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Study on Tick-Borne Pathogens and Tick-Borne Diseases Using Molecular Tools with Emphasis on *Anaplasma* spp. and *Ehrlichia* spp. in Ticks and Domestic Ruminants in Ethiopia

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LIST OF ABBREVIATIONS

BLAST	Basic Local Alignment Search Tool
CSA	Central Statistical Authority
EBs	Elementary Bodies
ELISA	Enzyme Linked Immuno-Sorbent Assay
GDP	Gross Domestic Product
HGA	Human Granulocytic Anaplasmosis
IFA	Immuno Fluorescent Antibody
ILRI	International Livestock Research Institute
ITS	Internal Transcribed Spacer
LMP	Livestock Master Plan
MAP	Major Antigenic Protein
MSP	Major Surface Protein
PCR	Polymerase Chain Reaction
RBs	Reticulated Bodies
RLB	Reverse Line Bloat Hybridization
RFLP	Restriction Fragment Length Polymorphism
rtPCR	real time Polymerase Chain Reaction
TBDs	Tick-Borne Diseases
USA	United States of America

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CHAPTER 1

Introduction

Ticks are the predominant vectors of diseases of livestock and they are surpassed only by mosquitoes as vectors of human diseases (Parola and Raoult, 2001; Parola *et al.*, 2013). Several pathogens can be propagated and transmitted by ticks to animals and humans. Since ticks are found in many ecological conditions, the tick-borne pathogens and diseases show a worldwide distribution and they are responsible for annual losses of about US$ 7 billion, including direct (mortality, morbidity, production) and indirect costs (control and hindrance to genetic improvement) (Bianchin *et al.*, 2007).

Tick-borne pathogens and diseases are a major constraint to ruminant development throughout the tropics and sub-tropics (Kocan *et al.*, 2000, 2010). Several million domestic and wild ruminants are exposed to anaplasmosis, babesiosis and heartwater in sub-Saharan Africa each year (Mukhebi, 1996; Uys *et al.*, 2015), causing mortality up to 82% in susceptible cattle and 95% in susceptible small ruminants under certain situations (Allsopp, 2010). In addition to mortality, tick-borne diseases are responsible for reduction in live-weight gains and milk yield (Norval *et al.*, 1988). A 23% reduction in milk yield in crossbred cattle has been documented (Furlong *et al.*, 1996) and a 13 kg extra weight gain is reported in beef cattle in Brazil treated against tick-borne pathogens compared to untreated animals (Bianchin *et al.*, 2007). However, the exact economic impact of tick-borne diseases has not been quantified in many of the African countries. An estimate of the Southern African Development Community revealed a production loss of about US$ 48 million per year on average (Allsopp, 2015) whilst some 36,000 smallholder units were totally lost during 1983-1986 (Spickett and Heyne, 1988). In addition, tick-borne pathogens within the genera *Anaplasma, Ehrlichia, Bartonella, Borrelia* and *Rickettsia* cause human diseases (Parola and Raoult, 2001; Parola *et al.*, 2013; Chomel *et al.*, 2004). This doctoral study was carried out on the molecular detection of piroplasms, rickettsiales,

Bartonella spp. and *Borrelia* spp. in ticks and domestic ruminants in Ethiopia. However, the focus was on the differential diagnosis and epidemiology of *Anaplasma* spp. and *Ehrlichia* spp. in ticks and domestic ruminants. Ethiopia is a developing country where domestic ruminants contribute about 70% of the livelihood of the poor (Perry *et al.*, 2000). As in many parts of the developing world, in Ethiopia women and children are short of animal protein (Ethiopian Public Health Institute (Addis Ababa), 2003). The Ethiopian government has established dairy and meat institutes to circumvent this problem. To this end, the Ministry of Agriculture has identified the ruminant sector as a priority development area in its livestock development master plan. The target is to upgrade the average market weight of beef cattle from 280 kg to 375 kg (LMP-team, 2014). For dairy sector the target is to increase the national milk production from 495 million liters in 2014/15 to 1301 million liters in 2019/20 (LMP-team, 2014). To achieve this target, Holstein cattle and Boer goats were imported and are being distributed to the smallholder farmers. Elsewhere in Africa anaplasmosis and heartwater have been identified to be important constraints to genetic improvement and translocation of ruminants (Bekker *et al.*, 2001). Empirical evidence, including personal observations in the field, indicated the occurrence of anaplasmosis and heartwater in ranches and dairy farms. Secondly, eco-tourism and outdoor activities are growing businesses in Ethiopia. There are increasing numbers of wildlife conservation and game ranches in the country. Tick-borne pathogens can cause mortalities in game animals and negatively affect the sector (Uys *et al.*, 2015). Some of the tick-borne *Anaplasma* and *Ehrlichia* species are known to be zoonotic (Jongejan and Uilenberg, 2004). Therefore, tick-borne anaplasmosis and ehrlichiosis are important problems that can impair the achievement of food security, economic growth and public health in Ethiopia. Reliable and accurate diagnosis of *Anaplasma* spp. and *Ehrlichia* spp. has not been achieved in the country. The accurate detection of these pathogens in ticks and vertebrate hosts is an important aspect of livestock development and disease diagnosis. Molecular methods provide the advantage of sensitive and specific diagnosis of tick-borne pathogens. This study aims at molecular identification of tick-borne pathogens with emphasis on *Anaplasma* spp. and *Ehrlichia* spp. in ixodid ticks and domestic ruminants and attempts to elucidate the epidemiology of anaplasmosis.

CHAPTER 2

Literature Review

2.1 Ticks and their life cycle

Ticks belong to the order Ixodida, which is sub-divided into three families: Ixodidae (hard ticks), Argasidae (soft ticks) and Nuttalliellidae. By 2015, a total of 901 tick species had been described, including Ixodidae (707 spp.), Argasidae (193 spp.), and Nuttalliellidae with a single species (Estrada-Peña, 2015). Members of the family Ixodidae are by far the most important ticks transmitting pathogens to animals. They are categorised into one-host, two-host or three-host ticks depending on the number of hosts required to complete their development (Walker *et al.*, 2003).

In one host ticks all the three instars (larva, nymph and adult) feed on a single host to complete the life cycle. Male and female adults copulate on host during the feeding process (Walker *et al.*, 2003). The engorged female then drops to the ground to lay eggs (Jongejan and Uilenberg, 2004). Eggs are laid as a single large batch and the female afterwards dies. *Rhipicephalus* (*Boophilus*) species fall into this category (Walker *et al.*, 2003).

In two-host ticks, larvæ attach and complete development through to the nymphal stage, which feed on the same individual host. The replete nymph drops from the host, moults to the adult stage and later seeks a second host to complete development. *Rhipicephalus evertsi* and *Hyalomma marginatum* are examples of two-host ticks (Walker *et al.*, 2003).

In three-host ticks, the larvae hatch and quest for a host, feed on it for 3-7 days, drop off and moult into nymphs after 3-4 weeks in the vegetation. The nymph must then await another host, climb onto it, attach, engorge, drop off and moult into the adult stage on the ground after 2-8 weeks. Then the adult quests for a third host to feed on which takes 1-3 weeks. Finally it drops off and completes that cycle with oviposition on the ground (Latif and Walker, 2004). All *Amblyomma* species are three-host ticks (Walker *et al.*, 2003).

2.2 Major Tick-borne Diseases

2.2.1 Introduction

Tick-borne diseases are a major constraint to ruminant development throughout the tropics and sub-tropics (Kocan *et al.*, 2000, 2010). Some of the tick-borne pathogens also infect humans and thus pose a public health threat (Parola and Raoult, 2001; Parola *et al.*, 2013). Common tick-borne pathogens include protozoa, bacteria and viruses. The most commonly encountered protozoal and bacterial tick-transmitted pathogens are presented in Table 2.1. Important tick-borne diseases, not specifically dealt with in this thesis, are first discussed in this section. Detailed descriptions of *Ehrlichia* and *Anaplasma* infections are given in section 2.3.

Table 2.1: Summary of tick-borne diseases, host preference and vectors species

Pathogen sp.	Principal host(s)	Principal vector	Disease	Reference
Babesia bigemina	cattle	*Rhipicephalus* (*Boophilus*) spp.	babesiosis	(Simuunza, 2009)
Babesia bovis	cattle	*Rhipicephalus* (*Boophilus*) spp.	babesiosis	(Simuunza, 2009)
Theileria parva	cattle	*Rhipicephalus appendiculatus*	ECF	(Simuunza, 2009)
Theileria annulata	cattle	*Hyalomma* spp.	TT	(Simuunza, 2009)
Theileria mutans	cattle	*Amblyomma variegatum*	BT	(Simuunza, 2009)
Theileria ovis	small ruminants	*Hyalomma* spp.	OT	(Berggoetz, 2013)
		Rhipicephalus spp.	OT	(Berggoetz, 2013)
Theileria lestoquardi	small ruminants	*Hyalomma* spp.	ST	(de la Fuente *et al.*, 2008)
Anaplasma marginale	cattle	*Rhipicephalus* spp.	anaplasmosis	(Kocan *et al.*, 2010)
		Dermacentor spp.	anaplasmosis	(Kocan *et al.*, 2010)
		Rhipicephalus (*Boophilus*) spp.	anaplasmosis	(Kocan *et al.*, 2010)
Anaplasma centrale	cattle	*Rhipicephalus simus*	anaplasmosis	(Kocan *et al.*, 2010)
		Haemaphysalis longicornis	anaplasmosis	(Kocan *et al.*, 2010)
Anaplasma phagocytophilum	several spp.	*Ixodes* spp.	tick-borne fever/HE	(Woldehiwet, 2010)
		Amblyomma americanum	tick-borne fever/HE	(Woldehiwet, 2010)
		Dermacentor auratus	tick-borne fever/HE	(Woldehiwet, 2010)
		H. longicornis	tick-borne fever/HE	(Woldehiwet, 2010)
Anaplasma ovis	small ruminants	*Dermacentor* spp.	small ruminant anaplasmosis	(Rymaszewska and Grenda, 2008)
		Rhipicephalus spp.	small ruminant anaplasmosis	(Rymaszewska and Grenda, 2008)
Anaplasma bovis	cattle	*Haemophysalis* spp.	anaplasmosis	(Rymaszewska and Grenda, 2008)
		Rhipicephalus spp.	anaplasmosis	(Rymaszewska and Grenda, 2008)
		Amblyomma spp.	anaplasmosis	(Rymaszewska and Grenda, 2008)
Anaplasma platys	dogs	*Rhipicephalus sanguineus*	canine cyclic thrombocytopenia	(Rymaszewska and Grenda, 2008)
Ehrlichia ruminantium	ruminants	*Amblyomma* spp.	heartwater	(Allsopp, 2010)
Ehrlichia chaffensis	dogs, humans	*A. americanum*	HME	(de la Fuente *et al.*, 2008)
Ehrlichia canis	dogs	*R. sanguineus*	canine ehrlichiosis	(de la Fuente *et al.*, 2008)
Ehrlichia ewingii	humans	*A. americanum*	HME	(de la Fuente *et al.*, 2008)
Rickettsia africae	humans	*A. variegatum*	ATB fever	(Parola *et al.*, 2013)
Ricketssia conorii	humans	*R. sanguineus*	Mediterranean SGF	(Parola *et al.*, 2013)
Bartonella spp	dogs and humans	Lice	bartonellosis	(Parola and Raoult, 2001)
Borrelia burgdoferi s.l	humans	*Ixodes* spp.	lyme borreliosis	(Parola and Raoult, 2001)

ECF = East Coast fever; TT = tropical theileriosis; BT = benign theileriosis; OT = ovine theileriosis; ST = sheep theileriosis;

HME = human monocytic ehrlichiosis; HGE = human granulocytic ehrlichiosis; ATB = African tick bite; SFG = Spotted Fever Group

2.2.2 Babesiosis

Babesiosis is tick-transmitted a disease of worldwide economic, medical and veterinary importance caused by intra-erythrocytic protozoan parasites of the genus *Babesia*. Since its recognition in 1888 by Babes in Romania it is known to be distributed worldwide (Bock *et al.*, 2004). Of the more than 100 known species of *Babesia*, *B. bovis* and *B. bigemina* (Simuunza, 2009) are of greatest importance in cattle. *Babesia* infection is found in most tropical and sub-tropical countries between latitudes 40°N and 32°S (Bock *et al.*, 2004). *Babesia* spp. (*B. bigemina* and *B. bovis*) are transmitted vertically by one-host ticks

of the genus *Rhipicephalus* (*Boophilus*). The most recognised vectors of the disease are *Rhipicephalus microplus*, *Rhipicephalus annulatus* and *Rhipicephalus geigyi* for *B. bovis* and *B. bigemina*, while *Rhipicephalus decoloratus* exclusively transmits *B. bigemina* (Simuunza, 2009). *R. e. evertsi* is also incriminated in the transmission of *B. bigemina* (Bock *et al.*, 2004). The distribution and ecological preferences of the tick vectors in Africa have been described by Estrada-Peña *et al.* (2006). The minimum temperature and rainfall requirements are similar for both *R. microplus* and *R. decoloratus*. However, the two species do not occur together due to interspecies competition, with *R. microplus* replacing the other species if conditions are suitable for it (Estrada-Peña *et al.*, 2006).

2.2.3 Theileriosis

Theileriosis is a disease of domestic and wild ruminants caused by infection with several species of Theileria. Of the five species known to infect cattle, the two most economically important are *T. annulata*, the cause of tropical theileriosis, which is widespread throughout the Mediterranean basin, the Middle East and Asia, and *T. parva* which causes East Coast fever (ECF), a highly fatal disease of cattle in eastern, central and southern Africa (Norval *et al.*, 1991, 1992b). The other species of bovine *Theileria*, *T. mutans*, *Theileria taurotragi*, *Theileria buffeli/orientalis* and *Theileria velifera* are usually benign, although *T. mutans* and *T. taurotragi* can occasionally cause losses (Simuunza, 2009). Clinical theilerosis in small ruminants is caused *T. lestoquardi*. Hence, the three most important Theileria species, affecting ruminants are *T. parva*, *T. annulata* and *T. lestoquardi* (reviewed in Norval *et al.* (1992b)). With over one million animals dying each year from ECF in sub-Saharan Africa causing an economic loss of about US$ 300 million/annum, the disease presents one of the most important threats to livestock production in the tropics and is a major constraint for the livelihoods of millions of rural farmers (Minjauw and McLeod, 2003; Ouraa *et al.*, 2011).

Theileria buffeli/orientalis group is a benign bovine pathogen. However some authors such as Kamau et al. (2011) [cited by Berggoetz (2013)] described severe symptoms in cattle. It has been reported in Europe, Asia, Australia, America and Africa. This vast geographic distribution should coincide with tick vectors belonging to the *Haemaphysalis*, *Dermacentor* and *Amblyomma* genera. It is considered a parasite derived from the Asiatic Water Buffalo (*Bubalus bubalis*) that was introduced into Africa probably through cattle trade (Berggoetz, 2013).

Theileria velifera was first reported by Uilenberg (1964) [cited by Berggoetz (2013)] in Madagascar as a benign cattle parasite characterised by veil-like structures, which makes this parasite easy to identify. It has been shown to be apathogenic having a wide geographic distribution including eastern and southern Africa (Debeila, 2012; Muhanguzi *et al.*, 2010). It has also been reported from African buffalo. The occurrence of this piroplasm

is associated with several ticks from the genus *Amblyomma*, including *A. variegatum*, *Amblyomma hebraeum*, *Amblyomma lepidum* and *Amblyomma astrion* (Berggoetz, 2013). It was reported that some genotypes are limited to African buffalo, suggesting that this parasite evolved among wild African buffalo and adapted to cattle.

Theileria ovis, the cause of ovine theileriosis in small domestic ruminants, has been shown to cause mostly subclinical infection (Altay *et al.*, 2005). Some reports show that *T. ovis* occurs in the Middle East countries such as Iran [reviewed by Berggoetz (2013)] and in Turkey (Altay *et al.*, 2005). It has also been reported in central European countries like Spain and Asiatic countries like Southern Korea [reviewed by Berggoetz (2013)]. It also occurs in western Africa where it was observed in Ghana (Bell-Sakyi *et al.*, 2004a). According to Li et al. (2010) [reviewed by Berggoetz (2013)] *Hyalomma anatolicum*, *Hyalomma* and *Rhipicephalus* spp. such as *Rhipicephalus bursa* transmit *T. ovis*. So far *T. ovis* was only reported in small domestic ruminants and no mention is made in the literature that it infects wild ruminants.

2.2.4 Rickettsiosis

Tick-borne rickettsiosis (caused by obligate intracellular bacteria grouped in the order Rickettsiales) is a febrile illness of humans caused by a group of Rickettsia spp. collectively called spotted fever group or SFG (Kim *et al.*, 2006). Since 1910 when Rocky Mountain spotted fever was recognised for the first time only four tick-borne rickettsioses were described until 1974. These were *Rickettsia rickettsi* in USA, *R. conorii* in Africa, Europe and Asia, *Rickettsia sibirica* in Siberia and Russia and *Rickettsia australis* in Australia (Parola *et al.*, 2013). Since 1991 several new species of *Rickettsia* were identified both in areas where no *Rickettsia* spp. had been described previously and in areas where *R. conorii* was identified. Interestingly, the majority of the *Rickettsia* spp. recognised during this time period were shown to be pathogenic to humans. They were all associated with ticks for transmission. For example out of the nine tick-borne SFG rickettsiae found to be pathogens for people after 1984, six were first isolated from ticks and later found to be pathogenic to humans (Parola *et al.*, 2013)). This shows that rickettsial diseases are emerging zoonoses. In general the numbers of species of pathogenic *Rickettsia*, vector types and reservoirs have been increasing. This calls for continual research in the ecology and epidemiology of rickettsioses.

However, many questions remain unanswered concerning the eco-epidemiology and life cycle of many tick-borne rickettsiae. Besides tick-borne SFG rickettsioses, an emerging flea-borne spotted fever (also called cat flea typhus) caused by *Rickettsia felis*, is recognised although it is incompletely described. This *Rickettsia* was discovered in the mid-gut of *Ctenocephalides felis* by electron microscopic examination in 1990 (Parola *et al.*, 2013). The definitive cultivation and characterisation of the rickettsia was achieved in 2001. Now

it is considered one of the emerging SFG rickettsioses the epidemiology of which is not elucidated.

2.2.5 Lyme Borreliosis

Lyme disease (borreliosis) is considered one of the common tick-borne diseases since 1970s. It is caused by spirochete bacteria of the *Borrelia burgdorferi* sensu lato complex and transmitted by tick bites (Monaghan *et al.*, 2016). The disease can affect virtually any system in the body, producing a range of symptoms that are commonly shared with febrile diseases. The outcome of infection is variable even though mortality associated with *B. burgdorferi* infection is low (Hvidsten *et al.*, 2015). Interestingly the incidence of Lyme disease is increasing dramatically and being reported from various parts of the world (Monaghan *et al.*, 2016). The majority of Lyme disease cases are reported from USA and Europe where nymphs of *Ixodes* spp. serve as the primary vectors. The occurrence of the disease has been shown to be seasonal with the majority of human cases being reported during the months of June, July and August in Scandinavia (Hvidsten *et al.*, 2015) during which the nymph stage of *Ixodes* spp. are active. Eleven *Borrelia* spp. within the *B. burgdorferi* sensu lato complex have been described worldwide. Information on the occurrence of Lyme borreliosis in African countries does not abound in the literature.

2.2.6 Bartonellosis

Only two diseases (Carrion's disease due to *Bartonella bacilliformis* and trench fever due to *Bartonella quintana*) were recognised to be associated with *Bartonella* until 1990 (Jacomo *et al.*, 2002). There has been an increased knowledge about the occurrence and epidemiology of bartonellosis in dogs, cats and humans since that time (Chomel *et al.*, 2004). *Bartonella* organisms are considered to be emerging zoonotic agents. They are usually vector-borne, and the vectors vary with the *Bartonella* sp. involved (ranging from sand flies for *B. bacilliformis* to human body lice for *B. quintana*). Several *Bartonella* spp are now identified in domestic animals (including ruminants), wild felids, canids and rodents (Chomel *et al.*, 2004). There is an increasing number of reports of clinical cases of *Bartonella* infections in dogs and humans associated with rodents. Domestic cats and dogs appear to be important reservoirs of zoonotic *Bartonella* spp. For instance, domestic cats are considered main reservoirs of *Bartonella henselae* (the predominant causative agent of cat scratch disease), *Bartonella clarridgeiae* and *Bartonella kochelerae* (Jacomo *et al.*, 2002). Similarly *Bartonella vinsonii* subsp. *berkhoffii* (the agent of canine and human endocarditis), *B. clarridgeiae* (the cause of endocarditis and lymphocytic hepatitis), *B. henselae* (the agent of canine peliosis hepatitis) and *Bartonella elizabethae* have been isolated from dogs. In addition, a rodent-borne zoonotic *Bartonella* (*Bartonella washoensis*) was isolated from dogs (Angelakis *et al.*, 2010). This implies that various

animals including rodents could serve as reservoirs of zoonotic *Bartonella* organisms. Human cases of *Bartonella* infections are mostly associated with cats. Transmission of *Bartonella* spp. among cats appears to be due to flea bite (Chang *et al.*, 2002). However, transmission to humans is associated with cat scratch and bite. Dogs can be infected by a wide range of *Bartonella* spp. and therefore could serve as excellent sentinels for potential human exposure. In dogs, transmission of *Bartonella* spp. is associated with tick bite but no dog-to-human transmission has been reported (Chomel *et al.*, 2004). Fleas and ticks appear to play important role in the occurrence of *Bartonella* infections in humans. The involvement of ticks in the transmission of *Bartonella* spp. is particularly increasing in the recent years (Angelakis *et al.*, 2010). In general, various arthropods including mites, biting flies, sand flies, ticks, lice and fleas have been incriminated for transmission of *Bartonella* spp. However, the definite vectors and reservoirs of the different *Bartonella* species that are associated with various clinical cases in animals and humans remain to be elucidated.

2.3 Anaplasmosis and Heartwater

2.3.1 Taxonomy of *Anaplasma* spp. and *Ehrlichia* spp.

Both the agents of heartwater and anaplasmosis underwent changes of names since their recognition. According to the current taxonomy both agents belong to the family *Anaplasmataceae* of the order Rickettsiales (Bekker *et al.*, 2001; Allsopp *et al.*, 2004). They are obligate intracellular parasites, which are found exclusively within the host cell cytoplasm. The agent of heartwater was initially named *Rickettsia ruminantium* in 1925 by Cowdry and later Moshkovski renamed it as *Cowdria ruminantium* in 1947 (Allsopp, 2010) before the current *E. ruminantium* was coined for it. The prototype of *A. phagocytophilum*, the causative agent of tick-borne fever in ruminants also underwent a few nomenclature changes since its first description, including *Rickettsia phagocytophila*, *Cytoectes phagocytophila*, *Ehrlichia phagocytophila* and the currently *A. phagocytphilum* (Yoo-eam, 2012). The taxonomy of other *Anaplasma* species remained relatively stable. Based on concatenated alignments of the small and large subunit rRNA genes Ferla *et al.* (2013) reclassified the agents belonging to Rickttsiales and the current classification is depicted in Figure 2.1.

2.3.2 Historical background

Historically heartwater was first described in South Africa in 1838 as a fatal disease of sheep in the Louis Trichardt district [Neitz (1947) referred to in Allsopp *et al.* (2004)]. Later the disease was reported in sheep, goats and antelope in the King William's town district in 1876. At that time the then Commission of Livestock Diseases reported the occurrence of heartwater in Grahamstown. Coincidentally William Bowker described the

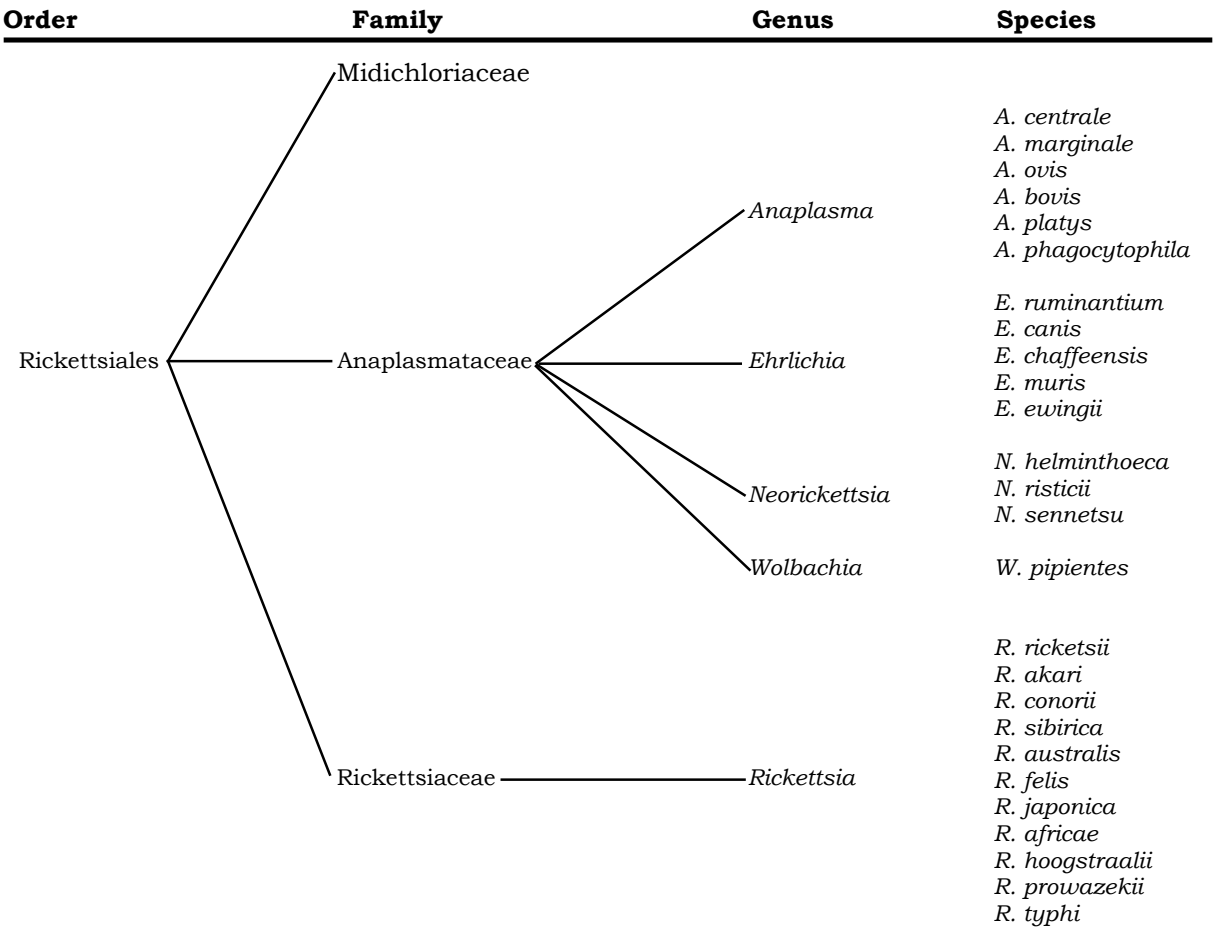


Figure 2.1: Current classification of the organisms under the family *Anaplasmataceae* [adapted from Ferla *et al.* (2013)]

A. hebraeum tick for the first time when he found a "bont" tick infesting a cow imported from Zululand (Provost and Bezuidenhout, 1987; Allsopp *et al.*, 2004; Camus *et al.*, 1996), but the vector role of the tick was not elucidated at that time. The transmissibility of the disease to susceptible animals via infected blood was proven during the late 19th century. Thereafter Lounsbury (1900) established that the disease is vectored by *A. hebraeum*.
The etiological agent of the disease, *E. ruminantium*, was first demonstrated in tissues of animals affected by heartwater in 1925 (Cowdry, 1925). During the 1950s a blood vaccine (Ball3) was developed at the Onderstepoort Veterinary Research Institute to control the disease (Provost and Bezuidenhout, 1987). A mouse model was established in the early 1970s to aid in the biological characterization of isolates of the agent (Du Plessis and Kümm, 1971) and cultures of the organism was established in vitro in 1985 (Yunker, 1995). Growth of *E. ruminantium* in tissue cultures was further improved by the development of a chemically defined medium (Zweygarth and Josemans, 2001) and cultivation of the organism in tick cell lines (Bell-Sakyi *et al.*, 2000, 2004b; Zweygarth *et al.*, 2008). Now the availability of large amounts of *E. ruminantium* led to a landmark discovery and completion of the genome sequence of the 'Welgevonden stock' (Collins *et al.*, 2005). The advancement in the application of molecular methods for epidemiological investigations

has led to the discerning of *Anaplasma* sp. Omatjenne, which was previously considered to be another *Ehrlichia* species often infecting ruminants (Allsopp, 2010).

Cases of tick fever in small ruminants and calves caused by *A. phagocytophilum* have been recognised since 1780 in Europe. Its occurrence in farm animals and dogs has been reported for many years without the knowledge of the causative agent. One hundred and fifty years later, the causative agent of this disease was described as small bacterium, and designated *E. phagocytophia* (Rymaszewska and Grenda, 2008). Soon after its recognition it was shown to cause tick-borne fever in sheep and pasture fever in cattle in UK and several European countries (Woldehiwet, 2006; Stuen *et al.*, 2013). Besides, it is known to cause equine and canine granulocytic anaplasmosis in USA and Europe (Woldehiwet, 2010). The initial documentation of human illness caused by *A. phagocytophilum*, now termed human granulocytic anaplasmosis, was made in Minnesota and Wisconsin in 1994. Now the bacterium appears to be a generalist infecting a wide range of domestic and wild animals and humans.

The first report of infection of cattle with *A. marginale* came from the reports of Salmon and Smith in USA as early as 1894 when it was described as inclusion bodies associated with babesiosis (Kocan *et al.*, 2010). But it was Sir Arnold Theiler, who provided a full description of *Anaplasma* as a distinct parasite of erythrocytes of cattle in South Africa. He coined the genus *Anaplasma* and discriminated between *A. marginale* and *A. centrale* in stained bovine erythrocytes in 1910 (Kocan *et al.*, 2010). Following Theiler's work, anaplasmosis was soon widely recognised in tropical, subtropical and many temperate zones throughout the world. The discoveries of other species of *Anaplasma* pathogenic to animals followed in the footsteps of Theiler's work. *Anaplasma ovis* was described for the first time in 1912 by Bevan. It has been found in different regions of the world including Italy, Turkey, Iraq, India, France and USA among others (Renneker *et al.*, 2013). At present the occurrence of anaplasmosis due to *A. ovis*, *A. bovis* and *A. platys* in domestic animals are well recognised (Rymaszewska and Grenda, 2008).

2.3.3 Morphology and Life Cycle

Ehrlichia ruminantium is Gram-negative bacterium that stains purple-blue with Giemsa stain. It grows only inside the intracellular vacuole bounded by a double layer of lipid membranes (Dumler *et al.*, 2001; Allsopp, 2010). This distinguishes *E. ruminantium* from most of *Anaplasma* species, which grow freely in the cytoplasm of the eukaryotic host cells. The organisms are coccoid in appearance and vary in size from 0.4 µm to 1.04 µm with occasionally larger organisms than 1.04 µm occurring (Pienaar, 1970). The organism forms colonies within the vacuole in the host cells (Figure 2.2). The colonies (called morula, Figure 2.3) vary from horseshoe shape to ring form and bacillary shaped. These forms of the organism have also been observed in the cells of tick vectors using stains such as

Löffler's methylene blue, basic aniline dyes, Unna-Pappenheim's methyl-green pyronin and Fuchsin staining red, and Mallory's phloxine-methylene staining blue (Allsopp *et al.*, 2004). Individual organisms from the colony have a double layered membrane: an inner plasma membrane and an outer membrane (Du Plessis, 1970). The electron-microscopic studies of *E. ruminantium* showed that there are two forms: elementary bodies (EBs) and reticulated bodies (RBs).

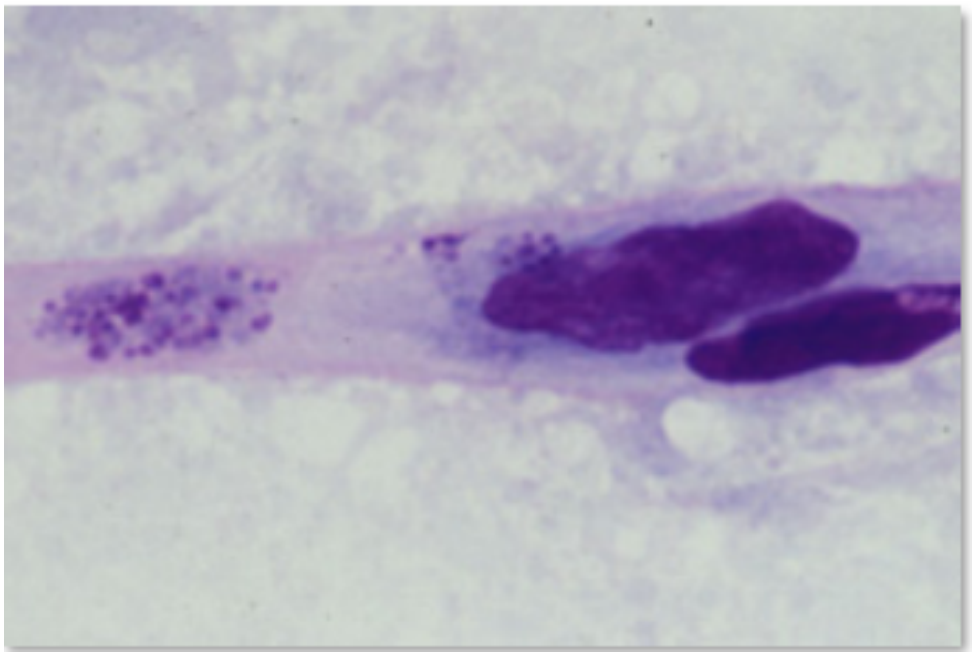


Figure 2.2: *Ehrlichia ruminantium* colonies in the cytoplasm of endothelial cells in the artery of a brain smear [adapted from: Allsopp *et al.* (2004)].

Ehrlichia ruminantium replicates in both mammalian and tick hosts. The replication is by binary fission of the reticulated bodies (Kocan *et al.*, 1987). The elementary bodies are the infective form of the organism in both hosts (Jongejan *et al.*, 1991). The latter authors demonstrated that reticulated bodies could be seen for two to four days post-infection. These are followed by the development of intermediated bodies about four to five days after infection before the endothelial cells rupture to release large number of elementary bodies six to nine days post infection. The initial replication of the organism takes place in the endothelial cells of reticulo-endothelial systems in lymph nodes. Rupture of these cells release elementary bodies. These are carried by the efferent lymph into the circulation and infect arterial endothelial cells (Du Plessis, 1970). In the endothelial cells each elementary body grows to form a colony within a vacuole. Rupture of the vacuole will disseminate the elementary bodies to the blood stream (Prozesky and Du Plessis, 1987). Once in the blood stream the EBs can infect new endothelial cells. This cycle repeats itself until the animal starts to develop fever. Ticks are infected with EBs during the release phase, while feeding on the animals (Figure 2.3).

Some species of *Anaplasma* (*A. marginale*, *A. centrale* and *A. ovis*) infect mature

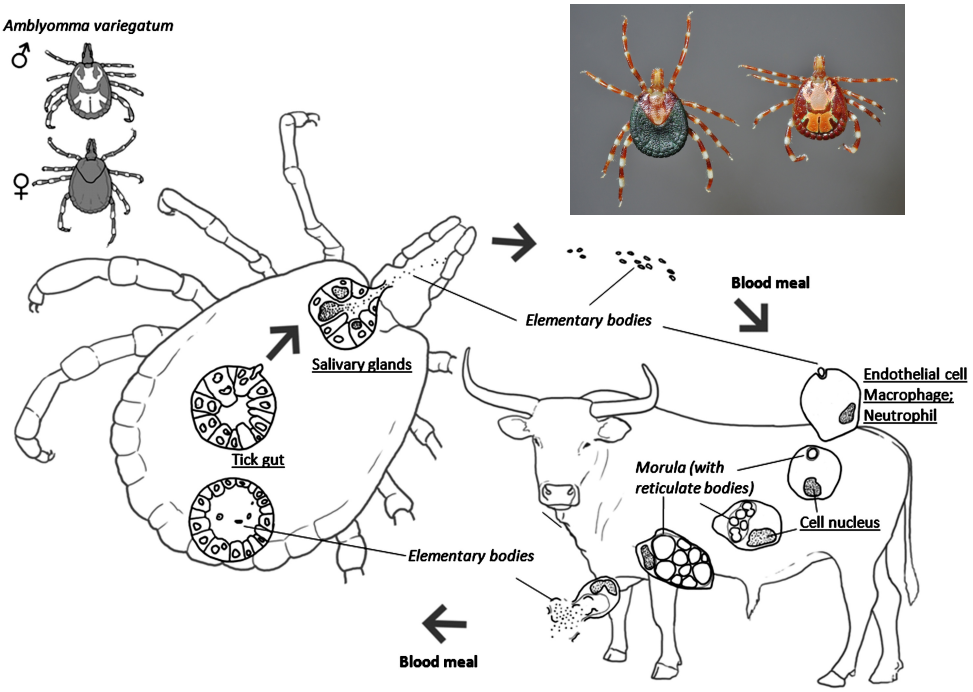


Figure 2.3: Pictorial description of the life cycle of *E. ruminantium* [adapted from Marcelino *et al.* (2012)]

erythrocytes of vertebrate hosts (Kocan *et al.*, 2010). In the erythrocytes membrane-bound inclusions containing 4 to 8 rickettsiae develop initially. The organisms released from initially infected erythrocytes invade naive ones by invagination of the cytoplasmic membrane and form vacuoles, containing the initial bodies of about 0.3-1 µm in size that appear purple-blue when stained by Giemsa stain (Kocan *et al.*, 2004). These initial bodies start a series of binary divisions resulting in inclusion bodies, which remain separated by a membrane from the cytoplasm. Such developmental process in the erythrocytes can involve as many as 70% of the erythrocytes. The number of infected erythrocytes increases geometrically and they are later removed by the reticuloendothelial cells. In those animals, which survive the acute phase of infection, persistent infection with low-level cyclic rickettisemia develops. The persistent infection serves as a source of infection for ticks. The *Anaplasma* inclusion bodies are taken up by ticks during their blood meal. The development of the rickettsiae begins in the gut cells of the ticks. After undergoing development process in the gut cells, the rickettsiae infect several types of the tick tissues (Minjauw and McLeod, 2003) including salivary glands, from which the rickettsiae find their way back to the erythrocytes of animals during feeding (Figure 2.4). At each site of infection in the ticks, the rickettsiae develop within membrane-bound vacuoles (Kocan *et al.*, 2004). The vegetative form (reticulate bodies) develops initially and eventually gives rise to the infective form (elementary bodies).

Few of the *Anaplasma* species such as *A. phagocytophilum* and *A. bovis* survive and replicate in leukocytes. During blood feeding the tick saliva stimulate neutrophil-associated

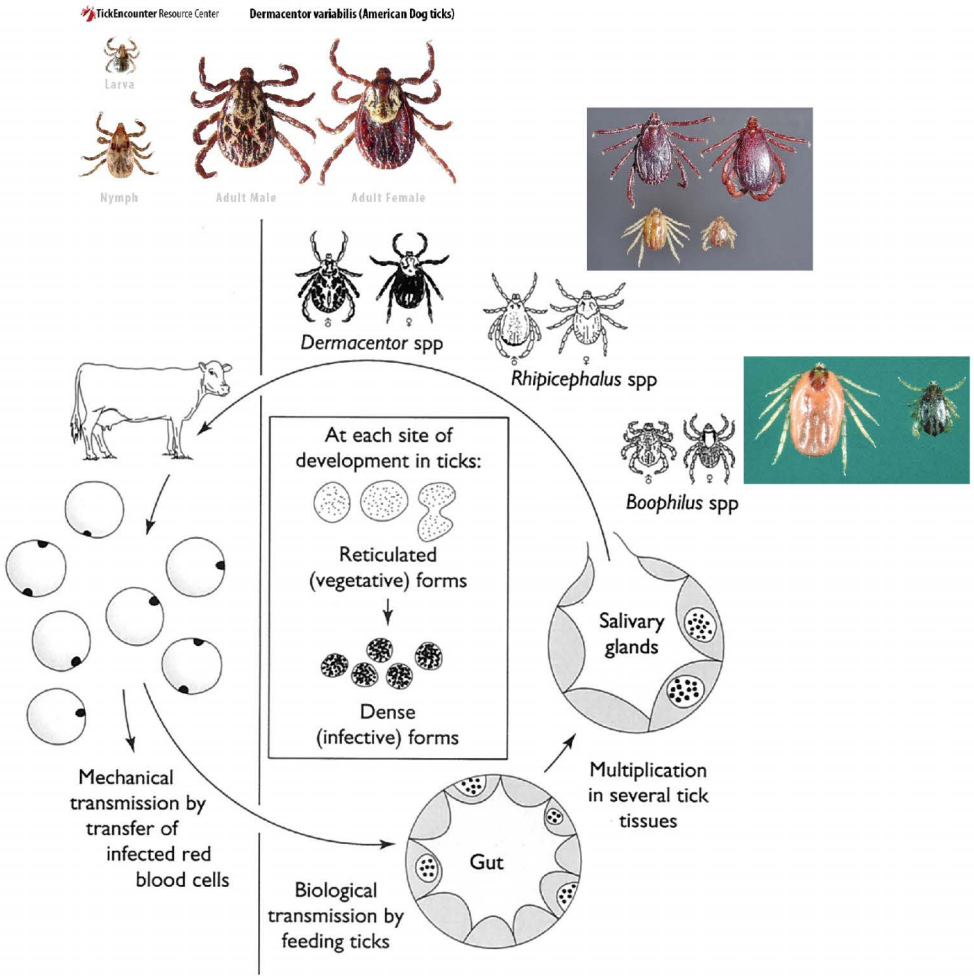


Figure 2.4: Life cycle of *Anaplasma marginale* [Adapted from Kocan *et al.* (2003)].

inflammatory responses. The immune response is orchestrated through the rickettsiae-tick interaction and has been suggested to favour the transmission of *A. phagocytophilum* to the host (Stuen *et al.*, 2013). It results in an increased number of infected circulating granulocytes at the end of the tick bite. Cyclical low-level bacteraemia with infected granulocytes has been documented (Woldehiwet, 2010), but full knowledge of cellular colonisation and establishment of infection of cells by Anaplasma species that have tropism to leukocytes is lacking at present.

2.3.4 Clinical signs of heartwater

Four different clinical forms of heartwater occur, depending on the susceptibility of the hosts and the virulence of the various strains of the agent. The peracute form of the disease is usually seen in Africa in non-native breeds of cattle, sheep and goats introduced into an heartwater-enzootic area. Heavily pregnant cows are especially prone to develop the peracute disease. Sudden death occurs, usually preceded only by a fever, severe respiratory distress and terminal convulsions. The acute form of the disease, by far the most commonly

observed syndrome, is seen in non-native and indigenous domestic ruminants. A sudden fever of up to 42℃ followed by inappetence, depression, listlessness and rapid breathing are usual signs. Nervous signs then develop, the most prominent being chewing movements, twitching of the eyelids, protrusion of the tongue and circling, often with high stepping gait. The animal may stand with its legs apart and head lowered. Galloping movements and opisthotonos are commonly seen before death. Hyperaesthesia is often observed in the terminal stages of the disease, as is nystagmus and frothing at the mouth. Diarrhoea is occasionally seen, especially in younger animals. The acute disease is usually fatal within a week after the onset of signs. Rarely, the disease may run a subacute course characterised by prolonged fever, coughing (a result of lung oedema) and mild incoordination; recovery or death occurs in one to two weeks. A mild or subclinical form of the disease, known as "heartwater fever" is seen in partially immune cattle or sheep, in calves less than three weeks of age, in antelope and in some indigenous breeds of sheep and cattle with high natural resistance to the disease. The only clinical sign in this form of the disease is a transient febrile response (Allsopp, 2010; Melaku *et al.*, 2014).

2.3.5 Clinical signs of anaplasmosis

The outcome of infection with *A. marginale* depends on the age of cattle at the time of infection and inoculum size. Cattle can become infected at any age. Generally, calves infected at less than one year of age will not show clinical signs of illness, while those between one and two years may have mild disease. Older cattle, especially those over two years of age, tend to have the most severe illness, and between 25 and 50 per cent of them die. The clinical signs include: anaemia, fever, rapid breathing and appear to have pneumonia but do not respond to conventional treatment. They may also be lethargic, have icterus or jaundice and a recent history of lower milk production and weight loss. Infected pregnant cows may abort and some will die. In many infected herds, the appearance of a sick mature cow is the first sign the disease is present in the herd (Simuunza, 2009). Similar clinical signs have been described in *A. ovis* infected sheep and goats including depression, debility, marked decline in body weight, fever and progressive anaemia, whose consequence is among other things a reduction in milk production. The infection is also potentially lethal. The symptoms of the disease depend on age, on the general condition of the animals and their breed (Rymaszewska and Grenda, 2008; Yasini *et al.*, 2012). In farm animals infected by *A. phagocytophilum* the disease usually follows subclinical course. But sometimes there are symptoms including fever, drowsiness, anorexia, abortions, drop in body weight and reduction in milk production (Rymaszewska and Grenda, 2008). Left unattended, especially in weaker individuals, it can lead to death (Stuen *et al.*, 2013).
Infection of human leukocytes with either *A. phagocytophilum* may result in an acute, febrile and systemic illness. Signs and symptoms reported for human granulocytic anaplasmosis

include fever, malaise, myalgia, headache, nausea, vomiting, diarrhoea, cough, arthralgia, rash, stiff neck and confusion (Woldehiwet, 2010).

2.4 Epidemiology of Heartwater and Anaplasmosis

2.4.1 Transmission and tick vectors

Heartwater is transmitted by members of the three-host tick genus *Amblyomma*. In South Africa, *A. hebraeum* is the major vector of the disease (Zweygarth *et al.*, 2005; Allsopp, 2010). *Amblyomma hebraeum* is the first tick species incriminated in the transmission of heartwater (Lounsbury, 1900). In the remaining parts of Africa *A. variegatum* is the main vector of the disease (Radostits *et al.*, 2007) even though other *Amblyomma* species can transmit it. Infections in *Amblyomma* ticks are transmitted trans-stadially, i.e. from larvae to nymph, from nymph to adults, and from larva through nymph to adults, and a single infected tick feeding on a susceptible animal can lead to fatal heartwater (Lounsbury, 1900). Andrew and Norval (1989b) reported that intra-stadial transmission by male ticks does occur. However, heartwater is not transmitted trans-ovarially (Lounsbury, 1900; Allsopp, 2010). Among the several species of *Amblyomma* ticks presently known, only 10 African species transmit *E. ruminantium* (Zweygarth *et al.*, 2005). Natural and proven experimental vectors of heartwater are summarised in Table 2.2. Dissemination of *E. ruminantium* from endemic areas could be effected through livestock trade. Besides, ticks are carried from island to island by migrating cattle egrets (*Bubulcus ibis*) (Barré and Uilenberg, 2010) and thus heartwater could be introduced into the USA, where large areas are climatically suitable for *A. variegatum*.

As far as New-World Amblyomma species are concerned, *Amblyomma maculatum*, is an efficient vector (Uilenberg, 1982), whereas *Amblyomma cajennense* is an inefficient vector (Uilenberg, 1983). *Amblyomma dissimile* (Jongejan, 1992) has been shown to transmit *E. ruminantium* experimentally, but being a tick of reptiles, it is not considered to play a significant role in the transmission of heartwater between ruminants, although it could maintain a rickettsial reservoir in the reptile population (Jongejan, 1992). Besides transmission through a vector tick, vertical transmission of *E. ruminantium* from cows to their calves has been demonstrated by xenodiagnosis and by a polymerase chain reaction assay. Furthermore, the transmission of *E. ruminantium* was shown after injection of viable colostral cells from dams living in a heartwater-endemic area into goats (Deem *et al.*, 1996).

The transmission of *Anaplasma* species among vertebrate hosts is achieved by several genera of ticks. The involvement of different tick species in the transmission of different *Anaplasma* species has been published from several countries of the world (Kocan *et al.*, 2004). Transmission of *Anaplasma* species is achieved by trans-stadial and intra-stadial

Table 2.2: African *Amblyomma* species able to transmit heartwater [adapted from Zweygarth *et al.* (2005)]

Amblyomma species	Reported mode of transmission
A. hebraeum	I, II, III
A. variegatum	I, II, III
Amblyomma pomposum	I, II
Amblyomma gemma	II
A. lepidum	I, II
Amblyomma tholloni	I, II, III
Amblyomma sparsum	I, III
A. astrion	I, III
Amblyomma cohaerens	I, III
Amblyomma marmoreum	I, III

I = from larva to nymph
II = from nymph to adult
III = from larva through nymph to adult

means in ticks. The transmission of those species which infect erythrocytes (particularly *A. marginale* and *A. ovis*) by biting flies and fomites has also been uncovered (Kocan *et al.*, 2010; Hornok *et al.*, 2011). This mechanical transmission is an efficient means of distribution of certain strains of *A. marginale*. For instance, the Florida strain of *A. marginale* has been shown to be more efficiently transmitted by biting flies than by ticks (Scoles *et al.*, 2005). In general, mechanical transmission of erythrocyte-infecting *Anaplasma* species can play an important role in areas where the tick vectors are absent. The known tick vectors of *Anaplasma* species are summarised in Table 2.3.

2.4.2 Host range and geographical distribution

Evidence of infection with heartwater has been detected in a large number of wild African and non-African ruminants, some of which are incriminated as possible reservoirs of the agent (Allsopp *et al.*, 2004). Domestic ruminants, notably cattle, sheep and goats are the most susceptible species in which clinical disease has been documented (Allsopp, 2010). In Africa, the distribution of heartwater coincides with the distribution of *Amblyomma* species (Faburay, 2005). It extends from West Africa through most of North-East Africa including Sudan to Ethiopia, Somalia, East and Central Africa. Most of southern African countries are also infected (Norval *et al.*, 1992a). The disease is reported from almost all African countries south of the Sahara, but also from Madagascar and three Caribbean islands (Guadeloupe, Marie Galante, and Antigua) and Mayotte in the Indian Ocean (Radostits *et al.*, 2007). The occurrence of heartwater outside sub-Saharan Africa is linked with the introduction of *A. variegatum* along with cattle from Senegal to Carribbean (Allsopp, 2010). The geographic distribution of heartwater vectors in Africa is depicted in

Table 2.3: Tick vectors of *Anaplasma* species from different parts of the world [adapted from Potgieter and Van Rensburg (1987); Rymaszewska and Grenda (2008); Kocan *et al.* (2010); Woldehiwet (2010); Stuen *et al.* (2013)]

Anaplasma species	Vectors	Region/Country
A. marginale	*R. decoloratus*	world wide
	R. microplus	world wide
	Dermacentor andersoni	USA
	Dermacentor variabilis	USA
	R. sanguineus	Israel
	R. simus	South Africa
	R. annulatus	Israel, Central America, South Africa, Mexico
	Ixodes species	Europe
A. bovis	*Haemaphysalis leporispalustris*	Asia
	H. longicornis	Asia
	Rhipicephalus species	Asia
	Amblyomma species	Asia
A. ovis	*Dermacentor* species	USA
	R. sanguineus	Europe
	R. bursa	Spain
A. centrale	*H. longicornis*	
	R. simus	Africa
A. phagocytophilum	*Ixodes ricinus*	Europe, Morocco, Tunisia
	Ixodes persulcatus	Europe
	Ixodes scapularis	USA
	Ixodes pacificus	USA
	A. americanum	USA
	D. auratus	USA
	H. longicornis	USA

Figure 2.5.

Anaplasmosis occurs in tropical and subtropical areas throughout the world where it is considered to be a major constraint to ruminant production. *A. marginale*, *A. ovis*, *A. bovis* and *A. centrale* are host-specific infecting only ruminants (Futse *et al.*, 2003; Kocan *et al.*, 2000), whereas *A. phagocytophilum* infects many animal species and humans throughout the world (Woldehiwet, 2010; Stuen *et al.*, 2013). Anaplasmosis is endemic in North America, Latin America, Africa, Asia and the Mediterranean region (Norval *et al.*, 1992a; Kocan *et al.*, 2010). The distribution of anaplasmosis due to *A. marginale* and *A. centrale* has been estimated in Africa. The estimate was based on the predicted values of the habitat suitability for the major vectors in the continent. It was found to be distributed throughout Africa with varying risks among different areas (Figure 2.6). Several studies have been carried out to determine the susceptibility of different breeds of cattle to infection with *A. marginale*. Results from these studies show that *Bos* taurus-type

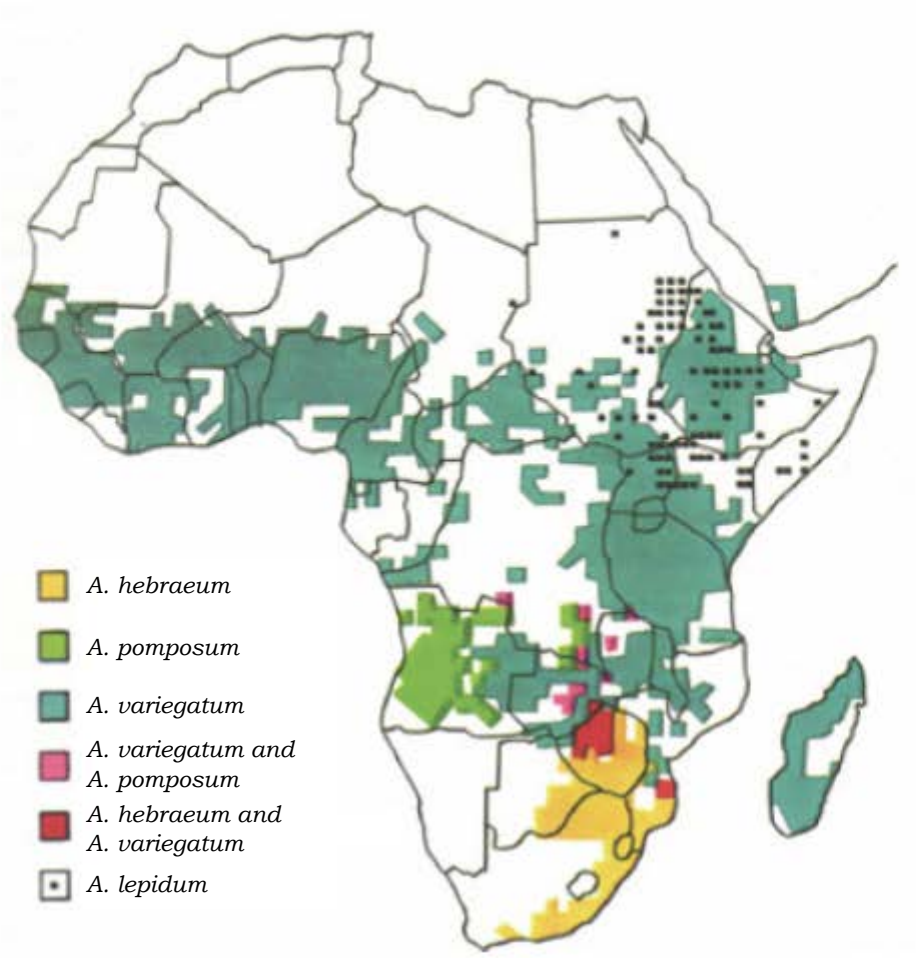


Figure 2.5: Geographic distribution of heartwater and its vector species in Africa (Allsopp *et al.*, 2004).

and *Bos* indicus-type are equally susceptible to the disease (Bock *et al.*, 1997, 1999; Wilson *et al.*, 1980). Calves are less susceptible to infection by *A. marginale* and when infected rarely develop clinical disease. Calves that recover from the disease develop life-long immunity (Guglielmone, 1995). *Anaplasma* species cause persistent infections in older animals. This situation results in the development of endemic stability in infected animals that end up in concomitant immunity (Kocan *et al.*, 2010). Therefore, it is possible to find Anaplasma infection in apparently healthy animals. The prevalence of infection increases with age as it has been demonstrated in the field by Swai *et al.* (2005).

2.4.3 Incidence and prevalence of heartwater

The reports of AU-IBAR (Anon., 2014) show that heartwater is present inmany African countries south of the Sahara and several island states. Infection of domestic ruminants and antelope species is documented. In 2014, thirteen countries reported to AU-IBAR the occurrence of heartwater with a total of 1376 outbreaks affecting 4250 ruminants whereby 1395 deaths were recorded (Table 2.4). Zimbabwe (1059) reported the highest number of

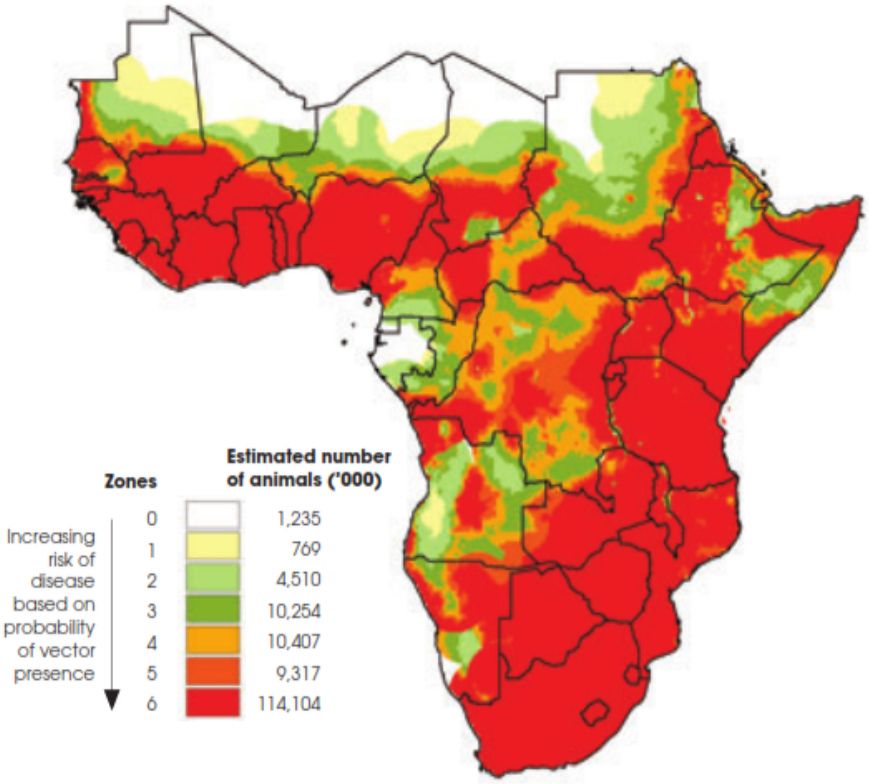


Figure 2.6: Estimated distribution and risk of anaplasmosis due to *A. marginale* and *A. centrale* in Africa. Taken from Minjauw and McLeod (2003).

outbreaks, followed by Botswana (102), Zambia (97), and South Africa (60).

Table 2.4: Countries reporting occurrence of heartwater to AU-IBAR in 2014

Country	Outbreak	Susceptible	Cases	Deaths	Slaughtered	Destroyed
Botswana	102	5212	368	249	0	0
Burkina Faso	1	150	1	0		
Cameroon	1	2	2	0	0	
Ghana	6	56	10	0	5	0
Kenya	2	23	5	0	0	0
Mozambique	4	116	6	5	0	
Somalia	7	719	24	4	2	2
South Africa	60	532	116	38		0
Sudan	1	80	14	4		
Swaziland	22	15690	393	144	10	0
Tanzania	14	39441	442	86	0	0
Zambia	97	9976	501	98	0	0
Zimbabwe	1059	627570	2368	767	12	26
Total	1376	699567	4250	1395	29	28

2.4.4 Prevalence and incidence of Anaplasmosis

Various studies showed that ruminant anaplasmosis is one of the most prevalent diseases reported from different countries across the world. It has been reported in almost all states of the USA (Kocan *et al.*, 2010), all Latin American countries, with the exception of deserts, certain mountain ranges, Mexico, central America and Caribbean islands (Guglielmone, 1995). It has also been reported from many African countries, southern Europe and Asia. The prevalence and incidence of anaplasmosis in ruminants vary widely among countries and within countries as summarised in Table 2.5. The prevalence of Anaplasma infection in ticks has also been shown to vary from place to place. For instance, Fyumagwa *et al.* (2009) reported a prevalence ranging from 0.7% to 13%, depending on tick species involved in Tanzania.

2.5 Impact of Heartwater and Anaplasmosis

2.5.1 Economic impact of heartwater

Heartwater is of considerable economic importance in sheep and goats. In endemic areas, morbidity and mortality are low, although the percentage of seropositive animals could be as high as 100% in adults, depending on the abundance of tick vectors. Case fatality can be as high as 100% in peracute cases in sheep and goats and as low as 10% in cattle. The severity of clinical disease and mortality depend on the species, breed and age of the host and the virulence and inoculum size (Faburay, 2005). Young animals immediately after birth have an innate resistance in all species and breeds, which lasts for about a month in calves (Du Plessis and Malan, 1987) and for two weeks in lambs and kids (Yunker, 1996). The disease is less severe in indigenous breeds and related game animals reared in enzootic areas, some of which may become symptomless carriers. Since most losses are in exotic animals, heartwater is a major constraint to livestock improvement in sub-Saharan Africa. It is in fact the most important rickettsial infection of ruminants in Africa and the second most important tick-borne disease after East Coast fever (Radostits *et al.*, 2007). It has a potential to spread to currently free areas and establish in the ruminant community if favourable conditions avail for the vector. This situation is responsible for detention of infected animals from export (Du Plessis, 1990). Besides, the disease is one of the hindrances to translocation of ruminant stock from marginal areas to endemic regions (Bekker *et al.*, 2001). Losses have occurred when high-yielding exotic breeds have been introduced to upgrade or replace local stock (Uilenberg, 1982). Introduction of susceptible animals from heartwater-free areas to endemic areas have been associated with heavy losses. In Botswana, such losses occurred when cattle were moved from heartwater-free areas in the drier western part of the country to endemic areas in the east [immediate mortality losses around 20% are reported in Windsor (1987)]; in Zimbabwe, it occurred when cattle

Table 2.5: Prevalence and incidence of ruminant anaplasmosis in the world

Host spp.	Country	Detection method	Magnitude	Reference
Cattle	Ghana	smear	60.0%*	Bell-Sakyi *et al.* (2004a)
Cattle	Kenya	smear	10.9%*	Muraguri *et al.* (2005)
Cattle	Kenya	serology	4.6-32.1%*	Okuthe and Buyu (2006)
Cattle	Kenya	serology	50.2%	Okuthe and Buyu (2006)
Cattle	South Africa	ELISA	87.0%	Mtshali *et al.* (2004)
Cattle	Sudan	PCR	6.1%	Awad *et al.* (2011)
Cattle	Sudan	ELISA	57.6%	Kivaria *et al.* (2012)
Cattle	Tanzania	smear	18.0%	Swai *et al.* (2009)
Cattle	Uganda	RLB	5.3%	Muhanguzi *et al.* (2010)
Cattle	Zambia	PCR	51.5-58.5%	Simuunza *et al.* (2011)
Cattle	Mongolia	IFA	0.0-76.5%	Sophia *et al.* (2012)
Cattle	Mongolia	PCR	13.0%	Sophia *et al.* (2012)
Cattle	Pakistan	smear	30.0%	Rajput *et al.* (2005)
Cattle	Turkey	RLB	9.0%	Aktas *et al.* (2011)
Cattle	Vietnam	ELISA	28.0%	Geurden *et al.* (2008)
Cattle	Brazil	smear	48.0%	Pohl *et al.* (2013)
Cattle	Brazil	rtPCR	70.2%	Pohl *et al.* (2013)
Cattle	Costa Rica	ELISA	37.2%	Oliveira *et al.* (2011)
Cattle	Italy	serology	26.0-78.0%	de la Fuente *et al.* (2005)
Cattle	Italy	PCR	0.0-50.0%	de la Fuente *et al.* (2005)
Cattle	Italy	IFA	16.8%	Ebani *et al.* (2008)
Goats	Ghana	smear	94.0%*	Bell-Sakyi *et al.* (2004a)
Goats	Mongolia	IFA	0.0-71.4%	Sophia *et al.* (2012)
Goats	Mongolia	PCR	47.2%	Sophia *et al.* (2012)
Goats	Italy	IFA	4.2%	Ebani *et al.* (2008)
Sheep	Ghana	smear	92.0%*	Bell-Sakyi *et al.* (2004a)
Sheep	Iran	smear	33.3%	Noaman (2013)
Sheep	Iran	PCR	87.4%	Jalali *et al.* (2013)
Sheep	Iran	smear	33.6%	Jalali *et al.* (2013)
Sheep	Mongolia	IFA	33.3-88.0%	Sophia *et al.* (2012)
Sheep	Mongolia	PCR	39.8%	Sophia *et al.* (2012)
Sheep	Italy	serology	25.0-75.0%	de la Fuente *et al.* (2005)
Sheep	Italy	PCR	0.0-87.0%	de la Fuente *et al.* (2005)
Sheep	Italy	IFA	12.7%	Ebani *et al.* (2008)
Sheep/goats	Sudan	PCR	41.7%	Renneker *et al.* (2013)
Sheep/goats	Iraq	PCR	66.7%	Renneker *et al.* (2013)
Sheep/goats	Turkey	PCR	31.4%	Renneker *et al.* (2013)
Sheep/goats	Portugal	PCR	82.5%	Renneker *et al.* (2013)

* = incidence, all others are prevalence

were moved from the heartwater-free Highveld to the Lowveld and in South Africa losses occurred when animals were moved locally to better grazing grounds (Norval *et al.*, 1992a). Losses have been reported in Mali when livestock was trekked from the Sahel to markets in areas where *A. variegatum* is common. It was also shown in Mozambique where mortality in goats due to *E. ruminantium* was responsible for rendering translocation of goats from north to the south of the country impossible (Bekker *et al.*, 2001). These all show that heartwater-associated mortalities constitute significant losses to ruminant producers and considerably hamper livestock amelioration efforts.

It is generally accepted that the impact of heartwater remains difficult to quantify accurately, because of underreporting (Allsopp *et al.*, 2004). The same authors provide two case studies. A first report pertained to the Eastern Cape Province of South Africa, where the Deputy Director of Veterinary Services estimated that 10% of all losses were due to heartwater (total losses estimated at US$ 30 million for the year 1998). The second study referred to Zimbabwe, where annual losses amounted to US$ 5.6 million. The latter amount included the cost of acaricides, treatment and losses in milk production (Allsopp *et al.*, 2004).

2.5.2 Economic impacts of anaplasmosis

As shown in Table 2.5, a significant proportion of ruminant populations in the tropical and sub-tropical parts of the world are infected by *Anaplasma* species (chiefly *A. marginale*, *A. ovis* and *A. centrale*). Animals that recover from infection retain the pathogens and remain immune for long periods of time (Jongejan and Uilenberg, 2004). In endemic areas, the local livestock has been exposed to the infections for a number of generations and become tolerant. Under such conditions young animals are more tolerant than adult ones. These conditions can achieve the state of endemic stability, in which large numbers of animals are infected in the absence of clinical diseases. Even though endemic stability can be achieved in endemic areas, infection with *Anaplasma* species is not without negative effect. It has been shown that erythrocyte-dwelling *Anaplasma* species culminate in anaemia and icterus due to phagocytosis of infected erythrocytes which could reach up to 70%. Besides, *Anaplasma* species have been known to cause weight loss, abortion and lethargy. Immunosuppression is the sequel of anaplasmosis. Death can also occur in weaker animals if left untreated (Rymaszewska and Grenda, 2008). Clinical anaplasmosis can occur in genetically improved dairy herds causing significant losses. For instance, incidence of clinical anaplasmosis ranging from 5.6% to 33.9% was observed in dairy calves in Kenya (Muraguri *et al.*, 2005). Oliveira *et al.* (2011) have also reported incidence of clinical anaplasmosis in dairy herds that vary from 5% (in the dry season) to 50% (in the rainy season). Similarly, Swai *et al.* (2009) recorded an incidence of confirmed clinical anaplasmosis equal to 27% in calves in Tanzania. These later authors reported mortality of 21.4% attributable to anaplasmosis in young dairy stock.

Anaplasma species infecting granulocytes have been shown to cause infection in up to 90% of the granulocytes during peak bacteraemia (Woldehiwet, 2010). Infected animals suffer from leucopenia. In sheep and cattle infected with *A. phagocytophilum*, marked reduction in the number of circulating neutrophils has been documented and this reduction has been shown to coincide with increased susceptibility to pyogenic bacterial infections (Woldehiwet and Scott, 1993). Anaemia has also been recorded in infected animals. Importantly, infection of ruminants with *A. phagocytophilum* has been shown to culminate

in immunosuppression. Such incidents have been observed in cattle herds and flocks of sheep in which infection with *A. phagocytophilum* is associated with a variety of clinical symptoms, such as respiratory disorders, pyogenic infections, abortion storms and drops in milk yield (Woldehiwet, 2010).

Restriction of live animal trade from endemic countries to free ones would escalate the economic losses associated with anaplasmosis. Although mortality due to anaplasmosis is low in animals, the economic losses associated with milk yield drop, abortion, infertility and reduced weight gain observed in pastured animals is considerable (Stuen *et al.*, 2013; Woldehiwet, 2010). For instance, Morley and Hugh-Jones (1989) quantified the financial loss associated with reduced milk production and culling in *Anaplasma* infected cattle in Louisiana (USA) to be US$ 0.5 million per year. The clinical outcome of infection with *Anaplasma* species have been shown to be more severe when there is co-infection with other pathogens (Renneker *et al.*, 2013). The financial loss incurred due to infection with different species of *Anaplasma* has not been determined but some studies showed that anaplasmosis due to *A. marginale* incurred a loss of approximately 300 and 800 million dollars per year in the USA and Latin America, respectively. Therefore, anaplasmosis can cause significant economic losses in the ruminant industry and such losses could be greater in Africa where ruminants are important sources of income.

2.5.3 Public health impact

Some reports suggest that *E. ruminantium* might be infective to humans. One report was based on the observation of four fatal cases of suspected ehrlichiosis in humans in South Africa (Kasari *et al.*, 2010). Diagnosis of *E. ruminantium* infection was made in three unrelated individuals who were overtly immunocompetent (Allsopp *et al.*, 2005). Two of the patients were children who died after an illness of about a week while the third was an adult patient who died after three weeks of illness. The patients presented a clinical picture of encephalitis with complications of severe headache, sleepiness and an unsteady gait. Molecular analysis based on 16S rDNA and pCS20 gene sequences carried out on samples from the three individuals indicated the involvement of *E. ruminantium* (Allsopp *et al.*, 2005). Besides detection of *E. ruminantium* DNA and the clinical picture, the presence of brain lesions that are typical of those seen in domestic ruminants infected with *E. ruminantium* was documented (Kasari *et al.*, 2010). These observations showed the need for further investigation into the public health importance of heartwater in endemic areas.

Zoonotic *Anaplasma* infections are among those tick-borne human diseases that are on the rise. The importance of *A. phagocytophilum* (the agent of human granulocytic anaplasmosis) is widely recognised since its discovery in USA (reviewed by Blanco and Oteo (2004)). Human Granulocytic Anaplasmosis (HGA) was first described in USA

in 1994 (Woldehiwet, 2010) and later reported in Europe and Asia (Stuen *et al.*, 2013). Although *A. phagocytophilum* has been known as a veterinary pathogen for over 70 years, its recognition as a cause of human disease in the USA and parts of Europe created a renewed interest in this bacterium. Human infection with the HGA agent is considered accidental and humans are 'dead end' hosts. The pathogen is maintained in tick-ruminant-rodent cycles but birds could also play a role in the epidemiology of the bacterium (Blanco and Oteo, 2004). Published data has also shown that a variant of *A. ovis* infects humans (Chochlakis *et al.*, 2010), which adds more public health importance to *Anaplasma* infections.

2.6 Control and prevention of heartwater and anaplasmosis

2.6.1 Vaccination against heartwater

Animals recovering from the natural disease or from artificial exposure to the organism are solidly immune for a variable period ranging from six months to eighteen months. Animals exposed to re-infection during this period of resistance will have their immunity reinforced and will remain immune as long as they are periodically re-infected. There is now conclusive evidence that immunity to heartwater is T-cell mediated (Du Plessis *et al.*, 1991) and that circulating antibodies play a minor role in immunity. Calves and lambs are very resistant to *E. ruminantium* in the first four weeks of life. This resistance seems to be a true age resistance and has successfully been used in the immunisation of cattle and sheep. Calves of less than four weeks of age, and lambs in the first week of life can be immunised by intravenous inoculation of heartwater infected blood. The infection that follows is usually mild and upon recovery animals are immune to re-infection because immunity is continuously stimulated by natural exposure to the organism. Older animals or very valuable calves should be examined daily after immunisation and should be treated with antibiotics as soon as the febrile response commences. A subcutaneous implant of doxycycline at the time of immunisation will eliminate the labor-intensive tetracycline treatment method. The immunity will not be affected by the antibiotic treatment. Flock immunisation of sheep and goats can be accomplished by inoculation followed by mass treatment at the end of the incubation period. Immunologically different strains of the organism do exist, but present evidence indicates that there is considerable cross-protection between different strains (Jongejan *et al.*, 1991), thus allowing successful immunisation. However, there are some strains between which there is little cross-protection.

2.6.2 Vaccination against anaplasmosis

The use of vaccines for control of anaplasmosis is thought to be most economical and effective way. Both killed and live vaccines have been developed and used for control of anaplasmosis. Both types of vaccines have mostly been prepared from *A. marginale* obtained from infected cattle while certain vaccines were formulated from *A. centrale* (Kocan *et al.*, 2010). Both killed and live *Anaplasma* vaccines have been shown to stimulate immunity that prevents development of clinical disease but does not prevent persistent infection of animals with field *Anaplasma* organisms. Even though clinical disease is prevented, vaccinated animals become persistently infected and further spread *Anaplasma* organisms since they are a source of the organisms for biological and mechanical vectors. Studies have been continued in search for effective vaccines against anaplasmosis although improved vaccines have been produced and made available on the market (Kocan *et al.*, 2010).

The practice of using live vaccines for the control of anaplasmosis dates back to the contribution of Sir Arnold Theiler at the beginning of 20th century(Kocan *et al.*, 2010). Live vaccines against anaplasmosis were prepared from erythrocytes infected with th *A. centrale* or with attenuated *A. marginale*. Vaccination against anaplasmosis involving infection of calves with virulent *A. marginale* and treating them with tetracyclines at the onset of the febrile reaction or the dete of parasitaemia has also been practiced. This method requires close monitoring of c and may not be applicable for use in large herds such as pastoral herds. Sometimes acute diwere then withdrawnsease can occur even if animals were timely treated with tetracyclines (Kuttler and Todorovic, 1973).

Killed vaccines that were developed in the USA in the 1960s were available on the market until 1999, when they were withdrawn (Kocan *et al.*, 2010). Killed vaccines were better than live vaccines in several aspects. They have a lower risk of being contaminated with extraneous agents, require inexpensive storage facilities and generally cause minimal post-inoculation reactions. They also have some disadvantages, including the need for annual administration of booster doses, a huge cost of purification of *A. marginale* from erythrocytes and the absence of cross-protection among various strains/isolates obtained from different geographic areas. Besides, the level of protection induced by killed vaccines is generally less than that induced by live vaccines (Kocan *et al.*, 2010).

2.6.3 Chemoprophylaxis

Cattle, sheep, and goats moving into heartwater and anaplasmosis enzootic areas can be protected from heartwater and anaplasmosis by prophylactic treatment with tetracyclines (short or long-acting) either by feeding (Maré, 1972) or by inoculation (Purnell, 1987). However, they should be kept under surveillance and individually treated if clinical signs are seen.

2.6.4 Tick Control

The principal mode of bringing heartwater and anaplasmosis into an area is thus through introduction of infected ticks or carrier animals. Andrew and Norval (1989a) have shown that experimentally infected sheep, cattle, and African buffalo can be a source of infection for nymphs of *A. hebraeum* for 223, 246, and 161 days, respectively. This prolonged carrier state needs to be considered when animals are moved from heartwater-enzootic to heartwater free areas. Careful dipping and hand-dressing followed by inspection to ensure the absence of ticks is recommended for animals in transit to free areas. A wide range of acaricides, including arsenical, chlorinated hydrocarbons, organophosphates, carbamates and synthetic pyrethroids are being used for controlling ticks on livestock (Beugnet and Franc, 2012). Direct application of acaricides to animals is the most popular method of controlling ticks on livestock. Applications of acaricide to tick-infested cattle via dipping or sprayer can be equally effective under ideal conditions with proper handling of equipments without injuring animals and subsequent dilution of a product (George *et al.*, 2008). The development of acaricide resistance has further complicated attempts at tick control. In enzootic areas, tick levels are now allowed to remain at levels high enough to permit re-infection of immune animals to booster the immunity.

2.7 Diagnosis of tick-borne diseases

Available methods for the diagnosis of tick-borne diseases (TBDs) include blood smear examination, serological tests and DNA-based assays. A comparison of these methods in terms of their sensitivity, specificity, costs and throughput is shown in Table 2.6. Blood smear examination is often considered to be the standard technique for routine diagnosis of TBDs. It is less time consuming than most other methods and is relatively inexpensive (Simuunza, 2009).

Microscopic examination of blood smear is less time consuming than other diagnostic methods and is also relatively cheap. However, it has some drawbacks: the accuracy of diagnosis relies on the training and experience of the laboratory personnel; it has low sensitivity and specificity. Another problem of microscopic diagnosis is that differentiation of parasite species based solely on morphology is difficult, and confusion may arise if mixed species infections occur (Almeria *et al.*, 2001).

Many serological methods standardised for the diagnosis of TBDs have been employed in epidemiological field studies (Swai *et al.*, 2005). Serological tests have a number of limitations that are common to all assays, based on the detection of antibodies, *i.e.* issues of stability, sensitivity, specificity and the objectivity of reading the results. Some serological techniques may be too cumbersome for testing large numbers of samples, *e.g.* a fluorescent antibody test (IFAT). They are often unable to differentiate recovered animals with sterile

immunity, carrier animals and clinical cases. In addition, antibody cross-reactions have been reported among closely related parasitic species. Serological tests are also unable to detect some chronically infected animals. Incorrect interpretation of test results may result in inappropriate control measures being indicated. Some serological diagnostic techniques such as IFAT are tedious, subjective and of low throughput.

Table 2.6: Comparison of various diagnostic methods of tick-borne disease (Simuunza, 2009)

Method	Sensitivity	Specificity	Cost	Throughput
PCR	++++	++++	++++	++++
Blood smear	+	+	+	++
IFAT	++	++	++	++
ELISA	+++	++	++	+++

PCR = Polymerase Chain Reaction; IFAT = Indirect Fluorescent Antibody Test; ELISA = Enzyme-linked Immunosorbent Assay; + = very low; ++ = low; +++ = high; ++++ = very high

The application of DNA-based methods for diagnosis and subsequent epidemiological investigation of TBDs is up surging (Faburay *et al.*, 2007b). The molecular methods have desirable characteristics of high sensitivity and specificity (Simuunza, 2009). Available DNA-based techniques for the detection of TBDs include the real-time PCR methods (which include standard PCR, Southern blotting and reverse line blot (RLB)), real-time PCR (qPCR) and the isothermal amplification methods (Georges *et al.*, 2001). The cost of DNA-based diagnostic techniques is relatively high when compared to serological and microscopic methods. This makes the wide-scale use of these methods among the resource-poor veterinary services not a viable prospect.

2.8 Molecular Diagnosis of Infection with *Anaplasma* spp. and *Ehrlichia* spp.

2.8.1 Introduction

Conventional diagnostic methods used for detection of tick-borne pathogens are not suitable for the detection of pathogens in vectors (Martinez *et al.*, 2004). Molecular methods, in addition to detection of the pathogens in ticks, can also aid in the understanding of the parasite-vector interactions (Prichard, 1997). Molecular techniques have a wide applicability besides epidemiologic investigations (Prichard, 1997; Zadoks and Schukken, 2006). In developed countries, molecular methods have been used in global, national, regional, local, farm-level and animal-level studies. In Africa, in contrast, the use of molecular

methods for epidemiological investigations is limited even though their application is valid. Currently, the techniques have been refined and have become simpler and more affordable. This allowed some of the molecular methods to become universal and find their way into veterinary diagnostic laboratories. Although their routine implementation still faces challenges, it is inevitable that molecular methods will be among the routine tools that are used in veterinary epidemiology for a better understanding of disease ecology. This will be crucial for decision making in order to improve animal productivity and safeguard public health.

The conventional diagnostic methods used for investigation of tick-borne infections of livestock have obvious drawbacks. They are less sensitive to detect chronically infected animals. They do not differentiate among species of the various pathogens involved and are not suitable for detection of mixed infections (Simuunza *et al.*, 2011). Furthermore, they are not suitable for use in tick vectors. Molecular techniques are nowadays becoming promising alternatives to the conventional (serological and microscopic) methods. Molecular techniques have been shown to be more sensitive and specific than the conventional methods and provide accurate differential diagnosis of the pathogens encountered (Martinez *et al.*, 2004; Peter *et al.*, 2005). They can be used in ticks and often detect mixed infections. Moreover, molecular techniques can be used to provide sound and reliable taxonomic evidences for better classification and understanding of the natural history of pathogens. Various molecular techniques have been developed and used in the epidemiological analysis of infection with *Ehrlichia* spp. and *Anaplasma* spp.

2.8.2 Molecular Analysis of Infections with *Ehrlichia* spp.

Molecular characterisation of *E. ruminantium* has been more commonly based upon 16S rRNA gene. This gene target has been used to differentiate between various isolates. Although differences based on 16S ribosomal DNA are relatively small, specific probes for the differentiation of *E. ruminantium* stocks have been reported (Allsopp *et al.*, 1997). Allsopp *et al.* (1997) used the 16S ribosomal RNA gene to design specific probes for the detection of *E. ruminantium* and proposed four genotypes based on the profiles observed. Allsopp *et al.* (2004) later extended this classification to eight genotypes (Figure 2.7), but the authors immediately cautioned that this was a preliminary classification, whereby it was not certain that all genotypes were correctly classified as *E. ruminantium* (see *e.g.* Debeila (2012) on the re-classification of *Ehrlichia* sp. Omatjenne).

The first molecular method used to detect infection of ruminants with *E. ruminantium* was made by Waghela *et al.* (1991) in *A. variegatum* using DNA probes targeting the pCS20 gene fragment (a locus with multiple copies in the DNA of *E. ruminantium*). The probes were also shown to detect *E. ruminantium* DNA extracted from sheep plasma collected before and during the febrile state of the disease (Mahan *et al.*, 1992). Recently, various

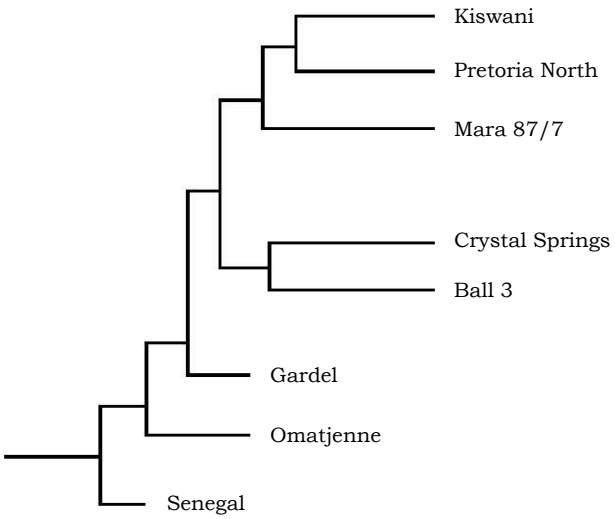


Figure 2.7: 16S rRNA genotypes of *E. ruminantium* [adapted from Allsopp *et al.* (2004)]

primers were designed to amplify a fragment of pCS20 and a PCR with these primers was shown to be a more sensitive method than one based on 16S rDNA and MAP1 for the detection of *E. ruminantium* in ticks and ruminants (Martinez *et al.*, 2004; Peter *et al.*, 2005). Molecular detection of *E. ruminantium* using this gene target has been carried out in the field both in tick vectors and animal hosts (Martinez *et al.*, 2004; Faburay *et al.*, 2007a,b).

Genes encoding for the major antigenic protein (MAP1) of *E. ruminantium* have also been used for molecular detection of the pathogen (Mahan *et al.*, 1994; van Vliet *et al.*, 1994). PCR assays targeting MAP1 have been used to detect the presence of *E. ruminantium* in blood and bone marrow samples from clinically normal animals (Kock *et al.*, 1995). Molecular detection of *E. ruminantium* using MAP1 gene was shown to be less sensitive compared to the pCS20 gene in both ticks and ruminants (Allsopp *et al.*, 1999; Martinez *et al.*, 2004; Faburay *et al.*, 2007b). However, this gene target is better situated for differentiation of genotypes of *E. ruminantium* due to its sequence polymorphisms between different strains or genotypes (Faburay *et al.*, 2007a; Allsopp, 2010).

Published results showed that pCS20 and MAP1 genes based molecular analysis have different performances in the field (Allsopp *et al.*, 1997). However, meaningful comparison between the results using these two different methods is difficult, because different isolates were used. Furthermore, reported differences between *E. ruminantium* stocks do not appear to correlate with phenotypic properties such as immunological differences determined in cross-protection tests in animals. The alternative is to explore other possible polymorphic loci that were used to differentiate other bacterial species. One such target gene is the groESL heat shock operon. This gene has been cloned from *E. ruminantium* stocks. The other important gene target is the internal transcribed spacer 2 (ITS 2) (Mahan *et al.*, 1994; Lally *et al.*, 1995; van Meer *et al.*, 1999). The groESL operon has been used to differentiate *Ehrlichia* species by using both groEL gene sequences as well as the intergenic

spacer sequence between groES and groEL (Sumner *et al.*, 1997, 2000). So far, only the groESL operon of the Welgevonden isolate has been sequenced. ITS 2 has been used to differentiate different *Wolbachia* strains but not that of *Ehrlichia* species (van Meer *et al.*, 1999).

An evaluation of the three different probes (16S, map1, and pCS20) for the detection of *E. ruminantium* showed that the pCS20 probe was the most sensitive indicator for heartwater associated pathogens except for the 'Omatjenne' isolate (Allsopp *et al.*, 1998). Since its inception the use of molecular techniques in the investigation of heartwater has continued and some improvements have been made. For instance, recently the sequencing and annotation of the *E. ruminantium* genome has been completed (Collins *et al.*, 2005), the first whole genome to be determined in Africa. This has provided a platform for the identification of the genes encoding for various proteins, which will be used for the development of diagnostic kits and improved vaccines. Besides their use in epidemiologic investigations, molecular methods have been serving important purposes in improving knowledge about taxonomy of tick-borne pathogens, including *E. ruminantium*. The recent reclassification of the order Rickettsiales for instance was based upon genetic analyses of 16S rRNA, groESL and genes encoding for surface protein (Bekker *et al.*, 2001).

2.8.3 Molecular Analysis of Infections with *Anaplasma* spp.

Various diagnostic methods have been used for many decades for the diagnosis of anaplasmosis in livestock. A characteristic feature of infection with *Anaplasma* spp. is that animals, which recover from a primary infection become carriers of the pathogen (Lew and Jorgensen, 2005). Traditionally, microscopic examinations of Giemsa stained blood smears are used to confirm acute disease while serological methods have been used mostly for the detection of antibodies in carrier animals. Molecular approaches such as DNA probes [based on the major surface protein (MSP)1b gene] have been developed for in situ hybridisation detection of *A. marginale* in both blood and ticks during the 1990s. Instead of microscopy, molecular methods, particularly PCRs, have increasingly been applied to detect *Anaplasma* spp. in both blood samples and tick vectors. Several field investigations have been carried out using PCR-based molecular diagnostic methods. The most commonly used gene target for molecular characterisation of *Anaplasma* spp. is 16S rDNA. PCR and sequencing based on this target have been used to detect *A. marginale* in various haematophagous arthropods in Hungary (Hornok *et al.*, 2008). It has also been used in the diagnosis of *Anaplasma* spp. in domestic carrier animals in various countries, e.g. in Turkey (Aktas *et al.*, 2011).

The major surface proteins genes are also widely used targets for molecular investigation of *Anaplasma* spp. infection in domestic animals. Importantly this gene target has been used for determination of the genetic diversity of *Anaplasma* spp. that were responsible for

outbreaks of anaplasmosis in endemic areas (Almazán *et al.*, 2008). Ruybal *et al.* (2009) have used this target to identify 15 different genotypes of *A. marginale* in outbreaks of bovine anaplasmosis in Argentina. Differences between isolates/genotypes in virulence, tick-transmissibility and cross-protection have been observed (Potgieter and Stoltsz, 2004). The variants of MSPs such as MSP1b, MSP2 and MSP3 are multigene family and are subjected to variation during multiplication of the *Anaplasma* spp. in persistently infected animals. Besides, some research results revealed that selection of MSP2 sequence variants occurs in persistently infected ticks (Kocan *et al.*, 2004). The other variants of MSP such as MSP4 have been used in the field to estimate the prevalence of different *Anaplasma* spp. carried by ticks (Fyumagwa *et al.*, 2009). A quantitative real-time PCR targeting MSP5 gene was recently developed (Bacanelli *et al.*, 2014). This method was shown to be more sensitive than endpoint PCR. It proved to be a suitable method for the detection of *Anaplasma* infection in endemic areas. Furthermore, in silico analysis showed that the real-time PCR was suitable for detection of *A. ovis* (Bacanelli *et al.*, 2014). Molecular methods with reasonable sensitivities have been used for field investigation of anaplasmosis. For example, Noaman *et al.* (2009) used PCR-RFLP targeting the 16S rDNA gene for the detection of *Anaplasma* infection in carrier cattle.

The emergence of certain *Anaplasma* spp. in humans and animals, thought to be due to genetic transformation of previously non-pathogenic strains or due to genetic exchange between non-pathogenic strains and related organisms, is an important aspect of the epidemiology of anaplasmosis. Such phenomena became evident mostly after the use of molecular techniques in the investigation of infection with *Anaplasma* spp. The use of molecular diagnostic methods provided an opportunity to discovery several new variants or novel strains of *Anaplasma* spp. For example, *Anaplasma* spp. strain WTD76 (GenBank: DQ007351.1), WTD81 (GenBank: DQ007352.1) and wz57 (GenBank: AY180920.1) have been reported in ticks (Katragadda, 2007) even though their pathogenic role has not been determined. Apart from the identification of the species or strains of *Anaplasma*, molecular methods have also been used in discerning of the competent vectors of various *Anaplasma* spp. In line with this certain isolates of *A. marginale* were shown not to be competently transmitted by Brazilian *R. microplus* (Patrícia *et al.*, 2005).

2.9 Ticks and TBDs as Problems of Ruminant Production in Ethiopia

2.9.1 Ruminant livestock production in Ethiopia

Livestock production is an important economic activity in Ethiopia. According to some conservative estimates ruminant livestock contributes to over 45% of the Agricultural Gross Domestic Product (AGDP). In monetary values, the sector contributes about US$

4.65 billion out of the total of US$ 10.29 billion contributed by the agriculture per annum (Behnke, 2010). The highest contribution is made by cattle (US$ 3.38 billion) followed by goats and sheep in that order. The total ruminant populations of the country are 56.7 million cattle, 29.3 million sheep and 29.1 million goats (CSA, 2015). Ruminant production is an integral part of life in all regions of the country but the highest numbers are found along a North-South transect including central Ethiopia and along the transect that connects Adama (Nazret) to Dire Dawa (Figure 2.8). Those areas with higher livestock density are also characterised by their higher human population density (Jabbar *et al.*, 2007). A lower density of ruminants is found in the pastoral areas. In general, ruminants contribute to natural, financial, human, physical and social capital in different ways and to different degrees within all production systems. Therefore, ruminants play a key role in the rural economy in particular and the entire nation's livelihood in general. The Ethiopian government considers development of the ruminant sector to be a way out to resolve the prevailing poverty.

The diversified agro-ecology of Ethiopia favours the occurrence of diverse ruminant species and tick vectors. The ruminant sector is one of the promising agribusiness areas to circumvent the prevailing poverty (Mohamed *et al.*, 2004). Ruminant livestock are integrated into the dominant smallholder or peasant farming systems throughout the country. Currently the Ethiopian government identified the ruminant sector to be a priority development area. The primary aim of sheep and goat production is meat. For small ruminants, a 20% live-weight gain with a 4% increase of the parturition rate is foreseen in the livestock masterplan of the country. The primary purpose of cattle rearing is milk and drought power production, but they are of course eventually also slaughtered. The government masterplan for meat production focuses on increasing off-take of youngstock (annual increase of 4-5%) in light of the expanding slaughterhouses and meat industries. The target is to specifically upgrade the average market weight of beef cattle to 375 kg while currently the average live-weight of adult local cattle is 280 kg (LMP-team, 2014). It has also been indicated that growth of the dairy sector, particularly the smallholder dairy operations is prioritised as the existing economic policy favours the transition of the smallholder dairy sector towards a more market-oriented system. The target according to the masterplan is to increase the average milk production of 1.9 litres per day / local cow to 19.2 litres per day / improved breed in 2019/20. During this period the number of cross-bred dairy cattle is projected from 453,000 to 4,044,000 head and this in rainfall-sufficient zones that practice mixed farming. In terms of national milk production, the volume is projected to increase from 495 million litres in 2014/15 to 1,301 million litres in 2019/20 (LMP-team, 2014). This will lift the contribution of the dairy sector in the GDP from about US$ 171 million in 2014/15 to US$ 457 million in 2019/20. The strategy identified is a large-scale upgrading of the genetic potential of the indigenous stock through crossbreeding for milk and meat production. In this regard, Holstein cattle and Boer

goats have been imported and raised in different breeding centres to be distributed to farmers. Translocation of livestock producers from drier areas to wetter areas is underway at present. Most of the drier areas are known for their lower incidence of tick-borne diseases while the moist areas are considered endemic. Disease control is limited and in most cases tick control is absent. Because of the extensive production system, herd intermixing and poor disease control, tick-borne diseases are prevalent and under-recorded in the traditional production systems (Mekonnen, 1996). The above long-term goals will probably fail without proper inventory of the most important impediments, such as ticks and TBDs, and without designing suitable control options.

Figure 2.8: Ethiopia: political map (`http://ontheworldmap.com/ethiopia/`)

2.9.2 Ticks and their distribution in Ethiopia

Ticks, especially ixodids, have been reported to be widespread in Ethiopia causing considerable damage to the livestock industry (Figure 2.9, Figure 2.10, Figure 2.11, Figure 2.12). The species composition and geographical distribution of ticks in Ethiopia have been studied previously (Morel, 1980; Pegram *et al.*, 1981; Gebre-Ab, 1983). The previous studies have identified the existence of more than 60 species of ticks (Morel, 1980) with varying geographical distribution and abundance. It was revealed that *R. decoloratus*,

A. variegatum and *R. e. evertsi* are the most widespread ticks. *Rhipicephalus pulchellus* and *A. gemma* were found to the east of the Rift Valley while *A. cohaerens*, *R. annulatus* and *Rhipicephalus bergeoni* were found in the humid and moist western highlands (Pegram *et al.*, 1981). The most important tick species are *Amblyomma* spp. and *Rhipicephalus* spp. They are widespread and have been incriminated to transmit or to be associated with the occurrence of various rickettsial, protozoal and bacterial pathogens in livestock (Mekkonen *et al.*, 2001). Later tick surveys, carried out through the Food and Agriculture Organisation (FAO) projects ETH/83/023, TCP/ETH/0053 and GCP/ETH/048/DEN in different eco-climatic zones of the country, were conform to the earlier findings. According to the later survey results the major tick genera recorded were *Amblyomma* (40%), *Rhipicephalus* (*Boophilus*) (21%), *Haemaphysalis* (0.5%), *Hyalomma* (1.5%) and other *Rhipicephalus* (37%) (Mekkonen, 1996). Two important ixodid species (*R. appendiculatus* and *R. microplus*) have not been found in Ethiopia. The temporal dynamics of the tick species studied appeared to vary depending on the eco-climatic conditions of the areas. In western Ethiopia ticks appear to infest livestock throughout the year while in other parts of the country tick infestation significantly fluctuates between the dry (lower infestation) and wet (higher infestation) seasons (Mekonnen, 1996). It is clear from those studies that the livestock industry of the country is at risk of considerable losses due to ticks and the pathogens they transmit. A conservative estimate has shown that a loss of over US$ 1 million was attributable to ticks through rejection and downgrading of hides and skins (Gebre-Ab, 1983). Heavy tick burdens cause sufficient irritation and stress such that affected animals become anorexic, which may lead to reduced productivity (Radostits *et al.*, 2007).

Currently, there is no tick control policy in Ethiopia, but acaricides are commercially available in the country. Owners are at liberty to use these acaricides, the most commonly used being Steladone (Chlorfenvinphos, Bayer). Generally speaking, only animals at research centres are regularly being sprayed against ticks.

2.9.3 Tick-borne diseases in Ethiopia

The first published evidence of tick-borne infection in Ethiopian livestock dates back to the report of Philip *et al.* (1966) who reported the occurrence of infection with *R. conorii* and *Coxiella burnetii* in sheep and goats. Since then several cases of tick-borne diseases have been suspected or empirically diagnosed in various parts of the country. At present tick-borne diseases, namely heartwater, babesiosis, anaplasmosis and theileriosis, are thought to infect ruminants in Ethiopia (Mekkonen, 1996). Only one published article described the occurrence of bovine tropical theileriosis due to *T. annulata* (Gebrekidan *et al.*, 2014) while East Coast fever caused by *T. parva* has not been reported so far. Infection of cattle by *A. marginale* is thought to be widespread in the country as do its

major vectors, *Rhipicephalus* spp. (Table 2.7). Serological studies carried out previously in some ecological zones of the country showed the prevalence of anaplasmosis to vary from 82.4% (Feleke *et al.*, 2008) to 99% (Mekonnen, 1996) in dairy cattle. The later author also reported a lower prevalence of 2.2% by Giemsa stained blood smear examination in dairy cattle. The seroprevalence of babesiosis has been shown to vary from about 60% (Mekonnen, 1996) to 87% (Feleke *et al.*, 2008). Those previous studies confirm the occurrence of babesiosis and anaplasmosis but the species of *Babesia* and *Anaplasma* involved, the epidemiology and importance of the infections in livestock of the country have not been investigated. Specifically, the differential diagnosis of the species of *Babesia* and *Anaplasma* has not been carried out. Knowledge of the occurrence and distribution of various species of pathogens belonging to these genera is crucial to counter their impacts on livestock development and public health.

Table 2.7: Suggested distribution of TBD in Ethiopia, currently based on vector distribution and clinical evidence (Mekkonen, 1996; Gebrekidan *et al.*, 2014)

Species	Principal vector	Distribution
A. marginale	*R. decoloratus*	Country wide
B. bigemina	*R. decoloratus*	Country wide
B. bovis	*R. annulatus*	South-west Ethiopia (Gambella)
E. ruminantium	*A. variegatum*, *A. gemma*	Country wide
T. mutans	*A. variegatum*	Country wide
T. buffeli/orientalis	Unknown	South-west Ethiopia
T. annulata	Not determined	North Ethiopia

Systematic investigation into heartwater has not been carried out in the country. Most cases of heartwater were reported on the basis of clinical observation and post-mortem examinations. It has nevertheless been thought to occur in different parts of the country based on the distribution of *Amblyomma* spp. (Mekkonen, 1996). Both proven natural vectors and experimentally confirmed vectors belonging to the genus *Amblyomma* are widespread in the country (Pegram *et al.*, 1981). The disease is reported in western, eastern, central and northern Ethiopia particularly in ranches and dairy farms where improved breeds are raised. It has been held responsible for several cases of mortality on premises where it was reported. Outbreaks have occurred in some dairy farms where mortality rates as high as 46% (Melaku *et al.*, 2014) and 25% (Mekkonen, 1996) were observed in dairy heifers in northern and central Ethiopia, respectively. In 1992 a devastating outbreak occurred at Habernosa ranch where Boran-Friesian crosses are kept (Solomon Gebre, personal communication, 2014). *E. ruminantium* was demonstrated in brain crush smears in 17 out of 40 dead cattle (42.5%). Its economic importance is recognised but not well documented. For years, heartwater has been known to be present in the country on the basis of clinical and pathological evidence. The disease is regarded as a problem in this ranch causing high losses, particularly in young animals (2-8 months old). Pegram *et al.*

(1981) isolated the agent and a suspected outbreak has been reported on several other ranches and farms. Molecular evidence of the presence of *E. ruminantium* in ticks and blood from livestock was reported in the pastoral areas of the Somali Region (Tomassone *et al.*, 2012). Hence, the occurrence of the disease in the country is confirmed but the extent of the infection and its epidemiology has not been studied.

Few studies have been carried out on zoonotic tick-borne pathogens in the country. For instance, the occurrence of the relapsing fever group (*Borrelia* spp.) has been reported (Boutellis *et al.*, 2013). Recent studies have shown the occurrence new *Borrelia* spp. in hard ticks from some parts of the country (Mediannikov *et al.*, 2013; Kumsa *et al.*, 2015a). Similarly, studies making use of molecular methods were conducted by some authors and revealed the occurrence of spotted fever group rickettsiae in ixodid ticks Kumsa *et al.* (2015b), *R. felis* and *B. henselae* in dog and cat fleas (Kumsa *et al.*, 2014b), *Acinetobacter* species DNA in lice and keds (Kumsa *et al.*, 2012b) and candidatus *Bartonella melophagi* in sheep keds (Kumsa *et al.*, 2014a). These findings suggest further investigation to uncover the domain and distribution of tick-borne pathogens and diseases in Ethiopia.

As shown in Table 2.7, the occurrence and distribution of tick-borne diseases of livestock have been empirically supposed to coincide with the distribution of tick vectors. Limited field surveys have been carried out using either microscopy or serology, which have obvious shortcomings (Simuunza, 2009). The results have shown the occurrence of infection with *Anaplasma* and *Babesia* without discriminating the various species (Mekonnen, 1996; Feleke *et al.*, 2008). Few studies carried out in different Regional States using molecular methods revealed the occurrence of *Anaplasma*, *Ehrlichia* and *Theileria* spp. in ticks (Tomassone *et al.*, 2012) and ruminants including *T. annulata* (Tomassone *et al.*, 2012; Gebrekidan *et al.*, 2014). Surprisingly, a positive case of *T. mutans* infection was reported in camels (Tomassone *et al.*, 2012). It has been known that microscopic diagnosis is suitable only for acute infections while serologic methods are used mostly to detect sub-clinical infections. Both diagnostic methods do not distinguish between pathogenic and non-pathogenic species. This means that previous studies did not provide accurate evidence on the occurrence of tick-borne pathogens. Understanding of the epidemiology of these infections is important to intercept the losses they mean for both the ruminant sector and the public health.

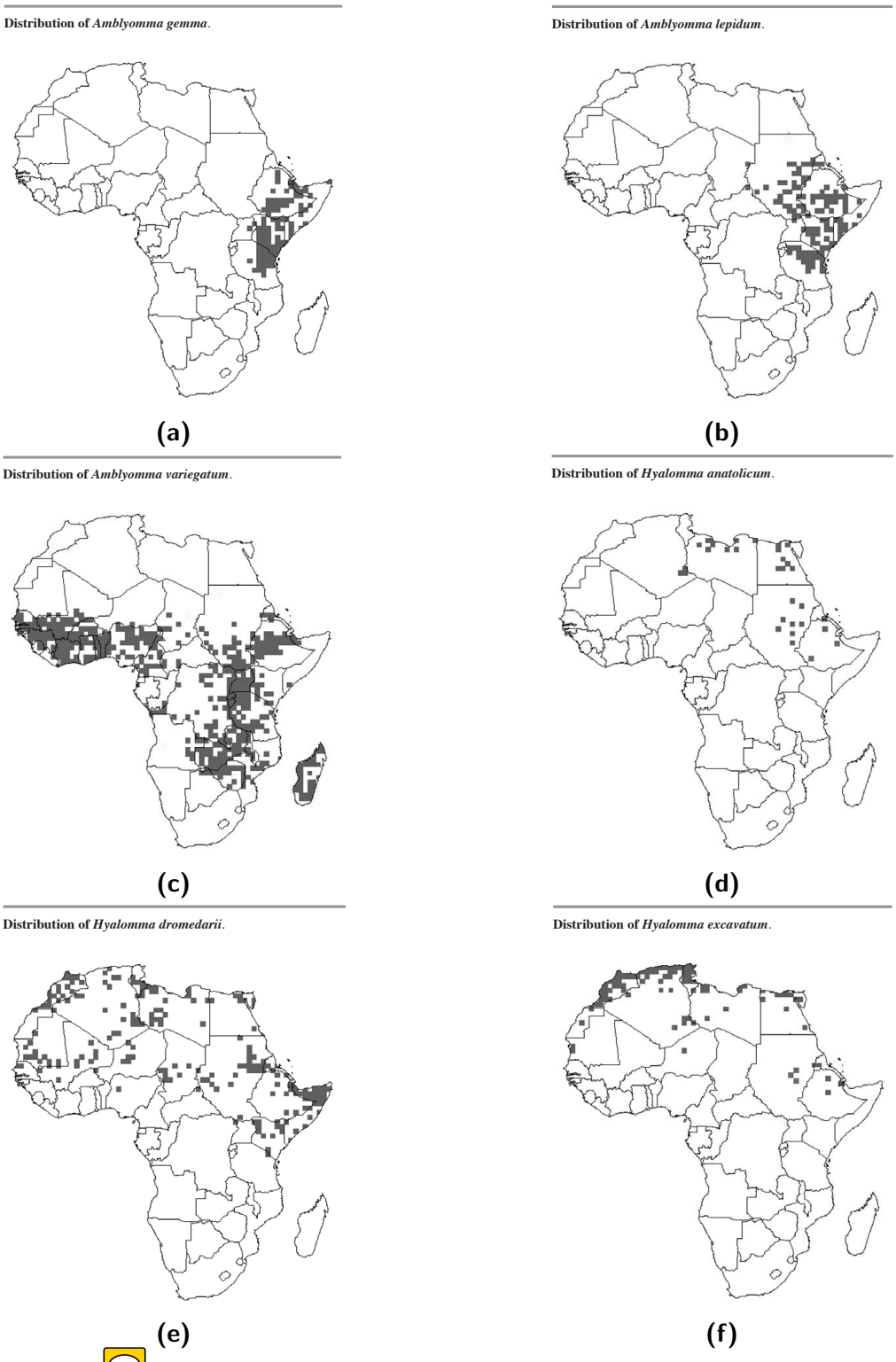


Figure 2.9: Ethiopia: tick distribution maps [extracted from Walker *et al.* (2003)]
(a) *Amblyomma gemma*; (b) *Amblyomma lepidum*; (c) *Amblyomma variegatum*
(d) *Hyalomma anatolicum*; (e) *Hyalomma dromedarii*; (f) *Hyalomma excavatum*

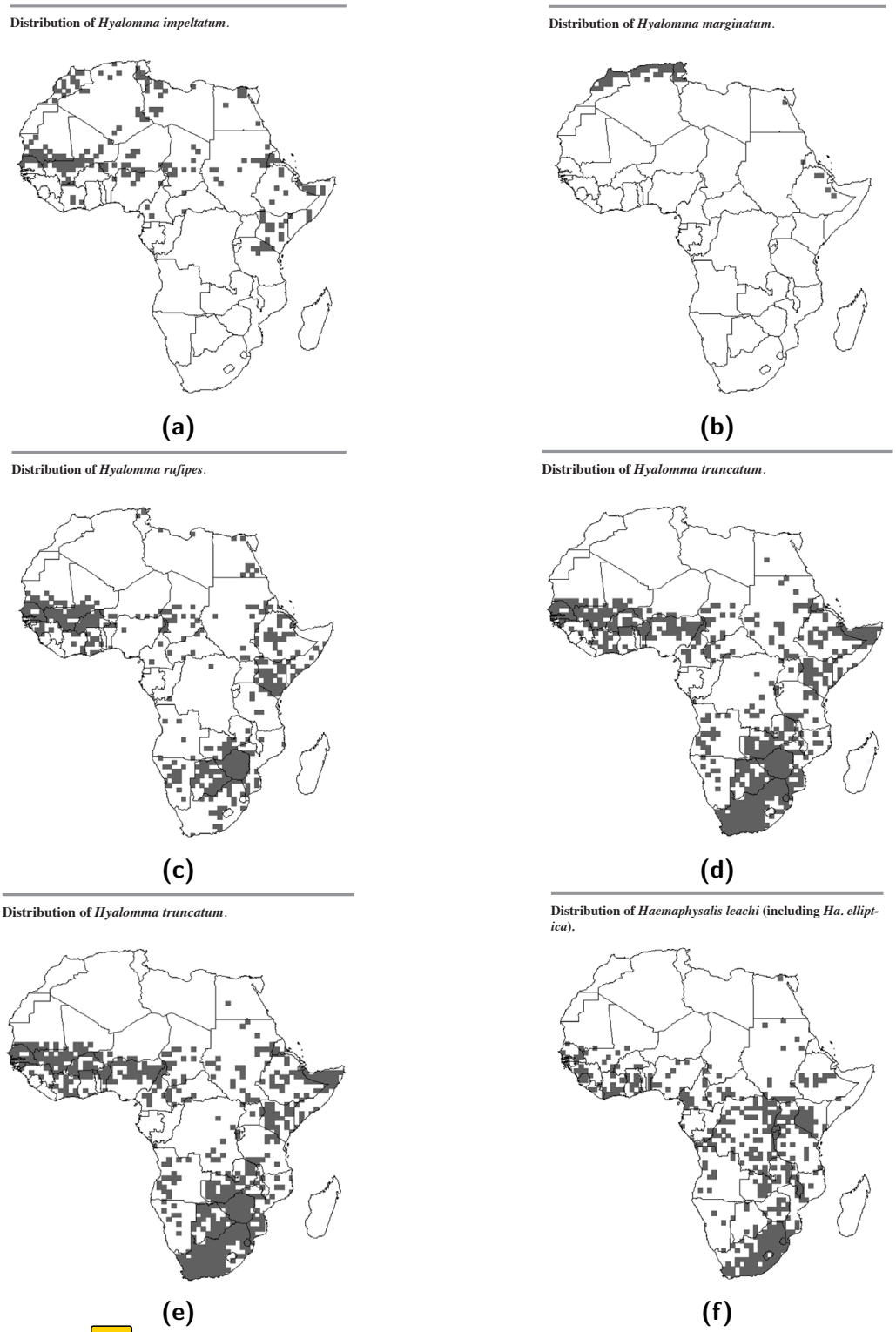
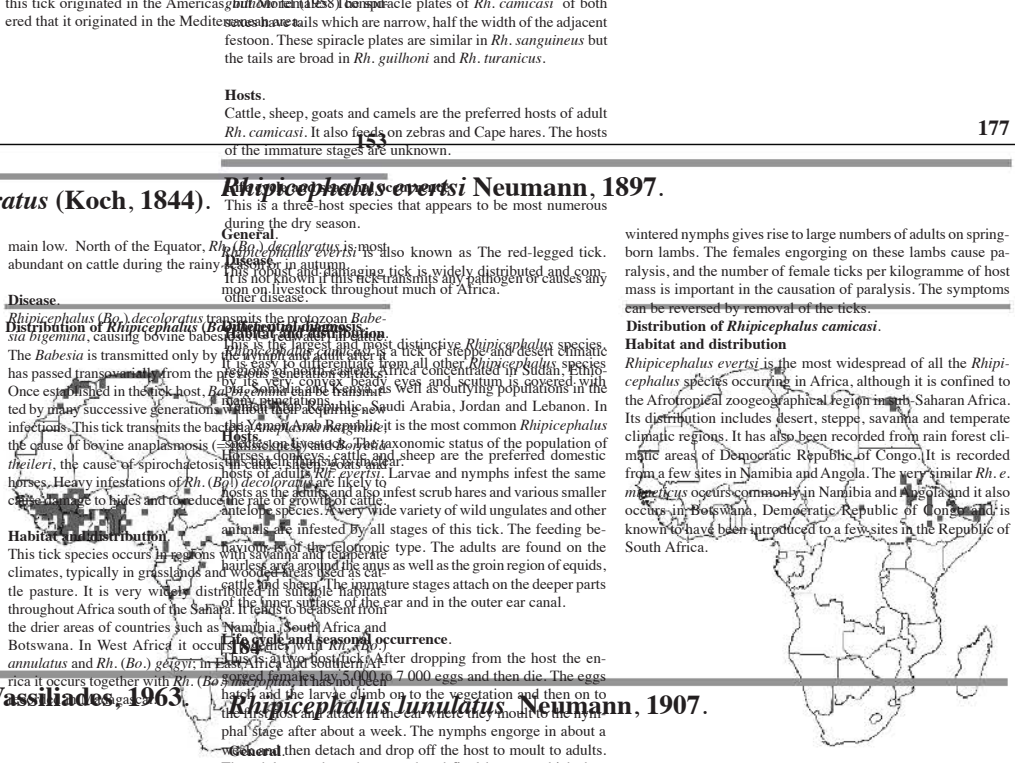
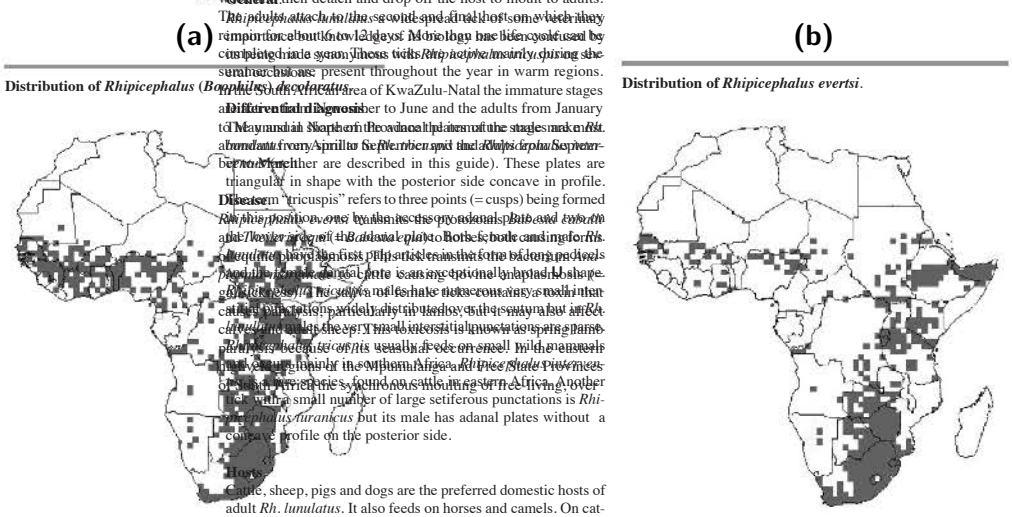


Figure 2.10: Ethiopia: tick distribution maps [extracted from Walker *et al.* (2003)]
(a) *Hyalomma impeltatum*; (b) *Hyalomma marginatum*; (c) *Hyalomma rufipes*
(d) *Hyalomma truncatum*; (e) *Hyalomma turanicum*; (f) *Haemaphysalis leachi*



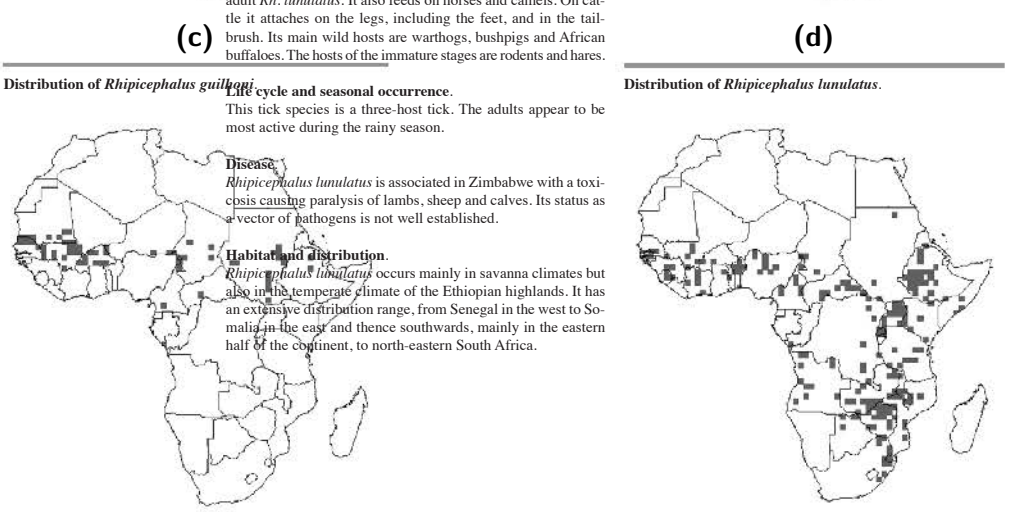
(a)

(b)



(c)

(d)



(e)

(f)

Figure 2.11: Ethiopia: tick distribution maps [extracted from Walker *et al.* (2003)]
(a) *Rhipicephalus annulatus*; (b) *Rhipicephalus camicasi*; (c) *Rhipicephalus decoloratus*
(d) *Rhipicephalus evertsi*; (e) *Rhipicephalus guilhoni*; (f) *Rhipicephalus lunulatus*

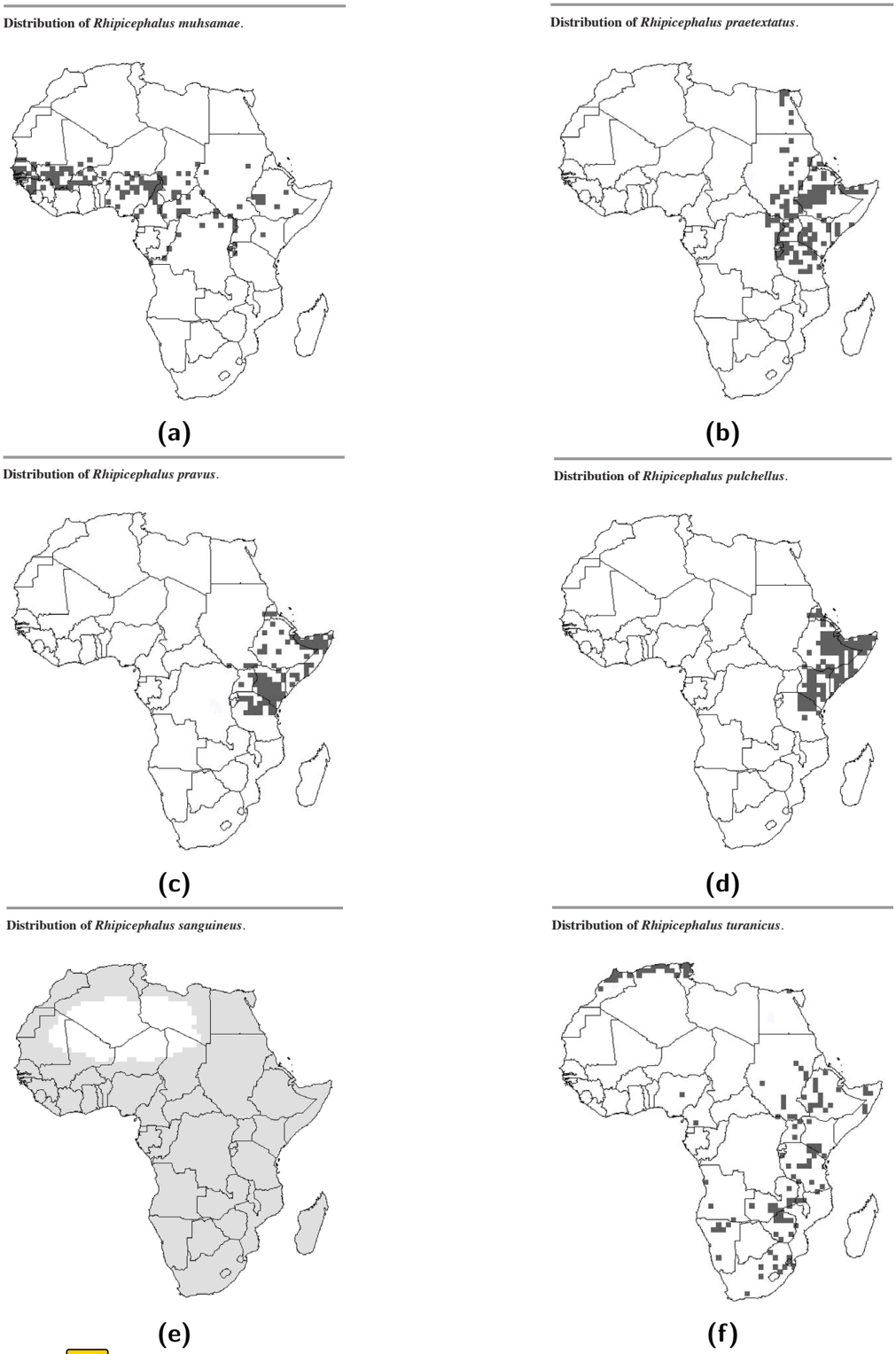


Figure 2.12: Ethiopia: tick distribution maps [extracted from Walker *et al.* (2003)]
(a) *Rhipicephalus muhsamae*; (b) *Rhipicephalus praetextatus*; (c) *Rhipicephalus pravus*
(d) *Rhipicephalus pulchellus*; (e) *Rhipicephalus sanguineus*; (f) *Rhipicephalus turanicus*

CHAPTER 3

Rationale and Objectives of the Study

The livestock sector is considered to be one of the important areas for poverty alleviation and achievement of food security. The Ethiopian government, therefore, has prioritised the ruminant sector in its livestock development masterplans. Due attention is given to expansion of smallholder milk and meat production, which need large-scale genetic upgrading of the indigenous stock. To this end, improved breeds such as Holstein cattle and Boer goats are imported and raised at various centres to be distributed to farmers. Tick-borne diseases are one of the important constraints that counter such ruminant improvement programs. Hence, there is a need for control of tick-borne diseases. Rational control of tick-borne diseases calls for a better knowledge of the type of tick-borne pathogens prevailing in the country under various agro-ecological conditions. The public health sector also requires reliable epidemiological information to understand and devise effective control of tick-borne zoonoses. The overall objective of this study was to generate scientific information that would contribute to the control of tick-borne infections of veterinary and medical importance in Ethiopia.

The specific objectives are:

1. To identify tick-borne piroplasm and bacterial species of veterinary and medical importance in Ethiopia (Chapters 4 and 5)
2. To improve molecular tools for simultaneous detection and identification of *Anaplasma* spp. and *Ehrlichia* spp. (Chapter 6)
3. To investigate the epidemiology of anaplasmosis and heartwater in domestic ruminants in central Ethiopia and selected African countries (Chapter 7)
4. To detect *Anaplasma* species in questing ticks (ixodid) using molecular methods (Chapter 8)

To this end, three cross-sectional surveys were conducted:

1. A survey of bacteria and piroplasms in ticks collected from cattle and sheep in Ethiopia (Objective 1, Chapters 4 and 5)
2. A survey of *Ehrlichia* and *Anaplasma* spp. in domestic ruminants in Africa, with special reference to Ethiopia (Objective 3, Chapter 7)
3. A survey of *Ehrlichia* and *Anaplasma* spp. in questing ticks in Ethiopia (Objective 4, Chapter 8)

CHAPTER 4

Molecular detection of piroplasms in ixodid ticks infesting cattle and sheep in western Oromia, Ethiopia

Abstract In Ethiopia, ticks and tick-borne diseases are widely distributed and contribute to important economic losses. Several studies investigated the prevalence and species composition of ticks infesting ruminants; however, data on tick-borne pathogens are still scarce. During the study period from October 2010 to April 2011, a total of 1,246 adult ticks and 264 nymphs were collected from 267 cattle and 45 sheep in Bako District, western Oromia, Ethiopia. The study showed infestation of 228/267 (85.4%) cattle and 35/45 (77.8%) sheep with adult ticks. Overall, eight tick species, belonging to three genera (*Amblyomma*, *Rhipicephalus*, *Hyalomma*), were identified and *Amblyomma cohaerens* (n=577), *Rhipicephalus evertsi evertsi* (n=290), *Rhipicephalus* (*Boophilus*) *decoloratus* (n=287), and *Amblyomma variegatum* (n=85) were the more prevalent species. A statistically significant host preference in *A. cohaerens* for cattle and *R. evertsi evertsi* for sheep was noticed. Molecular detection of piroplasms, performed only for adult ticks of two species of the genus *Rhipicephalus* (*R. evertsi evertsi* and *R. decoloratus*), revealed an overall prevalence of 4% (8/202) *Theileria buffeli/orientalis*, 0.5% (1/202) *Theileria velifera*, and 2% (4/202) *Theileria ovis*. The study showed that tick infestation prevalence is considerably high in both cattle and sheep of the area, but with a low intensity of tick burden and a moderate circulation of mildly pathogenic piroplasm species.

Adapted from:
Kumsa, B., Signorini, M., **Teshale, S.**, Tessarin, C., Duguma, R., Ayana, D., Martini, M., Cassini, R., 2014. Molecular detection of piroplasms in ixodid ticks infesting cattle and sheep in western Oromia, Ethiopia. Tropical Animal Health and Production 46, 27–31.

4.1 Introduction

Ticks are arthropods of great veterinary and medical importance. Recent reports show the growing risks associated with ticks and tick-borne diseases worldwide due to expanding tick populations, global warming, and increased contacts between humans, animals, and ticks (de la Fuente and Estrada-Peña, 2012). The most economically important tick-borne piroplasms infecting ruminants include species of the genera *Babesia* and *Theileria*, generally referred to as piroplasms. In tropical and subtropical countries, *Babesia bovis*, *Babesia bigemina*, *Theileria parva* and *Theileria annulata* are the most important species. Other less pathogenic species like *Theileria orientalis* (also referred to as *Theileria buffeli/orientalis* group), *Theileria mutans* and *Theileria velifera* also may infect animals, causing mild disease.

In Ethiopia, ticks are widely distributed in all agro-ecological zones and particularly affect ruminants (Pegram *et al.*, 1981). *Amblyomma*, *Rhipicephalus*, *Hyalomma* and *Haemaphysalis* are the major genera of ixodid ticks reported to exist in different agro-ecological zones in the country and previous investigations, using serologic tests reported the existence of *B. bigemina*, *T. mutans* and *T. velifera* in some parts of the country (Solomon *et al.*, 1998; Mekonnen *et al.*, 2007; Tomassone *et al.*, 2012).

Most of the previous studies on ticks of domestic animals in Ethiopia focused on the epidemiology and the impact on the commercial value of skin and hides of animals. For instance, several studies have been conducted in different parts of Ethiopia on prevalence and species composition of ticks infesting cattle (Pegram *et al.*, 1981; Tafesse, 1996; Bekele, 2002; Yacob *et al.*, 2008; Abera *et al.*, 2010; Tomassone *et al.*, 2012; Ayalew *et al.*, 2014) and small ruminants (Sertse and Wossene, 2007; Tomassone *et al.*, 2012; Kumsa *et al.*, 2012a; Beyecha *et al.*, 2014). Even though there are several recent reports on molecular detection of tick-borne pathogens from other African countries and the rest of the world, data on the role of ticks as vectors of pathogens of veterinary and medical importance are remarkably scarce in Ethiopia. Thus, the current study was aimed to estimate the prevalence, species composition, and presence of *Theileria* and *Babesia* spp. in ixodid ticks feeding on cattle and sheep in Bako Tibe District in western Oromia, Ethiopia.

4.2 Materials and Methods

4.2.1 Study area and animals

The study was carried out at Bako, the capital of Bako Tibe District. Bako is located 257 km to the west of Addis Ababa, latitude of 9°08'N and longitude of 37°03'E with an elevation of 1,743 m above sea level (a.s.l.). The average annual rainfall of the area is 1,320 mm and the long rainy season occurs from June to September. The district has a

warm humid climate with mean minimum, maximum, and average temperatures of 14, 28, and 21℃, respectively, and an average relative humidity of 67.2%. These environmental characteristics are typical of the midland agro-ecology of Ethiopia. The main types of livestock kept in the area include cattle, sheep, goats, horse, donkeys, and chickens. The farming system is mixed crop-livestock type (CSA, 2008).

4.2.2 Tick collection and identification

Tick collection was performed from October 2010 to April 2011 from a total of 267 cattle and 45 sheep kept in different Peasant Associations in the district. All the body surfaces and particularly the known tick feeding regions of each study animal were thoroughly examined visually in order to establish the presence or absence of ticks. All ticks attached to the skin of each animal were collected and placed into separate pre-labeled small plastic tubes containing 70% ethanol. The ticks were then brought to the laboratory and identified morphologically according to the standard identification keys described by Hoogstraal (1956) and by Walker *et al.* (2003). Adult ticks were identified at species level, whereas nymphs were identified only at genus level. Some of these ticks were transported to the Laboratory of Parasitology and Parasitic Diseases of the University of Padova (Italy) for molecular studies. Ticks genera are abbreviated as indicated by Dantas-Torres (2008).

4.2.3 DNA extraction from ticks

Prior to DNA extraction, each tick specimen was rinsed twice in sterile water for 15 min and then dried on sterile filter paper. Genomic DNA from each tick was extracted using NucleoSpin® Tissue (Macheray-Nagel, Dren, Germany) and samples were screened by PCR for the presence of piroplasms as previously described (Cassini *et al.*, 2012). The DNA from each tick was eluted in 100 µl of elution buffer and stored at -20℃ under sterile conditions to preclude any contamination until the sample was used for PCR.

4.2.4 Molecular detection of piroplasms

DNA samples from *Rhipicephalus* (*Boophilus*) *decoloratus* and *Rhipicephalus evertsi evertsi* were subjected to standard PCR in an automated DNA thermal cycler using the primers CRYPTOF (5'-AAC CTG GTT GAT CCT GCC AGT-3') (Herwaldt *et al.*, 2003) and RLB-R2 (5'-CTA AGA ATT TCA CCT CTG ACAGT-3') (Georges *et al.*, 2001) that amplify a fragment of approximately 800 bp of the 18S ribosomal RNA and allow the detection of all species belonging to *Babesia* and *Theileria* genera. The PCR products were cleaned of excess primers and nucleotides using a QIAquick Spin PCR Purification Kit (Qiagen) as per instructions of the manufacturer. Samples positive to piroplasms were sequenced on both strands by BMR Genomics (Padova, Italy). All obtained sequences

were assembled and edited using Chromas Pro1.34 (Technelysium Pty Ltd., Tewantin, Queensland, Australia). The obtained sequences were then subjected to Basic Local Alignment Search Tool (BLAST) analysis to determine similarities to those available in the GenBank.

4.2.5 Data analysis

Descriptive statistics like prevalence and mean burden were calculated to display the status of tick infestation. Relative abundances of tick species encountered in both host species were compared using Pearson's chi-square test, in order to investigate host preferences.

4.3 Results

4.3.1 Tick prevalence and species composition

An overall prevalence of 89.1% (278/312) tick infestation was recorded. The study revealed that a total of 228/267 (85.4%) cattle and 35/45 (77.8%) sheep were infested at least by one species of adult ticks. Besides, 101/267 (37.8%) cattle and 25/45 (55.6%) sheep were infested by *Amblyomma* spp. and *Rhipicephalus* spp. nymphs.

Overall, 1,246 adult ticks were collected from 228 cattle and 35 sheep of the study area. A total of eight tick species were identified: *Amblyomma cohaerens* (n=577), *Rhipicephalus evertsi evertsi* (n=290), *R.* (*Bo.*) *decoloratus* (n=287), and *Amblyomma variegatum* (n=85) in both cattle and sheep, whereas *Amblyomma gemma* (n=3), *Rhipicephalus pulchellus* (n=2), *Hyalomma marginatum rufipes* (n=1), and *Rhipicephalus praetextatus* (n=1) were collected only from cattle. Tick burden ranged from 1 to 21 ticks, with an overall mean burden of 4.4 adult ticks per animal in cattle, and from 1 to 22 ticks, with a mean burden of 6.9 in sheep. Prevalence of tick infestation in hosts and mean burden of ticks are reported in Table 4.1 for all species.

A total of 260 nymphs of *Amblyomma* spp. were collected from 98 cattle (range, 1-14; mean burden, 2.7) and 4 nymphs of *Rhipicephalus* spp. from three cattle. In sheep, only nymphs of *Amblyomma* spp. were collected: a total of 117 ticks from 25 animals (range, 1-12; mean burden, 4.7). Relative abundances of adult tick species in cattle and sheep are reported in Table 4.2. For *A. cohaerens* relative abundance was significantly ($p < 0.001$) higher in cattle, whereas *R. evertsi* showed significantly ($p < 0.001$) higher preference for sheep. For the other two tick species, no statistically significant difference in relative abundance between the two hosts was observed (Table 4.2).

Table 4.1: Species composition, prevalence, total number, and mean burden of adult ticks collected from cattle and sheep

host species	tick species	positive hosts	prevalence (%)	95% CI	N (ticks)	Mean burden
sheep (N=45)	*R. evertsi evertsi*	23	51.1	36.5-65.7	120	5.2
	A. cohaerens	20	44.4	29.9-58.9	59	3
	R. decoloratus	17	37.8	23.6-52.0	51	3
	A. variegatum	6	13.3	3.4-23.2	10	1.7
	total	35	77.8	65.7-89.9	240	6.9
cattle (N=267)	*A. cohaerens*	177	66.3	60.6-72.0	518	2.9
	R. decoloratus	102	38.2	32.4-44.0	236	2.3
	R. evertsi evertsi	69	25.8	20.6-31.0	170	2.5
	A. variegatum	46	17.2	12.7-21.7	75	1.6
	R. pulchellus	1	0.4	0-1.2	2	
	R. praetextatus	1	0.4	0-1.2	1	
	H. marginatum rufipes	1	0.4	0-1.2	1	
	A. gemma	1	0.4	0-1.2	3	
	total	228	85.4	81.2-89.6	1006	4.4

Table 4.2: Relative abundances of the predominant tick species in cattle and sheep hosts

Tick species	relative abundance (%) in host species: cattle (N=238)	sheep (N=40)	χ^2	p-value
A. cohaerens	51.9	24.6	57.96	< 0.001
A. variegatum	7.5	4.2	3.3	0.06
R. evertsi evertsi	17	50	117.6	< 0.001
R. decoloratus	23.6	21.3	0.575	0.44

4.3.2 Molecular detection of piroplasms

Molecular investigation of 118 *R. decoloratus* and 84 *R. evertsi evertsi* ticks collected from 94 cattle and 28 sheep showed the presence of piroplasm species DNA in 13 ticks (6.4%; 13/202). DNA of *T. buffeli/orientalis* was detected in eight ticks, all from cattle, one tick from a cow was found positive for *T. velifera* and four ticks (both from cattle and sheep) were positive for *T. ovis* (Table 4.3). The molecular investigation did not produce any positive results for *Babesia* species. A BLAST analysis of the 18S rRNA gene sequence obtained from the 13 positive ticks showed similarity ranging from 96 to 100% with the reference sequences deposited in the GenBank (Table 4.3).

4.4 Discussion

Tick infestation appears to be common in both cattle and sheep in the study area, but with low intensity. The overall prevalence values are considerably high both in cattle

Table 4.3: Percentage of piroplasm species detected by PCR in tick species collected from cattle and sheep and results of BLAST analysis of 18S RNA gene sequences obtained from ticks

Host	Tick	Ticks exam by PCR (n)	Ticks pos	Piroplasm species identified	Identity (%)	GenBank a.n.
sheep	Re	26	2	To	100	AY508460
(N=28)	Rd	17	0			
cattle	Re	58	1	Tb	96	AF236094
(N=94)			1	To	100	AY508460
	Rd	101	7	Tb	96-100	AF236094
			1	To	100	AY508460
			1	Tv	96	FJ869897

Re = *R. evertsi evertsi*; Rd = *R.* (*Bo.*) *decoloratus*
To = *T. ovis*; Tb = *T. buffeli/orientalis*; Tv = *T. velifera*

(89.1%) and sheep (88.9%), confirming the pattern of tick distribution in the country, where animals living in lowland and midland agro-ecologies are commonly affected by tick infestation and therefore potentially more threatened by tick-borne diseases than animals from the highland agro-ecology. This trend has been already demonstrated for other domestic species, such as goats (Sertse and Wossene, 2007; Beyecha *et al.*, 2014) and horses (Kumsa *et al.*, 2012c).

The percentage of cattle infested with adult ixodid ticks (85.4%) is similar to what was reported in a neighbouring midland area (1,400-2,000m a.s.l.; 1,400 mm average annual rainfall) of southwestern Oromia (Abera *et al.*, 2010). Also, species composition of ticks recorded in the present study is in agreement with the previous report (Abera *et al.*, 2010): *A. cohaerens* was the predominant species, followed by *R. decoloratus*, *R. evertsi evertsi*, and *A. variegatum*. A similar prevalence was reported also in nomadic cattle herds of a lowland area (200-1,300 m a.s.l.; $<$ 450 mm average annual rainfall) of Ethiopia (Tomassone *et al.*, 2012), but species composition differs totally with a predominance of *R. pulchellus*. Other authors (Yacob *et al.*, 2008) reported instead lower prevalence in the highland area of northern Ethiopia ($>$ 2,000 m a.s.l.; 1,500-2,000 mm average annual rainfall). The overall intensity of tick infestation in cattle (mean burden=4.4 adult ticks/host) seems to be low, if compared to values reported in other studies (Tafesse, 1996; Abera *et al.*, 2010; Tomassone *et al.*, 2012), recording, on average, more than 15 ticks for each host. This is most probably attributable to the fact that our study was performed during the dry months of the year.

The high prevalence (77.8%) of adult tick infestation in sheep of the present study is also in agreement with high values found by other authors in lowland (Tomassone *et al.*, 2012) and midland (Abera *et al.*, 2010; Kumsa *et al.*, 2012a) areas. For this host species, also the infestation intensity (mean burden=6.9 adult ticks/host) is similar to what was reported in the cited studies.

The prevalence and mean burden of nymphs are rarely investigated and it is impossible to make comparisons with similar studies in the country. The possible explanation for the nearly absence of *Rhipicephalus* nymphs both in cattle and sheep may depend on different factors. Firstly, as many species of this genus are three-host tick species, most of the larval and nymphal stages may prefer other hosts than domestic ungulates (Pegram *et al.*, 1981). Moreover, collection of *Rhipicephalus* nymphs was difficult due to their smaller size compared to that of *Amblyomma* nymphs and to their seasonality, with decreasing abundance during the dry season.

The detection of an overall percentage of 4% (8/202) of DNA of *T. buffeli/orientalis*, 0.5% (1/202) of *T. velifera*, and 2% (4/202) of *T. ovis* among ticks collected from cattle and sheep is comparable to the prevalence reported in the arthropod vectors by several investigators in many countries of the world. The absence of any isolates belonging to *Babesia* species is remarkable, since *R. decoloratus* is one of the known vector of *B. bigemina*, which was previously reported in the country. The presence of *T. mutans* and *T. velifera* in different parts of Ethiopia has been previously reported (Solomon *et al.*, 1998; Tomassone *et al.*, 2012). *Theileria mutans* can cause mild clinical signs, but pathogenic strains in eastern Africa are also reported to cause severe anaemia, icterus, and sometimes death, whereas *T. velifera* and the *T. buffeli/orientalis* group are generally regarded as nonpathogenic species in cattle. There is a general belief that local breeds of cattle and other ruminants in Ethiopia have some innate immunity especially against tick-borne piroplasms and mortality rarely occurs (Pegram *et al.*, 1981; Regassa, 2001; Mekonnen *et al.*, 2007). However, in view of the current escalating number of exotic breeds of cattle kept in increasing number of dairy farms in Ethiopia, tick-borne pathogens need to receive special attention (Ayalew *et al.*, 2014).

Finally, to our knowledge, this is the first study reporting the molecular detection of *T. ovis* in *R. decoloratus* from cattle and in *R. evertsi evertsi* from sheep and cattle. A potential vector competence for these species of ticks can be only suspected, since their capacity to transmit the pathogens has not been proved by our study. Similarly, *T. ovis* have been reported from *R. bursa* collected from sheep in Turkey (Aktas *et al.*, 2006) and in Ghana (Bell-Sakyi *et al.*, 2004a). The genus *Rhipicephalus* is generally considered a vector of this piroplasm species. *Theileria ovis* is considered less pathogenic species and is mostly reported from tropical and subtropical countries.

In conclusion, this study suggests a relevant impact of tick infestation and a moderate circulation of mildly pathogenic species of piroplasms among domestic ruminants of this area of Ethiopia. It is therefore very important to raise the farmers' awareness on prevention of tick infestation and tick-borne diseases transmission.

CHAPTER 5

Anaplasma, *Ehrlichia*, *Bartonella*, *Borrelia* and rickettsial pathogens in ixodid ticks infesting cattle and sheep in western Oromia, Ethiopia

5

Abstract Although ticks are widely distributed in all agro-ecological zones of Ethiopia, information on tick-borne pathogens is scarce. This study was conducted to determine the presence of *Anaplasma* spp., *Ehrlichia* spp., and Rickettsia spp. in *Rhipicephalus evertsi* and *Rhipicephalus* (*Boophilus*) *decoloratus* collected from cattle and sheep at Bako, western Oromia, Ethiopia, using polymerase chain reaction and sequencing. *Anaplasma ovis* and *Anaplasma* spp., *Ehrlichia ruminantium* and *Ehrlichia* spp. were detected in *Rh. decoloratus*, whereas only *A. ovis* was detected in *Rh. evertsi*. Both tick species were found to harbor DNA belonging to *Rickettsia* spp., and *Rickettsia africae*. Our findings highlight the risk of infection of animals and humans with these zoonotic tick-borne bacteria in Ethiopia.

Adapted from:
Teshale, S., Kumsa, B., Mondin, A., Cassini, R., Martini, M. 2016. *Anaplasma*, *Ehrlichia* and rickettsial pathogens in ixodid ticks infesting cattle and sheep in western Oromia, Ethiopia. Experimental and Applied Acarology, 70, 231–237.

5.1 Introduction

Ticks transmit a wide variety of pathogens including viruses, bacteria and protozoa to vertebrate hosts including humans (de la Fuente *et al.*, 2008). Rickettsiales (bacteria of the genera *Rickettsia*, *Ehrlichia* and *Anaplasma*), *Borrelia* spp. and *Bartonella* spp. are among important tick-borne pathogens with economic importance in animals and causing emerging, life-threatening zoonoses in humans worldwide (Kim *et al.*, 2006; de la Fuente *et al.*, 2008; Parola *et al.*, 2013). The continuing changes in biological, demographic and environmental factors are thought to be responsible for the growing importance of emerging tick-borne pathogens in animals and humans around the world (Paddock *et al.*, 2002). Ticks serve as vectors, reservoirs and/or amplifiers of some of these pathogens particularly those of spotted fever group Rickettsia species and *Borrelia* species (Parola *et al.*, 2013).

Although several tick species are common and widely distributed in all agro-ecological zones of Ethiopia with significant impact on the commercial values of skin and hides of animals (Pegram *et al.*, 1981; Tafesse, 1996; Bekele, 2002; Abera *et al.*, 2010; Tomassone *et al.*, 2012; Ayalew *et al.*, 2014; Kumsa *et al.*, 2012a; Beyecha *et al.*, 2014), little is known about the occurrence of tick-borne pathogenic bacteria in the country. Screening of ticks for such pathogens using molecular methods is one of the modern and highly sensitive techniques that could be used to determine their occurrence and distribution in a given geographical area.

Livestock industry is considered a priority sector for poverty alleviation in Ethiopia. There is increasing demand for livestock products from national and regional markets (LMP-team, 2014). Besides, ecotourism and outdoor activities are growing business in the country. That means apart from the losses incurred in livestock, infected animals may act as reservoirs of some of tick-borne pathogens for humans Kelly *et al.* (1991a,b). Therefore, tick-borne pathogens assume greater importance and there is increasing necessity for accurate detection and identification of these pathogens. Even though there are several recent reports on molecular detection of tick-borne pathogens elsewhere in the world, data on the role of ticks as vectors of pathogens of veterinary and medical importance are remarkably scarce in Ethiopia. Thus, the current study was aimed to detect the occurrence and species composition of *Anaplasma*, *Ehrlichia*, *Bartonella*, *Borrelia* and rickettsial pathogens in ixodid ticks feeding on cattle and sheep in Bako Tibe District in western Oromia, Ethiopia.

5.2 Materials and Methods

5.2.1 Study area and animals

The ticks used in this study were collected from cattle and sheep in Bako, the capital of Bako Tibe district, which is located in the western Ethiopia, at latitude of 9°08'N and longitude of 37°03'E. Environmental characteristics are typical of the midland agro-ecology of Ethiopia, with warm humid climate, average annual rainfall of 1,320 mm and average temperature of 21°C. Livestock kept in the area include cattle, sheep, goats, horse, donkeys and chickens. The farming system is mixed crop-livestock type (CSA, 2008).

5.2.2 Tick collection and identification

Tick collection was performed during the long dry season, from October 2010 to April 2011, from cattle and sheep raised by smallholder farmers in the whole district. All ticks attached to the skin were collected and placed into separate pre-labeled small plastic tubes containing 70% ethanol. The ticks were then brought to the laboratory of Parasitology and Parasitic Diseases of the University of Padova (Italy) for molecular studies and identified morphologically at species level, according to the standard identification keys described by Hoogstraal (1956) and by Walker *et al.* (2003).

5.2.3 Molecular detection of pathogens

Totally 118 *R. decoloratus* and 84 *R. evertsi evertsi* adult ticks collected from 94 cattle and 28 sheep were investigated by means of biomolecular analysis. Genomic DNA from each tick was extracted using NucleoSpin Tissue® (Macheray-Nagel, Dren, Germany) method. All DNA samples were tested for the presence of *Anaplasma* spp., *Ehrlichia* spp. and *Bartonella* spp. using a PCR amplifying a portion of 16S rRNA gene (Goodman *et al.*, 1996). The detection of *Rickettsia* spp. belonging to spotted fever group was performed with two different reactions producing 171bp and 267bp amplicons respectively: a PCR targeted to ompA gene (Kidd *et al.*, 2008) and a nested PCR targeted to ompB gene (Choi *et al.*, 2005). All reactions were carried out using reagents from Kapa Biosystems (Cape Town, South Africa) in a 2720 Thermal Cycler (Applied Biosystems, Carlsbad, CA, USA). For each reaction, at least one positive control and a blank (water instead of DNA) were always included. As positive controls we used DNA of various *Rickettsia* species (*Rickettsia conorii*, *Rickettsia rickettsii* and *Rickettsia slovaca*), *Ehrlichia canis* and *Anaplasma phagocytophilum* extracted by boiling cells fixed on multi-well slides for Immuno-fluorescence (Fuller Laboratories, Fullerton, CA; VMRD inc, Pullman, WA). The specific length of each amplicon was confirmed by 2% agarose gel electrophoresis. The identification of *Borrelia burgdorferi* s.l. was carried out using Real time PCR that

targeted 16S rDNA.

5.2.4 Sequencing and typing of PCR products

After purification with the Nucleospin Extract II (Macherey-Nagel) both strands of PCR products from positive samples were sequenced using the Big Dye Terminator v.3.1 Cycle sequencing Kit (Applied Biosystems) according to manufacturer's instructions with the primers used in PCR. Purified sequencing products were analyzed using a 3100 DNA Analyzer (Applied Biosystems). The quality of chromatograms was checked with Finch TV (`www.geospiza.com`) while consensus sequences were obtained using ChromasPro (Technelysium, Brisbane, Australia). The obtained consensus sequences were finally analysed by BLAST through the NCBI's Mega-BLAST algorithm.

5

5.3 Results

Of the total 202 *Rhipicephalus* spp. collected from 122 parasitised animals (94 cattle and 28 sheep) fourteen different bacterial species within the genera *Anaplasma*, *Ehrlichia*, *Bartonella*, *Borrelia* or *Rickettsia* were identified. The details of the number of ticks collected from cattle and sheep, the number of *Rhipicephalus* spp. examined and tested positive and the frequency of detection of the pathogen species is presented in Tables 5.1 to 5.4. In this study, 44.19% (19/43) and 45.91% (73/159) of the ticks collected from sheep and cattle, respectively were positive for *Anaplasma*, *Ehrlichia*, *Bartonella*, *Borrelia*, or *Rickettsia* spp. sequences.

5.3.1 Molecular detection of *Anaplasma/Ehrlichia* species

Out of the total 202 *Rhipicephalus* spp. tested 22 (10.9%) of them were positive for *Anaplasma/Ehrlichia* spp. DNA. *Anaplasma ovis* sequences were detected in *R. evertsi evertsi* collected from sheep and *R. decoloratus* collected from cattle. *Ehrlichia ruminantium* and *Ehrlichia* spp. sequences were identified in *R. decoloratus* collected from sheep whereas *Anaplasma* spp. and *Ehrlichia* spp. sequences were identified in *R. decoloratus* collected from cattle (Table 5.1).

5.3.2 Molecular detection of *Bartonella* species

Out of the total 202 *Rhipicephalus* spp. (84 *R. evertsi evertsi a d 118 R. decoloratus*) tested 20 (9.90%) of them were positive for *Bartonella* spp. sequences. Blast analysis of the sequenced PCR product identified six species of *Bartonella* including the zoonotic species. Sequences specific to *B. koehlerae* and *B. bovis* were identified in ticks collected from sheep

Table 5.1: *Anaplasma/Ehrlichia* spp. detected by PCR in tick species collected from cattle and sheep and results of BLAST analysis of 16S RNA gene sequences

Host species	Tick species	Ticks exam by PCR (n)	Ticks +ve at PCR (n)	Bacterial spp.	Identity (%)
sheep	*R. evertsi evertsi*	26	1	*A. ovis*	100
(N=28)	*R. decoloratus*	17	2	*E. ruminantium*	100
			1	*Ehrlichia* spp.	99
cattle	*R. evertsi evertsi*	58	0		
(N=94)	*R. decoloratus*	101	1	*A. ovis*	100
			6	*Anaplasma* spp.	97-100
			11	*Ehrlichia* spp.	100

and sequences belonging to *B. elizabethae*, *B. henselae*, *B. koehlerae*, *B. quintana* and *B. vinsonii berkhoffii* were detected in ticks collected from cattle (Table 5.2).

Table 5.2: *Bartonella* spp. detected by PCR in tick species collected from cattle and sheep and results of BLAST analysis of 16S RNA gene sequences

Host species	Tick species	Ticks exam by PCR (n)	Ticks +ve at PCR (n)	Bartonella spp.	Identity (%)
sheep	*R. evertsi evertsi*	26	2	*B. koehlerae*	100
(N=28)	*R. decoloratus*	17	1	*B. koehlerae*	100
			2	*B. bovis*	100
cattle	*R. evertsi evertsi*	58	1	*B. elizabethae*	100
(N=94)			1	*B. henselae*	100
			9	*B. koehlerae*	100
			1	*B. quintana*	100
	R. decoloratus	101	1	*B. henselae*	100
			1	*B. koehlerae*	100
			1	*B. vinsonii berkhoffii*	100

5.3.3 Molecular detection of *Rickettsia* species

Out of the total 202 *Rhipicephalus* spp. (84 *R. evertsi evertsi* and 118 *R. decoloratus*) tested 29 (14.36%) of them were positive for different *Rickettsia* spp. sequences. Sequences belonging to 3 *Rickettsia* spp. were identified in both *R. evertsi evertsi* and *R. decoloratus* collected from both host species. Among the nine tick samples that were positive for *Rickettsia* spp. sequences from sheep 2 were identified as the zoonotic *R. africae* (the agent of African tick bite fever) and 2 were identified as *R. felis* (an emerging agent of spotted fever) (Table 5.3). *Rickettsia africae* sequences were identified in 13 ticks collected from cattle where as sequences of *R. felis* were identified in 4 ticks collected from cattle.

Table 5.3: *Rickettsia* spp. detected by PCR in tick species collected from cattle and sheep and results of BLAST analysis of 16S RNA gene sequences

Host species	Tick species	Ticks exam by PCR (n)	Ticks +ve at PCR (n)	Rickettsia spp.	Identity (%)
sheep (N=28)	*R. evertsi evertsi*	26	2	*R. africae*	100
			2	*R. felis*	100
			2	*Rickettsia* spp.	98-100
	R. decoloratus	17	1	*R. felis*	100
			2	*Rickettsia* spp.	96-100
cattle (N=94)	*R. evertsi evertsi*	58	6	*R. africae*	100
			1	*Rickettsia* spp.	98-100
	R. decoloratus	101	7	*R. africae*	99-100
			3	*R. felis*	98-100
			3	*Rickettsia* spp.	99-100

5.3.4 Molecular detection of *Borrelia burgdorferi* s.l.

Both *R. decoloratus* and *R. evertsi evertsi* collected from cattle were found to carry *Borrelia burgdorferi* s.l. sequences with respective prevalence of 13.9% and 10.3%. In contrast, only a single specimen of *R. decoloratus* (5.9%) and none of the *R. evertsi evertsi* collected from sheep gave positive signal for this bacterium. The results of molecular analysis of ticks for *Borrelia burgdorferi* s.l. is presented in Table 5.4.

Table 5.4: Prevalence of *Borrelia burgdorferi* s.l. detected by Real time PCR in tick species collected from cattle and sheep

Host species	Tick species	Ticks exam by PCR (n)	Ticks +ve at PCR (n)	Prevalence (%)
sheep (N=28)	*R. evertsi evertsi*	26	0	0
	R. decoloratus	17	1	5.9
cattle (N=94)	*R. evertsi evertsi*	58	6	10.3
	R. decoloratus	101	14	13.9

5.4 Discussion

Although ixodids are important and widespread parasites of livestock and pets throughout Ethiopia (Kumsa *et al.*, 2012a), published evidence on ixodids infesting humans are not available. However, previous studies carried out elsewhere in Africa showed that *A. variegatum*, *A. cohaerens*, *R. praetextatus*, *R. pulchellus*, *R. muhsamae* and *H. marginatum rufipes* bite humans (Estrada-Peña and Jongejan, 1999). Recent report revealed that an American resident who returned from a trip to Ethiopia was infested by *H. truncatum* (Mathison *et al.*, 2015). In Ethiopia diagnosis of tick-borne infections in animals has been mainly based on microscopic examination of stained smears and occasionally serology.

Microscopic method is suitable only for acute infections (Friedhoff and Bose, 1994) and cannot distinguish between pathogenic and non-pathogenic species while serologic methods, which are used in sub-clinical infections, suffer from false results due to cross-reactions (Passos *et al.*, 1998). Diagnosis of tick-borne pathogens in ticks is not common practice in the country.

Thanks to a global effort on this subject, sensitive and specific molecular methods in both ticks and their hosts are becoming of common use (de la Fuente *et al.*, 2008; Parola *et al.*, 2013). This provides rational basis for inventory of tick-borne pathogens in ticks collected from cattle and sheep. The findings of this study uncovered the occurrence of *Anaplasma*, *Ehrlichia*, *Bartonella*, *Borrelia* and *Rickettsia* spp. in ticks infesting animals in Ethiopia. To our knowledge the occurrence of *B. elizabethae*, *B. bovis*, *B. koehlerae*, *B. quintana* and *B. vinsonii berkhoffii* in Ethiopia is made for the first time in this study. The absence of accurate diagnostic methods such as molecular techniques and culture could lead to misdiagnosis and inappropriate treatment of febrile diseases in humans and animals in Ethiopia and other African countries.

The occurrence of *E. ruminantium* (the causative agent of heartwater in ruminants) and *Ehrlichia* spp. in ticks collected from cattle and sheep was observed in this study. This represents the first report of *E. ruminantium* DNA in *Rhipicephalus* ticks in the country, but the epidemiological consequences of this finding need further investigation. Other authors (Biguezoton *et al.*, 2016) suggested a potential vectorial capacity for *R. microplus* in West Africa. However, in our study, ticks were collected from animals and the detection of *E. ruminantium* in *R. decoloratus* adults may be only a consequence of bactaeremia in the host. Previously the DNA of *E. ruminantium* was detected in *Amblyomma* ticks collected from cattle by Teshale *et al.* (2018) and Tomassone *et al.* (2012) in central and eastern Ethiopia, respectively. Thus, our finding provides confirmation of the occurrence of *E. ruminantium* and *Ehrlichia* spp. in ticks in the country. Suspected cases of heartwater have been reported on some farms and ranches with mortality of 46.25% (37/80) (Melaku *et al.*, 2014) and 25% of cattle (Mekonnen, 1996). The proportions of positive ticks were higher in this study than the previous reports made in Ethiopia. Moreover, the previous authors did not detect any of the *Ehrlichia* spp. in ticks collected from sheep. In agreement to our observation the occurrence of *Ehrlichia* spp. in ticks was also made elsewhere in Africa and other parts of the world. For instance, Sarih *et al.* (2005) have detected these pathogens in ticks collected from Tunisia and Morocco. The occurrence of DNA of *Ehrlichia* spp. has been also reported in ticks from Vietnam (Parola *et al.*, 2003) and Korea (Kim *et al.*, 2006) suggesting a widespread distribution of this group of pathogens. However, a precise identification of the species involved and their significance in causing diseases needs to be investigated.

In this study, we identified *A. ovis* (the cause of anaplasmosis in small ruminants) and *Anaplasma* spp. in ticks collected from cattle and sheep. Previously *A. ovis* was detected

in blood of small ruminants using molecular technique in the country (Teshale *et al.*, 2015). This observation further confirms its occurrence in the country and alerts the veterinary authorities to consider its economic importance on the small ruminant industry as it has been shown to cause clinical disease in sheep (Yasini *et al.*, 2012). The detection of *A. ovis* in *R. decoloratus* (one host tick) collected from cattle could indicate the occurrence of infection with this pathogen in cattle. Previously it was detected in cattle from Turkey (Aktas *et al.*, 2011). A variant of *A. ovis* has been recently detected in a human febrile case with history of tick bite in Cyprus (Chochlakis *et al.*, 2010). Further characterisation of the variants of this pathogen and elucidation of its public health significance is needed. *Anaplasma* species have been known to cause economic losses to cattle and small ruminant industries (Rymaszewska and Grenda, 2008). Hence, our finding bears important information to the ruminant breeders and producers in the area. Similar results have been reported elsewhere in the world (Parola *et al.*, 2003; Kim *et al.*, 2006; Sarih *et al.*, 2005).

The detection of *Bartonella* spp. in ticks has veterinary importance especially in pet animal practice. *Bartonella* spp. such as *B. v. berkhoffii*, *B. henselae*, *B. bovis* and *B. koehlerae* have been known to cause clinical diseases in dogs and cats (Chomel *et al.*, 2004; McGill, 2012). In particular *B. v. berkhoffii* has been associated with different clinical cases in dogs. Exposure to ticks was identified as a risk factor for the presence of this pathogen in serological surveys in dogs (Jacomo *et al.*, 2002). Moreover, *B. bovis* was identified as a cause of bovine endocarditis and long lasting bacteremia. In Europe, infection of cattle with *B. bovis* has been reported in France, Italy and Poland whereas *B. chomelii* is considered the most frequent species infecting cattle in Spain (Ebani *et al.*, 2015a). Therefore, the veterinary authorities in Ethiopia should be aware of these pathogens in their practices.

Our identification of *Bartonella* spp. in ticks is relevant from a public health point of view. The detection of *Bartonella* spp. in *R. decoloratus* collected from cattle and sheep should get attention. Since *R. decoloratus* is a one host tick, the detection of these pathogens in these ticks suggests the occurrence of infection in resident ruminants. Elsewhere it was shown that cattle constitute the reservoir of *Bartonella* species that usually do not cause clinical signs (Ebani *et al.*, 2015a). Furthermore, in USA researchers have recently shown that ungulates are infected with *Bartonella* spp. with prevalence of bacteremia being 15% in elk (*Cervus elaphus*), 90% in mule deer, and 50% in domestic cattle (*Bos taurus*) (Jacomo *et al.*, 2002). There is increasing evidence for the involvement of ticks in the transmission of *Bartonella* organisms (Billeter *et al.*, 2008; Ebani *et al.*, 2015b). Therefore, the identification of *Bartonella* spp. in *R. decoloratus* collected from ruminants raises question on the role of ticks and ruminants in the epidemiology of *Bartonella* infections.

Since *R. africae* and *R. felis* were identified in many ticks collected from cattle and sheep our findings bear further public health concern. Because *R. decoloratus* is a one host tick

the detection of these pathogens in these ticks suggests the occurrence of infection in host species. Cattle have been incriminated as reservoir of *R. africae* elsewhere (Kelly *et al.*, 1991a). The role of *Rhipicephalus* ticks in transmitting *R. africae*, instead, is still not clear; however, other authors reported infection rates ranging from 0.4% to 5% in *R. evertsi evertsi* and from 5 to 77% in *R. decoloratus*, in other Sub-Saharan African countries (Parola *et al.*, 2003). *Amblyomma variegatum*, which is a documented vector of *R. africae* and one of the predominant tick species found in Bako area (Kumsa *et al.*, 2014c), has been shown to feed on humans (Mediannikov *et al.*, 2010). Hence, the observation of *R. africae* in Bako area signals possible risk to resident humans. Recently, *R felis* was identified in fleas collected from dogs and cats in Ethiopia (Kumsa *et al.*, 2014b). The known vector of *R felis* is flea spp. but there is evidence of involvement of ticks recently (Parola *et al.*, 2013). In some of the sub-Saharan African countries seroprevalence of spotted fever group rickettsiae in humans and prevalence of *R. africae* in ticks is high (Sixl *et al.*, 1987; Parola *et al.*, 2003; Mediannikov *et al.*, 2010). However, in Ethiopia, febrile diseases are often empirically diagnosed as malaria and some as fever of unknown origin. The similarity in clinical manifestations of tick-borne infections in humans and malaria suggests the possibility of misdiagnosis in Ethiopia and elsewhere in Africa.

The occurrence of relapsing fever group *Borrelia* spp. has been reported in Ethiopia (Boutellis *et al.*, 2013). No study was carried out on Lyme borreliosis. Since empirical diagnosis of febrile diseases do not differentiate among causes of febrile illness, whether Lyme borreliosis occurs in the country has not been known. Recent studies have shown the occurrence of new *Borrelia* spp. in hard ticks from some parts of the country (Mediannikov *et al.*, 2013; Kumsa *et al.*, 2015a). Our finding of *Borrelia burgdorferi* s.l. in *R. decoloratus* collected from cattle and sheep highlights the possible occurrence of this *Borrelia* group in Ethiopia. This group of *Borrelia* is the causative agent of Lyme borreliosis, transmitted by ixodid ticks and most tick-borne disease in USA and Europe (Rudenko *et al.*, 2011) . Identification of this group of *Borrelia* in *R. decoloratus* collected from cattle and sheep highlights its occurrence in these ruminant species. Further investigation is needed, however, to confirm the identity of this Borrelia group and its distribution both in ticks and domestic ruminants.

In conclusion, the various tick-borne pathogens identified in this study suggests the possible emergence of tick-borne diseases in animals and humans in Ethiopia. This study provides the first report of *B. elizabethae*, *B. bovis*, *B. koehlerae*, *B. quintana*, *B. vinsonii berkhoffii* and *Borrelia burgdorferi* s.l. in the country. The demonstration of these pathogens in *R. decoloratus* (one-host tick) collected from cattle and sheep implies the possible role of domestic ruminants in the epidemiology of these zoonoses in the country. This fact needs to be considered by health authorities since there is risk of infection for rural residents and tourists.

CHAPTER 6

Improved molecular detection of *Ehrlichia* and *Anaplasma* species and its application in *Amblyomma* ticks collected from cattle and sheep in Ethiopia

Abstract Detection of *Ehrlichia* and *Anaplasma* species in animals and tick vectors is crucial for an understanding of the epidemiology of diseases caused by these pathogens. In this study, a pair of primers designated EBR2 and EBR3 was designed from the *Anaplasma* 16S rDNA sequence and was used along with a previously described primer EHR 16SD for the simultaneous detection of *Ehrlichia* and *Anaplasma* species by nested PCR. The primers were used to amplify 925 bp of DNA from known species of *Ehrlichia* and *Anaplasma*. Restriction with MboII and MspI enzymes allowed *Ehrlichia* and *Anaplasma* speciation. Restriction with MboII differentiated between *An. marginale*, *Anaplasma* (formerly *Ehrlichia*) sp. Omatjenne, and *An. centrale* with *An. marginale* and *Anaplasma* (formerly *Ehrlichia*) sp. Omatjenne yielding 2 distinct fragments each while *An. centrale* produced three distinct bands. *Ehrlichia ruminantium* and *An. phagocytophylum* remained undigested. Subsequent restriction with MspI differentiated *E. ruminantium* from *An. phagocytophylum* with 2 and 4 fragments, respectively. When used on tick samples from the field, 63 ticks (16.4%) out of 384 collected from cattle and sheep were positive for one or more species of *Ehrlichia* and *Anaplasma*. The positivity ranged from 6.3% at Andasa to 36.7% at Habernosa. Higher overall infection rates were found in *Amblyomma lepidum* than in *Amblyomma variegatum* ticks ($p = 0.009$). *Amblyomma lepidum* from Habernosa were more often infected with all detected species of *Anaplasma* and *Ehrlichia* than *Am. variegatum*. At Bako, however, *Anaplasma* (formerly *Ehrlichia*) sp. Omatjenne was detected only in *Am. variegatum*. A significantly higher proportion of ticks collected from cattle (20.6%) was found positive than in those collected from sheep (3.3%) ($p =$

0.003). Simultaneous detection of *Ehrlichia* and *Anaplasma* species and correct identification of mixed infections was possible. Since the ticks were collected from animals, the occurrence of the major species of *Ehrlichia* and *Anaplasma* in ruminants in the area is confirmed.

Adapted from:
Teshale, S., Geysen, D., Ameni, G., Asfaw, Y. and Berkvens, D. 2015. Improved molecular detection of *Ehrlichia* and *Anaplasma* species applied to *Amblyomma* ticks collected from cattle and sheep in Ethiopia. Ticks and Tick-Borne Diseases, 6, 1–7.

6.1 Introduction

Infections by *Ehrlichia* and *Anaplasma* species are the source of important tick-borne diseases of ruminants in Ethiopia. Heartwater, which is caused by *Ehrlichia ruminantium* has been known to result in heavy morbidity and mortality in exotic and improved (crossbred) cattle and has been confirmed microscopically in 42.5% of dead dairy cattle at Habernosa ranch (Mekonnen, 1996). This rickettsia is transmitted by ticks belonging to the genus *Amblyomma* (Allsopp, 2010).

Infections with *Anaplasma marginale*, *Anaplasma phagocytophilum* and *Anaplasma centrale* are known to be wide spread in occurrence (Dumler *et al.*, 2001; Okuthe and Buyu, 2006; Aktas *et al.*, 2011). *Anaplasma marginale* and to a lesser extent *A. phagocytophilum* are known to be pathogenic to domestic ruminants especially to high producing dairy cattle. The latter is also known to cause human granulocytic anaplasmosis (Woldehiwet, 2010). *Anaplasma marginale* and *A. centrale* are mainly transmitted by *Rhipicephalus* species even though other ticks such as *Hyalomma* spp., *Dermacentor* spp. and *Ixodes* spp. are also incriminated in transmission (Kocan *et al.*, 2004). *Anaplasma phagocytophilum* is mainly transmitted by Ixodes species in Europe (Telford *et al.*, 1996) and especially *I. scapularis* (Dawson *et al.*, 2001) in America. In tropical settings especially in Africa, *A. marginale* is known to cause serious economic losses in exotic and crossbred animals. They remain one of the serious impediments to genetic improvement of livestock in most sub Saharan African countries.

Anaplasma (formerly *Ehrlichia*) sp. Omatjenne is another species of *Anaplasma* known to infect ruminants (Allsopp *et al.*, 1999). It has been mostly considered nonpathogenic to cattle (Allsopp, 2010) but experimental infection produced a disease indistinguishable from heartwater in sheep (Du Plessis, 1990). Accurate identification of *Anaplasma* species is therefore important to understand their pathogenic role.

Microscopic examination of blood smears and serological tests are cheap and routine methods of diagnosis of TBDs. But these are associated with false positive and false negative results, often leading to misdiagnosis and wrong implementation of control measures. Besides, these cannot be used to detect the causative agents in ticks. Identification and quantification of pathogens in local tick populations is an important requisite to understand the risk posed to the livestock industry. The introduction of molecular techniques has allowed accurate detection of numerous pathogens. These have the advantage of detecting multiple pathogens, discriminating species and being able to detect low level infections (Oura *et al.*, 2004; Shayan and Rahbari, 2005). As the climatic conditions in Ethiopia are favourable for multiple vectors, the occurrence of co-infections in ruminants by different tick-borne pathogens is probably common.

The most frequently used gene targets for the development of diagnostic PCR for tick-transmitted pathogens is 16S rDNA (Allsopp *et al.*, 1999; Criado-Fornelio, 2007). This

is because this gene is ubiquitous, is of suitable size and a very large set of sequences from many organisms is available. In addition, it has regions of low and high sequence divergence between species (Van de Peer and De Wachter, 1997). Previously Simuunza *et al.* (2011) developed a duplex PCR using this target and was able to detect and identify *A. marginale* and *E. ruminantium* infections. This method however, was not tested for other *Anaplasma* and *Ehrlichia* organisms. It did not differentiate *A. marginale* from *A. centrale*. Another molecular method targeting this locus was the reverse line blot hybridisation developed by Bekker *et al.* (2002). This method efficiently differentiates species of *Ehrlichia* and *Anaplasma* but it requires a hybridisation step which is time consuming and expensive.

Simultaneous detection of *Ehrlichia* and *Anaplasma* species is helpful in reducing the cost of diagnosis and minimising time spent in analysing samples. By designing a primer pair that can amplify a DNA fragment common to all species of *Ehrlichia* and *Anaplasma*, it is possible to detect pathogens belonging to both genera in one PCR. This will provide a relatively easier method of analysis to use in epidemiological studies. In this study we designed a primer pair that can amplify about 925bp of 16S rDNA from both *Ehrlichia* and *Anaplasma* species and included one primer previously characterised by Hornok *et al.* (2008). PCR was followed by digestion with restriction enzymes for identification the pathogens up to species level. This method was used to detect *Ehrlichia* and *Anaplasma* species in ticks collected from cattle and sheep at three sites in Ethiopia.

6.2 Materials and Methods

6.2.1 Primer design

Our primary aim was to detect *Ehrlichia* and *Anaplasma* genera simultaneously and identify their respective species using semi-nested PCR followed by fragment length polymorphism. For this purpose we used the following primers: EHR 16SD, EBR2 and EBR3. The EHR 16SD ((5'-GGTACCYACAGAAGAAGTCC-3') was previously described by Hornok *et al.* (2008). This primer was shown to amplify 16S rDNA from all members of Anaplasmatacea. The other two primers used EBR2 (5'- TGCTGACTTGACATCATCCC-3') and EBR3 (5'-TTGTAGTCGCCATTGTAGCAC-3') were designed at the Institute of Tropical Medicine, Antwerp, Belgium for the purpose of this study. Since the EHR 16SD primer was already tested and proved to be suitable for *Ehrlichia* and *Anaplasma* species we used it as a forward primer in the first round of amplification and as forward external primer for the second cycle of amplification.

6.2.2 DNA samples used

DNA reference samples from animals with known infections of *E. ruminantium* (Gambia isolate), *A.* sp. Omatjenne (10 E8), *A. marginale* (3298), *A. phagocytophilum* (Belgian isolate) and *A. centrale* (South African isolate) were used to test the suitability of the newly designed primers. The DNA samples were amplified and the amplicons were restricted using MboII, HhaI and MspI enzymes to analyse the size polymorphism of the fragments so as to identify their species.

6.2.3 Field sites for collection of ticks

The ticks used in this study were collected from three locations in Ethiopia (Figure 6.1). Two of the locations (Bako Research Centre and Habernosa Ranch) are in Central Oromia while the other one (Andasa Ranch) is from the Amhara Region. Bako is located at 9°8'N, 37°5'E 250 km west of the capital. It has an average altitude of 1650 m above sea level. The area has a warm and moist climate with an average humidity of 85% and an annual rainfall of 1227mm. The mean annual minimum and maximum temperature is 13.5℃ and 27.9℃, respectively. The dominant vegetation of Bako area is dense forest with interspersed grassland. Integrated crop-livestock farming is the main economic activity in the area. Breeds at the research centre include indigenous Horro cattle and their crosses with Jersey and Holstein along with indigenous Horro sheep.

Figure 6.1: Map of Ethiopia showing study sites.
■ = Addis Ababa; 1 = Bako Research Centre; 2 = Habernosa Ranch; 3 = Andasa Ranch.

Habernosa is geographically situated in the mid-Rift valley of Oromia, Ethiopia at 7°58'N

latitude and 38°43'E longitude. It is located 170 km in the south-east direction of the capital. The average elevation of the area is 1500 m above sea level. This region is relatively drier with an annual rainfall of 650-750 mm and humidity of 60%. The mean annual minimum and maximum temperature is 12.7℃ and 27.2℃, respectively. The vegetation cover of the area is typically *Acacia* woodland. Livestock production with some integration with crop farming is widely practiced in the area. At the ranch indigenous Borana cattle and Holstein breeds are raised to produce crosses intended to be distributed to smallholder farmers in the area.

Andasa is a ranch in the Amhara regional state, north-west of the capital. It is located at 11°29'N, 37°29'E. The ranch is situated at an elevation of 1730 m above sea level with a rainfall of 1150 mm annually. The mean annual minimum and maximum temperature of the area is 8.8℃ and 29.5℃, respectively. The area and the ranch are characterised by grassland and bushy type vegetation. Mixed farming with integration of livestock and crops is the main agricultural avenue in the area. Indigenous Fogera cattle, Holstein breeds and their crosses are raised at the ranch with the intension of distribution of crossbred heifers to the surrounding farmers.

6.2.4 Tick sample collection

The ticks used in this study were collected from cattle raised at Mariyam Agro-industry (Habernosa) cattle ranch, Bako Agricultural Research Center and Andasa Research Center. At Bako ticks were also collected from sheep that were raised alongside cattle at the center. Each animal was inspected and carefully examined for the presence of ticks late in the afternoon when they came back from the pasture and when found infested, the ticks were collected. Animals of both sex and all age groups were included. The animals that were infested with ticks were restrained and adult and nymphal ticks were collected. The collection was done by gentle rotation of the ticks from their attachment sites using thumb and fingers. The collected ticks were transferred to universal bottles and plastic containers filled with 70% ethyl alcohol after variable period of allowing to digest the blood ingested. Each of the containers was properly labeled with date of collection, age, sex, breed and species of the host and transported to the Veterinary Parasitology laboratory of the School of Veterinary Medicine, for identification up to species level. During collection of ticks data were collected and recorded including animal identification, tick attachment sites, tick loads, body condition of animals and other risk factors.

6.2.5 Identification of ticks

The identification of ticks was done using Petri dish, forceps, filter paper and stereomicroscope. The ticks in the containers were transferred to a Petri dish, spread onto filter paper to absorb excess preservatives and examined under stereomicroscope for identification

using standard identification keys described by Walker *et al.* (2003).

6.2.6 DNA extraction

DNA extraction from the ticks was carried out using the boom extraction method as described previously (Herrmann and Frischauf, 1987) with modifications. Each tick was cut into pieces using scalpel and blade. The cut pieces were transferred into a 1.5 mL tube. To each tube 180 µL of ATL buffer and 20 µL proteinase K (20mg/mL) was added. The tubes were incubated at 56℃ overnight while shaking at 1400 rpm using a thermomixer compact heat block (Eppendorf®). The tubes were centrifuged briefly and 200 µL of AL buffer was added, vortexed for 15 seconds and incubated at 70℃ for 10 minutes at 800 rpm. Then the tubes were centrifuged briefly, 40µL of diatomaceous earth solution was added, mixed and incubated at 37℃ for at least 1 hour while shaking at 1200 rpm. Subsequently, the tubes were centrifuged for 20 seconds and the supernatant was discarded. The pellets were rinsed with 900 µL of 70% ethanol, centrifuged for 20 seconds and supernatants discarded. This step was repeated once. The resulting pellets were rinsed with 900 µL of absolute acetone, centrifuged for 20 seconds and supernatants discarded. There after the pellets were dried at 50℃ for 20 minutes using a thermoblock. The dried pellets were eluted with 90 µL of TE buffer and incubated at 60℃ for 20 minutes at 1000 rpm. They were centrifuged for 40 seconds and 50 µL of the supernatant was collected into new tubes, labeled and stored at -20℃ until analysed.

6.2.7 Polymerase chain reaction amplification of DNA

A semi nested PCR was used to amplify a fragment of about 925bp of the 16S rDNA gene. For this purpose the EHR 16SD and EBR3 primers were used for the first round PCR amplification. For the second round, EHR 16SD was used as forward external primer while EBR2 was used as internal primer. The reaction mix consisted of Y sub (Yellow Sub™ GENEO BIO Products, Germany), milli-Q water, buffer (20mM Tris-HCl, pH 8.4, Promega, Leiden, The Netherlands), 1.65mM MgCl2 (Triton X-100), 200M dNTP, primers (25 µmol/µL) and 1.0U of Taq polymerase (Promega, Maidson WI, USA). Five µL of the extracted DNA was used as a template. The PCR reaction was carried out in a total volume of 25 µL using a programmable thermocycler (T3 thermocycler Biometra®, Westburg, NL). For the first round the reaction consisted of initial heating at 92℃ for 3 minutes, followed by 40 cycles of denaturation at 92℃ for 30 seconds, annealing at 62℃ for 45 seconds, elongation at 72℃ for 1 minute and final extension at 72℃ for 10 minutes. For the second round of amplification 0.5 µL of the PCR products from the first reaction were used as template. The reaction consisted of 25 cycles with the same reaction conditions as the first round PCR. All the PCR products were visualised by gel electrophoresis in TAE buffer (0.04M Tris, 0.4mM EDTA, pH = 7.7-8.8) using 2% agarose at 100V for 20

minutes and staining 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 negative control while DNA from an in vitro culture of *E. ruminantium* was used as positive control.

6.2.8 Restriction enzyme fragment length polymorphism analysis of the amplified products

The amplified products were digested by using restriction enzymes MboII, HhaI and MspI to identify the species pathogen encountered. Restriction was done in a final volume of 15 µL consisting of 4 µL DNA (PCR product) and 11 µL RFLP mix (restriction enzymes, Biolabs, New England; 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 TAE buffer (0.04M Tris, 0.4mM EDTA, pH = 7.7-8.8) at 100V for 40 minutes and visualised under UV illumination after staining with ethidium bromide.

6.2.9 Cloning and sequencing of amplified DNA fragment

Since we did not get DNA of some important pathogens such as *Anaplasma ovis*, DNA samples were cloned and sequenced from PCR positive samples from sheep (SH11) and goats (SG6). Purification of the PCR products was done using QIAquick® purification kit (QIAGEN®, 2011). The PCR products were mixed with Buffer PB five times their volumes. The mixture was transferred to the QIAquick column and centrifuged for 40 seconds. The product was washed using 750 µL of Buffer PE by centrifuging for 40 seconds. Finally DNA was eluted from the QIA column by adding 50 µL of Buffer EB and centrifuging for 1 min. Cloning of the purified DNA was carried out in plasmid with *E. coli* using TOPO TA Cloning® kits (Invitrogen™, 2006). Four µL of the purified PCR product was mixed with 1 µL of salt solution and 1µL of TOPO® vector to make a reaction volume of 6 µL. They were mixed gently and incubated at room temperature for 15 min. The tubes were then placed on ice. One Shot® *E. coli* cells were taken out of deep freezer and thawed. Two µL of the TOPO® cloning reaction was added to each vials of One Shot® *E. coli* and mixed gently. The vials were incubated on ice for 15 min and heat shocked at 42℃ for 30s. Two hundred fifty µL of LB medium (room temperature) was added to each vial of *E. coli* cells and incubated at 37℃ for 1 hour with gentle shaking. The LB plates containing X-gal and ampicillin were prepared and IPTG was added to each. The plates were then spread with 70 µL of the transformation and incubated at 37℃ overnight. The next morning 10 white colonies were selected from each plate and plated onto LB medium containing ampicillin and incubated at 37℃ overnight. The presence of the cloned DNA in the vector was analysed by collecting some of the colonies into PCR tubes containing

50 µL of sterile water. Five µL of this sample was mixed with 20 µL of the PCR mix and amplified under and visualised following similar procedures described above.

6.2.10 Statistical analysis

The data recorded were analysed using R statistical software version 2.15.1 for windows and STATA version 11. Logistic regression analysis was used to identify factors associated with positivity of ticks for *Ehrlichia* and *Anaplasma* species. Five percent significance level was considered significant.

6.3 Results

6.3.1 Analysis of DNA samples

The primers were initially verified by amplification of part of the 16S rDNA from *E. ruminantium*, *A.* sp. Omatjenne, *A. marginale*, *A. centrale* and *A. phagocytophilum*. DNA from all these organisms was amplified and gave a clear single band at 925bp. Restriction of the amplicons with MboII differentiated *A. centrale* and *A.* sp. Omatjenne. These pathogens yielded fragments respectively of 344, 203 and 167bp, and 476 and 235bp while *A. marginale* yielded a fragment of 544 and 167 (Figure 6.2 **A**). *Ehrlichia ruminantium* and *A. phagocytophilum* were not restricted (Figure 6.2 **A**: lanes 1 and 3 and 4, respectively) but subsequent restriction with MspI gave different patterns. As shown in lane 8 of Figure 6.2 **A**, *E. ruminantium* yielded fragments of 485 and 216bp while *A. phagocytophilum* produced fragments of 398, 155, 86 and 64bp (lane 9).

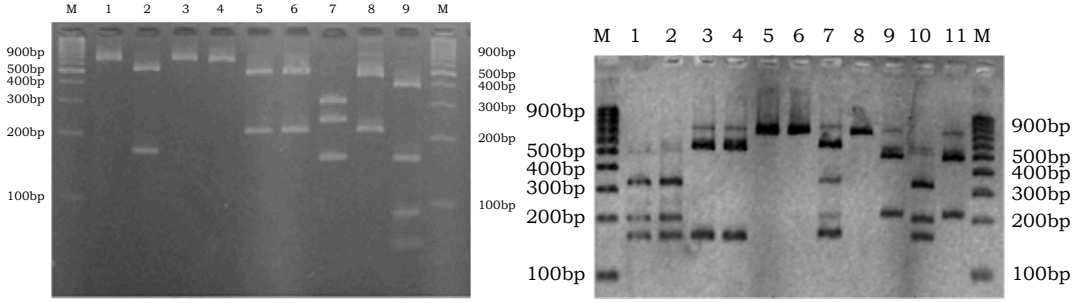


Figure 6.2: A. Results of restriction analysis using MboII and MspI enzymes on DNA samples used: M = DNA marker, lane 1 = *E. ruminantium* (MboII), lanes 3 and 4 = *A. phagocytophilum* (MboII), lane 2 = *A. marginale* (544 and 167bp), lanes 5 and 6 = *A.* sp. Omatjenne (476 and 235bp), lane 7 = *A. centrale* (344, 203 and 167bp), lane 8 = *E. ruminantium* (MspI) (485 and 216bp) and lane 9 = *A. phagocytophilum* (MspI) (398, 155, 86 and 64bp).
B. Results of restriction of DNA amplified from ticks with enzyme MboII. M= 100 bp DNA ladder, lanes 1, 2 and 10 = *A. centrale* (344, 203 and 167bp), lanes 3 and 4 = *A. marginale* (544 and 167bp), lanes 5, 6 and 8 = *A. phagocytophilum*/*E. ruminantium*, lanes 9 and 11= *A.* sp. Omatjenne (476 and 235bp) and lane 7 = mixed infection with *A. marginale* and *A. centrale*. The weaker bands are remainders of partly digested DNA.

6.3.2 Results of sequencing

Two representative samples (SH11 and BS132 from sheep and SG6 from goats) among PCR positive ones were cloned and sequenced (Figure 6.3). The results of the sequence showed that all of them were *A. ovis*. When restricted with MboII these isolate yield the same pattern as *A. marginale* (both producing fragments of 544 and 167 bp, Figure 6.3 **A**). Subsequent restriction HhaI differentiated the two species of *Anaplasma* as depicted in Figure 6.3 **B** *A. ovis* yielded two fragments of 183 and 539bp while *A. marginale* yielded three fragments which are 84, 99 and 538 bp.

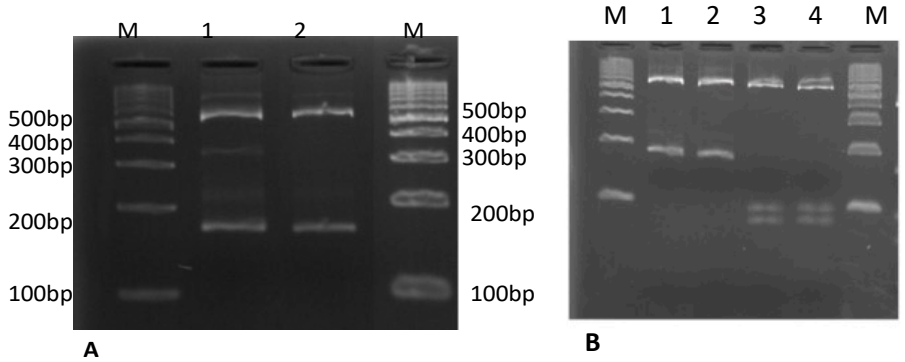


Figure 6.3: **A**. Results of restriction with MboII: M = 100bp DNA marker, lane 1 = *A. ovis* (167 and 544bp), lane 2 = *A. marginale* (167 and 544bp). **B**. Results of restriction with HhaI: M = 100bp DNA marker, lanes 1 and 2 = *A. ovis* (183 and 539bp), lanes 3 and 4 = *A. marginale* 88, 99 and 538bp).

6.3.3 Detection of *Ehrlichia* and *Anaplasma* species in *Amblyomma* ticks

Results of PCR analysis of the field ticks are presented in Table 6.1.
Overall 384 Amblyomma ticks (203 *A. variegatum* and 181 *A. lepidum*) collected from cattle and sheep were tested with PCR followed by RFLP analysis. Out of these 63 (16.40%, 95 CI: 12.69, 20.10) were positive for one or more *Ehrlichia* and/or *Anaplasma* species. The positivity ranges from 6.3% (95 CI: 3.86, 8.73) at Andasa to 36.7% (95 CI: 31.85, 41.49) at Habernosa. This variation was statistically significant ($p < 0.001$) in univariate analysis but not in a multiple logistic regression analysis. At Bako *A. marginale* and *A. centrale* were detected more in *A. lepidum* than in *A. variegatum* while *A.* sp. Omatjenne and *A. phagocytophilum* were detected only in *A. variegatum*. At this site *E. ruminantium* was detected in 3.2% and 1.6% of the *A. variegatum* and *A. lepidum*, respectively. More *A. lepidum* (11.7%) were found positive for *E. ruminantium* than *A. variegatum* ticks (3.3%) at Habernosa with an overall detection of 7.5% at this location. The detection of *A.* sp. Omatjenne was also higher in *A. lepidum* (13.3%) than in *A. variegatum* (3.3%). Similarly, *A. lepidum* showed higher positivity for *A. phagocytophilum* and *A. centrale* than

A. variegatum. But the same proportion of both tick species was positive for *A. marginale*. At Andasa only 0.9% of the *A. variegatum* were positive for *E. ruminantium*, *A. marginale* and *A. phagocytophilum* while 1.8% of them were positive for *A.* sp. Omatjenne and *A. centrale*. *Amblyomma lepidum* ticks were found to be more infected than *A. variegatum* with *Ehrlichia* and *Anaplasma* species. A statistically significantly higher proportion of *A. lepidum* was infected with these rickettsiae than *A. variegatum* ($P = 0.009$). At all the three sites mixed infection was detected in the ticks ranging from 0.65% at Bako to 3.33% at Habernosa.

Overall, more ticks from cattle were found to be more infected than ticks collected from sheep (Table 6.2). Out of 291 ticks collected from cattle, 60 (20.62%, 95 CI: 16.57, 24.67) were found to be positive for one or more of the *Ehrlichia* and *Anaplasma* species tested. On the contrary out of 93 ticks collected from sheep only 3 (3.23%, 95 CI: 1.46, 5.0) tested positive for one or more species of these pathogens. This difference was statistically significant ($p = 0.003$). Ticks collected from cattle are nearly six times more infected with *Ehrlichia* and *Anaplasma* than those collected from sheep (Table 6.3).

Table 6.1: Results of analysis of ticks using PCR for species of *Ehrlichia* and *Anaplasma* at three sites in Ethiopia.
Av = *A. variegatum*, Al = *A. lepidum*, Er = *E. ruminantium*, AO = *A.* sp. Omatjenne, Am = *A. marginale*, Ac = *A. centrale*, Ap = *A. phagocytophilum*

			Number (%) positive for					
Location	Tick species	Test	Er	AO	Am	Ac	Ap	mixed
Bako	Av	32	1 (3.12%)	1 (3.12%)	1 (3.12%)	0	2 (6.25%)	1 (3.12%)
	Al	121	2 (1.65%)	0	6 (4.96%)	1 (0.82%)	0	0
	Sub-total	153	3 (1.96%)	1 (0.65%)	7 (4.57%)	1 (0.65%)	2 (1.31%)	1 (0.65%)
Habernosa	Av	60	2 (3.33%)	2 (3.33%)	3 (5%)	4 (6.67%)	2 (3.33%)	1 (1.67%)
	Al	60	7 (11.67%)	8 (13.33%)	3 (5%)	7 (11.67%)	6 (10%)	3 (5%)
	Sub-total	120	9 (7.5%)	10 (8.33%)	6 (5%)	11 (9.17%)	8 (6.67%)	4 (3.33%)
Andasa	Av	111	1 (0.9%)	2 (1.8%)	1 (0.9%)	2 (1.8%)	1 (0.9%)	1 (0.9%)
	Gr. Total	384	13 (3.38%)	13 (3.38%)	14 (3.64%)	14 (3.64%)	11(2.86%)	6 (1.56%)

Table 6.2: Proportions of ticks collected from cattle (n=291) and sheep (n=93) that tested positive for species of *Ehrlichia* and *Anaplasma*

	Prevalence		
Pathogen	Cattle	Sheep	Total
E. ruminantium	4.12	0	4.12
A. sp. Omatjenne	3.78	0	2.78
A. marginale	4.47	2.15	3.90
A. phagocytophilum	3.78	0	3.78
A. centrale	4.47	1.07	3.65
Overall	20.62	3.22	16.4

Table 6.3: Results of multivariable logistic regression analysis

Variable	Odds ratio	Std.err	z	p-value	95% CI
Location					
Bako	2.25	1.18	1.54	0.123	0.80–6.29
Habernosa	6.68	2.98	4.26	<0.001	2.78–16.01
Animal species					
Cattle	6.92	3.96	3.38	0.001	2.26–21.22
Tick species					
A. lepidum	2.28	0.74	2.54	0.011	1.21–4.31
Constant	0.01	0.007	-6.61	<0.001	0.003–0.43

6.4 Discussion

6.4.1 Simultaneous detection and identification of *Ehrlichia* and *Anaplasma* species

The value of the 16S rDNA gene for the detection of *Ehrlichia* and *Anaplasma* in ticks and blood was proven previously (Bekker et al., 2002), and has been widely used for the diagnosis, taxonomy and phylogenetic analysis of *Ehrlichia* and *Anaplasma* organisms (Dumler *et al.*, 2001; Allsopp, 2010). However, the identification of species of pathogens belonging to these genera depends on the hybridisation of the PCR products with species specific probes designed in the variable region of the V1 loop of this gene. It is a preferable method for the detection of mixed infections and novel species (Faburay *et al.*, 2007b).
In this study we used 16SD EHR primer whose specificity and sensitivity has previously been tested and published (Hornok *et al.*, 2008). We used this primer as external and internal primer during PCR amplification. This avoids the need to do the assessment of sensitivity and specificity of the method. We designed two new primers so as to amplify a longer fragment of 16S rDNA gene to be able to identify the species of *Ehrlichia* and *Anaplasma* by restriction enzyme fragment polymorphism. This avoids the need to use a technically demanding and expensive hybridisation technique, an important consideration when planning field epidemiological studies in resource poor countries. The more sensitive pCS20 PCR developed for epidemiological study of *E. ruminantium* was reported to be specific but did not detect co-infecting pathogens. It could not even differentiate *E. ruminantium* from *A.* sp. Omatjenne (Allsopp *et al.*, 1999) unless a further hybridisation step is used. The current semi-nested 16S assay however differentiated these two species using RFLP. Faburay *et al.* (2007b) have shown that the level of detection of infection with *E. ruminantium* in field *Amblyomma* ticks was lower for 16S rDNA using reverse line blot hybridisation technique compared to pCS20 and map1 PCR assays. Similar findings were also published elsewhere (O.I.E., 2008). It is known that pCS20 is a multi copy gene making the assay more sensitive than those based on 16S rDNA

which is single gene in rickettsiae (Simuunza *et al.*, 2011). Notwithstanding the lower sensitivity of the current method, our study shows that this molecular assay is preferable for epidemiological studies as it detects multiple pathogens simultaneously as mixed infections in field ticks (Figure 6.2 **B**: lane 7).

6.4.2 Detection of *Ehrlichia* and *Anaplasma* species in field ticks

Two *Ehrlichia* and three *Anaplasma* species were found in ticks during this study, but the overall prevalence is relatively low. Particularly the prevalence of *Ehrlichia* in ticks from cattle and sheep is lower than reported previously by authors such as Peter *et al.* (2000), Martinez *et al.* (2004), Molia *et al.* (2008), Christova *et al.* (2003) and Allsopp *et al.* (1999). This difference might have arisen from the use of different gene targets for molecular detection of these pathogens. For instance some of the previous authors used pCS20 for the detection of *E. ruminantium*, which is a multi-copy gene. Another important factor is the differences in tick species analysed and the geographical origin of these ticks. However, our findings are comparable with the results of Street and Gilfroy (2002), Muhanguzi *et al.* (2010) and Aktas *et al.* (2011), but higher than that of Fyumagwa *et al.* (2009) who have used rtPCR as detection method.

Higher overall *Ehrlichia* and *Anaplasma* infections were observed in *Amblyomma* ticks at Habernosa than at Bako and Andasa. Similarly, each species of *Ehrlichia* and *Anaplasma* had a higher prevalence in ticks at Habernosa. This difference could be explained by the strategic application of acaricides at Bako and Andasa while no tick control activity was practiced at Habernosa. This might have resulted in a reduction in the circulation of pathogens between host and vector. The higher infection of *Anaplasma* species in ticks collected from Habernosa could also be due to higher numbers of *Rhipicephalus* ticks at this site. Ticks belonging to the genus *Rhipicephalus* account for more than half of the total ticks collected from this site while low numbers were found at Bako (Mekonnen *et al.*, 2001). *Hyalomma* ticks were also found infesting livestock at Habernosa but not at Bako. Although *R. decoloratus* ticks are considered the main vectors for anaplasmosis, other ticks belonging to *Rhipicephalus*, *Hyalomma* and even other biting arthropods have been incriminated in transmission (Kocan *et al.*, 2004). It could be due to the widespread occurrence of ticks belonging to the *Rhipicephalus* and *Hyalomma* groups that *Anaplasma* organisms are more prevalent at Habernosa than at other sites.The first evidence of rickettsial pathogens in Ethiopia dates back to the work of Philip *et al.* (1966) who detected the agent of Q-fever and *Rickettsia conorii* in ticks collected from cattle. Since then, the occurrence of tick-borne rickettsial infections has been suspected on several occasions on the basis of clinical observations and serological evidence (Mekonnen, 1996; Feleke *et al.*, 2008). But confirmation by detection of these agents either in ticks or blood

samples was lacking. Only one study (Tomassone *et al.*, 2012) detected *E. ruminantium* in ticks in the Somali region. We report here the detection of *E. ruminantium* in ticks in all the three sites although in varying degrees. At Habernosa ranch the outbreak of heartwater has been confirmed by positive brain squash smears in 17 out of 40 samples (42.5%) in dairy cattle (Mekonnen, 1996). The detection of *E. ruminantium* in ticks collected from cattle on the same ranch now provides molecular evidence of the presence of this pathogen. This stresses the importance of *E. ruminantium* in the area when attempting to improve the livestock industry. At Bako the occurrence of cases of heartwater was suspected on the basis of clinical symptoms and postmortem lesions in sheep particularly in youngsters. The presence of *E. ruminantium* in this area is also confirmed now. Even though serological evidence of anaplasmosis in Ethiopian livestock has been available, this study provides the first molecular evidence of these pathogens, as our assay could differentiate between *A. marginale* and *A. centrale*. We are also reporting for the first time the detection of *A.* sp. Omatjenne and *A. phagocytophilum* in Ethiopian ticks.

The pathological role of *A.* sp. Omatjenne is still under discussion (Allsopp, 2010; Du Plessis, 1990). But the detection in ticks collected from animals suggests the presence of this rickettsia in the resident ruminant population. The role of this species in causing disease in livestock needs to be investigated. The ability to give cross-reactions with *E. ruminantium* antigens in serological tests presents a serious limit to the application of serological assays in epidemiological surveys (Allsopp, 2010). On the other hand, *A. phagocytophilum* has been known to produce clinical disease in high producing dairy cattle (Woldehiwet, 2010). The epidemiology and vector(s) of this pathogen remains to be investigated but its occurrence in ticks also suggest its presence in livestock in the area. Moreover, this pathogen is known to constitute a risk to public health as some of the strains can cause a zoonotic infection. The two widespread and important *Amblyomma* species were *A. variegatum* and *A. lepidum*. But a higher proportion of *A. lepidum* was found to harbour *Ehrlichia* and *Anaplasma* species. Although *A. variegatum* has been known for its widespread occurrence and transmission of *E. ruminantium*, it has been suggested that *Amblyomma* species such as *A. lepidum* can play an important local role in vectoring of these pathogens (Jongejan and Uilenberg, 2004). In that respect, our results suggest that *A. lepidum*, since it is a widespread *Amblyomma* species, might be the key species in transmitting *Ehrlichia* species in Habernosa and its surrounding.

In conclusion, the newly developed 16S rDNA assay was able to detect and identify simultaneously the major ruminant pathogens belonging to *Ehrlichia* and *Anaplasma*. It was also shown that mixed infections could be diagnosed in ticks collected from livestock in Ethiopia. In general, the presence of these pathogens in Ethiopian ticks was relatively low. Our results describe for the first time molecular evidence of these pathogens in central and western Ethiopia and could be used as preliminary inventory information for epidemiological investigation and disease management.

CHAPTER 7

Survey of *Anaplasma phagocytophilum* and *Anaplasma* sp. Omatjenne infection in cattle in Africa with special reference to Ethiopia

Abstract Evidence of infection of domestic animals by *Anaplasma phagocytophilum* and *Anaplasma* sp. Omatjenne is becoming available at present but the epidemiological and ecological significance of infection with these agents has not yet been elucidated. Understanding the epidemiology of infection by these pathogens, however, is important to quantify the clinical and socio-economic impact of diseases they cause. The aim of this study was therefore first to analyse the occurrence of *A. phagocytophilum* and *A.* sp. Omatjenne in bovine samples collected from selected African countries using a polymerase chain reaction and restriction enzyme fragment length polymorphism. In total, 695 samples from cattle in six African countries were analysed: overall, 45 positive results were obtained for *A.* sp. Omatjenne (6.47%) and 19 for *A. phagocytophilum* (2.73%). *Anaplasma* sp. Omatjenne was detected in all countries except Tanzania while *A. phagocytophilum* was detected only in samples from Ethiopia. The proportion of samples tested positive for *A.* sp. Omatjenne ranged from 1.2% in Morocco to 16% in Rwanda. The occurrence of both agents is now confirmed in African cattle.

The second part of this study was aimed at molecular identification of *Ehrlichia* spp. and *Anaplasma* spp. infection in ruminants raised under different production systems in selected sites in central Ethiopia. A semi-nested 16S rDNA polymerase chain reaction followed by restriction fragment length polymorphism was used for the identification of *Ehrlichia* spp. and *Anaplasma* spp. in blood samples. Randomly selected samples were also analyzed by pCS20 polymerase chain reaction for the detection of *E. ruminantium*. Positive results were obtained for *E. ruminantium* and five species of *Anaplasma* including the zoonotic species.

A first report of infection of domestic ruminants with *A. phagocytophilum*, *A. ovis* and *A.* sp. Omatjenne in Ethiopia is provided. In addition, occurrence of *A. phagocytophilum* together with *A.* sp. Omatjenne outside Europe and South Africa is made for the first time.

Livestock improvement plans such as smallholder dairy development schemes through introduction of high yielding breeds should be aware of the importance of these diseases and take the necessary precautions to avoid losses. The public health authorities should also be aware of the occurrence zoonotic *Anapasma* spp. Further epidemiological investigation of infections with *E. ruminantium* and *Anaplasma* spp. are recommended.

Adapted from:

Teshale Sori Tolera, Dirk Geysen, Gobena Ameni, Pierre Dorny, Dirk Berkvens. Survey of *Anaplasma phagocytophilum* and *Anaplasma* sp. 'Omatjenne' infection in cattle in Africa with special reference to Ethiopia. Parasites & Vectors. Accepted.

7.1 Introduction

Anaplasma phagocytophilum is a recently emended species of bacteria that comprises *Ehrlichia phagocytophila*, *Ehrlichia equi* and the agent of human granulocytic ehrlichiosis (Dumler *et al.*, 2001). The bacterium appears to be a generalist infecting a wide range of domestic and wild animals (causing tick-borne fever) and humans (causing human granulocytic anaplasmosis) (Yoo-eam, 2012). Most outbreaks of tick-borne fever have been recorded in sheep flocks and cattle herds but isolated outbreaks have also been documented in goats. Although mortality and morbidity due to *A. phagocytophilum* infection is low in animals, economic losses due to drop in milk yield, abortion, infertility and reduced weight gains have been observed in pastured animals (Stuen *et al.*, 2013; Woldehiwet, 2010). Death can also occur in weaker animals if left untreated (Rymaszewska and Grenda, 2008). Since its recognition, an escalating number of human cases of *A. phagocytophilum* has been reported in the USA, Europe, Middle East and Asia with an hospitalisation rate of 36% in the USA and a mortality of 26.5% in China (Stuen *et al.*, 2013) Although tick attachment is thought to be the main route of human infection, contact with infected animal blood and prenatal infection has been reported (Santos *et al.*, 2004).

Despite its veterinary and public health importance, data on the occurrence of *A. phagocytophilum* are lacking for the African continent. Only a few published articles reported its occurrence and detection of the bacterium was coincident with detection of other tick-borne pathogens. For instance, Muhanguzi *et al.* (2010) detected DNA of this pathogen in 2.7% of cattle blood in Uganda while Sarih *et al.* (2005) detected its DNA in two *Ixodes ricinus* specimen in Tunisia. Recently, the bacterium was detected in ticks in Ethiopia (Teshale *et al.*, 2015). Apart from these, no systematic and pre-designed epidemiological studies were undertaken to explore *A. phagocytophilum*. Empirically, several tick associated febrile diseases, whose etiologic agents remain obscure, have been reported frequently by field veterinarians. Elsewhere, it has been shown that 8.6% of such tick associated febrile diseases (15% in horses and 6% in dogs) are caused by *A. phagocytophilum* (Anderson *et al.*, 2005). The possibility of *A. phagocytophilum* being involved in such cases in Africa cannot be ruled out. Investigation into the occurrence of *A. phagocytophilum* from different areas of Africa is needed.

Anaplasma sp. Omatjenne, a rickettsia belonging to the family *Anaplasmatacaea*, was described for the first time by Allsopp *et al.* (1997) and was detected in healthy Boer goats in heartwater-free areas of South Africa using molecular methods. The distribution and epidemiology of this genotype of rickettsia has not been investigated in Africa. However, infection with it has been reported in wild and domestic animals in Africa and the Mediterranean. It was detected in 1.9% of cattle in Uganda (Muhanguzi *et al.*, 2010) and in Turkey (Aktas *et al.*, 2011). It has also been reported in Nyala (*Tragelaphus angasii*) in four game ranches sharing grazing areas with cattle in KwaZulu-Natal (Pfitzer *et al.*, 2011)

and African Buffaloes (*Syncerus caffer*) in Kruger National Park and in Hluhluwe-iMfolozi Park (Debeila, 2012). Apart from *Amblyomma* ticks (Teshale *et al.*, 2015), Teshale and colleagues also detected the bacterium recently in ruminants in Ethiopia (unpublished data). Since the role of this *Ricketssia* in producing clinical disease remains obscure, study on its occurrence in Africa is required.

The epidemiological and ecological significance of infection with *A. phagocytophilum* is not elucidated and the importance of *A.* sp. Omatjenne in causing disease has not been clarified despite its occurrence in ruminants. The first aim of this study was to analyse the occurrence of *A. phagocytophilum* and *A.* sp. Omatjenne in bovine samples collected from several African countries.

In the second place a detailed field survey was conducted trying to elucidate the occurrence of infection by species of *Erhlichia* and *Anaplasma* in cattle, sheep and goats in central Oromia (Ethiopia) using molecular methods. The information was used to answer the following questions: (i) Which *Ehrlichia* and *Anaplasma* spp. infect ruminants in central Ethiopia, (ii) what is the prevalence of infection of these pathogens in ruminants in in central Ethiopia and (iii) what are the risk factors associated with infection by *Anaplasma* spp. in ruminants? At the same time, all major tick species of the area were identified. Ticks are widespread found in all agro-ecological zones of Ethiopia (Pegram *et al.*, 1981; Abera *et al.*, 2010; Zeleke and Bekele, 2010) and empirical evidence shows heartwater and anaplasmosis problems in areas where *Amblyomma* and *Rhipicephalus* spp. are encountered. However, epidemiological investigation of tick-borne diseases is either absent or inadequate in the country (Feleke *et al.*, 2008). The available information itself is obsolete because the results are based on serology and microscopy with obvious limitations (Bose *et al.*, 1995). Only limited molecular studies have been carried out so far by Tomassone *et al.* (2012), Kumsa *et al.* (2014c) and Teshale *et al.* (2015), mostly in ticks. Since Ethiopia has diverse agro-climatic regions that support several vector species, mixed infections with *Ehrlichia* and *Anaplasma* spp. are common. Molecular characterisation is essential for accurate differential diagnosis and detection of mixed infections of species belonging to the two genera. This leads to a better understanding of the epidemiology in order to minimise losses incurred in livestock during genetic improvement and translocation programs. Making an inventory of tick-borne *Erhlichia* and *Anaplasma* spp. is an important pre-requisite to understand their role in constraining ruminant production.

7.2 Materials and Methods

7.2.1 International Study

Samples used in this study were DNA samples extracted from bovine blood collected from selected African countries. The samples (238 in total), originating from Ivory Coast

(53 samples), Morocco (81 samples), Rwanda (50 samples), Tanzania (17 samples) and Zambia (37 samples), were obtained from the Department of Biomedical Science, Institute of Tropical Medicine (Antwerp, Belgium) and were analysed using 16S rDNA PCR-RFLP.

7.2.2 Oromia survey

7.2.2.1 Study Areas

The study was carried out in five livestock premises: Bishoftu, Bako, Habernosa, Alage and Adami-Tullu. Figure 7.1 shows their geographical locations. They are distributed in three districts (Ada'a-Liban, Bako-Tibbe and Adami-Tullu-Jiddo-Kombolcha) in central Oromia (Ethiopia) (Table 7.1). Selection of the study sites was based on previous clinical and post-mortem reports of heartwater using microscopic examination of brain squash smears at the National Animal Health Research and Diagnostic Centre (Sabata, Ethiopia) Mekonnen (1996), serologic reports of anaplasmosis at the International Livestock Research Institute (Addis Ababa, Ethiopia) Feleke *et al.* (2008) and molecular evidence of infection in ticks in the area Kumsa *et al.* (2014c) and Teshale *et al.* (2015).

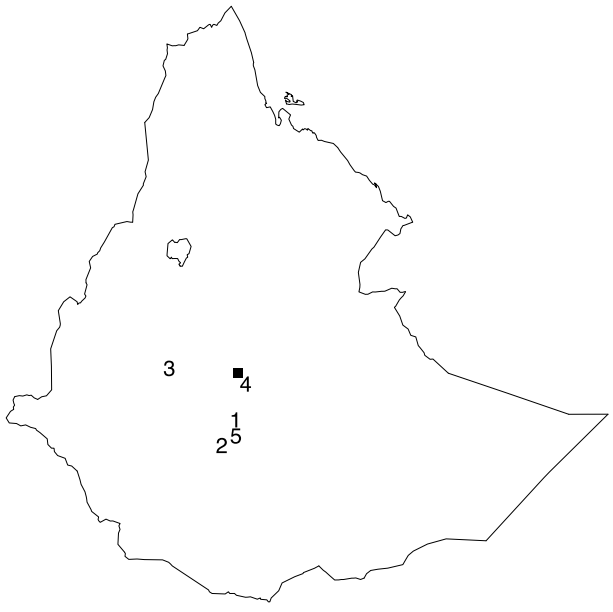


Figure 7.1: Map of Ethiopia showing the locations of the livestock premises included in the study. ■ = Addis Ababa; 1 = Adami-Tullu Research Centre; 2 = Alage; 3 = Bako Agricultural Research Centre; 4 = Bishoftu; 5 = Habernosa.

Heartwater was suspected to occur in exotic dairy cattle in Ada'a-Liban District, in and around Bishoftu on several farms. Bishoftu is one of the international tourist destinations, located in the Eastern Shewa Zone of the Oromia Regional State southeast of Addis Ababa. It is the main town of Ada'a-Liban district, situated on two international trade routes:

Table 7.1: Description of study districts.

Description	Ada'a Liban	Bako	Adami-Tullu-Jiddo-Kombolcha
Location	9°N and 4°E	9°08'N and 37°05'E	7°09'N and 38°07'E
Mean temperature	8.5℃-30.7℃	13.5℃-27.9℃	22℃-28℃
Mean annual RF	1156mm	1227mm	760.9mm
Mean humidity	61%	85%	63%
Mean altitude	1550m	1650m	1650m
Farming type	Mixed	Mixed	Livestock based
Main livestock	Cattle, sheep, goats	Cattle and sheep	Cattle and goats
Premises selected	Bishoftu farms	Bako Research Farms	Habernosa,Alage,A/Tullu Farm
Animals sampled	cattle (n=125)	cattle (n=149)	cattle (n=183)
	sheep (n=125)	sheep (n=164)	
	goats (n=51)		goats (n=125)
Samples collected	blood and ticks	blood and ticks	blood and ticks

(i) the Franco-Ethio-Djibouti railway and (ii) the Addis-Moyale-Nairobi international asphalted route. It is one of the main milk producing area for Addis Ababa and is known for its commercial livestock production. Commercial dairy farms raising exotic dairy cattle (Holstein cattle and their crosses with zebu) and smallholder farmers rearing zebu cattle as well as Menz sheep and local goats were included in the study. Data were collected in February 2012.

Bako Agricultural Research Centre with a history of clinical heartwater cases in lambs and calves, detected by pathology, was also selected for this study. Teshale *et al.* (2015) detected DNA of *E. ruminantium* and *Anaplsma* spp. in ticks collected from cattle raised on this premise. The Bako region has a warm and humid climate that supports survival and proliferation of several tick species. The main vegetation of the area is a forest type, which is favourable for wildlife. Mixed crop-livestock production is the economic mainstay for communities in the area. Data collection was carried out in December 2012.

The remaining three selected livestock premises (Habernosa, Adami-Tullu and Alage farms) are found in Adami-Tullu-Jiddo-Kombolcha district. The district is located in the mid Rift Valley of Ethiopia to the south of Addis Ababa. Data from this district were collected in January 2012. The main climate type of the district is semi-arid and livestock production is the dominant farming system. Dairy cattle are mostly reared in small-scale dairy operations under different levels of management. These three livestock premises were selected based on previous history of mortality of dairy cattle due to tick-borne diseases (Mekonnen, 1996). Habernosa is a private ranch where exotic (Holstein) animals and their crosses with Borana cattle are raised and distributed to nearby smallholder farmers. On this ranch heartwater was confirmed in 17 of 40 cattle that died after showing clinical signs of the disease (Mekonnen, 1996). The occurrence of *Ehrlichia* and *Anaplasma* spp. in ticks collected from this ranch was confirmed by molecular techniques (Teshale *et al.*, 2015). Currently the ranch has introduced goats of the Arsi breed obtained from the

south-eastern highlands. The goats are at risk for tick-borne infections since they are introduced from areas where tick challenge is low. Adami-Tullu is an agricultural research centre breeding mainly Arsi goats and cattle for distribution to nearby farmers. Alage is a small village with commercial dairy and swine farms. Samples were collected from dairy cattle as clinical cases of heartwater have been experienced on this farm (own unpublished observations, ST).

7.2.2.2 Sample Collection

Blood samples were collected from cattle, sheep and goats during the dry season (November 2011 to April 2012) using EDTA coated vacutainer tubes. In addition, information on the type of farms (commercial, ranch or smallholder) and the type and frequency of tick control was recorded. The tick burden (adult and nymph) on each animal was assessed as: none (no tick seen), few (1 to 20 ticks), moderate (21 to 50 ticks) and abundant (>50 ticks). Convenience sampling was used throughout with the primary aim to detect and describe. Statistical analysis was not envisaged as controlling for possible confounders proved impossible. Overall 922 blood samples (457 cattle, 289 sheep and 176 goats) were collected. Ticks infesting the study animals were also collected and identified based on morphological identification keys described in (Walker *et al.*, 2003). The identification was carried out in the Veterinary Parasitology Laboratory of the College of Veterinary Medicine and Agriculture of the Addis Ababa University (Bishoftu, Ethiopia).
DNA samples were extracted from whole blood using Gentra® Puregene® Bood Kit (QIAGEN®, 2010) as described by the manufacturer and stored at -18°C before transferring them to the Biomedical Sciences Department of the Institute of Tropical Medicine (Antwerp, Belgium) for molecular characterisation.

7.2.3 Molecular Analysis of the Samples

7.2.3.1 16S rDNA Polymerase Chain Reaction

A semi-nested PCR described by Teshale *et al.* (2015) was used to amplify a fragment of 16S rDNA gene. Primers designated EHR 16SD (Hornok *et al.*, 2008) and EBR3 (Teshale *et al.*, 2015) were used for the first round of PCR amplification. For the second round of amplification the same EHR 16SD was used as forward external primer while EBR2 (Teshale *et al.*, 2015) was used as internal primer. Details of the primers used are presented in Table 7.2. The reaction mix consisted of HotStartTaq Master Mix (2.5 units of DNA polymerase, PCR buffer containing 1.5 mM MgCl2 and 200 µM of each dNTP), 0.2 µM of each primer and PCR water. The PCR reaction was carried out in a total volume of 25µl using a programmable thermocycler (T3 thermacycler Biometra®, Westburg, NL). For the first round, the reaction consisted of initial heating at 92°C for 3 minutes, followed by 40 cycles of denaturation at 92°C for 30 seconds, annealing at 62°C for 45 seconds,

elongation at 72°C for 1 minute and final extension at 72°C for 10 minutes. For the second round of amplification 0.5µl of the PCR products from the first reaction were used as template. The reaction consisted of 25 cycles with the same reaction conditions as the first round PCR. All the PCR products were visualised by gel electrophoresis in TAE buffer (0.04M Tris, 0.4mM EDTA, pH = 7.7–8.8) using 2% agarose at 100V for 20 minutes and staining with ethidium bromide. Throughout the PCR procedures, a PCR mix without DNA template was used as negative control while DNA from in vitro cultures of *A. phagocytophilum*, *A.* sp. Omatjenne, *A. marginale* and *E. ruminantium* were used as positive control. Negative samples were retested at 1/10 dilution to check if high concentration of DNA inhibited the reaction.

Identification to species level of the detected *Anaplasma* was carried out by using restriction with MboII (5000U/mL, Biolabs, New England), HhaI (20,000U/mL, Biolabs, New England) and MspI (20,000U/mL, Biolabs, New England) as described earlier by Teshale *et al.* (2015). Restriction was carried out in a final volume of 15µl mix consisting of 4µl DNA (PCR product from positive samples) and 11µl reaction mix. Incubation was done overnight following the manufacturer's instructions. The restricted fragments were separated on 2% high resolution agarose gel by electrophoresis in TAE buffer (0.04M Tris, 0.4mM EDTA, pH = 7.7–8.8) at 100V for 40 minutes and visualised under UV illumination after staining with ethidium bromide (final concentration of 0.5µg/ml).

7.2.3.2 Identification of *E. ruminantium* using pCS20 PCR

A semi-nested pCS20 PCR assay was used to identify *E. ruminantium* DNA on randomly selected samples and samples collected from clinical cases of heartwater in Ethiopia. The assay was carried out as described by Martinez *et al.* (2004) and Faburay *et al.* (2007a) using primers AB129 and ITM130 as external reverse and forward primers and primers AB128 and AB129 as internal primers (Table 7.2). After initial denaturation of DNA at 94°C for 3min, the first round of amplification was carried out using 40 cycles of 30s denaturation at 94°C, 45s annealing at 62°C and 1 min elongation at 72°C and a final extension of 10 min at 72°C. For the second round amplification, 0.5µl of the PCR product of the first round amplification was used as template. The amplification process consisted of 25 cycles of the same PCR conditions as in the first round except that the annealing temperature was set at 58°C. The amplification process was carried out in the thermocycler described above. The presence of *E. ruminantium* DNA was analysed using a 2% agarose gel electrophoresis, giving a 280bp DNA fragment for a positive sample. A PCR mix without DNA template was used as negative control while DNA from *E. ruminantium* was used as a positive control.

Table 7.2: List of primers used for PCR amplifications and their sequences.

Primer	Sequence	Target gene	Size	Reference
HER 16SD	GGTACCYACAGAAGAAGTCC	16S rDNA		Hornok *et al.* (2007)
EBR2	TGCTGACTTGACATCATCCC	16S rDNA	925bp	Teshale *et al.* (2015)
EBR3	TTGTAGTCGCCATTGTAGCAC	16S rDNA	925bp	Teshale *et al.* (2015)
AB128	ACTAGTAGAAATTGCACAATCTAT	pCS20	280bp	Martinez *et al.* (2004)
AB129	TGATAACTTGGTGCGGGAAATCCTT	pCS20	280bp	Martinez *et al.* (2004)
ITM130	TCAATTGCTTAATGAAGCACTAACTCAC	pCS20	280bp	Faburay *et al.* (2007a)

7.3 Results

7.3.1 *Anaplasma phagocytophilum* and *A.* sp. Omatjenne survey

The results of the international survey and the results of the Ethiopian survey are combined for clarity. A total of 695 samples from cattle were assayed for the presence of DNA of *A. phagocytophilum* and *A.* sp. Omatjenne. Respectively 19 (2.7%) and 45 (6.5%) yielded positive signals (Table 7.3). *Anaplasma* sp. Omatjenne was detected in all countries except Tanzania while *A. phagocytophilum* was detected only in samples originating from Ethiopia.

Table 7.3: Results of molecular analysis of bovine samples from African countries for infection with *A.* sp. Omatjenne and *A. phagocytophilum*.

Country		Number tested	*A. phagocytophilum* Number	*A. phagocytophilum* Prev (95% CI)†	*A.* sp. Omatjenne Number	*A.* sp. Omatjenne Prev (95% CI)†
Ivory Coast		53	0	0.000 (0.000-0.055)	2	0.038 (0.004-0.130)
Morocco		81	0	0.000 (0.000-0.036)	1	0.012 (0.001-0.067)
Rwanda		50	0	0.000 (0.000-0.058)	8	0.160 (0.072-0.291)
Zambia		37	0	0.000 (0.000-0.078)	5	0.135 (0.045-0.288)
Tanzania		17	0	0.000 (0.000-0.162)	0	0.000 (0.000-0.162)
Ethiopia	Bako	149	16	0.107 (0.063-0.169)	13	0.087 (0.047-0.144)
	Bishoftu	125	3	0.024 (0.005-0.069)	11	0.088 (0.045-0.152)
	Habernosa	145	0	0.000 (0.000-0.020)	5	0.034 (0.011-0.079)
	Alage	38	0	0.000 (0.000-0.076)	0	0.000 (0.000-0.076)
	Overall	457	19	0.042 (0.025-0.064)	29	0.063 (0.043-0.090)
Total		695	19	0.027 (0.017-0.042)	45	0.065 (0.048-0.086)

† Prev (95% CI) = prevalence with 95% confidence interval

7.3.2 Oromia survey

7.3.2.1 Tick Species Encountered in the Study Area

Ticks belonging to three genera (*Amblyomma*, *Rhipicephalus* and *Hyalomma*) were encountered in the study areas (Table 7.4). *Amblyomma* spp. accounted for the largest proportion of the ticks (57.49%) collected at Bako. *Ablyomma lepidum* was the most abundant tick infesting animals at this locality followed by *Rh. e. evertsi* and *Rh. decoloratus*. At Habernosa and Alage the majority of the ticks collected were *Rhipicephalus* spp. (63.17% and 66.02%, respectively). *Rhipicephalus evertsi evertsi* was the predominant tick species followed by *A. variegatum* on both livestock premises. *Rhipicephalus* spp. (particularly *Rh. decoloratus*) predominated tick collections at Bishoftu. No ticks were collected from animals at Adami-Tullu.

Table 7.4: Tick species infesting ruminants on the five livestock premises in central Oromia, Ethiopia.

Tick Species	Bako	Habernosa	Bishoftu	Alage	Adami-Tullu
Amblyomma variegatum	121	588	170	570	-
Amblyomma lepidum	762	155	200	70	-
Amblyomma gemma	40	82	-	30	-
Amblyomma nymphs	236	11	-	5	-
Sub-total	1159	836	370	675	-
Proportion	57.5%	28.6%	15.5%	29.7%	-
Rhipicephalus decoloratus	275	16	1303	10	-
R. decoloratus nymph/larvae	-	10	14	-	-
Rhipicephalus evertsi evertsi	578	1283	252	1150	-
Rhipicephalus pulchellus	-	540	-	340	-
Sub-total	853	1849	1569	1500	-
Proportion	42.3%	63.2%	66.0%	66.0%	-
Hyalomma marginatum marginatum	-	179	440	82	-
Hyalomma marginatum rufipes	-	13	-	3	-
Hyalomma impressum	-	38	-	-	-
Hyalomma sp.	-	4	-	2	-
Unidentified nymphs	4	8	-	10	-
Sub-total	4	242	440	97	-
Proportion	0.2%	8.2%	18.5%	4.3%	-
Grand total	2016	2927	2379	2272	-

7.3.2.2 Identification of *Ehrlichia* spp. and *Anaplasma* spp. using molecular tools

Results of the molecular analysis are given in Table 7.5. Out of the 922 blood samples from cattle, sheep and goats from five different localities analysed by 16S rDNA PCR

523 (56.7%) tested positive. The lowest proportion of positive samples was observed at Habernosa (44%) while the highest was observed at Alage (79%). None of the samples collected from Adami-Tullu yielded a positive result. The variation in the proportion of positive samples among the locations could not be compared statistically as the species composition (and thus the resulting sample) in the five livestock premises was not the same.

The highest proportion of positive samples was observed in cattle followed by sheep and the lowest in goats. Overall 67.4% of cattle, 65% of sheep and 18% of goats tested positive for one or more *Anaplasma* spp. The difference in prevalence among the species could again not be tested statistically, as an effect of sampling location could not be excluded (*e.g.* a comparison at Bishoftu -the only location where all three species were sampled- was not significant). Mixed infections, mostly with *A. marginale* and *A. centrale* were detected at all four sites with proportion of 11% at Bishoftu, 13.4% at Bako, 15% at Habernosa and 15.8% at Alage.

Table 7.5: Number and percentage of samples tested positive by 16s rDNA in cattle, sheep and goats in central Oromia, Ethiopia (N=922).

Animal species	Bako	Alage	Adami-Tullu	Habernosa	Bishoftu
Cattle					
Number tested	149	38	-	145	125
Number positive	99	30	-	104	75
Proportion	66.4%	79%	-	71.7%	60%
Sheep					
Number tested	164	-	-	-	125
Number positive	124	-	-	-	63
Proportion	75.6%	-	-	-	48%
Goats					
Number tested	-	-	20	105	51
Number positive	-	-	0	5	26
Proportion	-	-	0%	4.8%	51%

Restriction fragment length polymorphism (RFLP) analysis resulted in the diagnosis of *A. marginale*, *A. centrale*, *A. phagocytophilum*, *A. ovis* and *A.* sp. Omatjenne (Table 7.6). This is the first report of infection of domestic ruminants with *A. phagocytophilum*, *A. ovis* and *A.* sp. Omatjenne in Ethiopia. Occurrence of *A. phagocytophilum* together with *A.* sp. Omatjenne outside Europe and South Africa is made for the first time. The proportion of samples tested positive for *A. marginale* was higher at Bako and Alage than at Habernosa and Bishoftu. The occurrence of *A. centrale* was highest at Alage and lowest at Bako. Only samples from Bako and Bishoftu gave positive results for *A. phagocytophilum* and *A. ovis* and the proportion of samples positive for both pathogens was higher at Bako than at Bishoftu). Almost the same numbers of positive samples

for *A.* sp. Omatjenne were found at Bako and Bishoftu, and half of these numbers at Habernosa.

Table 7.6: Frequency of infection with *Anaplasma* spp. in cattle, sheep and goats in five livestock premises in central Oromia, Ethiopia.

Location	Am	AspO	Aph	Ac	Ao
Bako					
Cattle (n=149)	67 (44.97%)	13 (8.72%)	16 (10.74%)	14 (9.40%)	0 (0%)
Sheep (n=164)	120 (73.17%)	10 (6.09%)	2 (1.22%)	3 (1.83%)	26 (15.85%)
Alage					
Cattle (n= 38)	23 (60.53%)	0 (0%)	0 (0%)	11 (28.95%)	0 (0%)
Habernosa					
Cattle (n=145)	67 (46.21%)	5 (3.45%)	0 (0%)	50 (34.48%)	0 (0%)
Goats (n=105)	3 (2.86%)	2 (1.90%)	0 (0%)	0 (0%)	0 (0%)
Bishoftu					
Cattle (n=125)	45 (36.00%)	11(8.80%)	3 (2.40%)	48 (38.40%)	0 (0%)
Sheep (n=125)	27 (21.60%)	5 (4%)	4 (3.20%)	7 (5.60%)	22 (17.60%)
Goats (n=51)	15 (29.41%)	1 (1.96%)	3 (5.88%)	5 (9.80%)	4 (7.84%)
Adami-Tullu					
Goats (n=20)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
Overall (n=922)	367 (39.8%)	47 (5.1%)	28 (3%)	138 (15%)	52 (5.6%)

Am = *A. marginale*, AspO = *A.* sp. Omatjenne , Aph = *A. phagocytophilum*, Ac = *A. centrale* and Ao = *A. ovis*

7.3.2.3 Results of pCS20 PCR Amplification

Since none of the 16s rDNA PCR-RFLP analysis yielded positive result for *E. ruminantium*, pCS20 PCR was used to analyse randomly selected samples. Thus, from the 922 DNA samples, 493 samples (271 cattle, 145 sheep and 77 goats) were randomly selected and tested for *E. ruminantium*. Three of the 75 cattle blood tested from Bako gave positive result giving an apparent prevalence of 4% in the area. Overall the prevalence of *E. ruminantium* in the cattle included in this study was 0.6%.

7.3.2.4 Active clinical search

Thirteen blood samples (ten from dairy farm in Bishoftu and three form dairy farm of Haramaya University) were collected from clinically affected animals and analysed by pCS20 PCR. The result of the assay is depicted in Figure 7.2. Three (30%, 95% CI: 6.7%, 65.2%) and two (66.7%, 95% CI: 9.4%, 99.2%) of the samples from Bishoftu and Haramaya University, respectively were tested positive for *E. ruminantium*. Overall 38.46% (95% CI: 13.86, 68.42) of the clinically affected cows were found positive.

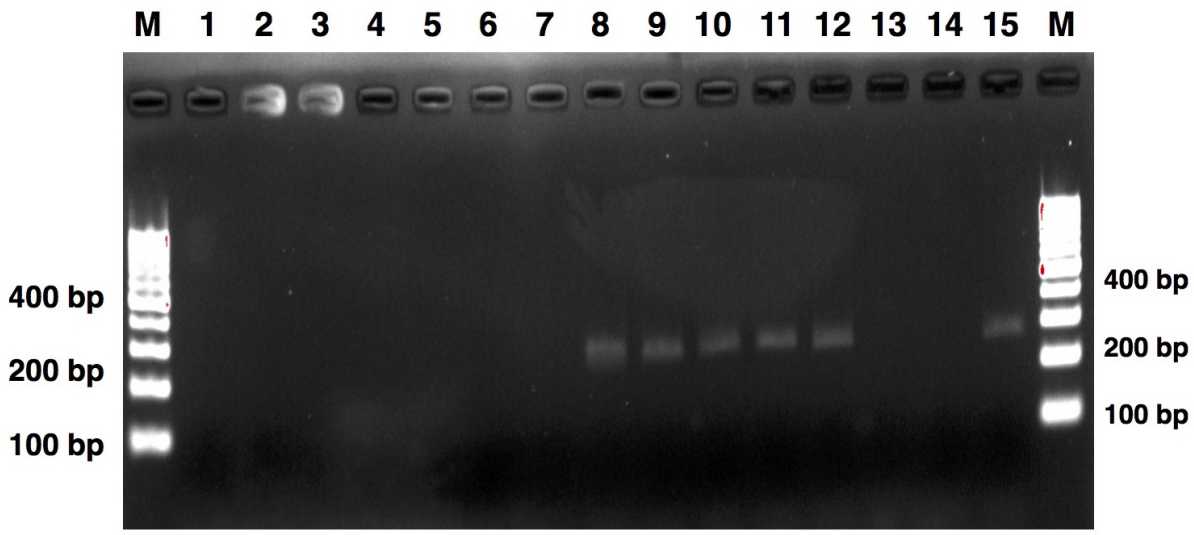


Figure 7.2: Results of pCS20 PCR assay on samples collected from clinical cases of heartwater. M = DNA ladder; lanes 1-10 = samples from dairy cattle in Bishoftu; lanes 11 13 samples from dairy farm of Haramaya University; lane 14 = negative control (PCR water) and lane 15 = positive control (*E. ruminantium*).

7.4 Discussion

Since it was first recognised, anaplasmosis caused by *A. phagocytophilum* is considered to have a world-wide distribution. However, studies about the extent of its occurrence in animals and humans have been limited to Europe and USA. Advances in molecular diagnosis result in new evidence for the wide occurrence of this pathogen in the northern hemisphere Rymaszewska (2011). In Africa, where many tick-borne diseases are encountered (Kocan *et al.*, 2000), data that support the occurrence of *A. phagocytophilum* is lacking, possibly due to lack of appropriate diagnostic tools: diagnosis of anaplasmosis, based on microscopic examination of stained bloodsmears and serologic tests, is unable to differentiate the various species and requires a high level expertise and skill (Simuunza, 2009; Stuen *et al.*, 2013). In this study we used molecular methods to investigate the occurrence of *A. phagocytophilum* in Africa.

So far no reports of clinical cases associated with *A. phagocytophilum* have been published in Africa. Very few studies reported its occurrence in animals and ticks within the continent. Overall, the proportion of infected animals reported by earlier authors is in consent with our findings. For example, the overall prevalence observed in this study (2.73%) agrees with the prevalence reported by Muhanguzi *et al.* (2010) in cattle (2.70%) from Uganda and Teshale and colleagues in sheep and goats (2.49%) from Ethiopia (unpublished data). The present result is higher than the prevalence reported by M'Ghirbi *et al.* (2012) in horses (0.90%) in Tunisia, where the same authors however report a prevalence of 13% in dogs (M'Ghirbi *et al.*, 2009). The apparent variability in the prevalence of *A. phagocytophilum* can in the first place be explained by the different host species examined and differences in

environmental conditions between Tunisia and Ethiopia also cannot be excluded: Tunisia is characterised by a mediterranean/subtropical climate while Ethiopia features tropical conditions, a difference that affects tick species, their numbers, distribution and infection rate with tick-borne pathogens (de la Fuente *et al.*, 2008). *Anaplasma phagocytophilum* has also been detected in ticks in Africa: in questing *Rhipicephalus pulchelus* in Ethiopia (Teshale *et al.*, 2015) and in *I. ricinus* in Tunisia (Sarih *et al.*, 2005). It must be noted that higher prevalence levels of *A. phagocytophilum* have been reported outside Africa, *e.g.* 16.7% cattle in Italy (Torina *et al.*, 2007). Furthermore, the prevalence, based on the detection of DNA, was shown to be as high as 51% in Guatemala, 20% in France, 13-19% in Spain and 1-80% in Japan (Stuen *et al.*, 2013).

In the present study no samples collected from Ivory Coast, Morocco, Rwanda, Tanzania and Zambia were positive for *A. phagocytophilum*, possibly due to the small sample sizes tested, collected only from cattle (95% confidence intervals shown in Table 7.3 all include expected prevalence levels). Secondly, it could be due to the low level of *A. phagocytophilum* circulating in the persistently infected hosts, characterised by intermittent periods of bacteraemia: earlier studies have shown that domestic animals are persistently infected with *A. phagocytophilum* causing cyclic bacteraemia featured by periodic peaks (Ladbury *et al.*, 2008). Hence, sampling done during the periods when there is no or low bacteraemia would yield negative results. The effect of distribution of vectors and reservoirs on the occurrence of *A. phagocytophilum* in domestic animals was discussed recently (Baráková *et al.*, 2014). Absence of positive samples in the above African countries thus does not rule out the occurrence of this pathogen. Further work involving larger samples is needed to clarify this situation.

Infection with *A.* sp. Omatjenne was recognised for the first time in cattle in an *Amblyomma*-free farm in Namibia. At that time it was shown that 81% of the cattle on the farm were seropositive to heartwater but no evidence of clinical cases of the disease was found. The extent of its occurrence and importance was then not explored, mainly due to lack of differential diagnosis (Holland *et al.*, 1987), a situation that has changed since. PCR-RFLP using 16S rDNA was able to differentiate between this pathogen and other *Anaplasma* spp. (Teshale *et al.*, 2015).

Infection with *A.* sp. Omatjenne was detected in samples collected from Ivory Coast, Morocco, Rwanda, Zambia and Ethiopia but not from Tanzania. The prevalence of samples tested positive ranged from 1.23% in Morocco to 16% in Rwanda with an overall average of 6.47%. This shows that infection with *A.* sp. Omatjenne is widespread in the countries from which samples were available. The proportion of samples from Morocco giving a positive signal is similar to those reported by Muhanguzi *et al.* (2010) and Aktas *et al.* (2011). In contrast, the proportion of samples from Ivory Coast, Rwanda, Zambia and Ethiopia testing positive is higher, agreeing with the results of Debeila (2012). Again, this variation may be due to differences in sampling strategy, sampling season and cattle

breeds sampled. The difference in the sensitivity of the assay methods used in this study, compared to Muhanguzi *et al.* (2010) and Debeila (2012), could also be another factor that contributes to the variation.

In this study we provide the first molecular evidence for the occurrence of infections with *E. ruminantium* and five *Anaplasma* spp. (*A. marginale*, *A. phaocytophilum*, *A. ovis*, *A. centrale* and *A.* sp. Omatjenne) in domestic ruminants in Ethiopia. To our knowledge this is the first report of the presence of *A. phagocytophilum*, *A. ovis* and *A.* sp. Omatjenne infection in domestic ruminants in the country. *Anaplasma* spp. were more prevalent than *Ehrlichia* spp. at all sites and in all animal species (7.6). Overall more than half of the animals examined tested positive for *Anaplasma* spp. and the proportion might even be higher during the wet season. Even though samples were collected from apparently healthy animals, infections with these pathogens are not without negative effects. *Anaplasma* spp. have been known to cause reduction in body weight, milk yield and abortions in pregnant animals (Rymaszewska and Grenda, 2008) and immune-suppression in infected animals (Larsen *et al.*, 1994). Moreover, it has been shown that the outcome of infection with *Anaplasma* spp. can be more severe in the presence of co-infection (Renneker *et al.*, 2013). In line with this we detected mixed infections at all sites during this study.

Results of earlier serologic surveys for anaplasmosis in cattle in Ethiopia showed a prevalence between 84% (Feleke *et al.*, 2008) and 99% (Mekonnen, 1996), which is higher than our observation. This may be attributed to the persistence of antibody at detectable levels among infected and immune animals for longer periods of time than rickettsiaemia. Differences in sampling season and management of animals might be other causes of variation in prevalence of infection. The previous studies were carried out during the rainy season while our study was done during the dry season. However, our study reports a much higher prevalence for anaplasmosis than the 2.2% reported by Mekonnen (1996) using microscopy. The overall prevalence of infection with *Anaplasma* spp. observed in this study is higher than the reports of Okuthe and Buyu (2006) in Kenya, Muraguri *et al.* (2005) in Kenya, Swai *et al.* (2009) in Tanzania, Muhanguzi *et al.* (2010) in Uganda and that of Aktas *et al.* (2011) in Turkey probably due to difference in environmental factors and management practices. Our finding is comparable to results reported by Simuunza *et al.* (2011) in Zambia and Oliveira *et al.* (2011) in Costa Rica.

The prevalence of infection with *A. marginale* reported in this study is comparable to the reports of Torina *et al.* (2007) in Italy and that of Bell-Sakyi *et al.* (2004a) in Ghana. But it is higher than the reports of Awad *et al.* (2011) in Sudan and that of Aktas *et al.* (2011) in Turkey. The prevalence of *A. ovis* reported in this study is lower that the prevalence reported previously (Hornok *et al.*, 2007; Torina *et al.*, 2007; Razmi *et al.*, 2006; Torina *et al.*, 2010). In Ethiopia, the agro-climatic conditions are favourable for many tick species and no tick or tick-borne disease control is practiced. Previous field studies showed that more than 60 species of ticks are present in the country (Morel, 1980).

The dominant production system is extensive resulting in free contact between livestock and wildlife. The absence of tick control allows infestation of domestic animals by ticks and free ranging domestic animals that share grazing areas with wildlife would increase the chance of circulation of tick-borne pathogens. Kocan *et al.* (2010) [but also Allsopp (2010), Woldehiwet (2010)] pointed out that the ultimate reservoir of tick-borne rickettsia is wildlife.

Within Ethiopia, higher prevalence of *Anaplasma* spp. were recorded in wetter, more humid localities, favouring higher densities of ticks of the genera *Amblyomma* and *Rhipicephalus* (e.g. Bako), and localities where exotic animals are kept (e.g. Alage). The effect of environmental factors and animal breeds on the occurrence and distribution of ticks and tick-borne diseases has been described (Kocan *et al.*, 2010). Simuunza *et al.* (2011) have also reported the variation in the prevalence of tick-borne diseases in different localities of Zambia. The higher prevalence of *A. ovis* at Bako and Bishoftu is most likely due to sampling of sheep in these areas while no sheep were sampled at other sites.

Anaplasma sp. Omatjenne was detected at three localities within Ethiopia. Although it has been suggested to be apathogenic to cattle by some authors (Allsopp, 2010), several samples collected from ranches and farms where clinical cases of heartwater has been suspected tested positive for *A.* sp. Omatjenne and gave negative results for *E. ruminantium*. *Amblyomma* spp. were widespread in these areas. The proportion reported for *A.* sp. Omatjenne in this study is higher than that of Muhanguzi *et al.* (2010) and Aktas *et al.* (2011). As explained before, such variability in the proportions could arise from differences in season of study, management of study animals and type and frequency of control activities.

Systematic investigations of heartwater are not done in Ethiopia. The occurrence of the disease has been suspected either on the basis of clinical signs or based on brain squash smear examination. We provided the first molecular evidence of infection with *E. ruminantium* in blood samples collected from ruminants. It was detected in 4% of samples from cattle at Bako where *Amblyomma* spp. are abundant and account for higher proportion of the tick collection. It was not detected in other sites even though we previously detected its DNA in *Amblyomma* spp. with a prevalence ranging from 0.9% to 11.67% (Teshale *et al.*, 2015). The proportion of samples that tested positive for *E. ruminantium* in this study is lower than that of Faburay *et al.* (2007a), Muhanguzi *et al.* (2010) and Simuunza *et al.* (2011), where molecular methods were used. It is also lower than the prevalence previously reported using serological methods (Awad, 1997; Bekker *et al.*, 2001; Koney *et al.*, 2004). Although ruminants remain the primary mammalian hosts of *E. ruminantium*, possible canine infection (Allsopp and Allsopp, 2001) and its association with several cases of rapidly fatal encephalitis in humans (Allsopp, 2010) in South Africa raises the question about the importance of this pathogen in causing disease in pets and humans. Hence this study provides preliminary information for the veterinary

and public health authorities for further investigation.

A. phagocytophilum has been shown to cause clinical disease and impair ruminant production and predispose them to other infections (Larsen *et al.*, 1994). It has also been shown to cause human granulocytic anaplasmosis (Woldehiwet, 2010). The occurrence of *A. phagocytophilum* in domestic animals and ticks raises the question of whether human granulocytic anaplasmosis occurs in Africa in general and in Ethiopia in particular. The absence of published clinical cases caused by *A. phagocytophilum* in the continent, however, could be due to lack of differential diagnosis of febrile diseases other than malaria.

Infection with *A.* sp. Omatjenne has been considered apathogenic to domestic ruminants (Allsopp *et al.*, 1997; Allsopp, 2010). But the detection of this pathogen in samples collected from five of six countries and especially from Ethiopia, which were collected from ranches where outbreaks of tick-borne diseases were previously reported on the basis of clinical and postmortem examination warrant further epidemiological studies. Besides, the detection of infection with *A.* sp. Omatjenne in African Buffalo (*Syncerus caffer*) (Debeila, 2012) and in nyala (*Tragelaphus angasii*) (Pfitzer *et al.*, 2011) confirms the occurrence of natural infection in these wildlife species. In general detailed epidemiology of infection with *A.* sp. Omatjenne needs to be investigated.

In conclusion the occurrence of both *A.* sp. Omatjenne and *A. phagocytophilum* in African cattle is confirmed. Although humans may be bitten by ticks or biting flies carrying zoonotic anaplasmas, the public health implication of *A. phagocytophilum* in Africa remains to be elucidated. Similarly the occurrence of *A.* sp. Omatjenne infection in cattle in Africa raises the question of whether or not it is involved in clinical diseases. Livestock improvement plans such as smallholder dairy development schemes through introduction of high yielding breeds should be aware of the importance of these diseases and take the necessary precautions to avoid losses. Further epidemiological investigation of tick-borne diseases is needed to fully understand their impact. Detailed studies on*A.* sp. Omatjenne infections and the public health role of *A. phagocytophilum* and *A. ovis* infection is recommended.

CHAPTER 8

Molecular detection of *Anaplasma* species in unfed ticks (ixodids) in Ethiopia: implications for their vector potential and public health with emphasis on *Anaplasma phagocytophilum* and *Anaplasma ovis*

Abstract Identification of ticks that transmit important pathogens such as *Anaplasma phagocytophilum* and *Anaplasma ovis* is important to understand the epidemiology of the diseases they cause. The aim of this study was to identify tick vectors of *A. phagocytophilum* and *A. ovis* in Ethiopia. Unfed ticks (n=240) were collected and analysed using molecular methods (polymerase chain reaction and restriction fragment length polymorphism). DNA of *A. ovis* was detected in *Rhipicephalus* species, *Amblyomma* species and *Hyalomma* species. Only two specimens of *Rhipicephalus pulchelus* gave positive result for *A. phagocytophilum*. Our finding identified potential vectors of *A. ovis* but extended study is needed to identify the potential vectors of *A. phagocytophilum*.

Adapted from:
Teshale, S., Geysen, D., Ameni, G., Ketema, B., Dorny, P. and Berkvens, D. 2016. Molecular detection of *Anaplasma* species in questing ticks (ixodids) in Ethiopia. Asian Pacific Journal of Tropical Diseases, 6, 449–452.

8.1 Introduction

Anaplasmosis caused by *Anaplasma marginale* is a worldwide disease of domestic ruminants especially cattle (Zhou *et al.*, 2016) and has been reported in all continents causing huge loss to cattle industry. Infection of domestic ruminants with *Anaplasma centrale* and *Anaplasma* sp. Omatjenne has been also known even though these two *Anaplasma* spp. are considered non pathogenic (Zhou *et al.*, 2016; Kasari *et al.*, 2010). *Rhipicephalus* spp. are known to be important vectors of *A. marginale* throughout the world (Cabezas-Cruz and de la Fuente, 2015; Kocan *et al.*, 2010). Other *Anaplasma* spp. infecting domestic ruminants such as *Anaplasma ovis* and *Anaplasma phagocytophilum* are either neglected (Renneker *et al.*, 2013) or less investigated, so that little is known about their epidemiology and vectors under African conditions (Stuen *et al.*, 2013).

According to (Dumler *et al.*, 2001) *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 multi-host bacterium infecting various species of wild and domestic animals and humans (Butler *et al.*, 2008; Woldehiwet, 2010; Gorna *et al.*, 2013; Ebani *et al.*, 2015a). 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 has been observed in pastured animals (Woldehiwet, 2006; Stuen *et al.*, 2013). Deaths have been also recorded in weaker animals if they are not treated (Rymaszewska and Grenda, 2008). 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 has been associated with human infection (Henningsson *et al.*, 2015). Human infections can result in severe clinical consequences with hospitalisation rate as high as 36% in USA and a mortality of over 26.5% in China (Stuen *et al.*, 2013).

Anaplasma ovis has been known to infect domestic and wild ruminants since 1912 (Renneker *et al.*, 2013). It is considered 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 (Renneker *et al.*, 2013). Mortality associated with infection due to *A. ovis* is not frequent (Yasini *et al.*, 2012), despite causing huge financial losses to farming stock due to reduced productivity (Rymaszewska and Grenda, 2008). It has now gaining more importance as a result of observations that suggest its zoonotic importance following detection of variant of *A. ovis* in human patients in Cyprus (Chochlakis *et al.*, 2010).

The vectors of *A. phagocytophilum* (Woldehiwet, 2010; Yoo-eam, 2012; Stuen *et al.*, 2013) and *A. ovis* (Renneker *et al.*, 2013) have been shown to vary among and within different continents and countries. It has also been shown that *A. ovis* can be transmitted by biting flies such as sheep ked (Hornok *et al.*, 2011). *Anaplasma ovis* and *A. phagocytophilum* were recently identified in Ethiopia (Teshale *et al.*, 2015). 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 this *Anaplasma* spp. requires the knowledge of their tick vectors. Identification of DNA of these *Anaplasma* spp. in questing ticks is first step in the identification of potential tick vectors of *A. ovis* and *A. phagocytophilum*. The main objective of this study was to identify questing ticks carrying *A. ovis* and *A. phagocytophilum* DNA in Ethiopia.

8.2 Materials and Methods

8.2.1 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 (Teshale *et al.*, 2015) were purposely selected for collection of unfed ticks from the field for molecular analysis. These are Bishoftu, Bako and Awash depression. Bishoftu is the main town of Ada'a district in East Shewa Zone, central Oromia, Ethiopia. It is located at a distance of 45 km east of Addis Ababa. Bako a district in the West Shewa Zone of Oromia State, Ethiopia which is 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 are closer and have similar conditions, Fantale was taken as representative location for Gari and Marti. The area is located in East Shewa Zone of Oromia State about 190 Km 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 are given in Table 8.1.

Table 8.1: Characteristics of the study sites where unfed ticks were collected

	Sites		
Characteristics	Bishoftu	Bako	Awash Depression
Location	9°N;4°E	9°8'N; 37°5'E	8.975°N - 8°58'30"N 39.93°E - 39°56'0"E
Mean temperature	8.5℃- 30.7℃	13.5℃- 27.9℃	29℃- 38℃
Rainfall (annual)	1156mm	1227mm	560mm
Humidity	61.30%	85%	NA*
Vegetation type	Woody vegetation	Forest type	Acacia woodland
Altitude	1550m	1650m	955-2007m
Climate type	Intermediate	Wet, warm and humid	Arid and semi-arid
Farming type	Mixed	Mixed	Livestock based
Main livestock	Cattle, sheep, goats	Cattle, sheep	Cattle, goats, camels
Production system	Commercial, smallholder	Smallholder	Smallholder

* Data on humidity not available.

8.2.2 Collection and identification of ticks

Sampling of the ticks was carried out in September and October 2013. The ticks were unfed, actively questing and hunting. The ticks were collected by flagging vegetation on pastures and wooded areas bordering farms and homesteads as described by ICTTD-3 (2007). Most of the collections were carried out during morning hours. Some of the ticks were collected just while actively moving near the 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. Identification of ticks was done based on their morphological characteristics using standard identification keys described by Walker *et al.* (2003).

8.2.3 Extraction of DNA from ticks

DNA extraction from the ticks was carried out using the boom extraction method as described previously (Herrmann and Frischauf, 1987) with modifications described previously by Teshale *et al.* (2015).

8.2.4 Amplification of DNA with polymerase chain reaction

A semi-nested PCR was used to amplify a fragment of about 925bp of the 16S rDNA. Amplification was carried out using EHR 16SD (5'-GGTACCYACAGAAGAAGTCC-3') (Hornok *et al.*, 2008) and EBR3 (5'-TTGTAGTCGCCATTGTAGCAC-3') (Teshale et al., 2015) primers for the first round of amplification and EHR 16SD and EBR2 (5'-TGCTGACTTGACATCATCCC-3') ((Teshale *et al.*, 2015) for the second round of reaction. The reaction mix consisted of HotStartTaq Master Mix (2.5 units of DNA polymerase, PCR buffer containing 1.5 mM MgCl2 and 200 µM of each dNTP), 0.2 µM of each primer and PCR water. The PCR reaction was carried out in a total volume of 25 µL using a programmable thermocycler (T3 thermocycler Biometra®, Westburg, NL). The PCR procedures were described by Teshale *et al.* (2015).

All the PCR products were visualised by gel electrophoresis in TAE buffer (0.04M Tris, 0.4mM EDTA, pH = 7.7 - 8.8) using 2% agarose at 100V for 20 minutes and staining 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 negative control while DNA from an in vitro culture of *E. ruminantium*, *A. marginale* and *A. phagocytophilum* was used as positive control.

8.2.5 Restriction enzyme fragment length polymorphism analysis of the amplified products

The amplified products from positive samples were digested by using restriction enzymes MboII, HhaI and MspI to identify the species of Anaplasma detected as described by Teshale *et al.* (2015). 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, Biolabs, New England; 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 TAE buffer (0.04M Tris, 0.4mM EDTA, pH = 7.7 - 8.8) at 100V for 40 minutes and visualised under UV illumination after staining with ethidium bromide (final concentration of 0.5 µg/ml).

8.3 Results

A total of 240 questing ixodid ticks (*Rhipicephalus evertsi evertsi* n=61, *Rhipicephalus pulchelus* 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* n=53) were collected from the field and analysed for *Anaplasma* spp. DNA targeting 16S RNA gene. All ticks were tested by PCR and positive ones were subsequently analysed by RFLP to identify the species of *Anaplasma* detected. DNA belonging to five *Anaplasma* spp. (*A. marginale*, *A. centrale*, *A. ovis*, *A.* sp. Omatjenne and *A. phagocytophilum*) was detected (Table 8.2).

Anaplasma marginale was identified in 2.3% (95% CI: 0.1%, 12%) of *H. marginatum* collected from Bishoftu, 12.5% (95% CI: 0 .3%, 52.6%) of *R. pulchelus* from Fantale and in 13% (95% CI: 4.9%, 26.3%) of *R. pulchelus* from Marti. *A. centrale* was detected in 4.5% (95% CI: 0 .1%, 22.8%) of *R. evertsi* specimen collected from Bishoftu, 12.5% (95% CI: 0 .3%, 52.6%) and 8.7% (95% CI: 2.4%, 20.8%) of *R. pulchelus* collected from Fantale and Marti, respectively and 50% (95% CI: 1.3%, 98.7%) of *H. marginatum* collected from Gari. Only two (22.2%; 95% CI: 2.8%, 60%) of *A. lepidum* collected from Bako gave positive signal for *A.* sp. Omatjenne.

The DNA of *A. phagocytophilum* was detected only 4.3% (95% CI: 0.53%, 14.8%) of *R. pulchelus* specimens collected and tested at Marti while no other ticks were found to contain DNA belonging to this pathogen. The DNA of *A. ovis* was detected in 20% (95% CI: 7.7%, 38.6%) of *R. evertsi*, 11.1% (95% CI: 0.3%, 48.2%) of *A. lepidum* and 66.7% (95% CI: 22.3%, 95.7%) of *Amblyomma* nymphs collected from Bako. All together *A. ovis* was detected in 24.4% (95% CI: 12.9%, 39.5%) of the ticks collected from Bako area. Among ticks collected and tested from Marti *A. ovis* was detected in 25% (95%

Table 8.2: Results of molecular analysis of unfed ticks for *Anaplasma* species from selected sites in Ethiopia

Site	Tick Species	N tested	Stage	N positive
Bishoftu	*R. evertsi evertsi*	22	adult	1 *A. centrale*
	A. variegatum	17	adult	-
	A. lepidum	20	adult	-
	H. marginatum	44	adult	1 *A. marginale*
Bako	*R. evertsi evertsi*	30	adult	6 *A. ovis*
	A. lepidum	9	adult	1 A. ovis, 2 *A.* sp. Omatjenne
	Amblyomma	6	nymph	4 *A. ovis*
Awash depression				
Fantale	*R. pulchelus*	8	adult	1 *A. marginale*, 1 *A. centrale*
	H. margiunatum	7	adult	-
	R. evertsi evertsi	4	adult	-
	A. variegatum	1	adult	-
Marti	*R. pulchelus*	46	adult	2 *A. phagocytophilum*, 4 *A. centrale*, 6 *A. marginale*
	A. lepidum	6	adult	1 *A. ovis*
	R. evertsi evertsi	4	adult	1 *A. ovis*
	R. decoloratus	1	larva	-
	A. variegatum	4	adult	-
	A. gemma	7	adult	-
Gari	*A. lepidum*	1	adult	-
	R. evertsi evertsi	1	adult	-
	H. margiunatum	2	adult	1 *A. ovis*, *A. centrale*

CI: 0.6%, 80.6%) of *R. evertsi evertsi* and 16.7% (95% CI: 0.4%, 64.1%) of *A. lepidum* specimens. It was identified in 50% (95% CI: 1.3%, 98.7%) of *H. marginatum* collected from Gari. No ticks collected from Bishoftu contained DNA of *A. ovis*. *Ehrlichia* spp. were not detected in any of the ticks tested.

8.4 Discussion

Anaplasmosis has been well recognised in many countries of the world resulting in enormous economic losses in ruminant industry despite infrequent mortalities (Rymaszewska and Grenda, 2008; Kocan *et al.*, 2010; Woldehiwet, 2010). Variants of *Anaplasma* species have been also associated with human illness (Chochlakis *et al.*, 2010; Stuen *et al.*, 2013). The occurrence of zoonotic *Anaplasma* spp. such as *A. ovis* and *A. phagocytophilum* was recently reported in domestic ruminants in Ethiopia (Teshale *et al.*, 2015). However, no study has so been carried out on unfed ticks in Ethiopia. To the best of our knowledge this is the first report of the occurrence of *Anaplasma* species in unfed ticks collected from the field in the country. Knowledge of the local tick vectors is required to get better insight into the ecology of these pathogens. In this study we screened unfed ixodid ticks for the DNA of *Anaplasma* species emphasising on *A. ovis* and *A. phagocytophilum* and identified ticks with potential for transmission of *A. ovis* under Ethiopian conditions.

In this study unfed *Rhipicephalus* spp., *Hyalomma* spp. and *Amblyomma* spp. were found to carry *A. ovis* DNA. This suggests that these ticks could be involved in the transmission of this *A. ovis*. The occurrence of *A. ovis* infections in resident small ruminant population was confirmed recently (Teshale *et al.*, 2015). Now the presence of *A. ovis* in unfed tick population in the country is proven. The vector role of ticks that belong to the genus *Hyalomma* has already been documented elsewhere (Jafarbekloo *et al.*, 2014), in which unfed *Hyalomma* spp. were found to be infected with *A. ovis*. *Rhipicephalus* spp. (*R. evertsi evertsi* and *R. pulchelus*) have been also shown to be competent vectors of *Anaplasma* spp. Kocan *et al.* (2010) reviewed that *R. evertsi* had been shown to be competent vectors of *A. marginale*. Therefore, the detection of DNA of *A. ovis* in unfed *Hyalomma* spp. and *Rhipicephalus* spp. in this study is in consent with the previous reports made elsewhere in the world. In this study *A. ovis* was also detected in two *A. lepidum* and four *Amblyomma* nymphs. *Amblyomma* spp. are mostly recognised for their role in the transmission of *Ehrlichia* spp. and *T. mutans* (Estrada-Peña and Salman, 2013). Rymaszewska and Grenda (2008) have previously documented that *Amblyomma* spp. are vectors of *A. bovis* in Asia. The detection of *A. ovis* DNA in adults and nymphs that did not attach to animals suggests the possible involvement of *Amblyomma* spp. in the transmission of this bacterium. The actual role of the tick spp. found positive with *A. ovis* DNA in this study in the transmission of the agent to resident ruminants needs to be further confirmed by experimental studies. However, our result showed that *A. ovis* can be transmitted by several genera and species of ticks including *Amblyomma* spp. This is supported by earlier findings reported elsewhere, showing that *A. ovis* is transmitted by many tick species and several biting flies (Hornok *et al.*, 2011; Stiller *et al.*, 1999).

Two DNA specimens from *R. pulchelus* gave positive results for *A. phagocytophilum*. This tick species was collected only from the Awash depression and surrounding, which has a typical semi-arid climate. Previous studies also showed that this tick species is found to the east of Rift Valley, which has a drier weather. The occurrence of infection with *A. phagocytophilum* was confirmed in ruminants in wetter and humid areas such as Bako where *R. pulchelus* is not found (Teshale *et al.*, 2015). This suggests the involvement of other tick spp. in the transmission of this rickettsia. An extended study covering larger area, more tick species and quantity is needed to identify the potential vectors of *A. phagocytophilum* in the country.

In conclusion, the findings of this study underline the potential for *Rhipicephalus*, *Hyalomma* and *Amblyomma* to transmit *A. ovis* among livestock in Ethiopia. This is useful finding for the small ruminant sector and could be considered during the genetic improvement and translocation programs. The risk of human infection could also be highlighted as ecotourism and outdoor activities are growing in the country. Therefore, our findings could be used as preliminary information by the public health authorities. Further investigations are warranted in order to identify the vectors associated in the transmission of these

Anaplasma spp. and elucidate the pathogenic role of *A. ovis* and *A. phagocytophilum* on human and animal health burden in the country.

CHAPTER 9

General Discussion

9.1 Tick-borne diseases in Ethiopia and implications for livestock and public health

As explained in subsection 2.9.1, the Government of Ethiopia has made the development of the dairy sector, particularly the smallholder dairy operations, a priority, including an increase of the number of improved crossbred animals, setting a goal of 4,044,000 dairy cows by 2019/20 (LMP-team, 2014).

Simultaneously the Ethiopian Government has been advised to adopt a "low emissions development" for its commercial dairy industry. This entails (*i*) the introduction of genetically improved animals (together with changes in animal feeding techniques) to reduce the quantity of greenhouse gases emitted per litre of milk produced and (*ii*) the reduction of so-called "unproductive emissions" related to animal mortality and morbidity (FAO & New Zealand Agricultural Greenhouse Gas Research Centre, 2017). This report furthermore specifically lists tick-borne diseases in this respect.

Development and application of a suitable, optimum strategy for the control of animal diseases in any country start with an understanding of the epidemiology of the diseases in question, of necessity based on knowing which diseases are present, which in turn implies being able to catalogue the potential pathogens present within the country. This knowledge, together with information on effects and consequences of infection by the respective pathogens and costs incurred in the control of them, is essential to rank and prioritise any interventions envisaged.

In Ethiopia, the occurrence of infection with tick-borne pathogens used to be estimated based on the distribution of their tick vectors (Mekonnen, 1996) or microscopic and

serologic examination of blood samples from vertebrate hosts (Mekonnen *et al.*, 2001; Feleke *et al.*, 2008), both of which have limitations in the detection and identification of specific pathogens (Simuunza, 2009). The present study was based on a molecular analysis and provides reliable and accurate differential diagnosis of potential pathogen species. The results of this study made additions to the list of tick-borne pathogens identified so far in the country. The occurrence of *Theileria ovis* and *Theileria buffeli/orientalis* is reported in addition to the species of *Theileria* already reported (Solomon *et al.*, 1998; Tomassone *et al.*, 2012; Gebrekidan *et al.*, 2014). Although only mildly pathogenic *Theileria* spp. were detected, their occurrence could cause cross-reactions in a serological survey of pathogenic species. Their pathogenic role can furthermore not be ruled out in light of the increasing number of exotic breeds of ruminants in the country. Therefore, the veterinary authorities should take this into account when performing surveys and setting up systems of livestock health monitoring.

A general remark should be added here to address possible criticisms on the relevance and/or extrapolation of the results obtained during the different studies, reported in this thesis. It is understood that sampling was not random, but rather convenient and that because of various constraints, the sampling was not always carried out at the right time of the year (correct season). It must thus be remembered that the results obtained are 'minimum' results in that they only reflect presence of the pathogen in the samples obtained. Any measure of prevalence/incidence is thus only an indication, and the most relevant conclusion is when the presence of a pathogen was confirmed. Lastly, some of the results reported here should be confirmed through repeated studies.

The identification of pathogenic species of *Anaplasma* and *Ehrlichia* has significant implications. They are among important pathogens that need attention from veterinary, livestock and wildlife authorities for their negative impact on ruminant development. They are likely to be important constraints to genetic improvement in ruminants. For instance, active clinical search during this study revealed the occurrence of heartwater in 13 clinically affected exotic and crossbred cattle, of which 5 (38.46%) were confirmed to be positive for *Ehrlichia ruminantium*. Similarly, *E. ruminantium* was responsible for 30 (41.67%) clinical cases out of 72 Boer goats with a case-fatality of 50%. No clinical cases of heartwater were encountered in indigenous ruminants during the survey. Previous studies also revealed mortality of 46.25% due to *E. ruminantium* in exotic dairy cattle (Melaku *et al.*, 2014). Therefore, *E. ruminantium* remains a serious constraint for ruminant genetic improvement programs. The Ministry of Agriculture should take this into account during the implementation of its livestock development masterplan. The detection of various *Anaplasma* spp. has similar consequences for the ruminant industry. Even though indigenous ruminants are resistant to anaplasmosis, infections with these pathogens are not without negative effects. *Anaplasma* spp. have been known to cause reduced body-weight increase, reduced milk yield, higher abortion rates in pregnant females (Rymaszewska and

Grenda, 2008) and immune-suppression in infected animals (Larsen *et al.*, 1994). The economic losses resulting from these tick-borne infections could also extend to wildlife. Wildlife conservation and game ranching are recent innovations in Ethiopia, where they serve as a tourism destination. Elsewhere it was shown that high levels of mortality due to tick-borne diseases have endangered a number of wildlife species, mainly Sable and Roan Antelope (Uys *et al.*, 2015). The consequences of tick-borne pathogens infections could even be higher when there are co-infections (Renneker *et al.*, 2013).

This study showed widespread occurrence of anaplasmosis in ticks and domestic ruminants in Ethiopia. Cattle and sheep were more frequently infected with *Anaplasma* spp. than goats. This also has important economic implication for Ethiopia. The majority of the livestock of the country is accounted for by cattle and sheep. As described in Chapter 2 these two ruminant species make the largest contribution to the national GDP. Hence, the loss incurred due to anaplasmosis in these ruminant species could be considerable. The negative effect of anaplasmosis on the ruminant industry is further justified by the high prevalence of infection in animals kept in commercial farms and ranches, compared to animals raised in smallholder farms. Commercial farms and ranches keep mostly exotic and crossbred animals, which are generally more susceptible than indigenous breeds to ticks and tick-borne diseases. The ruminant improvement program by the Ministry of Agriculture might be hindered by tick-borne diseases such as anaplasmosis. Therefore, integrated control needs to be considered with ultimate aim of establishing endemic stability (Jongejan and Uilenberg, 2004; Ghosh *et al.*, 2007). Because of growing interest for the pathogenicity of *Anaplasma marginale*, *Anaplasma phagocytophilum*, and *Anaplasma ovis* in farm animals and also in humans, this study provides ample base-line information for the veterinary and public-health sectors in Ethiopia. Several factors may be responsible for the widespread occurrence of anaplasmosis in Ethiopia. The absence of tick and tick-borne disease control is an important factor favouring widespread occurrence of *Anaplasma* infection. Similar observation on the occurrence of tick-borne disease in the absence of control practice has been described elsewhere (Kocan *et al.*, 2010; Simuunza *et al.*, 2011).

The present study did not record the presence of *Babesia bovis* or the presence of its vector *Rhipicephalus microplus*. It is however recommended to continue a vigilance for this pathogen/vector pair as cattle brought into Ethiopia together with their Sudanese refugee owners were found to harbour the pathogen (Mekonnen, 1996).

Bartonella spp. have been known to be associated with infections of dogs and cats (Chomel *et al.*, 2004; McGill, 2012). That means the detection of *Bartonella* spp. in ticks in this study reveals the importance of these pathogens in the differential diagnosis of clinical disorders in pets in Ethiopia. Interestingly detection of *Bartonella* spp. has public health significance. Since *Rhipicephalus decoloratus* is a one-host tick, detection of these pathogens in these ticks suggests the occurrence of infection in the host species. This raises questions on the role of ruminants in the epidemiology of diseases caused by *Bartonella*

spp. One previous study by Pappalardo *et al.* (1997) revealed that exposure of dogs to cattle appeared to be associated with seropositivity to *Bartonella* spp. in dogs. The knowledge about the vectors and reservoirs of *Bartonella* spp. is incomplete (Chang *et al.*, 2001; Rar *et al.*, 2005; Angelakis and Raoult, 2010). The demonstration of *Bartonella* spp. in *R. decoloratus* collected from cattle and sheep in this study suggests that ruminants may be reservoirs of these zoonotic bacteria. Our observation highlights the possible emergence of these zoonoses in hitherto unknown animal reservoirs in Ethiopia. This fact needs to be considered by health authorities since there is risk of infection for rural residents.

Detection of *Rickettsia africae* and *Rickettsia felis* (the emerging spotted fever group rickettsias) is of public health concern. Ticks and domestic animals, e.g. cattle, have been incriminated as reservoirs of these zoonoses (Parola *et al.*, 2003, 2013; Kelly *et al.*, 1991a). Since the ticks used in this study were collected from cattle and sheep, our results indicate that *R. africae* occurs in cattle and sheep in Ethiopia. *Amblyomma variegatum*, the known vector of this *Rickettsia* species (Sixl *et al.*, 1987), is one of the predominant tick species in Ethiopia (Kumsa *et al.*, 2014c) and is known to feed on humans (Mediannikov *et al.*, 2010). Hence, the observation of *R. africae* in the area signals a possible risk to humans residing in the area. Recently *R. felis* was identified in fleas collected from dogs and cats in Ethiopia (Kumsa *et al.*, 2014b). This *Rickettsia* is now recognised as a human pathogen in sub-Saharan Africa. It was confirmed in several cases of febrile disease in Kenya and Senegal (Parola, 2011). However, in Ethiopia, differential diagnosis of febrile diseases in humans is often lacking, with the exception of malaria: most of the febrile diseases other than malaria are classified as 'fever of unknown origin'. The similarity in clinical manifestations of spotted fever group rickettsioses and malaria in humans suggest the possibility of misdiagnosis of these zoonoses in Ethiopia. The role of ticks in the transmission of *R. felis* and the question whether domestic animals serve as reservoirs of *Rickettsia* spp. need to be investigated.

Our finding of *Borrelia burgdorferi* s.l. in *R. decoloratus* collected from cattle and sheep highlights the possible occurrence of this *Borrelia* group in Ethiopia. This group of *Borrelia* is the causative agent of Lyme borreliosis, transmitted by ixodid ticks and most common tick-borne disease in USA and Europe (Rudenko *et al.*, 2011). Further investigation is needed, however, to confirm the identity of this *Borrelia* isolate and its distribution both in ticks and domestic ruminants.

Our observation highlights the possible emergence of these zoonoses in hitherto unknown animal reservoirs in Ethiopia. This fact needs to be considered by health authorities since there is risk of infection for rural residents. The medical practionners should consider these TBDs in the differential diagnosis of febrile and debilitating diseases in individuals exposed ticks. As the public health importance of tick-borne zoonoses is recognized worldwide, inclusion of the major tick-borne zoonoses in the curricula is needed.

9.2 Diagnostic challenges of tick-borne diseases

Diagnostic methods that are sensitive, specific and cost-effective are an important component in the investigation and control of TBDs, as they are essential for both epidemiological investigations and clinical diagnostic purposes.

The routine diagnosis of infections of *Anaplasma* spp. using microscopic examinations of stained blood films is suitable only for acute cases. Serologic assays can be used for the detection of chronic carriers but suffer from lack of specificity. Both methods do not provide an accurate differential diagnosis of infection with various *Anaplasma* spp. (Rymaszewska and Grenda, 2008; Noaman, 2013). Previously developed molecular techniques accurately differentiate among the species of Anaplasma but are either expensive or require multiple analytic steps (Simuunza, 2009). The improved molecular method described in chapter 6 of this document provides accurate diagnosis of infection with *Anaplasma* spp. and avoids the need for running the test several times. It was shown to be suitable for detection of multiple infections by means of a single test. The method was tested both in ticks and ruminants and can be used for epidemiological investigations in developing countries where both laboratory facilities and expertise are limited. The method has the potential to be used for the identification of carrier animals and reservoir hosts, information that is vital for a better understanding of the epidemiology of anaplasmosis. This improved method is suitable for detection of *E. ruminantium* in ticks and in cattle and goats with clinical symptoms. However, it did not detect the sequence of *E. ruminantium* in carrier animals suggesting the need for further improvement in this regard. Extending new technological advances into the field is a crucial step in dissemination of scientific knowledge. This improved molecular method demonstrated a real progress on how new scientific advancements bring an impact on lives of resource-poor people in Africa. By helping animal health professionals in their decision making regarding the diagnosis and control of tick-borne anaplasmosis and heartwater, the molecular method described will help the government in establishing a disease monitoring program in dairy and meat industries. It can be used alongside the routine diagnostic methods such as microscopic examinations of stained blood films for quick confirmation of clinical cases and serological tests for infection without differentiation of species of pathogen. Such examination in live animals infected with *E. ruminantium* does not exist. For confirmation of clinical cases of heartwater, molecular tests are the only options.

The 16s rDNA PCR - RFLP described in chapter 6 was used for the epidemiological study of infection with *Anaplasma* spp. in Ethiopia. The reason to use this assay method rather than others is mainly due to the suitability of the method for field investigations. We used two new primers in order to amplify a longer fragment of 16S rDNA gene from all members of the genera Anaplasma, which enabled us to identify their respective species by restriction enzyme fragment polymorphism. This avoids the need to use a technically

demanding and expensive hybridisation technique (Bekker *et al.*, 2002), an important consideration when planning field epidemiological studies in resource-poor countries. The method also saved time to complete the species identification of the pathogen detected and was able to detect mixed infections. The results of the PCR - RFLP analysis of blood samples collected from domestic ruminants showed that infection with *Anaplasma* spp. is prevalent in all sites surveyed in central Ethiopia. In general, the prevalence of infection with *Anaplasma* spp. was high.

In Ethiopia there two national laboratories (National Animal Health Diagnosis Centre, Sebeta, and National Veterinary Institute, Bishoftu), which are equipped with molecular facilities. Furthermore, some of the University laboratories have molecular units. These laboratories can use the diagnostic method, developed here, when monitoring *Anaplasma* and *Ehrlichia* infections.

9.3 Challenges for the control of tick-borne diseases

If and when required, control of tick-borne diseases, should proceed by means of combinations of control strategies, depending on the husbandry (in particular animal breeds, exotic or indigenous) and ecological conditions (suitability for ticks). Points deserving special attention in this respect are of course cost, but also maintenance of endemic stability, if present. Herdsmen and veterinarians should aim to manage tick populations and tick-borne diseases within economically acceptable limits ensuring that animals are immune.

Any control program should therefore aim to provide sufficient tick control to increase productivity, whilst not reducing tick numbers to the extent that endemic stability, if present and important, is adversely affected. A combination of strategic and threshold acaricide application should be the recommended practice in this case, whereas continuous application may be necessary in the case of exotic, fully susceptible animals.

Although no reports are available thus far on acaricide resistance in ticks, this aspect also remains important as it has been a major concern elsewhere (Abbas *et al.*, 2014).

To this end programmes to train farmers are required in order to acquaint them with the need for adequate tick control and to highlight the consequences of inadequate tick control policies. This has to be accompanied by a close monitoring for the occurrence of TBDs and possible administration of chemotherapy as required. Although highly controversial, the use of antibiotics for effective treatment of acute cases of TBDs may be helpful.

Immunisation undoubtedly has a role to play, but a lot of work remains to be done in Ethiopia, since cross-protection of currently available vaccine strains with regards to local strains still has to be elucidated. These studies must therefore be envisaged as soon as possible. The integration of immunisation within a general tick and TBD control programme also remains to be clarified.

9.4 Conclusion

In conclusion, this doctoral study identified tick-borne pathogens of veterinary and public health importance. An improved molecular method was developed for simultaneous detection of Anaplasma spp. and Ehrlichia spp. and was used for epidemiological studies in domestic ruminants and ticks. Accordingly, high prevalence of anaplasmosis and low prevalence of heartwater were observed. The occurrence of infection of cattle with *Anaplasma* sp. Omatjenne was identified in selected African countries. On top of this, potential vectors of *A. ovis* were identified. Therefore, ruminant improvement programs such as those devised by Ministry of Agriculture should take the occurrence of tick-borne pathogens and diseases into account. The presence of zoonotic *Rickettsia* spp., *Bartonella* spp. and *Anaplasma* spp. highlights the risk of human infections. Further studies on the medical significance of the zoonotic pathogens identified, search for vectors of *A. phagocytophilum* and experimental studies for the confirmation of the vectors of *A. ovis* are warranted. Moreover, improvement of molecular methods is needed for the detection of carriers of *E. ruminantium*.

9.5 Future research areas

1. Further investigation into epidemiology of zoonotic *Rickettsia* spp. and *Bartonella* spp.
2. Studies to elucidate the association of *Rickettsia* spp. and *Bartonella* spp. to human illness
3. Investigation into the dynamics of infection with *Anaplasma* spp. and *Ehrlichia* spp. in ruminants
4. Continued search for vectors of *A. phagocytophilum*
5. Improvement of molecular diagnostic methods for better detection of carriers of *E. ruminantium*

CHAPTER 10

Back Matter

10.1 Bibliography

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10.2 Summary

Although tick-borne pathogens and tick-borne diseases are among the most important constraints in the domestic ruminant industry causing huge economic losses through impaired genetic improvement and translocation programs, wildlife development, eco-tourism and public health, little information is available on their occurrence and distribution in Ethiopia. Within this doctoral study, three cross-sectional epidemiological studies using molecular techniques were conducted, one on ticks collected from cattle and sheep, one on domestic ruminants and one on unfed ticks collected from the field. The survey conducted on ticks collected from cattle and sheep using a molecular analysis of 18S rDNA identified the occurrence of *Theileria buffeli/orientalis*, *Theileria velifera*, and *Theileria ovis* in *Rhipicephalus evertsi evertsi* and *Rhipicephalus decoloratus*. According to the 16s rDNA PCR and sequencing, six species of *Bartonella*, three species of *Rickettsia* (*Rickettsia africae*, *Rickettsia felis* and *Rickettsia* sp.), *Anaplasma ovis*, *Ehrlichia ruminantium*, *Ehrlichia* spp., *Anaplasma* spp. and *Borrelia burgdorferi* s.l. were identified in *Rhipicephalus* spp. This is the first report of the occurrence of *A. ovis*, *Bartonella elizabethae*, *Bartonella bovis*, *Bartonella koehlerae*, *Bartonella quintana* and *Bartonella vinsonii berkhoffii* in Ethiopia. Our finding highlights the risk of infection of animals and humans with zoonotic tick-borne bacteria in Ethiopia.

At the end of the first survey, we focused on the molecular identification of *Ehrlichia* spp. and *Anaplasma* spp., in which we developed a semi-nested PCR amplifying 925bp of 16S rDNA. A pair of primers designated EBR2 and EBR3 was designed from the *Anaplasma* 16S rDNA sequences for simultaneous detection of *Ehrlichia* spp. and *Anaplasma* spp. Individual species of *Ehrlichia* and *Anaplasma* were identified by restriction with MboII, HhaI and MspI enzymes. The method was suitable for the identification of *Ehrlichia* spp. and *Anaplasma* spp. including detection of mixed infections. Analysis of *Amblyomma* spp. from various parts of Ethiopia for bacterial pathogens using this molecular method revealed the occurrence of *Anaplasma marginale*, *Anaplasma phagocytophilum*, *Anaplasma centrale*, *Anaplasma* (formerly *Ehrlichia*) sp. Omatjenne and *E. ruminantium*. By helping animal health professionals in their decision-making regarding the diagnosis and control of anaplasmosis and heartwater, this improved molecular method will help the government in establishing a disease-monitoring program in the dairy and beef industries.

The second part involves survey of *A. phagocytophilum* and *A.* sp. Omatjenne infection in cattle in Africa and that of *Anaplasma* spp. and *Ehrlichia* spp. in cattle, sheep and goats in Ethiopia. In the international survey, respectively 19 (2.7%) and 45 (6.5%) samples yielded positive signals for *A. phagocytophilum* and *A.* sp. Omatjenne. *Anaplasma* sp. Omatjenne was detected in all countries except Tanzania while *A. phagocytophilum* was detected only in samples originating from Ethiopia. Out of the 922 blood samples from cattle, sheep and goats from five different localities in Ethiopia analyzed by 16S

rDNA PCR, 523 (56.7%) tested positive. Overall, 67.4% of cattle, 65% of sheep and 18% of goats tested positive for one or more *Anaplasma* spp. No positive result was observed for *Ehrlichia* spp. using the 16S rDNA analysis. RFLP analysis identified *A. marginale*, *A. ovis*, *A. phagocytophilum*, *A. centrale* and *A.* sp. Omatjenne. We conclude that infection of domestic ruminants with *Anaplasma* spp. is widespread in Ethiopia. Livestock improvement plans through introduction of improved breeds should be aware of this and take the necessary precautions to minimize losses associated with anaplasmosis. First reports of infection of domestic ruminants with *A. phagocytophilum*, *A. ovis* and *A.* sp. Omatjenne are provided for Ethiopia. In addition, the occurrence of *A. phagocytophilum* together with *A.* sp. Omatjenne outside Europe and South Africa is made for the first time. Random samples of 493 animals were tested with a pCS20 PCR for the identification of *E. ruminantium*. Three samples from 75 (4%) cattle gave a positive result. *Ehrlichia ruminantium* was identified in five of thirteen clinical cases of heartwater in dairy cows (38.46%) and 30 of 72 (41.67%) clinically affected Boer goats.
Identification of ticks that carry pathogens of veterinary and public-health importance, belonging to the genus *Anaplasma*, is an initial step to identify tick vectors that play a role in the epidemiology of the diseases they cause. A molecular investigation was carried out to identify *Anaplasma* spp. carried by unfed field ticks with an emphasis on *A. phagocytophilum* and *A. ovis*. A total of 240 unfed ticks (adults and nymphs) were collected and analyzed using PCR-RFLP. *Anaplasma ovis* was identified in *R. evertsi*, *Amblyomma* spp. and *Hyalomma* spp. *Anaplasma phagocytophilum* was detected only in *Rhipicephalus pulchellus*. Our finding identified potential vectors of *A. ovis* to be further confirmed by experimental study.
In conclusion, this doctoral study identified tick-borne pathogens of veterinary and public-health importance in Ethiopia. An improved molecular method was developed for simultaneous detection of *Anaplasma* spp. and *Ehrlichia* spp. and it was used in epidemiological studies in domestic ruminants and ticks. A high prevalence of anaplasmosis and a low prevalence of heartwater were observed. The occurrence of infection in cattle with *A.* sp. Omatjenne was identified in certain African countries. Furthermore, candidate vectors of *A. ovis* were identified. Further studies on the medical significance of the identified zoonotic pathogens, search for vectors of *A. phagocytophilum* and experimental studies for the confirmation of the vectors of *A. ovis* are warranted. Lastly, an improvement of molecular methods is needed for the detection of carriers of *E. ruminantium*.

10.3 Samenvatting

Zeer weinig informatie is beschikbaar betreffende het voorkomen en de verspreiding van door teken overgedragen pathogenen en ziekten in Ethiopië, ondanks het feit dat ze tot de belangrijkste belemmeringen behoren voor de herkauwer industrie, waar ze verantwoordelijk zijn voor zware verliezen omwille van de onmogelijkheid tot genetische verbetering, translocatie programma's, wildvee ontwikkeling en ecotoerisme en omwille van hun gevolgen voor de volksgezondheid. Binnen het kader van deze thesis werden drie moleculaire studies georganiseerd, een eerste bij teken gevonden op rundvee en schapen, een tweede bij de herkauwers zelf en een derde bij questende, niet-gevoede teken in het wild. De eerste studie maakte gebruik van 18S rDNA en identificeerde het voorkomen van *Theileria buffeli/orientalis*, van *Theileria velifera* en van *Theileria ovis* in *Rhipicephalus evertsi evertsi* en *Rhipicephalus decoloratus*. Een 16S rDNA PCR gevolgd door sequeneren bracht het voorkomen van zes *Bartonella* soorten, drie *Rickettsia* soorten (*Rickettsia africae*, *Rickettsia felis* en *Rickettsia* sp.), *Anaplasma ovis*, *Ehrlichia ruminantium*, *Ehrlichia* spp., *Anaplasma* spp. en *Borrelia burgdorferi* s.l. aan het licht in *Rhipicephalus* spp. Dit werk is het eerste verslag van het voorkomen van *A. ovis*, *Bartonella elizabethae*, *Bartonella bovis*, *Bartonella koehlerae*, *Bartonella quintana* en *Bartonella vinsonii berkhoffii* in Ethiopië. Onze bevindingen onderlijnen het risico op infectie van zowel dier als mens door potentieel zoönotische, door teken overgedragen bacteria in Ethiopië.

Op het einde van de eerste studie werd de focus verlegd op de moleculaire identificatie van *Ehrlichia* spp. en *Anaplasma* spp., waarbij een "semi-nested PCR" 925bp van het 16S rDNA amplificeerde. Een stel primers, met name EBR2 en EBR3, werden geconstrueerd vanuit 16S rDNA sequenties van *Anaplasma* soorten voor de gezamenlijke detectie van *Ehrelichia* en *Anaplasma* soorten. Individuele *Ehrlichia* en *Anaplasma* soorten werden geïdentificeerd door restrictie met MboII, HhaI en MspI enzymes. Deze methode was geschikt voor de identificatie van individuele *Ehrlichia* en *Anaplasma* soorten alsmede voor de detectie van menginfecties. Toepassing van deze methode op verschillende *Amblyomma* soorten afkomstig uit verschillende streken van Ethiopië toonde het voorkomen aan van: *Anaplasma marginale*, *Anaplasma phagocytophilum*, *Anaplasma centrale*, *Anaplasma* (voorheen *Ehrlichia*) sp. Omatjenne en *E. ruminantium*. Deze verbeterde moleculaire methode is een bijdrage tot het stellen van een ondubbelzinnige diagnose, wat de diergeneeskundige specialisten zal helpen bij de diagnose en controle van anaplasma en heartwater en aldus de regering zal toelaten ziekte-monitoring programma's op te stellen voor de melkvee en mestvee sectoren.

Het tweede deel van dit werk bevat een studie over het voorkomen van *A. phagocytophilum* en *A.* sp. Omatjenne infectie bij runderen in Afrika en over de verspreiding van *Anaplasma* en *Ehrlichia* soorten in runderen, schapen en geiten in Ethiopië. Bij de internationale studie werden bij respectievelijk 19 (2.7%) en 45 (6.5%) stalen positieve signalen voor

A. phagocytophilum en *A.* sp. Omatjenne. *Anaplasma* sp. Omatjenne werd gevonden in alle landen behalve Tanzanië en *A. phagocytophilum* kwam alleen voor in stalen genomen in Ethiopië. Van de 922 bloedstalen van runderen, schapen en geiten afkomstig van vijf verschillende plaatsen in Ethiopië, onderworpen aan de 16S rDNA PCR, gaven 523 (56.7%) een positief resultaat. Globaal gesproken werden respectievelijk bij 67.4%, 65% en 18% van de runderen, schapen en geiten postieve resultaten gevonden voor een of meerdere *Anaplasma* soorten. Voor wat betreft *Ehrlichia* werd met de 16S rDNA analyse geen positief resultaat opgetekend. RFLP analyse identificeerde *A. marginale*, *A. ovis*, *A. phagocytophilum*, *A. centrale* en *A.* sp. Omatjenne, waarbij dit voor de tweede, derde en laatste soort om de eerste aanwijzing in Ethiopië betreft en voor de derde en de laatste soort om de eerste documentatie buiten Europa en Zuid Afrika gaat. De conclusie is dat infectie door *Anaplasma* wijdverspreid is bij herkauwers in Ethiopië. Programma's, die verbetering van het vee nastreven via introductie van exotische rassen, dienen hiermee rekening te houden en de vereiste voorzorgen te voorzien teneinde verliezen ten gevolge van anaplasmose tot een minimum te herleiden. *Ehrlichia ruminantium* werd opgespoord bij 493 dieren door middel van een pCS20 PCR. Drie runderstalen op 75 (4%) gaven een positief resultaat. De bacterie werd gevonden in vijf op dertien klinische heartwater gevallen bij melkkoeien (38.5%) en bij 30 op 75 klinisch aangetaste Boer geiten (41.7%).
De identificatie van teken die *Anaplasma* soorten herbergen, die van belang zijn voor de diergeneeskunde en de volksgezondheid, is een eerste stap om de epidemiologie van de ziekten uit te klaren. Daarom werd een moleculaire studie uitgevoerd om *Anaplasma* op te sporen bij niet-gevoede teken met een nadruk of *A. phagocytophilum* en *A. ovis*. In totaal werden 240 niet-gevoede teken (volwassen en nymph) verzameld en onderworpen aan een PCR-RFLP. *Anaplasma ovis* werd gevonden in *Amblyomma* spp. en *Hyalomma* spp., terwijl *A. phagocytophilum* enkel teruggevonden werd in *Rhipicephalus pulchellus*. Deze studie identificeerde uiteraard potentiële vectoren van *A. ovis* en experimentele studies zijn nodig om deze bevindingen te confirmeren.
In conclusie kan gesteld worden dat dit doctoraat door teken overgedragen pathogenen, van diergeneeskundig belang en met mogelijke consequenties voor de volksgezondheid, aan het licht bracht in Ethiopië. Een verbeterde moleculaire methode voor de gelijktijdige opsporing van *Anaplasma* en *Ehrlichia* soorten werd ontwikkeld en aangewend in epidemiologische studies op herkauwers en veldteken. Een hoge anaplasmose en een lage heartwater prevalentie werden waargenomen. Infectie met *A.* sp. Omatjenne werd genoteerd voor verscheidene Afrikaanse landen. Potentiële vectoren van *A. ovis* werden geïdentificeerd. Deze dienen experimenteel geconfirmeerd te worden, terwijl vectoren van *A. phagocytophilum* dienen gezocht te worden. Tenslotte is een verbetering nodig voor de opsporing van latente dragers van *E. ruminantium*.

10.4 About the author

Teshale Sori holds a DVM (1999) from Addis Ababa University and MSc (2010) degree from Institute of Tropical Medicine, Belgium and has attended several face-to-face and online postgraduate courses in veterinary epidemiology and biostatistics.

After working for two years (2000-2001) as assistant researcher at Bako Agricultural Research Centre (Ethiopia) and two and have years (2002-2004) as junior research officer at the National Veterinary Institute at Bishoftu (Ethiopia), he joined the College of Veterinary Medicine and Agriculture, Addis Ababa University in 2004 where he has since worked as a lecturer. He teaches Analytical Veterinary Epidemiology, Large Animal Medicine and Infectious diseases of poultry.

Besides his teaching assignments he has been involved in several research activities and advised MSc and DVM students in the area of Veterinary Epidemiology and Infectious diseases of poultry. His research focuses on the impact of ticks and tick-borne diseases on the national economy and public health, the dynamics of tick-borne diseases in various epidemiological situations. Recently he has been involved in the design, implementation and monitoring of intervention studies of Newcastle Disease and Infectious Bursal Disease and was asked to strengthen the interaction of research and clinical aspects of Newcastle disease.

He has produced several publications in peer reviewed international journals.