POTENTIALLY – ZOONOTIC VIRUSES IN THE STRAW-COLORED FRUIT BAT (*EIDOLON HELVUM*) IN GHANA

BY

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THIS THESIS IS SUBMITTED TO THE UNIVERSITY OF GHANA, LEGON IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE AWARD OF PhD ZOOLOGY DEGREE

JULY 2015

DECLARATION

I, Richard Dery Suu-Ire declare that this thesis and the work presented in it are my own and have been generated by me as the result of my own original research. I certify that this work, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

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ACKNOWLEDGEMENT

I express my sincere appreciation to the excellent guidance I received from my supervisors: Prof. Yaa Ntiamoa – Baidu, Department of Animal Biology and Conservation Science, University of Ghana, Prof. Andrew A. Cunningham (Institute of Zoology, UK) and Prof. James L. N. Wood (University of Cambridge). The support from Staff of the Veterinary Services and Forestry Commission, Accra Zoological Gardens, Staff of Department of Animal Science and Conservation Biology, Center for African Wetlands and Dr David Hayman, Sinead Sheerin of Cambridge University is appreciated. I acknowledge the support from Noguchi Memorial Institute for Medical Research for granting me access to the Level 3 laboratory and animal experimentation laboratory for the experiment (Necropsy and Storage of bat samples). My appreciation to Prof. Anthony R. Fooks and Dr. Ivan kuzmin for reading and providing valuable comments that helped improve on the work. The financial support from the European Union Antigone Project and Dynamic Drivers of Disease in Africa Consortium (DDDAC) is much appreciated.

Specific Contributions

- David Selden, Priya Das, Emma Wise and Hooman of APHA help in the laboratory analyses of bat samples
- Accra Zoological Garden staff cared for the captive bats
- Dr. Meyir Yiryele Ziekah, Kofi Amponsah Mensah and Silke Riesle-Sbarbaro helped in the routine bat sampling
- Andrew Cunningham, James Wood, Dan Horton and Tony Fooks co-devised the bat experiments.
- Dr. Ivan Kuzmin, Silke Riesle-Sbarbaro, Meyir Ziekah, Ashley Banyard and Andrew Cunningham helped conduct the bat experiments

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LIST OF ABBREVIATIONS

ABLV	Australian bat lyssavirus
ACDP	Advisory committee on dangerous pathogens
APHA	Animal and Plant Health Agency
Approx	Approximately
ARAV	Aravan virus
BBLV	Bekeloh bat lyssavirus
BHK	Baby hamster kidney
cDNA	Complementary deoxyribonucleic acid
CNS	Central neuvous system
CSF	Cerebrospinal fluid
DDDAC	Dynamic Drivers of Disease in Africa Consortium
DFA	Direct fluorescent antibody test
dRIT	Direct rapid Immunohistochemical Test
DUVV	Duvenhage virus
EBLV - 1	European bat lyssavirus type 1
EBLV - 2	European bat lyssavirus type 2
ELISA	Enzyme-linked immunosorbent assay
FAO	Food and Agriculture Organisation of the United Nations
FAT	Fluorescent Antibody test
FAVN	Flourescent antibody virus neutralization
Fig.	Figure
FITC	Fluorescein isothiocyanate Conjugate
GT	Genotype
GMT	Greenwich Mean Time

Hn-PCR	Hemi-nested Polymerase Chain Reaction
IC	Intracerebral Inoculation
IKOV	Ikoma virus
IM	Intramuscular Inoculation
IRKV	Irkut virus
IV	Intravenous
Juv	Juvenile
Kg	Kilogram
KHUV	Khujand virus
LBV	Lagos Bat virus
LLEBV	Lleida bat lyssavirus
MEM	Minimum Essential medium
MERS	Middle East Respiratory Syndrome
mFAVN	Modified fluorescent antibody virus neutralization test
Mg	Miligram
MIT	Mouse Inoculation test
Ml	Milliliter
M-MLV RT	Moloney murine leukemia virus reverse transcriptase
MOKV	Mokola virus
MSA	Multiple sequence alignment
NTC	No template control
^{o}C	Degree Celsius
OIE	World Organisation for Animal Health
PCR	Polymerase chain reaction
Pi	Post inoculation

RABV	Rabies virus
RFFIT	Rapid Fluorescent Focus Inhibition test
RIDT	Rapid Immunodiagnostic test
RNA	Ribonucleic Acid
RT	Room temperature
RTCIT	Rabies tissue culture inoculation test
RT-PCR	Reverse transcriptase polymerase chain reaction
SA	Sub-adult
SAPO	Specific animal pathogen order
SARS	Severe Acute Respiratory Syndrome
SC	Subcutainous
SHIBV	Shimoni bat virus
SM	Sexually mature
TCID	Tissue culture infective dose
µg	Microgram
µl	Microlitre
VNA	Viral Neutralising Antibody
VNT	Viral neutralization test
WCBV	West Caucasian bat virus
WHO	World Health Organisation

ABSTRACT

Bats are reservoirs for many emerging zoonotic RNA viruses, including rabies virus (RABV), SARS-like coronavirus, henipaviruses and filoviruses. Several bat species have been associated with Lagos bat virus (LBV) infection. They include Wahlberg's epauletted fruit bat (*Epomorphorus wahlbergi*), the straw-colored fruit bat (*Eidolon helvum*), the Egyptian fruit bat (*Rousettus aegyptiacus*), and an insectivorous bat, the Gambian slit-faced bat (*Nycteris gambiensis*). Bats are important for the maintenance and transmission of zoonotic viruses. The role of bats in the maintenance and evolution of lyssaviruses is complex and poorly understood. Fruit eating bats, including *E. helvum*, often inhabit and feed in agricultural areas, and this brings them into closer contact with humans and domesticated animals. LBV and other non-rabies lyssaviruses have not been well studied and the risk for humans and animals in Ghana is uncertain. Lyssavirus epidemiology is influenced by the host species distribution, abundance, demographics, behavioral ecology, dispersal, and interactions with humans. *E. helvum* occur in cities and rural areas of Ghana. The species is comestible and hugely harvested for bushmeat by both urban and rural folk. *E. helvum* feeds on fruits from farm plantations and roosts within or near residential areas. The species thus has close interaction with people. The pathobiology and route of transmission of LBV is not known in *E. helvum* bats. This study is one of the few studies of LBV in fruit bats in Ghana and the experimental infection study is the first conducted on LBV in one of its natural reservoir species. Using *E. helvum* as a model, we hypothesize that there is endemic circulation of LBV in this bat species in Ghana. The circulation of Lagos bat virus in the bat population is facilitated by certain pathobiology of the infection including susceptibility to the virus, incubation and clinical periods for different doses and routes of LBV infection, behavioral changes of rabid bats, virus occurrence in various bat tissues and excreta. Inoculation of LBV will

result in clinical disease (rabies), although not all inoculated bats will develop the disease, especially when inoculated peripherally. The aim of this PhD study was dual. Firstly, it investigated infection dynamics of LBV in *E. helvum* in free ranging populations at the 37-Military hospital in Accra and Tano Sacred grove and in a captive population of *E. helvum* kept at the Achimota Forest. Secondly, the study conducted experimental investigation of the pathogenesis of Lagos bat virus, in *E. helvum* in captivity at the Achimota Forest in Accra, Ghana. To evaluate these hypotheses, *E. helvum* bats were repeatedly sampled from two major roost locations in Ghana from 2012 - 2014. Longitudinal sampling was also undertaken quarterly on captive bats at the Achimota Forest. Dead bats were collected from the roost sites and predetermined tissues collected at necropsy. Serum samples were subjected to virus neutralization tests against LBV. Tissues from dead bats were tested by FAT and PCR for LBV antigen and RNA respectively. To investigate the pathobiology of LBV in fruit bats, we conducted experimental intracranial (i.c) and peripheral (i.m) inoculation in captive bred straw-colored fruit bats (*Eidolon helvum*) which were seronegative for LBV, in Accra, Ghana. The study found high seroprevalence (0.447; CI=0.391-0.503) against LBV in Ghanaian *E. helvum* bats. There was, however, clear seasonal variation with a higher proportion of bats testing negative during the rainy season (Wild caught bats p-value = 1.06). None of the brain samples from bats found moribund or dead in this study showed presence of lyssavirus antigen or RNA. The experimental study confirmed that LBV can cause disease in *E. helvum*. Bats that were infected by either route of inoculation showed clinical signs similar to animals infected with RABV, though with some variation between isolates in the IC inoculation experiment. There was detection of virus in the brain, salivary gland and saliva. There was seroconversion in peripherally inoculated bats. A high seroprevalence against LBV is reported in *E. helvum* and other African megacheroptera. No published infection study exists of LBV in one of its

reservoir hosts, such as the straw-colored fruit bat, with a gap in scientific knowledge of the disease. This study demonstrated that LBV is endemic and circulates among *E. helvum* in Ghana. The experimental study confirms that LBV is pathogenic to *E. helvum* by IC and peripheral inoculation and that there is seroconversion in bats before death and in surviving bats. This is the first ever experimental infection study of LBV in *E. helvum* bats. It is concluded that *E. helvum* is a suitable animal model for studies of the pathobiology of lyssaviruses. The species is abundant and widely distributed and adapts well to captivity. Comparative studies should be initiated with other LBV host species and viral isolates to compare the influence of viral origin, dose and route of inoculation, host demographics, and environmental conditions on infection dynamics. This will develop further our understanding of the dynamics of pathogen adaptation and host responses, as well as help to identify public health risk factors for emerging infections among the Chiroptera.

CHAPTER ONE: GENERAL INTRODUCTION

1.1 Introduction

Natural systems such as forests, grasslands, wetlands and oceans provide ecological services that all life depends on. Approximately 70 percent of the 1.5 billion poorest people depend on livestock and natural resources (Newman *et al.*, 2011). The exponential growth of the human population, from ≈1 billion in 1900 to 6.5 billion in 2006, has led to major ecologic changes and drastic wildlife habitat reduction.

Domestic and wild animals, and their ecosystems, contribute to the health and well-being of people. Wildlife is a major source of income, either directly for consumptive or productive use value or indirectly for touristic and scientific values. Alongside the many benefits people derive from animals, the latter can also contribute to public health and veterinary risks that emerge at the human, animal and ecosystem interface (Mackenzie *et al.*, 2013).

Wildlife populations constitute a large and often unknown reservoir of infectious agents, some of which are zoonotic (Daszak *et al.*, 2000; Hayman *et al.*, 2012). Zoonoses from wildlife represent a significant, growing threat to global health. Emerging zoonotic pathogens have been identified in ungulates, carnivores, rodents, primates, bats other mammals and non-mammalian species (Newman *et al.*, 2011). More than 60% of the newly identified infectious agents that have affected people over the past few decades have been caused by pathogens originating from animals or animal products. Of these zoonotic infections, 75% originate from wildlife (Takayama-Ito *et al.*, 2006).

Infectious pathogens that originate in wild animals have become increasingly important throughout the world in recent decades, as they have had substantial impacts on human health, agricultural production, wildlife-based economies and wildlife conservation. Most

infectious diseases exist within a balanced host –agent equilibrium within their natural ecological niches but may spill over and emerge in populations of domestic animals and humans (Patz *et al.*, 2005). Human populations encounter animals with varying frequency depending on their occupation, geographical location and the prevailing culture of the people.

The emergence, re-emergence, and spread of pathogens are driven by complex factors, most of them linked to the sharp and exponential rise of global human activity. Anthropogenic factors lead to alterations of contact rates between humans, domestic animals, and wildlife hosts. Some of these factors are: increase in global human population, increased global movement of human and animals (including animal importation and trade), intensification of agricultural practices and deforestation that expose man to wildlife and change the existing ecological niches of pathogens and their natural hosts, and environmental changes leading to the same (Bengis *et al.*, 2004).

Bats (Chiroptera) represent a large group of mammals. Approximately 20% of the 4,600 mammalian species are bats (Boulger & Porterfield, 1958). Bats are reported to be associated with many viral pathogens of livestock and humans. They have been implicated in numerous emerging infectious disease events and are increasingly recognised as important reservoir hosts for viruses that can cross species barriers to infect humans and other domestic and wild mammals (Luis *et al.*, 2013). About 66 different species of viruses have been isolated from bats or detected within their tissues, and there is serological evidence for many others (Luis *et al.*, 2013)

Over 90% of bat-associated zoonotic pathogens are RNA viruses. These include Nipah and Hendra viruses (Chua *et al.*, 1999), SARS and MERS coronaviruses, Chikungunya virus, Japanese and St. Louis encephalitis viruses, Hantaan virus (a relative of the Sin Nombre hantavirus), Rift Valley fever virus, Ebola and Marburg haemorrhagic fever

viruses, Rabies and rabies related Lyssaviruses, among others (Newman *et al.*, 2011). More viruses with undetermined zoonotic potential also have been documented in bats, including Rhabdoviruses, Paramyxoviruses, Polyomaviruses, Hepaciviruses and Hepadnaviruses. Many species of bats have peridomestic habits, roosting in houses and other buildings, as well as trees in dense urban areas, leading to frequent human contact with bat excreta. Bat–human contact is also increasing in recent decades owing to habitat encroachment and increased use of bats as bush meat (Kamins *et al.*, 2011).

Lyssaviruses (family *Rhabdoviridae*, genus *Lyssavirus*) are the aetiological agents for rabies – acute, progressive viral encephalitis. Lyssaviruses are transmitted directly between mammals, usually via a bite, causing the disease known as rabies; an acute progressive encephalitis, with nearly a 100% case fatality rate. All mammals are susceptible to lyssaviruses although bats and carnivores are the major natural reservoirs. Bats are the primary reservoir hosts for lyssaviruses from at least 12 of the 14 identified species. In contrast, carnivores are reservoir hosts for RABV only (Rupprecht *et al.*, 2011). Lyssaviruses are highly neurotropic.

Bats host a range of lyssaviruses depending on their species and geographical locations (Hayman *et al.*, 2008). Human rabies has the highest case-fatality rate ever reported. Besides rabies virus, viruses belonging to all other known lyssavirus genotypes can cause rabies-like lethal encephalitis in human beings (WHO, 2011).

Lyssavirus infection of bats occurs across much of the globe, although different virus species are present in different regions and tend to infect particular bat species. Whereas rabies virus (RABV) appears to be restricted to bats of the New World, related lyssavirus species have not been detected in the Americas and have only been detected in bat populations across Africa, Eurasia, and Australia (Banyard *et al.*, 2011). Bats, being a

reservoir of lyssaviruses and other dangerous zoonotic diseases, pose a significant public health risk.

High seroprevelance against lyssaviruses have been reported in bats, including for Lagos bat virus in the straw-colored fruit bat (*Eidolon helvum*) in Ghana (Hayman *et al.*, 2008). However, the pathogenesis and dynamics of Lagos bat virus (LBV) and other related lyssaviruses in bats are not known. This study focuses on the dynamics of Lagos bat virus in its reservoir host, the straw-colored fruit bat (*E. helvum*).

1.2 Justification and Hypothesis of Proposed Study

Rabies has a case fatality rate approaching 100%, one of the highest of any infectious disease. Bats and carnivores are the principal reservoir hosts. The majority of Lyssavirus spill-over events are to "dead end" hosts, from which onward transmission does not occur. Several bat species have been associated with LBV infection, including Wahlberg's epauletted fruit bat (*Epomorphorus wahlbergi*), the straw-colored fruit bat (*Eidolon helvum*) (Fig 2.1), the Egyptian fruit bat (*Rousettus aegyptiacus*), and an insectivorous bat, the Gambian slit-faced bat (*Nycteris gambiensis*). Spill-over events of LBV from bats into other mammals have been reported, albeit infrequently (Markotter *et al.*, 2006) with infection of human beings never having been demonstrated (Markotter *et al.*, 2008).

In non chiropteran hosts, infection with lyssaviruses generally leads to the development of disease, and ultimately death, with seroconversion either occurring late during the symptomatic phase or not at all. Seroconversion may also result from an exposure to the virus (peripheral abortive infection) where the virus does not reach the CNS and does not cause disease. If it does reach the CNS, LBV causes fatal rabies encephalitis in mammals.

Research in Ghana by Hayman *et al.* (2012), has confirmed the endemicity of LBV in *E. helvum.* It is currently unknown whether seropositive animals have ever been infectious and recovered from a neurological infection or whether the seropositivity is evidence of peripheral infection that has been cleared by the host's immune response before reaching the CNS. In addition, the difficulties of aging adult wild animals and the problems of undertaking capture-mark-recapture studies in such large populations of flying wild animals currently limit knowledge regarding age-specific seroprevalence and antibody titre decay rates in this virus–host system (Hayman *et al.*, 2010; Hayman *et al.*, 2008)

Typically, lyssaviruses are transmitted in infectious saliva delivered via a bite. Studies with lyssaviruses in a wide spectrum of animal models have shown that both the route of exposure and viral dose play important roles in the outcome of infection. Experimental studies in insectivorous bats have attempted to explain the importance of route of inoculation in the natural host, but a clear trend with regard to how virus is transmitted between bats has not yet been fully defined (Freuling *et al.*, 2009).

There are few reports of experimental work with lyssaviruses in bats. Most of the work using bat variant rabies and bat host species was done using vampire bats in the 1930s (Pawan, 1936) or insectivorous bats around the 1960s (Constantine & Woodall, 1966)

There have been very few virological (as opposed to serological) investigations (observational studies or experimental studies) of Lagos bat virus in African bats despite them being the presumed natural hosts of this virus. Hayman *et al.* (2008), reports on antibody detection in some species of Ghanaian fruit bats in limited geographic locations of Ghana and provide limited seroprevalence data. There have been no records of LBV detection in either domestic or wild animals in Ghana. The transmission and pathogenesis of this virus in its natural reservoir hosts under natural conditions, therefore, is not known.

Using *Eidolon helvum* as a model, I hypothesize that:

1. Endemic circulation of Lagos bat virus exists in this bat species in Ghana.
2. Circulation of Lagos bat virus in bat populations is facilitated by certain pathobiological and transmission patterns of the infection.
3. Inoculation of LBV results in clinical disease (rabies), although not all inoculated bats will develop the disease, especially when inoculated peripherally.
4. Infected bats will exhibit certain disease patterns and virus excretion which will shed light on virus circulation at the population level and on the likelihood of cross-species transmission.

In this study, I investigated the basic pathobiology of LBV in *E. helvum*, including susceptibility to the virus, incubation and clinical periods for different doses and routes of LBV infection, behavioral changes of rabid bats, virus occurrence in various bat tissues and excreta. For this research, I experimentally infected *E. helvum* using 3 different West African LBV isolates (lineage A: Ghana 2013, Senegal 1985 & lineage B: Nigeria 1956) to examine their pathogenesis in one of LBV's natural reservoir host species. This work will help to bridge the scientific knowledge gap in the pathogenesis of LBV in bats.

Initially, three different isolated strains of LBV were used for intracranial challenge, after which the strain which produced the most-appropriate outputs for the purposes of this study (i.e. causes clinical disease when inoculated intracranially) was used for peripheral infections.

By using *E. helvum* as the animal model in this study, I aim to obtain a better understanding of the ecology of LBV infection within one of its main reservoir hosts. This species has a high anti-LBV seroprevalence (approx. 40%) in the wild, and there is a history of LBV isolation from this species. Also, as it has a wide sub-Saharan distribution,

a propensity to form roosts in urban areas, and is widely used as a source of bushmeat (Kuzmin *et al.*, 2008), the results are likely to have important implications for public health.

1.3 Aim of study

General Objectives

The aim of this PhD study is dual. First, I will investigate infection dynamics of a potentially-zoonotic virus in *Eidolon helvum* in free ranging populations at the 37-Military hospital in Accra and at Tano Sacred grove, and in a captive population of *E. helvum* at the Achimota Forest. Second, I will conduct experimental investigations of the pathogenesis and transmission patterns of Lagos bat virus in *Eidolon helvum*.

Specific Objectives

The questions this study seeks to address are as follows:

1) Can LBV cause disease in *E. helvum* and if so, is this always fatal or can recovery occur? Is this affected by route of inoculation?
    a. Intracerebral
    b. Peripheral
2) What is the difference in the disease pathobiology for different routes of experimental exposure
    a. Susceptibility
    b. Duration of incubation periods
    c. Period to virus excretion
    d. Period to illness and outcomes.
    e. Presence of the virus in various tissues and excreta of infected bats.

CHAPTER TWO: LITERATURE REVIEW

2.1 Bats and Their Behaviour

*Bats (*Chiroptera*)*

Bats are mammals of the order Chiroptera. Their forelimbs form webbed wings making them the only mammals naturally capable of true and sustained flight. Bats are greatly diverse both in habitat and in feeding strategies. About 70% of bats are insectivorous and most of the rest are frugivorous. There are also carnivorous, omnivorous, nectar eating, fish-eating and blood-eating bats (Schmidt, 1985).

There are 1,116 recognised species of bats (Chiroptera) worldwide (Happold and Happold, 2013). These species are placed in 18 families and 202 genera, of which 42% are monotypic and 16% have only two species (Happold and Happold, 2013). The Order Chiroptera is divided into two Suborders, the Yangochiroptera and Yinpterochiroptera, the latter including the super family Pteropodidae, the old world fruit bats, with Rhinolophoidea (Giannini & Simmons, 2003). Bats account for 20% of the 4,600 known mammal species in the world (Hutson & Mickleburgh, 2001). They are second only to rodents in number of genera and species. Of the 221 species of bats recognized in Africa, 28 species are Megabats of the suborder Megachiroptera, and 193 belong to the suborder Microchiroptera (Happold and Happold, 2013).

Microchiroptera: Microchiropteran bats are found throughout the world, except for the Arctic and Antarctic and on some isolated oceanic island (Hutson & Mickleburgh, 2001). Microchiropteran bats use a variety of habitats both for roosting and feeding. 75% of microchiroptera feed on insects (Hutson & Mickleburgh, 2001).

Microchiropterans' echolocate. Bat echolocation is a perceptual system where ultrasonic sounds are emitted specifically to produce echoes. By comparing the outgoing pulse with

the returning echoes, the brain and auditory nervous system can produce detailed images of the bat's surroundings. This allows bats to detect, localize and even classify their prey in complete darkness.

Megachiroptera: Megachiropteran bats are generally bigger than their *microchiropteran* cousins; they constitute a quarter of all bats. Megachiropterans (Fruit and Nector eating bats) occur in the tropics and sub-tropical regions of the Old World, and 120 species have been documented in West Africa (Kankam & Oduro, 2009). *E. helvum* is a Megachiropteran bat in the family, Pteropodidae; this family is made of 42 genera and 186 species of which 14 genera and 28 species are found in Africa (Happold and Happold, 2013).

Roosting behaviour

Bats have variable roosting behaviors, roosting requirements and habits. Some species roosts in large colonial groups, some roost in small groups and others roost solitarily. For instance, *Eidolon helvum* forms colonies numbering many thousands, tens of thousands or even hundreds of thousands of bats, often clustering together in one large tree or group of trees. Some of the largest and densest aggregations of mammals are those formed by the Mexican free-tailed bats *(Tadarida brasiliensis).* Their average roosting density is approximately 1800 adults per square meter (Jones, 1972).

Vampire bats can be found roosting in tree hollows, humid caves, and old mineshafts, abandoned wells and man-made structures. Roosting preference and colony size may vary. Vampire bats may roost alone, in small groups or in very large colonies of many hundreds of individuals.

Generally, bats roost during the day and feed at night. They travel varied distances to feeding sites in the night in search for food and water.

The location of bat roosts varies. Some roost in, or close to, human habitation, while others roost far from human disturbance. It is generally observed that *E. helvum* roosts in trees near villages and towns as well as the outskirts of these areas (Ayensu, 1974). This species is also known to roost in large numbers in the middle of African megacities, such as Accra, Abidjan and Dar es Salaam (Fahr *et al.*, 2015), as well as in rural areas, such as Kasanka National Park in Zambia and Tano Sacred Grove in Ghana. These bats (*Eidolon helvum*) appear not to fear humans. Their roosts have been observed above noisy markets and in busy city centres. In such locations, there can be direct contact with people, such as through defaecation and urination on human belongings and directly on people under the roost, posing a risk of human exposure to bat-borne pathogens.

The structure of bat populations may influence the dynamics of disease infection (Luis *et al.*, 2013). Some gregarious bat species have a metapopulation structure consisting of spatially discrete subpopulations with seasonal interactions amongst these (Jesse *et al.*, 2008)

Migration

Some bats exhibit seasonal migratory behavior. In temperate regions, cold winters force bats to migrate, enter torpor, or hibernate. Migrating bats that are seeking a more favorable climate require energy storage and rationing for the intense demands of flight, given their small body size. Seasonal shifts in rain patterns trigger migration in some species of African bats (Thomas, 1983). For instance, *E. helvum* migrates distances of 1,500 kilometers each year following rains into the Niger River basin (Thomas, 1983). Migration of Australian Flying Foxes has also been documented. Nelson (1965)describes regular

seasonal migrations, associated with food and climate, carried out by the grey-headed flying fox (*Pteropus poliocephalus*).

Bats & people

Throughout the history of humankind, bats have had an unchallenged notoriety in the realm of superstition and mythology. As the only flying mammals, and with preference for nocturnal activities, bats have, for centuries, been associated with various mysteries. In West Africa, as in other regions of the world, several mythical stories about bats have been handed down from one generation to the next (Ayensu, 1974). In some cultures, bat meat is a delicacy whilst in others it is an important source of protein, contributing to the sustainability of livelihoods (Bourhy *et al.*, 1992). For example, *E. helvum* is widely eaten as bushmeat throughout much of West and Central Africa (Mickleburgh *et al.*, 2008) including Ghana (Kamins *et al.*, 2011).

Bats & ecosystem function

Bats play a vital role in ecosystem functioning (Taylor, 1999). Fruit eating bats are key in the dispersal of many types of seeds and they play an important role in pollination of many plants. Fruit bats help maintain the diversity of forests by dispersing seeds across different ecosystems, often introducing novel plant species into previously disturbed landscapes and to oceanic islands. Similarly, nectarivorous bats that visit flowers provide valued ecosystem services by pollinating plants, dispersing pollen, and, thus, helping to maintain genetic diversity of flowering plants.

Insectivorous bats are important in the control of insects in the ecosystem. In doing so, they suppress both naturally occurring and anthropogenically-generated insect populations (such as agricultural pest species and insects that annoy or transmit specific pathogens to

humans and other mammals. They thus contribute to the maintenance of ecosystem stability.

In addition to suppressing insect populations, pollinating flowers, and dispersing seeds, insectivorous (Gonsalves *et al.*, 2013) nectarivorous, and frugivorous (Kankam & Oduro, 2009) species of bats may redistribute nutrients and energy through their guano to sustain terrestrial, aquatic, and cave ecosystems.

Collectively, bats are responsible for the consumption of millions of insects and the pollination of thousands of plants, flowers and fruit trees, and play a major role in seed dispersal in the forest and in other habitat where they occur. Bats thus play an important role in providing key ecosystem services.

2.2 Bats, Emerging and Re-emerging Zoonotic Diseases

Animals, particularly wild animals, are known to be the source of over 71.8% of all emerging infections in people (Jones *et al.*, 2008). The significance of bats as reservoirs of such emerging infectious diseases (EIDs) has been increasingly appreciated (Calisher *et al.*, 2006). There are diverse species of bat with worldwide distribution. They therefore have diverse species of pathogens which they have co-evolved with over millions of years. These may highlight the role of bats in the maintenance and transmission of viruses of public health concern.

Many emerging RNA viruses of public health concern have recently been detected in bats. These include lyssaviruses, coronaviruses (CoVs), filoviruses, paramyxoviruses, astroviruses (AstVs), influenza viruses, and others. DNA viruses, including herpesviruses and adenoviruses (AdVs), have also been detected in bats. Bats have been implicated as the source of fatal Ebola virus outbreaks in African apes (Leroy *et al.*, 2005) and a source

of Marburg virus in Uganda, Kenya and other African countries (Towner *et al.*, 2009), of fatal Hendravirus infection of horses and humans in Australia (Murray *et al.*, 1995), Nipah virus spilled over from bats into domestic pigs and further to humans in Malaysia, or directly to humans in Bangladesh (Chua *et al.*, 1999), indirect source of SARS and MERS coronaviruses via unidentified amplifying hosts.

In Africa, *E. helvum* has been shown to have neutralizing antibodies against henipaviruses (Hayman *et al.*, 2008). Serological evidence of infection of bats with potentially-zoonotic viruses, including Lagos bat virus, paramyxoviruses and Ebola virus, has been found within a single colony of *E. helvum* in Accra, Ghana (Hayman *et al.*, 2010; Hayman *et al.*, 2008).

Bats are known to be the principal reservoirs for 12 of the 14 recognized lyssavirus species. The principal reservoirs for MOKV (Kuzmin *et al.*, 2009) and for the newly reported Ikoma virus (Marston *et al.*, 2012) are unknown. Classical rabies virus (RABV) circulates in New World bats and worldwide in other mammals (predominantly carnivores) (FAO, 2011).

Bat species involved in the transmission of lyssaviruses to people are largely restricted to the following genera: *Eptesicus, Myotis, Lasiurus, Lasionycteris, Pipistrellus, Tadarida, Miniopterus, Desmodus* and *Nycteris.* Insectivorous bats species including *Eptesicus, Miniopterus* and *Nycteris* are widely distributed throughout Africa, but their role in the transmission of lyssaviruses to people is not known (McColl *et al.*, 2002).

A. Roost on trees in the hospital compound B. Roost over a hospital Ward

Fig 2.1: **Colony of *E. helvum* roost at the 37-Military hospital in Accra, Ghana.**

2.2A Microbat roost in a cave 2.2B. bat excreta on Log 2.2C. Bat Feed remains

Fig 2.2: **Bat behaviors and activities.**

Bats have special features that can contribute to the persistence and spread of pathogens compared to other mammals (Luis, *et al*., 2013). These characteristics include the unique characteristic of flight. In addition, bats are abundant, widely distributed, and highly gregarious and thus can maintain infections. Many bat species roost together in very large and dense colonies (such as *E. helvum* (Fig. 2.1). This dense clustering of individuals provides ample opportunities for viral exchange amongst individuals within bat populations. Bats with high levels of interspecies contact, such as *Myotis* spp., have been

found to harbor a diverse range of lyssaviruses, suggesting that increased contact between species increases viral transmission amongst them. Several infectious agents, including Nipah virus, have been isolated from the urine of fruit bats and during mutual grooming. Fur contaminated by urine may allow for viral transmission between individuals (Middleton *et al.*, 2007).

Regarding ecological flexibility, bats inhabit a wide variety of ecological niches (Fig. 2.1). Some species are flexible in roost preferences, including caves, trees, and many man-made structures, others are more restricted to specific types of roosting sites. The ability of bats to occupy man-made structures and roost near human habitations is of particular importance, because it increases the opportunities for interactions between bats, domestic animals, and humans. For example, the big brown bat (*Eptesicus fuscus*) and the serotine bat (*Eptesicus serotinus*), (Eurasian and Nothrth American bats) both of which are known to harbor lyssaviruses, commonly roost in man-made structures, such as churches, hospitals, schools, or houses (Constantine & Blehert, 2009).

Not only are bats able to inhabit a variety of diverse locations, but they also have a number of trophic specializations. Three bat species, all found in Central and South America, are hematophagous (vampire bats – which feed on blood). These dietary habits affect rabies transmission risks. Vampire bats, due to depletion of environmental resources caused by anthropogenic and other related factors, may have increasing contacts with man and are switching feed preferences to humans and domestic animals due to scarce natural sources (Kuzmin *et al.*, 2011).

Fruit eating bats often inhabit and feed in agricultural areas, which brings them into closer contact with humans and domesticated animals. In the tropics, frugivorous bats can be found roosting in urban areas and feeding on fruit trees in plantations. Fruit remains

contaminated with bat saliva (Fig.2. 2C) might be eaten by people or livestock, and thus expose them to bat associated pathogens. It is hypothesised that the consumption of half-eaten fruits may have caused the transmission of Nipah virus infection from fruit bats to pigs and humans, as well as infected bats sharing of the raw date palm sap with people (FAO, 2011). Similarly, insectivorous bats will discard contaminated insect parts, which can then be consumed by foraging animals (FAO, 2011), although mechanisms for such route of pathogen transmission from insectivorous bats has not been corroborated to date. Such specific trophic feeding behavior may also lead to increased diverse species of bats within an area, enhancing the opportunities for multiple species to interact and share infectious pathogens. During periods of resource limitation, diverse species of animals may be brought together at a particular spot. During the dry season, primates and bats may come into closer contact as they search for limited food supplies, enhancing opportunities for cross-species transmission of filoviruses (Leroy *et al.*, 2005)

Beside their mobility, sociality, and ability to inhabit a variety of niches likely to influence the emergence of infectious diseases in bats, there are some additional characteristics of bats that may contribute to this phenomenon:

- Microchiroptera possess the ability to echolocate, to produce laryngeal vocalizations for navigational purposes. Echolocation may cause aerosolization of viral particles in the nasal mucosa and saliva, enhancing transmission to other individuals (Calisher *et al.*, 2006).

- When adjusted for body mass, Chiroptera are the longest-living mammalian order (Calisher *et al.*, 2006), this is favorable for the development of long-lasting persistent infections.

The long evolutionary history of bats may also play a role in their association with emerging infectious diseases (EIDs), because of long co-evolution between bats and their viruses. Pathogens could have evolved to utilize cellular receptors that are conserved across a wide range of animal species, providing a mechanism for interspecific infections. For example, henipaviruses are capable of infecting species in six mammalian orders (Kuzmin *et al.*, 2011) and SARS-CoV uses an enzyme receptor that is conserved among mammals (Chua *et al.*, 1999).

2.3 Lyssaviruses:

Lyssaviruses are classified in the *Rhabdoviridae* family, which belongs to the order of *Mononegavirales*. These viruses are non-segmented, negative-strand RNA viruses (Delmas *et al.*, 2008). The *Mononegavirales* also includes the *Paramyxoviridae*, *Filoviridae*, and *Bornaviridae* families. The *Rhabdoviridae* are characterized by a typical rod- or bullet-shaped morphology (http://www.cdc.gov/rabies/ transmission/virus.html). Rhabdoviruses are currently divided into six genera, with the *Ephemerovirus, Tibroviruses, Tupaviruses, Spriviviruses, Perhabdoviruses* and *Vesiculovirus,* together with about 130 unclassified viruses, forming the dimarhabdovirus super group (dipteran mammal-associated rhabdovirus).

Within the rhabdovirus family, there are viruses infecting plants, arthropods, fish, bird, reptiles, marsupial and placental mammals (Fu, 2005). Rhabdoviruses of mammals also include economically important livestock pathogens, such as vesicular stomatitis viruses, tibroviruses and ephemeroviruses. Notably, although rhabdoviruses span all continents, relatively few are known to cause human infections. Rabies virus (RABV) and related viruses from the Lyssavirus genus, and Chandipura virus (CHPV) from the Vesiculovirus genus, are known to cause acute encephalitis syndromes in human beings. Other viruses

from the genus Vesiculovirus cause vesicular stomatitis (mucosal ulcers in the mouth) and ‘‘flu-like’’ syndromes in both cattle and humans.

The majority of rhabdoviruses is transmitted by invertebrate vectors and for a long time was not thought to cause a human disease. The outstanding exception is rabies, caused by the members of the *Lyssavirus* genus, which is probably the only mammalian rhabdovirus group that is not transmitted by an insect vector (Jackson, 2011). All lyssaviruses (Fig.2.3) share certain characteristics, including a negative-sense single-stranded RNA genome about 12 kb long, common genome organization, antigenic properties, virion morphology, two structural units (an internal helical nucleocapsid, about 50 nm in diameter, and a lipid envelope which is derived from the host cytoplasmic membrane during budding), as well as physicochemical and physical properties.

The lyssavirus genome encodes genes for five viral proteins (Fig.3A): nucleoprotein (N), phosphoprotein (P), matrix protein (M), glycoprotein (G) and polymerase (L). The bullet-shaped lyssavirus particle is 100–300 nm in length and 75 nm in diameter. It is composed of two structural and functional units (http://www.who.int/rabies/ ExpertConsultationOnRabies.pdf) (Fig.2.3B)

1. . The lipid bilayer from the host cell builds the outer envelope. It is protruded with spike of G trimers, which recognise and bind cell receptors and is responsible for the induction of humoral immune response.

2. The internal ribonucleocapsid (RNP) is of helical structure and is composed of the genomic RNA intimately associated with protein N, polymerase L and its co-factor protein P (formerly named M1). The ribonucleocapsid complex ensures genome transcription and replication in the cytoplasm.

The matrix protein M (formerly named M2) occupies an intermediate position between the ribonucleocapsid and the envelope, and is responsible for virus budding and the bullet-shaped morphology. (http://www.cdc.gov/rabies/transmission/virus.html).

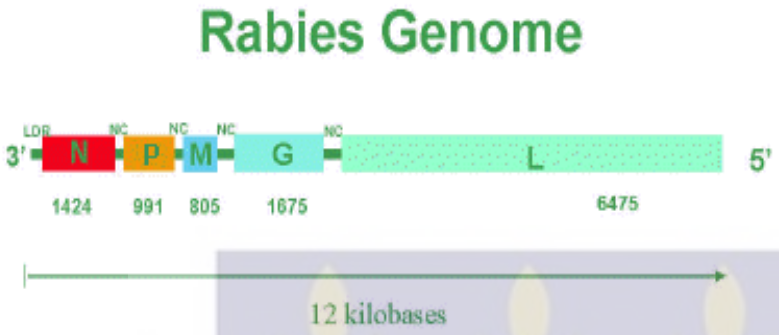

Fig. 2.3A. Structure of the rabies virus genome showing the five protein encoding regions of the genome.

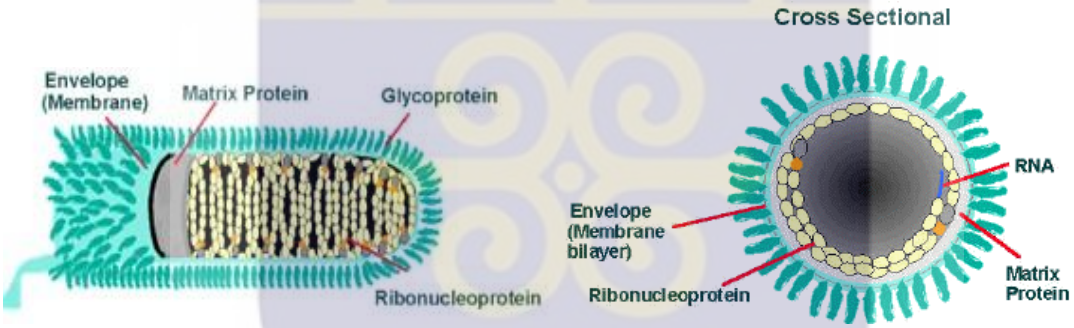

Fig. 2.3B Structural diagram of a lyssavirus Fig 2.3C cross section of the lyssavirus

Fig 2.3: Structure of a generic lyssavirus. Adapted from *CDC* (www.cdc.gov/rabie

Table 2.1: Taxonomy of Lyssaviruses

SPECIE	PHYLOGROUP	GENOTYPE	GEOGRAPHICAL ORIGIN	VECTOR	REFERENCE
Rabies Virus (RABV)	I	1	Worldwide except some islands	Carnivorous (worldwide),Bats (America)	(WHO, 2013)
Lagos Bat Virus (LBV)	II	2	Sub - Saharan Africa	Frugivorous bats	(WHO, 2013)
Makolo Virus (MOKV)	II	3	Sub - Saharan Africa	Unknown	(WHO, 2013)
Duvenhage Virus (DUVV)	I	4	South Africa	Insectivorous bats (*Eptesicus serotinus)*	(WHO, 2013)
Shimoni Bat Virus (SHIBV)	II	Undetermined	Africa	Insectivorous bat (*Hipposideros commersoni*)	(WHO, 2013)
Ikoma Lyssavirus (IKOV)	Unclassified	Undetermined	Africa	Africa Civet Cat (*Civettictis civetta*)	(WHO, 2013)
West Caucassian bat Lyssavirus (WCBV)	III	Undetermined	Eurasia/Africa	Insectivorous bat (*Miniopterus Schreibersii*)	(WHO, 2013)
Aravan Virus (ARAV)	I	Undetermined	Eurasia	Insectivorous (Myoitis blythi)	(WHO, 2013)

Table 2.1 Continued

SPECIE	PHYLOGROUP	GENOTYPE	GEOGRAPHICAL ORIGIN	VECTOR	REFERENCE
Khujand Virus (KHUV)	I	Undetermined	Eurasia	Insectivorous (*Myotis Mystacinus*)	(WHO, 2013)
Irkut Virus (IRKV)	I	Undetermined	Asia	Insectivorous (*Murina leucogaster*)	(WHO, 2013)
European Bat Lyssavirus 1 (EBLV-1)	I	5	Europe	Insectivorous bat (*Myotis Sp*)	(WHO, 2013)
European Bat Lyssavirus 2 (EBLV-2)	I	6	Europe	Insectivorous bat (*Myotis Sp*)	(WHO, 2013)
Bokehlo Bat Lyssavirus (BBLV)	I	Undetermined	Europe	Insectivous bat (*Myotis nattereri*	(WHO, 2013)
Lleida Bat Virus (LLEBV)	Unclassified	Undetermined	Europe	Insectivorous bat (*Miniopterus Schreibersii*)	(Ceballos et al., 2013)
Australian Bat Lyssavirus (ABLV)	I	7	Australia	Frugivorous bats/ Insectivorous bats	(WHO, 2013)

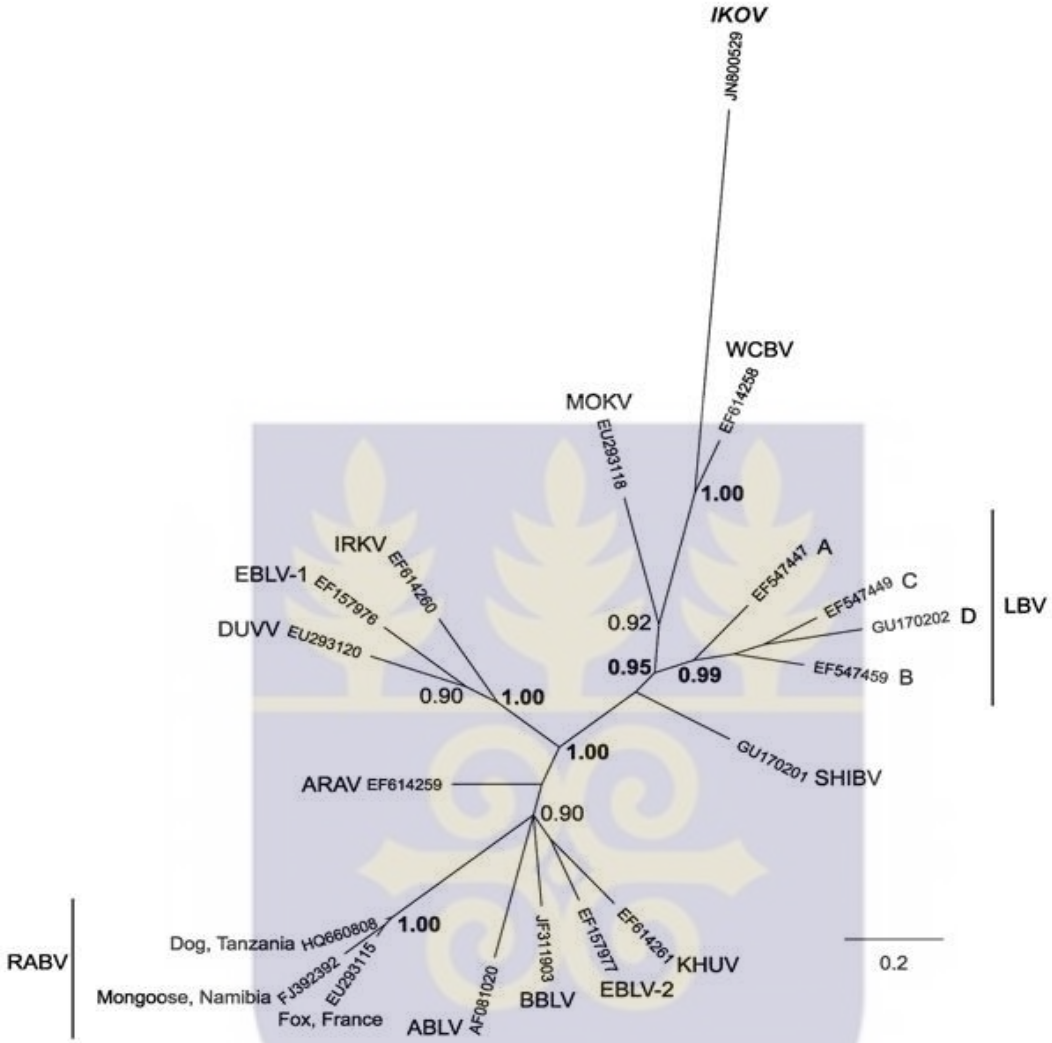

Fig 2. 4: **Phylogenetic tree of lyssaviruses showing the relations of LBV to other lyssaviruses and the strains of LBV (Adapted from Marston *et al.*, 2012)**

Six lyssaviruses species are reported in mammals in Africa – RABV, DUVV, LBV, MOKV, SHIBV, and Ikoma Virus (IKOV) to date (Marston *et al.*, 2012). However, LBV, DUVV, MOKV, SHIBV and the IKOV are the only true indigenous lyssaviruses on the African continent (Markotter *et al.*, 2008). The distribution of African lyssaviruses is shown in Fig. 6. Of these, LBV, DUVV and SHIBV are known to circulate in bats. Africa possesses the greatest known lyssavirus diversity, both genetically and serologically. Given that such diversity exists in Africa, it has been hypothesised that early evolution and divergence of lyssaviruses occurred in African bats (Winkler *et al.*, 1985). The actual impact of bat lyssaviruses in Africa is not known as there is little systematic surveillance for bat lyssaviruses in Africa. There is limited epidemiological and disease ecology data available on African lysaviruses.

Lyssaviruses are highly pathogenic agents, being the aetiological agents of rabies disease. Lyssaviruses in bats present a public health risk and are classified as 'category C emerging infectious disease threats'. Twelve (12) of the fourteen (14) currently known viruses of the Lyssavirus genus have been isolated from bats (Marston *et al.*, 2012). Only the reservoir hosts of MOKV and Ikoma virus have yet to be determined (Marston *et al.*, 2012). Literature on phylogenetic analyses and virus – host association indicate that all lyssaviruses, including RABV, originated from bats and some were eventually transmitted to terrestrial species, principally carnivores (Banyard *et al.*, 2011). Genetic analysis of glycoprotein sequences from viruses circulating in both bat and terrestrial carnivore species suggest that host switching of lyssaviruses from bats to other mammals have occurred repeatedly and successfully in history (Badrane & Tordo, 2001)

The type of lyssavirus hosted by bats is dependent on host species and location. Genomic analyses have differentiated lyssaviruses into 14 genetically distinct species including: Rabies virus (RABV, genotype 1), Lagos bat virus (LBV, genotype 2), Mokola virus (MOKV, genotype 3), Duvenhuge virus (DUVV, genotype 4), European bat lyssavirus type 1 (EBLV-1, genotype 5) and type 2 (EBLV-2, genotype 6) and Australia bat lyssavirus (ABLV, genotype 7) (Calisher & Ellison, 2012) (Table 1). In addition, Irkhut virus (IRKV), Aravan virus (ARAV), Khujand virus (KHUV), West Caucassian bat virus (WCBV) , Shimoni virus (SHIBV), Bekeloh bat virus and Ikoma virus (IKOV) (Marston *et al.*, 2012), are recognised as distinct species, but these have yet to be assigned to a genotype within the lyssavirus genus (Kuzmin *et al.*, 2005). A 15th lyssavirus species, Lleida bat lyssavirus has recently been reported in bats in Spain and further study is required to classify the virus.

Unlike classical rabies virus, the other lyssaviruses have restricted geographical distributions and are associated only with particular bat species. These differences may be due to reduced ability to infect other host species when compared to RABV, perhaps due to the co-evolutionary history of these viruses with particular reservoir species. Figure 2.5 shows the known global distribution of lyssaviruses.

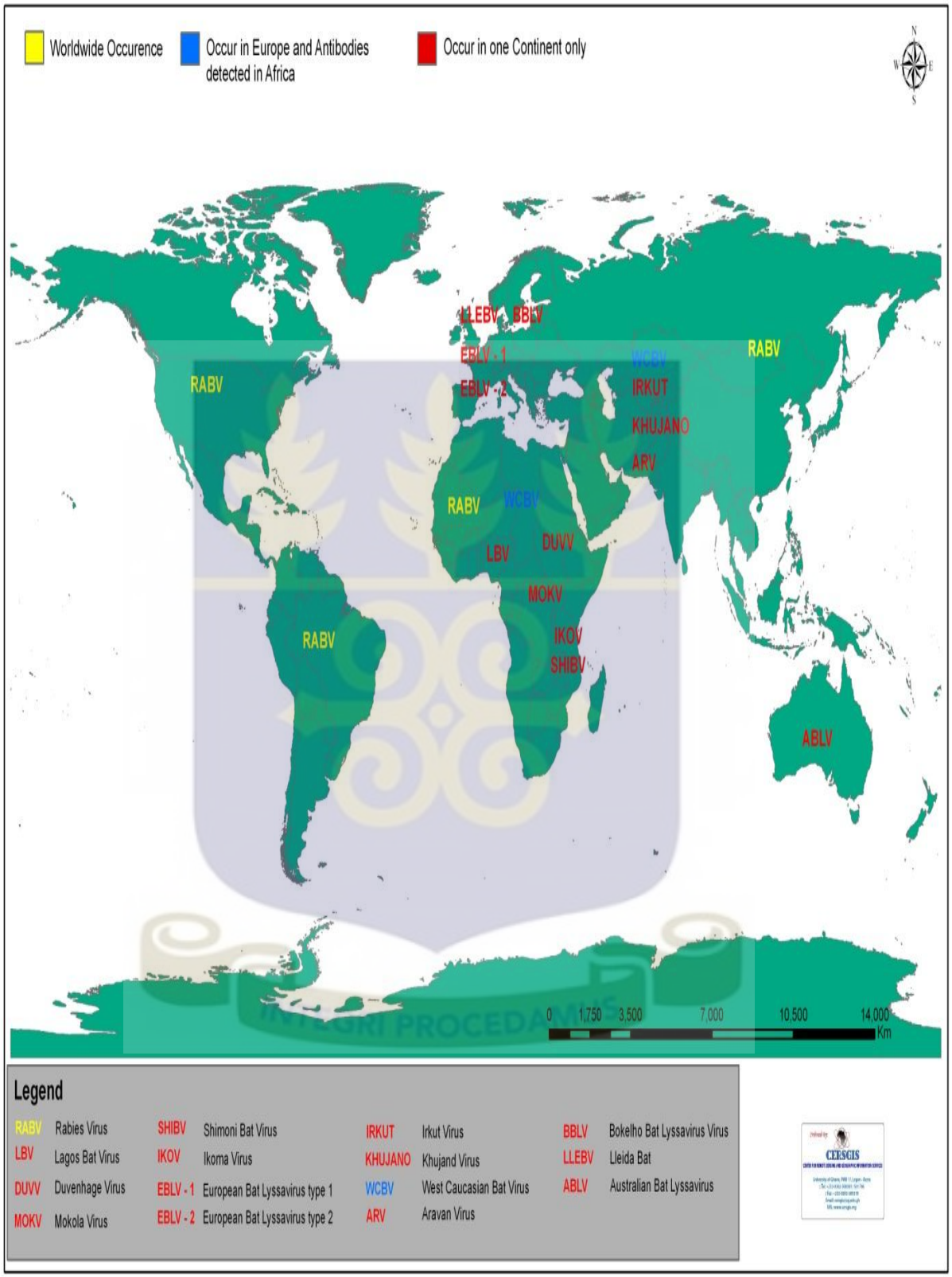

Fig 2.5: **Global Distribution of Lyssaviruses. Adapted from (Nel & Rupprecht, 2007)**

Lyssavirus species are distinguished by their genetic diversity, serological profile, pathogenicity, host species and geographical distribution. Phylogenetically, the lyssaviruses are classified into two Phylogroups: Phylogroup I consist of RABV, DUVV, EBLV1, EBLV2, ABLV, ARAV, KHUV, IRKV and BBLV; Phylogroup II consists of MOKV, SHIBV and LBV; WCBV, IKOV and LLEBV which cannot be included in either of these Phylogroups (WHO, 2013). The ectodomain of the G protein differ among phylogroups and thus cross-reactivity exists among viruses within the same phylogroup, but not between phylogroups (www.who.int/rabies/Expert ConsultationOnRabies.pdf). It is important to consider, therefore, that while commercial vaccines derived from classical RABV strains provide cross-protection with other Phylogroup I lyssaviruses, they have little or no protection against Phylogroup II lyssaviruses, WCBV, IKOV and LLEBV.

Initial experimental studies suggested that Phylogroup I viruses are more pathogenic to mice than viruses of Phylogroup II (MOKV and LBV), although there is a large range in the pathogenicity of different LBV and MOKV isolates (Lumio *et al.*, 1986). Similarly, there is variance in the pathogenicity of different strains, or variants, of classical rabies virus. For example, a canine RABV variant is considered to be less pathogenic in raccoons than raccoon RABV variants: the canine RABV causes paralytic rabies in raccoons, while the raccoon RABV cause furious rabies in raccoons (Bingham, 2005).

In temperate climates, lyssavirus disease in bats is evident only when the bats are active, i.e. during the warmer times of the year. In tropical climates, where the weather favours abundant food resource and bats are active throughout the year, lyssavirus disease in bats may occur all year-round. Infected bats may serve as source of the disease that can spill-over to other animals including humans, domestic and wild animals (Aghomo *et al.*, 1990). The ecology of lyssaviruses in colonial bats is characterized by a relatively low

prevalence of the virus, which varies from < 1 to 4%, and by a much higher seroprevalence of virus-neutralizing antibodies. For example, in Brazilian free-tailed bats (*Tadarida brasiliensis mexicana*), a seroprevalence of 65%-70% has been recorded (Dimitrov *et al.*, 2007). In the majority of cases spill-over events to non-bat species are dead-end infections (Badrane & Tordo, 2001).

Numerous serosurveillance initiatives have reported serological positivity for virus exposure for several representatives of lyssavirus species (Bussereau *et al.*, 1988; Kuzmin *et al.*, 2006). Indeed, the presence of virus neutralizing antibodies in healthy bats remains an interesting aspect of lyssavirus biology (Turmelle *et al.*, 2010). In non chiropteran hosts, infection with lyssaviruses generally leads to the development of disease, and ultimately death, with seroconversion either occurring late during the symptomatic phase or not at all.

Bats can experience either a lethal or non-lethal rabies virus infection. A lethal infection consists of typical disease progression where the virus replicates and migrates through neural tissue to the central nervous system; for some, virus presents in saliva facilitating transmission, and clinical signs manifest, including death. In other cases, an exposure leads to peripheral abortive infection with serologic response (Kuzmin and Rupprecht, 2007). In theory, seroprevalence may suggest evidence of recovery from clinical rabies or the existence of non-clinical "carrier" state. The documentation of EBLV-1viral RNA in the saliva of healthy bats (Echevarria *et al.*, 2001) and the recovery of rabies virus from the saliva of experimentally-infected vampire bats that did not succumb to the disease (Umoh & Blenden, 1982) also suggest a possible subclinical carrier state. However, majority of experimental studies the bats that developed CNS disease (rabies) succumbed, and virus excretion occurred only a few days before the clinical onset. Similarly, no LBV

RNA was detected in thousands of oral swabs from apparently healthy fruit bats whereas the virus was always present in the saliva of dead bats with virus in the brain. From anatomical standpoint, the only way for a virus to reach neurotropically the salivary glands is the spread from the brain stem. Hence, no explanation exists for the presence of a lyssavirus in bat saliva in the absence of CNS infection. Finally, the present practice accepted in many countries regarding human exposure, that includes rabies testing of the brain of biting bat and non-administration of post-exposure prophylaxis if the brain is negative, never failed.

The mechanisms of maintenance, transmission, and evolution of lyssaviruses in bats are complex and generally poorly understood. There is currently a lack of knowledge at both the pathogen and the host level.

A study of the big brown bat species, indicated that the juvenile mortality rate and viral incubation period in the main transmission season, along with the case fatality rate, were the most important parameters driving bat population and pathogen dynamics (George *et al.*, 2011). These parameters affect viral maintenance in two different ways: (i) through effects on bat population viability and (ii) through effects on viral maintenance within a viable bat population. The study by George *et al.* (2011) demonstrated an interaction between disease incubation period and hibernation, and showed that the annual birth pulse is required to maintain this complex dynamic. The longer the incubation period, the more likely infected bats will survive long enough to enter hibernation and provide infectious contacts in the subsequent main transmission season. The combination of long incubation periods and the metabolic effect of cold temperatures that suppresses viral activity during hibernation, combine to make a temporal maintenance reservoir, preserving rabies virus until the birth pulse provides a new supply of immunologically naïve bats.

The duration or half life of lyssavirus antibodies in bats is not known. Age-stratified analysis of serological testing would assist in determining the timing of the infection in relation to the life history of the reservoir host. The inclusion of juveniles, whose age can be approximated, in any sampling studies would be helpful in interpreting serological results (Epstein *et al.*, 2008).

Cross-sectional studies have revealed high seroprevalence of antibodies against LBV in two colonial fruit-bat species, *Eidolon helvum* and *Rousettus aegyptiacus* (Dzikwi *et al.*, 2010; Hayman *et al.*, 2008; Kuzmin *et al.*, 2008).Therefore, if the serological findings are evidence of endemic transmission, mechanisms other than the use of extended torpor must exist that allow infection persistence within these tropical bat populations. The maintenance of lyssavirus in natural populations of bats is likely influenced by the ecology and sociality of the reservoir species. In large colonies, such as those of *E. helvum*, the bats roost in close proximity to one another; it is thus proposed that there would be increased opportunity for a rabies-infected individual to infect conspecifics through bites, licking of wounds and mucus surfaces (Correa-Giron *et al.*, 1970). Exposure to low doses of virus through such inoculation may result in abortive peripheral infections, thus explaining a high seroprevalence in the apparent absence of CNS infection (rabies).

Transmission, Pathogenesis and Clinical signs of Lyssaviruses:

Lyssaviruses are transmitted between mammals, including bats, through the bite inoculation of the infected saliva. Virus is inoculated through the skin into muscle and subcutaneous tissues. Transmission of lyssaviruses can also occur by exposure of a scratch, abrasion, open wound or mucous membrane to saliva or brain material of an infected animal. There are also rare reports of transmission via other routes; a few cases

have been reported after transplantation of organs, particularly corneas but also pancreas, kidneys and liver (WHO, 2013).

Lyssaviruses cannot, however, penetrate through intact skin. Transmission in human beings via tissue transplantation (cornea, internal organs, and a vascular segment) from donors with unrecognized rabies has been reported (van Thiel *et al.*, 2009). Airborne natural infection is possible in exceptional circumstances, for example in caves harboring large numbers of bats carrying the virus (Constantine & Woodall, 1966). Lyssaviruses are not transmitted by arthropods (Calisher & Ellison, 2012).

Natural transplancental infection has been reported in a cow and a skunk on the basis of fluorescent antibody detection of rabies virus antigen in the foetal calf and a skunk embryo, respectively (Howard, 1981), and by virus isolation from foetal calf tissues (Martell *et al.*, 1973).

Experimental infection studies using EBLV-2 indicate that intracranial inoculation leads to the rapid development of disease in *M. daubentonii* (Johnson *et al.*, 2008). However, peripheral inoculation routes, such as intramuscular and intranasal, did not lead to infection or seroconversion of the animals challenged (Johnson *et al.*, 2008). One of seven Daubenton bats (*M. daubentonii*) inoculated with EBLV-2 by the subdermal route developed disease (Johnson *et al.*, 2008). This suggests that the most effective route of transmission is through biting; as virus was detected in oral swabs of the experimentally infected daubenton's bat (Johnson *et al.*, 2008). Scratches or bites might explain the infection with EBLV-2 virus of two bat biologists with histories of encounters with *M. daubentonii*.

All lyssaviruses cause rabies. All warm blooded animals are susceptible to infection with rabies virus, and this usually causes acute encephalitis. Immediately after infection, there

is local replication of virus at the site of inoculation. The rabies virus enters an eclipse phase during which it cannot be detected. During this phase, it replicates in non-nervous tissue such as muscle. After several days or months, the virus enters the peripheral nerves and is transported to the central nervous system by retrograde flow in the axons. The virus disseminates within the CNS resulting in development of clinical signs as the neurons are infected. From the CNS, the virus is distributed to highly innervated tissues via the peripheral nerves. The virus is found in nervous tissue, salivary glands, saliva, which should all be handled with extreme caution. Virus has also been detected in other tissues and organs, including the lungs, adrenal glands, kidneys, bladder, heart, ovaries, testes, prostate, pancreas, intestinal tract, cornea, germinal cells of hair follicles in the skin, sebaceous glands, tongue papillae and the brown fat of bats (http://www.cdc.gov/rabies/m).

Despite the dramatic clinical course of lyssavirus-induced disease, there are no obvious lesions in the CNS during gross post-mortem examination. Histopathological changes produced by lyssavirus infection occur in the central nervous system and ganglia and are similar in humans and animals.

The pathognomic histological lesion in rabies is the presence of Negri bodies. Negri bodies are eosinophilic intracytoplasmic inclusions within neurons. These bodies are most often observed in animals infected with classical rabies. The presence or absence of Negri bodies, however, cannot be used to differentiate between infections with rabies virus and the other lyssaviruses. The role of Negri bodies in lyssavirus infections remains unknown.

The recognition of species-specific variants of lyssaviruses suggests that the natural pathogenesis of lyssaviruses involves unique interactions between the host species and virus variant that at this time are not understood.

The clinical signs of rabies can be highly variable, and many cases do not fit neatly into either the classic furious or paralytic presentation. The most reliable diagnostic signs are behavioral changes and unexplained paralysis, but rabies should be a consideration in all cases of unexplained neurological disease. In the beginning of clinical rabies, 50% of infected people reported pain, burning, numbness, itching or tingling at the site of exposure during the prodromal stage of the disease (Hemachudha, 1994), but no characteristic gross or histological lesions have been identified at these sites. The incubation period of RABV is accepted to be between 20 and 90 days; however, this can vary and there have reports of incubation periods of 14 and 19 years (Fishbein, 1991).

There are no species – specific pathognomonic symptoms of rabies in animals. The symptoms are similar to that of rabies in human. There are two forms of animal infection – dumb and furious. Paralytic rabies in both domestic and wild animals starts with a clinical presentation of lethargy and a lack of coordination. This leads to weakness in the hind limbs and subsequent motor paralysis that spreads eventually to the mandible, which results in cessation of the swallowing reflex. Furious rabies is the classic "mad-dog syndrome," although it may be seen in all species. There is rarely evidence of paralysis during this stage. The animal becomes irritable and, with the slightest provocation, may viciously and aggressively uses its teeth, claws, horns, or hooves. The posture and expression is one of alertness and anxiety, with pupils dilated. Noise may invite attack.

The outbreaks of rabies in bats have not been documented. This might be due to many factors. The relatively high seroprevalence of virus neutralizing antibodies that are often

detected in bat populations may decrease their susceptibility to disease, thereby reducing the number of rabid bats in a given population. The virulence of a lyssavirus variant at the population level will, thus, depend on the proportions of naïve and seroconverted bats (Davis *et al.*, 2012).

It has been suggested that colonial bats tend to show dumb or paralytic symptoms of rabies more often than furious symptoms, with the opposite trend in solitary species (Constantine & Blehert, 2009).

The incubation period for lyssavirus infections of bats can range from weeks to months. It is rarely more than a year. The incubation period varies with the amount of virus transmitted, virus strain, site of inoculation (bites closer to the head have a shorter incubation period), pre-existing host immunity and nature of the wound (WHO, 2013). In addition, incubation may be influenced by the ambient temperature (George *et al.*, 2011). There is delay in the onset of clinical signs of the disease in bats that are already infected before hibernation. However, the disease continues to develop very slowly in hibernating bats (Fooks *et al.*, 2003).

Baer and Bales (1967) also suggested that longer incubation periods were generally associated with higher titres of virus in the salivary glands of bats.

The incubation period of rabies in insectivorous bats varies between 6 days to over 6 months (Constantine & Blehert, 2009). The duration of the disease in insect-eating bats is reported to be between 1 day to 20 days. Although in rare cases infected bats can transmit rabies viruses as early as 12 days before the appearance of clinical signs and 24 days before death of an infected bat, in the majority of cases the virus appears in bat saliva at the clinical onset or at the very end of disease (Constantine & Blehert, 2009). The

excretion is intermittent, and a large proportion of rabid bats does not have virus in the saliva at all (Constantine & Blehert, 2009).

Clinical signs of sick bats are non-specific and change as the disease progresses. The clinical picture may also be affected by environmental stimuli. In bats, clinical signs include: aggression or intolerance to humans, and fighting with other bats; isolation of infected bats from the roost, increased biting and vocalization. There have been unprovoked attacks on humans and other moving objects by rabid bats. Since fruit bats require to hunt for food (insects or fruits), sick bats, therefore, cannot feed and gradually depreciate in condition (Muller *et al.*, 2004). Clinically sick bats are often observed hanging on low tree branches or on the ground (FAO, 2011). When touched, rabid bats respond by biting objects, flapping the wings and by muscle tremors. Sometimes there is incoordination of movement. Affected bats generally cannot fly. When they do fly, they may collide with objects and can be found in strange places. The general presentation of lyssavirus infection in bats is that of completely altered behaviour and nervous dysfunction.

2.3.1 Rabies Virus (RABV)

Rabies virus (RABV) belongs to the Rhabdovirus family and lyssavirus genus. It is classified as serotype1/genotype 1. The rabies virus (gt 1) (RABV) is the prototype lyssavirus and the other species are known as the rabies-related lyssaviruses (or non-rabies lyssaviruses). All mammals are susceptible to RABV. There are many strains of the rabies virus; each strain is maintained in particular reservoir host(s). Important maintenance hosts include members of the Canidae (dogs, jackals, coyotes, wolves, foxes and raccoon dogs), Mustelidae (skunks, martens, weasels and stoats), Viverridae (mongooses and meerkats),

and Procyonidae (raccoons), and the order Chiroptera (bats). Cat-adapted rabies variants have not been seen, although cats are often infected with rabies viruses from other hosts.

Factors that may affect the outcome of exposure include the virus variant, dose of the virus, route and location of exposure, and host factors such as age and immune status. The incubation period varies with the amount of virus transmitted, virus strain, site of inoculation (bites closer to the head have a shorter incubation period), host immunity and nature of the wound. In dogs and cats, the incubation period is 10 days to 6 months; most cases become apparent between 2 weeks and 3 months (WHO, 2013).

There is some speculation that ingestion could play a role in rabies transmission among wild animals (OIE, 2013). One epizootic among kudu may have spread between animals when they fed on thorn trees (Barnard & Hassel, 1981). There are no records of human disease acquired by this route.

Although rabies can infect and be transmitted by a wide range of mammals, reservoirs comprise only mammalian species within the Orders *Carnivora* (e.g. dogs, raccoons, skunks, foxes, jackals) and Chiroptera (bats) (Passos *et al.*, 1998). Rabies is an underreported disease that is present on every continent, except Australia and Antarctica. The disease causes over 55,000 human deaths annually (Bengis *et al.*, 2004). Of the mammalian RABV, those that circulate in dogs (*Canis lupus familiaris*) are responsible for more than 99% of the human cases worldwide (Knobel *et al.*, 2005)

According to the WHO (2011), 30% - 50% of the 55,000 rabies victims estimated each year are individuals under 15 years of age. More than 99% of all known human rabies occurs in the developing world (Africa and Asia): 56% of these deaths occur in Asia, with 44% occurring in Africa. The impact of rabies on people is estimated to cost US$583.5

million (estimated US$563 million cost within Asia and only US$20.5 million cost in Africa) (www.who.int/rabies/ExpertConsultationOnRabies.pdf).

Control of Rabies

Although effective control measures are available, rabies remains a neglected disease throughout most countries. Control in dogs is by vaccination. Oral vaccination is used in wildlife. Strategies to control vampire-bat-transmitted rabies in Latin America include vaccination of humans and livestock and the reduction of bat populations by culling. However, it has recently been shown that culling of vampire bats may be counter-productive (Streiker *et al*., 2012). Vaccination of livestock is effective, but poses economic and logistical challenges that limit its practicality in the developing countries where the vampire bat rabies problem is most severe. Culling in wildlife disease systems can increase disease prevalence either by increasing recruitment of susceptible individuals or increasing host dispersal. In the vampire bats, culling resulted in immigration of bats from neighboring colonies to fill vacant roost space. Adult bats have developed protective immunity and so culling of adult bats results in removal of bats with protective immunity. Culling also results in availability of resources and space. This naturally stimulates increase in birth and hence increases in susceptible bats (Streiker *et al*., 2012). RABV is readily destroyed by ultra-violet light (including sunlight), and heat. It is killed by fat solvents and detergents, such as soap, ether, chloroform, acetone, iodine, and quarternary ammonium compounds. The virus is stable at pH 5-10 (Keep, 1982).

Epidemiology of dog Rabies

Lyssavirus epidemiology is influenced by the host species distribution, abundance, demographics, behavioral ecology, dispersal and interactions with human. Classical rabies caused by rabies virus (RABV) occur worldwide (Fig 2.5). RABV is adapted to persist in species with large populations and high intrinsic growth rates. Such species are capable of recovering rapidly after an epizootic wave has reduced the population density to a level at which the reproductive rate of the disease falls below unity. Red foxes, jackals, domestic dogs, striped skunks and raccoons are species with a wide distribution and population of relatively high density. They have high reproductive rates that permit rapid population recovery. The *Canidae* family represent the principal reservoir species for classical rabies and maintains the infectious cycle in most parts of the world. In the Americas, however, several bat species are also important reservoir hosts for RABV.

While cats are susceptible to the disease, no cat-to-cat transmission of rabies has been recorded and no feline variant of the virus is known. Natural rabies infection in all species generally causes an acute fatal illness, but rabies antibody has been detected in apparently healthy vector species including mongooses, skunks, raccoons, foxes, hyenas, jackals, fruit bats, vampire bats, insectivorous bats, and domestic dogs in Ethiopia (Warrell & Warrell, 2004). Antibodies to RABV have also been detected in the blood of carrion eating birds (Keep, 1982). However, rabies virus binds to mammalian, but not avian, neurotrophin receptors, thus explaining the lack of pathogenesis of rabies in avian species (Warrell & Warrell, 2004).

Several cycles of rabies may exist at the same time within a geographic region (Keep, 1982):

- The urban cycle of rabies is maintained in domestic dogs. In the urban rabies cycle, dogs are the main reservoir host. This cycle predominates in areas of Africa, Asia, and Central and South America where the proportion of unvaccinated and semi-owned or stray dogs is high (OIE, 2013). It has been virtually eliminated in North America and Europe; although sporadic cases occur in dogs infected by wild animals, the urban cycle is not perpetuated in the canine population.
- The sylvatic (or wildlife) cycles of rabies is maintained in one or several species of wildlife. The sylvatic cycles are predominant in areas where canine-mediated rabies has been controlled. The sylvatic cycle is the predominant cycle in Europe and North America. It is also present simultaneously with the urban cycle in some parts of the world (OIE, 2013). The epidemiology of this cycle is complex; factors affecting it include the virus strain, the behavior of the host species, ecology and environmental factors.

Two main modes of spread have been documented for rabies:

- human-assisted migration
- viral dispersal by gradual spatial spread of the virus through intra- and interspecific transmission (e.g., canid RABV movements within Africa)

In nature, rabies virus represents an assemblage of phylogenetic lineages, associated with specific mammalian host species. RABVs can be grouped into seven major clades designated according to their geographical distribution as follows:

- American indigenous – This strain includes all viruses associated with insectivorous and hematophagous bats of the Americas as well as a small number

of viral strains associated with non-flying mammals. This clade, which has often been designated the American bat clade, is more accurately referred to as the American indigenous clade since it includes not only all bat viruses of the Americas, but also strains associated with skunks (south central skunk strain of the southern United States and Mexican skunk strain), raccoons in eastern North America, and a marmoset species in Brazil (Jackson, 2011).

- India lineage - This strain includes mostly dog viruses from southern India and Sri Lanka;
- Asia lineages - These lineages comprise dog viruses from China and the countries of southeast Asia, the Philippines, and Indonesia;
- Africa 2 lineage- canid viruses from western and central Africa;
- Africa 3 lineage - viruses of the mongoose biotype of southern Africa;
- Arctic-related lineage - viruses harbored by red and arctic foxes from circumpolar areas of the northern hemisphere as well as by dogs in several countries of central/ western Asia;
- Cosmopolitan lineage - The cosmopolitan clade, which includes the group previously referred to as ‘‘Africa 1’’. It is believed to have been widely distributed as a result of human-assisted movement of diseased animals from Europe to many parts of the world during colonial activities (Botvinkin *et al.*, 2003; Bourhy *et al.*, 1992) Thus, this clade includes viruses from several parts of the Americas and the Caribbean and large areas of northern, eastern, and southern Africa and those of the Middle East and Europe. This clade is also harbored by wild canines, skunks and mongooses.

African Canine Rabies Lineages

Canine rabies predominates in Africa, Asia and Middle East (Mays & Aiello, 1998). Rabies virus (RABV) is enzootic throughout Africa, with the domestic dog (*Canis lupus familiaris*) being the principal vector.

Four genetically distinct lineages of the rabies virus have been documented in Sub-Saharan Africa - Africa 1, 2, 3 and 4 (Bourhy *et al.*, 1992). Africa 1 and 4 lineages are parts of the "cosmopolitan" lineage and were introduced at various times from European sources.

Africa 1 and 2 lineages have been detected in a range of domestic and wild carnivore species. A sub-lineage, Africa 1a, dominates northern and eastern Africa, but has also been detected in Nigeria, Gabon and Madagascar, suggesting a very broad distribution. Another sub-lineage Africa 1b is found in eastern, central and southern Africa (Bourhy *et al.*, 1992). Africa 3 is thought to be maintained within viverrid species in southern Africa. Africa 4 lineage has been identified only very recently, having been found in northern Africa (Turmelle *et al.*, 2010). While domestic dogs appear to be the only population essential for maintenance of canid variants in some parts of Africa (Sodja & Matouch, 1973), wild canids have been suggested to contribute to sustaining canine rabies cycles in specific geographic loci in South Africa and Zimbabwe (Tignor *et al.*, 1973).

Two sub-Saharan African RABV lineages have been detected in West Africa: Africa 1 & 2 (Albas *et al.*, 2011). Lineage 2 is present throughout West Africa, whereas Africa 1a predominates in northern and eastern Africa, but has been detected in Nigeria and Gabon, Africa 1b was considered to be absent from West Africa, but rabies diagnostics within West Africa are limited to non-specific staining techniques, including the Sellers' stain

and FAT (Hayman *et al.*, 2011). Hayman *et al.* (2011) reported, for the first time, the detection of Africa 1b in dogs in Ghana.

Bat Rabies

The common vampire bat is the principal wildlife reservoir of rabies in tropical and subtropical Latin America. The vampire bat is also one of the most important vectors of rabies to humans in this region. It was the first bat-associated rabies virus discovered since European colonization of America (Carini, 1911). Rabies virus in non-haematophagous bats was discovered first in a fruit bat (*Artibeus lituratus*) in Brazil (Correa-Giron *et al.*, 1970) and later from several species of phyllostomid bats in Trinidad (Cunha *et al.*, 2006). The virus has been reported in insectivorous bats in North America since 1953. Through increased awareness and surveillance rabies infection has been reported in the majority of insectivorous bat species throughout the USA and Canada.

Variants of RABV have been identified in bats in America: two closely related RABV variants (previously considered as one), occur in the silver-haired bat (*Lasionycteris noctivagans*) and eastern tri-colored bat (*Perimyotissub flavus*) (Kuzmin *et al.*, 2011). Other variants are the big brown bat RABV variant, the *Myotis* RABV variant, and Mexican free-tailed bat RABV variant (Kuzmin *et al.*, 2011). New World bats are reservoirs of rabies virus with evidence of species-specific variants (Correa-Giron *et al.*, 1970), which suggests that enzootic foci in bats are maintained by intraspecific transmission. However, studies highlight significant cross-species transmissions of rabies among some species of North American bats (Crawford-Miksza *et al.*, 1999). With elimination of dog rabies in North America through vaccination (Velasco-Villa *et al.*, 2006), bat associated variants of the virus are the important source of rabies in reported

human cases in North America. Among the bat reservoirs, the common vampire bat (*Desmodus rotundus*) causes the greatest rabies burden in other species owing to its unique habit of feeding on mammalian blood. Although all species of mammals are susceptible to rabies virus infection, only a few species are important as reservoirs for the disease. In the United States, several distinct rabies virus variants have been identified in terrestrial mammals, including raccoons, skunks, foxes, and coyotes (http://www.cdc.gov/rabies/exposure/animals/wildlife_reservoirs). In Europe the red fox (*Vulpes vulpes*) is the main reservoir species. In parts of Asia the racoon dog (*Nyctereutes procyonoides*) is also considered reservoir species for rabies. In Eastern Europe, introduced raccoon dogs may be implicated in sustaining the chain of infection, too (http://www.who-rabies-bulletin.org/about_rabies/Epidemiology). In Africa, rabies diseases has been found only in southern Africa, where wild canids, such as jackals (*Canis adustus* and *C. mesomelas*) and bat-eared foxes (*Otocyon megalotis*) are assumed to be primary hosts of rabies virus (Zulu *et al*., 2009). Additionally, members of the Herpestidae family (e.g. mongooses) appear to be responsible for transmission of a distinct variant of rabies virus in southern Africa (Van Zyl *et al*., 2010). Rabies has been reported to cause significant mortality among kudus (*Tragelaphus strepsiceros*) in Namibia (Scott *et al*., 2012).

2.3.2 Lagos Bat Virus (LBV)

Lagos bat virus (LBV) belongs to genotype (gt) 2 of the lyssavirus genus in the family *Rhabdoviridae*, order *Mononegavirales*. The first isolation of LBV was in 1956 in Nigeria from a frugivorous bat (*Eidolon helvum*) (Boulger & Porterfield, 1958) but it was not identified as a lyssavirus until 1970 (Shope *et al*., 1970). LBV reportedly contains at least two genetically distant clades that are divergent enough to suggest they may represent

different viral species (Markotter *et al*., 2008; Delmas *et al*., 2008). This virus causes fatal rabies encephalitis in vertebrate animals and has only been reported from the African continent with exception of an imported case from African origin identified in France. To date, there have been 28 reports of LBV disease throughout Africa, from which virus was isolated from only 16 cases (Table 2.2). One isolate was obtained in France (thought to be Togo or Egyptian origin) from an Egyptian fruit bat (*Rousettus aegyptiacus*) which had been displaying signs of aggression (Markotter *et al.*, 2008).

Fruit bats of several species serve as reservoir hosts for LBV (Table 2.2), with infrequent spillover infections documented in dogs, cats, and a mongoose (Markotter *et al.*, 2006).

Two LBV isolations were made in 1985, one from a frugivorous bat, *Eidolon helvum*, in Dakar (Senegal) and another from an insectivorous bat, *Nycteris gambiensis*, in Kindia, Guinea (Institute Pasteur, 1985).This was the only report of LBV from an insectivorous bat and both these isolates were shown to be related to the LBV isolate from the Central African Republic through complement fixation and neutralization tests (Institute Pasteur, 1985). In 1986 LBV was isolated from a rabies-vaccinated cat in Dorowa, Zimbabwe after the cat displayed abnormal behavior (Foggin & Zimbabwe, 1988).

A virus neutralization study of sera from *E. helvum* from Ghanaian fruit bats reported a very high and sustained seroprevalence of LBV in *E. helvum* (Hayman *et al*., 2008). Of the total samples screened, 56% neutralized LBV. The sustained seroprevalence in the study populations (Hayman *et al.*, 2012) provides evidence of the endemicity or circulation of the LBV in Ghanaian bat population. The maintenance of elevated seroprevalence in juvenile bats in Hayman *et al*. (2012) study is consistent with LBV exposures during the first year of life, similarly to that reported for RABV in vampire bats by Streicker *et al*. (2012).

Very little is known about lyssavirus LBV diversity and the relationships between different LBV isolates, or between LBV and other lyssaviruses. Genotype 2 viruses have not been well studied to date and the true risk of LBV for humans and animals is uncertain. The pathogenicity of LBV *in vivo* is thus debated within the scientific community. Different experimental studies have reported varied pathogenicity results for LBV. While some reported that the virus has a reduced pathogenicity, compared to RABV, others have provided very different results (Markotter *et al.*, 2009). Experimental infection studies using LBV (Lagos, Nigerian isolate) failed to produce disease in guinea pigs (i.m), rabbits (i.m. &i.c), monkey (*Cercocebus torquatus*) subcutaneous (s.c) (Boulgerand Porterfield, 1958). This, and other similar studies (Badrane *et al.*, 2001), led to the suggestions that phylogroup II viruses (LBV and MOKV) have a reduced pathogenicity compared to phylogroup I viruses (Badrane *et al.*, 2001). However, some strains of LBV caused 100% mortality in mice (Markotter *et al.*, 2009). Markotter *et al.*, (2009) further argued that the earlier studies were based on a single isolate of LBV and its low pathogenicity was essentially attributed to an amino-acid substitution at position 333 of glycoprotein ectodomain. Arginine 333 on the glycoprotein is responsible for the virulence of lyssaviruses (Takayama-Ito *et al.*, 2006). Phylogroup I has the arginine amino acid. Arginine 333 is replaced by aspartic acid in Phylogroup II (in all LBV isolates, MOKV and SHIBV) (Kuzmin *et al.*, 2010). This might account for the variation of pathogenicity among the various isolates of LBV. The absence of an unpassaged source of the original Nigerian isolate makes it impossible to confirm the real pathogenicity of this isolate.

Recent studies show that LBV (LBVCAR1974, LBVSA1982, LBVSEN1985, LBVZIM1986, LBVAFR1999, LagSA2003, LagSA2004, Mongoose2004 and

LBVSA2006) is pathogenic to mice when a high dose of the virus is inoculated intramuscularly (Markotter *et al.*, 2009). There are also reports that inflammatory changes in murine studies using LBV, MOKV and DUVV were more severe than those produced by RABV (Shope *et al.*, 1970). The pathogenicity of LBV might thus be underestimated. The virus, however, has not yet been detected in cases of human illness (Calisher & Ellison, 2012).

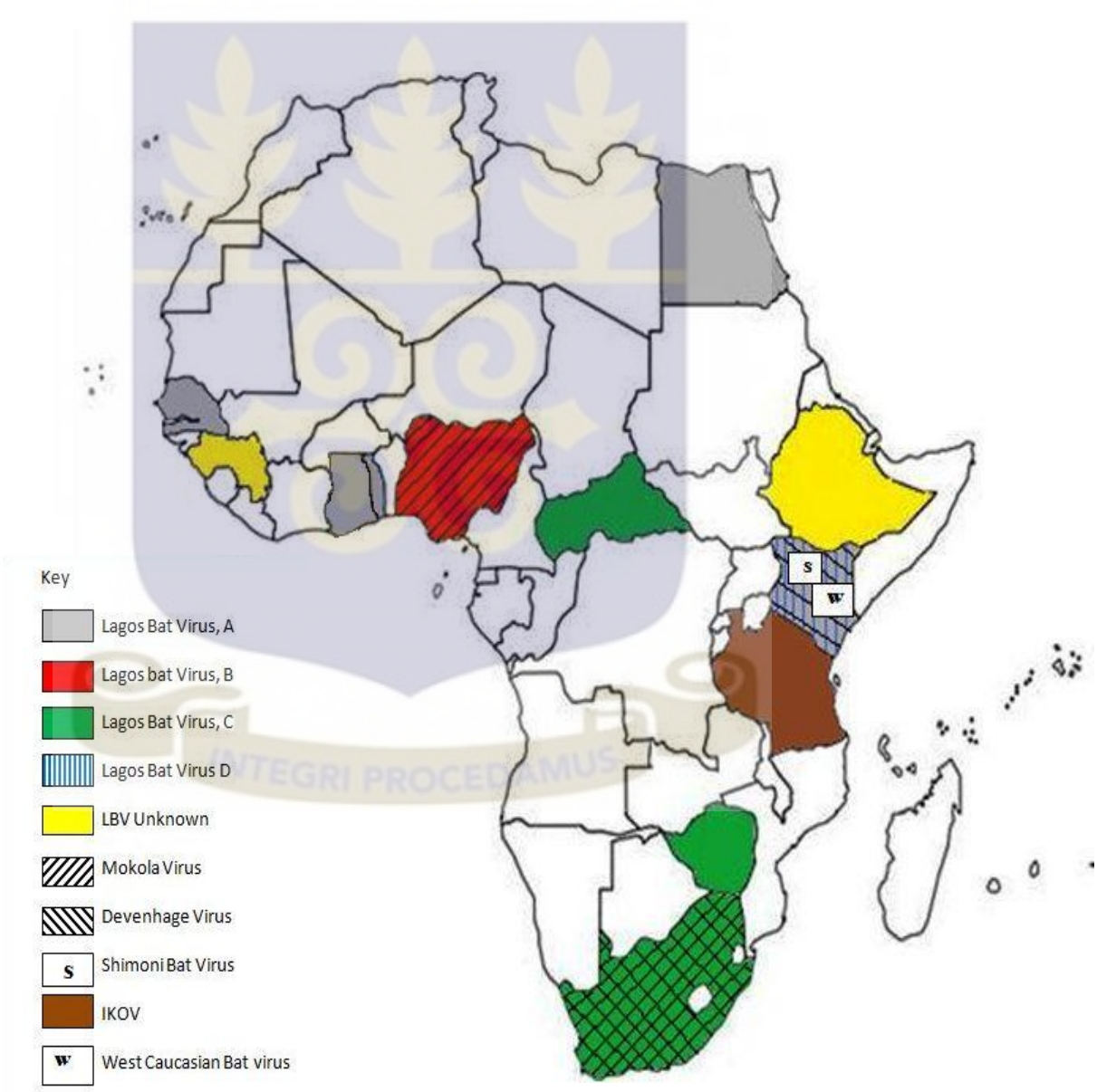

Fig 2.6: Distribution of lineages of LBV and other lyssaviruses of Africa. (**Adapted from Jackson, 2011)**

Recent studies by Kuzmin *et al*. (2010) have shown that LBV has a more complex phylogeny and said to have four divergent lineages including:

- Lineage A – Senegalese (1985), *E. helvum*; Kenyan (2007) , *E. helvum*, and French isolate (1999), presumable *R.aegyptiacus*; Ghana, ex-FLI (2013), *E. helvum*. Lineage A consisted of three isolates; Two from Senegal (both in 1985), one from Kumasi, Ghana (2013) and one from an unknown exact location in Africa, believed to be either Togo or Egypt (1999).
- Lineage B – Lineage B consisted of one isolate from Nigeria (1956). It was isolated in 1956 (Boulger and Porterfield, 1958) from the brain of a Nigerian fruit bats (*Eidolon helvum*) at Lagos Island, Nigeria, but not characterized until 1970. It also include 3 cases in domestic animals initially diagnosed as rabies, but weak immunofluorescence led to suspicion of "rabies-related" virus. It was later confirmed by typing with monoclonal antibodies or nucleotide sequence analysis (Wellenberg *et al.*, 2002).
- Lineage C – Lineage C is made of three distinct groups consisting of; (a) eight isolates from South Africa (1980-2005), (b) an isolate from Zimbabwe (1986) and (c) an isolate from the Central African Republic (1974) (Lumio *et al.*, 1986).
- Lineage D – Kenyan Isolates (Kuzmin *et al.*, 2010), *R. aegyptiacus*.

The isolate from Ethiopia is of unknown lineage and date of isolation.

The distribution of the lineages of LBV is shown and shaded grey in Fig. 2.6 and Table. 2.2.

Table 2.2: Lagos Bat Viruses isolated up to date

Geographic origin	Year isolated	Animal (species)	Reference
Lagos, Nigeria	1956	Bat (*E. helvum*)	Boulger and Porterfield, 1956
Bozo, Central African Republic	1974	Bat (*M. pusillus*)	Markotter *et al*., 2006
Pinetown, South Africa (3 isolates)	1980	Bat (*E.Wahlbergi*)	Markotter *et al*., 2006
Stanger, South Africa	1982	Cat (*Felis catus*)	Crick *et la*., 1982
Kindia, Senegal	1985	Bat (*N. cambiensis*)	Institute Pasteur 1985
Dakar, Senegal	1985	Bat (*E. helvum*)	Mebatsion *et al*., 1992
Dorowa, Zimbabwe	1986	Cat (*Felis catus*)	Foggin, 1988
Durban, South Africa	1990	Bat (*E. wahlbergi*)	Swanepoel, 1994
Ethiopia	Before 1992	Dog (*Canis lupus familiaris*)	Mebatsion, 1992
France	1999	Bat (*R. aegyptiacus*,)	Aubert, 1999
Durban, South Africa	2003	Bat (*E. wahlbergi*)	Markotter *et al*., 2006
Durban, South Africa	2003	Dog (*Canis lupus familiaris*)	Markotter *et al*., 2008
Durban, South Africa	2004	Bat (*E. wahlbergi*)	Markotter *et al*., 2006
Durban, South Africa	2004	Mongoose (*Atilax paludinosus*)	Markotter *et al*., 2008
Ghana (Ex-FL, Germany)	2013	Bat (*E. helvum*)	Freuling (unpublished)

2.3.3 Other Lyssaviruses

Mokola Virus (MOKV)

Mokola virus is the genotype 3 member of the Genus *Lyssavirus* within the Family *Rhabdoviridae*. Mokola virus was first isolated in Ibadan, Nigeria, in 1968 from shrews (*Crocidura* spp.) (Warner *et al.*, 1997). The virus was isolated also from Nigerian children with central nervous system disease in Nigeria in1968 (Calisher & Ellison, 2012). Subsequently, MOKV was detected in shrews from Nigeria and Cameroon, domestic cats in Zimbabwe, Ethiopia and South Africa, a domestic dog in Zimbabwe and a rodent (*Lophuromys sikapusi*) from the Central Africa Republic (Charlton *et al.*, 1984). Mokola virus infection has to date been reported from the African continent only.

Mokola virus is the only lyssavirus that has not been isolated from bats, but it has been found in a diverse host range (supposedly spill-over infections) and the principal reservoir remains unknown. Infection with the Mokola virus produces clinical signs compatible to rabies. It causes acute encephalitic diseases in human very similar to rabies virus infection (de Mattos *et al.*, 2000). In the two human cases reported, both young girls, the symptoms were fever and convulsion (with full recovery) in one case and drowsiness, paralysis and terminal coma in the second case (Sasaki *et al.*, 1992). One distinction between typical rabies infection and Mokola virus infection in domestic cats, seem to be the lack of unprovoked aggression in the latter. However, as with rabies, unusual behaviour, neurological disturbance, hypersensitivity, dehydration and salivation have been most commonly reported in cases of Mokola virus infection of domestic animals (de Mattos *et al.*, 2000). MOKV infections are rarely documented; only 23 isolates are known (Sabeta *et al.*, 2007). Most MOKV isolates have been found in South Africa. Lack of isolates from

other regions of Africa may be due to lack of active surveillance and limited diagnostic capabilities in many African laboratories.

Poor cross-neutralization in mouse serum neutralization tests and mouse vaccination trials suggested neither rabies vaccine nor antiserum would be protective (Fekadu *et al.*, 1988). This is supported by the fact that a dog vaccinated with a potent inactivated rabies vaccine (Rabisin; Institut Merieux) six months previously (Foggin, 1982) got infected by Mokola virus, developed clinical disease and died.

Duvenhage Virus (DUVV)

The Duvenhage virus (DUVV) belongs to Phylogroup II and is classified as genotype 4. The virus causes fatal rabies encephalitis in humans and mice (Bussereau *et al.*, 1988). Various studies have shown that DUVV is most closely related phylogenetically to EBLV-1.

Phylogenetic analysis indicated that the DUVV isolates constitute two different lineages. The southern African isolates together form one lineage and the more recent isolate from Kenya constitutes a new, second lineage (Van Eeden *et al.*, 2011).

The virus is exclusively associated with insectivorous bats of unknown species. DUVV has been identified as the cause of three human fatalities: in South Africa in 1970 and 2006, and in Kenya in 2007 (Meredith *et al.*, 1971). All of these were linked to contact with small bats, but the bats were never found or positively identified.

European Bat Lyssavirus type 1 and type 2 (EBLV-1 and EBLV-2)

The first report of a rabid bat in Europe came from observations in 1954 (Banyard *et al*., 2011). In Europe, lyssaviruses have been reported in eight bat species since 1985: *E. serotinus*, *Nyctalus noctula*, *Myotis daubentonii*, *Myotis dasycneme*, *Myotis myotis*, *Pipistrellus nathusii*, *Pipistrellus pipistrellus* and *Plecotus auritus*. RT-PCR studies have detected viral RNA in four additional species: *Barbastella barbastellus*, *Myotis nattereri*, *Miniopterus schreibersii*and *Rhinolophus ferrumequinum* (Echevarria *et al.*, 2001). There are at least four reported species of lyssaviruses that are circulating in bats in Europe: *European bat lyssavirus 1* (EBLV-1), *European bat lyssavirus 2* (EBLV-2), Bokeloh bat lyssavirus (BBLV), and *West-Caucasian bat virus* (WCBV).

EBLV-1 is antigenically and genetically more closely related to Duvenhage virus than it is to EBLV-2 (Bourhy *et al.*, 1992) and is found exclusively in insectivorous bats. EBLV-1 has only been reported in Europe, although it might also be in North Africa due to bat species range overlaps between Europe and North Africa (Freuling *et al.*, 2009).

Two lineages of EBLV-1 have been defined. EBLV-1a occurs in northern Europe, mostly in France, the Netherlands, Germany, and Poland. EBLV-1b has been reported from Southern Germany, France, and Spain. However, the distribution of EBLV-1b might be greater than currently known (Amengual *et al.*, 1997). Both EBLV-1 lineages are mainly associated with the serotine bat (*Eptesicus serotinus*) with 99% of cases being associated with this species. In Spain, however, EBLV-1 has been reported from a range of species including the greater mouse-eared bat (*Myotis myotis*), the Natterer's bat (*Myotis nattereri*), the greater horseshoe bat (*Rhinolophus ferrumequinum*), and the common bent-winged bat (*Miniopterus schreibersii***)** (Amengual *et al.*, 2007).

EBLV-2 has been detected only in the United Kingdom, The Netherlands, Finland and near the border between Switzerland and Germany (Whitby *et al.*, 2000). It is associated with bats of the genus Myotis, pond bats (*Myotis dasycneme*) and Daubenton's bats (*Myotis daubentonii).* The virus has been isolated sporadically across a number of countries of Northern Europe, but there is a knowledge gap in the persistence of EBLV-2 in its natural host. The 1 and 4% seroprevalence suggest a low level of perpetuation of the virus in bat populations (Harris *et al.*, 2009).

EBLV-2 has caused rare, geographically scattered but fatal human infections. It was isolated from a zoologist who died in Finland with what was diagnosed as rabies (Lumio *et al.*, 1986), and in a bat rehabilitator in Scotland in 2002 (Fooks *et al.*, 2003).

EBLV-1 was reported to infect terrestrial species, including sheep, cats and marten (Fooks *et al.*, 2003). The infection in these species causes clinical disease similar to classical rabies virus (RABV). These viruses have also caused at least three cases of human rabies, in Finland, Russia and the UK (Kuzmin *et al.*, 2011). A few cases of spillover of EBLV-1 infections were documented in terrestrial mammals, including domestic cats (Dacheux *et al.*, 2009), and they represent a potential exposure risk for humans. EBLV-1 was isolated from several Danish sheep in 1998 and 2002 (Harris *et al.*, 2009), from a German stone marten in 2001 (Muller *et al.*, 2004) and from a cat in France in 2007 (Dacheux *et al.*, 2009). One human case of EBLV-1 infection was reported from Russia (Selimov *et al.*, 1989).

EBLV-1 appears to be more virulent than EBLV-2 in certain animal models. Disease caused by EBLV-1 in bats is indistinguishable from that observed with RABV infection. Direct inoculation of EBLV-1 into the brain of Egyptian flying foxes (*Rousettus aegyptiacus*) caused neurological disease and death in 5 of 8 (63%) inoculated animals.

Similar mortality was also observed in experimental studies of EBLV-1 in the North American Big Brown Bat (*E. fuscus*) (Franka *et al*., 2008). The virus in i.m inoculation of big brown bats results in 50% morbidity. In another study, using the proposed EBLV-1 reservoir host, *E. serotinus*, and 100% induction of rabies by intracranial inoculation was demonstrated (Freuling *et al.*, 2009).

West Caucasian bat Virus (WCBV)

West Caucasian bat virus (WCBV) was first isolated in 2002 in southeastern Europe from Schreiber's long-fingered bat, *Miniopterus schreibersii* (Kuzmin *et al.*, 2005), and only 1 isolate is available to date. WCBV-neutralizing antibodies have also been reported in Africa (Kenya) in *Miniopterus* insectivorous bats; suggesting the circulation of WCBV or other serologically-related virus in African bats (Kuzmin *et al.*, 2008). WCBC is one of the most phylogenetically divergent lyssaviruses. It also exhibits limited relatedness to genotype 2 and 3 viruses. WCBC does not cross - react serologically with any viruses in Phylogroup I or II. WCBV awaits classification with regards to phylogroup. However, it has been proposed as a new phylogroup III (Kuzmin *et al.*, 2005). It has now been shown to be related to the most recent lyssaviruses identified: Ikoma virus from Tanzania (Marston *et al.*, 2012) with which there is serological cross reactivity with Lleida virus from Spain (Ceballos *et al.*, 2013). It might be, therefore, that the bats seropositive to WCBV in Africa are actually seropositive for Ikoma virus or a closely-related, as-yet-undiscovered, virus (Marston *et al.*, 2012).

Australian Bat Lyssavirus (ABLV)

Australian bat lyssavirus was first isolated from a brain sample of a black flying fox (*Pteropus alecto*) showing nervous signs which was collected in New South Wales, Australia in 1996 (Fraser *et al.*, 1996). The virus has now been shown to occur in all flying fox species (black flying fox, gray-headed flying fox (*Pteropus poliocephalus*)), little red flying fox (*Pteropus scapulatus*) and spectacled flying fox (*Pteropus conspicillatus*) found in Australia. The virus has also been isolated from an insectivorous bat species, the yellow- bellied sheath-tailed bat (*Saccolaimus flaviventris*). Phylogenetic analyses have indicated that ABLV forms a monophyletic group which differentiates into two distinct clades. One associated with the four *Pteropus* species, and the other one with the insectivorous bat species (Calisher & Ellison, 2012). The two clades have a nucleotide divergence of up to 18.7% (John *et al*., 2008). There are thus two lineages with established separate transmission cycles in Australia. Serological evidence of infection has been found in a number of other bat genera in Australia, indicating that the ecology and diversity of this virus is yet to be fully understood.

ABLV has caused two human deaths in Australia (John *et al*., 2008). The first case involved a bat rehabilitator who had been scratched by a yellow-bellied sheath-tailed bat 5 weeks previously.The second death occurred in a woman, also a bat rehabilitator, and 2 years after she had received a bite from a flying fox. ABLV has been isolated, or infection demonstrated, in both insectivorous and fruit bats (flying foxes) from: New South Wales, Northern Territory, Queensland, Victoria and Western Australia. The rabies vaccine is effective in protecting against ABLV. Australia's rabies-free status has not changed as a result of the Australian bat lyssavirus discovery based on the present OIE determination of

a rabies-free territory, but from the WHO standpoint, a territory where any lyssavirus is present (Including Australia) is not rabies-free (WHO, 2013).

Shimoni Bat Virus

Shimoni bat virus (SHIBV) was isolated from the brain of a dead Commerson's leaf-nosed bat (*Hipposideros commersoni*), found in a cave in the coastal region of Kenya. Kuzmin *et al*. (2011) confirmed this bat species as the reservoir host for Shimoni virus. Genetic distances and phylogenetic reconstructions demonstrated that SHIBV cannot be identified with any of the other viral species (Kuzmin *et al.*, 2010). SHIBV demonstrates similarity to MOKV and LBV, but is considered an independent species within phylogroup II. The significance of SHIBV to public health is unknown (Kuzmin *et al.*, 2010). However, as in the case of WCBV, it is pathogenic to laboratory animals, which develop rabies and die after intracranial or peripheral inoculation.

Irkut Virus

The source of this lyssavirus were insectivorous bats, greater tube-nosed bat (*Murina leucogaster*) captured in the Irkuts region of eastern Siberia in 2002 (Botvinkin *et al.*, 2003) and further in China (Lius *et al*., 2013). One human fatal illness caused by this virus has been documented in Russia after a bat bite (Belikov *et al*., 2009).

Aravan Virus (ARV)

The Aravan virus was isolated from a Lesser Mouse-eared Bat (*Myotis blythi*) in the Osh region of Kyrghyzstan, central Asia, in 1991 (Kuzmin *et al*., 2003). The virus demonstrates phylogenetic relatedness to EBLVs and DUVV but cannot be included in any of these species.

The lesser mouse-eared bat, from which the Aravan virus was isolated, is widely distributed in northern Africa, the Mediterranean, southern Europe, Crimea, Caucasus, Palestine, southwest Asia, and parts of central and eastern Asia. This information should be considered in the discussion of lyssavirus classification and evolution, as it suggests the possibility of a broader geographic distribution of the Aravan virus.

Khujand Virus (KHUV)

Khujand virus was isolated in northern Tajikistan in 2001 (Kuzmin *et al.*, 2003) from a whiskered bat (*Myotis mystacinus*). KHUV is related phylogenetically to EBLV-2 more than to other lyssaviruses. Phylogenetic analysis and comparison of different parts of the genomes generally suggested that Khujand virus is mainly related to genotype 6.

Bokeloh Bat Virus (BBLV)

Bokeloh bat virus was first isolated from Natterer's bat (*M. nattererii*) (*www.cdc.gov/eid*) in Bokeloh, Lower Saxony, Germany in 2010. It belongs to lyssavirus phylogroup I. BBLV is pathogenic: it caused a fatal disease in the Natterer's bat that was similar to the clinical picture of rabies seen in other bats. Since only three isolate of the virus has been obtained and no longitudinal serological studies specific for this virus have been conducted, the natural reservoir host of the virus has yet to be determined.

Ikoma Virus (IKOV)

Ikoma virus was first isolated from the brain of a sick African civet cat (*Civettictis civetta*) in the Serengeti National Park in Tanzania in 2009 (Marston *et al.*, 2012). The civet displayed signs of rabies, bit a child and was shot by rangers. Sequencing and Bayesian reconstruction analysis showed IKOV virus to be unique and related to WCBV albeit with

a long genetic distance. However, more research is required to determine the phylogenetic groupings and antigenic properties of IKOV, natural reservoir and circulation patterns of IKOV.

Lleida bat Virus (LLEBV)

Lleida bat virus is a new tentative lyssavirus reported in a bent-winged bat (*Miniopterus schreibersii*) in Spain (Arehiga-Cebellos *et al.*, 2013). LLEBV does not belong to phylogroups I or II. It is proposed to be accepted as a new lyssavirus and called Lleida bat lyssavirus (LLEBV). Only a limited N gene sequence available to date shows that LLEBV is related to WCBV and IKOV, although via long genetic distances to both (Ceballos et al., 2013).

No human exposure to LLEBV has been reported as yet. However, because of the divergence exhibited by LLEBV and IKOV, and the growing evidence of inadequate protection/cross-neutralization against viruses outside phylogroup I, the effectiveness of current rabies vaccines for these viruses remains a concern (Arehiga-Cebellos *et al*, 2013).

2.3.4 Diagnosis of Lyssavirus Infection

Diagnosis of rabies based upon clinical presentation or gross lesions is unreliable. Clinical signs of the disease are not specific and may vary greatly from one animal to another; therefore, confirmation of infection can only be achieved by laboratory techniques. Laboratory diagnosis of rabies virus antigen was historically done by Negri body examination, but now direct FAT using rabies antinucleocapsid antibody conjugate on brain impression smears is used most often along with other rapid and reliable antigen-detection methods, such as dRIT. Validated diagnostic tests that confirm the presence of

rabies virus or a lyssavirus variant have been the foundation of rabies control strategies in many countries. Historically, histopathological techniques such as the Sellers Stain technique were used to determine the presence of Negri bodies as rabies virus-specific antigen, however due to poor sensitivity and specificity this technique is no longer recommended by the World Health Organization (OIE, 2011).

The development of techniques to detect lyssavirus infection and previous exposure is fundamental to understanding both the risks to humans posed by lyssaviruses and in studying virus epidemiology. Monoclonal antibodies have been developed for the identification of rabies virus variants and other lyssaviruses, and these have been used for the demonstration of viral antigen in tissue culture, frozen tissue sections, and in fixed impression smears of the brain. Lyssaviruses exhibit cross-reactivity at the neucleocapside level due to conservation of the N protein. This allows the use of similar reagents for diagnosis by nucleoprotein antigen detection (www.who.int/rabies/ ExpertConsultationOnRabies.pdf) for infection with all lyssaviruses. The following methods are used in the diagnosis of rabies and non- rabies lyssaviruses:

1. *Antigen-detection methods.*

These methods include direct and indirect Fluorescent antibody test (FAT), Immunochemical test, Enzyme-linked immunosorbent assay (ELISA), direct Rapid immunodiagnostic test (dRIT).

The most widely used test for rabies diagnosis is the FAT, which is recommended by both WHO and OIE (WHO, 2013) as the gold standard test for lyssavirus antigen detection. FAT is considered to be a standard rabies virus diagnostic test against which other diagnostic approaches are to be compared (www.who-rabies-

bulletin.org/about_rabies/Diagnosis.aspx). The FAT relies on the ability of a detector molecule (usually fluorescein isothiocyanate) coupled with a rabies specific antibody forming a conjugate to bind to and allow the visualisation of rabies antigen using fluorescent microscopy techniques. Microscopic analysis of samples is the only direct method that allows for the identification of rabies virus-specific antigen in a short time and at a reduced cost, irrespective of geographical origin and status of the host. It has to be regarded as the first step in diagnostic procedures for all laboratories where luminescent microscope is available. Depending on antibodies used (e.g. combinations of monoclonal antibodies or polyclonal antibody) FAT can detect all lyssaviruses known to date (Kuzmin *et al.*, 2010; Marston *et al.*, 2012). The FAT gives reliable results on fresh specimens within a few hours in more than 95–99%. The sensitivity of the FAT depends on the specimen (the degree of autolysis and how comprehensively the brain is sampled) (Barrat and Aubert, 1995), on the type of lyssavirus and on the proficiency of the diagnostic staff. Pen – side test, for field testing of rabies, have been developed using lateral flow devices coated with RABV antigen – trapping antibodies (Markotter *et al.*, 2009; Servat *et al.*, 2012).

2. *Virus isolation Test.*

Two methods – the Mice Inoculation Test (MIT) and the Rabies Tissue Culture Isolation Test (RTCIT) are employed. Virus isolation is used to confirm the results of an antigen detection and for further characterization of the isolate (Bourhy *et al.*, 1989), and for amplification of the virus for further studies. The OIE guidance for rabies diagnosis is that FAT on postmortem material be performed and subsequent virus isolation undertaken to confirm the diagnosis. These tests detect the infectivity of a tissue suspension in cell cultures or in laboratory animals. They should be used if the FAT gives an uncertain result

or when the FAT is negative in the case of known human exposure. Another diagnostic test, the Rapid Rabies Enzyme Immunodiagnosis (RREID) Test is able to detect RABV in brain tissue, even tissue that is partially decomposed, without the use of microscope. Sensitivity of this method is however low.

3. *Molecular Methods*.

Modern molecular techniques such as RT-PCR are fast and highly sensitive. Due to their high sensitivity, molecular methods can be applied as confirmatory or alternative tests on poor quality brain tissue samples. RT-PCR is also useful, particularly when the sample is small (e.g., saliva) or when large numbers of samples must be tested in an outbreak or epidemiological survey (www.efsa.europa.eu/en/supporting/doc/67e). However, because of the high specificity, PCR-based methods are recommended by WHO and OIE as confirmatory tests. There are new molecular techniques for the detection of rabies virus in humans. They include microarrays for lyssavirus detection. The advantages in the use of reverse transcription PCR (RT-PCR) is the detection of viral RNA in a range of biological samples, including skin biopsy and saliva. RT-PCR is also able to differentiate between viral species in real time, which makes diagnosis rapid and accurate. Sequensing of PCR products is a useful tool for characterisation to differentiate between lyssavirus species and also phylogenetically characterize isolates (Hoffman, *et al*., 2010). Modern molecular techniques such as RT-PCR and Microarrays may not however be applicable in the developing rabies –endemic countries (Nunez *et al.*, 1987) due to lack of required biosafety level-3 containment and bat-borne virus cell lines for the isolation of bat lyssaviruses.

4. Serology Methods.

The measurement of neutralising and binding antibodies has been commonly used for many years to assess the level of immunity to rabies in animals and humans. The WHO expert committee on rabies considers that a level of antibodies equal to or greater than 0.5 international units/ml (IU/ml) of serum indicates adequate protection. Serology is very effective in monitoring vaccination status in susceptible populations. It has been proposed as a measure that could replace or alleviate quarantine controls (Aubert, 1992). Serology is very useful for epidemiological studies. It is not useful in ante-mortem diagnosis in animals due to late seroconversion and high mortality rates of host species. However, in human rabies diagnosis the serology is one of the necessary tests to detect the infection and to monitor its progression during the clinical course. The level of antibodies in vaccinated and non-vaccinated animals can be measured using serological methods including: Viral Neutralising Test in mice (VNT; mainly historical test), Rapid Fluorescent Focus Inhibition Test (RFFIT), Fluorescent Antibody Virus Neutralization (FAVN) Test, Indirect Fluorescent Antibody (IFA) Test, and ELISA (http://www.cfsph.iastate.edu/Factsheets/pdfs/rabies.pdf). RFFIT and FAVN tests determine the amount of virus-neutralizing antibodies. In contrast, IFA determines mainly binding (non-neutralizing) antibodies against viral nucleocapsid. ELISA, though employed for wildlife serology, may lack sensitivity or give inconsistent results (Knoop and Freuling, 2010). Pseudo typed lentiviral vectors consist of vector particles bearing glycoproteins (GPs) derived from other enveloped viruses. Neutralisation assays using pseudotypes containing lyssaviruses in developing African countries including LBV, DUVV, MKV, may offer a safe alternative to the use of pathogenic viruses in neutralization assays (Fooks *et al.*, 2009).

Of the methods listed above, three principal routine methods of laboratory diagnosis of rabies are recommended both by WHO and OIE, i.e. Florescent Antibody Test (FAT) for antigen detection, Molecular test, and the RTCIT or MIT for virus isolation. Both international organisations recommend that in-vivo test (MIT) should be replaced by in vitro methods (RTCIT) (http://www.efsa.europa.eu/en/supporting/doc/67e.pdf) if possible.

2.2.6 Maintenance and Persistence of Lyssaviruses in Bat Populations

Bats have a long evolutionary history compared to most other mammals. The ancient origins deduced for certain zoonotic viruses maintained in bats such as lyssaviruses, suggest a long history of co speciation (Calisher *et al.*, 2006). A number of viruses can establish persistent infection in a host; for example Bovine viral diarrhoea virus (BVDV) has a unique capacity to cause persistent infections of fetuses (Brock, 2003). Persistence encompasses the concept of long-term and continuous presence of disease within a metapopulation (Bingham, 2005). Successful persistence requires that a virus is maintained in ≥1 local population at a time (Bingham, 2005).

RABV in mammals causes acute, lethal encephalitis. However, some bats occasionally survive abortive infections that may provide some naturally acquired immunity (Streicker *et al.*, 2012).

Active infections and antibodies to RABV variants have been reported in numerous bat species in North and South America (Belotto *et al.*, 2005) but not in bats on other continents. In the Old World there are many more species of lyssaviruses, the majority of which have been isolated from bats (Kuzmin *et al.*, 2005; Kuzmin *et al.*, 2010). Our understanding of how viruses persist in different bat populations is very limited, particularly outside the Americas. Bats have unique behaviour that enables persistence of

pathogens within bat populations. Kuzmin (2011) noted that, some agents of bats also possess traits that enable them to evade host immune system. Streicker *et al*. (2012) reports that RABV perpetuates in vampire bat populations through the recruitment of naïve bats via births and immigration.

Bat behavior factors that enhance pathogen persistence:

Unique anatomy and ability of bats to fly: Bats are the only mammals that fly. All bats cover varied distance mostly at night in search for food and water. Long distance dispersal of bats through seasonal migration and daily feeding behavior is common in both micro- and mega chiropterans. Although most tropical bats will travel distances <200 km during a season when shifting roosts in response to the availability of fruit production by tropical trees, a few species, such as the pteropid bat, *Eidolon helvum,* will seasonally travel~1500 km in one-way migrations from forest habitats to savannahs in Africa . The long-distance migratory behaviour of bats may influence the geographic distribution and genetic variability of lyssaviral variants associated with specific bat species (Table 2.1). Long-distance movements of bats may also lead to regular, but not constant, contact between individual bats from different subpopulations allowing partial connectivity between colonies of bats and hence sharing of bat pathogens ensuring their persistence in these species.

Large colonial population size and roosting behaviour: Maintenance is the notion of indefinite transmission of virus through members of a host population (Bingham, 2005). Maintenance of RABV in natural populations of bats is likely influenced by the ecology and sociality of reservoir species. Given the small colony sizes or solitary lifestyle of most non synanthropic tree roosting bats, such as the *lasiurines*, there may be differences in

transmission dynamics when compared to more gregarious species, due to less opportunity for intra-colonial transfer between individuals. Opportunity for the virus to spread in a solitary mammal population is limited. Individual local populations are also unable to maintain rabies continuously; the disease is normally reintroduced from other infected local populations of a metapopulation (Bingham, 2005). In large colonies, bats roost in close proximity to one another, increasing the opportunity for a rabies-infected individual to bite and infect conspecifics (Klug *et al.*, 2011).

Based on current research, there is very little evidence to suggest that any of the major bat-associated emerging infectious diseases persist within the host, and it is therefore most likely that these viruses are maintained in nature by perpetuation within and between bat colonies and through multiple spillover events into other hosts due to the extreme mobility and highly social nature of the bat hosts. Among these various agents, lyssaviruses have been most thoroughly characterized. For example, RABV, the type species of this genus, perpetuates through bite transmission between infected animals. In essence, RABV is characterized by a rather low basic reproductive rate and a short infectious period. It is also reported that host species which exist in large population sizes and in high densities are capable of acting as reservoirs for a greater number of viruses than smaller, low density populations (Moya *et al.*, 2004). Large colonies also provide naive infants, the recruitment of which supports the persistence of pathogens. The close proximity of numerous individuals packed into dense concentrations can also facilitate virus transmission by direct contact, such as biting or licking and other means, such as through respiratory transmission or contact transmission by transfer of infectious secreta and excreta. It is in caves harboring millions of closely packed free-tailed bats that airborne rabies virus transmission was documented (FAO, 2011).

Hibernation: An important trait of temperate bats of the families *Vespertilionidae* and *Rhinolophidae* is their ability to enter into daily torpor and seasonal hibernation to conserve energy during cool nights and absence of food sources during winter.

The ecology of bat rabies and other lyssavirus infection is closely associated with the ecology of bats. In temperate areas, the disease fluctuates with the activities of bats. Streiker *et al*. (2012) suggested that RABV in tropical bats evolves faster than in temperate bats probably because the bats are active year around and viruses has more times time to evolve.

Most, if not all, temperate bat species are capable of entering into regulated torpor whereby their body temperature is allowed to fall, and many species enter hibernation during winter. Additionally, some tropical microchiropteran and megachiropteran species reduce temperature, but whether this is regulated torpor or caused by extreme peripheral vasoconstriction remains to be determined. Although the impact of torpor and hibernation on the pathogenesis and maintenance of viral infections in bats has not been studied extensively, literature reports that viruses may overwinter in bats (Constantine & Blehert, 2009). Infected bats may shed viruses, such as lyssaviruses (family *Rhabdoviridae*) or flaviviruses (family *Flaviviridae*) for extensive periods without evidence of disease (Sulkin and Allen, 1974). The impact of torpor and hibernation on the immune response and the persistence of viral infections among experimentally infected bats has been investigated for Japanese encephalitis virus (JEV) and rabies virus. Bats may experience abortive infection by rabies virus or unusually long incubation or latency periods. Factors responsible for long delay in incubation require some further investigation. However George *et al.* (2011), indicated that long incubation period is vital for the maintenance of rabies virus while short incubation results in fading out of the disease in bats.

Cross-sectional studies have revealed high seroprevalence of antibodies against LBV in two colonial fruit-bat species, *Eidolon helvum* and *Rousettus aegyptiacus*. Therefore, if the serological findings are evidence of endemic transmission, mechanisms other than the use of extended torpor must exist that allow infection persistence within these populations (Hayman *et al.*, 2012).

Viral neutralizing antibodies have been reported in apparently healthy bats in population of bats globally (Constantine & Blehert, 2009). Infection with classical rabies (RABV) in terrestrial mammals leads to the development of rabies disease. Seroconversions with detection of viral neutralizing antibodies in these animals are detected only in the final stages of the disease. However, the basic immunological status of bats especially relating to exposure to lyssavirus is poorly known.

Colonial bats are associated with close contact. Transmission of disease in colonial bats is by introduction of virus from infective saliva through bites, licking of wounds and mucosal surfaces or droplet aerosol. There have been experimental studies in insectivorous bats to explain the importance of the route of infection in bats, there has not been a clear trend as to the transmission of viruses between bats in their natural bat roost. Intranasal route failed to cause disease, however seroconversion was reported. Intracranial Infection has been reported to result in disease and death. Intramuscular and Intradermal inoculation have been reported to give mixed success in different animal models (Banyard *et al.*, 2011). There are therefore suggestions that seroprevelances in bats might be due to low dose of virus inoculated during bites in bat colony or through aerosol in caves. It is also postulated that young bats may acquire temporal passive maternal antibodies through the milk or during the antenatal period. The passive immunity thus acquired protects them from developing a disease from a bite inoculation from infected bats. However, such

young bats develop lifelong immunity from such exposure (FAO, 2011). Although the virus immunizes some bats in the population, some juvenile bats from non-immune mothers are killed by the virus to maintain disease within the colony so that the virus persist (Constantine & Blehert, 2009). There have not been any studies on persistence to date of LBV in *E. helvum*. However, the several reports of high LBV seroprevalences in this species could be due to the above listed reasons.

Immunosuppression during hibernation: The role of the environment, including temperature, humidity, food availability, parasite load and infection with other bat pathogens may all play diverse roles in the outcome of an infection with lyssaviruses. Cooler temperatures has been reported by George and others 2011 to slow viral development rates in bats and thus to prolong the incubation period by the duration of the torpor. However, there are reports that temperature has influence on the manifestation of diseases including rabies (Calisher *et al.*, 2006).

Many species of small temperate bats of the suborder Microchiroptera have life spans that exceed 25 years, with the greatest longevity, of 35 years, documented for a little brown bat. Virus isolation and antibody studies suggest that many viruses can cause persistent infections in bats (Kuno, 2001).The extreme longevity of bats, together with the possibility that they might develop persistent infections with certain viruses, may help maintain the viruses and transmit them to other vertebrates.

Echolocation: Insectivorous bats echolocate, and thus in the process could cause aerosolisation of rabies virus particles in the saliva that can enhance transmission to other animals including man (Kuzmin *et al.*, 2011). Echolocation signals are produced by the larynx, are powered by the muscles of the abdominal wall of bats, and are emitted through

the mouth or nostrils. Production of such loud sounds also could generate droplets or small-particle aerosols of oropharyngeal fluids, mucus, or saliva, enabling transmission of viruses between individuals in close proximity. The hypothesis that rabies virus could be expelled from the nostrils of echolocating bats was supported by the isolation of rabies virus from mucus obtained from naturally infected Mexican free-tailed bats (Constantine *et al.*, 1972).

Long lasting immunity of bats after infection with lyssavirus as reported in the study of temporal dynamics of EBLV-1 in *M. myotis* by Amengual *et al.* (2007)provide information on the protection of bats with a longer immunity against re-infection. An experimental study on effect of repeated infection of bats with RABV also concluded that Long-term repeated infection of bats in a colony induce in them an immunological memory and reduce the susceptibility of bats to RABV infection (Turmelle *et al.*, 2010).

Bats have different Interferon

Recent studies have examined innate, antiviral, and interferon genes from several species of bats, and suggest that certain alleles may be associated with increased parasite burden (Wibbelt *et al.*, 2010). Interferon alpha and beta are homologous to other mammalian interferons, but there is low homology of these interferons specifically between bats and humans, which could indicate different antiviral activity between the two and contribute to the high pathogenicity of bat agents in humans (Omatsu *et al.*, 2008). In addition, recent sequencing of genome fragments to infer genes within the interferon alpha family in both *Pteropus* and *Myotis* bats has revealed that both have up to 24 IFNW genes, while humans, mice, and pigs have only one (Kepler *et al.*, 2010). The enormous size of this gene family within bats compared to other mammals suggests that it may still be involved

in host immune defense, even though its function may have been lost in other vertebrates. He *et al*. (2010) in a separate research indicated that the bat interferon alpha gene family is positively selected through evolution with its agents. This evolutionary adaptation or co-evolution of bats and their pathogens enable pathogens to block immune recognition although host immune systems respond and generate an effective immunity to these pathogens.

Traits of bat pathogens that enable them evade bat immune system surveillance:

RABV has selected multiple strategies (collectively known as neuroinvasiveness) to achieve its virus cycle into the host nervous system from the site of entry up to the salivary glands, where it will be excreted to infect a new host. After entry into the nervous system, the virus promotes neuronal survival and avoids premature cell death of the neurons it infects by triggering survival pathways. RABV seems to minimize the inflammation in the nervous tissues it infects and thus reduce other inflammatory response. These survival signaling activities of the G protein of infected neurons and other under listed traits of lyssaviruses enable them to evade immune system of their host.

Ability of Lyssaviruses to Evade Host Immune Response.

Pathogenic RABV may limit its replication rate and produce fewer infectious particles to completely evade, or minimally activate, the peripheral host response. Single exposures do not always confer protection against successive infections, leading to perpetuation in bat populations, but repeated exposure has been shown to provide long-term immunity (up to a year) and reduced susceptibility (Turmelle *et al.*, 2010).

The essential pathobiology of bat agents contributes inherently to their persistence or perpetuation in reservoir individuals, colonies, and populations. The ability to reach the CNS from a peripheral infection site, referred to as ‘‘neuroinvasiveness,’’ largely determines the virulence of the virus. A key strategy of the virus must therefore be to avoid direct apoptosis, innate and adaptive immunity, and inflammation to conserve the integrity of the neuronal network and to gain time to reach the CNS (Dietzschold *et al.*, 1983).

The rabies virus glycoprotein (G) is the target of virus-neutralizing antibodies (VNA) and is generally expressed at relatively low levels in cells infected with wild-type rabies viruses (Chopy *et al.*, 2011). The low level of G expression likely enables the virus to escape immune recognition during an infection. RABV seems to minimize the inflammation in the nervous tissues it infects. The more pathogenic the virus strain is, the less acute is the inflammatory response.

CHAPTER THREE: GENERAL MATERIALS AND METHODS

3.1 Dynamics of Lagos Bat Virus in Wild and Captive Straw-colored Fruit Bats (*Eidolon helvum*)

3.1.1 Study Sites

This study was undertaken at three sites in Ghana: Tano Sacred Grove in the Techiman North District of the Brong Ahafo Region, 37-Military hospital in Accra and the Achimota Forest where a captive colony of bats was kept (Fig. 3.1).

The Republic of Ghana is on the southern coast of West Africa and lies along the Gulf of Guinea. Ghana is located between latitude 4^{o} 44'N and 11^{o} 11'N; and longitude 3^{o} 11' W and 1^{o} 11^{o}E. It shares borders with Togo (East), Ivory Coast (West), and Burkina Faso (North). Ghana has a population of 24.66 million (2010 national population and house census) (Ministry Of Food and Agriculture [MOFA], 2012).

The country has five main agro-ecological zones defined on the basis of climate, reflected by the natural vegetation and influenced by the soils. These are: Rain Forest, Deciduous Forest, Transitional Zone, Coastal Savanna and Northern Savanna (Guinea and Sudan Savanna). The highest point in Ghana is only 885 m above sea level along the eastern border, however, the world's largest artificial lake, Lake Volta, separates much of eastern Ghana from the rest.

The tropical eastern coastal belt is warm and comparatively dry, the south west corner is hot and humid, and the north is hot and dry. Annual average temperatures range from 26.1^{0}C in places near the coast to 28.9^{0}C in the extreme north.

Two colonial populations (250,000–1,000,000 bats each) of *E. helvum* in Ghana were sampled: one in Accra and the other in Tano Sacred Grove (approx.400km North). The

Accra population is urban, roosting in trees over a city center hospital (the 37-Military Hospital). The Tano sacred grove population is rural, roosting in a protected forest area. The captive *E. helvum* bats are kept and bred in the Accra Zoo, located in the Achimota forest, Accra. The 37-Military Hospital (05°35.192'N, 000°11.053'W) is located in the capital city of Accra in the Greater Accra region of Ghana. The roost at the 37 Military Hospital is the second largest and is located in a densely populated area of Accra. The hospital is the second most attended hospital in Accra. There is also residential accommodation for military personnel within the area. The 37-military Hospital has high human traffic resulting in daily interaction of bats with the hospital workers, patients and also military personnel living within the hospital area and along the main city highway.

Achimota forest (05°37.569'N, 000°12.086'W) (Fig. 3.1) is the only remaining urban park in Accra. The forest has many wild birds and reptiles, some small antelopes (Maxwell Duikers, *Philantomba maxwellii*) and some primates (White-nosed monkeys, *Cercopithecus petaurista* Mona monkeys, *Cercopithecus mona* and Green monkeys, *Chlorocebus sabaeus*).

The third site, Tano sacred grove is located in Tanoboase (N07° 39.942' W001o 51.449'). The sacred grove, with an area of 300 acres, is the site of one of the earliest Bono settlements. The grove is also the site of the annual apoo festival, which is a time of spiritual cleansing (held in April-May). The sacred grove was developed for ecotourism in 1996 under a community based ecotourism project. Tanoboase is a small farming community located 15km from Techiman along the Techiman – Kintampo road in the Brong Ahafo Region of Ghana.

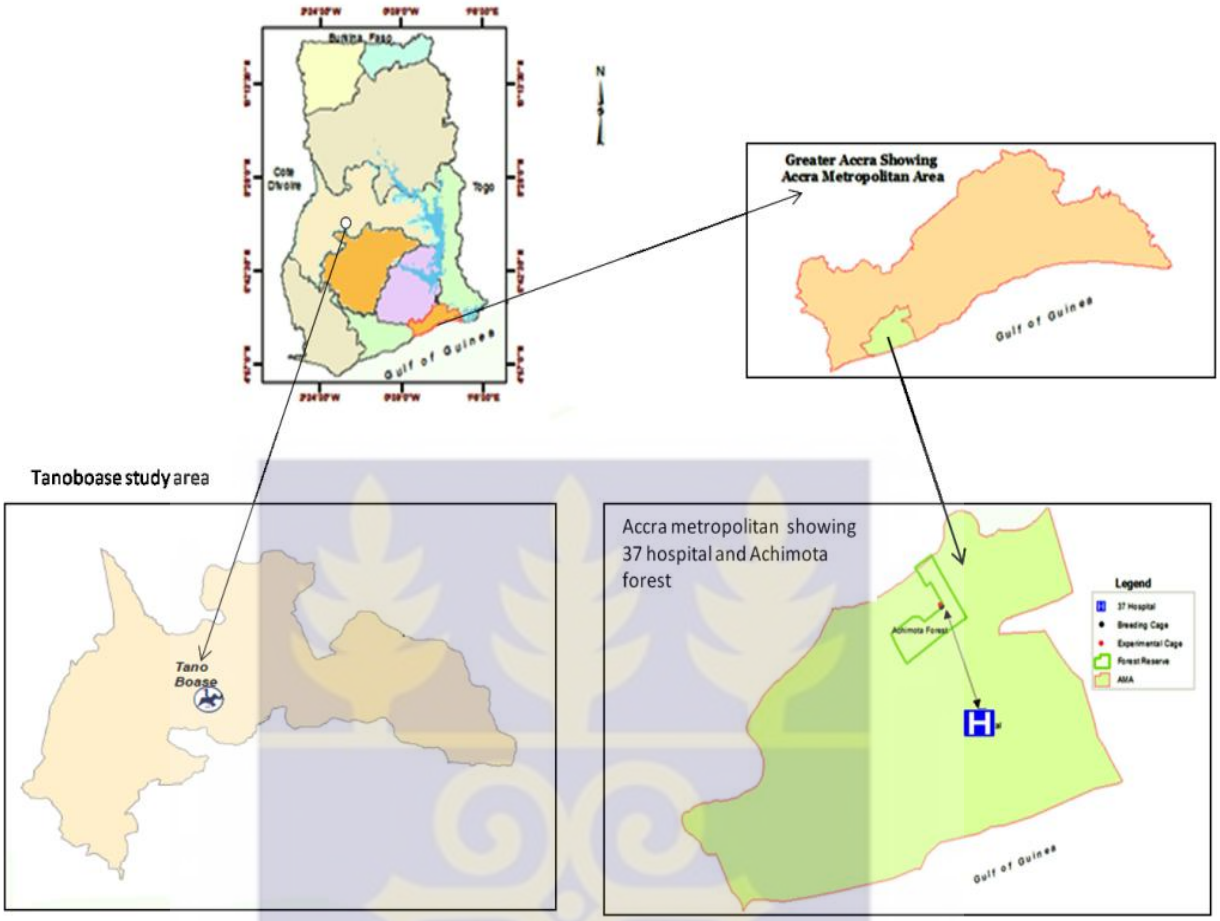

Fig 3.1: **Map showing location of study roosts in Tanoboase, 37 Military hospital and of the captive bat colony in Achimota forest.**

It is under the Techiman North District. Tanoboase has three (3) settler communities under its traditional area including Baafi, Tanokrom and Forest. Together, these communities have a total estimated population of Five thousand five hundred people of varying ethnic origin. The sacred grove is located within a semi-deciduous forest and encloses a cluster of striking rock formations. The major activity of the town is small holder agriculture, employing traditional system of farming. Main crops cultivated include yam, maize, plantain, cashew, tomato and vegetables. Livestock including sheep, goats, pigs and chickens, but not cattle, are kept semi-free range. Wildlife within the area is observed

mainly in the sacred grove and includes birds, monkeys, small mammals (Duikers, *Cephalophinae* and bushbucks, *Tragelaphus scriptus*), and bats (Chiroptera). The sacred grove hosts the largest colony of straw-colored fruit bats (*Eidolon helvum*) in Ghana, with the highest estimated population of over 2 million bats recorded. The rural livelihood (hunting, farming, fruit harvesting, etc) results in close interactions between domestic livestock, humans and bats in Tanoboase and adjoining communities.

The 37-hospital and Tano sacred grove sites were therefore considered to be ideal sites for research into zoonotic disease at the interface between the straw-colored fruit bats, domestic animals, and humans in urban and rural settings.

3.1.2 Study Animal (Eidolon helvum)

Eidolon helvum, the straw-colored fruit bat is classified in the order Chiroptera, suborder Megachiroptera and family Pteropodidae. *Eidolon* is a polytypic genus with two species: *E. helvum* in mainland Africa and *E. dupreanum* on the Island of Madagascar.

E. helvum is the second largest of the West African fruit bats, exceeded in size only by the hammer-headed fruit bat, *Hypsignathus monstrosus* (Okon, 1974).

It weighs approximately 250–310 grams and has a wingspan of 80 cm (Taylor, 2006). The straw-colored fruit bat is widely distributed throughout sub-Saharan Africa (DeFrees & Wilson, 1988), however, the tropical forests of Africa are presumed to represent its primary range, with the species migrating annually, perhaps to savannah regions in a north-south axis (Richter & Cumming, 2008; Thomas, 1983). It is thought that food availability may be the driver of this migratory pattern (Richter and Cumming, 2006). It is a gregarious, socially roosting species. While this species is abundant and forms vast

colonies in those areas where there is a year-long abundance of fruit, in less favorable areas it forms smaller colonies or occurs only as a visitor during seasonal migrations.

Eidolon helvum is commonly acknowledged as a migratory species, but the details of its movements are unknown. West African colonies are reported to undertake an annual seasonal migration of some 931 miles (1,500 km). Although the timing and direction of migrations generally reflect shifting patterns of food availability, the predominant driving forces, routes and distances of their migrations are largely unknown (Hayman *et al.*, 2008). The Accra colony migrates annually from Accra, leaving around March and returning around September.

Different studies have found different age structures for *E. helvum* (Nokak and Paradisco, 1983; Hayman *et al.*, 2012). They can live closer to twenty years in captivity. The record lifespan was recorded at 21 years and 10 months (Nokak and Paradisco, 1983) Hayman *et al.* (2012) also described longevity data using tooth rings that show that these bats have a mean life-span of ~5 years, with individuals surviving up to 14 years in the wild.

It is generally observed that straw-colored fruit bats have the tendency to roost on trees near villages and towns as well as the outskirts of towns (Ayensu, 1974). Roosts are usually situated in groves and thickets close to human habitation, local shrines and marshy areas, which offer sanctuary to the bats. The straw-colored fruit bat (*Eidolon helvum*) has been observed to roost in a number of African megacities such as Accra (Fig. 3.2), Abidjan and Dar es Salaam, as well as in rural areas and in some protected areas such as Kasanka National Park (Sørensen & Halberg, 2001) in Zambia and Tano Sacred Grove in Ghana. Roosting clusters are located 6 to 20 m above ground on sturdy branches.

Fig. 3.2A *Eidolon helvum* roost on trees within 37-Military Hospital compound.

Fig. 3.2B Cluster of *E. helvum* colony. (Curtesy Hayman)

Fig 3.2: *Eidolon helvum* roost at 37-Military Hospital in Accra. E. helvum roost in large colony and urban centres as shown here of the 37-military hospital in Accra, Ghana.

Within colonies they form tight clusters of up to 100 animals (Fig 3.2B). At temporal roosting sites at feeding areas, smaller colonies and even single bats may be seen feeding or roosting. There are no reported detailed studies of social structure in colonies. It is however reported that there is no significant sex bias observed in the roost structure of *E. helvum* (Ansell, 1986). Bats within roosts relocate regularly and thus continually come into contact with new bats. Nursing mothers and their young are the most active members of *E. helvum* colonies throughout the day. Regular activities include grooming, nursing, flight practice, and weaning.

Within colonies and on feeding grounds, bats interact with each other in many ways. They communicate to each other through volcalisation. Bats that belong to a group may roost and forage together. Individuals that are not recognised may be threatened with volcalisation, swiped by the claws of a thumb or pursued attacked and bitten. Fighting is common in colonies and often consists of wing battering. The author has observed a baby

bat that fell off the mother attacked by other bats. Many bat species defend roost sites or feeding territories (Kingdon *et al*., 2013).

E. helvum is important in the local economy as its roosting activities cause extensive mechanical damage to some economically important trees like oil palm (*Eleasis guineensis*) and Iroko (*Chlorophora excelsa*). Consumption of fruits including mango, pawpaw, guava, plantain and banana cause yield losses and qualitative devaluation of many fruits. Feces from thousands of bats cause much filth and stench in the environment of some roosts and beneath feeding trees while the clamour of bats in roosts or while feeding near human habitation can be very exasperating and pose a health threat to the people (Funmilayo, 1979).

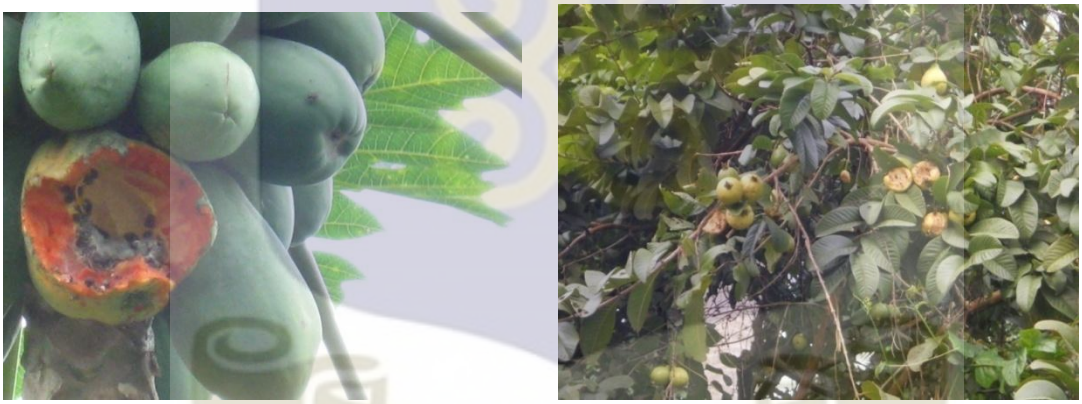

Fig. 3.3A Over ripe pawpaw (*Asimina triloba)* partially eaten by *E. helvum*.

Fig. 3.3B Guava fruits eaten by *E. helvum.*

Fig 3.3: Bats Feeding activities. Bats are a menace as they feed on fruit plantations. This could post a public health risk if such probably contaminated fruit remains are freshly eaten by man.

Fig 3.4: *E. Helvum* roasted meat for sale in the street of Kumasi, Ghana. (Curtesy: Abedi-Lartey)

3.1.3 Bat Trapping (Mist Netting)

Sampling at the sites was carried out every three months (2012 to 2014). Samples were collected in late January and early February, and late March and early April corresponding to early and late pregnancy periods. Samples from July and November correspond to periods when the majority of the colony had migrated and returned from migration respectively. A minimum number of 60 bats (been estimated sample size for an infinite population) were trapped and sampled each time (sampling section) from each location. Bats were trapped by a team of licensed trappers, using mist nets (Fig. 3.5).

Fig 3.5: Field Capture of bats using mist nets. Bats are trapped in a Mist net for sampling

Mist nets can be used to capture large or small bats in open spaces near roost or feeding sites. The nets are typically attached to two poles either in a static assembly, where the net remains in a fixed position, or on a rope and pulley system, where the net can be elevated to capture the bats, and lowered to remove them. Regardless of which technique is used, it is important that field personnel monitor the nets or traps regularly to prevent bat injury or death, which can occur as a result of extensive struggling, entanglement, predation or exposure to harsh environmental variables (heat, sunlight, water). It is recommended that mist nets are actively monitored throughout the trapping session and that bats are extracted from the net as soon as possible after capture.

The sites for setting nets were identified during the day. Potential bat travel corridors typically were the most effective places to net. The nets were usually placed approximately perpendicular across the corridor. It was best that nets filled the corridor

and were raised to the level of the tree top as *Eidolon helvum* flies higher than other fruit bats. The actual height depended on the height of the tree used by the bats. Two nets were sometimes joined to cover a bigger space. A cord pulley system allowed nets to be lowered when bats entered. Nets were erected and lowered (left closed) during the day. The nets were opened after the bats had left to feed in the night. Bats were caught between the hours of 0300 and 0700 when the bats returned to the roost from foraging. Nets were continually monitored so that bats were removed immediately on capture to avoid unnecessary distress or injury.

Once removed, the captured bats were placed in individual cotton holding bags or a larger bat cage. The bags were hung in a prepared site with adequate shade and were sampled either immediately or early morning. In June 2013 we caught two (2) wild nursing *E. helvum* mother bats (ID: 900 and ID: 905) carrying the pups (ID: 901 and 904 respectively) (form part of the wild caught bats) at the 37-Military hospital during field sampling section. We identified the mothers and pups and sera were collected from the mothers and pups for serological testing.

Trapping of Captive/Cage Bats

Bats in the captive colony were captured by partitioning their large flight cage using a tarpaulin. The bats were then captured using a loop mist net on a long pole**.** Bats were kept either in holding bags or in a smaller cage prior to sampling.

3.1.4 Bat Sampling

A field camp for sampling was prepared at a shaded area at the sampling site. Each bat to be sampled was weighed together in the bag using a spring balance. It was carefully taken out of the bag by a handler, using leather gloves. The bag was then weighed empty, and

the weight of the bat was determined as the difference. Wing span length, forearm length and other measurements were taken using calipers. Each bat was assessed for health, sex, age, and reproductive status. The age of each bat was determined and classified as neonate (suckling), junvenile (male or female), sub-adult or sexually immature (male or female) and adult or sexually mature (male or female). Evidence of suckling young by females (and therefore categorisation as sexually mature (SM)) was determined from nipple shape and size. Males were determined to be SM by testicular development. Pregnancy was determined by abdominal palpation, allowing estimation of pregnancy rates for the population.

Captive bats were marked individually with a microchip (Travan Electronic Identification System, Germany), inserted subcutaneously under the scruff of the neck and number identified and recorded using a microchip reader. This information for each bat was recorded in a data sheet (Table 3.1). Personal Protective Equipment (PPE) including a coat, latex gloves, face mask, and protective goggles were worn before sampling bats. All trapping and sampling staff had been provided with the full prophylactic vaccination for rabies and tetanus, prior to the commencement of the study. Booster vaccinations were taken as was recommended by the physician.

Throat and faecal swab samples were taken from the restrained animal using sterile cotton tipped swabs **(**Fig. 3.6A**)**. Throat swabs and faecal swabs were placed separately in an Eppendorf tube with 1ml RNALater® Ambion, USA. Each sample was labeled with the bat identification number and this was recorded on the data sheet. Samples were stored at 4℃ in the field and transferred to a -70^{o}C freezer until use. These samples will be analysed at a later date.

Blood Collection

The propatagial vein was occluded with a cloth peg and the bleeding site was disinfected using alcohol before blood was withdrawn from the vein using a 28-gauge needle and a 1ml syringe **(**Fig. 3.6B**)**. The quantity of blood taken was 0.2-1.0 mL (approximately 0.2-0.5% of the bat body mass). The blood sample was transferred to a citrate tube to prevent clotting and to allow for collection of a buffy coat.

All samples were placed in a cooled box with ice packs. They were transferred to the National Veterinary Laboratory in Accra for processing. In Tanoboase, blood was processed in the field using field centrifuge or at the Techiman Veterinary Investigation farm.

In both cases, samples were kept for about 2-3 hours to allow the blood to clot before processing. Serum was separated from the blood clot by centrifugation at 6000 rpm for 15 min, and collected in a separate labeled tube. All serum samples were heat-inactivated at 56 ^{0}C for 30 min prior to archival storage.

Fig. 3.6B. Blood Collection from the propatagial vein Fig.3.6A. Throat swabbing from a bat.

Fig 3.6: Bat Sample collection in a field camp. Blood, throat swabs and faecal swabs are taken from restrained bats and stored in cool box for further investigation

3.1.5 Necropsy of dead Bats:

Injured bats and sick bats (during experiment) that were judged to be suffering were euthanised and submitted for full postmortem. Such bats for pathogen discovery were anaesthetised with an excess of ketamine and medetomidine and euthanised by exsanguinations via cardiac puncture, prior to necropsy. Dead bats were collected during field survey and also occasionally submitted by the general public. Carcasses of dead bats were immediately stored in a freezer at -20 ^{o}C until necropsy. Oropharyngeal, urogenital, and faecal swabs were taken. Full necropsy was performed on dead bats and tissues taken and stored for further viral diagnosis. Tissues collected in 3 tubes labeled appropriately (Fig. 3.7) plain tube, tube with RNA later, and tube containing neutral-buffered 10% formalin. Tissues collected included brain, salivary gland, heart, lung, lymph nodes, kidney, liver, spleen and urogenital tract. Canine teeth were removed for aging by cementum rings (Hayman *et al*., 2008; Divljan *et al*., 2006). Tissues in plain tubes were immediately stored in a –70°C freezer All RNALater® (Ambion) samples were frozen at -70°C following overnight storage to allow fixation. Formalin samples were stored at room temperature (RT).

3.7A Bat being postmortemed 3.7B Tissues samples in tubes for storage

Fig 3.7: Dead bat necropsy. Dead bats are postmortemed (3.7A) and predetermined tissues sampled into tubes, for further laboratory investigation

3.2. Straw-Colored Fruit bat Infection Study

To assess dynamics including incubation period, clinical signs, routes of transmission and pathogenesis of LBV in *E. helvum*, captive bats were infected with LBV. Two (2) experimental studies were conducted (between 2012 – 2014) to study the dynamics of LBV in the straw -colored fruit bats in a specially constructed facility in the Achimota Forest in Accra, Ghana.

Breeding and Care of E. helvum

The captive bats were kept in a 27.5m diameter, 3.5m height flying cage (Fig. 3.8) that is furnished with branches and brushes. The cage entrance had a double door gate and the cage was double fenced with a solid tin roof. The outer mesh and tin roof provided a second separating fence that ensured there was no contact of bats with external free ranging wildlife (including wild bats). An aluminium foil fence on the ground of the cage excluded reptiles and rodents from entering the cage. Mixed sex group of seventy-eight bats were caught from the 37-Military Hospital and kept in the breeding cage in between July, 2009 and January 2010, after which the enclosure was maintained as a closed colony. Good husbandry management and veterinary care was provided to ensure breeding of the bats. The bats bred successfully with offspring added to the enclosure once annually. The stock provided captive bats for various studies.

The diet for the captive bats comprised a mixture of fruit, vegetables, and supplements and included banana, pawpaw, mango and leafy greens. The animals were provided with fresh clean water at all times. Food supplements included well balanced vitamin supplement, calcium supplement and other vitamin –mineral supplements. In the captive mixed breeding cage, food was served on manufactured wooden platforms that could be raised from the ground and water was served in suspended bowls.

3.8A Captive bat cage at Achimota forest

3.8B. Bats in captive cage.

Fig 3.8: **Captive bat breeding cage. It is double fenced. The cage had internal natural vegetation to ensure the welfare of bats and has external aluminium foil to exclude rodents and reptiles from entering the cage.**

Experimental Facility

The experimental facility consisted of triple-enclosed cages within each other to provide total security for the infection study. The main house (middle fence) consisted of steel and mesh fence. It had an aluminum roof. The outer external fence is made of smaller size mesh. This outer layer excluded the inner cages from contact with the external environment including birds and small mammals. Two double door entrances enforce the security within the experimental facility. The inner cages consist of 20 individual cages (80cm x 80 x 80cm) hanging from the roof and made of wire mesh. These were used for housing bats for the study. Two doors on each individual cage enabled easy capture of bats in the individual cage. The doors to individual cages were secured with locks. Drinkers and feeders were used to serve individual bats. Tin baffles hung between the individual cages prevented droplet spread of pathogens between bats.

There was a bench station inside the cage for sampling bats. The station has a concrete floor that is easily disinfected and provided with a tap for running water. Fig 3.9 shows the experimental cage with the work station and the individual cages hanging inside.

Fig 3.9: **Experimental bat cage at the Achimota Forest, Accra.**

3.2.1 Intracranial inoculation of *E. helvum* with Lagos Bat Virus

Study design

Bats in the Breeding cages were tested for LBV antibody by Florescent Antibody Neutralisation test (FAVN). Sixteen bats that had been bred in captivity and which were known to have always been seronegative for Lagos bat virus (LBV) were transferred from the breeding colony at Achimota forest to the experimental cage. They were kept in the individual cages for a month and randomly selected for the i.c inoculation experiment. Details of the bats ID, Cage number (indicated on padlock) and morphometrics of each bat are provided in Table 3.1.

Table 3.1: **Demography data on bats selected for IC inoculation**

Date Inoculated	Cage No.	Chip ID	Sex	Age	Bat Weight	Transponder ID	Inoculated Virus Isolate
12/10/2013	1	1311	M	J	185	IC1311BIC01	(CONTROL)
12/10/2013	2	721	F	A	190	IC0721BIC02	(CONTROL)
12/10/2013	3	9113	M	J	155	IC9113BIC03	(CONTROL)
12/10/2013	5	6179	F	J	175	IC6179BIC05	Senegal(RV41)
12/10/2013	6	1191	M	SA	180	IC1191BIC06	Senegal(RV41)
13/10/2013	7	1711	M	J	180	IC1711BIC07	Nigeria (RV1)
13/10/2013	8	7193	F	A	225	IC7193BIC08	Nigeria (RV1)
13/10/2013	9	5368	M	J	220	IC5368BIC09	Nigeria (RV1)
13/10/2013	10	4146	F	A	175	IC4146BIC10	Ghana (FL1)
13/10/2013	11	8308	F	A	160	IC8308BIC11	Ghana (FL1)
13/10/2013	12	4741	M	A	190	IC4741BIC12	Ghana (FL1)
14/10/2013	13	4099	F	A	230	IC4099BIC13	RV41

Key: A – Adult, **SA** – Sub adult, **J** - Juvenile

Study Viruses

The three LBV isolates were used for the study were:

- Lineage B (RV1 10e5 TCID50/ml Lagos Nigeria 1956). The virus was isolated in Lagos Island, Nigeria in 1956 (Boulger & Porterfield, 1958). It has been subjected to an indefinite number of passages in mice and cell culture in several laboratories

- Lineage A (RV41, Senegal 1985): 10e7.25 TCID50/ml). It was isolated from *E. helvum* in Dakar, Senegal in 1985 (Markotter *et al.*, 2006). The virus has been subjected an unknown number of passages.

- Lineage A (FLI from Germany, ex Kumasi, Ghana). The virus was isolated from *E. helvum* in Kumasi in Ghana in 2013 and had undergone a small number (4) of passages.

For each of the three lineages, three bats were inoculated; three bats were inoculated also with Minimum Essential Medium (a virus culture medium (MEM)), Gibco®, U.K.). This last group served as a control.

Method of IC inoculation

The bats infected with each of the three virus isolates and the three negative control bats (born in captivity and serologically naïve) were housed in individual cage for this study. The bats were anaesthesised using a combination of Ketamine (Ketamine hydrochloride 115.36mg/ml, Fort dodge animal Health ltd, U.K.) (5mg/Kg body weight) and Meditomidine (Medetomidine hydrochloride, Laboratories SYVA S. A., Spain (0.05mg/kg body weight) and immobilized in a stereotactic frame. Skin was cut on the center of calvaria with a sterile scalpel, and a dental drill (0.3 mm) was used to make a hole in the skull bone, 2-3 mm to the right from the central line. Then the meninges (dura mater and arachnoid) were perforated using a 28-gauge syringe needle. The bats were inoculated intracranially (IC) each with the $10^{3.5}$ $TCID_{50}$ of each LBV isolate (30 ul volume) using Hamilton syringe with a 36-gauge needle (Fig. 3.10). The inoculum was injected 5 mm into the brain to ensure that it would reach sub-cortical brain tissue, at a speed of 5 ul/min to avoid causing brain trauma. The needle was removed slowly after the injection (during

1 minute) to avoid a reflux of the inoculum. After removal of the needle, the skin was stitched and the surgery area was treated with antiseptics.

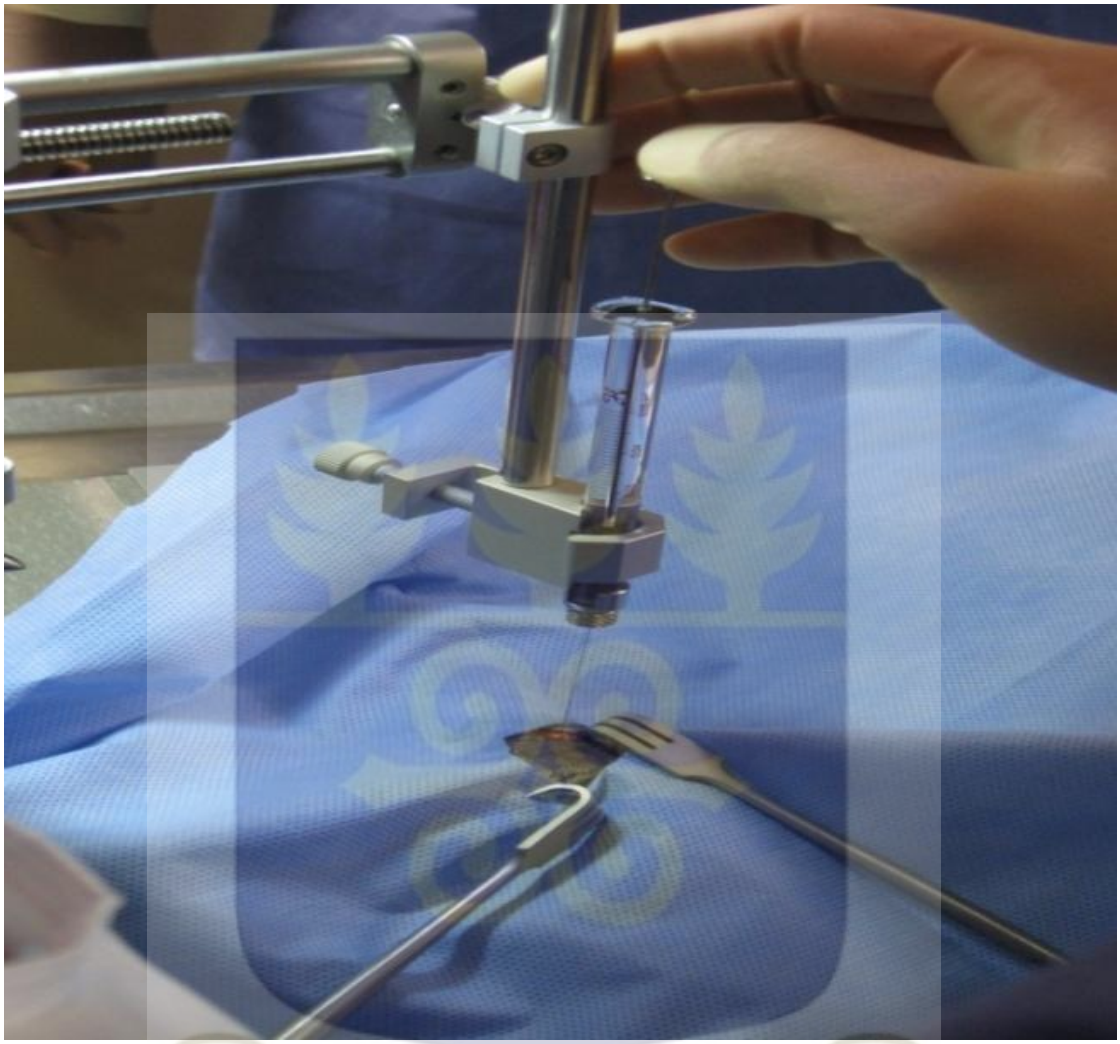

Fig 3.10: **Intracerebral Inoculation of bat. Anaesthetised bats were mounted on a stereotactic frame and inoculated intracerebrally with virus using Hamilton syringe.**

Each bat was implanted with a transponder chip (Bio Medic Data system Inc.) for identification and also to assist in recording daily body temperature. A transponder reader was used to identify bats and to record daily body temperature of bats as in Fig. 3.11. The anaesthesia was reversed using Atipamezole, 0.1 mg/kg (Veterinary Essentials, UK).

Fig 3.11: **Monitoring temperature of infected bats with a transponder reader in individual bat cage. Infected bats were housed in individual cages and monitored. Temperature of bats were recorded using a transponder reader**

After the surgery, all bats were put on a pain relieving medication, Meloxicam 2mg/ml (Boehringer Ingelheim Vet medica GmbH, Germany) and antibiotics, Baytril 2.5% (Bayer plc, U.K.) for 3 days.

Bats were returned to individual cages and observed closely for 2 hours until full recovery. Detail of the anaesthesia and inoculation protocol is provided in Appendix 1. Demographic data on bats and isolates used are detailed in Table 3.1.

Observation/sampling protocol

- Bats were observed twice daily in the morning at 07.00 and evening at 16.00GMT.
- Body temperature was registered daily in the morning with a transponder reader (Table 3.11).
- Blood samples were taken on day 0 and at euthanasia/death.
- Blood, oral swab, urine swab and faecal swab sample were taken at a frequency of twice a week.
- Daily saliva swabs were taken after onset of clinical signs, sedating if necessary.
- Bats were euthanised by using pentobarbitone euthanizing drug or by using ketamine/meditomidine and cardia exsanguination, if adjudged appropriate on welfare grounds, following daily observation by suitably qualified veterinary personnel.
- Disease was allowed to run its course if welfare permited as above. This was essential to establish whether recovery was possible as this is one of the major outstanding scientific questions in the field of lyssavirus ecology in bats.
- Control bats were euthanized after the last infected bat succumbed to the disease.

Found-dead or euthanized bats were transported from the Achimota Forest to the Animal experimentation laboratory of the Noguchi Memorial Institute for Medical Research, University of Ghana, on frozen ice packs for postmortem sampling. Post mortem examination and pre-determined tissues were collected for PCR, virus culture and

serologic tests. Cross-contamination was avoided by using a clean set of instruments for the removal of each individual tissue from each fresh carcass.

3.2.2 Peripheral Inoculation of E. helvum with Lagos bat virus

Method

The objective of this study was to determine if the infectious dose of the virus is related to the incubation period of the disease, and what is the pathobiology of LBV infection in *E. helvum* following the more natural (as compared to intracranial) inoculation route. This experiment used only one LBV isolate FL1 (ex Kumasi, Ghana) at different doses.

For this experiment captive-bred bats (seronegative for LBV) were transferred from the common breeding cage to experimental cages and housed individually. Five groups of 4 bats each were used for each virus dose:

Group 1: $10^{4.1}$ $TCID_{50}$/0.03 mL

Group 2: $10^{3.1}$ $TCID_{50}$/0.03 mL;

Group 3: $10^{2.1}$ $TCID_{50}$/0.03 mL;

Group 4: $10^{1.1}$ $TCID_{50}$/0.03 mL;

Group 5: $10^{0.1}$ $TCID_{50}$/0.03 mL.

Viral dilutions were prepared in MEM immediately before the inoculation. The inoculation was performed in the left masseter muscle. Bats were implanted with transponders to allow individual identification and temperature reading.

Observation /sampling protocol

- Bats were observed twice daily in the morning at 07.00 and evening at 16.00GMT.
- Body temperature was registered daily (morning) with a transponder reader (**Table 5**).
- Blood, oral swab, urine swab and faecal swab samples were taken per the following schedule:

    - Day 0 – 3weeks - twice a week
    - 3 – 6 weeks – Once weekly
    - After 6weeks - Fortnightly
    - Daily saliva swabs were taken after onset of clinical signs, sedating if necessary.
    - Dead or euthanised animal were examined post-mortem and predetermined tissues sampled for laboratory investigation.
    - Bats were euthanised by using ketamine / meditomidine prior to exsanguination, if adjudged appropriate on welfare grounds, following daily observation by suitably qualified veterinary personnel.
    - Experiment duration was 90 days after the last death due to rabies

Table 3.2: **Demography data on bats selected for peripheral inoculation**

Cage number	Microchip	Transponder	Sex	Age	Bat Weight	Virus titre
1	3072	EXP2EH001	Male	SA	220	Neat virus
2	3158	EXP2EH002	Male	SA	195	Neat virus
3	8615	EXP2EH003	Male	A	196	Neat virus
4	3647	EXP2EH004	Male	A	227	Neat virus
5	1492	EXP2EH005	Male	A	229	-1
6	8009	EXP2EH006	Male	SA	267	-1
7	9186	EXP2EH007	Male	SA	201	-1
8	3428	EXP2EH008	Male	SA	201	-1
9	6929	EXP2EH009	Male	A	215	-2
10	2183	EXP2EH010	Male	A	160	-2
11	4968	EXP2EH011	Male	SA	202	-2
12	7531	EXP2EH012	Male	SA	185	-2
13	6544	EXP2EH013	Male	A	200	-3
14	6826	EXP2EH014	Male	SA	235	-3
15	3767	EXP2EH015	Male	A	248	-3
16	7817	EXP2EH016	Male	A	220	-3
17	6349	EXP2EH017	Male	SA	165	-4
18	7114	EXP2EH018	Male	A	185	-4
19	7554	EXP2EH019	Male	A	210	-4
20	982	EXP2EH020	Male	A	210	-4

Key: A – Adult, **SA** – Sub adult, **J** – Juvenile

3.3 Laboratory Investigation

The study samples were potentially infectious tissues, therefore the investigations, including neutralization assays, RNA extractions and tissue cultures, were performed under category III containment conditions in a dedicated Advisory Committee on Dangerous Pathogens (ACDP) 3/Specified Animal Pathogens Order (SAPO) IV high-security unit at the World Health Organisation (WHO) and World Organisation for Animal Health (OIE) rabies reference laboratory at the AHVLA Weybridge, UK.

3.3.1 Sample Processing

Blood from field sampling was processed at the National Veterinary laboratory in Accra. Blood from the infection study was processed at the animal experimentation laboratory at Noguchi Memorial Institute for Medical Research (NMIMR), University of Ghana. Samples from wild bats were stored in -70 freezers at the Veterinary Laboratory, Accra. Samples from the infection study were stored in -80 freezers at a P3 Laboratory in the virology department of the NMIMR. They were transported in Bio-freeze bottles for viral and serologic investigations to the World Reference Laboratory for lyssaviruses in the UK.

Swabs:

Oropharyngeal and faecal swabs were taken and placed immediately in RNALater® (Ambion). They were kept in a fridge in the field and transferred into a freezer at -70°C within 2 days. Faecal and oral swabs from the infection study were kept in RNAlater and MEM and kept in -80 freezer at Nuguchi. These samples are kept frozen and woud be analysed later.

Blood:

Whole blood was centrifuged (Eppendorf – Netherler-Hinz, GmbH, Germany) (6000g for 15minutes) and plasma and buffy coat separated by micropipette. The Buffy coat was stored at -70°C. The plasma was heat-treated at 56°C for 30 minutes to heat inactivate pathogens and to remove complement before freezing at -70°C.

3.3.2 Rabies Tissue Culture Isolation Test (RTCIT)

The rabies tissue culture isolation test (RTCIT) is an OIE prescribed confirmatory method for rabies diagnosis. Lyssaviruses can be isolated by rabies tissue culture isolation (RTCIT). Baby hamster kidney (BHK) cells were trypsinised using Antibiotic Trypsin Versine (ATV), re-suspended in 40-60ml of fresh medium to the required concentration of ~2 x 10^5 cells/ml. 200µl of cells were placed into 96 well micro titre tissue culture plate and incubated at 37°C in 5% CO2 for 30 minutes to allow the cells to attach. The test sample was clarified by centrifugation for 5 minutes at 500*g* and 20µl added to the media. A challenge virus standard (CVS) positive control was included and the plate incubated at 37°C in 5% CO2 overnight. The media was changed after 24 hours and incubated for a further 3 days. Plates were fixed in 80% acetone solution, air-dried and 50µl of 1:40 FITC anti-rabies monoclonal globulin added, before incubation at 37°C in 5% CO2 for 30 minutes. Finally, plates were rinsed in 0.1M buffer solution (PBS) and read with a 488nm fluorescent microscope. The result is qualitative with any apple green fluorescence deemed a positive result.

3.3.3 The Fluorescent Antibody Test (FAT)

In rabies virus infected cells the specific inclusions consist of nucleocaspid aggregates. Murine antibodies, conjugated to Flourescein Isothiocynate (FITC), will specifically stain

these inclusions by direct immunofluorescence. This assay involves testing using an FITC conjugated virus specific antibody (Dean *et al*., 1996).

Impression smears of brain sample (hippocampus, cerebellum or medulla) material were made by placing tissues onto filter card, and slides are then inverted onto the tissue. The slides were then blotted to remove excess material, and allowed to dry. A positive mouse brain was used as an internal control. The microscope slides were placed in 100% acetone (-20^{o}C) (fixative) for 20 minutes. The slides were then removed and allowed to dry. A drop of working strength conjugate (Centocor 1/20) was added to each of the samples under test. The slides were then incubated at 37^{o}C in a humidity chamber for 30 minutes. Following incubation, the slides were removed and washed in 0.1M Buffer solution (PBS) 7.2 twice for 2 minutes with fresh PBS. The slides were rinsed briefly with distilled water and allowed to air dry. The slides were read using the fluorescent microscope, the positive control slide first. Specific fluorescence is denoted by bright 'apple' green fluorescence generally in the peri-nuclear area of cells. A second person must check and confirm the results.

3.3.4 Serology (Fluorescent Antibody Virus Neutralisation Test, FAVN)

The Neutralisation test analyses the ability of an antibody to block virus infectivity. Serum neutralization is the gold standard for the detection and quantification of antiviral antibodies.

The presence of antibodies against LBV in the bat blood collected was determined using the mFAVN, as described by (Cliquet *et al.*, 1998). The mFAVN method allows the evaluation of sero-neutralising antibody levels by incubating a constant dose of titrated virus with serial dilutions of sera. Briefly, the LBVNig56 isolate used for the mFAVN was

prepared to a working strength of 100 tissue culture infective doses (TCID)50/50 µl. Negative control sera [a pool of naïve (unvaccinated) dog sera] and two positive anti-LBVNig56 rabbit sera was used.

The sera to be tested were heat inactivated for at least 30 minutes at 56°C. It was tested in duplicate. Baby hamster kidney (BHK) minimum essential medium (MEM) was dispensed into a 96-well plate to allow threefold dilutions of each test sera with each sample tested in duplicate, starting with a 1/9 dilution, with a total of 100µl of serum/media in the well. Threefold dilution of each reference sera was performed. Similarly, BHK MEM was dispensed to allow for the virus titration, cell and media controls. 50µl of 100 TCID50/50µl LBV virus was dispensed into the first well of the virus titration plate and the virus titrated, 50µl dispensed into each test well, except media and cell controls. Plates were incubated at 37°C with 5% CO2 for 1 hour. BHK cells were trypsinised and a suspension made to obtain approximately 5 x 105 cells/ml, before addition of 50µl of the cell suspension to all wells excluding the media controls. Plates were incubated at 37°C with 5% CO2 for 48 hours. Plates were fixed in 80% acetone and stained with Centocor fluorescein-labelled (FITC) anti-rabies monoclonal globulin conjugate (diluted to 1:40 in 0.1M PBS pH 7.2). Plates were incubated for 30 minutes at 37°C, before washing with 0.1M phosphate buffered saline (PBS) at pH 7.2 and examined using a 488nm fluorescent UV microscope. The result is qualitative with any apple green fluorescence deemed a positive result. Animals were considered seropositive if the serum sample neutralised LBV at a greater or equal to a 1 in 10 serum dilution, over three times the background neutralization titre measured against RABV CVS (Wright *et al.*, 2010).

3.3.5 PCR Molecular studies

Molecular methods apply PCR technology to detect *lyssavirus* RNA. It is more sensitive and can detect a very low amount of RNA and also in decomposed tissues. A nested PCR (RT-PCR) can detect RNA in samples 1000 fold diluted beyond the level of detection using cell culture.

Samples of brain and salivary glands were tested for lyssavirus using SYBR green and Hemi-nested PCR as described. Details described as in (Hayman *et al.*, 2011). Viral RNA was extracted from samples and quantified for molecular analyses.

RNA Extraction techniques

RNA was extracted from 100 µL of sample using three methods: MagMAX™ 96 Microarray (Ambion) with the KingFisher 96® (Thermo Electron Corporation) technology system, High Pure™ RNA Isolation Kit (Roche Applied Science) and TRIZOL (Invitrogen™).

High Pure™ RNA extraction.

The High Pure™ RNA Isolation Kit is designed for the purification of total RNA using guanidine with an integrated DNase digestion step. Blood Cells or tissues were re-suspended in 200µl PBS or 200µl of sample used. 400µl Lysis/Binding Buffer was added to the 200µl sample and vortexed for 15s. This was placed in the upper reservoir of the filter tube and centrifuged for 15s at 8,000*g*. After discarding the flow through liquid, 100µl of 90µl DNase incubation Buffer and 10µl DNase I was mixed, added to the reservoir and incubated for 15min at room temperature (RT). This was washed with 500µl Wash Buffer I and centrifuged for 15s at 8,000*g*. Washing was repeated with 500µl Wash

Buffer II and centrifuged as above. A further wash with 200µl Wash Buffer II was performed and centrifugation for 2 minutes at maximum speed (approx. 13,000*g*) removed any residual wash buffer. Elution was in 50 Elution Buffer with centrifugation for 1 minute at 8,000*g*.

Isolation of RNA using TRIZOL™

TRIZOL ™ (Invitrogen™) is a monophasic solution of phenol and guanidine isothiocyanate for the isolation of total RNA from tissue and cells. For each 200µL of liquid sample, 600µL (3 times the volume of sample) of TRIZOL™ was added, mixed and incubated for 5 minutes at RT to liquid samples. For tissue samples 1ml of TRIZOL™ reagent per 50-100 mg of tissue was used, and the sample homogenised. 160uL (liquid) or 200uL (tissue) chloroform was added and shaken vigorously by hand for 15 seconds, before incubation at room temperature (RT) for 3 minutes to allow complete dissociation of nucleoprotein complexes. Centrifugation was for 15 minutes at +4°C and <12,000g, followed by transfer of the top clear aqueous phase to a fresh tube and 400µl (liquid) or 500µl (tissue) of isopropanol added. Mixing was by inverting the tube, followed by incubation for 10 minutes at RT. RNA was pelleted by centrifugation as above, the supernatant (SN) removed and pellet washed with 20µL 75% Ethanol. Centrifugation and SN removal was followed by air-drying the pellet for 5-10 minutes and re-suspension in 10-20µl of RNase free DEPC-treated water. RNA was stored at -80°C.

MagMAX™-96 Microarray RNA extraction.

MagMAX™-96 Microarray RNA (Ambion) extraction employs TRI Reagent® and the Ambion® MagMAX™ magnetic bead-based RNA purification technology. Briefly, 1ml TRI reagent was added to 100µL sample, mixed with 100µL bromochloropropane (BCP)

and incubated at RT for 5 minutes. The aqueous phase was removed and added to the 96-well Processing Plate following centrifugation at +4°C and <12,000*g* for 10 minutes. 50µL isopropanol was added, before shaking for 1 min on an orbital shaker. Using the KingFisher 96® (Thermo Electron Corporation) robot that is designed to automate the preparation processes of nucleic acids in a 96-plate format using magnetic-particle-based methods, 10µL of RNA Binding Beads was added, shaken for 3 min, magnetically captured and the SN discarded. The plates were washed twice with 150µL of Wash Solution 2, dried for 2 min and RNA eluted in 50µL of Elution Buffer. Because this was the first time this technique was used at the VLA, eluted product was tested by RTCIT to ensure the product was inactivated.

MELTTM total nucleic acid isolation and the KingFisher96® extraction robot

The MELT™ Total Nucleic Acid Isolation System (Ambion) uses an enzymatic digestion to process fresh or frozen tissue per sample and irreversibly destroy RNases. A 10mg tissue section (approx 3mm cube) was mixed with 100µl of the MELT™ master mix and vortexed for 10 minutes until no tissue fragments were present. 100µl of the binding solution (containing β- mercaptoethanol) was added and mixed with 100µl of 100% ethanol and 10µl of the binding beads per sample. 210µl of the binding bead master- mix was added into each well of a 96 Thermofisher deep well plate (Thermo Scientific) and then 100µl of sample added to each well and incubated for 3 minutes at room temperature. This was run on the KingFisher 96® with four wash steps, two each with 300µl of Wash Solution 1 and 300µl of Wash Solution 2. Add 30-50µl of Elution Buffer to each well of a 96 well plate and run on the appropriate KingFisher 96® programme.

Nucleic acid (RNA) quantification by UV spectrophotometry using the Nanodrop ND-2000

Extracted RNA were diluted (1:10) by adding 1ul of RNA template to 9ul of HPLC water. This was then quantified using nanodrop method.

Quantification of extracted samples, such as Lyssavirus RNA samples is used for a number of purposes including:

- To ensure sufficient amount of nucleic acid is present for input into a PCR assay
- To obtain the dilution factor for production of dilution series and positive controls and
- Quantification of purified PCR products prior to DNA sequencing.

An ND-2000 UV-Vis Spectrophotometer was used to measure sample absorbance in the UV-Visible light range (220-750nm). Microvolume amounts of sample (0.5-2.0ul) are measured. For the ND-2000, the machine was allowed to initialise without applying any liquid to the detector. The machine was blanked by Pipette 0.5 to 2µl of the solution (HPLC water) that the sample is eluted onto the lower pedestal, close the sampling arm and for the ND-2000 select 'BLANK'. This will initialise the spectrophotometer. The Nanodrop will now use this solution to make a blank reference reading. For the ND-2000, once this has been done, 0.0 will appear in the ng/µl field.

Once initialised open the sampling arm and thoroughly dry both the upper and lower pedestals using a clean dry mediwipe and 70% ethanol. For diagnostic samples, before loading the desired sample, ensure that the Sample ID field has been completed with the necessary information to identify samples. Place a sample on the detector, close the pedestal arm and press 'Measure'. Results of readings are recorded, or exported and saved locally.

TaqMan® RT-PCR

TaqMan® RT-PCRs were performed in 50µL reaction volumes comprised of 21.75µL of nuclease-free water, 5µL of 10X Magnesium-free PCR buffer (Promega Corporation), 12µL of 25 mM MgCl2, 1µL of deoxynucleoside triphosphates (a 10 mM concentration of each), 1µL each of the PCR primers (a 20 ρM concentration of each), 1µL each of the TaqMan® probes (a 5 ρM concentration of each), 1µL of Triton X-100 (10% [vol/vol]), 0.25µL of RNasin (20 to 40 U/ µL) (Promega Corporation), 0.5µL MMLV-RT (200 U/µL) (Promega Corporation), and 0.5µL of *Taq* polymerase (5 U/µL) (Promega Corporation). Two µL of total RNA were added to this mixture at a concentration of 1 µg/µL. The reactions were carried out in Thermo-Fast 96-well PCR plates or Thermo-tube strips with Ultra Clear caps (ABgene®, Thermo Scientific) in an MX3000P multiplex quantitative PCR system (Stratagene). The RNAs were reverse transcribed and amplified according to the following heating and cooling program: 1 cycle of 42°C for 30 min and 94°C for 2 min followed by 45 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 20 s. Positive RABV control assays were run using CVS RNA. For each RT-PCR, a critical threshold cycle number (*CT*) was determined corresponding to the PCR cycle number at which the fluorescence of the reaction exceeded a value determined to be statistically higher than the background by the software associated with the MX3000P system (Stratagene).

SYBR® Green PCR

SYBR® Green I is a double- stranded DNA intercalating dye, that fluoresces once bound to the DNA. RNA was reversed transcribed using MMLV-RT as published previously (Heaton, Johnstone *et al*., 1997; Wakeley, Johnson *et al*., 2005). SYBR® Green Hot Start (Applied Biosystems) PCRs were performed using 20µL SYBR® Green Hot Start, 13µL nuclease-free water, 1µL JW12 (20pmol/µL) primer, 1µL N165-147 (20pmol/µL) primer,

and 5µL cDNA. Details of SYBR green reaction mix and Amplification program are in Table 3.3. Reactions were performed with the same plates, caps, and PCR systems as above.

Table 3.3: **PCR reagents and cycling program for molecular testing of bats**

Reagent	**Per Reaction (ul)**	**16 samples (ul)**
Water	As supplied 19.00	304
SYBR Green PCR reaction mix	As supplied 25.00	400
JW12	20uM 1.50	24
N165-146	20uM 1.50	24
iScript RT enzyme mix	As supplied 1.00	16
Total	**48**	**768**

B-actin mix

Reagent	**Per reaction**	**16samples (ul)**
Water	As supplied 19.00	304
SYBR Green PCR reaction mix	As supplied 25.00	400
BatRatBetaAct Intronic	20uM 1.50	24
BatRatBetaAct Rev	20uM 1.50	24
iScript RT enzyme mix	As supplied 100	16
Total	**48**	**768**

SYBR Green cycling program

Phase	Cycles	Temp(oC)	Time
cDNA synthesis	1x	50	10min
RT inactivation	1x	95	5min
PCR cycling and detection	40x	95	10sec
		60	30sec
Melt curve analysis	80x	95	1min
		55	1min
		55-95	10sec

Hemi-nested RT-PCR

The RNA samples were reverse transcribed and analysed using a hemi-nested PCR incorporating pan-lyssavirus primers (JW6/12, JW10/12), with a standard 18S ribosomal RNA internal control where necessary, as previously described (Heaton *et al.*, 1997).

Reverse Transcription of Lyssavirus RNA.

Reverse transcription (RT) is used for the RNA viruses, since cDNA must first be synthesized from extracted viral RNA before PCR can be attempted. Reverse transcription is an enzyme capable of synthesizing single-stranded DNA from RNA in the 5' to 3' direction. In the case of lyssavirus, the universal primer JW12 (Marston *et al* 2007) was used. In brief, RNA was reverse transcribed by Moloney murine leukemia virus reverse transcriptase (MMLV-RT) and the primer JW12, plus random hexamer primers if 18S controls are used, at 42°C for 60 minutes. A RT master-mix was prepared.

In a template room, 2ul of the RNA template to be transcribed was added to a tube containing the master mix and incubated in a water bath at 42^{o}C for 1hr. After the incubation, the cDNA was diluted 1:10 in nuclease-free water (80ul HPLC water), and

5µL of the mixture was used in a first-round hn- PCR (JW6-JW12 PCR) of 46 cycles. Unused cDNA is stored at -20^{o}C .

Table 3.4: Components of Reverse Transcription Master-mix formula.

Reagent	Batch No.	Per reaction (ul)	16 reactions (ul)
HPLC grade water		6	96
RT buffer (5x)		4	64
dNTPs		1	16
DTT		1	16
Random Hexamers		2	32
JW12		2	32
RNAsin		1	16
MMLV-RT		1	16
Total		**18**	**108**

First Round PCR (JW6-JW12 PCR)

For the 1st round PCR, a JW6-JW12 PCR master mix is used. Add 5ul of cDNA to the master mix (or water PCR negative or CVS PCR positive) below the surface to minimize aerosol.

Normally, the 18S PCR is run alongside the JW6/12 PCR, as they have identical cycling parameters. If 18S is to be run, then the cDNA being tested must contain Random Hexamers. Tables 3.5 details of PCR master-mix for the 1st, 2nd, 18S PCR and the cycling parameters of the Hemi-nested PCR

Table 3.5: **Hemi-nested PCR Programme**

1st round (JW6/12) PCR Mix:	**Volume Added per Reaction**	**Final Concentration**
HPLC water	33.075 µl	
10 x amplification buffer	5 µl	1 x amplification buffer
10 mM dNTPs	1 µl	200 µM each dNTPs
7.5 pmoles/µl JW12	1 µl	7.5 pmoles/50µL
7.5 pmoles/µl JW6 UNI	1 µl	7.5 pmoles
Amplitaq Gold	0.25 µl	2.5 U
2.5 mM TMAC	1 µl	0.05 mM
DMSO	0.675 µl	1.35%
Total Volume	**45 µl**	

18S PCR Mix:	**Volume Added per Reaction**	**Final Concentration**
HPLC water		35 µl
10 x amplification buffer	5 µl	1 x amplification buffer
10 mM dNTPs	1 µl	200 µM each dNTPs
18s primers	2.25 µl	As provided
18s competemers	1.5 µl	As provided
Amplitaq Gold	0.25 µl	2.5 U
Total Volume	**45 µl**	

1st round PCR Cycling Parameters:			
Denature	10 mins	95°C	
Denature	1 min 30 sec	95°C	x 5 cycles
Anneal	1 min	45°C	
Pause	20 sec	50°C	
Extend	1 min 30 sec	72°C	
Denature	30 sec	95°C	x 40 cycles
Anneal	1 min	45°C	
Pause	20 sec	50°C	
Extend	1 min	72oC	
Denature	30 sec	5°C	x 1cycle
Anneal	1 min	45°C	
Pause	20 sec	50°C	
Extend	10 min	72°C	
Chill	pause	4°C	

One µL of the first-round PCR product was then subjected to the second-round heminested (JW10-JW12) PCR for 36 cycles.

2nd PCR Master mix:		
Contents of a JW12/JW10 mastermix RT PCR Mix	**Volume Added per Reaction**	**Final Concentration**
HPLC water		38.065 µl
10x amplification buffer	5 µl	1 x amplification buffer
10 mM dNTPs	1 µl	200 µM each dNTPs
7.5 pmoles/µl JW12	1 µl	7.5 pmoles/50µL
7.5 pmoles/µl JW10 UNI	0.65 µl	7.5 pmoles
Amplitaq Gold	0.25 µl	2.5 U
2.5 mM TMAC	1 µl	0.05 mM
DMSO	0.735 µl	1.47%
Total Volume	**49 µl**	

2nd Round cycling programme			
Denature	10 mins	95oC	
Denature	1 min 30 sec	95oC	x 5 cycles
Anneal	1 min	45oC	
Pause	20 sec	50oC	
Extend	1 min 30 sec	72oC	
Denature	30 sec	95oC	x 30 cycles
Anneal	1 min	45oC	
Pause	20 sec	50oC	
Extend	1 min	72oC	
Denature	30 sec	95oC	x 1cycle
Anneal	1 min	45oC	
Pause	20 sec	50oC	
Extend	10 min	72oC	
Chill	pause	4oC	

The products of both the first and second rounds of PCR were analyzed in 1.8% agarose gels stained with ethidium bromide. Negative mouse brain was used for negative extraction control. HPLC water is used as a 'No Template Control' (NTC). A laboratory adapted RABV (CVS-11) RNA and cDNA were used as positive controls.

Gel Electrophoresis and visualization of the lyssavirus RT-PCR products

Agarose gels (1.8%) were made using 1xtris-Acetate-EDTA 9TAE) as the buffer. The mixture was heated until the agarose dissolved and was then left to cool to approximately 37^{o}C . Stock ethidium bromide (Bio-Rad) was added (2ul) and the contents of the flask mixed thoroughly. Gel cast was made by pouring molten agarose into a stoppered gel tray with a casting comb in place. On setting, the gel was placed in an electropophoresis tank containing 1xTAE buffer and the gel comb removed. Unless otherwise stated, 20ul of the product to be analysed was added to 3ul gel-loading buffer and the samples loaded into the wells. Electrophoresis was used by applying 120v across the tank until the gel loading buffer band was approximately 2cm from the end of the gel.

The DNA bands were visualized usig UV illumination and the image recorded using a gel documentation system (Biod-Rad, USA). The electrophoresed gels were caliberated via the inclusion of 5ul DNA marker.

CHAPTER FOUR: INFECTION DYNAMICS OF LAGOS BAT VIRUS IN STRAW-COLORED FRUIT BATS (EIDOLON HELVUM) IN GHANA

4.1 Introduction

The emergence and re-emergence of a number of viruses from bats that impact human and animal health has resulted in a resurgence of interest in bat immunology. Understanding how bats co-exist with viruses in the absence of apparent disease is essential if we are to develop intervention strategies targeting viruses in humans, livestock and companion animals. Many bat species are gregarious. The persistent viral infections occurring among long-lived bats, coupled with their often gregarious roosting habits, could greatly increase the potential for intra- and inter-species transmission of pathogens.

Fruit bats of several species serve as reservoir hosts for LBV with infrequent spillover infections documented in dogs, cats, and a mongoose (Markotter *et al.*, 2006). The LBV virus circulates among fruit bats in sub-Saharan Africa. LBV has been isolated in Nigeria and Senegal in West Africa (Boulger and Porterfield, 1956; Pasteur Institute, 1985; Mebatsion *et al*., 1992), Ethiopia and Kenya in East Africa (Kuzmin *et al.*, 2010; Kuzmin *et al.*, 2008; Mebatsion *et al.*, 1992), and Zimbabwe and South Africa in Southern Africa (Coetzer *et al.*, 1994; Markotter *et al.*, 2009; Markotter *et al.*, 2006). Cross-sectional studies have revealed high seroprevalence of antibodies against LBV in two colonial fruit-bat species, *Eidolon helvum* and *Rousettus aegyptiacus* (Hayman *et al*., 2008; Kuzmin *et al*., 2008; Dzikwi *et al*., 2010). Kuzmin *et al*. (2008) also reported that 931 oral swabs collected from healthy bats tested by RT-PCR were negative for LBV RNA. However, this included only 107 from *E. helvum* and 106 *R. aegyptiacus.* Viral RNA was detectable from one dead *E. helvum* bat from which LBV was isolated from the brain. The low

pathogenicity and long incubation period of lyssaviruses (Boots & Sasaki, 1999), roosting ecology and reproductive activity of colonial bats (Turmelle *et al.*, 2010) are proposed as important factors that might account for sustained perpetuation of lyssaviruses in colonial bats.

There are limited epidemiological and disease ecology data available on African lyssaviruses due to lack of systematic surveillance. The impact of LBV and other African lyssaviruses is not known. Longitudinal models have been used in Spain to investigate the dynamics of EBLV-1 in naturally infected maternal *M. myotis* colonies (Amengual *et al.*, 2007). In Ghana, there has been limited longitudinal serological and mortality survey of LBV in bats.

Studies of infectious disease ecology rely on robust surveillance of pathogens in reservoir hosts, often based on serology, which is the detection of specific antibodies in the blood and is used to infer exposure history. Antibody prevalence data are often used to elucidate infection dynamics in animal populations. This study investigated infection dynamics of LBV in *E. helvum* in a wild population of *E. helvum* in two of the biggest roosting sites in Ghana (37-Military hospital in Accra and a rural population at Tano Sacred grove) using serological method. The study also investigated LBV dynamics in a captive population of *E. helvum* at the Achimota Forest. It investigated the implication of lyssaviruses in the mortality of bats through active survey and postmortem of dead bats at these sites. The study provided vital information on the dynamics and epidemiology of LBV both in free-ranging *E. helvum* and captive population of *E. helvum* bats in Ghana.

4.2 Materials and Methods

Permission was obtained from the Ghana Wildlife Division of the Forestry Commission for this research, and permission was renewed on a yearly basis, as required.

4.2.1 Wild bat Study Sites

Two colonial populations (about 250,000–1,000,000 bats each) of *E. helvum* in Ghana were selected for the study: one roost in Accra and the second roost in Tano Sacred Grove (approx.400km North). The Accra population is urban with the bats roosting in trees over a city central hospital. The Tano Sacred Grove population is rural; the bats roost in a protected forest area. The roost at the 37- Military Hospital is the largest among the known roosts in Ghana. The 37-military hospital has high human traffic resulting in daily interaction of bats with the hospital workers, patients and also military personnel living within the hospital area and along the main city highway. Tano sacred grove is located in Tanoboase a small farming community located 15km from Techiman along the Techiman – Kintampo road in the Brong Ahafo Region of Ghana.

The population density of *E. helvum* at the 2 sites fluctuated with bats migrating (Thomas, 1983) out of Ghana during the rainy season (April – November) and return during the dry season (December - March). There are reported interaction between the populations of *E. helvum* in Ghana (Peel *et al.*, 2012).

4.2.2 Bat Trapping and Sampling

Bats were trapped in the early hours of morning using mist nets (set in the night) when they return from feeding to daytime roosting place. Nets were continuously inspected regularly to avoid trapped bats from staying long in mist nets. Bats were kept individually

in cloth bags (Fig. 4.1) for sampling which was carried out in make –shift sampling stations.

Blood serum samples of bats from the two field sites (Tanoboase and 37-Military hospital) and of the captive bats were collected between 2012 and 2014 to monitor dynamics of LBV antibodies. A minimum of 60 wild *E. helvum* bats were trapped during each sampling expedition, and throat swabs, faecal swabs and blood were collected. The age and sex of bats were assessed based on body size and observation of the reproductive organs. Bats were classified as adults (sexually mature bats) or juveniles (sexually in-mature bats). Bats that had prominently developed sexual organs were considered adult. Juvenile bat was classified as a bat that had comparatively smaller weight and less developed sexual organs.

Fig 4A Bats kept in cotton bags in a shade awaiting sampling

Fig. 4.1B

Fig 4.1: Field Sampling of bats. Fig 4.1A. Bats kept in cotton bags. Fig 4.1 B Blood sampling from a trapped bat.

Standard measurements were taken on all bats, including mass, forearm length and age estimated. Trapped bats were micro-chipped for individual identification before release. The blood (under 1ml) was taken from the propatagial vein as described (Hayman *et al.*, 2008) into a cryovial. Blood was centrifuged (Eppendorf – Netherler-Hinz, GmbH, Germany) for serum separation at 6000g for 15minutes. Sera were stored at -70^{o}C until use. Injured and dead bats were submitted for necropsy.

4.2.3 *E. helvum* Mortality Survey for Lagos Bat Virus.

During 2012 to 2014, fifty-five (55) dead and sick bats were collected from the 3 research sites (37-hospital - 40; Tano Sacred grove – 7 and Accra Zoo – 8) as part of LBV disease surveillance. Decomposed bats were not included. Sick and dead bats were collected

through daily active search at the *E. helvum* roosting site at the 37-military hospital area in Accra. A few dead bats were submitted by the public. Bats accidentally injured or killed during sampling were mostly from Tano Sacred Grove. Accra zoo captive bat breeding programme also provided dead bats. Nine (9) sick bats from the 37-Military Hospital demonstrated neurological clinical signs. They were found on the ground unable to fly. Such bats were anaesthetized with an excess of ketamine and medetomidine and euthanased by exsanguination via cardiac puncture. Carcasses of dead bats were collected in zip-lock plastic bags and transported in cooler boxes from the field site. Bats were assigned PM numbers and pathological findings and other data captured in a PM data sheet. Necropsy of bat carcasses was either performed on the same day, or the carcasses were stored in a freezer (at -70^{o}C) at the Veterinary Laboratory in Accra. Predetermined tissues, including brain (cerebellum, medulla, rostrum and hippocampus), salivary glands, mandibular lymph nodes, heart, kidney, liver, and various parts of the digestive tract were collected at necropsy. Oral swabs were taken from sick bats, where possible, before such bats were euthanized. Tissue samples were placed in plain tubes and in tubes containing RNALater® (Ambion, USA). In addition, pooled organ samples from each bat were placed in formalin. Whole blood and cerebrospinal fluid were taken where possible. All samples were stored at (-70^{o}C) at the Accra Veterinary Laboratory, Ghana and further shipped in liquid nitrogen to VLA, UK for laboratory testing.

4.2.4 Captive breeding of bats at the Achimota Forest, Accra zoo.

Seventy-eight Straw-colored fruit bats (*E. helvum*) of mixed sex and ages were collected in 2009 for captive breeding in the Achimota Forest, Accra zoo, Ghana. Bats were kept in a 595m Octagonal cage with a diameter of 27.5m and height of 3.5m constructed for this purpose (Material and Method section for details). Bats were fed with assorted fruits on

raised wooden platforms. Water was provided at all the time for drinking. Vitamin supplement was included in food or drinking water when ever judged appropriate and based on professional advice.

4.2.5 Laboratory Investigation

The presence of lyssavirus antigens and RNA in the dead bats was determined using fluorescent antibody test (FAT) and real-time RT-PCR (SYBR® Green PCR).

Virus detection by FAT

The fluorescent antibody test (FAT) was performed on brain samples from dead bats following standard protocols (Anderson *et al.*, 1980; Cliquet *et al.*, 2010; Dean *et al.*, 1996) as detailed in Chapter 3 of this thesis.

Lagos Bat Virus diagnoses using molecular methods

Molecular method was used for detection of LBV RNA in postmortem bat samples. Total RNA was extracted from organ samples using Trizol (Invitrogen) following the manufacturer's protocol. For detection of virus genome, a RT-PCR, Pan-lyssavirus SYBR Green real-time (q) RT-PCR assay described by Wakeley *et al.* (2005) and Hemi-nested-PCR was utilized.

The lyssavirus SYBR Green real-time (q)RT-PCR was modified using primers Jw12 (5'-ATGTAACACCYCTACAATG- 3') and N165-146 (5'-GCAGGGTAYTTRTACTACTCATA-3') where indicated, to incorporate SYBR Green using the SYBR Green JumpStart TaqReadyMixassay (Sigma) to enable quantification of

genome copies as described by Johnson *et al*. (2006). *B*-actin primers (Wakeley *et al.*, 2005) were used to detect host mRNA within samples.

Detection of Anti-LBV Virus Neutralising antibodies (mFAVN Test)

Serological testing for anti-LBV antibodies was conducted using a modified fluorescent antibody virus neutralization test (mFAVN), as described previously (Hayman *et al.*, 2008; Wright *et al.*, 2010) using the LBVNig56 isolates (Boulger & Porterfield, 1958). Negativecontrol sera [a pool of naive (unvaccinated) dog sera] and two positive anti-LBVNig56 rabbit sera were included as negative and positive controls respectively. A full protocol of the modified mFAVN is detailed in the Research Method section of this thesis (Chapter 2). In brief, a constant dose of titrated LBV was incubated with serial dilution of the sera. For this analysis, two-fold dilutions of sera were used to provide greater sensitivity to changes in antibody titres. An initial starting dilution of 1:8 (rather than 1:9) was used in order to ensure all samples could be tested in duplicate. After 90 min incubation at 37^{o}C, a suspension of BHK-21 cells was added into each well with the virus-serum mixture for following incubation during 20 hours at 37^{o}C, acetone fixation and FAT staining.

4.2.5 Ethical Clearance

Wildlife Division of the Forestry Commission of Ghana provided approval and permission certificates for the research and also collection of bats for captive breeding. Ethical permissions were granted by the Institute of Zoology of UK and the Noguchi Memorial Institute for Medical Research. Laboratory investigation including virus antigen detection (FAT), RNA detection (RT-PCR) and serology (mFAVN) was carried out in a biosafety

level 3 laboratory (Animal and Plant Laboratory Agency) in U.K. Details of these protocols are provided in this section and also in the methodology section of this thesis.

4.2.6 Data Handling and Analysis

Seroprevalence patterns in various groupings of bats are described; non-parametric statistical tests (chi-squared test) is used to determine significant difference in the distribution of age and class of bats which tested either positive or negative to the serum.

4.3 Results

A total of 596 sera of the wild and captive bats from the 3 sites were tested for LBV-neutralizing antibodies between 2012 and 2014. Of this number, 232 sera tested positive and 364 tested negative (Seroprevalence –0.389, CI= 0.350-0.428).

4.3.1 Influence of of sex and age of bats on seroprevalence in wild caught bats.

A total of 304 (37-Military Hospital in Accra – 213, and Tano Sacred Grove – 94) wild caught bat sera were tested for LBV-neutralizing antibodies using the mFAVN method at the Rabies laboratory at the AHVLA in U.K. during the period 2012 to 2014. This comprised 236 adults, 58 juveniles; 201 males and 84 females. The sexes of ten bats were not determined and therefore were not included in the analyses of age and sex seroprevalence. One hundred and thirty-six of the three hundred and four bats sera tested were positive (Seroprevalence of 44.73%). From the chi-square test it was observed that there was no significant difference in the different age groups that tested positive (p-value=0.78. Seroprevalence in Adult bats (0.46, n=236) was slightly higher than in Juvenile bats (0.43, n=58) (Table 4.1).

Similarly, there was no significant difference between the sexes of bats that were tested. Slightly more males tested negative to the serum than females [seroprevalence in females was slightly higher than in the males (p-value= 0.95) as in Table 4.2].

Table 4.1: Proportion of seroprevalence in wild caught bats by age

2X2 TABLE

		SEROSTATUS			
F		**Positive (+ve)**	**Negative (-ve)**	**Totals**	**Rates p(D+/F)**
A	**Adult**	109	127	136	0.462
C					
T	**Juvenile**	25	33	58	0.431
O					
R	**Totals**	134	160	294	
	Rates (p(F+/D)				

The corrected chi-square statistic is : 0.076

Odds Ratio : 1.133

Relative Risk : 1.072

P = 0.7828

Table 4.2: Proportion of seroprevalence in wild caught bats by sex

2X2 TABLE

		SEROSTATUS			
FACTOR		**Positive (+ve)**	**Negative (-ve)**	**Totals**	**Rates p(D+/F)**
	Male	95	115	210	0.452
	Female	39	45	84	0.464
	Totals	134	160	294	
	Rates (p(F+/D)	0.709	0.719		

The corrected chi-square statistic is :	0.003		
Odds Ratio :	0.953		
Relative Risk :	0.974	P =	0.9563

4.3.2 Site difference in seroprevalence in wild caught bats

Comparing the two sites, 119 out of a total of 213 bats tested negative (seroprevalence of 44%) at 37 - Military Hospital compared to 49 out of 91 bats that tested negative (seroprevalence of 46%) at Tano Sacred Grove (p-value = 0.84). Table 4.3 and Fig 4.2 indicate the proportion and graphs of bats at the two field sites.

Table 4.3: **Proportion of seroprevalence in wild caught bats by location**

2X2 TABLE

		SEROSTATUS			
F		**Positive(+ve)**	**Negative(-ve)**	**Totals**	**Rates p(D+/F)**
	Tano Sacred				
A	**Grove**	42	49	91	0.462
C					
T	**37-Mil. Hospital**	94	119	213	0.441
O					
R	**Totals**	136	168	304	
	Rates (p(F+/D)	0.309	0.292		
	The corrected chi-square statistic is :	0.04		RR=1.046	
	Odds Ratio :	1.085			P = 0.8415

4.3.3 Seasonal differences in seroprevalence in wild caught bats

There are two major seasons in Ghana – Dry Season (December - March) and the Rainy Season (April - November). There was no difference in seroprevalence (p-value = 0.83). (**Table 4.4**).

Table 4.4: Proportion of seroprevalence in Wild caught bats by season

2X2 TABLE

		SEROSTATUS			
		Positive	**Negative**		
F		**(+ve)**	**(-ve)**	**Totals**	**Rates p(D+/F)**
A	**Dry Season**	29	33	62	0.468
C					
T	**Rainy Season**	107	135	242	0.442
O					
R	**Totals**	136	168	304	
	Rates (p(F+/D)	0.213	0.196		
	The corrected chi-square				
	statistic is :		0.048		
	Odds Ratio :		1.109	P =	0.8266
	Relative Risk :		1.058		

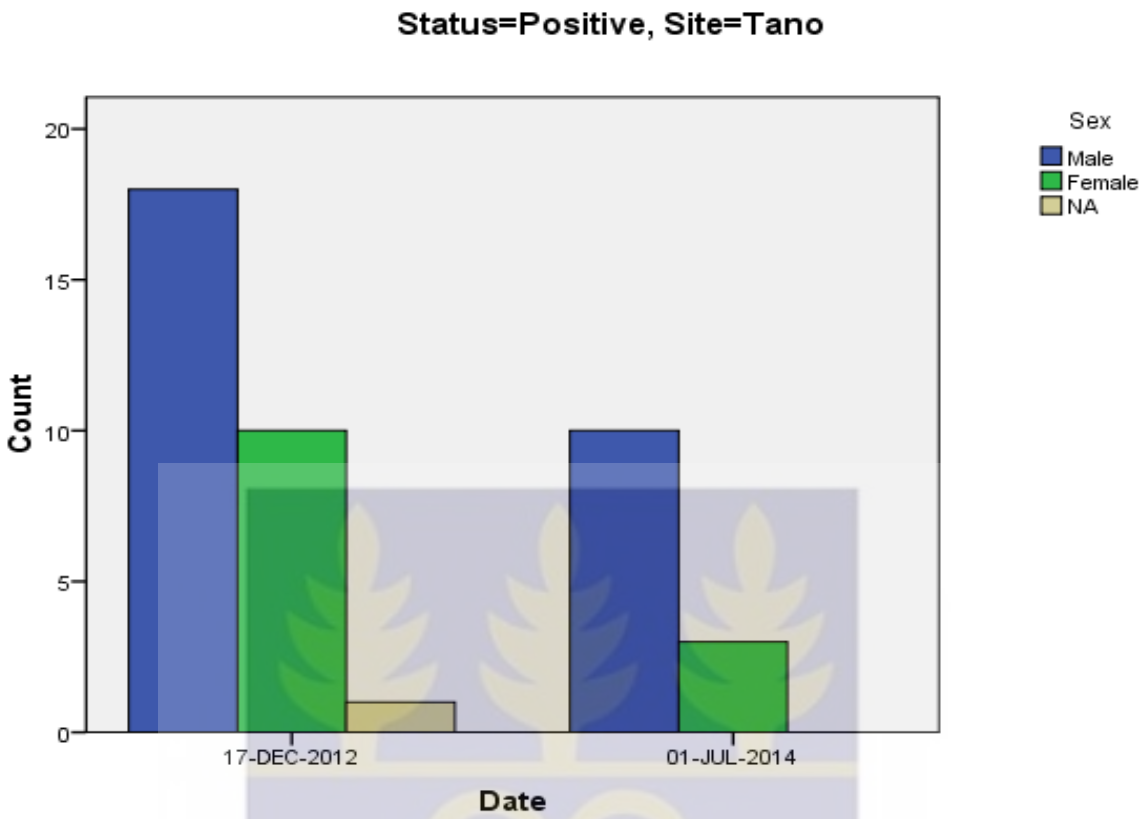

Fig 4.2A Bats seropositive bats at Tano Sacred Grove.

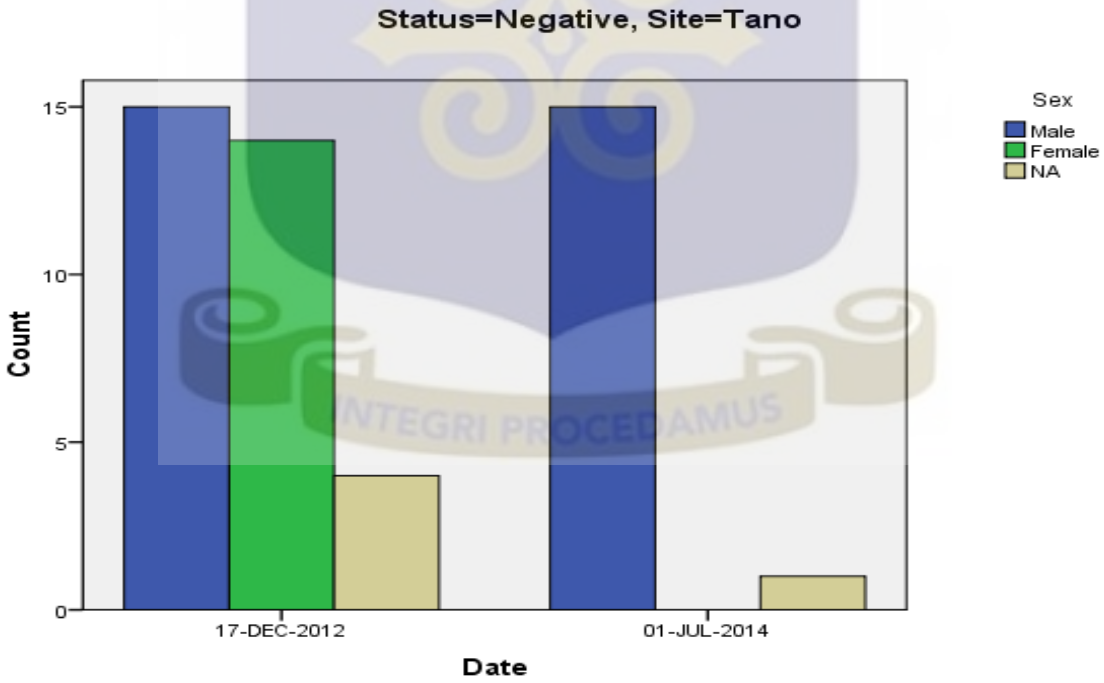

Fig 4.2B Bats seronegative bats at Tano Sacred Grove.

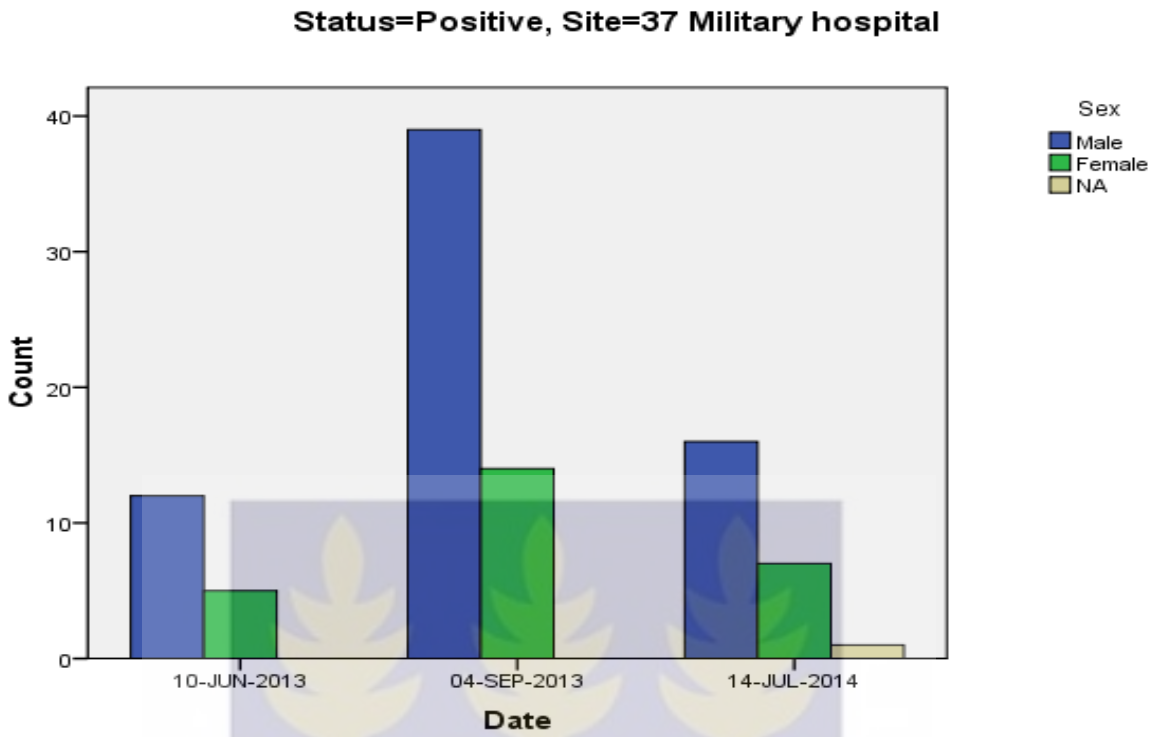

Fig 4.2C Seropositive bats at the 37-Military Hospital

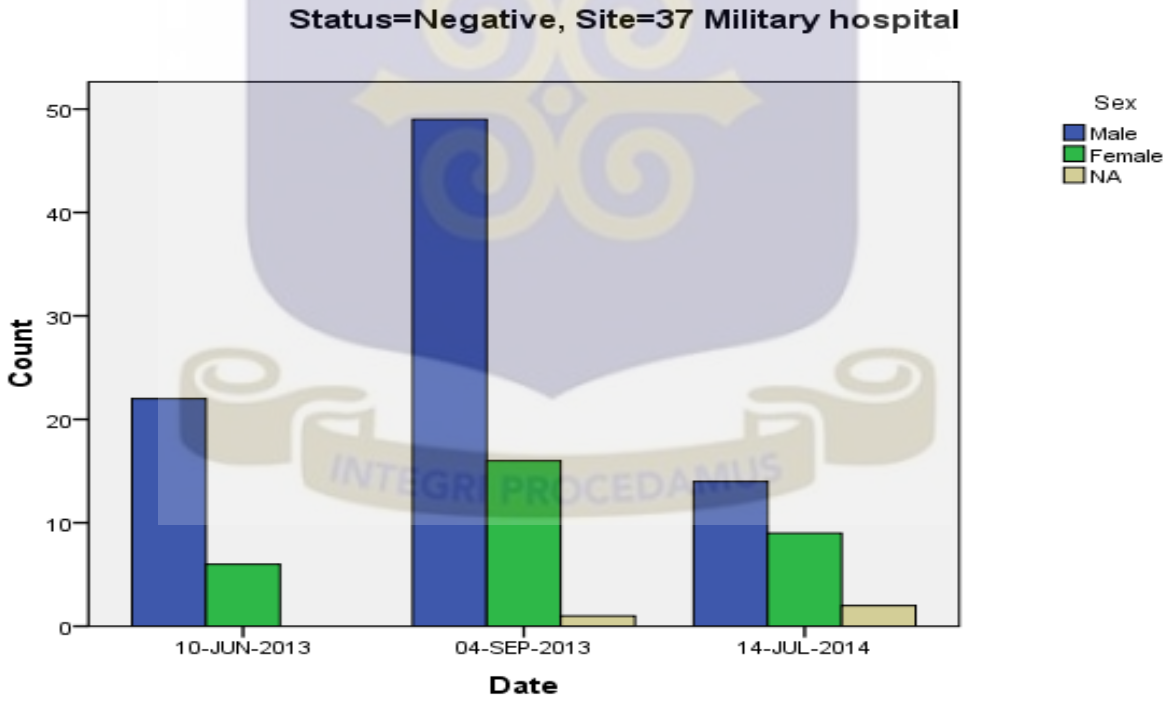

Fig 4.2D Seronegative sampled at 37-Military Hospital

Fig 4.2: Graph indicating seroprevalence at Tano (A-positive status; B-negative status) and 37-Military hospital (C-positive status; D – ngative status).

4.3.4 Lagos Bat Virus seroprevalence in captive bats

In the captive bats the seroprevalence did not vary significantly with age (p-value = 0.87) or sex (p-value = 0.75). Prevalence in antibody positive Adult bats was slightly higher (0.34, n=197) than juvenile bats (0.32, n=81).

We detected variation in antibodies in individual bats in the captive bat colony over the 2year study period from our serological data. The re-sampling of bats (3 times, with 7-8 months' interval) allowed us to follow the seroneutralisation titre of fifty (50) bats sampled consecutively. Nine (9) bats had maintained their positive status (had detectable antibodies above cut-off level) for 10 months and 16 bats had waned antibodies below detectable level. At the next sampling sixteen months from the first sampling (September 2013); eight (8) bats were recorded to have gained seropositive status with detectable antibody. Though most bats had marginal variation in antibodies, 2 bats recorded high antibody increase from the previous levels (Bat 4194 (A146) titre level increased from 9.00 to 729.00 and Bat 5678 (A123) from 14.00 to 140.30). Four bats (9675; 3660; 1760 and 0691) maintained a positive status persistently for the 2 years. Eight (8) of the captive bats died during the period of study. Their brain samples were tested rabies negative by FAT and SYBR®Green qRT-PCR. Eighty-one (81) animals were born in captivity, 26 of which were seropositive.

Table 4.5: **Seroprevalence of captive bats tested**

Date of Sampling	Total bats Tested	Total Positive	Percentage Positive
Jun-12	82	40	49
Feb-13	103	29	28
Sep-13	107	27	25
Total	**292**	**96**	**33**

Table 4.6: Seroprevalence of captive bats by Age

Date Sampled	Total Adults	Total Positive (% Positive)	Total Juveniles	Total Positive (% Positive)
Jun-12	59	28 (48%)	20	12 (60%)
Feb-13	75	22 (29%)	22	4 (18%)
Sep-13	63	17 (27%)	39	10 (25%)
Total	197	67 (34%)	81	26 (32%)

Table 4.7: **Seroprevalence by Month and Sex in Captive *E. helvum* bats tested**

Date Sampled	Total Male	Total Positive (%)	Total Female	Total Positive (%)
Jun-12	27	13 (48%)	52	27 (51%)
Feb-13	46	9 (19%)	52	17 (32%)
Sep-13	58	15 (25%)	44	12 (27%)
Total	131	37 (28%)	148	56 (37%)

4.3.5 Post –mortem findings in E. helvum bats

A total of 55 *E. helvum* bats were post-mortemed during the period with the highest number coming from the 37-Military Hospital (40/55). Carcasses of *E. helvum* at hot and humid ambient environment decompose very fast. Most bats were found already at an advance stage of autolysis and unacceptable for virological testing. Major gross post-mortem finding was injury, probably due to hunting. Other findings included abcesses, haemorrhages, skin infection (from captive bats (2)) and enlarged spleen (1bat). Table 25 gives the post-mortem findings. Most of the bats with neurological symptoms were found during January and February 2014. It was thus considered as an outbreak of a kind disease. One bat (Bat A38) which was reported to have fought with a dog in the night before was found dead the following morning. We followed to the location in Achimota area in Accra and observed the bat had caused injury to the face of the dog. The affected dog was quarantined for 90 days. After receipt of negative FAT test results from the National Veterinary laboratory on brain of the bat, the dog was vaccinated against rabies and released to the owner. The dog remains healthy till date.

Table 4.8: **Pathological findings in dead/sick *E. helvum* bats at necropsy**

BAT LOCATION	TOTAL NECROPSY	COMMENTS
37-Hospital	40	1. Found dead (19); 2. Injury (Fractured body parts) (euthanized) (12) 3. Behavioral signs (Euthanised) (9).
Tano Sacred grove	7	1. Euthanasia due to Injury/ Fractured body parts – (7)
Accra Zoo, Achimota Forest	8	1. Found dead – 6 bats; 2. Euthanasia due to disease (Skin infection and Poor body condition) – 2 bats.
TOTAL	**55**	

Fluorescent Antibody Test (FAT) for Post-mortem bats

Brain samples of all bats and a patas monkey that died after earlier contact with bats were tested by FAT at the Accra Veterinary laboratory. All samples tested negative by FAT. Samples from nine (9) wild bats (A6, A10, A14, A16, A20, A21, A22, A23, A24) that were showing neurological signs (Table 4.8), bat A38 that attacked the dog and the brain taken from a dead patas monkey were shipped to AHVLA (WHO and OIE rabies reference laboratory) for further laboratory investigation. The samples were tested by FAT

and SYBR®- Green PCR. All bat samples and the monkey sample also tested negative using the FAT at the AHVLA, U. K.

Virus Prevalence in Postmortem bats

Total RNA was extracted from the brains of the 9 bats with neurological symptoms, using trizol® and RNA diluted to 1ug/ul i.e 1:10 (1ul neat RAN + 9ul water) for RT-PCR. The samples were run on PCR protocol described in section 3 of this thesis using B-Actin and lyssavirus N-gene primers (CVS +ve and No template control, NTC added to the plate. All samples of dead and sick bats (A6, A10, A14, A16, A20, A21, A22, A23, A24, A38) collected tested negative for lyssavirus (N-gene), NTC was negative and *B*-actin was positive.

4.4 Discussion

Isolated small captive breeding population of *E. helvum* bats (80 – 150 bats) kept at the Accra Zoo and also a wild population of bats from two large roosts (37-Military Hospital and Tano Sacred Grove) with 250,000 to 1,000,000 bats each were investigated. The study focuses on testing the hypothesis that the presence of LBV neutralization antibodies in serum of bats is indicative of circulation of LBV. First the presence of LBV neutralisation antibodies in the populations of *E. helvum* bats at the two sites and in the captive colony was investigated and tested along with variation in LBV antibody seroprevalence based on sex, age, season of study (dry or rainy season), and roosting colony. Secondly, the longitudinal variability in the presence of LBV antibodies in identified bats in the captive colony is described. The purpose of this study was to provide further understanding of dynamics of LBV in E. helvum, the straw-colored fruit bat, one of the virus's potential reservoir hosts.

Wild caught bats

A total of 294 sera from wild caught bats were tested during the period of study (2012 – 2014). A total of 134 bats sera representing 45% of sera were positive (Table 4.1). In the wild caught bats, there was insignificant difference in seroprevalence in age groups (p-value = 0.78). There was however a higher proportion of seropositivity in adult bats (0.46) than in juvenile bats (0.43). Similar findings are reported elsewhere in hoary bats (*Lasiurus cinereus*) (O'Shea *et al.*, 2014; Bowen *et al.*, 2013).

The serological test of two healthy mothers with pups did not detect any anti-LBV antibodies in the two mothers (seronegative, with 5.20 titre in both parents). However, the two (2) pups had detectable anti-LBV antibodies with antibody titre of 46.77 for bat 901 and 81.00 for bat (ID) 904). The difference in proportion of seroprevalence among age groups and the two seropositive juvenile bats may be due to maternal antibodies. It is possible that antibody titres in the 2 mothers were too low to be detected, but the concentration of antibodies in pups was not. Naïve juvenile and youngs bats may also be getting abortive infection from infected adult bats in the population (Hayman *et al.*, 2011).

Similarly, there was no statistically difference in seroprevalence between the sexes of bats that were tested (p-value= 0.95).

There was no difference in seroprevalence comparing the two (2) field sites (p-value = 0.297). The seroprevalence ranged from 0.44 (37-Military Hospital) to 0.46 (Tano Sacred Grove).

Peel et. al., (2013) reported continent-wide panmixia of *E. helvum* bats that facilitate transmission of potential zoonotic disease. Hayman (2011) also reported movement of *E. helvum* between roost and colonies. The findings in this thesis support these reports.

There was no difference in seroprevalence (p-value = 0.83) when compared by seasons. However, fewer bats were caught during the dry season (62) compared to that caught during rainy season (242) and this possibly affected the results.

That fewer bats were caught in the dry season. There is an increase in bat population in these 2 sites due to addition of bats migrating into the country during the dry season in Ghana. We therefore expected an increased prevalence of LBV due to interaction of infected bats and naïve bats and possible sharing of pathogens (Hayman *et al.*, 2012; Peel *et al.*, 2012). We proposed a reduced interaction during the rainy season and thus therefore reduced chances of infection and seroconversion. There was no difference in seroprevalence. This may be due to circulation of the virus in the population. Since the lifespan of LBV antibodies in bats is not known, it could also be persistence of antibodies in some captured bats.

Many scientific studies have reported similarly high seroprevalence for LBV in *E. helvum* of 14 – 44% (Nigeria), 37% (Ghana), and 40–67% (Kenya) (Dzikwi *et al.*, 2010; Hayman *et al.*, 2008; Kuzmin *et al.*, 2008) respectively as found in this study. These findings are not surprising in a gregarious bat species such as *E. helvum* in which a high contact rate between individuals is facilitated in the roost, where bats are highly concentrated. Two pups had detectable antibodies, while their mothers had undetectable antibodies. This might support the fact that birth pulse provides opportunity for circulation of viruses in a population and maintenance of infection in a population (Amengual *et al.*, 2007; George *et al.*, 2011; Gilbert *et al.*, 2013). The mechanism of maintenance for RABV in bat species with very high roost densities is hypothesized to be due to population size, contact rates, susceptibility, seroprevalence, the proximity of animals within large roosts, and other variables that facilitate virus circulating at low levels of transmission (Luis *et al.*, 2013).

The ppups might have acquired the antibodies from other seropositive mothers (allomothering) or survived from infection and thus acquired immunity.

Captive bats

Two hundred and ninety-two (292) sera samples from an isolated captive *E. helvum* bats were collected during 3 sampling sessions between 2012 and 2014. LBV infection was reported in this population by Hayman *et al.* (2011). The purpose of the study was to monitor LBV infection dynamics in the captive population by serological method. Our study is the first longitudinal serological study of infection dynamics of LBV in a captive population of *E. helvum* bats.

We recorded mean seroprevalence of 33% (representing 96 of the 292 captive bats) In this study, antibody prevalence in captive E. *helvum* bats was higher in females (37.83%; range 27.27% - 51.92%) than in male (28.24%, range 19.56% - 48.14%). A similar pattern of seroprevalence in males and females *E. helvum* was recorded in wild caught bats (Turmelle *et al.*, 2010). However, serpositivity among age groups (p-value = 0.25) and sex (p-value = 0.26) groups was statistically insignificance in the captive's population of bats. There was insignicant temporal variation in seroprevalence observed in the captive bats (p-value = 0.18) (Turmelle *et al.*, 2010).

We observed in this work antibody variation in individual captive bats. It was observed in this study that eight (8) bats seroconverted in captivity. Nine (9) bats in the captive population maintained positive status for ten months and four bats maintained a positive status persistently for at least 2 years. It should be noted here that the titres of those bats that became seropositive were marginally low, and therefore this may be due to error in sample handling, and other intrinsic and extrinsic factors including the use of dynamic

testing, such as the mFAVN, rather than true seroconversion due to viral challenge. There is however need to continue monitor serconversion in these bats as few were reported with some high seroconversion. Four (4) bats maintained a positive antibody level for the period of the project. Similar persistence of of antibodies for more than one year in some individual bats was reported by Amengual *et al.* (2007).

Rabies virus neutralizing antibodies were shown to wane in experimentally-infected bats within six months after an initial inoculation with RABV, but persisted for longer (6–12 months) after a second inoculation of surviving bats (Turmelle *et al.*, 2010). In a study reported by Sohayati *et al.* (2011), one individual bat was initially seropositive, became seronegative within 1–2 months and remained seronegative for 11 months before displaying a gradual increase in neutralizing antibody and viral excretion.

LBV prevalence in dead bats

None of the brain samples from the bats found sick or dead in this study showed presence of lyssavirus antigens or RNA. Similar studies did not succeed in detecting lyssavirus antigen or RNA in bats elsewhere (Amengual *et al.*, 2007; Dzikwi *et al.*, 2010; Reynes *et al.*, 2004; Wellenberg *et al.*, 2002). Infection prevalence of RABV in gregarious colonial bat species is reported usually to be significantly less than 1%, whereas seroprevalence may be as high as 70% (Steece & Altenbach, 1989).

The high percentage of seropositive bats after infection is indicative of efficient virus transmission between individuals and rapid circulation of the virus in the colony. These findings are not surprising in a gregarious bat species such as *E. helvum* in which a high contact rate between individuals is facilitated in the roost, where bats are concentrated. It was quite interesting to detect antibody in juveniles with a history of having been born

from serologically naïve and apparently healthy mothers. Such juveniles could influence the perpetuation of LBV in the captive population and there is the need for a further investigation in this direction.

It was not surprising that no LBV or antigen was detected in this study. Some of the bats are from bats euthanizing due to injury during capture, thus affecting samples size. A much higher sample size of dead or sick bats (more than the 49 investigated here) is required to improve the chances of detecting LBV. This can assist in the investigation of the various strains of LBV that might be circulating in Ghanaian Straw-colored fruit bats (*E. helvum*). The high seroprevalence of LBV reported here and elsewhere (Hayman *et al.*, 2008) in the Ghanaian *E. helvum* colonies may be evidence of the host clearing the infection, but it is unknown if this infection is due to exposure of sub-lethal doses of LBV RNA via routes other than biting, such as from aerosol, grooming, or food sharing.

E. helvum is known for its close contact with human populations in continental Africa. Ongoing ecology studies by our team indicate close interaction between *E. helvum*, domestic animal and human populations. Our results, therefore, could have important public health implications, but more information is required on the viruses involved, their infection dynamics within the bat populations, potential spillover routes, and bat population dynamics before any risk can be assessed. However, we advise post exposure rabies vaccination should be provided after an exposure to Ghanaian bats. However, it should be borne in mind that rabies vaccine is less efficient against lyssavirus belonging to the phylogroup 2, including LBV (Markotter *et al.*, 2006).

CHAPTER FIVE: STRAW-COLORED FRUIT BAT INFECTION STUDY

5.1 Introduction

Very little is known about Lagos bat virus (LBV) diversity and the relationships between different isolates or between LBV and other lyssaviruses (Markotter *et al.*, 2009). The true risk of LBV infection for humans and animals is uncertain (Markotter *et al.*, 2009). There is little data to indicate minimum dose of lyssaviruses required to cause clinical disease. A murine study has suggested that for the development of clinical disease in mice, as few as four infectious virus particles may be sufficient when inoculating via peripheral route (Banyard *et al.*, 2014). It is unclear if LBV causes fatal encephalitis (rabies) in natural hosts or primates although it is known that such disease occurs in some carnivores infected via spill-over infections (Markotter *et al.*, 2006).

The ecology, pathogenesis and pathology of rabies in animals and humans have been thoroughly studied (Iwasaki, 2002; Jackson, 2002; Niezgoda *et al.*, 2002). However, the mechanisms of viral invasion and spread in a single host are still not completely clear. Although intramuscular (i.m.) inoculation is believed to be the most common route of lyssavirus transmission between terrestrial mammals, it may be different among chiropterans. The usual method of viral introduction into a susceptible host occurs typically by inoculation of infectious virus present in saliva through a bite. Non-bite exposures including inhalation of highly concentrated aerosols, licks, scratches, contamination of wounds and mucous membrane are thought rarely to cause rabies (Rupprecht, 1996).

Development of rabies disease depends on the location of infection, severity of bite, and the strain of the rabies virus. Duration of the incubation period may depend on the site of

the bite and proximity to the CNS, severity of the bite and quantity of virus introduced, host age and immune status. Incubation can vary, from several weeks to years, without sufficient explanation of virus persistence in tissues of the infected animal or human during this time (Briggs & Organization, 2011).

Experimental infection studies using European bat lyssavirus type 2 (EBLV-2) indicated that intracranial inoculation of bats had led to the rapid development of disease in *M. daubentonii*, a natural host for this virus (Johnson *et al.*, 2008). The pre-clinical period has been reported to vary. Experimental I.C inoculation of Serotine bats with a high dose of EBLV-1 led to clinical disease after 7 to 13 days; subcutaneous (S.C.) inoculation had a 17 to 18 days incubation period and i.m. 26 days (Banyard *et al.*, 2014). The excretion of RABV is reported to be intermittent and a large proportion of rabid bats does not secrete virus in the saliva (Constantine & Blehert, 2009; Kuzmin *et al.*, 2008).

Clinical signs in sick bats are non-specific and change as the disease progresses (FAO, 2011). The clinical picture may also be affected by environmental stimuli. In bats, clinical signs include: aggression or intolerance to external irritation, fighting with other bats, the isolation of infected individual from other bats in a common roost, increased vocalization and movements which progress to the development of paralyses and death. Clinically sick bats are often observed hanging on low tree branches or lying on the ground (FAO, 2011). When sick bats are touched or disturbed, they respond by biting, flapping the wings and by muscle tremors. Affected bats generally cannot fly., in an experimental infection of *Artibeus jamaicensis*, reported that bats developed signs of rabies including leg spasticity and uncoordinated wing movements beginning 4 days' post inoculation (p.i). This advanced to ataxia, leg paresis, and inability to fly by 7 days p.i. Bats became moribund between 5 and 7 days p.i.

Experimental studies with EBLV-1 in *E. fuscus* with various doses suggest that a low dose of virus inoculum is more efficient for virus spread in infected animals (Franka *et al.*, 2008).

In infected bats, the virus distribution has been reported in the brain, salivary glands and other extra neural tissues; the virus is reported to be associated with innervations and ganglia. The presence of infectious virus in the reproductive tract and in a vaginal swab implies a possibility of an alternative transmission opportunity (Kuzmin *et al.*, 2008). EBLV-2 has been detected in oral swab material from both naturally and experimentally infected bats and this has suggested that EBLV-2 may be transmitted through biting or scratching (Banyard *et al.*, 2014).

On most occasions, lyssavirus exposure in bats is not thought to lead to development of productive CNS infection (rabies) but to restricted peripheral infection with the development of immunity. Although domains important in pathogenicity have been indicated in several lyssavirus proteins, the G protein remains the most important (Kgaladi *et al.*, 2013). The G protein is the only antigen that induces neutralizing antibodies and is able to confer immunity against a further lethal challenge.

There is no experimental study of LBV in its reservoir host, *E. helvum*, *R. aegyptiacus* or other pteropodid bats. While some studies suggested that the virus has a reduced peripheral pathogenicity in mouse and carnivore models compared to RABV, others have provided different results (Markotter *et al.*, 2009). This component of the study involved experimental investigation of the pathobiology of LBV in *E. helvum*. The study seeks to address key questions in lyssavirus infection dynamics in bats including infectivity of the virus in bats, susceptibility of bats to different routes of exposure and different doses of inoculum, tissue distribution, clinical course and excretion patterns.

5.2 Materials and Methods

Experimental *E. helvum* bats used for the experiments were bred in captivity since 2009 and were tested seronegative for anti-Lagos bat virus antibodies. Bats used were of different ages and sex in experiment 1. Only 1-2-year-old males were used for experiment 2.

5.2.1 Viruses

Intracrainial inoculation: Three LBV isolates of lineage A and B were used for the intracranial study, namely:

- Lineage B (RV1 10^5 $TCID_{50}$/ml Lagos Nigeria (1956). The virus was isolated in Lagos Island, Nigeria in 1956. It has been subjected to an undefined number of passages in mice and cell culture in several laboratories (Boulger & Porterfield, 1958).

- Lineage A (RV41, Senegal, 1985; $10^{7.25}$ $TCID_{50}$/ml) was isolated from *E. helvum* in Dakar, Senegal in 1985. The virus had been subjected to four (4) passages (Markotter *et al.*, 2006).

- Lineage A (FLI, Germany, ex Kumasi, Ghana; $10^{5.7}$ $TCID_{50}$/ml) was isolated from *E. helvum* collected by Professor Drosten's research group in Kumasi, in 2013 and was grown in cell culture by Professor Müller at Frederick Luthan Institute, Germany (FLI).

5.2.2 Experiment 1: Intracranial Inoculationof bats

All three LBV isolates of lineage A and B were used for the intracranial inoculation. Seronegative, mixed sex bats of various age groups from the breeding colony at the Achimota forest were selected for experiment 1. For experiment 2, 1-2-year-old male bats were selected in order to avoid any confounding variables that might be introduced by pregnant females as the experiment was to run for several months. Bats for the experiments were acclimatised for two weeks, housed individually in cages within a biosecure experimental facility. After infection, in both experiments, bats were observed twice daily (Morning and evening) to ensure closer monitoring of infected bats. A security staff was detailed 24 hours' duty to record observations and to immediately inform the team of night deaths for early postmortem.

The individually housed bats were segregated in three groups (3 bats in each group). Each group was inoculated intracranially with one of the three LBV isolates, and the last group of three control bats was inoculated with Minimum Essential Medium (MEM), Gibco®, U.K).

Before inoculation, the bats were weighed, forearm measured and baseline blood, throat and faecal swabs taken. The bats were anaesthesised using a combination of ketamine (Ketamine hydrochloride 115.36mg/ml, Fort Dodge Animal Health ltd, U.K.), at a dose of 5mg/kg of body weight and Meditomidine (Dorbene vet 1mg/ml as Medetomidine hydrochloride, Laboratories SYVA S. A., Spain), at 0.05mg/kg of body weight. The bats were inoculated intracranially (I.C) with $10^{3.5}$ $TCID_{50}$ of each LBV isolate in 30µl volume.

The procedure was performed surgically, with fixation of each bat on a stereotactic frame, drilling of skull 2-3 mm lateral from the center and 3-4 mm behind the eye, and penetration of meninges with a 26-gauge needle. The entire inoculation was performed via

insertion of 45-gauge needle to the depth of 5 mm into bat brain, and slow infusion of the inoculum at a rate of 5µl / min. The needle was removed slowly over 1 minute to avoid reflux of the inoculum. The wounds were disinfected and sutured. Bats were implanted (S.C) with individual transponder chips (Bio Medic Data system Inc.) for identification and also to assist in recording daily body temperature.

After completion of surgery, anesthesia was reversed with Atipamezole (Veterinary Essentials, UK). After full recovery from anaesthesia, bats were returned to the individual cages. They were put on a 3 days treatment with analgesia, Meloxicam 2mg/ml (BoehringerIngelheim Vet medica GmbH, Germany) and antibiotics, Baytril 2.5% (Bayer plc, U.K.). Table 3.1 gives details of bats and LBV isolates used. It indicates the bats ID (cage number indicated on padlock) and morphometrics of each bat.

5.2.3 Experiment 2: Peripheral Inoculationof bats

The Ghana isolate (FLI), in serial dilution was used for the peripheral challenge. Individually caged bats were subdivided into five groups, (4 animals each). These groups were inoculated with different doses of LBV isolate FL: group 1, $10^{4.1}$ $TCID_{50}$; group 2, $10^{3.1}$ $TCID_{50}$; group 3, $10^{2.1}$ $TCID_{50}$; group 4, $10^{1.1}$ $TCID_{50}$; and group 5, $10^{0.1}$ $TCID_{50}$. All bats were inoculated intramuscularly (i.m) in the masseter muscle with 30ul of the inoculum. Bats were implanted with transponder chips for identification and digital recording of daily temperature of bats. Details are presented in Table 3.2 in chapter 3.

Table 5.1: **Incubation periods of bats inoculated peripherally (into masseter muscle) with serial dilutions of LBV**

Inoculation Date	Group	Bat ID	Age	Bat Weight	Virus dose ($TCID_{50}$)	Date Died	Incubation Period days
5/3/2014	1	EXP2EH001	SA	220	$10^{4.1}$	13/3/2014	8
5/3/2014	1	EXP2EH002	SA	195	$10^{4.1}$	12/8/2014E	H
5/3/2014	1	EXP2EH003	A	196	$10^{4.1}$	12/8/2014E	H
5/3/2014	1	EXP2EH004	A	227	$10^{4.1}$	14/3/2014	9
5/3/2014	2	EXP2EH005	A	229	$10^{3.1}$	12/8/2014E	H
5/3/2014	2	EXP2EH006	SA	267	$10^{3.1}$	12/8/2014E	H
5/3/2014	2	EXP2EH007	SA	201	$10^{3.1}$	13/3/2014	8
5/3/2014	2	EXP2EH008	SA	201	$10^{3.1}$	18/3/2014	13
5/3/2014	3	EXP2EH009	A	215	$10^{2.1}$	15/3/2014	10
5/3/2014	3	EXP2EH010	A	160	$10^{2.1}$	18/3/2014	13
5/3/2014	3	EXP2EH011	SA	202	$10^{2.1}$	22/3/2014	17
5/3/2014	3	EXP2EH012	SA	185	$10^{2.1}$	20/3/2014	15
5/3/2014	4	EXP2EH013	A	200	$10^{1.1}$	17/3/2014	12
5/3/2014	4	EXP2EH014	SA	235	$10^{1.1}$	12/3/2014	7
5/3/2014	4	EXP2EH015	A	248	$10^{1.1}$	12/8/2014E	H
5/3/2014	4	EXP2EH016	A	220	$10^{1.1}$	12/8/2014E	H
5/3/2014	5	EXP2EH017	SA	165	$10^{0.1}$	13/8/2014E	H
5/3/2014	5	EXP2EH018	A	185	$10^{0.1}$	13/8/2014E	H
5/3/2014	5	EXP2EH019	A	210	$10^{0.1}$	3/6/2014	61
5/3/2014	5	EXP2EH020	A	210	$10^{0.1}$	13/8/2014E	H

Key: E- Euthanised**; H-**Healthy at end of observation**.**

5.2.4 Observations and Sampling

Observation: In both experiments, bats were observed twice daily and any signs of disease were recorded. When clinical signs occurred, bats were observed every two hours, and euthanized following the development of clinical disease or at the end of observation with

intracardiac injection of Pentoject (Pentabarbitone Sodium 20% w/v solution for injection, Animalcare Ltd, York, UK). The disease was allowed to run its course as far as welfare considerations permitted. This was essential to establish whether recovery was possible as this is one of the major outstanding scientific questions in the field of lyssavirus ecology in bats.

Sampling: Dead or euthanized bats were transported on ice for postmortem sampling. Cross-contamination was avoided by using a clean set of scalpels to carefully remove each individual tissue from the fresh carcass.

Bats in both experiments were weighed, bled, and throat and faecal swabs taken on the day of inoculation to provide baseline antibody titre and other information on bats enrolled in the experiment. Temperature was measured daily, blood, saliva and faecal swabs were taken twice a week during 3 weeks post challenge along with body weight determination. The same specimens were collected once weekly from 3 to 6 weeks, and fortnightly after six weeks.

When clinical signs compatible with rabies were observed in a bat of any group, oral swabs were taken daily during a week from all group members. Two oral swabs were taken each time. One was placed in a tube containing MEM for virus isolation and the other throat swab placed in a tube with RNAlater for RNA extraction. Swabs and tissue samples were put in a flask containing ice and transported for storage at -80°C at Noguchi Memorial Institute for Medical Research (NMIMR), University of Ghana.

The experiment was terminated 90 days after the death of the last bat. Terminal blood was collected from dead or euthanized bats, and necropsy was performed immediately at the Animal Experimentation Department of NMIMR.

Predetermined set of tissues were collected and they included: heart, lung, liver, spleen, kidney, duodenum, ileum, large intestine, salivary gland, lymph node, brain (cerebellum, medulla, rostrum and hippocampus). Several specimens of each tissue were taken in plain tubes, RNAlater, and formalin. Tissues in plain tubes were immediately stored at -80°C. Those in RNAlater were stored at +4°C for 4 days to soak tissues. Tubes were emptied of RNAlater before transferring to -80°C. Formalin samples were stored at room temperature with the replacement of the first portion of formalin after 72 hours of storage.

5.2.5 Laboratory Analysis of Bat Samples

Bat samples from the i.c and i.m experiments were analysed using Flourescent Antbody Virus Neutralization Test (FAT), Rabies Tissue Culture Inoculation Test (RTCIT) and Molecular PCR Methods (SYBR Green PCR and Hemi-Nested PCR) at the AHVLA laboratory in U.K. Details of each methodology are provided in the material and method section (Chapter 3) of this thesis.

Direct fluorescent antibody test (FAT)*:* FITC monoclonal antibody conjugate was used in an FAT to stain impressions of 3 regions of the brain including the hippocampus, cerebellum and medulla. The procedure was performed as described by Dean *et al.* (1996)Dean (1996). Impression smear of brain sample was fixed with acetone and airndry for 20-30 minutes. It was stained with FITC monoclonal antibody conjugate and incubated at 37°C for 30 – 45minutes. The slids were washed in 0.1M PBS 7.2, rinsed with distil water and air dry. Slides were observed using flourescent microscope. Details is in appendix 3 and mthod section of this thesis.

Rabies tissue culture isolation test (RTCIT): The RTCIT involves the isolation and culture of lyssavirus from a homogenate of a suspect test specimen in mouse neuroblastoma cells. Bat brain tissue samples were homogenized by adding brain (cerebellum) tissue of bats to Dulbecco PBS and antibiotics to make a homogenate of approximately 10%. Samples were inoculated in MNA cells and incubated at 37°C in 5% CO2 for a minimum of 30 minutes to allow the cells to attach. The presence of lyssavirus antigens in the cells was detected by FAT.

Serology: The presence of antibodies against LBV in the bat blood collected was determined using mFAVN, as described previously (Cliquet *et al.*, 1998; Hayman *et al.*, 2008) using an anti-rabies FITC monoclonal antibody conjugate with fluorscein isothiocyanate (Centecor, USA) and a Challenge Virus standard rabies virus (CVS-11).

Detection of viral RNA: SYBR green PCR and Hemi-nested PCR were used to detect viral RNA in the samples. The total RNA was extracted from tissue homogenates by Trizol reagent as described in the methods section (chapter 3) of this work. The RNA was diluted 1:10 in HPLC water before being quantified by using Nanodrop 2000 Spectrophotometer (Thermo Scientific, UK). The RNA was diluted to 1µg/µl before use as template in the PCR.

SYBR Green real time RT-PCR

The RNA was reversed transcribed using MMLV-RT as published previously (Heaton, *et al.*, 1997; Wakeley, *et al.*, 2005). SYBR® Green Hot Start (Applied Biosystems) PCRs were performed using 20µL SYBR® Green Hot Start, 13µL nuclease-free water, 1µL of JW12 (20pmol/µL) primer, 1µL of N165-147 (20pmol/µL) primer, and 5µL of cDNA template.

The iScript one-step RT-PCR kit with SYBR Green is a highly sensitive assay in which cDNA synthesis and PCR amplication are carried out in single tube. The assay includes two major phases: amplification and dissociation. The amplification phase results in the generation of dsDNA which is detected by SYBR Green. In the dissociation phase, the dsDNA product is melted into ssDNA by a stepwise increase in temperature, with fluorescence data being collected at each step. This dissociation phase gives an indication of the amplicon size.

Hemi-nested PCR

The RNA samples were reverse transcribed and amplified using a hemi-nested PCR incorporating pan-lyssavirus primers (JW6/12, JW10/12), with a standard 18S ribosomal RNA internal control where necessary, as previously described (Heaton *et al.*, 1997). RNA was reverse transcribed by Moloney murine leukemia virus reverse transcriptase (MMLV-RT) (Ambion). A laboratory adapted RABV (CVS-11) RNA and cDNA were used as positive controls. Reverse Transcription Master-mix, Hemi-nested 1st and 2nd PCR Master-mix used in PCR for the bat samples are described in the methodology section of this thesis.

Sequencing of PCR Products

PCR product from one of each of the three isolates used in the i.c study was sequenced. A partial Ngene was sequenced to compare the virus used for the inoculation and the virus isolated from the infected bats. Direct consensus DNA sequencing of the amplified 405bp region of the nucleoprotein (N) gene was undertaken as previously described (Johnson, *et al*. 2003). Sequences produced were edited using SeqMan (DNASTAR Lasergene®) and aligned with published sequence data from Gen Bank using multiple sequence alignment

(MSA) (DNASTAR Lasergene®). The phylogenetic relationship predicted by the MSA was visualised in Megalign following bootstrapping with 1000 trials.

Serology

Serum neutralization is the gold standard for the detection and quantification of antiviral antibodies. The presence of antibodies against LBV in the bat blood collected was determined using the mFAVN, as described previously (Cliquet *et al.*, 1998). The mFAVN method allows the evaluation of sero-neutralising antibody levels by incubating a constant dose of titrated virus with serial dilutions of sera. Briefly, the LBVNig56 isolate used for the mFAVN was prepared to a working dose of 100 tissue culture infective doses (TCID)50/50 µl. Negative control sera (a pool of naïve, unvaccinated dog sera) and two positive anti-LBVNig56 rabbit sera were used. Plates were fixed in 80% acetone and stained with Centocor fluorescein-labelled (FITC) anti-rabies monoclonal globulin conjugate (diluted to 1:40 in 0.1M PBS pH 7.2). Plates were incubated for 30 minutes at 37°C, before washing with 0.1M phosphate buffered saline (PBS) at pH 7.2 and examined using a 488nm fluorescent UV microscope.

Ethics Statement: The study, experimental design and animal care were approved by the Noguchi ethics committee (Ghana) and Institute of Zoology of the Zoological Society of London. Permission for collection of bats for breeding was obtained from the Ghana Wildlife Division of the Forestry Commission.

5.3 Data Handling and Statistical Analyses

In the I.C inoculated bats, data were analysed using R – Software (R Core Team, 2014). The differences in the incubation periods of the isolates used for experiment 1 (intracranial) were determined to decide on the isolates to select for experiment 2.

5.4 Results

5.4.1 Results of Experiment 1- Intracranial inoculation.

5.4.1.1 Incubation Period using Isolates (i.c inoculation)

The incubation period recorded with the various isolates are given in table 5.2 and Fig 5.1

Table 5.2: Mean incubation period of Lagos Bat Virus isolates (used for I.C inoculation) in straw-colored fruit bats inoculated intracranially.

Isolate	Mean Incubation Period
Ghana (FLI)	5 ± 0.6 days
Nigeria (RV1)	8 ± 0 days
Senegal (RV41)	5 ± 1.0 days
Control	>10days

The difference in incubation period between the different isolates was significant (Kruskal-Wallis chi-squared = 9.85, df = 3, p-value = 0.019).

The results of a post-hoc test used to determine any significant differences in the means of incubation periods of the isolates are provided in Table 5.3**.**

From Table 5.3 and Fig 5.1, the means of incubation periods between the various isolates as well between the isolates was all significantly different with the exception of means of FL1 and RV41 that were not significantly different (P>0.05).

We used clinical symptoms, source and knowledge of passage history to decide on the isolate to use for the experiment 2. The Ghana isolate with symptoms closer to classical furious rabies was the best choice for experiment 2

Table 5.3: Post-hoc Test for difference in means of incubation periods of the isolates used in I.C inoculations.

	Control	FL1	RV1	RV41
Control	-	P=4.3 E-5	P=0.012	P=2.6 E-5
FL1		-	P=0.0021	P=0.89
RV1			-	P=9.8E-4
RV41				-

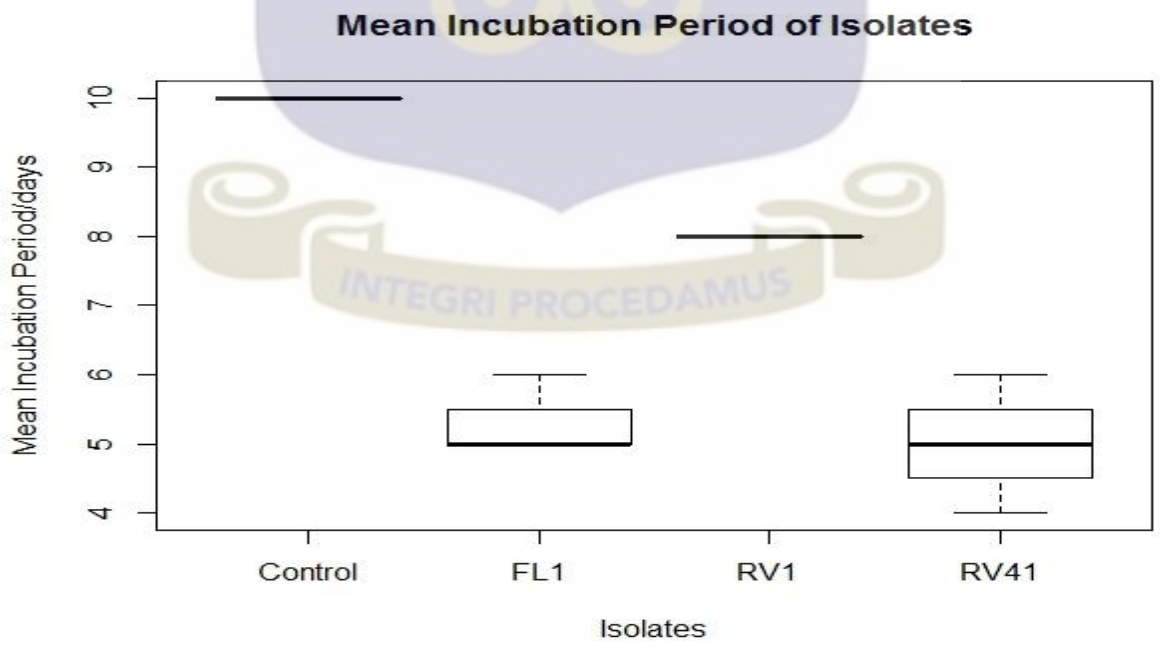

Fig 5.1: Mean Incubation period of LBV Isolates used in the i.c inoculated bats

5.4.1.2 Clinical observation of bats with Intracranial Inoculation:

All 9 bats inoculated IC with LBV developed clinical signs of rabies. Major clinical observations included irritability, hyperactivity, aimless movement in the cage, increased vocalization with flapping wings while hanging on the cage sealing, significant excretion of foaming saliva, aggression with unprovoked biting of cage wires, muscle tremors and spasms, self- urination and self-defecation. The disease progressed rapidly. Within a few hours these signs generally progressed to paresis of limbs and further to general paralysis. Sometimes the animals that were observed apparently healthy on the previous evening were found dead at the bottom of the cage in the morning. The bats did not eat during the clinical disease but retained the ability to drink water from their bowls until the terminal stage. The bats inoculated with the FL1 isolate demonstrated "furious" clinical signs compared to bats inoculated with the RV41 and RV1 isolates. None of the bats experienced significant changes of body temperature except the terminal stage of the disease (paralysis) when body temperature was decreased to 2-3°C compared to non-infected individuals. Weight measurements were not performed given the short duration of incubation and clinical periods.

5.4.2.3 Clinical outcome of Intracranial Inoculation

Clinical observations and outcome of infection following IC are detailed in Table 5.4. All these bats survived over 48 hours post inoculation. All bats inoculated IC with the three LBV isolates developed clinical signs compatible with rabies on days 4 to 8 (mean 6.11) post inoculation. The disease progressed rapidly. In a few hours bats developed generalized paralysis and were euthanized. Two bats were found dead in the morning

although they had not demonstrated clinical signs the previous evening. There was 100% mortality in bats inoculated IC with LBV, but all control bats survived.

Table 5.4: **Clinical Observation in i.c. inoculated bats.**

Bat ID	LBV Strain	Clinical Observations	Outcome	Incubation Period (days)
IC1311BIC01	MEM (CONTROL)	No clinical sign observed	Euthanasia, 22/10/2013	N/A
IC0721BIC02	MEM (CONTROL)	No clinical sign observed	Euthanasia, 22/10/2013	N/A
IC9113BIC03	MEM (CONTROL)	No clinical sign observed	Euthanasia, 22/10/2013	N/A
IC6179BIC05	RV41	Weak, muscle spasm, Depression and foaming muscle	Euthanasia, 18/10/2013	6
IC1191BIC06	RV41	tremors	Died 17/10/2013	5
IC1711BIC07	RV1	Severe Depression, muscle Spasm.	Died 21/10/2013	8
IC7193BIC08	RV1	Depression, Spasm, foaming in mouth	Euthanasia, 21/10/2013	8
IC5368BIC09	RV1	Depression, Spasm, foaming in mouth	Euthanasia, 21/10/2013	8
IC4146BIC10	FL1	Aggression (biting cage wire), Vocalization, lip smacking	Euthanasia, 18/10/2013	5
IC8308BIC11	FL1	Aggression (biting cage wire), Hindlimb paresis (drop in drinking water)	Euthanasia, 18/10/2013	5
IC4741BIC12	FL1	Aggression (biting cage wire), agitation, Vocalization, Hindlimb paresis	Euthanasia, 19/10/2013	6
IC4099BIC13	RV41	Depression, tremors	Euthanasia, 18/10/2013	4

5.4.1.4 Laboratory findings of i.c Infected bats

Results of virus detection by FAT: The impressions of hippocampus, cerebellum and medulla sections of the brain from all bats that were inoculated I.C were positive for viral antigens by FAT (Table 5.4|). As expected, the 3 control (IC1311BIC01, IC0721BIC02, IC9113BIC03) bats in this experiment tested negative.

Table 5.5: FAT Results for I.C inoculated bats was positive for all bats except control bats

Bat ID/	Inoculated Solate	Cerebellum	Hippocampus	Medulla
IC1311BIC01	MEM	-	-	-
IC0721BIC02	MEM	-	-	-
IC9113BIC03	MEM	-	-	-
IC6179BIC05	RV41	+	+	+
IC1191BIC06	RV41	+	+	+
IC1711BIC07	RV1	+	+	+
IC7193BIC08	RV1	+	+	+
IC5368BIC09	RV1	+	+	+
IC4146BIC10	FL1	+	+	+
IC8308BIC11	FL1	+	+	+
IC4741BIC12	FL1	+	+	+
IC4099BIC13	RV41	+	+	+

Key:MEM – Virus medium; RV41 –Senegal Isolate; RV1-Nigeria Isolate; FL1- Ghana Isolate

Results of Molecular Testing in intracranial inoculated bats: SYBR green Real Time PCR and Hemi-nested PCR were undertaken with samples of bats from Experiment 1.

*SYBR green PCR: Six (*6) out of the twelve infected bat samples from bats inoculated intracranially tested positive by qPCR. The 6 positive samples were from the three (3) bats that were infected with the Senegal isolate (RV4) (IC1311BIC05, IC1179BIC06 and IC4099BIC13) and the three bats infected with the German isolate (FLI) (IC4146BIC10, IC8308BIC11 and IC4741BIC12). No virus RNA were detected in any of the samples from three (3) bats that were inoculated with the Nigeria isolate (RV1). Challenge Virus Standard (CVS) was used as the positive control. All 12 samples were positive for B-actin as expected. Table 5.6 gives details of the SYBR green PCR results.

Table 5.6: **SYBR green PCR Results for intracranially inoculated bats**

Well Name (Bat ID)	**Inoculated Isolate**	**Ct Value**	**Results**
IC1311BIC01	MEM	No Ct	-
IC0721BIC02	MEM	No Ct	-
IC9113BIC03	MEM	No Ct	-
IC6179BIC05	RV41	25.26	+
IC1191BIC06	RV41	25.24	+
IC1711BIC07	RV1	No Ct	-
IC7193BIC08	RV1	No Ct	-
IC5368BIC09	RV1	No Ct	-
IC4146BIC10	FL1	24.14	+
IC8308BIC11	FL1	25.14	+
IC4741BIC12	FL1	23.75	+
IC4099BIC13	RV41	25.37	+
CVS552(Control)		20.87	+

Key:MEM – Virus medium; RV41 –Senegal Isolate; RV1-Nigeria Isolate; FL1- Ghana Isolate; Ct cutoff point.

Hemi-nested PCR: PCR products were run on 1.8% agarose gel. 6ul PCR product +4ul blue loading dye was used. 10ul was loaded on the gel and run at 120V. All brain samples, from i.c. inoculated bats, except samples IC1311; 0721; 9113 from control bats were positive in the 1st round PCR (Table 5.7). Results of the 2nd round PCR matched the 1st round PCR results. Controls were positive and negative as expected. Fig 2 presents the agarose result of the second PCR results.

All salivary gland samples were negative in the 1st round of PCR. Positive controls were positive and water alone no template control (NTC) negative. Four bat samples IC6179BIC05, IC1191BIC06, IC8308BIC11 and IC4099BIC13 were positive after the 2nd

round of PCR. These were 2 of the 3 bats infected with RV41 isolate and 2 of the 3 bats infected with FL isolate. All 3 bats infected with RV1 isolate did not show viral RNA in the salivary gland. The obtained PCR results were confirmed by direct sequencing. Positive controls were positive and NTC negative.

Table 5.7: Hemi-nested PCR Results. All infected bat cerebellum was positive, while some salivary glands were negative

1st Round hn- PCR Results			2nd Round hn-PCR Results	
Bat ID	**Cerebellum**	**Salivary gland**	**Cerebellum**	**Salivary gland**
IC1311BIC01	-	-	-	-
IC0721BIC02	-	-	-	-
IC9113BIC03	-	-	-	-
IC6179BIC05	+	-	+	+
IC1191BIC06	+	-	+	+
IC1711BIC07	+	-	+	-
IC7193BIC08	+	-	+	-
IC5368BIC09	+	-	+	-
IC4146BIC10	+	-	+	-
IC8308BIC11	+	-	+	+
IC4741BIC12	+	-	+	-
IC4099BIC13	+	-	+	+

.Key: - negative; + positive

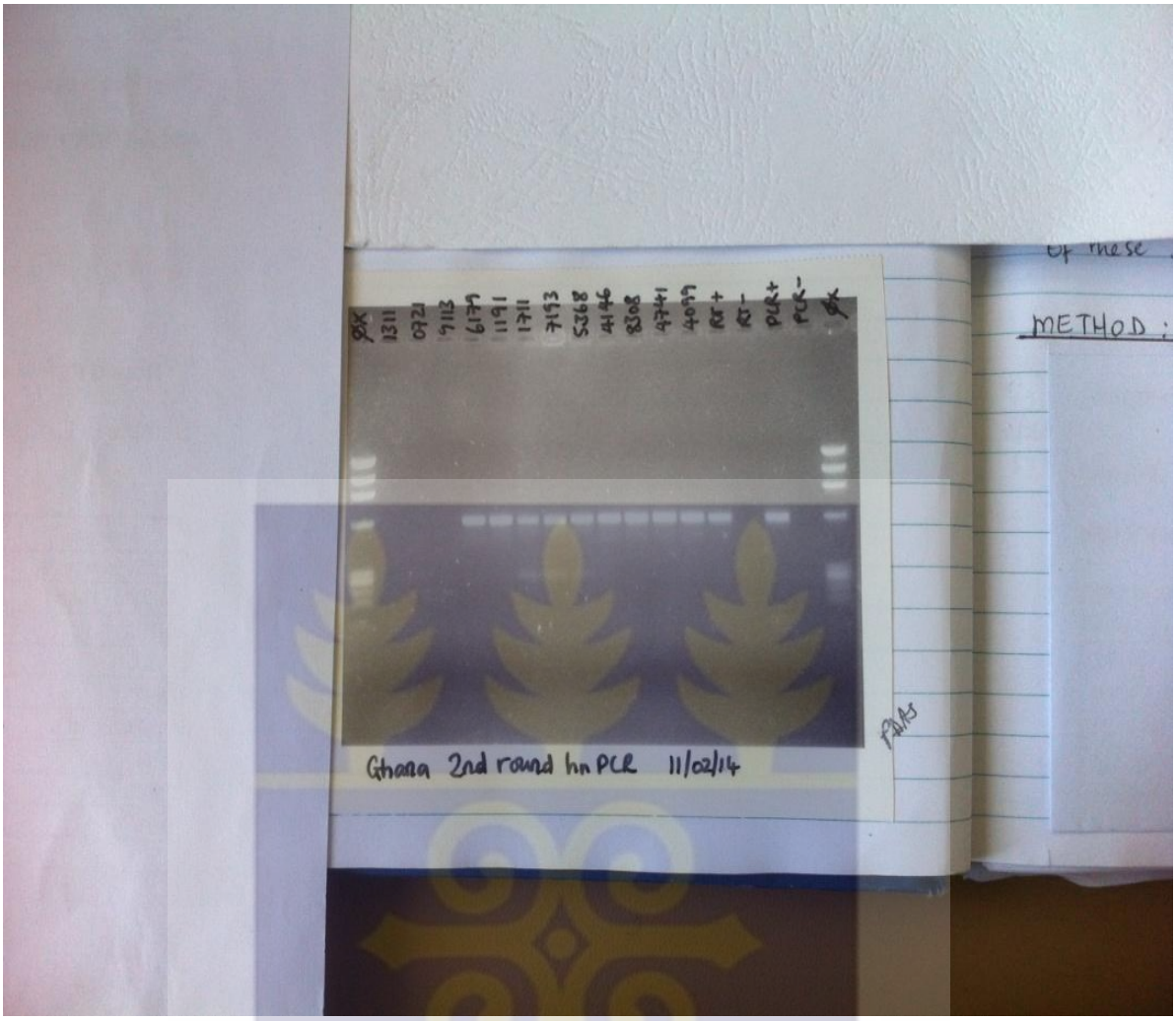

Fig 5.2: **Agarose gel results for 2nd round hn-PCR for Cerebellum. All samples are shown positive**

Results of sequencing of virus from I.C inoculation: Sequence results of the PCR product of a sample each from the 3 isolates used for the I.C experiment matched with Ngene of viral isolates used (Table 5.8) except Bat IC4146BIC10 which has 99.30% probably due to handling or PCr error or due to passage history of virus.

Table 5.8: **LBV Ngene partial sequence results.**

Bat ID	Closest BLAST Alignment	% Identity
IC6179BIC05	EF547448.1 (LBVSEN1985 Isolate)	100%
IC1711BIC07	EF547459.1 (LBVNIG1956 isolate)	100%
IC4146BIC10	EU259198 (LBVKE131 isolate)	99.30%

5.4.2 Experiment 2 – Peripheral inoculation:

5.4.2.1 Incubation period of dose groups:

Fig 5.3 presents the mean incubation periods of the groups. The mean incubation period ranged from 8 days to 61 days in the experimental animals. There was no significant difference in the means of the various virus dose (Kruskal-Wallis chi-squared = 3.30, df = 4, p-value = 0.51).

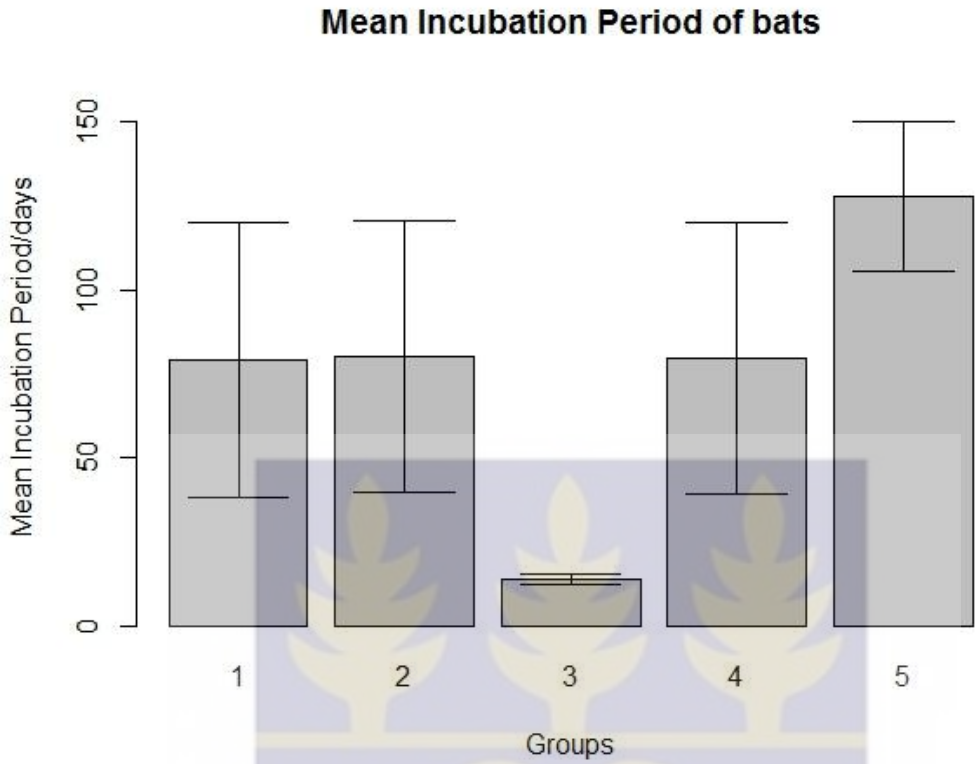

Fig 5.3: Mean Incubation Period of the group of bats peripherially inoculated

5.4.2.2 Clinical Observation of Peripheral Inoculation

Clinical presentation in bats that had peripheral (i.m) inoculation with serial dilutions are presented in Table 5.9. Fig. 5.4 showed some of the clinical signs observed in diseased bats.

Among the 11 bats that developed clinical disease, eight (8) died and three were euthanized at terminal stage. It appeared that susceptibility of bats to a relatively low virus dose might be greater than susceptibility to high doses (4/4 versus 2/4 bats succumbing) although this was not statistically significant (p=0.43). Incubation periods were 7-61 days, longer than those observed in the intracranial inoculated bats. The incubation period did not appear to depend on the virus dose, although the one bat which was found dead 61

days post inoculation was from the group infected with the lowest virus dose, $10^{0.1}$ $TCID_{50}$. This was FAT-positive. The duration of clinical period was also similar: the disease progressed to terminal stage during several hours, and in one bat (EXP2EH008) it was longer than 24 hours. Clinical signs were compatible with those observed in the intracranial infected bats. The biting behavior was very prominent. The bats hanging on the cage ceiling demonstrated periods of increased anxiety, disoriented movement, flapping wings and vocalization, frequently bending up and biting the cage wires with "mortal grip". In addition, similarly to the bats inoculated intracranially, these bats did not demonstrate changes in body temperature except the very end of the clinical course when it decreased

Fig 5.4: **LBV infected bat showing salivary discharge and aggression (biting fence).**

5.4.2.3 Clinical outcome in Peripheral Inoculation

Eleven (55%) bats from the 5 serial dose groups (2 from group 1; 2 from group 2; 4 from group 3; 2 from group 4; and 1 from group 5) developed clinical signs compatible to rabies. Nine (9) of the sick bats died and two (2) sick bats were euthanized based on welfare judgment. Nine bats (45%) survived the infection and were euthanized at the end of the experiment which was 90 days after the last death. Diagnoses of all bats were confirmed by FAT. Remarkably, 100% mortality was observed in the group 3, inoculated with $10^{2.1}$ $TCID_{50}$ of the virus, whereas bats inoculated with higher and lower doses demonstrated 25-50% mortality rates.

Table 5.9: Clinical Outcome of bats inoculated with Ghana Isolate peripherally.

Bat ID	Virus titre	Inoculation date	Clinical Observation	Clinical Outcome	Clinical duration	Incubation period
EXP2EH001	Neat virus	05-03-14	Not sick, found dead	Died,13/3/2014	Nil	8 days
EXP2EH002	Neat virus	05-03-14	Survive	Euthanasia,12/8/2014	N/A	N/A
EXP2EH003	Neat virus	05-03-14	Survive	Euthanasia,12/8/2014	N/A	N/A
EXP2EH004	Neat virus	05-03-14	Not fed for 1 night, aggression, non-motivated movement, and vocalization.	Died, 14/3/2014	1day	9 days
EXP2EH005	-1	05-03-14	Survived	Euthanasia,12/8/2014	N/A	N/A
EXP2EH006	-1	05-03-14	Survived	Euthanasia,12/8/2014	N/A	N/A
EXP2EH007	-1	05-03-14	Non-motivated motion, volcalization, wing flapping and aggression (biting cage mesh) on 12/3/14 and died same day	Died, 13/3/2014	1day	8 days
EXP2EH008	-1	05-03-14	Not fed for 1 day, 17/3/2014 sick - ataxia, self-urination, aggression(biting cage)	Died, 18/3/2014	2days	13 days
EXP2EH009	-2	05-03-14	Not fed for 1 day, non-provoked movement, and spasm, very weak.	Euthanasia, 15/3/2014	1day	10 days
EXP2EH010	-2	05-03-14	Not fed for 1day, Self urination, and defaecation, wing flapping, vocalization, aggression (cage biting).	Died, 18/3/2014	1day	13 days

Table 5.9 continued

Bat ID	Virus titre	Inoculation date	Clinical Observation	Clinical Outcome	Clinical duration	Incubation period
EXP2EH011	-2	05-03-14	Wing flapping, non-motivated motion, aggression (biting cage), paralysis of hind limbs	Euthanasia, 22/3/2014	□ 1day	17 days
EXP2EH012	-2	05-03-14	Not sick, Found dead, enlarged lymph node at PM	Died, 20/3/2014	Nil	15 days
EXP2EH013	-3	05-03-14	Not fed for 1 day, self-urination, wing flapping, prostration	Died, 17/3/2014	□ 1day	12 days
EXP2EH014	-3	05-03-14	Mutilation (tongue), fractured finger	Died, 12/3/14	□ 1day	7 days
EXP2EH015	-3	05-03-14	Survived	Euthanasia,12/8/2014	N/A	N/A
EXP2EH016	-3	05-03-14	Survived	Euthanasia,12/8/2014	N/A	N/A
EXP2EH017	-4	05-03-14	Survived	Euthanasia,13/8/2014	N/A	N/A
EXP2EH018	-4	05-03-14	Survived	Euthanasia,13/8/2014	N/A	N/A
EXP2EH019	-4	05-03-14	Emaciation, found dead	Died, 5/5/2014	Nil	61 days
EXP2EH020	-4	05-03-14	Survived	Euthanasia,13/8/2014	N/A	N/A

Key: N/A – Not applicable; □ - Less than; Nil – No clinical duration

Results of virus detection by FAT (i.m inoculated bats)

In the experiment 2 (peripheral inoculation), all bats that developed clinical signs and died or were euthanized, tested positive for lyssavirus infection of the brain (hippocampus, cerebellum and medulla) by FAT. All bats that survived till the end of observation period were FAT-negative (Table 5.10).

Table 5.10: **FAT Results for peripheral inoculated bats. All bats that died tested positive for LBV while all surviving bats tested negative**

Bat ID	Hippocampus	Cerebellum	Medulla
EXP2EH001	+	+	+
EXP2EH002	-	-	-
EXP2EH003	-	-	-
EXP2EH004	+	+	+
EXP2EH005	-	-	-
EXP2EH006	-	-	-
EXP2EH007	+	+	+
EXP2EH008	+	+	+
EXP2EH009	+	+	+
EXP2EH010	+	+	+
EXP2EH011	+	+	+
EXP2EH012	+	+	+
EXP2EH013	+	+	+
EXP2EH014	+	+	+
EXP2EH015	-	-	-
EXP2EH016	-	-	-
EXP2EH017	-	-	-
EXP2EH018	-	-	-
EXP2EH019	+	+	+
EXP2EH020	-	-	-

Molecular test for Peripherally Inoculated bats

To investigate the presence of viral RNA in samples, the brain (hippocampus, cerebellum and medulla portions), and salivary glands were tested by SYBR Green and Hemi-nested RT-PCR techniques.

Eight bat brains tested positive by SYBR green PCR. Results by hemi-nested PCR were inconsistent in both the 1st and 2nd round test. Bat 019 tested positive by Hn-PCR in the 1st round while 5 bat brains (bats 010;011; 012; 013; and 014) tested PCR positive only in the 2nd round of PCR. All bat saliva glands except bat no.008 tested negative by SYBR green PCR and Hemi-nested PCR. Bat no.008 salivary gland tested positive in the 2nd round of Hemin-nested PCR. Details are presented in Table 5.11.

Table 5.11: SYBR Green and Hn-PCR results for peripheral inoculated bats.

	RT-PCR (real-time SYBR Green)		RT-PCR (hemi-nested)	
BAT ID	**Brain (H/C/M)**	**Salivary gland**	**Brain (H/C/M)**	**Salivary gland**
EXP2EH001	+	-	-	-
EXP2EH002	-	-	-	-
EXP2EH003	-	-	-	-
EXP2EH004	+	-	-	-
EXP2EH005	-	-	-	-
EXP2EH006	-	-	-	-
EXP2EH007	-	-	-	-
EXP2EH008	+	-	-	+ 2nd round
EXP2EH009	-	-	-	-
EXP2EH010	-	-	+ 2nd round	-
EXP2EH011	+	-	+ 2nd round	-
EXP2EH012	+	-	+ 2nd round	-
EXP2EH013	+	-	+ 2nd round	-
EXP2EH014	+	-	+ 2nd round	-
EXP2EH015	-	-	-	-
EXP2EH016	-	-	-	-
EXP2EH017	-	-	-	-
EXP2EH018	-	-	-	-
EXP2EH019	+	-	+ve 1st round	-
EXP2EH020	-	-	-	-

Rabies Tissue Culture Inocuation Test for i.m peripherally inoculated bats:

Virus culture was performed to evaluate the presence of infectious virus in the brain and salivary glands of the experimentally-infected bats. The virus was detected in the brain of 10 out of the 11 FAT-positive bats (Table 5.12).

Table 5.12: Rabies Tissue culture inoculation Test (RTCIT). There was positive culture of virus in ten brain samples, while no virus was cultured in the Salivary glands.

BAT ID	Brain (H/C/M)	Salivary gland
EXP2EH001	-	-
EXP2EH002	-	-
EXP2EH003	-	-
EXP2EH004	+	-
EXP2EH005	-	-
EXP2EH006	-	-
EXP2EH007	+	-
EXP2EH008	+	-
EXP2EH009	+	-
EXP2EH010	+	-
EXP2EH011	+	-
EXP2EH012	+	-
EXP2EH013	+	-
EXP2EH014	+	-
EXP2EH015	-	-
EXP2EH016	-	-
EXP2EH017	-	-
EXP2EH018	-	-
EXP2EH019	+	-
EXP2EH020	-	-

Serology (mFAVN) of Experiment bats (peripheral inoculation):

The titre of sera were evaluated for the presence of antibodies using mFAVN. All bats sera with antibody titre 15.59 and above are considered to be seropositive. All sera taken from bats before experiment did not show seroconversion (Table 5.12). A total of seven bats (35%) seroconverted, five of the bats that seroconverted survived while 2 died.

Table 5.13: Experiment 2 Serology Results. A total of Seven (7) bats Seroconverted: Five (5) Surviving bats and Two that died of rabies

	TITRE OF VNA AT DAYS POST INOCULATION										
BAT ID	**0**	**7**	**10**	**14**	**17**	**24**	**31**	**46**	**59**	**74**	**88**
EXP2EH001	5.20	9.00									
EXP2EH002	5.20	9.00	81.00	9.00	5.20	46.77	5.20	27.00	9.00	9.00	5.20
EXP2EH003	5.20	5.20	5.20	5.20	5.20	15.59	5.20	5.20	5.20	5.20	5.20
EXP2EH004	5.20										
EXP2EH005	5.20	5.20	5.20	5.20	9.00	9.00	5.20	9.00	9.00	5.20	5.20
EXP2EH006	5.20	46.77	2187.00	243.00	420.89	46.77	140.89	243.00	81.00	81.00	27.00
EXP2EH007	5.20										
EXP2EH008	5.20	5.20	27.00								
EXP2EH009	5.20	9.00									
EXP2EH0010	5.20	5.20									
EXP2EH0011	5.20	No sample	9.00	5.20							
EXP2EH0012	5.20	5.20	5.20								
EXP2EH0013	5.20	9.00									
EXP2EH0014	5.20	5.20									

EXP2EH0015	5.20	5.20	9.00	5.20	5.20	5.20	5.20	9.00	5.20	5.20	5.20
EXP2EH0016	5.20	5.20	5.20	5.20	9.00	5.20	5.20	5.20	5.20	5.20	5.20
EXP2EH0017	5.20	81.00	5.20	9.00	5.20	5.20	5.20	5.20	5.20	5.20	5.20
EXP2EH0018	5.20	9.00	15.59	5.20	5.20	5.20	5.20	5.20	5.20	5.20	5.20
EXP2EH0019	5.20	5.20	9.00	5.20	5.20	5.20	5.20	9.00	729.00		
EXP2EH0020	5.20	5.20	5.20	5.20	5.20	9.00	5.20	5.20	5.20	5.20	5.20

KEY: Green - Surviving bats Red - Antibody positive

5.4.3 Pathological findings in infected bats

No gross pathological findings were found in any bats inoculated i.c. route by external observation and at necropsy. There were a few gross pathological observations during necropsy of the bats that had the peripheral (i.m.) inoculations. One bat (bat EXP2EH012) had visibly enlarged mandibular lymph nodes. Bat EXP2EH014 had lost half of her tongue (Fig. 5.5) and had a finger amputated probably due to self-mutilation. Bat EXP2019 was noticeably emaciated and had lost weight when died (Base weight before experiment 210g and after experiment 157g).

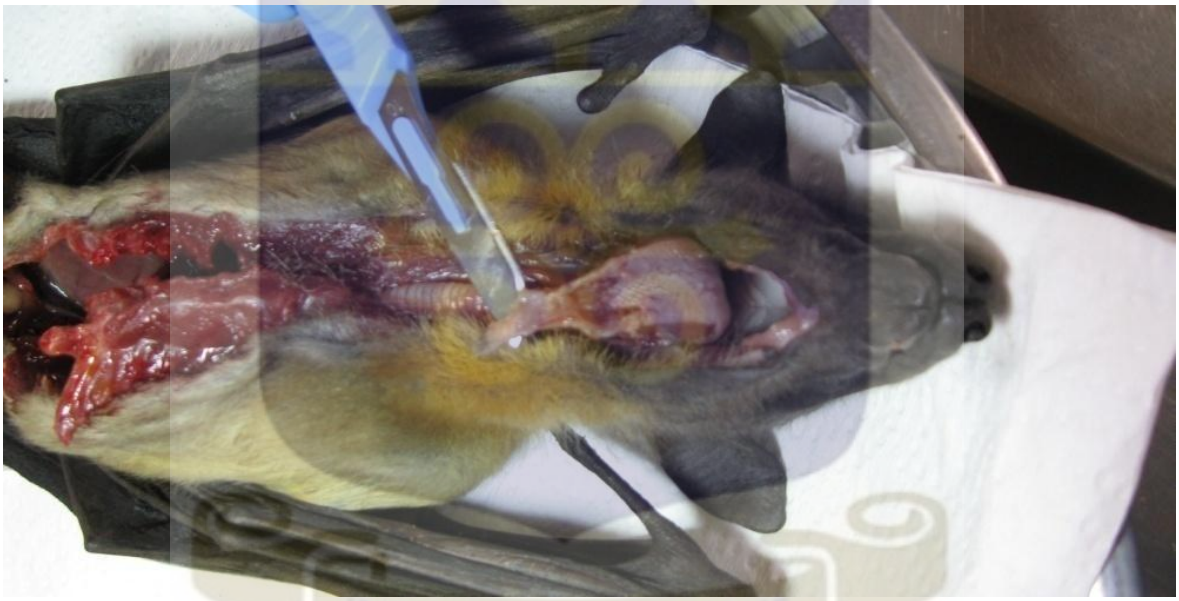

Fig 5.5: Pathological observation in infected bats. Self-Mutilation (Tongue mutilated by an infected rabid bat).

5.5 Discussion

The pathogenesis of LBV continues to present a challenging enigma to scientists; the transmission and pathogenesis of this virus in its natural reservoir hosts under natural conditions is not known.

This study employed two experimental designs to bridge the knowledge gap in the pathobiology of LBV in the natural host, the African straw-colored fruit bat, *Eidolon helvum* (*E. helvum*). Both experimental studies (intracranial and intramuscular inoculations) employed naïve *E. helvum* bats that were bred in a biosecure captive facility established at the Achimota forest, Accra Zoo, Ghana.

In the IC inoculated bats, using 3 different isolates of LBV (RV1 =LBV Nigeria 1956; RV41=LBV Senegal 1985; FL1=LBV Germany Ex Kumasi 2013), 100% mortality was recorded. Bats inoculated IC died within 24 hrs of onset of observed clinical signs. Incubation period varied from 4 days (lowest, Senegal isolate) to 8 days (longest, in the Nigeria isolate). Bats inoculated with the Ghana, 2013 isolate showed furious form of rabies with hyper excitation, aggression (with bats biting cage wire), and excessive salivation / froth at mouth, lip smacking and hind limp paresis. There was hyper aesthesia, hyperactivity and agonal excessive salivation / froth at mouth and limb paresis in the Senegal 1985 isolate. There was general weakness and depression; with some having limb / wing paralysis in bats infected with Nig 1956 isolate.

The LBV RNA was detected in the brain samples from all bats infected via IC inoculation and in salivary gland from 3/3 bats inoculated with the Senegal isolate, 1/3 inoculated with the Ghana isolate and 0/3 inoculated with the Nigeria isolate.

To characterise and differentiate between lyssavirus species and also to phylogenetically characterize isolates (Hoffmann *et al.*, 2010), we sequenced isolates from each of the 3

groups of infected bats. The sequences of isolated virus from IC inoculated bats revealed a high similarity with the viruses used for the experiment, with 99 –100% homology.

A similar pattern of clinical observations in behaviour, with some exceptions, was observed in animals developing signs of the disease in experiments conducted by Franka *et al.* (2008). (Franka *et al.*, 2008) study was to determine the susceptibility of insectivorous bats (using the big brown bat, *Eptesicus fuscus,* as a model) to infection with European bat lyssavirus type 1a (EBLV-1a) and also to assess the dynamics of host immune responses and to evaluate the opportunity for horizontal viral transmission within colonies. Franka *et al.* (2008) reported that the morbidity period lasted 1–2 days, but sudden death, without any other apparent signs, also occurred. Initially, sick animals either separated themselves or were excluded from the rest of the group. Paresis and paralysis later restricted the movement of sick bats and they remained at the bottom of the cage. Observed clinical signs associated with rabies after EBLV-1 infection included tremors, irritability, aggressiveness and paralysis, but sudden death without any apparent signs of disease also occurred in rare cases (Franka *et al.*, 2008). In another study, using the proposed EBLV-1 reservoir host, *E. serotinus*, 100% induction of rabies by intracranial inoculation was demonstrated (Freuling *et al.*, 2009).

In the peripherally inoculated bats, 11 of 20 (55%) bats were infected, had rabies disease and died, either naturally or by euthanasia. This indicates that i.c. is a more efficient route of virus transmission than i.m. inoculation. We recorded 100% mortality (by i.m inoculation) for the relatively low virus dose of $10^{2.1}$ $TCID_{50}$ whereas bats inoculated with higher and lower doses demonstrated less susceptibility to LBV. Studies indicated that LBV isolates are pathogenic to mice when a high dose of the virus is inoculated intramuscularly (Markotter *et al.*, 2009). Davis *et al.* (2012), also reported that 100% of *E. fuscus* developed rabies following i.m. inoculation with 10^3 $TCID_{50}$ whereas 40% develop

rabies following inoculation with the same RABV at 10^2 $TCID_{50.}$ A limited susceptibility to high virus doses may be explained by the phenomenon of significant triggering of the innate immune response in bats (Faber *et al*., 2002) whereas low doses may be cleared by the existing innate immune system without extensive triggering. Perhaps the $10^{2.1}$ $TCID_{50}$ is the "just right" dose to develop productive infection in *E. helvum* in a balance between low activation of immune response and replicative ability of the virus.

Incubation periods in peripherally inoculated bats ranged from 7 to 61days. Means of dose group were not statistically significantly different. Clinical observations in peripheral inoculation were similar to that of the IC inoculation for the Ghana 2013 isolate of LBV. Disease progression was accompanied by weight loss in most of the i.m. inoculated bats**.** There was weight lost in 9 bats of the 11 that developed rabies, with average loss of 10.9% (n = 9, range 5 to 53 weight loss). Healy *et al.* (2013) reported similar findings in RABV infected mice, with an average loss of 11.8% (n = 11) of their overall body weight between the prodromal and clinical phases of disease. The wtRABV infected mice lost on average 12.8% (n = 8) of their overall body weight, while the EBLV-1 and EBLV-2 infected mice lost on average 17.6% (n = 7) and 16.6% (n = 5), respectively, during disease progression.

Our gross pathological findings of enlargement of lymph nodes support that of inflammatory changes in murine studies using LBV, MOKV and DUVV which were more severe than those produced by RABV (Shope *et al.*, 1970). However, no microscopic examination was done and given that only one out of bats had enlarged nodes could be considered non-specific.

We reported detectable antibody levels in 7 out of the 20 bats infected (35% seroconvertion). Of the bats that did not seroconvert, 7 bats died. The short incubation

period might explain the lack of immune response in some of the bats, however four (4) bats survived till the end without detectable antibodies. Two bats (Bat 8 and 19) developed high antibody titre prior to death. Five (5) surviving bats developed immunity. One of these (bat 6) maintained detectable antibody throughout the experiment (88 days p.i) and the other four (4) bats lost their immunity with a decreased antibody titre to undetectable levels in about 10 days. One bat (Bat 2) had fluctuating antibody with detectable antibodies on days 10, 24 and 46. This was not expected.

The outcome of lyssavirus exposure depends on the virus variant, dose of inoculum, route of inoculation and individual host factors including age, and immune status. Experimental inoculations of lyssaviruses in bats, rodents, carnivores and other animals have reported varied clinical signs in animals that got infected, or absence of apparent disease with the development of seroconversion (Davis *et al.*, 2012; Turmelle *et al.*, 2010). Duration of incubation and clinical periods in the infected animals was reported to vary. In dogs and cats, the incubation period varies from 10 days to 6 months; most cases become apparent between 2 weeks and 3 months (WHO, 2013). This provides the medical and veterinary community with recommendations for a 10-days observation period and 6-months quarantine for suspected dogs, cats and ferrets (Davis *et al.*, 2012). Incubation periods of lyssavirus infection in bats have not been extensively studied. The incubation period in bats depends on the dose of the inoculum (Turmelle *et al.*, 2010), virus strain (Healy *et al.*, 2013), site of inoculation, pre-existing host immunity and nature of the wound. In temperate bats, the incubation period is influenced by the seasonality and life cycles that include hibernation (George *et al.*, 2011). Experimental studies using constant dose of rabies virus via different routes also have reported varying incubation periods. Johnson *et al.* (2008) reported incubation periods of i.c. inoculation to be between 7 to 13 days; i.m. 17 to 18 days and s.c.26 days in serotine bats. Franka *et al.* (2008) reported an incubation

period of 9 to 12 days by i.c. route and 12 to 33 days by i.m. inoculation in bats. The incubation period of rabies in insectivorous bats is reported by Constantine and Blehert (2009) to range between 6 days to 6 months. In this study, the incubation periods in bats that received LBV through i.c. route ranged from 4 to 8 days and i.m from 7 to 61 days.

In our studies, clinical signs associated with LBV infection of *E. helvum* are compatible with rabies observed in other bat species and various animal models (FAO, 2011; Franka *et al*., 2008). The reported clinical findings included ataxia, muscle spasms, muscle tremor, paresis and paralyses, behavioral changes such as hyper excitation, aggression, salivation / froth at mouth, vocalization, wing flapping and self mutilation (FAO, 2011). In this study some bats died without any apparent signs of disease observed during the previous check (i.e. 12 hours earlier). All 3 control bats survived till the end of i.c. inoculation experiment. FAO (2011) suggested that the clinical signs in rabid bats may be affected by environmental stimuli. Clinical signs exemplified by FAO (2011) include: aggression or intolerance to humans, fighting with other bats, the isolation of infected bats from the roost, increased biting and vocalization. Clinically sick bats are often observed hanging on low tree branches or on the ground. When sick bats are touched or disturbed, they respond by biting objects, flapping the wings and by muscle tremors. Sometimes there is incoordination of movement. We observed similar clinical presentations in this study (Table 5.9).

Although there is evidence that virus can be transmitted among bats by bite, aerosol, and transplacental infection (Constantine, 1966; 1967; Constantine *et al*., 1968) the mechanism of virus transmission between bats has not been studied in detail (Kuzmin & Rupprecht, 2007).

Centrifugal spread of virus from CNS to the salivary glands is thought to be integral for the maintenance of RABV in nature and thus lack of dissemination through saliva would

result in a dead end. In our study we demonstrated that both i.c and i.m inoculations can cause rabies disease in bats with dissemination of the virus in the brain (hippocampus, cerebellum and medulla) in all (i.c and i.m.) infected bats and salivary glands (3 i.c. inoculated bats and 2 i.m (7 and19). Although i.m. inoculation is believed to be the most common route of RABV transmission between terrestrial mammals, it may be different among chiropterans. Davis *et al.* (2012) reported that i.m. inoculation is more likely to result in clinical infection yet is less likely to result in dissemination of the virus to the salivary glands. Dissemination was inconsistent with dose and incubation period in this experiment. However, Davis *et al.* (2012) indicates that extensive centrifugal spread was evident in bats with the longest incubation periods or following inoculation with the putatively more virulent RABV.

Virus Neutralisation Antibody productions have been reported in experimentally infected bats (Nathanson and Gonzalez-Scarano 1991; Franka *et al*., 2008; Kuzmin *et al*., 2008; Davis *et al*., 2012b; Turmelle *et al*., 2010). Nathanson and Scarano (1991) reported that anti-rabies VNA titers increased throughout infection, reaching the highest levels during the terminal phase of infection (Nathanson and Gonzalez-Scarano 1991). Similarly, (Kuzmin *et al*., 2008), reported VNA 12 months following experimental inoculation with West Caucasian bat virus and Irkut virus. Davis *et al.* (2012) reported that Anti-rabies VNAs were maintained in experimentally exposed animals between 90 and 150 dpi. Our data (Table 5.12) slightly differ from these reports. The detection of measurable antibodies in bats that survived infection might explain the seroprevalence of LBV reported in wild caught bats (Dzikwi *et al*., 2010; Hayman *et al*., 2008; Kuzmin *et al*., 2008). Although rabies virus–infected animals often seroconvert in the terminal phase of disease, many remain antibody-negative throughout the incubation and clinical phase of disease (Jackson *et al*., 2008).

We confirm that LBV is pathogenic to *E. helvum* by i.c and peripheral inoculation. This is the first ever experimental infection study of LBV in *E. helvum* bats. We suggest that *E. helvum* is a suitable animal model for studies of the pathobiology of lyssaviruses. The species is abundant and widely distributed and accommodates well to captivity. Comparative studies should be initiated with other LBV host species and viral isolates which attempt to compare the influence of viral origin, dose and route of inoculation, host demographics, and environmental conditions on infection dynamics. This will help all to further develop our understanding of the dynamics of pathogen adaptation and host response, as well as the public health significance of emerging infections among the Chiroptera. The 100% mortality detected for the relatively low virus dose of $10^{2.1}$ $TCID_{50}$ while bats inoculated with higher and lower doses demonstrated less susceptibility to LBV and the absence of immune response in none of the surviving bats in the peripheral challenged experiment were surprising results that may require further investigation. The results have confirmed that LBV can cause disease in *E. helvum*, and bats that got infected showed clinical symptoms similar to animals infected with RABV, though with some variation with isolates in the i.c. inoculation.

CHAPTER SIX: GENERAL DISCUSSION

Wildlife populations constitute a large and often unknown reservoir of infectious agents, some of which are zoonotic (Daszak *et al.*, 2000; Hayman *et al.*, 2012). About 75% zoonotic infections originate from wildlife (Takayama-Ito *et al.*, 2006). These include influenza viruses, Nipah and Hendra viruses (Chua *et al.*, 2000), Severe Acute Respiratory syndrome (SARS) and Middle East Respiratory Syndrome (MERS) coronaviruses, Chikungunya virus, Japanese and St. Louis encephalitis viruses, Hantaan virus, Rift Valley fever virus, Ebola and Marburg haemorrhagic fever viruses, Rabies and rabies related Lyssaviruses, among others (FAO, 2011). More viruses that post potential, but yet undetermined, zoonotic risk also have been documented in bats, including Rhabdoviruses, Paramyxoviruses, Polyomaviruses, Hepaciviruses and Hepadnaviruses.

Many species of bats such as *Eidolon helvum* have peridomestic habits, roosting in houses and other buildings, as well as trees in dense urban areas, leading to frequent human contact with bat excreta and secretions. Bat–human contact has also increased in recent decades owing to habitat encroachment and increased use of bats as bushmeat (Luis *et al.*, 2013; Kamins *et al.*, 2011). There are 1,116 recognised species of bats (Chiroptera) worldwide (Happold, 2013). The diversity of bat species alone, along with their worldwide distribution, contributes to the variety of their pathogens, which have co-evolved with their hosts over millions of years.

Rabies virus (RABV) is the most significant zoonotic pathogen of bat origin. Lyssaviruses (family *Rhabdoviridae*, genus *Lyssavirus*) are the aetiological agents for rabies – acute, progressive viral encephalitis. Rabies is an under-reported disease that is present on every continent, except Antarctica. The disease causes over 55,000 human deaths annually (Hayman *et al.*, 2012). The actual impact of bat lyssaviruses in Africa is not known as there is little systematic surveillance for bat lyssaviruses in Africa (Markotter *et al.*, 2008).

Several species of Fruit bats serve as reservoir hosts for Lagos Bat Virus (LBV), with infrequent spillover infections documented in dogs, cats, and a mongoose (Markotter *et al.*, 2006).

A number of studies have confirmed the endemicity of LBV in *E. helvum* in Ghana (Hayman *et al.*, 2008; Hayman *et al.*, 2010); but there has been very few virological (as opposed to serological) investigations (observational studies or experimental studies) of LBV in African bats despite they being the presumed natural hosts of this virus. Another area of lyssavirus infection dynamics that remains unclear is the role of lyssavirus seropositivity in healthy bats (Hayman *et al.*, 2008). Several fundamental aspects of lyssavirus infection require further examination to develop a better understanding of the maintenance and transmission of these viruses within bat colonies.

The objective of this PhD study is dual. First, it investigated infection dynamics of potentially-zoonotic viruses in *Eidolon helvum* in free ranging populations at the 37-Military hospital in Accra and Tano Sacred grove and a captive population of *E. helvum* at the Achimota Forest. Second, the study conducted experimental investigation of the pathogenesis and transmission patterns of Lagos bat virus, in *Eidolon helvum*.

Lagos Bat Virus Dynamics in Wild and captive E. helvum population in Ghana

Studies of infectious disease ecology rely on robust surveillance of pathogens in reservoir hosts, often based on serology, which is the detection of specific antibodies in the blood and is used to infer infection history. The measurement of antibodies in blood is a critical disease surveillance tool because antibodies are typically easier to detect and persist longer than the inciting infectious agents. Incidence and prevalence are the most frequently used measures to describe the epidemiology of infection in natural populations.

Seroprevalence in wild bats was 44.09% and ranged between 42.94% (37-Military Hospital) and 46.15% (Tano Sacred Grove). In the wild caught bats, two nursing E. *helvum* mothers from the wild bats (ID: 900 and 905) were antibody-negative, while their pups (IDs 901 and 904 respectively) were both tested positive for anti-LBV antibodies. As was documented for RABV, infection prevalence in gregarious colonial bat species is usually significantly less than 1% (Steece and Altenburg, 1989), However, seroprevalence may be as high as 70% for rabies in Brazilian free-tailed bats, *Tadarida brasiliensis*, and 14 – 44% (Nigeria), 37% (Ghana), and 40–67% (Kenya) for LBV in *E. helvum* (Constantine 1967; Steece *et al*., 1989; Kuzmin *et al*., 2008; Dziwi *et al*., 2010; Hayman *et al*., 2008).

None of the brain samples from the postmortem bats in this study showed evidence of lyssavirus antigen or infectious particles. Similar studies elsewhere did not succeed in detecting lyssavirus antigen or RNA in bats (Dzikwi *et al*., 2010; Amengual *et al*., 1997; Van der Poel *et al*., 2000, Wellenberg *et al*., 2002; Reynes *et al*., 2004). It was not surprising that no LBV antigen or RNA was detected in this study. It was surprising no virus was isolated from the dead/sick bats, especially from the captive population too. However, a much higher sample size (Kuzmin *et al*., 2008) and a long-term sampling of natural colonies is needed to provide additional insight into how roosting and behavioral ecology affect enzootic LBV infection in *E. helvum* in Ghana.

The mechanisms by which bats maintain lyssaviruses within roosts remains undefined. In the case of RABV strains circulating in bat populations across the New World, it is hypothesized that population size and the proximity of animals within large roosts could feasibly maintain virus circulating at low levels (Luis *et al*., 2013). Other factors such as low pathogenicity and long incubation period (Boots & Sasaki, 1999), roosting ecology

and reproductive activity are reported as important factors affecting rabies seroprevalence in colonial bats (Turmelle *et al.*, 2010).

The captive population of *E. helvum* had a mean seroprevalence of 32.87%, ranging from 25.15% (September, 2013) to 48.78% (June, 2012). Antibody prevalence in captive bats was higher in females (37.83%; range 27.27% - 51.92%) than in males (28.24%, range 19.56% - 48.14%). A higher seroprevalence was recorded in wild caught female *E. helvum* 61.90% compared to 45.23% males. Adult bats in this study recorded higher seroprevalence (46.18%; range 45.09% - 48.19%) than juvenile (43.10%; range 42.85% - 50%). Similar findings are reported elsewhere in hoary bats (*Lasiurus cinereus*) (O'Shea *et al.*, 2014; Bowen *et al.*, 2013). However, few juvenile bats were caught during the trapping in this study and the result should be interpreted with caution.

There is limited literature on the ecology of *E. helvum*, especially on roosting behavior. Elsewhere, it was reported that *Eptesicus fuscus* females live in large maternity colonies, whereas males were more typically dispersed and solitary (Kurta & Baker, 1990). The high percentage of seropositive bats could be an indication of efficient virus transmission between individuals and circulation of the virus in the colony. These findings were not surprising in a gregarious bat species such as *E. helvum* in which a high contact rate between individuals is facilitated in the roost, where bats are highly concentrated.

The study recorded a bat that had maintained a positive status persistently for at least 2 years, eight bats that seroconverted in captivity and nine (9) bats in the captive population maintained positive status for ten months. Amengual *et al.*, (2007) reported persistence of immunity for more than one year in some individual bats. The serological study in the captive bats suggest that cyclic lyssavirus infections occurred with periodic oscillations in

the number of susceptible, immune and infected bats as reported by Amengual *et al*., 2007.

Persistence of an infectious disease in a structured population has been reported by several authors. It is reported that reservoir host population size and density play a critical role in the ability of a species to maintain viruses that cause acute or immunising infections (Bartlett, 1957). Studies indicate that pathogens causing acute immunising infections require large host population sizes to maintain an adequate supply of susceptible individuals to maintain transmission, unless birth rates are very high (Peel *et al.*, 2012).

A study by Peel *et al*. (2012) has reported the persistence of henipaviruses in *E. helvum* in a small population on Annobon Island. Given the isolation of fruit bats on Annobon and their lack of connectivity with other populations, it was expected that the population size on the island would be too small to allow persistence of viruses that are thought to cause acute and immunising infections. The captive population of bats in the Achimota forest that was used in this study were captured in 2009 and has been monitored to date. The duration or half life of lyssavirus antibodies in bats is not known (Epstein *et al.*, 2008) and antibodies may persist from weeks to years, depending on the host–pathogen system and individual variation (Gilbert *et al.*, 2013). One study indicates that Nipah virus (NiV) antibodies may persist in individual adult bats for at least 14 months, whereas juvenile antibody levels wane over a period of up to 7 months (Sohayati *et al.*, 2011)(Sohayati *et al*., 2011). Rabies virus neutralising antibodies were shown to wane in experimentally-infected bats within 6 months after an initial inoculation, but persisted for longer (6–12 months) after a second inoculation of surviving bats (Turmelle *et al*., 2010). Evidence for viral recrudescence has also been reported in a captive *P. vampyrus*, which displayed changes in neutralizing antibody to Nipah virus, providing evidence of the maintenance of virus in bats in a manner that does not sustain an antibody response.

It was quite interesting that such a small captive bat population could maintain circulation of the virus for as long as two years and also to detect antibody in juveniles with a history of having been born from serologically naïve and apparently healthy mothers. Such juveniles could influence the perpetuation of LBV in the roosting population and there is the need for a further investigation in this direction.

It was not surprising that no LBV or antigen was detected in the dead necropsied bats in the study. A much higher sample size of dead or sick bat samples (more than 49 investigated here) is required to improve the chances of detecting LBV. The isolation of more LBV viruses from the population will assist in the investigation of the various strains of LBV that might be circulating in Ghanaian Straw-colored fruit bats (*E. helvum*).

Experimental Infection study

Very little is known about LBV diversity and the relationships between different isolates, or between LBV and other lyssaviruses. The true risk of LBV infection for humans and animals is uncertain (Markotter *et al*., 2009). There is little data to indicate minimum dose of lyssaviruses required to cause a clinical disease. Experimental infection studies using LBV (Lagos, Nigerian isolate) failed to produce disease in guinea pigs (i.m), rabbits (i.m &i.c), monkey (*Cercocebus torquatus*) subcutaneous (s.c) (Boulger *et al*., 1958). This, and similar other studies, led to the suggestions that phylogroup II viruses (LBV and MOKV) have a reduced pathogenicity compared to phylogroup I viruses (Badrane *et al*., 2001). However, some strains of LBV were reported to have caused 100% mortality in mice (Markotter *et al*., 2009). There has been no study of LBV in *E. helvum*.

The risk of LBV for human and animal is uncertain (Markotter *et al*., 2009). The study of RABV in natural host such as bats can be challenging due to lengthy quarantine requirements, age variation, husbandry concerns, genetic differences between animals, and

unknown history of previous exposure to RABV. To date, all RABV studies in bats have been accomplished using wild caught animals. This study was the first of its kind in one of LBV's reservoir bat species. We used naïve anti-LBV antibody negative bats kept in a purpose built biosecure cage for the study. The highly biosecure housing had a structural design to prevent interaction of captive bats with external environment (free-living bats, rodents and other wildlife) and hence eliminated possible interchange of possible pathogens.

In the i.c inoculated bats, using 3 different isolates of LBV (RV1 =LBV Nig, 1956; RV41-LBV Senegal 1985; FL1-LBV Germany Ex Kumasi 2013) 100% mortality was recorded in this experiment and there was no difference between incubation periods of the 3 isolates. Clinical symptoms were used to select candidate isolate for the peripheral (i.m.) inoculation. In the peripherally inoculated bats, 11 of 20 (55%) bats were infected, had rabies disease and died, either naturally or by euthanasia based on account of welfare considerations. There was no significant difference in the means of the various virus dose (Kruskal-wallis chi-squared = 3.3069, df = 4, p-value = 0.51). There have been varying reports from similar experiments elsewhere: for example direct inoculation of EBLV-1 into the brain of Egyptian flying foxes (*Rousettus aegyptiacus*) caused neurological disease and death in 5out of 8 (63%) inoculated animals (Van der Poel *et al*., 200). Similar mortality rate was observed in experimental studies of EBLV-1 in the North American Big Brown Bat (*E. fuscus*) (Franka *et al*., 2008). Experimental inoculations of lyssaviruses in bats, rodents, carnivores and other animals have reported varied clinical signs in animals that were infected with the virus and had rabies disease, or absence of apparent disease with the development of seroconversion (Davis *et al*., 2013; Turmelle *et al*., 2010). Duration of incubation and clinical periods in the infected animals was reported also to

vary. In dogs and cats, the incubation period is usually between10 days to 6 months, with most cases becoming apparent between 2 weeks and 3 months (OIE, 2013).

In the i.c study, clinical signs associated with LBV infection of *E. helvum* were compatible with rabies observed in other bat species and various animal models. Clinical signs observed in infected bats included ataxia, muscle spasms, muscle tremor, paresis and paralyses, behavioral changes such as hyper excitation, aggression, salivation / froth at mouth, vocalization, wing flapping and self mutilation. Some bats died without any apparent signs of disease observed during the previous check (i.e. 12 hours earlier). All 3 control bats survived till the end of i.c. inoculation experiment. The incubation period for the i.c inoculated ranged from 4 - 8 days. Most i.c inoculated bats died within 24 hours of Clinical manifestation. The virus antigen (by FAT) or RNA (by PCR) was detected in the brain and salivary gland, an indication that the virus can be transmitted in *E. helvum* through saliva by bites or contamination through an opening in the skin or membrane.

In the i.m study serial dilution of LBV virus isolated from Ghanaian *E. helvum* was used for inoculation of bats via peripheral route (i.m.). Infected bats showed clinical signs similar to rabid animals. No dose-dependent difference in the duration of incubation periods was detected between groups inoculated with $10^{1.1}$ – $10^{4.1}$ $TCID_{50}$ virus doses. The only exception was a single bat that succumbed in the group inoculated with the lowest virus dose, $10^{0.1}$ $TCID_{50}$ that developed the considerable longer incubation period of 61 days.

Disease progression was accompanied by weight loss in most of the i.m. inoculated bats**.** There was weight loss in 9 bats of the 11 that develop rabies, with average loss of 16.33g (n = 9, range 5 to 53 weight loss). Healy *et al.* (2012) reported similar findings in RABV infected mice, with an average loss of 11.8% (n = 11) of their overall body weight between the prodromal and clinical phases of disease. The wtRABV infected mice lost on

average 12.8% (n = 8) of their overall body weight, while the EBLV-1 and EBLV-2 infected mice lost on average 17.6% (n = 7) and 16.6% (n = 5), respectively, during disease progression.

Detectable antibody levels were recorded in 7 of the 20 bats infected (35% seroconvertion). Four (4) bats survived till the end of the experiment without detectable antibodies. Two bats (Bat 8 and 19) developed high antibody titre prior to death. Five (5) surviving bats developed immunity. One of these (bat No. 6) maintained detectable antibody throughout the experiment (88 days p.i) and the other four (4) bats lost their immunity with a decrease in antibody titre to undetectable levels in about 10 days. Bat No.2 had fluctuating antibody with detectable antibodies on days 10, 24 and 46. This was not expected. Virus Neutralisation Antibody production has been reported in experimentally infected bats (Nathanson and Gonzalez-Scarano, 1991; Franka *et al*., 2008; Kuzmin *et al*., 2008; Davis *et al*., 2012). Nathanson and Gonzalez-Scarano (1991) reported that anti-rabies VNA titers increased throughout infection, reaching the highest levels during the terminal phase of infection. Similarly, Kuzmin *et al*. (2008) reported VNA 12 months following experimental inoculation with West Caucasian bat virus and Irkut virus (Kuzmin *et al*., 2008). Davis *et al*. (2012) reported that Anti-rabies VNAs were maintained in experimentally exposed animals between 90 and 150 days post inoculation (dpi). Jackson *et al*. (2008) indicated that, although rabies virus–infected animals often seroconvert in the terminal phase of disease, many remain antibody-negative throughout the incubation and clinical phase of disease. The inability to detect antigen and antibodies in bats inoculated with LBV suggest that previous exposure to LBV cannot be determined by the presence or absence of VNA.

We confirm that LBV is pathogenic to *E. helvum* by i.c and peripheral inoculation. This is the first ever experimental infection study of LBV in *E. helvum* bats. We suggest that *E. helvum* is a suitable animal model for studies of the pathobiology of lyssaviruses. The 100% mortality detected for the relatively low virus dose of $10^{2.1}$ $TCID_{50}$ whereas bats inoculated with higher and lower doses demonstrated less susceptibility to LBV and the absence of immune response in none of the surviving bats in the peripheral challenged experiment were surprising results that may require further investigation. The significance of this study is also demonstrated by the lack of LBV infection and LBV antibodies among naïve bats inoculated with the LBV.

It was interesting to have confirmed that LBV can cause disease in *E. helvum*, and bats that infected showed clinical symptoms similar to animals infected with RABV. The question as to when, where and how LBV transmission occurs in a natural environment require answers. There is the need for further experimental investigation to enlighten the scientific community on this important epidemiological gab in LBV transmission in a natural population.

CHAPTER SEVEN: SUMMARY, RECOMMENDATIONS AND CONCLUSIONS

7.1 Summary of the study of viral zoonoses in Ghanaian fruit bats

Eidolon helvum is widely distributed across sub-Saharan Africa where it forms large, dense colonies in close proximity to both man and domestic animals. The species is migratory and satellite telemetry studies have demonstrated that individuals can migrate over 1500 km. The species is harvested for bushmeat, particularly in West Africa (Kamins *et al.*, 2011): we recently estimated that at least 128,000 *E. helvum* are harvested annually in southern Ghana alone, and it is unknown if current harvest rates are sustainable (Kamins *et al.*, 2011). Bats including *E. helvum* are major seed dispersal agents. Bats play diverse roles in ecosystem services. A team of international collaborators, of which I was the local collaborator, are investigating zoonotic viruses of fruit bats in Ghana since 2007. They include: The Veterinary Services of Ghana, Wildlife Division of the Forestry Commission and The University of Cambridge and Institute of Zoology, both of U.K. Their findings are presented here.

Findings:

Within the period of our research since 2007 - 2016, the following findings were made:

1. *Lagos bat virus infection in fruit bats.* It was detected that a high prevalence of antibodies exists against Lagos bat virus (LBV) in *E. helvum* and other bat species in Ghana (Hayman *et al.*, 2008). Further follow-up studies confirmed the endemicity of LBV in *E. helvum* in Ghana (Hayman *et al.*, 2012). This study has reported high antibody prevalence that supports earlier findings with a clear temporal variation of LBV in *E. helvum* in Ghana. We detected high seroprevalence of 44.09% (44.13 at 37-Military Hospital and 46.15 at Tano Sacred Grove) in the wild population of bats in Ghana. The captive population of *E. helvum* had seroprevalence of 32.87%.We documented in this work seroconvertion, persistent antibodies in individual bats, and oscillation of antibody levels in some captive bats.

2. We conducted infection study of LBV in *E. helvum* and confirmed that LBV is pathogenic to *E. helvum* by i.c and peripheral inoculation. This is the first ever experimental infection study of LBV in *E. helvum* bats. We evaluated antibody levels of sera collected and this enable us reported detectable antibody levels in 7 of the 20 peripherally inoculated bats (35% seroconvertion). Five (5) surviving bats develop immunity. One of these (bat 6) maintained detectable antibody throughout the experiment (88days p.i) and the other four (4) bats lost their immunity with a decrease antibody titre to undetectable levels in about 10days. One bat (Bat 2) had fluctuating antibody with detectable antibodies on days 10, 24 and 46. This was not expected. The detection of measurable antibodies in bats that survived infection might explain the seroprevalence of LBV reported in wild

caught bats (Kuzmin *et al*., 2008); Hayman *et al*., 2008; Dziwi, Kuzmin *et al*., 2010).

3. *Henipavirus infection in fruit bats.* In the same population we also found antibodies against henipaviruses in *E. helvum* (Hayman *et al.*, 2008). Spill over investigation led to detection of antibodies to henipaviruses in pigs and found henipavirus in pigs in Ghana (Hayman *et al.*, 2011). Phylogenetically – related paramyxoviruses including Achimota virus have been detected in these bats using next generating sequencing technology (Baker *et al*., 2012).

4. *Ebolavirus infection in fruit bats in Ghana*. There are suggestions of bats being the reservoir of filoviruses including Ebola and Marburg viruses (Leroy *et al.*, 2005). An antibody to Ebolavirus in a single long surviving bat was detected in *E. helvum* in the Accra population during our survey (Hayman *et al.*, 2010). We thus expanded our survey and undertook investigations to look for serologic evidence of Ebola virus infection in bats in Ghana using samples from previous work and also samples of Egyptian fruit bats (*Rousettus aegyptiacus*) that live in the caves at Buoyem near Techiman. These investigations detected antibodies to Ebola Zaire virus in *Epomops fraqueti*, *Epomophorus gambianus* and *Hypsignathus monstrosus* (Hayman *et al*., 2012).

5. Other investigations included the following:

    - We investigated Bartonella infection dynamics in fruit bats and reported Bartonella species in bat flies (Diptera: Nycteribiidae) from western Africa in *E. helvum* in Ghana (Billeter *et al*., 2012). There is also on-going study on *bartonella* infection dynamics of Bartonella in *E. helvum* in a Captive population of *E. helvum* bats in Accra, Ghana.

- Because of all these findings, we decided to conduct a close study of the dynamics of *henipaviruses, paramyxoviruses* and other viruses in *E. helvum* in the captive population of *E. helvum* being bred at the Accra zoo in the Achimota forest, where further longitudinal studies are carried out to determine the perpetuation of the infection in small close bat populations.

Lagos bat virus seroprevalence in E. helvum fruit bats.

This study found high antibody prevalence and documented in this work seroconversion, persistent antibodies in individual bats, and fluctuation of antibody levels in some captive bats. The study did not detect LBV RNA or lyssavirus antigen in sick / dead bats. The high seroprevalence of LBV reported here and elsewhere (Hayman *et al*., 2008) in the apparently healthy Ghanaian *E. helvum* colonies and the antibody variation reported here in the captive bats may be evidence of past infection or circulation of LBV in bats, but it is unknown if this infection is due to exposure of sub-lethal doses of LBV via routes other than biting, such as from aerosol, grooming, or food sharing.

Ongoing ecology studies by our team indicate close interaction between *E. helvum*, domestic animal and human populations. Human consumption of bats is practiced in many parts of Ghana. Our results, therefore, could have important public health implications, but more information is required on the viruses involved, their infection dynamics within the bat populations, potential spillover routes, and bat population dynamics before any risk can be assessed. Surveillance to identify circulating non-rabies lyssaviruses is advised, as existing rabies biologics do not provide reliable protection against some African non-rabies lyssaviruses including LBV, MOKV, and West Causcasian bat virus (WCBV) (Hanlon *et al*., 2005). We advise post exposure rabies vaccination should be provided

after an exposure to Ghanaian bats, however, keeping in mind that rabies vaccine is less efficient against lyssavirus belonging to the phylogroup 2, including LBV (Markotter *el al*., 2006).

Lagos bat virus infection study in E. helvum fruit bats.

The infection study confirmed that LBV is pathogenic to *E. helvum* by i.c and peripheral inoculation. This is the first reported experimental infection study of LBV in *E. helvum* bats. In Ghana and most developing African countries, there is no laboratory capacity to diagnose phylogroup 2 viruses. There is therefore poor rabies surveillance for African rabies related lyssaviruses. The true picture of rabies in Ghana cannot therefore be true. I therefore recommend that laboratories be provided necessary resources to develop capacities to diagnose African lyssaviruses. All rabies outbreak investigations and surveillance will then include phylogroup 2 viruses. This would help provide the true picture of rabies in the country.

There was no antibody detected in the i.c inoculated bats. The antibody levels in sera collected from i.m infected bats were evaluated and found detectable antibody levels in 7 of the 20 peripherally inoculated bats (35% seroconversion). I therefore speculate that there was not enough infection duration to allow development of antibodies in the i.c. inoculated bats while those in i.m inoculated bats had enough time. The absence of LBV infection and failure of some infected bats to develop LBV antibodies is surprising. The development of antibodies in healthy bats infected with LBV support the seroprevalence in both wild and captive bat study in this thesis. These findings indicate there are gaps in bat rabies immunology that require further scientific research.

7.2 Conclusion

The current study has reported high antibody prevalence levels that support earlier findings with a temporal variation of LBV in *E. helvum* in Ghana. The continued high seroprevalence of LBV reported previously and in this study in apparently healthy *E. helvum* colonies may be evidence of circulation of LBV in the bat population. Surveillance to identify circulating non-rabies lyssaviruses is advised, as existing rabies biologics do not provide reliable protection against some African non-rabies lyssaviruses

The experiment inoculation studies in this thesis confirm that LBV is pathogenic to *E. helvum* by i.c and peripheral inoculation. This is the first ever experimental infection study of LBV in *E. helvum* bats. The 100% mortality detected for the relatively low virus dose of $10^{2.1}$ $TCID_{50}$ while bats inoculated with higher and lower doses demonstrated less susceptibility to LBV and the absence of immune response in none of the surviving bats in the peripheral challenged experiment were surprising results that may require further investigation. The results have confirmed that LBV can cause disease in *E. helvum*, and bats that got infected showed clinical symptoms similar to animals infected with RABV, though with some variation with isolates in the i.c. inoculation.

E. helvum population level is near threatened (http://www.Africanbats.org) and any disease that impact on their population (through mortality) of this specie should be of concern.

Ongoing ecology studies have indicated the potential for close interactions between chiropteran, domestic animal and human populations. Human consumption of bats is practiced in many parts of Ghana and across Africa. These results, therefore, could have important public health implications, but more information is required on LBV infection dynamics within the bat populations, potential spillover mechanisms, and bat population dynamics.

7.3 Recommendation

From the study, the following are recommended in order to expand our knowledge on the epidemiology of LBV in bats:

1. There are many roosts and populations of *E. helvum* in Ghana. The field study focused on *E. helvum* roost at only two locations. Further surveillance is needed in other populations of *E. helvum* and other Ghanaian bats to evaluate the distribution, circulation patterns, and ecology of LBV.
2. Public awareness and education must be increased, and additional surveillance (including sex- and age-specific seroprevalence and longitudinal studies) is needed for a better understanding of the epizootic situation and threat of lyssavirus emergence in Ghana. Awareness of both the general public and healthcare providers regarding the potential transmission of rabies from bats must be increased, surveillance must be enhanced, and new broader biologics need to be developed to provide adequate protection against all lyssaviruses.
3. Although the burden of LBV and other African non rabies lyssaviruses for veterinary and public health is largely unknown, this lack of knowledge is primarily based on the absence of an adequate surveillance system in the majority of African countries (Markotter *et al*. 2008). There are reports indicating that the circulation of these viruses occur naturally. LBV spill-over infections have been sporadically documented in various mammals, which is an indication of its pathogenicity. LBV is proven to be highly pathogenic for non-human primates by peripheral routes (Tignor *et al*., 1973), and their significance for public health should not be underestimated. There is a need to investigate possible spill-over of LBV to other animals especially in rural areas where human and livestock closely interacts with bats.

4. Current rabies diagnoses in Ghana do not consider non-rabies lyssaviruses. It is recommended that the Veterinary Services and Ghana Health Services, Research and Academic Institutions employ the use of molecular diagnostics that can detect non rabies lyssaviruses so that it will be possible to investigate the types of lyssaviruses involved in rabies cases in Ghana.

5. The experimental study described here employed a limited number of bats in each group and infection studies did not consider subcutaneous inoculation and transmission from an infected bat to a co-housed bat. These are possible natural transmission pathways of lyssaviruses. Future research studies using a larger number of bats in the experiment and including subcutaneous transmission routes would add additional knowledge on the pathogenesis of LBV in bats.

6. The study has confirmed that LBV is pathogenic to *E. helvum* by i.c and peripheral inoculation. This is the first ever experimental infection study of LBV in *E. helvum* bats. We suggest that *E. helvum* is a suitable animal model for studies of the pathobiology of lyssaviruses.

7. Captive bats were monitored for three years duration of this work and the study documented serocoversion, persistent antibodies in individual bats, and fluctuation of antibody levels in some captive bats. For detailed knowledge of the dynamics of anti – LBV antibodies in *E. helvum*, there would be a need to continue with the monitoring LBV dynamics in the captive population for a longer period.

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www.who-rabies-bulletin.org/about_rabies/Diagnosis.aspx

APPENDICES:

Appendix 1. Protocol use for Bat Intracranial Inoculation

1. Surgery should be done under aseptic conditions in accordance with your institution's biosafety and animal use guidelines. Use only sterile surgical tools.
2. All persons involved must fully done PPE including using scrub cloths, cap, mask, and examination gloves.
3. Clean the surgical area with 70% alcohol and dry the surface with absorbent lab bench paper. Cover the surgical area with absorbent lab bench paper.
4. Choose an area adjacent to the surgical area that will be dedicated to placing the loaded syringes with virus, and cover it with absorbent lab bench paper.
5. Setup a waste container of 10% bleach in the dedicated virus handling area for disposing of materials that come in contact with the virus.
6. Setup the Stereotactic frame in the covered area of the surgical table.
7. Retrieve one syringe from storage and place one syringe in a container of ice in this area allowing the virus to thaw on ice as the surgery is being performed.
8. Anesthetize the bat with a mixture of Medetomidine (0.05 mg/kg)/Ketamine HCL (5 mg/kg) via IV injection with a 50/50 mix. Start with an induction dose of M/K 0.03/3 mg/kg. (Having a loaded syringe with Atipamezole dose of 0.25 mg/kg) (*5 min)*
9. Inject the bat subcutaneously with Butorphanol at a dose of 0.2 - 2 mg/kg (*1 min)*
10. Inject the bat with preemptive antibiotics and analgesia (Baytril 2,5% = 0.1 ml per 500 g; Metacam 0.2 mg/kg SC; Buprenorphine 0,05 mg/kg SC) (*1 min)*
11. Place the bat in the Stereotactic frame, assuring that the skull is correctly positioned by immobilization of the head, and remove the hair from the top of the head with a small electric hair trimmer, making sure to leave margins large enough

to prevent hair from entering the incision. (*15 - 20 min)*

12. Place lubricant eye gel on each eye of the bat to keep the eyes moist during surgery. (*1 min).*
13. Place the surgical drape in position and setup sterile surgical instrumental and prepare the scalp by swabbing several times with a piece of sterile gauze dipped in chlorhexidine solution (*6 min)*
14. Put on the surgical loupes and arrange it to the desire augmentation. Put on surgical gloves and gown (*5 min)*
15. Position the surgical field leaving exposed the desire area, using a sterile scalpel, complete a sagittal incision over the parieto-occipital bone, approximately 1 cm long, pull the skin back to expose the skull using Skin retractor or atraumatic tissue forceps (*3 min)*
16. Carefully remove the fascia from the skull using forceps. Remove blood excess with a sterile cotton swab (*5 min)*
17. Locate the area to be injected using stereotaxic coordinates (A/P= X; Lat= X; D/V= X) and mark the skull making a small dent using a dental drill with a 0.8 mm burr (*3 - 5 min)*
18. Drill the marked spot carefully until the skull layer looks darker, and start pressing with a sterile needle to assess the perforation depth (*15 - 20 min)*
19. Throughout this procedure, keep the skull moist with applications of sterile saline.
20. Once the skull is perforated remove excess blood absorbing it with sterile cotton swabs, place the syringe loaded with virus into the Stereotactic frame and apply a drop of mineral oil to the tip of it to prevent clogging as the needle is lowered into the brain (*7 min)*
21. Very slowly lower the syringe with the pre-setup coordinates and start inoculating

the virus at a rate of **X** ul per minute, once the solution is completely injected leave the syringe to rest for 4 minutes to prevent efflux of virus during removal. After this period, very slowly remove the syringe from the brain (*5 + **X** min).*

22. Remove excess blood with cotton swabs and proceed to suture the scalp (*10 min).*
23. Inject Atipamezole 0.1 mg/kg and carefully place the bat inside a bag into its cage. Monitor the animal before performing the next procedure (*20 min).*
24. Discard lab bench paper into biohazard bag and wipe down all surfaces and instruments that may have come in contact with the animal and virus with Hibiscrub.
25. Maintain with Baytril 2,5% Oral solution (0,3 ml per 500 g/ q24hr) and Metacam 0.1 mg/k q24hr PO for 3 days.

Appendix 2 Dosage of Anaethetic drugs and Antibiotics used for Intrcranial Inoculation of bats

ID	Weight (Kg)	Ketamine IV	Medeto-midine IV	Combo IV	Dopram SC	Baytril SC	Metacam SC	Buprenorp hine SC	Atipame-zole IV
IC1311BIC01	0.185	0.01	0.01	0.02	0.02	0.04	0.02	0.03	0.02
IC0721BIC02	0.09	0.01	0.01	0.02	0.01	0.02	0.01	0.02	0.02
IC9113BIC03	0.155	0.01	0.01	0.02	0.01	0.03	0.02	0.03	0.02
IC6179BIC05	0.175	0.01	0.01	0.02	0.01	0.035	0.02	0.03	0.02
IC1191BIC06	0.18	0.01	0.01	0.02	0.02	0.04	0.02	0.03	0.02
IC1711BIC07	0.18	0.01	0.01	0.02	0.02	0.04	0.02	0.03	0.02
IC7193BIC08	0.225	0.01	0.01	0.02	0.02	0.04	0.02	0.04	0.02
IC5368BIC09	0.12	0.01	0.01	0.02	0.01	0.03	0.01	0.02	0.02
IC4146BIC10	0.75	0.01	0.01	0.02	0.01	0.03	0.01	0.02	0.02
IC8308BIC11	0.16	0.01	0.01	0.02	0.01	0.03	0.02	0.03	0.02
IC4741BIC12	0.19	0.01	0.01	0.02	0.02	0.04	0.02	0.03	0.02
IC4099BIC13	0.23	0.01	0.01	0.02	0.02	0.04	0.02	0.04	0.02

Appendix 3. Rabies Fluorescent antibody Test (FAT) used for Surveillance and Infected bat samples.

This method involves preparation of slides and reading results on a fluorescents microscope. Ensure sample is received, given an ID and recorded appropriately. A 20°C freezer is required for fixation of impression / smear slides and storage of acetone and other reagents; long term sample storage requires a freezer at -70°C. A complete cross section of the brain stem is required for FAT test.

Preparation of the Slides

Preparation of the slides are done in a safety cabinet.

- Set up safety cabinet – Clean and disinfected, and transfer all required equipment and bat sample in cabinet
- Label microscope slide with the ID for the bat
- **Slide Smear**: Take labeled slide and invert onto the tissue and make an impression smear. Blot excess if necessary. Impressions/smears must dry completely at room temperature prior to the fixation step. This may take 15 to 30 minutes.
- **Fixation of Smear**: Place the slides in a coplin jar containing acetone (which has been stored in -20oC) for 20 – 45 minutes.
- Remove slides, air dry and place them in a humid chamber
- **Staining and Rinsing**: Add a drop of working strength conjugate (sufficient to cover the smear) to the top of the sample smear.
- Incubate by placing in 37oC incubator for 30 – 45 minutes

- Wash twice in 0.1M PBS pH 7.2 for 5 minutes. After the 1st wash, the PBS is discarded and replaced and the slides soaked for a second 3 to 5 minute interval.
- Rinse briefly with distilled water
- Allow to air dry.

Mounting and Reading of Slides

- Slides should be read within 2 hours of mounting. Rabies-specific staining should be stable for at least 2 hours, and stained slides can be preserved for reference for weeks to months at refrigerator temperature or below.
- Slides are mounted by dropping a small amount of 20% glycerol - Tris buffered saline pH 9.0 onto coverslips arranged on absorbent paper.
- Read control slide first, each impression is observed for rabies specific fluorescence using the 10x objective. Positive fluorescence is denoted by bright "apple" green fluorescence in peri-nuclear area of cells.
- Then read the sample slides, looking for similar fluorescence as in the control slide, if necessary return to the control slide for comparison.
- Sore remainder of sample for archive use. Disinfect cabinet as appropriate.
- Record Results in the appropriate lab sheets.
- A second operator should cross-check to confirm results of slides

Appendix 4. Protocols used for Rabies Virus Molecular Diagnosis (RT-PCR)

i. Protocol for Lyssavirus RNA extraction using Trizol

Diagnostic specimens for rabies include brain tissues, skin biopsies, saliva, and cerebral spinal fluid. This extraction procedure is used for the extraction of viral RNA from any diagnostic tissue material, or cell monolayer and the method provides viral RNA from diagnostic tissue sample or cell monolayer for RT-PCR test and subsequent DNA sequencing.

TRIZOL™ is a ready use mixture of phenol, guanidine, isothiocyanate, red dye and other proprietary components used to isolate RNA in 1 hour in a single step.

A tissue samples is homogenize in 1ml of TRizol reagent per 50 – 100mg of tissue in a homoginising tube using disposable pestle and cordless motor and incubate at room temperature for 5 minutes permitting complete dissociation of nucleoprotein complexes. Add 0.2ml of chloroform per 1ml of TRIZOL reagent used. Shake vigorously for 15 seconds. Incubate at room temperature for 2-3 minutes. Centrifuge the sample at 10,000 rpm at 4°C for 15 minutes. Transfer the upper aqueous phase, to 0.5 ml isopropanol per 1 ml TRIZOL reagent used. Mix by inversion and incubate the sample at room temperature for 10 minutes. Centrifuge at 10,000 rpm at 4°C for 10 minutes. Remove the supernatant. Wash the pellet with 20µl 75% ethanol per 1 ml TRIZOL reagent used for the initial homogenisation. Remove supernatant and leave the pellet to air dry for 5-10 minutes maximum. Re-suspend the RNA pellet in 10-20 µl RNase free HPLC grade water. Store at –80°C

For liquid samples, add 0.75ml Trizol LS reagent for each 0.25ml of sample. If sample is less than 0.25, then add water to make up and stored at –80°C.

ii. Protocol for Reverse Transcription of Lyssavirus Viral RNA

Lyssavirus is an RNA virus. For RNA viruses, a complementary DNA (cDNA) must first be synthesised from the extracted viral RNA using reverse transcriptase enzyme which is capable of synthesising single-stranded DNA from RNA in the 5' to 3' direction. This synthesis is achieved using the rabies specific primer JW12 (Heaton *et al*., 1997). The procedure is outlined in the Reverse Transcription master-mix

Reverse Transcription Mastermix

Keeping all reagents on ice, prepare a master-mix with the following volumes per reaction:

Reagents	**Batch No.**	**Per reaction (ul)**	**N (e.g for 18* test reactions) reactions (ul)**
HPLC grade water		6	108
RT buffer (5x)		4	72
dNTPs		1	18
DTT		1	18
Random hexamers		2	36
JW12 primer		2	36
RNAsin		1	18
MMLV-RT		1	18
Total		**18**	**324**

*Number of reaction mix (18) is usually prepared to be more than the actual number of samples to test (16 in this case)

- Vortex the master – mix (SciQuip vortex Fixmix, UK)

- Aliquot 18 ul into number tubes equivalent to the number of test samples.
- Add 2µl of test RNA (1µg/µl) below the surface of the prepared reaction mix,
- Add 2µl of CVS RNA (positive control) control and water to the NTC (last 2 tubes). Mix gently with a pipette.
- Incubate at 42°C for 60 minutes. Chill the cDNA on ice for 1 minute and add the required volume of HPLC water to each reaction mix (usually so the final volume is 100µl). Maintain on ice or store at (-20°C).

iii. Reverse Transcription of RNA from bat saliva

This reverse transcription mastermix is used for samples with low levels of RNA (such as RNA derived from bat saliva). RNA from bat saliva would normally be extracted using the High Pure™ method.

Keeping all reagents on ice, prepare a master mix with the following volumes per reaction at the same concentrations as above:

Mastermix

Reagents	Batch No.	Per reaction (ul)	N reactions (ul)
HPLC grade water		0	
RT buffer (5x)		8	
dNTPs		2	
DTT (0.1M)		3.0	
Random hexamers		2	
JW12 primer		2	
RNAsin (28U/ul)		1	
MMLV-RT (200U/ul)		4.0	
Total		**22ul**	

Mix and add 18µl of approximately 1µg/µl RNA to the mastermix. Incubate at 42°C for 60 minutes. Chill the cDNA on ice for 1minute. Leave the cDNA undiluted, and maintain on ice for PCR or store at -20°C.

iv. Pan-Lyssavirus hemi-nested PCR

Hemi-nested PCR is validated to detect all established lyssaviruses (Gt1-7) at an annealing temperature of 45oC. Alteration of temperature may impair the detection of rare African lyssaviruses (Gt 2,3 4). Two rounds are involved in the procedure; the first and second round amplication of complementary DNA (cDNA). The 1st and 2nd round PCR products are then run on agarose gel separately for the result.

- Aliquot 45 of master-mix into tubes according to test samples and controls

  e.g Tubes; 1 2 3 4 5 6 7 8…… 9 10

- Add 5ul of cDNA to each tube containing master-mix as above and. NTC (-ve control) CVS (+ve control).

- Transfer tubes to a hn-PCR machine (2720 hermal cycler, Applied biosystems, life technologies) and cycle on the PCR machine using cycling programme.This can be run together with the 18S PCR if its done.

1st Round PCR Master-mix (JW6/12).

Reagents	Batch No.	Per reaction (ul)	N … reactions (ul)
HPLC grade water		35.075	
GAB (10xbuffer)		5	
dNTPs		1	
JW6 UNI		1	
JW12		1	
Amplitaq gold		0.25	
Tmac		1	
DMSO		0.675	
Total		**45**	

18S PCR

The 18s PCR can be used to confirm the presence of RNA in samples derived from tissues with low cellula content, such as saliva. This provide confidence that a rabies negative result by JW1/12 is a true negative (not poor RNA extraction). This can be run alongside the hn-PCR as they have same temperature.

18S master-mix

Reagents	**Batch no.**	**Per reaction (ul)**	**N reactions (ul)**
HPLC H2O		35	
Gab		5	
dNTPs		1	
18s primers		2.25	
18s competemers		1.5	
Amplitaq Gold		0.25	
total			

Cycling Parameters for 1st round Hn-RT - PCR (and 18S PCR):

Hemi-nested PCR and 18S PCR if run together, is done using the following parameter setup for the PCR machine:

1 cycle

Denature ……..10 mins 95°C

5 cycles

Denature ……..1 min 30 sec 95°C

Anneal ……..1 min 45°C

Pause ……20 sec 50°C

Extend ……1 min 30 sec 72°C

40 cycles

Denature ……30 sec 95°C

Anneal ……1 min 45°C

Pause ……20 sec 50°C

Extend ……1 min 72°C

1 cycle

Denature ……30 sec 95°C

Anneal ……1 min 45°C

Pause ……..20 sec 50°C

Extend ……10 min 72°C

Chill pause ……4°C

2nd Round Hemi-nested PCR Master-mix (JW10/12)

Reagents	Batch No.	Per reaction (ul)	N … reactions (ul)
HPLC grade water		39.015	
GAB (10xbuffer)		5	
dNTPs		1	
JW10 UNI		1	
JW12		1	
Amplitaq gold		0.25	
Tmac		1	
DMSO		0.675	
Total		**45**	

- Aliquot 49ul into tubes already label with sample numbers as in the first round.
- Add 1ul of 1st round PCR product into the 49ul of the master-mix ensuring product are aliquot into master-mix with corresponding numbers. This is done in a cabinet in the template room.

Transfer the tubes with content to the PCR cycler. The same cycling parameters as in 1st cycle PCR is used to the 2nd PCR.

Gel Electrophoresis and Visualisation of Lyssavirus hemi-nested and Taqman PCR Products.

- Seel 2 ends of the agarose plate (moult)
- Insert metal combs to make holes
- Prepare agarose (200ml TAE + 3.6g agarose) in a flask. The percentage of agarose required depends on the size of PCR products; hn-PCR use about 100ml of 1.8% gel. Taqman RT-PCR, minimum of 2% gel prepared in 150ml should be used.
- Melt agarose in microwave (30min or untilno agarose settlement in glass is observed)
- Cool molten gel by gently turning flask under running cold water.
- Add 5ul SBYR safe solution per 100ml of gel. Swell until evenly mixed.
- Pour into the agarose into the plate/moult.
- Check for small bubbles and remove bubbles to side of moult. Wait for gel to solidify
- To enable loading of DNA (or RNA) sample it must be mixed with a suitable value of gel loading buffer. 2-3ul of loading buffer into a PCR plate wells. Prepare extra plate wells for a DNA ladder/marker (This assist to read the distance moved by the DNA or RNA)
- Add 7ul of 1st hn-PCR product to the dye in the PCR plate well.
- Remove combs from agarose when gel solidifies (looks cloudy).

- Place agarose gel in Tank containing TAE buffer, ensuring gel is covered with TAE buffer. If not add TAE buffer to cover gel. NB; TAE must be changed every week.

- Load 10ul solution and marker (ensuring no spill into tank TAE) in the 1st row of gel holes near the black cord (negative pole) end. Usually the DNA ladder/marker solution with dye is added to first gel hole. NB: Ensure you note on paper the order of samples added to holes as prepared in the plate.

- Repeat for 2nd Round hn-PCR product (3ul dye = 7ul 2st PCR product) by putting in the second row of gel holes usually in the middle of the gel block. Again 1st gel hole should be use for the 2nd DNA reader

- Immediately put lid on ensuring –ve = black cord; +ve – red cord

- Connect machine through electrophoresis power supply (PowerPacTM, Bio Rad Laboratories, USA). There should be some bubbles/current) at the the –ve end

- Run for 45minutes to 1hour observing that sample has moved a reasonable distance but not getting to the end of gel.

- Remove plate with gel from the tank. Put gel only with 1st product and later 2nd product in a Gel imager machine (Universal Hood, Bio Rad, USA). Read results from the monitor connected to the machine.

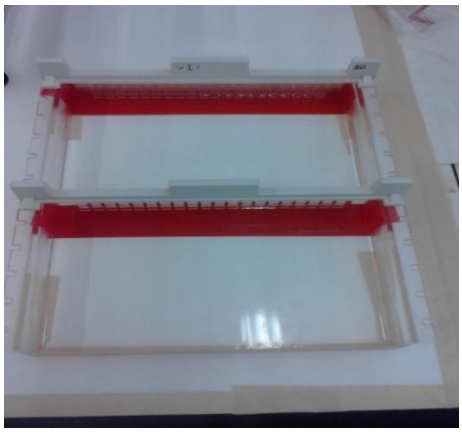


Fig. 27A. Electrophoresis Gel with gel-forming holes comb in the plate

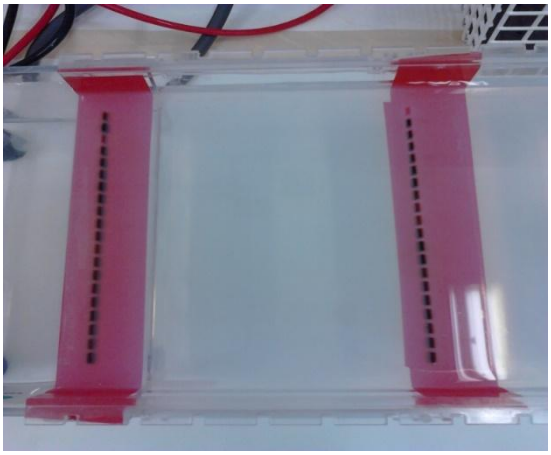


Fig. 27B. DNA products loaded in gel

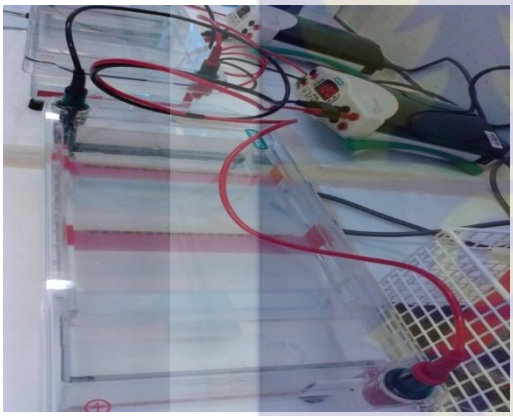


Fig. 27C. Plate in Tank of buffer and (Universal GEL connected Powerpac™.

Fig. 27D. hn-PCR Result reading using imager machine Hood)

Fig. 27 Gel Electrophoresis and Visualisation of Lyssavirus hemi-nested PCR Products.

v. Lyssavirus SYBR Green real-time PCR assay for molecular diagnosis

This method is able to detect all lyssavirus species and may be used as an alternative to differential Taqman RT-PCR especially if genotypes 1, 5, or 6 are suspected. The protocol includes a separate RT-PCR assay containing SYBR Greeen for amplification of the housekeeping control, mRNA for Beta-actin.

The iScript RT-PCR kit with SYBR Green is very sensitive with cDNA synthesis and and PCR amplification is carried out in a single tube. SYBR green assays have 2 phases: The Amplification phase correspond to the PCR portion resulting in generation of dsDNA. In the second dissociation phase, dsDNA is melted into ssDNA. This phase give an indication of the amplicon size.

Mastermix was prepared in the clean room to avoid contamination:

Mastermix:

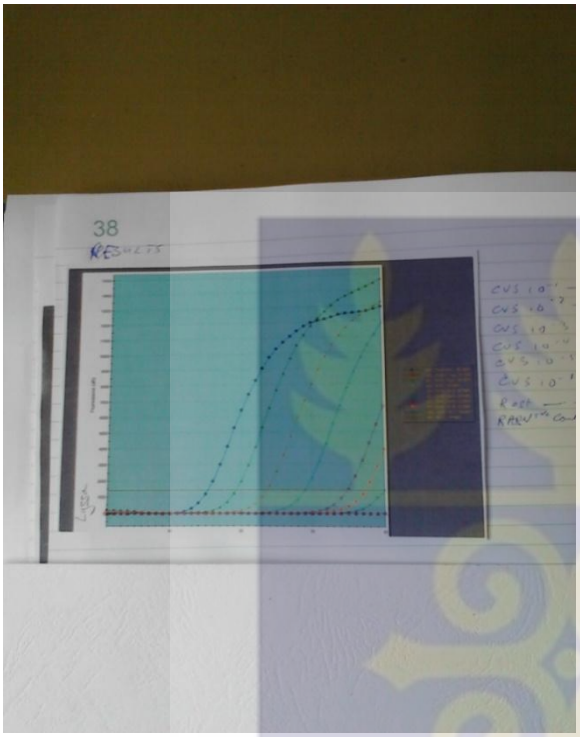
Reaction mastermix reagent	**concentration**	**Volume per reaction(ul)**
Water	As supplied	19
SYBR Green RT-PCR reaction mix	As supplied	25
JW12	20pmol/ul	1.5
N165-146	20pmol/ul	1.5
iScript RT enzyme mix	As supplied	1.0
Total Volume		**48**
B-Actin mix		
water	As supplied	19
SYBR Green RT-PCR reaction mix	As supplied	25
BatRatAct intronic	20pmol/ul	1.5
BatRatAct Reverse	20pmol/ul	1.5
iScript RT enzyme mix	As supplied	1
Total Volume		**48**

- The mastermix was vortex and
- Aliquoted 48ul into the relevant wells 8-well strips (according to the plate setup sheet).

Addition of template (extracted RNA). This is done in the template room.

- Add 2ul of total RNA to each of the mastermix tube (reagent mix and b-actin mix)
- Cover with lid and press down
- Transfer the PCR plate to the machine for thermal cycling

Results, generated automatically by the PCR soft ware, were obtained from the amplification curve and confirm result with a dissociation curve as in the graph (**Fig 28**). In the event of an inconclusive assay, an agarose gel can be run to confirm presence or absence of an amplicon.



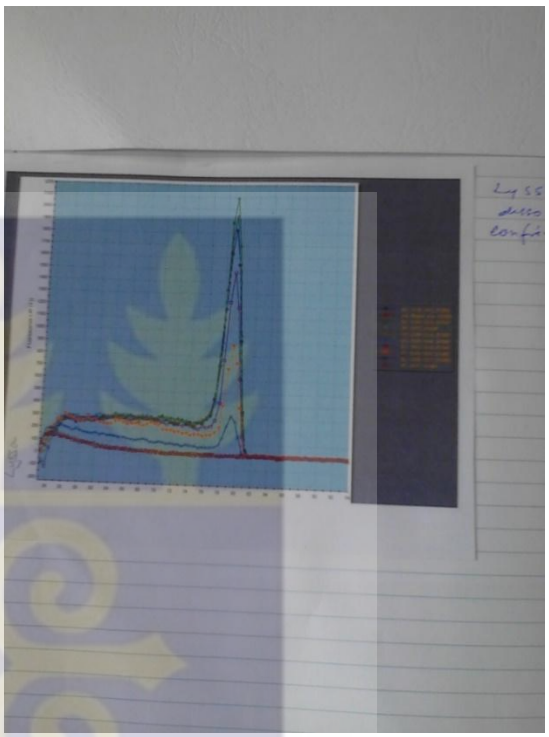


Fig. 28A PCR Amplification curve **Fig. 28B PCR dissociation curve**

Fig. 28 Lyssavirus PCR Curves (Results) **Fig 28A** show a amplification curve and **Fig. 28B** show a dissociation. Positive samples has an exponential amplification curve and a cycle number. A negative sample has a flat amplification curves and no cycle number.

Cycling Parameters for SYBR Green PCR

1 cycle

cDNA synthesis 10 min at 50oC

1cycle

Reverse ranscription inactivation 5min at 95oC

40 cycles;

PCR cycling and detection	10 sec at 95oC
	30sec at 60oC

1 cycle

Melt curve analysis	1 min at 95oC
	1 min at 55oC
	10sec at 55-95oC

Appendix 5 Publications (On subject Area)

Peer-Reviewed Journals:

Richard D. Suu-Ire,Yaa Ntiamoa-Baidu, Andrew A. Cunningham and James Wood. Lyssavirus surveillance in fruit bats in Ghana, 2008 – 2012. Epizootiology and Animal Health in West Africa 9 (2013: 106 – 112.

Billeter, S. A., Hayman, D. T. S., Peel, A. J., Baker, K., Wood, J. L. N., Cunningham, A. A., **Suu-Ire, R.**, Dittmar, K. & Kosoy, M. Y. (2012) Bartonella species in bat flies (Diptera: Nycteribiidae) from western Africa. Parasitology, 139, 324–329 – Undertook experiment

Hayman, D.T.S., **Suu-Ire, R.**, Breed, A.C., McEachern, J.A., Wang, L., Wood, J.L.N. & Cunningham, A.A. (2008) Evidence of Henipavirus Infection in West African Fruit Bats. *PLOS One* **3** (7); e2739 – Field Sampling, Processing of samples

Hayman, D.T.S., Fooks, A.R., Horton, D., **Suu-Ire, R.**, Breed, A.C., Cunningham, A.A. & Wood, J.L.N. (2008) Antibodies against Lagos Bat Virus in Megachiroptera from West Africa. *Emerging Infectious Diseases* **14**, 926-8 - Field Sampling, Processing of samples

Hayman DTS, Emmerich P, Yu M, Wang L-F, **Suu-Ire R**, *et al*. (2010) Long-Term Survival of an Urban Fruit Bat Seropositive for Ebola and Lagos Bat Viruses. PLoS ONE 5(8): e11978. doi:10.1371/journal.pone.0011978 - Field Sampling, Processing of samples

Baker KS, Todd S, Marsh G, Fernandez-Loras A, **Suu-Ire R**, Wood JLN, Wang LF, Murcia PR, Cunningham AA (2012) Co-circulation of diverse paramyxoviruses in an urban African fruit bat population. J Gen Virol. 2012 Apr;93(Pt 4):850-6 - Field Sampling, Processing of samples

Hayman DTS, Yu M, Crameri G, Wang L-F, **Suu-Ire R**, Wood JLN, *et al*. Ebola virus antibodies in fruit bats, Ghana, West Africa [letter]. Emerg Infect Dis [serial on the

Internet]. 2012 Jul [*date cited*]. DOI: 10.3201/eid1807.111654 - Field Sampling, Processing of samples

D.T.S. Hayman, A.R. Fooks, J. M. Rowcliffe, R. McCrea, O. Restif, K.S. Baker, D.L.Horton, **R. Suu-Ire**, A.A.Cunningham,and J.L.N. Wood. Endemic Lagos Bat virus infection in Eidolon helvum. Epidmiology Infection Jan. 2012 – Field sampling and processing of samples

Conference Presentations:

Richard D. Suu-ire, Yaa Ntiamoa-Baidu, James L. N. Wood, Andrew, A. Cunningham. LAGOS BAT VIRUS INFECTION STUDY**.** Twentieth (20th) Congress of the Ghana Veterinary Medical association, October 2014, Accra, Ghana – Wrote and presented paper

Suu-Ire R. Bats, Rabies and Lyssaviruses. Oral presentation at the 19th Congress of the Ghana Veterinary Medical Association, Accra, Ghana. October 2012

Richard D. Suu-ire, Yaa Ntiamoah-Baidu, James L. N. Wood, Andrew, A. Cunningham. LAGOS BAT VIRUS INFECTION STUDY**.** Oral presentation at the Twentieth (20th) Congress of the Ghana Veterinary Medical association, October 2014, Accra, Ghana

Richard D. Suu-Ire, David T.S. Hayman**,** Yaa Ntiamoa-Baidu, James L. N. Wood, Andrew, A. Cunningham. SEROLOGICAL INVESTIGATION OF EBOLA VIRUS IN FRUIT BATS IN GHANA. A paper presented at the 6th Pan Commonwealth Veterinary Association and 27th Malaysia Veterinary association conference in Kuala Lumpur, Malaysia, 23rd to 27th March 2015.