Aus dem Institut für Parasitologie und Tropenveterinärmedizin
des Fachbereichs Veterinärmedizin
der Freien Universität Berlin

Molecular detection and characterization of tick-borne pathogens of cattle in Southwestern Ethiopia

Inaugural-Dissertation
zur Erlangung des Grades eines
PhD in Biomedical Sciences
an der
Freien Universität Berlin

vorgelegt von
Zerihun Hailemariam Negasi
aus Addis Abeba, Äthiopien
DVM, Veterinary Medicine; MSc, Immunology

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Dedicated to

my dear wife and my lovely daughters

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List of abbreviations

BLAST	Basic Local Alignment Search Tool
CI	Confidence interval
CSA	Central Statistical Agency
DAAD	German Academic Exchange Service
DFG	German Research Foundation
DNA	Deoxyribonucleic acid
dNTPs	Deoxynucleotide Triphosphates
ECF	East Coast fever
EDTA	Ethylenediaminetetraacetic acid
FRET	Fluorescence resonance energy transfer
FTA	Flinders Technology Associates
GDP	Gross domestic product
GTP	Growth and transformation plan
LAMP	Loop-mediated isothermal amplification
m.a.s.l	Meter above sea level
OR	Odds ratio
PA	Peasant associations
PCI	phenol–chloroform-isoamyl alcohol
PCR	Polymerase chain reaction
PCV	Packed cell volume
RFLP	Restriction Fragment Length Polymorphism
RLB	Reverse line blotting
rRNA	Ribosomal ribonucleic acid
RT	Room temperature
SPS-LMM	Sanitary and Phytosanitary standards and Livestock and Meat Marketing Program
SSU	Small subunit
TBDs	Tick-borne diseases
TBPs	Tick-borne pathogens

Preface

Recent reports show the growing risks associated with ticks and tick-borne diseases (TBDs) worldwide due to expanding tick populations, global warming, and increased contacts between humans, animals, and ticks (de la Fuente and Estrada-Pena, 2012). The Ethiopian government recognizes the importance of livestock in poverty alleviation and has increased its emphasis on modernizing and commercializing the livestock sub-sector in recent years. In effect, the genetic potential of local breeds is being upgraded to achieve significantly higher milk production through crossbreeding with exotic dairy breeds (SPS-LMM, 2008). The majority of cattle imported are of Friesian type and they are normally chosen from countries where there is strict tick control regime or countries where tropical ticks and TBPs do not occur. In this way, they are highly susceptible to ticks and tick-borne diseases. Reliable information regarding the prevalence and species composition of bovine tick-borne pathogens (TBPs) based on diagnostic techniques that are both sensitive and specific is of paramount importance for development, management and implementation of efficient control strategies and reducing the economic losses from TBDs. Hence, the principal aim of this thesis was to investigate and thereby establish the prevalence and species composition of bovine TBPs of veterinary significance in Southwestern Ethiopia.

The cumulative thesis is structured into chapters as follows:

The first chapter intends to provide general background on ticks and TBDs in Ethiopia, problem statement and justification of this thesis' research. Moreover, the chapter also highlights the scope and objectives of the thesis.

The second chapter provides a review on the current state of knowledge of ticks and TBDs in Ethiopia. In addition, the etiology, vectors, life cycle, epidemiology, pathogenesis and clinical signs of major bovine TBDs and advances in the molecular detection and differentiation of TBPs are reviewed in this chapter. This chapter also briefly addresses contribution and challenges of livestock production in Ethiopia and strategies proposed towards improved contribution of this sector to the national economy.

The third chapter deals with the evaluation of six DNA extraction methods from blood spotted on Flinders Technology Associates (FTA) cards, to determine the optimal protocol for the subsequent molecular detection of TBPs by PCR and reverse line blot (RLB) hybridization. The detection limit by PCR and RLB was compared for six DNA extraction methods from FTA card and also for DNA isolated from whole blood dilutions using a commercial kit. The number of 3 mm discs punched from FTA cards was used as starting material for DNA extraction with FTA purification reagent followed by Chelex® resin.

The fourth chapter elucidates the prevalence and species composition of bovine TBPs of veterinary significance in local cattle populations of Illubabor zone in Southwestern Ethiopia. The findings in this chapter reveal a very high burden of infection of cattle with TBPs and a high frequency of co-infections with different TBPs. Moreover, the prevalence and phylogenetic characterization of three novel *Anaplasma* species identified from bovine blood is presented in this chapter.

The fifth chapter presents a summarizing discussion drawn primarily from the findings of the previous chapters of this thesis and supplemented with information from existing scholarly works. Furthermore, the strengths and limitations of this thesis are considered

and suggestions for further research are indicated. This chapter concludes with recommendations for how the findings of this work can be applied in the diagnosis and control of ticks and TBDs in the study area in particular and in Ethiopia in general.

1 CHAPTER 1: Introduction

Tick-borne diseases affect 80% of the world's cattle population, hampering livestock production throughout the world (Marcelino et al., 2012). In 1997, the annual global losses associated with ticks and TBDs in cattle was estimated to amount between US$ 13.9 billion and US$ 18.7 billion (de Castro et al., 1997). Four groups of TBDs are primarily of importance to livestock production: theileriosis, babesiosis, anaplasmosis and heartwater (also called cowdriosis).

In Ethiopia, government policy has recently been changed to encourage commercial farming. The genetic potential of local breeds is being upgraded to achieve higher milk production through crossbreeding with exotic dairy breeds through artificial insemination and estrus synchronization. As part of the growth and transformation plan II (GTP II) of the federal government of Ethiopia, it is planned to increase the number of crossbred dairy cattle to 3.6 million by the end of 2019/20 or almost eight times the base-year number (2014/15) (Shapiro et al., 2015). Bovine babesiosis, anaplasmosis, heartwater and theileriosis have all been reported to occur in Ethiopia. Generally, local breeds of cattle are believed to have some innate immunity against tick-borne pathogens (Pegram et al., 1981; Regassa, 2001; Mekonnen et al., 2007). However, susceptibility of exotic breeds to TBDs presents a major obstacle to the improvement of cattle production. Therefore, although the introduction of *Bos taurus* dairy breeds may increase milk production in Ethiopia, it will require good management and adequate control measures against ticks and TBDs, which in turn depends upon accurate information regarding the epidemiology of infection of cattle with TBPs.

1.1 Scope and objectives of the thesis

DNA extraction forms an essential step in the molecular detection of tick-borne pathogens. In low resource settings, commercial DNA extraction kits and infrastructures for the storage and transport of blood samples are not always available (Port et al., 2014). FTA cards are useful for sample storage and transportation in areas where infrastructure for the cold storage and transport of blood samples is not available and in situations where the transport of frozen forms of biohazardous agents from one geographical region to another is complicated by international regulations (Michaud et al., 2007). However, in addition to common PCR inhibitors in the blood, effectiveness and efficiency of DNA extraction from FTA filter cards are affected by localized trapping of the genomic material (Ahmed et al., 2011).

Several methods have been described for DNA extraction from blood samples collected on filter paper. These include phenol–chloroform-isoamyl alcohol based extraction (PCI) (Cardoso et al., 2010), the use of FTA purification reagent (Oura et al., 2004; Simuunza et al., 2011), Saponin followed by Chelex® (Devos and Geysen, 2004), Saponin combined with PCI (Tani et al., 2008) and commercial solid-phase extraction kits (Salih et al., 2007). The availability of these methods with different attributes makes the choice difficult. The paucity of data in this regard set the objective for the first part of the thesis, which was:

- ❖ The evaluation of six different DNA extraction methods from blood spotted on FTA cards, to determine the optimal protocol in terms of sensitivity and cost-effectiveness for subsequent molecular detection and epidemiological investigation of TBPs by PCR and RLB.

Over the years a number of different molecular diagnostic assays have been developed to detect TBPs. Moreover, the occurrence of mixed infections of TBPs in both mammalian hosts and tick vectors makes the discrimination of benign and pathogenic forms of TBPs as well as tick species responsible for disease outbreaks increasingly important, both for diagnostic and epidemiological purposes (Mans et al., 2015). Increased sensitivity and specificity of molecular tools to detect TBPs was achieved by combining PCR with specific hybridization by means of RLB. This macroarray is capable of simultaneously detecting multiple TBPs in a single sample. Hence, currently this technique has found widespread adaptation for the simultaneous detection and differentiation of tick-borne pathogens (e.g. Gubbels et al., 1999; Bekker et al., 2002; Schnittger et al., 2004; Berggoetz et al., 2014).

In East Africa, a combination of PCR and RLB has been applied in a field situation for the identification of tick-borne haemoparasites including *Theileria*, *Anaplasma*, *Babesia* and *Ehrlichia* species from indigenous and crossbred cattle in Uganda to identify carrier animals (Oura et al., 2004). Similarly, this technique was applied in the epidemiological study of certain tick-borne protozoal diseases with special reference to East Coast Fever (ECF) in Central Equatorial State, Southern Sudan (Salih et al., 2007).

Tick-borne diseases constitute an important constraint on the livestock production in Ethiopia. Several studies have investigated the prevalence and species composition of ticks infesting ruminants. However, detailed epidemiological studies and data on the distribution of TBPs based on sensitive and specific molecular detection methods are scarce in Ethiopia. Accordingly, based on the above background information, the second part of the thesis aims:

- to investigate and thereby establish the prevalence and species composition of bovine TBPs of veterinary significance in Southwestern Ethiopia. Due to the existence of diverse species of TBPs known to be transmitted by hard ticks in Ethiopia, this study relied on a combination of PCR and RLB for the simultaneous detection of TBPs from bovine blood.

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2 CHAPTER 2: Literature review

2.1 Livestock production in Ethiopia: contribution, challenges and strategies

Ethiopia is believed to have the largest livestock population in Africa. The livestock sector has contributed considerably to the economy of the country, and is still pivotal to the economic development of the country (CSA, 2013). Between 1996 and 2006, the share of the livestock sub-sector averaged 24% of agricultural gross domestic product (GDP) and 11% of national GDP, with the highest shares recorded at 27% and 13%, respectively, at its peak (Negassa et al., 2011).

Livestock fulfills an important function in conferring a certain degree of security in times of crop failure, as they are a “near-cash” capital stock, accumulating wealth, and serving as a store of value in the absence of formal financial institutions and other missing markets. In the case of smallholder mixed farming systems, livestock provides nutritious food, additional cash income and fuels for cooking food. On the other hand, draught animals provide power for the cultivation of the smallholdings and for crop threshing and are also essential modes of transport to take holders and their families long-distances, to convey their agricultural products to the market places and bring back their domestic necessities. In the case of pastoralists, livestock represents a sole means to support and sustain their livelihoods (Negassa et al., 2011; CSA, 2013).

Compared to its potential, the contribution of livestock sector to the national economy is disproportionately low. One of the main causes of the mismatch between herd population size and production output from livestock in Ethiopia is undoubtedly the widespread occurrence of a multitude of infectious and parasitic diseases, causing morbidity, mortality

and market restrictions, which drastically reduce animal production. One of the strategies proposed towards increasing the contribution of the livestock sector to the national economy is broad-based interventions and improvements in animal health services and further exploitation of improvement in productivity of the herd/flock with better feeding and genetic improvement that aims at crossbreeding, but also at the strategic use of the genetic niche in some local breeds for the taste and quality of their meat (Shapiro et al., 2015).

2.2 Ticks and tick-borne diseases in Ethiopia

2.2.1 Distribution of Ixodid ticks infesting cattle in Ethiopia

Ixodid or hard ticks (Acari: Ixodida: Ixodidae) are blood-feeding ectoparasites with worldwide occurrence and are capable of transmitting a broad range of human and animal pathogens (Guglielmone et al., 2014).

In Ethiopia, ticks are commonly found in all agro-ecological zones of the country (Pegram et al., 1981). However, the distribution and abundance of tick species infesting domestic ruminants vary greatly amongst different locations and is influenced by factors such as host, climate and vegetation cover. More than 30 tick species were reported from different locations across the country, belonging to the genera *Amblyomma* (8 spp.), *Haemaphysalis* (4 spp.), *Hyalomma* (9 spp.) and *Rhipicephalus* (17 spp.) (Mekonnen et al., 2007). The most common ixodid ticks infesting domestic ruminants reported were *Amblyomma cohaerens*, *Am. variegatum*, *Rhipicephalus decoloratus*, *Rh. pulchellus*, *Rh. evertsi*, *Rh. praetextatus* and *Hyalomma rufipes*.

Of the genus *Amblyomma*, four species that commonly infest cattle are known to exist in Ethiopia. These include *Am. variegatum*, *Am. cohaerens*, *Am. gemma* and *Am. lepidum* (Mekonnen et al., 2007; Abera et al., 2010). While *Am. variegatum*, *Am. gemma* and *Am. lepidum* were reported to have a wider distribution in southern Ethiopia (Pegram et al., 1981; Regassa, 2001), *Am. variegatum* and *Am. cohearens* are widely prevalent in southwestern Ethiopia (Abera et al., 2010; Abunna et al., 2012). In eastern Ethiopia, *Am. variegatum* and *Am. gemma* are the two most common species (Bekele, 2002).

Rhipicephalus decoloratus is the most common and widespread tick in Ethiopia collected in all regions except Afar (Mekonnen et al., 2007; Abera et al., 2010). *Rh. evertsi* has also widespread distribution (Sinshaw, 2000; Gebre et al., 2004). *Rh. lunulatus* species were encountered in Central Ethiopia (Mekonnen et al., 2001; Tsegaye et al., 2013) and *Rh. muhsamae* from Borena (Southern Ethiopia) (Regassa, 2001) and wetter western parts of the country (Pegram et al., 1981; De Castro, 1994). *Rh. pulchellus* is distributed widely in the northeastern (Seyoum, 2001), eastern (Abebe et al., 2010) and southern part of the country (Regassa, 2001). *Rh. simus* are reported from eastern (Abebe et al., 2010) and central (Mekonnen et al., 2001) parts of the country.

In Ethiopia, about eight species of *Hyalomma* that affect cattle were identified, which includes *Hyalomma rufipes*, *Hy. dromedarii*, *Hy. truncatum*, *Hy. marginatum*, *Hy. impeltatum*, *Hy. excavatum*, *Hy. anatolicum* and *Hy. albiparmatum* (Regassa, 2001; Bekele, 2002; Mekonnen et al., 2007; Tsegaye et al., 2013). Species of ticks in the genus Haemaphysalis are also known to infest Ethiopian cattle. *Haemaphysalis aciculifer*, *Ha. parmata* and *Ha. leachi* are distributed in many parts of the country (Mekonnen et al., 2001; Seyoum, 2001).

2.2.2 Pathogenic roles of ticks

In Ethiopia, ixodid ticks are responsible for a diversity of livestock health problems. Losses attributed to ticks are caused either directly, through tick worry, blood loss, damage to hides and udders and the injection of toxins or indirectly through mortality or debility caused by the diseases transmitted (anaplasmosis, babesiosis, heartwater and theileriosis). Exports of hides and skins yield foreign earnings, but these products are consistently downgraded or rejected because of damages caused to hide and skin (Mekonnen, 2014).

2.2.3 Tick-borne diseases in Ethiopia

The major cattle tick-borne diseases in Ethiopia are anaplasmosis, babesiosis, heartwater and mild forms of theileriosis.

Infection by *A. marginale* is widespread in the country as is its major vector *Rh. decoloratus*. Babesiosis is mainly a disease of cattle in Ethiopia. Infection with *B. bigemina* is widespread in the country. *B. bovis* has been detected serologically in sera collected from Sudanese refugee cattle in Gambella region southwest Ethiopia (Radley, 1980; Mekonnen, 1996).

The presences of *Theileria mutans*, *T. velifera*, *T. orientalis* and *T. ovis* have been previously reported from some parts of Ethiopia in tick specimens and blood collected from cattle (Mekonnen et al., 2007; Tomassone et al., 2012; Kumsa et al., 2015). Recently, infection of cattle with *T. annulata* was reported for the first time in Ethiopia in a location called Humera, close to the Sudanese border (Gebrekidan et al., 2014). Taking into consideration the location where this pathogen is reported and the prevailing

uncontrolled cattle movement across border, there is a possibility that the infected cattle might have originated from Sudan where the disease is known to exist.

Heartwater is considered to be the most important tick-borne disease of exotic and cross-bred cattle in Ethiopia. Recently, *E. ruminantium* has been molecularly detected from ticks in Ethiopia (Tomassone et al., 2012; Teshale et al., 2015). Outbreaks have occurred in some dairy farms in which mortality rates have reached as high as 25% (Mekonnen, 1996).

Previous studies have also documented the presence of *Rickettsia* spp. in ixodid ticks. *Rickettsia* spp. detected include *R. africae*, *R. aeschlimannii* and *R. massiliae.* These pathogens were detected in different species of *Amblyomma, Hy. rufipes* and *Rh. decoloratus* ticks collected from various locations in the country (Mura et al., 2008; Pader et al., 2012; Kumsa et al., 2015).

Generally, local breeds of cattle and other ruminants in Ethiopia are believed to have some innate immunity against TBPs (Pegram et al., 1981; Regassa, 2001; Mekonnen et al., 2007). However, in view of the increasing number of exotic breeds of cattle kept in dairy farms in Ethiopia, tick-borne pathogens warrant due attention. On the other hand, studies conducted on detection of *Rickettsia* spp. in ixodid ticks have indicated that the transmission of spotted fever group rickettsiae through ixodid ticks is a potential risk for human health in different parts of Ethiopia (Kumsa et al., 2015).

2.2.4 Control of ticks and TBDs in Ethiopia

The conventional method of controlling tick infestations in Ethiopia is application of acaricide either by hand spraying, by hand dressing or using spray races. Traditional tick

control methods such as hand picking, burning with a hot iron or application of plant juice are also used in rural areas (Mokenen, 1998). Chemicals used as acaricides are grouped into organophosphates, carbamates, cyclic amidines and synthetic pyrethroids (Mekonnen et al., 2001). In dairy farms and big ranches where regular tick control program is practiced, synthetic pyrethroids group of acaricide are mostly used to control ticks (Mekonnen, 2014). Control of TBDs in Ethiopia is also based mainly on intensive tick control using acaricides and treatment of cattle with antibiotics and antiparasitic drugs when cases of TBDs are suspected or diagnosed.

The use of acaricides is constrained by their high costs, concerns with toxicity, ticks developing resistance against acaricides, and residues in food and in the environment (Mekonnen, 1996). Integrated control strategies are the ultimate options in tick and TBDs control which requires integration of many technologies into specific tick management systems. Individual components include new acaricide formulation and delivery systems, biological control, ecological control, immunological control, genetic control, regulatory control and models based on ecological database (George *et al.,* 2002).

2.3 Major bovine tick-borne diseases

2.3.1 Babesiosis

Babesiosis is a vector-borne protozoal disease caused by intraerythrocytic protozoan parasites of the genus *Babesia* (Uilenberg, 1995). The genus *Babesia* belongs to the phylum Apicomplexa, family Babesiidae (Allsopp et al., 1994). Cattle babesiosis is caused mainly by the tick-borne apicomplexan parasites *B. bovis*, *B. bigemina* and *B. divergens*.

Because *B. bovis* and *B. bigemina* are the most significant species infecting cattle, they will be the main focus of this review.

2.3.1.1 Vectors of *B. bigemina* and *B. bovis*

Both *B. bovis* and *B. bigemina* are most commonly transmitted between cattle by the tick vectors *Rhipicephalus* microplus and *Rhipicephalus annulatus, Rhipicephalus decoloratus* only transmits *B. bigemina*. *B. bovis* and *B. bigemina* are primarily found in tropical and subtropical regions of the world including Australia, Africa, Asia and the Americas (Bock et al., 2004; Chauvin et al., 2009).

2.3.1.2 Life cycle

Babesia bovis and *B. bigemina* exhibit a typical apicomplexan life cycle. The life cycle is characterized by merogony, gametogony, and sporogony; have erythrocytes as the only single cell target in the bovine host; and are transovarially transmitted (Chauvin et al., 2009; Suarez and Noh, 2011). The *Babesia* life cycle was reviewed in depth by Chauvin et al. (2009) and Suarez and Noh (2011). Briefly, *Babesia* parasites enter susceptible cattle hosts with the saliva of the infected feeding tick larvae (*B. bovis*) or nymphs (*B. bigemina*). Sporozoites invade erythrocytes, where they divide asexually by binary fission to become merozoites that are released into the circulation upon rupture of the host RBC. Upon release, merozoites invade new erythrocytes, where they transform into trophozoites that divide by binary fission (merogony) to produce a pair of merozoites that perpetuate the cycle of erythrocyte invasion upon exiting the erythrocyte.

When babesia-infected erythrocytes are ingested by ticks, some intracellular trophozoites develop into gametocytes starting sexual reproduction. The gametes fuse in the lumen of

the digestive tract of the tickto form an elongated zygote 8 to 10 μm in length bearing a spike-like arrowhead organelle, which facilitates cell penetration. Once the babesia zygotes have been internalized, they transform into kinetes that can gain access to the hemolymph in the haemocoel of the tick vectors, where they invade the eggs in the ovaries of the ticks. This process allows transmission of *Babesia* parasites to the next generation of ticks, a mechanism known as transovarial transmission. Once in the larvae, the kinetes invade the cells in the salivary gland, where they become sporozoites. An important expansion in the number of sporozoites occurs in the salivary glands through sporogony. Sporogony takes place at each tick stage and the babesia infection acquired during one life stage is passed on to the next (transtadial transmission). Sporozoites are transmitted to the vertebrate hosts after at least 2 or 3 days following attachment of *B. bovis*-infected tick larvae, whereas transmission occurs through tick nymph stages for *B. bigemina* (Chauvin et al., 2009; Suarez and Noh, 2011).

2.3.1.3 Epidemiology

The prevalence of infection and the occurrence of tick-borne diseases are determined by complex interactions among the hosts, the environment, the vectors and the parasites. Passively acquired immunity from colostrum lasts two months but this is followed by innate immunity from 3 to 9 months of age. Therefore, calves exposed to babesiosis early in life rarely show clinical signs but develop long lasting immunity (Mahoney et al., 1973). *Bos taurus* cattle are more susceptible to *B. bovis* than *Bos indicus* breeds (Bock et al., 1997). Breeds of cattle that are indigenous to *Babesia*-endemic regions often have a certain degree of natural resistance to these diseases and the consequences of infection are not as serious as those for exotic *Bos taurus* breeds. In addition, in tropical areas with

a large vector population, natural exposure usually occurs at an early age, when these animals are naturally protected, allowing acquired immunity to develop. These cattle are therefore immune to subsequent challenge as adults (Bock et al., 2004). Under natural tick challenge, due to continued exposure to infected ticks, a large number of older cattle remain seropositive. This reduces the proportion of susceptible individuals in the population (Magona et al., 2008). This scenario represents a state of endemic stability, defined as the situation where all calves below the age of 6 months have been in contact with the parasite and where clinical disease is rare (Yeoman, 1966).

The interaction between vector, parasite and host gives rise to a wide range of situations due to different husbandry management systems. For instance, the introduction of European breeds into tropical and subtropical regions of South America often failed because of high losses due to ticks and babesiosis (Wilkins, 1986). The use of acaricides, alone or in combination with pasture spelling, was responsible for enzootic instability (Johnston et al., 1981). Regarding the epidemiology of babesiosis, the biggest difference between acaricides is the duration of their residual effect, which prevents the transmission of *Babesia*. Pyrethroids can persist for 15-21 days (Taylor and Elliott, 1987) while organophosphates persist for only three days (Barnett, 1961). Ivermectin at a single dose of 0.2 µg/kg protects cattle for 21-28 days (Pegram and Lemche, 1985). The grazing system is also important in such a way that in some rotational grazing, pastures can remain without cattle for two to three months, thus reducing the population of *Babesia* (Wilkinson and Wilson, 1959).

There is a seasonal variation in the prevalence of clinical babesiosis, the greatest incidence occurring soon after the peak of tick population. Of the climatic factors, air temperature is the most important because of its effect on tick activity; higher temperatures increase its occurrence. Heaviest losses occur in areas where the tick population is highly variable depending on the environmental conditions (Radostits et al., 2007).

2.3.1.4 Pathogenesis and clinical signs

The pathogenesis of *B. bigemina* infection is principally linked with induction of intravascular haemolysis and associated anaemia, jaundice and haemoglobinuria. Whilst similar symptoms occur later in the course of disease associated with *B. bovis* infections, there is an earlier, more complicated and severe reaction associated with the induction of cytokines and other pharmacologically active agents. *Babesia bovis* also has the ability to dramatically alter the structure and function of the infected red blood cell (RBC). In *B. bovis*, this is accompanied by the accumulation of parasitised RBCs in the microvasculature in a number of organs including the brain and lungs, and the subsequent development of often fatal clinical complications such as cerebral babesiosis, respiratory distress and multi-organ failure (Bock et al., 2004; Gohil et al., 2013).

2.3.2 Theileriosis

Bovine theileriosis is a disease caused by infection with protozoan parasites of the genus *Theileria.* The genus *Theileria* encompasses a number of protozoan species that infect a wide range of both domestic and wild animals and are transmitted by ixodid ticks of the genera *Amblyomma*, *Haemaphysalis*, *Hyalomma* and *Rhipicephalus* (Bishop et al.,

2004). They are classified in the phylum Apicomplexa along with *Babesia*, *Eimeria*, *Plasmodium* and *Toxoplasma*. A broad classification, based on the ability of these parasites to transform the leukocytes of host animals, divides *Theileria* into two groups, consisting of transforming and non-transforming species. This classification is based on the ability of some of *Theileria* species to transform host leukocytes in a way that enables the infected cells to proliferate indefinitely along with the parasites occupying them. Non-transforming *Theileria* parasites do not induce this type of host-cell proliferation. Although the parasites in the latter category are considered to be relatively benign, disease outbreaks and economic losses related to the farm animals affected are not uncommon as a result of anaemia induced by the piroplasm stage (Sivakumar et al., 2014). The following species have been described as transforming *Theileria*: *Theileria parva*, *Theileria annulata*, *Theileria lestoquardi*, and *Theileria taurotragi* (Sugimoto and Fujisaki, 2002; Dobbelaere and Küenzi, 2004). However, recent studies added *Theileria* sp. (buffalo), a benign *Theileria* parasite in African buffaloes, to the list of transforming parasite species (Zweygarth et al., 2009; Chaisi et al., 2011). Species of non-transforming parasites include *Theileria orientalis*, *Theileria mutans*, *Theileria velifera*, and *Theileria cervi* (Aparna et al., 2011; McFadden et al., 2011; Eamens et al., 2013; Sivakumar et al., 2014). Among the *Theileria* species known to infect cattle, the two most economically important ones 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 ECF, a highly fatal disease of cattle in Eastern, Central and Southern Africa (Norval et al., 1992). Since there are no clinical or serological reports of the presence of

either bovine tropical theilerioses or ECF in Ethiopia, this review focuses on the non-transforming *Theileria.*

2.3.2.1 Vectors of the non-transforming Theileria

Theileriosis caused by *T. orientalis* is predominantly transmitted by ticks belonging to the genus *Haemaphysalis*, whereas *T. mutans* and *T. velifera* are both transmitted by ticks belonging to the genus *Amblyomma* (Sugimoto and Fujisaki, 2002; Coetzer and Tustin, 2004).

2.3.2.2 Life cycle

The life cycle of *Theileria* parasites in the ruminant host and tick vector has been reviewed (Bishop et al., 2004; McKeever, 2009). Briefly, the life cycle involves asexual reproduction of the blood-stage parasites in the host animal, and sexual reproduction of the parasites in a tick vector. A generalized life cycle for the *Theileria* genus includes secretion of infective sporozoites during tick feeding into the feeding site. Sporozoites then infect leukocytes and multiply by merogony, after which merozoites are released, which invade RBCs and thereby establish the piroplasm stage. During a next feeding cycle, larval or nymphal vector ticks ingest piroplasms and the released parasites undergo syngamy in the tick gut, forming a zygote, the only diploid stage. The zygote divides into motile kinetes that infect the tick gut epithelial cells and migrate to the haemolymph and subsequently infect the salivary glands. After molting and commencement of feeding by the tick, sporogony results in the multiplication of sporozoites in the salivary gland acini before injection into the feeding site by nymphs or adult ticks (McKeever, 2009).

Theileria parasites are trans-stadially transmitted by the tick vectors; therefore, the known transmission vectors are usually 2- or 3-host tick species (Bishop et al., 2004).

2.3.2.3 Epidemiology

Theileria epidemiology considers parasite and vector distribution, mortality and morbidity of disease outbreaks, disease outbreak risk assessment and disease control measures, socio-economic factors, climate change, host resistance and susceptibility (Gachohi et al., 2012). However, the epidemiology of non-transforming *Theileria* parasites is not very well established and it depends upon the causative parasites under this group. Regarding *T. mutans*, this species is known to be confined to eastern, western and Southern Africa and to the Caribbean Islands. It has been shown that in an endemic area 99% of calves became infected by *T. mutans* when they reached six months of age (Coetzer and Tustin, 2004). *A. cohaerens* develops high infection rates with *T. mutans*, will frequently feed on cattle and is likely to promote high level of *T. mutans* transmission. It is probable that stress caused by poor nutrition, intercurrent disease or other factors predisposes cattle to anemia when infected with *T. mutans*. Field and experimental evidence suggests that strains of *T. mutans* derived from buffalo may be more pathogenic to cattle than those derived from cattle (Coetzer and Tustin, 2004). *T. velifera* on the other hand is widespread in eastern and southern Africa and also in the Caribbean. It is becoming increasingly apparent that *T. orientalis* is widely distributed in cattle around the world (Sugimoto and Fujisaki, 2002; Coetzer and Tustin, 2004).

2.3.2.4 Pathogenesis and clinical signs

The pathogenic effect of non-transforming *Theileria* parasites results from the invasion and proliferation of piroplasms in circulating erythrocytes. Pathogenic strains are presumed to cause intravascular hemolysis associated with a heavy piroplasm parasitemia resulting in anemia and icterus (Coetzer and Tustin, 2004).

Infection with non-transforming theleriosis can easily be overlooked owing to the fact that they cause predominantly subclinical infections in endemic areas (Sugimoto and Fujisaki, 2002). Clinical signs of infection of cattle with these pathogens may be absent or range from mild febrile reactions, inappetance, slight enlargement of superficial lymph nodes (especially the parotid lymph nodes), pale mucous membranes, reduced milk production to sometimes even death. Clinical illness usually occurs in animals subjected to stress such as poor nutrition and concurrent infection with other parasites, bacteria and viruses (Robson et al., 1977; Sugimoto and Fujisaki, 2002; Coetzer and Tustin, 2004).

2.3.3 Anaplasmosis

Bovine anaplasmosis is a tick-borne disease of cattle caused by the intra-erythrocytic rickettsia, *Anaplasma marginale* (Theiler, 1910). *Anaplasma marginale* is classified in the genus *Anaplasma* belonging to the family Anaplasmataceae of the order Rickettsiales (Dumler et al., 2001). *Anaplasma marginale* subsp. *centrale*, commonly referred to as *Anaplasma centrale*, causes a milder form of anaplasmosis (Theiler, 1911). Originally it was described as *A. marginale* “variety” *centrale;* but has been renamed *A. marginale* subsp. *centrale* in keeping with current taxonomic conventions (Herndon et al., 2013).

2.3.3.1 Vectors of *A. marginale*

Mechanical transmission of *A. marginale* occurs when infected blood is transferred to susceptible cattle via blood-contaminated fomites or the mouthparts of biting flies (Ewing, 1981). Biological transmission of *A. marginale* is effected by ticks and approximately 20 species of ticks have been incriminated as vectors worldwide. In general, tick vectors of *A. marginale* include selected *Dermacentor* spp., *Ixodes ricinus* and *Rhipicephalus* spp. (Kocan et al., 2004). Biological transmission can occur from one tick life cycle stage to the next (trans-stadial) or when a tick feeds on more than one host in the same life cycle stage (intra-stadial). *Anaplasma marginale* can also be transmitted from cow to calf transplacentally (Kocan et al., 2003).

2.3.3.2 Life cycle

Infected erythrocytes taken into the tick with the blood meal provide the source of *A. marginale* infection in the gut cells. After development of *A. marginale* in the gut cells, many other tissues become infected, including the salivary glands, from where the rickettsiae are transmitted to vertebrates during tick feeding (Kocan et al., 2004). In the vertebrate host, the only known site of replication is the bovine erythrocyte. Within the erythrocytes, membrane bound inclusion bodies contain from 4 to 8 rickettsiae, and as many as 70 % or more erythrocytes may become infected during acute infection or disease (Kocan et al., 2004).

2.3.3.3 Epidemiology

Anaplasma marginale is the most prevalent TBP of cattle worldwide with endemic regions in North, Central, and South America, as well as Africa, Asia, and Australia (Brayton et

al., 2009). Among domestic livestock, *A. marginale* infects ruminants, but is principally pathogenic only in cattle. The pathogen has a wide occurrence in different hosts including various wild animals. With regards to the epidemiology of *A. marginale*, information the contribution of domestic and wild animals towards the prevalence of the disease is incomplete due to paucity of published reports, validation of tests and cross reactivity of *Anaplasma* species antibodies (Kuttler, 1984; Aubry and Geale, 2011).

In wildlife-livestock interface settings, disease problems are often bidirectional (Bengis et al., 2002). Ticks and TBDs are one of the major concerns in areas with a high wild-livestock interface and wild animals are often blamed to serve as reservoirs (Wesonga et al., 2006; Ocaido et al., 2009). Molecular studies based on PCR and RLB have recognized wide occurrence of *A. marginale* in wild animals. In sub-Saharan Africa, *A. marginale* is known to infect various wild animals including African buffalo (*Syncerus caffer*), common eland (*Taurotragus oryx*) and gemsbok (*Oryx gazella*). All have been proven to be carriers using these molecular techniques (Tonetti et al., 2009; Oura et al., 2011a; Oura et al., 2011b; Berggoetz et al., 2014). There is limited knowledge of how and if wildlife-livestock interface affects the occurrence of *A. marginale*. However, Kabuusu et al. (2013) showed that *A. marginale* significantly increased in livestock with close proximity to a wildlife-livestock interface.

Several studies have been carried out to determine if there is a difference in susceptibility for *A. marginale* infection between local breeds (*Bos indicus*), European breeds (*Bos taurus*) and their crosses. A study by Bock et al. (1997) reported that all breeds of cattle, including pure *Bos indicus* breeds and pure *Bos taurus* breeds and their crosses are susceptible to development of severe disease if exposed to a virulent strain of *A.*

marginale. This study evaluated animals inoculated with a strain of *A. marginale* under laboratory conditions and did not account for the role of *R. microplus* in the transmission of the organisms. However, a follow-up study using *R. microplus* ticks infected with *A. marginale*, reported that innate resistance of purebred *Bos indicus* and crossbred (50%, F1 generation), cattle was not significantly different. Even though *A. marginale* affects both species similarly, under field conditions *Bos indicus* are not as commonly affected as *Bos taurus*, presumably due to their relative resistance to heavy tick infestation (Bock et al., 1997).

Endemic stability is characterized, among several things, by a high seroprevalence (>70 %) resulting in little fluctuation in disease incidence over time (Rubaire-Akiiki et al., 2004). In an endemic stable area, the young animals become infected at an early age, when there is a significant passively acquired immunity and consequently they usually do not develop severe symptoms. This way the young animals become immune to challenge later in life and the incidence of clinical cases becomes low despite high level of infection (de Vos, 1992). In contrast, in endemic instable areas the young animals do not encounter the pathogen frequently enough and subsequently do not become immune, which is reflected in a low seroprevalence (Alonso et al., 1992). For anaplasmosis the seroprevalence has been used as an indicator of the existents of endemic stability and it thus serve as a biological indicator for the need of interventions, as for example immunization (Perry and Young, 1995).

The distribution of bovine anaplasmosis, being caused by a vector-borne pathogen, may be expected to change in part as a result of climate change, which may influence the movement of tick populations (Jonsson and Reid, 2000) as well as the survival of A.

marginale in overwintering ticks. However, predicting the impact of climate change on vector-borne disease epidemiology is not a trivial or straightforward exercise (Aubry and Geale, 2011). This is highlighted by Tabachnick (2010), who indicated that 'predicting and mitigating the effects of future changes in the environment like climate change on the complex arthropod–pathogen–host epidemiological cycle requires understanding of a variety of complex mechanisms from the molecular to the population level'.

Dipping may have little effect on the prevalence of the disease as it has been shown that vectors other than ticks, such as *Stomoxys calcitrans* (Potgieter et al., 1981) and *Tabanus taenida* (Wiesenhütter, 1975), may play an important role in the epidemiology of bovine anaplasmosis.

2.3.3.4 Pathogenesis and clinical signs

Following initial infection and an incubation period of 7–60 days (Kocan et al., 2003), *A. marginale* invades erythrocytes and undergoes cycles of replication, removal of infected erythrocytes by the reticuloendothelial system and subsequent reinvasion of erythrocytes within the ruminant. Destruction of erythrocytes by the reticuloendothelial system results in development of icterus without haemoglobinaemia and haemoglobinuria. Acute anaplasmosis, caused by *A. marginale*, is characterised by a progressive haemolytic anaemia associated with fever, weight loss, abortion, decreased milk production and in some cases death of the infected cattle (Kocan et al., 2000). 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. Cattle that

survive acute disease develop persistent infection characterized by cyclic low-level rickettsaemia but life-long immunity. These animals serve as a reservoir of *A. marginale,* providing a source of infective blood for both mechanical and biological transmission (Guglielmone, 1995; Kocan et al., 2003).

2.3.4 Heartwater

Heartwater (or cowdriosis) is a tick-borne disease caused by an intracellular rickettsial pathogen known as *Ehrlichia ruminantium* (Dumler et al., 2001). *Ehrlichia ruminantium* is an obligate intracellular bacterium that parasitises vascular endothelial cells, neutrophils and macrophages of the mammalian host (Peter et al., 1995). The genus *Ehrlichia* belongs to the order Rickettsiales within the family *Anaplasmataecoe* (Dumler et al., 2001).

2.3.4.1 Vectors of *E. ruminantium*

Heartwater is transmitted transtadially by all ticks in the genus *Amblyomma* and the distribution of the disease in Africa coincides with that of the two most important vector species, *A. variegatum* and *A. hebraeum* (Bezuidenhout, 1987; Uilenberg, 1996). Only nymphs and adults are able to transmit the disease after being infected as larvae and nymphs, respectively (O'Callaghan et al., 1998).

2.3.4.2 Life cycle

The larvae and nymphs acquire infection by feeding on *E. ruminantium*-infected domestic or wild ruminants. The major site of development of *E. ruminantium* in ticks appears to be in the midgut epithelial cells (Kocan et al., 1987; Kocan, 1995). Subsequent stages invade and develop in the salivary gland acini cells of the vector. A major mode of transmission

of *E. ruminantium* to mammalian hosts occurs during feeding by an infected tick. In the vertebrate host, it has been proposed that the initial development of the organism occurs mainly, but not exclusively, in reticulo-endothelial cells. Then the parasitized reticulo-endothelial cells rupture and the organism is released into the general circulation where it invades endothelial cells (Du Plessis, 1970; Prozesky and Du Plessis, 1987). Depending on the host, the organism seems to have a predilection for endothelial cells in certain organs. In ruminants, the highest concentrations of bacterial organisms are found in the brain followed by kidneys (Prozesky and Du Plessis, 1987).

2.3.4.3 Epidemiology

Heartwater occurs only where its vectors are present and 10 *Amblyomma* spp. capable of transmitting the organism occur in Africa. An increased prevalence of heartwater usually occurs when peak numbers of ticks are present, and good rains are often followed by a transient increase in the occurrence of the disease. Ticks retain their infectivity for life, so a small number of infected ticks could presumably maintain the infection in a particular herd or area. When a pathogenic genotype of *E. ruminantium* infects a susceptible vertebrate host, either inapparent or overt disease may develop depending on the virulence of the organism and on the breed, age, degree of natural resistance and immune status of the host. Young calves possess a reverse age resistance, which is independent of the immune status of the cow. The susceptibility of different breeds of cattle varies, *Bos indicus* (Zebu) breeds being in general more resistant than European (*Bos taurus*) breeds. The resistance of local zebu breeds is probably due to an inherited resistance acquired through years of natural selection. This resistance does not prevent

the establishment of infection but reduces the severity of clinical disease (Coetzer and Tustin, 2004).

2.3.4.4 Pathogenesis and clinical signs

The pathogenesis of heartwater is not well understood. In domestic ruminants *E. ruminantium* most readily infects endothelial cells of the brain and this coincides with the onset of febrile reaction (Du Plessis, 1970). Increased vascular permeability with transudation is responsible for effusion into body cavities and tissue edema, and this is particularly noticeable in the lungs, the pericardial sac and the pleural cavity. Edema of the brain is responsible for the nervous signs; hydropericardium contributes to cardiac dysfunction during the terminal stages of the disease. Progressive pulmonary edema and hydrothorax result in eventual asphyxiation (Owen et al., 1973). Damage to the endothelial cells and alveolar capillaries is limited and the often mild cytopathic changes seen in parasitized endothelial cells suggest that the organism itself may not be the cause of the increased vascular permeability. It has been suggested that endotoxin and increased cerebrospinal fluid pressure play a role in the development of lung edema (van Amstel et al., 1988; Brown and Skowronek, 1990). Clinical signs of heartwater range from mild to transient fever in subclinical cases, to death without premonitory signs in peracute cases. The acute form of the disease is characterized by sudden onset of fever, tachycardia, inappetance and neurological signs (hyperaesthesia, high-stepping gait, twitching eyelids, chewing, abnormal tongue movement and tremor of individual muscles). Haemorrhagic diarrhoea is commonly reported (Van de Pypekamp and Prozesky, 1987).

2.4 Molecular diagnosis of TBDs

New molecular technologies have greatly advanced the knowledge of ticks and TBPs, and developed or refined diagnostic tools have increased the efficacy and accuracy of identifying TBPs (de la Fuente and Estrada-Pena, 2012).

The molecular diagnostic assays targeting specific genes and species of TBPs include conventional PCR followed by agarose gel electrophoretic analysis and several other PCR-based methods such as loop-mediated isothermal amplification (LAMP) assays, RLB, Restriction Fragment Length Polymorphism (RFLP), a nested fluorescence resonance energy transfer (FRET), real-time PCR based pan-FRET assays and high-resolution melting analysis. In all cases, detection by molecular methods allow for direct confirmation of the presence of parasite genomic material, with the inference that live parasites are present in the animal at the moment of sampling (reviewed in Mans et al. (2015) and Criado-Fornelio (2007)). Development from conventional to nested- and real-time PCR has allowed for improvements in sensitivity, quantification and speed of detection. LAMP assays have gained popularity in resource-poor settings because of their ease of use, amplification of DNA under isothermal conditions, inexpensive instrumentation, and fairly short turnaround time (Rodrigues et al., 2016). Methods such RLB, bead arrays, pan-FRET assays and high-resolution melting analysis hold the promise of detection of multiple species or genotypes at the same time (Mans et al., 2015).

PCR combined with RLB is a robust technique, where multiple samples can be analyzed against a variety of probes to enable simultaneous detection of different tick-borne

pathogens. This technique has gained widespread adaptation in the detection and differentiation of TBPs (Berggoetz et al., 2014). In RLB, PCR products are hybridized in a miniblotter apparatus to oligonucleotide probes immobilized on a membrane (Kong and Gilbert, 2006). Successful hybridization of complementary sequences is subsequently visualized using chemiluminescence, which makes this macroarray capable of identifying mixed infections from single samples with high sensitivity and specificity (Gubbels et al., 1999; Schnittger et al., 2004).

The RLB primers for *Theileria* and *Babesia* target the 18S rRNA regions flanking the V4 hyper-variable region, which has been found to be conserved in all members of these genera (Gubbels et al., 1999). The RLB primers for *Anaplasma* and *Ehrlichia* target the 16S rRNA gene to amplify the hypervariable V1 loop by PCR (Bekker et al., 2002). The probes used in RLB are assumed to be species-specific and to be able to detect all members of a species, based on the assumption that the 18S hyper-variable region is conserved within the species of *Theileria* and *Babesia* whereas species-specific oligonucleotide probes designed in the conserved domains within the 16S rRNA gene were used to amplify the hypervariable V1 loop allow species-specific detection of species of *Anaplasma* and *Ehrlichia*. It is therefore also useful to detect new species if only a catch-all probe is detected (Bekker et al., 2002; Oosthuizen et al., 2009; Chaisi et al., 2014).

Due to these benefits and based on a thorough review of its application to the diagnosis of TBPs in different parts of the world, in this study, RLB was selected as the method of choice for studying the TBPs circulating in cattle in Ethiopia.

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3 CHAPTER 3: Publication I

A comparison of DNA extraction protocols from blood spotted on FTA cards for the detection of tick-borne pathogens by Reverse Line Blot hybridization

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3.1 Abstract

An essential step in the molecular detection of tick-borne pathogens (TBPs) in blood is the extraction of DNA. When cooled storage of blood under field conditions prior to DNA extraction in a dedicated laboratory is not possible, the storage of blood on filter paper forms a promising alternative. We evaluated six DNA extraction methods from blood spotted on FTA Classic® cards (FTA cards), to determine the optimal protocol for the subsequent molecular detection of TBPs by PCR and the Reverse Line Blot hybridization assay (RLB). Ten-fold serial dilutions of bovine blood infected with *Babesia bovis*, *Theileria mutans*, *Anaplasma marginale* or *Ehrlichia ruminantium* were made by dilution with uninfected blood and spotted on FTA cards. Subsequently, DNA was extracted from FTA cards using six different DNA extraction protocols. DNA was also isolated from whole blood dilutions using a commercial kit. PCR/RLB results showed that washing of 3mm discs punched from FTA cards with FTA purification reagent followed by DNA extraction using Chelex® resin was the most sensitive procedure. The detection limit could be improved when more discs were used as starting material for the DNA extraction, whereby the use of sixteen 3 mm discs proved to be most practical. The presented best practice method for the extraction of DNA from blood spotted on FTA cards will facilitate epidemiological studies on TBPs. It may be particularly useful for field studies where a cold chain is absent.

Keywords

DNA extraction, FTA cards, tick-borne pathogens, Reverse Line Blot hybridization, FTA purification reagent, Chelex

3.2 Introduction

Over the years, a number of different molecular diagnostic assays have been developed to detect tick-borne pathogens (TBPs) with high sensitivity and specificity (Criado-Fornelio, 2007). One assay which has found widespread adaptation in the detection and differentiation of tick-borne pathogens is the Reverse Line Blot (RLB) hybridization assay. This macroarray is capable of simultaneously detecting multiple TBPs in a single sample (Bekker et al., 2002; Gubbels et al., 1999).

An essential step in the molecular detection of TBPs in blood is the extraction of DNA. For most DNA extraction methods, the use of a functional molecular biology laboratory is essential, but the cold or frozen storage of blood samples prior to analysis in a dedicated laboratory may be difficult in field situations where a cold chain is not readily available.

In the absence of a functional cold chain, the storage of blood spotted on filter paper forms a promising alternative. Flinders Technology Associates (FTA®) technology (Whatman) has improved filter paper based systems, as it protects the sample from spoiling and degradation, allowing for long-term storage and archiving of nucleic acids at room temperature (RT) (Ahmed et al., 2011). In addition, potential pathogens are lysed and become inactivated on FTA cards, making the samples safe to handle and transport (Abdelwhab et al., 2011).

Several methods to extract DNA from blood spotted on filter paper for the molecular detection of TBPs have been described. These include phenol–chloroform-isoamyl alcohol based extraction (PCI) (Cardoso et al., 2010), the use of FTA purification reagent (Simuunza et al., 2011), Saponin followed by Chelex® (Devos and Geysen, 2004),

Saponin combined with PCI (Tani et al., 2008) and commercial solid-phase extraction kits (Salih et al., 2007). Here we report on the evaluation of six different DNA extraction methods from blood spotted on FTA cards, to determine the optimal protocol in terms of sensitivity and cost-effectiveness for subsequent molecular detection and epidemiological investigation of TBPs by PCR and RLB.

3.3 Materials and methods

3.3.1 Parasite stocks

Four blood samples, stored in liquid nitrogen and infected with *Babesia bovis* (6% parasitemia), *Theileria mutans* (unknown parasitemia), *Anaplasma marginale* (20% parasitemia) and *Ehrlichia ruminantium* (unknown parasitemia) were used in this study. The samples were thawed at RT and subsequently diluted ten-fold with freshly collected whole blood from a non-infected calf to a final dilution of 10^{-10}.

3.3.2 Preparation of samples for DNA extraction

For each TBP, 125 µl of infected blood was spotted on FTA cards, in quadruplicate. After overnight air drying at RT, 3 mm diameter discs were punched out using a Harris Micro-Punch (Whatman) and placed together into Eppendorf tubes for DNA extraction. In order to avoid carryover contamination between samples, discs from FTA cards containing the highest dilution were punched out first and five discs were cut from a blank filter paper after each sample. As negative extraction controls, discs were punched from blank FTA cards and processed together with the samples to be analysed.

3.3.3 DNA extraction

DNA was extracted from FTA cards using the following six DNA extraction protocols:

I. Whatman FTA Protocol - FTA discs were prepared for PCR using FTA purification reagent following Whatman Protocol BD08 with some modifications. Briefly, the discs were washed twice for 15 min with 500 µl FTA purification reagent (Whatman) followed

by two rinses of 15 min with 1.0 ml TE^{-1} buffer (10 mM Tris–HCl, 0.1 mM EDTA, pH 8.0). The discs were left to dry at RT and then transferred to PCR tubes as template for the PCR reaction.

II. Standard phenol-chloroform-isoamyl alcohol (PCI) protocol - The standard PCI method was carried out according to the protocol adapted from Ausubel et al., (1995). Briefly, discs were placed in a 1.5 ml Eppendorf tube. Then 400 µl extraction buffer (10 mM NaCl, 20 mM Tris-HCl, pH 8.0, 1 mM EDTA) and 10 µl proteinase K (20 mg/ml) were added and the mixture was vortexed and incubated at 56 °C for a minimum of 2 hours with agitation. An equal volume of phenol/chloroform/isoamyl alcohol (25:24:1, Sigma-Aldrich) was subsequently added, after which the samples were vortexed briefly and centrifuged for 10 minutes at 13,000 × g. The upper aqueous layer containing the target DNA was preserved and mixed with 50 µl of 3M sodium acetate (pH 5.2) by vortexing. Then, 800 µl of ice-cold 100 % ethanol was added to the mixture and precipitated at -20 °C for at least 90 min. Next, the precipitate was pelleted by centrifugation at 13,000 × g for 10 min. Following removal of the supernatant, 1 ml of 70% ethanol was added to the tube and it was centrifuged at maximum speed for 4 minutes. The supernatant was then completely discarded followed by air drying until no visible liquid remained. Pellets were finally rehydrated with 100 µl of distilled water.

III. Saponin - PCI method - A Chelex-based method in combination with saponin washing and PCI extraction (Tani et al., 2008) was adapted follows: the FTA discs were eluted with 1 ml of 0.5% saponin in PBS, briefly shaken, incubated at 4 °C for 2 h and centrifuged at 8,200 × g for 5 min. The supernatant was removed and replaced with 1 ml

PBS and centrifuge once again at 8200 x g for 5 min. The supernatant was again removed and replaced with 300 µl of a 10% Chelex® 100 (Bio-Rad Laboratories, Hercules, California, USA) solution. The tubes were then incubated in a heating block at 95 °C for 10 min and centrifuged at 8200 × g for 5 min. The supernatant was transferred to a new tube and centrifuged at 13,000 × g for 5 min. The supernatant was used, mixed with an equal volume of PCI after which DNA was extracted as described in the PCI protocol above.

IV. NucleoSpin® Tissue kit - a NucleoSpin® Tissue Kit support protocol for dried blood spots was followed according to the manufacturer's instructions (Macherey-Nagel, Düren, Germany). The concentrated DNA was eluted with 100 µl of elution buffer prewarmed to 70 °C.

V. FTA purification reagent and extraction using Chelex® 100 resin - for this method, a protocol previously used for the detection of *Trypanosoma* species (Ahmed et al., 2011) was adapted as follows: the FTA discs were washed and prepared using FTA purification reagent following Whatman Protocol BD08 as described above under I. After drying at 45°C for at least 60 minutes, discs were incubated for 30 minutes at 90°C in 100 µl of 5% (w/v) aqueous suspension of Chelex® 100 resin. This was followed by centrifugation of the sample for 3 min at 20,000 × g. The supernatant was subsequently transferred to a new sterile pre-labeled microcentrifuge tube, without disturbing the Chelex® 100 resin pellet.

VI. Microwave irradiation - Microwave irradiation based DNA extraction was modified after a recently published method (Port et al., 2014). Briefly, 50 µl of direct whole blood sample dilutions or 8 dried FTA discs were assessed as starting materials. The dried discs from FTA cards were moisturized in 30 µl sterile PBS. Both types of samples were transferred into 0.5 ml tubes and treated at 900 W for 2 minutes in a microwave (Model R-939IN-A, Sharp, Thailand) until precipitated and condensed droplets were visible on the tube walls. After spinning down the condensed droplets, 2.5 µl of the clear precipitated watery solution containing DNA was used as a template for the PCR.

VII. DNA extraction from whole blood samples

For comparison purpose, DNA was also extracted directly from 50 µl of infected whole blood sample in a final elution volume of 100 µl using the NucleoSpin® Blood kit (Macherey-Nagel, Düren, Germany), according to the manufacturer's instructions.

3.3.4 Quantification of DNA

The extracted DNAs were quantified by using Epoch™ Multi-Volume Spectrophotometer System (BioTek, Winooski, VT, USA). A volume of 2 µL was placed in duplicate on a Take 3™ plate and absorbance was measure at 260 nm. The data was collected and analysed using the Gen5 software. Normalization of DNA amounts was conducted for the dilution series of *B. bovis*, to rule out an effect of varying DNA amounts associated with the efficiency of the different extraction methods on the sensitivity of the assay.

3.3.5 Comparison of different number of 3 mm FTA discs

From serially diluted blood infected with *B. bovis*, DNA was extracted from different numbers of 3 mm FTA card discs (1, 2, 4, 8 and 16 discs) using protocol V.

3.3.6 PCR

Amplification of a fragment of 460–540 bp from the 18S SSU rRNA gene spanning the V4 region of *Babesia* and *Theileria* species was carried out with forward primer, RLB-F2 and reverse primer RLB-R2 (Gubbels et al., 1999; Nijhof et al., 2003). The *Ehrlichia/Anaplasma* PCR was performed using forward primer Ehr-F2 (5′-AGA GTT TGA TCC TGG CTC AG-3′) and reverse primer Ehr-R (5′-biotin-GAG TTT GCC GGG ACT TYT TCT-3′) amplifying a fragment of 460–500 bp from the V1 hypervariable region of the 16S SSU rRNA gene. The PCR reactions were performed in a 25-µl reaction volume consisting of 0.5 U Phusion Hot Start II High-Fidelity DNA polymerase (Thermo Fisher Scientific), 5 µl of 1×HF buffer, 200 µM of each dNTPs, 10 pmol of each primer and 2.5 µl of the template DNA or, in case of the Whatman FTA protocol, 1 FTA disc. PCR cycle parameters included an initial denaturation at 98 °C for 30 s, followed by 10 cycles of 98 °C for 5 s, 68 °C for 5 s, 72° C for 7 s, with lowering of the annealing step after every second cycle with 2 °C. The reaction was then followed by 40 cycles of denaturation at 98 °C for 5 s, annealing at 58 °C for 5 s and extension at 72 °C for 7 s. PCRs were performed in a C1000™ thermal cycler (Bio-Rad Laboratories, Hercules, California, USA). PCR products (5 µL) were analysed by gel electrophoresis on 1.5% (W/V) agarose gels stained with GR Green (Labgene Scientific, Châtel-St-

Denis, Switzerland). Gel images were obtained using a G:box imaging system (Syngene, Cambridge, UK).

3.3.7 Reverse line blot hybridization (RLB)

RLB oligonucleotide probes

A list of RLB probes and their sequences used for detecting pathogen DNA used in this study are presented in Table 1. The RLB was performed as previously described (Nijhof et al., 2005). Chemiluminescent signals were detected using an electronic documentation system (ChemoCam, Intas, Göttingen, Germany).

Table 1 RLB probes used in this study.

Oligonucleotide probe specificity	Sequence (5'→3')	Reference
Anaplasma and *Ehrlichia* catch-all	GGG GGA AAG ATT TAT CGC TA	(Bekker et al., 2002)
Anaplasma marginale	GAC CGT ATA CGC AGC TTG	(Bekker et al., 2002)
Babesia and *Theileria* catch-all	CTG TCA GAG GTG AAA TTC T	(Gubbels et al., 1999)
Babesia bovis	ATG GAA TAA CCT TGT ATG ACC C	This study
Babesia catch-all	ATT AGA GTG TTT CAA GCA GAC	(Bhoora et al., 2009)
Bacteria catch-all	CTA CGG GAG GCA GCA GT	This study
Ehrlichia ruminantium	AGT ATC TGT TAG TGG CAG	(Bekker et al., 2002)
Theileria mutans	CTT GCG TCT CCG AAT GTT	(Gubbels et al., 1999)

3.4 Results

3.4.1 Comparison of different DNA extraction protocols

Different methods for the DNA extraction from FTA cards were evaluated using serial dilutions of *B. bovis* infected blood spotted on FTA cards. DNA extraction directly from whole blood sample using a commercial NucleoSpin® Blood kit (protocol VII) was used as a reference in order to compare different extraction protocols from FTA cards. For this protocol, the detection limit for the PCR and RLB was $1x10^{-4}$ and $1x10^{-5}$ respectively. When six different DNA isolation protocols from FTA cards were compared, the best outcome was achieved when discs were washed with FTA purification reagent followed by extraction with 5% Chelex® 100 resin, with a detection limit of $1x10^{-3}$ (Table 2). Normalization of DNA concentrations among the *B. bovis* dilution series did not result in a change in the detection limits mentioned above (data not shown).

Table 2 RLB detection limits of different DNA extraction protocols using serially diluted blood infected with *Babesia bovis*.

Protocol	Method	Sample type	No. of 3 mm discs (amount of blood)	Detection limits
I	Whatman FTA Protocol	FTA card discs	1[a]	10^{-1}
II	Standard PCI method	"	8	10^{-2}
III	Saponin - PCI method	"	8	10^{-2}
IV	NucleoSpin® Tissue kit	"	8	10^{-2}
V	FPR + Chelex[b]	"	1	10^{-2}
V	FPR + Chelex[b]	"	2	10^{-2}
V	FPR + Chelex[b]	"	4	10^{-2}
V	FPR + Chelex[b]	"	8	10^{-3}
V	FPR + Chelex[b]	"	16	10^{-4}
VI	Microwave irradiation	"	8	10^{-2}
VI	Microwave irradiation	whole blood	50 µl	10^{-3}
VII	NucleoSpin® Blood kit	"	50 µl	10^{-5}

[a] Only one FTA card disc was used since it is practically not feasible to use more discs directly in the PCR mix; [b] Washing with FTA purification reagent followed by extraction with 5% Chelex® 100 resin. PCI: phenol–chloroform-isoamyl alcohol based extraction; FPR: FTA purification reagent.

The maximum detection limit recorded for investigation of whole blood samples by microwave irradiation was 1×10^{-3}. It was only possible to process a maximum of eight FTA cards by microwave irradiation since the Eppendorf tube exploded when a higher number of discs were used. With eight FTA card discs, the maximum detection limit for this technique was 1×10^{-2}, equivalent with that of both PCI methods (protocols II and III) and the commercial kit (protocol IV).

3.4.2 Comparison of different numbers of FTA discs

Following the selection of the optimal DNA extraction procedure, the use of different numbers of 3 mm FTA card discs spotted with serially diluted *B. bovis* infected blood as starting material for the DNA extraction was evaluated. The use of one, two or four 3 mm FTA card discs as template for the PCR reaction did not result in differences in the

detection limit (Fig. 1). An increase in the detection limit could be observed when DNA extracted from eight or sixteen discs was used as a template, with positive signals up to 1,000 and 10,000 fold dilutions respectively, corresponding to parasitemias of 0.006 and 0.0006%.

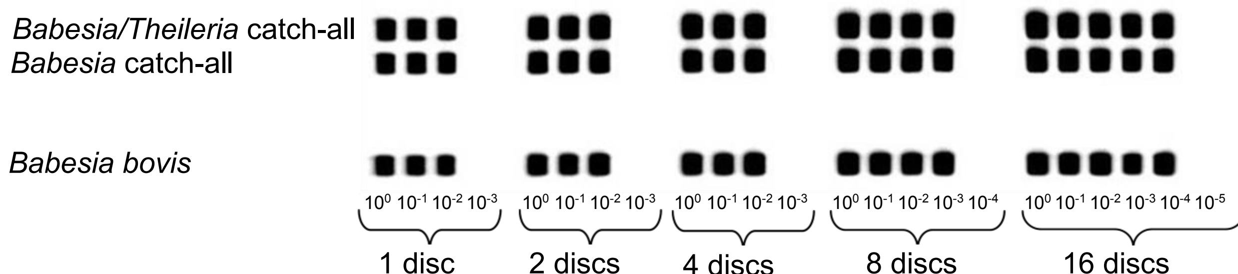

Figure 1 Reverse Line Blot of PCR products performed on different numbers of 3 mm FTA card discs with serially diluted *Babesia bovis* infected blood. DNA was extracted using FTA purification reagent, followed by elution with Chelex® 100 resin (protocol V).

3.4.3 Detection limits for different TBPs

After the use of FTA purification reagent and extraction with 5% Chelex® 100 resin was established as the best approach for DNA extraction from FTA cards using serially diluted blood infected with *B. bovis*, the sensitivity of this extraction method was determined for serial dilutions of three other TBPs: *A. marginale*, *E. ruminantium* and *T. mutans.* The detection limit for DNA extracted from 16 FTA discs with a 3 mm diameter was found to be $1x10^{-4}$ for *T. mutans*, $1x10^{-6}$ for *A. marginale* and $1x10^{-3}$ for *E. ruminantium* (Table 2). The sensitivity of the RLB for both protozoa (*T. mutans* and *B. bovis*) was 10 times higher for DNA extracted from whole blood compared to DNA extracted from 16 FTA discs. This difference was not observed for both rickettsial samples (*A. marginale* and *E.*

ruminantium). In most cases, detection of TBPs by RLB analysis was found to be approximately ten times more sensitive than by gel electrophoresis (Table 3).

Table 3 Detection limits of PCR/gel electrophoresis and RLB for DNA extracted directly from whole blood dilutions and from FTA cards using FTA purification reagent and Chelex® with (FPR + C) for blood infected with different TBP.

Tick-borne pathogen	Detection limits			
	Direct DNA extraction from Whole blood dilutions		DNA extraction from FTA cards with FPR +C (16 discs)	
	PCR	RLB	PCR	RLB
Babesia bovis	10^{-4}	10^{-5}	10^{-3}	10^{-4}
Theileria mutans	10^{-4}	10^{-5}	10^{-3}	10^{-4}
Anaplasma marginale	10^{-6}	10^{-6}	10^{-5}	10^{-6}
Ehrlichia ruminantium	10^{-2}	10^{-3}	10^{-2}	10^{-3}

FPR: FTA Purification reagent, C: Chelex®100 resin

3.5 Discussion

FTA cards are useful for sample storage and transportation in areas where infrastructure for the cold storage and transport of blood samples is not available and in situations where the transport of frozen forms of biohazardous agents from one geographical region to another is complicated by international regulations (Michaud et al., 2007). In this study, we aimed to identify the most sensitive method among six commonly used methods for DNA extraction from FTA cards for subsequent molecular detection of TBPs.

DNA extraction using only FTA purification reagent as described in the Whatman FTA Protocol (without elution with 5% Chelex® 100 resin), where the washed FTA disc was directly used as a template for the PCR mix, yielded detection up to $1x10^{-1}$. This is likely to be caused by the use of only one FTA card disc since it is practically not feasible to use a higher number of discs directly in the PCR mix. Additionally, the use of a washed FTA disc without the use of a chelating agent might have caused carryover of PCR inhibitors from the blood, resulting in poor detection limit observed by PCR and RLB. This could explain the observed difference in detection limit between protocols I and V when one FTA disc was used as starting material (Table 1). The use of Chelex helps to overcome the inhibitory effects of blood components such as heme, lactoferrin and IgG, and also favors dissociation of DNA into single strands thereby facilitating downstream PCR amplification (Becker et al., 2004; Walsh et al., 2013). Another advantage of using FTA purification reagent in combination with 5% Chelex® 100 resin is its safety, since it does not require the use of organic toxic solvents such as phenol.

The detection limit for *B. bovis* remained constant at $1x10^{-2}$ level when 1, 2 or 4 discs were used for DNA extraction and increased to 10^{-3} when 8 discs were used. When the number of discs further increased to 16 discs, the detection limit also improved further to 10^{-4}. This maximum detection limit was only observed when DNA was extracted from FTA cards using FTA purification reagent combined with elution with 5% Chelex® 100 resin. A similar trend was reported in a study for application of FTA cards in the diagnosis of *Trypanosoma brucei* (Ahmed et al., 2011). It was not practically feasible to use more than 16 discs. The improved detection limit with increasing number of discs considered as starting material examined can be explained by an increasing chance of obtaining parasite DNA (Cox et al., 2010). This will eventually lead to an improved sensitivity in downstream molecular detection techniques.

Using microwave irradiation (protocol 6), it was possible to detect *B. bovis* DNA until a parasitemia of 0.06 % and 0.006 % in DNA samples extracted from blood applied on FTA cards and from whole blood sample, respectively. Even though the detection limit of this technique is less compared to that of the commercial DNA extraction kit used for DNA extraction directly from whole blood or the optimized protocol for the extraction of DNA from FTA discs, it does offer a cheap, easy and fast alternative method of DNA extraction and may find use in resource-poor settings.

Comparison of evaluation of detection limits of RLB and PCR was performed for the four major tick-borne pathogens of cattle. This showed that the sensitivity of DNA extraction

from FTA card may differ between different TBPs. The sensitivity of the RLB was in nearly all instances 10x higher compared to gel electrophoresis. Similar observations were reported regarding higher sensitivity of RLB in comparison to PCR for the detection of different pathogens (Abbasi et al., 2009; Niu et al., 2012; Xiang et al., 2007; Xiong et al., 2006).

In this study, we evaluated different DNA extraction methods using freshly prepared FTA cards. Although DNA from blood preserved on FTA cards is rather stable over a long time period, DNA concentration and integrity was recently shown to be affected by prolonged storage of up to 16 years (Rahikainen et al., 2016). This should be taken into account when conducting studies in which the use of high quality DNA is important.

3.6 Conclusions

DNA extraction from blood spotted on FTA cards is an attractive alternative to the use of whole blood in studies on TBPs, particularly in the absence of facilities for the cold storage of blood samples and for instances where the samples are transported across borders. Amongst the protocols tested in this study, the use of FTA purification reagent in combination with Chelex® resin was the most sensitive method for the extraction of DNA from FTA cards for downstream applications such as the RLB. Besides the choice of DNA extraction protocol from FTA cards, the amount of filter paper material has shown to have marked impact on sensitivity.

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4 CHAPTER 4: Publication II

Molecular detection of tick-borne pathogens in cattle from Southwestern Ethiopia

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4.1 Abstract

Tick-borne diseases (TBDs) cause significant losses among livestock and impact the livelihoods of resource-poor farming communities worldwide. In Ethiopia, detailed studies on the epidemiology of tick-borne pathogens (TBPs) in cattle using sensitive molecular detection methods are scarce. The objective of this study was to determine the prevalence and species composition of bovine TBPs of veterinary significance in local cattle populations. A cross-sectional epidemiological study was conducted in cattle populations of Illubabor zone in Southwestern Ethiopia from June to August 2013. For this purpose, blood samples were collected from 392 cattle. A combination of polymerase chain reaction (PCR) and a Reverse Line Blot (RLB) hybridization assay was employed for the detection of TBPs in these samples. The PCR/RLB results of the 392 blood samples indicated a high overall prevalence of 96.9% for TBPs, including *Theileria mutans* (66.1%), *Theileria orientalis* (51.8%), *Anaplasma* sp. Omatjenne (25.5%), *Anaplasma marginale* (14.5%), *Babesia bigemina* (14.0%) and *Theileria velifera* (13.0%) and minor occurrences of *Ehrlichia ruminantium* (0.5%) and *Ehrlichia minasensis* (0.26%). Moreover, three novel *Anaplasma* genotypes were detected in bovine blood samples. A phylogenetic analysis revealed that they most likely represent three, but at least two, new species. The prevalence of the three novel *Anaplasma* species, preliminary designated as *Anaplasma* sp. Hadesa, *Anaplasma* sp. Saso and *Anaplasma* sp. Dedessa, was 12.5%, 14.3% and 5.6%, respectively. Overall, a total of 227 cattle (57.9%) were found to be co-infected with two or more TBPs simultaneously and 86 different species combinations were observed. The findings show a very high burden of infection of cattle with TBPs in Ethiopia. The high frequency of co-infections suggests that

clinical manifestations might be complex. Further research is required to determine the pathogenicity, host cell types and vector of the three novel *Anaplasma* species identified in this study.

4.2 Introduction

With approximately 54 million heads of cattle, Ethiopia is considered to have the largest livestock population in Africa. The majority of the population is comprised of local zebu breeds, but the numbers of imported *Bos taurus* breeds and their crosses are increasing (CSA, 2013). In Ethiopia, cattle are infested by several species of hard ticks (Acari: Ixodidae) including *Amblyomma cohaerens*, *Amblyomma variegatum*, *Rhipicephalus decoloratus*, *Rhipicephalus pulchellus*, *Rhipicephalus evertsi evertsi*, *Rhipicephalus praetextatus* and *Hyalomma rufipes* (Mekonnen et al., 2007). These ticks can act as vectors for a variety of pathogens with veterinary and zoonotic importance and several tick-borne diseases (TBDs) are known to be endemic in Ethiopia. This includes bovine babesiosis caused by *Babesia bigemina* and *Babesia bovis*, bovine anaplasmosis caused by *Anaplasma marginale* and heartwater caused by *Ehrlichia ruminantium.* In addition, a number of mildly pathogenic *Theileria* species such as *Theileria mutans, T. velifera*, and *T. orientalis* have also been reported to occur (Tomassone et al., 2012; Kumsa et al., 2014; Kumsa et al., 2015; Teshale et al., 2015). In indigenous breeds of cattle, the course of these TBDs is usually subclinical. However, they pose a greater challenge to susceptible exotic breeds of cattle, thus representing a major constraint in the upgrading and development of cattle production in Ethiopia (Mekonnen et al., 2007).

Given the importance of cattle husbandry in Ethiopia, information regarding the prevalence and species composition of bovine tick-borne pathogens (TBPs) is of paramount importance. However, studies on the epidemiology of these pathogens in Ethiopia are remarkably scarce. Accordingly, this study aimed to investigate the occurrence of bovine TBPs of veterinary significance in Southwestern Ethiopia. Due to

the existence of diverse species of TBPs known to be transmitted by hard ticks in Ethiopia, this study relied on a combination of PCR and a Reverse Line Blot (RLB) hybridization assay for the simultaneous detection of TBPs from bovine blood samples.

4.3 Materials and methods

4.3.1 Study area

The study was conducted in Illubabor zone of Oromia Regional State, Southwestern Ethiopia (Fig. 1). Illubabor zone is located at 7°27′40″ to 9°2′10″ North and 34°52′12″ to 41°34′55″ East. The zone has 1.6 million hectares of land with altitudes ranging from 500 to 2575 meter above sea level (m.a.s.l), 10% of which is highland (2500–2575 m.a.s.l.), 67% is midland (1500–2500 m.a.s.l.) and 23% is lowland (500–1500 m.a.s.l.). The temperature varies from 18 °C to 24 °C and annual precipitation ranges from 1500-2200 mm, with 6 to 9 months of rainfall. Illubabor zone is divided into 24 administrative districts of which 2 are urban and 22 are rural. Approximately 1.6 million persons live in the region, of which 88% live in the rural areas. Agriculture is the mainstay of the economy with mixed farming system practiced at subsistence level. The size of the cattle population in the zone is approximately 1.3 million (IZOARD, 2013).

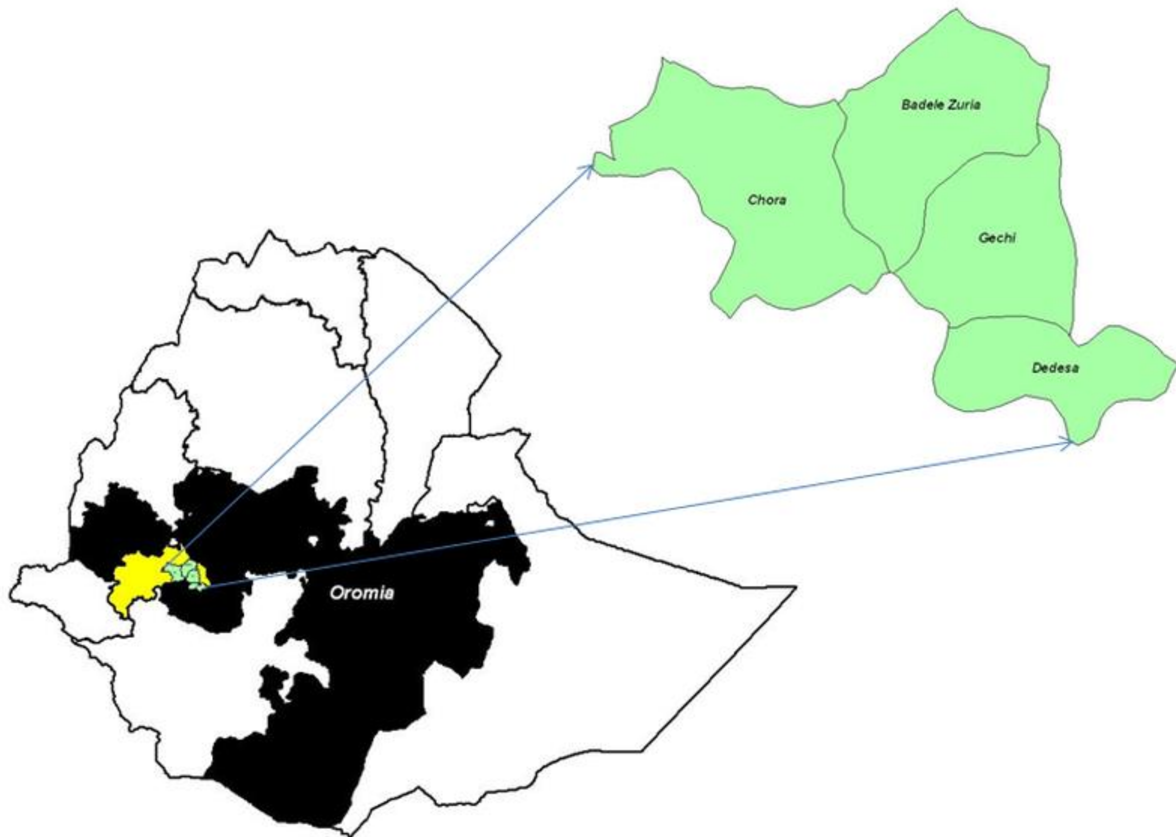

Fig 1. Map of Ethiopia with the study area. Districts studied indicated in green color and Illubabor zone of Oromia region indicated in yellow color.

4.3.2 Study design, sample size and sampling strategies

A cross-sectional study design was employed to address the objective of this study. The sample size was determined using the online Epitools epidemiological calculators (Sergeant, 2009). The level of confidence and absolute precision desired were set to 95% and 0.05, respectively while the expected (unknown) prevalence was set to 50%. Having stratified the study population by districts (4) and peasant associations (PAs) (12), proportional allocation was utilized to determine the number of cattle to be sampled per

stratum. Combinations of stratified, multistage and purposive sampling methods were applied following previously published guidelines (Putt, 1987; Toma et al., 1999). Four districts in the Illubabor Zone were selected purposively based on previous history of occurrence of TBDs (first stage). Then a list of PAs within districts was compiled from data obtained from the districts' agricultural offices (second stage) and three sampling PAs from each district were randomly selected using a lottery system. Villages in each PA were selected in collaboration with the respective district's animal health personnel by purposive sampling based on farmers' cooperation, logistics and share of communal grazing land (third stage). From selected villages, herds grazing within the same grazing land were considered as primary sampling unit. Then cattle were sampled randomly from each grazing herd.

4.3.3 Sample collection and processing

The sampling was carried out in July and August 2013. All cattle included in the study were selected and sampled with the consent of their owners and chiefs of the villages. Approximately 4 ml of blood was collected from each selected animal by jugular venipuncture in ethylenediaminetetraacetic acid (EDTA) containing Vacutainer tubes (BD Biosciences, Franklin Lakes, NJ, USA). Blood samples were also collected from an ear vein pierced with a lancet after which blood was drained into 75 mm × 1.5 mm heparin-treated haematocrit capillary tube for the measurement of packed cell volume (PCV). A drop of blood from the ear vein was also used to prepare thin blood smears. Ethical approval for this study was obtained from the Research and Ethics Committee of the

College of Veterinary Medicine, Haramaya University, Ethiopia (Ref.: CVM/76/13). Informed consent was obtained from all livestock owners included in the study.

A standardized questionnaire was used to obtain information regarding farm management practices and possible risk factors associated to infection with TBPs. The questionnaires comprised questions regarding the type of acaricide used, timing and frequency of acaricide application, drug administered to treat clinical cases of TBDs and the farmers' knowledge about ticks and TBDs.

Initial sample processing was conducted at Bedelle regional veterinary laboratory, Ethiopia. Upon reaching the laboratory, 125 µl of EDTA blood was spotted on Flinders Technology Associates (FTA) Classic® cards (FTA cards, Whatman Biosciences, Cambridge, UK) and allowed to air-dry over night at room temperature.

Air-dried thin blood smears were fixed in methanol for 5 min and stained with 10% Giemsa's stain (Merck, Darmstadt, Germany) for 45 min. At least 50 microscopic fields were examined per slide at 1000× magnification (oil immersion). Presence of tick-borne haemoparasites was recorded; identification was carried out to the genus and, where possible, the species level (Norval et al., 1992).

Blood samples in microhematocrit capillary tubes were centrifuged using a microhaematocrit centrifuge (Hawksley, Sussex, UK) at 12,000 rpm for 5 min. The PCV was measured using a microhaematocrit reader (Hawksley, Sussex, UK). Animals with a PCV lower than 24% were considered anemic.

4.3.4 DNA extraction from FTA cards

DNA was extracted from FTA cards following a previously described protocol (Hailemariam et al., 2017). Briefly, sixteen 3 mm diameter discs of each sample were punched out using a Harris Micro-Punch (Whatman) and placed into 1.5 ml Eppendorf tubes. In order to avoid carryover contamination between samples, discs were cut from a blank filter paper after each sample. As negative extraction controls, discs were punched from blank FTA cards and processed together with the other samples. The FTA discs were washed and prepared using FTA purification reagent following Whatman Protocol BD08. After drying at 45 °C for at least 60 min, discs were incubated at 90 °C in 100 µl of 5% (w/v) aqueous suspension of Chelex® 100 resin for 30 min. This was followed by centrifugation of the sample at 20,000 × g for 3 min. The supernatant was subsequently transferred to a new sterile pre-labelled microcentrifuge tube and used as a template for the PCR.

4.3.5 Molecular detection of tick-borne pathogens

Amplification of a fragment of 460-540 bp from the 18S SSU ribosomal ribonucleic acid (rRNA) gene spanning the V4 region of *Babesia* and *Theileria* species was carried out with forward primer, RLB-F2 and reverse primer RLB-R2 (Nijhof et al., 2003). A second PCR was performed using forward primer Ehr-F2 (5′-AGA GTT TGA TCC TGG CTC AG-3′) and reverse primer Ehr-R2 (5′-biotin-GAG TTT GCC GGG ACT TYT TCT-3′) amplifying a fragment of 460–500 bp from the V1 hypervariable region of the rickettsial 16S rRNA gene (Hailemariam et al., 2017). The PCR reactions were performed in a 25

µl reaction volume consisting of 0.2 mM dNTPs (Thermo Fisher Scientific), 0.5 µM of each primer, 0.02 U/µl Phusion Hot Start II High Fidelity DNA Polymerase (ThermoFisher Scientific) and 2.5 µl template DNA in 1 × Phusion HF buffer (Thermo Fisher Scientific). The *Babesia/Theileria* PCR cycle parameters included an initial denaturation at 98 °C for 30 s, followed by 10 cycles of 98 °C for 10 s, 68 °C for 20 s, 72° C for 15 s, with lowering of the annealing step after every second cycle by 2 °C until an annealing temperature of 58°C was reached. The reaction was followed by 40 cycles with annealing at 58 °C for 20 s before a final extension at 72 °C for 8 min was conducted. The annealing temperature in the *Ehrlichia/Anaplasma* PCR cycle started at 71°C and was gradually lowered to 61°C, all other conditions were similar to the *Babesia/Theileria* PCR protocol. Gel electrophoresis and RLB were performed as previously described (Hailemariam et al., 2017). A list of RLB probes used for detecting pathogen DNA in this study is presented in Table 1.

Table 1. List of RLB probes used in this study.

Species	**Sequence (5’-3’)**	**Reference**
Anaplasma bovis	GTAGCTTGCTATGAGAACA	(Bekker et al., 2002)
Anaplasma centrale	TCGAACGGACCATACGC	(Bekker et al., 2002)
Anaplasma marginale	GACCGTATACGCAGCTTG	(Bekker et al., 2002)
Anaplasma sp. Dedessa	ACGGATTATATTTGTAGCTTGCT	this study
Anaplasma sp. Hadesa	AGCTTGCTACAGAAGTAATTAGTGG	this study
Anaplasma sp. Omatjenne	CGGATTTTTATCATAGCTTGC	(Bekker et al., 2002)
Anaplasma sp. Saso	GTCGAACGGATTTTTATCATAGC	this study
Babesia bigemina	CGTTTTTTCCCTTTTGTTGG	(Gubbels et al., 1999)
Babesia bovis	CAGGTTTCGCCTGTATAATTGAG	(Gubbels et al., 1999)
Babesia caballi	GTGTTTATCGCAGACTTTTGT	(Butler et al., 2008)
Babesia genus specific 2	ACTAGAGTGTTTCAAACAGGC	(Bhoora et al., 2009)
Babesia genus-specific 1	ATTAGAGTGTTTCAAGCAGAC	(Bhoora et al., 2009)
Bacteria catch-all	CTACGGGAGGCAGCAGT	

Species	**Sequence (5'-3')**	**Reference**
Ehrlichia / Anaplasma genera specific	TTATCGCTATTAGATGAGCC	(Schouls et al., 1999)
Ehrlichia canis	TCTGGCTATAGGAAATTGTTA	(Schouls et al., 1999)
Ehrlichia chaffeensis	ACCTTTTGGTTATAAATAATTGTTA	(Schouls et al., 1999)
Ehrlichia minasensis	CGGACAATTATTTATAGCTTTTGGC	this study
Ehrlichia ruminantium	AGTATCTGTTAGTGGCAG	(Bekker et al., 2002)
Midichloria genus-specific	GCGAAATAACAGTTGGAAGCAAT	this study
Rickettsia aeschlimanni	ATATTATACTGTATGTAGCCCC	(Jado et al., 2006)
Rickettsia africae	ACTAATTTTTGGGGCTTGCTC	this study
Rickettsia catch-all	TAGCTCGATTGRTTTACTTTG	(Jado et al., 2006)
Rickettsia conorii	GTTATATACTGTAGCCCTG	(Jado et al., 2006)
Rickettsia massiliae	CCGCCACGATATCTAGAAAAATTA	this study
Theileria / Babesia genera specific	CTGTCAGAGGTGAAATTCT	(Gubbels et al., 1999)
Theileria annulata	CCTCTGGGGTCTGTGCA	(Georges et al., 2001)
Theileria equi A1	TTGGCGTTTGTCATCGTTGC	this study
Theileria equi A2	GTTGTGGCTTAGTTGGGGCAT	this study
Theileria equi B	CTGTATCGTTATCTTCTGCTTGACA	this study
Theileria genus-specific	ATTAGAGTGCTCAAAGCAGGC	(Bhoora et al., 2009)
Theileria lestoquardi	ATTGCTTGTGTCCCTCCG	(Schnittger et al., 2004)
Theileria mutans	CTTGCGTCTCCGAATGTT	(Gubbels et al., 1999)
Theileria orientalis	GGCTTATTTCGG(AT)TTGATTTT	(Gubbels et al., 1999)
Theileria ovis	TTGCTTTTGCTCCTTTACGAG	(Schnittger et al., 2004)
Theileria parva	TCGGACGGAGTTCGCTTTG	this study
Theileria separata	GGTCGTGGTTTTCCTCGT	(Schnittger et al., 2004)
Theileria sp. (buffalo)	CAGACGGAGTTTACTTTGT	(Oura et al., 2004)
Theileria sp. (sable)	GCTGCATTGCCTTTTCTCC	(Nijhof et al., 2005)
Theileria taurotragi	TCTTGGCACGTGGCTTTT	(Gubbels et al., 1999)
Theileria velifera	CCTATTCTCCTTTACGAGT	(Gubbels et al., 1999)

4.3.6 DNA purification and confirmation of RLB positive samples by sequencing

The 16S or 18S rRNA genes of selected RLB-positive samples were amplified and sequenced to verify the RLB results. For *Babesia* species, a ~550 bp fragment of the 18S rRNA gene was amplified using primers Babesia specific-F (5'-CCA TCA GCT TGA CGG TAG GG-3') and RLB-R2 with the Babesia/Theileria RLB PCR protocol described above.

The same protocol and cycle parameters were also used for the amplification of a ~560 bp fragment of the *Theileria* 18S rRNA gene with primers Theileria specific-F (5'-CTA TCA GCT TTG GAC GGT AGG G-3') and RLB-R2. For confirmation of RLB positive *Anaplasma* and *Ehrlichia* samples, a ~1438 bp fragment of 16S rRNA gene was amplified using forward primer Ehr-F2 and reverse primer AnaEhrl full (5′-CCC TAG TCA CTR ACC CAA CCT TA-3′). PCR cycle parameters included an initial denaturation at 98 °C for 60 s, followed by 40 cycles of 98 °C for 10 s, 61.5 °C for 10 s and 72 °C for 45 s before a final extension at 72 °C for 10 min. The *Anaplasma* and *Ehrlichia* samples were sequenced with reverse primer Ehr-R4 (5′-GAG TTW GCC GGG RCT TYT TCT-3′).

DNA was cleaned directly from the PCR reactions with the DNA Clean & Concentrator™-5 Kit (Zymo Research Corporation, Irvine, USA) or from excised gel bands by the Zymoclean™ Gel DNA Recovery Kit (Zymo Research Corporation) according to the manufacturer's instructions. Purified products were sequenced by LGC Genomics GmbH (Berlin, Germany).

4.3.7 DNA cloning, sequencing and phylogenetic analysis

For characterization of novel *Anaplasma* species, amplification products from positive samples were analyzed by agarose gel electrophoresis and expected bands were excised and purified by the Gel DNA recovery kit (Zymo Research). Purified PCR products were cloned into the StrataClone blunt-end PCR cloning vector 'pSC-B-amp/kan' supplied in the StrataClone Blunt PCR cloning kit (Agilent Technologies, CA, USA) and recombinant plasmid vectors were transformed into Solopack® competent cells (Agilent Technologies,

CA, USA) according to the manufacturer's instructions. Following plasmid DNA isolation using the Plasmid Mini Prep Kit EasyPrep® Pro (Biozym, Oldendorf, Germany), clones with inserts were sequenced by LGC Genomics (Berlin). The 16S rRNA clones were sequenced bidirectionally. The obtained sequences were analyzed by BLASTn search (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

Selected small ribosomal (16S) rRNA sequences from representative members of the family Anaplasmataceae were aligned using MAFFT 7 with the Q-INS-i iterative refinement method which considers RNA structure predictions (Katoh and Toh, 2008). Phylogenetic maximum-likelihood analysis was conducted with RAxML 8.2.9 (Stamatakis, 2014) on the CIPRES gateway (Miller et al., 2010). The GTRGAMMA model was chosen with 25 substitution rate categories and a rapid bootstrap analysis (1000 replicates) with identification of the tree with the highest likelihood in the same run was performed. The resulting tree was used as additional input in a second run to constrain the tree topology and calculate alternative node support values using the Shimodaira–Hasegawa modification of the likelihood ratio test. The tree was visualized using Mega 7 (Kumar et al., 2016) and rooted using the sequences from species outside of the genus *Anaplasma*. Novel *Anaplasma* 16S rRNA sequences obtained in this study were deposited in the GenBank database with accession numbers KY924884 - KY924886.

4.3.8 Statistical analysis

Software used for statistical analysis in this study were IBM SPSS Statistics Version 23.0 and OpenEpi Version 3.03. The prevalence of several pathogens and 95% confidence

intervals were calculated as Wilson Score intervals. Univariate analysis of associations using the Chi-squared test was carried out for each exposure variable, with the RLB based infection prevalence by *B. bigemina* and *A. marginale*, infections considered as a binary outcome (positive or negative). We tried to perform multivariate analysis for risk factor analysis but that was not applicable due to the co-linearity of many variables. The main consideration taken into account to perform risk factor analysis only for *B. bigemina* and *A. marginale* were their economic significance and availability of sufficient data to perform a risk factor analysis. P values less than 0.05 were considered significant. The exposure variables considered were age, sex, breed, management system, acaricide used and frequency of acaricide applications.

4.4 Results

4.4.1 Demography of the study population

Blood samples were collected from a total of 392 apparently healthy cattle in Illubabor zone, Southwestern Ethiopia. Samples were collected from 12 PAs in four districts. The majority of the animals were local Zebu breeds (*Bos taurus*) (371/392; 94.6%), and the rest (21/392; 5.4%) Holstein Friesian x Zebu crossbreds. While the local zebu breeds of cattle were kept under extensive managements system, all cross-bred cattle was managed semi-intensively, i.e. they received supplementary feed in addition to grazing. The study population comprised more females (60.5%, 237/392) than males 39.5% (155/392) (Table 2).

Table 2. Descriptive statistics and univariable analysis of risk factors associated with *B. bigemina* and *A. marginale* infections detected by RLB in cattle from Southwestern Ethiopia.

Variable	Categories	Total No. (%)	*B. bigemina*		*A. marginale*	
			No. + ve (%)	p-value	No. + ve (%)	p-value
Breeds	Zebu	371 (94.6)	50 (13.5)	0.194	47 (12.7)	<0.001*
	Cross-bred	21 (5.4)	5 (23.8)		10 (47.6)	
Sex	Male	155 (39.5)	25 (16.1)	0.37	19 (12.3)	0.38
	Female	237 (60.5)	30 (12.7)		38 (16.0)	
Age	Calf	51 (13.0)	9 (17.6)	0.70	5 (9.8)	0.34
	Young	120 (30.6)	17 (14.2)		15 (12.5)	
	Adult	221 (56.4)	29 (13.1)		37 (16.7)	
Management system	Semi-intensive	21 (5.4)	5 (23.8)	0.19	10 (47.6)	<0.001*
	Extensive	371 (94.6)	50 (13.5)		47 (12.7)	
Acaricide used	Diazinone	170 (43.4)	26 (15.3)	0.28	32 (18.8)	0.06
	Cyper/deltamethrin	52 (13.3)	4 (7.7)		7 (13.5)	
	Amitraz	144 (36.7)	19 (20.2)		18 (12.5)	
	Ivermectin	26 (6.6)	6 (23.1)		0 (0)	
Frequency of acaricide application	1-3× per year	329 (83.9)	48 (14.6)	0.09	40 (12.2)	<0.001*
	4-6× per year	42 (10.7)	2 (4.8)		7 (16.7)	
	6-8× per year	21 (5.4)	5 (23.8)		10 (47.6)	

*As all the 21 cattle managed semi-intensively and sprayed four to six times per year with acaricide are the same 21 cross-bred animals in the breed category, the statistically significant association observed here was not taken as a valid association. No.: Number, + ve: Positive

4.4.2 Microscopic identification of tick-borne haemoparasites

Microscopic examination of Giemsa-stained blood smears revealed that 67 out of 392 (17.1%) samples were positive for at least one TBP. The most frequently observed haemoparasites were *Theileria* spp. (39/392; 10.0%), followed by *A. marginale* (18/392; 4.6%), *B. bigemina* (5/392; 1.3%), mixed infection of *Theileria* spp. and *A. marginale* (3/392; 0.8%) and mixed infection of *B. bigemina* and *A. marginale* (2/392; 0.5%).

4.4.3 RLB based prevalence of hemoparasites

Out of 392 blood samples applied on FTA cards examined for TBPs by PCR/RLB, 380 samples (96.9%) were positive for at least one hemoparasite. DNA from eleven different TBPs including three novel *Anaplasma* species was detected. Among the TBPs detected were *T. mutans* (259/392; 66.1%), *T. orientalis* (203/392; 51.8%), *Anaplasma* sp. Omatjenne (100/392; 25.5%), *A. marginale* (57/392; 14.5%), *B. bigemina* (55/392; 14.0%) and *T. velifera* (51/392; 13.0%), with minor occurrences of six other haemoparasites including the highly pathogenic *E. ruminantium*. Another recently identified pathogenic *Ehrlichia* species, *Ehrlichia minasensis* was detected for the first time in Ethiopia (1/392; 0.3%). The *E. minasensis* sequence generated in this study was 100% (420/420) identical with the *E. minasensis* genotype UFMG–EV 16S rRNA gene sequence deposited in GenBank (JX629805).

When a number of the PCR products were sequenced to confirm the RLB results, three samples showed a mixed sequence content, indicating the presence of multiple sequences. These PCR products were subsequently cloned and re-sequenced. A

preliminary BLASTn analysis of these sequences revealed the presence of three novel 16S rRNA sequences that did not show 100% identity to any known GenBank entry. They were preliminary designated as *Anaplasma* sp. Hadesa, *Anaplasma* sp. Saso and *Anaplasma* sp. Dedessa. Novel RLB probes were developed based on these sequences and all Anaplasma/Ehrlichia positive samples were screened again by RLB on a membrane that included these new probes. The prevalence of the three novel *Anaplasma* genotypes was thus determined to be 49/392 (12.5%), 56/392 (14.3%) and 22/392 (5.6%), respectively (Table 3).

Table 3. Prevalence of tick-borne pathogens in cattle blood samples from Southwest Ethiopia as determined by RLB.

Species	Total (n = 392)	Prevalence (%)	95% CI
A. marginale	57	14.5	11.40 - 18.37
Anaplasma sp. Omatjenne	100	25.5	21.45 - 30.05
E. ruminantium	2	0.5	0.14 - 1.84
E. minasensis	1	0.3	0.05 – 1.43
B. bigemina	55	14.0	10.94 - 17.82
T. mutans	259	66.1	61.25 - 70.58
T. orientalis	203	51.8	46.85 - 56.69
T. velifera	51	13.0	10.04 - 16.70
Anaplasma sp. Hadesa	49	12.5	9.60 - 16.14
Anaplasma sp. Saso	56	14.3	11.17 - 18.10
Anaplasma sp. Dedessa	22	5.6	3.74 - 8.35

CI: confidence interval

4.4.4 Phylogenetic analysis of novel *Anaplasma* spp.

Samples containing *Anaplasma* sp. Hadesa, *Anaplasma* sp. Saso and *Anaplasma* sp. Dedessa were used as templates for the PCR-amplification of a ~1438 bp 16S rRNA gene fragment, followed by cloning, sequencing and a phylogenetic analysis. BLASTn searches showed highest 16S rRNA sequence similarity for *Anaplasma* sp. Hadesa to

Anaplasma phagocytophilum strain HN (KC470064, 1369/1441 bp, 95.0%). *Anaplasma* sp. Saso showed most 16S rRNA sequence similarity with uncultured *Anaplasma* species isolated from canine blood in the Philippines (KP006398, 1372/1440 bp, 95.3%). *Anaplasma* sp. Dedessa showed highest sequence similarity to *Anaplasma* sp. BL099-6 (KJ410247, 1406/1417 bp, 99.2%) originally isolated from *Hyalomma* ticks in China. Comparisons between the three novel *Anaplasma* genotypes revealed 99.1% identity between *Anaplasma* sp. Hadesa and *Anaplasma* sp. Saso. In contrast, identity of *Anaplasma* sp. Dedessa to the other two genotypes was only 95%. In comparison, identity between the three closely related species *A. marginale*, *A. centrale* and *A. ovis* was between 99.3 and 99.5%. For the phylogenetic analysis, only the 16S rRNA gene was included since all three novel *Anaplasma* genotypes occurred as mixed infections and it was not possible to unequivocally pair the 16S rRNA genes with additional genes belonging to the same *Anaplasma* species. The analysis identified all three new genotypes with high support values as members of the cluster formed by the genus *Anaplasma*. *Anaplasma* sp. Dedessa could be identified with high confidence as a closely related relative of *Anaplasma* sp. BL099-6. In contrast, the position of *Anaplasma* sp. Hadesa and *Anaplasma* sp. Saso, which are more closely related to each other than to any other member of the genus *Anaplasma* included in the analysis, could not be ascertained with confidence (Fig. 2). Branch lengths between these genotypes (0.012 substitutions/site) are nearly twice as long as between *A. marginale* and *A. ovis* (0.00637) and 33-45% longer than for the comparisons of *A. centrale* with *A. ovis* (0.00818 substitutions/site) and *A. centrale* with *A. marginale* (0.00899 substitutions/site)

suggesting that they might represent two closely related species and not only genotypes of the same species.

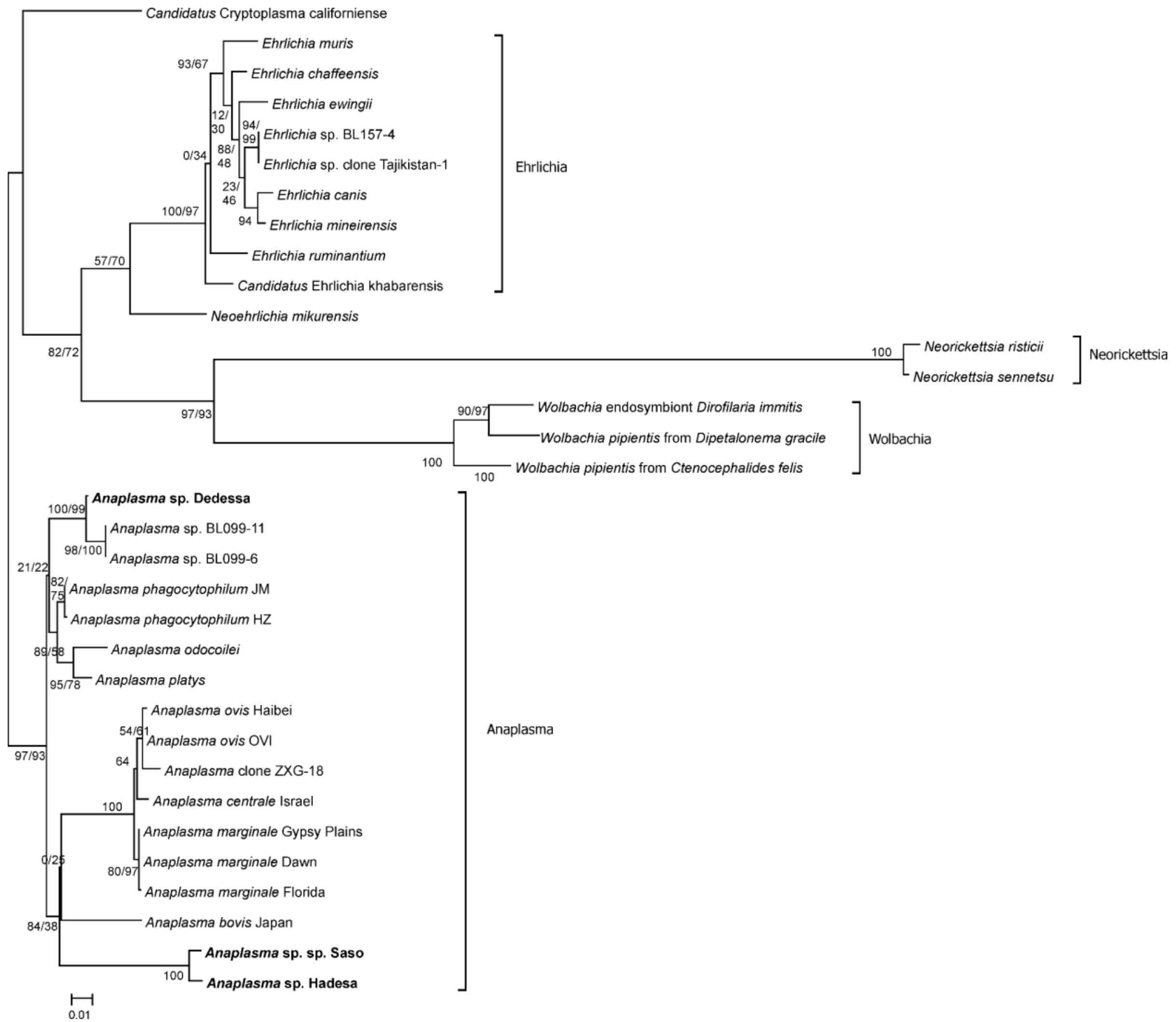

Fig 2. Phylogenetic analysis of *Anaplasma* species identified in this study. Maximum-likelihood phylogenetic analysis of Anaplasmataceae using 16S rRNA sequences. The sequences from other genera in the family Anaplasmataceae were included to serve as outgroup. Numbers before and after the slashes represent node support values obtained by the Shimodaira–Hasegawa likelihood ratio test and bootstrapping, respectively. The scalebar represents 0.01 substitutions per side. The sequences obtained in the present study are highlighted in bold.

4.4.5 Co-infections analysis

A total of 227 cattle (57.9%) were found to be simultaneously co-infected with two or more TBPs. Overall, 86 different species combinations were observed. The level of co-infections ranged from double to sextuple. The majority of the mixed infections occurred as double infections (97/227; 42.7%). The most frequent co-occurrences included *T. orientalis* and *T. mutans, B. bigemina* and *T. mutans, T. orientalis* and *T. velifera,* and *Anaplasma sp. Omatjenne* and *T. orientalis* (Table 4 and S1 Table).

Table 4. Observed level and frequency of co-infections by different species of tick-borne pathogens.

Level of co-infections	Frequency	%	No of species combinations
Double	97	42.7	12
Triple	56	24.7	26
Quadruple	38	16.7	22
Quintuple	30	13.2	20
Sextuple	6	2.6	6
Overall	**227**	**100**	**86**

S1 Table. Combinations of mixed infections observed in FTA cards examined for TBPs by PCR/RLB.

	Nature of infections											
	Single		**Double**		**Triple**		**Quadruple**		**Quintuple**		**Sextuple**	
	Sp.	**Total**	**Spp.**	**Total**	**Spp.**	**Total**	**Spp.**	**Total**	**Spp.**	**Total**	**Spp.**	**Total**
1.	To	80	ToTm	43	EoToAh	6	EoToTmAs	5	AmEoToTmAs	6	AmBbToTmAdAs	1
2.	Tm	65	BbTm	12	EoBbTm	5	AmEoBbTm	4	EoToTmTvAs	3	AmEoBbToTmTv	1
3.	Bb	6	TmTv	9	EoToTm	5	ToTmAhAs	3	AmBbTmTvAs	2	AmEoTmTvAhAs	1
4.	Am	1	EoTo	8	AmTmAh	3	AmEoTmAh	2	EoBbToTmAs	2	EoBbTmTvAdAh	1
5.	Eo	1	EoTm	6	AmToTm	3	AmEoToTm	2	EoToTmAhAs	2	EoBbTmTvAhAs	1
6.			AmTm	5	BbTmTv	3	AmToTmAd	2	AmBbToTmAs	1	EoToTmAdAsAh	1
7.			TmAh	4	EoTmAs	3	EoTmTvAh	2	AmEoBbTmAs	1		
8.			AmTo	3	EoToAs	3	EoToTmAh	2	AmEoTmTvAd	1		
9.			BbTo	3	AmEoTm	2	EoToTmTv	2	AmEoTmTvAs	1		
10.			EoBb	2	AmTmAs	2	TmTvAdAh	2	AmTmTvAhAs	1		
11.			AhAd	1	AmTmTv	2	AmBbTmAs	1	AmToTmAdAs	1		
12.			ToAh	1	AmToAs	2	AmEoTmAs	1	AmToTmTvAs	1		
13.					EoTmTv	2	AmErEoTm	1	EoBbTmAhAs	1		
14.					TmTvAs	2	AmTmTvAh	1	EoBbTmTvAd	1		
15.					ToAhAs	2	AmToTmTv	1	EoBbTmTvAh	1		
16.					AmBbTm	1	BbTmTvAd	1	EoBbTmTvAs	1		
17.					AmEoTo	1	EoBbTmAh	1	EoTmTvAdAh	1		
18.					EoBbAs	1	EoBbTmAs	1	EoTmTvAhAs	1		
19.					EoTmAd	1	EoBbToTm	1	EoToTmTvAd	1		
20.					EoTmAh	1	EoTmTvAs	1	EoToTmTvAh	1		
21.					EoToAd	1	TmTvAhAs	1				
22.					ErToTm	1	ToTmAhAd	1				
23.					TmAhAs	1						
24.					TmTvAh	1						
25.					ToTmAh	1						
26.					ToTmTv	1						
Total		**153**		**97**		**56**		**38**		**30**		**6**

Abbreviations: Am, *A. marginale;* Eo, *Anaplasma* sp. Omatjenne; Bb, *B. bigemina;* Tm, *T. mutans;* To, *T. orientalis;* Tv, *T. velifera;* Ah, *Anaplasma* sp. Hadesa; As, *Anaplasma* sp. Saso; Ad, *Anaplasma* sp. Dedessa.

Summary: Total combinations of mixed infections: 86; Proportions of samples based on nature of infections: negative samples: 12 (3%); single infection: 153/392 (39.0%); mixed infections: 227/392 (57.9%)

4.4.6 Risk factor analysis

Univariate analysis of potential risk factors revealed that breed of cattle was significantly associated with *A. marginale* infection ($p < 0.05$) (Table 2). Cross-bred cattle (47.6%, Odds ratio [OR] = 6.23, 95% CI [2.52; 15.56]) were more likely to be infected with *A. marginale* than local Zebu breed cattle. The same 21 animals were also the only one kept under a semi-intensive management system (OR = 6.23) and that were regularly treated with acaricides. Due to this fact, these variables were in the same or a similar way associated with an increased odd to be positive for *A. marginale*. Although the difference in prevalence of *B. bigemina* infection did not significantly differ compared to local zebu breeds ($p > 0.05$), a higher proportion of infection with *B. bigemina* was also observed in cross-bred cattle.

4.5 Discussion

In this study, a combination of PCR and a RLB hybridization assay was employed for the simultaneous detection of TBPs from bovine blood samples collected on FTA cards. The use of FTA cards facilitated the collection, storage and shipment of the blood samples and was also in compliance with German customs import regulations, which prohibits the import of whole blood samples from Foot and Mouth Disease endemic countries such as Ethiopia. A thorough evaluation of six DNA extraction methods from blood spotted on FTA cards was previously conducted to ensure the use of an optimal extraction method for the analysis of the field samples (Hailemariam et al., 2017). However, direct DNA extraction from 200 µl of whole blood using a commercial spin column based method was shown to slightly increase the detection limit of the RLB compared to the use of DNA extracted from sixteen 3 mm diameter FTA discs (Hailemariam et al., 2017). It is therefore possible that the results reported here still underestimate the true prevalence of TBPs in Ethiopia.

A complex pattern of co-infections was observed in this study. Mixed infections were detected in 226 samples (57.65%) and overall 86 different species combinations were observed. The highest frequency of co-infection was recorded for *T. mutans* and *T. orientalis* (Additional file 2), which were also the most frequently encountered TBPs, with RLB-based prevalence of 66.1% and 51.8%, respectively. In endemic areas, infections with these mildly pathogenic *Theileria* species are usually acquired by calves early in their lifes, after which they remain life-long carriers (Sugimoto and Fujisaki, 2002). This may explain the high prevalence found for both species. At a more generic level, the high frequency of occurrence of mixed infections may increase or decrease the pathogenicity of existing infections. Results of a recent study suggested heterologous protection by

mildly pathogenic *Theileria* species against East Coast Fever caused by *T. parva*, reducing the severity of infection (Woolhouse et al., 2015). However, interactions between different TBP can be much more complex and involve ecological, epidemiological and also clinical aspects (Swanson et al., 2006; Baneth, 2014; Diuk-Wasser et al., 2016). For instance, one pathogen might enhance transmission of the other in the ecosystem as observed for *Borrelia burgdorferi* and *Babesia microti* or mutually enhance disease processes and promote disease severity as reported for *B. burgdorferi* and both, *B. microti* and *Anaplasma phagocytophilum*. Clinical symptoms in co-infected hosts can considerably deviate from typical patterns observed in mono-infected animals, which hampers diagnosis and can lead to treatment failures since only one of the two diseases was recognized.

Besides protozoa, DNA from several bacteria was detected by RLB, including *A. marginale* (14.5%) and *Anaplasma sp. Omatjenne* (25.5%). *Anaplasma marginale* is known to be pathogenic to domestic ruminants, especially in high producing dairy cattle. Under mixed farming systems, serological investigations elsewhere in East Africa showed seroprevalences of 58% for Mbeere District, Kenya (Gachohi et al., 2010), 57% for Soroti District, Uganda (Kabi et al., 2008) and 50% in Central Equatoria State, South Sudan (Malak et al., 2012). The prevalence of *A. marginale* reported in this study is lower compared to the above mentioned results. This is presumably due to the fact that RLB detects active infections or carrier animals, whereas serology cannot differentiate between active and past infection. Cross-reactions between pathogens of the *Anaplasma* genus on serological assays have also been reported and may lead to an over-estimation of the true prevalence when performing a serological screening only (Dreher et al., 2005).

This study also confirms the occurrence of *Anaplasma* (formerly *Ehrlichia*) sp. Omatjenne in Ethiopian cattle. This bacteria was recently also identified in *Am. variegatum* and *Am. lepidum* ticks collected from two locations in Central Oromia and one in the Amhara Region of Ethiopia (Teshale et al., 2015). Its pathogenicity is still poorly understood.

Only 2 blood samples from cattle (0.5%) tested positive for *E. ruminantium*, which is the first molecular detection of *E. ruminantium* in bovine blood samples in Ethiopia. The low apparent prevalence detected here might be attributed to the biology of *E. ruminantium*, as it mainly resides in endothelial cells and is only periodically found in the bloodstream (Andrew and Norval, 1989; Lorusso et al., 2016). DNA of *E. ruminantium* was previously also detected in *Amblyomma* ticks from Ethiopia (Tomassone et al., 2012; Teshale et al., 2015). The presence of pathogenic TBPs such as *E. ruminantium*, *A. marginale* and *B. bigemina* together with vector tick species (Mekonnen, 1996) should be taken into account when attempting livestock improvement through the introduction of exotic cattle breeds, such as highly productive, taurine (i.e. *Bos taurus*) and other naive breeds in the area.

A new *Ehrlichia* genotype, *Ehrlichia* sp. UFMG-EV was identified in *Rhipicephalus microplus* ticks in Brazil (Cruz et al., 2012). In 2014, another genotype, *Ehrlichia* sp. UFMT-BV was also detected in cattle, and was subsequently shown to cause clinical symptoms similar to those of canine ehrlichiosis in an experimentally infected calf (Aguiar et al., 2014). The genetic characterization of 16S ribosomal RNA (rRNA) and thio-disulfide oxidoreductase (*dsb*) genes showed that both *Ehrlichia* sp. UFMG-EV and *Ehrlichia* sp. UFMT-BV genotypes represent a single species phylogenetically close to *E. canis*. Recently, the name *Ehrlichia minasensis* was proposed for this recently identified

Ehrlichia species (Cabezas-Cruz et al., 2016). In this study, we have detected *E. minasensis* DNA in a bovine blood sample for the first time outside of the Americas. The *E. minasensis* sequence generated in this study exhibited 100% (420/420) identity with the *E. minasensis* genotype UFMG–EV 16S rRNA gene sequence deposited in GenBank (JX629805). Since this species was previously identified only from *R. microplus*, a tick species that to the best of our knowledge has not been reported to occur in Ethiopia, it suggests transmission of *E. minasensis* by ticks other than *R. microplus*. The significance of this pathogen for bovine health and identification of the vector responsible for its transmission in Ethiopia requires further investigation.

Remarkably, in the present study, three novel *Anaplasma* genotypes were identified from naturally infected cattle from Ethiopia that most likely represent three, but at least two new species. The prevalence of these *Anaplasma* genotypes ranged from 5.6% - 14.3%. Sequence analysis indicated that these organisms are phylogenetically distinct from known *Anaplasma* species. Two of the species, *Anaplasma* sp. Hadesa and *Anaplasma* sp. Saso, are closely related and appear to be distant to any of the other members of the genus *Anaplasma*. The branches separating *Anaplasma* sp. Hadesa and *Anaplasma* sp. Saso are short, but longer than those separating e.g. *A. marginale*, *A. centrale* and *A. ovis,* three bacterial species that also share 99.3-99.5% 16S rRNA sequence identity. *Anaplasma centrale* was initially described as a subspecies of *A. marginale* (Theiler, 1911) but even comparison of whole genome sequences could not resolve whether *A. centrale* should be considered a subspecies of *A. marginale* or a separate species (Herndon et al., 2010). In contrast, validity of the species status for *A. ovis* is usually not questioned. The biology of the three species differs in terms of morphology/position of the

inclusion bodies in the erythrocyte, vertebrate host spectrum, pathogenicity and tick vector (Battilani et al., 2017) suggesting that they represent independently evolving species. It is therefore likely that *Anaplasma* sp. Hadesa and *Anaplasma* sp. Saso also represent independent, closely related species and not simply two genotypes within one species. It was unfortunately not possible to resolve the phylogenetic position of these bacteria in the genus *Anaplasma* solely on the basis of the 16S rRNA sequence. Additional sequences from other genes and multi-locus phylogenetic analysis using material from mono-infected animals will be required to unravel the questions if these are truly separate species and what the exact phylogenetic position of these species within the genus might be.

Based on the 16S rRNA sequence, *Anaplasma* sp. Dedessa appears to be a previously unrecognized species and a close relative of Anaplasma BL099-6, which was recently characterized as *Candidatus* Anaplasma boleense (Guo et al., 2016). Analysis of samples from mono-infected cattle was again not possible due to high prevalence of mixed infection by various *Anaplasma* species.

Certain members of the genus *Anaplasma* are recognized to be important human and animal pathogens (Kocan et al., 2015). Recently, in addition to *Anaplasma phagocytophilum* and *Anaplasma ovis* that have been recognized as pathogens of human anaplasmosis (Chen et al., 1994; Chochlakis et al., 2010), a novel *Anaplasma* species designated “*Anaplasma capra*” has been identified in goats, ticks and humans in northern China (Li et al., 2015). Moreover, it is assumed that additional *Anaplasma* species remain undiscovered and contribute to human and/or animal diseases (Yang et al., 2016). It is unknown whether the *Anaplasma* species detected in this study are

pathogenic to humans or livestock animals. Isolation and further characterization of these *Anaplasma* species from infected animals as well as their zoonotic potential need to be further investigated. Moreover, it would be interesting to screen questing ticks in the study area to ascertain tick vectors transmitting these bacteria.

A significant association was observed between cross-breed (in comparison to zebu) cattle and a high prevalence of *A. marginale* infection. However, the same or a similar positive effect on *A. marginale* prevalence were observed for a semi-intensive management system and highly frequent (six to eight times per year) use of acaricides since these three categories of the variables were completely collinear, i.e. they included the same 21 animals from a single farm. This co-linearity also prevented the use of multi-variate analyses methods and exclusion of the 21 animals identified no significant effects of the remaining variables. Although statistically not significant, compared to local zebu breeds, a higher proportion of infection by *B. bigemina* was also observed in the same 21 cross-bred cattle. In this regard, the limitation of this study is that a lower percentage of crossbred cattle were studied compared to local zebu breed. However, this represents the locally available cattle breeds since the system to identify the animals that were included did not consider the breed and was largely random. Future case control studies with equal or at least representative numbers of crossbred cattle are necessary to address higher susceptibility of cross-bred animals to *A. marginale* and other TBP. Such studies will also allow to statistically evaluate the effects management practices and acaricide treatment in relation to the breed in the epidemiological situation in Ethiopia and probably many similar regions in the tropics.

In conclusion, this study revealed a very high prevalence of tick-borne pathogens close to 100% in the study area and co-infections were more common than single infections. This might have implications for potential interactions of pathogens and the patterns of clinical symptoms. The significance on animal health, zoonotic potential and vectors responsible for transmission of the novel *Anaplasma* species identified in this study as well as of *E. minasensis* need to be further investigated. The epidemiological data from this study will provide significant information on tick-borne diseases in the area and will serve as scientific basis for planning future control strategies.

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4.7 References

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5 CHAPTER 5: General discussion and recommendations

Tick-borne diseases continue to be a hindrance to the development of the livestock industry in many countries of the world. Apart from causing high morbidity and mortality, they are also a significant impediment to the improvement of indigenous breeds of cattle as they prevent the introduction of more productive exotic breeds to TBDs endemic regions (Simuunza et al., 2011). Despite the wide distribution of several species of hard ticks and pathogens causing TBDs, there is scarce information on the epidemiology of infections with TBPs in Ethiopia. Therefore, in this dissertation, the aim was to investigate and provide baseline data regarding the prevalence and species composition of bovine TBPs of veterinary significance in Southwestern Ethiopia.

Since the transport of whole blood samples collected from cattle in Ethiopia to Germany was not possible due to the German sanitary regulations which prohibit the importation of such samples from Foot and Mouth Disease endemic countries such as Ethiopia, a thorough evaluation of six DNA extraction methods from blood spotted on FTA Classic® cards was conducted to ensure the use of an optimal extraction method for the analysis of the field samples (Chapter 3). Then in Chapter 4, the best method for the extraction of DNA from blood spotted on FTA cards was employed for the molecular investigation of the occurrence of bovine TBPs of veterinary significance in Southwestern Ethiopia. Due to the existence of diverse species of TBPs known to be transmitted by hard ticks in Ethiopia, this study relied on a combination of PCR and a RLB hybridization assay for the simultaneous detection of TBPs from bovine blood samples.

5.1 Comparison of DNA extraction methods from blood spotted on FTA cards

FTA cards are useful for sample storage and transportation in areas where infrastructure for the cold storage and transport of blood samples is not available and in situations where the transport of frozen forms of biohazardous agents from one geographical region to another is complicated by international regulations (Michaud et al., 2007). However, overcoming obstacles associated with the localized trapping of the genomic material in the card matrix and the inhibitory effects of blood components such as haem, lactoferrin and IgG is essential for efficient DNA extraction from FTA cards. Multiple methods to extract DNA from blood spotted on filter paper for the molecular detection of TBPs have been described. The research described herein aimed to identify the most sensitive method among six commonly used methods for DNA extraction from FTA cards for subsequent molecular detection of TBPs. The result showed that washing of 3 mm discs punched from FTA cards with FTA purification reagent followed by DNA extraction using Chelex® resin was the most sensitive procedure. The use of FTA discs washed with FTA purification reagent and treated with a chelating agent (Chelex®) helps to overcome the inhibitory effects of blood components and favours dissociation of DNA into single strands (Becker et al., 2004; Walsh et al., 2013), facilitating downstream PCR amplification and eventually leading to an improved detection limit by RLB.

Attempts were also made to evaluate the detection limit of the best method described above with regard to number of punches used. The detection limit was improved when more discs were used as starting material for the DNA extraction, whereby the use of sixteen 3 mm discs proved to be most practical. Furthermore, comparison of detection limits of RLB and PCR using DNA extracted from whole blood with the best practice

method to extract DNA from FTA cards was performed for four major TBPs of cattle. It is worth mentioning that direct DNA extraction from whole blood using a commercial spin column based method slightly increased the detection limit of the RLB for some pathogens compared to the use of DNA extracted from sixteen 3 mm diameter FTA discs. This limitation should be taken into account in the application of the best practice method presented here when estimating the prevalence of TBPs and diagnosis of carriers with very low parasitemia of these pathogens.

5.2 Epidemiology of infection with TBPs in Southwest Ethiopia

Given the importance of cattle husbandry in Ethiopia and the favourable environmental conditions providing a multi-tick species ecology where TBPs can co-infect cattle, studies on the epidemiology and species composition of bovine TBPs are remarkably scarce in Ethiopia. In Chapter 4 of this thesis, a combination of PCR and RLB was employed for the detection of TBPs in blood samples collected from local cattle populations of Illubabor zone in Southwestern Ethiopia. This represents a survey of TBPs carried out in Ethiopia by means of broad-scale molecular methods. This study revealed very high prevlences in the overall cattle population, with a broad diversity of haemoparasite species detected, in presence of a complex scenario of multiple infections.

In endemic areas, infections with mildly pathogenic *Theileria* species are usually acquired by calves early in life, after which they remain life-long carriers (Sugimoto and Fujisaki, 2002). The high prevalence recorded for *T. mutans* (66.1%) and *T. orientalis* (51.8%) certainly suggests the existence of a constant challenge to cattle in southwestern Ethiopia by their tick vectors and likely occurrence of steady immunity in animals. Most of the

animals surveyed in this study did not present any clinical signs referable to theileriosis. This may suggest that a scenario of 'endemic stability' might exist in the surveyed cattle population for these pathogens (Coleman et al., 2001). However, the prevalences recorded for the remaining TBPs (especially TBD-causing pathogens), does not lead to confident assumption of stability in the region. This is of epidemiological relevance as it poses a high risk for a potential outbreak of clinical diseases in the event of the introduction of exotic breeds to this region. Therefore, the presence of pathogenic TBPs such as *E. ruminantium*, *A. marginale* and *B. bigemina* and integrated control strategies with their vector tick species should be taken into account when attempting livestock improvement through the introduction of exotic cattle breeds, such as highly productive, taurine (i.e. *Bos taurus*) and other naive breeds in the study area in particular and in the country in general.

Despite a previous 49% seroprevalence report of *B. bigemina* in Southwestern Ethiopia (Mekonnen, 1996), our data showed a relatively low prevalence of *B. bigemina* (14.0%). Calves exposed to babesiosis early in life rarely show clinical signs but develop long lasting immunity after recovery (Mahoney et al., 1973). This would explain the prevalence reported in this study for *B. bigemina*, which might have been affected by lower infection doses and/or presence of low parasitemia below the detection limit of RLB. It is also possible that the above mentioned comparative limitation of DNA extraction method from FTA cards employed in this study might have underestimated the true prevalence of *B. bigemina* and other TBPs studied in this thesis. In a broader sense, it has been stated that TBPs densities in carrier animals appear to fluctuate over time and periodically fall below the levels detectable by PCR (Herrero et al., 1998; Geysen, 2000). For this reason,

while offering the opportunity to detect animals with active infections, PCR does not provide information on herd immunity (Simuunza et al., 2011). On the other hand, even though serology cannot allow multiple screenings and the results might also be affected by cross-reactions (Passos et al., 1998), serological tests are able to detect antibodies in animal in which the parasitaemia is below the detection limit of PCR and in those that have cleared the infection. Therefore, combining molecular tools with serological tests for certain infections would improve the accuracy of the estimated prevalence and may provide useful information concerning herd immunity to these diseases.

The present study provides the first molecular detection of *E. ruminantium* in bovine blood samples from Ethiopia. Only 2 blood samples from cattle tested positive for *E. ruminantium*. Considering the fact that none of the two *E. ruminantium*-positive cattle displayed any clinical sign referable to heartwater, it is likely these animals were carriers of this rickettsia. After recovery from the acute phase, low numbers of this microorganism can still reproduce in the endothelial cells of the capillaries, being released only periodically into the bloodstream (Andrew and Norval, 1989). This would explain the very low prevalence of *E. ruminantium* reported in this study and why not all carrier animals can be diagnosed as positive from screening of blood samples.

Interestingly, this study provides the first report on the detection of *E. minasensis* DNA in a bovine blood sample for the first time outside of the Americas. Since this species was previously identified only from *R. microplus* (Cruz et al., 2012), a tick species, which to the best of our knowledge has not been reported to occur in Ethiopia, this suggests transmission of *E. minasensis* by ticks other than *R. microplus*.

Certain members of the genus *Anaplasma* are now recognized to be important human and animal pathogens (Kocan et al., 2015). In connection with the identification of novel *Anaplasma* species designated "*Anaplasma capra*" in goats, ticks and humans in northern China (Li et al., 2015), it is assumed that additional *Anaplasma* species remain undiscovered and contribute to human and/or animal diseases (Yang et al., 2016). In the present study, three novel *Anaplasma* genotypes (designated as *Anaplasma* sp. Hadesa, *Anaplasma* sp. Saso, and *Anaplasma* sp. Dedessa) were identified from naturally infected cattle from Ethiopia. Sequence and phylogenetic analysis indicated that these organisms are phylogenetically distinct from known *Anaplasma* species and most likely represent three, but at least two, new species. However, multi-locus phylogenetic analysis was not possible due to high prevalence of mixed infection by various *Anaplasma* species. Consequently, it was not possible to resolve the phylogenetic position of these bacteria in the genus *Anaplasma*. Further analysis is required with additional sequences from other genes and multi-locus phylogenetic analysis using material from mono-infected animals to determine the exact phylogenetic position of these genotypes within the genus *Anaplasma*. It is also unknown whether the *Anaplasma* genotypes detected in this study are pathogenic to humans or livestock animals and tick vectors transmitting these genotypes.

5.3 Conclusion and areas of future study

In conclusion, the best practice method for the extraction of DNA from blood spotted on FTA cards presented in this thesis will facilitate epidemiological studies on TBPs and other haemoparasites and it may be particularly useful for field studies where a cold chain is absent. The findings from the epidemiological study on the other hand show a very high

burden of infection of cattle with TBPs in Ethiopia. High frequency of co-infections with different pathogens suggests that clinical manifestations might be complex and can hardly be used for diagnostic purposes. The present thesis contributes to a better understanding of the epidemiology of infection of cattle with TBPs and serves as a scientific basis towards integrated and strategic control of ticks and TBDs in Ethiopia which should be designed taking into account host resistance, breed, production system, seasonal dynamics of tick infestation, the availability of medicines and vaccines against ticks and TBDs and cost/benefit analysis of the strategies.

Future studies would be needed to address the following points:

- The study showed both the usefulness and versatility of RLB towards the sensitive detection of multiple tick-borne haemoparasitic infections in cattle in Ethiopia. As there is no information available on the epidemiology of ticks and TBPs in wildlife of Ethiopia, future studies are also recommended in this aspect to investigate whether wildlife act as a reservoir for TBPs that are transmissible to livestock. It would also be useful to investigate the occurrence of these and other zoonotic TBPs of medical importance caused by spotted fever group rickettsiae in tick vectors to deepen the understanding and expand the epidemiological knowledge of ticks and TBDs in the country.

- With regard to the three novel *Anaplasma* genotypes identified and characterized by phylogenetic analysis, in future, it would be worthwhile to isolate and further characterize these species from infected animals and determine the pathogenicity, host cell types and their zoonotic potential. Moreover, it would also be useful to

screen questing ticks in the study area to ascertain tick vectors transmitting these species.

- In this study, we have detected *E. minasensis* DNA in a bovine blood sample for the first time outside of the Americas. The significance of this pathogen for bovine health and identification of the vector responsible for its transmission in Ethiopia requires further investigation.

- This study has revealed a significant association between crossbred (in comparison to zebu) cattle and a high prevalence of *A. marginale* infection. However, since the system of sampling employed in this study was largely random without consideration to the breed of cattle and cattle production system, a lower percentage of crossbred cattle was studied compared to local zebu breed. Future case-control and cohort studies with equal or at least representative numbers of crossbred cattle and cattle production systems are necessary to address risk factors associated with TBPs versus breed of cattle and associated cattle production parameters especially in urban and peri-urban areas of the country where commercial dairy cattle productions are concentrated. Such studies will also allow to statistically evaluate the effects management practices and acaricide treatment in relation to the breed in the epidemiological situation of TBDs in Ethiopia and probably many similar regions in the tropics.

5.4 References

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Summary

Tick-borne diseases (TBDs) affect 80% of the world's cattle population, hampering livestock production throughout the world. Bovine babesiosis, anaplasmosis, heartwater and theileriosis have all been reported to occur in Ethiopia. However, little is known about the extent and prevalence of these diseases in the local cattle population. The principal aim of this thesis was to estimate the species composition and prevalence of bovine tick-borne pathogens (TBPs) in Southwestern Ethiopia.

The first paper of this cumulative thesis deals with the evaluation of six DNA extraction methods from blood spotted on Flinders Technology Associates (FTA) cards, to determine the optical protocol for the subsequent molecular detection of TBPs by PCR and reverse line blot (RLB) hybridization. Ten-fold serial dilutions of bovine blood infected with *Babesia bovis*, *Theileria mutans*, *Anaplasma marginale* or *Ehrlichia ruminantium* were made by dilution with uninfected blood and spotted on FTA cards. Subsequently, DNA was extracted from FTA cards using six different DNA extraction protocols. Additionally, DNA was also isolated from whole blood dilutions using a commercial kit.

The findings from this study showed that (i) among the methods compared, the highest detection limit was observed when DNA was extracted from FTA cards using FTA purification reagent combined with elution with 5% Chelex® 100 resin. Secondly, the detection limit was improved when more discs were used as starting material for the DNA extraction, whereby the use of sixteen 3 mm discs proved to be most practical, (iii) comparisons of the detection limits of RLB and PCR for the four TBPs of cattle showed that the sensitivity of DNA extraction from FTA card vary between different TBPs and the

sensitivity of the RLB was in nearly all instances 10x higher compared to gel electrophoresis.

The second study aimed to determine the prevalence and species composition of bovine TBPs of veterinary significance in local cattle populations of Illubabor zone in Southwestern Ethiopia. The best method for the extraction of DNA from blood spotted on FTA cards as described above and a combination of PCR and a Reverse Line Blot (RLB) hybridization assay were employed for the detection of a broad-spectrum of TBPs (i.e. *Babesia*, *Theileria*, *Anaplasma* and *Ehrlichia* spp.) in blood samples collected from 392 cattle. The findings showed a high prevalence of several bovine TBPs in Southwest Ethiopia, some which are of great veterinary importance. Individual prevalences of these pathogens included *Theileria mutans* (66.1%), *Theileria orientalis* (51.8%), *Anaplasma* sp. Omatjenne (25.5%), *Anaplasma marginale* (14.5%), *Babesia bigemina* (14.0%) and *Theileria velifera* (13.0%) and minor occurrences of *Ehrlichia ruminantium* (0.5%) and *Ehrlichia minasensis* (0.26%). *Ehrlichia minasensis* was until now only reported from North and South America. In addition, three novel *Anaplasma* genotypes were detected in bovine blood samples. A phylogenetic analysis revealed that they most likely represent three, but at least two, new species. A total of 227 cattle (57.9%) were found to be co-infected with two or more TBPs simultaneously and 86 different species combinations were observed. The high frequency of co-infections observed in this study suggests that clinical manifestations might be complex and complicate their diagnosis. The data presented in this thesis provides a more informed picture of the epidemiology of infection of cattle with TBPs and contributes towards integrated and strategic control of ticks and TBDs in Ethiopia.

Zusammenfassung

Molekularbiologischer Nachweis und Charakterisierung von durch Zecken übertragenen Pathogenen bei Rindern im Südwesten Äthiopiens

Zecken-übertragene Krankheiten (TBDs), welche 80 % der weltweiten Rinderpopulation betreffen, haben einen negativen Einfluss auf die Nutzviehwirtschaft auf der ganzen Welt. Das Vorkommen der Babesiose, Anaplasmose, Herzwasserkrankheit und Theileriose bei Rindern in Äthiopien wurde beschrieben. Allerdings ist wenig bekannt über die Bedeutung und die Prävalenz dieser Krankheiten in den lokalen Rinderpopulationen Äthiopiens. Ziel dieser Dissertation war es daher, die Spezieszusammensetzung und die Prävalenz der von Zecken übertragenen Pathogene (TBP) in Rindern im Südwesten von Äthiopien zu bestimmen.

Die Dissertation gliedert sich in zwei Studien. Die erste Studie beschäftigt sich mit der Evaluierung von sechs DNS-Extraktionsmethoden. Eine Zehner-Verdünnungsreihe von Rinderblut, das mit *Babesia bovis*, *Theileria mutans*, *Anaplasma marginale* oder *Ehrlichia ruminantium* infiziert war, wurde durch Verdünnung mit nicht infiziertem Blut hergestellt und auf FTA-Karten („Flinders-Technology-Associates“ cards) übertragen. Anschließend wurde die DNS unter Verwendung von sechs verschiedenen DNS-Extraktionsmethoden aus den FTA-Karten extrahiert. Zusätzlich wurde vergleichend DNS aus den Proben mit einem handelsüblichen Extraktions-Kit isoliert. Ziel war es, das optimale Protokoll für eine sich anschließende molekulare Erfassung der von Zecken übertragenen Pathogene durch PCR und „Reverse-Line-Blot-Hybridisierung“ (RLB) zu bestimmen.

Die Ergebnisse dieser Studie zeigten, dass (i) unter den verglichenen Methoden die höchste Nachweisempfindlichkeit für die Erreger erreicht wurde, wenn die DNS aus FTA-Karten unter Verwendung der FTA-Reinigungslösung, kombiniert mit der Elution mit 5% Chelex® 100-Harz, extrahiert wurde. Zweitens wurde die Nachweisgrenze verbessert, wenn mehrere Stanzproben von den FTA-Karten der gleichen Probe als Ausgangsmaterial für die DNS-Extraktion verwendet wurden, wobei die Verwendung von sechzehn 3 mm-Stanzproben sich als am praktischsten erwies. (iii) Vergleiche der Nachweisgrenzen von RLB und PCR für die durch Zecken übertragenen Pathogene für Rinder zeigten, dass die Empfindlichkeit der DNS-Extraktion aus den FTA-Karten zwischen verschiedenen TBPs variierte und die Empfindlichkeit der RLB-Hybridisierung in fast allen Fällen 10x höher war als im Vergleich zur Gelelektrophorese.

In der zweiten Studie sollte die Prävalenz und Spezieszusammensetzung der TBPs in den lokalen Rinderpopulationen in der Region von Illubator, im Südwesten von Äthiopien, bestimmt werden. Die Extraktionsmethode mit der höchsten Nachweisempfindlichkeit (FTA-Reinigungslösung kombiniert mit 5% Chelex® 100-Harz) wurde in Kombination mit der PCR und einem RLB-Hybridisierungsassay für die Detektion eines breiten Spektrums von TBPs angewandt. Insgesamt wurden Blutproben von 392 Rinder auf die Erreger aus vier TBP-Gattungen untersucht (*Babesia* spp., *Theileria* spp., *Anaplasma* spp. und *Ehrlichia* spp.). Die Ergebnisse zeigen eine hohe Prävalenz mehrerer Rinder-TBPs in Südwest-Äthiopien, von denen einige eine große veterinärmedizinische Bedeutung haben. Die Prävalenz dieser Pathogene betrug für *Theileria mutans* (66,1%), *Theileria orientalis* (51,8%), *Anaplasma* sp. *Omatjenne* (25,5%), *Anaplasma marginale* (14,5%), *Babesia bigemina* (14,0%), *Theileria velifera* (13,0%), *Ehrlichia ruminantium* (0,5%) und

Ehrlichia minasensis (0,26%). *Ehrlichia minasensis* war zuvor ausschließlich in Rinderproben aus Nord- und Südamerika nachgewiese worden. Zusätzlich wurden drei neue Anaplasma-Genotypen in Rinderblutproben identifiziert. Eine phylogenetische Analyse zeigte ferner, dass diese höchstwahrscheinlich drei, zumindest aber zwei, neue Arten darstellen. Insgesamt waren 227 Rinder (57,9%) mit zwei oder mehreren TBPs gleichzeitig infiziert. Dabei wurden 86 verschiedene Spezieskombinationen beobachtet. Die in dieser Studie beschriebene Häufigkeit von Ko-Infektionen deutet darauf hin, dass klinische Manifestationen komplex sein können und daher eine Diagnose verkomplizieren können.

Die in dieser Arbeit präsentierten Daten ergeben einen Überblick über das Artenspektrum und die Prävalenz der von Zecken übertragenen Pathogene bei Rindern im Südwesten von Äthiopien. Die epidemiologischen Daten liefern eine wichtige Voraussetzung für eine strategische und nachhaltige Kontrolle der Zecken und der durch sie im Untersuchungsgebiet übertragenen Erreger.

List of publications

Peer-reviewed journal articles

1. **Hailemariam, Z.**, Krücken, J., Baumann, M., Ahmed, J. S., Clausen, P. H., & Nijhof, A. M. (2017). Molecular detection of tick-borne pathogens in cattle from Southwestern Ethiopia. PLoS ONE 12(11), S. e0188248.
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11. **Hailemariam, Z.**, Omar, A. R., Hair-Bejo, M. & Giap, T. C. (2008). Detection and characterization of chicken anemia virus from commercial broiler breeder chickens. Virology Journal 5(1), 128.

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1. **Hailemariam, Z.**, Krücken J.; Ahmed, J., Clausen, P.H., Baumann, M., & Nijhof, A. M. (2017): Molecular diagnosis and epidemiology of tick-borne diseases in southwestern Ethiopia. In: Deutsche Veterinärmedizinische Gesellschaft (DVG) - Fachgruppe Parasitologie und parasitäre Krankheiten, Hannover, Germany - 12.06.-14.06.2017. Abstract band ISBN 978-3-86345-372-5, pages 68-69.
2. **Hailemariam, Z.**, Baumann, M., Ahmed, J., Clausen, P. H., & Nijhof, A. M. (2016): Epidemiology of bovine tick-borne diseases in Southwestern Ethiopia as determined by reverse line blot hybridization assay. In: First Joint International Conference of the Association of Institutions for Tropical Veterinary Medicine (AITVM) and the Society of Tropical Veterinary Medicine (STVM), Berlin, Germany – 04.09.-08.09.2016. Programme & Abstract band ISBN 978-3-86345-338-1, page 256.
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Disclosure of contribution to the intellectual content

Jabbar Ahmed, Ulrike Seitzer and Peter-Henning Clausen were lead applicants on the grant awarded by DFG that supported this study. Dr. Ard Menzo Nijhof was responsible for overseeing the study and made significant contributions to the design, acquisition and interpretation of data. Zerihun Hailemariam Negasi designed and carried out the field and laboratory procedures, compiled, analyzed and interpreted the data and drafted both manuscripts. Dr. Maximilian Baumann contributed in sample design and sampling strategies. Dr. Jürgen Krücken contributed to data and phylogenetic analysis. All co-authors contributed to revising the manuscripts for intellectual content and approved the published articles.

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Statement of authorship

Except where reference is made in the text, this thesis contains no material published elsewhere or extracted in whole or in part from any previous work presented by me for another degree or diploma. No other person's work has been used without due acknowledgement in the main text of the thesis. This thesis has not been submitted for the award of any other degree or diploma in any other tertiary institution.

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