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Molecular Detection of *Ehrlichia* Species in *Amblyomma* Ticks Collected from Ruminants in Abernosa Ranch, Ethiopia

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Abstract: Heartwater or cowdriosis is an acute, infectious, non-contagious and tick-borne rickettsial disease of ruminants caused by *Ehrlichia ruminantium* and transmitted by *Amblyomma* ticks. It occurs in nearly all sub-Saharan countries of Africa and affects domestic and wild ruminant species. The PCR-based molecular genetics revolution in diagnostic techniques has provided the only reliable methods for *E. ruminantium* diagnosis in carrier animals or ticks. This research was conducted to identify species of *Amblyomma* carrying *E. ruminantum* in ruminants of Abernosa ranch from November 2011 to April 2012. After DNA extraction from the ticks was carryout using modified boom extraction method, a semi-nested PCR reaction was used to amplify a fragment of about 900bp 16s RNA gene. From 120 *Amblyomma* ticks examined 10 (8.3%) of them were positive for *E. ruminantium* while 10 (8.3%) of them were positive for *E. omatijene*. *E. ruminantium* was detected in 5.8 % of *A. lepidum* and 2.5% of *A. variegatum*. Similarly *E. omatijene* was detected in 6.6% of *A. lepidum* and 1.7% of *A. variegatum*. In both cases *A. lepidum* was found to carry more *Ehrlichia* species than *A. variegatum*. Overall 20 (16.6%) of ticks were positive for *Ehrlichia* species. The results of this study provide molecular evidence for the presence of *E. ruminantium* and *E. omatijene* in *A. variegatum* and *A. lepidum* ticks of ruminants in Abernosa ranch. So it was concluded that different ticks can act as a vector of *Ehrlichia* species in different geographical settings. These findings need to be taken into consideration when future livestock upgrading programs are implemented.

Key words: DNA · Heartwater · PCR · Prevalence

INTRODUCTION

Tick-borne diseases and their vectors are most important constraint to livestock development in Africa [1]. In Ethiopia, for the vast majority of the community, ruminant livestock is the source of animal proteins and income. The population growth has resulted in increased demand for animal protein but the livestock productivity specially that of ruminants does not commensurate with the demand in Ethiopia. The domestic ruminant species dwell diverse agro-climatic zones and are raised under various production systems [2]. This in turn results in abundance of various vector species and vector-borne diseases especially ticks and tick-borne infections [3]. Particularly, information from literature shows that in areas where favorable conditions exist for *Amblyomma* ticks there is problem of heartwater [4].

Heartwater or cowdriosis is an infectious, non-contagious and tick-borne disease caused by an intracellular rickettsial pathogen previously known as *Cowdria ruminantium* but reclassified as *Ehrlichia ruminantium* [5]. The disease is usually an acute disease and may be fatal within days of the onset of clinical signs [6]. It is characterized by pyrexia, anorexia, malaise, nervous symptoms, dyspnoea, gastro-enteritis, hydrothorax and hydro pericardium. The latter, a common pathological lesion, is probably the origin of the name heart-water [7]. The rates of mortality resulting from this disease range from 20 to 90% in susceptible livestock [6]. Heartwater affects domestic ruminant species, cattle, sheep, goats and several wild ruminant species such as buffalo, giraffe and antelope as well as some wild rodents. Domestic ruminants are more susceptible than wild ones. Indigenous domestic ruminants are more

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resistant compared to exotic high producing breeds. Wild ruminants are usually the reservoir of the agent of heartwater [8].

Heartwater is transmitted by many ticks of the *Amblyomma* genus that carry the agent from carrier animals to susceptible ones. About 150, 000, 000 domestic ruminants are at risk of *Amblyomma* challenge in sub-Saharan Africa of which 114, 000, 000 (76%) are at greatest risk. Among the several species of *Amblyomma* capable of transmitting *E. ruminantium*, *A. variegatum* and *A. hebraeum* are most important [8] globally. However, several other species of *Amblyomma* have been known to play role in a given locality. *A. variegatum* is more widespread in Sub-Saharan Africa and in that it has the widest distribution in Ethiopia. It accounts for 40% of the Ethiopian tick population. Heartwater is assumed to occur throughout the country where ever the tick occurs but it not well documented. Certain outbreaks with significant mortality (25%) were reported in Abernosa cattle ranch [4] and several other suspected cases were observed in sheep and cattle in various parts of the country. Infection in ticks is transmitted transstadially and possibly transovarially.

The disease is a serious constraint to livestock improvement programs throughout sub-Saharan Africa and through its occurrence on some islands in the Caribbean, poses a potential threat to ruminant species in mainland North, Central and South America. It is the second most important tick-borne disease after East Coast Fever in Africa [9]. In countries or regions where there is endemic stability, losses from heartwater are minimal until new animals are introduced. So that the disease is a major obstacle to the introduction of high-producing animals into sub-Saharan Africa to upgrade local stock and is of particular importance when susceptible animals are moved from heartwater-free to heartwater-infected areas [10]. Furthermore, it has the potential to spread from endemic areas to free areas such as from the Caribbean to the American. The economic impact of the disease is therefore difficult to quantify, although estimates which have been made indicate that the losses can be enormous [11].

There is lack of information on the epidemiology of the disease and studies into the genetic diversity and characteristics of the causative agent, *E. ruminantium*, have until recently been hampered by the lack of sensitive and specific diagnostic tools that are particularly suitable for use in countries in Africa. 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 [12]. Therefore, simple, sensitive and specific discriminating techniques are required at species or strain level. Successfully, Van Viliet *et al.* [13] 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 [14]. It included DNA hybridization [9], polymerase chain reaction (PCR) [15] and reverse line blot (RLB) [16].

Extraction of genomic DNA allowed the development of PCR-based techniques for identification of pathogen DNA in ticks and in hosts and to genetically characterize different isolates from geographically diverse areas [17]. The PCR-based molecular genetics revolution in diagnostic techniques has provided the only reliable methods for *E. ruminantium* diagnosis. Three families of probes have been used, targeting the pCS20 genetic region, the 16s RNA gene and the map1gene. The pCS20 genetic region was the first genetic target to be identified especially for *E. ruminantium* diagnosis [18] and it has proved to be specific for *E. ruminantium*, giving no cross-reactions with other *Ehrlichia* species [19]. The 16s RNA probes are difficult to use, because the sequence variations are small and they do not provide such sensitive detection as the pCS20 probe, so they are not used for routine diagnosis. They are particularly useful, however, when previously unknown *Ehrlichia* species are encountered, allowing them to be phylogenetically identified as *E. ruminantium* or else to be assigned to other groupings. The map1 gene, which is extensively polymorphic, has also been used as a diagnostic target for *E. ruminantium* in order to characterize different antigenic variants of the parasite [14].

In general understanding of the distribution of vectors of *E. ruminantium* in a given area is a crucial prerequisite for effective control. Therefore, the objective of this research was to identify species of *Amblyomma* carrying *E. ruminantum* in ruminants of Abernosa ranch.

MATERIALS AND METHODS

Study Area: The study was conducted in Abernosa ranch. The ranch is located at 7.58°N latitude and 38.43°E longitude in the mid rift valley of central Oromia 170

kilometers south of Addis Ababa. Agro-ecologically, the ranch is characterized as semi arid with minimum mean temperature of 12.7°c and maximum mean temperature of 27.2°c. The ranch has relative humidity of 60%. Its altitude ranges from 1500-2000 meters above sea level. The average annual rainfall ranges from 650-750mm and the distribution is highly variable between and within the year. Cattle and goats are animal species reared in the ranch. Natural pasture, the major feed resources of the livestock, is composed of predominantly grass (Pennisetum, Cenchurus, Sporobulus, Arestiola and Hyperhene and legumes (Crotalaria spp.). Weeds are also found with varying proportion. In the ranch, Irregular rainfall and shortage of feeds coupled with shortage of veterinary service gives livestock production a high degree of risk and uncertainty.

Study Population and Design: The study population included in this study was indigenous and cross breed cattle breeds reared by the ranch. The animals were managed under semi-intensive production system. During sampling ruminants of different age, sex, body condition and different level of tick infestation were included. The study design was a cross-sectional study in which the elementary unit was selected randomly.

Methodology

Collection of Ticks: The animals that were infested by ticks were restrained and all observed ticks (adults and nymphs) were collected. The collection was done by gentle rotation using thumb and fingers from their attachment sites. The collected ticks were transferred to universal bottles and plastic containers containing 70% ethyl alcohol. Each of the containers were properly labeled with date of collection, age, sex, breed and species of the host and transported to Addis Ababa University, College of Veterinary Medicine and Agriculture, Veterinary Parasitology laboratory for identification of their genera and species. During collection of ticks data sheet with animal identification, tick attachment sites, tick loads, body conditions of animals and other risk factors was prepared and filled appropriately.

Identification of Ticks: To identify ticks Petri dish, forceps, filter paper and stereomicroscope were used. The ticks in the containers were transferred to Petri dish. Then they were spread onto filter paper to absorb excess preservatives. Finally, they were examined under stereomicroscope for morphological identification of the ticks to their genus and species level according to standard identification keys described by walker *et al*. [20] and Okello_Onen *et al*. [21].

DNA Extraction: DNA extraction from the ticks was carried using modified boom extraction method. Each ticks sample was cut into pieces using scalpel and blade. The pieces were transferred into 1.5mL tube. To each of the tubes 180µL of ATL buffer and 20µL proteinase K (20mg/mL) was added. The tubes were incubated at 56°C overnight while shaking at 1400rpm using thermomixer compact-eppendorf. The tubes centrifuged briefly and 200µL of AL buffer was added. They were vortexed for 15 seconds and incubated at 70°C for 10 min at 800rpm. The tubes were centrifuged briefly, 40µL of diatomaceous earth solution was added and mixed and incubated at 37°C for at least 1 hour while shaking at +1200rpm. The tubes were centrifuged for 20 seconds and the supernatant was discarded. The pellets were rinsed with 900µL of 70% ethanol, centrifuged for 20 seconds and the supernatants were discarded. This step was repeated once. The resulting pellets were rinsed with 900µL of absolute acetone, centrifuged for 20 seconds and the supernatants were discarded. The resulting pellets were dried at 50°C for 20 minutes using thermoblock. The dried pellets were rinsed with 90µL of TE buffer and incubated at 60°C for 20 minutes at 1000rpm. They were centrifuged for 40 seconds and 50µL of the supernatant was collected into new tubes, labeled and stored at +4°C until analyzed.

Polymerase Chain Reaction Amplification of DNA: A semi nested PCR reaction was used to amplify a fragment of about 900bp 16s RNA gene. For this purpose two sets primers that amplify 16s RNA genes from all *Anaplasma* and *Ehrlichia* species were used. They were HER 16SD (5'-GGTACCYACAGAAGAAGTCC-3') and EBR3 (5'-TTGTAGTCGCCATTGTAGCAC-3') as external primers for the first round PCR amplification. For the second round amplification HER 16SD was used as external primers while EBR2 (5'-TGCTGACTTGACATCATCCC-3') was used as internal primers. The reaction mix consisted of Y sub (Yellow Sub™ GENEO BIO Products, Germany), milli-Q-PCR water, buffer (20mM Tris-HCl, pH 8.4), 1.65mM $MgCl_2$ (Triton X-100), dNTP, primers (25umol/µL) and Taq polymerase (Silverstar DNA polymerase, Eurogentec, Belgium). The PCR reaction was carried out in a total volume of 25µL in programmable thermocycler (T3 thermacyclerBiometra®, Westburg, NL). For the first

round amplification the reaction consisted of 40 cycles of denaturation at 92°c for 30 seconds, annealing at 62°c for 45 seconds, elongation at 72°c for 1min and final extension at 72°c for 10 min. For the second round of amplification 0.5µL of the PCR products from the first reaction were used as a template. The reaction consisted of 25 cycles with the same reaction conditions as the first round PCR. All the PCR products were visualized by gel electrophoresis using 2% agarose at 100V for 20 min and staining with ethidium bromide. Throughout the PCR procedures PCR mix with no DNA template was used as negative control while DNA from *Cowdria* (*Ehrlichia*) was used as positive control.

RESULTS

In this study a total of 120 *Amblyomma* species collected from both indigenous and cross breed cattle reared by the Mariam Agro-industry, Ranch, were examined for the presence of *Ehrlichia* species using semi-nested PCR. Of these 60 were *A. variegatum* and the rest 60 were *A. lepidum*. From 120 *Amblyomma* ticks examined 10 (8.3%) of them were positive for *E. ruminantium* while 10 (8.3%) of them were positive for *E. omatijene* (Table 1 and 2). *E. ruminantium* was detected in 5.8 % of *A. lepidum* and 2.5% of *A. variegatum* (Table 1). Similarly *E. omatijene* was detected in 6.6% of *A. lepidum* and 1.7% of *A. variegatum* (Table 2). In both cases *A. lepidum* was found to carry more *Ehrlichia* species than *A. variegatum*. Overall 20 (16.6%) of ticks were positive for *Ehrlichia* species.

During the period of sample collection, it was observed that most animals of Abernosa ranch were infested by ticks. Amongst the ticks observed there were nymphs and adults of *Amblyomma* (*A. variegatum* and *A. lepidum*), *Rhepicephalus*, *Boophilus*, *Hayaloma* and some unidentified.

DISCUSSION

Diagnosis of active infection of *Ehrlichia* 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 *Ehrlichia* species in blood of infected animals at the febrile stage of infection, in carrier animals and in ticks [14,15, 22]. According to Allsopp *et al*. [19], DNA-based diagnostic assays targeting three DNA regions were developed for the amplification and detection of

Table 1: Prevalence of *E. ruminantium* on the species of *Amblyomma* ticks

Species of ticks	No. of ticks tested	No. of positive animals	Percentage of positive cases
A. variegatum	60	3	2.5%
A. lepidum	60	7	5.8%
Total	120	10	8.3%

Table 2: Prevalence of *E. omatijene* on the species of *Amblyomma* ticks

Species of ticks	No. of ticks tested	No. of positive animals	Percentage of positive cases
A. variegatum	60	2	1.7%
A. lepidum	60	8	6.6%
Total	120	10	8.3%

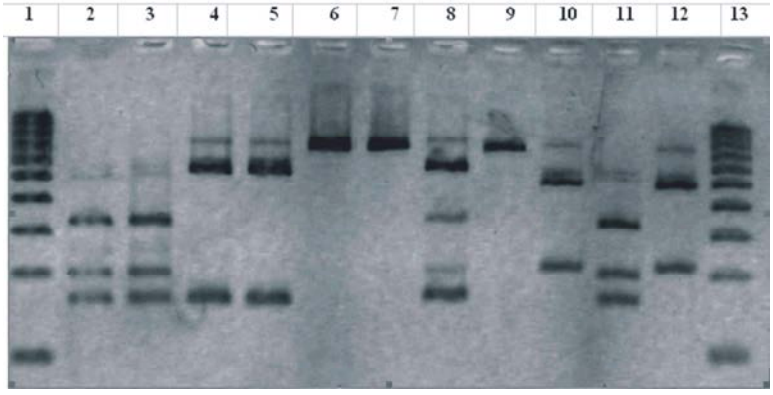

Fig 1: PCR detection of *E. ruminantum*, *E. omatijene* and *Anaplasma*, based on their different molecular weight calibrated on the DNA marker, from *Amblyomma* ticks (1 and 13 DNA markers, 2, 3 and 11 *Anaplasma*, 4, 5 and 8 *E. ruminantium*, 6, 7 and 9 mixed infections and 10 and 12 *E. omatijene*).

E. ruminantium, but pCS20 DNA region was found of most superior. Waghela *et al.* [18] first described the use of cloned DNA probes to detect the presence of *E. ruminantium* in *A. variegatum* ticks.

In this study PCR was used for detection of *Ehrlichia* species in *Amblyomma* ticks from Abernosa ranch. The set of primers (HER 16SD, EBR3 and EBR2) used in this assay was targeting a fragment of 900bp in the 16s RNA gene of *E. ruminantium*. Out of 120 DNA extracts, twenty (16.6%) were positive for *Ehrlichia* species. Of these ten (8.3%) were positive for *E. ruminantium*. The result also shows that *A. lepidum* is playing more roles in the transmission of *E. ruminantium* as well as *E. omatijene*. This means in the ranch *A. lepidum* is more important than *A. variegatum* in transmission of *E. ruminantium* to the ruminants. But in *A. lepidum* ticks, a prevalence of 1.9 % was found from Gedarif State and 8.2% in *A. variegatum* from Juba [23]. Also, a prevalence of 1.8% was reported in *A. lepidum* [12]. This shows that different ticks can act as a vector of *Ehrlichia* species in different geographical settings.

The prevalence of the infection using pCS20-PCR in some other African countries was more or less similar with the one found here. In Zimbabwe, prevalence 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 [24].

From the epidemiological observation in the areas investigated in this study and from previous reports, the dominant *Amblyomma* tick species in this area are *A. variegatum* and *A. lepidum* [25]. Cattle are rare hosts of *A. lepidum* nymph but sheep and goats are important hosts [26, 27]. Local breeds of domestic ruminants may become resistant to *E. ruminantium* infection if exposure has been observed elsewhere [7]. It was observed that animals of Abernosa ranch receive less care from their owner in terms of tick control and use of oxytetracycline for different pathological conditions. Oxytetracycline is the most effective treatment of heartwater [28]. During sampling (collection of tick) no animal was found clinically infected. So, the PCR method used was able to detect the organism in the ticks. This is in line with the report of [14], who said that the PCR assay is of high sensitivity and specificity for detection of *Ehrlichia* species in blood of infected animals at the febrile stage of infection, in carrier animals and in ticks. The PCR assay has the potentiality to detect low level of infection (70 to 200 organisms) [15].

The occurrence of heartwater was confirmed long ago in Ethiopia by Pegram *et al.* [29]. But the epidemiology of the disease including the infection rate in vectors and the major vector in each agro-ecology was not been elucidated. Certain outbreaks of heartwater in dairy cattle with significant mortality (25%) were reported at Abernosa ranch [4]. The current study has shown that both *A. variegatum* and *A. lepidum* play role in vectoring *E. ruminantium*. *A. lepidum* seen to be more important than *A. variegatum*.

E. omatijene was previously considered as strain of *E. ruminantium.* Now it is considered separate species infecting ruminants. Initially it was isolated from apparently healthy cattle and at that time it was considered non-pathogenic to cattle and goats [8]. However, this species has been considered pathogenic to sheep. It has been reported in South Africa [8] and Uganda [30] both in ticks and blood of ruminants. It has been shown that disease indistinguishable from cowdrosis was produced in sheep by this species [31]. Therefore, its pathogenic effect under natural conditions cannot be ruled out in sheep. Besides, its importance as of contributes to sero-reactivity to *E. ruminantium* making the results of sero-diagnosis unreliable. In Ethiopia where molecular diagnosis is not routine, the presence of *E. omatijene* will complicate diagnosis and treatment of heartwater.

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