MOLECULAR DETECTION AND PHYLOGENETIC ANALYSIS OF TICK-BORNE PHLEBOVIRUSES FROM TICKS INFESTING CATTLE IN SOUTHERN PROVINCE, ZAMBIA

By

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Thesis submitted to the University of Zambia in Fulfilment of the Requirements for the Award of the Degree of Master of Science in Infectious Diseases

THE UNIVERSITY OF ZAMBIA

LUSAKA

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DEDICATION

This work is dedicated to my son Ian Walusungu Mbambara, my father and mother, for their spiritual support and guidance, who, for their love, shared with me the challenges and hardships during my studies at the University of Zambia. Many thanks are due to my dearest wife and best friend, Muleya Michelo Mbambara, for her immeasurable support and understanding, especially when I could not avail myself as much as I should have done during the course of my studies.

To my late sister, Janet Mbambara, on whose shoulders my academic journey rests, she still remains a source of inspiration and drives throughout my life. She always encouraged me to go further so that I could see furthest and led the way for me to walk through. My memories of her words and guidance throughout my formative years remain fresh even in this present day; this work is for Janet too.

My dedication is further extended to my brothers and sisters, nieces and nephews and grandnephews, all for your love, care and support during this journey. I am grateful to you all, and this is the reward of your patience and endurance.

DECLARATION

This Thesis is the original work of **SAIDON MBAMBARA** and represents his own work. It has not been previously submitted for any degree, diploma or any other qualification at this or any other University.

Signature ……..…….

Date ……..

CERTIFICATE OF APPROVAL

This Thesis of **Saidon Mbambara** has been approved as fulfilling the requirements for the award of the Degree of Master of Science in Infectious Diseases by the University of Zambia.

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Chairperson, Board of Examiners

…..……..…… **Date……**

ABSTRACT

Although novel tick-borne pathogens have emerged to cause severe disease in humans in many parts of the world, information on tick-borne viruses is scanty in many countries in sub-Saharan Africa. The Shibuyunji virus, a novel tick-borne phlebovirus (TBPV) was recently identified in ticks in Zambia. To gain insights into the prevalence, distribution, and genetic diversity of TBPVs in Zambia, 363 ticks infesting cattle were collected from Namwala and Livingstone districts in Southern Province and screened for TBPVs RT-PCR. The L-gene of TBPVs was detected by PCR from 19 (5.2%) *Rhipicephalus* ticks in Namwala District and confirmed by sequence analysis. Shibuyunji virus was the only TBPV detected. The detected isolates at the nucleotide, and amino acid level shared 92.8-100% and 98.1-100% similarity, respectively. Phylogenetic analysis showed that the Shibuyunji viruses detected in Zambia were closely related to American dog tick phlebovirus and formed a well bootstrap-supported distinct cluster. This study highlights the possible role of *Rhipicephalus* ticks as the potential host for the Shibuyunji virus and that this virus may be present outside the area of initial discovery. Furthermore, the study stresses the need for more studies to be conducted to assess the potential impact of TBPVs on animal and human health.

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LIST OF ABBREVIATIONS AND ACRONYMS

ANTV	Antigone Virus
ASFV	African Swine Fever Virus
BHAV	Bhanja Virus
CCHFV	Crimean-Congo Haemorrhagic Fever virus
cDNA	Complementary DNA
DNA	Deoxyribonucleic Acid
DUGV	Dugbe Virus
GTV	Guertu Virus
HRTV	Heartland Virus
KOD	*Thermococcus kodakaraensis*
KFV	Kyasanur Forest Virus
mRNA	Messenger RNA
NGS	Next Generation Sequencing
NSDV	Nairobi Sheep Disease Virus
ODWV	Odaw Virus
PCR	Polymerase Chain Reaction
RdRp	RNA-dependent RNA-polymerase

RNA	Ribonucleic Acid
RNP	Ribonucleoprotein
RRI	Recombinant RNase Inhibitor
RT-PCR	Reverse transcription PCR
RVFV	Rift Valley Fever virus
SFTS	Severe Fever with Thrombocytopenia Syndrome
SFTSV	Severe Fever with Thrombocytopenia Syndrome Virus
SSIV	Superscript four
$SSIV_{RT}$	Superscript four reverse transcriptase
TBDs	Tick-borne diseases
TBEV	Tick-borne Encephalitis virus
TBPVs	Tick-borne Phleboviruses
TBPs	Tick-borne Pathogens

CHAPTER ONE: INTRODUCTION

1.1 Background

Ticks are obligate ectoparasites that are known to transmit a wide variety of pathogens such as bacteria, viruses and protozoa. They are haematophagous parasites impacting human and animal health. Their potential as vectors of pathogens depends on the selection of the host and feeding mechanism (Tokarz, Williams and Sameroff, 2014; Shi *et al.*, 2018; Sameroff *et al.*, 2019). A close relationship occurs between ticks and pathogens they carry, and this results in a balanced life cycle between the vector (ticks) and pathogens (Vayssier-taussat *et al.*, 2015; Bartíková *et al.*, 2017; Kazimírová, Thangamani and Bartíková, 2017). Ranked second from mosquitoes in terms of transmission of pathogens, ticks account for about 25% of all arthropod transmitted pathogens (Sang *et al.*, 2006).

Tick-transmitted pathogens causing diseases in humans and animals have been reported from different geographical locations globally. About 16 diseases transmitted by ticks affect humans, while 19 affect livestock and companion animals (Zhang *et al.*, 2011; Kelly *et al.*, 2014; Li *et al.*, 2016; Maes *et al.*, 2018; Zhao *et al.*, 2018). Tick-borne diseases (TBDs) that have been associated with infections in humans include encephalitis caused by tick-borne encephalitis virus (TBEV) and haemorrhagic infections caused by viruses such as Crimean-Congo Haemorrhagic fever virus (CCHFV). Livestock and companion animals have also been affected by TBDs caused by viruses such as the Nairobi sheep diseases virus (NSDV) and African swine fever virus (ASFV) (Labuda and Nuttall, 2004). In India, the Kyasanur forest disease virus (KFDV) was reported in 1957 during an outbreak that caused a high mortality among monkeys and local residents (Shi *et al.*, 2018). In 1945 in the Crimean Peninsula, a febrile illness accompanied by severe haemorrhagic manifestation was reported, and the etiologic agent was isolated (Q. Zhang *et al.*, 2011). In Belgium, Congo, now known as the Democratic Republic of Congo (DRC), another febrile illness

was reported in 1950. The aetiological agents of the two illnesses were serologically related. Further analyses revealed that the aetiological agents were different strains of the same virus termed as CCHFV (Zivcec, Safronetz and Feldmann, 2013; Johnson and Johnson, 2017).

Among the different pathogens transmitted by ticks, tick-borne viruses (TBVs) constitute the majority group of pathogens with considerable diversity and public health concern. TBVs form the largest group of arthropod-borne (arboviruses) viruses and are distributed worldwide. Approximately 160 TBVs have been reported globally, and these spread over 12 genera, with some as yet still unclassified (Qin *et al.*, 2014; Bartíková *et al.*, 2017; Harvey *et al.*, 2019). In the last decade, a number of novel TBVs with as yet unknown impact on the health of humans and/ or animals have been reported across the world (Matsuno *et al.*, 2013; Xia *et al.*, 2015; Papa *et al.*, 2016; Fuente *et al.*, 2017; Torii *et al.*, 2018; Zhao *et al.*, 2018). The increase in the number of identified TBVs in the last decade has also coincided with the emergence of new tick-borne infectious diseases spread across different locations globally (Palacios *et al.*, 2013; Yu *et al.*, 2015; Fuente *et al.*, 2017).

In the sub-Saharan African region, epidemiological information on tick-borne viral pathogens, their prevalence and distribution have been limited (Kobayashi *et al.*, 2017). TBVs have been detected and identified in some parts of Africa, particularly East and West African regions. In the East African region in Kenya, a member of the *Nairovirus* family, NSDV was first detected and isolated in 1910 near Nairobi. The virus is known to be transmitted by *Ixodid* ticks (*Rhipicephalus appendiculatus*) and reported to be highly pathogenic to sheep and goats, and has spread to other countries within East Africa (Marczinke and Nichol, 2002). In West Africa, in Ghana, a novel TBV, *Dugbe* virus, was detected in the Accra region. *Dugbe* virus is transmitted by a member of *Ixodidae* ticks, *Amblyomma variegatum* (Kobayashi *et al.*, 2017). Serological evidence of the

Dugbe virus had previously been detected in South Africa from a patient suffering from severe thrombocytopenia (Burt *et al.*, 1996). This virus has also been reported in Nigeria, the Central African Republic and Ethiopia (Burt *et al.*, 1996).

Studies on tick-borne viral pathogens have been reported in Zambia. The recently published reports of TBVs in Zambia include ASFV and *Shibuyunji* virus in ticks (Matsuno *et al.*, 2015; Chambaro *et al.*,2020). ASFV has been extensively studied in Zambia, and previous reports have highlighted the epidemiology of ASFV in Zambia (Wilkinson *et al.*, 1988). However, information on TBVs in Zambia remains scanty to date.

The importance of this study in Zambia is to provide information on the current and circulating TBPVs detected in ticks. Information on the ecology and epidemiology of TBPVs, and their evolutionary characteristics will help understand the properties of novel TBPVs in Zambia.

1.2 Statement of the Problem

Ticks and other arthropods play a major role in the transmission and epidemiology of diseases of humans and animals globally. Their distribution and abundance determine the prevalence of tick-borne pathogens (TBPs). Emerging and re-emerging diseases caused by TBPs remains a public health concern (Kamani *et al.*, 2013). In Livingstone and Namwala Districts, farming involving cattle is the local people's major activity practiced. Some cattle used in farming are often infested with ticks which pose a veterinary and public health problem. Ticks may transmit pathogens from these domestic animals to humans during pastoral activities. Cattle infested with ticks may serve as competent reservoir hosts for several TBPs. These animals become sentinels for tick infestation in the environment and thus influences the distribution of pathogens carried by ticks during pastoral activities from one place to another. The presence of tick-infested animals in the surrounding environments contributes to the transmission of TBPs, hence the need to implement

control measures to prevent further spread of TBDs.

1.3 Justification of the Study

Knowledge on TBPVs is scanty in many countries, including sub-Saharan Africa. In Zambia, there is paucity of information on the ecology and epidemiology of TBPVs. As a result of this knowledge gap, little is known about circulating TBPVs in the country and their potential threat to veterinary and public health. This study could help gain more insights into the genetic diversity, prevalence and distribution of TBPVs in Zambia. Knowledge of TBPVs will help inform strategies for the control of TBPs. Disease surveillance and screening for TBPVs will help in monitoring tick species and pathogens they carry. This will also help to understand the diversity of viruses in ticks and their potential to cause diseases of human and veterinary concern.

1.4 Research Question

What is the prevalence and genetic characteristics of TBPVs in ticks infesting cattle in the Southern Province of Zambia?

1.5.0 Objectives

1.5.1 General Objective

To detect and phylogenetically analyze TBPVs from ticks infesting cattle in the Southern Province of Zambia.

1.5.2 Specific Objectives

1.5.2.1 To detect the presence of TBPV genome in ticks infesting cattle in Namwala and Livingstone districts of Southern Province using Reverse-Transcriptase Polymerase Chain Reaction (RT-PCR).

1.5.2.2 To determine the phylogenetic relationships among TBPVs in ticks infesting cattle in Namwala and Livingstone districts of Southern Province.

CHAPTER TWO: LITERATURE REVIEW

2.1 Overview and Classification of TBVs

TBVs are a group of arthropod-borne viruses (arboviruses) that are transmitted by ticks. Arboviruses belong to different families and genera of viruses that are spread by ticks and mosquitoes (Bartíková *et al.*, 2017; Kazimírová, Thangamani and Bartíková, 2017; Dehhaghi *et al.*, 2019). Tick-borne arboviruses rank second from mosquito-borne viruses in terms of pathogen transmission. Mosquitoes have been reported to transmit about 50% of arboviruses, whereas ticks transmit 25% of all known arboviruses (Sang *et al.*, 2006). TBVs are the most diverse group of viruses taxonomically and are distributed among five families (*Asfarviridae, Flaviridae, Nairoviridae, Orthomyxoviridae* and *Phenuiviridae*) and two orders, the *Bunyavirales* and *Mononegavirales* (Brites-neto *et al.*, 2015; Rochlin and Toledo, 2020).

The International Committee on the Taxonomy of viruses (ICTV) re-classified the family *Bunyaviridae* into an order *Bunyavirales* (Maes *et al.*, 2018, 2019). The order *Bunyavirales* is the latest order which contains the largest group of arboviruses infecting humans and animals (Albornoz *et al.*, 2016; Rochlin and Toledo, 2020). Approximately 530 viruses with diverse properties are known to belong to the order *Bunyavirales* (Kuhn *et al.*, 2016; Maes *et al.*, 2018). Different families have been assigned within this order together with different genera within each family to species level based primarily on serological cross-reactivity, characteristic genus-specific genome, segment termini sequences, host association and transmission pathways (Kuhn *et al.*, 2016; Maes *et al.*, 2018). The order currently has nine families, of which eight are new and one renamed family, *Peribunyaviridae* (Matsumoto *et al.*, 2018; Maes *et al.*, 2019). Of the known 200 TBVs, about 80% are members of *Flavivirus, Nairovirus, Orbivirus,* and *Phlebovirus* genera (Labuda and Nuttall, 2004). Included in the *Phlebovirus* group are viruses known to cause diseases

of humans and animals. These viruses are vectored by arthropods, including Phlebotomine sand-flies, mosquitoes and ticks (Matsuno *et al.,* 2015; Blitvich *et al.*, 2018; Maes *et al.*, 2019).

2.2 Tick-borne Phleboviruses

TBPVs belong to the arboviruses group, which is the largest biological group of viruses. They have been reported in different geographical locations worldwide, and this has been attributed to the spread of ticks and also due to anthropogenic activities (Crispin *et al.,* 2014). About 70 arboviruses reported belong to the genus *Phleboviruses,* and these are distributed worldwide. They are significant to public health and are zoonotic in nature. *Phleboviruses* are endemic in most parts of Africa and the Middle East and cause diseases with atypical symptoms both in humans and animals. Some infections caused by TBPVs are clinically difficult to identify and detect (Crispin *et al.*, 2014; Matsuno *et al.*, 2015).

TBPVs are members of the family *Phenuiviridae* in the order *Bunyavirales.* The *Phenuiviridae* family has four genera (*Goukovirus*, *Phasivirus*, *Tenuivirus* and *Phlebovirus*) transmitted by different vectors (ticks, mosquitoes, midges) (Bartíková *et al.*, 2017; Ohlendorf *et al.*, 2019). The genus *Phlebovirus* is taxonomically divided into two groups: a monophyletic group of dipteran-transmitted phlebovirus group and a paraphyletic group of TBPV. The dipteran-phlebovirus group are found in eponymous phlebotomies, biting midges, mosquitoes and sand flies, and this forms the sand fly/mosquito group transmitted by sandflies and mosquitoes (*Aedes* and *Culex* mosquitoes) with Rift Valley fever virus (RVFV) representing the group (Wuerth and Weber, 2016; Ohlendorf *et al.*, 2019). The TBPV group is transmitted by ticks of the *Ixodidae* and *Agarsidae* groups. The *Ixodidae* ticks transmit two human-pathogenic viruses: severe fever with thrombocytopenia syndrome virus (SFTSV) and the Heartland virus (HRTV). Both SFTSV and HRTV are associated with haemorrhagic fever-like illnesses in humans. The *Argasidae* groups are

known to transmit viruses such as ASFV which infect both domestic and wild pigs with *Ornithodoros moubata* been the primary vector (Mansfield *et al.*, 2017).

Other groups of the TBPVs include the Uukuniemi, Bhanja and Kaisodi groups. The Uukuniemi and Kaisodi groups have not yet been recognized to contain human pathogens, while the Bhanja group that includes *Bhanja virus* (BHAV) has been associated with sporadic febrile illnesses (Matsumoto *et al.*, 2018; Matsuno *et al.*, 2018; Ohlendorf, Marklewitz, Kopp and Yordanov, 2019; Ohlendorf, Marklewitz, Kopp, Yordanov, *et al.*, 2019). Matsuno *et al* (2018) showed serological evidence for SFTSV and HRTV to infect domestic animals, including sheep, goats, cattle, pigs, dogs and chickens, and rodents (Matsuno *et al.*, 2018). However, these viruses have been reported to cause disease in humans, and epidemiological information about the infections they cause has been reported in different regions of the globe, such as the China, Japan, South Korea and the USA (Chen *et al.*, 2014; Liu *et al.*, 2014).

2.3 Genome Organization of TBPVs

TBPVs have a resemblance to *Nairoviruses* in terms of genomic organization. The virion of *Phleboviruses* is spherical with a diameter of 100nm and enveloped by a host-derived lipid membrane that comprises two viral glycoproteins, Gn and Gc, on the surface of the virion particle (Hornak et al., 2016). Three distinct single-stranded RNA genome segments are packaged in the ribonucleoprotein (RNP) particles by the nucleocapsid protein N and associated with the RNA-dependent RNA polymerase (RdRp) (Wuerth and Weber, 2016). The tripartite genome of *Phleboviruses* consists of the single-stranded negative sense (ambisense) RNA segments with the large or L-segment that encodes for the viral polymerase (RNA-dependent RNA polymerase), the medium or M-segment, glycoprotein precursors Gn and Gc and in some viruses, an accessory NSm protein, the small or S-segment and non-structural proteins NSs (Hornak, Lanchy and Lodmell,

2016; Klimentov *et al.*, 2016). The S-segment uses an ambisense coding strategy, i.e. it contains two genes with opposite polarities. The nucleocapsid protein N is thereby translated from mRNA that is directly transcribed from the genomic S-segment, whereas the non-structural proteins NS, mRNA is transcribed from the antigenomic S-segment (Briese, Calisher and Higgs, 2013; Matsuno *et al.*, 2013; Elliott and Brennan, 2014; Spiegel and Plegge, 2016; Wuerth and Weber, 2016; Bartíková *et al.*, 2017). For most TBPVs, the viral L-segment is the most conserved region, and this has been used as a target for PCR amplification in the screening and detection of new and/ or novel TBPVs. Matsuno *et al* (2015) designed degenerate primers targeting the conserved region of the L-segment of *Phleboviruses* and reported a novel virus, the Shibuyunji virus. Kobayashi *et al* (2017) used the same primer sets previously described by Matsuno *et al* (2015) to screen for TBPVs and also reported a novel virus, the Odaw virus. Both of these novel TBPVs were detected using RT-PCR targeting the partial L-gene in the L-segment of TBPVs.

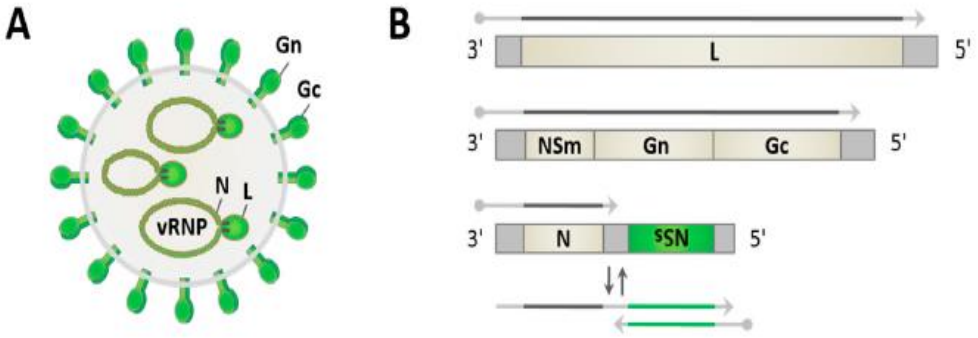

Figure 2.1: Prototypic phlebovirus virion and genome organization

(**A**) Virus particles contain the pseudo circularized tripartite single-stranded RNA genome, packaged into virus-sense RNPs (vRNPs) by nucleocapsid protein N and associated with the viral RNA-dependent RNA polymerase (RdRp) L, within a lipid envelope covered by heterodimers of glycoproteins Gn and Gc. (**B**) The three viral genome segments, large (L), medium (M) (both being purely negative-sense), and small (S) (ambisense), code for the structural proteins L, the Gn and Gc, and N, respectively. Viral mRNAs contain a 51-cap (dot) and short heterogenous host-derived sequences. mRNAs transcribed from genomic RNAs are shown as grey arrows. The non-structural protein NSs mRNA (green arrow) is synthesized from antigenomic RNA (two-coloured arrow). Dipteran-borne Phleboviruses also encode a non-structural protein on the M segment (NSm).
Source: Wuerth and Weber, 2016**.**

2.4 Replication of TBPVs

Replication of the virus occurs in the cytoplasm of the host cell. Following attachment of the virus particle, uncoating is mediated by the fusion of the viral envelope with host membranes of the endocytic system (Wuerth and Weber, 2016). Then the incoming RNPS serve as templates for primary transcription. Translation of the viral protein in the cytoplasm and endoplasmic reticulum is mediated by cleavage of the polyprotein encoded by the M segment into Gn and Gc. Replication of the viral genome occurs when the viral polymerase switches to a primer-independent synthesis of full-length antigenomic RNA, which serves as a template for the synthesis of progeny genomic RNA (Wuerth and Weber, 2016). The newly generated genomes then produce more viral mRNAs in secondary transcription. Both genomic and antigenomic RNA segments carry a 5'-triphosphate moiety and are then packaged into RNPs. Assembly and budding of the viral particles occur at the membranes of the Golgi apparatus, followed by the release of the virions via secretory pathway (Wuerth and Weber, 2016). The synthesis of viral proteins, and the virus replication depends on the cellular host machinery (Amroun *et al.*, 2017).

2.5 Epidemiology of Diseases caused by TBPVs

Newly emerging TBPVs have complicated the epidemiological landscape of tick-borne infections. Emerging and re-emerging infectious diseases caused by TBPVs have become public health concerns in some endemic and non-endemic regions of the world (Matsuno *et al.*, 2018). TBPVs have been known to cause diseases of humans and animals in different geographical settings of the world. They cause a range of symptoms, from mild febrile disease to haemorrhagic fever (Wuerth and Weber, 2016). On the global scale, TBPVs haven't yet been reported or associated with global pandemics. However, they have been known to cause diseases in certain regions of geographical description, such as Northern America and South East Asia, where they have been associated with diseases such as Severe Fever with Thrombocytopaenia Syndrome (SFTS) and

HRTV in humans (Q. Zhang *et al.*, 2011; Elliott and Brennan, 2014; Li, 2015; Mansfield *et al.*, 2017). In China, an emerging infectious disease occurring in humans and classified as SFTS was first documented in 2007. The aetiological agent of the infection was isolated from the patients' blood, and the virus was named SFTSV. SFTSV was detected in ticks of the genus *Haemaphysalis longicornis* that were collected from livestock in places where the patients originated from (Q. Zhang *et al.*, 2011; Zhang *et al.*, 2019). The patients were admitted to the hospital in Henan Province and presented with acute fever, thrombocytopaenia, leukocytopaenia, neurological disorders and multiple organ failure, muscular and gastrointestinal symptoms. Between 2008 and 2010, further reports of this disease were documented in Hubei Province with a case fatality rate of 10%. Epidemiological characteristics of SFTS was associated with tick activities present in endemic areas (Li, 2015). In 2009, HRTV a member of TBPVs was reported in North America from two farmers in Missouri.

The patients had clinical presentations similar to SFTS, and the source of the infection was tick-bites from ticks belonging to the genus *Amblyomma americanum*. Sequencing and phylogenetic analysis of HRTV showed that the virus clustered closely to SFTSV (Q. Zhang *et al.*, 2011). The sequence identity of HRTV and SFTSV revealed a 73% similarity by alignment of the polymerase gene. HRTV and SFTSFV are genetically related viruses in the genus *Phlebovirus* and causing infections in humans (Elliott and Brennan, 2014; Mansfield *et al.*, 2017). In Zambia, although reports of ticks and tick borne infections have been reported, most of these are associated with parasites and/ or bacteria. TBPVs infections in humans haven't yet been identified or reported. In domestic pigs, cases of ASF have been reported and documented (Wilkinson *et al.*, 1988).

2.6 Novel TBPVs and Shibuyunji virus

In the recent years, there has been a worldwide emergency of several novel tick-associated viruses with unexplained human and animal health risks (Dinçer *et al.*, 2017). Sequences of novel, genetically-related strains and those with unique molecular and biological signatures and not previously reported have been characterized from a wide range of geographical locations, including Africa, America, Asia and Europe and around the Mediterranean region. This has been aided by the advancement of molecular biology techniques like PCR and new technologies such as Next Generation Sequencing (NGS) (Tokarz, Williams and Sameroff, 2014; Papa *et al.*, 2016; Sameroff *et al.*, 2019). Shen *et al* (2018) reported a first study that identified a novel TBPV named Guertu virus (GTV) isolated from *Dermacentor nuttalli* ticks in Guertu County, Xinjiang Province of China in 2018. *Dermacentor nuttalli* is a dominant tick species in Xinjiang Province of China and an important vector of TBDs. Genome sequence and phylogenetic analyses showed a close relationship between GTV and SFTSV, and HRTV. GTV is a classified member of the *Phlebovirus*, in the family *Phenuiviridae* and order *Bunyavirales*.

In sub-Saharan Africa, few studies have been documented on novel or circulating TBPVs. Kobayashi *et al* (2017) reported two novel putative TBPVs known as *Odaw* virus (ODWV) and *Dugbe* virus (DUGV) in Ghana. These viruses were detected by reverse transcriptase PCR (RT-PCR) using primers targeting the conserved region of the viral L-gene. The viruses were detected in *Amblyomma variegatum* tick species infesting cattle and dogs in Ghana. Although human infection associated with the DUGV is not known, the virus was known to cause a febrile illness in children (Kobayashi *et al.*, 2017). In South Africa, Burt *et al* (1996) showed serological evidence of DUGV virus from a patient with severe thrombocytopenia. The patient had exposure to tick bites, and ticks were collected from the surrounding of where the patient was coming from. The

virus was detected in *Ambylomma variegatum* ticks. Previous isolation and detection of the DUGV virus have been reported from Nigeria, the Central African Republic and Ethiopia (Burt *et al.*, 1996).

In Zambia, Matsuno *et al* (2015) first reported and documented the *Shibuyunji* virus as a novel TBPV discovered in Southern Africa. Conventional RT-PCR using primers targeting the conserved region of the L-gene of the viral genome was used to detect the virus from *Rhipicephalus* tick species infesting cattle in Shibuyunji District of Central Province in Zambia. Viral sequences of the *Shibuyunji* virus first detected in Shibuyunji District were deposited into the GenBank (accession numbers KM370976 CZCZT 13-14 to KM370979 CZCZT 13-23). Human or animal disease associated with *Shibuyunji* virus is not yet known (Matsuno *et al.*, 2015).

Information from this study will help in understanding the importance of TBPVs in Zambia and how ticks play a major role as potential vectors of TBPVs. An understanding of the properties of TBPVs, and their occurrence in nature will be useful in documenting information about TBPVs, which to the present day remains insufficient not only in Zambia but in many parts of the world. This study will also generate information that will be added to existing knowledge about TBPVs.

CHAPTER THREE: MATERIALS AND METHODS

3.1 Study Design

This was a cross-sectional study carried out in Namwala and Livingstone Districts of Southern Province of Zambia. Samples used in this study were conveniently accessed following a previous study on Rickettsial and TBVs.

3.2 Study Sites

This study was conducted in two districts of the Southern Province of Zambia, namely Namwala and Livingstone Districts. In Namwala and Livingstone districts, the study was conducted in Nakamboma and Simoonga areas, respectively. In Namwala district, farming, including livestock production, is the main activity and a source of income for communities. The district is known to have the highest cattle population in the country. In Livingstone district, the tourism industry is the economic activity, and the district is also known to have abundant wildlife. Besides tourism and wildlife, livestock farming is also an important activity, though the district has a lower cattle population than Namwala. In Southern Province, livestock is regarded as a source of income and livelihood (Tembo *et al.*, 2014).

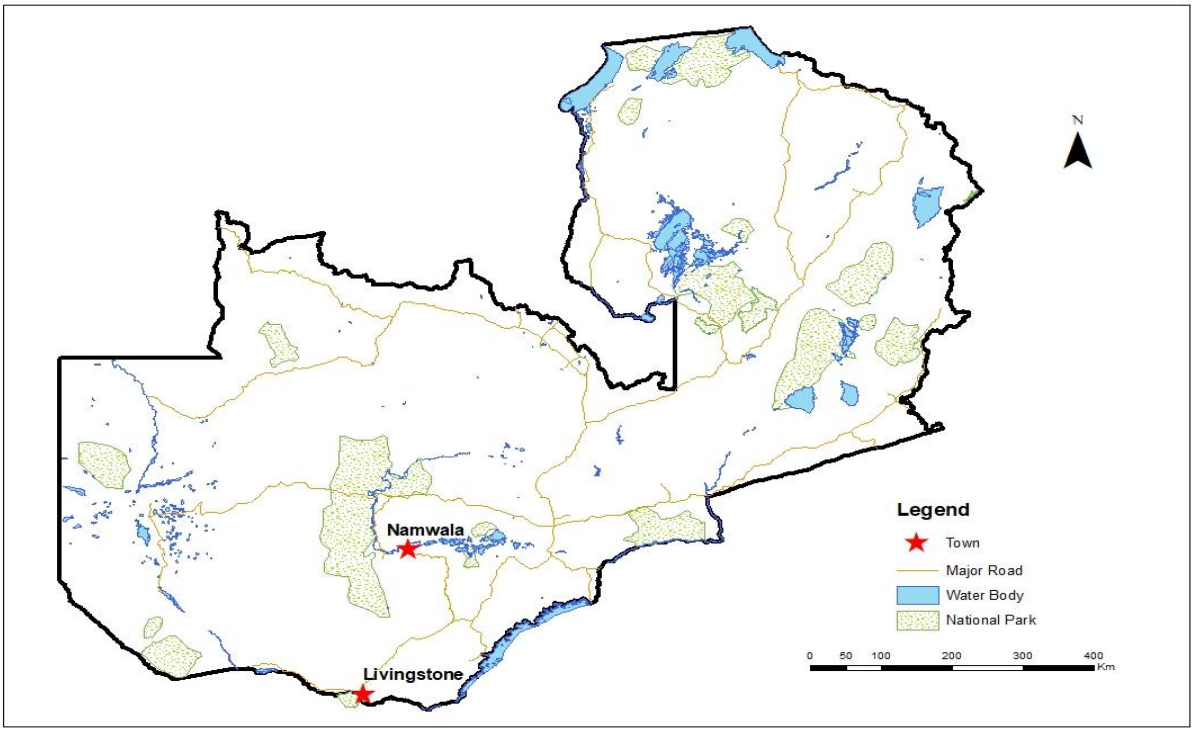

Figure 3.1: Map showing the location of Namwala and Livingstone study sites
Source:(http://www.distancesfrom.com/zm/map-from-Namwala-to-Livingstone-Zambia/MapHistory/9687694.aspx**).**

3.3 Study Frame

3.3.1 Inclusion criteria

The inclusion criteria was that cattle infested with ticks were sampled for tick collection.

3.3.2 Exclusion criteria

The exclusion criteria was that cattle that were known to suffer from any diseases were excluded and not sampled for tick collection.

3.4 Sample Size Determination

The sample size of the population was calculated using the formula below (Wilkinson *et al.*, 1988):

$$n = \frac{Z^2 P\,(1-P)}{d^2}$$

Where: *Z=1.96*, the value of the standard distribution corresponding to a significant value of α *(1.96 at* α *=0.05), P* is the known prevalence taken to be at 80% *d* is the absolute value at 0.04

Where: $$n = \frac{(1.96)^2 0.8(1-0.8)}{(0.04)^2}$$

$$n = 384.16$$

Therefore, n = 384 was the required sample size. However, only 363 ticks, representing 94.5% of the total number of required ticks, were collected from cattle in the two districts.

3.5 Detection of TBPVs in Ticks

3.5.1 Tick Collection, Transportation and Identification

A total of 363 ticks were collected from cattle in the two districts of Namwala (n=199) and Livingstone (n=164) in the Southern Province of Zambia. Ticks were picked from animals using a pair of forceps and placed into individual Eppendorf tubes that were perforated and containing a fresh leaf for moisture. Ticks were then transported live to the Virology Laboratory in the Department of Disease Control in the School of Veterinary Medicine. They were identified morphologically using taxonomic keys and stored at -80ºC until further processing. The ticks used in this study were collected between 2018 and 2019.

3.5.2 RNA Extraction

RNA was extracted from individual ticks using the TRIzol™ (Sigma Aldrich, Dorset, UK) kit according to the manufacturer's instructions. Briefly, individual ticks were put into 1.5mL Eppendorf tubes, and two stainless steel beads added to each tube. Then 100µL of the lysis buffer was added to the tube, and the mixture was placed on a homogenizer and homogenized at 3500xg speed for 30seconds. Approximately 500µl of TRIzol™ reagent was added to each tube and the mixture vortexed briefly. Samples were incubated at room temperature for 5 minutes. About 100µL of chloroform was added to each tube, and the tubes were vigorously shaken and vortexed

for 15 seconds to ensure complete mixing and lysis. The tubes were then incubated for 3 minutes at room temperature and centrifuged at 12000xg for 15 minutes at 4ºC. The aqueous layer was pipetted into a clean 1.5mL Eppendorf tube leaving behind the interphase and the Phenol-chloroform phase. Three hundred microlitres of 100% ethanol were added to each tube. To precipitate the RNA, 250µL of 100% Isopropanol was added to each tube containing the aqueous phase per 0.75ml TRIzol LS reagent used for homogenization. The mixture was incubated for 10 minutes and then centrifuged at 12000xg for 10 minutes. The supernatant from each tube was discarded, leaving behind only the RNA pellet. The pellet was then washed with 1mL of 75% ethanol per 0.75mL TRIzol LS reagent used in the initial homogenization. Samples were vortexed briefly to mix completely. The samples were centrifuged at 7500xg for 5 minutes at 4ºC and supernatant discarded. The tubes were air-dried for 5-10minutes, and the RNA was resuspended in 50µl of RNase free water. Tubes were incubated in a water bath for 15 minutes, and RNA was extracted. The extracted RNA was pooled (n=37) by tick species and sampling area (table 3.1). The number of tick pools varied, with some pools having less than ten ticks per pool. About 3µL of RNA from individual ticks was used to make each pool. Samples were stored overnight at -30ºC and then used the following day for downstream application.

Table 3.1: Pools of RNA showing the number of ticks per individual pool, tick species and sampling area.

Pool number	Number of ticks	Tick species	Site
1	10	*Amblyomma sp.*	Livingstone
2	6	*Amblyomma sp.*	Livingstone
3	10	*Rhipicephalus sp.*	Livingstone
4	10	*Rhipicephalus sp.*	Livingstone
5	10	*Rhipicephalus sp.*	Livingstone
6	10	*Rhipicephalus sp.*	Livingstone
7	10	*Rhipicephalus sp.*	Livingstone
8	12	*Rhipicephalus sp.*	Livingstone
9	11	*Hyalomma sp.*	Livingstone
10	10	*Hyalomma sp.*	Livingstone
11	10	*Hyalomma sp.*	Livingstone
12	12	*Hyalomma sp.*	Livingstone
13	10	*Hyalomma sp.*	Livingstone
14	13	*Hyalomma sp.*	Livingstone
15	10	*Hyalomma sp.*	Livingstone
16	10	*Hyalomma sp.*	Livingstone
17	10	*Amblyomma sp.*	Namwala
18	10	*Amblyomma sp.*	Namwala
19	10	*Amblyomma sp.*	Namwala
20	11	*Amblyomma sp.*	Namwala
21	10	*Rhipicephalus sp.*	Namwala
22	10	*Rhipicephalus sp.*	Namwala
23	10	*Rhipicephalus sp.*	Namwala
24	8	*Rhipicephalus sp.*	Namwala
25	8	*Rhipicephalus sp.*	Namwala
26	10	*Rhipicephalus sp.*	Namwala
27	10	*Rhipicephalus sp.*	Namwala
28	8	*Rhipicephalus sp.*	Namwala
29	10	*Rhipicephalus sp.*	Namwala
30	10	*Rhipicephalus sp.*	Namwala
31	10	*Rhipicephalus sp.*	Namwala
32	10	*Rhipicephalus sp.*	Namwala
33	10	*Rhipicephalus sp.*	Namwala
34	10	*Rhipicephalus sp.*	Namwala
35	10	*Rhipicephalus sp.*	Namwala
36	6	*Rhipicephalus sp.*	Namwala
37	8	*Rhipicephalus sp.*	Namwala

3.5.3 cDNA Synthesis

For cDNA synthesis, SuperScript® IV reverse transcriptase kit (Invitrogen, Carlsbad, CA, USA) was used. The protocol was followed as per the manufacturer's instructions. The reaction mixture of 25µL contained 5µL of 5X SuperScript IV buffer, 1µL of 50µM random hexamers, 1µL of Oligo (dT) 12-18 primer (0.5µg/µL), 1µL of 10mM deoxynucleotide triphosphate (dNTP) mixture, 1µL of 100mM dithiothreitol, 1µL of RNase inhibitor (40U/µL), 1µL of SuperScript IV reverse transcriptase (200U/µL), 4.0µL of nuclease-free water, and 10µL of pooled RNA. The reaction mixtures were incubated at 23ºC for 10minutes, and then 50ºC for 10 minutes and finally 80ºC for 10minutes. The samples were then stored at -20˚C prior to PCR.

3.5.4 PCR Detection of the L gene in Tick Samples

To screen for TBPVs, KOD One PCR Master Mix Blue (Toyobo Co. Osaka, Japan) was used to amplify an approximately 500bp fragment using degenerate primers that binds to the conserved regions of the L-gene. The primers used in this study were designed as previously described by Matsuno *et al* (2015). Primers TBPVL2759F (5'-CAGCATGGIGGICTIAGAGAGAT-3') were used as the forward primer and TBPVL3267R (5'-TGIAGIATSCCYTGCATCAT-3') as the reverse primer, respectively. The use of positive controls in this study was not applicable as screening for all TBPVs was based on the targeted L-gene based on the primers described by Matsuno *et al* (2015). First, pooled samples were tested for TBPVs and positive pools had the individual ticks from each pool tested for TBPVs using the same primers and assay conditions. The 20µL reaction mixture had 6µL nuclease-free water, 10µL KOD one master mix, 1µL of forward primer (10µM), 1µL of reverse primer (10µM), and 2µL of cDNA. The PCR cycling conditions were 35 cycles each consisting of denaturation at 98ºC for 10s, annealing at 55ºC for 5s and an extension at 68ºC for 1s.PCR products were subjected to electrophoresis on a 1.5%

agarose gel (Invitrogen, USA) stained with ethidium bromide (0.625mg/ml) and viewed under ultraviolet light in a UV transilluminator to check for amplification.

3.6 Determination of Phylogenetic Relationships amongst TBPVs Detected

3.6.1 Purification of PCR Products

PCR products of approximately 500bp size were purified by Wizard® SV Gel and PCR Clean-Up System (Promega, Madison, WI, USA) according to the manufacture's instruction. Equal volumes of membrane binding solution and PCR products were placed in an SV Mini Column inserted in a collection tube. The mixture was incubated at room temperature for 1 minute. Then the mixture was centrifuged at 16000xg for 1 minute after incubation, and the flow-through was discarded. The Mini Column was reinserted into the collection tube, and 700µL of the membrane wash solution was added and centrifuged at 16000xg at room temperature for 1 minute. The flow-through was discarded, and the Mini Column was reinserted into the collection tube. This step was repeated with 500µL of the membrane wash solution and centrifuged at 16000xg for 5 minutes. The collection tube was emptied and column assembly centrifuged with the lid open at 16000xg for 1 minute to allow for evaporation of residual ethanol. The Mini Column was transferred to a clean 1.5mL centrifuge tube. Then 50µL of nuclease-free water was added to the Mini Columns and incubated at room temperature for 1 minute and later centrifuged at 16000xg for 1 minute. The Mini Column was discarded, and purified DNA was stored at -30ºC.

3.6.2 DNA Sequencing of PCR Products

The purified PCR products were sequenced directly in both directions using the forward and reverse primers in the reaction. Sanger sequencing of the purified PCR products was done using the BrilliantDye™ v3.1 Terminator Cycle Sequencing Kit (NimaGen BV, Nijmegen, The Netherlands). Briefly, 2µL of Sodium acetate and 2µL of EDTA were added to each PCR tube after the sequencing reaction. The 50µL of 100% alcohol was added to each of the tubes and mixed

by tapping. The mixture was incubated at room temperature for 15 minutes while protecting it from sunlight by covering it with aluminium foil. The mixture was centrifuged at 15000rpm for 20 minutes at room temperature. The supernatant was then pipetted out, leaving the pellet. Next, 70µL of 70% alcohol was added and centrifuged at 15000xg for 15 minutes. The supernatant was removed, leaving behind the pellet. While open, the tubes were vacuumed for 5 minutes to dry. Then 15µL of formadide HiDi was added, and the mixture was vortexed thoroughly. The mixture was then denatured at 95ºC for 2 minutes. The samples were then transferred on ice and loaded into the AB 3500 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) for analysis. Assembly and editing of nucleotide sequences were performed using GENETYX ATGC software, version 7.5.1 (GENETYX Co., Tokyo, Japan). The sequences obtained in this study were deposited in the GenBank under accession numbers LC567391-LC567409.

3.6.3 Phylogenetic Analysis of TBPV sequences

Phylogenetic analysis was conducted using the Maximum Likelihood method based on the GRT+G model (Tamura-Nei parameter model). The analysis was conducted using the Molecular Evolution and Genetic Analysis (MEGA) software version 6.06, with the bootstrap (1000 replicates) method being used to determine tree reliability. Nucleotide and Amino Acid similarities of the sequences obtained were calculated using Sequence Demarcation Tool (SDT) software version 1.2.

3.7 Data Analysis

Data analysis to determine the pooled prevalence of the Shibuyunji virus in this study was performed using an online Epidemiological calculator (EpiTools, Sergeant, ESG, 2018) software available at: http://epitools.ausvet.co.au. The pooled prevalence calculator was used for estimating prevalence with uncertain sensitivity and specificity.

3.8 Ethics Consideration

Ethical approval to conduct the study was granted by ERES Converge (IRB-00005948, FWA-00011697), under reference number 2017-Jul-021. Permission to collect ticks from the animals was sought and granted by the livestock owners as well as the Ministry of Fisheries and Livestock, Department of Veterinary Services.

CHAPTER FOUR: RESULTS

4.1 Detection of TBPVs in Ticks

4.1.1 Tick Identification

In this study, ticks were identified according to species and site of collection. *Amblyomma sp* ticks collected in Namwala and Livingstone Districts were 41 and 16, respectively. *Hyalomma sp* ticks were only collected in Livingstone (n=86). *Rhipicephalus sp* ticks collected in Namwala and Livingstone study sites were 158 and 62, respectively (Table 4.1).

Table 4.1: Tick genera and prevalence in Namwala and Livingstone districts.

Tick genus	**Number of ticks analyzed (Prevalence)**		**Total number of ticks (Prevalence)**
	Namwala	Livingstone	
Amblyomma sp	41 (0%)	16 (0%)	57 (0%)
Hyalomma sp	0 (0%)	86 (0%)	86 (0%)
Rhipicephalus sp	158 (12 %)	62 (0%)	220 (8.6 %)
Total	**199**	**164**	**363 (5.2%)**

4.1.2 PCR Detection of the L gene Sequences in Ticks

Rhipicephalus ticks were positive for TBPVs with a pooled prevalence of 8.1%. Of the 158 individual ticks tested, 19 were positive for TBPVs, and this gave a prevalence of 5.2% (19/363) Out of the 37 pooled samples, only three pools from *Rhipicephalus* ticks (3/37), all from Namwala district, were found to be positive for TBPVs (Figure 4.1).

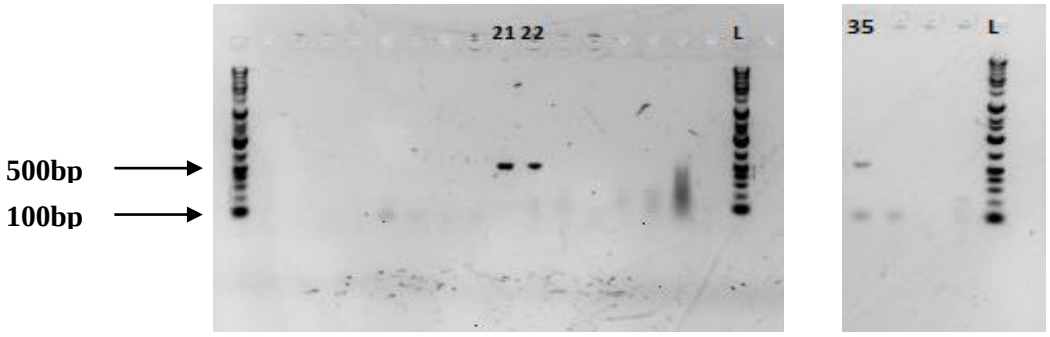

Figure 4.1: Gel images of fragments amplified by PCR. Samples 21, 22and 35 are positive samples, and L is the 100bp DNA ladder. Five microliters of each DNA sample was loaded into each well on the 1.5% agarose gel containing ethidium bromide stain.

4.2 Determination of Phylogenetic Relationships amongst TBPVs

4.2.1 Percent similarity of the amplified 500bp L-gene

Percent similarity analysis of the amplified approximately 500bp revealed that all the TBPVs detected in this study showed a close similarity to *Shibuyunji* virus isolates (table 4.2) that were detected in *Rhipicephalus spp.* collected in 2013 in Zambia. The *Shibuyunji* virus isolates with the highest similarity to those investigated in this study as determined by BLAST analysis showed a 94.1%-99.8% nucleotide sequence identity. Among those detected in this study, at the nucleotide and amino acid levels, the virus shared 92.8% -100%and 98.1%-100% sequence identity, respectively.

Table 4.2: Shibuyunji viruses with the highest nucleotide sequence similarity to those investigated in this study as determined by BLAST analysis.

Shibuyunji virus investigated (GenBank accession no.)	Virus with the highest degree of sequence identity (GenBank accession no.)	% Identity	GenBank Accession No.
Zam1-19 (LC567391)	Shibuyunji virus isolate CZCZT13-14	99.16	KM370976
Zam2-19 (LC567392)	Shibuyunji virus isolate CZCZT13-14	99.37	KM370976
Zam3-19 (LC567393)	Shibuyunji virus isolate CZCZT13-14	99.37	KM370976
Zam4-19 (LC567394)	Shibuyunji virus isolate CZCZT13-14	99.79	KM370976
Zam5-19 (LC567395)	Shibuyunji virus isolate CZCZT13-14	99.79	KM370976
Zam6-19 (LC567396)	Shibuyunji virus isolate CZCZT13-14	99.79	KM370976
Zam8-19 (LC567397)	Shibuyunji virus isolate CZCZT13-18	99.58	KM370977
Zam10-19 (LC567398)	Shibuyunji virus isolate CZCZT13-14	99.79	KM370976
Zam11-19 (LC567399)	Shibuyunji virus isolate CZCZT13-14	99.58	KM370976
Zam12-19 (LC567400)	Shibuyunji virus isolate CZCZT13-14	99.37	KM370976
Zam13-19 (LC567401)	Shibuyunji virus isolate CZCZT13-22	94.11	KM370978
Zam15-19 (LC567402)	Shibuyunji virus isolate CZCZT13-14	99.16	KM370976
Zam16-19 (LC567403)	Shibuyunji virus isolate CZCZT13-14	99.79	KM370976
Zam18-19 (LC567404)	Shibuyunji virus isolate CZCZT13-14	98.73	KM370976
Zam19-19 (LC567405)	Shibuyunji virus isolate CZCZT13-14	99.58	KM370976
Zam20-19 (LC567406)	Shibuyunji virus isolate CZCZT13-14	99.79	KM370976
Zam21-19 (LC567407)	Shibuyunji virus isolate CZCZT13-14	99.58	KM370976
Zam33-19 (LC567408)	Shibuyunji virus isolate CZCZT13-14	98.73	KM370976
Zam35-19 (LC567409)	Shibuyunji virus isolate CZCZT13-14	99.37	KM370976

4.2.2 Phylogenetic analysis

Phylogenetic analysis of the Shibuyunji viruses detected in the country showed genetic diversity, with ZAM13-19 being the most distantly related to others (Figure 4.2). Compared to other reference sequences from GenBank, the phylogenetic analysis revealed a close relationship of the Zambian Shibuyunji virus to American dog tick phlebovirus, with those from Zambia forming a well-supported distinct cluster (Figure 4.3).

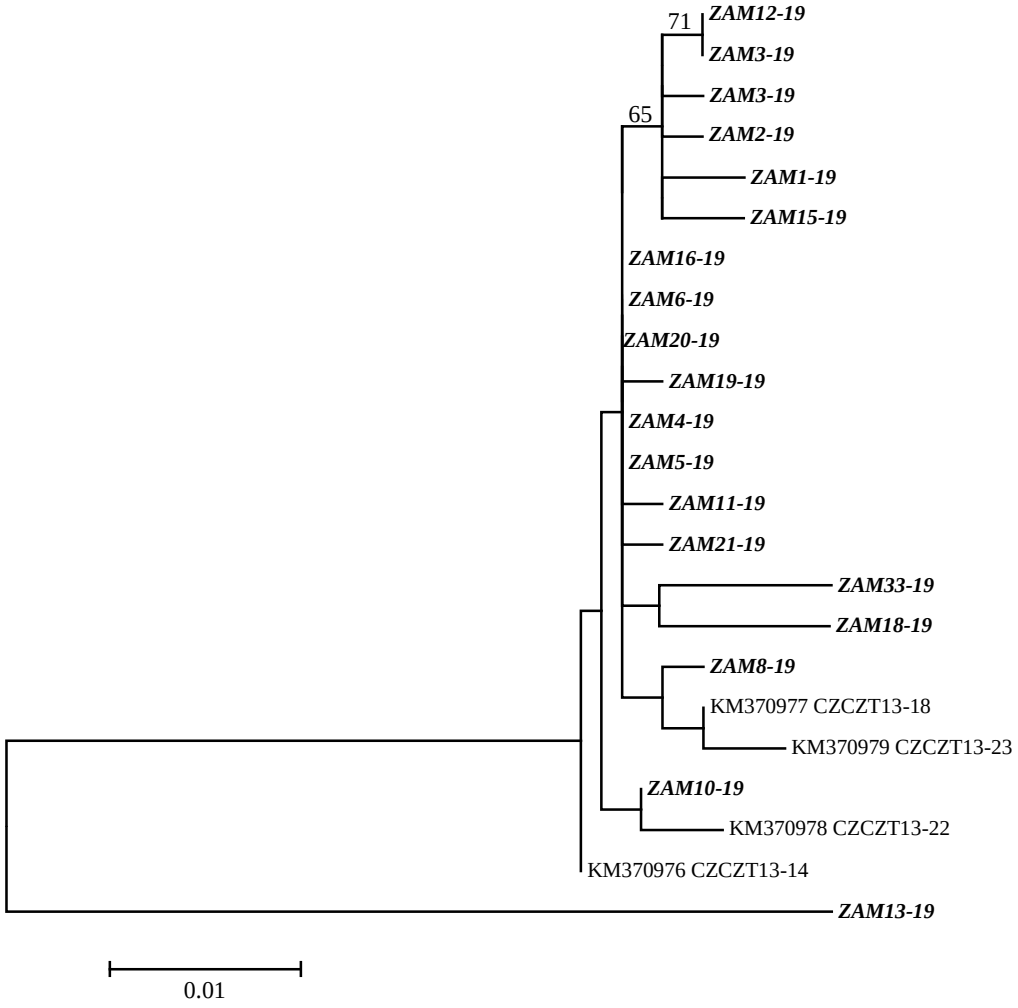

Figure 4.2: Phylogenetic relationship of the Shibuyunji virus isolates detected in Zambia based on the L-gene. The tree was constructed using the maximum likelihood method based on the GRT+G model with 1000 bootstrap replications in MEGA 6.06. The viruses detected in this study were all from Namwala District and are bold and italicized. Isolates collected from Shibuyunji District are represented by GenBank accession numbers. The analysis included 23 nucleotide sequences and 475 positions in the final data set.

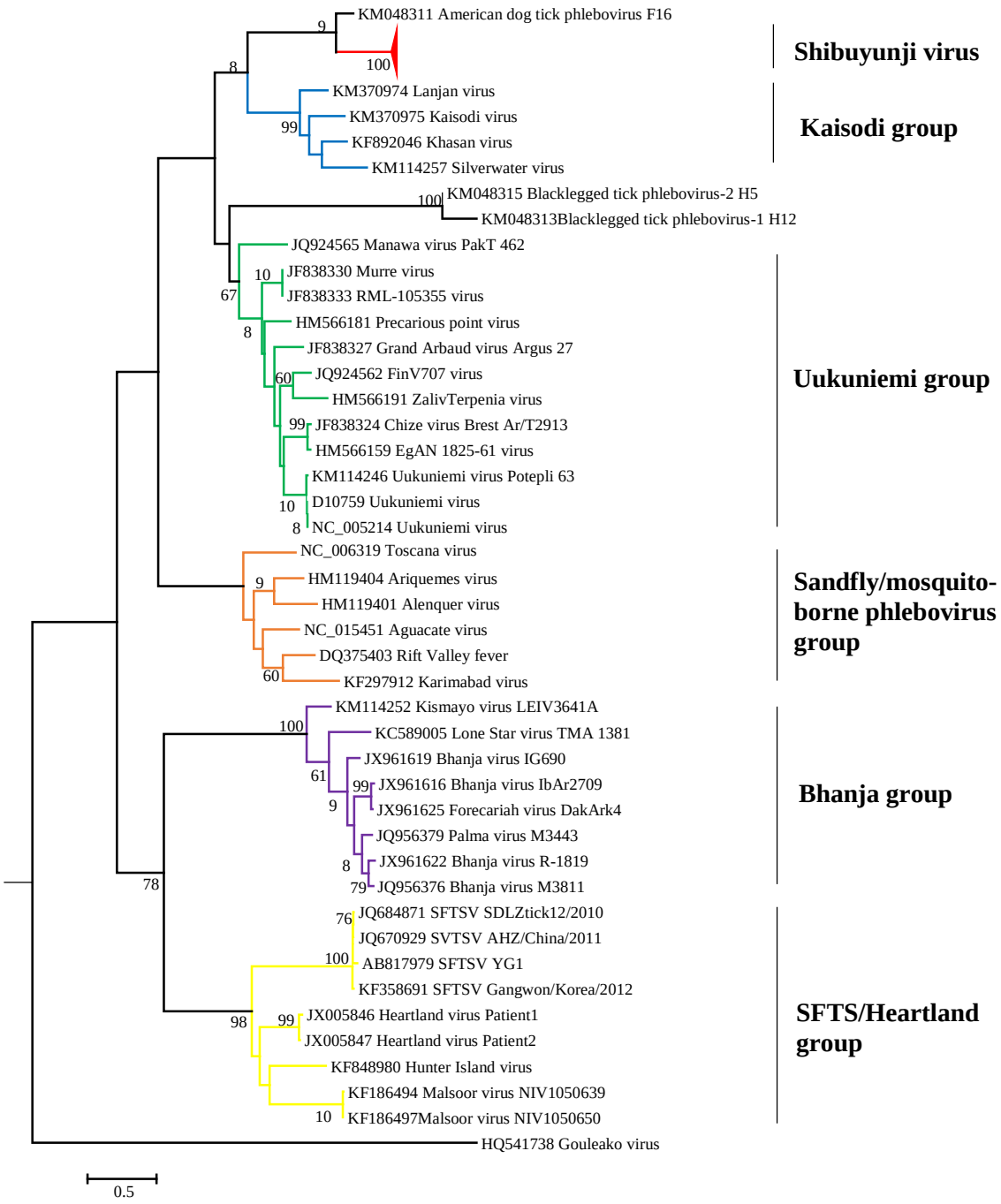

Figure 4.3: Phylogenetic tree showing evolutionary relationships of the Shibuyunji virus isolates and reference sequences. The tree was constructed using the maximum likelihood method based on the GRT+G model with 1000 bootstrap replicates. The analysis included 66 nucleotide sequences with a total of 436 positions in the data set. All Shibuyunji virus isolates formed a distinct cluster and are compressed in colour red, while reference sequences are indicated by their GenBank accession numbers and strain names. The different branch colours indicate the distinct genetic groups of the TPBVs. Bootstrap values ≥60% are shown at branch nodes. The scale indicates the number of substitution per site.

CHAPTER FIVE: DISCUSSION

5.1 Discussion

This study screened for TBPVs in *ixodid* ticks from the Southern Province of Zambia. A prevalence of 5.2% of the Shibuyunji virus was reported in this study. Matsuno *et al* ((2015) first reported the Shibuyunji virus at a prevalence of 34.8% (8/23) from ticks collected in the Shibuyunji district in Central Province of Zambia (Matsuno *et al.*, 2015). Considering the difference in the numbers of sampled ticks and the lack of information on the prevalence in *Rhipicephalus* ticks specifically, it is difficult to compare the prevalence between the two studies. In agreement with Matsuno *et al* (2015) study, only *Rhipicephalus* species were positive for the Shibuyunji virus, with the *Amblyomma* and *Hyalomma* species being negative. Therefore, it may seem that *Rhipicephalus* ticks might be the potential vectors of Shibuyunji virus in the country. More vector competence studies are needed to support the potential aspect of *Rhipicephalus* ticks been vectors of the Shibuyunji virus.

Shibuyunji virus is a novel TBPV first isolated and detected in Shibuyunji District in Central Province. In this study, the Shibuyunji virus was detected after screening for TBPV from ticks collected in Namwala and Livingstone Districts with the virus been present only in Namwala district. The primers used in this study were previously described (Matsuno *et al.*, 2015), targeting the partial L-gene in the conserved region of the L-segment RNA. Pathogenicity of the Shibuyunji to cause human or animal infections is not yet known. Considering that TBPVs may be classified under the SFTS/HRTV group, the mosquito/ Sandfly group, the Kaisodi and Bhanja virus groups (Matsuno *et al.*, 2015), taxonomically, the Shibuyunji virus belong to the unclassified group of TBPVs. According to the phylogenetic analysis of TBPVs from a previous study conducted by Kobayashi *et al* (2017), Shibuyunji virus was shown to cluster next to American dog tick phlebovirus and both viruses belonged to the unclassified group of TBPVs.

This study sampled ticks from two different geographical regions, with the Shibuyunji virus being found only in Namwala District. Viral sequences from this study were compared with sequences from a previous study conducted by Matsuno *et al* (2015) in Shibuyunji District. The findings show that the virus could be present beyond the initial area of detection previously reported (Matsuno *et al.*, 2015). This emphasizes the need to conduct countrywide surveillance studies of the Shibuyunji virus to better understand its distribution.

The RT-PCR model was used to screen for TBPVs using degenerate primers as previously described (Matsuno *et al.*, 2015) that binds to the conserved regions of the viral L-gene. The viral L-segment in TBPVs is the largest of the three ssRNA and codes for the L-protein (RdRp). Studies done by Kobayashi *et al* (2017) and Matsuno *et al* (2015) used the RT-PCR model to screen for TBPVs targeting the L-gene, and this revealed the detection of novel *Phleboviruses*, Odaw and Shibuyunji viruses and the same set of primers described by Matsuno *et al* (2015) were used in both studies. The L-genome segment is the most conserved among most TBPVs and therefore this assay identified Shibuyunji virus as the only TBPV based on the amplification of the targeted L-gene (Matsuno *et al.*, 2015). Findings of this study suggest that the Shibuyunji virus may be the only TBPV circulating in the areas that were sampled in this study (detected in Namwala district only), in addition to Shibuyunji district (Matsuno *et al.*, 2015). The conventional RT-PCR assay used in this study was sufficient enough for first-line screening of all TBPVs based on the primers targeting the partial L-gene in the Phlebovirus L-segment RNA prior to NGS.

Sequence and phylogenetic analysis of the nucleotide sequences based on the L-genome segment of the virus revealed considerable genetic diversity of *Shibuyunji* virus detected in Namwala district compared to sequences obtained from Shibuyunji district. In this study, the viral isolate ZAM8-19 (detected in this study) was closely related to the isolates KM370977 CZCZT 13-18

and KM370979 CZCZT 13-23 (isolates from Shibuyunji district), while the isolate ZAM10-15 was closely related to the isolate KM370978 CZCZT 13-22 previously reported by (Matsuno *et al.*, 2015). The isolate ZAM13-19 detected in this study was the most genetically diverse isolate among the Shibuyunji virus sequences from both Namwala and Shibuyunji districts. As some of the strains detected in this study clustered with those detected previously (Matsuno *et al.*, 2015), it seems probable that the movement of tick-infested cattle through local trade may play a role in disseminating ticks harbouring genetically similar viruses between the two areas. Phylogenetic analysis of the Shibuyunji virus with reference sequences obtained from the GenBank showed the Shibuyunji virus forming a bootstrap cluster that was closely related to the American dog tick phlebovirus. The finding of the Shibuyunji virus to be closely related to American dog tick phlebovirus (Matsuno *et al.*, 2015) remains intriguing considering the lack of apparent Spatio-temporal connections (Matsuno *et al.*, 2015).

Within Africa, two other TBPVs have been reported and these are Bhanja virus (Johnson, 1980; Estrada-Peña, Hubálek and Rudolf, 2013; Vayssier-taussat *et al.*, 2015)and *Odaw* virus (Kobayashi *et al.*, 2017). These TBPVs have been reported and identified based on the L-genome segment, which is highly conserved in all *Phleboviruses.* Based on the findings from this study and findings by Kobayashi *et al* (2017) and Matsuno *et al* (2015), it can be said that the L-genome segment of most *Phleboviruses* which is highly conserved, is an important *Phlebovirus* genome for the screening and detection of all novel TBPVs that are yet to be reported. According to some studies conducted in Africa on TBPVs, it would seem that *Rhipicephalus* species play an important role as either vectors or reservoirs as they are the most common ticks from which the viruses have been found (Sang *et al.*, 2006). The only other tick species from which TBPVs have been confirmed in Africa is *Amblyomma variegatum* (Sang *et al.*, 2006).

The potential of this data to public health and veterinary medicine is that it will help understand the evolution, taxonomy and ecological distribution of novel TBPVs and their epidemiological characteristics. Data obtained will also be useful in informing policy on the surveillance and control of ticks and TBPs in the environment.

5.2 Conclusions

In this study, the Shibuyunji virus was the only TBPV detected in one of the two sampled areas (Namwala district). Since no other TBPVs were detected, the findings of this study showed a low diversity of circulating TBPVs in ticks. A prevalence of 5.2% reported in this study indicates the presence of the Shibuyunji virus in other parts of the country other than the initial area of its first detection in the Shibuyunji district. Furthermore, *Rhipicephalus* ticks could play a role either as vectors or reservoir hosts as they are the only ticks in which the Shibuyunji virus was detected. Since isolates detected in this study clustered together with those previously detected in the Shibuyunji district, the movement of tick-infested cattle through local trade or pasture may have contributed to the spread of the virus.

5.3 Limitations of the Study

In this study, only two sampling sites, Namwala and Livingstone Districts, were used, and samples were conveniently accessed following a study on Rickettsia and TBVs. Also, whole genome sequencing analysis was not done to understand the molecular epidemiology of the virus fully.

5.4 Recommendations

Based on the information emerging from this study, the recommendations made are that control measures to address tick infestation in cattle needs to be implemented through farmer training and veterinary extension services. This will minimize TBDs in animals as well as reduce zoonotic transmissions to humans. Further studies with a large sample size covering an extensive geographical area are needed to help understand the diversity and distribution of the Shibuyunji

virus in Zambia. This will provide a better understanding of the ecology, evolution and epidemiology of TBPVs and their potential impact on veterinary and public health. This may be done through tick surveillance studies in different parts of the country.

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