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

Molecular diagnosis and characterization of ticks and tick-borne pathogens infecting cattle in Mymensingh district of Bangladesh

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

vorgelegt von
Babul Chandra Roy
Tierarzt aus Thakurgaon, Bangladesh

Berlin 2018
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Cattle; *Rhipicephalus microplus*; disease vectors; vector-borne diseases; anaplamoses; babesiosis; epidemiology; Bangladesh.

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This PhD dissertation is dedicated to my beloved spiritual master, His Holiness Jayapataka Swami Gurumaharaj, Lord Sri Sri Radha-Madhava, Pancha-Tattva, Jagannatha Baladeva Subhadra and Nrisimhadeva.

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List of Abbreviations

A.	*Anaplasma*
Am.	*Amblyomma*
AIC	Akaike Information Criterion
B.	*Babesia*
BLAST	Basic Local Alignment Search Tool
Bp	Base Pair
°C	Degree Celsius
CAT	Card Agglutination Test
cELISA	Competitive Enzyme Linked Immunosorbent Assay
CF	Complement Fixation
CI	Confident Interval
COI	Cytochrome Oxidase Subunit 1
DAAD	Deutscher Akademischer Austauschdienst
DAMBE	Data Analysis in Molecular Biology and Evolution
DLS	Department of Livestock Service
DLO	District Livestock Officer
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
dNTPs	Deoxyribonucleotide Triphosphates
E.	*Ehrlichia*
ECF	East Coast Fever
ECL	Enhanced Chemiluminescence
EDTA	Ethylenediaminetetraacetic Acid
ELISA	Enzyme Linked Immunosorbent Assay
FTA	Flinders Technology Associates
G	Gravitational constant
GDP	Gross Domestic Product
Hy.	*Hyalomma*
H.	*Haemaphysalis*
H	Hour
IFAT	Indirect Fluorescent Antibody Test
ITS2	Internal Transcribed Spacer 2
kDa	Kilo Dalton
km^2	Kilometer squared
LAMP	Loop Mediated Isothermal Amplification
LRT	Likelihood Ratio Test
M	Molar
MAFFT	Multiple Alignment using Fast Fourier Transform
MEGA6	Molecular Evolutionary Genetics Analysis 6
Min.	Minute
Ml	Milliliter
mM	Millimolar
MSP	Major Surface Protein
MUSCLE	Multiple Sequence Comparison by Log-Expectation

List of Abbreviations

NCBI	National Center for Biotechnology Information
OR	Odds Ratio
PCR	Polymerase Chain Reaction
pH	Potential of Hydrogen
Pmol	Pico-molar
q-PCR	Quantitative Real-Time Polymerase Chain Reaction
R.	*Rhipicephalus*
RaxML	Randomized Axelerated Maximum Likelihood
RBC	Red Blood Cell
RLB	Reverse Line Blot
rRNA	Ribosomal Ribonucleic Acid
RNA	Ribonucleic Acid
S	Second
SH	Shimodaira Hasegawa
SME	Scanning Electron Micropcopy
spp.	Species
sp.	Species
SSU	Small Subunit
T.	*Theileria*
TBDs	Tick-Borne Diseases
TPBs	Tick-Borne Pathogens
Tris-HCL	Tris hydrochloride
ULO	Upazila Livestock Officer
UK	United Kingdom
USA	United State of America
USDs	United States Dollars
µl	Microliter
VS	Veterinary Surgeon
W/V	Weight/Volume

Preface

Ticks and tick-borne pathogens (TBP) affect the majority of the global cattle population, causing significant losses to the livestock industry. Tick-borne diseases (TBDs), in particular anaplasmosis, babesiosis and theileriosis are considered to be the major impediments in the health and productive performance of livestock in the tropics and subtropics, impacting the livelihoods of resource-poor farming communities. In Bangladesh, the livestock sector is considered one of the important factors for poverty alleviation and socio-economic development. In general, the productivity of livestock industry is increased through crossbreeding with exotic dairy cattle. However, the improvement and genetic upgrading of cattle is hindered by several factors, including ticks and tick-borne pathogens (TBPs) as exotic cattle breeds are usually highly susceptible to TBDs. Since ticks and TBPs constraint the productivity and improvement of the livestock industry, reliable information on their epidemiology is essential and may contribute to the development and implementation of effective control strategies. The main objective of this work was to determine the prevalence of TBPs in blood samples collected from cattle in Mymensingh district, Bangladesh using the Reverse Line Blot (RLB) assay. This assay allows for the simultaneous detection of up to 42 different TBPs in a single sample.

This cumulative dissertation is divided into the following chapters:

The first chapter provides general information on ticks and TBPs occurring in Bangladesh, outlines the rationale of the study and highlights the objectives of this thesis.

In the second chapter, a general introduction to Bangladesh, its livestock sector and current knowledge of ticks and TBPs occurring in the country is presented. In addition, the etiology,

geographical distribution, life cycle, clinical manifestations, diagnosis, epidemiology and chemotherapy of major TBDs are highlighted.

In the third chapter, a comprehensive epidemiological survey of TBPs in the local cattle population of Mymensingh district, Bangaladesh is presented. Samples were collected from cattle following a cross-sectional study outline and subsequently analyzed using a RLB assay. For this assay, an in-house chemiluminescence solution was adapted to replace a commercial alternative, by which a significant cost reduction could be obtained. Results of this study revealed a high prevalence of ticks and various TBPs. Two novel *Anaplasma* and *Babesia* ribosomal gene sequences and a genetic variant of *T. orientalis* 18S rRNA were detected and phylogenetically analysed.

The fourth chapter provides a detailed picture on the phylogenetic relationship between *R. microplus* ticks collected from cattle in Bangladesh, Pakistan and Myanmar with other one-host *Rhipicephalus* spp. based on their morphology using Scanning Electron Microscopy (SEM) and analysis of gene sequences to elucidate the structure of the *R. microplus* species complex. The phylogenetic analysis revealed that the *R. microplus* complex consists of five taxa: *R. annulatus, R. australis, R. microplus* clade A sensu Burger *et al.* (2014), *R. microplus* clade B sensu Burger *et al.* (2014) and *R. microplus* clade C sensu Low *et al.* (2015). All analysed *R. microplus* ticks from Bangladesh, Pakistan and Myanmar belonged to *R. microplus* clade C. The SEM results revealed large morphological intraspecific variations which complicate the unambiguous identification of species within the *R. microplus* complex based on morphological features only.

In the fifth chapter, the major results of the performed studies are summarized, placed in perspective and future lines of investigations are proposed.

CHAPTER 1

1. Introduction

Tick-borne diseases (TBDs) affect the majority of world's cattle population and are widely distributed throughout the world, particularly in tropical and subtropical countries (Minjauw and McLeod 2003). TBDs are considered to be the major constraint to the profitable livestock production and productivity in many countries of the world and pose a significant impact on the livelihood of resource-poor farming communities due to high morbidity and mortality, loss of draught power and milk production, increased cost for diagnosis and control measures (Minjauw and McLeod 2003). Bovine babesiosis caused by *Babesia bovis* and *Babesia bigemina,* anaplasmosis caused by *Anaplasma marginale* and theileriosis caused by *Theileria parva*, *Theileria annulata,* and other *Theileria* spp., are the economically most important and widely distributed TBDs affecting cattle (Jongejan and Uilenberg 2004). *Theileria orientalis* has previously been considered as benign theileriosis; however, a number of clinical outbreaks of oriental theileriosis caused by *T. orientalis* have recently been reported from the Asia Pacific countries, resulted in huge production losses to cattle industry (McFadden et al. 2011; Perera et al. 2014). The production losses attributed to TBDs varies widely due to different husbandry and management system, cattle breed and disease control programs (Jonsson et al. 2008), but the global estimated annual production losses associated with ticks and TBDs in cattle ranges between 13.9 and 18.7 billion US dollars (de Castro 1997).

1.1 Problem statement and objectives of the thesis

In Bangladesh, the livestock sectors play an important role in the socio-economic development and poverty reduction by providing nutrition and cash income to smallholder rural farmers. The growing demand for livestock products requires a further increase in the productivity of the livestock industry, to fulfill food security and the nutritional requirement

of the ever-growing population in Bangladesh. However, the improvement and genetic upgrading of the local cattle production is constrained by several factors, including tick infestations and tick-borne pathogens (TBPs). Bovine babesiosis caused by *Babesia bovis* and *Babesia bigemina,* anaplasmosis caused by *Anaplasma marginale* and theileriosis caused by *Theileria annulata,* and other *Theileria* spp. e.g *Theileria orientalis* have been reported to occur in Bangladesh, resulting in production losses to the cattle industry. The susceptibility of highly productive exotic cattle breeds (*Bos taurus*) to these TBDs impedes their local use to improve livestock production (Kocan et al. 2003). The majority of the TBPs reported to occur in Bangladesh were diagnosed by the visual examination of blood smears in combination with clinical symptoms from infected animals (Chowdhury et al. 2006; Alim et al. 2012; Al Mahmud et al. 2015; Belal et al. 2015). Although the microscopic examination of blood smears is very useful for diagnosing clinical infections, it is also considered to be insensitive and non-specific compared to molecular diagnostic methods such as PCR (Salih et al. 2007). The employment of molecular diagnostic techniques for the detection of tick-borne pathogens has been limited, with only a few recent reports describing the use of conventional and multiplex PCRs for the diagnosis of TBPs (Karim et al. 2012; Rahman et al. 2015; Qiu et al. 2016).

A tool that has proven to be particularly useful for the detection and differentiation of TBPs in carrier animals is the Reverse Line Blot (RLB) hybridization assay. With this macro-array, it is possible to simultaneously detect and differentiate up to 42 TBPs in a single sample and the RLB has found use in a number of epidemiological surveys (Gubbels et al. 1999; Georges et al. 2001; Oura et al. 2004; Nijhof et al. 2007). Use of this sensitive and specific molecular technique would result in the improved detection, identification and characterization of previously known, as well as novel TBPs in Bangladesh. This would help to improve the understanding of the local epidemiology of TBPs and the implementation of cost-effective

control strategies. The first part of the cumulative thesis therefore aimed to determine the prevelance of TBPs in blood samples collected from local cattle population of Mymensingh district of Bangaladesh using PCR followed by RLB.

One-host cattle ticks previously referred to as *Boophilus* species were synonomized with the genus *Rhipicephalus* based on molecular and morphological evidence (Murrell and Barker 2003). They are found in subtropical and tropical areas including Bangladesh and are considered to be the most important ectoparasites of livestock globally. These ticks cause considerable economic losses to the livestock industry by causing irritation and blood loss in cattle, decreasing leather quality and acting as vectors for economically important bovine diseases such as anaplasmosis and babesiosis (Guerrero et al. 2006). Their control is severely hampered by the occurrence of acaricide resistance (Abbas et al. 2014).

Recent phylogenetic analyses of mitochondrial genome sequences shows that the *R. microplus* is part of a species complex that consists of five taxa, namely *R. annulatus*, *R. australis*, *R. microplus* clade A sensu Burger *et al.* (2014), *R. microplus* clade B sensu Burger *et al.* (2014) and *R. microplus* clade C sensu Low *et al.* (2015). The economically most important representative species of this complex is *R. microplus.* Its clades shares many morphological similarities and show large intraspecific morphological variations which complicates their morpho-taxonomical identification and differentiation (Lempereur et al. 2010; Barker and Walker 2014). A reliable identification and differentiation of species belonging to the *R. microplus* complex is however needed to reliably monitor the spread of these ticks and that of acaricide resistant populations. Molecular characterization is currently considered to be the only alternative way to distinguish the *R. microplus* complex species. *Rhipicephalus microplus* is the most common and predominant tick species in Bangladesh and has been reported from different regions (Islam et al. 2006; Ghosh et al. 2007; Kabir et al. 2011; Fuehrer et al. 2012). Since little is known about the morphology, distribution and

genetic background of *R. microplus* from Asia, the second part of the thesis focused on the morphological and phylogenetic analysis of *R. microplus* ticks from Bangladesh, Pakistan and Myanmar using Scanning Electron Microscopy (SEM) and genetic markers.

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CHAPTER 2

2. Literature Review

2.1 Geographical and climatic characteristics of Bangladesh

Bangladesh is a low-lying, delta plain, riverine and agro-based developing country in Southern Asia situated between 20°34′N to 26°38′N latitude and 88°01′E to 92°41′E longitude. It is one of the most densely populated countries in the world, with 160 million people living in an area of 147,570 km^2 (Anonymous 2014). Bangladesh has a sub-tropical monsoon climate with wide seasonal variations in rainfall, temperature and humidity. The country has four distinct seasons, namely (i) a pre-monsoon hot summer season from March to May (ii) the monsoon rainy season from June to September (iii) the post-monsoon autumn season from October to November and (iv) the dry winter season from December to February. The average temperature of the country ranges from 17 to 20.6 °C during winter and 26.9 to 31.1 °C during summer and the average relative humidity ranges from 70.5% to 78.1%. Rainfall mostly occurs during the monsoon season, which varies from 1400 mm in the west to more than 4300 mm in the east of the country (Shahid 2010).

2.2 Livestock production in Bangladesh

Livestock production is considered to be an important factor for socio-economic development and poverty alleviation in Bangladesh as it provides nutrition and cash income to rural households. It is an integral component of the mixed farming agricultural system that is widely practiced in Bangladesh and significantly contributes to the national economy. In the fiscal year 2012-13, livestock contributed 16.95% to the agriculture sector, and 2.07% to the national gross domestic product (GDP) (Anonymous 2014). Cattle, buffaloes, sheep, goat and poultry constitute the livestock population in the country, with population sizes of 23.6 million for cattle, 1.46 million buffaloes, 25.6 million goats, 3.2 million sheep and 312.2

million poultry (DLS 2014). More than 75% of the population lives in villages and their subsistence principally depends on agriculture and livestock production. They rear livestock in an extensive system with limited disease control measures and allow their animals to graze on the roadside, homestead areas, riverbank and fallow lands (Islam et al. 2013). The zebu breed (*Bos indicus*) is predominant, but there is also small number of exotic cattle (*Bos taurus*) and their crosses. In general, the productivity of local cattle is lower compared to exotic cattle and their crosses. Crossbreeding of zebu cattle with exotic cattle breeds can be done through artificial insemination and estrus synchronization to improve milk and meat production and fulfill the nutritional demands and food safety of ever-growing population in Bangladesh (Khan et al. 2009). Around 70–80% of the total milk production in the country is produced by smallholder dairy farmers (Uddin et al. 2011). Although the density of livestock is, with 142 large ruminants/km^2, relatively high in Bangladesh compared to other countries of Southeast Asia (Teufel et al. 2010), livestock production is hampered by prevalent endemic diseases including ticks and TBDs and limited livestock research facilities.

Figure 1. Subsistence farmers and their animal (zebu cattle) in Mymensingh district, Bangladesh.

2.3 Important tick species of domestic animals and their distribution in Bangladesh

A number of studies have been conducted in different regions of Bangladesh to investigate the prevalence of ticks infesting domestic animals. The most important ixodid tick species reported from domestic animals in Bangladesh are *Rhipicephalus microplus, Haemaphysalis bispinosa, Rhipicephalus sanguineus, Hyalomma anatolicum, Hyalomma truncatum* and *Amblyomma testudinarium* (Islam et al. 2006; Ghosh et al. 2007; Rony et al. 2010; Kabir et al. 2011; Fuehrer et al. 2012). Among them, *R. microplus* is the predominant species that infests mainly cattle and lesser extend to other animals e.g. goats and buffaloes and has also been reported to occasionally infest human beings e.g. animal attendants (Islam et al. 2006; Ghosh et al. 2007). *Haemaphysalis bispinosa* mostly parasitizes goats, cattle, buffaloes; *R. sanguineus* principally infests dogs, and to a lesser extend of cattle and goat, *Hy. anatolicum* mainly infests cattle and *Am. testudinarium* infests both cattle and pigs. *R. microplus, H. bispinosa* and *R. sanguineus* are widely distributed tick species in Bangladesh and found throughout the year, whereas *Hy. anatolicum* is confined to the northwestern dry regions (Rajshahi, Rangpur, and Dinajpur districts) and *Am. testudinarium* is restricted to the northeastern and southeastern hilly areas of the country (Islam et al. 2006; Ghosh et al. 2007). *Rhipicephalus microplus* is considered the economically most important tick from a global perspective with estimated economic losses in the range of be approx. 2.5 billion US dollars annually (Lew-Tabor et al. 2014). Recently, *R. microplus* was reported to be a cryptic species, and the need for further detailed molecular and morphological studies from Asian *R. microplus* ticks to elucidate the phylogenetic relationships within the *R. microplus* complex was emphasized (Burger et al. 2014).

2.4 Economic importance of ticks

Ticks are thought to be second only to mosquitoes as important vectors of disease affecting both humans and animals worldwide (Ahmed et al. 2007; de la Fuente et al. 2008). They

cause considerable economic losses to cattle by reducing productivity and fertility, thereby affecting the livelihood of resource-poor farming communities in tropical and subtropical countries (Jabbar et al. 2015). The direct effects of ticks on animals' health and productivity may include blood loss, debilitation, anemia, reduced weight gain and milk production, damaged hides, irritation, allergy, tick toxicosis and tick paralysis. Indirectly, they play an important role as vectors for a wide range of infectious pathogens including bacteria, protozoa and viruses (Jongejan and Uilenberg 2004; de la Fuente et al. 2008).

2.5 Current status of tick-borne diseases in Bangladesh

Anaplasmosis, babesiosis and theileriosis are the most important and widely distributed TBDs affecting cattle and ranked high in terms of their impact on the livelihood of resource-poor farming communities in developing countries, including Bangladesh (Jongejan and Uilenberg 2004). The causative agents of the aforementioned TBDs have also been reported from cattle from different parts of Bangladesh (Chowdhury et al. 2006; Alim et al. 2012; Karim et al. 2012; Al Mahmud et al. 2015; Belal et al. 2015; Qiu et al. 2016). However, the majority of the researchers diagnosed the TBDs by examining thin blood smears from clinically infected animals (Chowdhury et al. 2006; Alim et al. 2012; Al Mahmud et al. 2015; Belal et al. 2015) and only a few studies report the use of conventional and multiplex PCR for the detection of TBDs in Bangladesh (Karim et al. 2012; Rahman et al. 2015; Qiu et al. 2016). The status and distribution of the important TBPs infecting bovine and other animals in Bangladesh is given in Table 1. So far, there appears to be an absence of information on the seroprevalence of TBPs test in Bangladesh.

Table 1. Studies performed to determine the prevalence of TBDs in bovine and other animals from different regions of Bangladesh.

Study areas	Tick-borne pathogens	Study duration	Detection methods	Host	References
Different districts of Bangladesh	*Babesia* sp *Theileria* sp *Anaplasma* sp	January 1983-December 1986	Microscopic examination	Cattle	(Samad et al. 1989)
Sirajgonj district	*Anaplasma* sp *Babesia* sp	September to October 2004	Microscopic examination	Cattle	(Chowdhury et al. 2006)
Chittagong district	*Anaplasma* sp *Babesia* sp *Theileria* sp	Information missing	Microscopic examination	Cattle	(Siddiki et al. 2010)
Tangail District	*Anaplasma* sp *Babesia* sp *Theileria* sp	Information missing	Microscopic examination and multiplex PCR	Cattle	(Karim et al. 2012)
Chittagong division	Anaplasmosis Babesiosis Theileriosis	2009-2010	Microscopic examination	Cattle	(Alim et al. 2012)
Bandarban, Rangamati and Khagrachari	*Anaplasma marginale* *Babesia bigemina*	July 2007 to June 2008	Microscopic examination	Cattle and Gayal	(Mohanta and Mondal 2013)
Sirajganj district	*Anaplasma* spp.	December 2013 to November 2014	Microscopic examination	Cattle	(Belal et al. 2015)
Rangpur district	*Anaplasma* sp *Babesia* sp	January to September 2014	Microscopic examination and multiplex PCR	Cattle	(Rahman et al. 2015)
Sirajganj district	*Babesia* spp. *Theileria* spp.	December 2013 to November 2014	Microscopic examination	Cattle	(Al Mahmud et al. 2015)
Mymensingh district	Spotted fever group (SFG) rickettsia and *Anaplasma bovis*	May 2012	PCR and real-time PCR	Stray dogs and ticks	(Qiu et al. 2016)
Mymensingh district	*Babesia gibsoni*	May 2012	PCR, nested PCR and RFLP	Stray dogs	(Terao et al. 2015)

2.6 Control of ticks and TBDs in Bangladesh

Control of ticks is important to prevent or limit production losses and to minimize the transmission of TBPs to susceptible animals (de la Fuente et al. 2007). In Bangladesh, control of tick infestations is usually practiced conventionally with the application of chemical acaricides. Since acaricides tend to be expensive and are not available everywhere, in particular in rural village areas, resource-poor farmers traditionally used local remedies or sprays prepared from the seeds and leaves of the Neem tree (*Azadirachta indica*) or Ata tree (*Annona reticulata*). These are applied topically to tick-infested animals (Rahman et al. 2009; Razu et al. 2010; Islam et al. 2014). Ticks are also often removed manually by farmers during milking. Frequent use of acaricides for controlling ticks may lead to the development of acaricide resistance, environmental contamination and residues of acaricides in milk or meat may endanger food safety (Graf et al. 2004; Willadsen 2006) and so far no information is available regarding the acaricide resistance in Bangladesh. In general, the control of TBDs in Bangladesh is mainly practiced by controlling tick infestations using synthetic acaricides and treating clinically infected animals with antibiotics or antiparasitic drugs based on a tentative diagnosis of the TBDs. Organized ticks or TBD control programs do not exist in Bangladesh.

2.7 Important tick-borne diseases of livestock

2.7.1 Anaplasmosis

2.7.1.1 Etiology

Anaplasmosis is a tick-borne disease of domestic and wild ruminants caused by the rickettsial parasites of the genus *Anaplasma* (Rickettsiales: Anaplasmataceae). The genus *Anaplasma* currently includes the pathogens of ruminants, namely *A. marginale*, *A. centrale (subspecies of A. marginale), A. bovis* (formerly *Ehrlichia bovis*), and *A. ovis*. It also includes *A. phagocytophilum* (formerly *E. phagocytopylium*, *E. equi* and the agent of human granulocytic

ehrlichiosis), and *A. platys* (formerly *E. platys*) which infects dogs (Dumler et al. 2001; Kocan et al. 2004).

2.7.1.2 Geographical distribution

Anaplasma marginale is the most prevalent TBP infecting cattle, endemic in six continents and the causal agent of bovine anaplasmosis (Futse et al. 2003). This pathogen infects red blood cells and inclusion bodies are found in the periphery of the infected cells. Bovine anaplasmosis causes considerable economic losses to the cattle production globally and is widely distributed in tropical and subtropical regions (Kocan et al. 2004; Aubry and Geale 2011). Although clinical disease is mostly notable in cattle, other ruminants including water buffalo, bison, African antelopes, and deer can also become persistently infected (Aubry and Geale 2011). *Anaplasma centrale* is a less pathogenic species which is closely related to *A. marginale* and is used as a live vaccine against *A. marginale* in Israel, South Africa, South America and Australia (de la Fuente et al. 2005). *A. bovis* infects the monocytes in ruminants and causes a subclinical infection (Ooshiro et al. 2008).

2.7.1.3 Transmission and life cycle

Anaplasmosis can be transmitted mechanically by biting flies (e.g. *Tabanus, Stomoxys)* or blood-contaminated fomites (needles, ear tagging devices, and dehorning saws) and biologically by ticks (Kocan et al. 2004). Biological transmission is considered the most important mechanism and around 20 different ticks species have been reported as vectors worldwide, including *Rhipicephalus (Boophilus)* spp., *Rhipicephalus* spp., *Hyalomma* spp., *Dermacentor* spp. and *Ixodes* spp. (Kocan et al. 2004; Kocan et al. 2010). Biological transmission can occur from one life stage of the tick to the next stage (transstadial) or within a stage (intrastadial). Transovarial transmission from one generation to the next does not appear under natural conditions (Samish et al. 1993). Intrastadial transmission of *A.*

marginale mainly occurs in male ticks (Kocan et al. 2004). In addition, *A. marginale* can also be transmitted transplacentally from cow to newborns (Norton et al. 1983; Zaugg and Kuttler 1984). Infected erythrocytes are ingested by the tick during feeding and *A. marginale* starts to develop within the tick gut epithelium. After that it develops into a vegetative (reticulated) form within membrane bound vacuoles and infects many other tissues, including the salivary glands from where they are transmitted to vertebrate hosts during feeding (Kocan et al. 1992; Ge et al. 1996). The reticulated form thereby divides by binary fission and changes into the dense form, which is the infective stage and cattle become infected with *A. marginale* when the infective stage is transmitted via the salivary glands during feeding and complete the life cycle (Kocan et al. 2004).

2.7.1.4 Pathogenesis and clinical signs

Anaplasma marginale invades mature erythrocytes and replicates intracellularly by binary fission (Palmer et al. 2000) and the major surface proteins (MSPs) play an important role in the interaction with both vertebrate host and ticks (Kocan et al. 2004; Brayton et al. 2005; de la Fuente et al. 2005). The pre-patent period of *A. marginale* infection varies from 7 to 60 days (average 28 days) depending on the load of infective stages and the number of infected blood cells increases geometrically. Depending upon the strains of *Anaplasma* spp. and susceptibility of the host, approximately 10% to 90% of the erythrocytes may be infected during acute infection (Aubry and Geale 2011) and the number of infected cells may be as high as 10^9 cells per milliliter of blood (Palmer et al. 1999). Infected blood cells are subsequently phagocytized by the reticulo-endothelial cells, which leads to anemia and icterus without hemoglobinemia and hemoglobinuria (Kocan et al. 2000). Clinical signs may include fever, weight loss, anorexia, lethargy, icterus, abortion and often death in animals older than 2 years (Potgieter and Stoltsz 2004). Milk production significantly reduces in lactating cows and pregnant cows may abort (Aubry and Geale 2011).

2.7.1.5 Epidemiology

Anaplasma marginale can infect cattle of all age groups; however severity of disease depends mainly on their age at first infection. Calves below six months of age are less susceptible and rarely show clinical signs and animals between six to twelve months usually develop mild disease. Animals from one to two years old suffer from acute infection but rarely death occurs and animals are over two years old suffer more of acute disease which often leads to death (Kocan et al. 2003; Aubry and Geale 2011). Zebu cattle (*Bos indicus*) appear to pose a greater resistance to *A. marginale* infection than exotic breeds (*Bos taurus)* (Callow 1984). Recovered animals from acute infection may develop persistent infection and act as carrier of infection (Kocan et al. 2003; Aubry and Geale 2011).

2.7.1.6 Chemotherapy

Antimicrobial therapy for bovine anaplasmosis included the use of tetracycline, imidocarb drugs and a variety of chemotherapeutic agents including arsenicals, antimony derivatives and dyes (Potgieter and Stoltsz 2004). Tetracycline drugs (e.g. chlortetracycline, tetracycline, oxytetracycline) are the most extensively used chemotherapeutic agents for the treatment of bovine anaplasmosis (Kuttler 1979; Rogers 1979) and inhibit the multiplication of *Anaplasma* infection effectively in the erythrocytes (Brock et al. 1953). Gloxazone and the derivatives of carbanilide like amicarbalide and imidocarb also have chemotherapeutic effects against *Anaplasma* infection (Kuttler 1972; De Vos et al. 1978); however, gloxazone is unavailable commercially due to causing severe toxicity in lactating cows (McHardy et al. 1980).

2.7.2 Babesiosis

2.7.2.1 Etiology

Babesiosis is caused by intra-erythrocytic protozoan parasites of the genus *Babesia* which belongs to the phylum apicomplexa, order piroplasmida and family babesiidae (Bock et al. 2004). *Babesia* parasite infects a wide range of domestic and wild animals and occasionally humans and transmitted by ixodid ticks (Bock et al. 2004). Bovine babesiosis is caused mainly by *B. bovis, B. bigemina* and *B. divergens,* while others species include *B. major, B. ovata* and *B. occultans* (Bock et al. 2004; Uilenberg 2006).

2.7.2.2 Geographical distribution

Bovine babesiosis caused by *B. bovis* and *B. bigemina* is widely distributed and found mostly in tropical and sub-tropical countries between latitudes 40^{o}N and 32^{o}S where it affects more than a billion heads of cattle (Bock et al. 2004). *Babesia bovis* and *B. bigemina* are found in Asia, Africa, Australia, Central and South America and Southern Europe, *B. divergens* in North-West Europe, Spain, Great Britain, and Ireland whereas, *B. major* found in Europe, North West Africa, and Asia (Bock et al. 2004).

2.7.2.3 Transmission and life cycle

Bovine babesiosis is principally transmitted vertically by the one-host ticks of the genus *Rhipicephalus* (Bock et al. 2004). *Rhipicephalus microplus*, *R. annulatus* and *R. geigyi* transmit both *B. bovis* and *B. bigemina* and *R. decoloratus* transmits only *B. bigemina*. *Rhipicephalus evertsi evertsi* transmits *B. bigemina* while *B. divergens* is transmitted exclusively by *Ixodes ricinus* (Zintl et al. 2003; Bock et al. 2004). In all species, sporozoite development usually begins when the infected tick attaches to the vertebrate host (Bock et al. 2004). *Babesia bigemina* is mainly transmitted by nymph and adult ticks whereas *B. bovis* infection transmitted through larval stages (Riek 1964; Bock et al. 2004).

Bovine babesiosis exhibits a typical apicomplexan life cycle characterized by merogony, gametogony, and sporogony; it infects the host erythrocytes and is transmitted transovarially (Mehlhorn and Schein 1985; Hunfeld et al. 2008; Chauvin et al. 2009). Both *B. bovis* and *B. bigemina* follow similar patterns of development in adult *Rhipicephalus* spp. (Potgieter et al. 1976; Mehlhorn and Schein 1985). Cattle became infected by the inoculation of sporozoites during feeding of blood meal through saliva, invade the RBC and replicates asexually by binary fission (merogony) and produces merozoites in the erythrocytes. The merozoites invade new erythrocytes by disrupting the membrane and continue the intra-erythrocytic cycle until the death of the host or recover from the infection. After feeding on infected host, gametocytes (gametes) develop to micro and macro-gametes in the tick gut epithelium, fuse to form zygote by gamogony and transforms into a kinetes (vermicules). Subsequently the kinetes leave the gut epithelium, enter into the hemolymph and invade other cells including the ovaries where successive cycles of schizogony take place (trans-ovarian transmission). After hatching of egg, the kinetes migrate to the salivary gland, transforms to multi-nucleated stages (sporogony), forms the sporozoite and infects the susceptible host by sucking of blood (Uilenberg 2006; Chauvin et al. 2009).

2.7.2.4 Pathogenesis and clinical signs

Babesia bovis is considered more pathogenic than *B. bigemina* (Zintl et al. 2005). The pre-patent period of *B. bovis* is usually 6–12 days with peak parasitaemia and clinical signs manifest between 3–5 days, the over-production of cytokines and other pharmacologically active agents following infection contributes the pathogenesis progress (Ahmed 2002; Bock et al. 2004). However, the parasitaemia remains very low in acutely infected cattle, and infected erythrocytes undergo sequestration by attachment to the capillary endothelium, resulting in allergic reactions, organ damage, neurological dysfunction, respiratory distress and edema (Brown and Palmer 1999; Zintl et al. 2005). Clinical signs include fever (>40 °C),

inappetence, depression, anaemia, haemoglobinuria, jaundice, increased respiratory rate, reluctance to move and abortion (Bock et al. 2004). Abortion occurs in pregnant cows and fertility may be reduced in bulls (Bock et al. 2004). On the other hand, the pre-patent period of *B. bigemina* infection is usually 12 to 18 days with high parasitaemia (Bock et al. 2004). The pathogenesis of *B. bigemina* infection is characterized by the rapid and massive destruction of erythrocytes, leading to severe anaemia, jaundice leading to death (Bock et al. 2004; Schnittger et al. 2012). More than 40% of erythrocytes may be infected in acutely infected animals, causing anaemic anoxia (Bock et al. 2004; Zintl et al. 2005). Haemoglobinuria is present more consistently than in *B. bovis* infections, but fever is lower in general. Death may occur in acutely infected cattle due to shock and respiratory distress (Brown and Palmer 1999).

2.7.2.5 Epidemiology

Both *B. bovis* and *B. bigemina* have a high degree of host specificity. In endemic areas, zebu cattle often have a certain degree of natural resistance to disease and exotic cattle are known to be more susceptible to both *B. bovis* and *B. bigemina* than zebu cattle (Bock et al. 1999). Clinical signs of bovine babesiosis mainly occur in mature animals when they exposed for the first time. Calves less than 9 months old possess an innate immunity to disease and rarely show clinical signs. They are protected by maternal antibodies early in life in endemic areas, while the young animals remain resistant for period beyond the persistence of passively transferred antibodies (Zintl et al. 2005). In endemic areas with a high tick challenge, the exposure to infection usually occurs at an early age when the animals are naturally protected, allowing acquired immunity to develop and providing them immunity against subsequent challenge as adults (Bock et al. 2004).

2.7.2.6 Chemotherapy

Although a number of drugs have been used for the treatment of babesiosis, only a few of them are commercially available. Currently, diminazene aceturate and imidocarb dipropionate (imidocarb) are the most commonly used drugs against babesiosis (Bock et al. 2004). Treatment with imidocarb provides protection from *B. bovis* for 1 month and *B. bigemina* for 2 months while diminazene treatment protect cattle from both diseases for 2 and 4 weeks, respectively (Taylor 1979). The protective activity of diminazine is comparatively less than that of imidocarb and both drugs have an inhibitory effect on vaccination efforts (Combrink et al. 2002). However, problems with residual effects in the food chain and limited supply have affected the use of babesiacidal drugs in many countries. Furthermore, at high dose imidocarb eliminates the infection with *B. bovis* and *B. bigemina* from carrier hosts and animals treated with long-acting oxytetracycline significantly reduces the parasitaemia (Jorgensen et al. 1993).

2.7.3 Theileriosis

2.7.3.1 Etiology

Theileriosis is a disease caused by infection of intracellular protozoan parasites of the genus *Theileria* (Phylum Apicomplexa; order Piroplasmida and family Theileriidae), which are transmitted by various ixodid ticks (Perera et al. 2013). *Theileria* parasites affect a wide range of mammals, including domestic and wild ruminants, and cause diseases with varying degrees of severity (Sivakumar et al. 2014). *Theileria* parasites are broadly categorized into two groups based on their ability to transform the leukocytes of host animals: the transforming group (*T. parva*, *T. annulata*, *T. lestoquardi*, and *T. taurotragi*) and non-transforming group (*T. orientalis*, *T. mutans*, *T. velifera*, and *T. cervi*) (Sivakumar et al. 2014). The taxonomy of the benign *Theileria sergenti*/*buffeli*/*orientalis* group is

controversial. Species within this group were previously designated according to their geographical origin, with *T. sergenti* in Japan, *T. buffeli* in Australia and *T. orientalis* in Europe and elsewhere (Jeong et al. 2010). Taxonomically, *T. sergenti* is considered to be an invalid name since it has been previously used to describe a parasite of sheep (Morel and Uilenberg 1981). Based on serological and morphological identities, *Theileria* parasites of this group are now referred to as *T. orientalis* (Uilenberg et al. 1985). Among the aforementioned *Theileria* parasites, *T. parva* (the causative agent of East Coast Fever) and *T. annulata* (the causative agent of tropical theileriosis) cause lymphoproliferative diseases and are considered to be the most pathogenic and economically important *Theileria* spp. infecting bovines (Yusufmia et al. 2010). Other species such as *T. orientalis* and *T. mutans* are considered previously as benign theileriosis and thought to cause milder and/or asymptomatic infections (Sivakumar et al. 2014); however, a number of clinical outbreaks of oriental theileriosis caused by *T. orientalis* have recently been reported from cattle in Asia Pacific countries, causing considerable production losses to cattle industry (McFadden et al. 2011; Perera et al. 2014; Gebrekidan et al. 2016).

2.7.3.2 Geographical distribution

The geographical distribution of *Theileria* parasite is confined to tropical and subtropical regions where suitable tick vectors occur. *Theileria parva* is the causative agent of East Coast Fever (ECF) and is found in Eastern, Central and Southern Africa whereas *T. annulata*, the causal agent of tropical theileriosis, is widely spread throughout the Mediterranean basin, the Middle East and Asia (Norval et al. 1992; Bishop et al. 2004). Other *Theileria* spp. such as *T. mutans*, *T. velifera* and *T. taurotragi* are distributed in Sub-Saharan African countries, and *T. orientalis* in Europe, Australia, New Zealand, Africa, USA, and Asia (Bishop et al. 2004).

2.7.3.3 Transmission and life cycle

Generally, *Theileria* parasites are transmitted by various ixodid ticks of the genera *Rhipicephalus, Amblyomma, Hyalomma* and *Haemaphysalis* (Mans et al. 2015). *Rhipicephalus* tick species (mainly *R. appendiculatus*) are responsible for transmission of *T. parva* as well as *T. taurotragi*, while *T. annulata* is transmitted by several *Hyalomma* tick species. *Theileria mutans* and *T. velifera* are transmitted by *Amblyomma* ticks, whereas *Haemaphysalis* ticks are responsible for transmission of *T. orientalis* (Bishop et al. 2004).

The life cycle of *Theileria* parasite is relatively complex, involving morphologically distinct phases in the tick vector (sexual reproduction) and mammalian host (asexual reproduction) (Shaw and Tilney 1992; Bishop et al. 2004). The asexual lifecycle begins with the inoculation of sporozoites with tick saliva during feeding. The sporozoites then infect nucleated blood cells, where they transform into multinucleated schizonts. In the case of transforming *Theileria* parasites, the schizont-infected leucocytes proliferate exponentially and disseminate throughout the lymphoid system within the host body (Dobbelaere and Heussler 1999). Subsequently, the merozoites are released into blood stream by lysis of the infected leukocytes, progress to invade erythrocytes where they develop into piroplasms, thus completing the asexual life cycle. In case of non-transforming *Theileria* parasites, there is no intra-lymphocytic multiplication and the parasites exclusively multiply within the erythrocytes (Kawamoto et al. 1990). The sexual life cycle starts within the gut of ticks. Circulating piroplasms with gametes are ingested by ticks during feeding and released in the gut lumen, where they develop to macro and micro-gametes which fuse to form zygotes. The resulting zygotes enter the gut epithelium and develop into motile kinetes. Following tick molting, kinetes are liberated into the body cavity and migrate to the salivary glands through the haemolymph. In the salivary glands, they transform into sporozoites (infective stage)

during feeding (Katzer et al. 2006). *Theileria* parasites are usually transmitted transstadially by 2- or 3-host tick species (Bishop et al. 2004).

2.7.3.4 Pathogenesis and clinical signs

The pathogenic effect of various *Theileria* spp. results from the invasion and proliferation of schizonts in lymphocytes and piroplasms in circulating erythrocytes. The transforming group of *Theileria* spp. produces induces more pathogenic effects whereas non-transforming *Theileria* spp. rarely produce schizonts but may cause varying degrees of intravascular haemolysis associated with a high parasitaemia in the circulating blood, resulting in anaemia and icterus (Radostits et al. 2007). Piroplasms can be detected in the erythrocytes at approximately 10 days post inoculation, during which time a transient pyrexia may develop (Sugimoto and Fujisaki 2002) but the pathogenesis of anaemia as consequence of infection is still unclear and may be multifactorial (Stockham et al. 2000). An immune-mediated process for erythrocyte destruction involving an oxidative mechanisms may be involved (Shiono et al. 2001). The major clinical manifestations of theileriosis (transforming group) includes fever, anorexia, dyspnea, increased respiration and heart rate, swelling of superficial lymph nodes and respiratory distress due to sever pulmonary oedema (Lawrence et al. 2004), whereas oriental theileriosis (non-transforming group) showing clinical signs such as pyrexia, haemolytic anaemia, jaundice, lethargy, weakness, reduces milk production and abortion in female animals (Izzo et al. 2010; McFadden et al. 2011).

2.7.3.5 Epidemiology

The epidemiology of theileriosis is largely depends on the distribution of its tick vectors and its ability to complete the development cycle. *Theileria parva* is prevalent throughout the wetter areas of Africa but absent in dry highland (Radostits et al. 2007). The disease is transmitted transstadially and mostly occurs during rainy season (Lawrence et al. 2004). The

morbidity and fatality is very high and affect approximately 90-100% in recently introduced exotic breed and unexposed naïve indigenous cattle. *Theileria annulata* affects cattle and is transmitted transstadially by *Hyalomma* ticks: the main vector in Asia and northeastern Africa is *Hy. anatolicum* and the 2-host tick *Hy. detritum* acts as the main vector in the Mediterranean basin and Middle East (Radostits et al. 2007). Calves are highly susceptible to disease and calve mortality rates reach up to 10-20% (Lawrence et al. 2004). This disease occurs mainly in summer season when the tick abundance is high. Recovered animals possess solid, long lasting immunity and act a carrier host (Radostits et al. 2007). *Theileria orientalis* is widely distributed around the world and infects cattle, Asiatic and African buffaloes (Uilenberg et al. 1985). It is mainly transmitted by *Haemaphysalis* ticks (Fujisaki 1992). This disease is usually considered as benign theileriosis, but a number of clinical outbreaks were recently reported in the Asia pacific region, thus posing a significant economic threat to cattle production (Izzo et al. 2010; McFadden et al. 2011; Perera et al. 2014). *Theileria orientalis* infection is assumed to often occur in mixed infections with other *Theileria* species (Uilenberg et al. 1985).

2.7.3.6 Chemotherapy

Theileriosis in cattle is treated by parvaquone, buparvaquone (hydroxynaphthoquinone derivatives), halofuginone (quinazolinone derivatives) and tetracycline drugs. Parvaquone and buparvaquone are effectives against both schizonts and piroplams stage of *Theileria parva*, while halofuginone is only effective against the schizont stage (Lawrence et al. 2004). Based on recovery rate, recrudescence of infections and therapeutic index, parvaquone and buparvaquone are more effective compared to halofuginone treatment (McHardy et al. 1983; McHardy et al. 1985). However, these drugs have disadvantage of being relatively expensive and this reduces their application in the field conditions (Lawrence et al. 2004).

2.8 Economic impact of tick–borne diseases

Tick-borne diseases cause substantial economic losses to the livestock production sector in tropical and subtropical countries, not only by direct losses through morbidity, mortality, abortions, decreased milk and meat production, but also as a consequence of costs incurred the treatment, prevention and control measures (Minjauw and McLeod 2003). The global economic loss caused by ticks and TBDs was estimated between 13.9 to 18.7 billion US dollars annually, where 80% of the cattle population are at risk (de Castro 1997). Cattle production losses due to TBDs have been estimated annually around 26 million US dollars in Australia (Sackett et al. 2006), 500 million US dollars in Brazil (Grisi et al. 2002), 168 million US dollars in Africa (Mukhebi et al. 1992) and 499 million US dollars in India (Minjauw and McLeod 2003).

2.9 Diagnostic methods for tick-borne diseases

The diagnosis of TPB may be based on characteristics clinical signs, geographic features, and necropsy findings observed in infected animals. However, clinical signs and pathology may not be reliable source of diagnosis because of many clinical signs associated with disease are not pathognomonic and other infectious and non-infectious disease may show similar signs to that of TBDs (Lawrence et al. 2004). Laboratory diagnostic tests are required to confirm the diagnosis based on clinical signs, necropsy findings and epidemiological information, with the inference that live parasites are present in the animal body during sampling (Mans et al. 2015).

2.9.1 Microscopic examination

Microscopic examination of blood smear is often considered to be the standard technique for the routine diagnosis of TBDs. The method is relatively cheap, quick and easy to perform (Minjauw and McLeod 2003). A major drawback of this technique is its low sensitivity and

specificity which make it unsuitable to detect parasites in carrier animals due to relative low parasitaemias (Salih et al. 2007). Although commonly used in laboratories for clinical diagnosis, the differentiation between parasites is difficult and requires trained and experienced personnel (Almería et al. 2001).

2.9.2 Serological methods

Serological methods have been used for the diagnosis of TBDs in many epidemiological studies e.g. seroprevalence of *Anaplasma marginale* was estimated from dairy cattle of Tanzania using an indirect enzyme-linked immunosorbent assay and bovine babesiosis from Zambia using the indirect fluorescent antibody test (IFAT) (Jongejan et al. 1988; Swai et al. 2005). Commonly used serological tests for indirect detection of TBDs include the indirect fluorescent antibody test (IFAT), enzyme-linked immunosorbent assay (ELISA), competitive enzyme-linked immunosorbent assay (cELISA) (de Echaide et al. 1998), complement fixation (CF) test, card agglutination test (CAT) and capillary agglutination assay (Aubry and Geale 2011). Although serological tests can be more sensitive than microscopic examination, they do have a number of limitations. These tests are often unable to differentiate between previous and current infections and carrier status of infection (Oura et al. 2004; Salih et al. 2010). In addition, antibody cross-reactions have also been reported among closely related species and incorrect interpretation of results may lead to inappropriate control measures (Edelhofer et al. 2004). The competitive enzyme-linked immunosorbent assay (cELISA) (de Echaide et al. 1998) and indirect immunofluorescence antibody technique (IFAT) are widely used for diagnosis of anaplasmosis (Goff et al. 1985) whereas, ELISA and IFAT are commonly used for diagnosis of babesiosis and theileriosis (Bakheit et al. 2004; Darghouth et al. 2004).

2.9.3 DNA based molecular methods

DNA based molecular techniques are mostly depend on specific primers and/or probes that targeted the small region of genes and thought to be conserved for all members of a species or genus of a parasite (Mans et al. 2015). Molecular methods have been developed to accurately diagnose TBPs and have found to use in numerous epidemiological studies e.g. *Babesia* and *Theileria* species infecting cattle is identified using a reverse line blotting method (RLB) in Portugal (Silva et al. 2010). Molecular techniques can diagnose acute infections as well as latent infections in carrier animals with very low parasitaemias, whereas serological tests detect exposure to infection (Sibeko et al. 2008; Salih et al. 2010). However, molecular diagnostic techniques are relatively expensive compared to serological and microscopic examinations, which hampered their adoption in resource-poor developing countries (Jabbar et al. 2015). Several DNA-based molecular diagnostic techniques are used for identifying TBPs in epidemiological surveys include conventional polymerase chain reaction (PCR) assays (Oliveira-Sequeira et al. 2005; Criado-Fornelio 2007) and various other PCR based techniques such as Restriction Fragment Length Polymorphism (RFLP) methods (Zaeemi et al. 2011), nested-PCR (Odongo et al. 2009), real-time PCR (Criado-Fornelio 2007; Sibeko et al. 2008; Steyn et al. 2008), loop-mediated isothermal amplification (LAMP) assays (Criado-Fornelio 2007; Salih et al. 2008; Xie et al. 2013), fluorescence resonance energy transfer (FRET) real-time PCR based assays (pan-FRET) (Yang et al. 2014), high-resolution melt analysis (Salim et al. 2013) and PCR followed by the Reverse Line Blot hybridization (RLB) assay (Gubbels et al. 1999; Oura et al. 2004; Nijhof et al. 2007). The development of PCR from conventional to nested to real-time has improved the sensitivity, specificity, quantification and speed of detection, while other methods like RLB, pan-FRET assays and high-resolution melt analysis allowed to detect multiple pathogens species or genotype at the same time (Mans et al. 2015).

The RLB is suitable for the simultaneous detection and differentiation of TBPs. The technique is based on the PCR amplification of related microorganism species in a single reaction, i.e. the PCR uses primers that are based on conserved regions of ribosomal RNA genes, instead of specific PCR reactions for each individual species. The individual species can subsequently be differentiated through hybridization with oligonucleotides specific for each species (species-specific probes) in a line-blotter apparatus (Gubbels et al. 1999; Nijhof et al. 2003). The first step is PCR amplification of the hypervariable V1 region of the rickettsial 16S rRNA gene (*Ehrlichia, Anaplasma* and *Rickettsia* spp.); the 18S rRNA gene spanning the V4 region (*Babesia* and *Theileria*) and the 5S and 23S rRNA genes (*Borrelia* spp.) using three sets of biotinylated primers located within the conserved parts of these genes (Rijpkema et al. 1995; Gubbels et al. 1999; Schouls et al. 1999; Bekker et al. 2002). In a second step, the successful PCR products are hybridized in a miniblotter to known species-specific oligonucleotide probes which are covalently linked to the membrane by a 5' terminal amino-linker (Gubbels et al. 1999). The PCR products are applied in a perpendicular way to the species-specific oligonucleotides probes. In this way, the PCR products can hybridize at the cross-sections with the specific oligonucleotide probes. Successfully hybridized PCR products are then visualized by incubation with peroxidase-labeled streptavidin and enhanced chemiluminescence (ECL) detection reagents on the membrane. Sites where spots appear represent hybridization between the species-specific oligonucleotide probes and PCR products and micro-organisms in the samples can subsequently be identified (Gubbels et al. 1999; Nijhof et al. 2003).

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CHAPTER 3

Publication I

Molecular identification of tick-borne pathogens infecting cattle in Mymensingh district of Bangladesh reveals emerging species of *Anaplasma* and *Babesia*

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CHAPTER 4

Publication II

Morphological and phylogenetic analyses of *Rhipicephalus microplus* ticks from Bangladesh, Pakistan and Myanmar

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CHAPTER 5

5. General discussion and recommendations

Tick-borne diseases (TBDs) are considered a major constraint for profitable livestock production and productivity in many developing countries of the world and pose a significant impact on the livelihood of resource-poor farming communities due to the associated high morbidity and mortality (Minjauw and McLeod 2003). They are also an important hindrance for the genetic improvement of indigenous cattle breeds by preventing the importation of highly productive exotic cattle breeds from temperate countries to TBDs endemic regions of the tropics and sub-tropics as these animals are highly susceptible to TBDs (Kocan et al. 2003; Simuunza et al. 2011). Since multiple TBPs are known to be transmitted by various ixodid ticks that are endemic in Bangladesh, a comprehensive cross-sectional study was performed by screening bovine blood samples collected from the local cattle population of Mymensingh district, Bangaladesh by PCR and a RLB assay. This study is highlighted in the Chapter 3 of this thesis. The *Rhipicephalus microplus* complex consists of several species which share many morphological similarities and thus difficult to distinguish. The most important representatives of this complex is *R. microplus*, the southern cattle tick, which causes multi-billion dollar economic losses to the cattle industry around the world. This one-host tick is also common in Bangladesh and known to be responsible for the transmission of bovine anaplasmosis and babesiosis. Since there is a paucity of information regarding the distribution and molecular background of *R. microplus* in Asia including Bangladesh, Chapter 4 of this thesis intended to fill this gap by studying the morphology and genetics of *R. microplus* tick specimens from Bangladesh, Myanmar and Pakistan in more detail.

5.1 Epidemiology of TBPs infesting cattle in Bangladesh

The geo-climatic conditions in Bangladesh provide a suitable environment for various ixodid tick species. Many of these may act as vectors for variety of TBPs that can infect the cattle population, thereby constraining the productivity of the local cattle industry. However, information on the occurrence of TBPs using sensitive and specific molecular diagnostic techniques is scarce for Bangladesh. Therefore, in Chapter 3 of this thesis, a combined PCR and RLB assay was performed using in-house prepared ECL detection reagents for the simultaneous detection and differentiation of TBPs in bovine blood samples spotted on FTA cards collected in Mymensingh district of Bangladesh. This study revealed overall high infection rates with ticks (60.4%) and TBPs (62.2%) in local cattle populations with a complex scenario of multiple infections in general. Several risk factors associated with the PCR/RLB-based prevalence of TBPs were also identified through a multivariate logistic regression model which may help to improve our understanding of the TBP prevalence and possible interventions.

Microscopic examination of thin blood smears is the standard technique for the diagnosis of clinical infection. Results from the present study revealed that eighteen smears were positive (4.7%, 18/384) for *Anaplasma* spp. and none of the smears were found to be positive for *Babesia* and *Theileria* species. This is illustrative for the relatively low sensitivity and specificity of classical diagnostic methods compared to molecular diagnostic techniques such as the PCR/RLB, which complicates the diagnosis of TBDs by microscopic examination in particular in healthy carrier animals with low parasitemias (Almería et al. 2001; Salih et al. 2007).

Theileria orientalis has previously been reported from cattle from all the major continents of the world, including neighbouring countries of Bangladesh such as India (Aparna et al. 2011; Kakati et al. 2015), Pakistan (Gebrekidan et al. 2017) and Myanmar (Bawm et al. 2014). This

study represents the first evidence for the presence of *T. orientalis* infection in cattle of Bangladesh. *Theileria orientalis* has previously been considered as benign theileriosis, however, the pathogenic genotypes (*ikeda* and *chitose*) caused considerable clinical outbreaks in cattle of Asia-pacific regions and encountered significant production losses to the cattle industry in these regions (Izzo et al. 2010; Islam et al. 2011; McFadden et al. 2011; Perera et al. 2014; Gebrekidan et al. 2016). Clinical diseases as a result of *Theileria orientalis* infection in Bangladesh have not been reported. *Haemaphysalis* spp. ticks are considered the principal vectors for transmission of *T. orientalis* infection (Fujisaki 1992) and *Haemaphysalis bispinosa* reported in this study may act as a vector for this pathogen in Bangladesh. In general, cattle become infected with theileriosis as calves and thereafter remain carriers, thus forming as an animal reservoir in endemic areas (Sugimoto and Fujisaki 2002). All the sampled animals in the present study were apparently healthy and did not show any clinical signs of theileriosis, suggesting the existence of endemic stability in the surveyed cattle population. The present study revealed high prevalence of *A. bovis* (35.7%), which is also reported for the first time from bovine blood samples in Bangladesh. This finding is consistent with previous reports describing the detection of *A. bovis* in canine blood and ticks (Qiu et al. 2016). In general, this bacterium causes a subclinical form of anaplasmosis and it is most likely that this pathogen has previously been overlooked due to lack of using molecular diagnostic tools in Bangladesh. Moreover, the epidemiology of this bacterium is still unclear in mammalian hosts (Ooshiro et al. 2008) and requires further investigations.

Anaplasma marginale, B. bigemina and *B. bovis* are widely distributed TBPs affecting cattle globally and are thought to be the most economically important and prevalent TBPs causing high morbidity and mortality in subtropical and tropical countries (Jongejan and Uilenberg 2004; Kocan et al. 2010). These pathogens are also known to be endemic in Bangladesh and cause production losses to the cattle industry (Chowdhury et al. 2006; Karim et al. 2012; Al

Mahmud et al. 2015; Belal et al. 2015; Rahman et al. 2015). The present study showed a relatively lower prevalence of *A. marginale* (4.2%), *B. bigemina* (1.0%) and *B. bovis* (0.5%) compare to the previous findings reported in Bangladesh. In general, zebu cattle (*Bos indicus*) is the mostly dominant cattle breed in Bangladesh and are used by resource-poor, small householders farmers for draft power, milk and meat production. It has been reported previously that zebu cattle (*B. indicus*) are generally more resistant to tick infestations and infection with TBPs (Bianchin et al. 2007; Jonsson et al. 2008) and this would most likely explain the lower prevalence of TBPs as the majority of the sampled animals in the present study were zebu cattle. In addition, animals that recover from TBP infection, often remain persistent carriers and may serve as lifelong reservoirs (Aubry and Geale 2011). Parasitemias in healthy carrier animals are generally low and fluctuate over time, periodically falling below the detection limit of PCR (Herrero et al. 1998). It is also possible that the sensitivity of PCR was compromised due to limitations associated with the DNA extraction since blood samples were collected and spotted on FTA filter cards. DNA was extracted from sixteen 3 mm FTA filter card punches, containing a limited amount of pathogen DNA compared to DNA extracts from a larger whole blood sample volume, which may have led to an underestimation of the actual prevalence of these pathogens in this study. FTA filter cards are useful in regions where a cold chain for the storage of biological samples is not available. Since FTA filter cards inactivate infectious micro-organisms and lyses cellular material upon contact with the matrix, it is also useful for the shipment of biological samples that may otherwise pose a biohazard risk (Michaud et al. 2007; Abdelwhab et al. 2011) FTA cards have previously been used effectively for the detection of various infectious organisms including bacterial pathogens (Lampel et al. 2000), viral pathogens (Picard-Meyer et al. 2007), protozoan parasites (Orlandi and Lampel 2000) and tick-borne pathogens (Hailemariam et al. 2017). In this study, it found use in the export of samples from

Bangladesh to Germany, since the importation of whole blood samples from Bangladesh, where Foot and Mouth Disease is endemic, into Germany is not allowed.

The RLB is a versatile diagnostic micro-array tool that allows for sensitive and simultaneous detection and discrimination of up to 42 different pathogens in a single sample. The RLB is based on the simultaneous PCR amplification of related microorganism species; here *Anaplasma/Ehrlichia/Richettsia* spp. in one PCR and *Babesia/Theileria* spp. in a second PCR using biotinylated primers. The individual species can subsequently be differentiated by hybridization of the biotinylated PCR products to oligonucleotides specific for each species (species-specific probes) in a line-blotter apparatus (Gubbels et al. 1999; Nijhof et al. 2003). Successful binding of the PCR products to complementary oligonucleotide probes is subsequently visualized using peroxidase-labeled streptavidin and luminol-based chemiluminescence. RLB makes the economic use of resources due to the possibility of re-using the membrane containing the oligonucleotides and the limited number of PCR amplifications required. This method has previously been effectively used for the identification and characterization of various novel tick-borne pathogens (Nijhof et al. 2003; Nijhof et al. 2005). In our study, some samples displayed a positive *Anaplasma/Ehrlichia* or *Babesia/Theileria* catch-all signal without species-specific signals, indicating that a novel species or a genetic variant of a known TBP was present. Subsequent sequence analysis of the 16S rRNA and 18S rRNA genes confirmed the presence of two new *Anaplasma* and *Babesia* species, designated as *Anaplasma* sp. Mymensingh and *Babesia* sp. Mymensingh, as well as a novel *T. orientalis* genotype, designated as *Theileria orientalis* BR-BDH3. New specific RLB probes for these novel pathogens were designed and included on a new RLB membrane and all samples were retested to screen for the presence of these pathogens. The combined phylogenetic analysis (ML) of 16S rRNA and *groEL* sequences demonstrated that *Anaplasma* sp. (Mymensingh) is closely related to *A. platys* and the phylogeny of 18S rRNA

sequences revealed that *Babesia* sp. (Mymensingh) is closely related to *B. bigemina* and *Theileria orientalis* BR-BDH3 clustered together with *T. orientalis* type buffeli from Australia and *Theileria buffeli* from China, respectively.

Since it is unknown whether this newly discovered *Anaplasma* species is pathogenic to humans or livestock, as certain other *Anaplasma* species do (Kocan et al. 2015), further studies are required to isolate, cultivate and characterise the newly detected *Anaplasma, Babesia* species and *T. orientalis* from infected animals and determine their pathogenicity, questing tick vector and reservoir hosts.

The multivariate logistic regression model demonstrated that access to grazing land and cattle housing made from mud showed a strong positive association with *T. orientalis* infection in cattle. It is plausible that access to grazing land increases the probability of cattle coming into contact with ticks and therefore with the TBPs. Mud-made houses are likely to conserve more humidity and often have cracks and crevices in walls that may provide shelter where ticks can hide and maintain their lifecycle (Muhammad et al. 2008). It has also been reported previously that *H. bispinosa*, responsible for transmission of *T. orientalis*, has a predominant endophilic nature and is known to breed in cattle sheds (Geevarghese and Mishra 2011). Keeping cattle together with other livestock species was identified as a potential risk factor for *A. marginale* infection whereas, concurrent infection with other TBPs and tick infestation were risk factors for *Anaplasma* sp. Mymensingh infection. Acaricide usage was found to have a protective effect against both *A. marginale* and *Anaplasma* sp. Mymensingh infections.

In this study, we evaluated the use of a previously described non-commercial, inexpensive and in-house prepared ECL solution for Western blots (Haan and Behrmann 2007), adapted it for the RLB and subsequently used for the screening of the collected samples. The in-house

made ECL solution showed similar signal intensity and background to commercial ECL solution and it could reduce the costs of the RLB assay, making the technique more accessible for laboratories in resource-poor settings. Further efforts to further reduce the costs of the RLB could focus on the development of a robust multiplex PCR, in which the amplification of two or more target sequences, for instance of the 16S rRNA gene from (i) *Anaplasma, Ehrlichia,* and *Rickettsia* and the 18S rRNA gene from (ii) *Babesia* and *Theileria*, are combined into a single reaction. Other multiplex PCR-based RLB (mPCR/RLB) assays have previously been described for several other targets (Kong and Gilbert 2006) including the identification and classification of staphylococcal cassette chromosome mec (SCCmec) types of methicillin-resistant *Staphylococcus aureus* (MRSA) (Cai et al. 2009; O'Sullivan et al. 2010) and the simultaneous detection of urogenital microorganisms (McKechnie et al. 2009).

The findings from this epidemiological survey show a high burden of infection with multiple TBPs in cattle in Mymensingh district of Bangladesh. It is conceivable that the occurrence of concurrent infections with multiple TBPs may play a role in the clinical outcome of disease. Recently, it was reported that East African shorthorn zebu calves that were co-infected with *T. parva* (the causal agent of East Coast Fever) and a low pathogenic *Theileria* species (*T. mutans*), resulted in decreased calve mortality associated with *T. parva* infection in western Kenya (Woolhouse et al. 2015). In contrast, co-infections with *Borrelia burgdorferi* (Lyme disease) and *Babesia microti* were reported to cause greater disease severity and duration in humans (Diuk-Wasser et al. 2016). Further studies are required to investigate the clinical outcomes of such co-infections in the study area and its impact on cattle production in Bangladesh in general.

5.2 Morphological and phylogenetic analyses of *Rhipicephalus microplus* ticks

The southern cattle tick, *Rhipicephalus microplus* is considered to be the most economically important ticks, distributed widely in tropical and subtropical countries. It causes considerable impact to the cattle industry directly by causing blood and weight loss, irritation, damaging hides and skin, and indirectly by transmitting various pathogens such as *Babesia* spp. and *Anaplasma* sp. causing babesiosis and anaplasmosis (Guerrero et al. 2006). Currently, there are six valid species in the subgenus *Rhipicephalus* (*Boophilus*): *Rhipicephalus annulatus* (Say, 1821), *Rhipicephalus decoloratus* Koch, 1844, *Rhipicephalus microplus* (Canestrini, 1887), *Rhipicephalus australis* Fuller, 1899, *Rhipicephalus kohlsi* (Hoogstraal & Kaiser, 1960), and *Rhipicephalus geigyi* (Aeschlimann & Morel, 1965) and among them, *Rhipicephalus microplus* is thought to be the most widely distributed species from a global prospective (Guglielmone et al. 2014). The multi-locus analysis from the present study demonstrated that the *R. microplus* species complex consists of at least five independent taxa: *R. annulatus*, *R. australis* and three *R. microplus* clades: *R. microplus* clade A sensu Burger *et al.* (2014), *R. microplus* clade B sensu Burger *et al.* (2014) and *R. microplus* clade C sensu Low *et al.* (2015). These results are in agreement with previous phylogenetic studies from Australia and Malaysia (Burger et al. 2014; Low et al. 2015). In general, we found that *cox1* marker was more variable and informative in resolving the evolutionary relationships among the closely related species of the *R. microplus* complex than 12S rRNA and ITS-2 markers. The results of this present study were concordant with the previous reports where it has been demonstrated that the *coxI* candidate had a strong support to resolve the phylogenetic relationships among the apparent clades within the *R. microplus* complex (Burger et al. 2014; Low et al. 2015). Our present findings were also in line with a phylogenetic analysis of the *R. sanguineus* complex where the *cox1* gene was found to be more suitable in discriminating *Rhipicephalus* ticks at species level compared to the nuclear

ITS-2 gene (Latrofa et al. 2013). Unfortunately, the number of available *R. microplus cox1* gene sequences in public databases, is currently not as extensive compared to other markers such as 12S or 16S rRNA. There is thus a need for a broader geographic sampling for the cox1 gene, to provide a more detailed picture of the *R. microplus* complex distribution.

The *Rhipicephalus microplus* species complex is composed of several species with intraspecific morphological variations that complicate their identification and discrimination. The morphological characteristics of *R. microplus* clade A and clade C adults observed by SEM in our study confirm that it is very difficult, if not impossible, to unambiguously differentiate closely related species of the *R. microplus* species complex without background knowledge on the geographical origin of the tick, a finding which is in concordance with a previous report for *R. australis* and *R. microplus* (Barker and Walker 2014). An analysis of morphological differences between juveniles representing different clades of the *R. microplus* complex might be useful to see whether these life stages can be discriminated based on morphology alone. Currently, and in the absence of a morphological description for *R. microplus* clade B adults, *Rhipicephalus annulatus* is the only species within the *R. microplus* complex that can be discriminated unambiguously with relative ease, since a caudal appendage is absent in male ticks. However, caution should be taken as the caudal appendage may occasionally also be absent in some males specimens of *R. microplus* (Uilenberg 1962). It is also possible that a variation in morphological features is caused by the hybridization between different species of the *R. microplus* complex and further genotyping will be required, in particular in regions where multiple species of the *R. microplus* complex are sympatric such as peninsular Malaysia, southern China and Indonesia. Here, detailed morphological, molecular and crossbreeding studies would be required to elucidate the taxonomic status of the tick species.

The data presented in this cumulative thesis have raised several issues that require further investigation:

i) The present study showed the advantages and versatility of the RLB assay for the sensitive and simultaneous detection of multiple TBPs in cattle of Bangladesh. However, its adaptability and applicability are limited in developing countries like Bangladesh where the laboratories are poorly set up, possibly due to associated higher costs. One of the more costly consumable used in RLB assay is the commercial ECL detection reagent, which can be substituted with a non-commercial, inexpensive and in-house prepared ECL solution as shown in this study. Future efforts to make the RLB assay more cost-effective could focus on the development and optimization of a multiplex PCR, in which two or more TBP target sequences can be amplified simultaneously in a single reaction instead of two or more PCRs.

ii) Since it is unknown whether the newly discovered and characterized *Anaplasma* and *Babesia* species from this study are pathogenic to humans and/or livestock, future studies are required to isolate and further characterize these pathogens and to determine their pathogenicity and impact on the cattle production as well as their zoonotic importance in Bangladesh. Moreover, it needs to be determined which tick species could act as a vector for these TBPs.

iii) The present study was performed to investigate the prevalence of TBPs in local cattle population in Mymensingh district only. Additional studies could focus on different regions, seasons and management systems to address the risk factors associated with the prevalence of ticks and TBPs. There is also a paucity of information on the prevalence of ticks and TBPs in wildlife such as wild pigs, foxes, deer and monkeys in Bangladesh.

iv) Ticks are obligate hematophagous arthropods having great veterinary and medical importance. The growing risks associated with the emerging tick-borne pathogens in human and animals are increasing around the world due to increase tick populations, global warming, changes in biology and increased contacts between humans, animals and ticks (de la Fuente and Estrada-Pena 2012). Available information on the occurrence of human zoonotic diseases in particular the spotted fever group (SFG) rickettsia transmitted by various ixodid ticks is scarce in Bangladesh. Recently, so far one study was performed in this country where two genotypes of rickettsiae were identified from *Rhipicephalus microplus* and *Haemaphysalis bispinosa* (Qiu et al. 2016). Since large numbers of ticks were collected from domestic animals in the study area, where farmers live in a close association with animals. It would seem to have a higher chance to get human infection; therefore, future studies should focus to detect various rickettsial pathogens from ixodid ticks feeding on animals to investigate their potential zoonotic significance in Bangladesh.

v) Although resource-poor farmers treated their animals with acaricides, high prevalence of ticks and TBPs were detected from the sampled animals in this study area, which might be indicative for the occurrence of acaricide resistance. Therefore, further studies could focus on testing *R. microplus* ticks in particular for the presence of acaricide resistance against different classes of chemicals that are regularly used as acaricides in Bangladesh.

vi) Since the occurrence of intraspecific variations complicates the morphological discrimination of closely related species belonging to the *R. microplus* complex, further detailed morphological, genomic and crossbreeding studies are recommended to elucidate the species status of *R. microplus* clades.

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Summary

Tick-borne diseases (TBDs) affect the majority of world's cattle population and are considered a major constraint to the health and productive performance of profitable livestock production throughout the world. Bovine anaplasmosis, babesiosis and theileriosis are the most economically important and widely distributed TBDs affecting cattle and are also endemic in Bangladesh. However, detailed information on the prevalence of bovine tick-borne pathogens (TBPs) determined using sensitive and specific molecular diagnostic tools is scarce for this country. The main objective of this cumulative thesis therefore was to investigate the prevalence of TBPs from blood samples collected from local cattle in Mymensingh district of Bangladesh using a Reverse Line Hybridization (RLB) assay.

The first study aimed to estimate the prevalence of TBP´s in cattle of Mymensingh districct of Bangladesh. In total, 384 blood samples from apparently healthy cattle from 12 sub-districts (Upazilas) of Mymensingh district were spotted on FTA cards in the field and subsequently screened using a Reverse Line Blot (RLB) hybridization assay for the presence of *Anaplasma, Ehrlichia, Rickettsia, Babesia* and *Theileria* spp.. *Rhipicephalus microplus* and *Haemaphysalis bispinosa* were the main tick species infecting the examineed cattle. In the blood samples, *Theileria orientalis* infections were most frequently found (212/384, 55.2%) followed by infections with *Anaplasma bovis* (137/384, 35.67%), *Anaplasma marginale* (16/384, 4.17%), *Babesia bigemina* (4/384, 1.04%) and *Babesia bovis* (2/384, 0.52%). Two previously uncharacterized tick-borne pathogens (TBPs) were also found and further characterized. This included a novel *Anaplasma* sp. (*Anaplasma* sp. Mymensingh) and *Babesia* sp. (*Babesia* sp. Mymensingh) with estimated prevalences of (50/384, 13.0%) and (1/384, 0.3%). A phylogenetic analysis demonstrated that these newly described pathogens are genetically closely related to *Anaplasma platys* and *Babesia bigemina,* respectively. Several key risk factors e.g. access to grazing land, mud-made cattle house, tick infestation

and presence of other TBDs were also identified on the prevalence of *T. orientalis*, *A. marginale* and *Anaplasma* sp. Mymensingh. A previously described inexpensive and in-house prepared ECL solution for Western blots was successfully adapted for use within the RLB assay and used in this study. These findings contribute to our knowledge on the occurrence of TBPs in Bangladesh and provide us with a better understanding of the epidemiology of ticks and TBPs, which will be helpful in the development of control strategies to limit the impact of TBPs.

The second study aimed to elucidate the phylogenetic relationship between *R. microplus* ticks collected from Bangladesh, Myanmar and Pakistan with other *Rhipicephalus* spp. using Scanning Electron Microscopic (SEM) imaging and mitochondrial genome sequence analyses.

Boophilid ticks are classified in the genus *Rhipicephalus* and are widely distributed in subtropical and tropical countries. *Rhipicephalus microplus,* also known as the common cattle tick, is the most important representative of the *Rhipicephalus microplus* complex. This complex is composed of several species that share many morphological similarities, complicating their identification. This one-host tick account for a significant economic losses to the livestock industry by causing irritation and blood loss, decreasing leather quality and acting as vectors for potentially fatal diseases such as bovine anaplasmosis and bovine babesiosis. However, little is known about the morphology and genetic background of *R. microplus* from Asia.

Phylogenetic analyses of cytochrome oxidase 1 (cox1), internal transcribed spacer 2 (ITS-2) and 12S rRNA gene sequences confirmed that the *R. microplus* complex consists of at least five independent taxa. This includes *R. annulatus, R. australis, R. microplus* clade A sensu Burger *et al.* (2014), *R. microplus* clade B sensu Burger *et al.* (2014) and *R. microplus* clade C sensu Low *et al.* (2015). *Rhipicephalus microplus* ticks from Bangladesh, Myanmar and

Pakistan were all assigned to *R. microplus* clade C. Furthermore, we confirmed that the *cox1* gene was the preferred genetic marker for resolving the evolutionary relationships among closely related species within the *R. microplus* complex. SEM images of adult specimens from *R. microplus* clade C revealed a wide range of intraspecific morphological variations, including the features previously identified as critical for distinguishing *R. microplus* clade A from *R. australis* ticks, which is illustrative for the complications in identifying species within the *R. microplus* complex solely relied on the morphology. The results from this study highlight the need for more genetic sampling of the *R. microplus* complex from a wider geographical range and crossbreeding experiments between the different taxa or comparison of populations using nuclear genome data after deep sequencing to elucidate the species status of *R. microplus* clades.

Zusammenfassung

Molekularbiologische Diagnose und Charakterisierung von Zecken und durch Zecken-übertragene Erreger bei Rindern im Distrikt von Mymensingh, Bangladesch

Von Zecken übertragene Krankheiten (TBDs) betreffen den Großteil der weltweiten Rinderpopulation und werden als einer der größten negativen Einflüsse auf die Gesundheit und die produktive Leistungsfähigkeit der wirtschaftlichen Nutztierproduktion weltweit gesehen. Die Bovine Anaplasmose, Babesiose und Theileriose sind die wirtschaftlich am wichtigsten und am weitesten verbreiteten TBDs, die Rinder beeinträchtigen können. Sie sind auch endemisch in Bangladesch. Es fehlen jedoch detaillierte Informationen zur Prävalenz von Zecken übertragenen bovinen Pathogenen bei Rindern in Bangladesch.

In der ersten Studie dieser Dissertation sollte daher die Prävalenz von TBP´s in Blutproben von Rindern im Distrikt von Mymensingh bestimmt werden. Insgesamt wurden 384 Blutproben von klinisch gesunden Rindern von 12 Sub-Distrikten (Upzilas) des Mymensingh Distrikts im Feld auf FTA-Karten aufgetragen und anschließend mittels eines Reverse Line Blot (RLB) Hybridisierung Assays auf das Vorhandensein von *Anaplasma, Ehrlichia, Rickettsia, Babesia* and *Theileria* spp. getestet werden. *Rhipicephalus microplus* und *Haemaphysalis bispinosa* waren die Hauptzeckenarten, die die untersuchten Rinder befallen hatten. In den Blutproben wurden am häufigsten *Theileria orientalis*-Infektionen nachgewiesen (212/384, 55.2%), gefolgt von Infektionen mit *Anaplasma bovis* (137/384, 35.67%), *Anaplasma marginale* (16/384, 4.17%), *Babesia bigemina* (4/384, 1.04%) und *Babesia bovis* (2/384, 0.52%). Auch zwei zuvor nicht bestimmte von Zecken übertragene Pathogene (TBPs) wurden gefunden und weiter charakterisiert. Dazu gehörten zwei neue Spezies, *Anaplasma* sp. (*Anaplasma* sp. Mymensingh) und *Babesia* sp. (*Babesia* sp. Mymensingh), mit geschätzten Prävalenzen von (13.0 %, 50/384) und (0.3 %, 1/384). Eine

phylogenetische Analyse zeigte, dass diese neu beschriebenen Pathogene genetisch nah verwandt mit *Anaplasma platys*, beziehungsweise *Babesia bigemina* sind. Mehrere Risikofaktoren für das Auftreten von *T. orientalis*, *A. marginale* und *Anaplasma* sp. -Infektionen in Mymensingh wurden ebenfalls identifiziert, z.B. der Zugang zu Weiden, die Aufstallung in Lehmhäusern/-stallungen, hoher Zeckenbefall und das Vorhandensein anderer TBDs.

Eine zuvor beschriebene kostengünstige und hausintern hergestellte ECL-Lösung für Western Blots wurde erfolgreich für den Gebrauch im RLB Assay angepasst und in dieser Studie benutzt. Diese Befunde tragen zu unserem Wissen über das Vorkommen von TBPs in Bangladesch bei und verschaffen uns ein besseres Verständnis über die Epidemiologie der Zecken und der TBPs, was hilfreich für die Entwicklung von Bekämpfungsstrategien sein wird, um die Auswirkungen von TBPs einzuschränken.

Ziel der zweiten Studie war es, die phylogenetische Beziehung zwischen *R. microplus*-Zecken, die in Bangladesch, Myanmar und Pakistan gesammelt wurden, und anderen *Rhipicephalus* spp. mittels rasterelektronenmikroskopischer (REM) Bildgebung und mitochondrialen Genomsequenzanalysen zu erklären.

Zecken des Subgenus‘ *Boophilus* werden unter dem Genus *Rhipicephalus* eingeordnet und sind weit in den subtropischen und tropischen Ländern verbreitet. *Rhipicephalus microplus*, auch als die gemeine Rinderzecke bekannt, ist der wichtigste Stellvertreter des *Rhipicephalus microplus*-Komplexes. Dieser Komplex besteht aus mehreren Spezies, die viele morphologische Gemeinsamkeiten haben, was ihre Identifikation erschwert. Diese einwirtige Zecke ist der Grund für signifikante wirtschaftliche Einbußen in der Nutztierindustrie, indem

sie Irritation und Blutverlust hervorrufen und die Lederqualität reduzieren kann und indem sie als Vektor für potentiell tödliche Erkrankungen wie die bovine Anaplasmose und Babesiose agiert. Über die Morphologie und den genetischen Hintergrund von *R. microplus* aus Asien ist jedoch wenig bekannt. Phylogenetische Analysen der Cytochromoxidase 1 (Cox 1), des internal transcribed spacer 2 (ITS-2) und der 12S rRNA Gensequenzen bestätigen, dass der *R. microplus*-Komplex aus mindestens fünf unabhängigen Taxa besteht. Diese beinhalten *R. annulatus, R. australis, R. microplus* Klade A sensu Burger *et al.* (2014), *R. microplus* Klade B sensu Burger *et al.* (2014) and *R. microplus* Klade C sensu Low *et al.* (2015). *Rhipicephalus microplus*-Zecken aus Bangladesh, Myanmar und Pakistan wurden alle der *R. microplus* Klade C zugeordnet. Darüber hinaus haben wir bestätigt, dass das Cox 1-Gen der bevorzugte genetische Marker war, um die evolutionären Beziehungen zwischen den nah verwandten Spezies innerhalb des *R. microplus*-Komplexes zu erklären. REM-Bilder von adulten Exemplaren der *R. microplus* Klade C zeigten eine große Bandbreite intraspezifischer morphologischer Variationen, die auch Merkmale beinhalteten, die zuvor als ausschlaggebend für die Unterscheidung der *R. microplus* Klade A von *R. australis*-Zecken identifiziert worden waren. Dies veranschaulicht noch einmal die Komplikationen, die sich bei der nur auf Morphologie basierenden Identifikation der Arten innerhalb des *R. microplus*-Komplexes ergeben. Die Ergebnisse der Studie unterstreichen die Notwendigkeit, den *R. microplus*-Komplex aus einem größeren geographischen Bereich genetisch zu beproben und Kreuzungsversuche zwischen den verschiedenen Taxa durchzuführen oder Populationen mittels nuklearer Genomdaten nach einhergehender Sequenzierung zu vergleichen, um den Spezies-Status der *R. microplus*-Kladen aufzuklären.

List of publications in peer-reviewed journals

Roy BC, Krücken J, Ahmed JS, Majumder S, Baumann MP, Clausen P-H, Nijhof AM (2018) Molecular identification of tick-borne pathogens infecting cattle in Mymensingh district of Bangladesh reveals emerging species of *Anaplasma* and *Babesia*. Transbound Emerg Dis. 65(2):e231-e242.

Roy BC, Estrada-Peña A, Krücken J, Rehman A, Nijhof AM (2018) Morphological and phylogenetic analyses of *Rhipicephalus microplus* ticks from Bangladesh, Pakistan and Myanmar. Ticks Tick Borne Dis. 9(5):1069-1079.

Published conference proceedings:

Roy BC, Ahmed JS, Baumann MP, Clausen P-H, Nijhof AM (2016) Molecular Epidemiology of Ticks and Tick-borne Disease of Cattle in Mymensingh, Bangladesh. In: Tagung der Deutschen Veterinärmedizinischen Gesellschaft (DVG), Fachgruppe Parasitologie und parasitäre Krankheiten. May 2-4, 2016 Berlin, Germany. Abstract band ISBN: 978-3-86345-311-4, page No.72.

Roy BC, Ahmed JS, Baumann MP, Clausen P-H, Nijhof AM (2016) Molecular epidemiology of ticks and tick-borne diseases of cattle in Mymensingh district of Bangladesh. In: The first Joint International Conference of the Association of Institutes for Tropical Veterinary Medicine (AITVM) and the Society for Tropical Veterinary Medicine (STVM). September 4-8, 2016 Berlin, Germany. Abstract band ISBN: 978-3-86345-338-1, page No.233.

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Roy BC, Ahmed JS, Baumann MP, Clausen P-H, Nijhof AM (2016) Molecular epidemiology of ticks and tick-borne disease of cattle in Mymensingh, Bangladesh In: The 9th Doktorandensymposium and DRS Präsentationsseminar "Biomedical Sciences", Freie Universität Berlin (FU). September 16, 2016 Berlin, Germany. Abstract band ISBN: 978-3-86387-744-6, page No.19.

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Disclosure of contributions to the intellectual content

Prof. Dr. Jabbar S. Ahmed, Prof. Dr. Ulrika Seitzer and Prof. Dr. Peter-Henning Clausen were lead applicants on the grant awarded by DFG which supported this study. Dr. Ard Menzo Nijhof was responsible for overseeing the whole study and made considerable contributions to the design, acquisition and interpretation of data of this thesis. Babul Chandra Roy carried out the field study and molecular procedures in the laboratory, compiled, analyzed and interpreted the data and drafted both manuscripts. Dr. Maximilian Baumann contributed in designing the sample and sampling strategies. PD Dr. Jürgen Krücken assisted to phylogenetic analysis. Prof. Shankar Majumder contributed on data analysis. All co-authors contributed to revise the both manuscripts for the intellectual content and approved the published articles.

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Declaration of author

I declare that this dissertation, which I hereby submit for the degree of **"Doctor of Philosophy"** (PhD) at the Freie Universitaet Berlin, Germany, is solely my own research work and contains no material published elsewhere except the reference is made in the text. No other persons´ work is used without due acknowledgement in the main text and has not previously been submitted by me for an award or degree at any other university or institution.

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