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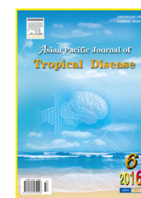

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# Molecular detection of *Anaplasma* species in questing ticks (ixodids) in Ethiopia

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ABSTRACT

**Objective:** To identify *Anaplasma* spp. in questing ticks with emphasis on *Anaplasma phagocytophilum* (*A. phagocytophilum*) and *Anaplasma ovis* (*A. ovis*) in Ethiopia.
**Methods:** DNA extracted from 237 questing ticks [*Rhipicephalus evertsi* (*R. evertsi*) (*n* = 61), *Rhipicephalus pulchellus* (*R. pulchellus*) (*n* = 54), *Rhipicephalus decoloratus* (*n* = 1), *Amblyomma variegatum* (*n* = 22), *Amblyomma lepidum* (*n* = 36), *Amblyomma* nymphs (*n* = 6), *Amblyomma gemma* (*n* = 7) and *Hyalomma marginatum* (*Hy. marginatum*) (*n* = 53)] were tested by PCR-RFLP assay.
**Results:** Overall 32 (13.33%; 95% confidence interval: 9.8%–18.3%) of the ticks were positive for *Anaplasma* spp. DNA. *Anaplasma marginale* was detected in *Hy. marginatum* and *R. pulchellus*. *Anaplasma centrale* was identified in *R. evertsi*, *R. pulchellus* and *Hy. marginatum*. *A. ovis* was detected in *R. evertsi*, *Amblyomma* spp. and *Hyalomma* spp. *A. phagocytophilum* was detected only in *R. pulchellus* and *Anaplasma* sp. *omatijenne* was detected only in *Amblyomma lepidum*. *Ehrlichia* species were not detected in any of the tick species examined.
**Conclusions:** The results demonstrated the presence of several *Anaplasma* spp. including the zoonotic *A. phagocytophilum* and potentially zoonotic *A. ovis*. Our finding identified potential vectors of *A. ovis* to be further confirmed. However, an extended study is needed to identify the potential vectors of *A. phagocytophilum*. The variety of *Anaplasma* spp. indentified in this study suggests risks of anaplasmosis in animals and humans in the country.

## 1. Introduction

Anaplasmosis caused by *Anaplasma marginale* (*A. marginale*) is a worldwide disease of domestic ruminants, especially cattle, and has been reported in all continents causing huge loss to cattle industry[1]. The infection of domestic ruminants with *Anaplasma centrale* (*A. centrale*) and *Anaplasma.* sp. *omatijenne* (*A.* sp. *omatijenne*) has been also known even though these two *Anaplasma* spp. are considered non-pathogenic[1,2]. *Rhipicephalus* spp. are known to be important vectors of *A. marginale* throughout the world[1,3]. Other *Anaplasma* spp. infecting domestic ruminants such as *Anaplasma phagocytophilum* (*A. phagocytophilum*) and *Anaplasma ovis* (*A. ovis*) are either neglected or less investigated so that little is known about their epidemiology and vectors under African conditions[4,5].

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According to Dulmer *et al.*[6], *A. phagocytophilum* is a recently emended species of bacteria that comprises *Ehrlichia phagocytophila, Ehrlichia equi* and the agent of human granulocytic anaplasmosis. It is a multihost bacterium infecting various species of wild and domestic animals and humans[7-10]. The mortality associated with *A. phagocytophilum* infection is low in animals, but significant economic losses associated with drop in milk yield, abortion and infertility and reduced weight gain have been observed in pastured animals[5,11]. Deaths have been also recorded in weaker animals if they are not treated[12]. The number of human cases associated with *A. phagocytophilum* infection has been increasing in USA, Europe, Middle East and Asia since its recognition as a human pathogen. Tick attachment, contact with infected animal blood and prenatal infection have been associated with human infections[13]. Human infections can result in severe clinical consequences with a hospitalization rate as high as 36% in USA and a mortality rate of over 26.5% in China[5].

*A. ovis* has been known to infect domestic and wild ruminants since 1912[14,15]. It is considered to be endemic in tropical and subtropical regions, but it is frequently reported in temperate

regions. It has been detected in small ruminants in Europe, USA, Africa and Asia[14]. The mortality associated with the infection of *A. ovis* is not frequent, even though it causes huge financial losses to farming stock because of the reduced productivity[14,16]. It has now gained more importance as a result of observations that suggest its zoonotic importance following detection of the variant of *A. ovis* in human patients in Cyprus[17].

The vectors of *A. phagocytophilum* and *A. ovis* have been shown to vary among and within different continents and countries[5,8,17,18]. It has been also shown that *A. ovis* can be transmitted by bites of flies such as sheep ked[19]. *A. ovis* and *A. phagocytophilum* are recently identified in Ethiopia[20]. This has significant implications for Ethiopia where open range animal farming and ecotourism are the main sectors to alleviate poverty. Understanding the epidemiology of anaplasmosis caused by these *Anaplasma* spp. requires the knowledge of their tick vectors. Identification of the potential tick vectors of *A. ovis* and *A. phagocytophilum* in Ethiopia is the main objective of this study.

## 2. Materials and methods

### 2.1. Field sites for collection of ticks

Three sites where the occurrence of *Anaplasma* spp. including *A. phagocytophilum* and *A. ovis* was confirmed in domestic ruminants previously were purposely selected for collection of unfed ticks from the field for molecular analysis[20]. They were Bishoftu, Bako and Awash Depression. Bishoftu is the main town of Ada'a District in the east of Shewa Zone, Central Oromia, Ethiopia. It was located at a distance of 45 km east of Addis Ababa. Bako was a district in the west of Shewa Zone of Oromia State, Ethiopia, which was located at about 225 km away from the capital. Samples from Awash Depression were collected from three different localities (Fantale, Gari and Marti). Since these three sites were closer and had similar conditions, Fantale was taken as a representative location. The area was located in the east of Shewa Zone of Oromia State, about 190 km from the east of Addis Ababa. The Awash Depression is one of the irrigated areas in the mid-rift valley of Ethiopia. The detailed characteristics of the study sites were given in Table 1.

**Table 1**

Characteristics of the study sites where unfed ticks were collected.

| Characteristics | Sites | | |
|---|---|---|---|
| | Bishoftu | Bako | Awash Depression |
| Location | 9° N, 4° E | 9°8' N, 37°5' E | 8°58'30" N–8°58'3" N, 39°55'48" E–39°56'00" E |
| Mean temperature | 8.5–30.7 °C | 13.5–27.9 °C | 29–38 °C |
| Annual rainfall | 1 156 mm | 1 227 mm | 560 mm |
| Humidity | 61.30% | 85.00% | Not obtained |
| Vegetation type | Woody vegetation | Forest type | *Acacia* woodland |
| Altitude | 1 550 m | 1 650 m | 955–2 007 m |
| Climate type | Intermediate | Wet, warm, humid | arid, semi-arid |
| Farming type | Mixed | Mixed | Livestock based |
| Main livestock | Cattle, sheep, goats | Cattle, sheep | Cattle, goats, camels |
| Production system | Commercial, smallholder | Smallholder | Smallholder |

### 2.2. Collection and identification of ticks

Sampling of the ticks was carried out in September and October, 2013. The ticks were unfed, actively quested and hunted. Tick collection was carried out by flagging vegetation on pastures and wooded areas bordering farms and homesteads as described by Uys *et al*.[21]. Most of the collections were carried out during morning hours. Some of the ticks were collected just while actively moving near kraals late in the afternoon when animals came back from pastures. The ticks were preserved in 70% ethanol and transported to the Veterinary Parasitology Laboratory of the College of Veterinary Medicine and Agriculture, Addis Ababa University, Bishoftu. The identification of ticks was done based on their morphological characteristics using standard identification keys described by Walker *et al*.[22].

### 2.3. Extraction of DNA from ticks

DNA was extracted from the ticks using the boom extraction method as described previously[23] with modifications described previously by Teshale *et al*.[20].

### 2.4. Amplification of DNA with PCR

A semi-nested PCR was used to amplify a fragment of about 925 bp of the 16S rDNA. Amplification was carried out using EHR 16SD (5'-GGTACCYACAGAAGAAGTCC-3')[24] and EBR3 (5'-TTGTAGTCGCCATTGTAGCAC-3')[20] primers for the first round of amplification and EHR 16SD and EBR2 (5'-TGCTGACTTGACATCATCCC-3')[20] for the second round of the reaction. The reaction mix consisted of HotStartTaq Master Mix (2.5 IU of DNA polymerase, PCR buffer containing 1.5 mmol/L $MgCl_2$ and 200 μmol/L of each deoxyribonucleotide triphosphates), 0.2 μmol/L of each primer and PCR water. The PCR reaction was carried out in a total volume of 25 μL using a programmable thermocycler (T3 thermacycler Biometra®, Westburg, NL). The PCR procedures were described by Teshale *et al*.[20].

All the PCR products were visualized by gel electrophoresis in Tris-acetate-EDTA buffer (0.04 mol/L Tris, 0.4 mmol/L EDTA, pH = 7.7–8.8) using 2% agarose at 100 V for 20 min and stained with ethidium bromide. Negative samples were retested at 1/10 dilution for any possible inhibition effect. Throughout the PCR procedures, PCR mix with no DNA template was used as a negative control while DNA from an *in vitro* culture of *Ehrlichia ruminantium*, *A. marginale* and *A. phagocytophilum* was used as the positive control.

### 2.5. Restriction fragment length polymorphism (RFLP) analysis of the amplified products

The amplified products from positive samples were digested by using restriction enzymes, *Mbo* II, *Hha* I and *Msp* I, to identify the species of *Anaplasma* detected as described by Teshale *et al*.[20]. The restriction was done in a final volume of 15 μL consisting of 4 μL DNA (PCR product) and 11 μL RFLP mix (0.3 μL per final volume of restriction enzymes, Milli-Q water and buffer for each enzyme, Biolabs, New England). Incubation was done overnight at temperatures specific for each enzyme. The restricted fragments were separated on a 2% high resolution agarose gel by electrophoresis in Tris-acetate-EDTA buffer (0.04 mol/L Tris, 0.4 mmol/L EDTA, pH = 7.7–8.8) at 100 V for 40 min and visualized under UV illumination after staining with ethidium bromide (final concentration of 0.5 μg/mL).

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