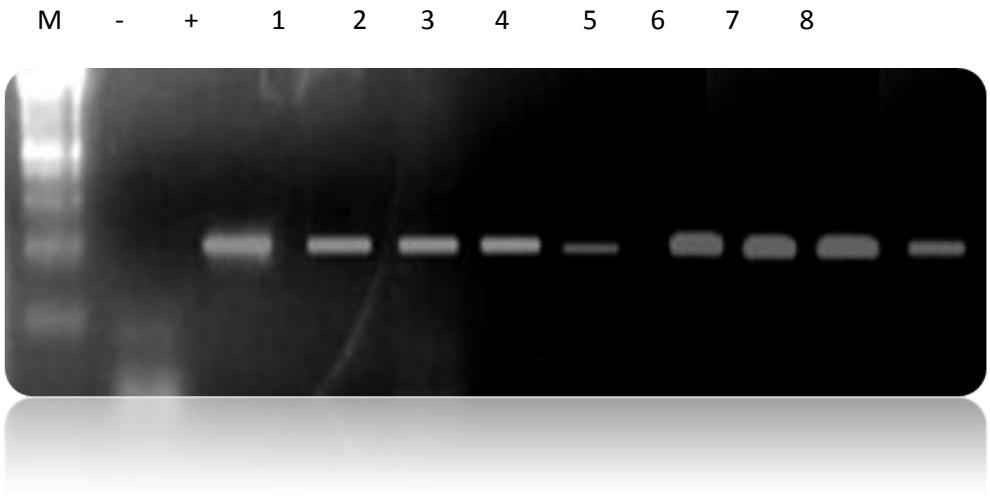

Figure 2: Amplification of *E. ruminantium* genomic DNA isolated from *Amblyomma lepidum* using the Wizard® SV Genomic DNA Purification System. Lane (M) 100 bp molecular marker. Lane (-) PCR negative control. Lane (+) positive control lanes 1, 2, 3, 4, 5, 6, 7 and 8: PCR products obtained from *A. lepidum* collected from Singa, Dinder, Abonama, Elhoush, Nyala, Aidelfersan, Elnihoud and Um Rawaba, respectively, showing single specific band at approximately 226 bp.

Detection of E. ruminantium in A. variegatum

A total of 100 *A. variegatum* adults were tested with pCS20 PCR. Fourteen out of 100 (14%) were positive for *E. ruminantium* (Table 3 and Figure 3). The highest (20%) infection rate was at Aidelfersan followed by Kass (17.5%) and the lowest (7.5%) in Nyala (Table 3).

Table 3: Infection rate of *E. ruminantium* in *A. variegatum* using pCS20 PCR in South Darfur State during the wet season in 2007

Locality	Source of ticks	No. tested	No. +ve	Infection rate%
Nyala	Cattle	40	3	7.5
Aidelfersan	Cattle	20	4	20
Kass	Cattle	40	7	17.5
Total		100	14	14

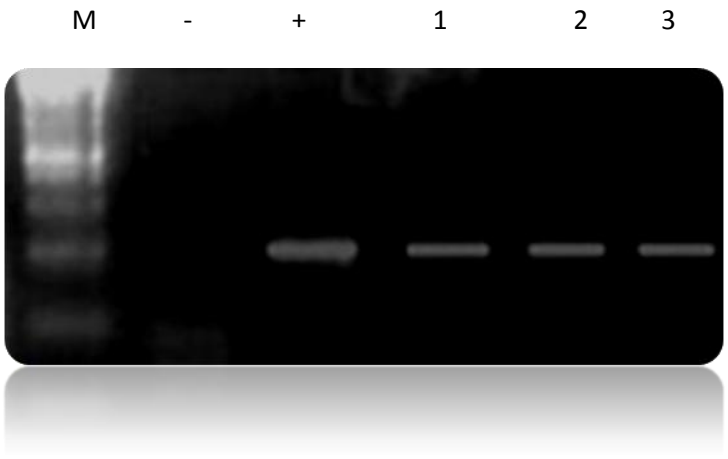

Figure 3: Amplification of *E. ruminantium* genomic DNA isolated from *Amblyomma variegatum* feeding on cattle using the Wizard® SV Genomic DNA Purification System. Lane (M) 100 bp molecular marker. Lane (-) PCR negative control. Lane (+) positive control lanes 1, 2 and 3: PCR products obtained from *A. variegatum* collected from Nyala, Aidelfersan and Kass, respectively, showing single specific band at approximately 226 bp.

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%) blood samples were positive for *E. ruminantium* (Table 4). In cattle 4/6 blood samples were positive (6.7% - Figure 4). The highest prevalence (10%) in cattle was in Tamboul and Elnihoud followed by (5%) in Singa and Nyala. Two out of 50 (4%) blood from sheep were positive for *E. ruminantium*. The highest prevalence (5%) in sheep was in Tamboul and Singa and the lowest prevalence (0%) was in Elnihoud (Table 4; Figure 5). Five out of 60 (8.3%) blood of

goats were positive. The highest prevalence (15%) in goats was in Nyala followed by (5%) in Singa and Tamboul (Table 4; Figure 5).

Table 4: Prevalence of *E. ruminantium* in blood samples from domestic ruminants using pCS20 PCR in some localities in the Sudan during the wet seasons in 2007 to 2009

Locality	Cattle			Sheep			Goats			Over all Prevalence
	No. tested	No. +ve	%	No. tested	No. +ve	%	No. tested	No. +ve	%	
Singa	20	1	5	20	1	5	20	1	5	5
Tamboul	10	1	10	20	1	5	20	1	5	6
Nyala	20	1	5	Nt	0	0	20	3	15	10
Elnihoud	10	1	10	10	0	0	Nt	0	0	5
Total	60	4	6.7	50	2	2.4	60	5	8.3	6.5

Nt: not tested

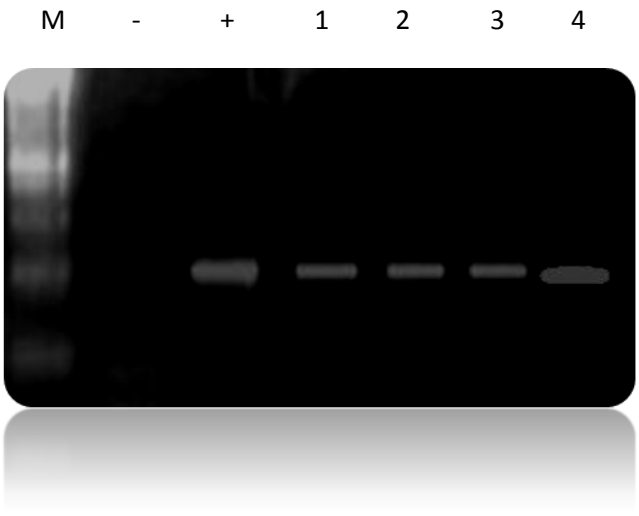

Figure 4: Amplification of *E. ruminantium* genomic DNA isolated from blood of cattle using the Wizard® SV Genomic DNA Purification System. Lane (M) 100 bp molecular marker. Lane (-) PCR negative control. Lane (+) positive control lanes 1, 2, 3 and 4: PCR products obtained from Cattle blood collected from Singa, Tamboul, Nyala and Elnihoud, respectively, showing single specific band at approximately 226 bp.

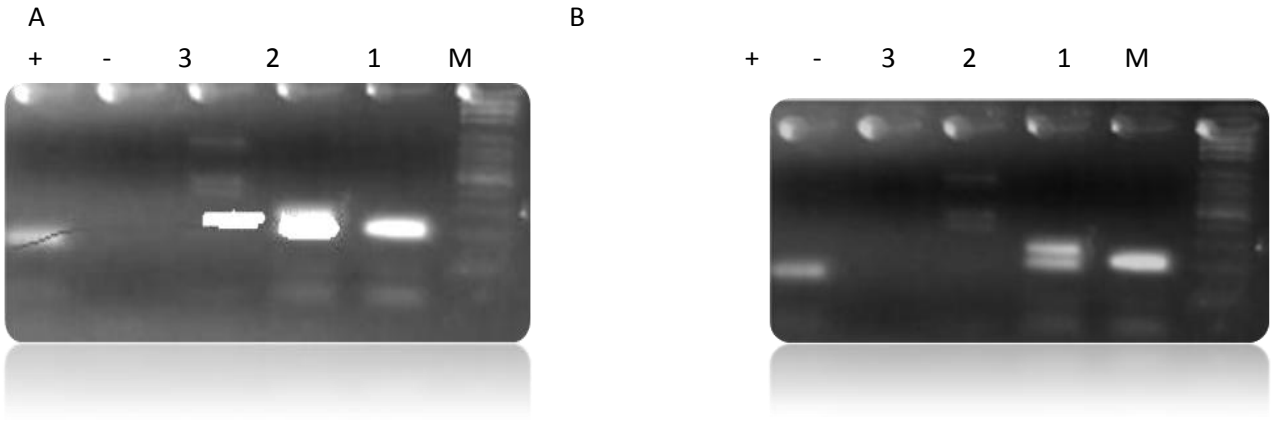

Figure 5: Amplification of *E. ruminantium* genomic DNA detected in blood of sheep and goats using the Wizard® SV Genomic DNA Purification System. Lane (M) 100 bp molecular marker. Lane (-) PCR negative control. Lane (+) positive control. (A) Lanes 1, 2, and 3: PCR products obtained from goats' blood 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.

DISCUSSION

Accurate estimates of prevalence of tick infestation are essential in the development and validation of models of heartwater disease transmission dynamics (Deem *et al*., 1996). These models could be used to evaluate the effectiveness of different disease control strategies, such as vaccination and acaricide treatment, and assist in the formulation of cost-effective control programmes (OĆallagham *et al*., 1998). For epidemiological surveys and clinical diagnostic purposes, the polymerase chain reaction offers an ideal combination of sensitivity and specificity. 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), indicating that a DNA-based technique is useful even for the diagnosis of latent infection.

This study targeted the pCS20 PCR-based estimates of *E. ruminantium* prevalence in adults of *A. lepidum* and *A. variegatum*, the principal 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 some localities in the Sudan. The overall infection rate of *A. lepidum* was 15.3% (Table 2). This indicated that approximately one in seven adult *A. lepidum* was infected with *E. ruminantium*. This infection rate was higher than 1.9% and 1.8% previously reported by Muramatsu *et al.* (2005) and Abdel Rahman (2006), respectively. It was, also, higher than that reported in *A. hebraeum* in South Africa (5.4%) by Du Plessis (1981) and Zimbabwe (1.7%) by Peter *et al.* (1999). However, the findings obtained in this study were in line with those recorded for *A. hebraeum* in Zimbabwe (10-40%) by Norval, *et al.* (1990). however, the results obtained were in line with those recorded in

A. hebraeum in Zimbabwe 10-40% and 11.5% by Norval, *et al*. (1990) and Peter *et al*. (1997) respectively. The high prevalence (25%) reported in Abonama, was by far the highest value in the study where one in four ticks was infected with *E. ruminantium*.

The overall prevalence of *E. ruminantium* in *A. variegatum* collected in South Darfur State (Western Sudan) was 14%. The highest prevalence (20%) was reported in Aidelfersan where one in five ticks was infected with *E. ruminantium*. The prevalence estimates for *A. variegatum* (14%) fall within the wide range found in heartwater endemic regions in Senegal (11%) by Gueye *et al.* (1994) and were higher than those previously recorded in the South of Sudan (8.2%) by Muramatsu *et al*., (2005) and the Caribbean Islands (2%) by Camus and Barré (1987).

The exact reasons for the high infection rates in this study were difficult to explain, but a wide range of results were similarity recorded within the one country by previous investigators. In South Africa, Du Plessis and Mahan (1987) recorded prevalence rates of infected ticks up to 30%, while Peter et al. (1999) reported only 1.7% prevalence. 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 husbandry. This high infection rate in ticks is not surprising because, as *Amblyomma* spp. On being three host-ticks were capable of transmitting infection transstadially, through 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 chance of this tick to become infected with heartwater is high. On the other hand, the prevalence of *E. ruminantium* detected in cattle (6.7%), sheep (4%) and goats (8.3%) in the different localities in the Sudan was higher than that recorded in South Africa (3.9%) by Steyn *et al.* (2008) who reported prevalence ranging from 0.2 to 3.9% and lower than 43.3% and 45.5% reported by Jongejan *et al.*, (1988) in Zambia. The prevalence rates recorded in this study were also lower than the sero-prevalence reported in the Sudan in cattle (38.7%), sheep (69%) and goats (75.2%) by Abdel Rahman (2006), and, also, lower than those reported in Cameroon (61%) by Awa (1997), Kenya (61%) by Maloo *et al.*, (2001), Tanzania (50.3%) by Swai *et al*., (2008) and Ghana (70%) by Bell- Sakyi *et al*., (2003). However, the PCR detection of *E. ruminantium* in ticks and blood from field animals is more accurate and specific than the sero-prevalence employed by previous invistigators.

CONCLUSION

This study proved that the PCR assay is an effective tool for determining *E. ruminantium* infection prevalence. Hence, pCS20 PCR is a powerful tool not only for the epidemiological study of heartwater but also for the rapid and sensitive diagnosis of infected animals in the disease-endemic areas.

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REFERENCES

Abdel Rahman, M. B.**, (**2006). Studies on *Ehrlichia ruminantium* in domestic ruminants in the Sudan. PhD Thesis, University of Khartoum, Sudan.

Awa, D. N., (1997). Serological survey of heartwater relative to the distribution of the vector *Amblyomma variegatum* and other tick species in north Cameroon. *Veterinary Parasitology* **68**: 165-173.

Bell-Sakyi, L., Koney, E. B. M., Dogbey, O., Sumption, K. J., Walker, A. R., Bath, A., and Jongejan, F., (2003). Detection by two enzyme-linked immunosorbent assays of antibodies to *Ehrlichia ruminantium* in field sera collected from sheep and cattle in Ghana. *Clinical and Diagnostic Laboratory Immunology* **10**: 917-925.

Camus, E. and Barré, N., (1987). Epidemiology of heartwater in Guadeloupe and in the Caribbean. *Onderstepoort Journal of Veterinary Research* **54**: 419-426.

Camus, E., Barré, N., Martinez, D., and Uilenberg, G., (1996). Heartwater (Cowdriosis) a review. 2nd edition, O.I.E., Paris, 177pp.

Deem, S. L., Norval, R. A. I., Yonow, T., Peter, T. F., Mahan, S. M. and Burridge, M. J., (1996). The epidemiology of heartwater: Establishment and maintenance of endemic stability. *Parasitology Today*, **12** (10): 402-405.

Dreher, U. M., de la Fuente, J., Hofmann-Lehmann, R., Meli, M.L., Pusterla, N., Kocan, K. M., Woldehiwet, Z., Braun, U., Regula, G., Staerk, K. D. C. and Lutz, H., 2005. Serologic cross-reactivity between *Anaplasma marginale* and *Anaplasma phagocytophilum*. *Clinical and Diagnostic Laboratory Immunology*. **12**: 1177–1183.

Du Plessis, J. L., (1981). The application of the indirect fluorescent antibody test to the serology of heartwater, In: Proc. International Conference on 'Tick biology and control'. Grahams town, South Africa, 47-52.

Du Plessis, J. L. and Malan, L., (1987). The non-specific resistance of cattle to heartwater. *Onderstepoort Journal of Veterinary Research*. **54**: 333-336.

Dumler, J. S., Barbet, A. F., Bekker, C. P. J., Dasch, G. A., Palmer, G. H., Ray, S. C., Rikihisa, Y. and Rurangirwa, F. R. (2001). Reorganisation of genera in the families *Rickettsiaceae* and *Anaplasmataceae* in the order *Rickettsiales*: Unification of some species of *Ehrlichia* and *Ehrlichia*

with *Neorickettsia*, description of six new species combinations and designation of *Ehrlichia equi* and 'HGE agent' as subjective synonymous of *Ehrlichia phagocytophila*. International Journal of Systematic Evolutionary Microbiology **51:** 2145-2165.

Gueye, A., Mbengue, M., and Diouf, A., (1994). Ticks and haemoparasites of livestock in Senegal. VI. The Soudano-Sahelian zone. *Revue d'elevage et de medecine veterinaire des pays tropicaux*. **47**: 39-46.

Jongejan, F., Uilenberg, G., Franssen, F. F. J., Gueye, A. and Nieuwenhuijs, J., (1988). Antigenic differences between stocks of *Cowdria ruminantium*. *Research in Veterinary Science*. **44**: 186-189.

Maloo, S. H., Thorpe, W., Kioo, G., Ngumi, P., Rowlands, G. J. and Perry, B. D., (2001). Seroprevalence of vector-transmitted infections of small-holder dairy cattle in coastal Kenya. *Preventive Veterinary Medicine* **52**(1): 1-16.

Muramatsu, Y., Ukegawa, S., Elhussein, A. M., Abdel Rahman, M. B., Gabbar, K. M. A. A., Chitambo, A. M., Komiya, T., Mwase, E. T., Morita, C. and Tamura, Y., (2005). *Ehrlichia ruminantium,* Sudan. *Emerging Infectious Diseases* **11**: 1792-1793

Norval, R. A.I., Yunker, C. E., Duncan, I. M. and Peter, T. (1990). Phermone acaricide mixtures in the control of the tick *Amblyomma hebraeum*. *Experimental and Applied Acarology*. **11**: 233-240.

Norval, R. A.I., Andrew, H. R. and Yunker, C. E., (1991). Infection rates with *Cowdria ruminantium* of nymphs and adults of the bont tick *Amblyomma hebraeum* collected in the field in Zimbabwe. *Veterinary Parasitology*. **36**: 277-282.

O'Callaghan, C. J., Medley, G. F., Peter, T. F. and Perry, B. D., 1998. Investigating the epidemiology of heartwater (*Cowdria ruminantium* infection) by means of a transmission dynamics model. *Parasitology* **117**: 49-61.

Peter, T. F., Deem, S. L., Barbet, A. F., Norval, R. A. I., Simbi, B. H., Kelly, P. J. and Mahan, S. M., (1995). Development and evaluation of PCR assay for detection of low levels of *Cowdria ruminantium* infection in *Amblyomma* ticks not detected by DNA probe. *Journal of Clinical Microbiology*. **33**: 166-172.

Peter, T. F., Perry, B. D. and O'Callaghan, C. J., (1997). The distribution of heartwater in the highveld of Zimbabwe: 1980±1997. *Onderstepoort Journal of Veterinary Research*. **65**: 177-187.

Peter, T. F., Perry, B. D., O'Callaghan, C. J., Medley, G. F., Mlambo, G., Barbet, A. F. and Mahan, S. M., (1999). Prevalence of *Cowdria ruminantium* infection in *Amblyomma hebraeum* ticks from heartwater-endemic areas of Zimbabwe. *Epidemiology and Infection*. **123**: 309–316.

Peter, T. F., Barbet, A. F., Alleman, A. R., Simbi, B. H., Burridge, M. J. and Mahan, S. M., (2000). Detection of the agent of heartwater, *Cowdria ruminantium*, in *Amblyomma* ticks by PCR: Validation and Application of the assay to field ticks. *Journal of Clinical Microbiology*. **38:** 1539-1544.

Sawi, E. S., Muti, P. F., Changả, A. K. and Machange, G. E., (2008). The prevalence of serum antibodies to *Ehrlichia ruminantium* infection in ranch cattle in Tanzania: A cross section study. *Journal of the South African Veterinary Association*. **79** (2): 71-75.

Steyn, H. C., van Heerden, H., Allsopp, M. T. E. P. and Allsopp, B. A., (2003). Variability of pCS20 gene sequence among different *Ehrlichia ruminantium* isolates. *Annals of the New York Academy of Sciences*, **990**:723-725.

Steyn, H. C., Pretorius, A., McCrindle, C. M., Steinmann, C. M. and van Kleef, M., (2008). A quantitative real-time PCR assay for *Ehrlichia ruminantium* using pCS20. *Veterinary Microbiology* **131:** 258-265.

Uilenberg, G., (1983). Heartwater (*Cowdria ruminantium* infection): Current status. *Advances in Veterinary Science and Comparative Medicine*. **27**: 427-480.

van Heerden, H., Steyn, H. C., Allsopp, M. T. E. P., Zweygarth, E., Josemans, A. I., and Allsopp, B. A., (2004). Characterisation of the pCS20 region of different *Ehrlichia ruminantium* isolates. *Veterinary Microbiology* **101**: 279-291.

Waghela, S. D., Rurangirwa, F. R., Mahan, S. M., Yunker, C. E., Crawford, T. B., Barbet, A. F., Burridge, M. J. and McGuire, T. C., (1991). A cloned DNA probe identifies *Cowdria ruminantium* in *Amblyomma variegatum* ticks. *Journal of Clinical Microbiology*. **29**: 2571–2577.

Walker, A. R., Bouattour, A., Camicas, J-L., Estradapeńa, A., Horak, I.G., Latif, A. A., Pegram, R. G. and Preston, P. M., (2003). *Ticks of domestic animals in Africa: A guide to identification of species*. Bioscience reports

Walker, J. B. and Olwage, A., (1987). The tick vectors of *Cowdria ruminantium* (Ixodoidea, Ixodidae, genus *Amblyomma*) and their distribution. *Onderstepoort Journal of Veterinary Research* **54**: 353–379.