Detection and Characterization of Tick-borne Pathogens of Livestock in Egypt, Kenya and Benin

(エジプト・ケニア・ベナンの家畜におけるマダニ媒介病原体の検出と解析)

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Abbreviations

ATBF: African tick bite fever

DDW: double distilled water

DNA: deoxyribonucleic acid

dNTP: deoxynucleotide triphosphate

ECF: East Coast fever

EDTA: ethylenediaminetetraacetic acid

ILRI: International Livestock Research Institute

MEXT: Ministry of Education, Culture, Sports, Science and Technology

MPSP: major piroplasm surface protein

Msp5: major surface protein 5

nPCR: nested PCR

NRCPD: National Research Center for Protozoan Diseases

OIE: Office International Des Epizooties

PCR: polymerase chain reaction

p104: *Theileria parva* p104 gene

Rap-1a: rhoptry-associated protein 1a

RLB: reverse line blot hybridization

RNA: ribonucleic acid

rompA: rickettsial outer membrane protein

SBP-4: spherical body protein 4

SFG: Spotted fever group

Semi nPCR: semi nested PCR

TBDs: tick-borne diseases

16S rDNA: gene coding for the small subunit of the ribosomal ribonucleic acid of bacteria and archaea

18S rRNA: component of the small subunit of the ribosomal ribonucleic acid

Unit abbreviations

bp: base pair

^{o}C: degree Celsius

′E: east

kDa: kilo Dalton

km: kilometer

min: minute

ml: milliliter

mm: millimeter

s: seconds

′S: south

µl: microliter

µM: micromolar

%: percentage

General introduction

Ticks transmit a large variety of pathogens to animals as well as human, causing harmful infections referred to as tick-borne diseases (TBDs). In Africa, the TBDs of veterinary and public health importance include babesiosis, theileriosis anaplasmosis and zoonotic Spotted fever group (SFG) ricketsioses.

Babesiosis, theileriosis and anaplasmosis are TBDs of cattle. Babesiosis and theileriosis are caused by the protozoan parasites, *Babesia* and *Theileria*, respectively, while anaplasmosis is caused by rickettsial organism known as *Anaplasma*. In Africa, bovine Babesiosis is caused by *Babesia bovis* and *Babesia bigemina* and the disease is transmitted by *Rhipicephalus* ticks (13). Though, *B. bigemina* is more widespread, *B. bovis* infection is the most critical and fatal due to its neurological symptoms (113).

Bovine theileriosis is another tick-borne disease found in many African countries. Tropical theileriosis and East Coast fever (ECF) are the most severe forms of the disease. Tropical theileriosis caused by *Theileria annulata* and transmitted by *Hyalomma* ticks is distributed in Northern Africa. ECF is probably the most important tick-borne disease in Eastern, Central and Southern Africa. The disease is caused by *Theileria parva* and transmitted mainly by *Rhipicephalus appendiculatus*. *Theileria parva* natural host is the African Cape buffalo (*Syncerus caffer*), which

serves as source of infection for cattle. Some variants of the parasite are transmitted solely from buffalo to cattle whereas others can spread from cattle to cattle. The other *Theileria* species reported in Africa are *T. mutans*, *T. taurotragi*, *T. sergenti/buffeli/orientalis* (referred to as *T. orientalis* complex) and *T. velifera.* These species are considered to be either less pathogenic or nonpathogenic, and only cause benign, moderate to asymptomatic theileriosis (45). For bovine anaplasmosis, *Anaplasma marginale* is one of the causative agents and this pathogen is transmitted biologically by approximately twenty tick species, and mechanically by biting flies and blood-contaminated fomites (55).

Babesia bovis, *B. bigemina, T. parva, T. annulata* and *A. marginale* cause mortalities and morbidities leading to losses in production of milk, meat, and other livestock by-products. Control measures directed to these pathogens are costly. In addition, because of these pathogens, farmers are reluctant to adopt highly productive exotic breeds as they are more susceptible to clinical infections than indigenous breeds. Consequently, they cause severe economic losses to livestock farmers involved in dairy and beef production in tropical and sub-tropical regions (44).

SFG rickettsioses are febrile illnesses caused by obligate, intracellular, gram negative bacteria that are transmitted to human by tick bite and known as SFG

rickettsiae. Although, animals can be infected with SFG rickettsiae, in contrast to humans, they are asymptomatic carriers and therefore serve as reservoir for ticks to acquire and transmit the bacteria. In Africa, nine SFG rickettsiae, namely *Rickettsia africae*, *R. conorii*, *R. sibirica mongolitimonae*, *R. slovaca*, *R. helvetica*, *R. aeschlimannii*, *R. massiliae*, *R. monacensis* and *R. raoultii* are considered to be human pathogens (16). *Rickettsia africae*, particularly is an emerging public health problem in rural areas in Africa. The bacteria is harbored by ticks feeding on livestock and causes African tick bite fever (ATBF) (50, 97) which is one of the most common causes of flu-like illness in international travelers to sub-Saharan Africa (42).

In spite of extensive researches and increasing body of knowledge on the aforementioned TBDs of cattle, there are no fully safe and effective vaccines (38, 45, 55, 107). Currently, assessment of pathogen prevalence, genotypes and risk factors such as animal species, breed, age, and ticks vectors are the key elements for effective control. With regard to zoonotic rickettsioses, although effective drugs exist, their usage requires the identification of the disease. SFG rickettsioses symptoms which include fever, headache, myalgia, eschars, rash, and lymphadenopathy, are not observed in all patients, and even completely absent in some cases (31, 42, 43). The broad range of symptoms makes the diagnosis difficult and misdiagnosis with other

febrile tropical diseases often occurs. Reports of SFG rickettsiae occurrence in the area visited by a febrile patient is important information for the diagnosis and effective treatment of the disease. Thus, the identification and molecular characterization of *B. bigemina, B. bovis, Theileria* species*, A. marginale* and SFG ricketsiae occurring in particular geographic area is a prerequisite for risk level evaluation and choosing appropriate strategies for the control and prevention of related diseases. Unfortunately, adequate information on pathogens genotypes or even knowledge on their molecular epidemiology are lacking in many African countries including Egypt, Kenya and Benin (Figure 1), because of limited resources for performing molecular-based studies.

Therefore, given this general background, the overall objective of this study was to improve the understanding of the epidemiology of babesiosis, theileriosis, anaplasmosis and SFG rickettsioses in Africa by using molecular biology tools to detect their etiological agents, analyze their prevalence in Egypt (Chapter 1), Kenya (Chapter 2) and Benin (Chapter 3); assess the genetic diversity and establish the phylogenies of these pathogens in relation to other regions of the world (Chapter 4).

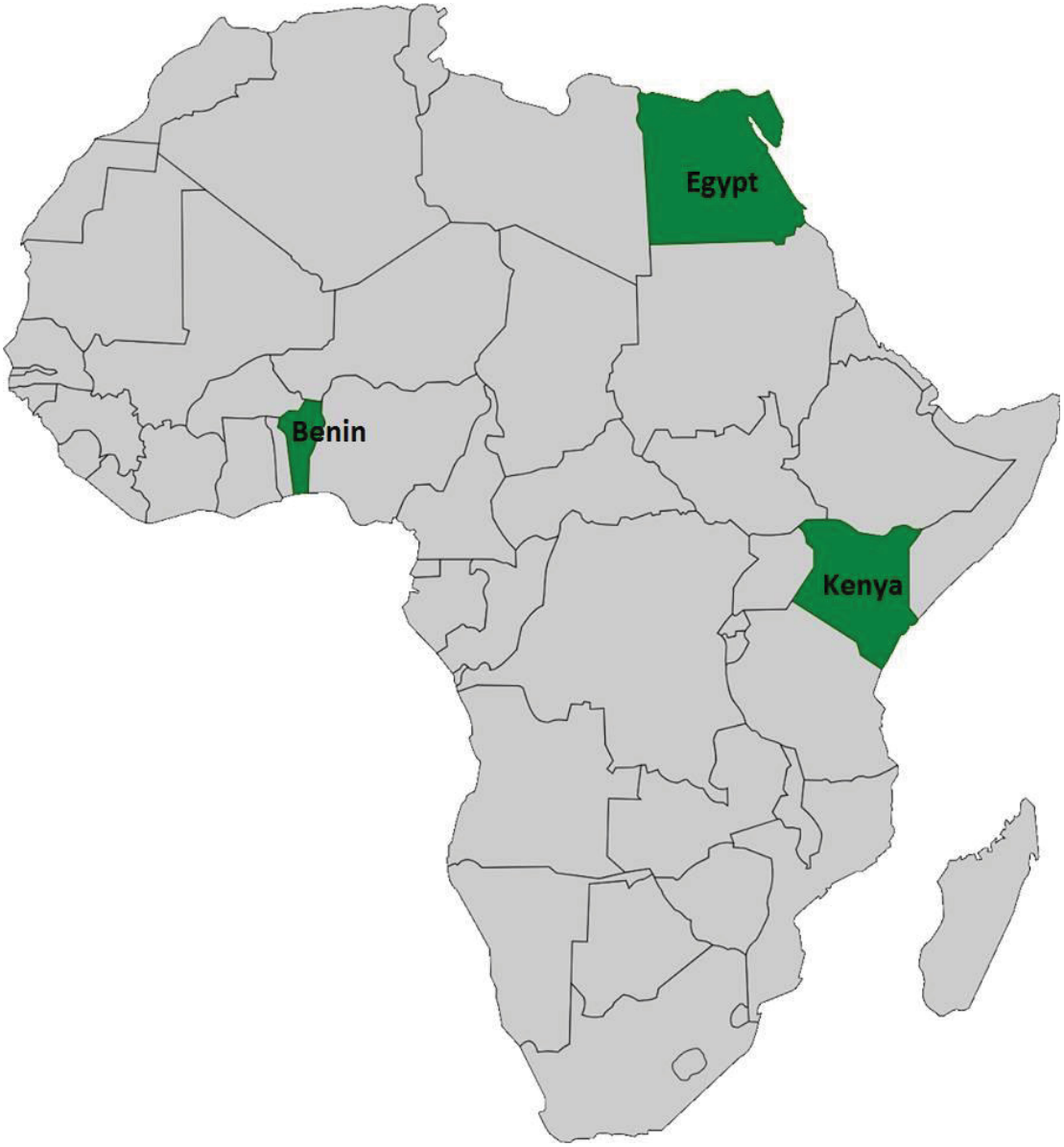

Figure 1. **Map of Africa showing the countries in which this study was carried out.**

Chapter 1

Molecular detection of *Babesia bovis*, *Babesia bigemina*, *Anaplasma marginale* and SFG *Rickettsia* species in cattle and water buffalos blood samples collected in Egypt

1.1 Introduction

Babesia bovis, *Babesia bigemina*, *Theileria annulata*, *Anaplasma marginale* and SFG *Rickettsia* species are endemic in Egypt. Although several studies have identified these pathogens in cattle (98, 24, 25, 26, 39, 80), water buffalos (*Bubalis bubalis*) (26, 62, 61), or ticks (1, 2, 23, 58), some features of the epidemiology of these pathogens have not yet been investigated. In particular, the characteristics of *B. bovis*, *B. bigemina* and *A. marginale* infections occurring in small-scale dairy farms and the situation of dairy animals with regards to SFG *Rickettsia* spp. infections remain poorly understood.

An epidemiological survey using molecular approach and including the livestock species used by Egyptian small holders will therefore provide highly desirable information for adequate control and prevention of babesiosis, anaplasmosis and SFG rickettsiosis in Egypt. The present study aimed to investigate

the prevalence of *B. bigemina*, *B. bovis*, *A. marginale* and SFG *Rickettsia* species infections in cattle and water buffalos in Beheira and Faiyum Provinces of Egypt through a cross sectional survey based on species-specific nested PCR (nPCR).

1.2 Materials and methods

1.2.1 Study areas and blood samples collection

A total of 247 blood samples from randomly selected Friesian Holstein cross-bred cattle (*n*=151) and water buffalos (*n*=96) were collected from open public markets in Beheira and Faiyum Provinces (Figure 2), respectively. The sampling was carried out during January-July, 2011. Beheira Province lies north to Cairo between 31° North latitude and 30° East longitude whereas Faiyum Province lies south to Cairo between 29° North latitude and 30° East longitude. The climate in Beheira is humid, warm and rainy while in Faiyum it is drier, hotter with less rain.

The cattle and water buffalos of this study belong to small-scale dairy farmers. The animals are usually moved to fields in daytime and returned back to farmer's stables in the evening. Cattle from 4 to 8 years old were divided into two groups based on their age; young (less than 5 years) and aged (5-8 years). Water buffalos from 5 to 10 years old were divided into two groups based on their age; young (5-7 years) and aged (8-10 years). Based on their body temperature at the sampling time cattle were divided into two groups: fevered and normal. The blood samples were collected from the jugular vein of individual cattle or water buffalo in EDTA-vacutainer tubes (Venoject, Terumo, Belgium), transported on ice to the laboratory and stored at -20 °C prior to DNA extraction.

1.2.2 Extraction of genomic DNAs

The genomic DNA was extracted from the whole blood using a commercial kit (QIAamp DNA Blood Mini-Kit, Germany) according to the manufacturer's instructions. The extracted DNA samples were transported to the National Research Center for Protozoan diseases in Obihiro, Japan, where they were stored at -30 °C pending further genetic analysis.

1.2.3 Detection of tick-borne pathogens

Specific primers targeting *B. bovis* spherical body protein-4 (SBP-4), *B. bigemina* rhoptry-associated protein-1a (RAP-1a) and *A. marginale* major surface protein 5 (Msp5) genes were used to amplify the respective genes by using previously described nPCRs (14, 109, 112, 118). The detection of SFG rickettsiae was performed using a PCR assay that amplifies *Rickettsia* species 16S rDNA (Table 1) (64).

Initial PCR amplifications were done in a 10 *µl* -reaction mixture having 1 *µl* of DNA template, 1 *µl* (10 µM) of each primers, 1 *µl* of 10x Ex buffer, 1 *µl* of dNTP (200 µM each), 0.1 *µl* of Ex Taq polymerase (Takara, Japan) and 4.9 *µl* of double distilled water (DDW). A second PCR was done using 1 *µl* of DNA template obtained from the first PCR amplification. Primers and thermocycling conditions

were as described in referenced papers (Table 1) except for *A. marginale* and SFG rickettsiae. For *A. marginale*, the thermocycling conditions were changed to a touchdown PCR and for SFG rickettsiae the PCR conditions were as follow: initial denaturation (5 min, 95 °C) followed by 35 cycles of denaturation (1 min, 94 °C) annealing (1 min, 56 °C) and extension (1 min, 72 °C) then a final extension step (10 min, 72 °C). The following samples were used as positive controls; DNAs of *B. bigemina* and *B. bovis* (112); DNA of *Rickettsia africae* isolated from *Amblyomma variegatum* collected in Tororo district, Uganda (78) and *A.marginale*-Msp5 plasmid (118). DDW was used as a negative control. The PCR products of each of the assays were electrophoresed on a 2% FastGene agarose gel (Nippon Genetics, Japan), which was subsequently stained in ethidium bromide solution (Nacalai tesque, Japan). PCR results were visualized and photographed under UV transilluminater (Printgraph AE- 6905CF, Atto, Japan) and samples showing bands of the expected size were declared positive.

1.2.4 Statistical analysis

For each of the pathogens detected, the proportions of DNA samples positive per study area and the confidence intervals were computed employing the EPI INFO™ software (CDC, USA, version 7.1.1). The chi-square test was used to

evaluate the differences of infection rate in animals of different age and health status using SPSS version 11 (SPSS Inc., USA). Statistically significant differences were determined at $P<0.05$.

1.3 Results

Detection of tick borne pathogens in cattle and water buffalos

A total of 247 blood samples collected from water buffalos and crossbred cattle belonging to small scale farmers were tested for *B. bovis, B. bigemina*, *A. marginale* and SFG *Rickettsia* species using molecular assays. *Babesia bovis, B. bigemina* and *A. marginale* were identified in 10 (4.1%), 18 (7.3%) and 25 (10.1%) samples, respectively, whereas SFG *Rickettsia* species were not identified in the samples. *Babesia bigemina* (5.3%) was the most prevalent hemoparasite in cattle samples whereas in water buffalos samples, the most frequent was *A. marginale* (20.8%) (Table 2). Statistically significant ($P<0.05$) difference was observed for *A. marginale* prevalence in the two animal species. Most of the positive samples were infected with only one pathogen. Mixed infections were observed only among water buffalos and included one case of co-infection with *B. bigemina* and *B. bovis*, and 3 cases of *B. bigemina* - *A. marginale* co-infection. Based on the results obtained, the confidence interval of the prevalence of *B. bovis* in Beheira cattle and Faiyum water buffalos under small-scale dairy farming is 1.5–8.4% and 1.1–10.3%, respectively, whereas for *B. bigemina* is 2.3–10.2% and 5.1–18.3%, respectively. For *A. marginale,* the prevalence is 1.1–7.6% and 13.2–30.3% in Beheira cattle and Faiyum water buffalos, respectively.

Assessment of relationship between ages, health status and infection level

To further understand the epidemiology of babesiosis in the study areas, the prevalences of *Babesia* spp. and *A. marginale* on the basis of the age and health status were assessed. *B. bigemina* prevalence in aged water buffalos (8–10 years) was significantly higher than observed in the young (5–7 years) (11.3% versus 6.3%). Moreover, chi square values indicated that the prevalence of *B. bigemina* was significantly ($P<0.05$) higher in fevered cattle (9.7%) compared to normal healthy cattle (4.2%). None of the cattle detected with *B. bovis* and none of the *Babesia* spp. or *A. marginale*-infected water buffalos were having fever at the sampling time.

1.4 Discussion

In Egypt, water buffalos and crossbred cattle (Egyptian Native cattle breed x Holstein-Friesian) are important in strategies for improvement of milk and meat production (61). The current study provides additional information of the epidemiology of *B. bovis, B. bigemina* and *A. marginale* in these two types of livestock under small scale farming and assesses their potential as reservoirs for SFG *Rickettsia* species. Except for SFG rickettsiae, all the tick-borne pathogens mentioned above were detected in the samples. Overall prevalence of *B. bigemina* and *B. bovis* were not significantly different ($P>0.05$). However, *B. bigemina* infection seemed to be slightly more frequent in accordance with previous works noting that in Africa *B. bigemina* is more prevalent than *B. bovis* due to the tick distribution (117). In Mediterranean countries like Egypt, *Rhipicephalus* (*Boophilus*) *annulatus* is known to be the most prevalent tick and the main vector transmitting *B. bigemina* and *B. bovis* (2, 24, 41). Since Adham *et al*. reported that prevalence of *B. bigemina* is higher than *B. bovis* in *R. annulatus* (2), the absence of significant difference of prevalence between the two parasites in this study may be due to sampling time, season, and geographic location. Relating to animal species, water buffalos from Faiyum had higher infection rates (for *B. bigemina* ($P>0.05$), *B. bovis* ($P>0.05$) and *A. marginale* ($P<0.05$)) than cattle from Beheira. Conversely, previous

studies in Egypt (26) and Thailand (112) reported that tick-borne hemoparasites prevalence in water buffalos is generally lower than in cattle. According to Terkawi *et al.* water buffalos spend much of their time submerged in muddy waters what could reduce tick infestation (112). Hence, the prevalence observed in Faiyum water buffalos might be explained by the habitat in which they live. The prevalence recorded in cattle in this study is consistent with the earlier epidemiological investigations of *B. bigemina* and *B. bovis* infections in Egypt (25, 26, 80) and in other Mediterranean countries (59, 109). In regard to water buffalos, infection rates recorded are comparable to those reported in Menoufia province, Egypt (26) and in Thailand (112) . However, in Argentina Ferreri *et al.* detected higher infection rate for *B. bovis* in water buffalos living in a *Rhipicephalus microplus*-endemic area (29). For *A. marginale*, the prevalence in Beheira cattle is lower than observed in Dakahlia Governorate, Egypt (25) and the prevalence in Faiyum water buffalos is higher than the figures reported in northern Brazil (7). Tick distribution, micro-climate pattern, breeds, farm management and the sampling condition may explain the fluctuation of prevalence between countries. The results of this study suggest that investigations on management practices, ticks distribution, tick infestation level in Egyptian cattle and water buffalos are needed to further understand exposure to infection.

The findings regarding the risk factors associated with *Babesia* spp.

infections were in agreement with previous studies: Terkawi *et al.* reported that aged animal was associated with increased *Babesia* spp. prevalence in Syrian cattle (109) and Safieldin *et al*. showed that fever had a strong relationship with the occurrence of *Babesia* infection in Sudanese dairy cattle (101). Despite the detection of *Babesia* and *Anaplasma* parasites in the circulating blood suggesting an ongoing infection, some of the cattle and all the water buffalos did not show any clinical signs. The absence of fever in infected cattle is probably due to low parasitemias and may indicate subclinical infections or carrier state. Subclinical infections with *A. marginale* have also been reported in Holstein Friesian cattle from Dakahlia Governorate, Egypt (25). In regard to water buffalos, similar asymptomatic infections have been observed in Sharkia Province, Egypt (61), in Argentina (29) and in Thailand (112). According to Mahmmod the clinical symptoms of *B. bovis*-infected water buffalos are rare and less severe than those of *B. bovis*-infected cattle (61). One the other hand, clinical anaplasmosis do not occur in water buffalos (55). My results compiled with previous reports (29, 55, 61) indicate that Egyptian water buffalos may serve as unapparent carrier of *Babesia* spp. and *A. marginale* and are therefore a potential source of infection for ticks and cattle. Thus, water buffalos should be included in the strategies to control bovine babesiosis and anaplasmosis.

In conclusion, this study provided information on the prevalence and risk

factors of bovine babesiosis and anaplasmosis with regard to cattle and water buffalos reared by Egyptian small scale farmers. The current data is valuable for the control and prevention of these diseases in Egypt.

1.5 Summary

In order to determine the molecular prevalence of *B. bigemina, B. bovis*, *A. marginale* and SFG *Rickettsia* species a total of 247 blood samples were collected from cattle and water buffalos in Beheira and Faiyum Provinces in Egypt and examined by standard and nPCR. Except for SFG rickettsiae, all the tick-borne pathogens mentioned above were detected in the samples. In cattle, the prevalence of *B. bovis*, *B. bigemina*, and *A. marginale* was 4.0%, 5.3% and 3.3%, respectively, whereas those of water buffalos were 4.2%, 10.4% and 20.8%, respectively. *B. bigemina* prevalence in aged water buffalos (8–10 years) was significantly higher than observed in the young (5–7 years) (11.3% versus 6.3%). Among the cattle examined, around 10% of the samples were infected with *B. bigemina* and showed fever whereas 4% were healthy but positive for the parasite. In contrast, all the water buffalos in this study were clinically healthy although they were positive to tick-borne hemoparasites. Taken together, these results indicate that water buffalos are important reservoir of tick-borne pathogens, and may act as source of infection for cattle. Therefore, water buffalos should be included in strategies for controlling babesiosis and anaplasmosis in Egypt.

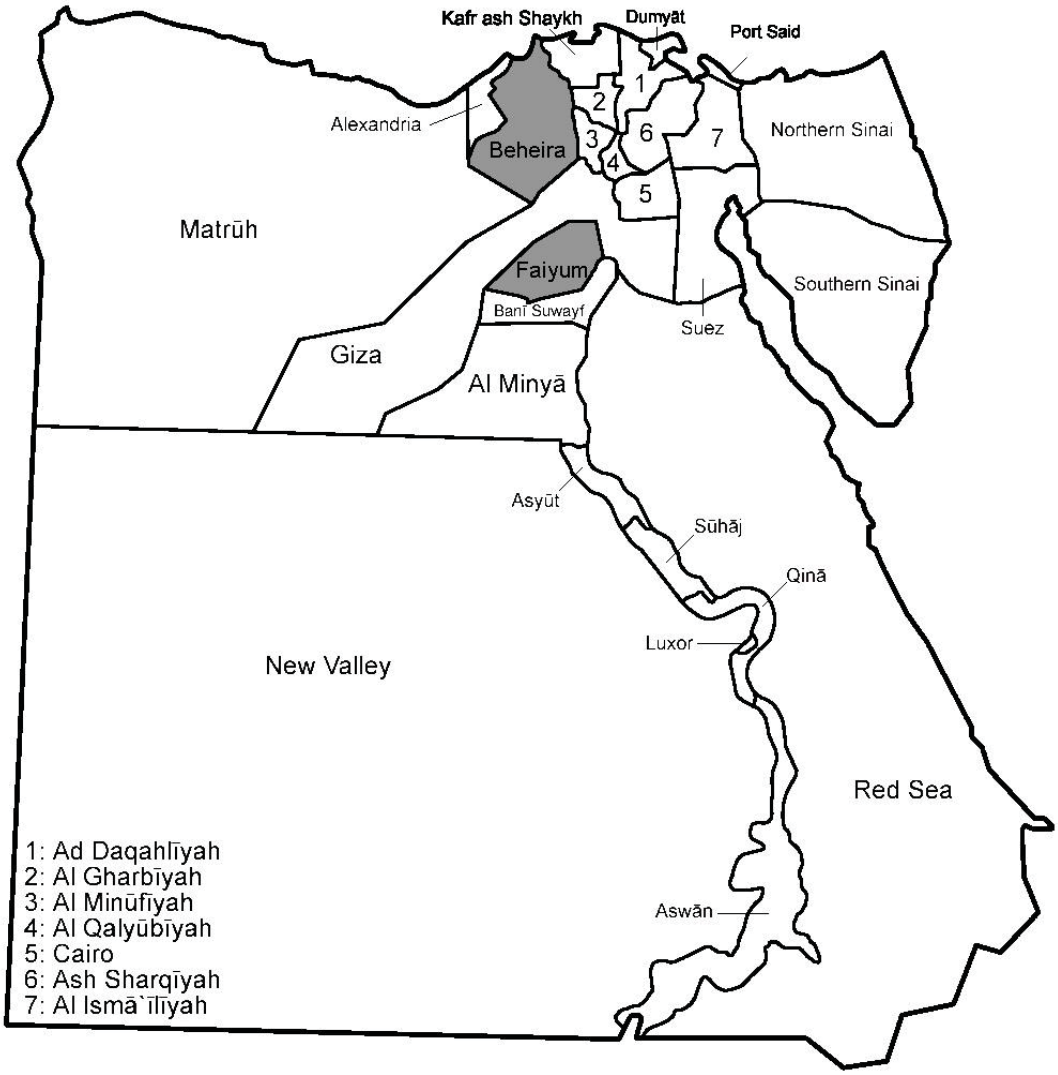

Figure 2. **Map of sampling area.** Blood samples were collected from cattle in Beheira and water buffalo in Faiyum Provinces, Egypt.

Table 1. **Sequences of primers set used for pathogen detection**

Pathogen Target gene	Assays	Oligonucleotide sequences (5'>3')	Product size (bp)	Reference
B. bovis *SBP-4*	First PCR	AGTTGTTGGAGGAGGCTAAT TCCTTCTCGGCGTCCTTTTC	907	(112)
	Second PCR	GAAATCCCTGTTCCAGAG TCGTTGATAACACTGCAA	503	
B. bigemina *RAP-1a*	First PCR	GAGTCTGCCAAATCCTTAC TCCTCTACAGCTGCTTCG	879	
	Second PCR	AGCTTGCTTTCACAACTCGCC TTGGTGCTTTGACCGACGACAT	412	
A. marginale *Msp5*	First PCR	GTGTTCCTGGGGTACTCCTATGTGAACAAG AAGCATGTGACCGCTGACAAACTTAAACAG	547	(118)
	Second PCR	AAGCACATGTTGGTAATATTCGGCTTCTCA AATTCTCGCATCAAAAGACTTGTGGTACTC	195	
Rickettsia spp. *16S rDNA*	PCR	AGAGTTTGATCCTGGCTCAG AACGTCATTATCTTCCTTGC	426	(64)

Table 2. **Prevalence of *B. bovis*, *B. bigemina* and *A. marginale* infections in cattle and water buffalos from Beheira and Faiyum Provinces, Egypt**

Regions	Tested samples		Positive samples (%)			
	Animal species	Number	*B. bovis*	*B. bigemina*	*A. marginale*	SFG *Rickettsia* spp.
Beheira	Cattle	151	6 (4.0)	8 (5.3)	5 (3.3)[a]	0
Faiyum	Water buffalo	96	4 (4.2)	10 (10.4)	20 (20.8)[b]	0
Total		247	10 (4.1)	18 (7.3)	25 (10.1)	0

Unlike superscript letters (a, b) denotes significant difference between samples ($P<0.05$)

Chapter 2

Molecular detection of *Babesia bovis*, *Babesia bigemina*, *Theileria* species, *Anaplasma marginale* and SFG *Rickettsia* species in cattle blood samples collected in Kenya

2.1 Introduction

Theileriosis, anaplasmosis, babesiosis and zoonotic rickettsiosis are among the most important tick-borne diseases in Kenya. Theileriosis, anaplasmosis and babesiosis account for economic losses in the dairy and beef industries (47, 75, 116) while SFG rickettsioses is a growing health problem among travelers to Kenya (99, 119). The diagnosis of tick-borne hemoparasites in Kenyan cattle have relied mostly on clinical signs (47), microscopic examination of blood smears (47, 48, 57, 76, 89) and antibody detection (34, 53, 89). A few studies employing molecular methods (PCR, reverse line blot hybridization (RLB), real time PCR) and genome sequencing have been exploited in Kenya (37, 85, 86, 87). However, these studies have been limited to a few tick-borne diseases with most of the studies lacking adequate information on their molecular epidemiology, which is critical for the control and prevention of these diseases. Concerning rickettsiosis, although rickettsial infections

have been detected in Kenyan cattle (77), very little is known about their prevalence.

Therefore, this study was carried out with the main objective of determining and understanding the molecular epidemiology of some species of *Babesia, Theileria*, *Anaplasma* and *Rickettsia* pathogens infecting cattle in Kenya. In particular, blood samples of cattle raised in farms located in Machakos and Ngong districts of Kenya were screened for specific target genes of *B. bovis*, *B. bigemina, Theileria* spp., *A. marginale* and SFG *Rickettsia* spp.

2.2 Materials and methods

2.2.1 Study areas and blood samples collection

Two separate dairy cattle farms were investigated in Kenya with one farm located in Ngong district of Kajiado County and the other farm is located in Machakos district of Machakos County. The farm in Ngong district (1° 22′S, 36° 38′E) lies 22 km Southwest of Nairobi whereas the other one in Machakos district (1° 14′S, 37° 23′E) lies 63 km Southeast of Nairobi (Figure 3). The average annual temperatures and rainfalls are 16.7 °C, 865 mm and 19.0 °C, 830 mm for Ngong and Machakos, respectively (http://en.climate-data.org/). Cattle in both farms are kept under a semi-extensive system, characterized by free grazing on pastures. The cattle were kept under semi-enclosed system at night and allowed to graze on pastures where the animals mixed with Masaai cattle grazing in the same area. The grazing together with Masaai cattle was particularly seen in case of cattle kept in Ngong farm.

A total of 154 and 38 blood samples were collected in EDTA-vacutainer tubes from cattle in Ngong and Machakos farms, respectively, during a cross-sectional survey done in August 2011. The samples were collected from randomly selected male and female crossbred cattle including adults and yearlings; all of which were apparently healthy. The samples were then transported on ice to

the Central Veterinary Laboratory in Nairobi, Kenya and stored at -20 °C prior to DNA extraction.

The genomic DNA was extracted at the Central Veterinary Laboratory in Nairobi, Kenya using a commercial DNA extraction kit according to the manufacturer's instructions (QIAamp DNA Blood Mini-Kit, Germany). The extracted DNA samples were transported to the National Research Center for Protozoan Diseases in Obihiro, Japan, where they were stored at -30 °C pending further genetic analysis.

2.2.2 Detection of tick-borne pathogens

The detection of *B. bovis*, *B. bigemina, A. marginale* and SFG *Rickettsia* spp. was carried out as described in Chapter 1. *Theileria* species were detected using a previously described nPCR which amplifies *Theileria* spp. 18S rRNA (15). Primers, reaction setup and thermocycling conditions were as in referenced article (Table 3). *Theileria parva* (Muguga G6, ILRI), *T. annulata* (Ankara C9, Edinburgh University) and cattle DNA sample positive for *T. orientalis* (5) were used as positive controls while DDW was used as the negative control. Amplification products were visualized as described in Chapter1.

To detect co-infection with *T. parva* and *T. orientalis* complex, samples positive for *Theileria* spp. 18S rRNA were further amplified by nPCR using primers targeting *T. parva* p104 (p104) gene (56) for *T. parva* and those targeting *T. orientalis* major piroplasm surface protein (MPSP) gene (90) for *T. orientalis* (Table 3).

2.2.3 Identification of *Theileria* species

All *Theileria* spp.-positive samples were used as templates for sequence-based identification of *Theileria* species. Nested PCR amplicons of *Theileria* spp. 18S rRNA were purified using QIAquick Gel Extraction Kit (QIAGEN GmbH, Germany) and directly sequenced using the nPCR primers, the Big Dye Terminator Cycle Sequencing Kit (Applied Biosystems, USA) and an ABI PRISM 3100 genetic analyzer (Applied Biosystems, USA). DNA sequences with heterozygous base-calling were analyzed using Mixed Sequence Reader web-based program (http://msr.cs.nthu.edu.tw) and identified as two distinct sequences. The sequenced DNAs were analyzed by BLASTn tool of NCBI GenBank database. The correct species identity was established by comparing the query sequences with those available in the GenBank database. Species confirmation was done when the closest BLASTn match has a ≥98% identity to the homologues found in the

GenBank. The *Theileria* spp. 18S rRNA sequences identified in this study are available in the GenBank of the NCBI database under the accession numbers KP347567 to KP347575.

2.2.4 Statistical analysis

Proportions of DNA samples positive for respective pathogens per farm were computed and a comparison of pathogen prevalence was done using the chi-square test employing the EPI INFO™ software (CDC, USA, version 7.1.1) and VassarStats (http://vassarstats.net/). Statistically significant differences were determined at $P<0.05$.

2.3 Results

Detection of tick-borne pathogens in cattle

A total of 192 blood samples (154 in Ngong and 38 in Machakos) were analyzed by nested and single PCRs to be able to detect infection of cattle with *B. bovis, B. bigemina*, *Theileria* spp., *A. marginale* and SFG *Rickettsia*. *Babesia bovis, B. bigemina*, *Theileria* spp., and *A. marginale* were detected in the two farms. In contrast, no samples had detectable levels of *Rickettsia*. In Ngong farm, *B. bigemina* (65 samples-42.2%) was more prevalent than *B. bovis* (19 samples-12.3%). On the other hand, *Theileria* spp. and *A. marginale* DNAs were detected in 52 (33.8%) and 50 (32.5%) blood samples, respectively. In Machakos farm, 9 (23.7%) samples were positive for *B. bovis* while *B. bigemina* was detected in 5 (13.2%) samples. *Theileria* spp. and *A. marginale* were detected in 15 (39.5%) and 6 (15.8%) samples, respectively. There was significant difference in prevalence observed in the two farms ($P<0.05$) for *B. bigemina* and *A. marginale* (Table 4).

Identification of *Theileria* species

Genetic analysis of the sequenced *Theilera* spp. 18S rRNAs revealed homologues that belonged to seven distinct *Theileria* species including *T. parva*, *T. taurotragi*, *T. mutans*, *T. velifera, T.orientalis* complex, *T. ovis* and *Theileria* sp. (buffalo) (Table 5). In particular, *T. parva*, *T. taurotragi*, *T. mutans* and *T. velifera*

were detected in samples from cattle in both farms while *T. orientalis* complex, *T. ovis* and *Theileria* sp. (buffalo) were detected in Ngong farm-cattle only (Table 6). Co-infections with *T. parva* and *T. taurotragi*, and with *T.mutans* and *T. taurotragi* were observed in some blood samples from Ngong farm-cattle. Other samples with *T. parva* and *T. velifera* co-infections were seen in the same farm (Table 7). In contrast, co-infections with *T. taurotragi* and *T. velifera* were detected only in cattle from Machakos farm (Table 8). All the samples that were positive for *T. parva* and *T. orientalis* complex 18S rRNAs were also positive for *T. parva* p104 and *T. orientalis* MPSP genes, respectively. Thirty five samples from Ngong farm and 11 samples from Machakos farm that were negative for *T. parva* 18S rRNA were found to be positive for *T. parva* p104 DNA. *Theileria parva* was the most prevalent with the parasite DNA being detected in 46 (29.9%) and 12 (31.6%) samples from cattle in Ngong and Machakos farms, respectively (Table 6). Infections with *T. velifera, T. taurotragi* and *T. mut*ans were also observed though less frequently. Few cases of *T. orientalis* complex, *T. ovis* and *Theileria* spp. (buffalo) infections were detected.

Mixed infections with *Babesia*, *Theileria* and *Anaplasma*

Of the 192 samples, at least one hemoparasite was detected in 135 (70%) samples. In Ngong farm, 110 (71%) samples had hemoparasites DNAs (Table 7) whereas 25 (66%) samples were positive for at least one hemoparasite in Machakos

(Table 8). More than half of the positive samples were infected with at least two hemoparasites, which generally belong to different genus. Twenty nine different types of mixed infections were seen in Ngong farm with some cattle having up to five pathogens co-infecting the same cattle (Table 7). In Machakos farm, 11 different types of mixed infections were seen with up to three pathogens simultaneously being detected in samples (Table 8). Single infections with *B. bovis*, *T. velifera* and *A. marginale* were observed in both farms, while single infections with *B. bigemina* and *T. parva* were seen only in Ngong farm. In most cases, mixed infections rather than single infection were detected for all hemoparasites in both farms except for *B. bovis* for which most of positive samples in Machakos farm were single infections.

2.4 Discussion

This study was done to determine the molecular epidemiology of *B. bovis*, *B. bigemina, Theileria* spp., *A. marginale* and SFG *Rickettsia* spp. infecting cattle raised in Machakos and Ngong districts of Kenya. Such information is critical for controlling and preventing infections caused by these pathogens, which leads to loss of livelihoods of many livestock owners (69) and threatens the lives of international travelers (43). Although rickettsial infections had previously been reported in cattle from Kajiado and Machakos counties (77), none of the study samples was positive for the bacteria. The rickettsial infection rates recorded in the anterior study (166/1019; 16.3%) suggest that my results could be explained by the number of farms investigated (two farms), samples size (192 cattle) and low rickettsiemia levels. The causative agents of babesiosis, theileriosis, and anaplasmosis; however, were prevalent in the two farms surveyed. These findings are consistent with previous reports ranking tick-borne pathogens as important causes of diseases in Kenyan cattle (17, 47, 74, 75, 116). Though *Theileria* spp. have always been reported to be the most prevalent hemoparasite in Kenya (76, 89, 116), this was not the case in Ngong farm as *Babesia* species were the most prevalent pathogens. However, *Theileria s*pecies were more prevalent in Machakos farm consistent with the previous studies in Kenyan cattle (76, 89, 116). *Anaplasma marginale* was the least

prevalent in both farms suggesting that it may not be one of the most prevalent tick-borne diseases in these regions.

The prevalence of *B. bovis* was higher than that of *B. bigemina* in Machakos and vice-versa in Ngong farm indicating that the epidemiology of babesiosis in the two farms may be different. The prevalence of *B. bigemina* may reflect the distribution of its vectors, *Rhipicephalus evertsi* and *Rhipicephalus decoloratus* (13) which are both present in Machakos and Ngong districts (18, 116). *Babesia bigemina* is the main cause of bovine babesiosis in Kenya (66, 116) and its predominance in Ngong farm is not surprising. The low prevalence of *B. bigemina* in Machakos farm could be explained by lower exposure to tick vectors. The detection of *B. bovis* was unexpected since none of its vectors namely, *Rhipicephalus microplus, R. geigyi* and *R. annulatus* (13) have been reported in the two districts (18, 116). My results, therefore, indicate that *B. bovis* has been overlooked or is becoming endemic in Machakos and Ngong districts. The competition between *R. microplus* and *R. decoloratus* may explain why *B. bigemina* is more widespread than *B. bovis* in Africa (13). Hence, the higher prevalence of *B. bovis* in Machakos farm suggests changes in tick distribution. Uncontrolled animal movement that is common in Kenya (66) or changes in ecological pattern may have contributed to the thriving of *B. bovis* tick vectors. The prevalences of *B. bovis* and *B. bigemina*

revealed by this study are higher than those previously observed in Western Kenya (85, 86). This may not be surprising as the studies were done in different ecological areas. In regard to *A. marginale*, the detection of its DNA in both farms is consistent with previous studies which reported antibodies to *A. marginale* in Kenyan cattle (34, 53, 89, 116).

Although this study detected *Theileria* species in both farms, the samples analyzed here were not large enough and elaborate studies will be needed to determine the full extent of the infections in Kenya. Many *Theileria* species including *T. parva*, *T. taurotragi, T. mutans* and *T. velifera* were detected in both farms. However, *T.orientalis/sergenti/buffeli*, *T. ovis* and *Theileria* sp. (buffalo) were detected only in Ngong farm. Cattle are the natural host of *T. parva*, *T. taurotragi*, *T. mutans* and *T. velifera. Rhipicephalus appendiculatus,* which transmits *T. parva* and *T. taurotragi* as well as *Amblyomma variegatum,* the vector for *T. mutans* and *T. velifera* are known to exist in the districts surveyed (18, 116). Therefore, the presence of these tick vectors may explain the occurrences of the hemoparasites in the farms. *Theileria ovis* is known to infect small ruminants (11) while *T. buffeli* and *Theileria* sp. (buffalo) infect African Cape buffalo (*Syncerus caffer)* (92). The detection of buffalo-*Theileria* sp. in Ngong farm-cattle may be attributed to their transmission by ticks and interaction of these cattle with nomadic Maasai cattle.

These Maasai cattle usually graze together with wildlife including buffaloes. Likewise, Maasai cattle are generally kept with flocks of small ruminants and therefore can be a source of *T. ovis* infected ticks for Ngong farm-cattle. Such accidental *Theileria* infections in cattle have previously been reported in wildlife-domestic animals interface and in areas where there is high animal movement in Kenya (37, 85, 86).

Theileria parva was the most frequent *Theileria* in the two farms. Among the *Theileria parva* -positive samples, 35 from Ngong farm and 11 from Machakos farm were positive for both *Theileria* 18S rRNA and *T. parva* p104 nPCRs but sequencing of their *Theileria* 18S rRNA-amplicons did not show a sequence specific to *T. parva*. This is probably due to low levels of *T. parva*-parasitemia and to several *Theileria* species co-occurring in the same animal. In the current study, genus specific primers were used to amplify *Theileria* 18S rRNA and obtained products therefore contained amplicons from several species. Only amplicons derived from species with abundant DNA were reflected in the sequencing chromatogram. Hence, *T. parva* 18S rRNA was not identified in some samples because it was outnumbered by the 18S rRNA gene of other *Theilera* species. ECF caused by *T. parva* is the most important tick-borne disease in Kenya and "immunization" against it is common (33, 36, 47). The carrier state is particularly important for this parasite as it contributes to and

may be necessary for maintenance of immunity against overt disease (11, 87). Most of *T. parva*-positive animals in this study seemed to be carriers and previous studies (91, 102, 103) suggested that such status could have been induced by previous "immunization" as well as natural infection. *Theileria parva* prevalences obtained were lower than the 67% observed in Marula, Rift Valley (37), similar to the values recorded by Odongo *et al* (87) but higher than the RLB data from Western Kenya (85, 86).

All the cattle in this study appeared healthy, although pathogenic hemoparasites (*B. bovis*, *B. bigemina*, *A. marginale* and *T. parva*) were detected in their blood. This absence of clinical disease in infected cattle may be attributed to a state of enzootic stability as described in previous reports (13, 45, 55). The high rate of multiple infections in both farms, sometimes involving hemoparasites belonging to different genus may be explained by the presence of a range of tick-vectors that exist in the same ecosystem. Benign *T. taurotragi*, and *T. mutans* detected in this study have been previously associated with morbidity and mortality in calves in Kenya (71, 70). Perhaps, further studies should explore the importance of these hemoparasites in Kenya with regard to *Theileria* infections in calves.

In conclusion, this study has confirmed the occurrences of diverse tick-borne hemoparasites in farms located in two districts of Kenya. The detection, prevention

and control of these hemoparasites in cattle should consider the co-infective nature of these pathogens and the role of wildlife in the transmissions of the tick-borne parasites. Therefore, this study will provide a basis for further research on tick-borne hemoparasitic diseases and their molecular epidemiology in Kenya and other regions of the world.

2.5 Summary

Infections with *B. bovis*, *B. bigemina, Theileria* species, *A. marginale* and SFG *Rickettsia* spp. are endemic in Kenya yet there is a lack of adequate information on their epidemiology. This study established the molecular epidemiology of the above tick-borne pathogens infecting cattle in Kenya. Nested and standard PCRs were used to determine the prevalence of the above pathogens in 192 cattle blood samples collected from Ngong and Machakos farms. *Rickettsia* species were not detected in any of the samples examined. *Babesia bovis*, *B. bigemina*, *T. parva*, *T. velifera*, *T. taurotragi*, *T. mutans* and *A. marginale* were prevalent in both farms, whereas *T. ovis, Theileria* sp. (buffalo) and *T. orientalis* were found only in Ngong farm. Co-infections were observed in more than 50% of positive samples in both farms. These findings point out that, in Kenya, it is necessary to consider co-infection of cattle with tick-borne hemoparasites and the role of wildlife in pathogens transmission when devising interventions related to the diagnostic, treatment and prevention of TBDs.

Table 3. **Sequences of primers set used for *Theileria* species detection**

Pathogen Target gene	Assays	Oligonucleotide sequences (5'>3')	Product size (bp)	Reference
Theileria spp. *18S rRNA*	First PCR	GAAACGGCTACCACATCT AGTTTCCCCGTGTTGAGT	778	(15)
	Second PCR	TTAAACCTCTTCCAGAGT TCAGCCTTGCGACCATAC	581	
T. parva *p104*	First PCR	ATTTAAGGAACCTGACGTGACTGC TAAGATGCCGACTATTAATGACACC	496	(56)
	Second PCR	GGCCAAGGTCTCCTTCAGATTACG TGGGTGTGTTTCCTCGTCATCTGC	277	
T. orientalis *MPSP*	PCR	CTTTGCCTAGGATACTTCCT ACGGCAAGTGGTGAGAACT	776	(90)

Table 4. **Results of tick-borne pathogens detection in cattle from Ngong and Machakos farms, Kenya**

Farm	No. of samples	Positive samples (%)				
		B. bovis	*B. bigemina*	*Theileria* spp.	*A. marginale*	SFG *Rickettsia* spp.
Ngong	154	19 (12.3)	65 (42.2) [a]	52 (33.8)	50 (32.5) [a]	0
Machakos	38	9 (23.7)	5 (13.2) [b]	15 (39.5)	6 (15.8) [b]	0
Total	192	28 (14.6)	70 (36.5)	67 (34.9)	56 (29.2)	0

Unlike superscript letters (**a,b**) in the same column, denotes significant difference between samples ($P<0.05$).

Table 5. **Identification of *Theileria* species by BLASTn analysis of the 18S rRNA sequences of the isolates from cattle in Kenya.**

Accession numbers	Sequence length (bp)	Highest BLASTn match	Accession number of match	% identity
KP347567	486	*T. mutans*	AF078815	99
KP347568	493	*T. orientalis*	AB520955	100
		T. sergenti	JQ723015	100
		T. buffeli	DQ287959	100
		Theileria spp. JW-2014	KJ020546	100
KP347569	497	*T. ovis*	KM924444	100
KP347570	515	*T. parva*	HQ684067	100
KP347571	512	*T. taurotragi*	L19082	99
KP347572	512	*T. taurotragi*	L19082	100
KP347573	488	*T. taurotragi*	L19082	98
KP347574	514	*T. velifera*	JN572705	100
KP347575	469	*Theileria* sp. (buffalo)	HQ895982	100

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Table 6. ***Theileria*** **species detected in cattle from Ngong farm and Machakos farm in Kenya**

Theileria species detected	Number of positive samples	
	Ngong-farm	Machakos-farm
T. parva	11[a] + 35[b]	1[a] + 11[b]
T. velifera	16	9
T. taurotragi	11	4
T. mutans	11	3
T. orientalis/buffeli/sergenti	3[c]	0
T. ovis	3	0
Theileria spp. *ex Syncerus caffer*	1	0

[a]: Number of samples in which *T. parva* 18S ribosomal RNA and *T. parva* p104 genes were detected.

[b]: Number of samples that were negative for *T. parva* 18S rRNA (*T. parva* 18S rRNA gene not detected in sequencing) but found to be positive for *T. parva* p104 DNA.

[c]: Number of samples in which *T. orientalis* 18S ribosomal RNA and *T. orientalis* MPSP genes were detected.

[No letter]: Number of samples in which the 18S ribosomal RNA gene of the corresponding *Theileria* species (species on the same line of the table) were detected.

Table 7. **Tick-borne hemoparasites detected in cattle (*n*=154) from Ngong-farm in Kenya**

	Pathogen species detected	Positive cattle (%)
One pathogen	*B. bovis*	8 (5.2)
	B. bigemina	22 (14.3)
	T. parva	3 (1.9)
	T. velifera	2 (1.3)
	A. marginale	12 (7.8)
Two pathogens	*B.bigemina* + *T. parva*	3 (1.9)
	B.bigemina + *A. marginale*	11 (7.1)
	A. marginale + *T. parva*	1 (0.6)
	A. marginale + *Theileria* sp. (buffalo)	1 (0.6)
	A. marginale +*T. taurotragi*	1 (0.6)
	B.bovis + *B. bigemina*	2 (1.3)
	B.bovis + *A. marginale*	1 (0.6)
	T. parva+ *T.velifera*	5 (3.2)
	T. parva + *T.mutans*	1 (0.6)
Three pathogens	*B.bovis* + *B. bigemina* + *A. marginale*	2 (1.3)
	B. bovis +*T. parva* +*T. ovis*	1 (0.6)
	B.bigemina + *T. parva* +*T. mutans*	3 (1.9)
	B. bigemina + *T. parva* +*T. velifera*	4 (2.6)
	B. bigemina + *T. parva* +*T. ovis*	1 (0.6)
	B. bigemina + *T. taurotragi* + *T. mutans*	1 (0.6)
	B. bigemina + *T. taurotragi* + *T. parva*	4 (2.6)
	B.bigemina + *A. marginale*+ *T. taurotragi*	1 (0.6)
	B.bigemina + *A. marginale*+ *T. parva*	1 (0.6)
	A. marginale + *T.parva* +*T.taurotragi*	2 (1.3)
	A. marginale + *T.parva* +*T.velifera*	4 (2.6)
	A. marginale + *T.parva* +*T.orientalis*	2 (1.3)
	A. marginale + *T.parva* +*T. mutans*	1 (0.6)
Four pathogens	*B. bigemina* + *A. marginale*+ *T. parva* +*T. taurotragi*	1 (0.6)
	B. bigemina + *A. marginale*+ *T. parva* +*T. ovis*	1 (0.6)
	B. bigemina + *A. marginale*+ *T. parva* +*T. mutans*	3 (1.9)
Five pathogens	*B.bovis* + *B. bigemina*+ *A. marginale* +*T. parva* +*T.orientalis*	1 (0.6)
	B.bovis + *B. bigemina*+ *A. marginale* +*T. parva* +*T. mutans*	2 (1.3)
	B.bovis + *B. bigemina*+ *A. marginale* +*T. parva* +*T. taurotragi*	1 (0.6)
	B.bovis + *B. bigemina*+ + *A. marginale* +*T. parva* +*T. velifera*	1 (0.6)
	Total	110 (71.4)

Table 8. **Tick-borne hemoparasites detected in cattle (*n*=38) from Machakos-farm in Kenya**

	Pathogen species detected	Positive cattle (%)
One pathogen	*B. bovis*	6 (15.8)
	T. velifera	1 (2.6)
	A. marginale	3 (7.9)
Two pathogens	*B. bigemina* + *T. parva*	1 (2.6)
	B. bigemina + *T. taurotragi*	1 (2.6)
	B. bovis + *B. bigemina*	1 (2.6)
	T. parva+ *T.velifera*	2 (5.3)
	T. taurotragi + *T.velifera*	1 (2.6)
	T. parva + *T. taurotragi*	1 (2.6)
Three pathogens	*B. bovis* +*T. parva* +*T. mutans*	2 (5.3)
	B. bigemina + *T. parva* +*T. velifera*	2 (5.3)
	A. marginale + *T.parva* +*T.velifera*	2 (5.3)
	A. marginale +*T.parva* +*T. mutans*	1 (2.6)
	T. parva +*T. taurotragi* + *T.velifera*	1 (2.6)
	Total	25 (65.8)

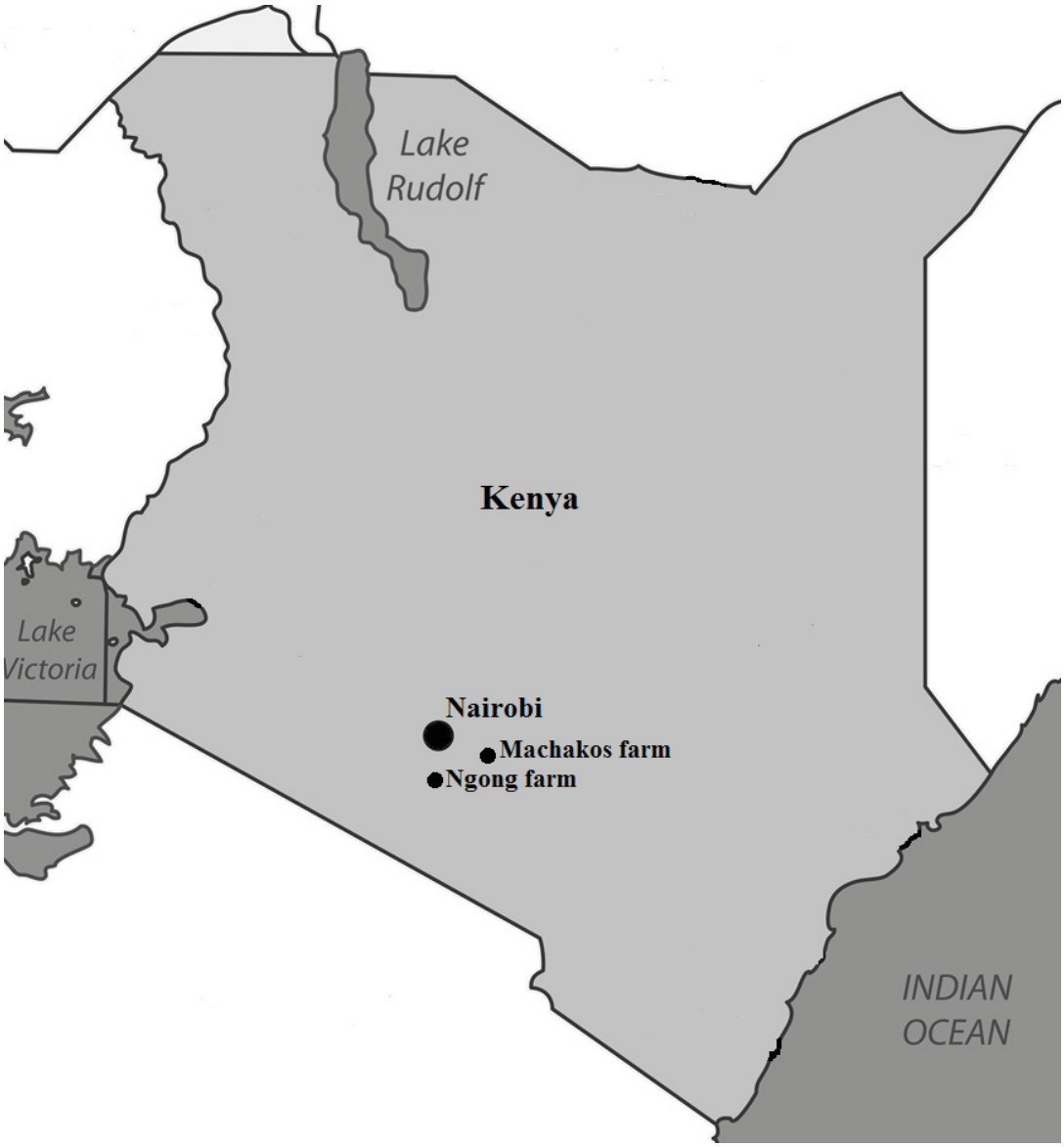

Figure 3. **Map of Kenya showing the location of the two study farms.**

Chapter 3

Molecular detection of *Babesia bovis*, *Babesia bigemina*, *Theileria* species, *Anaplasma marginale* and SFG *Rickettsia* species in *Amblyomma variegatum* ticks from Benin Republic

3.1 Introduction

The tick fauna in Benin Republic is diverse, with around 17 tick species that are known to feed on cattle (20, 114). Yet, information on pathogens associated with Benin cattle-ticks is scant. In particular, there are little or no data on the epidemiology of *Babesia* spp., *Theileria* spp., *Anaplasma* spp. and SFG *Rickettsia* species.

Molecular methods are among the most sensitive and specific techniques for pathogens detection in hosts and tick vectors. Ticks, compared to animal blood samples, are easy to obtain as herd owners generally do not oppose to their collection. The molecular detection of pathogens in ticks is currently a well-established approach to elucidate the epidemiology of tick-borne diseases (106). In the present study, *Amblyomma variegatum* ticks were used as templates for the molecular detection of tick-borne pathogens of veterinary and medical importance. In detail,

feeding *Amblyomma variegatum* ticks were collected in 8 locations in North Eastern Benin and screened for *B. bovis*, *B. bigemina*, *Theileria* spp., *Anaplasma marginale* and SFG *Rickettsia* spp. using genus-specific or species-specific PCR assays.

3.2 Materials and methods

3.2.1 Ticks samples collection

During May-June 2011, feeding ticks were collected in cattle farms located in four divisions of Borgou District, Benin Republic. Eight different locations were surveyed: Beterou (Tchaourou division), Nikki centre, Suya and Tasso (Nikki division), Dunkassa and Bouka (Kalale division), Sekere and Sikki (Sinende division) (Figure 4). The ticks were collected on indigenous cattle with the consent of cattle owners and care was taken to minimize discomfort for the animal. Species identification and morphological classification of the tick samples were conducted by Dr Kozo Fujizaki (National Agriculture and Food Research Organization, Japan) using microscopic examination and standard taxonomic keys according to the guidelines of Walker *et al* (115). A total of 180 herds were visited and 910 adult *Amblyomma variegatum* ticks (789 males and 121 females) in various state of engorgement were obtained. They were individually stored in 1.5 *ml* tube (Toho, Japan) containing 70% ethanol (Wako, Japan), labeled and kept at room temperature until processing.

3.2.2 DNA extraction

The DNA extraction was performed on individual tick samples. Each *A.*

variegatum tick was washed in three 70% ethanol baths, rinsed in double distilled water; air dried and collected in sterile 1.5 *ml* tubes. The tick-containing tubes were plunged in liquid nitrogen for 3-5 minutes and frozen ticks were crushed using sterile pellet mixers (Toho, Japan). The tissues of crushed ticks were digested using the ISOHAIR kit (Nippon Gene, Japan) according to the manufacturer's protocol and the DNA was extracted using the phenol method described by Barker (2005). The DNAs were then precipitated using the Dr. GenTLE precipitation carrier kit (Takara, Japan) according to the manufacturer's instructions; the resulting pellets were dissolved in 100 *µl* of DDW and stored at -30 ^{o}C until molecular analysis.

3.2.3 Detection of tick-borne pathogens

Amblyomma variegatum ticks were analyzed for the presence of *B. bovis*, *B. bigemina, T. mutans, T. taurotragi, T. annulata, T. orientalis, T. parva, A. marginale* and SFG rickettsiae. The *Babesia* species, *A. marginale* and *T. orientalis* were detected using PCR assays described in Chapter 1 and 2, respectively. *Theileria annulata* was identified using a nPCR assay targeting *T. annulata* major merozoite surface antigen while *T. mutans, T. taurotragi* and *T. parva* were detected using semi-nested PCRs amplifying the 18S rRNA gene of the respective parasites (Table 9). The detection of SFG rickettsiae was performed using PCR assays targeting

Rickettsia 16S rDNA and rickettsial outer membrane protein (rompA) genes, respectively. Tick samples were first screened for the presence of rickettsial bacteria by amplifying *Rickettsia* 16S rDNA as described in Chapter 1. Upon visualization of amplification products, all the samples positive for *Rickettsia* 16S rDNA were tested for the rompA gene which encodes for a 190-kDa protein specific to SFG rickettsiae. A 632 bp fragment at the 5' end of the gene was amplified in the samples using the primer pair 190-70 (5'-ATG GCG AAT ATT TCT CCA AAA-3') and 190-701 (5'-GTT CCG TTA ATG GCA GCATCT-3') (30). The thermo cycling conditions of the reaction consisted of initial denaturation (5 min, 94 °C) followed by 35 cycles of denaturation (30 s, 94 °C), annealing (1 min, 60 °C) and extension (2 min, 72 °C) then a final extension step (5 min, 72 °C). All the PCRs were carried out on individual ticks DNA samples and each reaction was conducted in a total volume of 13 *µl* composed of 2 *µl* of DNA template, 1 *µl* (10 µM) of each primers, 5 *µl* of 2x Ampidirect plus (Shimadzu, Japan), 0.075 *µl* of Ex Taq polymerase (Takara, Japan) and 3.925 *µl* of distilled water. DNAs of *B. bigemina* and *B. bovis* (112), *T. parva* (Muguga G6, ILRI), *T. annulata* (Ankara C9, Edinburgh University), DNA of *R. africae* isolated from *A. variegatum* collected in Tororo district, Uganda (78), cattle DNA samples positive for *T. orientalis*, *T. mutans, T. taurotragi* (4) and *A. marginale*-Msp5 plasmid (118) were used as positive controls and DDW as the

negative control. The visualization of PCR results was performed as described in Chapter 1.

3.2.4 Identification of SFG *Rickettsia* species

The SFG rickettsiae detected in Benin were identified based on the sequences of *Rickettsia* 16S rDNA and rompA genes. Briefly, amplification products of randomly selected positive samples were purified from agarose gel using QIAquick Gel Extraction Kit (QIAGEN GmbH, Germany) and used directly in sequencing reactions. When obtained DNA sequences were of low quality, the amplicons were cloned in pGEM-T Easy Vector (Promega, USA). Initially, three positive clones per template were randomly selected and sequenced with pGEM-T Easy Vector-primers (pUC/M13). In cases where sequences of the clones were different, additional clones were examined. Only sequences recovered at least two times were retained for further analysis. Sequencing assays were carried out as described in Chapter 2. Obtained DNA sequences were compared using the multiple sequence alignment feature of the GENETYX version 7.0 software (GENETYX Corporation, Japan) and the MUSCLE algorithm of MEGA version 6 software (108). Representative nucleotides sequences were analyzed by BLASTn tool of NCBI for species identification. The sequences obtained in this study were registered in the GenBank

under the accession numbers KT633238 to KT633259 for 16S rDNA gene sequences and KT633260 to KT633270 for rompA sequences.

3.2.5 Statistical analysis

Proportions of tick samples positive for each of amplified genes were computed. A comparison of prevalences on the basis of tick gender was done using the chi-square test employing the EPI INFO™ software (CDC, USA, version 7.1.1) and VassarStats (http://vassarstats.net/). Statistically significant differences were determined at $P<0.05$.

3.3 Results

Detection of tick-borne pathogens in *A. variegatum* ticks

A total of 910 *A. variegatum* ticks were tested for tick-borne pathogens of veterinary and public health importance. The pathogens identified included *B. bovis, B. bigemina, T. mutans, A. marginale* and SFG rickettsiae. All the ticks were negative for *T. parva*, *T. taurotragi*, *T. annulata* and *T. orientalis*. *Babesia bigemina* was detected in a single tick, collected in Nikki centre whereas *B. bovis* and *T. mutans* were found in 10 (5 males and 5 females) and 4 (3 males and 1 female) ticks from Beterou, respectively. *Babesia bovis*-positive ticks were found in three different herds while those positive for *T. mutans* were all from the same herd. Meanwhile *A. marginale*-positive ticks were found in all the study areas except in Suya (Table 10). Overall, 142 ticks (15.6%) harbored the bacteria and the prevalence per areas ranged from 5.5% in Dunkassa to 36.5% in Tasso. The percentages of *A. marginale* -positive female and male ticks were similar. In total, 24.4% (44/180) of herds had at least one infected tick.

Rickettsiae were the most prevalent pathogens. Out of 910 ticks examined, 585 (499 males and 86 females) were positive for Rickettsia 16S rDNA (Table 11). The highest prevalence rate was in Beterou (92.9%) and the lowest were observed in Suya (37.5%) and Bouka (37.6%). In the SFG rickettsiae- specific PCR, the rompA

gene was successfully amplified in 267 ticks (204 males and 63 females) and amplicons of various length were observed. Most of the samples (222/267) showed a single amplification product of expected size (632 bp), some had simultaneously the 632 bp product and an approximately 800 bp product (34/267) and fewer (11/267) showed only a 800 bp band. The prevalence of SFG rickettsiae ranged from 92.9% in Beterou to 17.8% which was recorded in Sekere (Table 11). The percentage of SFG rickettsiae-positive female ticks (52.1%) was significantly higher ($P<0.001$) than male ticks (25.8%). In 72.2% (130/180) of herds, SFG rickettsiae were detected in ticks. More than half of the herds were infected, in all the study areas.

Mixed infections

SFG rickettsiae plus *A. marginale* was the most frequent mixed infections detected in the ticks. It was observed in 6.2% (56/910) of the ticks and recorded in all the study areas. Other pathogen combinations found only Beterou areas, included *B. bovis* plus *T. mutans* plus *A. marginale* plus SFG rickettsiae (1/910), *B. bovis* plus *T. mutans* plus SFG rickettsiae (3/910), and *B. bovis* plus SFG rickettsiae (6/910).

Identification of SFG rickettsiae

Twenty six samples positive for both 16S rDNA and rompA PCRs, including 8, 3, 3, 2, 4 and 4 samples from Beterou, Tasso, Nikki centre, Sekere, Sikki, Dunkassa and Bouka, respectively, were subjected to 16S rDNA sequences analyses.

Species identification was achieved through direct sequencing for 16 samples and through cloning-sequencing for the remaining 10 samples. Two or three different sequences types were obtained from each of the cloned PCR products. Overall, 22 unique sequences were obtained from the 26 samples analyzed. The multiple sequences alignment revealed three sequence clusters designated group A, B and C, and characterized by specific nucleotides substitution and indels (Table 12). The BLASTn searches showed that all the sequences shared high similarity with *R. africae* isolates. The sequence in the group A was 100% identical to *R. africae* strain ESF-5 (NR074527), while group B and C sequences shared 98-97% identity with the reference strain. The closest BLASTn match for group B and C-sequences was a *R. africae* variant isolated in Nigeria (JF 949789) (Table 13). In order to confirm these results, 14 sequenced samples which included the three types of products identified during rompA amplification, were subjected to analysis of rompA sequences. In detail, 10 samples that showed only 632 bp product, 2 samples that showed only 800 bp products and 2 samples that showed both types of products were examined. Ten samples were subjected to direct sequencing and four required cloning-sequencing. Two or three different sequences were obtained from each cloned amplicons. From sequencing the 632 bp amplicons, we identified nine unique sequences. One sequence (KT633262) was 100% identical to *R. africae* strain ESF-5 (CP001612)

and *R. africae* ESF 2500 (U83436) whereas the others showed 99% nucleotides identity with the aforementioned strains (Table 14). Out of the nine nucleotides sequences, three (KT633264, KT633266, KT633267) and two others (KT633260, KT633261) had the same amino acid sequences while the others had different amino acid sequences. Two unique 799 bp sequences were recovered from the remaining amplicons. These sequences shared 98% identity with *R. africae* reference strain (ESF-5 (CP001612)) (Table 14) but were 99% similar to isolates from Kenya (AF 548339, AF 548341), Niger (AF311961) and Mali (AF 311959, AF 311960). The multiple sequence alignment revealed that the main difference between the 799 bp and 632 bp amplicons was a unique 167 bp insertion which was identical in the two 799 bp-sequences. Noteworthy, amino acid sequences derived from the 799 bp-sequences contained premature stop codons. For all samples examined, results from the analysis of rompA gene corroborated the results of 16S rDNA analysis.

3.4 Discussion

The detection of pathogens in livestock and human-biting ticks, in order to understand tick-borne pathogens epidemiology and elucidate disease risk is an approach that has been successfully applied in several studies (64, 88, 100). In this study, *A. variegatum* ticks were targeted because it is one of most important cattle-tick species in Benin (27, 28, 114) and because it is known to transmit diseases to cattle as well as humans beings (44). Except for *T. parva*, *T. taurotragi*, *T. annulata* and *T. orientalis* for which no competent vectors are reported in Benin (114), all the pathogens investigated were detected. Pathogens infection rates, however, were different. As expected, the infection rates for *B. bigemina* and *B. bovis* were among the lowest. *Amblyomma variegatum* ticks are not known for transtadially maintaining these parasites and because the ticks were collected while feeding on cattle, these pathogens probably originated from the blood meal ingested prior to collection. *Theileria mutans* is vectored by *A. variegatum* and therefore the low prevalence recorded should be attributed to the infection levels in the cattle of surveyed herds. The detection of *Anaplasma marginale* in *Amblyomma variegatum* was surprising as it has not been observed in previous studies (32, 88, 100). However, *Anaplasma marginale* has previously been detected in *Amblyomma gemma* ticks collected from cattle (32). The bacteria probably originated from the hosts and the

relatively high infection rates mirror the prevalence of the pathogen in the study areas. In contrast to protozoan parasites, *A. marginale* can be maintained in mechanical vectors (55). Thus, a probable role of *A. variegatum* as mechanical vector cannot be ruled out. Studies on the ability of *A. variegatum* ticks to transmit *A. marginale* will surely clarify the epidemiology of anaplasmosis in Africa. Nevertheless, this study is the first molecular detection of tick-borne hemoparasites in Benin. The results reported here, corroborate previous studies (28, 94) which identified *B. bigemina, B. bovis, A. marginale* and *T. mutans* in Benin cattle herds using microscopic methods. Elaborate molecular and serological investigations in cattle will be needed to determine the full extent of these infections in Benin.

SFG rickettsiae have been detected in several West African countries including Niger, Mali (95), Liberia, Guinea (68) and Nigeria (88, 100) but have never been investigated in Benin. This study demonstrated the presence of SFG rickettsiae in Benin. The infection rates recorded were similar to previous surveys (51, 60, 72, 79). Cattle are suspected to serve as source of rickettsial infection for ticks (52) which get infected during blood meal. Hence, the differences of infection rates between locations are probably related to rickettsiemia levels in the farms where the ticks were collected. The higher percentage of SFG rickettsiae positive-female ticks is probably due to their blood feeding habit and to the role of

female *A. variegatum* in the transovarial transmission of rickettsial (104). Tick blood meals contain PCR inhibitors (21, 93) which have previously been suspected of hindering the detection of SFG rickettsiae in engorged female ticks (51). However, it was not the case in this study. The use of a PCR buffer (Ampidirect plus) which according to the manufacturer can neutralize biological samples inhibitory substances, may explain my results. The detection of SFG rickettsiae-positive herds in all the study areas reflects the pathogens endemicity in North Eastern Benin. The mixed infections recorded may originate from accumulation of pathogens collected during different blood meals or from the ticks feeding on a co-infected host. The high frequency of *A. marginale* and SFG rickettsiae co-infections should be related to the bacterial nature of both pathogens and to the role of the ticks in their transmission. Similarly, Reye *et al.* noted that the combinaison *Rickettsia africae*-like species and *Coxiella burnetii* was the predominant mixed infection in nigerian cattle ticks (100).

The 16S rDNA and rompA sequences analyses performed on some SFG rickettsiae-positive samples, showed that they were infected with *R. africae,* which is known to cause ATBF (50, 97) in humans. Most of the *R. africae* sequence types obtained in this study was reported for the first time. The similarity between the 16S rDNA sequence of *R. africae* isolated in *A. variegatum* ticks from Benin and from

Nigeria should be attributed to geographic proximity and uncontrolled animal movement between the two countries. The diversity in length and nucleotides substitutions observed among the 16S rDNA sequences have also been reported in Nigeria (88). Likewise, the polymorphism of *R. africae* rompA gene sequences has previously been reported in Kenya (60, 63), Uganda (78) and Sudan (79). The rompA gene belongs to the "surface cell antigens" family which are characterized by frequent degradation resulting in different subsets of the genes being expressed among *Rickettsia* species (12). The variations in length of the rompA fragment amplified have also been observed in Kenyan *A. variegatum* ticks (63). However, the sequences identified in Kenya showed a 173 bp-insertion while in this study; it was a 167 bp-insertion. In addition, Maina *et al* (63) sequences were 99% identical to sequences of *R. africae* ESF-5 (CP001612) and uncultured *Rickettsia* species (AB 822475, AB 822463) from Uganda (78) whereas obtained 799 bp sequences were similar to *R. africae* isolates from West Africa (95) and Kenya (60). These unexpected amplicons are probably immature forms of the gene as evidenced by the presence of introns in the translated version of the sequences. The diversity observed among the 16S rDNA and rompA sequences suggests that the ticks harboured several *R. africae* genotypes/variants. *Amblyomma variegatum* ticks are biological vector and life-long reservoir of *R. africae* (42) due to transovarial and transstadial

transmission (104). The presence of multiple genotypes in the ticks is probably due to genetic materials modifications relevant to tick and SFG rickettsiae interactions (105). In addition, *A. variegatum* as three-host ticks which feed on a wide variety of vertebrates (44) could have contributed to genetic recombination between the various *R. africae* isolates that they may collect during feeding. Further researches on the extent of the genetic diversity of Benin *R. africae* strains will definitely aid in understanding the epidemiology of the bacteria.

Rickettsia africae is believed to be present in rural areas of more than 30 African countries. However, the infection has been confirmed in ticks or humans in only 15 countries (16). Benin Republic is one of the countries in which the prevalence of SFG rickettsiae and the occurrence of *R. africae* were not yet documented. The identification of *R. africae* in Benin extends the known range of the bacteria and emphasizes the need to consider ATBF among the causes of febrile illnesses contracted in the country. Though ATBF cases are rarely reported among indigenous population, the presence of antibodies to *R. africae* and the detection of its DNA in febrile patients in Cameroon (81, 82, 83) indicate that Benin cattle owners and rural dwellers can also suffer from SFG rickettsiosis. The country is in malaria endemic region and therefore febrile illnesses are often attributed to *Plasmodium* parasites. Clinicians in Benin and those treating travelers should be

aware that SFG rickettsiae infections are among the potential causes of febrile illnesses contracted in the country. Although *R. africae* is the SFG rickettsiae most frequently harboured by *A. variegatum* ticks, the presence of other species in the study samples cannot be ruled out. Further studies covering ticks, livestock as well as livestock owners and based on cost-effective, high-throughput methods such as the multispacer typing (78) are of interest for a better understanding of the epidemiology of SFG rickettsiae in Benin.

In conclusion, the present study reports the first identification of *B. bigemina, B. bovis, T. mutans, A. marginale* and SFG ricketsiae in *A. variegatum* ticks collected in Benin and demonstrates the presence of *R. africae* in the country. These findings confirm that several tick-borne pathogens that are threats to of humans and animal health are prevalent in Benin. Physicians and veterinarians in Benin should be aware of these infections when investigating health problems in humans and livestock.

3.5 Summary

In the present study, 910 *Amblyomma variegatum* ticks collected from 8 different locations in North Eastern Benin were tested fo*r B. bigemina, B. bovis, Theileria* species (*T. taurotragi, T. annulata, T. orientalis, T. parva, T. mutans), A. marginale* and SFG ricketsiae. Pathogens identified in the ticks included *B. bigemina* (1/910), *B. bovis* (10/910), *T. mutans* (4/910), *A. marginale* (142/910) and SFG ricketsiae (267/910). *B. bigemina*, *B. bovis* and *T. mutans* were detected in only one location whereas *A. marginale* and SFG ricketsiae were found in 7 and 8 locations, respectively. The prevalence of SFG rickettsiae varied according to the sampling sites and was significantly higher in the female than in the male ticks. The sequences analyses demonstrated the presence of *Rickettsia africae* and/or closely related species in Benin. These findings reaffirm the presence of tick-borne hemoparasites in Benin and extend the geographic distribution of *R. africae* and spotted fever rickettsioses in Africa. Clinicians in Benin and those treating travelers should be aware of the possibility of SFG rickettsiae infection when they are treating patients with febrile illness.

Figure 4. **Map of Borgou district showing tick collection sites.** The ticks were collected from 4 different divisions of Borgou district in North Eastern Benin. The black stars indicate tick collection sites.

Table 9. **Sequences of primers set used for *T. annulata, T. parva, T. taurotragi* and *T. mutans* detection in *A. variegatum* ticks**

Pathogen Target gene	Assays	Oligonucleotide sequences (5'>3')	Product size (bp)	Reference
T. annulata 30-kDa major merozoite surface antigen	First PCR	GTAACCTTTAAAAACGT GTTACGAACATGGGTTT	721	(65)
	Second PCR	CACCTCAACATACCCC TGACCCACTTATCGTCC	453	
Theileria/Babesia spp. *18S rRNA* V4 region	PCR	GACACAGGGAGGTAGTGACAAG CTAAGAATTTCACCTCTGACAGT	385 to 429	
T. parva	Second PCR	GACACAGGGAGGTAGTGACAAG CATGCAGAGACCCCGAAGGGACAC	224	(102)
T. taurotragi	Second PCR	GACACAGGGAGGTAGTGACAAG GAACCGTCCGAAAAAAGCCACG	243	
T. mutans	Second PCR	GACACAGGGAGGTAGTGACAAG AACATTCGGAGACGCAAGCGAG	258	

Table 10. **Results of *Anaplasma marginale* detection in *Amblyomma variegatum* ticks collected in Borgou district, Benin**.

Samples			Positive ticks			Infected herds (%) [c]
Location	No. of herds surveyed [a]	No. of ticks collected [b]	Male (%)	Female (%)	Total (%)	
Beterou	6	28 (13/15)	1 (7.7)	1 (6.7)	2 (7.1)	2 (33.3)
Suya	4	16 (13/3)	0	0	0	0
Tasso	42	200 (169/31)	64 (37.9)	9 (29)	73 (36.5)	17 (40.5)
Nikki center	14	82 (71/11)	9 (12.7)	1 (9.1)	10 (12.2)	2 (14.3)
Sekere	38	213 (195/18)	21 (10.8)	1 (5.6)	22 (10.3)	4 (10.5)
Sikki	16	87 (71/16)	7 (9.9)	3 (18.8)	10 (11.5)	2 (12.5)
Dunkassa	24	127 (106/21)	6 (5.7)	1 (4.8)	7 (5.5)	5 (20.8)
Bouka	36	157 (151/6)	17 (11.3)	1 (16.7)	18 (11.5)	11 (30.6)
Total	180	910 (789/121)	125 (15.8)	17 (14)	142 (15.6)	44 (24.4)

[a] Number of herds from which the ticks were collected

[b] Total number of ticks (Male/Female).

[c] Number of herds in which *A. marginale* positive ticks were found

Table 11. **Results for the detection of spotted fever group rickettsiae in *A. variegatum* ticks from Borgou district, Benin using 16S rDNA and rompA PCRs**

Samples			16S rDNA PCR positive samples			rompA PCR positive samples [c]			Infected herd (%) [d]
Location	No. of herds surveyed [a]	No. of ticks collected [b]	Male (%)	Female (%)	Total (%)	Male (%)	Female (%)	Total (%)	
Beterou	6	28 (13/15)	12 (92.3)	14 (93.3)	26 (92.9)	12 (92.3)	14 (93.3)	26 (92.9)	6 (100)
Suya	4	16 (13/3)	5 (38.4)	1 (33.0)	6 (37.5)	3 (23.1)	1 (33.3)	4 (25.0)	3 (75.0)
Tasso	42	200 (169/31)	151 (89.0)	29 (93.6)	180 (90.0)	74 (44.1)	22 (70.9)	96 (48.2)	38 (90.5)
Nikki centre	14	82 (71/11)	49 (69.1)	5 (45.5)	54 (65.9)	16 (22.5)	3 (27.3)	19 (23.2)	7 (50.0)
Sekere	38	213 (195/18)	133 (68.2)	13 (72.2)	146 (68.5)	30 (15.4)	8 (44.4)	38 (17.8)	25 (65.8)
Sikki	16	87 (71/16)	33 (46.4)	7 (43.8)	40 (45.9)	15 (21.1)	4 (25.0)	19 (21.8)	11 (68.8)
Dunkassa	24	127 (106/21)	61 (57.6)	13 (61.9)	74 (58.3)	24 (22.6)	10 (47.6)	34 (26.7)	17 (70.8)
Bouka	36	157 (151/6)	55 (36.4)	4 (66.7)	59 (37.6)	30 (19.9)	1 (16.7)	31 (19.8)	23 (63.9)
Total	180	910 (789/121)	499 (63.2)	86 (71.1)	585 (64.3)	204 (25.8) [e]	63 (52.1) [e]	267 (29.4)	130 (72.2)

[a] Number of herds from which the ticks were collected.

[b] Total number of ticks (Male/Female).

[c] Only samples positive for 16S rDNA were tested.

[d] Number of herds in which rompA PCR positive ticks were found

[e] Significant difference between male and female ticks ($P<0.001$).

Table 12. **Alignment of substitutions and indels observed in Rickettsia spp. 16S rDNA sequences recovered from A. *variegatum* ticks collected in Benin.** Identical nucleotides are indicated by a dot (.) and indels by a hyphen (-). Features defining each cluster are shown in bold and blue fonts. Numbers above the alignments represent the nucleotide position after the sequence type KT633242.

		Rickettsia spp. 16 S ribosomal RNA gene (16S rDNA)																																					
Sequences of Benin isolates		2	2	2	3	3	4	6	6	6	8	9	9	1 0	1 1	1 1	1 2	1 3	1 3	1 4	1 4	1 4	1 4	1 6	1 7	1 7	1 7	1 7	1 7	1 8	1 9	2 0	2 4	2 8	3 1	3 5	3 7	3 8
Cluster	GenBank ID	2	4	5	3	7	3	0	2	7	5	5	9	6	0	7	6	7	9	6	7	8	9	9	1	2	3	4	6	4	8	5	1	1	2	7	6	4
Group A	KT633255	A	G	A	C	A	A	C	A	A	A	T	A	T	T	T	A	C	G	T	T	-	T	C	-	-	-	-	A	T	T	T	A	A	A	A	A	C
Group B	KT633238	G	A	.	T	.	.	.	.	.	.	.	.	.	.	C	.	.	.	G	G	A	A	.	-	-	T	A	G	.	.	.	.	.	G	.	.	.
	KT633239	.	.	.	T	.	.	.	.	.	.	.	.	.	.	C	.	.	.	G	G	A	A	.	-	-	T	A	G	.	.	.	.	.	A	.	.	.
	KT633240	G	A	.	T	.	.	.	.	.	.	.	C	.	.	C	.	.	.	G	G	A	A	.	-	-	T	A	G	.	.	.	.	.	.	.	.	.
	KT633241	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	G	G	A	A	.	-	-	T	A	.	.	.	.	.	.	A	.	.	.
	KT633244	.	.	.	.	G	.	.	.	.	.	.	.	.	.	C	.	.	.	G	G	A	A	.	-	-	T	A	.	.	.	.	.	.	A	C	.	T
	KT633247	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	G	G	A	A	G	-	-	T	A	.	.	.	.	.	.	A	.	.	.
	KT633248	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	G	G	A	A	.	-	-	T	A	.	.	.	.	.	.	A	C	.	T
	KT633249	.	.	.	.	G	G	.	.	.	.	.	.	C	.	C	.	.	.	G	G	A	A	.	-	-	T	A	.	.	C	C	.	.	A	C	.	T
	KT633250	.	.	.	.	G	.	.	.	.	.	.	.	.	.	C	G	.	C	G	G	A	A	.	-	-	T	A	.	.	.	.	.	G	A	C	.	T
	KT633251	.	.	.	.	G	.	.	.	.	.	.	.	.	.	C	G	.	.	G	G	A	A	.	-	-	T	A	.	.	.	.	.	.	A	.	.	.
	KT633252	.	.	.	.	G	.	.	.	.	.	.	.	.	.	C	G	.	.	G	G	A	A	.	-	-	T	A	.	.	.	.	.	G	A	C	.	T
	KT633253	.	.	.	.	G	.	.	.	.	.	.	.	.	.	C	G	.	.	G	G	A	A	.	-	-	T	A	.	C	.	.	.	G	A	C	.	T
	KT633256	.	.	.	.	.	.	.	G	.	.	.	.	.	.	C	.	T	.	G	G	A	A	.	-	-	T	A	.	.	.	.	.	.	A	.	.	.

	KT633257	.	.	.	.	.	.	.	G	.	.	.	.	.	.	C	.	T	.	G	G	A	A	.	**-**	**-**	**T**	**A**	.	.	.	.	.	.	A	.	.	.
	KT633258	.	.	.	.	G	.	.	.	.	.	.	.	.	.	C	.	.	.	G	G	A	A	.	**-**	**-**	**T**	**A**	.	.	.	.	.	.	A	.	.	.
	KT633259	.	.	.	.	.	.	.	.	.	.	C	.	.	.	C	.	.	.	G	G	A	A	.	**-**	**-**	**T**	**A**	.	.	.	.	.	.	A	.	.	.
Group C	KT633242	.	.	.	.	.	.	**T**	.	.	.	.	.	.	.	C	.	.	.	G	G	A	A	.	**T**	**A**	**T**	**A**	.	.	.	.	.	.	A	.	.	.
	KT633243	.	.	.	.	.	.	**T**	.	C	.	.	.	.	C	C	.	.	.	G	G	A	A	.	**T**	**A**	**T**	**A**	.	.	.	.	.	.	A	.	.	.
	KT633245	.	.	.	.	.	.	**T**	.	.	G	.	.	.	.	C	.	.	.	G	G	A	A	.	**T**	**A**	**T**	**A**	.	.	.	.	.	.	A	.	.	.
	KT633246	.	.	G	.	.	.	**T**	.	.	.	.	.	.	.	C	.	.	.	G	G	A	A	.	**T**	**A**	**T**	**A**	.	.	.	.	.	.	A	.	G	.
	KT633254	.	.	.	.	.	.	**T**	.	.	.	.	.	.	.	C	.	.	.	G	G	A	A	.	**T**	**A**	**T**	**A**	.	.	.	.	G	.	A	.	.	.

Table 13. **BLASTn results for *Rickettsia* spp. 16S rDNA sequences recovered from *A. variegatum* ticks collected in Benin.**

Rickettsia spp. 16S rDNA sequences			Highest BLASTn match			%Identity* with reference strain[a]
Cluster	GenBank ID	Length (bp)	Species	GenBank ID (origin)	%Identity*	
Group A	KT633255	439	*R. africae*	NR074527(Ethiopia) JF949787 (Nigeria)	100 (439/439)	100 (439/439)
Group B	KT633238	442	*R. africae*	JF 949789 (Nigeria)	98 (436/446)	98 (431/442)
	KT633239	442	*R. africae*	JF 949789 (Nigeria)	98 (439/446)	98 (434/442)
	KT633240	442	*R. africae*	JF 949789 (Nigeria)	98 (435/446)	97 (430/442)
	KT633241	442	*R. africae*	JF 949789 (Nigeria)	99 (441/446)	98 (435/442)
	KT633244	442	*R. africae*	JF 949789 (Nigeria)	98 (438/446)	98 (432/442)
	KT633247	385	*R. africae*	JF 949789 (Nigeria)	98 (383/389)	98 (377/385)
	KT633248	442	*R. africae*	JF 949789 (Nigeria)	98 (439/446)	98 (433/442)
	KT633249	442	*R. africae*	JF 949789 (Nigeria)	97 (434/446)	97 (428/442)
	KT633250	442	*R. africae*	JF 949789 (Nigeria)	98 (435/446)	97 (429/442)
	KT633251	442	*R. africae*	JF 949789 (Nigeria)	98 (439/446)	98 (433/442)
	KT633252	442	*R. africae*	JF 949789 (Nigeria)	98 (435/446)	97 (429/442)
	KT633253	442	*R. africae*	JF 949789 (Nigeria)	98 (436/446)	97 (430/442)
	KT633256	442	*R. africae*	JF 949789 (Nigeria)	98 (439/446)	98 (433/444)
	KT633257	442	*R. africae*	JF 949789 (Nigeria)	98 (438/446)	98 (432/444)
	KT633258	403	*R. africae*	JF 949789 (Nigeria)	99 (401/407)	98 (395/403)

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	KT633259	442	*R. africae*	JF 949789 (Nigeria)	99 (440/446)	98 (434/442)
Group C	KT633242	444	*R. africae*	JF 949789 (Nigeria)	99 (444/446)	98 (434/444)
	KT633243	444	*R. africae*	JF 949789 (Nigeria)	99 (442/446)	97 (432/444)
	KT633245	444	*R. africae*	JF 949789 (Nigeria)	99 (443/446)	98 (433/444)
	KT633246	444	*R. africae*	JF 949789 (Nigeria)	99 (442/446)	97 (432/444)
	KT633254	444	*R. africae*	JF 949789 (Nigeria)	99 (443/446)	98 (433/444)

* percentage of identity (number of matched/number of nucleotides compared).

[a] *R. africa*e reference strain : strain ESF-5 (NR074527).

Note: The fragment of *Rickettsia* spp. 16S rDNA amplified in this study corresponded to 439 bp sequence for NR074527 and JF949787, and 446 bp-sequence for JF 949789.

Table 14. **Comparison of *Rickettsia* ompA gene (rompA) nucleotide sequences recovered from *A. variegatum* ticks collected in Benin to *R. africae* type specimen (strain ESF-5)**.

rompA sequences from Benin		%Identity* with
GenBank ID	Length (bp)	*R. africae* strain ESF-5[a]
KT633262	632	100 (632/632)
KT633263	632	99 (625/632)
KT633264	632	99 (629/632)
KT633265	632	99 (627/632)
KT633260	632	99 (626/632)
KT633261	632	99 (625/632)
KT633266	632	99 (628/632)
KT633267	632	99 (627/632)
KT633268	632	99 (626/632)
KT633269	799	98 (627/799)
KT633270	799	98 (626/799)

*percentage of identity (number of matched/number of nucleotides compared).

[a] GenBank ID of ompA gene sequence of *R. africae* strain ESF-5: CP001612.

Chapter 4

Molecular characterization and phylogenetic analyses of *Babesia bovis*, *Babesia bigemina*, *Anaplasma marginale*, *Theileria parva* and *Theileria orientalis* isolated in Egypt, Kenya and Benin

4.1 Introduction

The strains/genotypes of tick-borne pathogens occurring in an area is an important information because it determines pathogens virulence and the ability of vaccine strains to protect against overt disease (38, 45, 55, 107). The molecular characterization of pathogens is therefore a requirement for the design of effectives diagnostic and disease prevention tools. Unfortunately, studies on the genetic diversity of tick borne pathogens in African countries have been limited to a few pathogens, few countries or have exploited genus specific rather that species specific genes. Therefore, this study, aims to determine and understand the genetic diversities of *B. bovis*, *B. bigemina* and *A. marginale* isolated in Egypt, Kenya and Benin, and *T. parva* and *T. orientalis* isolated in Kenya. *Babesia bovis* spherical body protein 4, *B. bigemina* RAP-1a, *A. marginale* Msp5, *T. parva* p104 and *T. orientalis* MPSP were used as the marker genes. The sequences generated from these target genes were

used to establish phylogenies to aid in the understanding of their molecular epidemiology in Egypt, Kenya and Benin in relation to other regions of the world.

4.2 Materials and methods

4.2.1 Study samples

Cattle and water buffalos samples from Egypt (Chapter 1), cattle samples from Kenya (Chapter 2) and ticks samples from Benin (Chapter 3); infected with *B. bovis, B. bigemina*, *A. marginale, T. parva* or *T. orientalis* were used to characterize the respective pathogens.

4.2.2 Cloning and sequencing of hemoparasites DNAs

Randomly selected positive samples of *B. bovis, B. bigemina, T. parva, T. orientalis* and *A. marginale* (three samples for each parasite per study area in each country) were used as templates for genetic characterization of the hemoparasites. Nested PCR amplicons of *B. bovis* SBP-4 (Table 1) and *T. parva* p104 (Table 3) ; PCRs amplicons of *A. marginale* Msp5 (Table 1) and *T.orientalis* MPSP (Table 3); and the products of *B. bigemina* RAP-1a-semi-nPCR (Table 15) were purified by using QIAquick Gel Extraction Kit (QIAGEN GmbH, Germany). The purified DNA templates were first sequenced with the amplification primers to identify heterozygous base-calling positions and then cloned in pGEM-T Easy Vector (Promega, USA). Initially, two positive clones per template were randomly selected and sequenced with pGEM-T Easy Vector-primers (pUC/M13). When the sequences

of the clones did not include all the genotypes identified during direct sequencing, other two clones from the same template were sequenced. All sequencing analysis assays were performed using the Dye Terminator Cycle Sequencing Kit (Applied Biosystems, USA) and an ABI PRISM 3100 genetic analyzer (Applied Biosystems, USA).

4.2.3 BLAST analysis, sequence alignment and phylogenetic analysis

DNA sequences identities were computed using the pairwise alignment by EMBOSS NEEDLE software (http:// www.bioinformatics.nl/cgi-bin/emboss/needle). The comparison to previously published sequences was performed by using the BLASTn tool of NCBI GenBank database. Multiple sequence alignments were done using MUSCLE and GUIDANCE algorithms (http://guidance.tau.ac.il/). Phylogenetic analyses were inferred by the maximum likelihood method using MEGA version 6 software (108).

4.2.4 Nucleotide sequences accession numbers

The nucleotide sequences of all the genes sequenced are available in the GenBank of the NCBI database under the accession numbers outlined in Table 16.

4.3 Results

BLAST analysis and sequence alignment

To establish the genotypes of these tick-borne pathogens, sequences of SBP-4, RAP-1a, Msp5, p104 and MPSP genes found in *B. bovis, B. bigemina, A. marginale, T. parva* and *T. orientalis*, respectively were genetically characterized. The nucleotides sequences of *B. bovis* SBP-4 identified in Egyptian cattle (KF192806, KF192807 and KF192808) were highly conserved (99.8% identity). Surprisingly, the sequences obtained in Egyptian water buffalos (KF192805) were 18 nucleotides shorter than cattle isolates (503 bp against 521 bp). The identity values among Kenyan isolates' nucleotides sequences (KP347555, KP347556 and KP347557) ranged from 99.6 to 99.8%. Two nucleotides sequences (KU042085 and KU042086) sharing 99.8% identity were identified in Benin ticks samples. The comparison of isolates between countries showed that Egyptian water buffalos isolate was the most distant genotype with *B. bovis* T2Bo strain from USA (XM_001610418) as closest match (100%). Except for one Kenya isolate (KP347555) and one Benin isolate (KU042085) sharing 100% identity, the pair-wise identities of the SBP-4 sequences identified in Egypt cattle, Kenyan cattle and Benin ticks was 99%. BLASTn analysis revealed that one Kenyan isolate (KP347556) shared 100% sequence identity with the isolates from South Africa (KF626630 and

AB569303) and Ghana (AB569301). The multi-sequence alignment of *B. bovis* SBP-4 amino acid residues revealed that the Egyptian cattle, Kenya and Benin isolates contained additional amino acid residues and a specific pattern of substitutions unique to isolates obtained from African cattle (Table 17). For *B. bigemina*, the analysis of partial sequences of RAP-1a from Egyptian cattle and water buffalos (KF192809, KF192810, KF192811 and KF192812) revealed 99.4% identities whereas Kenyan sequences (KP347558, KP347559 and KP893330) had identities ranging from 99.6 to 99.9%. A further BLASTn analysis revealed that the *B. bigemina* Kenyan isolates shared between 99% and 100% nucleotide identities with the sequence of an Egyptian isolate (KF192811). The isolate from Benin was 99% identical to sequences from Kenya (KP347558 and KP347559) and from Egypt (KF192809 and KF192811). The RAP-1a sequences identified in this study shared ≥98% similarity with previous sequences available in the GenBank database. Two types of *A. marginale* Msp5 sequences, sharing 99.8% identity with each other, were identified. The two sequences were found in Egyptian cattle (KU042080 and KU042081), Egyptian water buffalo (KU042082 and KU042083) and in Kenyan cattle (KP347553 and KP347554) but only one was found in Benin ticks (KU042079). These sequences were homologous to the sequences of isolates from Australia (CP006847), the Philippines (AB704328) and China (EF546443).

The identities among the *T. parva* p104 sequences ranged from 97.8 to 98.9%. The Kenyan *T. parva* isolates detected were genetically different from *T. parva* isolates previously reported in Kenya. One of the *T. parva* isolates (KP347564) shared 99% nucleotide identity with a previously published sequence from Kenya (AY034071). However, the other two isolates (KP347565 and KP347566) shared 99% nucleotide sequence identity with isolates from Zambia (AB739676 and AB739678), Zimbabwe (AY034070) and Kenya (AY034069). Only one *T. parva* isolate (KP347565) was prevalent in one of the study farm (Machakos farm in Chapter 2) while all the three isolates found in this study were detected in samples from the second farm (Ngong farm in Chapter 2). The identities among the four *T. orientalis* MPSP sequences of this study ranged from 86.5 to 99.5%. The nucleotide sequences of three *T. orientalis* isolates (KP347560, KP347562 and KP347563) were conserved and shared 99% sequence identity with *T. buffeli* (AB016278), a hemoparasite, previously isolated in Kenya. The other isolate (KP347561) shared 99% sequence identity to the isolates from China (KJ020560 and AB571974), Thailand (AB562563) and Japan (AB218444).

Phylogenetic analysis

Phylogenetic analyses were done to determine whether the tick-borne pathogens are genetically diverse within different geographical regions of the world.

Analysis based on SBP-4 gene grouped Egyptian cattle, Kenyan and Beninese *B. bovis* isolates in the same clade (Clade 1) as Ghanaian and South African isolates. Egyptian water buffalos isolate, however, was found in a separate clade (Clade 3) grouped with *B. bovis* isolates from Thailand, Syria, Mexico, Brazil, Mongolia and United States of America (USA) (Figure 5). The *B. bigemina* isolates of this study belonged to the same clade as the isolates from Thailand, Syria and Mexico (Figure 6). A further phylogeny using the Msp5 gene grouped Egyptian, Kenyan and Beninese *A. marginale* isolates in the same clade as the isolates from China, Australia, Brazil and The Philippines (Figure 7). However, isolates from USA and Cuba were grouped in a different clade. For *T. parva*, the KP347565 and KP347566 isolates were closely related to the cattle-derived genotypes while KP347564 was related to the buffalo-derived *T. parva* genotypes (Figure 8). The phylogenetic analysis based on MPSP gene of *T. orientalis/sergenti/buffeli* grouped three of the isolates of this study (KP347560, KP347562 and KP347563) in the same clade and these isolates were classified as MPSP type 3. The divergent isolate KP347561 belonged to a separate clade and was identified as MPSP type 5 (Figure 9).

4.4 Discussion

The molecular characterization of pathogens isolates provides data that are required for understanding their epidemiology and for selection of disease control measures. On the other hand, understanding the extent of genetic diversity of a particular gene is important for assessing its value as diagnostic marker or vaccine target. In this study, using species-specific genes, I characterized pathogenic hemoparasites isolated in three African countries and assessed the genetic diversity of the target genes within and between countries. *Babesia bovis* SBP-4, *B. bigemina* RAP-1a, *A. marginale* Msp5, *T. orientalis* MPSP genes, and the corresponding PCR assays were exploited for the first time in Egypt, Kenya and Benin. My results confirm the value of these assays (46, 90, 112, 118) and suggest that they can be used to improve hemoparasites detection in these countries. *Babesia bovis* and *B. bigemina* isolated in the three countries were genetically conserved and closely related to isolates from other African countries. The multisequence alignment of *B. bovis* SBP-4 showed that *B. bovis* isolated from African cattle can be distinguished from other isolates by specific pattern of amino acid substitutions. Such findings suggest that *B. bovis* identified in water buffalos and cattle may be different genotypes and this sequence could be used as fingerprint for identification of infection origin. However, *B. bovis* SBP-4 phenogram showed the existence of an

"intermediate" clade (Clade 2, Figure 5) hosting sequences from South Africa which despite being isolated from cattle were not bearing the discriminatory "fingerprint". Although the function of *B. bovis* SBP-4 is not yet elucidated (107) the protein is known to shed in the cytosol of infected red blood cells at late stage of infection and might be involved in modification of membrane and function of erythrocytes (111). Further studies on *B. bovis* SBP-4 gene in other African countries are needed for clarification of the scope, the origin and impact of this "fingerprint" on the parasite pathogenicity or resistance. In addition, phylogenic studies based on polymorphic genes markers are necessary to further characterize *Babesia* parasite isolated in Egypt, Kenya and Benin.

The sequence identity of the two *A. marginale* genotypes detected in this study suggests that the parasite isolates circulating in Egypt, Kenya and Benin may be genetically conserved. However, further studies are required to unravel the genetic diversity of the parasite isolates.

Theileria parva p104 molecular analyses results were in agreement with previous studies (35, 40) which reported that *T. parva* p104 antigen loci exhibits moderate polymorphism. *Theileria parva* p104 phenogram indicates that cattle and buffalo derived *T. parva* isolates are prevalent in Ngong farm, Kenya (Chapter 2) whereas in Machakos farm, Kenya (Chapter 2) cattle are only exposed to the

cattle-derived *T. parva.* The identification of buffalo-derived genotypes in Ngong farm corroborates previous reports of the occurrence of buffalo-derived *T. parva* in cattle in Kenya (18; 66). Immunity to a *T. parva* strain is not always protective against heterologous challenge (45). Hence, complete characterization of strains present in Kenya will help in assuring successful immunization and reduce the impact of this much feared pathogen.

Before this study, only one MPSP allele of *T. sergenti/buffeli/orientalis* had been isolated in Kenya (49, 84). The polymorphism of the *T. orientalis* MPSP type 3 isolates identified and the description for the first time of *T. orientalis* MPSP type 5 indicate that at least two strains of this benign *Theileria* parasite are present in Kenya. None of the *T. orientalis* MPSP alleles identified in Kenya have ever been associated to disease outbreaks. However, in Ethiopia (9), Burundi (54), India (6), Australia (19, 22, 46) and New Zealand (67), some *T. orientalis* strains have caused disease outbreaks. Hence, *T. orientalis* complex should not be ruled out as a probable cause of disease, particularly for crossbred and exotic breeds dairy cattle reared in Kenya.

The present study characterized and compared *B. bovis*, *B. bigemina* and *A. marginale* detected in Egyptian cattle and water buffalos, Kenyan cattle and cattle ticks from Benin. *Theileria parva* and *T. orientalis* detected in Kenyan cattle were also analyzed and compared to isolates from other countries. The current findings

extend the understanding of the genetic diversity of the above hemoparasites and pave the way for a standardization of pathogens detection tools employed in Africa.

4.5 Summary

The genetic diversities of *B. bovis*, *B. bigemina*, *A. marginale* infecting livestock in Egypt, Kenya and Benin; *T. parva* and *T. orientalis* occurring in Kenya were addressed in this study. The partial sequences of *B. bovis* SBP-4, *B. bigemina* RAP-1a, *A. marginale* Msp5, *T. parva* p104 and *T. orientalis* MPSP genes were amplified in cattle and water buffalo samples from Egypt, cattle from Kenya and *A. variegatum* ticks from Benin. The resulting amplicons were sequenced and compared. *B. bigemina* Rap-1a and *A. marginale* Msp 5 sequences were conserved and showed high homology among the isolates from these countries. *B. bovis* SBP-4 sequences from Egyptian cattle, Kenyan cattle, and *A. variegatum* ticks from Benin were similar. However, *B. bovis* SBP-4 sequences from Egyptian water buffalos were 18 nucleotides shorter (503 bp against 521 bp). *T. parva* and *T. orientalis* isolated in Kenyan cattle farms were polymorphic. Cattle-derived *T. parva* was detected Machakos farm, whereas cattle and buffalo–derived strains were detected in Ngong farm suggesting interactions between cattle and wild buffaloes. The *T. orientalis* genotypes identified were classified as MPSP Type 3 and MPSP Type 5. Noteworthy, sequences derived from the hemoparasites isolates in this study were genetically related to the other African isolates. These findings shade a light on the genetic diversity of tick-borne pathogens in Egypt, Kenya and Benin.

Figure 5. **Unrooted phylogenetic tree of *Babesia bovis* SBP-4 gene**. The tree was constructed with the maximum likelihood method using the Kimura 2 parameter model in the MEGA ver.6. The sequences determined in this study are shown in bold-font. Numbers on internodes indicate percentages of 1000 bootstrap replicates.

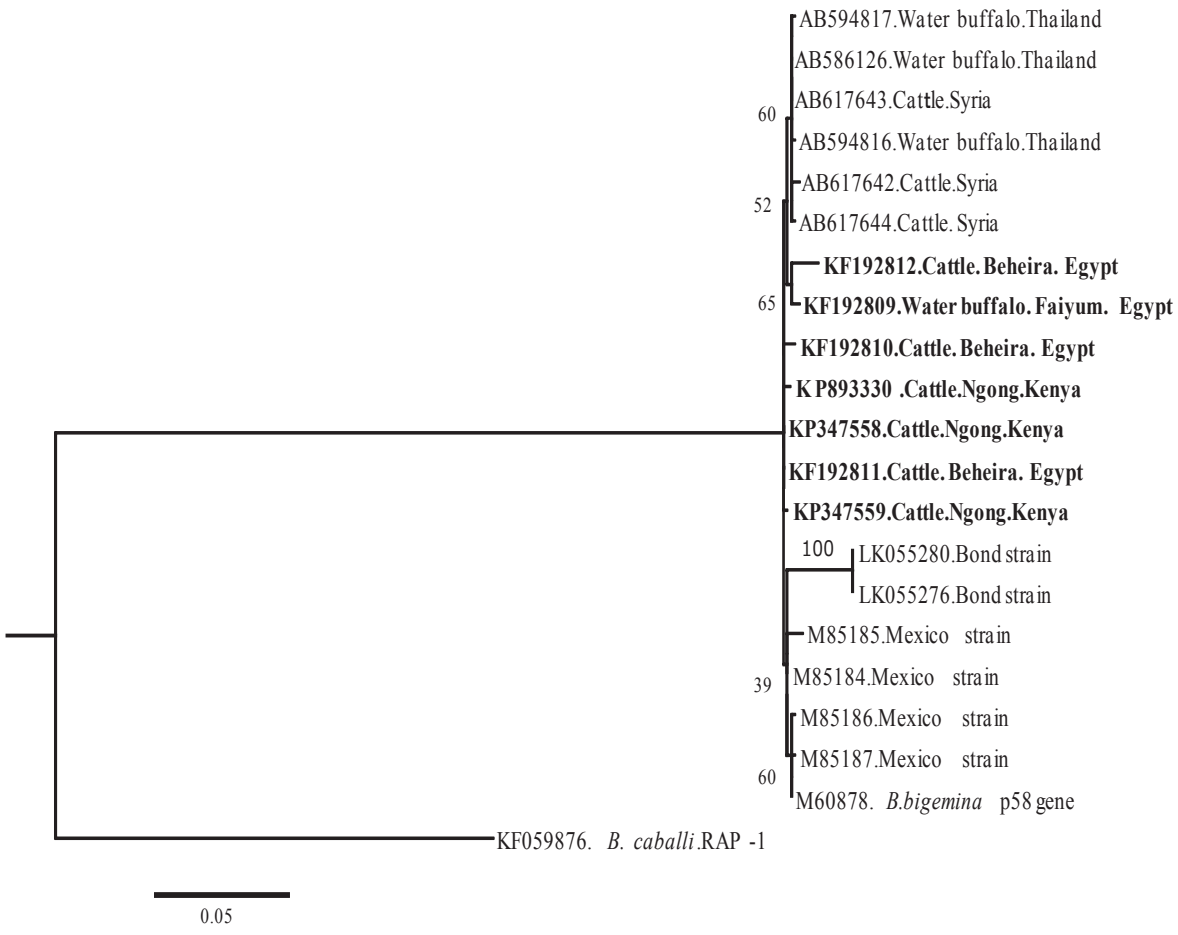

Figure 6. **Phylogenetic analyses of *B. bigemina* RAP-1a gene sequences obtained from Egyptian and Kenyan livestock**. *B. caballi* Rhoptry-associated protein-1(RAP-1) gene was used as out group. The tree was constructed with the maximum likelihood method using the Kimura 2 parameter model in the MEGA ver.6. The sequences determined in this study are shown in bold-font. Numbers on the branches show percentages of 1000 bootstrap replications. The scale bar indicates estimated number of substitutions per site.

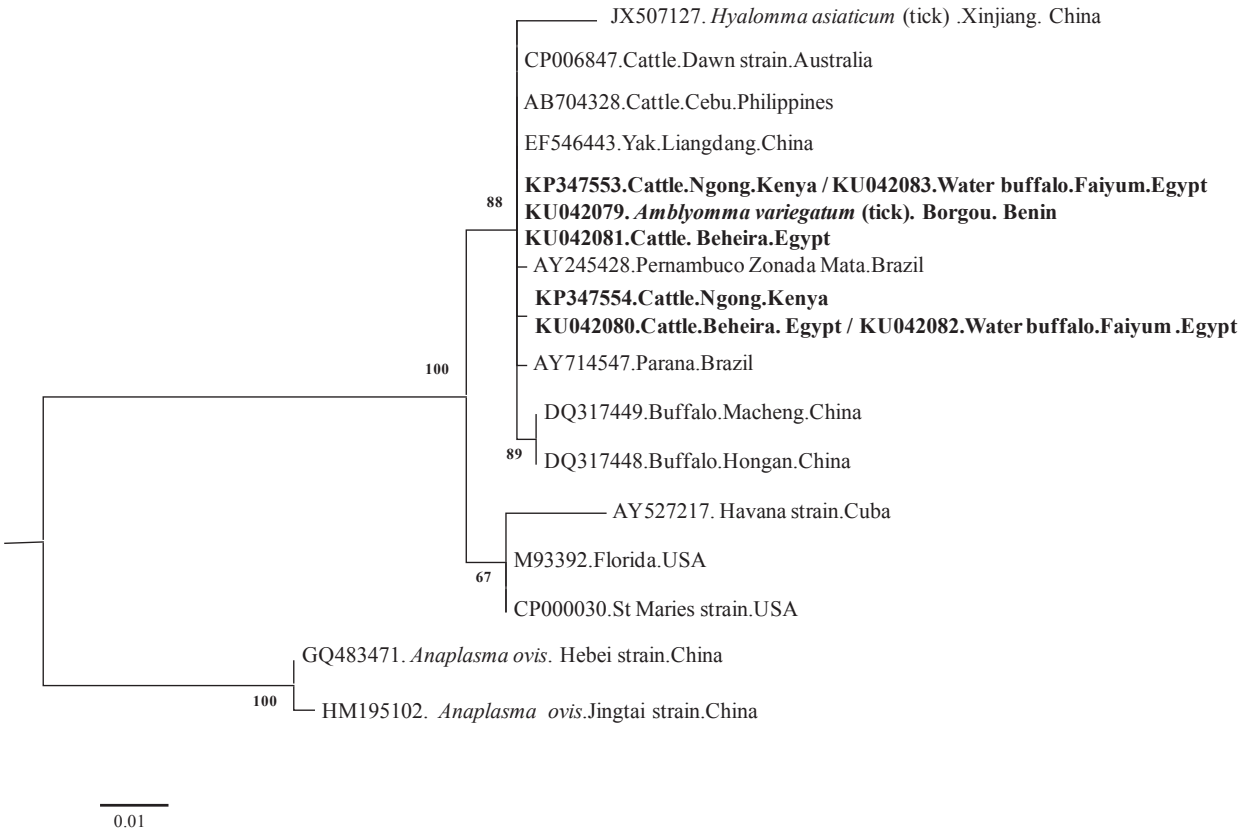

Figure 7. **Phylogenetic analyses of *A. marginale* Msp5 gene sequences obtained from Egyptian, Kenyan and Beninese livestock**. *Anaplasma ovis* was used as out group. The tree was constructed with the maximum likelihood method using the Kimura 2 parameter model in the MEGA ver.6. The sequences determined in this study are shown in bold-font. Numbers on the branches show percentages of 1000 bootstrap replications. The scale bar indicates estimated number of substitutions per site.

Figure 8. **Unrooted phylogenetic tree showing relationships between *T. parva* isolates based on p104 gene**. The tree was constructed with the maximum likelihood method using the John-Taylor Thornton with Gamma distribution (JTT+G) model in the MEGA ver.6. The sequences determined in this study are set in bold-font. Numbers on internodes indicate percentages of 1000 bootstrap replicates. The scale bar indicates estimated number of substitutions per site.

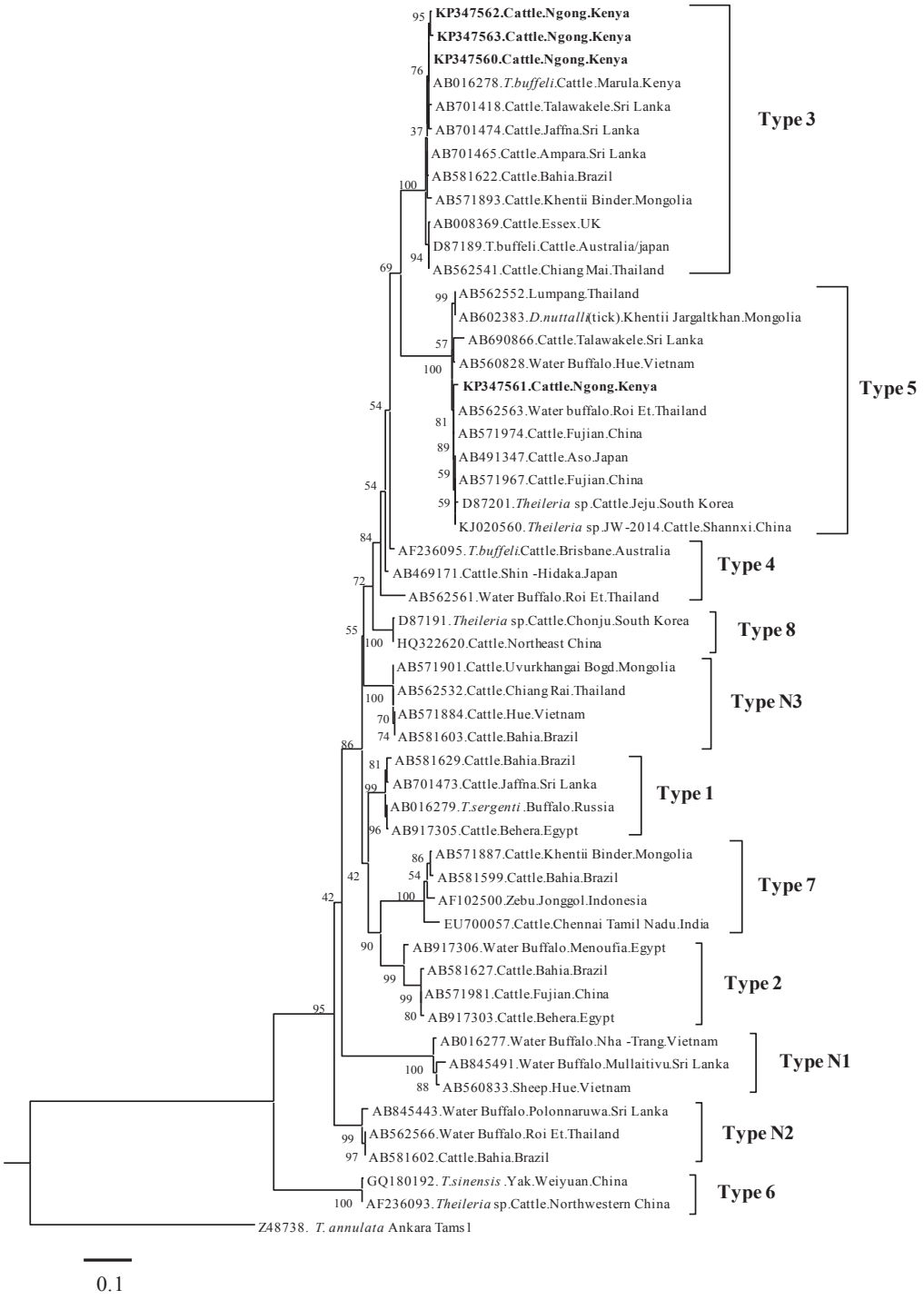

Figure 9. **Phylogenetic analyses of *T. orientalis* MPSP gene sequences obtained from Kenyan cattle**. The tree was constructed with the maximum likelihood method using the Tamura 3 parameter with Gamma distribution (T92+G) model in the MEGA ver. 6. *T. annulata* (Ankara strain) merozoite surface antigen 1 gene (Tams1) was used as outgroup. The sequences determined in this study are shown in bold-font. Numbers on the branches show percentages of 1000 bootstrap replications. The scale bar indicates estimated number of substitutions per site.

Table 15. **Sequences of primers set used for *B. bigemina* RAP-1a characterization.**

Pathogen / Target gene	Assays	Oligonucleotide sequences (5'>3')	Product size (bp)	Reference
B. bigemina RAP-1a	First PCR	GAGTCTGCCAAATCCTTAC TCCTCTACAGCTGCTTCG	879	(112)
	Second PCR	GAGTCTGCCAAATCCTTAC TTGGTGCTTTGACCGACGACAT	690	

Table 16. **Accession numbers of DNA sequences deposited in GenBank**

Parasite	Target genes	Isolate			
		Accession numbers	Sequence length (bp)	Country	Source
B. bovis	*SBP-4*	KF192805	503	Egypt	Water buffalo
		KF192806	521	Egypt	Cattle
		KF192807	521	Egypt	Cattle
		KF192808	521	Egypt	Cattle
		KP347555	521	Kenya	Cattle
		KP347556	521	Kenya	Cattle
		KP347557	521	Kenya	Cattle
		KU042085	521	Benin	Tick
		KU042086	521	Benin	Tick
B. bigemina	*RAP-1a*	KF192809	690	Egypt	Water buffalo
		KF192810	690	Egypt	Cattle
		KF192811	690	Egypt	Cattle
		KF192812	690	Egypt	Cattle
		KP347558	690	Kenya	Cattle
		KP347559	690	Kenya	Cattle
		KP893330	690	Kenya	Cattle
		KU042084	411*	Benin	Tick
A. marginale	*Msp5*	KU042080	576	Egypt	Cattle
		KU042081	576	Egypt	Cattle
		KU042082	510*	Egypt	Water buffalo
		KU042083	493*	Egypt	Water buffalo
		KP347553	576	Kenya	Cattle
		KP347554	576	Kenya	Cattle
		KU042079	469*	Benin	Tick
T. parva	*p104*	KP347564	278	Kenya	Cattle
		KP347565	278	Kenya	Cattle
		KP347566	278	Kenya	Cattle
T. orientalis	*MPSP*	KP347560	776	Kenya	Cattle
		KP347561	776	Kenya	Cattle
		KP347562	776	Kenya	Cattle
		KP347563	776	Kenya	Cattle

***** DNA sequences obtained through direct sequencing

Table 17. **Alignments of amino acid substitutions observed in the SBP-4 gene of Egypt, Kenya and Benin *B. bovis* isolates in comparison with sequences from other geographic areas**. Identical residues are indicated by stars (*). Numbers above the alignments represent the amino acid position taking *Babesia bovis* T2Bo (XM_001610418) as reference sequence. The sequences determined in this study are shown in bold-font.

			Babesia bovis Spherical body protein 4 (*SBP-4*) gene																						
Isolate			1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	2	2	2	2	2	2
			1	1	2	3	3	3	3	3	3	3	4	4	5	7	7	7	9	0	2	2	5	6	7
GenBank ID	Country	Source	0	8	9	2	3	4	5	6	7	8	2	8	9	2	3	8	2	4	1	8	4	3	0
XM_001610418	USA	Cattle	E	T	G	-	-	-	-	-	-	A	D	I	D	V	S	G	D	F	L	A	F	T	A
AB569300	Brazil	Cattle	*	*	*	-	-	-	-	-	-	*	*	*	*	*	*	*	*	*	*	*	*	*	*
AB569302	Mongolia	Cattle	K	*	*	-	-	-	-	-	-	*	*	*	*	*	*	*	*	*	*	*	*	*	*
AB571871	Thailand	Cattle	K	*	*	-	-	-	-	-	-	*	N	*	*	*	T	*	*	L	*	*	*	*	*
AB586125	Thailand	Water buffalo	*	*	*	-	-	-	-	-	-	*	*	*	*	*	*	*	*	*	F	*	*	*	*
AB617641	Syria	Cattle	*	*	*	-	-	-	-	-	-	*	*	*	E	*	*	*	*	*	*	*	*	*	*
KF192805	Egypt	Water buffalo	*	*	*	-	-	-	-	-	-	*	*	*	*	*	*	*	*	*	*	*	*	*	*
KF626638	South Africa	Cattle	*	A	*	-	-	-	-	-	-	*	*	V	*	*	*	*	D	*	*	S	*	*	*
KF626636	South Africa	Cattle	*	*	*	-	-	-	A	E	G	T	*	*	*	*	*	*	N	*	*	S	*	A	G
KF626634	South Africa	Cattle	*	*	*	-	-	-	A	E	G	*	*	*	*	*	*	*	N	*	*	S	*	A	G
KF626635	South Africa	Cattle	*	*	*	-	-	-	A	E	G	*	*	*	*	I	*	*	N	*	*	S	*	A	G
AB569301	Ghana	Cattle	*	*	*	A	E	G	A	E	G	*	*	*	*	I	*	*	N	*	*	S	*	A	G
AB569303	South Africa	Cattle	*	*	*	A	E	G	A	E	G	*	*	*	*	I	*	*	N	*	*	S	*	A	G
KF192806	Egypt	Cattle	*	*	R	A	E	G	A	E	G	*	*	*	*	I	*	*	N	*	*	S	*	A	G
KF192807	Egypt	Cattle	*	*	*	A	E	G	A	E	G	*	*	*	*	I	*	*	N	*	*	S	S	A	G

KP347555	Kenya	Cattle	*	*	*	A	E	G	A	E	G	*	*	*	*	I	*	*	N	*	*	S	*	A	G
KP347556	Kenya	Cattle	*	*	*	A	E	G	A	E	G	*	*	*	*	I	*	*	N	*	*	S	*	A	G
KP347557	Kenya	Cattle	*	*	*	A	E	G	A	E	G	*	*	*	*	I	*	*	N	*	*	S	*	A	G
KU042085	Benin	Ticks	*	*	*	A	E	G	A	E	G	*	*	*	*	I	*	*	N	*	*	S	*	A	G
KU042086	Benin	Ticks	*	*	*	A	E	G	A	E	G	*	*	*	*	I	*	S	N	*	*	S	*	A	G

General discussion

Babesiosis, theileriosis and anaplasmosis caused by *B. bovis* and *B. bigemina*, *Theileria* spp. and *A. marginale*, respectively, are important tick-borne diseases of cattle and a threat to the livelihoods of many livestock owners in Africa (69). Although, they are endemic in Africa, the epidemiological information pertaining to their prevention and control is insufficient in many countries. SFG rickettsioses are another important tick-borne infection. These zoonoses caused by SFG *Rickettsia* species, are an emerging public health problem worldwide including in Africa (16, 96). The bacteria are harboured by livestock and ticks, and transmitted to humans via tick bite. Most of cattle farmers in Africa practice extensive/ semi-extensive farming systems characterized by close contact with cattle and therefore exposure to tick bite. Although SFG *Rickettsia* species are believed to be present in almost all the African countries, the infection has been confirmed in ticks or humans in very few countries. Furthermore, little is known about the prevalence of the pathogens in African cattle. On this ground, this study aimed to increase the body of knowledge on aforementioned diseases in Africa. Specific and sensitive molecular assays were performed to assess the epidemiology of *B. bovis, B. bigemina*, *Theileria* spp., *A. marginale* and SFG *Rickettsia* spp. in Egypt (**Chapter 1**), Kenya (**Chapter 2**) and Benin (**Chapter 3**). Subsequently, sequences of species-specific

genes were used to compare isolates from the three countries to those from other areas (**Chapter 4**). These countries were selected as representative of three different tick ecosystems of Africa; Egypt served as representative for North Africa while Kenya and Benin represented East and West Africa, respectively. The samples examined were chosen based on the characteristics of cattle industry in each country. In Egypt, blood samples from cattle and water buffalos under small-scale dairy farming were analyzed because these animals are important source of incomes for Egyptian farmers. In Kenya, cattle kept under semi-extensive semi-enclosed system but sharing pastures with pastoralists cattle were selected in order to investigate the impact of co-grazing on herd health. In Benin, more than 95% of herds belong to pastoralist and agro-pastoralists (3). These farmers are sometimes reluctant to blood sampling on their cattle, therefore, *A. variegatum* ticks collected on their animals were used as alternative for investigating tick-borne pathogens in the country.

The assessment of epidemiological situation, risk factors and genetic variation of genes that are crucial for pathogen live cycle or subunit vaccine targets are key elements for effective control of babesiosis. The identification of *B. bovis* and *B. bigemina* infecting livestock in Egypt, Kenya and Benin contributes to a better comprehension of the parasites molecules, in this case, *B. bovis* SBP-4 and *B. bigemina* RAP-1a, which

are promising targets for detection and control of babesiosis (14, 107, 109, 110, 111, 112). *Babesia* species were detected in samples from all the study areas; however, the epidemiological features were not the same. In Beheira and Faiyum (Egypt) and Ngong (Kenya), *B. bigemina* was more frequent than *B. bovis* as expected. However, in Machakos (Kenya), a high *B. bovis* prevalence was recorded. *B. bovis* - *B. bigemina* co-infections were frequent in Kenya but rare in Egypt samples. In Kenya, the geographical location influenced *Babesia* spp. infections rates while in Egypt, it was animal age. Infected Egyptian cattle showed fever while all Kenyan cattle were asymptomatic carrier. Taken together, these results point out the usefulness of monitoring *Babesia* spp. infections features and ticks distribution as a step towards designing effective control strategies. Prior to this study, there was no molecular evidence of *Babesia* spp. infections in Benin. Microscopic observation is the diagnostic method commonly used in the country, but this assay has many limitations (73). The data reported here should encourage the use of molecular assays for surveys in Benin cattle. The similarity of the *B. bovis* SBP-4 and *B. bigemina* RAP-1a sequences obtained in the three countries suggests that the corresponding PCR assays can be employed in other African countries.

Theileria species were investigated in Kenya and Benin but not in Egypt

because data about the pathogens in Egypt were already available. Although each *Theileria* species is believed to be distributed in a specific area of Africa (11), this geographical distribution may not be static but rather dynamic. Climate change and the resulting expansion of ticks species habitat, uncontrolled animal movement, and pathogens exchanges between domestic stocks and wildlife reservoirs may modify the distribution of *Theileria* species. Hence, the identification and close monitoring of *Theileria* species existing in each area is necessary. In this study, genus, species-specific molecular assays and sequencing of conserved and moderately polymorphic genes (5, 35, 90) were combined to assess the diversity of *Theileria* species in the two countries. The role of wildlife and multi-host ticks in the transmission of pathogens to Kenyan cattle was depicted in the results. In Benin only *T. mutans* has been identified. Although considered nonpathogenic, this species have previously caused diseases in crossbred cattle in Kenya (71, 70) and therefore should be included among the potential threat to animal health in Benin.

A. marginale was detected in the three countries and was the least prevalent hemoparasites in Egyptian and Kenyan cattle suggesting that anaplasmosis may not be highly prevalent in these regions. However, the high prevalence observed in Egyptian water buffalos and *A. variegatum* ticks from Benin uncovered their role in the

epidemiology of the infection. *A. marginale* in contrast with Piroplasmid (*Babesia* spp., *Theileria* spp.) can be mechanically transmitted by biting flies and blood-contaminated fomite (55). The identification of water buffalos and *A. variegatum* ticks as reservoirs of *A. marginale* is novel and will aid in effort to control disease transmission to cattle. According to Kocan *et al. A. marginale* genes such as the major surface proteins family may be useful for strain comparison and information about the evolution of host-pathogen and vector-pathogen relationships (55). *A. marginale* Msp5 gene is a conserved gene (10, 118) and its variations may indicate selections and apparition of numerous strains, genotypes. Sequence analyses suggest that *A. marginale* isolates in the three countries did not undergo numerous antigenic changes and exhibit a stable host-pathogen-vector relationship.

Although livestock are suspected to be reservoirs of SFG *Rickettsia*, neither the cattle nor the water buffalos examined were positive to the bacteria. This probably portrays the minor role of livestock in the epidemiology of the infection. In contrast, most of ticks from Benin were positive. With the development of outdoors activities the frequency of tick bite in human has increased, not only farmers but all people visiting rural areas in Benin are at risk. The current findings are therefore linked to public health concerns in Benin.

In conclusion, in this study, tick-transmitted pathogens that are health threatening infection to livestock and humans were investigated in Egypt, Kenya and Benin. Novel molecular detection assays were applied for the first time and hemoparasites genotypes occurring in Africa were identified. These data should aid in educating veterinarian and clinicians, and serve in the practical diagnostic, prevention and control of tick-borne diseases.

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Summary

This dissertation describes studies on the epidemiology of tick-borne pathogens of livestock in three African countries and the use of molecular tools for their diagnosis. Ticks are known to transmit a large variety of pathogens to animals as well as human, causing harmful infections referred to as tick-borne diseases (TBDs). TBDs of livestock cause mortalities and morbidities leading to losses in production of milk, meat, and other livestock by-products. On the other hand, infected livestock and ticks are important reservoirs of zoonotic microorganisms. In Africa, bovine babesiosis, theileriosis, anaplasmosis and zoonotic Spotted fever group (SFG) rickettsioses caused by *Babesia bovis* and *Babesia bigemina*, *Theileria* spp., *Anaplasma marginale* and SFG *Rickettsia* spp., respectively, have emerged as animal and human health threatening TBDs. Therefore, the necessity to find strategies for controlling these infections is a major concern for livestock industry and public health.

Despite an increasing body of knowledge on the aforementioned TBDs of cattle, there are no fully safe and effective vaccines. Currently, assessment of pathogen prevalence, genotypes and risk factors such as animal species, breed, age, and ticks vectors are the key elements for effective control. With regard to zoonotic rickettsioses, the broad range of its symptoms makes the diagnosis difficult and misdiagnosis with

other febrile tropical diseases often occurs. Reports of SFG rickettsiae occurrence in the area visited by a febrile patient are crucial for the diagnosis and effective treatment of the disease. Although bovine babesiosis, theileriosis, anaplasmosis and SFG rickettsioses are endemic in Africa, the epidemiological information pertaining to their control is insufficient in many countries. Therefore, this study was carried out to gain a better insight into the epidemiology of TBDs in Africa. Cattle blood samples from Egypt and Kenya; water buffalos blood samples from Egypt and feeding *A. variegatum* ticks from Benin were examined for the presence of *B. bigemina, B. bovis, Theileria* species*, A. marginale* and SFG ricketsiae. Genetic diversities and pathogens phylogenies in relation to other regions of the world were also assessed. *B. bovis* spherical body protein 4 (SBP-4), *B. bigemina* rhoptry-associated protein 1a (Rap-1a), *A. marginale* major surface protein 5 (Msp5), *Rickettsia* 16S rDNA, *Theileria* spp. 18S rRNA, *T. parva* p104 (p104) and *T. orientalis* major piroplasm surface protein (MPSP) were used as the marker genes. The sequences generated from these target genes were used to confirm the identity of the pathogens and for molecular evolutionary genetic analyses.

In chapter 1, the molecular prevalence of *B. bovis*, *B. bigemina, A. marginale* and SFG *Rickettsia* species in Egyptian livestock under small-scale dairy farming was

assessed. A total of 247 blood samples collected from cattle (*n*=151) and water buffalos (*n*=96) in Beheira and Faiyum Provinces were examined by standard and nested PCR. Except SFG rickettsiae, all the above pathogens were detected in the samples. In cattle, the prevalence of *B. bovis*, *B. bigemina*, and *A. marginale* was 4.0%, 5.3% and 3.3%, respectively, whereas those of water buffalos were 4.2%, 10.4% and 20.8%, respectively. *B. bigemina* prevalence in aged water buffalos (8–10 years) was significantly higher than observed in the young (5–7 years) (11.3% versus 6.3%). Among the cattle examined, around 10% of the samples were infected with *B. bigemina* and showed fever whereas 4% were healthy but positive for the parasite. In contrast, all the water buffalos in this study were clinically healthy although they were positive to tick-borne hemoparasites. Taken together, these results indicate that water buffalos are important reservoir of tick-borne pathogens, and may act as source of infection for cattle. Therefore, water buffalos should be included in strategies for controlling babesiosis and anaplasmosis in Egypt.

In Chapter 2, the impact of co-grazing on the epidemiology of tick-borne infections was investigated in Kenya. The prevalence of *B. bovis*, *B. bigemina, Theileria* species, *A. marginale* and SFG *Rickettsia* spp. were determined in 192 cattle blood samples collected from Ngong and Machakos farms. *Rickettsia* species were not

detected in any of the samples examined. *B. bovis*, *B. bigemina*, *T. parva*, *T. velifera*, *T. taurotragi*, *T. mutans* and *A. marginale* were prevalent in both farms, whereas *T. ovis, Theileria* sp. (buffalo) and *T. orientalis* were found only in Ngong farm. Co-infections were observed in more than 50% of positive samples in both farms. These findings point out that, in Kenya, it is necessary to consider co-infection of cattle with tick-borne hemoparasites and the role of wildlife in pathogens transmission when devising interventions related to the diagnostic, treatment and prevention of TBDs.

In Benin, pastoralist and agro-pastoralists who own most of herds are sometimes reluctant to blood sampling on their cattle, therefore, in Chapter 3, feeding *A. variegatum* ticks were used as alternative for investigating tick-borne pathogens in the country. A total of 910 *Amblyomma variegatum* ticks collected from 8 different locations in North Eastern Benin were tested fo*r B. bigemina, B. bovis, Theileria* species (*T. taurotragi, T. annulata, T. orientalis, T. parva, and T. mutans), A. marginale* and SFG ricketsiae. Pathogens identified in the ticks included *B. bigemina* (1/910), *B. bovis* (10/910), *T. mutans* (4/910), *A. marginale* (142/910) and SFG rickettsiae (267/910). *B. bigemina*, *B. bovis* and *T. mutans* were detected in only one location whereas *A. marginale* and SFG ricketsiae were found in 7 and 8 locations, respectively. The prevalence of SFG rickettsiae varied according to the sampling sites and was

significantly higher in the female than in the male ticks. The sequences analyses demonstrated the presence of *Rickettsia africae* and/or closely related species in Benin. These findings confirm the endemicity of tick-borne hemoparasites in Benin and extend the geographic distribution of *R. africae* and spotted fever rickettsioses in Africa. Clinicians in Benin and those treating travelers should be aware that SFG rickettsiae infections are among the potential causes of febrile illnesses contracted in the country.

In Chapter 4, the genetic diversities of *B. bovis*, *B. bigemina*, *A. marginale* infecting livestock in Egypt, Kenya and Benin; *T. parva* and *T. orientalis* occurring in Kenya were investigated. The partial sequences of *B. bovis* SBP-4, *B. bigemina* Rap-1a, *A. marginale* Msp 5, *T. parva* p104 and *T. orientalis* MPSP genes were amplified sequenced and compared. *B. bigemina* Rap-1a and *A. marginale* Msp 5 sequences were conserved and showed high homology among the isolates from these countries. *B. bovis* SBP-4 sequences from Egyptian cattle, Kenyan cattle, and *A. variegatum* ticks from Benin were similar. However, *B. bovis* SBP-4 sequences from Egyptian water buffalos were 18 nucleotides shorter (503 bp against 521 bp). *T. parva* and *T. orientalis* isolated in Kenyan cattle farms were polymorphic. Cattle-derived *T. parva* was detected Machakos farm, whereas cattle and buffalo–derived strains were detected in Ngong farm suggesting interactions between cattle and wild buffaloes. The *T. orientalis*

genotypes identified were classified as MPSP Type 3 and MPSP Type 5. Noteworthy, sequences derived from the hemoparasites isolates in this study were genetically related to the other African isolates.

In conclusion, the use of identical, sensitive molecular assays for detection and characterization of tick-borne pathogen in different African countries provides insights into epidemiological features specific to each area/countries and favors the comparison of isolates. These data set a basis for a regional coordination of strategies for tick-borne infections control in Africa.

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