Molecular epidemiological study of selected infectious diseases of livestock in Zambia.

(ザンビアの家畜における感染症の分子疫学的調査)

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Contents

List of abbreviations

CBPP	Contagious bovine pleuropneumonia
CD	Corridor disease
CI	Confidence interval
CTL	Cytotoxic T-lympocytes
CVRI	Central Veterinary Research Institute
DFAT	Direct fluorescence antibody test
DFID	Department For International Development
DNA	Deoxyribonucleic acid
DRC	Democratic Republic of Congo
DUVV	Duvenhage virus
ECF	East Coast fever
EP	Eastern Province
ELISA	Enzyme linked immunosorbent isothermal amplification assay
FMD	Foot and mouth disease
FITC	Flourescein isothiocyanate
GDP	Gross domestic product
HS	Hemorrhagic septicemia
IFAT	Immuno flourescence antibdoy test
ITM	Infection and treatment method
Kb	Kilo base pair
kDa	Kilo dalton
Km	kilometer
LAMP	Loop-mediated isothermal amplification assay
LBV	Lagos bat virus
LD	Linkage disequilibruim
LE	Linkage equilibrium
LIAN	Linkage analysis
LNP	Lukusuzi National Park
MHC	Major histocompatibility complex
mL	Milliliter
MLG	Multilocus genotypes

MOKV	Mokola virus
NALEIC	National Livestock Epidemiology and Information Center
ng	Nano gram
NJ	Neighbor joining
NLNP	North Luangwa National Park
NP	Northern Province
PCA	Principle component analysis
PCR	Polymerase chain reaction
PIM	Polymorphic immunodominant membrane protein
RABV	Rabies virus
RAPD	Random amplification of polymophic DNA
RFLP	Restriction fragment length polymorphism
RLB	Reverse line blot
RT-LAMP	Reverse transcription loop-mediated isothermal amplification assay
RT-PCR	Reverse transcription polymerase chain reaction
RT-qPCR	Real time quantitative PCR
RNA	Ribonucleic acid
RNP	Ribonucleoprotein
R. appendiculatus	*Rhipicephalus appendiculatus*
R. zambeziensis	*Rhipicephalus zambeziensis*
SADC	Southern African Development Community
SD	Standard deviation
SLNP	South Luangwa National Park
SNP	Single nucleotide polymorphism
SP	Southern Province
Tpr	*Theileria parva* repeat
T. parva	*Theileria parva*
UNZA	University of Zambia
US	United States
USD	United States Dollar
WHO	World Health Organisation

1. General Introduction

Molecular epidemiological tools such as polymerase chain reaction (PCR), sequence analysis and phylogenetics, and population genetics using multilocus genotypes are very important aspects in the study of infectious diseases. These tools allow the identification of new and dominant strains in naïve areas, and for the study of disease spread across different regions thus providing important data for the control and prevention of infectious diseases. In Zambia, a variety of infectious diseases are present. These diseases have diverse effects on the livelihood of indigenous people through the destruction of livestock (loss of property due to low growth rates or high mortality rates), a major source of income for rural people. However, important epidemiological data on most of these diseases is lacking. Acquisition of this important information will enable the country to improve its livestock sector which is an important social-economic activity and a major contributor to the country's annual Gross Domestic Product (GDP) (Anon, 2003). It is for this reason that I employed molecular epidemiological tools such as population genetics and phylogenetics on two selected diseases namely theileriosis, a disease of prime economic importance, and rabies, an important zoonotic disease, respectively. This study was structured in two parts with each part answering specific questions. The first part addresses the population genetics of *Theileria parva (T. parva)* while the second part looks at the phylogenetics of rabies virus (RABV) in Zambia, all in all, providing valuable data on the dynamics and characteristics of both diseases.

2. Social background

Geography of the Republic of Zambia

The republic of Zambia lies between 22^{o} E and 34^{o} E and 8^{o} S and 18^{o} S, in Southern Africa, and covers an area of 752, 614 km^2 out of which 740, 724 km^2 is land and 11, 890 km^2 water. It is a land locked country, bordered by Tanzania, the Democratic Republic of Congo (DRC), Angola, Namibia, Botswana, Zimbabwe, Mozambique and Malawi (Fig. 1). For administrative purposes, the country is divided into nine provinces (Fig. 1). It has a population of 11,262,000. Zambia experiences a pleasant tropical climate, which except in valleys tends to be unpleasantly hot. She has three seasons namely; cool dry season from April to August, hot dry season from August to November and, warm wet season from November to April which is also usually the hottest time of the year. Frost occurs in some areas in the cool season. The Luangwa and Zambezi valleys in October experience excessive heat and humidity in the wet season. The movement of the inter-tropical convergence zone has a main effect on seasonal change in Zambia.

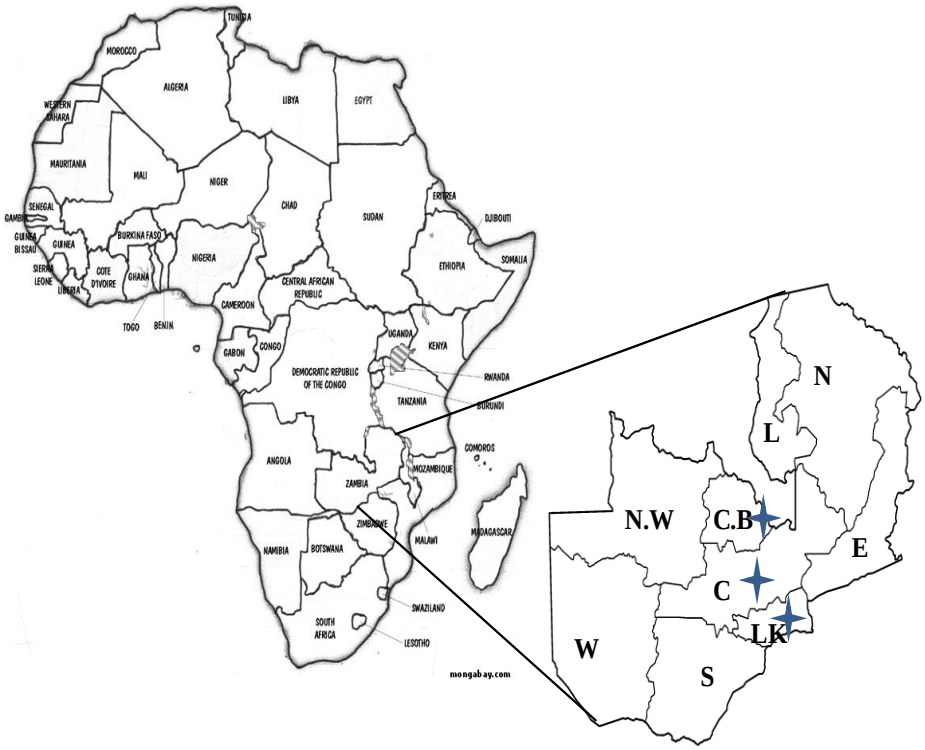

Figure 1. Map of Africa showing the location of the republic of Zambia with its provinces. The samples used in this study were obtained from Lusaka, Central and Copperbelt provinces (blue stars). N, Northern, E, Eastern, LK, Lusaka, L, Luapula, C, Central, C.B, Copperbelt, N.W, North-western, W, Western, S, Southern provinces.

Agriculture industry in Zambia

The agriculture labour force in Zambia is estimated at 3.4 million, with 85% of this comprising of subsistence farmers. Commercial agricultural farming mainly consists of a small number of large farms. In the rural areas, especially among women who comprise the higher proportion of the rural labour force and population (65%), agriculture tends to be the main source of income [Deparment for International Development (DFID), 2002]. Within the informal sector and with an unemployment rate of about 50%, agriculture often presents the only source of income or livelihood. The industrial growth rate of Zambia in 2004 was estimated at 6.9%, GDP (purchasing power parity), $ 9,409 billion, GDP – real growth rate 4.6%, GDP – per capita, purchasing power parity – $ 900; GDP – composition by sector; agriculture: 14.9% industry: 28.9%, services: 56.1%; labour force, 4.63 million and Labour force – by occupation, agriculture 85%, industry 6%, services 9%. About 90% of Zambia's foreign earnings come from copper, but because copper is a wasting asset and with the presence of vast resources such as land, labour and water, Zambia's motivation to shift from dependency on copper to agricultural production has greatly increased. Out of a total land area of 75 million hectares (752,000 km^2) Zambia possesses, 20.3 million hectares is grazing land. With the grazing land being approximately four times more than arable land, Zambia's potential lies heavily on livestock production than the arable cropping sector which currently receives the majority share of the government agriculture support.

Livestock production in Zambia

The commercial livestock areas in Zambia occur in the Southern Province, Central

Province, Lusaka Province, Copperbelt Province and Eastern Province. The main livestock reared includes; cattle, goats and sheep (Table 1) (Aregheore, 1994). The traditional agricultural sector accounts for 83%, 64% and 97% of cattle, sheep and goat production, respectively. The main livestock reared however still remains to be cattle with the commercial agricultural sector rearing exotic breeds such as Afrikander, Boran, Hereford, Friesian and Jersey, while the traditional sector rears other local breeds such as Zebu and Sanga types i.e. Tonga, Ngoni and Barotse. Cross-breeds are also promoted in the dairy industry so as to improve milk production and disease resistance. The cattle population in 2001 was estimated at 2.5 million head and is currently estimated at 2.9 million (Anon, 2007). Zambia's animal products' exports to neighbouring countries in 1995, 1999 and 2001 were US$ 1.4 million, US$ 4.4 million and US$ 3.1 million, respectively. The traditional sector while contributing 83% of cattle production is heavily characterized by low reproductive efficiency, slow growth rates and high rates of mortality exceeding 15% in some areas. It is estimated that approximately US$ 380 million is marketable meat. However, only 7% is the slaughter off-take, of which 75% is sold at a value of US$ 25 million. The low off-take consequently results in higher consumer prices of livestock as compared to other Southern African Development Community (SADC) countries. Cattle production in certain regions is limited by diseases such as foot and mouth disease (FMD), contagious bovine pleuropneumonia (CBPP), theileriosis, hemorrhagic septiceamia (HS), anthrax and clostridial infections. Cattle production is important but productivity is low, due in part to the poor nutritive value of natural pastures (Kulich, 1988). The livestock sector in Zambia is becoming an important component of Zambia's economy with a contribution of 6.4% in 1996 and 6.5% in 1997, to the National Gross Domestic Product (GDP). This was approximately

Table 1: Approximate number of livestock in the different provinces in Zambia

Province	Cattle	Goat	Sheep
Central	363, 000	195, 000	3, 000
Copperbelt	57, 000	6, 000	3, 000
Eastern	251, 000	125, 000	6, 000
Luapula	11, 000	19, 000	8, 000
Lusaka	75, 000	16, 000	1, 000
Northern	11, 000	15, 000	10, 000
North-western	58, 000	10, 000	10, 000
Southern	1, 100, 000	224, 000	11, 000
Western	500, 000	4, 000	ND

ND, data not available

35% of total agricultural production. In the year 1997, 33% of agricultural exports were from the livestock sector with 23% of the per capita supply of protein originating from animal products, with beef being the most preferred and cattle contributing 61% of the meat and milk produce (Daka, 2002). The increase in the output from the traditional sector has been due to the increase in animal numbers instead of increase in productivity. A steady increase of 3.5% per annum in cattle numbers in the traditional sector is due to the increase in the number of traditional farmers who are taking up livestock farming (Daka, 2002). However, this steady increase is threatened or limited in certain regions by the presence of infectious diseases such as theileriosis (east coast fever and corridor disease) which results in low productivity, and partly confounded by the poor nutritive value of natural pastures (Kulich, 1988). Prevention and control of infectious diseases in the livestock sector of Zambia is expected to result in; provision of food security for the majority of households, increase its contribution to the total foreign exchange earnings, increased contribution to GDP, and an increase in the income of people working in the livestock sector and the nation as a whole.

3. Infectious diseases in Zambia

In Zambia, a variety of infectious diseases of livestock occur ranging from; (1) protozoan diseases (theileriosis, trypanosomiasis, and babesiosis); (2) viral diseases (rabies, FMD, and lumpy skin disease), (3) bacterial diseases (anthrax, clostridial infections, HS, and tuberculosis), and parasitic diseases (liver flukes, cysticercosis, and hydatidosis). In an effort to control and understand the dynamics and impact of infectious diseases on livestock production in Zambia, acquisition of molecular data and the establishment of molecular diagnostic tools cannot be overemphasized. This general

introduction will focus on two infectious diseases, theileriosis and rabies. Theileriosis will be reviewed with respect to its vectors, *Rhipicephalus appendiculatus (R. appendiculatus) and Rhipicephalus zambenziensis (R. zambenziensis)*, risk factors and disease transmission, and its economic impact on livestock production. Rabies, an important infectious disease of both humans and animals, will also be reviewed with respect to its transmission, and clinical signs.

A. Theileriosis

Theileriosis is caused by a tick-borne protozoan parasite called *T. parva*. It causes East Coast fever (ECF), a major livestock disease in cattle occurring in the Eastern, Central and Southern Africa. In Zambia, it manifests as ECF in the Northern and Eastern Provinces and Corridor Disease (CD) in the Southern Province. Theileriosis (ECF) occurring in the eastern and northern parts of Zambia is assumed to have spread from neighboring countries like Malawi, Mozambique and Tanzania, while the origin of CD in Southern Province has several hypotheses including spread from ECF infected areas of Zambia (Eastern and Northern provinces) and Zimbabwe. It is also believed to spread from buffaloes. The first case of theileriosis recorded in Zambia based on clinical signs, was in 1922 in Nakonde district of Northern Province (Nambota et al., 1994). According to the annual reports of the Veterinary Department of Zambia, the first microscopically diagnosed case of the disease occurred in 1946 in Mbala district in Northern Province, and Chipata district of Eastern Province in 1947 and since then, it has spread and is believed to have reached enzootic stability within the Northern and Eastern Provinces. A malignant form of the disease was detected in the Hufwa area of Monze district in the Southern Province in 1977 (Nambota et al., 1994) and it was

diagnosed as CD. This form of theileriosis differed from the classical ECF in that: (1) the mortality of cattle in the buffalo-inhabited "corridor" area caused when cattle were moved outside the buffalo zone, (2) in infected cattle, few macroschizonts of small dimensions were found in the tissue smears, (3) recrudescence of piroplasm parasitemia was caused by splenectomy, and (4) the presence of a carrier state in buffalo in contrast to *T. parva* infection in cattle. Considering the above outlined characteristics of the buffalo parasite, a new species called *T. lawrencei* and separate from *T. parva* was proposed. However, this was later abandoned as the data from molecular genetic characterisation and cross immunity did not substantiate this claim (Conrad et al., 1987 and 1989; Allsopp et al., 1989). As such, *T. parva* parasites are classified simply as either cattle- or buffalo-derived. It is believed that southern province was disease free before 1977. At present, the disease is endemic in this region and because this is an important cattle-raising area containing about 1.2 million heads of cattle (approx 45% of the national herd), the disease is of high economical importance.

Transmission of theileriosis

T. parva is transmitted transtadially by the nymphs of *R. appendiculatus,* a brown ear tick after becoming infected as larvae and nymphs (Konnai et al., 2006; Mulumba et al., 2001). *R. appendiculatus* populations cover an area stretching from Southern Sudan in the north, to South Africa in the south, it has also been recorded in large areas of Eastern, Central and Southern Africa (Perry et al., 1990). The timing period of the adult stage largely determines the seasonal occurrence of *R. appendiculatus* coupled with the combined influences of temperature, humidity and day length (Short and Norval, 1981). There is an association between the adult tick activity and onset of rain in parts of

Africa (Chaya in Zambia and Lilongwe in Malawi) where a defined rainy season occurs. In these regions, only a single adult tick generation per annum is observed. In Zambia, adults ticks appear from December to April, larvae from March to May and nymphs from May to September (Pegram et al., 1986). As a consequence of the seasonal effect on the tick vector, in Zambia, theileriosis shows a cyclic occurrence with cases occurring more frequently during and shortly after the wet season lasting from October/November to April/May.

Life cycle of the *Theileria parva*

Theileria spp. having a complex life cycle, involves several morphologically distinct developmental stages occuring in the tick and mammalian host (Fawcett et al., 1982; Shaw and Tilney, 1992). The sporozoite, the infectious stage of the parasite, is first introduced into the bovine host through the saliva of feeding ticks at the nymphal or adult stage. The sporozoites then enter lymphoid cells and develop into schizonts, inducing the proliferation of the host cells. These cells are then disseminated throughout the body through the normal circulation of the host's lymphoid cells. The schizont gives rise to numerous uninucleate merozoites, after a period of growth and division in the lymphoid cells. The merozoites then leave the lymphoid cells to invade erythrocytes, where they develop into the intraerythrocytic stage called piroplasms. The piroplasms are then ingested through the blood meal by feeding ticks. Once in the tick gut, infected red blood cells are digested releasing piroplasms in the gut lumen. In the tick gut, they divide into macro- and microgametes which then fuse to form a zygote (Schein et al., 1977). The zygotes then enter the gut epithelial lining and develop into motile kinetes. As the tick vector moults, the kinetes migrate into the haemocoele, through the gut wall

and are transported to the salivary glands and once there, they develop intracellularly into sporoblasts (Fawcett et al., 1982). These sporoblasts are then introduced into the mammalian host as sporozoites by the feeding tick, starting the life cycle all over again. Genetic exchange has been shown to occur when 2 or more stocks of *T. parva* are used to infect the tick vector under laboratory conditions (Nene et al., 1998).

Epidemiology

The occurrence and distribution of the tick vector (*R. appendiculatus)* determines the occurrence of *T. parva*. Differences in climatic conditions influence the dynamics of tick population and hence, the transmission abilities of *T. parva*, creating a wide range of epidemiological situations in different areas (Lessard et al., 1990; Norval et al., 1991). For example, in East Africa, adult ticks are present all year round and as such, all year-round transmission occurs. In places where climatic conditions are not favourable for the all year-round presence of adult ticks i.e. in transitional zones such as Zambia, adult tick transmission may peak during the rainy and dry season. (Norval et al., 1991). In the southern part of Africa i.e. Zambia, where only one tick generation per year occurs, an unstable epidemiological situation characterized by a reduced adult tick transmission period of four months, coinciding with the rainy season occurs (Norval et al.,1991). In these areas, the nymphal transmission stage occurring from May to September plays an important role in the epidemiology of the theileriosis (Mulumba et al., 2001). In years characterized by reduced rainfall, high ECF sero-prevalences in September as a result of nymphal transmission have been reported in Southern Zambia (Fandamu et al., 2005). Experiments on the nymphal stage have shown that they are able to induce a lethal infection, provided a large number of nymphs are attached to the

host (Marcotty et al., 2002).

The host type and density are also important factors to consider in the epidemiology of theileriosis. In Africa, different cattle breeds show varying levels of parasite susceptibility and tick resistance (Norval et al., 1988). For example, indigenous breeds such as Sanga and Zebu types of cattle reared in endemic areas show a higher degree of resistance to the parasite as compared to the *Bos taurus* breeds which are more susceptible to tick infestation and *T. parva* infection (Ndungu et al., 2005). However, in these endemic areas, animals that recover from the disease, either through natural means or following treatment, remain carriers, which then act as reservoirs of parasite infection for feeding ticks (Kariuki et al., 1995).

Clinical signs and pathogenesis

Upon the introduction of sporozoites into a susceptible host (cattle) by the feeding tick, they invade the host lymphocytes, rapidly differentiating into schizonts (Fawcett et al., 1982) and consequently inducing the infected cells to proliferate uncontrollably. Through the parasite's associating with the host cell's mitotic spindle, it divides in synchrony with the host cell, consequently resulting in each daughter cell inheriting the infection from the mother cells (Rocchi et al., 2006). This phase of parasitic and host cell division is responsible for the severity of *T. parva* i.e. clinical sign and pathology. During the proliferation of the parasites i.e. the acute phase of *T. parva* infection, an up-regulation of cytokines thought to contribute to the severity of the disease has been observed (Yamada et al., 2009). The disease is first characterized by the swelling of the lymph nodes draining the ear surface, a predilection feeding site for ticks. It has an incubation period of 7 to 10 days following natural infection, and fever (39.5^{o}C or

higher), which is a consistent feature of the disease, develops from about day 10. Host cells infected with schizonts disseminate to lymph nodes, interstitial tissues of the lungs, gastro-intestinal tract and the kidneys. The major symptoms of ECF include fever, swelling of lymph nodes, anorexia, and dyspnoea. Death of the host is as a result of pulmonary oedema resulting from the severe destruction of the lung tissues due to inflammatory infiltration (Gwamaka et al., 2004).

Diagnosis

Diagnosis of theileriosis include: blood smear examination, serological tests and deoxyribonucleic acid (DNA)-based assays.

1. Microscopic examination

Blood smear examination is the standard technique for routine diagnosis of theileriosis. It is cheaper and faster than most other methods (Salih et al., 2007). However, it has the following drawbacks; it has low sensitivity and specificity, and its accuracy depends heavily on the experience of the laboratory personnel. In this technique, a lack of standardization of the amount of blood used to make each smear results in smears of different thickness and thus affecting the sensitivity of the assay (Quintao-Silva et al., 2007). Another drawback presented by microscopic diagnosis is that confusion is most likely to arise in cases were mixed species infections occur (Almeria et al., 2001).

2. Serological methods

A variety of standardized serological test for the diagnosis of tick borne diseases such

as theileria are available (Billiouw et al., 1999; Jongejan et al., 1988; Swai et al., 2005). Some of these tests include; immunoflourescence antibody test (IFAT), enzyme-linked immunosobent assay (ELISA). A number of limitations common to serological assays based on the detection of antibodies include; issues of stability, sensitivity, specificity and the objectivity of reading the results. Serological assays such as IFAT are difficult to apply to a large sample size without affecting sensitivity and preventing cross contamination. Serological assays have also been shown to fail to differentiate recovered animals from sterile immunity, carrier animals and clinical cases (Bose et al., 1995; Goff et al., 2008). In addition, antibody cross-reactions have been reported among closely related parasitic species (Edelhofer et al., 2004; Salih et al., 2007). Serological tests are also unable to detect some chronically infected animals (De Wall, 2000). Serological assays used in the diagnosis of theileriosis involve preparing crude parasites (used as a source of antigen) leading to lack of reproducibility. Recombinant antigen preparations free from host proteins have been used in diagnosing tick-borne diseases (Katende et al., 1998; McGuire et al., 1991). In as much as these antigens are cheaper to produce and have minimal batch-to-batch variations, they tend to be strain-specific as such failing to diagnose other strains (Bose et al., 1995).

3. DNA-based methods

DNA-based techniques such as LAMP, standard PCR, real time quantitative PCR (RT-qPCR), reverse line blot (RLB), (Criado-Fornelio, 2007) are available for diagnosing theileriosis. These diagnostic techniques tend to be relatively expensive i.e. need special equipments, as compared to serological and microscopic methods, thus preventing their full implementation in resource-poor countries. They are however

highly sensitive and specific.

a) Loop-mediated isothermal amplification assay (LAMP)

LAMP is a novel DNA amplification method. It employs the synthesis of a continuous strand-displacement DNA primed by a set of target-specific primers (Notomi et al., 2000). The basic LAMP method uses the *Bst* DNA polymerase and primer pairs. Results can be read visually through the observation of white precipitates (magnesium pyrophosphate) in the reaction mixture or through the use of gel electrophoresis to visualize the amplified DNA (Criado-Fornelio, 2007; Notomi et al., 2000). LAMP has a higher sensitivity than conventional nested PCR (Iseki et al., 2007). In the detection of *Theileria annulata* (*T. annulata*) in Sudan, it was shown that the sensitivity of LAMP was higher than that of conventional PCR, but equal to that of RLB (Salih et al., 2008b). LAMP is cheaper to utilize and amplified products can be obtained faster.

b) PCR

PCR is sensitive, specific and is able to detect a variety of infections. It is also able to detect more than one pathogen simultaneously (Bekker et al., 2002; Figueroa et al., 1993b). These types of PCR reduce the cost and time of performing the assay. A nested PCR assay for diagnosis of *T. parva* using the p104 gene is available (Skilton et al., 2002)

c) RT-qPCR

RT-qPCR is based on the use of fluorescent reporter molecules or sequence-specific probes to monitor the reaction as it progresses. Both resource and operator time are

saved as no post reaction processing is required. The use of qPCR is limited in resource poor countries due to the high cost of the equipment (Criado-Fornelio, 2007).

d) RLB

In this technique, PCR is combined with hybridization of amplified products to one or several species-specific oligonucleotide probes bound to a support membrane (Tait and Oura, 2004). Several probes can be combined to allow the discrimination and identification of well-characterised species. It can still detect related genotypes different from reference strains (Criado-Fornelio, 2007). The main weakness of this technique is that it requires specialized equipment and also the production of membranes limits its use in developing countries.

Prevention and control

Prevention and control of theileriosis is based on tick control, immunization (infection and treatment) and chemotherapy. The geography, socio-economy, cost of application of the method has a great influence on the type of prevention and control program implemented (Kocan et al., 2000; Mugisha et al., 2008).

1. Tick control

Tick control involves the use of acaricides, tick resistant breeds and immunization against ticks. This aims at preventing transmission of disease, reducing direct losses such as damage to hides, low weight gains and reduced milk production (Minjauw et al., 1997).

a) Chemical control

Chemical control of ticks involves plunge dipping, spraying of animals and the use of spot on acaricides. However, prolonged and intensive use of chemicals to control ticks may result in tick resistance, environmental pollution and residues in animal products (Ghosh et al., 2007). It also interferes with enzootic stability, resulting in a susceptible population of animals which are at risk to disease epidemics if disruptions in control programmes occur (Kocan et al., 2000; Lawrence et al., 1980).

b) Tick resistant breeds

Animals with a heritable ability to become immunologically resistant to tick infestation are prime in the control of ticks (Willadsen, 2004). These animals once identified can then be crossbred with susceptible animals. The Zebu cattle have some resistance to both ticks and *T. parva*. However, these animals tend to have a reduction in productivity i.e. reduced milk production.

c) Anti-tick vaccines

Allen and Humphreys (1979) were the first to demonstrate the feasibility of controlling tick infestations through immunization of hosts with selected tick antigens. This involved ticks feeding on immunized hosts, consequently ingesting antibodies specific for antigens within the tick's saliva, digestive tract and reproductive organ. This would then have deleterious effects on the feeding and reproductive behavior of the ticks. A number of tick antigens have been identified (De la fuente et al., 2007; Imamura et al., 2008; Labuda et al., 2006; Mulenga et al., 1999; Willadsen, 2004). There are two distinct types of antigen explored in vaccine development (Nuttall et al.,

2006). The first are conventional antigens are called exposed antigens. These are secreted in saliva during tick attachment and feeding on the host. From the tick feeding site, they are up taken by dendritic cells of the host, processed and presented to T lymphocytes resulting in a cell- or antibody-mediated immune response (Larregina and Falo, Jr. 2005; Nithiuthai and Allen, 1985; Willadsen, 2001). A 15 kilo dalton (kDa) protein from *R. appendiculatus,* a putative cement protein required for attachment and feeding has been used for vaccination of cattle. This reduced the infestation rates of nymphs and adults by 48 and 70% respectively (Labuda et al., 2006). Tick control by vaccination is cost effective, prevents the emergence of acaricide-resistant ticks and reduces environmental pollution.

2. Vaccination against theileriosis

a) Live and inactivated vaccines

The principle of immunization of cattle against theileriosis using live vaccines is been based on the hypothesis that animals that recover from disease acquire some level of immunity to subsequent challenge or infection (De Waal and Combrink, 2006; Morrison and McKeever, 2006). This is known as the infection and treatment method against ECF (Radley et al., 1975a), and the use of attenuated vaccines against tropical theileriosis (De Waal and Combrink, 2006; Pipano and Shkap, 2000). Theiler showed that cattle that had been previously infected and had recovered were immune to subsequent challenge (Theiler, 1911). Spreull also demonstrated that 70% of the cattle infected with *T. parva* using spleen and lymph node cell suspensions survived consequent challenge with infection (Spreull, 1914). The infection and treatment method (ITM) involving the administration of long-acting tetracycline and doses of

cryo-preserved sporozoites was found to induce immunity against *T. parva* (Radley et al., 1975). Immunization experiments using a mixture of three parasite isolates (Muguga, Kiambu 5 and Serengeti), called the Muguga cocktail, produced immunity against a range of heterologous isolates (Radley et al., 1975b). The Muguga vaccine has been extensively used in Tanzania and Uganda but it has been less utilized in Malawi and Zambia (McKeever, 2007). Theileriosis control in Zambia is based on a combined system of (i) vector control (using acaricides), (ii) cattle movement control, (iii) chemotherapy, and (iv) immunization (infection and treatment) using the local *T. parva* Katete and Chitongo stocks in Eastern and Southern Provinces, respectively (Makala et al., 2003). Studies have shown that protection following vaccination is mediated by major histocompatibility complex (MHC) class I-restricted cytotoxic T-lymphocytes (CTL) (McKeever et al., 1994; Morrison et al., 1987).

b) Subunit vaccines

Candidate antigens for vaccination against *T. parva* have been identified (Morrison and McKeever, 2006). Subunit vaccines utilizing the sporozoite surface antigen (p67) (Musoke et al., 2005), 6 schizont antigens (Graham et al., 2006) and the polymorphic immunodominant molecule (PIM), expressed by both the sporozoite and schizont stages of *T. parva* have been reported to produce a reduction in the severity of ECF (Musoke et al., 2005) and also the ability of the schizont antigens to induce protective immune responses (Graham et al., 2006). Immunization of cattle with recombinant PIM has been shown to induce antibody, $CD4^+$ T-cell responses and $CD8^+$ cytotoxic *T. parva* specific T-cells in the absence of other CTL dominant *T. parva* antigens (Ververken et al., 2008).

3. Chemotherapy and chemoprophylaxis

Theileriosis is mainly treated by using the following chemical products: tetracyclines, halofuginone, parvaquone and buparvaquone. Halofuginone (Terit®) has low efficacy in the incubation period and it is only active against the schizont stage of the parasite (Peregrine, 1994). Moderate to severe recrudescence has also been reported following the use of halofuginone (Kiltz and Humke, 1986; Morgan and McHardy, 1982). Tetracyclines have very little effect on clinical ECF (Peregrine, 1994) and as such, are mainly used in the ITM for prophylaxis (Radley et al., 1975a). Parvaquone (Clexon®, Parvexon®) and buparvaquone (Butalex®) are hydroxynaphthaquinone derivatives. These are more effective in the treatment of ECF compared to halofuginone with respect to recovery rate, recrudescence of infections and therapeutic indices (McHardy et al., 1983; McHardy et al., 1985; Morgan and McHardy, 1982). Combining of parvaquone and furosemide (a diuretic drug) greatly enhances recovery from ECF were pulmonary signs are present (Mbwambo et al., 2002; Musoke et al., 2004). Although buparvaquone and parvaquone are highly effective in treatment of clinical ECF, their high cost prevents their wide-scale use among small-scale farmers (D'Haese et al., 1999; Muraguri et al., 1999).

Economic impact of theileriosis in Zambia

The presence of theileriosis has a negative impact on livestock production and consequently on the economy as a whole. Theileriosis causes losses of up to 10,000 cattle annually and approximately 1.4 million of the 3 million cattle population is at risk of theileriosis (Nambota et al., 1994). Theileriosis also causes losses through damage of hides by ticks, morbidity, and treatment and control expenditure i.e. acaricides,

construction of dipping or spraying facilities and their maintenance, and variable costs such as those for water and labour. The vector control economics depend on the size of the herd and the method of acaricide application. Correct and intensive vector control measures can greatly reduce the losses caused by morbidity, mortality and tick damage, however, this is expensive and usually out of reach of the rural farmer. Vector control expenditure in Zambia is estimated at US$ 8.43, 13.62 and 21.09 per animal per year for plunge dipping, hand spraying and pour-on, respectively (D'Hasebe et al., 1999). Immunization costs US$ 9.5 per animal, and limits losses caused by *T. parva*, but not by ticks as they may still reduce the animal's productivity by transmitting other tick-borne pathogens. This type of expenditure entails that valuable resources have to be diverted to the control of theileriosis by both the farmer and the government. Immunization has shown to reduce the losses due to morbidity or mortality. However, unavailability of important molecular data on *T. parva* may complicate the implication of vaccination programs resulting in increased in expenditure on control programs. The high level of mortality in *taurine* breeds also impedes the introduction of highly productive exotic breeds in endemic areas, further preventing the improvement of cattle production in the affected areas. Theileriosis is thus a constraint to the development of the livestock industry with a final negative impact on the economy of the country.

B. Rabies

Globally rabies is one of the oldest, recognized viral diseases. It is a disease of both veterinary and public health concern because rabies has one of the highest mortality rates among infectious diseases (Krebs et al., 1995). With post-exposure treatment being administered to thousands of people bitten by rabid or suspected rabid animals annually,

rabies has emerged to be a costly disease to manage (Meltzer and Rupprecht, 1998a, 1998b). Rabies surveillance is involved in the identification of viruses originating from new and old reservoir species (De Mattos et al., 1993; Matter et al., 1998).

Aetiology

Rabies virus (RABV) is a cylindrical, bullet-shaped single stranded virion belonging to the family *Rhabdoviridae* and genus *Lyssavirus*. It comprises of a 12-kilobase pair (kb) ribonucleic acid (RNA) genome containing the N, P, M, G, and L genes coding for the nucleoprotein, phosphoprotein, matrix protein, glycoprotein, and polymerase protein, respectively (Tordo et al., 1986; Wunner, 1994).

Rabies viral proteins

1. Nucleoprotein (N)

The nucleoprotein is a 450 amino acid long protein with a molecular weight of 50 kDa encoded by the 1,350 base pair (bp)-long nucleoprotein gene (Tordo et al., 1986a; 1986b; Tordo et al., 1992; Kissi et al., 1995). It is phosphorylated and packages the RNA genome (Kouznetzoff et al., 1998; Yang et al., 1998; Tordo et al., 1986a). The nucleoprotein gene is highly conserved among different strains of RABV. Studies have also shown that it is involved in immunity against RABV (Dietzschold et al., 1987) particularly through T helper and cytotoxic cells induction (Ertl et al., 1989; Lafon, 1994).

2. Glycoprotein (G)

The glycoprotein comprised of a cytoplasmic domain, transmembrane domain, and

an ectodomain, is a 505 amino acid protein with a molecular weight of 57 kDa (Gaudin et al., 1992; Anilionis et al., 1981). It appears on the external surface of the virus as 10-nm long-peplomers. The ectodomain is more conserved than the transmembrane and cytoplasmic domains (Fodor et al., 1994; Baer et al., 1991; Tordo et al., 1992). It is also responsible for viral attachment and induction and binding of virus-neutralizing antibodies.

3. Matrix protein (M)

The matrix protein is the major structural protein of rabies virus located in the inner surface of the viral envelop. It has a molecular weight of 23 kDa and comprises of 202 amino acids and is encoded by the matrix gene. It facilitates the interaction of the membrane anchored glycoproprotein and the associated membrane viral proteins. It covers and keeps the ribo-nucleoprotein in a condensed form (Mebatsion et al., 1999)

4. Polymerase (L)

The polymerase is a multifunctional RNA-dependant RNA polymerase comprising of 2,142 amino acids with a molecular weight of 200 kDa. It is the largest protein and occupies 54% of the rabies genome (Tordo et al., 1988). The L protein is responsible for activities involved in RNA synthesis such as polymerase activity, capping, methylation, and polyadenylation.

5. Phosphoprotein (P)

The phospoprotein contains 297 amino acids and is encoded by the phosphoprotein gene. It has a molecular weight of 33 kDa and it is part of the ribonucleoprotein (RNP)

viral complex. It is thus a major component during transcription and replication of the single stranded RNA genome (Larson and Wunner, 1990; Tordo et al., 1992). The first 19 amino acids of the phosphoprotein interact with the nucleoprotein (Chenik et al., 1998; Schoen et al., 2001; Jacob et al., 2001). This interaction might indicate the possible involvement of the phosphoprotein in virus assembly.

Transmission and clinical signs

Dogs become infected with rabies after being bitten by a rabid animal, which might be a dog or other carnivores such as the jackal. The disease in dogs is characterized by an incubation period ranging from two to eight weeks followed by a prodromal phase which is only noticed in closely monitored pets. During this phase, a change in temperament is noticed, together with dilation of pupils, slowed papillary reflexes, and photophobia. The acute neurologic or furious phase follows and is characterized by nervousness, restlessness, increased responses to auditory and visual stimulation, injury to the teeth and mouth as a result of biting cages and straining chains, biting at imaginary flies, self inflicted injury at the bite site and swallowing of objects such as stones and wood. The animal is disorientated, wanders, moves aimlessly and bites any moving object. It develops a fixed stare, paralysis of the laryngeal muscles, drool saliva, convulsive seizures, inco-ordination followed by progressive paralysis, comatose and death (Tiekel et al., 1975). The dumb or paralytic form occurs in a few cases and is characterized by drooling of saliva, paralysis of masticatory and deglutination muscles, and a hoarse cough. Paralysis of other parts of the body, coma and death follow shortly afterwards (Swanepoel et al., 2004; Tiekel et al., 1975). Rabid dogs are able to excrete the virus up to seven days before the onset of clinical signs (Fekadu et al., 1992).

In human beings, rabies presents itself in the following stages: incubation, prodromal, acute neurological, coma phase and finally death. The incubation period usually lasts about 90 days or less, although in some cases it has been shown to last several years. The prodromal phase is uneventful with only a tingling, itchy painful sensation being felt at the bite site in a third of cases (Hemachudha et al., 1994). The acute phase is mostly manifested as the furious or encephalitic form with the following signs; hydrophobia, aerophobia, fluctuating consciousness, inspiratory spasms, autonomic nervous system stimulation signs, periods of lucidity, bladder dysfunction, loss of sensory function in the bite site and a persistent fever. In a fewer cases, the acute form is presented as the dumb or paralytic form without any distinct signs. The furious or encephalitic acute phase usually lasts about 7 days or less while the dump or paralytic phase is about 14 days. The pathological changes associated with the different presentations of the disease are not currently understood. The acute phase is followed by coma and death. Human rabies is incurable and nothing much can be done to avoid the inevitable result once the clinical signs set in. There has been one exceptional case were a patient survived after intense medical management with an intense anti-excitatory strategy without the use of immune prophylaxis (Rodney et al., 2005).

Epidemiology

Rabies, a notifiable disease, is present in all provinces of Zambia and mainly affects dogs with spill over infections to livestock and human beings. The first outbreak of rabies in Zambia is believed to have occurred in 1901 in the Western Province, where it is said that the local chief Lewanika ordered the destruction of all dogs in an effort to control the disease. However, the first confirmed diagnosis of the disease was only done

in 1913 (Swanepoel, 2004). It is believed that both the urban and sylvatic cycles of rabies occur in Zambia but currently there is not any information to support the sylvatic cycle. A study carried out in 1978 on wildlife rabies in Zambia on the Selous mongoose (*Paracynictis selousi*), which is the most common species, gave negative results thus nullifying it as a reservoir of rabies (Rottcher et al., 1978). A follow-up study ten months later, showed that at the time, rabies in wildlife was not a serious problem, however with the breakdown of the biological equilibrium due to human activities, the jackal might emerge as the reservoir and source of infection in wildlife (Rottcher et al., 1978). Jackal rabies has previously been reported in the Southern and Central parts of Zambia (Zyambo et al., 1985). However, dogs are more likely to be the reservoir and source of infection in humans and livestock in both urban and interface areas with the current reduction in the jackal population in southern and central parts of the country (Munang'andu et al., 2010).

Diagnosis of rabies in Zambia

1. Microscopic examination

Miscroscopic examination employs the use of fluorescent labeled antibodies (Direct or indirect fluorescent antibody test) and heamatoxylin and eosin stained biopsies.

a) Direct fluorescent antibody test (DFAT)

In this assay, the rabies antibody directed against the nucleoprotein (antigen) of the virus is used. The replication of rabies virus which occurs in the cell cytoplasm leaves large round or oval inclusions containing nucleoprotein and these appear as dust-like fluorescent particles. The hippocampus area of the brain is used for diagnosis. The

fluorescent labeled antibody is incubated with a rabies-suspect brain tissue to allow the antibodies to bind to the rabies antigen. The unbound antibody are then washed away. The bound antibodies are then visualized as fluorescent-apple-green areas.

b) Histological examination

Histological examination of biopsy tissues stained with hematoxylin and eosin in order to assess the presence of encephalomyelitis is occasionally used in the diagnosis of rabies. This method is based on the observation of mononuclear infiltration, perivascular cuffing of lymphocytes or polymorphonuclear cells, lymphocytic foci, babes nodules consisting of glial cells, and Negri bodies. However, this method is nonspecific and subject to the experience of the personnel.

The other diagnostic techniques that can be used in rabies diagnosis include nested PCR (Kalmovarin et al., 1993) and reverse transcriptase loop-mediated isothermal amplification (RT-LAMP) assay (Notomi et al., 2000; Parida et al., 2005; Ushio et al., 2005; Okafuji et al., 2005; Boldbaatar et al., 2009; Hayman et al., 2011).

Treatment of rabies

There is no effective treatment against rabies once the clinical signs manifest. However a treatment regime based on induced coma has been reported (Rodney et al., 2005). Suspected rabies cases in humans diagnosed on the basis of history of a recent dog bite are treated with post exposure vaccines.

Prevention and control of rabies

Vaccination remains the most effective control measure against rabies. In countries that have been able to implement effective vaccination campaigns, rabies has been erradicated. Vaccination of domestic animals and oral vaccination of wildlife coupled with educational programs conducted by public health authorities have greatly reduced the number of human cases (Schneider et al., 1995; Krebs et al., 1995; Meslin et al., 1994; Haupt et al., 1999). An ideal rabies vaccine is immunogenic, genetically stable, cheap, thermostable, and effective when orally administered to wide range of mammalian species and should provide long lasting immunity (Rupprecht and Kenny, 1988; Wandeler, 1991; Kilm et al., 1992; Artois et al., 1993; 1997). The types of rabies vaccines in use include: (1) inactivated virus preparations (inactivated nervous tissue origin and inactivated cell culture preparations) used in humans and domestic animals, (2) recombinant vaccines such as VR-G (vaccinia-rabies glycoprotein recombinant virus), (3) subunit vaccines based on specific viral proteins, and (4) DNA based vaccines designed for use in pre- and post-exposure treatment (Bunn et al., 1988; Rupprecht and Kenny, 1988; Wunner et al., 1983; Drings et al., 1999; Perrin et al., 2000; Lodmell and Ewalt, 2001). The cost of production of inactivated vaccines has hindered the full implementation in many developing countries. Subunit vaccines may not contain sufficient variety of epitopes for T-cells and as such may not elicit desired immune responses in the host population (Brown et al., 1993). Although some countries have had relatively good success in rabies control through the use of vaccines for dogs and dog registration, most developing countries in Africa and Asia have not been able to achieve the same level of success. This is mainly due to failure to implement the recommended procedures for rabies control [World Health Organisation (WHO) report.

1989], endemicity of the disease, lack of proper infrastructure, limited access to preventive medicine, and also because rabies is not a high priority disease in these countries (WHO report, 1984; Joshi and Bogel, 1988). In Zambia, rabies control is mainly based on the use of vaccines, both locally produced (Balmoral strain) and imported (Rabisin), as well as stray dog population control.

4. Molecular statistical tools for examination of pathogens

Population genetics

Population genetics is the study of the distribution and change of allele frequency under the influence of natural selection, genetic drift, mutation and gene flow with respectt to factors such as recombination, population subdivision and population structure. Population genetics attempts to explain phenomena such as adaptation and speciation. A population genetic analysis employing the use of microsatellite data, analyses the population structure of an organism through the determination of similarities, levels of genetic differentiation, and genetic linkage among individuals in a population.

1. Similarity analysis.

Similarity analysis provides a statistical test to assess whether significant difference between two or more groups of sampling units occurs. It can be performed using various computer-based software i.e. Excel microsatellite toolkit and GenAlEx 6. Excel microsatellite toolkit uses an allele sharing co-efficient (Bowcock et al., 1994) to calculate similarities among genetic data (Park, 2001) and GenAIEx 6 uses a similarity matrix to generate a graphical representation of this similarity (Peakall., 2006) known as

principal component analysis (PCA). PCA is defined as a mathematical algorithm that reduces the dimensionality of the data by identifying directions (principle components) along which the variation in the data is maximum while retaining the majority of the variation in the data set (Jollife, 2002). The plotting of samples provides the possibility to visually determine the similarities and differences between samples, and whether these samples can be grouped.

2. Genetic differentiation

Genetic differentiation is defined as the accumulation allelic frequency differences between completely or partially isolated populations as a result of evolutionary forces such as selection or genetic drift. Species are naturally subdivided into herds, flocks, colonies, etc. Environments greatly influence subdivision, which in turn decreases heterozygosity i.e. the mean percentage of heterozygous individuals per locus, generating genetic differentiation through natural selection and genetic drift. Wright's F index is used to determine the level of genetic differentiation. It is the equivalent of the reduction in expected heterozygosity with random mating at one level of the population hierarchy relative to another level that is more inclusive. Wright's F index can be calculated using F_{STAT} computer package version 2.9.3.2.

3. Genetic linkage analysis

Genetic linkage is the ability of some genes located proximal to each other on a chromosome to be inherited together during meiosis. Genes on loci located near to each other i.e. genetically linked, are not likely to separate onto different chromatids through chromosomal crossover. Genetic linkage analysis thus aims at assessing the frequency

of occurrence of shared and unique genes or alleles in a population, with low frequency indication possibility of a sub-structuring population and a high frequency indicating a homogenous, panmictic population. Genetic linkage analysis is calculated using a computer based online software called linkage analysis (LIAN). LIAN tests the null hypothesis of linkage equilibrium (LE) by calculating a quantification of linkage equilibrium/linkage disequilibrium called the standardized index of association (I_A^S) (Haubold et al., 2000). The statistical independence of alleles at all pairwise combinations of loci under study characterizes LE and this independent assortment was initially tested by LIAN by determining the number of loci at which each pair of multilocus genotypes (MLG) differs. The mis-match values from this distribution were then used to calculate the variance (V_D) which was then compared to the variance expected (V_E) for LE. Monte Carlo (MC) computer simulation was used to test the null hypothesis that $V_D = V_E$. The computer software calculates a 95% confidence limit L (L_{MC}). When V_D is greater than L, the null hypothesis of LE is discarded.

Through the study of population genetics, assessment of population structures of various organisms such as *Babesia bovis* (Simuunza et al., 2010) *Plasmodium falciprum* (Macleod et al., 2000), and *T. annulata* (Weir et al., 2007), have provided a variety of valuable insights

Phylogenetic analysis

Phylogenetic analysis is defined as the study of the evolutionary relationships among groups of organisms i.e. species. It involves the analysis of molecular sequencing data and morphological data matrices (Felsenstein, 1988; Miyamoto and Cracraft, 1991). A majority of phylogenetic studies are centered on the analysis of conserved genes in

organisms. Genetic relationships among organisms once clearly understood can provide valuable information on the geography, origin and lineage of the organism. This information might then be used to determine transmission patterns (Brown, 1994). Phylogenetics also provides information on the evolutionary history of specific genes and species (Fitch, 1995; Nei, 1994; Li and Grauer, 1991). Phylogenetic studies aim at estimating the time divergency between organisms from the point of the last shared ancestor and reconstruct the correct genealogies between organisms (Smith, 1998). It involves analyzing nucleotide or amino acid sequence data using multiple sequence alignment, distance methods and character based methods.

1. Multiple sequence alignment

This is one of the most utilized tools in molecular biology (Thompson et al., 1994). Sequence alignments are used to infer homology and functional relationships between sequences thus assisting in the construction of phylogenetic trees, predicting PCR primer sequences, and also sequences for restriction enzyme sites (Thompson et al., 1994). Several computer based softwares are employed in aligning sequences such "clustal W" program which uses pairwise alignment to calculate distance matrices (measure of similarity of the sequences), which are then used to find clusters of similar sequences. The jukes and cantor 1-parameter model and the kimura 2-parameter model are used to study the dynamics of nucleotide substitution (Li and Grauer, 1991; Page and Holmes, 1996)

2. Distance methods

This method involves the calculation of pairwise genetic distances for all the species

under study in a similarity matrix. An algorithm is then used to construct a tree based on the functional relationships among the distance values. Clustering commences with two sequences or species under study with the smallest distance and then gradually adding more distantly related sequences. The neighbor joining (NJ) method is an example of a distance method. In the NJ method, the nearest pairs of sequences are sought resulting in the shortest overall length of an unrooted tree. This tree can be rooted by assuming a degree of constancy in the molecular clock by placing the root along the longest branch and also by including a sequence called an out group, which completely branches outside the tree.

3. Boot strapping

The topology of the phylogenetic tree is an estimate of the relationships amongst the sequences. However it does not give information on the statistical significance of their structure. In this regard, jackknifing and bootstrapping are used to estimate the variability of a true estimate (Felsenstein, 1985; Dopazo. 1994). Bootstrapping is a non-parametric statistical procedure that involves re-sampling the data used to generate the tree. Using this data, a distribution of data sets is then generated and from which a new tree is produced (Felsenstein, 1985). It basically evaluates the significance of a particular topology. This method assumes that the sites in the alignment have independently evolved on the same phylogeny and are identically distributed.

Through phylogenetic studies of rabies viral genes i.e. Nucleoprotein and glycoprotein genes, several lineages of rabies virus have been reported (Sacramento et al., 1991; Kissi et al., 1995; Bourhy et al., 2008; Kissi et al., 1995; David et al., 2007).

5. Aim of this study

The main aim of this study was to demonstrate how molecular analytical tools can employed in the study of infectious diseases. I therefore, employed the use of two molecular analytical tools: (1) population genetic analysis to study thieleriosis, and (2) phylogenetic analysis to study rabies virus in Zambia. To date, no studies have been undertaken to investigate the population structure of *T. parva* and the phylogenetics of rabies virus in Zambia. In an attempt to achieve the main aim of the study, the following objectives were addressed:

As regards to theileriosis;

(i) To determine the population structure of *T. parva* in eastern and northern Zambia.

(ii) To determine whether gene flow occurs between *T. parva* populations sampled from each area.

(iii) To determine whether the *T. parva* populations from the sampled areas consist of a single or multiple distinct populations.

And with respect to rabies;

(i) To establish the lineage of field rabies viruses in central Zambia by analyzing the nucleotide sequences of the N gene of viruses from Zambia and the rest of the world.

(ii) To determine genetic diversity of rabies virus, based on the G gene analysis of field rabies samples, in different host species and regions of central Zambia,

(iii) To design appropriate primers for the RT-LAMP assay for diagnosis of RABV in Zambia using the nucleoprotein gene sequences.

It is believed that acquisition of the above information will lead to a better understanding of the dynamics of the two diseases under this study. This information could further contribute to the proper implementation of effective disease control measures.

Chapter I

Population genetic analyses and sub-structuring of *Theileria parva* in the northern and eastern parts of Zambia.

Introduction

Theileriosis is an economically important disease of cattle in Eastern, Central and Southern Africa. The disease, caused by the protozoan haemoparasite *T. parva*, is transmitted by the 3-host tick *R. appendiculatus* (Wallade et al., 2003). The severity of the disease manifests differently in various breeds of cattle, with the zebu cattle (*Bos indicus*) being more resistant than the exotic breeds (*Bos taurus*) against both the parasite (Guilbride et al., 1993) and the vector (Jongejan et al., 1989; Fivaz et al., 1990). Theileriosis causes high levels of mortality in *taurine* breeds (Uilenberg et al., 1995) and both high morbidity and mortality in indigenous 1- 6 month old calves (*Bos indicus*). Full-scale epidemics affecting all age groups of indigenous breeds may occur (Berkvens et al., 1991), resulting in reduction in productivity.

In natural parasite populations, sexual recombination plays a major role in producing genetic diversity and as such, a sound understanding of the levels of parasite genetic diversity is prime in the design of vaccines and chemotherapeutics. Publication of the *T. parva* genome sequence has facilitated the development and use of microsatellite markers in the study of population genetics of these species. Previously, most studies were more concerned with identifying diversity in field isolates. Geysen et al (1999), using a combination of restriction fragment length polymorphism (RFLP) techniques was able to carry out a molecular epidemiological study of *T. parva*. In Zambia, a previous study using Southern blotting with restriction digested DNA probed with '*Tpr'*

(*T. parva* repeat) locus and telomere probes reported *T. parva* was relatively homogenous, in contrast with the high level of heterogeneity observed in a previous Kenyan study (Bishop et al., 1997; Conrad et al., 1987). Through RFLP-PCR encompassing three loci (*PIM, p104,* and *p150*) showed that *PIM* was the most polymorphic and as such might be used in the differentiation of isolates from two geographical areas in Zambia. The *p104* and *p15*0 genes were monomorphic in these isolates. The study also indicated the presence of a homogenous, epidemic structure characterized by clonal expansion of one of the vaccine introduced components dominating the population. A panel of polymorphic markers, 11 micro- and 49 mini-satellite has been identified (Oura et al., 2003) following the publication of the *T. parva* genome sequence (Gardner et al., 2005). Using subsets of these markers, the population structures of *T. parva* in Uganda and Kenya have been analyzed. A study in the Kenya (Odongo et al., 2006), using 30 micro-and mini-satellite markers (Oura et al., 2003) to genotype thirty six samples showed a high genetic diversity with a lack of geographical sub-structuring. Significant linkage disequilibrium (LD), through the analysis of allele association at all pair-wise combinations of loci, was observed both when all samples were treated as one population and when each area was treated separately, suggesting a limited level of genetic exchange. The reason for this LD might be due to the fact that sampling was performed over a range of time points (1968 – 2005) and some samples were isolated during vaccine trials. The majority of samples were also cultured before genotyping and as such, selection during *in vitro* expansion and temporal sub-structuring might have led to the LD observed. The study in Uganda (Oura *et al.* 2005) was carried out in three geographically separate populations (Lira, Mbarara and Kayunga). In this study (Oura et al., 2005), samples were obtained from

animals between the ages of 3 to 9 months in order to overcome the problem of the presence of multiple genotypes in a high proportion of samples and as such making it possible to determine the predominant genotype directly. The majority of these samples contained a single predominant allele at each locus. These samples were then genotyped using twelve micro- and mini-satellite markers, evenly dispersed across the four chromosomes. With very few common multi-locus genotypes (MLG) among the three areas, a high level of genetic diversity was observed within each of the three areas. Limited genetic differentiation was observed between Lira and Mbarara populations in contrast to the moderate differentiation observed between Lira and Kayunga populations, and between Mbarara and Kayunga. Evidence of sub-structuring with a sub-group of six MLGs, dissimilar to any other group was observed in the Mbarara population. Omission of these six MLGs from the analysis showed little or no genetic differentiation among the three populations. Combining the three areas as one population produced LD attributed to geographical or genetic isolation. This LD persisted even when each area was analyzed separately. However an epidemic structure in Lira was observed, characterized by a disappearance of LD when identical isolates from this area were treated as one population. When both the subgroup and a set of identical isolates were removed from the analysis, linkage equilibrium (LE) was observed in the Mbarara population. LD persisted in Kayunga population despite the treatment of isolates with similar MLGs as one group, implying the presence of infrequent genetic exchange in this population. This study ultimately demonstrated the occurrence of significant level of genetic exchange.

Population genetic analysis of *T. parva* (Oura et al., 2005) and *T. annulata* (Weir et al., 2007) have shown the presence of frequent genetic exchange within populations

providing strong evidence of the existence of a sexual stage in the life-cycle of these parasite species i.e. evidence of genetic exchange and recombination in the vector. The high levels of recombination between loci also suggest frequent crossing-over during meiosis. Recombination and allelic assortment have been demonstrated using micro- and mini-satellite genotyping of cloned *T. parva* parasite lines derived from mixed isolates after transmission through ticks. Also the analysis of loci encoding antigens recognised by cytotoxic T-cells has provided evidence for allelic assortment resulting in the generation of parasites with different antigen repertoires, highlighting recombination as a mechanism for generating antigenic diversity (Katzer et al. 2006).

Micro- and mini-satellite markers have been used to genotype several species of apicomplexan parasites and their vectors, revealing different population structures (Oura et al., 2005; Simuunza et al., 2011; Macleod et al., 2000; Anderson et al., 2000; Samb et al 2012; Melachio et al., 2011; Ouma et al., 2011). For example, a study on the population structure of *T. parva* in Uganda reported a mixture of genotypes in many isolates and LD in 3 populations isolated from different areas (Oura et al., 2005). When multiple isolates with identical genotypes were treated as a single isolate, the presence of an epidemic structure was seen in 2 of the populations, suggesting an intermediate between extreme clonality and panmixia (Oura et al., 2005).

A crucial and important aspect in population genetic studies is to determine the effect of random and non-random mating on the population structures of disease-causing agents and consequently on the epidemiology of the diseases (Tibayrenc et al., 2002). Information on the genetic exchange in *T. parva* populations has practical implications in disease control and prevention. For instance, populations with a high degree of genetic diversity arise when high levels of recombination occur. This information is

very important in vaccine development as it is easier to develop a vaccine against a clonal pathogen than a highly diverse pathogen. A panel of polymorphic micro-satellite markers was used to genotype *T. parva* positive cattle blood DNA in order to answer the following questions: (i) What is the population structure of the *T. parva* in eastern and northern Zambia? (ii) Does gene flow occur between *T. parva* populations sampled from each area? (iii) Do the *T. parva* populations from the sampling areas consist of a single or multiple distinct populations? To my knowledge, this is the first study on the population genetics of *T. parva* in Zambia.

Materials and methods

Sample collection and DNA preparation

About 10 milliliter (mL) of whole blood samples (n=142) were collected in heparinized tubes from indigenous and mixed breeds of cattle from Kanyelele (n = 62) and Kalembe (n=34) areas in Isoka district (n=96) of the Northern province (NP) and from Saukani area in Petauke district (n=46) of the Eastern province (EP) (Fig. 2) of Zambia in May 2008, after the wet season. Kanyelele is located approximately 20 kilometer (km) from Kalembe. Isoka and Petauke districts are approximately 600 km apart (straight line distance) and are separated by the North Luangwa, South Luangwa and Lukusuzi National Parks (NLNP, SLNP, and LNP, respectively, in Fig. 2).

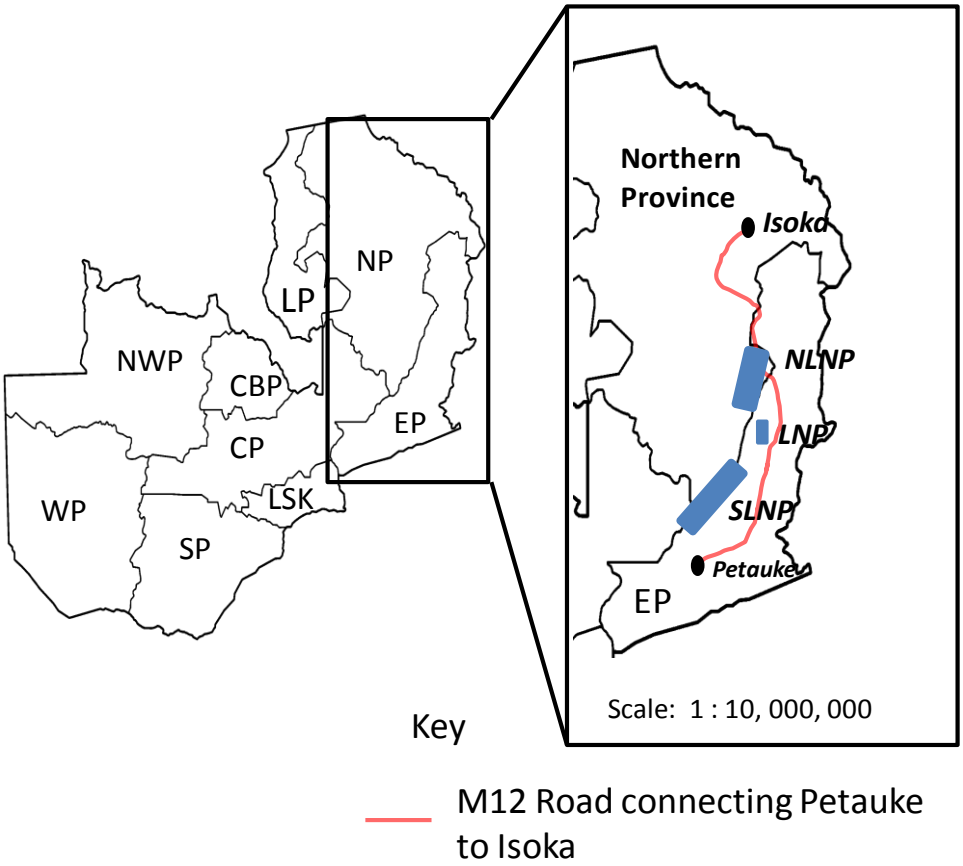

Figure 2 Map of the Republic of Zambia showing its nine provinces namely; the Northern (NP), Eastern (EP), Luapula (LP), Central (CP), Copperbelt (CBP), Lusaka (LSK) Southern (SP), Western (WP) and North-western (NWP) provinces. The *Theileria parva* samples used in this study were obtained from Isoka (NP) and Petauke (EP) districts. Isoka district is separated from Petauke district by the North Luangwa National Park (NLNP), Lukusuzi National Park (LNP), and the South Luangwa National Park (SLNP) (indicated by blue bars in the figure) and connected by the M12 commercial road (indicated in red).

Petauke and Isoka districts are connected by a commercial road (Fig.2) covering a distance of about 795 km. Whole genome DNA was extracted using DNAzol (Molecular Research Center, OH) following the manufacturer's instructions and stored at -20°C. A panel of 16 polymorphic microsatellite markers (Oura et al., 2003, Katzer et al., 2006, Katzer et al., 2010), representing the 4 chromosomes, was initially selected (Table 2) for genotyping of the samples. The following *T. parva* parasite laboratory stocks were used for the initial marker screening to determine which markers amplified more isolates and were sufficiently polymorphic for use in the genotyping of field samples from Isoka and Petauke districts: Onderstepoort (South Africa, year of isolation unknown), Serengeti (Tanzania, 1978), Muguga (Kenya, year of isolation unknown), Nyakizu (Rwanda, 1979), Entebbe (Uganda, 1980), Katumba (Burundi, 1981), Kiambu Z464/C12 and B8 (Kenya, 1972), Katete B2 (Zambia, 1989) and Buffalo Z5E5 (both origin and year of isolation unknown). These stocks were also used as Genescan control samples during the analysis of field samples. The recombinant DNA experiments were approved by the Hokkaido University.

T. parva screening

Whole blood DNA field samples were screened for *T. parva* DNA by using *T. parva*-specific p104 gene primers (Skilton et al., 2002). Template DNA (1 μL) was amplified in a 20 microliter (μL) reaction mixture as prescribed by the manufacturer using ExTaq polymerase (Takara, Japan). The PCR conditions were as follows: denaturation at 95°C for 5 minutes; 35 cycles at 95°C for 60 seconds, 63°C for 30 seconds and 72°C for 1 minute, followed by a final extension step of 5 minutes at 72°C. The amplified products were analyzed on 2% ethidium bromide pre-stained agarose gel.

Table 2: Panel of microsatellite markers used to genotype *Theileria parva* samples from Isoka and Petauke districts.

Marker	**Chromosome**	**Amplicon size (bp) of Muguga strain**	**Used in final analysis**	**Reference**
MS1	1	235-368	No	Oura *et al*., 2003
MS62	1	271	No	Katzer et al., 2010
MS48	**1**	223	**Yes**	Katzer et al., 2006
MS5	1	206-444	No	Oura et al., 2003
MS66	1	266	No	Katzer et al., 2010
MS67	1	245	No	Katzer et al., 2010
MS77	2	270	No	Katzer et al., 2010
MS71	**2**	252	**Yes**	Katzer et al., 2010
MS74	**2**	246	**Yes**	Katzer et al., 2010
MS75	**2**	244	**Yes**	Katzer et al., 2010
MS72	**2**	230	**Yes**	Katzer et al., 2010
MS51	**3**	161	**Yes**	Katzer et al., 2006
MS53	**3**	208	**Yes**	Katzer et al., 2006
MS57	**4**	118	**Yes**	Katzer et al., 2006
MS58	4	300	No	Katzer et al., 2006
MS59	**4**	111	**Yes**	Katzer et al., 2006

PCR amplification and analysis of microsatellite loci

PCR was performed using primers (Table 2) designed to amplify each of the 16 repeat regions on each of the *T. parva* stocks isolated from different geographical areas. The forward primer in each primer marker set was fluorescently labeled. The 20 µL PCR mixture used comprised of 10 ng of template DNA, 10 µL of 2x Amplitaq Gold master mix (Invitrogen, CA), 0.25 µM of each primer and distilled water. The PCR conditions were as follows: denaturation at 95°C for 5 minutes; 35 cycles at 95°C for 15 seconds, 50 - 58°C for 60 seconds and 72°C for 60 seconds, followed by a final extension step of 5 minutes at 72°C. The amplified products were observed on 1.5% ethidium bromide pre-stained agarose gels to determine the success of PCR amplification. To achieve high genotyping resolution of field samples, microsatellite PCR products were denatured and then capillary electrophoresed in an ABI 3130 genetic analyzer (Applied Biosystems, CA). DNA fragment sizes were analyzed relative to the ROX-labeled GS600 LIZ size standard (Applied Biosystems) using Gene Mapper software (Applied Biosystems). This facilitated the resolution of multiple products with 1 bp difference in a single reaction. Multiple products from a single PCR reaction indicated the presence of mixed genotypes. The output data from the genetic analyzer were provided as the area under the peak of each allele (quantitative measurement), with the predominant allele possessing the greatest peak area. In this way, the predominant allele at each locus was identified for each sample, and this data was combined to generate a MLG representing the most abundant genotype in each sample. Only the alleles with the prescribed base pair range were used to generate the MLG and samples from the same area were electrophoresed and genescaned on the same plate. Separate PCRs were also carried out for different regions.

Data analysis

Excel microsatellite toolkit (http://animalgenomics.ucd.i.e/sdepark/ms-toolkit/) was used for the similarity comparison of the MLGs (Park, 2001) and PCA was constructed using the Excel plug-in software GenAIEx6 (http://www.anu.edu.au/BoZo/GenAIEx/) (Peakall et al., 2006). F_{STAT} computer package version 2.9.3.2 was used to calculate estimates of F statistics for population genetic analysis (http://www2.unil.ch/popgen/softwares/fstat.htm). LIAN (http://adenine.biz.fh-weihenstephan.de/lian/) was used to calculate a quantification of linkage equilibrium/linkage disequilibrium called the standardized index of association (I_A^S) (Haubold et al., 2000) in order to test the null hypothesis of linkage equilibrium.

Multiplicity of infection

The majority of the samples in this study possessed several alleles at one or more loci, representing a mixed infection. The mean number of alleles for the nine loci in each sample was calculated and this index value represented the multiplicity of infection within each sample. The overall mean for the index values for each sample was then calculated to provide the multiplicity of infection for each region.

Results

PCR screening

The screening of cattle blood samples (n=142) using the p104 gene primers showed a combined *T. parva* positive percentage of 54.9% [95% confidence interval (CI): 46.7–63.1%, 78/142] from both districts. The percentage of positive samples within each district was 44.8% (95% CI: 34.8–54.8%) (23 from Kanyelele and 20 from

Kalembe, 43/96) and 76.1% (95% CI: 63.9–88.4%, 35/46) for Isoka and Petauke (Saukani area), respectively.

Marker diversity and allelic variation

A panel of 9 polymorphic microsatellite markers out of the initial 16 markers (Table 2), representing the 4 chromosomes of *T. parva*, was used to genotype 61 *T. parva* positive samples, that is 33 (14 from Kalembe and 19 from Kanyelele areas) from Isoka district and 28 from Petauke district (Saukani area). Seven markers that either failed to produce signals on gel electrophoresis or exhibited reduced polymorphism were excluded from the final analysis (Table 2). Seventeen samples were excluded from the final analysis because most of the markers failed to produce signals in them on gel electrophoresis. The reason for the failure of amplification of the target regions in these samples (n=17) was probably due to the poor quality of the DNA.

The maximum number of alleles identified by each marker in each population ranged from 3 (MS72) to 12 (MS71) for Isoka and 4 (MS57 and MS75) to 12 (MS53) for Petauke (Table 3). Marker MS53 was the most polymorphic, identifying 19 alleles in both populations whereas MS57 was the least polymorphic, identifying only 6 alleles. Similar gene diversities across all loci were observed between Isoka and Petauke populations except for markers MS48, MS59 and MS72 that exhibited slightly larger differences between the 2 populations (Table 3).

Allele frequencies at each locus for Isoka and Petauke populations were assessed with both populations showing high levels of diversity. Markers MS48 and MS53 showed only unique alleles in both populations (Fig. 3A). Of the 12 alleles identified by MS48, 5 were from Isoka and 7 from Petauke, while out of the 19 alleles identified by MS53, 7

were from Isoka and 12 from Petauke (Fig. 3A). The remaining markers showed both unique and shared alleles. For example, MS71 showed 11 and 5 alleles specific to Isoka and Petauke populations, respectively, with a single shared allele between Isoka and Petauke (Fig. 3A). MS51 also showed 5 and 3 unique alleles specific to Isoka and Petauke, respectively, with 3 shared alleles (Fig. 3A). Marker MS59 showed the lowest frequency of unique alleles (data not shown). A total of 91 (46 from Isoka and 45 from Petauke districts) unique alleles and 15 shared alleles were observed (Fig. 3B).

Table 3: Allelic variation among *Theileria parva* from Petauke and Isoka districts of Zambia

		N	MS48	MS51	MS53	MS57	MS59	MS71	MS75	MS72	MS74
Alleles within population	Petauke	28	7	6	12	4	6	6	4	6	9
	Isoka	33	5	8	7	5	5	12	5	3	11
	Overall	61	12	11	19	6	9	17	7	8	17
Gene diversity	Petauke	28	0.820	0.794	0.833	0.648	0.571	0.783	0.696	0.743	0.706
	Isoka	33	0.502	0.813	0.752	0.534	0.331	0.837	0.604	0.504	0.695

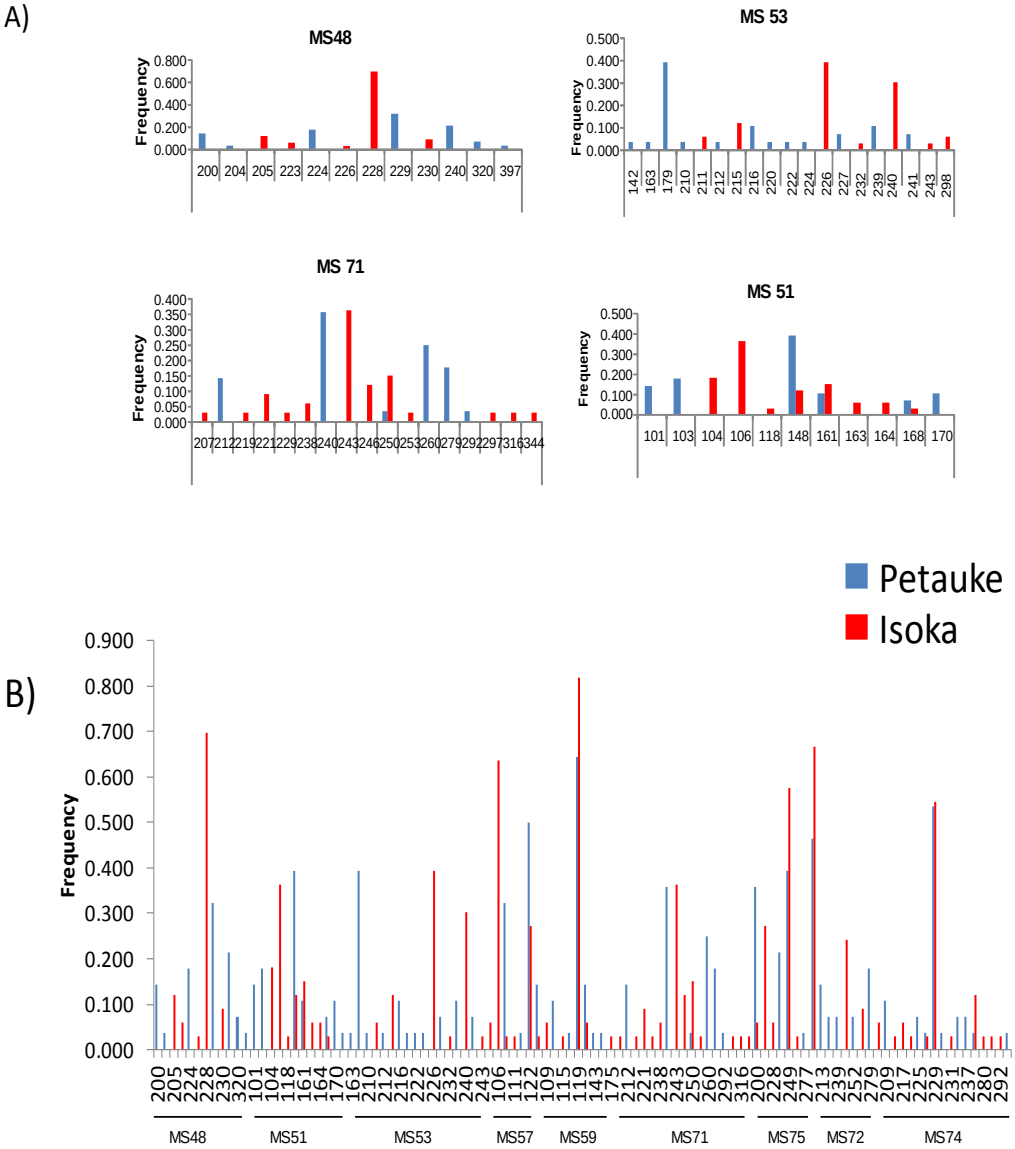

Figure 3 The allele frequencies of alleles in field populations of *Theileria parva* from Petauke and Isoka districts of Zambia. The frequency of each predominant allele in the samples from the Petauke and Isoka populations was calculated and presented in the form of histograms. (A) The results for MS48 and MS53 show the presence of unique alleles and the absence of shared alleles. The remaining loci generally show a greater proportion of unique alleles as compared to shared alleles, as represented by MS 71 and MS 51. (B) The overall allele frequency in both populations shows a greater number of unique alleles and a reduced number of shared alleles. Multi-locus genotype (MLG) data was used to generate the histograms. The frequency of each predominant allele was calculated as a proportion of the total of each marker.

Similarity analysis

Evidence of sub-structuring was assessed by using PCA. Independent clustering of samples from Isoka and Petauke districts was observed, suggesting a state of geographical sub-structuring (Fig. 4A). To determine the levels of sub-structuring on a finer scale, a separate PCA was constructed for samples from each district. Most of the samples from Isoka district, despite originating from 2 different areas (Kalembe and Kanyelele), occupied the same quadrants, except for four samples from Kanyelele which can be seen in the upper right quadrant of the PCA (Fig. 4B). The samples from Petauke district (Saukani area) occupied all quadrants. Five samples in the upper left, 4 in the upper right and 2 in the lower left quadrants, appeared to exhibit some level of independence from the rest of the samples that had congregated mainly in the lower right quadrant, lower parts of the upper left and right quadrants and the left part of the lower left quadrant but due to their reduced number i.e. low contribution rate (Fig. 4C), further investigation into these possible differences was not carried out.

Population diversity and differentiation

Estimated heterozygosities ($H_{e)}$ were calculated for samples from each district (Fig. 4). The (H_e) and mean number of genotypes per locus for Kanyelele and Kalembe areas in Isoka were 0.675 and 5.67, and 0.503 and 3.67, respectively, with the population from Kanyelele being more diverse than that from Kalembe. The overall estimated heterozygosity (H_e) and mean number of genotypes/locus for Isoka district was 0.619 and 6.78, respectively (Table 4). The H_e and mean number of genotypes/locus for Petauke district was 0.733 and 6.67, respectively (Table 4). The overall estimated heterozygosity for Petauke district showed that samples from this district were more

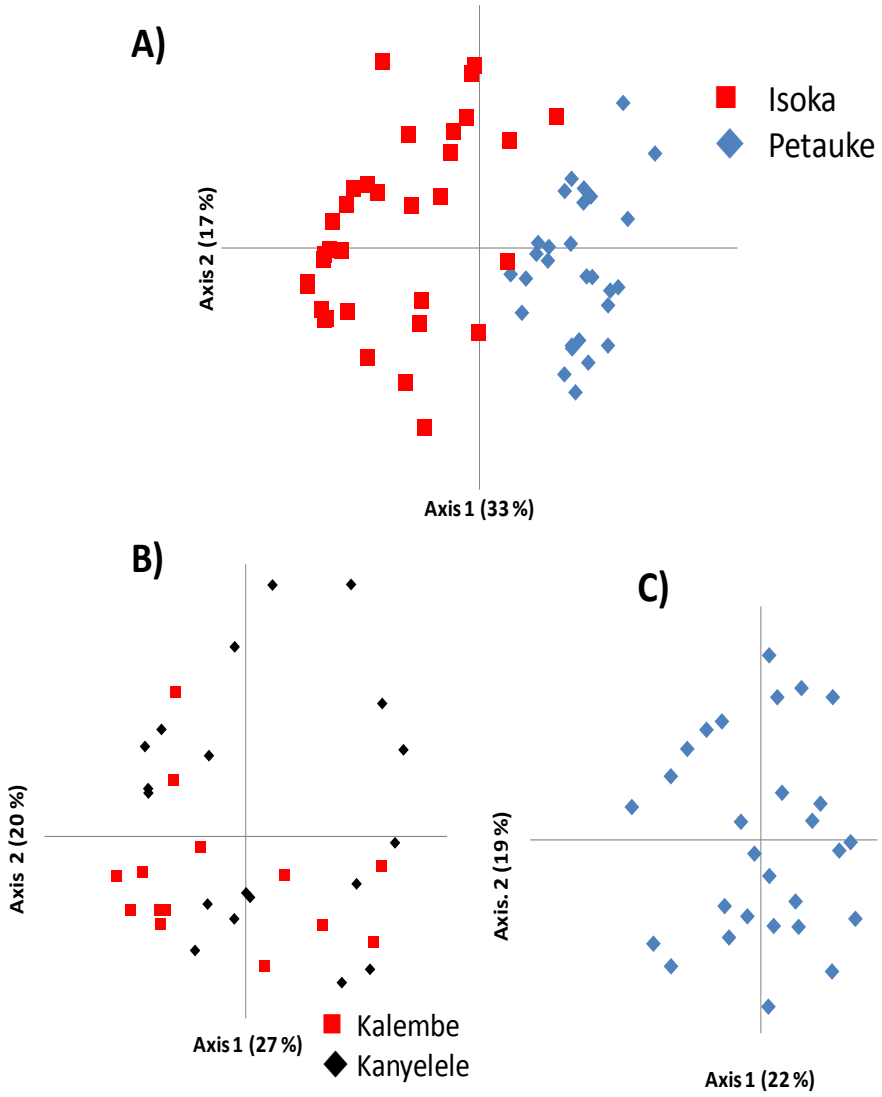

Figure 4 Principal component analysis (PCA) of *T. parva* populations from Petauke and Isoka districts of Zambia. The proportion of variation in the population dataset explained by each axis is shown in parentheses. PCA was performed using multi-locus genotype data from Petauke and Isoka districts. (A) Geographical sub-structuring between populations from Petauke and Isoka districts. (B) Homogenous population from Isoka district with the Kanyelele population showing more diversity by occupying all 4 quadrants while the Kalembe population occupies only 3 quadrants. (C) Saukani population from Petauke district.

Table 4: Population genetic analyses of *Theileria parva* from Petauke and Isoka districts of Zambia

Population	N	H_e	Mean numbers genotypes/locus	I_A^S	V_D	L	P-value	Linkage	F_{ST}
Between districts	61	0.745	11.78	0.078	2.457	1.677	< 0.001	LD	0.178
Isoka district									
Kanyelele area	19	0.675	5.67	0.075	2.637	2.066	< 0.001	LD	
Kalembe area	14	0.503	3.67	0.243	5.763	2.859	< 0.001	LD	
Isoka overall	33	0.619	6.78	0.104	3.418	2.254	< 0.001	LD	0.049
Petauke district									
Saukani area	28	0.733	6.67	0.027	1.979	1.910	0.015	LD	0.154

N: number of samples, H_e: estimated heterozygosity, I_A^S: standard index of association, V_D: mismatch variance (linkage analysis), L: upper 95% confidence limit of Monte Carlo simulation (linkage analysis) and LD: linkage disequilibrium and LE: linkage equilibrium.

diverse than those from Isoka (Table 4) despite both districts having a similar number of genotypes per locus. The overall heterozygosity for the two districts was 0.745 with a mean number of genotypes/locus of 11.78.

To measure the levels of genetic differentiation, Wright's F index was calculated between Isoka district and Petauke district populations, for the Isoka sub-populations and also within Petauke district (Table 4). An F_{ST} value of 0.178 was observed between Isoka and Petauke district populations (Table 4), showing significant genetic differentiation. The F_{ST} value for the Isoka populations (Kanyelele and Kalembe) was 0.049, implying moderate differentiation between the 2 populations (Table 4). The F_{ST} value for Petauke (Fig. 4C) was 0.154, showing significant differentiation.

Genetic analysis

In order to determine whether the parasite populations within the two districts comprised a single panmictic population with a high degree of genetic exchange, the level of LE of the alleles at pairs of loci was measured using the standard index of association (I_A^S). The I_A^S measures the association between alleles at pairs of loci, with I_A^S values close to 0 or negative indicating panmixia and those significantly greater than 0 indicating non-panmixia (Haubold et al., 2000). The variance of pairwise differences (V_D) between the data and that predicted for panmixia (V_E) and L, the 95% confidence interval for V_D relative to the null hypothesis, were calculated in order to test the hypothesis of panmixia. Using this analytical method, when the V_D value exceeds the L value, LD is indicated and the null hypothesis of panmixia is discarded. When the V_D is less than L, LE is indicated and the null hypothesis of panmixia is accepted. When Isoka and Petauke samples were treated as a single population, a I_A^S value of 0.078 ($p<$

0.001) and a V_D value greater than the value of L was obtained, suggesting LD (Table 4). To test the hypothesis of geographical sub-structuring, the I_A^S, V_D, and L values were calculated separately for samples from each district. The overall I_A^S value for Isoka district was 0.104 ($p< 0.001$) and the V_D value was greater than the L Value, indicating a state of LD. The I_A^S values for Kanyelele and Kalembe populations within Isoka were 0.075 ($p< 0.001$) and 0.243 ($p< 0.001$), respectively, and both areas showed a V_D value greater than the L value, suggesting LD (Table 4). An overall I_A^S value of 0.027 ($p< 0.015$) and a V_D value greater than the L value indicating LD were obtained for the Petauke population.

Multiplicity of infection

The multiplicity of infection for Isoka district was 2.16 while that of Petauke district was 2.44. Kanyelele and Kalembe areas of Isoka district showed multiplicity of infection of 2.22 and 2.06, respectively (Table 5).

Discussion

In order to establish effective control measures as well as to assess the effectiveness of the current control measures, information on the population structure of *T. parva* with regard to its epidemiology is important. In this study, I analyzed samples from Kanyelele and Kalembe areas in Isoka district (NP) and from Saukani area in Petauke district (EP) of Zambia, which are approximately 600 km (straight line distance) apart and are separated by the NLNP, SLNP and LNP (Fig. 2). To achieve this, I employed micro-satellite analysis, an effective way of studying population structures of a wide range of species.

Table 5: Multiplicity of infection in Petauke and Isoka districts

	N	Mean	SD	Minimum	Maximum
Petauke district					
Petauke overall	28	2.44	0.44	1.22	3.77
Isoka district					
Kanyelele	19	2.22	0.26	1.77	3.00
Kalembe	14	2.06	0.15	1.88	2.33
Isoka overall	33	2.16	0.23	1.77	3.00
Petauke and Isoka districts (overall)	61	2.28	0.36	1.22	3.77

SD: Standard deviation.

Micro-satellite analysis enables direct genotyping of parasite isolates directly from host blood samples by using specific primers. Recently, a panel of polymorphic micro- and mini-satellite markers for *T. parva* was identified (Oura et al., 2003; Katzer et al., 2006; Katzer et al., 2010). A panel of 16 of these microsatellite markers was initially chosen and only 9 were used in the final analysis (Table 2). All the markers (locus pairs) on the same chromosome were over 182 kbp apart. It is therefore unlikely that any of the markers could have been physically linked. To perform the population genetic study of *T. parva*, a haploid organism, an MLG was constructed for each sample, by assigning a single predominant allele for each marker at each locus for each sample. This method of selecting the most predominant allele at each locus from mixed infections is currently fairly standard although it has limitations (Oura et al., 2005; Simuunza et al., 2011; Morrison et al., 2008). There are several potential short comings of selecting predominant alleles from mixed populations to form an MLG: (i) each particular marker does not always amplify the predominant allele from all strains (ii) the same strain is not always amplified by a particular marker in all the samples, (iii) target regions in different strains that have the highest sequence homology with markers are easily amplified whether they are representative of the predominant strain or not and (iv) all markers in the selected panel of markers most likely do not amplify the same strain in different samples. To avoid these short falls in mixed infections cloning out samples is one solution or chosing samples with only a single allele and then proceeding with analysis and generation of an MLG. In this study, however, I used a quantitative method to generate an MLG and by so doing, I assume that this MLG presented the closest representative of the dominant strains across the whole sample size.

Allele frequencies at each locus from the Isoka and Petauke populations showed a

high number of unique alleles in each population (Fig. 3). The data obtained using markers MS48 and MS53 indicated the possibility of genetic differences between Isoka and Petauke populations as evidenced by the complete lack of shared alleles at these 2 sites (Fig. 3A). The separation of alleles on loci MS48 and MS53 might also be due to artifacts and as such the data on these loci should be treated with caution; however analysis of the overall data with and without loci MS48 and MS53 produced similar results (data not shown). The increased number of unique alleles versus shared alleles observed when Isoka and Petauke populations were treated as a single population (Fig. 3B) suggests a state of genetic and geographical sub-structuring. Genetic and geographical sub-structuring was also observed on PCA where samples from Isoka and Petauke districts clustered separately (Fig. 4A). A few samples (n=3) from Isoka district occupied the same quadrants as those from Petauke (Fig. 4A). This state of genetic and geographical sub-structuring indicated by allele frequencies (Fig. 3C) and PCA (Fig. 4A) was further confirmed by the significant differentiation (F_{ST} = 0.178) observed between the population from Isoka and Petauke districts. Furthermore, a state of LD with a I_A^S value greater than 0 was also observed when the Isoka and Petauke populations were treated as a single population (Table 4), indicating the absence of random mating between *T. parva* from the 2 populations and consolidating the state of genetic and geographical sub-structuring. The population from Isoka exhibited less diversity than that from Petauke. These observations therefore, suggests that the parasite populations from these 2 areas, while comprising a similar number of genotypes per locus, exist as separate populations because there is little or no movement of animals between these 2 districts, which in turn is caused by their separation by physical geographical barriers i.e. the national parks (Fig. 2). These findings are in agreement

with a study on a *T. parva* population wherein geographical sub-structuring was observed between the population from 2 areas separated by a distance of 300 km and a lake (Oura et al., 2005).

The population from Kanyelele occupied all four quadrants while that from Kalembe only occupied three quadrants (Fig. 4B). Although the Kanyelele population was more diverse than the Kalembe population in Isoka district, only moderate genetic differentiation (F_{ST} = 0.049) was observed between the 2 populations (Table 4). This was indicative of the existence of similar genotypes of *T. parva* in both areas. However, the ability of the parasites to randomly mate was restricted as shown from the state of LD, both at the district level and within each sub-population (Table 4). In the Petauke population, significant genetic differentiation (F_{ST} = 0.154) and a state of LD was also observed. This state of LD in Isoka (overall and in sub-populations) and Petauke might be due to the lack of mixing or movement of animals resulting in a lack of gene flow between the populations that can likely be attributed to the relative isolation of the sample areas from each other and the resulting restricted circulation of genotypes. Despite Kanyelele and Kalembe being far apart, animals from the two areas tend to share grazing grounds during the dry period because of the scarcity of pastures and thus it can be hypothesized that the sharing of grazing grounds allows the introduction of genotypes indigenous to one area into another area. However, due to the availability and close proximity of moderate vegetation for animal grazing during the month of May and the preceding months, it is unlikely that animals from Kanyelele and Kalembe areas would have mixed or shared grazing grounds as they will tend to graze pastures closer to their villages. This lack of mixing of animals would have prevented the introduction of genotypes from one area into another and vice-versa, hence the state of restrictive

circulation. The tick vector population is also low at this particular time of the year, thus reducing the challenge levels of infection (i.e. low levels of transmission). It can further be hypothesized that the low challenge levels of infection is also likely to give rise to the presence of fewer genotypes of *T. parva* which may result in the state of LD and non-panmixia observed in the majority of areas/regions. This is in agreement with a report in which *Plasmodium falciparum* populations showed strong LD, extensive population differentiation and low diversity in regions of low transmission (Macleod et al., 2000). The state of LD in these populations might also be due to the presence of epidemic strains (Maynard et al., 1993). A large sample size, coupled with tick vector ecology data of a wider geographical area in Petauke and Isoka districts, is required to completely validate the LD states observed in this study.

The majority of samples analyzed in this study comprised mixed infections, with Petauke district showing a higher multiplicity of infection than Isoka district (Table 5). Within Isoka district, Kanyelele area also showed a higher multiplicity of infection than Kalembe area (Table 5). Several reasons may be advanced for this situation including (i) transmission intensity reflected by the tick burden on cattle, (ii) the level of infection of the parasite in the ticks, (iii) cattle host factors such as age and breed, (iv) farming systems and (v) the geography of the respective regions. However, this data was inconclusive and as such the effect of these factors could not be further investigated.

Chapter II

Molecular epidemiology and a loop-mediated isothermal amplification method for diagnosis with rabies virus in Zambia

Introduction

Rabies is a fatal and devastating zoonotic disease of humans and other warm-blooded vertebrates and is caused by a RABV, which belongs to the genus *Lyssavirus* and the family *Rhabdoviridae*. This disease has a worldwide distribution and is responsible for 55,000 human deaths annually (WHO, 2005). Furthermore, 44% of all human cases occur in Africa (WHO, 2005). Although most African countries have functioning veterinary and medical departments, they generally lack the gold standard test, DFAT, for confirmation. With other human diseases such as malaria taking precedence, the African governments are unable to provide the necessary funding for monitoring and control of rabies, which remains problematic (Swanepoel, 2004).

The Republic of Zambia is located south of the equator in south-central Africa (Fig. 1). Rabies, a notifiable disease, is present in all parts of Zambia. About 1,088 DFAT-confirmed rabies cases were reported in Zambia from 1985 to 2004 (Munang'andu et al., 2010). Furthermore, the National Livestock Epidemiology and Information Center in Zambia reported 268 DFAT-positive rabies cases from 1999-2009 (Table 6). The DFAT, using fluorescein isothiocyanate (FITC)-labeled anti-rabies monoclonal immunoglobulin (Centocor, PA), is the main diagnostic tool for rabies in Zambia. Generally, molecular diagnostic methods (PCR) for amplification of viral RNA, are easier and more reliable. These methods, however, have not been established in Zambia because the equipment required is not readily available, particularly in the rural areas.

Table 6: Dog rabies cases in Zambia for the period from 2004-2009*.

Province	Area (Sq.Km)	Human Population	Dog population	Dog cases§
Northern	147, 826	1, 258, 696	41, 330	10
Luapula	50, 567	775, 353	63, 933	6
Eastern	69, 109	1, 306, 173	65, 000	4
Central	94, 394	1, 012, 257	56, 355	38
Copperbelt	31, 328	1, 581, 221	44, 363	11
Lusaka	21, 896	1, 391, 329	51, 663	30
N/Western	125, 826	583, 350	17, 854	4
Western	126, 386	765, 088	50, 772	6
Southern	85, 283	1, 212, 214	92, 358	23
Total	752, 615	2, 885, 681	483, 628	132

*Rabies cases in domestic dogs as reported by the National Livestock Epidemiology and Information Center (NALEIC) of the republic of Zambia. Information on the human population and area of each province was adapted from the central statistics office of the republic of Zambia (Main Zambia census report, vol 10, 2000) and the dog population was estimated by NALEIC. §Number of DFAT positive dog rabies cases.

RT-LAMP assay is a cheap and alternative diagnostic technique for RABV diagnosis in Zambia, because of its simplicity and its lack of expensive equipment. Various LAMP assays for the detection of other pathogens have been previously established (Notomi et al., 2000; Parida et al., 2005; Ushio et al., 2005; Okafuji et al., 2005; Boldbaatar et al., 2009). Rabies diagnosis through the use of RT-LAMP assay has recently been reported (Saitou et al., 2010; Hayman et al., 2011).

The genus *Lyssavirus* comprises 11 species (http://www.ictvonline.org/virusTaxonomy.asp?version=2009). Four species of *Lyssavirus*, i.e., classical RABV, Lagos bat virus (LBV), Mokola virus (MOKV), and Duvenhage virus (DUVV), have been documented in Africa (WHO, 2005; Ngoepe et al., 2009). The classical RABV is the most common and comprises the following lineages: Africa 1, 2, 3, and 4 (Kissi et al., 1995; Bourhy et al., 2008; David et al., 2007). Africa 1 lineage, divided into Africa 1a and 1b, is present in North Africa and in eastern and southern Africa, respectively (Kissi et al., 1995). Africa 2 occurs in western and central Africa (Talbi et al., 2009), while Africa 3 and 4 are found in southern Africa and in Egypt, respectively (Kissi et al., 1995; Bourhy et al., 2008; Johnson et al., 2004; Lembo et al., 2007).

The RABV *N gene* (1,353 nt) is highly conserved, protects genomic RNA during morphogenesis (Patton et al., 1984), and also elicits a strong immune response capable of protecting animals from peripheral challenge (Fu et al., 1991; Lodmell et al., 1991; Fekadu et al., 1992). The highly conserved nature of the N gene has allowed several studies on lineage analysis to be carried out (Hyun et al., 2005; Kamolvarin, 1993; Lembo, et al., 2007; Nagarajan et al., 2009; Nel et al., 2005; Sacramento et al., 1991; Susetya et al., 2008; Yamagata et al., 2007). For example, a study on field rabies viruses

from Uganda based on the *N gene* categorized the viruses into Africa 1a and 1b lineages (Hirano et al., 2009). The glycoprotein encoded by the *G gene* (1,575 nt) is the major antibody-stimulating antigen that interacts with the host immune system. It is responsible for viral attachment to host cells i.e. the virulence of the virus (Dietzschold et al., 1983; Seif et al., 1985), is less conserved (Seif et al., 1985) thus offers more genetic diversity making it suitable for the phylogenetic analysis of closely related viruses. This genetic diversity has been shown to produce distinct virus strains in regions that are separated by physical barriers such as mountainous ranges, rivers, and valleys (Badrane et al., 2001; Guyatt et al., 2003; Hyun et al., 2005).

In this study, I used nested RT-PCR (Heaton et al., 1997; Kamolvarin et al., 1993) followed by direct sequence analysis of selected *N* and *G gene* fragments (Hyun et al., 2005; Johnson et al., 2004; Nagarajan et al., 2009; Yamagata et al., 2007) to establish (i) the lineage of field rabies viruses in central Zambia by analyzing the nucleotide sequences of the *N gene* of viruses from Zambia and the rest of the world, (ii) the genetic diversity, based on the *G gene* analysis of field rabies viruses, in different host species and regions of central Zambia, and (iii) by using the *N gene* nucleotide sequences obtained in this study together with the reference sequences from Tanzania and Mozambique, the design for appropriate primers for the RT-LAMP assay for diagnosis of RABV.

Materials and methods

Sample collection

A total sample size of 87 DFAT-positive brain specimens (cows, dog and monkeys) was included in this study. This comprised samples stored at the School of Veterinary

Medicine, University of Zambia (UNZA) (n = 57) and samples (n = 30) from NALEIC (Table 6) whose year of isolation, origin, availability and moderate condition of the brain specimen could be validated because most specimens were either missing or had putrefied. The samples from the School of Veterinary Medicine, UNZA were collected from 1998 – 2009, while the samples from NALEIC were collected from 2004 – 2009. The samples (n = 87) in this study originated from Copperbelt, Lusaka, and Central Provinces, which represent the central part of Zambia (Fig. 1). The brain samples were sent to the diagnostic laboratory, i.e. The School of Veterinary Medicine, UNZA, and other laboratories for confirmation, after the animals showed signs of rabies.

RNA extraction and RT-PCR

Total RNA was extracted and purified from each brain sample using the TRIzol (Invitrogen) and Purelink RNA extraction kits (Ambion, CA), according to the manufacturers' instructions. Complementary DNA (cDNA) of the rabies virus genome was synthesized by reverse transcription using 1 μg of RNA, random hexamer primers, and M-MLV RTase (Promega, WI) at 37°C for 90 minutes. The RABV *N* and *G gene* fragments were amplified using the ExTaq polymerase (Takara, Japan) and the PCR mixture specified by the manufacturer. Nested RT-PCR was performed using outer and inner primer sets, RN1 (5′-CTACAATGGATGCCGAC-3′) and RN2 (5′-GAGTCACTCGAATATTGC-3′), and RN3 (5′-GACATGTCCGGAAGACTGG -3′) and RN4 (5′-GTATTGCCTCTCTAGCGGTG-3′) (Kamolvarin et al., 1993) for the N gene, respectively. The inner and out primers for the G gene were RAVGF (5′-CAAGGAAAGATGGTTCCTCAG-3′) and RAVGR (5′-TCACAGTCTGGTCTCACCTCCAC-3′) (Hyun et al., 2005), and GF

(5′-CCATCATGACCACCAAGTC-3′) and GR (5′-TTACAGCTTGGTCTCACC-3′) (Yamagata et al., 2007), respectively. The primers RN1, RN2, RN3, and RN4, and RAVF, RAVG, GF, and GR correspond to the sequences at positions 66–82, 1402–1419, 319–337, and 823–842, and 3309–3329, 4870–4892, 923–941, and 1558–1575 of the N and G genes of the Pasteur virus genome sequence (NC_001542.1), respectively. Mouse cDNA was used as a negative control to eliminate contamination. The first PCR program was as follows: denaturation at 95°C for 5 minutes, 35 cycles of 95°C, 50°C, and 72°C for 1 minute each, and extension at 72°C for 5 minutes. The products of the first PCR were diluted 100-fold and used as templates for the second PCR using the same program as the first reaction. The amplified products were analyzed on 1.2% agarose gel coated with ethidium bromide.

Direct sequencing

Nested PCR products were purified using the Monofas PCR Purification Kit (GL Sciences, Japan). Cycle sequencing reactions were performed using the Big Dye Terminator v3.1 system (Applied Biosystems) with the *N gene* (RN3 and RN 4) and *G gene* (GF and GR) inner primers. The labeled ddNTPs were removed from the cycle sequence products by ethanol precipitation and subjected to electrophoresis in the ABI 3130 genetic analyzer (Applied Biosystems).

Phylogenetic analysis

The referenced and obtained sequences were aligned using the ATSQ application software (Genetyx Ver.9, Japan). The *N gene* (position 349–810, 462 nt) and *G gene* (position 4260–4870, 611 nt) fragments of the Pasteur virus genome (NC_001542.1)

were used for phylogenetic analysis. Nucleotide sequences with 100% homologous nucleotide identity were placed in 1 group and a representative sequence from each group was used for the generation of the phylogenetic tree. Multiple sequence alignments were performed using ClustalW1.6 with a MEGA file format created using MEGA ver.4.0 (Tamura et al., 2007). Phylograms were generated using the NJ method (Saitou et al., 1987) with a confidence level of 1000 bootstrap replicates (Felsenstein, 1982). The Maximum Composite Likelihood method was used to compute the evolutionary distances with the number of base substitutions per site as units (Tamura et al., 2004). Rabies isolates from 1 different lineages across Africa (Africa 1, 2, 3, and 4) and other continents (Asia, Europe, the Middle East, North America, and South America) were included in the generation of the phylogenetic trees based on the *N* and *G gene* to determine the lineage and genetic diversity of the rabies virus in central Zambia, respectively.

RT-LAMP

The RT-LAMP primer sets (Table 7) were designed using Primer Explorer Ver. 4 (Eiken Chemical, Japan [http://primerexplorer.jp/lamp4.0.0/index.html]), from the consensus sequence of the *N gene* fragment sequences obtained in this study and those of the reference sequences (RVU22484_Mozambique, RVU22647_Tanzania, RVU22648_Tanzania, DQ900566_Tanzania, DQ900558_Tanzania, RVU22649_Namibia and RVU22638_Democratic republic of Congo).

Table 7: RT-LAMP primers sequences and positions.

Primer	Position (5'- 3')	Sequences (5'- 3')
F3	363 – 382	GAAAAGGAGACAAGATCACC
B3	528 – 545	CCGGTGTTTTGTCCTGAT
FIP	383 – 460	CCTTGTCAGCTCCATGCCTC-CCGGACTCTCTAGTGGAAAT
BIP	461 – 524	ACCCCACTGTCTCTGAGCAT-TGCTCAACCTATACAGACTCA

Primer sequence positions correspond to the *N gene* sequence of the Pasteur virus strain, accession number NC_001542.

F3, Forward outer primer, B3, Reverse outer primer, FIP, Forward inner primer, BIP, Reverse inner primer.

The RT-LAMP assay was performed in a total reaction volume of 20 μL using the Loopamp RNA Amplification Kit (Eiken Chemical, Japan) with a yellow fluorescent detection reagent (Eiken Chemical), according to the manufacturer's instructions. The reaction mixture was incubated at 63°C for 60 minutes and stopped by incubation at 95°C for 2 minutes. Four individual RT-LAMP reactions were performed using 100 ng of RNA from 12 clinical specimens as templates. The yellow fluorescent detection reagent was present in three individual reactions and absent in the fourth. A no-template reaction was used as a negative control. Reaction tubes with green fluorescence after isothermal amplification were classified as positive, whereas those with yellow fluorescence were classified as negative. The intensity of the fluorescence was measured in three individual RT-LAMP reactions using the VersaDoc MP 4000 imaging system (Bio-Rad Laboratories, USA). The fourth reaction lacking the fluorescent detection reagent was used to determine the RT-LAMP electrophoresis pattern using agarose gel (1.5%) coated with ethidium bromide.

Results

Nested RT-PCR

To examine the lineages of RABV in Zambia, *N* and *G gene* fragments were amplified from complementary DNA isolated from DFAT-positive brain samples. RNA was extracted from 87 DFAT-positive specimens, and 35 (31 dogs, 3 cows, and 1 monkey for *N gene* and 32 dogs and 3cows for the *G gene*) of these specimens were positive by nested RT-PCR results and produced sequences of sufficient quality for analysis, with serial accession numbers AB572927 toAB572961 for the *N gene* and AB572962 to AB572996 for the *G gene*.

Phylogenetic analysis

From the sequences obtained, the *N* and *G genes* were divided into 13 (n=33) and 14 (n=35) groups, respectively (Table 8). The origins of samples with accession numbers AB572929 and AB572936 could not be verified and as such were not included in the phylogenetic analysis of the *N gene* making a total sample size of 33. The groups of the *N gene* were as follows: Groups 1, 3, 4, 6, and 9–13 were obtained from brain specimens from Lusaka Province, 2 and 5 from Lusaka and Central Provinces, and 7 and 8 from the Copperbelt Province. The groups of the *G gene* were as follows: Groups 1, 2, 5–12, and 14 were obtained from Lusaka Province, 3 and 13 from Lusaka and Copperbelt Provinces, and 4 from Central and Lusaka Provinces (Table 8). Phylogenetic analysis of the nucleotide sequences of the *N gene* fragment revealed that all the samples analyzed from Zambia belonged to the Africa 1b lineage (Fig. 5). In this lineage, Zambian strains were divided into 2 clusters. While cluster 1 exclusively consisted of Zambian strains, cluster 2 included Zambian (Copperbelt and Lusaka Provinces), Tanzanian (DQ900566, DQ900558, RVU212647, and RVU22648), and Mozambican (RVU22484) strains. Furthermore, clusters 1 and 2 showed 98% - 99% and 96% - 99% intra-cluster nucleotide identities, respectively, and 96% - 98% inter-cluster nucleotide identity (data not shown). Phylogenetic analysis of the nucleotide sequences of the *G gene* fragment showed 2 clusters, A and B (Fig. 6), with 99% – 100% inter-group nucleotide identity (data not shown). Cluster B comprised 5 minor clusters, namely, 1, 2, 3, 4, and 5 (Fig. 6). Samples from minor clusters 1, 2, and 4 originated from Lusaka Province, 3 from Central and Lusaka Provinces, and 5 from Lusaka and Copperbelt Provinces. Cluster A only consisted of samples from Lusaka Province.

Table 8: List of summarized sequences that are identical over 460 and 610 bp of the nucleoprotein and the glycoprotein genes, respectively

Gene	Phylogroup	Samples # in Phylogroup	Representative Sample	Samples in Phylogroup (Accession numbers)	Origin (Province)	Year of isolation	Host
N gene	1	6	66_02_zambia_dog_02 (AB572940)	AB572940,AB572943, AB572950,AB572941, AB572961,AB572951	Lusaka	2002-2004, 2009	Dog
	2	5	21_07_zambia_dog_07 (AB572930)	AB572930,AB572956, AB572954,AB572959, AB572955	Central, Lusaka	1999, 2006-2007	Dog
	3	2	153_05_zambia_dog_05 (AB572952)	AB572952, AB572942	Lusaka	2005, 2009	Dog
	4	9	358_99_zambia_dog_99 (AB572933)	AB572933,AB572938 AB572946,AB572948, AB572939,AB572949, AB572937,AB572935, AB572947	Lusaka	1999-2001	Dog, Cow
	5	3	149_01_zambia_dog_01 (AB572957)	AB572957,AB572958, AB572945	Central, Lusaka	2000-2001	Dog
	6	1	117_04_zambia_dog_04 (AB572960)	AB572960	Lusaka	2004	Dog
	7	1	01_08_zambia_cow_08 (AB572927)	AB572927	Copperbelt	2008	Cow
	8	1	02_08_zambia_cow_08 (AB572928)	AB572928	Copperbelt	2008	Cow
	9	1	27_07_zambia_dog_07 (AB572932)	AB572932	Lusaka	2007	Dog
	10	1	410_00_zambia_dog_00 (AB572934)	AB572934	Lusaka	2000	Dog
	11	1	143_01_zambia_dog_01 (AB572931)	AB572931	Lusaka	2001	Dog
	12	1	59_08_zambia_dog_08 (AB572953)	AB572953	Lusaka	2008	Dog
	13	1	109_06_zambia_dog_06 (AB572944)	AB572944	Lusaka	2006	Dog

G gene	1	3	73_07_zambia_dog_07 (AB572980)	AB572980,AB572979, AB572981	Lusaka	2006-2007, 1999	Dog
	2	1	T163_99_zambia_dog_99 (AB572994)	AB572994	Lusaka	1999	Dog
	3	2	153_05_zambia_dog_05 (AB572969)	AB572969,AB572962	Lusaka, Copperbelt	2005, 2008	Dog, Cow
	4	3	149_01_zambia_dog_01 (AB572966)	AB572866,AB572964, AB572965	Central, Lusaka	2001	Dog
	5	15	140_00_zambia_cow_00 (AB572982)	AB572982,AB572975, AB572995,AB572983, AB572984,AB572976, AB572972,AB572971, AB572970,AB572974, AB572990,AB572973, AB572989,AB572991, AB572977	Lusaka	1998-2002, 2004, 2008	Cow, Dog
	6	1	55_09_zambia_dog_09 (AB572985)	AB572985	Lusaka	2009	Dog
	7	1	59_09_zambia_dog_09 (AB572967)	AB572967	Lusaka	2009	Dog
	8	1	109_06_zambia_dog_06 (AB572978)	AB572978	Lusaka	2006	Dog
	9	1	410_00_zambia_dog_00 (AB572988)	AB572988	Lusaka	2000	Dog
	10	1	14_07_zambia_dog_07 (AB572996)	AB572996	Lusaka	2007	Dog
	11	2	65_03_zambia_dog_03 (AB572993)	AB572993,AB572992	Lusaka	2003, 2004	Dog
	12	1	167_03_zambia_dog_03 (AB572987)	AB572987	Lusaka	2003	Dog
	13	2	02_08_zambia_cow_08 (AB572963)	AB572963,AB572986	Copperbelt, Lusaka	2002, 2008	Cow, Dog
	14	1	39_99_zambia_dog_99 (AB572968)	AB572968	Lusaka	1999	Dog

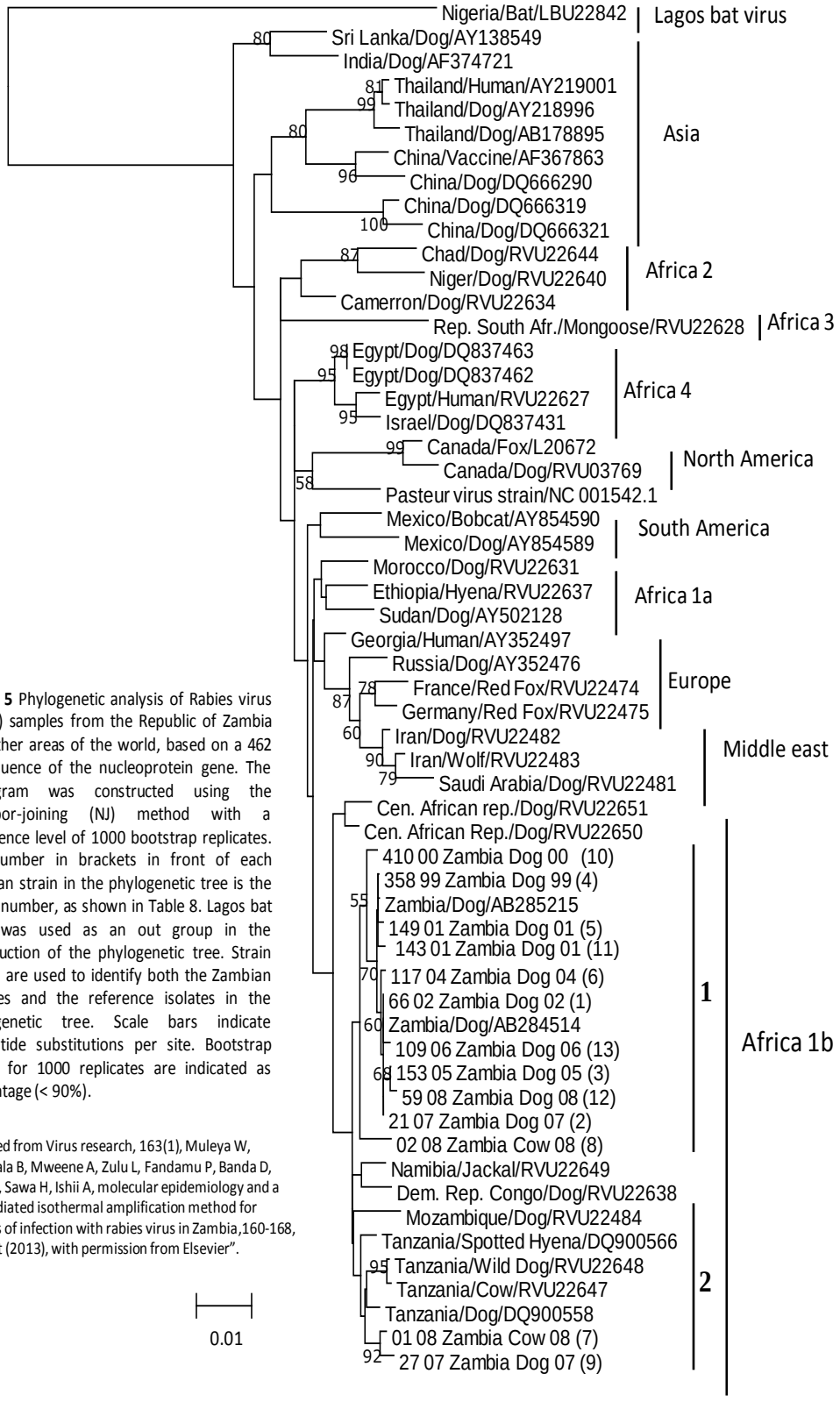

Figure 5 Phylogenetic analysis of Rabies virus (RABV) samples from the Republic of Zambia and other areas of the world, based on a 462 nt sequence of the nucleoprotein gene. The phylogram was constructed using the neighbor-joining (NJ) method with a confidence level of 1000 bootstrap replicates. The number in brackets in front of each Zambian strain in the phylogenetic tree is the group number, as shown in Table 8. Lagos bat virus was used as an out group in the construction of the phylogenetic tree. Strain names are used to identify both the Zambian samples and the reference isolates in the phylogenetic tree. Scale bars indicate nucleotide substitutions per site. Bootstrap values for 1000 replicates are indicated as percentage (< 90%).

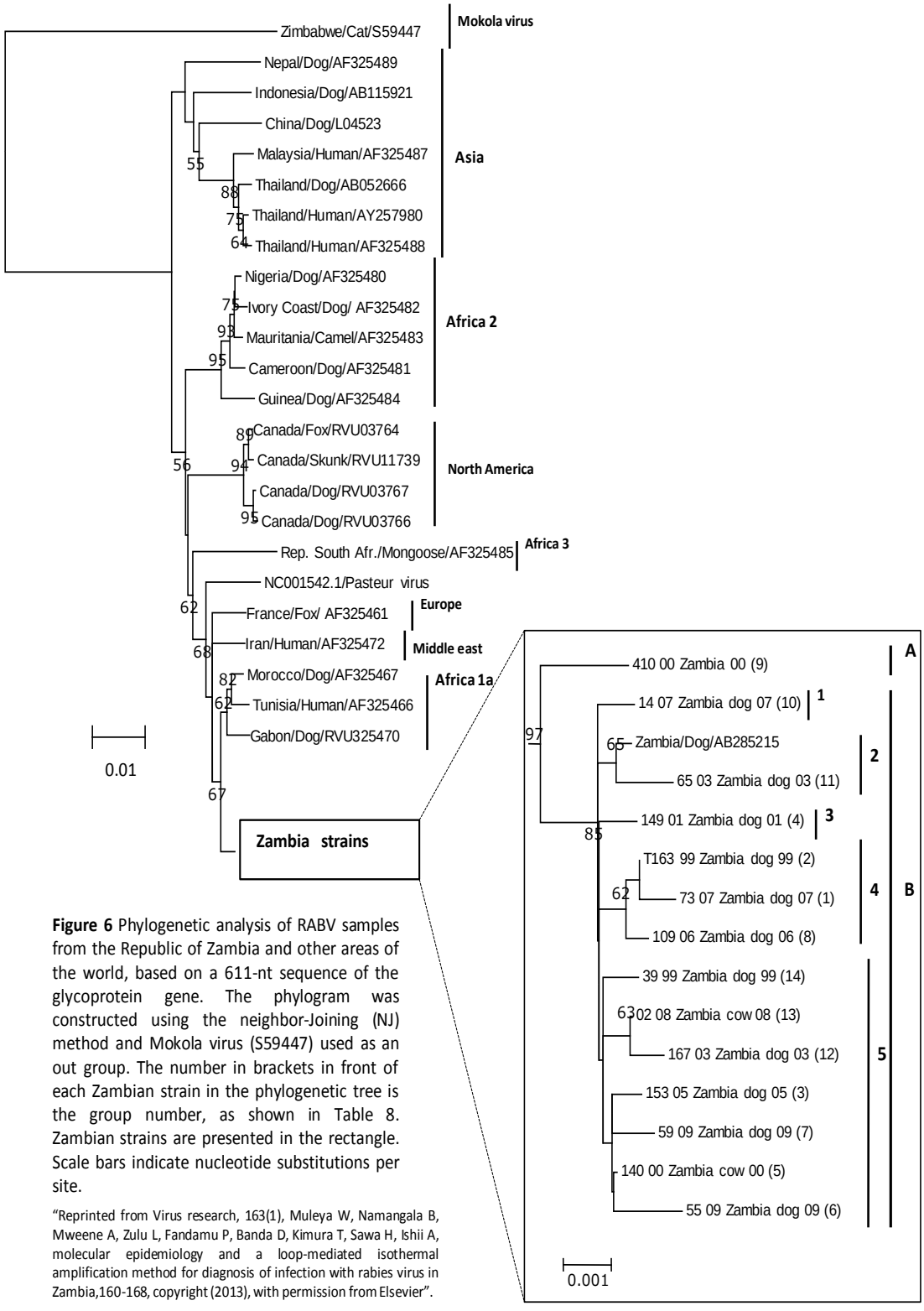

Figure 6 Phylogenetic analysis of RABV samples from the Republic of Zambia and other areas of the world, based on a 611-nt sequence of the glycoprotein gene. The phylogram was constructed using the neighbor-Joining (NJ) method and Mokola virus (S59447) used as an out group. The number in brackets in front of each Zambian strain in the phylogenetic tree is the group number, as shown in Table 8. Zambian strains are presented in the rectangle. Scale bars indicate nucleotide substitutions per site.

"Reprinted from Virus research, 163(1), Muleya W, Namangala B, Mweene A, Zulu L, Fandamu P, Banda D, Kimura T, Sawa H, Ishii A, molecular epidemiology and a loop-mediated isothermal amplification method for diagnosis of infection with rabies virus in Zambia,160-168, copyright (2013), with permission from Elsevier".

Establishment of RT-LAMP for RABV in Zambia

RT-LAMP assay does not require expensive equipment and as such could easily be applicable in Zambia, especially in rural areas. In view of this, I aimed to introduce RT-LAMP as a more useful rabies diagnostic method in Zambia than DFAT and nested RT-PCR. Primers were designed from the sequences obtained for the *N gene*. To confirm the specificity of the RT-LAMP primers, I compared the results of the assay with those of nested RT-PCR (Figs. 7A and B). RNA extracted from 12 clinical specimens stored at UNZA was used for the assays. Nested RT-PCR analysis was performed with 100 ng of the RNA template, according to the protocol described in Materials and Methods. Nested RT-PCR showed that 9 samples (1, 3 - 7, 9 - 11) were positive and 3 (2, 8, and 12) were negative (Fig. 7A). A non-template negative control (NC) did not amplify any specific cDNA band (Fig. 7A). RT-LAMP assay was also performed with 100 ng of RNA template, and green fluorescence was observed in positive sample tubes (1, 3–7, and 9–11) and yellow fluorescence in negative tubes (2, 8, 12, and NC) (Fig. 7B). The intensities of the fluorescence in 3 individual trials were measured (Fig. 7B). The calculated relative fluorescent intensities of the positive samples were 3.5-fold higher than the negative control and 2.5-fold higher than that of the negative samples (Fig. 7B). Gel electrophoresis showed a similar pattern of bands for each of the 9 positive samples (Fig. 7B). These results show that RT-LAMP assay has a high specificity and reproducibility for RABV diagnosis.

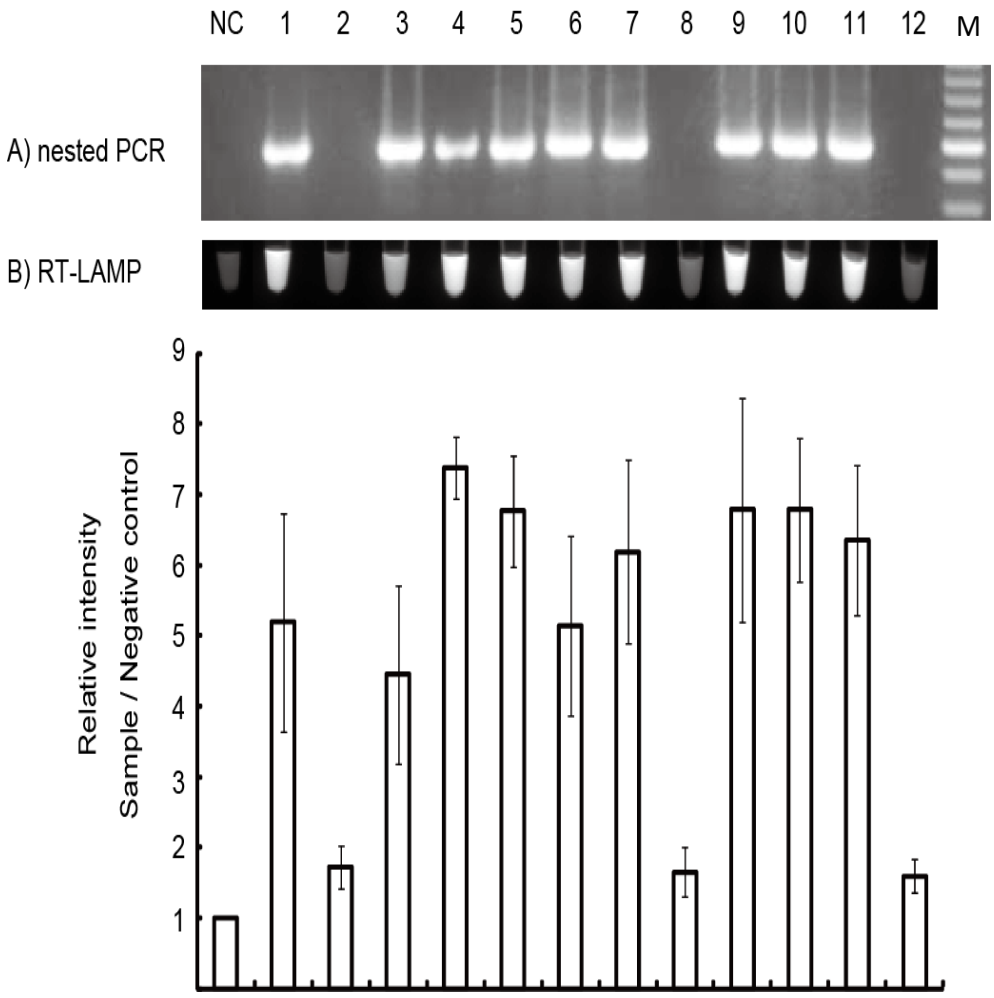

Figure 7 (**A**) Nested PCR results showing positive reactions for samples 1, 3–7, and 9–11. (**B**) (upper panel) Visual RT-LAMP results showing fluorescence in positive reaction tubes 1, 3–7, and 9–11. (middle panel) A bar graph indicating the relative fluorescence intensity of the RT-LAMP reaction assay. The relative fluorescent intensity of the examined samples was calculated as follows: The intensity of the examined sample was divided by the average intensity of samples without the RNA template. The 2-fold increase in signal intensity compared to that of the negative control represents the threshold of this assay. The relative fluorescence intensities were measured using the VersaDoc MP 4000 imaging system (Bio-Rad Laboratories, USA). (lower panel) Gel electrophoresis showing similar band patterns of the RT-LAMP reaction. 1-12 represents rabies clinical samples, M: the 100 bp molecular marker, NC: the negative control.

Discussion

RNA samples (n = 87) were successfully extracted from the DFAT-positive brain samples collected for this study. Thirty-seven (40%) of these samples were positive by nested RT-PCR results and 33 samples out of these produced sequences for phylogenetic analysis of the *N gene* of RABV. As for the *G gene*, 35 out of 37 samples produced sufficient sequence data for phylogenetic analysis. The low number of nested RT-PCR-positive samples does not imply low sensitivity of the nested RT-PCR as compared to DFAT, but rather indicates inappropriate storage of brain samples in 50% glycerol saline at ambient temperature, resulting in decomposition of samples over time. A similar problem was experienced in Uganda (Hirano et al., 2009). The 35 sequences represent samples collected from dogs, cows, and a monkey over a total period of 11 years (1998–2009) both by School of Veterinary Medicine, UNZA and NALEIC representing the Copperbelt, Central, and Lusaka Provinces of Zambia (Fig. 1). The phylogenetic analysis of sequences of the RABV *N gene* fragment from central Zambia revealed the existence of 2 phylogenetic groups (clusters 1 and 2) belonging to the Africa 1b lineage present in eastern and southern Africa (Fig. 5). Cluster 2 strains might have evolved separately during their circulation in Zambia or may have been introduced into Zambia (Copperbelt and Lusaka Provinces) by (i) cross-border trade of animals and (ii) free movement of animals across the borders between Zambia and its neighbors. Phylogenetic analysis of the *G gene* (Fig. 6) implied that similar virus strains were recognized in different animals in Lusaka, Copperbelt, and Central Provinces of Zambia. There was no evidence of separation of strains according to regions separated by physical barriers such as mountainous ranges, rivers, and valleys, as previously reported in other studies (Badrane et al., 2001, Guyatt et al., 2003, Hyun et al., 2005). This could

be due to the fact that the Copperbelt, Central, and Lusaka Provinces are located on the busiest trade route in the country, which overcomes physical barriers such as mountainous ranges and perennial rivers, which may impede the movement of animals and promote localized viral evolution in specialized host and geographical niches (Bourhy et al., 1999). In view of this, easy spread and mixing of viruses between these regions and in different hosts could have occurred resulting in the emergence of similar strains. Rabies strains from Zambia could not be compared to other strains from the Africa 1b lineage based on *G gene* sequences because the targeted gene area lacks Africa 1b reference sequences. The Zambian strains were however independent but closer to the Africa 1a lineage.

I established an RT-LAMP assay by designing the primers targeting the *N gene* of the RABV (Table 7). The RT-LAMP assay showed high specificity as evidenced by its ability to detect all the positive clinical brain specimens used in the study and was also reproducible (Fig. 7). RT-LAMP is a novel RNA amplification method which is as sensitive and specific as nested RT-PCR in the diagnosis of rabies. The assay in this study is based on *N gene* sequences of Africa 1b lineage, thus further research is needed to broaden the level of diagnostic ability to other lineages of RABV. RT-LAMP, unlike RT-PCR and DFAT, does not require sophisticated equipment (all it requires is a heating block), is simpler to use, and may conveniently be applied in remote rural Zambia, hence enhancing the rapid diagnosis of rabies cases at the local laboratory. In addition, the cost of running the RT-LAMP assay is approximately $1 USD per sample, being much cheaper than DFAT or RT-PCR and less labour-intensive. Furthermore, unlike RT-PCR, amplification of viral RNA during RT-LAMP reactions occurs at constant temperature (63°C) within an hour, producing large amounts of viral nucleic

acids (Notomi et al., 2000) that can even be visualized with naked eyes. The incorporation of the fluorescent dye in the initial reaction mix used in my study is advantageous over the assay described by Hayman et al. (2011) in which results were read by placing the RT-LAMP reaction products on a lateral flow device, which may contaminate the working environment. Thus RT-LAMP assay described in this study may be a better alternative cost-effective routine molecular diagnostic method for rabies diagnosis in certain field conditions or at work stations in the field in resource-poor countries such as Zambia.

In Zambia, rabies mainly affects dogs with spill-over infections in livestock and humans (Zyambo et al., 1985; Munang'andu et al., 2010). A previous study showed that the incidence of rabies in dogs was higher than that in humans, livestock, domestic cats, jackals, primates, and other wildlife, which implied that rabies is mainly transmitted from dogs (Munang'andu et al., 2010). In this study, I showed direct evidence by using phylogenetic analysis that the strains present in livestock are similar to those in dogs; therefore, the dog is the main reservoir or source of infection in both livestock and humans in Central Zambia. Dogs are well tolerated and live in close proximity to humans. Unfortunately, most of these dogs are unvaccinated and roam freely in communities, particularly in rural areas. This close association, together with human population growth, and ineffective stray dog and rabies control, has increased human contact rates and dog bites and, consequently, human rabies cases. There is a positive correlation between human population density and the prevalence of rabies (Mork et al., 2004). The steady increase in the Zambian population has thus increased contact rates between humans and dogs; therefore, the number of outbreaks in these populations has increased. The close association between dogs and humans, coupled with the high

prevalence of rabies in dogs, presents a serious public health concern, particularly in rural areas where monitoring and control strategies are inconsistent. It should, therefore, be stressed that many cases go unreported. This is attributed to the lack of surveillance measures owing to erratic allocation of funds by the government. Ineffective reporting is also compounded by a lack of specialized equipment, i.e., microscopes and thermal cyclers, in rural areas; therefore, the need for a simple, cheap, and rapid diagnostic technique (RT-LAMP) cannot be overemphasized.

Chapter III

General summary

General Discussion

Infectious diseases continue to hinder the development of the livestock industry in Zambia and indeed many countries. Molecular epidemiological data coupled with sensitive, specific and cost-effective diagnostic methods are an important component in the study and control of these diseases. Molecular techniques such as PCR are sensitive and specific and due to this fact, are of prime importance in epidemiological surveys and clinical diagnosis of infectious diseases. PCR is also an important aid in population genetic analytical studies as well as phylogenetic studies of a whole range of pathogens. With the potential contribution that livestock can make to the nation's GDP, it is imperative that these diseases be controlled.

Population genetics of *T. parva*

The population genetic structure of *T. parva* in northern and eastern Zambia was analyzed based on the use of microsatellite markers, PCR technique, ABI genetic analyzer and a range of computer-based softwares. PCR screening of cattle blood samples showed that theileriosis is prevalent in Northern and Eastern Zambia.

A panel of 16 micro-satellite markers was used to screen 16 *T. parva* isolates from a wide geographical range out-lined in chapter I (materials and methods-sample collection and DNA preparation). Using a stringent criterion to evaluate the suitability of each candidate marker locus, 7 markers were discarded from the final analysis. Only loci that were polymorphic, did not produce multiple bands, and produced few null alleles were used to genotype field isolates (Table 2).

Capillary electrophoresis allowed the determination of the exact allele sizes, easy discrimination of multiple alleles where present and the resolution of PCR products to 1 bp. In order to ensure the correct mapping of Genescan measurements to discrete allele's sizes, the electrophoresis patterns of all the alleles called by the software were also visually confirmed. By visual confirmation, all peaks on the size standard were correctly labeled and miscalled peaks were manually corrected. Thereafter, the size of the allele called by the software was compared with the one observed on the electrophoresis pattern. This was done in addition with an optimized PCR technique employing reduced number of cycles. A panel of isolates comprising of multiple infections indicated by the presence of more than one peak on most loci was used as control samples. Only the alleles with the prescribed base pair range were used to generate the MLG. Also for the binning criteria, allele ranges were created using our laboratory stock as calibration. In order to determine the consistency of the results, the multiple tube approach would have been required. Although the approach was performed on some loci, it was not carried out on all samples for locus MS48 due to the limited amount of our DNA samples. In the absence of capillary electrophoresis, the markers used in this study were of limited use. However a high resolution gel-based system such as the one used for *T. parva* genotyping in Uganda (Oura et al. 2005) may be used as an alternative.

The type of genetic markers used greatly influences the quality of the data produced in any population genetic study. Different genotyping methods (MacLeod et al. 2001), each with its own advantages and disadvantages have been used. Methods such as Isoenzyme electrophoresis (Ben Miled et al. 1994; Schweizer et al. 1988) and monoclonal antibody profiles (Ben Miled et al. 1994; Minami et al. 1983; Shiels et al.

1986) have facilitated the use of techniques such RFLP (Paindavoine et al. 1989) and random amplification of polymorphic DNA (RAPD) (Stevens and Tibayrenc, 1995). This is because these approaches allowed direct access to information on genotypes as compared to requiring inference from phenotypic data. The drawback to these methods is that a degree of selection and loss of genotypes may occur during amplification of parasite material in rodents or in cultures because these methods require large amounts of parasite material. In cases were a high proportion of isolates comprise of a mixture of genotypes, the determination of genotype frequencies becomes impossible resulting in the possible identification of mixed infections as new genotypes and thus confounding the results. Recent genotyping methods such as micro- and mini-satellite (Biteau et al., 2000) and single nucleotide polymorphism (SNP) (Coates et al., 2009) have produced similar estimates of population genetic parameters (Coates et al., 2009). However, identifying individual alleles in samples with mixed genotypes with respect to SNPs still remains a challenge. However, micro- and mini-satellite analysis coupled with capillary electrophoresis allows easy identification of samples with mixed genotypes. It is also easy to perform and does not require amplification of isolates in rodents or cultures but it also has its own short-comings as stated in the discussion section of chapter I, and in as much as cloning out each isolate would provide a solution to this problem, this system is time consuming and expensive.

This study determined that in Northern and Eastern Provinces of Zambia, *T. parva* occurred as two discrete populations, geographically and genetically sub-structured with the absence of random mating between them. The two populations also showed a high level of genetic diversity. The absence of similar genotypes in the *T. parva* populations across the two provinces suggested a state of limited self-fertilisation. The population

from Isoka exhibited less diversity than that from Petauke. The presence of physical geographical barriers i.e. the national parks (Fig. 2) between these areas impedes free movement of animals between these 2 districts, hence the sub-structuring observed. A study on *T. parva* populations from Uganda showed similar results (Oura et al., 2005). The geographical and genetic sub-structuring observed between Petauke and Isoka districts also implies that *T. parva* cocktail vaccines derived from Petauke district may not be appropriate for use in Isoka district of Northern Province. The use of these cocktail vaccines may introduce new genotypes into Isoka and the resulting recombination might bring about stable and more virulent strains. The presence of these new strains might completely hinder the improvement of livestock, adversely impacting the local economy. The sub-structuring also suggests that vaccine antigens in the districts under study might evolve independently in different populations.

The moderate genetic differentiation in Isoka district indicated the existence of similar genotypes of *T. parva* between the populations (Table 4), as such clonality could not be inferred. The high genetic diversity and the absence of duplicate genotypes implied that the population structure of *T. parva* is unlikely to be clonal or epidemic. By definition, a clonal population is one which is genetically homogenous, and has identical genotypes deriving either from a parental genotype through clonal propagation or from parasite populations where the occurrence of inbreeding and self-fertilisation is strong, i.e. the existing sexual reproduction (out-crossing) is not sufficient to break the clonal pattern (Tibayrenc and Ayala, 2002). *T. parva* has been shown to undergo an obligate sexual phase. The population structure of *Plasmoduim falciparum* is panmictic in regions of high transmission and clonal in low transmission areas (Anderson et al., 2000) implying that the presence of a sexual phase, is by itself insufficient to produce

panmixia thus, a minimum transmission rate is required to produce a panmictic population structure. All individuals in a panmictic population are potential mating partners without any restriction on mating and genetic flow. The high level of genetic diversity and the high number of genotypes in individual samples (as demonstrated by the high multiplicity of infection) implies that *T. parva* undergoes sexual recombination in the tick. The parasites within Isoka district population were in a state of restricted circulation. The low adult tick vector population also inferred low challenge levels of infection. This provides evidence that farming practices also have a strong influence on the population structure of *T. parva* and also the effectiveness of the control measures implemented against theileriosis. In this regard, famer education on disease control and prevention through agriculture extension services cannot be overemphasized. The information produced in this study would be useful for assessing the diversity of vaccine candidate antigens as well as testing whether they are subject to diversifying selection. It can also provide a template for assessing the spread of genotypes from endemic areas into naïve areas, thus enabling the assessment of the effectiveness of control measures and because the prevalence of theileriosis is higher in the wet season than in the dry season, conducting this study both in the dry and wet season in Zambia will allow the effect of seasonality on *T. parva* population structure to be assessed. In general, tick burden on cattle during the wet season increases and most farmers are aware of the increased risk of disease caused by ticks. Almost all of these farmers thus, tend to treat their animals with acaricides in the wet season while in the dry season a proportion of them do not. In addition, inadequate tick control has been observed, therefore, in order to conclusively establish the population structure of *T. parva*, a new study, noting the above concerns coupled with a larger sample size and a wider

geographical spectrum must be undertaken.

Molecular epidemiology of rabies

The methods used to generate phylogenetic trees have both disadvantages and advantages. In view of this, efficiency (speed with which the tree is generated), consistency (ability to produce the desired results if enough data is available), power (amount of data required by the method in order to produce desired results), robustness (ability of the method to produce desired estimates of the phylogeny even when minor variations occur in the data set), and falsibility (ability of the method to notify the user when assumptions are violated) are important attributes of any tree building method (Page and Holmes, 1996). The current tree building methods have a tendency to emphasize one of the above criteria while ignoring the others. For example, the NJ method seems to be more efficient and faster than the maximum parsimony which tends to assume a constant rate of evolution. The maximum likelihood method is time consuming and limited to small data sets (Bourhy et al., 1995) but on average is better than the maximum parsimony method (Nei, 1994). When analyzing closely related organisms such as two rabies virus biotypes, targeting a rapidly evolving part of the genome i.e. glycoprotein is highly recommended, whereas a slowly evolving gene i.e. nucleoprotein is desirable for a relatively distantly related group. In this study, the NJ method was utilised for generating the phylogenetic relationships among rabies viruses.

The phylogenetic structure of rabies virus was analyzed from samples obtained from the Central, Copperbelt and Lusaka provinces of Zambia. The analysis of the nucleoprotein determined that rabies virus in Zambia belongs to the Africa 1b lineage. Two clusters of this lineage were observed, one comprising entirely of the Zambian samples while the second one included samples from Tanzania and Mozambique.

Analysis of the *G gene* showed the presence of similar strains circulating in different hosts and regions of the Copperbelt, Central and Lusaka provinces of Zambia implying that the dog is the main reservoir and source of infection for other animals and humans and as such control of this disease should be aimed at mandatory vaccinations of the dog. With regard to this, it was stipulated that easy transmission and flow of rabies virus along this region occurs because of the fact that this region lies on the busiest trade route in Zambia. Therefore, control measures aimed at animal movement control, efficient vaccination and education of the general population should be implemented. Using the obtained sequences from the *N gene*, a cheaper and easy to apply RT-LAMP assay was designed because the high cost of using PCR continues to hinder its application in poorly resourced laboratories. The development and optimization of RT-LAMP assay where infection with rabies virus can be diagnosed provides an opportunity to reduce costs and increase the use of the technology for clinical diagnosis and epidemiological studies in most countries. The assay designed in this study was as sensitive and specific as nested PCR. It also employed the use of a fluorescent dye to allow easy and faster diagnosis without opening the reaction tubes consequently removing the possibility of contaminating the working surroundings. It produced consistent results when run simultaneously with nested PCR on clinical and field samples and the results were consistent with DFAT results. This assay, being cheaper and easily applicable entails that rabies diagnosis can be carried out cheaply and quickly at the point of origin of the samples without the need to transport them to the Central Veterinary Laboratories in Lusaka. Without the need to transport samples to Lusaka entails that the rabies diagnosis system can be de-centralised, run efficiently and at the same time provide important epidemiological data which is prime in the control of the

disease.

Future studies will include the following;

1. Extending the population genetics study of *T. parva* to the Central, Lusaka, Copperbelt and Southern provinces where cattle rearing is a very important livestock activity, and using a large sample size with effective biological data on the hosts, farming practices and tick ecology.
2. Extending the phylogenetic study of rabies to all regions of Zambia since rabies is present in all provinces and incorporating a large sample size.
3. Assessing the RT-LAMP assay by implementing it in diagnostic laboratories in Zambia so as to determine its true potential and designing another RT-LAMP assay incorporating the Africa 1a strains so as to broaden the scope of the assay.

Summary in English

This study showed that molecular tools are useful in the studies on the epidemiology and basic biology of infectious diseases of livestock in the field. The tools employed in this study both produced entirely new information hence proving the importance of such tools in the study of infectious diseases of livestock.

In the chapter I, cattle blood samples from Isoka and Petauke districts of Zambia were screened for *T. parva* DNA. A cohort of *T. parva* positive samples were analyzed using a panel of 9 microsatellite markers. Significant differentiation between the Isoka and Petauke populations was observed. Linkage disequilibrium was also observed when Isoka and Petauke district were treated as a single population. Separate analysis produced linkage disequilibrium in Kanyelele and Kalembe areas in Isoka district, Isoka district overall and Petauke district. As such, the study on population genetics of *T.*

parva from Isoka and Petauke districts showed a low level of genotype exchange between the districts, a high level of gene diversity within each district population, absence of panmixia and consequently, genetic and geographic sub-structuring between the districts. A higher multiplicity of infection was observed in Petauke as compared to Isoka district based on the average number of alleles identified through the use of capillary electrophoresis. With respect to this, theileriosis control strategies based on the use of cocktail vaccines in either Northern or Eastern provinces should be delicately carried out so as to prevent the introduction of new genotypes in naïve areas.

In chapter II, the lineage of rabies virus (RABV) in Zambia, using phylogenetic analysis of the nucleoprotein gene was determined using samples collected over an 11 year period. The level of genetic diversity of RABV strains in different hosts was also determined using the phylogenetic analysis of the glycoprotein gene. Analysis of the *N gene* produced two phylogenetic clusters belonging to the Africa 1b lineage present in eastern and southern Africa. One cluster comprised exclusively of Zambian strains. The other was heterogeneous and included strains from Tanzania, and Mozambique. Analysis of the *G gene* showed the presence of similar RABV strains in different hosts and regions of Zambia. I also designed primers for RT-LAMP assay using the consensus sequence of the *N gene*. I then confirmed the specificity and reproducibility of the RT-LAMP assay using actual clinical specimens. The RT-LAMP assay proved to be useful for routine diagnosis of rabies in Zambia. In this study, I established that the dog is the most probable source of infection to livestock and human beings and as such vaccinating these dogs will prevent spread of rabies to livestock and thus preventing loss of livestock.

The molecular tools used in this study proved to be a valuable way of obtaining

epidemiological data on the infectious diseases under study. These tools can also be applied to other pathogens, which will greatly enhance the prevention of the spread of diseases. The prevention of the spread of these diseases will facilitate the improvement of livestock and consequently, the livelihoods of the general population. Further research, however is needed so that cost effective disease control strategies, based on concrete knowledge of their epidemiology should be designed and implemented.

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Summary in Japanese

感染症の研究において、分子生物学的方法は、疫学研究、基礎研究、および診断法の開発を含む応用研究で重要な方法である。分子生物学的手法は、家畜や野生動物の検体を用いた臨床応用の前に、その感度、特異度、および技術的ミスを生じさせない利便性を有することを確認することが必要である。本博士論文は、ザンビアを含むアフリカ諸国で公衆衛生学的、経済的に問題となっている感染症であるタイレリア症、および狂犬病を対象として、分子生物学的手法に着眼し実施した、疫学研究および基礎研究で得られた結果について報告した。

第 I 章では、ザンビアで採取した牛血液中に存在する *Theileria parva*（*T. parva*）原虫の DNA の存在を、PCR 法でスクリーニングした。さらに PCR 陽性の試料について、マイクロサテライトマーカーを用いた多様解析を実施し、その結果を記載した。

タイレリア p104 遺伝子を対象とした PCR スクリーニングでは、両地区で平均 54.9%（78/142）の検体が陽性であり、Isoka 地区で採集した検体の陽性率は 44.8% （43/96）、Petauke 地区で採集した検体の陽性率は 76.1% （35/46）であった。*T. parva* 遺伝子陽性の 61 検体（Isoka の検体 33 例、Petauke の検体 28 例）は、原虫ゲノムの 4 つの染色体中に存在する 9 つのマイクロサテライトマーカーを用いて多様性解析を実施した。得られたマイクロサテライトマーカーの情報は、（1）Microsatellite tool kit、（2） GenAlEx ver. 6、（3） Fstat ver. 2.9.3.2、（4）LIAN の 4 つのソフトウェアを用いて解析した。その結果、Isoka と Petauke の原虫群のゲノム間の近交度は Wright の F 統計法による解析から比較的低いことが示唆された（Wright's F index, FST = 0.178）。また、Isoka および Petauke の各群を一つの群として仮定すると、マイクロサテライトは連鎖不平衡であると示されることから、両地区の原虫は交雑度が低いことが考えられた。さらに、Isoka 内の 2 地区群、Petauke 群内でも連鎖不平衡となることが示されたことから、各地域の原虫は独立しており、地域間での原虫の遺伝子交換の頻度が低いことが示唆された。

本結果は、混合生ワクチンを用いたタイレリア原虫対策を行う際に、タイレリア非汚染地域への原虫の導入を防ぎ、各地域での新しい遺伝子型の原虫を導入を防ぐために、流行型の遺伝情報に留意すべきであることを示唆するものであり、今後のタイレリア症対策にとって重要な情報を得ることができた。

第 II 章では、ザンビアにおける狂犬病を対象とした研究を実施した。狂犬病はザンビアの全土にわたって発生が認められており、1901 年に西部地域で狂犬病が発生し、その地域の族長であった Lewanika 氏が犬の駆除を命じたと伝えられている。また、ザンビアでの最初の狂犬病の診断は 1913 年に実施されたと報告されている。ザンビアにおいては、以前はジャッカルが狂犬病の保有動物として問題となっていたが、現在ではイヌが主要な保有動物であると報告されている（Zoonoses Public Health, 2010）。

本研究では、10 年間にわたって、ザンビア大学にて診断・保管されていた検体を用いて、

ザンビアのイヌ、家畜、および野生動物に感染していた狂犬病ウイルス（RABV）の流行株について核タンパク質である nucleoprotein（N）および膜タンパク質である glycoprotein（G）の遺伝子を検索し、系統樹解析を実施した。

蛍光抗体法で陽性であった 87 例の動物の脳組織から RNA を抽出し、nested RT-PCR 法を用いて狂犬病ウイルスゲノムを確認した結果、35 例で陽性所見が得られた。陽性検体を用いて、N 遺伝子を対象とした系統樹解析（33 例）では、検体はアフリカ東部および南部地域に分布する Africa 1b lineage に属する 2 つの cluster に帰属しており、1 つの cluster はザンビアの検体のみが認められ、もう 1 つの cluster はザンビアの他、タンザニア、モザンビークの検体が含まれていた。G 遺伝子を対象とした、解析ではザンビアの複数の地域および、異なる動物種から検出される RABV が同じ cluster に属することが示された。

さらに、得られた N 遺伝子配列を基に reverse transcription loop-mediated isothermal amplification（RT-LAMP）法に用いるプライマーを設計し、実際の検体を用いて、有用性を確認した。その結果、RT-PCR 法の結果と本 RT-LAMP 法は特異性と再現性を有しており、臨床検体への有効性が確認された。

本研究では、ザンビアで実際に流行している *T. parva* および RABV の遺伝子型を明らかにした。得られた成果を基にして、研究だけでは無く、実際の臨床検体に応用可能な分子生物学的手法を開発した。

本研究は、家畜等の感染症対策に有用な結果を残したが、今後さらに疫学調査で得られる結果に基づいた、安価な対策法の開発が必要とされる。