Molecular epidemiological study of protozoan and other zoonotic diseases from two countries in Africa

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Dedication

This thesis is dedicated to my late father Mr. Wilson Eliab Lwangonga for his love, support and sacrifice towards my education. To the late Mr. Suleiman Kakooza, the Deputy headmaster Lubiri Secondary School, the pillar to my education. To my late brother, Douglous Mulindwa for his support and unconditional love, and to my late mother, Thekera Najjemba for her sacrifice.

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ABBREVIATIONS

AAT**:**animal African trypanosomosis
BLAST**:** Basic Local Alignment Search Tool
bp**:**base pair
*Bst***:***Bacillus stearothermophilus.*
CATT**:**card agglutination test for trypanosomosis
CFT**:**compliment fixation test
CNS: central nervous system
CSF: cerebrospinal fluid
DDBJ**:**DNA Data Bank of Japan
DDW**:**double distilled water
DG: dark ground buffy coat technique
DNA**:**deoxyribonucleic acid
EIDs: emerging infectious diseases
ELISA**:**enzyme-linked immunosorbent assay
FAO**:**Food and Agriculture Organization of the United Nations
HAT**:** human African trypanosomosis
HCT**:** haematocrit centrifugation technique
IFAT**:** indirect fluorescent antibody test
ITS 1**:**internal transcribed spacer 1
LAMP**:** loop mediated isothermal amplification
MEGA**:**molecular evolutionary genetics analysis
MEXT**:** Ministry of Education, Culture, Sports, Science and Technology
NASBA: nucleic acid sequence-based amplification
NJ**:**neighbour joining
NHPs**:**non-human primates
NTDs: neglected tropical diseases
PCR**:**polymerase chain reaction
rRNA**:**ribosomal RNA
RNA**:** ribonucleic acid
SFG: spotted fever group
TG: typhus group
UV: ultraviolet
VSG**:**variant surface glycoproteins
VAT**:**variable antigen type
WHO**:** World Health Organization
WBC**:** white blood cells
^{o}C**:**degree celcius
h**:**hour
min**:** minute
ml**:** milliliter
µl**:** microliter

GENERAL INTRODUCTION AND LITERATURE REVIEW

I. Taxonomy and distribution of Trypanosomes

Trypanosoma is a genus of kinetoplastids (class Kinetoplastida), a monophyletic group of unicellular parasitic flagellate protozoa (Hamilton *et al*., 2004; Hausmann *et al*., 2003). The name is derived from the Greek *trypano*- (borer) and *soma* (body) because of their corkscrew-like motion. All trypanosomes are heteroxenous (requiring more than one obligatory host to complete life cycle) and most are transmitted via a vector. The majority of species are transmitted by tsetse flies, blood-feeding invertebrates, but there are different transmission mechanisms among the varying species. In an invertebrate host, that can biologically tansmit the parasite, they are generally found in the midgut, salivary glands or proboscis (Table GI-1). The parasites normally occupy the bloodstream or intracellular environment in the mammalian hosts. The mitochondrial genome of the *Trypanosoma*, as well as of other kinetoplastids, packaged in an intracellular organelle known as the kinetoplast, is made up of a highly complex series of catenated maxicircles and minicircles (Melville *et al.*, 2004).

Table GI-1: Traditionally recognized species found in Africa (Adams *et al*., 2010a)

Subgenus Species	Developmental sites in tsetse fly
Trypanozoon *Trypanosoma brucei* sensu lato	Midgut and salivary glands
Nannomonas *T. congolense, T. simiae*	Midgut and proboscis
Duttonella *T. vivax, T. uniforme*	Proboscis and cibarial pump
Pycnomonas *T. suis*	Midgut, salivary glands and proboscis

The traditionally recognized species of African trypanosomes are transmitted by tsetse fly via the salivarian route (Hoare, 1972). *T.* (*Pycnomonas*) *suis* is included for completeness, although it has not been recorded for over 30 years, and *T.* (*Duttonella*) *uniforme* is rarely reported. All these species are known pathogens of humans (*T. brucei*) or livestock (Table GI-2). In addition, several genotypes within species are recognized (Table GI-3). Each of the genotypes has different host range, geographical distribution or pathogenicity.

Table GI-2: Distribution of trypanosoma species among vertebrate host (Ugochukwu, 2008)

Species	Host	Reference
T. congolense	Goats	Ugochukwu (1983)
T. vivax	Cattle, Sheep, Goats, Horses	Roderick *et al*. (2000)
T. simiae	Pigs, Monkeys	Killick-Kendrick and Geoffery (1963)
T. b. gambiense	Man	ILARD* (1990)
T. b. rhodesiense	Man	ILARD* (1990)
T. b. brucei	Dogs, Cats, Ruminants Monkeys	Mulligan and Potts (1969) Nantulya (1990)
T. suis	Pigs	Killick-Kendrick and Geoffery (1963)
T. evansi	Camels, Horse	Mulligan and Potts (1963)
T. equiperdum	Horse	Mulligan and Potts (1963), Nantulya (1990)

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Table GI-3: Novel trypanosome genotypes described in the past 30 years (Adams *et al*., 2010a)

Subgenus	Trypanosome	Vertebrate hosts	Known distribution	Ref.
Undetermined	*T. brucei*-like	Unknown	Coastal Tanzania	[1]
Nannomonas	*T. congolense* Savannah	Broad range including ungulates & carnivores	Tropical Africa	[2, 3]
Nannomonas	*T. congolense* forest	Recorded from pigs Goats, cattle, dogs.	Riverine-forest biomes in west and Central Africa	[2, 3]
Nannomonas	*T. congolense* Kilifi	Recorded from cattle, sheep, and goats	East Africa	[3, 4, 5]
Nannomonas	*T. godfreyi*	Only pigs infected experimentally	Tropical Africa	[6]
Nannomonas	*T. godfreyi*-like	Unknown	Coastal Tanzania	[7, 8]
Nannomonas	*T. simiae* Tsavo	Only pigs infected Experimentally	East Africa	[9, 10]
Duttonella	*T. vivax* A	Unknown	Tanzania	[11]
Duttonella	*T. vivax* B	Unknown	Tanzania	[11]
Duttonella	*T. vivax* C	Nyala Nyala Antelope and experimental Infection in goats	Mozambique	[12]

References: 1-Hamilton *et al*., 2008; 2-Young and Godfrey 1983; 3-Gashumba *et al*., 1988; 4-Knowles *et al*., 1988; 5- Majiwa *et al*., 1985; 6- McNamara *et al*., 1994; 7-Malele *et al*., 2003; 8- Adams *et al*., 2006; 9- Majiwa *et al*., 1993; 10- Gibson *et al*., 2001; 11-Adams, *et al*., 2010b; 12- Rodrigues *et al*., 2008.

II. Human and Animal Trypanosomiases

African animal trypanosomiasis (AAT) is a disease complex caused by tsetse-fly-transmitted *T. congolense*, *T. vivax*, or *T. brucei brucei*, or multiple infections with one or more of these *Trypanosoma*. AAT is most important in cattle but can cause serious losses in pigs, goats, sheep, camels and horses (Connor and Van den Bossche, 2004). In southern Africa the disease is widely known as nagana, which is derived from a Zulu term meaning "to be in low or depressed spirits"— a very apt description of the disease.

Human African trypanosomiasis (HAT), caused by *T. brucei gambiense* in West and Central Africa and *T. brucei rhodesiense* found in East and southern Africa is strictly a problem for rural communities in Africa since it is dependent for biological transmission on tsetse flies (*Glossina* spp.). The disease persists in areas which have suffered most acutely from general breakdown of infrastructure, including health care (Louis, 2001). Sleeping sickness occurs in Africa between the 15° North and the 20° South. WHO (1998) estimates put 60 million people at risk of HAT today with approximately 500,000 people currently with infections, however, with rigorous control efforts this figure has fallen to below 10,000 cases annually. The disease is discontinuously spread over 10 million square kilometers and affects populations across 37 sub-Saharan countries. In general, trypanosome infections that threaten livestock are over 100 to 150-fold more prevalent in *Glossina* than the trypanosome infections that cause human trypanosomiasis (Jordan, 1976; Simarro *et al.*, 2011).

Taken as a group, the trypanosomiases of livestock are responsible for severe losses in the agricultural sector (Kristjanson *et al*., 1999), and together with the human disease burden imposed by human sleeping sickness, the trypanosomiases more generally form a very significant group of parasitic infections (Picozzi *et al*., 2002).

III. Life cycle of African Trypanosomes

The lifecycle is mostly the same for *T. brucei* (including *T. b. gambiense* and *T. b. rhodesiense*), *T. vivax* and *T. congolense*. The lifecycle of African *Trypanosoma* species is similar except the post-midgut stage as shown in Table GI-1 (Adams *et. al* 2010a, Connor and Van den Bossche, 2004). *T. vivax* has become wide spread in South

America presumably because its simpler lifecycle in the tsetse fly has made it easier for it to adapt to different vectors in South America. The importance of mechanical transmission in Africa by flies other than tsetse flies is not known, but reported for *T. vivax* and *T. evansi* transmission (Nimpaye *et al*., 2011; Salim *et al*., 2011).

During a blood sucking on the mammalian host, an infected tsetse fly injects metacyclic trypomastigotes into skin tissue. The parasites enter the lymphatic system and pass into the bloodstream. Inside the host, they transform into bloodstream trypomastigotes, which are carried to other sites throughout the body, reach other body fluids (e.g., lymph, spinal fluid), and continue the replication by binary fission. The entire life cycle of African trypanosomes is represented by extracellular stages. The tsetse fly becomes infected with bloodstream trypomastigotes when taking a blood meal on an infected mammalian host. In the fly's midgut, the parasites transform into procyclic trypomastigotes, multiply by binary fission, leave the midgut, and transform into epimastigotes. The epimastigotes reach the fly's salivary glands and continue multiplication by binary fission. The cycle in the fly takes approximately 3 weeks (Connor and Van den Bossche, 2004). Humans are the main reservoir for *T. brucei gambiense*, but this species can also be found in animals. Wild game animals are the main reservoir of *T. b. rhodesiense* (Welburn *et al*., 2009).

IV. Tsetse distribution

Thirty one species of genus *Glossina* exist in the African continent, eleven of which are important for transmitting the infection to humans (Connor and Van den Bossche, 2004). Historically, classification of tsetse flies was based on morphological criteria which divide the species into three groups; fusca, palpalis and morsitans, having different habitat requirements that are thought to reflect their evolutionary history.

The fusca group flies (subgenus *Austenina*) with supposedly the most primitive male genitalia, occur mainly in the low-land rainforests of West and Central Africa; the palpalis group species (subgenus *Nemorhina*) occupy similar forest habitats throughout sub-Saharan Africa and also extend into riverine and lakeside forests or the moist areas between such forests; and finally the morsitans group of flies (subgenus *Glossina*) occurs in a variety of savannah habitats lying between the forest edges and deserts.

Tsetse flies live in habitats that provide shade for developing puparia (pupae) and resting sites for adults. Their development is temperature- and humidity-limited, like that of many invertebrates.

V. Clinical signs and diagnosis of human African trypanosomiasis

There are two recognized stages in the clinical presentation of HAT, namely the early hemolymphatic stage, and the late encephalitic stage when the central nervous system (CNS) is involved. However, the transition from the early to the late stage is not always distinct in *rhodesiense* infection (Atouguia and Kennedy, 2000). The clinical course of the disease is usually acute in *rhodesiense* disease. CNS invasion by the parasite occurs early, within a few months after initial infection whereas *gambiense* infection is usually a slower, chronic infection,with late CNS infection lasting months to years.

1. Early or hemolymphatic stage

The onset of this phase of *rhodesiense* disease is variable but usually occurs 1–3 weeks after the tsetse fly bite. Episodes of fever lasting 1–7 days occur together with generalized lymphadenopathy. The early symptoms tend to be non-specific: malaise, headache, arthralgia, generalized weakness, and weight loss (Apted, 1970). Multiple organs may then be infected (Atouguia and Kennedy, 2000; Duggan and Hutchington, 1966), including the spleen, liver, skin, cardiovascular system, endocrine system, and eyes. This involvement underlies the wide spectrum of systemic dysfunction that may occur (Atouguia and Kennedy, 2000).

2. Late or encephalitic stage

The onset of the late phase of *rhodesiense* disease is insidious and the potential clinical phenotype is wide (Atouguia and Kennedy, 2000; Duggan and Hutchington, 1966). The broad neurologic spectrum has been detailed by Atouguia and Kennedy (2000), and the reported features can be grouped into general categories such as psychiatric, motor, and sensory abnormalities, and sleep disturbances.

3. Disease staging of HAT

Accurate staging of HAT is critical because early stage drugs do not efficiently cross the blood-brain barrier, and treating early stage patients with the more toxic drugs should be avoided (Bouteille *et al*., 2003; Kennedy, 2004). According to current World Health Organization (WHO) criteria, HAT patients in late stage are those with trypanosomes present in the cerebrospinal fluid (CSF) and/ or an elevated leukocyte count (> 5 cells/mm^3) or an increase in protein content of the CSF (> 37 mg/100 mL) (WHO, 1998). Determination of white blood cell count and the presence of trypanosomes are based on microscopic examination of a sample of CSF drawn by lumber puncture. Additional criteria such as intrathecal IgM synthesis, CSF end-titres in LATEX/IgM and LATEX/*T. b. gambiense*-positive CSF have been shown to be associated with the occurrence of treatment failures and could be useful markers to help determine the correct chemotherapeutic approach (Lejon *et al*., 2002, 2003, and 2008; Bisser *et al*., 2002; Rodgers, 2010).

VI. Clinical signs and diagnosis of African animal trypanosomiasis

The clinical signs of disease caused by these organisms vary according to the trypanosome species, the virulence of the particular isolate and the species of host infected. Characteristics of acute disease include: anemia, weight loss, abortion and, if not treated, possibly death. Animals that survive are often infertile and of low productivity. In some instances, infected animals show no overt signs of disease but can succumb if stressed, for example, by work, pregnancy, milking or adverse environmental conditions (Lucking, 1986).

Disease diagnosis may be based on the clinical signs and symptoms, by demonstration of the causative organism or by reactions to diagnostic tests. In some situations, the clinical manifestations of trypanosomiasis, particularly anemia, when taken into consideration with ecological conditions, might provide sufficient grounds for a putative diagnosis. However, the clinical signs are so varied and the ecological conditions under which trypanosomiasis occurs so diverse that, in terms of identifying animals with active infections, clinical diagnosis is too imprecise a procedure to use as a basis for the control of trypanosomiasis, and other means of diagnosis must be employed. Therefore,

diagnosis hereby refers to methods for detecting infection, either by identifying the parasites themselves or by interpretation based on the results of immunological tests or molecular diagnostics.

The type of diagnostic test used for the detection of infections caused by the animal trypanosomiases will vary according to the epidemiological characteristics of the disease and the strategy for control.

VII. Laboratory diagnosis of HAT

1. Laboratory diagnosis tests

The diagnosis of African trypanosomiasis is made through laboratory methods, because the clinical features of infection are not sufficiently specific. The diagnosis rests on finding the parasite in body fluid or tissue by microscopy. The parasite load in *T. b. rhodesiense* infection is substantially higher than the level in *T. b. gambiense* infection.

Many tests have been developed for laboratory diagnosis of HAT, including: latex agglutation test (Büscher *et al.* 1999); ELISA tests (Büscher *et al.* 1999; Nantulya 1988; Nantulya *et al.* 1992); immune trypanolysis test (Van Miervenne *et al*., 1995); polymerase chain reactions (Kanmogne *et al.* 1996; Kyambadde *et al.* 2000; Penchenier *et al.* 2000). Nevertheless, some of these tests are not in use, either because the test is still under evaluation or requires specialized facilities that are unavailable in laboratories in endemic countries. In the endemic countries, primary diagnosis is done serologically by use of the card agglutination test for trypanosomosis (CATT) (Magnus *et al.* 1978), and confirmation done by parasitological test. This is then followed by the determination of the stage of disease. No field serological screening test is available for *T. b. rhodesiense*, therefore the detection of field cases relies on parasitological methods.

T. b. rhodesiense parasites can easily be found in blood. They can also be found in lymph node fluid or in fluid or biopsy of a chancre. Serological testing is not widely available and is not used in the diagnosis, since microscopic detection of the parasite is straightforward. The classic method for diagnosing *T. b. gambiense* infection is by microscopic examination of lymph node aspirate, usually from a posterior cervical node. It is often difficult to detect *T. b. gambiense* in blood.

2. Parasitological diagnosis

The basic diagnostic method for trypanosomiasis is examination of body fluids (blood, lymph node biopsy and celebrospinal fluid) by light microscopy which is also applicable in the field. Basically, the technique involves examination of fresh or stained blood films. Because of its low diagnostic sensitivity, concentration methods e.g Haematocrit centrifugation technique (HCT) or the dark ground buffy coat technique (DG) have been developed (Paris *et al*., 1982).

Some modifications (e.g the separation or removal of blood cells prior to centrifugation by anion exchange chromatography or hypotonic lysis) have been suggested but are of limited application (Nantulya, 1990). Freshly collected blood can also be inoculated into laboratory rodents which can then be examined for the periods of 30 to 60 days to determine if they have developed trypanosome infections.

Evaluation under experimental conditions yields detection limits of some of these techniques in relation to numbers of different species of trypanosomes in a blood sample. In order of decreasing sensitivity, the results were as follows: DG>HCT>thick film>thin film>wet film (Paris *et al*., 1982).

Field diagnostic methods depend on practical considerations that determine which technique can be used, i.e. remoteness of location could prevent the maintenance of rodents while a lack of generators and centrifuges may preclude the HCT and DG techniques.

Parasitological diagnosis has limited sensitivity and failure to detect trypanosomes in the case of low parasiteamia e.g in the case of chronic infections (Masake and Nantulya, 1991), hence the need for more reliable methods.

3. Immunodiagnostic techniques

Basically, direct demonstration of trypanosomes in the infected animal gives conclusive proof of infection, but, due to the limitations of parasitological diagnosis, extensive research has been invested into alternative techniques that provide indirect evidence of infection including immunodiagnostic techniques.

Immunodiagnostic techniques for diagnosis have mainly been used in retrospective surveys, intended to add further information rather than play an integral part in a control

programme. The one exception to this generalization is in the application of the complement fixation test (CFT) to the diagnosis of *T. equiperdum*, the cause of dourine in horses. Serology has always played a major role in diagnosis of this disease since trypanosomes are rarely found in blood or other body fluids. The CFT was used successfully in the control and eradication of dourine in North America (Watson, 1920) and was also used in the diagnosis of surra in buffalo in the Philippines (Randall and Schwartz, 1936). This assay, little changed, is still in use today in testing sera before the import and export of horses between different countries. The test has not been used extensively for the other animal trypanosomiases because of problems with antigen preparation, standardization of the assay and interference by anticomplementary activity in sera from several animal species. Problems in the control and standardization of another sensitive test, the indirect haemagglutination (IHA) test, have precluded its general use although it was used in the diagnosis of *T. evansi* in camels (Jatkar and Singh, 1971) and in a control programme for buffalo and cattle (Shen, 1974). In tests with *T. vivax* it was considered too unreliable (Clarkson *et al.,* 1971).

The breakthrough in immunological diagnosis came with the introduction of primary binding assays for the detection of trypanosomal antibodies. These tests directly measure the interaction between antigen and antibody rather than relying on a secondary reaction consequent upon the initial binding. The indirect fluorescent antibody test (IFAT) has been used extensively for the detection of trypanosomal antibodies in animals and humans. Antigens are usually prepared from blood smears which are fixed in acetone and then stored at a low temperature. The IFAT has been proven to have both high specificity and sensitivity in detecting trypanosomal antibodies in infected cattle (Luckins and Mehlitz, 1978; Wilson, 1969) and camels (Lucking *et al*., 1979).

However, cross-reactions between different trypanosome species do occur. Ashkar and Ochilo (1972) found that more than 85 percent of cattle infected with *T. vivax* or *T. congolense* reacted with *T. brucei* antigen in the IFAT. When sera were tested against all three pathogenic trypanosome species, between 45 and 66 percent of sera reacted in the assay, and only by combining all the results did the test detect 94 percent of infected animals. Hence, although there is considerable cross-reactivity, these results indicate a degree of species specificity that requires the use of all three antigens in order to obtain

maximum efficiency. Modifications in the preparation of antigens involving fixation of the parasites in acetone and formalin (Katende *et al*., 1987) have provided antigens which are stable even at 4°C, can be kept in suspension until required and are capable of discriminating between different trypanosome species.

The major drawback of the IFAT - apart from its requiring sophisticated microscopy - is its subjectivity, which can make comparison of results quite difficult. The introduction of enzyme-linked immunosorbent assays (ELISA) for use as diagnostic tests for animal trypanosomiases (Ferenc *et al.*,1990; Luckins, 1986; Lucking and Mehlitz, 1978; Luckins *et al*., 1979; Rae *et al*., 1989) enhanced interest in the possibility of a universally applied immunodiagnostic assay, and the modification and refinement of these assays improves their field application (Nantulya *et al*., 1989). The tests are carried out in 96-well polystyrene micro-ELISA plates on which trypanosomal antigen is adsorbed. An indirect assay is routinely used in which serum from test cases is reacted with the antigen, followed by incubation of the resulting antigen/antibody complex with an enzyme-conjugated antiglobulin to the IgG fraction of the particular host species. The test is visualized by the addition of enzyme substrate and chromogen, with the resulting colour change allowing a photometric interpretation. Tests using crude sonicated trypanosomal extracts showed that the ELISA had a sensitivity and specificity similar to the IFAT. However, where tsetse-transmitted trypanosomiases occurred, cross-reactions were a problem. As with the IFAT, to ensure that a high proportion of infected animals were diagnosed, sera had to be screened against all trypanosome antigens in order to obtain the highest diagnostic sensitivity. Fractionation of the crude trypanosomal antigen extracts has identified antigens that are species-specific, and this method should enable discrimination between *T. brucei*, *T. vivax* and *T. congolense* infections (Ijagbone *et al.*, 1989). In addition, species-specific monoclonal antibodies developed against *T. brucei*, *T. vivax* and *T. congolense* (Nantulya *et al*., 1989) and *T. evansi* (Lucking, 1991) will allow isolation and purification of defined specific antigens for use in indirect ELISA.

The tests described above rely on antigen/antibody reactions between common or species-specific antigens but one immunodiagnostic test relies on the presence of a widely distributed variable surface antigen. CATT uses the formalin fixed variable antigen types of *T. evansi* that are used in the agglutination test. The test, which is simple

to perform, has been used for the diagnosis of *T. evansi* (Bajyana-Songa *et al*., 1987) but is unlikely to be of use for *T. vivax* or *T. congolense* because of the difficulty of identifying suitable variable antigens of these species.

An antigen capture assay which is a modification of ELISA enables the detection of circulating trypanosomal antigen in the blood of infected animals. Antibody against trypanosomal antigen is used to coat ELISA plates and any antigen present in test sera binds. The complex so formed is then incubated with the same antibody, conjugated with enzyme and visualized with a suitable substrate. Early assays using polyclonal antibodies raised against crude trypanosomal antigen preparations were found to detect antigen in animals infected with *T. evansi* and *T. congolense* within ten to 14 days of infection, and after trypanocidal drug treatment they disappeared within 21 days (Rae and Luckins, 1984). Later, the species specificity of the assay was improved following the development of monoclonal antibodies as capture antibodies that recognized antigens present in *T. brucei*, *T. vivax* and *T. congolense*. Specific circulating antigens could be detected in cattle from eight to 14 days after infection, but within 14 days of treatment they were no longer detectable (Nantulya and Lindqvist, 1989). Currently, there is a promising use of recombinant antigens to improve on the available trypanosome cell lysate to detect antibodies (Goto *et al*., 2011; Nguyen *et al*., 2012).

4. Molecular methods

Molecular detection techniques have been developed for the diagnosis of infections with African trypanosomes in humans, animals and tsetse flies (OIE, 2013). Polymerase chain reaction (PCR) first performed in 1983 (Comfort, 1999) now has various primer sets available that can amplify different trypanosome subgenus, species and types (Desquesnes and Dávila, 2002; Masiga *et al*., 1992). Additionally, species-specific probes are now available to identify the known trypanosome species in both host and vector (Majiwa, 1998). PCR can detect infection as early as 5 days following an infective tsetse bite (Masake *et al*., 2002). Using the quantitative PCR rather than the conventional PCR confers an additional advantage of identification as well as establishing the parasite burden (Zarlenga and Higgins, 2001).

These DNA techniques are extremely sensitive and better diagnostic tools, but their adoption in developing countries in Africa is still low, being limited to the confines of well-established laboratories. However, their prospects for improvement are good especially as most genomes are getting completed.

Newer molecular techniques are being developed namely: Loop-mediated isothermal amplification (LAMP) and nucleic acid sequence-based amplification (NASBA). LAMP involves the amplification of the target sequence at a constant temperature of 60-65°C using either two or three sets of primers and a *Bst* DNA polymerase with high strand displacement activity in addition to a replication activity (Notomi *et al*., 2000; Thekisoe *et al*., 2007a). LAMP is a simple (using water bath / heating block), rapid (1h amplification), highly sensitive and specific in addition, cost effective molecular technique. It is now increasingly being explored in the detection of various infectious diseases such as viral (Pham *et al*., 2005) tuberculosis (Geojith *et al*., 2011; Iwamoto *et al*., 2003) malaria, (Poon *et al*., 2006) and human African trypanosomosis (Njiru *et al*., 2008) and has the potential to replace conventional gene amplification methods once it is validated (Thekisoe *et al*., 2007a and 2007b).

On the other hand, NASBA (Mugasa *et al*., 2008, 2009, 2014) is a novel amplification technique with comparable sensitivity and simplicity to LAMP which can be combined with oligochromatography (NASBA-OC). Both are isothermal tests, easy to perform, and yield results within a relatively short time, without the need for specialized heating equipment or complicated downstream detection of the products, such as the use of the ethidium bromide-stained agarose gel and the expensive real-time equipment. The major difference between the 2 tests is that NASBA amplifies RNA while LAMP amplifies DNA as starting material. In addition, targeted genomic regions are different (18S RNA versus RIME: repetitive insertion mobile element). From this angle, there could be differences in sensitivity arising from available template copies or even stability of the targeted nucleic acid (Mugasa *et al*., 2014).

VIII. Chemotherapy

1. Recommended treatment

Both forms of HAT are invariably fatal without chemotherapeutic intervention. To date only four drugs, suramin, pentamidine, melarsoprol and eflornithine, are licensed for the treatment of HAT (Barrett *et al*., 2007). Their efficacy is dependent on whether the infection has reached the CNS-stage or remains in the haemolymphatic early-stage.

The type of drug treatment used depends on the type and stage of African trypanosomiasis (Table GI-4).

Table GI-4: Medications recommended for treatment of human African trypanosomiasis (www.medicalletter.org).

Type of Trypanosomiasis	**Medications Stage 1 (Hemolymphatic Stage)**	**Medications Stage 2 (Neurologic Stage)**
East African trypanosomiasis (caused by *T. brucei rhodesiense*)	Suramin 100-200 mg IV test dose, then 1 g IV on days 1, 3, 7, 14, 21	Melarsoprol 2-3.6 mg/kg/d IV for 3 d; after 1 wk, 3.6 mg/kg/d for 3 d; after 10-21 d, repeat the cycle
West African trypanosomiasis (caused by *T. brucei gambiense*)	Pentamidine isethionate 4 mg/kg/d IM for 10 d or Suramin 100-200 mg IV test dose, then 1 g IV on days 1, 3, 7, 14, 21	Melarsoprol 2-3.6 mg/kg/d IV for 3 d; after 1 wk, 3.6 mg/kg/d for 3 days; after 10-21 d, repeat the cycle or Eflornithine 400 mg/kg/d IV in 4 divided doses for 14 d

2. Combination Therapy

Combination therapy may be more effective than monotherapy for the treatment of late-stage *T. brucei gambiense* trypanosomiasis. In clinical trials, relapses were more common in patients receiving monotherapy regimens and adverse effects were more common in patients who received eflornithine monotherapy.

Drug control of animal trypanosomiasis relies essentially on three drugs, namely: Homidium (Novidium; homidium chloride, Ethidium; homidium bromide), diminazine aceturate (Berenil) and isometamidium chloride (Samorin, Trypamidium). Recently, however, Quinapyramine sulphate (Antrycide) has been reintroduced because of the need to especially combat camel trypanosomiasis. After the introduction of isometamidium in 1961 (Berg *et al*., 1961) the development of new trypanocidal drugs has made little

progress. It is estimated that in Africa 25-30 million doses of trypanocidal drugs are used annually in the treatment of animal trypanosomiasis, but the population of animals at risk indicated that ten times of this figure were necessary. The former figures are based on single-dose treatment. Although restrictive, the single dose treatment requirement is particularly suited to the nomadic situation in the field.

The present strategy of chemotherapy and chemoprophylaxis is faced with the following technical drawbacks: a limited number of drugs for use; the emergent drug resistance; cross-resistance to the present drugs; and, toxicity of the drugs (Anene *et al.*, 2001). However, the research and development of more effective drugs which takes an average of 8-12 years, has not been pursued as is not profitable for pharmaceutical companies (Blake *et al*., 2009).

3. Follow up of patients

Follow up of all treated patients, both early and late stage, is essential but may be problematic for logistical reasons (Lejon and Büscher, 2001). Ideally, 6 monthly clinical and laboratory evaluations including blood and CSF analysis (for CNS cases) should be carried out for a period of 2 years. At that point the patient is considered cured if all the screening tests are negative or normal. However, one or more relapses of the disease may occur. The treatment with melarsoprol needs to be repeated if the CSF is active with trypanosomes and/or high WBC count (by WHO criteria), even if the patient is otherwise asymptomatic. The prognosis of patients with CNS disease is not as good as in those with only early stage disease who have been treated promptly. Unfortunately, even patients who have been treated for late-stage disease may suffer from long-term neurological impairments including psychiatric and cognitive abnormalities, personality change, alteration of libido, hemiplegia, ataxia, involuntary movements and epilepsy (Atouguia and Kennedy 2000).

IX. Control of human and animal trypanosomiases

Since there is no vaccine or drug for prophylaxis against African trypanosomiasis, preventive measures are aimed at minimizing contact with tsetse flies. Control of African trypanosomiasis rests on two strategies: reducing the disease reservoir and controlling the

tsetse fly vector. Because humans are the significant disease reservoir for *T. b. gambiense*, the main control strategy for this subspecies is active case-finding through population screening, followed by the treatment of the infected persons that are identified. Tsetse fly traps are sometimes used as an adjunct. Reducing the reservoir of infection is more difficult for *T. b. rhodesiense*, since there are a variety of animal hosts (Welburn *et al*., 2001 and 2009).

1. Trypanotolerant breeds of livestock

Taking advantage of the trypanotolerant trait of certain breeds of domestic livestock is essentially a 'passive' approach to the control of trypanosomiasis, requiring no direct intervention. It should be noted that although these breeds are primarily considered of importance because of their tolerance of trypanosomiasis, some, such as N'dama cattle, also show increased tolerance towards other diseases, such as helminthiasis, compared to breeds of cattle more susceptible to trypanosomiasis in those ecosystems where they predominate (Connor and Van den Bossche, 2004).

2. Treatment of infected animals

The use of trypanocidal drugs is the main method of controlling trypanosomiasis in all those countries in which the disease occurs. Ideally their application should be under the control of qualified veterinary authorities - either through government departments or, increasingly in favour, through private veterinary practices. In fact, this is rarely the case and most trypanocidal drugs are applied by the livestock owners themselves, usually in the absence of definitive diagnosis of the disease. This is an unsatisfactory situation, often resulting in inappropriate drug usage, under-dosing and the consequent development of drug resistance.

3. Vector control

Although tsetse control has great potential and has aroused strong feelings - both for and against - realistically it must be appreciated that, except in a few countries, it has had minimal impact on animal trypanosomiasis compared to trypanocidal drugs yet it requires highly specialized techniques and specially trained personnel. Emotions have

been especially aroused by campaigns aimed at the permanent eradication of the vector over large areas; some of these campaigns have been very successful. However, present day thinking is moving away from the concept of large-area eradication of the vector and towards a reduction of disease risk by control of the vector, often over relatively circumscribed areas. Use of traps or targets and insecticide treated livestock can be easily achievable (Phelps and Lovemore, 2004).

X. Zoonotic diseases

Zoonotic diseases are contagious diseases spread between animals and humans. These diseases are caused by bacteria, viruses, parasites, and fungi that are carried by animals and vectors (www.cdc.gov). About 75% of recently emerging infectious diseases affecting humans are diseases of animal origin, either from domestic or wild animal species, and approximately 60% of all human pathogens are zoonotic (Cunningham, 2005; Taylor *et al*., 2001; Woolhouse *et al*., 2005). Some zoonotic pathogens such as arboviruses, malaria parasites and trypanosomes require transmission by arthropod vectors. In aggregate, they have high impacts on human health, livelihoods, animals and ecosystems.

Zoonosis is a major concern integrating the emerging interdisciplinary fields of conservation medicine (wildlife health and nature conservation), human and veterinary medicine, and environmental sciences. This is because the rise in zoonotic diseases is driven by a complex interplay of environmental changes such as global warming, precipitation, air and water pollution, which results in ecological damages including habitat destruction and fragmentation of wildlife. Other human activities such as encroachment on wildlife areas especially due to increasing human densities and global movements of plants and animals also have impacts on epidemiology of zoonotic agents (Van Bressem *et al*., 2009; Bossart, 2011). Massive under-reporting of zoonotic diseases, in addition to non-zoonotic diseases in humans and animals in developing countries is a major impediment to understanding prevalence and impacts of disease and developing appropriate control.

CHAPTER I

Molecular epidemiological studies on animal trypanosomiases in Ghana

Abstract

African trypanosomes are extracellular protozoan parasites that are transmitted between mammalian hosts by the bite of an infected tsetse fly. Human African Trypanosomiasis (HAT) or sleeping sickness is caused by *Trypanosoma brucei rhodesiense* or *T. brucei gambiense,* while African Animal Trypanosomiasis (AAT) is caused mainly by *T. vivax, T. congolense, T. simiae, T. evansi* and *T. brucei brucei*. Trypanosomiasis is of public health importance in humans and is also the major constraint for livestock productivity in sub-Saharan African countries. Scanty information exists about the trypanosomiasis status in Ghana especially regarding molecular epidemiology. Therefore, this study intended to apply molecular tools to identify and characterize trypanosomes in Ghana.

A total of 219 tsetse flies, 248 pigs and 146 cattle blood samples were collected from the Adidome and Koforidua regions in Ghana in 2010. Initial PCR assays were conducted using the internal transcribed spacer one (ITS1) of ribosomal DNA (rDNA) primers, which can detect most of the pathogenic trypanosome species and *T. vivax*-specific cathepsin L-like gene primers. In addition, species- or subgroup-specific PCRs were performed for *T. b. rhodesiense, T. b. gambiense, T. evansi* and three subgroups of *T. congolense*.

The overall prevalence of trypanosomes were 17.4% (38/219), 57.5% (84/146) and 28.6% (71/248) in tsetse flies, cattle and pigs, respectively. *T. congolense* subgroup-specific PCR revealed that *T. congolense* Savannah (52.6%) and *T. congolense* Forest (66.0%) were the endemic subgroups in Ghana with 18.6% being mixed infections. *T. evansi* was detected in a single tsetse fly. Human infective trypanosomes were not detected in the tested samples.

Results in this study showed that there is a high prevalence of parasites in both tsetse flies and livestock in the study areas in Ghana. This enhances the need to strengthen control policies and institute measures that help prevent the spread of the parasites.

Introduction

African trypanosomes are extracellular protozoan parasites that are transmitted between mammalian hosts by the bite of an infected tsetse fly. Human African Trypanosomiasis (HAT) or sleeping sickness is caused by *Trypanosoma brucei rhodesiense* or *T. brucei gambiense.* The two subspecies are geographically distinct; the separation can be approximated to *T. b. gambiense* present west of the Great Rift Valley and *T. b. rhodesiense* to the east (Thomson *et al*., 2009). Livestock is a major reservoir of HAT caused by *T. b rhodesiense* (Fevre *et al*., 2006). Trypanosomiasis in livestock has a significant impact on agricultural productivity and is caused mainly by *T. congolense, T. vivax, T. simiae, T. evansi* and *T. brucei brucei* (Van den Bossche, 2001). *T. evansi*, which is most closely related to *T. b. brucei*, is not transmitted by tsetse flies but mechanically transmitted by biting flies (http://www.fao.org).

Scanty information exists about the trypanosomiasis status in Ghana, especially regarding molecular epidemiology. In 2003, a 10-month-old Ghanaian boy recovered from a *T. brucei* infection (Deborggraeve *et al*., 2008). The identity of the trypanosome was determined by DNA extraction from the archived stained blood slides followed by sequential application of PCR assays that are specific for the order, subgenus, species and subspecies. The epidemiology of bovine trypanosomosis was investigated in two districts (Savelugu and West Mamprusi) of Northern Ghana with different land use and environmental characteristics (Mahama *et al*., 2004). The land use intensity and environmental change was suspected to be higher in the Savelugu district. The parasitological and serological prevalence of bovine trypanosomoses was significantly higher in West Mamprusi (16% and 53%, respectively) than in Savelugu district (8% and 24%, respectively). A cross-sectional entomological survey conducted along the White Volta River and its tributaries confirmed the presence of only *Glossina palpalis gambiensis* and *G. tachinoides* (Mahama *et al*., 2004).

Prohibitive costs and widespread perception that diagnostic PCR technology is complex slows down its adoption. For instance, in the case of the species-specific diagnosis, five different PCR assays per sample would be required to screen for *T. vivax, T. brucei, T. congolense, T. simiae* and *T. evansi.* The tests would consume considerable

time, labour and costs. If the cost constraints are overcome, efforts should be directed towards minimizing sample handling and decreasing the possibility of contamination, at the same time raising the potential to function efficiently in the hands of moderately trained technical staff (Eisler *et al*., 2004). The use of the internal transcribed spacer one (ITS1) of ribosomal DNA (rDNA) based primers as a universal diagnostic test for all pathogenic trypanosomes considerably overcomes the above constraints. ITS1 PCR detects eleven pathogenic *Trypanosoma* species in a single PCR, thereby saving time and costs as compared to species-specific PCR. The expected products of ITS1 PCR are species-specific with size differences as indicated: members of subgenus *Trypanozoon* (*T. b. brucei*, *T. evansi*, *T. b. rhodesiense* and *T. b. gambiense*) a constant product of approximately 480 bp; *T. congolense* Savannah subgroup 700 bp, *T. congolense* Kilifi subgroup 620 bp, *T. congolense* Forest subgroup 710 bp, *T. simiae* 400 bp, *T. simiae tsavo* 370 bp, *T. godfreyi* 300 bp, and *T. vivax* 250 bp (Adams *et al*., 2006; Cox *et al*., 2005; Desquesnes *et al*., 2001; Njiru *et al*., 2005). By reducing the number of reactions per sample, the test effectively reduces the cost of PCR and time required for diagnosis.

Previous research on trypanosomiasis in Ghana (Mahama *et al*., 2004) has employed mainly parasitological and serological tools, which are less accurate. Progress in diagnosis, treatment and epidemiology of trypanosomiasis depends on the existence of specific and sensitive diagnostic tools. Inherent shortcomings of serologic and parasitologic diagnostic methods can be overcome by molecular techniques. Accurate and efficient identification of the trypanosome species present in the fly vectors and vertebrate hosts is vital to assess the disease risk in Ghana.

Materials and Methods

Study area

The study was conducted on tsetse flies, pig and cattle blood samples collected from Adidome (Latitude: 6° 4' 26.6592" and Longitude: 0° 29' 59.2512") and cattle blood from Koforidua (Latitude: 6° 5' 14.9316" and Longitude: 0° 15' 44.82") areas in Ghana (Fig. 1-1), following an outbreak of trypanosomiasis in livestock in these areas in 2010 (A. Alhassan, personal communication).

Tsetse flies

Tsetse fly samples were collected from Adidome in Ghana. Typically biconical traps were used to catch the tsetse and each trap was baited with two types of chemical attractant: acetone and 3-*n*-propylphenol 4-methyl-phenol and octenol at a ratio of 1:8:4 (Hargrove and Langley, 1990). Effort was made to situate the traps under the shade of trees to avoid undue fly mortality and flies were collected every morning. The tsetse flies were sexed and preserved in silica gel until DNA was extracted. A total of 219 tsetse flies were analysed.

Animal blood

Blood samples were collected from 248 pigs and 108 cattle from Adidome and 38 cattle from Koforidua. Approximately 10 ml of whole blood was withdrawn into a heparinised vaccutainer from the jugular vein. Subsequently 100 µl was applied directly onto Flinders Technology Associates filter paper FTA® Cards (Whatman International Ltd., Abington, Cambridge, UK), which were allowed to dry thoroughly prior to storage at room temperature. Ethical approval was obtained by the Research Centre for Zoonosis Control, Hokkaido University, Japan. This study was conducted adhering to this institution's guidelines for animal husbandry. Verbal informed consent was obtained from each owner of livestock prior to the extraction of blood samples by the field team.

DNA extraction

Tsetse flies were homogenized by Micro Smash MS-100R (TOMY, Tokyo, Japan) in the presence of stainless steel beads (1.0 mm in diameter) for 2 min at 2,500 rpm,

followed by DNA extraction with DNAzol (Invitrogen, Carlsbad, CA). The tsetse homogenate was mixed with 1 ml DNAzol reagent prior to the addition of 100% ethanol. The sample was shaken vigorously and left at room temperature for 5 min followed by two washes with 75% ethanol. DNA was precipitated by centrifugation at 12,000 g for 10 min, solubilised in 200 µl of 8 mM NaOH. The solution was then neutralised by adding 2 µl of 1 M HEPES and was stored at -20ºC prior to further processing. DNA was extracted from FTA cards by punching 2 mm discs from the sample-saturated cards using an FTA punch and washing three times with distilled water and eluting the DNA in 100 µl of PCR buffer (Promega, Madison, WI).

PCR

All PCR reactions were conducted using Amplitaq Gold® 360 reagent (Applied Biosystems, Foster City, CA) in a 20 µl reaction volume. The initial PCR screening was performed using ITS1 primers (Njiru *et al*., 2005) to detect multiple *Trypanosoma* species in a single reaction. However, this primer set was reported to have a low sensitivity against *T. vivax* (Desquesnes *et al*., 2001; Njiru *et al*., 2005; Thumbi *et al*., 2008) presumably because of the low level of sequence similarity between different *T. vivax* isolates. To overcome this problem, this study employed *T. vivax*-specific PCR using the primers TviCatL1 and DTO155 (Cortez *et al*., 2009). The samples positive for *Trypanozoon* by ITS1 PCR were further tested by species-specific PCR to characterize *T. b. brucei, T. evansi, T. b. gambiense* and *T. b. rhodesiense*. When ITS1 PCR generated the PCR products of approximately 700 bp, *T. congolense* subgroup-specific PCR were conducted to distinguish between Savannah, Forest and Kilifi. All the primer sets employed in this study are listed in Table 1-1. The PCR products were electrophoresed in a 1.5% agarose gel stained with Gel-Red ™ (Biotium, Hayward, CA) and were visualized under UV light.

Sequencing analysis

The amplified products of ITS1 PCR were randomly selected (3-5 samples per each amplicon size) and subjected to direct sequencing. The products were treated with ExoSAP-IT (USB Corporation, Cleveland, OH) and sequenced using the BigDye

Terminator version 3.1 Cycle Sequencing Kit (Applied Biosystems) and an ABI Prism 3130x genetic analyzer (Applied Biosystems) according to the manufacturer's instructions. The DNA sequences obtained were submitted to the DNA Data Bank of Japan (DDBJ) (http://www.ddbj.nig.ac.jp) under accession nos AB742529 to AB742533.

Results

ITS1 and *T. vivax*-specific PCR

A total of 613 samples including 219 tsetse flies, 146 cattle and 248 pigs blood samples were subjected to ITS1 PCR screening for different trypanosome species and the prevalence of the parasites are indicated in Table 1-2. Despite that ITS1 PCR detected only 36 positives for *T. vivax* (data not shown), *T. vivax*-specific PCR using cathepsin L-like primers detected 21, 37 and 47 positives in tsetse flies, cattle and pigs, respectively. The overall prevalence of trypanosomes were 17.4% (38/219), 57.5% (84/146) and 28.6% (71/248) in tsetse flies, cattle and pigs, respectively. The trypanosome prevalence was lower in the tsetse flies than in the vertebrate hosts. The predominant species in vertebrate hosts was *T. vivax*, while in tsetse flies *T. congolense* was detected as a predominant species followed by *T. vivax*. In all samples *T. simiae* was the least common. Figure 1-2 indicates the frequencies of mixed infection in each sample. The mixed infections with two different *Trypanosoma* species were observed in 9, 18 and 28 individuals of tsetse flies, cattle and pigs, respectively. Three different parasite species were detected in 6 cattle and 7 pig blood samples.

ITS1 sequencing analysis

A total of five different sequences (249, 387, 470, 475 and 725 bp in size) were recovered by sequencing analysis of ITS1 PCR products. These sequences were compared with those available in public databases by using nucleotide BLAST at NCBI website (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The sequences of the 249- and 475-bp products were 100% identical to *T. vivax* (GenBank accession number: HE573019) and *T. evansi* (AY912278), respectively. The sequences of the 387-, 470- and 725-bp products showed 99%, 98% and 96% identities with *T. simiae* (AB625446), *T. brucei* (AC159414) and *T. congolense* (TCU22319), respectively.

PCR for human infective trypanosomes

The samples positive for *Trypanozoon* by ITS1 PCR were subjected to PCR assays specific for *T. b. rhodesiense* and *T. b. gambiense*. All the reactions were negative and human infective trypanosomes were not detected in the tested samples.

***T. evansi*-specific PCR**

Having detected *T. evansi* in a tsetse fly by sequence analysis of ITS1 PCR product, *T. evansi*-specific PCR was conducted using RoTat 1.2 VSG gene primers for the samples positive for *Trypanozoon* by ITS1 PCR. However, all the samples, including one positive for *T. evansi* by sequencing analysis, were negative.

***T. congolense* subgroup-specific PCR**

The samples positive for *T. congolense* by ITS1 PCR were subjected to *T. congolense* subgroup-specific PCRs to ascertain the subgroups endemic in Ghana. Out of 97 samples, 51 and 64 samples were tested positive for subgroups Savannah and Forest, respectively (Table 1-3). The Kilifi subgroup was not detected in the tested samples. The Forest subgroup was predominant in tsetse flies and pigs, while the Savannah subgroup was predominant in cattle. Mixed infections between subgroups Savannah and Forest were detected in 3 cattle and 15 pig samples.

Discussion

There are 11 different pathogenic trypanosomes known to exist in Africa. The primers ITS1 CF and ITS1 BR, previously designed to amplify the ITS1 region of all pathogenic trypanosome species, give PCR products with species-specific sizes and thus enable multiplex detection of different parasite species (Njiru *et al*., 2005). This assay system was successfully applied to identify and characterize trypanosomes in Ghana with some limitations discussed below.

A total of 613 samples were screened for trypanosome infections by ITS1 PCR, of which 59 samples tested positive for *Trypanozoon*. These were further tested for the presence of human infective trypanosomes, but none of them were positive. Since the livestock and wild animals are known to act as reservoirs for HAT (Simo *et al*., 2006; Njiokou *et al*., 2006 and 2010), it is important to continue active surveillance in animals to understand the transmission cycle of HAT in endemic areas.

T. evansi was detected in a single tsetse fly sample despite being classified as non tsetse- transmitted trypanosomes (NTTT) (http://www.fao.org). This parasite is known to be mechanically transmitted by biting flies such as the genera *Tabanus and Stomoxys*, enabling a world-wide distribution even outside the tsetse belt of Africa. Thus, the detection of *T. evansi* in a tsetse fly might reflect the existence of carrier animals in the vicinity of tsetse flies. Since the ITS1 PCR product size of *T. evansi* is similar to one of *T. brucei*, sequencing analysis was key to differentiating between the two infections. Furthermore, *T. evansi*-specific PCR based on RoTat 1.2 VSG gene failed in this study and thus might not be reliable in epidemiological surveys and diagnosis of *T. evansi* as reported elsewhere (Salim *et al*., 2011). These problems make it difficult to assess the possibility of *T. evansi* endemicity in Ghana.

T. congolense subgroup-specific PCR did not detect Kilifi in Ghana (Table 1-3). This result seems natural considering that it is thought to be an East African subgroup first isolated from livestock in 1982 on a ranch at Kilifi on the Kenyan coast (Knowles *et al.*, 1988). Both Savannah and Forest were the endemic subgroups in Ghana and mixed infections between the two subgroups were recorded at 18.6%. The Forest subgroup could be the most prevalent in Ghana possibly because of the humid equatorial forest ecosystems

of West Africa. Considering that the Savannah subgroup was reported to be more virulent than the Forest subgroup (Bengaly *et al*., 2002b), a high prevalence of the Savannah subgroup in cattle may indicate that the parasites were introduced recently into the tested herds.

The high prevalence of *T. vivax* corroborates other findings in domestic animals (Kalu *et al*., 2001) as well as wild animals (Herder *et al*., 2002; Njiokou *et al*., 2004 and 2006). This may result from the level of pathogenicity of this trypanosome, which is generally low and better controlled by animals (Authié *et al*., 1999), and/or from the mechanical transmission, which has been reported in *T. vivax, T. evansi* and to a certain extent *T. congolense* (Nimpaye *et al*., 2011). The lower prevalence of *T. congolense* with respect to *T. vivax* in animals may result from higher parasitemia in *T. congolense* infections, accompanied by serious anaemia, which leads to the rapid death of the host animal (Bengaly *et al*., 2002a; Sidibe *et al*., 2002). The very low prevalence of *T. simiae* as previously reported in pigs (Penchenier *et al*., 1996; Simo *et al*., 2006) and wild animals (Herder *et al*., 2002; Njiokou *et al*., 2004) may indicate a low transmission of the parasite in the studied localities of Ghana and is also likely due to its high pathogenicity because pigs infected with this trypanosome species would not probably survive the acute, severe and fatal nature of this parasite.

In large-scale epidemiological studies, FTA cards are becoming increasingly popular for the rapid collection and archiving of a large number of samples. However, there are some difficulties in the downstream processing of these cards, which is essential for the accurate diagnosis of infection. It should be noted that the use of FTA cards for sample preparation has some limitations. The main one is that at low trypanosome intensities (i.e. animals with a very low level infection and chronic infections) there is an uneven distribution of parasite DNA on the cards and that some positive cards can give a negative result because the wrong part of the card was sampled (Ahmed *et al*., 2011; Cox *et al*., 2010). This has an implication that the results presented from the FTA cards may actually be underestimates of the true prevalence. Therefore, in order to decrease the probability of false negative results from using a single disc, examination of more discs (3-5) would give more accurate estimation of the disease prevalence (Ahmed *et al*., 2011; Cox *et al*., 2010).

The mixed infections with two or three different *Trypanosoma* species were commonly observed in both cattle and pigs (Fig. 1-2). These co-infections have also been documented previously with multiple trypanosome species (Malele *et al.*, 2011; Mekata *et al*., 2008). This molecular epidemiological work confirmed the abundance of mixed infections in the field, which could not have been detected by the classical parasitological methods. Mixed infections could be a result of high chances of trypanosome infections by tsetse flies and/or a case of chronic infection in susceptible hosts. This points towards the severity of AAT, exacerbating animal losses incurred by farmers such that deliberate efforts need to be in place to control tsetse flies in order to break the transmission cycle to domestic animals and thus improve livestock production and productivity in the endemic area. AAT risk is usually linked to the density of the vector and the trypanosome infection rates (Malele *et al.*, 2011).

Table 1-1: PCR primers used in the present study.

Organism	Target gene	Primer	Sequence (5' to 3')	Amplicon size (bp)	Annealing temperature (°C)	Reference
Trypanosoma spp.	ITS1 rDNA	ITS1 CF ITS1 BR	CCGGAAGTTCACCGATATTG TTGCTGCGTTCTTCAACGAA	Variable	58	[1]
T. congolense Kilifi	Satellite DNA monomer	TCK 1 TCK 2	GTG CCC AAA TTT GAA GTG AT ACT CAA AAT CGT GCA CCT CG	294	55	[2]
T. congolense Forest	Satellite DNA monomer	TCF 1 TCF 2	GGA CAC GCC AGA AGG TAC TT GTT CTC GCA CCA AAT CCA AC	350	55	[2]
T. congolense Savannah	Satellite DNA monomer	TCS 1 TCS 2	CGA GAA CGG GCA CTT TGC GA GGA CAA AGA AAT CCC GCA CA	316	55	[2]
T. vivax	Cathepsin L-like gene	DTO 155 TviCatL1	TTAAAGCTTCCACGAGTTCTTGATGATCCAGTA GCCATCGCCAAGTACCTCGCCGA	177	65	[3]
T. evansi	RoTat 1.2 VSG gene	TeRoTat 920F TeRoTat 1070R	CTGAAG AGGTTGGAAATGGAGAAG GTTTCGGTGGTTCTGTTGTTG TTA	151	58	[4]
T. b. rhodesiense	SRA gene	Forward Reverse	ATAGTGACAAGATGCGTACTCAACGC AATGTGTTCGAGTACTTCGGTCACGCT	284	68	[5]
T. b. gambiense	TgsGP gene	sense anti-sense	GCTGCTGTGTTCGGAGAGC GCCATCGTGCTTGCCGCTC	308	63	[6]

References:1- Njiru *et al*., 2005; 2- Masiga *et al*., 1992; 3- Cortez *et al*., 2009; 4- Konnai *et al*., 2009; 5- Radwanska *et al*., 2002a; 6- Radwanska *et al*., 2002b.

Table 1-2: Overall prevalence of trypanosomes.

Sample type	Total no. of samples tested	ITS1 PCR			*T. vivax*-specific PCR	No. of positives
		T. congolense	*T. simiae*	*Trypanozoon*		
Tsetse flies	219	23	0	3	21	47
Cattle	146	36	4	37	37	114
Pigs	248	38	9	19	47	113
Total	613	97	13	59	105	

Table 1-3: *T. congolense* subgroup-specific PCR results.

Sample type	Total no. of samples tested	Savannah	Forest	Kilifi	Mixed infection
Tsetse flies	23	0	23	0	0
Cattle	36	36	3	0	3
Pigs	38	15	38	0	15
Total	97	51	64	0	18

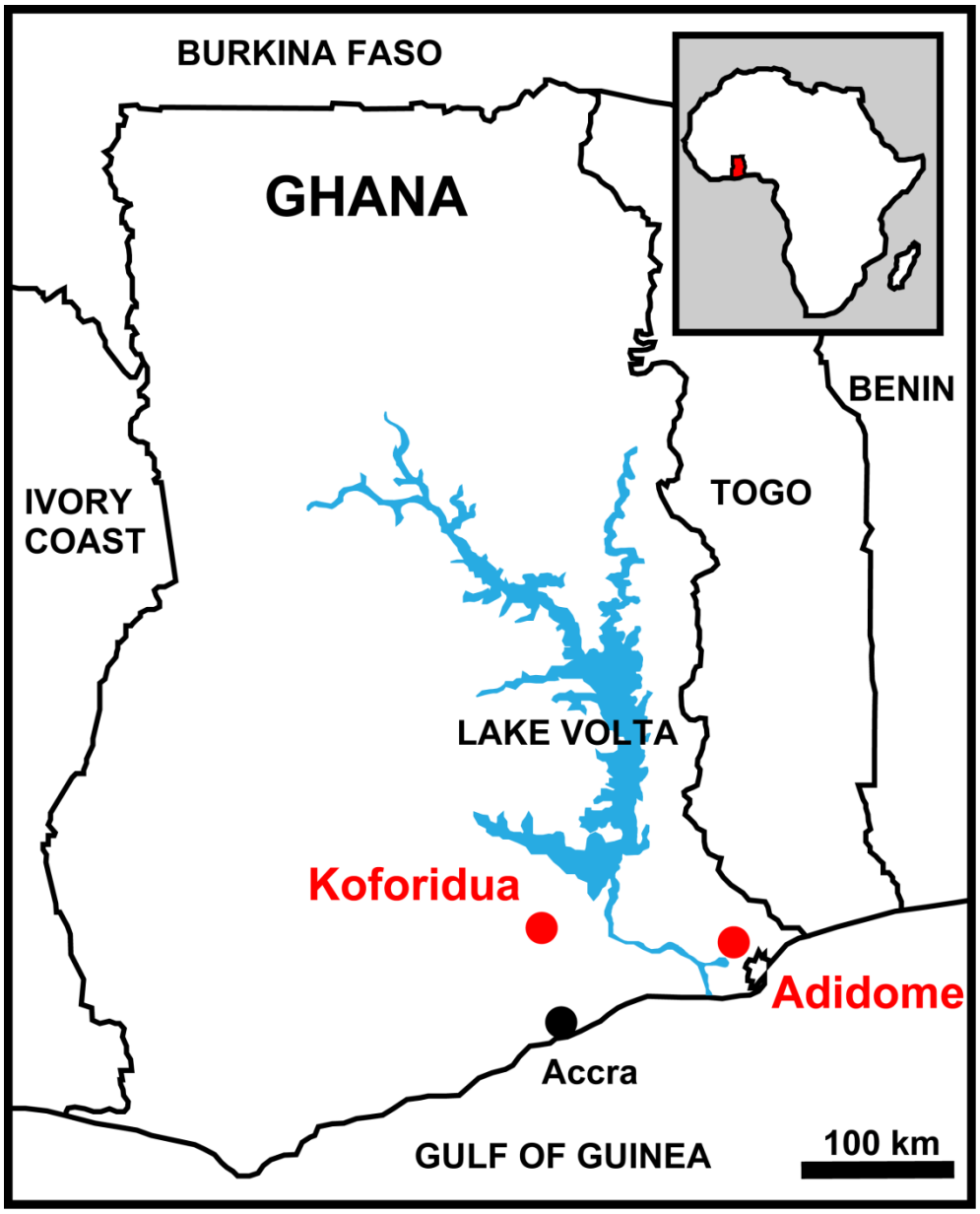

Figure 1-1: Map of Ghana showing the sites where sampling was conducted.
Tsetse flies, cattle and pig blood were collected from Koforidua and Adidome areas in the south of the country. Sample sites for this study are indicated in red.

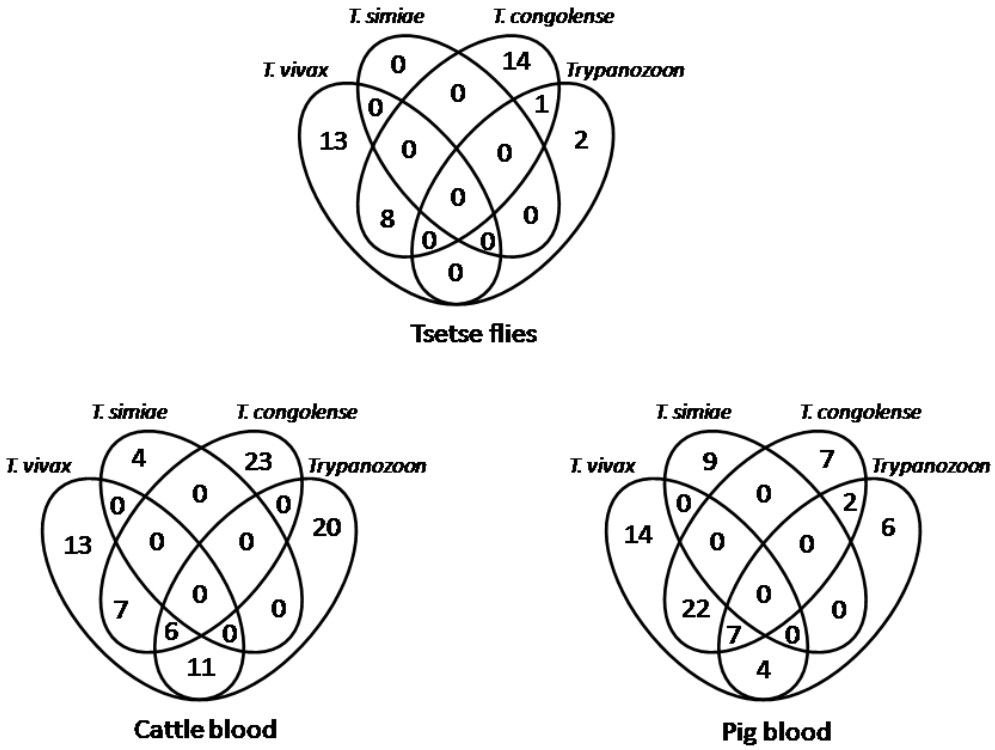

Figure 1-2: Status of mixed infections between different *Trypanosoma* spp.
Trypanozoon, *T. congolense* and *T. simiae* were screened using ITS1-PCR while *T. vivax* was screened using *T. vivax*-specific cathepsin L-like PCR.

CHAPTER 2

Genetic diversity among *Trypanosoma* (*Duttonella*) *vivax* strains from Zambia and Ghana, based on Cathepsin L-like gene

Abstract

Understanding the evolutionary relationships of *T. vivax* genotypes between West Africa and Southern Africa can provide information on the epidemiology and control of trypanosomosis. Cattle blood samples from Zambia and Ghana were screened for *T. vivax* infection using species-specific PCR and sequencing analysis. Substantial polymorphism was obtained from phylogenetic analysis of sequences of CatL-like catalytic domains. *T. vivax* from Ghana clustered together with West African and South American sequences, while *T. vivax* from Zambia formed one distinct clade and clustered with East African and Southern African sequences. This study suggests existence of distinct genetic diversity between *T. vivax* genotypes from West Africa and Zambia as per their geographical origins.

Introduction

Food and Agriculture Organization (FAO) has estimated that the problem of trypanosomosis costs Africa about US$4.5 billion per year, which includes losses in agricultural production, perennial expenditure on trypanocidal drugs and other local intervention schemes in attempts to control trypanosomosis (Eisler *et al*., 2003; Kabayo, 2002). Of major importance is *T. vivax* infection that predominantly affects cattle, buffalo, goats, sheep and wild bovids (Cortez *et al*., 2006; Hoare, 1972). *T. vivax* is a hemoprotozoan parasite found in Africa, Central and South America. In Africa the parasite is transmitted both cyclically by *Glossina* spp. and mechanically by tabanids and other biting flies, while in South America only mechanical transmission occurs (Applewhaite, 1990; Mele´ndez *et al*., 1993; Shaw and Lainson, 1972; Wells, 1984).

Certain cattle and goat breeds in Africa are trypanotolerant while others are highly susceptible to *T. vivax* infection. *T. vivax* infection in small ruminants from Africa and South America have been reported to result in a variety of clinical outcomes ranging from acute to chronic or subclinical disease; the course of infection varying depending on the parasite strain, endemicity and the species and breed of the ruminant host (Anosa, 1983; Desquesnes, 2004; Batista *et al*., 2008 and 2009). West African *T. vivax* strains are believed to be more pathogenic compared to East African strains, but, severe haemorrhagic outbreaks, with high mortality levels, have been periodically reported in East Africa (Catley *et al*., 2002; Magona *et al*., 2008). South American *T. vivax* strains have been shown to have close genetic similarity to West African strains (Fasogbon *et al*., 1990; Cortez *et al*., 2006), re-affirming the historical theory that *T. vivax* was imported into the New World through infected cattle from West Africa (Hoare, 1972).

Although *T. vivax* is considered to be an important salivarian trypanosome species because of its wide distribution, pathogenicity to cattle and its relatively high infection rates in tsetse, it remains highly neglected in the scientific literature. This is attributed to the species being notoriously difficult to work with as isolates are not easily adapted to culture or grown in standard laboratory animals (Gardiner *et al*., 1989).

Few studies that have investigated the genetic diversity in *T. vivax* have typically focused on comparison between isolates from across Africa and South America (Allsopp and Newton, 1985; Dirie *et al*., 1993a). The genomes of trypanosomes consist of multiple copies of CatL-like genes that vary depending on species. Cathepsin L-like (CatL-like) enzymes are cysteine proteases involved in the pathogenicity, immunity, cell differentiation, infectivity and metabolism of trypanosomes (Cortez *et al*., 2009). The potential role of cysteine proteases in the life cycle and pathogenesis of trypanosomes can be elucidated by knowledge of their evolutionary relationships and identification of species-specific molecules (Sajid and McKerrow, 2002; Lalmanach *et al*., 2002). In this study, sequences of CatL-like genes of *T. vivax* circulating in Ghana (West Africa) and Zambia (Southern Africa) were characterized and their relationship compared by phylogenetic analysis.

Materials and Methods

A total of 100 bovine blood samples from Petauke district in Zambia (Latitude: -14° 14' 28.5246" and Longitude: 31° 19' 11.4564"), were screened for *T. vivax* infection using the species-specific PCR (designated TviCatL-PCR, product size=177bp) employing primers TviCatL1 and DTO155 (Table 1-1, Cortez *et al*., 2009). Thereafter, *T. vivax*-positive samples (n=11) were randomly selected and subjected to PCR amplifying approximately 500 bp of the gene for catalytic domain of cathepsin L-like using primers DTO154 and DTO155 (Cortez *et al*., 2009). [DTO 154: ACAGAATTCCAGGGCCAATGCGGCTCGTGCTGG; DTO 155: TTAAAGCTTCCACGAGTTCTTGATGATCCAGTA (500 bp); TviCatL1: GCCATCGCCAAGTACCTCGCCGA (177 bp)]. The bovine blood samples from Adidome and Koforidua in Ghana, which were positive for *T. vivax* in the previous study (Chapter One, Nakayima *et al*., 2012), were also included in this study (n=11). PCR was conducted using Amplitaq Gold® 360 reagent (Applied Biosystems, Foster City, CA) with 35 cycles of amplification to minimize PCR errors. The amplified products were cloned into a pGEM-T vector (Promega, Madison, WI) and sequenced using the BigDye Terminator version 3.1 Cycle Sequencing Kit (Applied Biosystems) and an ABI Prism 3130x genetic analyzer according to the manufacturers' instructions. The DNA sequences obtained were submitted to the DNA Data Bank of Japan (DDBJ) (http://www.ddbj.nig.ac.jp) under accession nos. AB781071 to AB781078. Phylogenetic analysis was based on neighbor-joining method and ClustalW alignment, using CatL-like catalytic domain sequences from West African (Ghana) and Southern African (Zambia) isolates of *T. vivax* in comparison with CatL-like sequences of *T.*

vivax from Mozambique (southern Africa), Brazil, Venezuela (South America), Kenya (East Africa), and Nigeria, Burkina Faso (West Africa). All gene sequences used for the phylogenetic analysis were derived from cattle.

Results

Among 100 Zambian cattle tested, 32 samples were positive for *T. vivax* infection by TviCatL-PCR (data not shown). This infection rate (32%) was comparable to that observed in Ghanaian cattle (25.3%) in the previous study (Chapter One, Nakayima *et al*., 2012).

Rodrigues *et al*. (2008) corroborated the high complexity and the existence of distinct genotypes yet undescribed within the subgenus *Duttonella* based on phylogenetic analysis demonstrating that the clade *T. vivax* harbours a homogeneous clade comprising West African/South American isolates and the heterogeneous East African isolates. To evaluate the genetic polymorphism of CatL-like genes among several *T. vivax* isolates, a region of approximately 500 bp was amplified from the CatL (catalytic domain)-like genes of *T. vivax* from TviCatL-PCR-positive samples of Zambian and Ghananian cattle and sequenced the PCR products. The sequences of the amplicons generated using the CatL-like catalytic domain PCR were determined for isolates from Zambian cattle which were phylogenetically compared with Ghanaian, South American, southern African, West and East African strains.

Seven different sequences were obtained from 11 Zambian samples, while all the 11 samples from Ghana had an identical sequence. A phylogenetic analysis revealed that *T. vivax* from Ghana clustered together with those from West Africa (Burkina Faso) and South America (Brazil and Venezuela) (Fig. 2-1). *T. vivax* sequences from Zambia were assigned to three different clades, forming one distinct clade.

Discussion

Genetic diversity of *T. vivax* has been reported to be limited (Tait *et al*., 2011). It is, however, essential to know its true diversity considering the differences in disease outcome, diagnosis, response to drug and resistance to chemotherapeutic treatments (Hamilton, 2012). Clustering of Ghanaian *T. vivax* with those from West Africa and South America is in agreement with previous findings (Fasogbon *et al*, 1990; Dirie *et al*., 1993a and 1993b), corroborating the hypothesis that *T. vivax* was introduced into South America with cattle imported from West Africa (Hoare, 1972; Gardiner and Mahmoud, 1992; Dirie *et al*., 1993a and 1993b). *T. vivax* from Central Africa is reported to share molecular features with both the East and West African isolates (Fasogbon *et al*., 1990; Gardiner *et al*., 1989). Previously, other workers (Gardiner and Mahmoud, 1992; Cortez *et al*., 2006; Moloo *et al*., 1987; Vos and Gardiner, 1990; Fasogbon *et al*., 1990; Dirie *et al*., 1993a) illustrated a complex structure of *T. vivax* populations corroborating the high genetic divergence between West, and East African *T. vivax* strains. This has been revealed according to morphological, immunological, pathological, molecular features and behavior in the tsetse fly and mammalian hosts.

An attempt to characterize *T. vivax* using a panel of 8 microsatellite (Duffy *et al*., 2009) failed to amplify any positive alleles from *T. vivax*-positive samples in this study (data not shown). Such failure has been attributed to a population whose parasitaemia is below the threshold for the detection by single-locus PCR (Duffy *et al*., 2009). Additionally, there exist high levels of genetic diversity within this species, particularly in East Africa and the fact that primer sets used for microsatellite genotyping were designed from genomic sequences from a West African strain. The diverse genotypes in

East Africa and the need to identify novel genotypes require use of ‘generic’ primers, designed to amplify DNA from a wide range of trypanosome species (Hamilton, 2012). In conclusion, the genetic diversity of *T. vivax* genotypes from Ghana (West Africa) and Zambia (Southern Africa) was revealed.

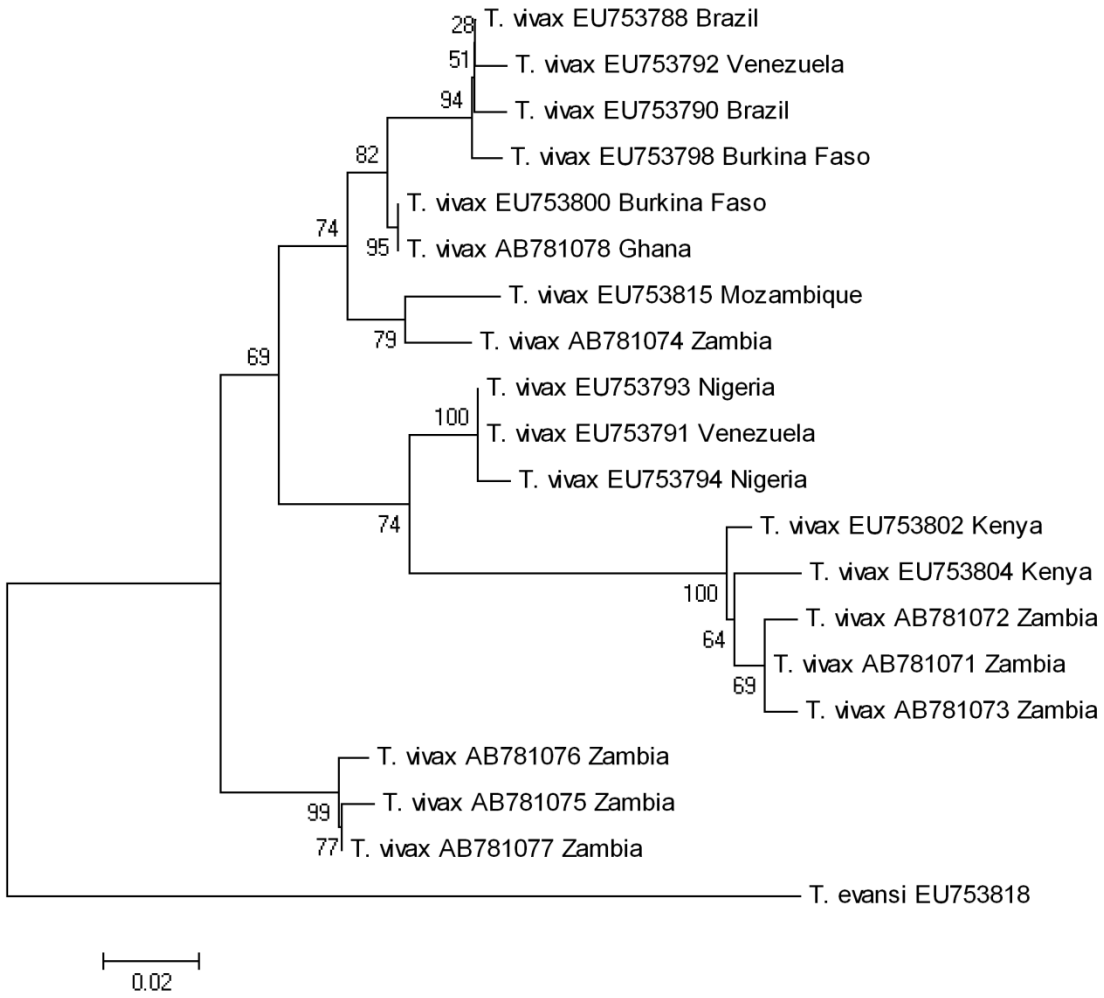

Figure 2-1. Phylogeny based on Neighbor-joining method using CatL-like catalytic domain sequences from West African (Ghana) and Southern African (Zambia) isolates of *T. vivax*. The numbers correspond to percentage of bootstrap support values derived from 1,000 replicates. Comparison with GenBank sequences with their accession nos. for South American, Southern African, East African, and West African *T. vivax* is indicated.

CHAPTER 3

Detection and characterization of zoonotic pathogens of free-ranging non-human primates from Zambia

Abstract

Wildlife may harbor infectious pathogens that are of zoonotic concern acting as a reservoir of diseases transmissible to humans and domestic animals. This is due to human-wildlife conflicts that have become more frequent and severe over recent decades, competition for the available natural habitats and resources leading to increased human encroachment on previously wild and uninhabited areas.

A total of 88 spleen DNA samples from baboons and vervet monkeys from Zambia were tested for zoonotic pathogens using genus or species-specific PCR. The amplified products were then subjected to sequencing analysis.

In this study, three different pathogenic agents, including *Anaplasma phagocytophilum* were detected in 12 samples (13.6%), *Rickettsia* spp. in 35 samples (39.8%) and *Babesia* spp. in 2 samples (2.3%).

The continuously increasing contacts between humans and primate populations raise concerns about transmission of pathogens between these groups. Therefore, increased medical and public awareness and public health surveillance support will be required to detect and control infections caused by these agents at the interface between human and wildlife.

Introduction

Wildlife pose a threat as a potential source of emerging infectious diseases (EIDs) to biodiversity conservation as well as human health. Three quarters of zoonotic EIDs are caused by pathogens in wildlife and the incidence of such diseases is increasing significantly in humans (Kooriyama *et al*., 2013; Bekker *et al*., 2012). Human activities have contributed to a closer contact between humans and wildlife due to a complex relationship between social and environmental factors causing a major threat both to human health and biodiversity conservation mainly through disease transmission between the two groups (Kaiser, 2003; Kondgen *et al*., 2008; Whitfield, 2003).

The Order Primates has traditionally been divided into two main groupings: prosimians and anthropoids (simians). Non-human primates (NHPs) are a diverse group of animals. Generally, Old World monkeys (Catirrhini) and apes (Hominoidea) are those found in Africa, the Indian sub-continent and in East Asia. New World or Neotropical NHPs (Platirrhini) are found in South and Central America. In Zambia, baboons and vervet monkeys are the major non-human primates not only in wildlife management regions, but even out of the management areas. Human-monkey conflicts in form of crop damage, grabbing of personal effects and direct injury are reported (Chomba *et al*., 2012).

Several hundred infectious diseases are classified as zoonotic diseases as they are caused by bacteria, viruses, fungi, prions or parasites that can be transmitted from animals to humans and vice versa (Lloyd-Smith *et al*., 2009). Transmission can be direct or indirect, via another organism, either a vector or an intermediate host. Invertebrates spread pathogens by two main mechanisms either through their bite, or

their feces, thus, transmission occurring mechanically or biologically. Tick-borne microbial pathogens, which cause human and zoonotic diseases such as Lyme disease, anaplasmosis, ehrlichiosis, babesiosis, Q ("query") fever, tick-borne encephalitis, Crimean–Congo hemorrhagic fever, Rocky Mountain spotted fever, Colorado tick fever, tick typhus and tularemia, have enormous negative impacts on human health and economic development worldwide. Other zoonotic disease vectors include tsetse flies (*Glossina* spp.) transmitting trypanosomiasis, sand flies (*Phlebotomus* spp.) transmitting leishmaniasis and mosquitoes (*Culicidae* spp.) transmitting malaria.

The hotspots of zoonotic disease transmission include livestock markets, urban and peri-urban wildlife and farming on fragments and edges of wildlife conservation areas and buffer zones. It was hypothesized that a possible interaction between human and simian pathogens coming from a zoonotic cycle cannot be disregarded because simians that live in the areas of the disease endemic foci of Africa could play a role as reservoir for urban cycle disease transmission.

Therefore, a study was undertaken of the sylvatic cycle zoonotic pathogens that can threaten humans in Zambia. The pathogens tested here included: *Anaplasma* spp., *Trypanosoma* spp., *Rickettsia* spp., *Coxiella burnetii*, *Leishmania* spp., *Babesia* spp., *Plasmodium* spp., *Ehrlichia* spp. and *Borrelia* spp.in African NHPs.

Materials and Methods

Sample collection and DNA extraction

Spleen samples were obtained from 48 yellow baboons (*Papio cynocephalus*) and 40 vervet monkeys (*Chlorocebus pygerythrus*) in 2008. The sampling was conducted at Mfuwe in South Luangwa National park, Zambia (13°14′42.00″ S, 31°38′54.07″ E) (Fig. 3-1). Eighty eight spleen DNA samples were analyzed in the current study for *Anaplasma* spp., *T. brucei rhodesiense* and *T. brucei gambiense*, *Rickettsia* spp., *Coxiella burnetii*, *Leishmania* spp., *Plasmodium* spp., *Babesia* spp., and *Borrelia* spp. DNA was extracted from these organs by using the QIAamp DNA Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions.

Ethical clearance

The culling was conducted under the permission from the Zambia Wildlife Authority (ZAWA) and the Institutional Ethical and Animal Care guidelines were adhered to during the culling and sampling exercise.

Molecular identification of pathogens-PCR amplifications

PCR reactions were conducted using Amplitaq Gold® 360 reagent (Applied Biosystems, Foster City, CA) in a 20 µl reaction volume. All the primer sets employed in this study and PCR conditions can be found in Table 3-1. The PCR products were electrophoresed in a 1.5% agarose gel stained with Gel-Red ™ (Biotium, Hayward, CA) and were visualized under UV light.

Sequencing of PCR products

The amplified PCR products for *Babesia* spp. *Rickettsia* spp. and *Anaplasma* spp. were subjected to direct sequencing and phylogenetic analysis. The amplicons were treated with ExoSAP-IT (USB Corporation, Cleveland, OH)**.** The sequencing reaction was carried out with the BigDye terminator kit version 3.1 and resolved with a 3130 ABI (Applied Biosystems) capillary sequencer. The DNA sequences obtained were submitted to the DNA Data Bank of Japan (DDBJ) (http://www.ddbj.nig.ac.jp) under accession nos. AB844434 to AB844437. Phylogenetic analysis of the pathogens (*R. africae*:A, 426 bp, 16S rRNA; *A. phagocytophilum*:B, 345bp 16S rRNA and *B. microti*:C, 238bp, 18S rRNA) detected in primates from Zambia was based on 16S rRNA or 18S rRNA sequences respectively. The tree was constructed using the neighbor-joining method and ClustalW alignment,

Results

PCR assays using genus- or species-specific primers for the selected zoonotic pathogens detected *Rickettsia* spp. in 35 samples (39.8%), *Anaplasma* spp. in 12 samples (13.6%) and *Babesia* spp. in 2 samples (2.3%). However, *Borrelia* spp., *Trypanosoma* spp., *Plasmodium* spp., *Leishmania* spp., and *Coxiella burnetii* were not detected. Importantly to note, *Babesia* spp. was only detected in baboons (Table 3-2).

Some of the positive samples with genus-specific primers were further subjected to direct sequencing and the BLAST sequence homology searches were performed. Two *Rickettsia* spp.-positive samples had 99% identity with *R. africae* from Nigerian ticks (Ogo *et al*., 2012). Two *Anaplasma* spp.-positive samples were sequenced and showed 100% similarity with *A. phagocytophilum* from various host species and geographical regions. Two *Babesia* spp.-positive samples from baboons showed the highest sequence similarity with *Babesia* spp. KMG-2009a from baboons with 100% identities, and also showed 98% similarity with *B. leo*-K8, the isolate from a domestic cat in South Africa, and 99% similarity with *Babesia* spp. from a laboratory baboon in USA (Bronsdon *et al*., 1999).

Phylogenetically, *Rickettsia* from baboons and vervet monkeys from Zambia formed a distinct clade with *R. africae* isolates from Nigeria, with some resemblance to an isolate from *Amblyomma variagetum* ticks from Ethiopia (Fig. 3-2A). *A. phagocytophilum* isolates from baboons and vervet monkeys from Zambia were monophyletic with the various isolates from different geographical origins (Fig. 3-2B). Phylogenetic analysis of *B. microti*-like parasite from various isolates of diverse geographical origin revealed multiple clades. African isolates from baboons (African

wild caught baboons, captive in USA primate facilities) *Babesia* spp. and *B. leo* from a domestic cat clustering with the Zambian isolates from baboon in this study (Fig. 3-2C).

Discussion

An investigation of pathogens in wild NHPs found in habitats close to human settlements is of importance in the control and eradication of probable human zoonotic pathogens.

Rickettsia spp. was detected in a total of 35 samples (39.8%). Further sequencing analysis revealed that some of the sequences were highly similar to that of *R. africae* (Fig 3-2). This is an agent of African tick bite fever, an acute and flu-like illness that is frequently accompanied by severe headache, inoculation eschars with regional lymphadenitis, vesicular cutaneous rash, and aphthous stomatitis to humans (Kelly *et al*., 1992; Raoult *et al*., 2001). The disease is transmitted in rural sub-Saharan Africa by ungulate ticks of the *Amblyomma* genus, mainly *Amblyomma hebraeum* in southern Africa and *Amblyomma variegatum* in west, central, and east Africa (Kelly *et al*., 1996). Phylogenetically, the *Rickettsia* genus is subdivided into the typhus group (TG), whose members are *R. typhi*, *R. prowazekii*, and *R. canada*; the spotted fever group (SFG), which includes about 20 different species; and the scrub typhus group, which includes *R. tsutsugamushi* (Raoult and Roux, 1997). Recent phylogenetic studies have demonstrated the evolutionary unity of the TG and SFG *Rickettsiae*. However, the position of *R. tsutsugamushi* has been found to be distinct enough to warrant transfer into a new genus *Orientia*, as *O. tsutsugamushi* (Tamura *et al*., 1995). *R. africae* from Zambian primates characterized in this study clustered together with Nigerian *R. africae* and an Ethiopian isolate. There was a divergence from other spotted fever group (SFG) *Rickettsiae* from diverse geographical origins, TG *Rickettsiae* and the genus *Orientia*. Phylogenetic comparisons between the obtained sequence and previous studies worldwide revealed a

close relationship between Zambian and Nigerian *R. africae* isolates, suggesting general occurrence of rickettsioses in African continent.

The sequences of *A. phagocytophilum* were also obtained from both baboons and vervet monkeys (Fig. 2). *A. phagocytophilum*, an obligate intracellular bacterium, is the agent of human granulocytic anaplasmosis, formerly known as human granulocytic ehrlichiosis (Dumler *et al*., 2001). This bacterium can infect humans and numerous animal species, including horses, cats, dogs, ruminants, and wildlife.

In the analysis of *A. phagocytophilum* 16S rRNA gene, the sequences of 16S rRNA were found to be very conserved not only between African isolates but also between the other isolates of world-wide origin and this was in agreement with previous studies (Zhang *et al*., 2013). The rodent parasite *B. microti* and the bovine pathogen *Babesia divergens* appear to be responsible for virtually all of the known human zoonotic *Babesia* cases (Piesman, 1987; Telford *et al*., 1993). A *B. microti*-like parasite was detected from Zambian primates at a prevalence of 2.3% from baboons. Because *B. microti* shares a vertebrate host reservoir, the white-footed mouse (*Peromyscus leucopus*) and tick vector (*Ixodes dammini*) with *B. burgdorferi*, it might be expected that the caseload for human babesiosis will parallel the rise in the number of cases of Lyme disease in endemic areas (Benach *et al*., 1985; Dammin *et al*., 1981; Spielman, 1976). The clinical course of human babesiosis often seen in asplenic hosts or elderly human individuals with fever, drenching sweats, hemolytic anemia and hemoglobinuria, shares many features with malaria (Benach and Habicht, 1981; Ruebush *et al*., 1977). Likewise, the many intraerythrocytic merozoite inclusions seen on a stained blood film may be likely mistaken for early ring-form trophozoites of *P. falciparum*. Therefore, clinicians in malaria endemic areas should consider *B. microti* differential diagnosis in

febrile patients. In addition, tick-borne bacteria such as *R. africae*, *Borrelia* spp., *A. phagocytophilum* and *C. burnetti* also run a febrile illness which could be confused for malaria in Africa.

Babesia microti, long considered on morphological grounds to be a single species found only in rodents, is now thought to consist of a complex of closely related subspecies, many of which are found in non-rodent hosts. Goethert and Telford III (2003), identified 3 clades based on the analysis of the 18S rRNA and beta-tubulin genes, with one (Clade 1) containing the majority of strains thought to be zoonotic. This clade includes the American zoonotic strains that have caused most babesiosis cases worldwide, but there are also separate zoonotic strains occurring in Japan ('Kobe' and 'Hobetsu') and Taiwan (Shih *et al*., 1997). Strains of unknown zoonotic potential but closely related to the zoonotic American strains, according to 18S rRNA or beta-tubulin gene analysis, have been isolated in Germany (Hannover), central and eastern Russia (Mis, near Berezniki, Perm region and Vladivostok), Japan, South Korea and north-east China (Xinjiang) (Goethert and Telford, 2003; Gray, 2006; Zamoto *et al*., 2004a and 2004b). The zoonotic potential of Zambian *B. microti*-like parasite found calls for further investigation.

To the best of my knowledge, this is the first report of these potential zoonotic pathogens detected in non-human primates in Zambia. Therefore, zoonotic infections namely: human Babesiosis, Anaplasmosis and Rickettsiosis are suspected to be endemic in Zambia in humans and cases could be simply misdiagnosed especially as malaria due to the febrile nature of the illnesses.

Borreliosis comprises of 37 known species of which 12 species are known to cause Lyme disease and are transmitted by ticks. *Borrelia burgdorferi* sensu *lato*

complex, which is related to Lyme disease, is classified into four genospecies on the basis of genetic, phenotypic, and immunological properties (Masuzawa *et al*., 1995). The four genospecies are *B. burgdorferi* sensu stricto, which has been isolated in North America and Europe, *Borrelia garinii* and *Borrelia afzelii*, which have been isolated in Europe, and *B. japonica*, which has been isolated from *I. ovatus* in Japan. On the other hand, relapsing fever is a recurrent febrile infection caused by various *Borrelia* spirochetes transmitted either by lice (epidemic relapsing fever) or by ticks (endemic relapsing fever). The endemic tick-borne relapsing fever spirochetes are transmitted through the bites of soft ticks of the genus *Ornithodoros*; *O. sonrai* serves as the principle vector for *Borrelia crocidurae* in West Africa, and *O. moubata* complex ticks effectively maintain these spirochetes in East Africa (Cutler *et al*., 2009). *Borrelia recurrentis* causes louse-borne relapsing fever and *B. duttonii* is the agent of East African tickborne relapsing fever (Cutler *et al*., 2010). Some cases of Lyme disease have been reported in Kenya (Jowi and Gathua, 2005), but no report in Zambia. In this study, *Borrelia* spp. were not detected.

Trypanosomes infect a wide range of wildlife species that constitute a reservoir of infection for both people and domestic animals. In Zambia, human African trypanosomiasis, caused by *T. brucei rhodesiense*, is endemic especially alongside the Luangwa Valley ecosystem (Anderson *et al*., 2011). Vervet monkeys are experimentally susceptible to African salivarian trypanosomes *T. b. rhodesiense* (Ngure *et al*., 2008; Thuita *et al*., 2008) and *T. b. gambiense* (Abenga and Anosa, 2005). Previous studies of trypanosome infections of neotropical NHPs from South America have reported high prevalence and diversity of natural infections (Ziccardi *et al*., 2000) in addition to experimental infections (Weinman *et al*., 1978; Ziccardi and Lourenco-de-Oliveira,

1997). However, active trypanosome infection was not demonstrated in sampled NHPs in this study, although the Luangwa Valley ecosystem is an active trypanosomiasis endemic focus with several human cases having been reported from the same area (Namangala *et al*., 2013). Several factors could elucidate this finding: self-cure has been observed in African green monkeys after experimental infection with *T. b. rhodesiense* (Gichuki *et al*., 1994). Additionally, the presence of trypanocidal factors in the serum of NHPs such as the baboon, which would lyse infecting trypanosomes before a demonstrable parasitemia develops (Baker, 1972; Poltera *et al*., 1985; Thomson *et al*., 2009).

Plasmodium spp. was not detected in the current study. So far, the transmission of *P. knowlesi*, a malaria parasite of Southeast Asian macaques occurs from monkeys to humans in South East Asia (Cox-Singh, 2012). Of recent, several additional *Plasmodium* species such as *P. cynomologi*, *P. inui*, *P. simium*, and *P. brasilianum* are considered to be the zoonotic parasites from monkey to human. Therefore, several authors hypothesized that monkeys may act as reservoirs for human malaria or vice versa (Yamasaki *et al*., 2011). Coexistence of humans and monkeys in the same habitat has been driven in some cases by ecological conditions as observed in the transmission of *P. knowlesi* to the human population in Southeast Asia (Yamasaki *et al*., 2011). In the wild, baboons harbour parasites closely related to *Plasmodium*, but they are not naturally susceptible to *Plasmodium* (Garnham, 1966). *Hepatocystis kochi* was first observed on the livers of AGMs in the form of macroscopic cysts (Garnham, 1966) and was formerly called *Plasmodium kochi*. *Hepatocystis* is the putative ancestor of present-day primate malaria parasites, a ubiquitous parasite of African monkeys and apes. This is a well-adapted, relatively benign parasite, which produces only

gametocytes in the circulation. Ubiquitous and high prevalence of *Hepatocystis* in non-human primates have already been reported elsewhere (Jeneby *et al*., 2008; Leathers, 1978; Turner *et al*., 1982). Several *Hepatocystis* species are pathogenic to non-human primates causing mild anemia, transmitted by the biting midge *Culicoides.*

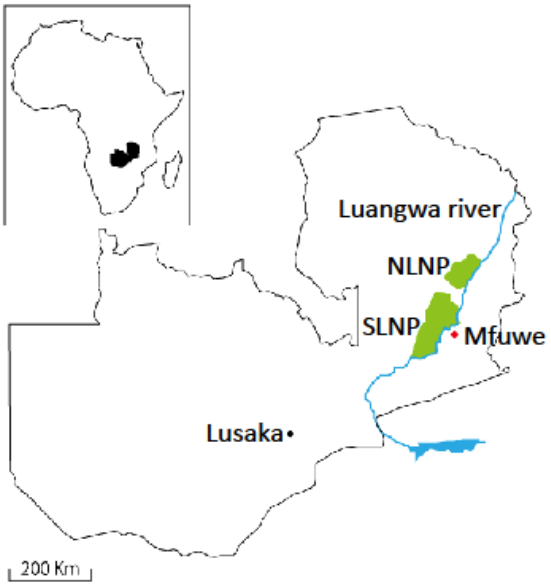

Fig. 3-1: Map of Zambia showing the sampling site. NLNP: North Luangwa National Park, SLNP: South Luangwa National Park of the Luangwa valley ecosystem a Human African Trypanosomiasis (HAT) focus.

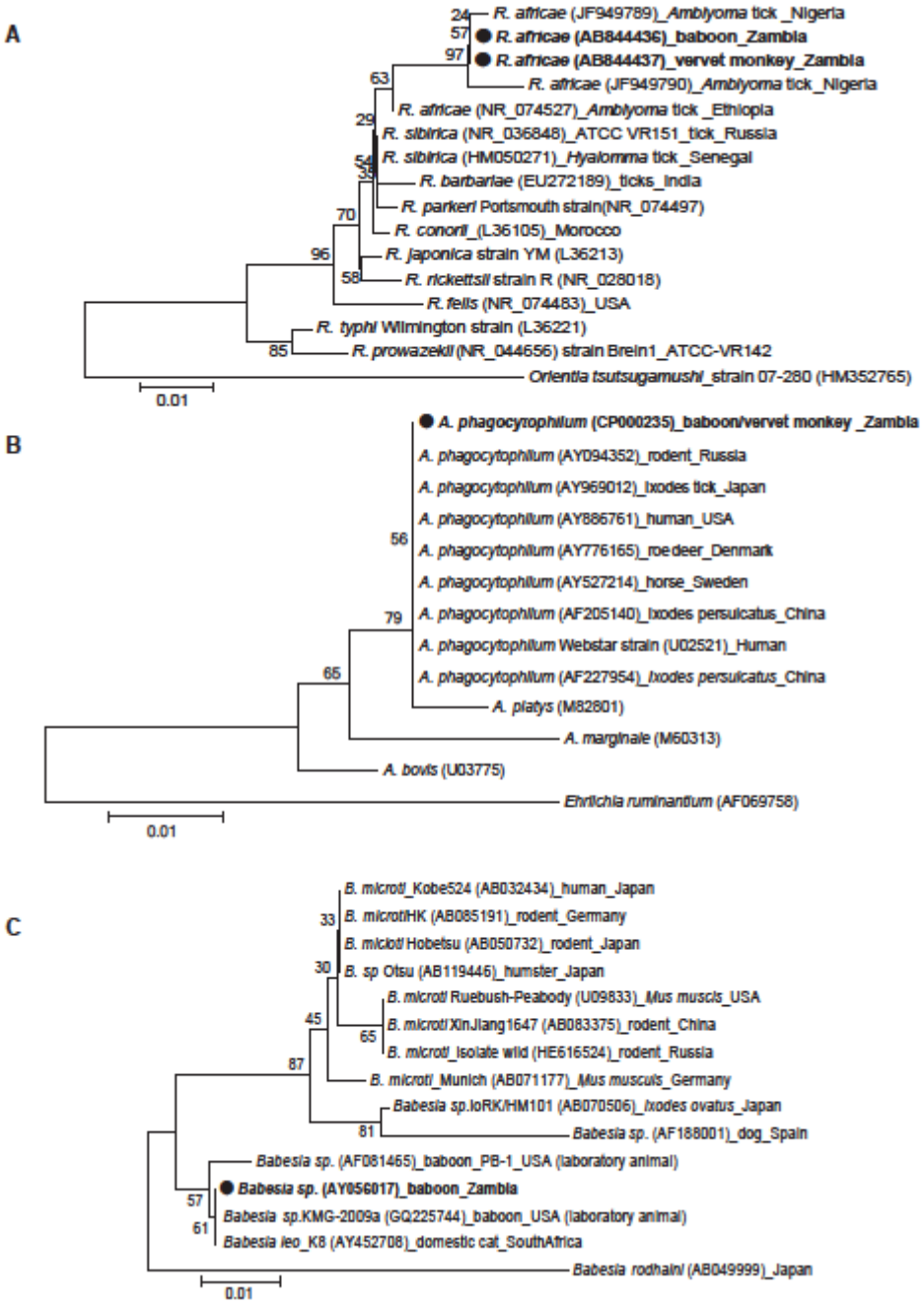

Fig. 3-2: Phylogenetic positions of the pathogens (*R. africae*:A, 426 bp,

16S rRNA; *A. phagocytophilum*:B, 345bp 16S rRNA and *B. microti*:C, 238bp, 18S rRNA) detected in primates from Zambia based on 16S rRNA or 18S rRNA sequences respectively.The tree was constructed using the neighbor-joining method and ClustalW alignment, and numbers on the tree indicate 1,000 bootstrap values for branch points. Accession numbers are indicated.

Table 3-1: Primers and conditions for PCR detection of pathogen DNA

Organism	Target gene	Primer	Sequence (5' to 3')	Amplicon size (bp)	Annealing temperature (°C)	Reference
Rickettsia spp.	*gltA*	RpCS.780p	GACCATGAGCAGAATGCTTCT	600	48	[1]
		RpCS.877p	GGGGACCTGCTCACGGCGG	480	54	
		RpCS.1273r	CATAACCAGTGTAAAGCTG			
Anaplasma spp.	16S rDNA	EHR16SD	GGTACCYACAGAAGAAGTCC	345	53	[2]
		EHR16SR	TAGCACTCATCGTTTACAGC			
Coxiella burnetii	IS1111	Trans 1	TATGTATCCACCGTAGCCAGTC	687	60	[3]
		Trans 2	CCCAACAACACCTCCTTATTC			
Borrelia spp.	fla	BflaPAD	GATCA(G/A)GC(T/A)CAA(C/T)ATAACCA(A/T)ATGCA		55	[4]
		BflaPDU	AGATTCAAGTCTGTTTTGGAAAGC			
		BflaPBU,nest	GCTGAAGAGCTTGGAATGCAACC	340	55	
		BflaPCR,nest	TGATCAGTTATCATTCTAATAGCA			
B. microti	18S rDNA	Babl	CTTAGTATAAGCTTTTATACAGC	238	55	[5]
		Bab4	ATAGGTCAGAAACTTGAATGATACA			
Trypanosoma spp.	ITS1 rDNA	ITS1 CF	CCGGAAGTTCACCGATATTG	Variable	58	[6]
		ITS1 BR	TTGCTGCGTTCTTCAACGAA			
Leishmania spp.	kDNA minicircle	L.MC-1S	CTRGGGGTTGGTGTAAAATAG-	700	55	[7]
		L.MC-1R	TWTGAACGGGRTTTCTG			
Plasmodium spp.	*Cytb*	DW2 &DW4	DW2; TAATGCCTAGACGTATTCCTGATTATCCAG DW4; TGTTTGCTTGGGAGCTGTAATCATAATGTG	1253	60	[8]
		Cytb1 & Cytb2	CYTb1; CTCTATTAATTTAGTTAAAGCACA Tb2; ACAGAATAATCTCTAGCACC	939	50	

References: 1- Ishikura *et al*., 2003; 2- Parola, 2000; 3- Parisi *et al*., 2006; 4- Takano *et al*., 2010; 5- Persing *et al*., 1992; 6- Njiru *et al*., 2005; 7- Kato *et al*., 2007; 8- Prugnolle *et al*., 2010.

Table 3-2: The prevalence of zoonotic pathogens in non-human primates in Zambia

	Baboon (n=48)			**Vervet monkey (n=40)**			
	Sex			Sex			
	M (39)	F (9)	Sub-total (%)	M (33)	F (7)	Sub-total (%)	
Anaplasma spp.	3	2	5 (10.4%)	6	1	7 (17.5%)	
Babesia spp.	1	1	2 (4.2%)	0	0	0	
Borrelia spp.			0			0	
Coxiella burnetii			0			0	
Leishmania spp.			0			0	
Plasmodium spp.			0			0	
Rickettsia spp.	14	2	16 (33.3%)	15	4	19 (47.5%)	
Trypanosoma spp.			0			0	

GENERAL DISCUSSION

This thesis addressed the epidemiology of protozoan diseases in selected African countries. A trypanosomiasis study was undertaken on tsetse fly samples and blood spotted on FTA cards from cattle and pigs from two sites in Ghana namely, Adidome and Koforidua. A second trypanosomiasis study was undertaken on cattle blood DNA from Petauke in Zambia for comparative analysis of the evolutionary relationships between *T. vivax* strains from different geographical regions. The third study was a detection of the zoonotic pathogens in non-human primates from Zambia.

African trypanosomes are transmitted by the tsetse fly to humans and livestock throughout sub-Saharan Africa and cause diseases known as sleeping sickness in humans and nagana in domestic animals. An estimated 70 million people are at risk of infection, distributed over 1.55 million km^2 in sub-Saharan Africa covering 36 countries (Simarro *et al*., 2012). *T. brucei gambiense* accounts for almost 90% of the infections in central and western Africa, the remaining infections being from *T. b. rhodesiense* in eastern Africa (Simarro *et al*., 2012). Rearing livestock in endemic areas is difficult to impossible and results in an economic loss in agricultural output of several billion U.S. dollars per year. Human infections are fatal if untreated, but tools for disease control are limited because it has not been possible to develop vaccines and current trypanocidal drug treatments result in undesirable side effects with growing reports of drug resistance.

The reduction or elimination of tsetse populations is an effective method for disease control. The tsetse belt covers a huge area of 10 million km^2 while animal trypanosoniases stretch over 25 million km^2 of the African continent given the fact that

some trypanosome species such as *T. vivax* and *T. evansi* can be mechanically transmitted outside the tsetse belt by biting flies of the genera *Tabanus* and *Stomoxys*. Additionally, *T. equiperdum* can be venereally transmitted in horses and it has scattered distribution in countries in southern Africa. These facts make erradication of human and animal trypanosomiases from African continent almost imposible.

Ghana was historically endemic to both HAT and AAT, but, according to WHO report, the last HAT case had been reported in 2000 from Takoradi (Western region). An active case-finding survey was subsequently performed in December 2005 in this area but no cases were detected. Control activities are a responsibility of the Disease Control Unit of the Ghanaian Health Service, which works in collaboration with and with the technical support of the West African Centre for International Parasite Control based in the Noguchi Memorial Institute for Medical Research (WHO, 2006). On the contrary, Nagana continues to constrain livestock production in several parts of Ghana and impedes introduction of improved breeds due to their high susceptibility. The riparian vegetation of the main river, which is locally referred to in Ghana as the "White Volta" and its tributaries are infested with *G. palpalis gambiensis* and *G. tachinoides* (Draeger, 1983). Contact between tsetse flies and cattle occurs when cattle are herded along the White Volta. Tsetse fly infestation in Ghana is a set back to livestock production as highly productive exotic livestock breeds that are more prone to trypanosomiasis cannot be kept in preference to the trypanotolerant indigenous low productive breeds.

T. vivax is the most prevalent trypanosome species given the fact that it can be transmitted both by tsetse flies and mechanical vectors such as biting flies, majorly due to, and /or in addition to this species having the simplest life cycle in tsetse, normally

developing in the proboscis, although infections can sometimes also be detected in the cibarium and the anterior gut. For this reason, it is endemic outside the tsetse belt, including Central and South America, where the parasite was introduced by cattle from West Africa. Because *T. vivax* develops only in the proboscis, it is less exposed to anti-trypanosomal factors in the gut of the fly than other trypanosome species. This is followed in abundance by *T. congolense. T. brucei* transmission in the field, however, is very low, whose life cycle is the most complicated. This was in agreement with my findings in addition to previous research.

T. evansi is cosmopolitan being transmitted outside the tsetse belt by biting flies. Diagnosis of *T. evansi* has been mainly based on CATT/*T. evansi* RoTat 1.2 VSG the most dominant variable antigenic type (VAT), but reports of RoTat-negative *T. evansi* in Kenya proves a challenge to differential diagnosis of *T. evansi* from other African trypanosomes

Any disease or infection that is naturally transmissible from vertebrate animals to humans and vice-versa is classified as a zoonosis. Zoonoses have been recognized for many centuries, and over 200 diseases have been described. They are caused by all types of pathogenic agents, including bacteria, parasites, fungi, and viruses. Reducing public health risks from zoonoses and other health threats at the human-animal-ecosystems interface is not straightforward. HAT caused by *T. b. rhodesiense* is one of important zoonoses in Africa. Management and reduction of these risks must consider the complexity of interactions among humans, animals, and the various environments they live in, requiring communication and collaboration among the sectors responsible for human health, animal health, and the environment. The WHO is engaging in an ever-increasing number of cross sectoral activities to address

health threats at the human-animal-ecosystem interface. These threats include existing and emerging zoonoses as well as antimicrobial resistance, food-borne zoonoses, and other threats to food safety.

Non-human primates from Mfuwe in Zambia were screened for zoonotic pathogens and *Rickettsia africae*, *Anaplasma phagocytophilum*, and *Babesia* spp were detected. This study site is located in South Luangwa National Park which is also a HAT endemic focus. This is a wildlife-livestock-human interface with human settlements and livestock farming. These zoonoses are endemic in the wildlife and could be transmitted to the human population, but due to lack of awareness of these zoonoses among veterinarians and physicians, most of them remain undiagnosed.

CONCLUSION AND RECOMMENDATIONS

Trypanosomiasis is an important disease in many countries both in sub-Saharan Africa and globally with public health and livestock productivity impacts. Wildlife serve as a reservoir for trypanosomiasis and vector control for both the tsetse flies and biting flies is a challenge. HAT is not entirely an African problem. Although human African trypanosomiasis (HAT) is uncommon among patients from non-endemic countries, there has been an increase in the number of cases reported in recent years. Between 1902 and 2012, HAT was reported in 244 patients. Most HAT cases were reported before 1920, and after the year 2000. In the colonial era the average age of patients was lower (32.5±7.8 vs. 43.0±16.1 years, $p<0.001$), the proportion of females was lower (10.0% vs. 23.9%, $p<0.01$], most cases were diagnosed in expatriates, missionaries and soldiers (74.3%), and Gambian trypanosomiasis accounted for 86/110, (78%) of cases. In the post-colonial era most patients 91/125 (72.8%) were short-term tourists to game parks in eastern and South-eastern Africa (mainly in Tanzania); Rhodesian trypanosomiasis accounted for 94/123 (76.4%) of cases. Between 1995 and 2010 there has been a constant linear increase in the number of incoming tourists to Tanzania, and HAT cases occurred in small outbreaks rather than following a similar linear pattern.

In recent decades HAT patients from non-endemic countries are older, and more likely to be tourists who acquired the disease while visiting game-parks in eastern and South-eastern Africa. While Rhodesian trypanosomiasis is relatively uncommon among Africans, it now accounts for most cases reported among patients from non-endemic countries. Returning febrile travellers without an alternative diagnosis should be evaluated for HAT. Cases among travellers may serve as sentinels for Rhodesian

trypanosomiasis ‘‘hot spots’’ in Africa. Most *T. b. rhodesiense* HAT cases are reported in tourists visiting Tanzania, Zambia and Malawi. Besides HAT, tick-borne zoonoses are also increasingly reported in tourists returning from African Safari. African tick-bite fever caused by *Rickettsia africae* is also on the rise.

In conclusion, there has been a change in the epidemiology of the disease among patients from non-endemic countries over the past century, and there has been an increase in the number of reported cases over the past two decades. In the colonial era patients were likely to be male Europeans in their thirties who had come to West or Central Africa as missionaries, soldiers, and settlers, and were afflicted with Gambian trypanosomiasis. In the postcolonial era, patients from non-endemic countries are somewhat older, are likely to be short-term tourists visiting the great nature reserves of East and South-East Africa, and commonly present with an acute febrile illness typical of Rhodesian trypanosomiasis. Elimination of HAT as a public health problem has now become a realistic goal in most African countries. Until that day, all returning travelers with a compatible febrile illness, and without an alternative diagnosis, will have to be evaluated for HAT. A timely diagnosis will not only ensure better care of the patient, but also aid in detecting Rhodesian trypanosomiasis ‘‘hot spots’’ in Africa

On the contrary, within the trypanosomiasis endemic regions in Africa, HAT and AAT control is mainly based on chemotherapy and chemoprophylaxis coupled with active case detection to block the transmission cycle. There is no effective vaccine for trypanosomiasis mainly because of the phenomenon of antigenic variation. This is coupled with the fact that there are few drugs which are quite old and challenged with drug resistance. Only four drugs are available for the chemotherapy of human African trypanosomiasis or sleeping sickness; Suramin, pentamidine, melarsoprol and

eflornithine. Suramin, pentamidine and melarsoprol were developed in the first half of the 20th century. Eflornithine, originally developed in the 1970s as an anti-cancer drug, became a treatment of sleeping sickness largely by accident. The two key problems are that none of them can be given orally, and all of them have potentially serious adverse effects. The greatest problem with melarsoprol is that it is followed by a severe post-treatment reactive encephalopathy in about 10% of cases and in about 50% of these this complication is fatal. Thus the drug kills about 5% of all those who receive it (Pepin *et al*. 1994; Kennedy, 2004). This is a remarkable figure. Although this drug complication rate is very high, it is of course preferable to the 100% death rate of untreated disease.

Despite affecting millions and killing tens of thousands each year, visceral leishmaniasis, sleeping sickness and Chagas disease garner little attention from drug developers, policy makers, or the mass media. Though the number of patients was substantial, those patients were poor, meaning pharmaceutical companies felt there was no profitable market in designing new drugs and diagnostics for their care. And national governments and donor countries alike did little to push an agenda that could lead to greater research and development on their behalf.

However, organizations such as Foundation for Innovative New Diagnostics**:** FIND, the Bill & Melinda Gates Foundation, have stepped forward to support Neglected Tropical Diseases (NTDs) control. Doctors Without Borders/Medecins Sans Frontieres has been working with a special focus on three life-threatening NTDs: Kala azar (or visceral leishmaniasis), and sleeping sickness (or Human African trypanosomiasis), which are both always fatal if left untreated; and Chagas Disease, which can lead to fatal complications. Sleeping sickness treatment, much of it spurred

by research that has involved the organization working with others, in particular the Drugs for Neglected Disease initiative - DNDi, which they helped found, are also on-board.

Therefore, all said and done, coupled with the various challenges, vector control proves to be the most promising method for trypanosomiasis control.

Control efforts are also hampered by civil unrest in many parts of Africa which cripples control activities leading to resurgence of the disease. In other areas e.g Uganda which hosts both forms of HAT, restocking of livestock in the North-eastern part of the country following livestock de-population by cattle rustling and civil unrest, resulted in movement of infected livestock from the *T. b. rhodesiense* HAT focus in South-eastern Uganda into HAT free regions hence the rapid spread of *T. b. rhodesiense* HAT northwards towards the *T. b. gambiense* focus. At present, there is a threat for the merger of the two HAT forms being separated merely by a disease free belt of less than <100 km just north of Lake Kyoga. This has implications to epidemiology and control of HAT as the two forms differ in pathology, diagnosis and treatment. In addition, political and military conflicts increase tsetse-breeding sites, prompt movement of cattle and people and lead to deterioration of public services.

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ABSTRACT

Molecular epidemiological study of protozoan and other zoonotic diseases from two countries in Africa

Human African trypanosomiasis (HAT), also known as sleeping sickness, is a neglected tropical disease that impacts 70 million people distributed over 1.55 million km^2 in sub-Saharan Africa. *Trypanosoma brucei gambiense* accounts for almost 90% of the infections in central and western Africa, the remaining infections being from *T. b. rhodesiense* in eastern Africa. Furthermore, the animal diseases caused by trypanosomes inflict major economic losses to countries already strained. The parasites are transmitted to the mammalian hosts through the bite of an infected tsetse fly. Additionally, zoonoses are infections or diseases that can be transmitted directly or indirectly between animals and humans. This study assessed the molecular epidemiology of human and animal trypanosomes, in addition to zoonotic pathogens in non-human primates in Zambia.

The first chapter of this thesis describes results of molecular epidemiological study on trypanosomiases which were carried out in two tsetse-infested areas of Ghana. The samples included tsetse flies, and cattle and pig blood, and were analyzed by using multiple polymerase chain reaction tests. *Trypanosoma vivax* was the most prevalent trypanosome species, followed by *T. congolense* and *T. brucei brucei*. Two subspecies causing HAT, *T. b. gambiense*, and *T. b. rhodesiense* were not detected in animals and flies in this study, which confirms that the country having been formally a HAT focus has been free of HAT since 2000. The results in this study may be reflected by the fact that *T. vivax* can be mechanically transmitted by biting flies in addition to biological

transmission by tsetse fly, hence its distribution outside the tsetse fly belt of Africa.

The second chapter describes results on the genetic characterization of *T. vivax* strains from different geographical regions based on sequence comparison of Cathepsin L-like gene. *T. vivax* from Ghana clustered with West African and South American strains while *T. vivax* from Zambia clustered with East and Southern African strains. These results revealed genetic diversity of *T. vivax* in Africa.

In the third chapter, molecular epidemiological studies on zoonotic pathogens in non-human primates in Mfuwe in South Luangwa National Park, Zambia were carried out. This area is a HAT endemic focus with wildlife-livestock-human interface, hence the risk for zoonotic disease transmission is very high. Three species of zoonotic pathogens, *Rickettsia africae*, *Anaplasma phagocytophilum* and *Babesia microti* were detected among 9 pathogenic species/genera tested by PCR. These zoonoses detected in Zambia could be endemic in Zambian primates and possibly transmitted to humans but simply misdiagnosed as malaria due to their febrile nature.

Zoonoses in Africa are not just an African problem, since recent studies reveal an increase in these zoonotic infections in non endemic countries, especially in returning tourists from African national parks. Therefore, zoonotic disease control requires a multi-sectoral approach involving participants from the health, veterinary, entomology and environment professions because zoonosis transmission involves interaction between the pathogen, host, vector and environment.

要旨

Molecular epidemiological study of protozoan and other zoonotic diseases from two countries in Africa

（アフリカ２カ国における原虫ならびにその他の人獣共通感染症の分子疫学的研究）

眠り病として知られているヒトアフリカトリパノソーマ症（Human African trypanosomiasis ; HAT)は、155 万 km^2 に及ぶサブサハラアフリカ諸国で発生があり、7000 万人に及ぶ人々の脅威となっている「顧みられない感染症」である。中部～西部アフリカでは原因の 90％以上が *Trypanosoma brucei gambiense* によるものである一方、東部アフリカでは *T. b. rhodesiense* が主原因となっている。本原虫はツェツェバエにより媒介される。これに加えて、動物からヒトに直接あるいは間接的に伝播する人獣共通感染症も存在する。本研究ではヒトと動物のトリパノソーマ症、ならびにザンビアに生息する非人類霊長類（Non human primate: NHP)における人獣共通感染症病原体の分子疫学的研究を実施し取りまとめた。

第一章では、ガーナにおける家畜のトリパノソーマ感染症について、2 か所の地域での調査研究の結果をとりまとめた。本研究では牛および豚血液 DNA ならびにツェツェバエ DNA を試料として、トリパノソーマ属原虫の検索を Polymerase chain reaction（PCR)で実施した。その結果、検出率は *T. vivax*、*T. congolense*、*T. brucei* の順であった。ガーナでは 2000 年以来 HAT の報告はないが、本研究でもヒトに感染性を有する *T. brucei rhodesiense, T. brucei gambiense* は検出されなかった。*T. vivax* はツェツェバエによる生物学的媒介に加えて、吸血バエによる機械的伝播もされることから、ツェツェバエが分布する地域以外でも高率に検出されるものと考えられた。

第二章では、*Trypanosoma vivax* の Cathepsin L 様遺伝子の塩基配列比較により、ザンビアおよびガーナに分布する株の遺伝学的多様性を明らかにした。その結果、ガーナ由来試料は全て同一の遺伝子配列を有しており、西部と南部アフリカで検出された株の遺伝子配列と同一クラスターに分類された。ザンビア由来検体では、東部と南部から検出される本種の遺伝子型と一致した。本研究の結果、アフリカ大陸に分布する *T. vivax* の遺伝的多様性が明らかになった。

第三章ではザンビアのサウスルアンガ国立公園ムフエ地域で捕獲された NHP 検体を対象に人獣共通感染症病原体を検索した。この地域ではヒト一家畜一野生動物が接触する機会が多く、HAT の流行地でもあることから、人獣共通感染症の感染リスクも非常に高い。本研究で検索した 9 種/属の病原体のうち、*Rickettsia africae, Anaplasma phagocytophilum* および *Babesia microti* の 3 種類が検出された。このことから、同地域ではこれらの病原体はザンビアの NHP に保有されており、ヒトへの感染の可能性もあり、患者は熱性疾患であるマラリアと誤診されている可能性もあると考えられた。

人獣共通感染症は、アフリカに止まる問題ではなく、特にアフリカの自然公園を訪れた観光客の感染例が報告されている様に、非流行地域での発生例も多くなってきている。人獣共通感染症の発生には、病原体に加えて宿主、媒介生物、環境等の要因が絡み合っていることから、保健衛生、獣医療、昆虫学、環境の専門家を含む多角的アプローチがその制圧に必要であると考えられる。