Investigation of Arenavirus and Filovirus Infections in Rodents and Non-Human Primates in the United Kingdom

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Abstract

Recent years have witnessed virus disease outbreaks that have caused considerable morbidity and mortality. As 80% of human viruses are zoonotic, outbreaks can be prevented by active surveillance of wildlife for pathogens with a potential for cross-species transmission. Rodents serve as important reservoirs for many zoonoses and epizooses. In this thesis, a cDNA library of 976 rodents, shrews, moles and birds was constructed. Molecular methods using inosine-containing degenerate primers were developed for the screening of novel viruses of the families, *Arenaviridae* and *Filoviridae*. Filovirus RNA was absent in all specimen screened. Lymphocytic choriomeningitis mammarenavirus (LCMV) RNA was detected in 4 *Mus musculus* (19%). Further screening of specimens obtained from a zoo outbreak resulted in the detection of LCMV RNA in a total of 20 *M musculus*, 2 Geoffroy's marmoset, 1 black and white colobus and 1 Black crested gibbon from two separate sampling sample sets. Viral RNA was detected in non-human primates (NHP) not previously reported. Rodents screened at the zoo after the outbreak were negative for LCMV RNA. Phylogenetic analyses of full-length glycoprotein precursor (GPC) of 4 NHP-derived and 4 rodent-derived LCMV revealed a clustering of the former with other lineage I LCMV GPC sequences. Molecular characterisation of the novel LCMV strains uncovered a consistency in amino acid substitutions across primate-derived and rodent-derived LCMV strains at position 211 of GPC which may be a possible determinant for cross-species transmission. Analysis of the alpha-dystroglycan receptor of rodents and NHPs revealed residues that might influence GPC binding affinity and therefore drive virus evolution. Pseudoviruses constructed from GPC of rodent-derived LCMV displayed higher infectivities in hepatocellular carcinoma cells than their NHP-derived LCMV counterpart which is indicative of the existence of another unidentified host receptor. Together, the results establish a framework for further investigation of the molecular basis for cross species transmission of LCMV between rodent and primate hosts.

Publications

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1. General Introduction

1.1. Emerging Infectious Diseases

Approximately fifty years ago, a considerable number of people believed that humans had finally won the fight against infectious diseases. This was partly due to breakthroughs in human therapies, vaccines and nutrition at that time. The celebration was cut short with the emergence of 'new' pathogens and diseases with devastating impacts on both plants and animals - a situation that remains unchanged (Lipkin, 2010; Mayer, 2000; National Institutes of Health, 2007). Emerging infectious diseases (EIDs) are those that have newly appeared in a population or would have existed in the past but presently either have increased incidence or change in geographic range (Morse, 1995; Rey, 2012). These diseases are caused by a variety of bacteria, fungi, protozoa and viruses (Table 1.1) and include Acquired Immunodeficiency Syndrome, hantavirus pulmonary syndrome, Severe Acute Respiratory Syndrome (SARS), Lassa fever, Zika virus infection, Lyme disease, and the haemolytic uremic syndrome (sometimes caused by *Escherichia coli*) to mention a few. Although some of these diseases can be transmitted to the human population through food and water, a significant number require the aid of vectors such as rodents and arthropods. The pathogens responsible for these diseases are not necessarily novel and may have been previously identified but have recently increased in significance as a result of a change in transmission dynamics brought out by factors such as drug resistance or introduction to new regions where they were not previously endemic (Rosenberg *et al.*, 2013).

Certain regions in the world are 'hotspots' for particular emerging diseases (Morse *et al.*, 2012). In 2013, The Centre for Disease Control and Prevention (Centres for Disease Control and Prevention, 2013b) confirmed a global increase in food-related diseases which has resulted in thousands of deaths, raising the costs of health care to $9 billion

annually. In addition, millions of water-borne illnesses are recorded each year in the United States of America. Diseases such as Dengue fever reported in over 100 endemic countries worldwide with 2.5 billion people at risk for contracting the virus annually.

Africa has had its fair share of reported outbreaks of a variety of infections including HIV/AIDS, Lassa fever, malaria and tuberculosis. These data just scrape the surface of the real statistics as most of these diseases in developing countries carry-on unreported (Centres for Disease Control and Prevention, 2013b). Nonetheless, countries where these diseases are not endemic may still be at risk as human activities including deforestation and reforestation can spread these diseases to areas that were not affected previously. This calls for a global and united effort.

1.1.1. Zoonoses

Zoonoses are infectious diseases that can be transmitted from animals to humans under natural conditions. They are of huge importance because they constitute the major percentage of 'novel' diseases. Over 70% of recently emerging infectious diseases affecting humans originate from animals and about 60% of all human pathogens are zoonotic (Centres for Disease Control and Prevention, 2013b; Slingenbergh *et al.*, 2004).

Interestingly, most warnings on cross-species transmission are given to prevent human exposure to animal diseases when the reality however is that transmission can also occur in the reverse order. This is referred to as reverse zoonoses (Cleaveland *et al.*, 2001; Wallis, 2000; Woolhouse & Gaunt, 2007).

Table 1.1: Some emerging/re-emerging infectious diseases, their causative agents and year of identification.

Disease	Infectious Agent	Year*	Source
Typhoid fever	*Salmonella typhi*	1880	(Marineli *et al.*, 2013)
Lassa fever	Lassa mammarenavirus	1969	(Shelokov, 1970)
Ebola Viral Disease	Ebolavirus	1976	(Emond *et al.*, 1977)
Lyme Disease	*Borrelia burgdoferi*	1982	(Burgdorfer *et al.*, 1982)
AIDS	Human Immunodeficiency virus	1983	(Barre-Sinoussi *et al.*, 1983)
Middle Eastern Respiratory Syndrome	Middle Eastern Respiratory Syndrome virus	2012	(Zaki *et al.*, 2012)
Zika virus infection	Zika virus	1947	(Attar, 2016)
Malaria	*Plasmodium falciparum*	1880	(Centres for Disease Control and Prevention, 2015b)
Haemolytic Uremic Syndrome	*Escherichia coli* (bacterium)	1983	(National Institutes of Health, 2007)

* Year of identification of disease/ isolation of causative agent

Although most zoonotic pathogens are either non-transmissible or minimally transmissible (Woolhouse & Gowtage-Sequeria, 2005), 25% can be transmitted from man-to-man as long as there are repeated reintroductions from a nonhuman reservoir. Global regions worldwide that are present sources of zoonotic disease are shown in Figure 1.1.

1.1.2. The Process of Disease Emergence

Various authorities have proposed theories on the process of emergence, splitting it into well-defined steps. Morse (1995) originally outlined a two-step process, the first involving the introduction of the pathogen into a new host population. Here the agent in question may have existed previously in the environment but in a totally different host species or as a variant of an existing human disease. The second step would then be the establishment of the pathogen in the new human host and further transmission within the new host population (Morse, 1995). Childs and colleagues (2007) agree with Morse's idea but see these two stages as the most important parts of a larger four-step process – the last two being the sustained transmission of the pathogen among the new host species (dependent on the mode of virus excretion from an infected host and social interactions within the new host population) and the adaptation of the pathogen - a function of its rate of replication and mutation (Childs *et al.*, 2007). Wolfe and colleagues proposed five stages in the evolution of a pathogen from one host to another before a final transition to humans exclusively. They explained that the completion of the process (Figure 1.2) is avoidable as pathogens may be stuck in one of the stages and is unable to move beyond it (Wolfe *et al.*, 2007).

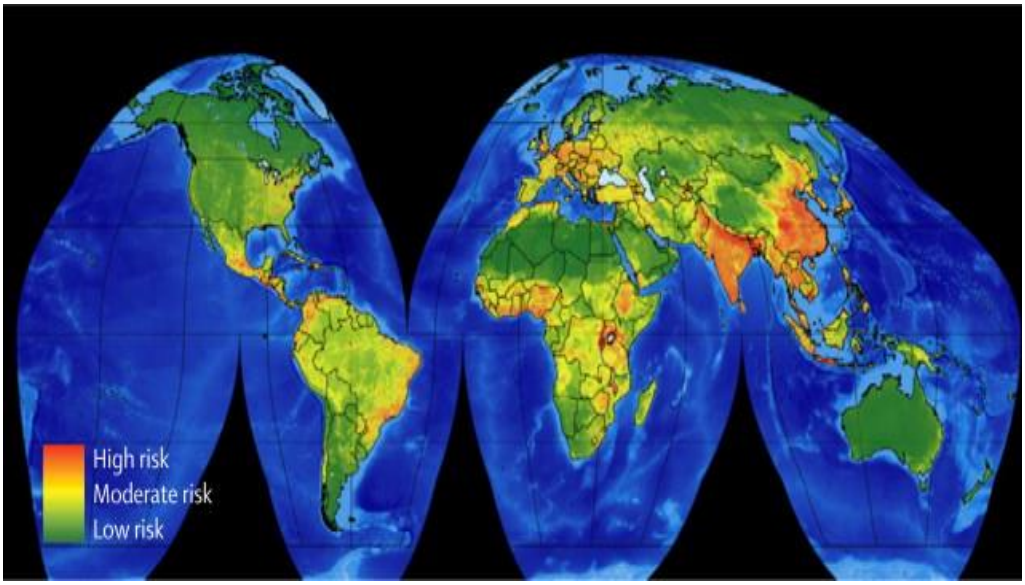


Figure 1.1: Global map showing regions where zoonotic diseases have emerged. Colours indicate regions of high risk, moderate risk and low risk (Morse et al., 2012).

More recently, Morse *et al.* (2012) also described this process, dividing it into three stages (pre-emergence, localised emergence and full pandemic emergence) shifting the focus from the properties of the pathogen to the dynamics of infection. In stage one; the pathogen is still present in its natural host. However, changes (ecological, social and socioeconomic) affect the pathogen's pattern of transmission, thus extending its infection to other host populations (including other non-human hosts), which increases the possibility of human contact. An example of this was observed during the herding of pigs to newly established farms in regions of Malaysia occupied by fruit bats. Fruit bats feeding on trees in the area of the new pig settlements, transmitted Nipah virus to the pigs, causing the emergence of the Nipah virus in Malaysia in 1997 (Chua *et al.*, 1999). Thus all activities that tend to draw humans and wildlife together encourages disease emergence. The second stage (localised emergence), involves the interactions between the humans and animals (exposure to fomites used by livestock farms) which causes an initial spread of the pathogen to people. Cases of infection at this stage are few and localised within the area of first exposure. The final stage is the continual pathogen transmission from one individual to the

next which is dependent on the pathogen's ability to maintain transmission (Morse *et al.*, 2012).

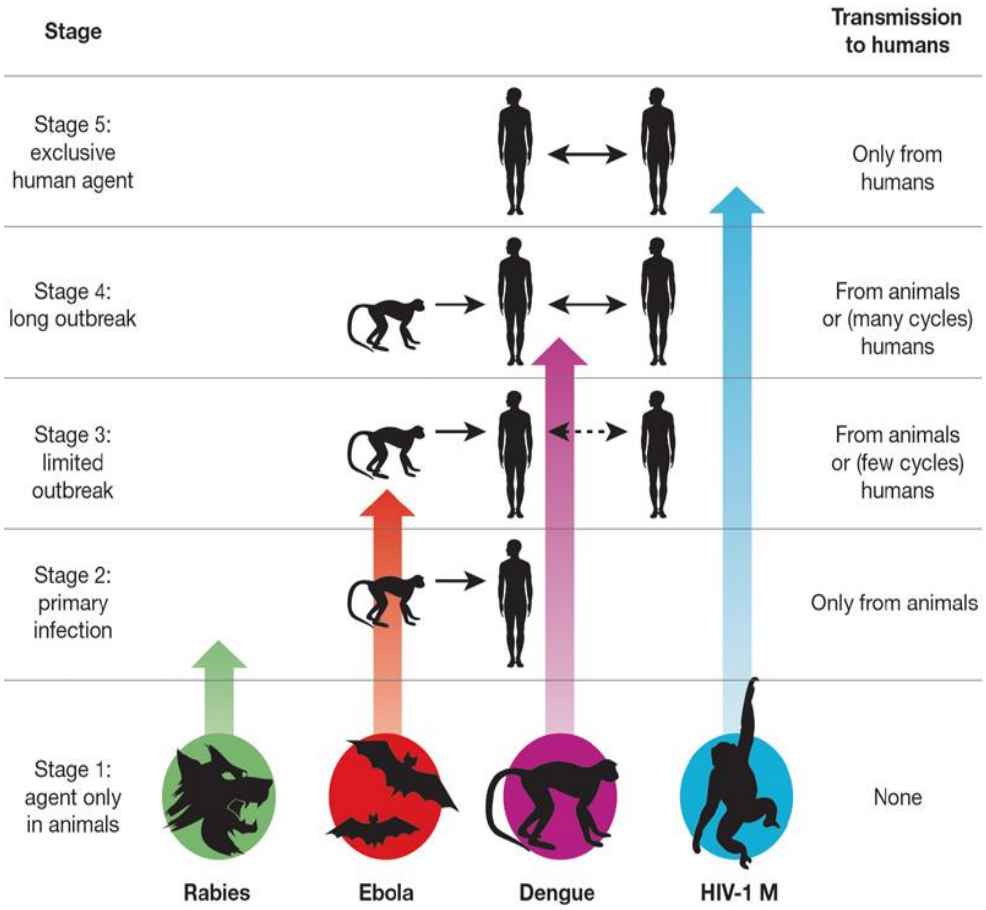


Figure 1.2: Five stages of pathogen evolution among species according to Wolfe and colleagues (Wolfe *et al.*, 2007).

Various factors cause disease emergence. These may include changes in the pathogen (as a result of antigenic drift or shift) and environment. Factors such as changes in human and animal demography and farming are also important. Other social and cultural factors such as food habits, religious beliefs and technologies also play significant roles (Haagmans *et al.*, 2009; Mather *et al.*, 2013; Mayer, 2000; Morse, 1995; Morse *et al.*, 2012; Slingenbergh *et al.*, 2004; World Health Organisation, 2013). The emergence of diseases such as Legionnaire's disease was brought about simply by changes in air-conditioning technologies (National Institutes of Health, 2007). The developments in transportation (e.g. air

travel and international trade) have also encouraged disease spread as natural and geographical barriers to international movement are overcome (Mather *et al.*, 2013). Natural disasters such as earthquakes and floods are also important factors. The probability of one single factor 'driving' a particular disease is very low. In most cases, a disease only emerges as a result of the combination of various factors (Woolhouse *et al.*, 2012).

The transmission routes through which new pathogens can be introduced into a population include direct contact (through inhalation, wounds and sexual contact), indirect contact (through water, food or an environmental reservoir) and vectors (mainly arthropods). In reality, a pathogen could be transmitted through a number of routes at the same time. Most emerging diseases however are transmitted via vectors (Taylor *et al.*, 2001; Woolhouse, 2002). Beyond crossing physical barriers however, pathogens may encounter host responses such as restriction factors that may inhibit replication. This determines disease outcome (Howard & Fletcher, 2012).

1.1.3. Host Range and Cellular Receptors

Cell tropism is an important requirement for cross-species transmission as the pathogens need to recognise the new host cellular receptors to allow for cell attachment and penetration. Typically, pathogens with a greater host range would be able to infect a variety of species. Host range however depends on the pathogen's ability to recognise receptors that have been conserved across a variety of species (Morse *et al.*, 2012; Woolhouse *et al.*, 2012). This is an important key to the prevention of emerging infections as it provides information on the potential zoonotic ability of novel viruses that have been isolated from reservoirs to date. Knowledge on host response to infection is also important in determining the reason for the variation in disease progression in reservoirs and new host species.

1.1.4. Vectors and Reservoirs

Vectors, in a broad sense can be described as anything (living or non-living) that allows, encourages, supports or creates an opportunity for the transmission of a pathogen. Such a broad definition includes fomites which are non-living. In this thesis a vector is examined from the biological (living) point of view - an organism that can transmit a pathogen between infected hosts or from one infected host to a healthy one. Living vectors have an ecological relationship with the pathogen and may be a significant part of the latter's life cycle (kurkjian *et al.*, 2010). Knowledge of the various vectors that act as vehicles for disease transmission and their behavioural pattern provides insight into methods for disease control as was observed between the period of 1955 and 1969 when the World Health Organisation took a stand against the *Anopheles* mosquito (the vector for malaria) and eliminated it from Asia, Europe and North America with the help of insecticides (Gravitz, 2012).

Living vectors are also called reservoirs. These can successfully harbour the pathogen after being infected and can spread it to other animals. They successfully harbour the pathogen (possibly throughout the animal's lifetime) usually without developing disease – a characteristic which is usually a consequence of the reservoir's evolution and subsequent adaptation to the pathogen. When these pathogens cross the species barrier however, disease develops (kurkjian *et al.*, 2010; Morse *et al.*, 2012; Woolhouse *et al.*, 2012). A reservoir can also be described as a species that sustains the pool of the virus (Peterson *et al.*, 2004b). The Sooty mangabey (SM) monkeys (*Cercocebus atys*) and chimpanzees are natural reservoirs of the simian immunodeficiency virus (SIV). They do not develop AIDS after infection with SIV while other non-natural hosts like the rhesus macaques do (Haagmans *et al.*, 2009; Martinot *et al.*, 2013; Woolhouse *et al.*, 2012). These non-human primates (NHPs) make up only a small fraction of the variety of non-

human hosts identified as reservoirs for many zoonoses. Bats, birds, carnivores, primates, rodents and ungulates are similarly important in harbouring viral diseases. Apart from HIV, other viruses suspected to have originated from primate-reservoirs include the hepatitis B virus, dengue fever virus, human T-lymphotropic virus (HTLV) and yellow fever virus. The avian influenza virus originated from the water fowl and wild birds while measles virus, mumps virus, smallpox and the human corona virus OC43 originated from livestock (Bean *et al.*, 2013; Woolhouse *et al.*, 2012).

1.1.5. Zoonotic Pathogens

About 60% of all human pathogens are zoonotic (Approximately 50% of bacteria, 40% of fungi, 95% of helminths, 70% of protozoa and 80% of viruses) and are the major causes of emerging infectious diseases (Anthony *et al.*, 2013b; Morse *et al.*, 2012; Taylor *et al.*, 2001). Some of these are exotic in that they have been introduced into new geographical regions where they didn't exist previously while others are endemic and have higher incidence or have started infecting local human populations for the first time. Zoonotic pathogens are spread across mainly bacteria, fungi, helminths and viruses. Viruses however make up more than two thirds of all newly emerging human pathogens (Woolhouse & Gaunt, 2007). It is interesting to note that none of the new viruses discovered so far belong to a 'new' family of viruses but are still within already identified viral families. These viruses have different infection rates and can shift from the strict infection of animals to man after periods as short as a few months to as long as decades as observed with HIV and influenza A) (Anthony *et al.*, 2013b; Morse *et al.*, 2012; Woolhouse & Gaunt, 2007; Woolhouse *et al.*, 2012).

Viruses with RNA genomes are more associated with emerging infectious diseases than their DNA counterparts. This is owed to the fact that RNA viruses have high error-prone replication (by viral polymerases)

and higher replication rates as well as frequent asymptomatic maintenance within their natural reservoir hosts (Cleaveland *et al.*, 2001; Johnson *et al.*, 2015; Mather *et al.*, 2013; Novella, 2003; Venkatesan *et al.*, 2010).

1.2. Filoviridae

This family of viruses consist of a few discovered species responsible for a variety of infections in humans and other animals. The name *Filoviridae* is from the Latin word 'filum' which means thread-like (Feldmann & Klenk, 1996; Wambani *et al.*, 2016; Yamamoto, 2015) based on the unique shape of filamentous virions possessed by members of the family (Burk *et al.*, 2016).

Members of the *Filoviridae* have non-segmented, negative-sense, single-stranded RNA packaged in a rod-like capsid (about 790nm long for marburgvirus and 970nm for Ebolavirus) (Figure 1.3). While other virus families in the order Mononegavirales have only the nucleocapsids shaped as filaments, the whole virion of the filoviruses are filamentous (Booth *et al.*, 2013). The linear RNA of each mature virion is about 18 to 19kb long and codes for at least seven structural proteins which include the nucleoprotein (NP), viral protein (VP) 35, VP40, VP30, VP24, polymerase (L) and glycoprotein (GP) (Elliott *et al.*, 1985). Intergenic regions of varying length and composition separate each gene from the next (Feldmann & Klenk, 1996). Transcription begins with attachment of RNA dependent DNA polymerase (RdRp) to the leader sequence on the 3' region of the RNA genome. The polymerase then transcribes each protein in regular succession recognizing the flanking start and stop codons.

1.2.1. Taxonomy and Classification

Filoviruses are assigned to the order Mononegavirales. The International Committee on the Taxonomy of Viruses (ICTV) currently recognises three genera containing seven discovered viral species (Figure 1.4) namely Cuevavirus (Lloviu cuevavirus), Ebolavirus (Bundibugyo ebolavirus, Reston ebolavirus, Sudan ebolavirus, Tai Forest ebolavirus and Zaire ebolavirus), Marburgvirus (Marburg marburgvirus) (International Committee on the Taxonomy of Viruses, 2015). Filoviruses are classified as hazard group 4 by the Advisory Committee on Dangerous Pathogens and as Tier 1 Selected Agents and Category A bioterrorism agents by the US Centres for Disease Prevention and Control regard filoviruses due to their high person-to-person transmission, mortality rate and absence of vaccines and chemotherapy. All laboratory investigations with infectious materials are carried out in maximum containment (BSL4) (Banadyga & Ebihara, 2015; Feldmann & Klenk, 1996).

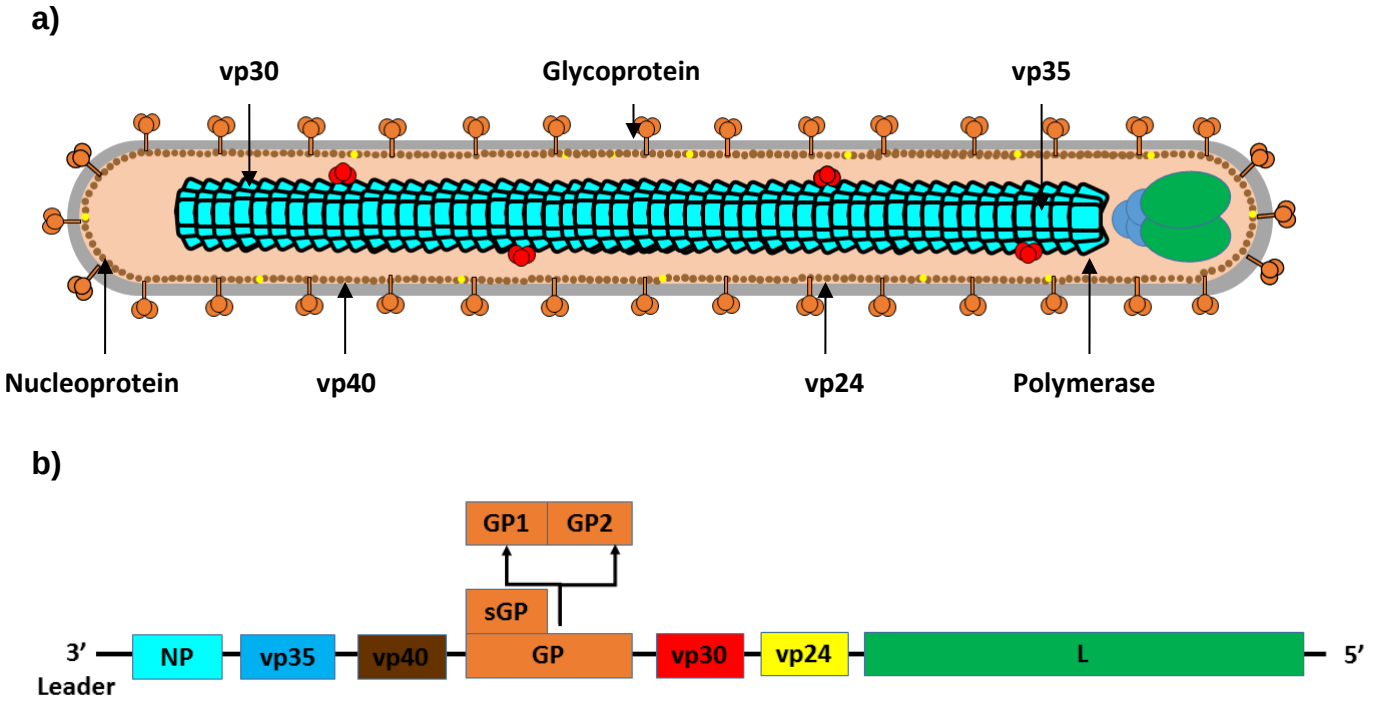


Figure 1.3: The molecular biology of filoviruses (a) Virion structure (b) Genome organisation.

1.2.2. Viral Proteins

The L protein is essential for replication and shares significant homologies with other non-segmented negative-stranded RNA viruses particularly at the N-terminal (Feldmann & Klenk, 1996).

The chief role of the nucleoprotein is the encapsidation of the viral genome. It determines nucleocapsid structure. It constitutes the major part of the ribonucleoprotein complex (RNP).

The glycoprotein (GP) is responsible for receptor binding and fusion (Elliott *et al.*, 1985). GP is expressed as a precursor protein after transcription. Proteolytic cleavage of GP with enzyme furin, splits the former into GP1 and GP2 subunits (Maruyama *et al.*, 2014). An additional soluble protein (sGP) is also encoded for by certain Ebolavirus species and is not included in mature virus particles during assembly but is secreted from infected cells (Muhlberger, 2007).

Together with the NP, vp35 is one of four proteins that make up the RNP. Its function has not yet been elucidated. However its genome position and association with RNP suggests that it plays a similar role to the phosphoprotein (P) in other non-segmented negative-stranded RNA viruses (Feldmann & Klenk, 1996).

The vp40 protein is a membrane-associated protein. It largely determines the filamentous shape of filoviruses and is the main structural protein. It is also the major driver for the assembly and budding of mature virus particles from infected host cells (Hartlieb & Weissenhorn, 2006).

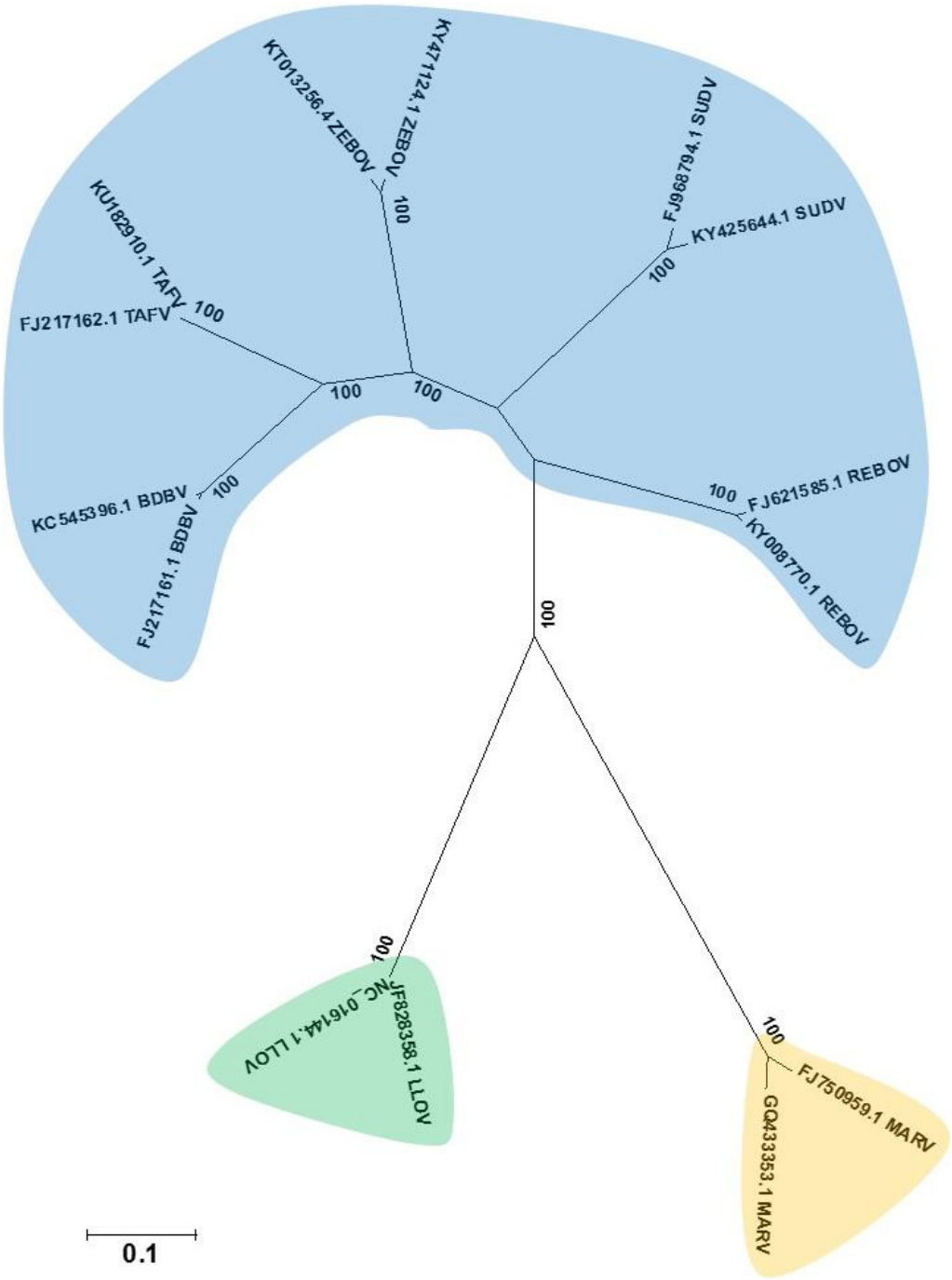


Figure 1.4: Unrooted phylogenetic tree showing relationships among filoviruses. The tree was computed using the complete L gene coding sequence using the Maximum Likelihood method. Reference sequences are indicated by their Genbank Accession Numbers. Coloured regions represent the following groups: Blue – ebolaviruses (Bundibugyo ebolavirus (BDBV), Reston ebolavirus (REBOV), Sudan ebolavirus, Tai Forest ebolavirus (TAFV) and Zaire ebolavirus (ZEBOV)); Green – Lloviu cuevaviruses (LLOV) and Yellow – Marburg marburgviruses (MARV). Branch lengths are drawn to scale: the bar indicates 0.1 nucleotide substitutions per site. Numbers above individual branches indicate the percentage that that branch was found in 1000 bootstrap replicates; only percentages >75% are shown.

VP30 is also a part of the RNP. It is an RNA-binding protein and is required for transcription initiation. It also possesses a zinc-binding domain which is possibly essential for interaction with viral nucleoprotein (John *et al.*, 2007; Modrof *et al.*, 2003).

VP24 is a membrane-associated protein. It is a protein unique only to filoviruses. Its function is not yet clear although but is suggested to play a role in virus assembly or budding. Bamberg and colleagues silenced vp24 using si-RNA and observed a reduction in the number of virions released. Transcription and replication were however not affected (Bamberg *et al.*, 2005).

1.2.3. Life Cycle

1.2.3.1. Virus Entry

Interaction of virus with host receptors is mediated by the trimeric glycoprotein (GP). GP1 and GP2 are receptor-binding and membrane fusion subunits respectively. The critical host receptor for the viral GP had remained elusive until recently Carette and colleagues put forward Niemann-Pick C1 (NPC1) as the likely candidate (Carette *et al.*, 2011) which is supported by other authorities (Cote *et al.*, 2011). NPC1 mediates the movement of intracellular cholesterol from the late endosomes and lysosomes, is widely-distributed and present in all cell types susceptible to virus infection.

After successful attachment of virus to host cells, entry commences leading to delivery of infectious particles to the late endosome, a process achieved through macropinocytosis (a form of endocytosis that is actin-dependent). Acidification of the endosome allows for cathepsin B and cathepsin L to act on GP exposing the domain sites for interaction with NPC1 which precedes fusion of viral and endosomal membranes (Cote

et al., 2011; Messaoudi *et al.*, 2015). After fusion, the ribonucleocapsid is released into the cytoplasm.

1.2.3.2. RNA Transcription and Replication

RdRp begins transcription by attaching to a single binding site at the 3' end of the RNA genome template. Each gene is transcribed by subsequent initiations and stops (sequential transcription) and the resultant mRNA is capped at the 5' end and polyadenylated at the 3' end. At the attainment of sufficient quantities of NP, replication commences where the RNA template is copied into a full-length compliment which serves as a template for the production of more viral RNA (Messaoudi *et al.*, 2015; Muhlberger, 2007).

1.2.3.3. Assembly and Budding

After viral proteins are translated, new virus particles are assembled at the plasma membrane. These interact with vp40 (the matrix protein) and budding follows shortly afterwards, releasing mature virions from the host cell (Messaoudi *et al.*, 2015). The life cycle of Ebolavirus, is represented in Figure 1.5.

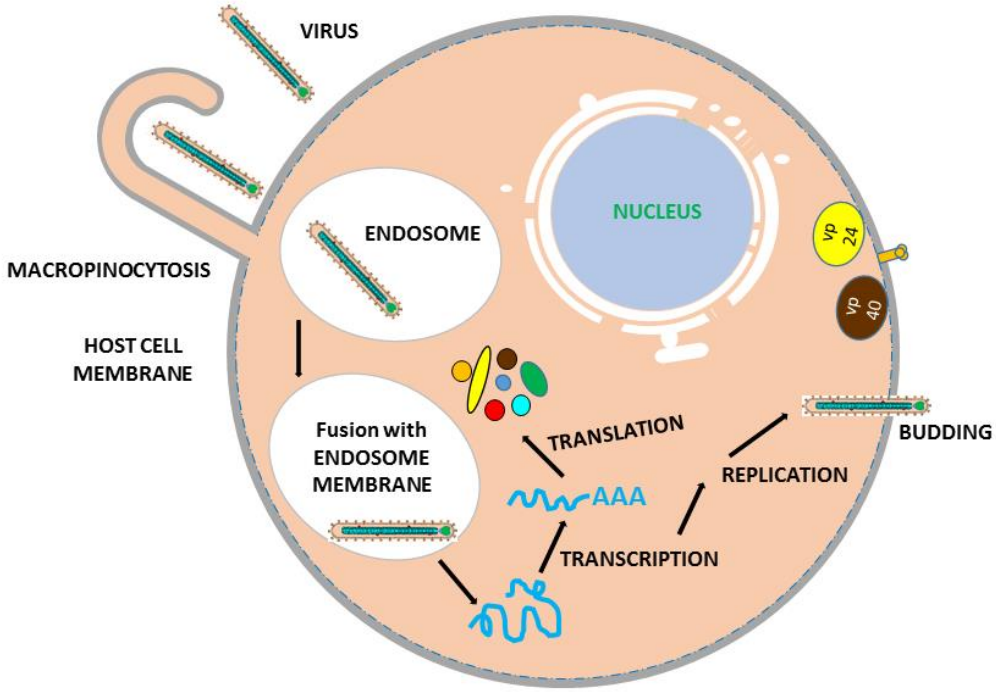


Figure 1.5: Life Cycle of Ebolavirus.

1.2.4. Reservoirs of Filoviruses

In the past, various plants and animals had been postulated as natural hosts for the virus. Investigations involved the experimental inoculation of vertebrates including bats, birds and rodents (Leirs *et al.*, 1999; Swanepoel *et al.*, 1996). A molecular screening of organs from 242 small animals (24 bats, 163 rodents and 56 shrews) obtained from the Central African Republic revealed the presence of DNA sequences with similarity to Ebolavirus genes in *Praomys sp* and *Mus setulosus* rodents (Morvan *et al.*, 1999). This finding is highly debatable however due to the non-reproducibility of results, failure to amplify larger segments of viral genome and failure to isolate the virus (Leroy *et al.*, 2007; Lõhmus *et al.*, 2013). Some outbreaks of Ebola have also involved several non-human primates, pigs and duikers and have led to a rapid decline in wildlife population (Barrette *et al.*, 2009; Le Guenno *et al.*, 1995; Leroy, 2004).

Bats are the second most diverse group of the mammalian class having over 1,200 species. They have been identified as natural hosts for a considerable number of viral pathogens including filoviruses, lyssaviruses, henipaviruses and coronaviruses. Their body size, tendency to live in large colonies and flight pattern over notable distances greatly enhances pathogen transmission and dissemination amongst various species (Luis *et al.*, 2013; Wynne & Wang, 2013).

Despite repeated outbreaks of filovirus infections spanning decades, the natural reservoirs remained a mystery until recently with the repeated isolation of Marburg marburgvirus (MARV) from Egyptian fruit bats (*Rousettus aegyptiacus*) located in two regions of Southwestern Uganda where miners and tourists contracted Marburg haemorrhagic fever (MHF) (Amman *et al.*, 2012; Towner *et al.*, 2009). Also the discovery of Lloviu cuevavirus in insectivorous bats in Spain (*Miniopterus schreibersii*) (Negredo *et al.*, 2011) and the subsequent detection of antibodies against Ebola viruses in other bat species (Leroy *et al.*, 2005; Olival *et al.*, 2013; Pourrut *et al.*, 2009; Taniguchi *et al.*, 2011), indicates that bats are the most probable culprits for filovirus transmission although Ebolavirus has not yet been isolated from any bat species.

1.2.5. Pathogenesis and Clinical Manifestation

All filoviruses (Except RESTV and possibly LLOV) cause severe and often fatal viral haemorrhagic fever upon infection in humans but the precise mechanism by which this is achieved is poorly understood. Insight into filovirus pathogenesis has been obtained from investigations of experimental infection of non-human primates (Messaoudi *et al.*, 2015). Transmission occurs through direct contact of contaminated sharp objects or body fluids from an infected animal or human (Centres for Disease Control and Prevention, 2014b; 2015a). Direct contact with broken human skin encourages viral entry into host systems and subsequent infection of a variety of cell types. Filoviruses appear to have

a preference for macrophages and dendritic cells. The reason for this preference is unknown. Endothelial cells are infected as the disease progresses. The infection of the macrophages and dendritic cells is advantageous as it enables the virus to be transported to the lymph nodes and thus allows for infection extension to kupffer cells (Geisbert *et al.*, 2003). Infection also cause the release of chemokines such as the macrophage inflammatory protein (MIP1α; also known as CCL3) and monocyte chemoattractant protein (MCP1; also known as CCL2) which can direct more macrophages to the site of infection causing further infection and spread of the virus (Messaoudi *et al.*, 2015).

The incubation period for Ebola virus disease is 2 to 21 days. Humans are not infectious until the manifestation of symptoms (Briand *et al.*, 2014). Symptoms of infection range from headaches and muscle pains to kidney and liver impairment. Headaches, myalgia, anorexia and fever constitute early symptoms while nausea, vomiting, abdominal pain and conjunctival vasodilation follow afterwards. Virus replication in parenchymal cells causes necrosis at affected sites (Feldmann & Klenk, 1996). Abnormalities in platelets and fluid distribution are an indication of damage to platelets and endothelial cells. Haemorrhaging (internal and external bleeding) may also occur. This may be caused by adrenocortical infection which affects blood pressure homeostasis (Geisbert *et al.*, 2003). The shock syndrome appears to be associated with the virus-mediated release of humoral factors (Feldmann & Klenk, 1996). Patients who eventually recover have fever of 5 to 7 days duration. Death from severe bleeding and shock usually occurs between day 6 and 16.

1.2.6. Filovirus Disease Outbreaks

The first recorded outbreak of filovirus infection took place in 1967 in Marburg, Germany (which is where the disease got its name from) and resulted in 7 deaths. The virus was transmitted to humans from monkeys (*Cercopithecus aethiops*) imported from Uganda for the purpose of

vaccine production (Slenczka & Klenk, 2007). The largest outbreak of Marburg Virus Disease took place between 2004 and 2005 in Angola. There were 252 reported cases of which 227 people died. The latest outbreak occurred in Uganda in 2017. Three cases were reported and all the infected individuals died (World Health Organisation, 2017a).

Also in 1976 there were two outbreaks of other filovirus infections, this time caused by the Sudan ebolavirus and Zaire ebolavirus which occurred in South Sudan and the Democratic Republic of the Congo (DRC) respectively (Johnson *et al.*, 1977; World Health Organisation, 1978). Since then about 34 other outbreaks have occurred involving various countries worldwide including England, USA and The Philippines (Centres for Disease Control and Prevention, 2016).

The 2014 West Africa outbreak is the largest recorded outbreak of filovirus infection. Multiple countries were affected. WHO reported a total of 28,616 suspected, probable, and confirmed cases with a total of 11,310 deaths in Guinea, Liberia, and Sierra Leone (Centres for Disease Control and Prevention, 2016; World Health Organisation, 2017b). The latest outbreak of Ebola viral disease took place in The Democratic Republic of Congo. There were 5 confirmed cases and 4 deaths (Centres for Disease Control and Prevention, 2017). The outbreak of Lloviu cuevavirus in Spain is the only reported of its kind so far being the only filovirus of European origin (Negredo *et al.*, 2011). Table 1.2 contains a list of all identified filoviruses and their global distribution.

Table 1.2: List of Filoviruses, reservoirs and worldwide distribution

Name	Reservoir	Global Distribution	Year*	Zoonoses	Source
Lloviu cuevavirus (LLOV)	Unknown	Spain	2011	Unknown	(Negredo *et al.*, 2011)
Bundibugyo ebolavirus (BDBV)	Unknown	Uganda, Democratic Republic of Congo,	2007	EVD (40% mortality)	(Towner *et al.*, 2008)
Reston ebolavirus (RESTV)	Unknown	Phillipines	1989	Asymptomatic human infections	(Jahrling *et al.*, 1990)
Sudan ebolavirus (SUDV)	Unknown	South Sudan, Uganda	1976	EVD (41 to 100% mortality)	(World Health Organisation, 1978)
Tai Forest ebolavirus (TAFV)	Unknown	Côte d'Ivoire	1994	EVD	(Le Guenno *et al.*, 1995)
Zaire ebolavirus (EBOV)	Unknown	Democratic Republic of Congo, Gabon	1976	EVD	(Johnson *et al.*, 1977)
Marburg Marburgvirus (MARV)	*Rousettus aegyptiacus*	Democratic Republic of Congo, Zimbabwe, Uganda, Kenya	1976	MHF	(Amman *et al.*, 2012; Siegert, 1972; Towner *et al.*, 2009)

* Year of identification of disease/ isolation of causative agent

1.2.7. Diagnosis, Prevention and Treatment

Various techniques are available for the diagnoses of filovirus infections. IgM Enzyme-linked Immunosorbent assay (ELISA), virus isolation and polymerase chain reaction (PCR) could be used at earlier stages of infection and when symptoms start to appear (Ksiazek *et al.*, 1999; Leroy *et al.*, 2000). Quantitative PCR (qPCR) was used extensively during the 2014 outbreak (Erickson *et al.*, 2016). As disease develops blood specimens can be screened for the presence of IgM and IgG antibodies. Samples from diseased patients can also be investigated by immunochemistry (Centres for Disease Control and Prevention, 2014b; 2015a; Negredo *et al.*, 2011).

Preventive measures for filovirus transmission generally involve the avoidance of direct contact with infected bats and non-human primates. Healthcare workers are required to put on protective clothing when caring for infected patients. Clothing includes gloves and masks. Equipment such as needles are properly sterilised before use and safely disposed of afterwards (Centres for Disease Control and Prevention, 2014b; 2015a).

There are various promising vaccine candidates but none have been approved by the US Food and Drug Administration for the prevention of filovirus infections. Live recombinant vesicular stomatitis virus-based (rVSV) vaccines have shown promise to provide post exposure protection of non-human primates from Marburg virus challenge and Zaire ebolavirus challenge (Daddario-DiCaprio *et al.*, 2006; Feldmann *et al.*, 2007; Garbutt *et al.*, 2004). The rVSV – based vaccine expressing Ebolavirus glycoproteins (rVSV-ZEBOV) have undergone phase 1 clinical trials and have been shown to be reactogenic but immunogenic at doses ranging between 300,000 to 50 million PFU in African and European volunteers (Agnandji *et al.*, 2016). Chimpanzee adenovirus serotype 3 (ChAd3) vaccine is another promising candidate to prevent

ebolavirus infection. It is a chimpanzee-derived replication defective adenovirus encoding ebolavirus glycoprotein (Stanley *et al.*, 2014). Recipients of the vaccine have only shown mild to moderate systemic reaction. Antibody response in most individuals were still significant after 6 months (De Santis *et al.*, 2016).

There is no specific treatment for filovirus infections. Supportive therapy is normally utilized. This includes the use of intravenous fluids and electrolytes to balance body salts. Others involve the maintenance of blood pressure and treatment of opportunistic infections (Cox *et al.*, 2014; Wolz, 2014). No licenced drug is available for the treatment of Marburg virus or Ebola virus infections. ZMapp, is a combination of three monoclonal antibodies directed against Ebola glycoprotein (Group *et al.*, 2016; Qiu *et al.*, 2014) and was first tested on humans in the 2014 Ebola outbreak (Wong & Kobinger, 2015) however its efficacy is still being investigated. Other promising drugs at several developmental stages include brincidofovir, an oral antiviral with in vitro activity against Ebola virus and favipivavir, a pyazinecarboxaminde derivative (Tully *et al.*, 2015).

1.3. Arenaviridae

The family *Arenaviridae* includes a number of zoonotic pathogens that cause significant morbidity and mortality and hence pose a threat to human existence. The name ‘arena’ is of Latin origin and means ‘sandy’ which is the general description of the appearance of unique grain-like ribosomes (acquired from infected host cells) observed in tissue cross-sections (Buchmeier *et al.*, 2013; Centres for Disease Control and Prevention, 2013a; Charrel & de Lamballerie, 2010).

Shared characteristics among members include an enveloped, spherical virion (50 to 300nm in diameter), housing a single-stranded, bi-segmented, negative-sense RNA (Figure 1.6). The small (S) segment

(3.5kb) encodes for the nucleocapsid protein (NP) and glycoprotein precursor (GPC), while the large (L) segment (7.5kb) encodes for the RNA-dependent RNA polymerase (L) and Z proteins (Albarino *et al.*, 2010b; Charrel & de Lamballerie, 2010; Charrel *et al.*, 2008; Fukushi *et al.*, 2012). The RNA is capped but not polyadenylated. Both segments employ an ambisense coding strategy and as such each segment has two non-overlapping open reading frames (ORF) where the RdRp (L protein) binds to transcribe messenger RNA (Bederka *et al.*, 2014) (Figure 1.6). Each segment also has a non-coding intergenic region (IGR) which encodes a hairpin that terminates gene transcription (Li *et al.*, 2014; Swiss Institute of Bioinformatics, 2015; Urata & Yasuda, 2012). At the start of each segment is also an untranslated region (UTR) which encourages the circularization of the genome forming a panhandle (Honko *et al.*, 2015; Loureiro *et al.*, 2012; Palmer *et al.*, 1977; Zapata & Salvato, 2013).

1.3.1. Taxonomy

Arenaviruses are currently not assigned to any order. Recently, the ICTV updated the previous classification of the *Arenaviridae* family based on recent discoveries of novel arenaviruses by varous independent groups (Bodewes *et al.*, 2013; Hetzel *et al.*, 2013; Radoshitzky *et al.*, 2015; Stenglein *et al.*, 2012). In the current taxonomy, 36 of all reported arenaviruses are recognized and conveniently classified under two genera based on genetic, serological and geographical similarities.

The first genus, *Mammarenavirus* (formerly '*Arenavirus*') consists of Old World and New World complexes. Arenaviruses indigenous to Africa are called the Old World arenaviruses while those indigenous to The Americas are called the New World arenaviruses. The only exception to this is the Lymphocytic choriomeningitis virus (LCMV) which is an Old World virus distributed globally due to its association with the house mouse, *Mus musculus* (International Committee on Taxonomy of

Viruses, 2015; Nunberg & York, 2012). The New World complex is larger than its Old World counterpart, having more viruses associated with it. It is further divided into three lineages, clades A, B, and C of which members belonging to Clade B cause the majority of haemorrhagic fevers in South America (Burri *et al.*, 2012).

The second genus, *Reptarenavirus* consists of three species isolated from boid snakes (Bodewes *et al.*, 2013; Hetzel *et al.*, 2013; International Committee on Taxonomy of Viruses, 2015; Stenglein *et al.*, 2012).

Arenaviruses that cause viral haemorrhagic fevers are classified as biosafety level four (BSL-4) pathogens. These include Machupo mammarenavirus, Sabia mammarenavirus and Lassa mammarenavirus (Centres for Disease Control and Prevention, 2013d). Generally neurotropic LCMV strains are placed in risk group 3 while non-neutrotropic strains are placed in risk group 2 (American Biological Safety Association, 2015; Health and Safety Executive, 2016). The Advisory Committee on Dangerous Pathogens (ACDP) place LCMV strain Armstrong in group 2 while other strains are placed in group 3. The CDC further recommends that laboratory safety and containment for potentially LCMV-infected samples should be handled under biosafety level 2 (BSL-2) while experiments that might cause aerosolization of particles are handled under biosafety level 3 (BSL-3) (United States Department of Health and Human Services, 2009).

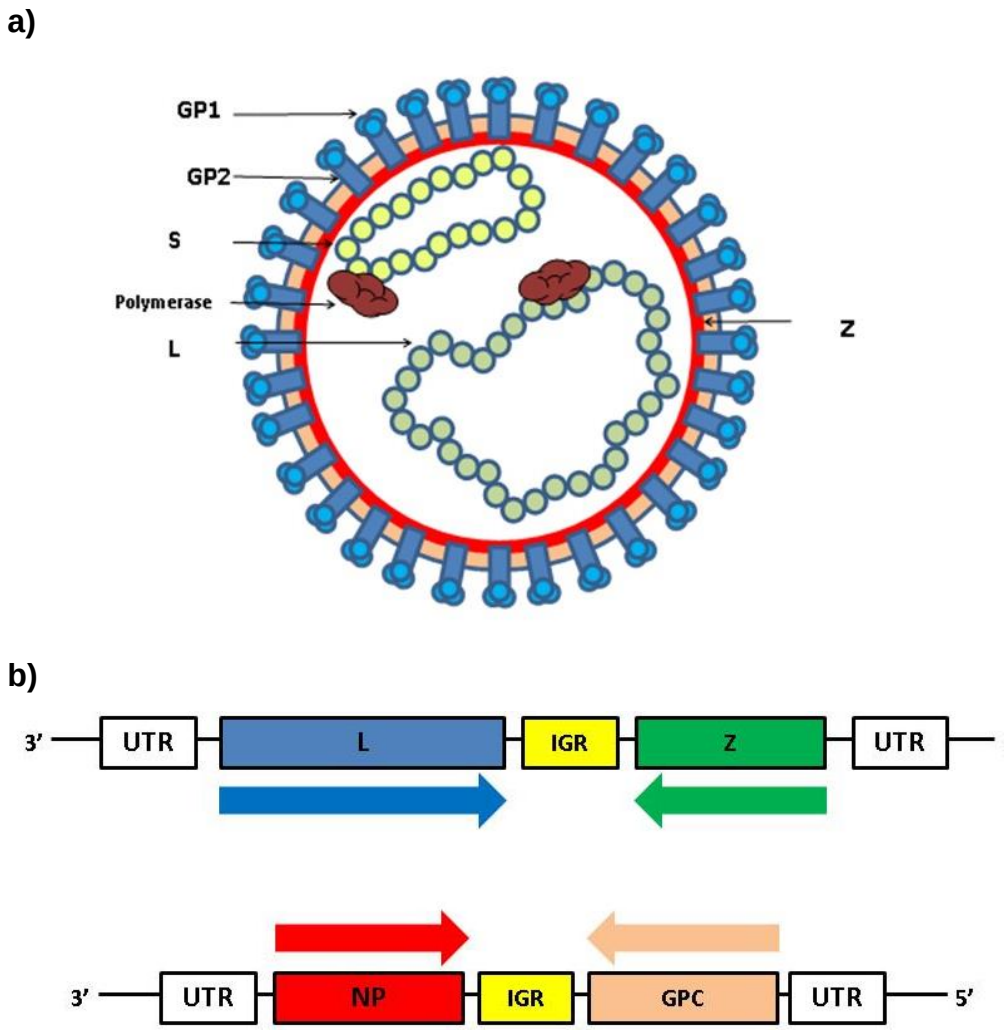


Figure 1.6: The molecular biology of arenaviruses (a) Virion structure (b) Genome organisation. Coloured arrows showing the ambisense coding strategy.

LCMV is generally susceptible to common household disinfectants. Bleach (Sodium hypochlorite) inactivates the virus (Centres for Disease Control and Prevention, 2005; Public Health Agency of Canada, 2011). LCMV is also sensitive to ultraviolet (UV) radiation and heat (55°C to 56°C for 20 minutes) (Armstrong & Lillie, 1934; Martin *et al.*, 2010). The virus is quickly inactivated if not preserved at - 80°C (Rousseau *et al.*, 1997).

1.3.2. Viral Proteins and Functions

The nucleocapsid protein is the most abundant of the four viral proteins produced in infected cells and virions. Its function includes the formation of viral capsid and ribonucleoprotein complexes as well as immune suppression of interferon induction through the exoribonuclease activity of the C-terminal domain (Honko *et al.*, 2015; Qi *et al.*, 2010).

RNA-dependent RNA Polymerase is the largest viral protein having a length of about 2200 amino acid residues. It has at least four domains which function independently and play important roles in viral RNA replication and transcription (Brunotte *et al.*, 2011; Hass *et al.*, 2008; Honko *et al.*, 2015; Lelke *et al.*, 2010; Vieth *et al.*, 2004).

The viral glycoprotein gene is first translated as a precursor protein before being cleaved within the Golgi apparatus and lumen of the endoplasmic reticulum into a 58 aminoacid-long stable signal peptide (SSP) by cellular signal peptidase (SPase) (Nunberg & York, 2012). The remaining part of the precursor is further cleaved to function as two units (GP1 and GP2) by the action of subtilisin kexin isozyme/site-1 protease (SKI-1/S1P)), an enzyme involved in the metabolism of cholesterol. After cleavage, SSP (6KDa) forms a complex with GP1 (44KDa) and GP2 (35KDa), SSP/GP1/GP2 which mediates attachment, fusion and entry of virus particles into infected cells (Eichler *et al.*, 2003; Gairin *et al.*, 1995; Honko *et al.*, 2015; Lenz *et al.*, 2001). A similar process also occurs with the Lassa fever mammarenavirus which has a structural glycoprotein similar to that of LCMV (Kunz *et al.*, 2003). The proteolytic cleavage of LCMV GPC does not affect cellular expression of GP on surfaces but is essential for infectivity (Beyer *et al.*, 2003). Borrow and Oldstone reported the successful inhibition of LCMV attachment to host cells by the blocking of GP1 not GP2, proving the exclusive role of the former in attachment (Borrow & Oldstone, 1992). SSP facilitates the movement of the GP-C complex through the endoplasmic recticulum and Golgi

apparatus during GP maturation (York & Nunberg, 2006). The proteins are spread around the virus surface and interact with host receptors. GP1 is highly glycosylated with 30% of its molecular mass composed of carbohydrates. Its function is the mediation of viral penetration into host cells. GP2 facilitates fusion within the endosome and is highly conserved among Old World arenaviruses. Only cleaved glycoproteins are incorporated into mature virus particles (Beyer *et al.*, 2003; Kunz, 2009; Lenz *et al.*, 2001).

The RING finger protein is the smallest of all four viral proteins and possesses a repertoire of functions including the formation of a matrix, lining the inside of the virion membrane (Kunz, 2009; Urata & Yasuda, 2012). It has also been demonstrated to inhibit viral RNA synthesis by hindering the catalytic activity of viral polymerase, forming a complex with the latter, and thus keeping it 'promoter bound' (Kranzusch & Whelan, 2011).

1.3.3. Life Cycle

1.3.3.1. Virus Attachment

Arenaviruses have a life cycle restricted to the cell cytoplasm. Replication of arenaviruses begins after a successful attachment to host cell receptors with the help of GP1 and GP2. Alpha-dystroglycan (α-DG) is reported as the major receptor for the Old World arenaviruses and the clade C New World arenaviruses (Buchmeier *et al.*, 2013; Cao *et al.*, 1998; Spiropoulou *et al.*, 2002). New World arenaviruses however use the human transferrin receptor (TfR1) as the primary receptor. This ability to recognise human TfR1 enhances zoonosis and is currently under investigation in the development of therapeutic agents (Flanagan *et al.*, 2008; Helguera *et al.*, 2012; Radoshitzky *et al.*, 2007). The Lassa mammarenavirus also uses TAM and C-type lectin family as receptors

for virus entry. These receptors may also play a role in LCMV virus entry (Urata & Yasuda, 2012).

1.3.3.2. Virus Entry

Virus attachment is followed by the fusion of viral membrane with host vesicle membrane. The nucleocapsid containing the viral genome is introduced into the host cytoplasm. This is a pH-membrane fusion step mediated by GP2 (Emonet *et al.*, 2011). The entry of Lassa mammarenavirus (LASV) and LCMV into host cells depends somewhat on membrane cholesterol but not on clathrin, caveolin, dynamin, flotillin, actin and ADP Ribosylation Factor VI (Arf6) (Kunz, 2009; Quirin *et al.*, 2008). With the New World arenaviruses however, the process of endocytosis is different as it is mediated by clathrin (Buchmeier *et al.*, 2013; Martinez *et al.*, 2007).

1.3.3.3. RNA Transcription and Replication

Transcription commences with the attachment of RNA polymerase to the leader sequence in the 3′ end of the viral RNA. Viral ribonucleoprotein and polymerase mRNAs are then synthesized from the S and L segments respectively. The synthesis of the glycoprotein and Z mRNAs begins after the successful generation of the full-length antigenome RNA (agRNA) (Buchmeier *et al.*, 2013; Swiss Institute of Bioinformatics, 2015).

1.3.3.4. Assembly and Budding

At the end of replication, virus assembly follows. The glycoprotein and Z protein play essential roles in the assembly and release of mature virus particles. The glycoproteins GP1 and GP2 are trimeric molecules that unite to form spikes that surround the cell surface. GP2 is

transmembranal and serves as the base of the spike while GP1 is positioned at the top. GP1 facilitates cell receptor binding. The Z protein facilitates budding from host cells. This molecule is unique only to arenaviruses and shares no homology with any known negative-stranded RNA virus (Buchmeier *et al.*, 2013; Urata & Yasuda, 2012).

The life cycle of LCMV, the prototype arenavirus is represented in Figure 1.7.

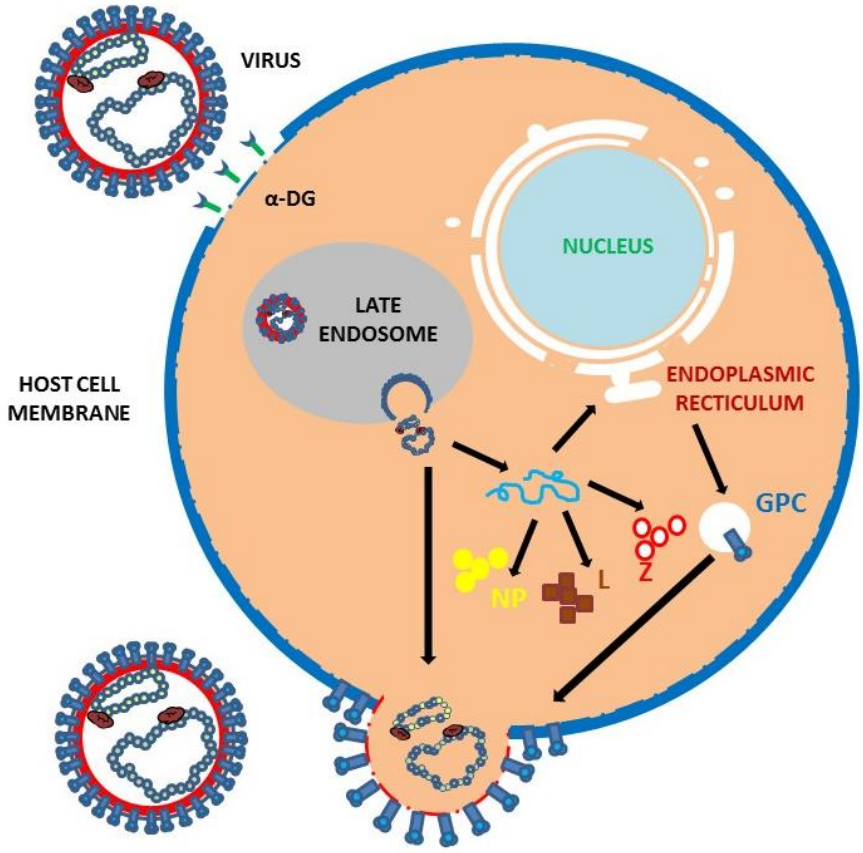


Figure 1.7: Life cycle of Lymphocytic Choriomeningitis Virus.

1.3.4. Reservoirs of Arenaviruses

Almost all the classified arenaviruses are associated with rodent-borne disease transmission. Exceptions to this include the *Tacaribe virus* (TACV) isolated from bats and ticks and other viruses isolated from boid

snakes (Downs *et al.*, 1963; Hetzel *et al.*, 2013; Li *et al.*, 2014; McLay *et al.*, 2014; Sayler *et al.*, 2014; Stenglein *et al.*, 2012).

1.3.4.1. Snakes

The identity of the etiological agent for the often fatal boid inclusion body disease (BIBD) remained elusive until Stenglein and colleagues detected viral RNA similar to previously identified arenaviruses. Marked by distinctive symptoms including pneumonia, regurgitation and neurological abnormalities, diagnosis in the past was based on the observation of intracytoplasmic inclusions in tissues in affected snakes. In some cases inclusions were present exclusively in the central nervous system (Bodewes *et al.*, 2013; Chang & Jacobson, 2010). Although these highly-divergent agents show typical ambisense genome orientation they possess a GP2 similar to that of the *Filoviridae* (Buchmeier et al., 2013; Koellhoffer et al., 2014; Stenglein et al., 2012). The arenaviruses discovered by Stenglein's group were discovered only in 75% of the snakes diagnosed with BIBD which suggests the presence of other more divergent arenaviruses that were undetectable by the assay used in the study (Darke et al., 2014). More recent discoveries of other highly-divergent arenaviruses in boid snakes have also been reported (Aqrawi *et al.*, 2015; Bodewes *et al.*, 2013; Hellebuyck *et al.*, 2015; Hetzel *et al.*, 2013). Aqrawi and colleagues were the first to detect viral genome in live snakes, including a green tree python, which were apparently healthy. This discovery hints at the possibility that other snake species can be infected with arenaviruses and also indicates that not all arenavirus infections lead to IBD and that certain snakes may possess the ability to clear infection (Aqrawi et al., 2015). It is inconclusive whether boid snakes are natural reservoirs for these newly discovered viruses. One suggestion is that infection is acquired as a result of fighting amongst snakes. Another possibility is through the direct feeding on rodents. This would not be impossible as an outbreak of callitrichid hepatitis (CH) in tamarins and marmosets was linked to the feeding of the latter with

LCMV-infected newborn mice (Montali et al., 1993; Stenglein et al., 2012). However so far, these viruses have not been isolated from rodents. Another possibility is transmission through mites (*Ophionyssus natricis*) reportedly associated with snakes having BIBD (Chang & Jacobson, 2010; Schumacher et al., 1994; Vancraeynest et al., 2006). However the substantial genetic differences between these novel viruses and the rodent-borne arenaviruses as well as among each other, hints at a distinct co-evolution with snakes (Hetzel et al., 2013). Studies carried out by Hepojoki et al. (2015) show that the University of Helsinki virus (UHV) can successfully infect mammalian cells cultured at 30°C. Cells however displayed limited growth and reduced cytoplasmic inclusion body (IB) formation when temperatures were raised to 37°C which suggests that the unidentified natural host most likely has a lower body temperature than mammals (Hepojoki *et al.*, 2015).

1.3.4.2. Insects

In 1956, Tacaribe mammarenavirus (TACV) was isolated from mosquitoes in Trinidad. However subsequent investigations, carried out serveral years later, on mosquitoes as potential hosts yielded negative results (Downs *et al.*, 1963). One of the five Amapari mammarenaviruses isolated in Brazil in the 1960s was obtained from a mite present on *Oryzomys* (Pinheiro *et al.*, 1966). The link between the virus and arthropod was not cemented until half a century later with the detection of TACV in lone star ticks (*Amblyomma americanum*) collected in Florida state park. Surprisingly, the newly isolated TACV bears a 98% amino acid similarity with the only remaining TACV (TRVL-11573) which was isolated decades ago from the artibeus bats (Sayler *et al.*, 2014). This is noteworthy considering the high mutation rates of single stranded RNA viruses. These developments have further changed the previous assumption that arenaviruses were restricted to mammalian hosts although the risk for human infection by these new arenavirus members has not yet been established. The successful replication of UHV in tick

cell lines (RAE/CTVM1, IRE/CTVM19, and BME/CTVM2) propagated at 30°C further proves the importance and likely role of arthropods in arenavirus cross-species transmission (Hepojoki *et al.*, 2015).

1.3.4.3. Bats

The first report of an arenavirus isolated from artibeus bats (*Artibeus jamaicensis* and *Artibeus lituratus*) was made in Trinidad in 1956 with the discovery of TACV. At the time of isolation, over 2000 mammals (including rodents) were examined. The evidence available at that time led to the conclusion that artibeus bats were the only possible reservoirs of the pathogen (Downs *et al.*, 1963; Price, 1978). The virus has however not been re-isolated from the species since then. Recent experimental infections of a colony of *Artibeus jamaicensis* to assess the possibility of persistent infection without substantial pathology revealed asymptomatic infection and virus clearance only at low-dose inoculations (10^4 $TCID_{50}$). High-dose (10^6 $TCID_{50}$) inoculations however caused considerable morbidity and mortality. Pathogen transmission to uninoculated cage mates also did not occur as evidenced in the failure of the latter to seroconvert as well as the absence of viral RNA in their tissues. These suggest that *A. jamaicensis* bats may not be a natural host for the virus (Cogswell-Hawkinson *et al.*, 2012).

1.3.4.4. Rodents

Rodents (Order *Rodentia*) identified by their distinct single pair of continuously growing incisors, are the largest and most diverse group of mammals (47% of all mammalian species). They are important reservoirs of various zoonotic pathogens and make up one of the three groups (inclusive of arthropods and bats) of reservoirs responsible for the transmission of viral haemorrhagic fevers (Hill & Brown, 2011; Luis *et al.*, 2013; Sunil-Chandra *et al.*, 2015; Wahl-Jensen, 2010; Woolhouse *et al.*, 2012). This is of particular significance because of close associations

between these small mammals and humans. They are often acquired as small pets for children because of their size, affordability and ease of care (Hill & Brown, 2011). In 2012, about 2.35 million households in the United States owned rodents including gerbils, guinea pigs and hamsters (American Veterinary Medical Association, 2012). The major route of rodent-to-human transmission is through contact with rodents or rodent-contaminated food (Ölschläger & Flatz, 2013). Aerosol transmission is also possible as humans can inhale particles mixed with rodent saliva or urine (Centres for Disease Control and Prevention, 2013a; Nunberg & York, 2012). In addition to the huge interaction with humans, their large taxonomic diversity and high reproduction rate (at the cost of a shorter life-span) make them successful viral reservoirs (Luis *et al.*, 2013).

Rodent reservoirs display certain traits that make them successful to habour disease. One such trait includes an increased rate of sexual maturity. Successful rodent reservoirs are those that are able to produce altricial offspring that attain sexual maturity within shorter periods than those of nonreservoirs. Rodent reservoirs also have mid-sized to large-sized litters which makes them more suitable to counter the negative effects caused by the introduction of lethal infections that will otherwise reduce the species population drastically. These reservoirs have perhaps developed an immune system that tolerates disease but does not hinder reproductive ability (Han *et al.*, 2015).

Members of the Old World complex (LASV-LCMV complex) of arenaviruses generally infect rodents of the *Murinae* subfamily. These include important pathogens such as Lassa mammarenavirus (LASV) and Lujo mammarenavirus (LUJV). Members of the New World complex (Tacaribe complex) on the other hand infect rodents of the *Sigmodontinae* subfamily (Buchmeier *et al.*, 2013; Zapata & Salvato, 2013). Phylogenic analyses of viruses and rodent hosts suggest that that virus-host co-evolution began after the split of the *Muroidea* superfamily

into the *Muridae* and *Cricetidae* families and subsequent spread and speciation of the rodent hosts across continents (Sauvage *et al.*, 2014).

Humans directly influence rodent population growth through the supply of resources and favourable environmental conditions including a reduction in the number of natural predators. Areas of poor hygiene usually experience local rodent overpopulation (Lõhmus *et al.*, 2013). As urban rodent communities usually have little or no contact with each other, construction activities such as the building of roads and bridges cause disturbances which can encourage inter-community interactions as well as contact with pets and humans (Patergnani *et al.*, 2010).

Young male rodents are primarily responsible for spreading pathogens between rodent communities as they instinctively leave their indigenous groups and move to new territories (Sauvage *et al.*, 2014). Transmission of pathogens within communities could be vertical, horizontal or both and appears to be species-dependent. With the drylands vesper mouse (*Calomys musculinus*) for instance, horizontal transmission is more common among males as evidenced with the presence of scars on the latter which is usually brought about by violent encounters with peers (Calisher *et al.*, 2001). Natural rodent hosts of LCMV transmit the virus horizontally and vertically and the means of transmission may affect disease outcome as vertical transmission may cause chronic infection while horizontal transmission may only result in acute infections (Skinner & Knight, 1974). A study on the dynamics of LCMV in yellow-necked mice (*Apodemus sylvaticus*) in Italy showed a direct correlation between sex and antibody prevalence when assessed at the individual level. Older males had a higher seroprevalence of LCMV infection hinting at the occurrence of horizontal transmission as a result of fighting (Tagliapietra *et al.*, 2009). Multimammate rats (*Mastomys spp.*) infected with LASV show no age or sex bias however indicating vertical transmission occurs more frequently. This may also be the situation in the spread of LCMV

among *Mus musculus* populations (Ostfeld & Mills, 2007; Zapata & Salvato, 2013).

Arenavirus infections in rodent reservoirs of arenaviruses are generally thought to be asymptomatic. The rodents successfully harbour the pathogen and continually serve as a medium through which the virus is reintroduced into the environment (Zapata & Salvato, 2013). In some cases however, the infection could have significant negative impacts on the natural host. This is dependent on various factors such as viral load. *Mastomys natalensis,* the rodent host for the East African Mopeia mammarenavirus (MOPV) displayed reduced weight, sexual maturity and body mass index owing to infection (Borremans *et al.*, 2011). A similar result was observed with the experimental inoculation of *C. musculinus* with the Junin mammarenavirus (JUNV) where infected rodents displayed low weight gain during lactation and reduced fertility (Vitullo *et al.*, 1987).

The association of arenaviruses with rodents initially suggested that they were restricted to the location of their natural host thus explaining the global distribution of LCMV as a consequence of its association with *Mus musculus*. The Natal multimammate rodent (*M. natalensis*) is a natural reservoir for LASV, MOPV and Luna mammarenavirus (LUNV). Although described as 'near Pan-African', outbreaks of LASV however remain localized within West Africa and have not spread to other areas of Sub-Saharan Africa. This can be due to lack of adequate prevalence studies carried out in unaffected regions of Sub-Saharan Africa. Surveillance investigations into factors responsible for pathogen restriction within geographical regions will provide valuable insight in the development of tools for disease prediction and control (Gryseels *et al.*, 2015; Lalis *et al.*, 2012; Sauvage *et al.*, 2014).

There is also a direct correlation between infection prevalence and rodent population density. In 2009, Tagliapietra and colleagues

compared the LCMV seroprevalence with rodent density in Northern Italy and reported an increase in overlaps between neighbouring rodent populations due to increases in population density and hence an increase in the potential for virus transmission (Tagliapietra *et al.*, 2009). One important factor that may have a direct effect on the number of rodent reservoir hosts and consequently affect viral transmission rates to humans is climate change. An increase in the number of Puumala virus-associated cases of *nephropathia epidemica* (epidemic nephropathy) in autumn due to bank vole abundance was directly linked to the increased production of seeds by mast-producing trees during the autumn season (Tersago *et al.*, 2009). An abundance of LCMV infection among *A. flavicolis* rodents was also reported to be directly associated with an increase in the number of mast-producing trees (Tagliapietra *et al.*, 2009).

Until recently, investigations on rodent-borne zoonoses have been approached from a reactive point – where surveillance efforts are carried out only after outbreaks have occurred. The number of cases may be drastically reduced if more proactive approaches are taken.. Han and colleagues used such variables to identify 58 rodent species potentially harbouring undiscovered zoonotic pathogens (Han *et al.*, 2015). The report further stated that future rodent-borne zoonoses will most likely emanate from regions with high richness of mammalian species and hotspots will include the United States of America, Middle East and Central Asia. The species with the highest probability of hosting undiscovered pathogens are *Microtus montanus*, *Onychomys leucogaster* and *Microtus gregalis*.

1.3.5. Arenavirus and Disease

Although a number of arenaviruses are an important cause of human and animal deaths, not all the members are zoonotic. The next session outlines the various arenaviruses recognised by the International

Committee on the Taxonomy of Viruses (ICTV). The focus of this thesis is on the viruses associated with zoonoses and epizooses. Figure 1.8 shows the phylogenetic relationship of these recognised viruses.

1.3.5.1. Reptarenaviruses

There are presently three reptarenaviruses recognized by the International Committee on the Taxonomy of viruses (Table 1.3). They are the Alethinophid 1 reptarenavirus (Golden gate virus), Alethinophid 2 reptarenavirus (California Academy of Sciences virus) and Alethinophid 3 reptarenavirus (Boa AV NL B3 and University of Helsinki viruses) (International Committee on Taxonomy of Viruses, 2015). Although these viruses cause severe disease in snakes, usually leading to death within weeks to months after the manifestation of symptoms (Hetzel *et al.*, 2013; Stenglein *et al.*, 2012), snake-to-human transmission has not been reported. Such transmission is highly unlikely as human cells successfully infected with BIBD-associated arenaviruses *in vitro* displayed limited inclusion body (IB) formation and reduced amounts of viral nucleoprotein and RNA at 37°C (Hepojoki *et al.*, 2015).

1.3.5.2. New World Mammarenaviruses (Tacaribe complex)

These are further divided into three clades A, B and C. These are listed on in Table 1.4. There are eight members in clade A. Members were isolated from South America (Allpahuayo mammarenavirus (ALLV), Flexal mammarenavirus (FLEV), Parana mammarenavirus (PARV), Pichinde mammarenavirus (PICV), Pirital mammarenavirus (PIRV-VAV)) and North America (Bear Canyon mammarenavirus (BCNV), Tamiami mammarenavirus (TAMV) and Whitewater Arroyo mammarenavirus (WWAV)) (King *et al.*, 2012).

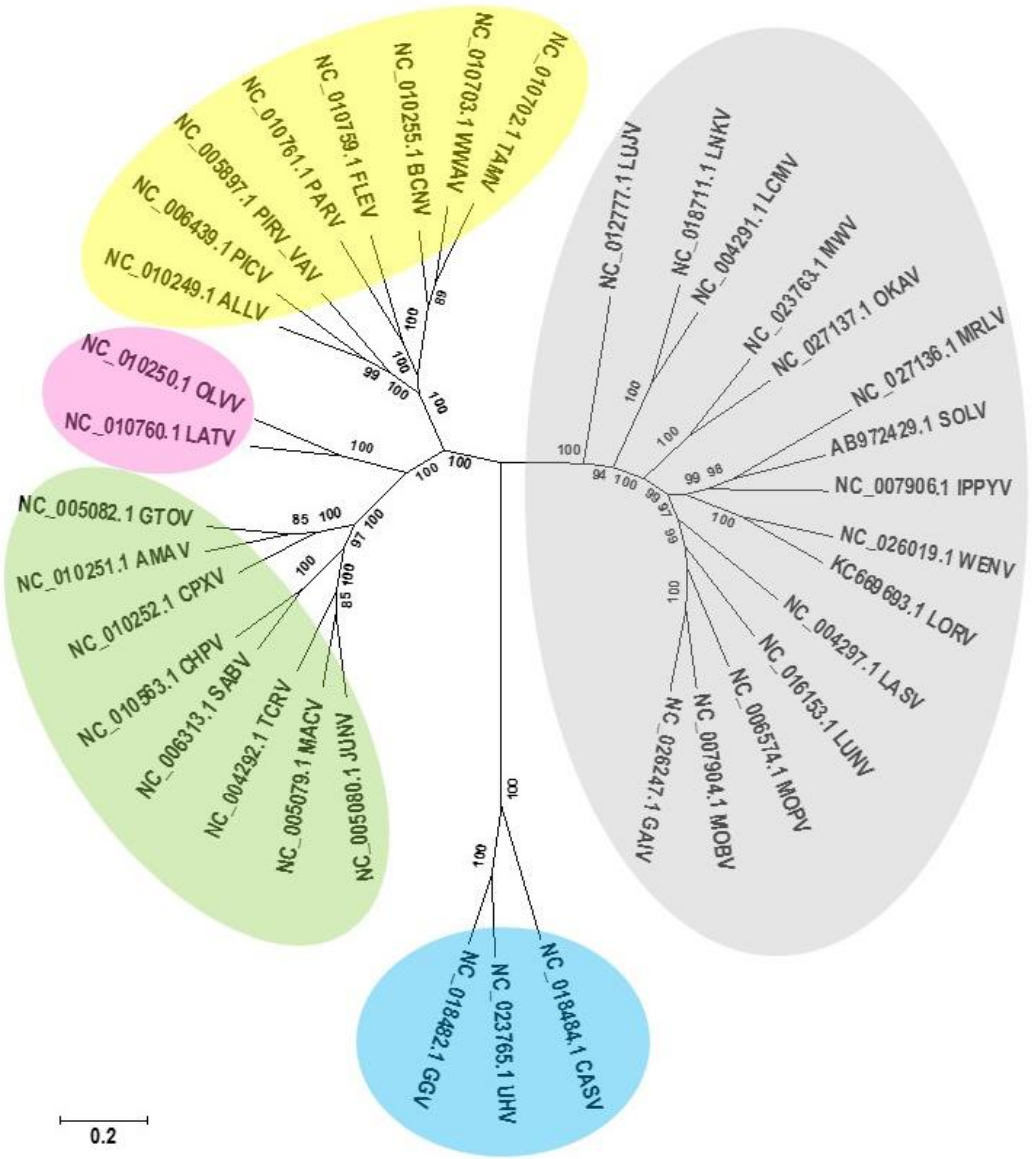


Figure 1.8: Unrooted Neighbour Joining phylogenetic tree showing relationships among arenaviruses. The tree was computed with complete L gene fragment using the Maximum Likelihood method. Reference sequences are indicated by their Genbank Accession Numbers. Individual arenaviruses are written as acronyms of full names in Table 1.3, Table 1.4 and Table 1.5. Coloured oval regions represent the following: Blue – Reptarenaviruses, Grey – Old World mammarenaviruses, Yellow – Clade A New World mammarenaviruses, Green – Clade B New World mammarenaviruses, Pink – Clade C New World mammarenaviruses. Branch lengths are drawn to scale: the bar indicates 0.1 nucleotide substitutions per site. Numbers above individual branches indicate the percentage that that branch was found in 1000 bootstrap replicates; only percentages >75% are shown.

Only FLEV, PICV and WWAV are known to cause human infections (Buchmeier *et al.*, 2013; Carlton *et al.*, 2012; Kumar *et al.*, 2012; Trapido & Sanmartin, 1971). Clade B viruses consists of the most dangerous of all the New World arenaviruses as most members of this cluster cause a range of viral haemorrhagic fevers in humans. Non-pathogenic members include the Amapari, Cupixi and Tacaribe mammarenaviruses as they have not been associated with any human infections. The Guanarito, Junin and Machupo mammarenaviruses cause the Venezuelan haemorrhagic fever (VHF), Argentine haemorrhagic fever (AHF) and Bolivian haemorrhagic fever (BHF) respectively (Patterson *et al.*, 2014; Radoshitzky *et al.*, 2014). The Chapare mammarenavirus was isolated from one fatal haemorrhagic case in Bolivia in 2008 making it the most recent of three arenaviruses to be isolated from the country. Nucleotide analyses of the virus revealed its close relationship to the Sabia mammarenavirus, having only 26% and 30% nucleotide difference in the S and L segments (Delgado *et al.*, 2008). The rodent reservoirs for the Chapare and Sabia mammarenaviruses are presently unknown. Only two members namely the Oliveros mammarenavirus (OLVV) and Latino mammarenavirus (LATV) belong to clade C. OLVV was discovered as result of a routine investigation for JUNV in rodents in Argentina (Bowen *et al.*, 1996). Although OLVV and JUNV are sympatric to Northern Argentina, the former is grouped with LATV due to their high nucleotide similarity (Charrel *et al.*, 2002).

Table 1.3: List of Reptarenaviruses, reservoirs and worldwide distribution

New Name	Old Name	Host	Global Distribution	Year*	Zoonoses	Source
Alethinophid 1 reptarenavirus	Golden Gate virus (GGV)	Annulated tree boa	USA	2012	Unknown	(Stenglein *et al.*, 2012)
Alethinophid 2 reptarenavirus	Carlifornia Academy of Sciences virus (CASV)	Boa constrictor	USA	2012	Unknown	
Alethinophid 3 reptarenavirus	Boa AV NL B3	Boa constrictor	Netherlands	2013	Unknown	(Bodewes *et al.*, 2013)
	University of Helsinki virus (UHV)	Boa constrictor	Germany	2013	Unknown	(Hetzel *et al.*, 2013)

* Year of identification of disease/ isolation of causative agent

Table 1.4: List of New World Mammarenaviruses, reservoirs and worldwide distribution

New Name	Old Name	Host	Global Distribution	Year*	Zoonoses	Source
Allpahuayo mammarenavirus	Allpahuayo virus (ALLV)	*Oecomys bicolor*, *Oecomys paricola*	Northeastern Peru	1997	Unknown	(Moncayo *et al.*, 2001)
Amapari mammarenavirus	Amapari virus (AMAV)	*Neacomys goeldi, Oryzomys guianae, Gamasidae*?	Brazil	1960s	Unknown	(Pinheiro *et al.*, 1966)
Bear Canyon mammarenavirus	Bear Canyon virus (BCNV)	*Peromyscus californicus*	USA	2002	Unknown	(Fulhorst *et al.*, 2002)
Chapare mammarenavirus	Chapare virus (CHPV)	Unidentified	Bolivia	2008	One fatal case	(Delgado *et al.*, 2008)
Cupixi mammarenavirus	Cupixi virus (CPXV)	*Oryzomys capito*	Brazil	1998	Unknown	(Sauvage *et al.*, 2014)
Flexal mammarenavirus	Flexal virus (FLEV)	*Oryzomys* spp.	Brazil	1975	Unknown	(Buchmeier *et al.*, 2013)
Guanarito mammarenavirus	Guanarito virus (GTOV)	*Zygodontomys brevicauda*	Venezuela	1989	VHF 34% mortality	(Buchmeier *et al.*, 2013; Salas *et al.*, 1991)
Junin mammarenavirus	Junin virus (JUNV)	*Calomys musculinus*	Argentine pampas	1958	AHF 15-30% mortality	(Buchmeier *et al.*, 2013; Rapp & Buckley, 1962)

New Name	Old Name	Host	Global Distribution	Year*	Zoonoses	Source
Latino mammarenavirus	Latino virus (LATV)	*Calomys callosus*	Bolivia	1965	Unknown	(Buchmeier *et al.*, 2013)
Machupo mammarenavirus	Machupo virus (MACV)	*Calomys callosus*	Bolivia	1959	25-30% mortality	(Aguilar *et al.*, 2009; Patterson *et al.*, 2014)
Oliveros mammarenavirus	Oliveros virus (OLVV)	*Bolomys obscurus*	Argentina	1990	Unknown	(Bowen *et al.*, 1996)
Parana mammarenavirus	Parana virus (PARV)	*Oryzomys buccinatus*	Paraguay	1965	Unknown	(Webb *et al.*, 1970)
Pichinde mammarenavirus	Pichinde virus (PICV)	*Oryzomys albigularis*	Colombia	1967	Unknown	(Trapido & Sanmartin, 1971)
Pirital mammarenavirus	Pirital virus (PIRV-VAV)	*Sigmodon alstoni*	Venezuela	1997	Unknown	(Fulhorst *et al.*, 1997)
Sabia mammarenavirus	Sabia virus (SABV)	Unidentified	Brazil	1990	Three reported cases	(Buchmeier *et al.*, 2013; Lisieux *et al.*, 1994)
Tamiami mammarenavirus	Tamiami virus (TAMV)	*Sigmodon hispidus*	USA	1970	Unknown	(Calisher *et al.*, 1970)

New Name	Old Name	Host	Global Distribution	Year*	Zoonoses	Source
Tacaribe mammarenavirus	Tacaribe virus (TACV)	*Artibeus jamaicensis, Artibeus lituratus, Amblyomma americanum*	Trinidad	1956	Unknown	(Downs *et al.*, 1963; Sayler *et al.*, 2014)
Whitewater Arroyo mammarenavirus	Whitewater Arroyo virus (WWAV)	*Neotoma albigula*	New Mexico	1996	87% identity to causative agent of three deaths in the USA.	(Fulhorst *et al.*, 1996)

* Year of identification of disease/ isolation of causative agent

Table 1.5: List of Old World Mammarenaviruses, reservoirs and worldwide distribution

New Name	Old Name	Host	Global Distribution	Year*	Zoonoses	Source
Gairo mammarenavirus	Gairo virus (GAIV)	*Mastomys natalensis*	Tanzania	2015	Unknown	(Gryseels *et al.*, 2015)
Ippy mammarenavirus	Ippy virus (IPPYV)	*Mastomys natalensis*	Zambia	2009	Unknown	(Ishii *et al.*, 2011)
Lassa mammarenavirus	Lassa virus (LASV)	*Mastomys* spp.	West Africa		Lassa fever 15% mortality, 5000 deaths yearly	(Buchmeier *et al.*, 2013; McLay *et al.*, 2014)
Loei River mammarenavirus	Loei River virus (LORV)	*Bandicota sp*	Thailand	2016	Unknown	(Blasdell *et al.*, 2016)
Lujo mammarenavirus	Lujo virus (LUJV)	Unidentified	Zambia	2008	80% mortality	(Paweska *et al.*, 2009)

New Name	Old Name	Host	Global Distribution	Year*	Zoonoses	Source
Lunk mammarenavirus	Lunk virus (LNKV)	*Mus minutoides*	Zambia	2012	Unknown	(Ishii *et al.*, 2012)
Lymphocytic choriomeningitis mammarenavirus	Lymphocytic choriomeningitis virus (LCMV)	*Mus musculus*	Worldwide	1933	<1% mortality.	(Centres for Disease Control and Prevention, 2013a)
Mariental mammarenavirus	Mariental virus (MRLV)	*Micaelamys na maquensis*	Namibia	2015	Unknown	(Witkowski *et al.*, 2015)
Merino Walk mammarenavirus	Merino Walk virus (MWV)	*Myotomys unisulcatus*	South Africa	1985	Unknown	(Palacios *et al.*, 2010)
Mobala mammarenavirus	Mobala virus (MOBV)	*Praomys* sp.	Central African Republic	1983	Antibodies detected in humans.	(Georges *et al.*, 1985; Gonzalez *et al.*, 1983)
Mopeia mammarenavirus	Mopeia virus (MOPV)	*Mastomys natalensis*	Mozambique	1977	Antibodies detected in humans.	(Georges *et al.*, 1985; Wulff *et al.*, 1977)

New Name	Old Name	Host	Global Distribution	Year*	Zoonoses	Source
Okahandja mammarenavirus	Okahandja virus (OKAV)	*Micaelamys na maquensis*	Namibia	2015	Unknown	(Witkowski *et al.*, 2015)
Solwezi mammarenavirus	*Arenaviridae* sp. 13ZR68	*Grammomys sp.*	Zambia	Unpublished	Unknown	Unpublished
Wenzhou mammarenavirus	Wenzhou virus (WENV)	*Rattus norvegicus, Rattus losea, Rattus tanezumi, Rattus rattus, Suncus murinus*	China	2015	Evidence of human infection	(Blasdell *et al.*, 2016; Li *et al.*, 2015)

* Year of identification of disease/ isolation of causative agent

1.3.5.3. Old World Mammmarenaviruses (Lassa complex)

Members of this group include LASV, LCMV, Ippy mammarenavirus (IPPYV), Lujo mammarenavirus (LUJV), Lunk mammarenavirus (LNKV), Mopeia mammarenavirus (MOPV), Mobala mammarenavirus (MOBV), Merino walk mammarenavirus (MWV) and Luna mammarenavirus (LUNV) (Table 1.5). Of these, only LASV, LCMV and LUJV cause disease in humans (McLay *et al.*, 2014). LUJV is the most recently discovered of the three but information on the virus is limited as the only outbreak reported so far took place in South Africa in 2008 and involved only five people (Centres for Disease Control and Prevention, 2013c).

Of all discovered arenaviruses, LASV is the most prevalent causing up to 300000 infections and 5000 deaths in West Africa annually (McLay *et al.*, 2014). These numbers are owed more to the seclusion of the regions affected as well as the unavailability of appropriate medical care, as such areas are poorly developed and far from suitable hospitals. LASV is globally recognised for its potential to be used in bioterrorism (Lõhmus *et al.*, 2013).

1.3.6. Lymphocytic Choriomeningitis mammarenavirus

LCMV is the prototypic arenavirus, and as its name suggests, is the cause of Lymphocytic choriomeningitis (LCM), a neurological disease in infants and immunocompromised adults (Albarino *et al.*, 2010b). Although not as severe as the haemorrhagic fevers caused by other arenaviruses, this often undiagnosed disease has gained increased recognition over recent years due to the substantial problems it presents particularly to child neurologists as infants infected congenitally by their mothers, could develop brain and eye defects which can be as serious as mental retardation and blindness (Asper *et al.*, 2001).

LCMV was isolated by Charles Armstrong in 1933 using monkey passaging experiments, from a patient who died during an encephalitis epidemic in St Louis that occurred in the same year. The strain was distinct from other viruses isolated in the epidemic as it had a shorter incubation period and showed negative results in cross neutralisation assays (Armstrong & Lillie, 1934; Beeman, 2007). A significant number of outbreaks have occurred since its discovery (Asper *et al.*, 2001; Lucke & Bennett, 1982; Montali *et al.*, 1993). These have happened within human and non-human primate populations. The largest recorded human outbreak took place between 1973 and 1974 in the United States involving 12 states with 181 infections reported although no death occurred (Gregg, 1975). Zoo outbreaks involving callitrichids have also been reported (Asper *et al.*, 2001; Ramsay *et al.*, 1989). The most recent LCMV outbreak occurred in 2012 at 3 rodent breeding facilities in the United States of America where 31 out of 97 employees tested positive for IgG and/or IgM antibodies to LCMV and 4 (4%) developed aseptic meningitis (Knust *et al.*, 2014). The precise prevalence of LCMV infection is difficult to estimate as cases can be mild or asymptomatic and only severe infections are reported.

1.3.6.1. Diagnosis of LCMV Infection

Challenges involved with the routine screening and detection of LCMV in tissue samples include the fact that only a few commercial laboratories test for the presence of the virus. Most outbreaks of LCMV were only identified because they were part of a larger infection outbreak (Asnis *et al.*, 2010; Perez-Ruiz *et al.*, 2012). Infection is routinely diagnosed by PCR or serology using ELISA and Indirect fluorescence antibody (IFA) due to the difficulties associated with virus culturing (Morita *et al.*, 1991c).

1.3.6.1.1. Serology

Serological tests are important tools used in the surveillance of viral diseases as they can detect and quantify antibodies produced by the host against viral antigens. They are of extreme importance in the evaluation of vaccination programs. These tests generally depend on the detection of antibodies produced by the host against the infectious particle which generally begins at 5 to 7 days post-infection and peaks at 10 to 20 days post-infection and lasts for many months (Compton & Riley, 2001). As antibodies can last for months after the initial introduction of the infectious agent, a positive serological result is not an indication of an acute infection. Traditional serological tests used in the diagnoses of LCMV infections include IFA or ELISA test (Becker *et al.*, 2007; Takimoto *et al.*, 2008). Both tests are safe and inexpensive ways to detect viral antibodies. ELISA has further advantages of rapid turnover as well as the potential for high throughput assays. One major limitation for both methods includes the cross-reactivity of antibodies produced against closely-related viruses (Mather *et al.*, 2013). Sensitivity is another challenge as such tests have not been able to detect antibodies in other animals screened to be positive by other tests such as virus isolation and immunochemistry staining of tissues (Morita *et al.*, 1991a). They also fail to detect antiviral antibodies directly in immunodeficient hosts that do not generate humoral response. Screening of immunocompetent hosts may also yield false-negative results if seroconversion has not yet occured (Besselsen *et al.*, 2003; Skinner *et al.*, 1976; Traub, 1936). Other serological tests that have been employed are the microplaque-reduction test for neutralizing antibody and complement fixation.

1.3.6.1.2. Mouse Antibody Production (MAP) Test

MAP was first reported in 1959 by Rowe and colleagues (Rowe *et al.*, 1959) as an alternative to tissue culture for the screening of Polyoma virus infection but has since been modified for the detection of other virus

agents as well. The assay typically involves the introduction of an infected biological material into mice through various inoculation routes followed by the isolation of the rodent for a minimum of 28days and the final testing of mouse serum for the presence of virus-specific antibodies (Blank *et al.*, 2004). A major disadvantage with this method is an increased turnaround time of 6 to 8 weeks. The assay is also liable to produce false positive results brought about by non-specific reactions of mouse serum in serological assays (Compton & Riley, 2001)

1.3.6.1.3. Polymerase Chain Reaction (PCR)

Park *et al.*, (2007) developed a reverse-transcriptase polymerase chain reaction assay (RT-PCR) for LCMV molecular detection which could successfully detect <1 $TCID_{50}$ virus particles in a specimen (Park *et al.*, 1997). Despite being sensitive, PCR assays generally have the disadvantages of being labour-intensive. Also the use of agarose gel electrophoresis in downstream processing limits its application as a high-throughput diagnostic assay. False positive results associated with contamination are also possible with end-point PCR assays. However PCR assays have the added advantages of having a reduced turnaround time as well as increased sensitivity when compared to other methods such as mouse or rat antibody production (MAP/RAP) tests (Besselsen *et al.*, 2003). Quantitative PCR assays however reduce the challenges associated with contamination and have been utilized to some degree in LCMV screening *in vivo* and *in vitro* (Cordey *et al.*, 2011; Emonet *et al.*, 2007; McCausland & Crotty, 2008). Finally, PCR assays also offer an alternative to whole-animal testing as MAP/RAP involve the introduction of the biological material into a rodent host and subsequent analyses of rodent serum for antibody production using ELISAs or IFAs.

1.3.6.1.4. Virus Isolation

LCMV present in the organs and tissues of infected animals has been successfully isolated in Vero cells (Asper *et al.*, 2001; Emonet *et al.*, 2007; Montali *et al.*, 1993; Stephensen *et al.*, 1991). In cases of acute human LCMV infection, virus can also be isolated from the Cerebrospinal fluid (CSF) (Bonthius, 2012; Park *et al.*, 1997; Schulte *et al.*, 2006)

1.3.6.2. Epidemiology

LCMV has achieved a global distribution status with reports of infection in Europe, the Americas, Australia, and Japan (Centres for Disease Control and Prevention, 2013a). It is generally accepted that the house mouse is grouped into 4 subgenera (*Mus*, *Coelomys*, *Nannomys*, and *Pyromys*) and 3 subspecies (*M. m. musculus, M. m. domesticus and M. m. castaneus*) of which LCMV infection has only been reported in *M. m. musculus* and *M. m. domesticus*. The subspecies are believed to have split from a common ancestor that originated in the North of the India subcontinent about 1 million years ago and a further differentiation into *M. m. musculus* and *M. m. domesticus* lineages occurred a few hundreds of thousands of years ago (Boursot *et al.*, 1996; Suzuki *et al.*, 2004).

Most rodents infected with LCMV congenitally show no symptoms which is most likely due to developed immune tolerance. These rodents become chronic carriers and shed large quantities of virus in their secretions throughout their lifetime (Klein, 2005). Although *Mus musculus* is the main reservoir for LCMV, other alternative hosts including bank voles (*Myodes glareolus*), wood mice (*Apodemus sylvaticus*), montane water vole (*Arvicola scherman*) and common vole (*Microtus arvalis*) have been identified (Charbonnel *et al.*, 2008a; Ledesma *et al.*, 2009; Tagliapietra *et al.*, 2009). Prevalence rates reported from different regions and periods vary significantly. Childs and colleagues (1992) reported 9% seroprevalence in rodents in urban

Baltimore between 1984 and 1989 (Childs *et al.*, 1992). In Europe and Japan, the prevalence rates are 4% to 9% and 7.0% to 25.9% respectively (Bonthius, 2012; Takagi *et al.*, 2012). The seroprevalence of LCMV among captured *M. domesticus* in North West England was reported to be 4% according to a small routine health screening (Becker *et al.*, 2007). Antarctica remains the only continent were the presence of *Mus musculus* is not reported.

Variations in LCMV prevalence in rodents can be owed to various factors including human activities such as the construction of buildings, bridges and dams as well as the elimination of competitors and predators of the reservoir. These may favour or discourage increase in rodent populations and also enhance interactions with humans and other potential hosts (Lõhmus *et al.*, 2013; Patergnani *et al.*, 2010).

LCMV infection in humans can occur throughout the year but infection rates are higher during the cold seasons due to increased interactions between rodents and humans in homes. Infection in infants, pregnant women, immunocompetent and immunocompromised individuals is well documented (Fischer *et al.*, 2006; Knust *et al.*, 2014; Perez-Ruiz *et al.*, 2012; Rousseau *et al.*, 1997; Schulte *et al.*, 2006). There is no evidence of human-to-human transmission. Disease prevalence in humans varies greatly and depends on exposure to mice and general living conditions of individuals which implies that rates could have higher in the past. Serological studies carried out in the United States suggest the prevalence of infection to be about 5 percent (Childs *et al.*, 1991; Stephensen *et al.*, 1992). In addition to immunocompromised individuals, laboratory and hospital personnel also have a high risk of acquiring infection as evidenced by reports of outbreaks in research institutes and rodent-breeding facilities (Knust *et al.*, 2014; Vanzee *et al.*, 1975). About 10-20% of acquired infection in immunocompetent individuals advance to aseptic meningitis (The Center for Food Security and Public Health, 2010). Most cases in infants go undiagnosed because the infection is

confused with that of cytomegalovirus or *Toxoplasma gondii* (Wright et al., 1997).

There is limited evidence on epidemiological studies to determine the incidence or effects of congenital LCMV infection. Recently a French group reported results of a study on the screening of 160 pregnant women over a three year period for LCMV infection. Although congenital neurological anomalies were diagnosed, serology for LCMV and TORCH (*Toxoplasma gondii*, Rubella, Cytomegalovirus, Herpes virus and HIV) pathogens were all negative. Although no molecular tests were carried out in the study, the results hint at the possibility of France having little or no recent cases of congenital transmission (Hannachi *et al.*, 2011). As LCMV is not among the TORCH pathogens routinely checked for in cases of congenital infection, and cases that do get reported have only reflected on infants with abnormal neurological development, it is impossible to determine if all congenitally infected infants typically display other less severe symptoms (Anderson *et al.*, 2014; Bonthius, 2012; van der Weiden *et al.*, 2011).

The morbidity and mortality rates for transplant patients are significantly higher. Fischer *et al.*, 2006 reported two separate clusters (In 2003 and 2005) where organ recipients (eight) were infected by donors. Seven out of the eight patients died within 11 weeks after transplantation. In the 2005 cluster, the source of infection was confirmed to be a pet hamster which was infected with an LCMV strain identical to those isolated from the organ recipients (Fischer *et al.*, 2006). A similar report was made in the USA where in four clusters from 2005 to 2011, eleven of the thirteen infected recipients died. One of the donors had been living in a home that had rodent infestation problems (MacNeil *et al.*, 2012). Information on the risk of LCMV in healthy blood donors is limited. De Lamballerie *et al.*, 2007 investigated the presence of IgG anti-LCMV in blood donors in Marseille, France. Two out of 600 serum samples were seropositive (0.33% prevalence) (de Lamballerie *et al.*, 2007). This prevalence value

suggests that the potential for LCMV transmission via blood transmission is very low.

The LCMV-associated symptoms manifested at any point in time depend on the stage of development of the host whether prenatal or postnatal.

1.3.6.3. Human LCMV Infection

1.3.6.3.1. Prenatal LCMV Infection

Congenital (prenatal) infections are transmitted to infants usually through the placenta. Other less common routes of transmission occur through contact with the mother's blood (high viraemia) or exposure to vaginal secretions.

Manifestations of infections at this stage of development are usually severe due to the poor immune status of the foetus and can cause premature death. Infected foetuses carried to full term typically manifest hydrocephalus and chorioretinitis. Chorioretinitis is marked by the inflammation of the choroid and retina and is a consequence of the development of chorioretinal scars and results in vision impairment (Ackermann *et al.*, 1974; Barton *et al.*, 1995; Bonthius, 2012; Sheinbergas, 1976). The prevalence of LCMV during pregnancy is unknown. The first recognised case of congenital LCMV infection was reported in England in 1955 (Bonthius & Perlman, 2007b; Komrower *et al.*, 1955). Congenital brain deformation due to LCMV infection is manifested as microcephaly or macrocephaly. In macrocephaly, the brain increases beyond the normal size as a result of non-communicating hydrocephalus (marked by the accumulation of fluid in the brain). One cause of hydrocephalus is the inflammation and blocking of the ventricles of the brain and requires surgical shunting to drain fluid out of the latter and relieve intracranial pressure (Bonthius, 2012; Larsen *et al.*, 1993). Microcephaly is a less common symptom where the brain is smaller than

normal. In LCMV infections, microcephaly is the result of reduced brain growth. It can also be caused by the destruction of infected brain tissue by the immune system. Although congenital LCMV infection has been linked to mental abnormalities in children, no investigations have been carried out on mentally retarded children to determine if the viral pathogen is a causative agent (Bonthius, 2012).

Genetic disorders such as Aicardi–Goutieres syndrome and pseudo-TORCH syndrome can mimic congenital LCMV infection which can lead to wrong diagnoses in early stages of the infection (Bonthius, 2012; Sanchis *et al.*, 2005). While Aicardi–Goutieres syndrome can manifest as neonatal encephalopathy and intracranial calcifications, pseudo-TORCH syndrome can present symptoms of the classic TORCH diseases (Rice *et al.*, 2007). Both diseases are genetic disorders and have no microbiological evidence of being associated with infection. The correct diagnoses of LCMV infection will be covered later in the chapter.

1.3.6.3.2. Postnatal LCMV Infection

Acquired (postnatal) infection occurs usually with the inhalation of infected aerosols and subsequent deposition in lungs. Viral replication then takes place within the lungs and moves into the blood stream before moving to other organs. Infection eventually spreads to the meninges causing inflammation which gives the virus its characteristic name. Although inflammatory response to infection eliminates the virus and protects from future re-infection, it also causes harm to the host in the process (Bonthius, 2012).

Acquired LCMV infections generally cause less severe infections than congenital LCMV infections. A major reason for this is the presence of a well-developed immune system in adults. Infections are usually self-limiting and rarely lead to death except in cases were the immune system has been compromised. Typically, disease is fully recovered from after a

brief fever (Bonthius, 2012). The manifestation of symptoms occurs in two stages between 8 and 13 days after exposure. Symptoms associated with the first stage include appetite loss, fever, malaise, myalgia, nausea and vomiting which last for about 7 days. The second stage consisting of meningitis, encephalitis or meningoencephalitis may follow afterwards (Bonthius, 2012; Centres for Disease Control and Prevention, 2014a). The course of disease usually ends after a 1-3 week period although symptoms may endure for much longer.

Postnatal LCMV infection has a broad clinical spectrum. While a third of infected individuals show no symptoms of disease, some others show neurological symptoms as well as complications including arthritis, orchitis and parotitis (Bonthius, 2012). Successful disease manifestation in immunosuppressed organ recipients has been linked to the depletion of T-cells which play a significant role in viral clearance (Bonthius, 2012).

1.3.6.4. Callitrichid Hepatitis

LCMV also causes severe disease in New World primates belonging to the family Callitrichidae. This family consists of marmosets and tamarins (Wissman, 2014). Infected animals develop CH after a 1-2 week incubation period. This severe disease is marked by dyspnea, fever, anorexia, lethargy and jaundice and is quite similar to Lassa fever in humans (Asper *et al.*, 2001; Montali *et al.*, 1995; The Center for Food Security and Public Health, 2010). Histopathological analysis show extensive liver degradation, inflammation and necrosis. LCMV displays a unique multi-organ tropism in these primates and has been isolated from a variety of tissues including the liver, adrenal gland, intestines and central nervous system (Montali *et al.*, 1995). Non-human primate deaths from callitrichid hepatitis have been reported in various zoos across the USA, United Kingdom and Germany between 1980 and 2001; ten of which resulted in the deaths of 57 marmosets and tamarins in North America alone (Asper *et al.*, 2001; Montali *et al.*, 1989; Ramsay, 1989).

One outbreak was the direct result of feeding the primates with LCMV-infected mice (Montali *et al.*, 1993)

There have been no reports on the transmission of LCMV from humans to NHPs. Antibodies were detected in veterinarians who had contact with NHPs. While one had necropsied an animal with callitrichid hepatitis (CH), the other was bitten by an infected animal (Montali *et al.*, 1989; Montali *et al.*, 1993).

1.3.6.5. Lineages, Strains and lethality

The genome replication for all RNA viruses is error prone leading to high mutation frequencies which results in the production of chimeric progeny. This is often driven by a variety of factors including immune pressure and drug therapy as well as host switching.

All identified strains of the virus have been grouped into four distinct lineages and are distributed globally (Figure 1.9). Lineages I and II are associated with *Mus musculus*. Lineages I, II and III are associated with severe human disease. European strains display the greatest diversity as they belong to lineages I, II, and IV whereas only lineages I and III have been detected in the United States. Lineage I comprises of the greatest variety of strains including the classic Armstrong (ARM 53b) and WE strains as well as the Makokou strain, the only strain isolated from Africa so far (Armstrong & Lillie, 1934; N' Dilimabaka *et al.*, 2015; Rivers & Scott, 1936). The M1 and M2 strains originated from Austria but were isolated in Japan from laboratory mice obtained from France. The Lyles strain is the only strain in lineage III and was obtained from a 58-year old patient with non-fatal aseptic meningitis. Albarino and colleagues investigated the genetic diversity of identified LCMV strains and reported nucleotide differences observed in the S segment between lineages to be 22% - 25% (Albarino *et al.*, 2010b). Although LCMV strains have also been isolated from the UK, no full-length sequences are available for

analysis. Partial sequences (283bp) from the non-functional glycoprotein gene place the UK strains in lineage I. Longer sequences are required to allow for definite conclusions (Blasdell *et al.*, 2008a).

Other common strains of lineage-1 LCMV include CL-13, Pasteur (CIPV 76001), Traub and UBC (Ahmed & Oldstone, 1988; Dutko & Oldstone, 1983; Hotchin & Cinits, 1958; Pulkkinen & Pfau, 1970; Riviere *et al.*, 1977; Traub, 1935). The clone13 (CL-13) strain was obtained from the spleen of mice infected at birth with the Armstrong strain.

Strains CABN, GR01 and SN05 were isolated from *Apodemus sylvaticus*. These were classified recently under a new lineage 4 due to the significant difference in the glycoprotein nucleotide and amino acid sequences between them and other identified LCMV sequences (15.9% - 19.7% and 23.4% - 27.7% respectively) (Ledesma *et al.*, 2009). The viral pathogenicity of these strains and their association with human LCMV infection is yet to be determined.

1.3.6.6. Virus Persistence in Mice

LCMV, like arenaviruses, can establish persistence in the infected host. The success of this depends on a number of factors including the inoculum size of the virus as well as the affinity of virus glycoprotein to alpha-dyatroglycan (Ciurea *et al.*, 1999; Kunz *et al.*, 2004a).

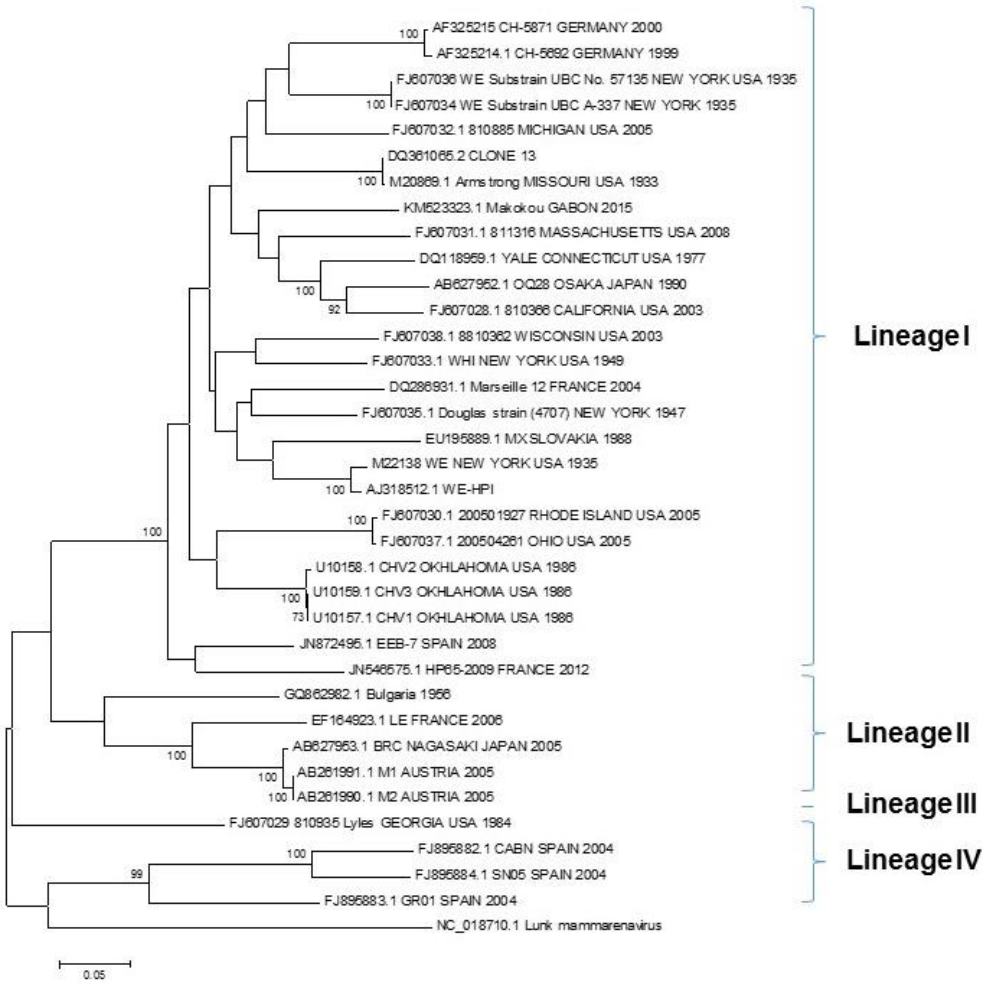


Figure 1.9: Maximum likelihood phylogenetic analysis of full LCMV GPC gene. The scale bar indicates 0.05 nucleotide substitutions per site. Reference sequences are indicated by their Genbank Accession Number. Branch lengths are drawn to scale: the bar indicates 0.05 nucleotide substitutions per site. Numbers above individual branches indicate the percentage that that branch was found in 1000 bootstrap replicates; only percentages >65 are shown.

In newborn mice, persistence is achieved with the inoculation of 10^3 virions. In immunocompetent mice, persistence takes place after inoculation with 10^6 virions while systemic infection of all organs (excluding the brain) can occur with 10^2-10^5 virus particles causing greater CD8+ Tcell expansion. CD4+ Tcell expansion also takes place but at a lesser level than CD8+. Antibodies are also detected within 7 days of infection but these are incapable of clearing the virus. The clearance of virions by the 14th day is dependent on the particular LCMV

strain. With the Armstrong and WE strains, virions are cleared or reduced to undetectable levels within 2-3 weeks of inoculation. With the CL-13 strain however, infected mice possess high virus titres in the blood, spleen, liver, and brain for over two months with the virus persisting indefinitely in the kidneys. The intracerebral inoculation of adult mice with 1-50 virions results in choriomeningitis leading to death between the 7th and 9th day (Ciurea *et al.*, 1999; Laposova *et al.*, 2013; Ng *et al.*, 2011; Zinkernagel, 2002). Strains that cause a generalized immunosuppression by T-cell dysfunction and the loss of virus-specific T-cells are described as $CTL^{-}P^{+}$ (CL-13 and MX strain). These strains induce CD8+ cytotoxic T-cells to the extent that they become 'exhausted' and vanish within a few days; a condition referred to as 'reduced ability' (Laposova *et al.*, 2013; Moskophidis *et al.*, 1993; Mueller & Ahmed, 2008; Ng *et al.*, 2011).

CL-13 and MX strains have a strong affinity for alpha-dystroglycan – a characteristic reportedly determined by the presence of leucine or isoleucine (aliphatic amino acids) at residue 260 of GP1. Such strains displace laminin from the glycosylated region of alpha-dystroglycan and this helps initiate immunosuppression (Laposova *et al.*, 2013). ARM 53b however has phenyl alanine (aromatic amino acid) at that same position and has a low affinity for alpha-dystroglycan. It does not establish persistent infection ($CTL^{+}P^{-}$). CL-13 and ARM 53b also have a difference in position 1079 of the polymerase with CL-13 having a glutamine while ARM 53b has a lysine at that position (Oldstone & Campbell, 2011; Smelt *et al.*, 2001).

1.3.6.1. Treatment and Prevention of LCMV Infection

1.3.6.1.1. Vaccine Development

Vaccination is an important strategy for the prevention of disease and can lead to reduced morbidity and mortality. Current licenced vaccines

are either live-attenuated, killed/whole or subunit. However the successful development of vaccines against most arenaviruses remains elusive. Candid 1, a live attenuated strain of JUNV is the only vaccine that has been tested on humans and is only approved for use in Argentina. The vaccine does not offer cross protection against LCMV and LASV however (Urata & Yasuda, 2012). The ML29 is another candidate that has shown promise for use as a live-attenuated vaccine against LASV. It was constructed from the L-segment of MOPV and S-segment of LASV (Lukashevich, 1992) and has been used successfully in the vaccination of rhesus macaques. All vaccinated animals produced specific humoral and cell-mediated immunity without the development of disease.

Although ML29 confers immunity in animal models, the mechanism of attenuation is still unknown. This raises concerns about its phenotypic stability when faced with additional mutations (Iwasaki *et al.*, 2015; Zapata *et al.*, 2013). There has also been considerable success with the development of LCMV vaccines. In 2012, Ammana and colleagues demonstrated the effectiveness of hydrogen peroxide (H_2O_2) as an inactivating agent for viruses. Results showed that H_2O_2 inactivated the pathogens but maintained their viral antigenicity. H_2O_2-inactivated LCMV (Armstrong) conferred protection to mice by eliciting the production of virus specific CD8+ T-cells which provided immunity against LCMV infection (Amanna *et al.*, 2012). Although these candidates are still under development and are yet to reach human trials, the success achieved thus far shows that the goal of developing protective immunity against disease-causing arenaviruses is within reach.

1.3.6.1.2. Therapy

Treatments for arenavirus infections involve the combination of supportive care and drug therapy. Supportive care involves the replenishment of reduced bodily fluids and correction of electrolyte

imbalances (Asnis *et al.*, 2010; Handy, 2004; Wendel *et al.*, 2009). The successful use of immune plasma therapy in the treatment of AHF has been reported (Enria *et al.*, 2008; Maiztegui *et al.*, 1979). A double-blind placebo study was conducted to assess the efficacy of immune plasma. Patients with AHF that had received the placebo had a higher mortality (16.5%) than those that received immune plasma within 8 days of the onset of the disease (1%). Some patients treated with immune plasma developed late neurological complications however. This has also been demonstrated in patients with Lassa fever. Patients that received Lassa fever convalescent plasma (LFCP) on or before the 10th day survived while all that received treatment after the 10th day died (Frame *et al.*, 1984). Treatment of LCMV-related diseases with immune plasma therapy has not yet been investigated.

The development of drugs for the treatment of arenavirus infection has been hugely based on the inhibition of the life cycle of the viruses. Viral load generally is a predictive factor for disease outcome and progression. Thus the immune system will be overwhelmed with a huge burden of high viral replication and spreading when there is a huge viral load. Antiviral drugs that hinder viral replication provide an avenue for the host immune system to control viral spread with the ultimate goal of eliminating the virus (Lee *et al.*, 2011a). Antiviral drug therapy is currently limited to the use of the ribavirin (Rib) (1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide), a guanosine analog. Its mode of action involves phosphorylation into ribavirin – 5'monophosphate which interacts with inosine monophosphate (IMP) dehydrogenase ultimately leading to a reduction in guanosine triphosphate (GTP) pools thus inducing mutations which may lead to the formation of non-functional viruses (Streeter *et al.*, 1973). It is the preferred drug of choice for the treatment and prevention of Lassa fever (McCormick *et al.*, 1986). However treatment is only effective if administered early in the course of the disease (usually before day 6). Early diagnosis is a problem as symptoms are often initially mistaken for malaria, influenza or typhoid fever (Ölschläger & Flatz,

2013). Although ribavirin has broad-spectrum antiviral activity, it has had only limited success in the treatment of arenaviruses with the manifestation of a number of side effects including thrombocytosis and anaemia (Damonte & Coto, 2002; Urata & Yasuda, 2012).

The treatment of LCMV infection is usually supportive. The recommended treatment for LCMV infection received through organ transplantation includes reduced immunosuppression and ribavirin. There is however limited information on the efficacy of ribavirin in such cases. It is not recommended in the treatment of pregnant women as it is a well-known teratogen (Asnis *et al.*, 2010; Damonte & Coto, 2002; Fischer *et al.*, 2006; Kerber *et al.*, 2015; Schafer *et al.*, 2014; Zapata *et al.*, 2011) .

1.4. General Aims

The emergence of many major human infectious diseases, including some now confined only to humans and absent from animals, has stirred up important questions including their origin and if future disease spread to humans can be prevented. As about 60% of human diseases are zoonotic, an active surveillance of wildlife for novel pathogens is required to assess the zoonotic potential of the latter (Jones *et al.*, 2008; Taylor *et al.*, 2001; Woolhouse & Gowtage-Sequeria, 2005). The cost involved in the discovery of novel pathogens in potential reservoirs will be far less than the economic impact caused by outbreaks if they occur (Anthony *et al.*, 2013a).

Arenaviruses and filoviruses are still a cause of significant morbidity and mortality despite being discovered decades ago (McLay *et al.*, 2014; Team *et al.*, 2014). More of these viruses are being discovered although not all have been confirmed to have zoonotic potential (Blasdell *et al.*, 2016; Gryseels *et al.*, 2015; Li *et al.*, 2015; Negredo *et al.*, 2011;

Stenglein *et al.*, 2012). Most arenaviruses have been identified in a rodent host. Although bats are the most likely culprits for the transmission of filoviruses, the ebolavirus genome has also been detected in rodents (Morvan *et al.*, 1999). The discovery of LLOV in from Spanish bats is evidence that filoviruses are present in continents beyond Africa and Asia (Barrette *et al.*, 2009; He *et al.*, 2015; Negredo *et al.*, 2011; Team *et al.*, 2014).

The prototype arenavirus, LCMV causes infections in humans and NHPs. LCMV infections in New World primates (Callitrichidae) is highly fatal (Asper *et al.*, 2001; Stephensen *et al.*, 1991). LCMV infections are still largely underdiagnosed. Commercial kits are not readily available for routine diagnoses (Perez-Ruiz *et al.*, 2012). Prevalence studies in the UK have either utilized assays which screen for antibodies or molecular methods for the detection of small fragments of the RNA genome. None of these provide sufficient information for further studies on phylogenetics as well as virus entry and host immune defence.

The aims of the present study were;

1. The detection of novel arenaviruses and filoviruses in rodents in the United Kingdom.
2. The detection, identification and molecular characterisation of strains associated with a reported outbreak of LCMV in a zoo in the United Kingdom.
3. The development of a pseudotype assay to investigate LCMV entry into host cells.

2. Materials and Methods

2.1. Materials

Study approval was obtained from the University of Nottingham Internal Ethics Committee (Nottingham, UK).

2.1.1. Sequence Data

Sequences used in this study were obtained from National Centre for Biotechnology Information (NCBI) database for primer design and phylogenetic analyses (Appendices 1 - 5).

2.1.2. Rodent, Shrews and Non-Human Primate Organs and Tissues

A total of 1033 animals were sampled in this thesis (Table 2.1). These originated from the East Midlands Region of the United Kingdom, the Mazury Lake District Region of Poland, and the Wadis in the Sanai Peninsula in Egypt.

Some of the animals were collected at two periods from during investigations of an outbreak of LCMV infection. The first set was collected between 2006 and 2008. These included 49 rodent and shrew liver organs donated by Dr. Rachael Tarlinton of the School of Veterinary Medicine and Science (Appendix 7) and 75 organs from a variety of animals donated by Public Health Engand (Appendix 8). The organs donated by Public Health England were part of a larger sample set involving a total of 179 animals. These are presented in Table 2.2.

The second set was obtained between 2013 and 2015 and were collected either as part of routine pest control, as a consequence of predation (*Felis catus*) or for other studies. Three *M. musculus* rodents

were obtained from Redmile, Leicestershire, United Kingdom (Appendix 9). These were donated by Dr. Patrick McClure of the School of Life Sciences, Nottingham. Ten *M. musculus* rodents were obtained from Nottingham, Nottinghamshire (Appendix 10). These were caught and donated by Professor Jan Bradley of the School of Life Sciences, University of Nottingham. Four hundred and fifty nine animal specimen were obtained from Twycross zoo (Appendix 11).

A total of 146 bank voles (*Myodes glareolus*) and 294 spiny mice (*Acomys dimidiatus*) were donated by Dr. Rachael Tarlinton. Information on the sampling dates for these animals were unavailable.

2.1.3. Cells

2.1.3.1. Competent cells for transformation

Stellar cells™, NEB® 10-beta cells and One Shot® BL (DE3) chemically competent cells were used for bacterium transformation assays. Stellar cells™ were purchased from Clontech. NEB® 10-beta cells were purchased from New England Biolabs® and One Shot® BL (DE3) chemically competent cells were purchased from Sigma-Aldrich.

2.1.3.2. Mammalian cell lines for cell culture

Human kidney epithelial (HEK 293T) (ATCC®CRL-3216™) cells were used as packaging material for pseudotype viruses and hepatocellular carcinoma (Huh7) cells were used in infection assays. Both cell lines were obtained from MRC-University of Glasgow Centre for Virus Research (Miletic *et al.*, 1999; Nakabayashi *et al.*, 1982; Nie *et al.*, 2004).

Table 2.1: Animals Screened for Viruses in this Thesis

Organism		Location	Sampling Period		Total
Common Name	**Scientific Name**		**2005-2006**	**2013-2015**	
House mouse	*Mus musculus*	United Kingdom	48	372	420
Brown rat	*Rattus norvegicus*	United Kingdom	8	88	96
Common vole	*Microtus arvalis*	United Kingdom	0	1	1
Field vole	*Microtus agrestis*	United Kingdom	11	0	11
Wood mouse	*Apodemus sylvaticus*	United Kingdom	6	3	9
Guinea pig	*Cavia porcellus*	United Kingdom	3	0	3
Pygmy shrew	*Sorex minutus*	United Kingdom	3	0	3
Bank vole	*Myodes glareolus*	Poland	-	-	146
Egyptian Spiny Mouse	*Acomys dimidiatus*	Egypt	-	-	294
Mole*	-	United Kingdom	0	5	5
Chimpanzee	*Pan troglodytes*	United Kingdom	1	0	1
Kloss Gibbon	*Hylobates klossii*	United Kingdom	1	0	1
Red Howler Monkey	*Alouata sara*	United Kingdom	5	0	5
Black Howler Monkey	*Alouatta caraya*	United Kingdom	3	0	3
Allen's Swamp Monkey	*Allenopithecus niviridu*	United Kingdom	1	0	1
Dusky leaf Langur	*Trachypithecus obscurus*	United Kingdom	3	0	3
Geoffroy's Marmoset	*Callithrix geoffroyi*	United Kingdom	3	0	3
Purple-faced Langur	*Trapithecus vetulus monticola*	United Kingdom	1	0	1
Black and White Colobus	*Colobus guereza occidentalis*	United Kingdom	3	0	3
Common Marmoset	*Callithrix jacchus*	United Kingdom	2	0	2

Organism Common Name	Scientific Name	Location	Sampling Period 2005-2006	2013-2015	Total
Emperor Tamarin	*Saiguinus imperator subgrisescens*	United Kingdom	2	0	2
Black-tailed Marmoset	*Callithrix argentata melanura*	United Kingdom	1	0	1
Golden - Headed Lion Tarmarin	*Leontopithecus rosalia*	United Kingdom	1	0	1
Cotton Top Tamarin	*Sanguinus Oedipus*	United Kingdom	1	0	1
Western lowland gorilla	*Gorilla gorilla*	United Kingdom	2	0	2
Agile Gibbon	*Hylobates agilis*	United Kingdom	2	0	2
Pygmy Marmoset	*Callithrix pygmaea*	United Kingdom	1	0	1
Northern white-cheeked Gibbon	*Nomascus leucogenys*	United Kingdom	1	0	1
Spectacled Langur	*Trachypithecus obscurus*	United Kingdom	1	0	1
Amur Leopard	*Panthera pardus orientalis*	United Kingdom	1	0	1
Seba's Short-tailed Bat	*Carollia perspicillata*	United Kingdom	1	0	1
Bat	-	United Kingdom	3	0	3
Striped Skunk	*Mephitis mephitis*	United Kingdom	1	0	1
Duckling	-	United Kingdom	0	3	3
Finch	-	United Kingdom	0	1	1
		Total	120	473	1033

M. glareolus was trapped between 1999 and 2010. *A. dimidiatus* was trapped in 2008. The bats, ducklings and skunk were not identified to the species level.

Table 2.2: Animals used in the Study Conducted by Public Health England

Organism		2005/2006 Sampling Period
Common Name	**Scientific Name**	
House mouse	*Mus musculus*	41
Brown rat	*Rattus norvegicus*	2
Guinea pig	*Cavia porcellus*	4
Chimpanzee	*Pan troglodytes*	14
Kloss Gibbon	*Hylobates klossii*	1
Red Howler Monkey	*Alouata sara*	6
Black Howler Monkey	*Alouatta caraya*	4
Allen's Swamp Monkey	*Allenopithecus niviridus*	3
Dusky leaf Langur	*Trachypithecus obscurus*	6
Geoffroy's Marmoset	*Callithrix geoffroyi*	8
Purple-faced Langur	*Trapithecus vetulus monticola*	3
Black and White Colobus	*Colobus guereza occidentalis*	7
Common Marmoset	*Callithrix jacchus*	9
Emperor Tamarin	*Saiguinus imperator subgrisescens*	3
Black-tailed Marmoset	*Callithrix argentata melanura*	1
Golden - Headed Lion Tarmarin	*Leontopithecus rosalia*	2
Cotton Top Tamarin	*Sanguinus Oedipus*	1
Western lowland gorilla	*Gorilla gorilla gorilla*	8
Agile Gibbon	*Hylobates agilis*	2
Pygmy Marmoset	*Callithrix pygmaea*	3
Northern white-cheeked Gibbon	*Nomascus leucogenys*	1
Unidentified primate	-	10
Javan Leaf Monkey	*Trachypithecus auratus auratus*	1
Variegated Columbian Spider Monkey	*Ateles belzebuth hybridus*	1
Linne’s Two-toed Sloth	*Choloepus didactylus*	2
Hamlyn’s Guenon	*Cercopithecus hamlyni*	3
Lesser Spot-nosed Guenon	*Cercopithecus petaurista*	2

Organism Common Name	Scientific Name	2005/2006 Sampling Period
Roloway Monkey	*Cercopithecus roloway*	1
Red-handed Tamarin	*Saguinus midas*	1
Langur*	-	4
Spectacled Langur	*Trachypithecus obscurus*	3
Diana Monkey	*Cercopithecus diana diana*	2
Woolly Monkey	*Lagothrix lagotricha*	1
Howler Monkey*	-	7
Spider Monkey	*Ateles sp*	4
Amur Leopard	*Panthera pardus orientalis*	1
Seba's Short-tailed Bat	*Carollia perspicillata*	2
Bat*	-	3
Striped Skunk	*Mephitis mephitis*	1
		179

* The information presented on the animal was insufficient to allow for proper identification.

2.1.4. Vectors

PcDNA 3.1^{TM}(+) vector and HIV-l luciferase reporter vector (pnl4.3 ***luc*** R-E-) were used for protein expression in tissue culture assays while pGEM®T Easy Vector was used for general cloning of Reverse Transcription PCR (RT-PCR) products. The pcDNA 3.1^{TM}(+) vector was purchased from Invitrogen. The HIV-l luciferase reporter (pnl4.3 ***luc*** R-E-), murine Leukaemia Virus and luciferase vectors were already available for use in the virology laboratory. The pGEM®T Easy Vector was purchased from Promega.

2.1.5. Chemicals and Reagents

All chemicals and reagents used in the study were commercially available. These included ethidium bromide (VWR International), Distel (Tristel Solutions Limited) and agar (Fluka). Ethanol, glycerol, fetal bovine serum (FBS), RNA*later,* Luria bertani (LB) broth and tris-acetate EDTA (TAE) buffer were purchased from Sigma-Aldrich. Nuclease free water and agarose were purchased from Fisher Scientific. Polyethylenimine (PEI), Penicillin Streptomycin (Pen Strep), reduced serum media (opti-MEM), Dulbecco's Modified Eagle Medium (DMEM) and Non-essential Aminoacids (NAA) were all purchased from Life Technologies.

2.1.6. Oligonucleotides

Primers for DNA amplification were either obtained from published literature or designed in-house using the Primer3, OligoAnalyzer 3.1 and Molecular Evolutionary Genetics Alignment (MEGA6) tools and then synthesized by Eurofins Genomics. Random hexamers for reverse transcription were synthesized by QIAGEN. See Appendi 6 for details of primers used.

2.1.7. Kits

RT-PCR reagents were obtained as follows: HotStarTaq® DNA Polymerase Kit was purchased from QIAGEN. LongAmp® Hot Start Taq DNA Polymerase Kit was obtained from New England Biolabs®. RevertAid™ Reverse Transcriptase Kit was purchased from Thermo Fisher Scientific. GenElute® Mammalian Total RNA Miniprep Kits, GenElute® Plasmid Miniprep Kits and GenElute® HP Plasmid Midiprep Kits were purchased from Sigma-Aldrich. Gibson assembly® master mix was obtained from New England Biolabs®. QIAquick® Spin kit was obtained from QIAGEN.

2.2. Methods

A flow chart listing the logical order of the processes conducted in this thesis is shown in Figure 2.1.

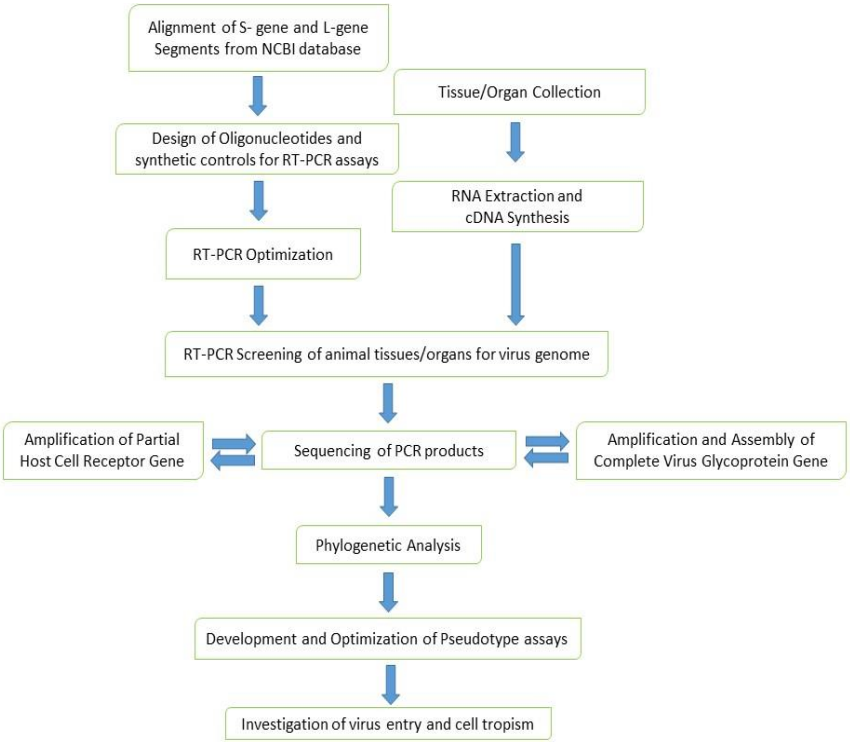


Figure 2.1: A Flow chart listing the logical order of the processes conducted in this thesis

2.2.1. Sample Collection, Processing and Storage

2.2.1.1. Rodent trapping and Killing

The method used in sampling has been previously described (Drexler *et al.*, 2012). Animals were identified by the veterinarians (Table 2.1). Rodents obtained during the zoo outbreak were caught and killed using traps. Some rodents obtained after the outbreak were first trapped and then euthanized using chloroform afterwards (Feldman & Seely, 1988). All trapping was carried out by employed animal attendants. Euthanization by chloroform was carried out by the doctoral student. In the case of the trap-killed rodents, the carcasses were stored at -25°C prior to organ harvesting. In the case of chloroform-euthanized rodents, organs were harvested immediately after the death of the animal and placed in 500µl RNA*later* (Sigma-Aldrich) solution before storage at -80°C (Sigma-Aldrich, 2014).

Liver sections from rodents trapped and killed between 2006 and 2008 were provided by Dr Rachael Tarlinton. Animal dissection and organ harvesting of all rodents trapped and killed between 2013 and 2015 was carried out by the doctoral student (Figure 2.2). Organs harvested include brain, liver, kidney, gut, spleen, heart and lungs.

Necropsies performed on the *Myodes glareolus* rodents of Polish origin were carried out by a separate group prior to the start of the present study. The liver tissue samples were stored at −70 °C in ethanol afterwards.

Information on the collection and processing of tissues from *Acomys dimidiatus* rodents obtained from Egypt was unavailable.



Figure 2.2: Rodents dissected in this thesis

2.2.1.1.1. Dissection of Shrews and Rodents

Frozen animals were thawed before dissection commenced. All dissections were conducted in a class one biosafety cabinet. Freshly-killed animals were moistened with 70% ethanol before opening them up to prevent animal hair from interfering with the dissection process as well as being mistakenly collected together with other organs and tissues. Forceps were used to hold the carcasses in place thus preventing direct contact. Cutting was achieved using scalpels (VWR International) or scissors. A midline incision was made from the mandibular section to the anus to expose the subcutaneous tissues thus allowing for its inspection. Exsanguination (removal of blood) was carried out before the harvesting of internal organs. Blood was obtained through cardiac puncture. Pieces of liver, kidney, heart, lung, spleen and intestine were collected. Brain tissue was obtained by dissecting the skull. Organs and tissues where either processed immediately after dissection or immersed in RNA*later*, and then stored indefinitely at -80°C afterwards.

2.2.1.2. Tissues from Non-Human Primates and Other Animals

Tissues from other animals including non-human primates affected by the outbreak were sourced from Public Health England (PHE). Information on the collection and processing of these tissues were unavailable. The tissues were supplied in labelled Bijou bottles. The labels contained insufficient information on the identity of the tissues within them. All tissues were stored at -80°C.

2.2.2. RNA Extraction

RNA was extracted from the sampled organs using the GenElute™ Mammalian Total RNA Miniprep Kit (Sigma-Aldrich). The extraction was carried out according to the protocol provided. About 40mg of liver organ from each sample was homogenised completely in lysis solution (To which mercaptoethanol was added) using a Hybaid ribolyser at speed 6 for 40 seconds prior to extraction. The lysis reaction was carried out immediately to minimise RNA degradation. Incomplete homogenization will reduce the quantity of RNA recovered. The lysate was then filtered in a filtration column (provided) using a centrifuge at ≥12,000 x*g* to remove cellular debris and shear DNA. The column was discarded and 500µl of 70% ethanol was added to the filtrate and the resultant solution was mixed thoroughly by pipetting and transferred to a binding column. This was followed by centrifugation for 15 seconds at ≥12,000 x*g*. Afterwards, the column was washed three times. The first wash was carried out using Wash Solution 1 (provided with the kit). The second and third washes were carried out using Wash Solution 2 (To which ethanol was added). Each of the three washes was followed by a centrifugation step; the first two centrifugations were carried out for 15 seconds at ≥12,000 x*g*. The centrifugation after the last wash was carried out for 2 minutes at ≥12,000 x*g*. The column was spun afterwards for 1 minute at ≥12,000 x*g* to dry it and remove any ethanol that may contaminate the RNA. RNA was finally eluted in Elution solution (Provided with the kit)

and used immediately in cDNA synthesis or stored at -80°C. The final concentration and quality of total mammalian RNA was determined by spectro-photometric analysis using a nanodrop spectrophotometry. The quality of RNA extracted was determined by measuring the ratio of absorbance at 260nm and 280nm. A ratio of ~2.0 is generally indicative of the absence of phenol, protein or other contaminants. A secondary measure of nucleic acid purity (the 260/230 ratio) was also determined.

2.2.3. Complementary DNA Synthesis.

Complementary DNA was synthesized from RNA using RevertAid™ Reverse Transcriptase in a two-step Reverse Transcription Polymerase Chain Reaction (RT-PCR). Random hexamers were used as primers in the first step reaction. Two master mixes for the two steps were made according to the manufacturer's instructions. In the first step, an RNA template-primer mix was incubated at 65°C for 5minutes and transferred to ice immediately afterwards. The second mix of 5X buffer, dNTPs and RevertAid transcriptase™ was then added and the resulting solution was incubated at 25°C for 10minutes. This was followed by a further incubation of 42°C for 60minutes. The reaction was terminated after a final heating at 70°C for 10minutes. Thermo Scientific RiboLock RNase Inhibitor was excluded from the second step reaction. The final PCR products were either used immediately or stored at 20°C.

2.2.4. RT-PCR amplification, DNA Modification and Processing

2.2.4.1. Primer Design for RT-PCR Assay

Species identification was conducted by the amplification of a partial segment of the cytochrome B gene. Primers (Cyt_421F/Cyt_840R) were constructed based on conserved regions across rodent and primate

sequences obtained from NCBI database. Primers were designed to amplify a target region of 419bp.

The quality of messenger RNA was inferred by the successful amplification of Glyceraldehyde 3-phosphate dehydrogenase (GAPDH), a housekeeping gene. Primers (GAPDH_F/GAPDH_R) were obtained from published literature (Tsoleridis *et al.*, 2016).

The primer pair ADG_460F/ADG_1297R were constructed based on conserved regions across rodent and primate sequences of the dystroglycan gene obtained from NCBI database. The primers were designed to amplify a target region of 836bp.

Chimeric primers were designed so their 3' ends were complementary to the E9 plasmid while the 5' end had sequences complementary to the specific filoviruses and arenaviruses.

Seven L-gene sequences from recognised filoviruses were obtained from NCBI database. These were aligned using the Molecular Evolutionary Genetics Analysis (MEGA) and conserved regions were used to design broadly-reactive primers to amplify the L-gene (Figure 2.3). Primers were designed with mixed degenerate bases. Gene-specific primers were also designed, each representing a recognised filovirus species. Figure 2.4 is a map of the filovirus L-gene showing the target position for the FILO_F/FILO_R primer set.

a)

Species	1787 * *** *** *** *** *** *** *** ** 1809
FILO_F1	... ..G CMT TYC CIA GYA AAY ATG ATG GTT ... .
Lloviu cuevavirus	CTA AAG CAT TTC CGA GTA AAT ATG ATG GTT GTT A
Tai Forest ebolavirus	CGA AAG CAT TCC CAA GCA AAT ATG ATG GTT GTG A
Bundibugyo ebolavirus	CTA AAG CCT TTC CTA GTA AAC ATG ATG GTT GTA A
Zaire ebolavirus	CTA AAG CAT TTC CTA GCA AAT ATG ATG GTA GTT A
Sudan ebolavirus	CTA AAG CCT TTC CAA GCA AAT ATG ATG GTT GTC A
Reston ebolavirus	CCA AGG CCT TCC CCA GTA AAC ATG ATG GTA GTA A
Marburg Marburgvirus	CAA AAG CAT TCC CTA GTA AAC ATG ATG GTT GTC A

b)

Species	2171 * *** *** ** *** *** *** * 2190
FILO_F1	... ..T GGA CIW CYA TIT CIT GTG C.. ...
Lloviu cuevavirus	AAT TAT GGA CAA GTA TTT CTT GTG CTC AGA
Tai Forest ebolavirus	AAC TCT GGA CAA GCA TCT CAT GTG CAC AGA
Bundibugyo ebolavirus	AAC TCT GGA CCA GCA TTT CAT GTG CAC AAA
Zaire ebolavirus	AAC TCT GGA CAA GTA TTT CAT GTG CTC AAA
Sudan ebolavirus	AGT TAT GGA CTA GTA TCT CAT GTG CTC AAA
Reston ebolavirus	AAC TGT GGA CGA GTA TAT CCT GTG CAC AAA
Marburg Marburgvirus	AAT TGT GGA CAT GCA TAT CAT GTG CCC AAA

Figure 2.3: Alignment of L-gene sequences of all recognised filoviruses showing the binding site of (a) Forward (FILO_F1) and (b) Reverse (FILO_R1) primers and their respective positions. The inosines (denoted by the letter 'I') compensate for sites of complete degeneracy.



Figure 2.4: Schematic of Filovirus L-gene (blue) showing the amplicon size of the FILO_F/FILO_R primer set (grey) their positions on the gene (in parenthesis).

2.2.4.1.1. Broadly-reactive Oligonucleotides used in the Detection of Arenavirus L-gene and S-gene

L-gene sequences from recognised arenaviruses were obtained from NCBI database. These were aligned using the Molecular Evolutionary Genetics Analysis (MEGA) and conserved regions were used to design two sets of broadly-reactive primers to amplify the L-gene. Primers were designed with mixed degenerate bases. The first set of primers (MAMF/MAMR) were constructed based on an alignment of L-gene sequences from twenty six of the currently recognised mammarenaviruses while the second set of primers (REPF/REPR) were constructed based on an alignment of L-gene sequences from three of the currently recognised reptarenaviruses.

Published primers were also used for the detection of arenaviruses (Vieth *et al.*, 2007). These were designed based on conserved regions within Lassa mammarenavirus, Lymphocytic choriomeningitis mammarenavirus, Pichinde mammarenavirus, Tacaribe mammarenavirus, Ippy mammarenavirus, Mobala mammarenavirus and Mopeia mammarenavirus L-gene sequences obtained from NCBI database. Gene-specific primers were also designed, each representing a recognised arenavirus species.

S-gene sequences were obtained from NCBI database. These were aligned using the Molecular Evolutionary Genetics Analysis (MEGA) and conserved regions were used to design broadly-reactive primers to amplify the GPC region of the S-gene. Primers for the amplification of the nucleoprotein were obtained from published literature (Perez-Ruiz *et al.*, 2012). Primers were designed with mixed degenerate bases. Figure 2.5 shows the maps of the arenavirus polymerase and glycoprotein genes and the target position for the various primer sets used.

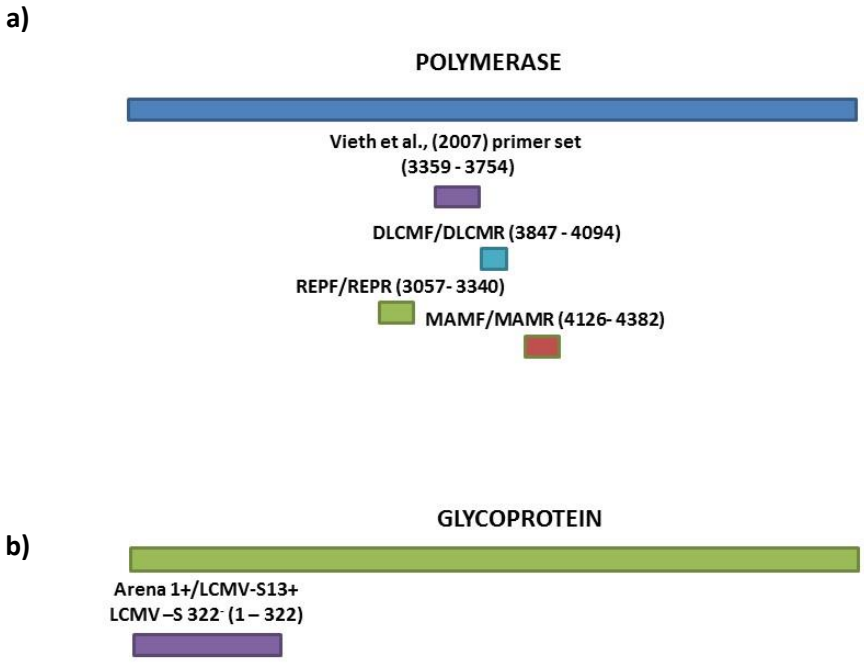


Figure 2.5: Schematic of Arenavirus L-gene (blue) showing the amplicon sizes of the various primer sets used and their positions on the a) Polymerase gene b) Glycoprotein gene.

2.2.4.2. Amplification of Full-length Glycoprotein gene

Various sets of primers were designed to detect the complete glycoprotein gene of LCMV (~1480bp). This was achieved by an alignment of full-length LCMV-glycoprotein sequences obtained from NCBI database. Full-length GPC was amplified using either a set of primers (GPF1/GPF8) or a combination of primers for 'primer walking'

(Figure 2.6). Oligonucleotides used for primer walking were GPF1, GPF2, GPF3, GPF4, GPF5, GPF6, GPF7, GPF7F, GPF7R, GPF8, GPF8R, GPF8R1, LCMV277 and LCMV1026. GPF1 and GPF8 Primers were designed with the complementary tags to facilitate insertion into pcDNA^{TM}3.1(+) vector.

2.2.4.3. Construction of Positive Plasmid Controls

Positive plasmid controls were constructed to monitor PCR efficiency. The DNA sequence used for plasmid design was obtained from the colicin E9 plasmid. The chimeric primer sets were used to amplify a segment of the colicin E9 plasmid. The successful amplicon had terminal ends complementary to the gene-specific and broadly-reactive primers. The amplicon was then incorporated into a pGEM®T Easy Vector (Figure 2.7).

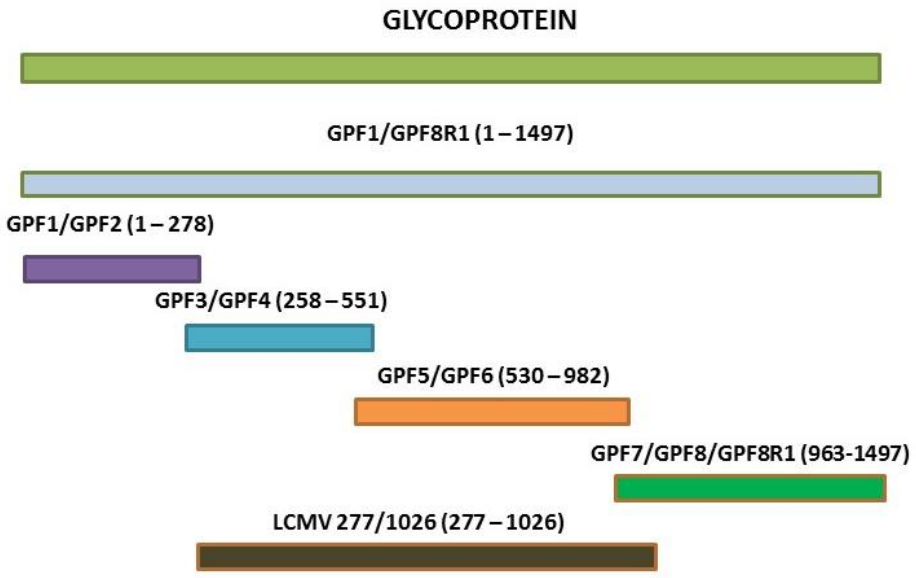


Figure 2.6: Schematic for primer walking procedure for the amplification and sequencing of complete GPC gene. The amplicon sizes of the various primers, combinations used and their positions on the glycoprotein gene are also shown (in parenthesis).

2.2.4.4. RT-PCR Amplification and Optimization

Amplification was carried out in a PCT-200 Peltier Thermal Cycler (MJ Research) using thin-walled reaction tubes (Axygen scientific). The basic formulation for 25µl PCR reactions used HotStar Taq Polymerase Kit with a combination of 2.5 µL PCR buffer, 200µM of each dNTP, 0.5µL of HotStar Taq DNA polymerase enzyme and 1uL of template DNA. To increase assay sensitivity and specificity, thermal cycling conditions were modified using gradient RT-PCR. Amplification generally involved 15 minutes at 95°C, 20 seconds at 95°C; and 50 cycles for 20 seconds at a range of annealing temperatures (46°C and 60°C), 20 seconds at 72°C and a final extension period of 60 seconds at 72°C.

2.2.4.5. Agarose Gel Electrophoresis and Purification of Amplified Products

Amplified products were loaded on agarose gels to observe band sizes. Smaller products (≤800bp) were run on 2% agarose gels at 90volts for 36minutes while larger products (>800bp) were run on 1% gels at 90volts for an hour or more.

Purification of amplified products was carried out using QIAquick® Spin. The processes were conducted according to the manufacturer's instructions. With direct purification, 5 volumes of buffer PB were mixed with 1 volume of the product. The resultant mixture was placed in a QIAquick spin column and centrifuged for 30 to 60 seconds at 13,000rpm. The flow-through liquid was discarded. The column was then washed with 750µl with buffer PE and centrifuged for 30 to 60 seconds at 13,000rpm and the flow-through liquid was discarded. The column was then centrifuged for an additional 1minute to remove any residual ethanol. Fifty microlitres of buffer EB was added to the centre of the column membrane. The column was then allowed to stand for 1minute.

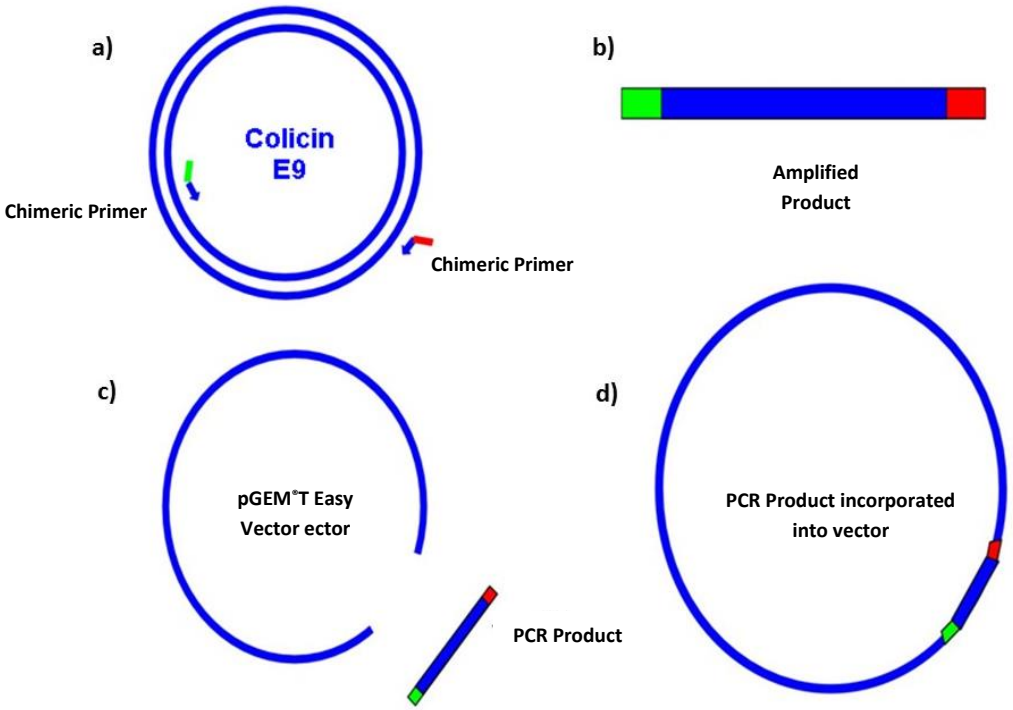


Figure 2.7: Construction of plasmid controls used in RT-PCR assay. Sequence of Colicin E9 is in blue. Complimentary sequences on chimeric primers are in blue. Partial sequences of the virus target in the chimeric primers are in green (forward primer) and red (reverse primer). a) Amplification of 240bp segment of colicin E9 plasmid using the chimeric primer sets. b) Final Amplified product containing sequence from colicin E9 (blue) and sequences from virus (red and green). c) Ligation of Amplified product into pGEM®T Easy Vector. d) Final plasmid vector containing ligated product.

This was followed by a final centrifugation step for 1minute at 13,000rpm. Purified DNA was analysed on a 1% gel for confirmation. With purification from gel extraction, the DNA fragment of the desired size was first excised from the gel using a clean, sharp scalpel and weighed. The weight was equated to volume (100mg = 100µl). Three volumes of buffer QG was added to one volume of gel. This was followed by incubation at 50°C for 10minutes. Isopropanol was then added to the sample at a volume equal to that of the gel slice. The resultant mix was then added to the QIAquick® Spin. This was followed by centrifugation at 13000rpm for 1minute. The flow-through liquid was discarded. Five hundred microlitres of buffer QG was added to the column and the latter was then centrifuged for 1minute. The column was washed with 750µl of buffer BE and the column was centrifuged for 1minute at 13000rpm after which the flow-through liquid was discarded. The column was then centrifuged for an additional 1minute to remove any residual ethanol. Fifty microlitre of buffer EB was added to the centre of the column membrane. This was then allowed to stand for 1minute. This was immediately followed by a final centrifugation step at 13000rpm for 1minute. The purified DNA was analysed on a 1% gel for confirmation.

2.2.4.6. DNA Sequencing and Basic Local Alignment Search Tool (BLAST)

Amplicons of the expected product length were sent for sequencing to determine the identity of the DNA. The DNA was analysed using the Applied Biosystems 3730XL (Life Technologies). Sequences obtained were then subjected to BLAST search to identify similar sequences stored in the NCBI database.

2.2.5. Preparation of Glycerol Stocks

Five hundred millilitres of bacterial culture was added to a sterile Eppendorf tube. Five hundred millilitres of 100% (v/v) sterile glycerol solution was then added to the culture. This was followed by mixing with pipetting action and storage at -80°C.

2.2.6. Preparation of Starter Cultures

Starter cultures were prepared by inoculating a single colony from a freshly streaked plate into 50ml of LB medium (containing ampicillin). The culture was then incubated at 37°C overnight with shaking at 250rpm.

2.2.7. Plasmid Cloning

2.2.7.1. Transformation of Competent Cells

Twenty five microlitres of competent cells were added to 2µl of ligation reaction and the resulting mixture was incubated on ice for 20minutes. This was immediately followed by a 'heat shock' of the cells for 45-50seconds at 42°C. The products were then transferred to ice and incubated further for 2minutes. Two hundred and fifty microliters of super optimal broth with catabolite repression (SOC) medium was then added to the reaction tubes. This was followed by incubation with shaking at 37°C and 220rpm. The culture was then inoculated on Luria Bertani (LB) agar medium (containing ampicillin) using spread-plate technique and incubated overnight at 37°C.

2.2.7.2. pGEM®T Easy Vector system

Ten microlitres ligation reactions were set up by mixing 1µl of amplified product with 5µl of 2X Rapid Ligation Buffer, 1µl pGEM®T Easy Vector (50ng) and 1µl of T4 DNA ligase. The mixture was made up to a final

volume of 10µl by the addition of deionized water. After pipetting to ensure proper mixing, the reactions were incubated for 1hour at 22°C. Competent cells were transformed using the DNA fragments. Colony RT-PCR was carried out afterwards to identify successful transformants using M13F/M13R primers. Amplified products were analysed on agarose gel and were sequenced afterwards. Successful transformants containing the desired plasmid were inoculated into 5ml of LB broth (containing ampicillin) and incubated overnight with shaking at 37°C and 220rpm. Plasmids were extracted and purified using GenElute™ Plasmid Miniprep Kit.

2.2.7.3. Fusion Cloning

2.2.7.3.1. Preparation of Plasmid and LCMV-GPC DNA Fragments

Uncut pcDNA3.1™(+) vector was linearized by double digestion with FastDigest BamHI and FastDigest HindIII restriction enzymes. Incubation was conducted at 37°C for 5minutes. This was followed by an inactivation step at 80°C for 10minutes. PCR products were column purified using QIAquick® Spin kit.

2.2.7.3.2. Gibson Assembly

Fusion cloning of full or partial LCMV GPC segments to pcDNA 3.1™(+) vector was achieved using the Gibson assembly® Cloning Kit. Competent cells were thawed on ice. The reaction was set up as follows; 0.02-0.5pmols of fragments were added to 10µl of Gibson Assembly Master Mix (2X). Water was then added to make the volume up to 20µl. this was followed by incubation at 50°C for 15minutes when 2 fragments (Full-length glycoprotein and pcDNA3.1™(+) vector) were assembled or 50°C for 60minutes when 5 fragments (4 partial fragments of GPC and pcDNA3.1™(+) vector) were assembled. The products were then used to transform competent cells. Plasmids were extracted and

purified from successful transformants. Glycerol stocks of transformed cells were also prepared and stored at -80°C. Colony PCR was carried out after transformation to identify successful transformants using T7F/BGHR primers. PCR products were analysed on agarose gel and were sequenced afterwards. Successful transformants containing the desired plasmid were inoculated into 5ml of LB broth (containing ampicillin) and incubated overnight with shaking at 37°C and 220rpm. Plasmids were extracted and purified using GenElute™ Plasmid Midiprep Kit.

2.2.7.4. Miniprep

Four millilitres of overnight cultures of transformed cells were centrifuged at ≥12000x*g* for 1minute to pellet cells. The supernatant was discarded afterwards. Bacterial pellets were re-suspended with 200µl of Resuspension Solution. Mixture was mixed by pipetting action to ensure complete resuspension of cells. Cells were lysed by the addition of Lysis solution. The contents were immediately mixed by gentle inversion until a clear solution was observed. Contents were not mixed by vortex action to avoid genomic shearing of DNA. The cell-lysing step did not exceed 5minutes to avoid denaturing of supercoiled plasmid DNA. The cell debris was then precipitated by the addition of the neutralization/binding Solution. Contents were mixed by gentle inversion of the reaction tube. This was followed by centrifugation at ≥12000x*g* for 10minutes to pellet cell debris. The cleared lysate was then transferred to a column and centrifuged at ≥12000x*g* for 1minutes. The flow-through liquid was discarded. Five hundred microliters of Optional wash was then added to the column. This was followed by centrifugation at ≥12000x*g* for 1minute. The flow-through liquid was discarded. Seven hundred and fifty microliters of diluted Wash Solution was added to the column and this was followed by centrifugation at ≥12000x*g* for 1minute. The flow-through liquid was discarded and the column was centrifuged at maximum speed for 2minutes without the addition of any Wash Solution

to remove any excess ethanol. The column was then transferred to a fresh collection tube. One hundred microliters of Elution Solution was added to the column. This was followed by a final centrifugation step at ≥12000x*g* for 1minute. The nucleic acid titre and 260/280 absorbance ratio were determined using the nanodrop photospectrometry.

2.2.7.5. Midiprep

Starter cultures were prepared. Fifty millilitre of overnight culture was centrifuged at 5000x*g* for 10minutes. The supernatant was discarded. Four millilitres of Resuspension solution was added to the bacterial pellet. Complete resuspension of pellet was achieved by pipetting action. Cells were lysed by adding 4ml of Lysis solution. The contents were immediately mixed by gently inverting the tubes for 6 to 8 times. Mixing was not carried out with vortexing to avoid contamination of plasmid DNA with sheared genomic DNA. Four millilitre of chilled Neutralization Solution was added to neutralize cell lysis. The mixture was inverted 4 to 6 times. Three millilitre of Binding Solution was then added and the solution was transferred to a syringe barrel. The contents were then filtered into a GenElute HP Midiprep Binding Column and the contents were passed through the column by vacuum action. Four millilitre of Wash Solution 1 was added to the column and the contents were allowed to pass through. Four millilitre of Wash Solution 2 was then added to the column and the contents were allowed to pass through. The column was left to stand while the vacuum pump was left running for 10 additional minutes to dry it and remove any residual ethanol. The Binding column was transfer to a collection tube. One millilitre of Elution Solution was added to the column. This was followed by centrifugation at 3000x*g* for 5minutes. The titre of the plasmid present in the eluate was determined using nanodrop spectrophotometry. The plasmid was stored at 4°C.

2.2.8. Phylogenetic Analyses

Alignments of the nucleotide sequences of the L and S segments were made using CLUSTAL W. Phylogenic analyses were then carried out with neighbour-joining trees (Bodewes *et al.*, 2013; Gascuel & Steel, 2006; Nei, 1996) using p-distance with 1000 bootstrap replicates and other default parameters in MEGA5.

Bayesian phylogenetic analyses of the sequence differences among the full GPC segments of LCMV strains were conducted using the BEAST v1.8.4, BEAUti v1.8.4, Tracer v1.6, Figtree v1.6 analysis software packages (Drummond & Rambaut, 2007; Drummond *et al.*, 2012). Analyses included Markov chain Monte Carlo chain lengths of 500,000,000 generations with sampling every 10,000,000 states using the GTR $+I+\Gamma_4$ nucleotide substitution model and a relaxed lognormal molecular clock and constant population size model were chosen.

2.2.9. Statistical Analyses

To compare the prevalence values of LCMV infection at the different periods of sampling, we used the Fisher's exact test with a 95% confidence interval. The simple formula presented below (Daniel, 1999; Naing *et al.*, 2006; Pourhoseingholi *et al.*, 2013) was used in determining the sample size needed for the study where n = sample size; Z = Z statistic for a level of confidence; P = expected prevalence or proportion; d = precision in proportion.

$$n = (Z^2P\ (1 - P))/d^2$$

Mean infectivity values of LCMV pseudotypes were compared using a one-way analysis of variance (ANOVA) using Bonferroni's method to control for p-values for multiple comparisons.

2.2.1. Pseudotyping

2.2.1.1. Pseudoparticle Production

2.2.1.1.1. Passaging of human kidney epithelial (HEK 293T) cells and hepatocellular carcinoma (Huh7) cells

The floor of the cabinet was disinfected with trigene. All materials and reagents were sprayed with ethanol prior to being placed in the hood. Spent media from previous cultures of HEK 293T cells and Huh7 cells were poured out of the flask and replaced with 8mls of Phosphate Buffered Saline (PBS) and gently rocked to aid mixing. The PBS was added to the non-growth side of the flask to avoid the cells from detaching from the flask. The PBS was then removed from the flask and replaced by trypsin and left for a moment to allow for complete detachment of cells from the flask. Do to the adherent nature of Huh7 cells; the passaging of cells required incubation for 5 minutes to allow for complete detachment. Eight microliters of DMEM was then added to the flask to inactivate the trypsin. The resulting mix was transferred to a 50ml falcon tube and DMEM was added to make up the total volume to 50ml. Ten microliter of the mixture was added to a haemocytometer and the total cell count was estimated. The supernatant was poured out after centrifugation and the cells were re-suspended in 10ml of DMEM. Seven millilitres of DMEM were added to each petridish followed by the addition of a total of 1.2 x 10^6 cells. The culture was then incubated overnight.

2.2.1.1.2. Transfection

The HEK 293T cells were checked to confirm confluency. Two microgram of each glycoprotein plasmid (VSVg, SB18, NA14 and NA29) was prepared for the transfection reaction. Two master mixes were also prepared. The first master mix was prepared to contain 300µl of reduced serum media (opti-MEM) and pnl4.3 *luc* R- E- provirus plasmid (Connor *et al.*, 1995; Hsu *et al.*, 2003; Urbanowicz *et al.*, 2016) for each transfection reaction. The second master mix was prepared to contain

300µl of opti-MEM and PEI for each transfection reaction. The PEI/opti-MEM and pnl4.3 *luc* R- E-/opti-MEM solutions were then combined with each of the glycoproteins plasmids to a total volume of approximately 600µl per reaction and mixed gently by pipetting. The resulting mix was left to incubate for one hour at room temperature.

The cell medium of the HEK 293T culture that was incubated overnight was removed and replaced with 7ml of OPTIMEM. The PEI + pnl4.3 plasmid + glycoprotein mix was then added in drops to the edge of the dish containing HEK 293T cells. The cells were gently mixed by rocking and the plate was incubated for 4 – 6hours. The medium was then replaced with fresh DMEM + penicillin-streptomycin (Gibco) 10% FBS (10ml) and was incubated for 72hours (between day2 and day5).

2.2.1.1.3. Seeding of Huh7 cells

Huh7 cells were seeded on a 96-well plate. Each well contained 1.5 x 10^4 cells in 100ul of DMEM + 10% FBS (10ml) + non-essential amino acids. Plate was incubated overnight afterwards.

2.2.1.1.4. Infection Assay

Day5: Collection of Pseudoparticles and Infection of Huh7 Cells
Overnight culture of HEK 293T cells with plasmids was observed under the microscope for confluency. Cultures were then filtered using 0.45µm filter to separate HEK 293T cells from supernatant. Supernatant was stored at 4°C (for 2-3weeks if not used immediately). The media in the seeded 96-well plate containing Huh7 cells was removed and replaced with the supernatants containing pseudoparticles. The 96-well plate was incubated for 4 – 6hours. Afterwards, 150µl of fresh DMEM + penicillin-streptomycin (Gibco) 10% FBS (10ml) and was added bringing the total volume in each well to 250µl. The plate was thereafter incubated for 72hours.

2.2.1.1.5. Lysis and Plate Reading

Huh7 cells were lysed at 48 hours post-infection. The leftover media was removed from the 96-well plate and replaced with 50µl of lysis buffer. This was left to rock at 15rpm for 20 minutes. The plate was then transferred to a micro shaker to dislodge the cells. Finally the luciferase activity was measured using the Fluorostar Omega plate reader. The experimental setup for LCMV pseudotype production is outlined in Figure 2.8.

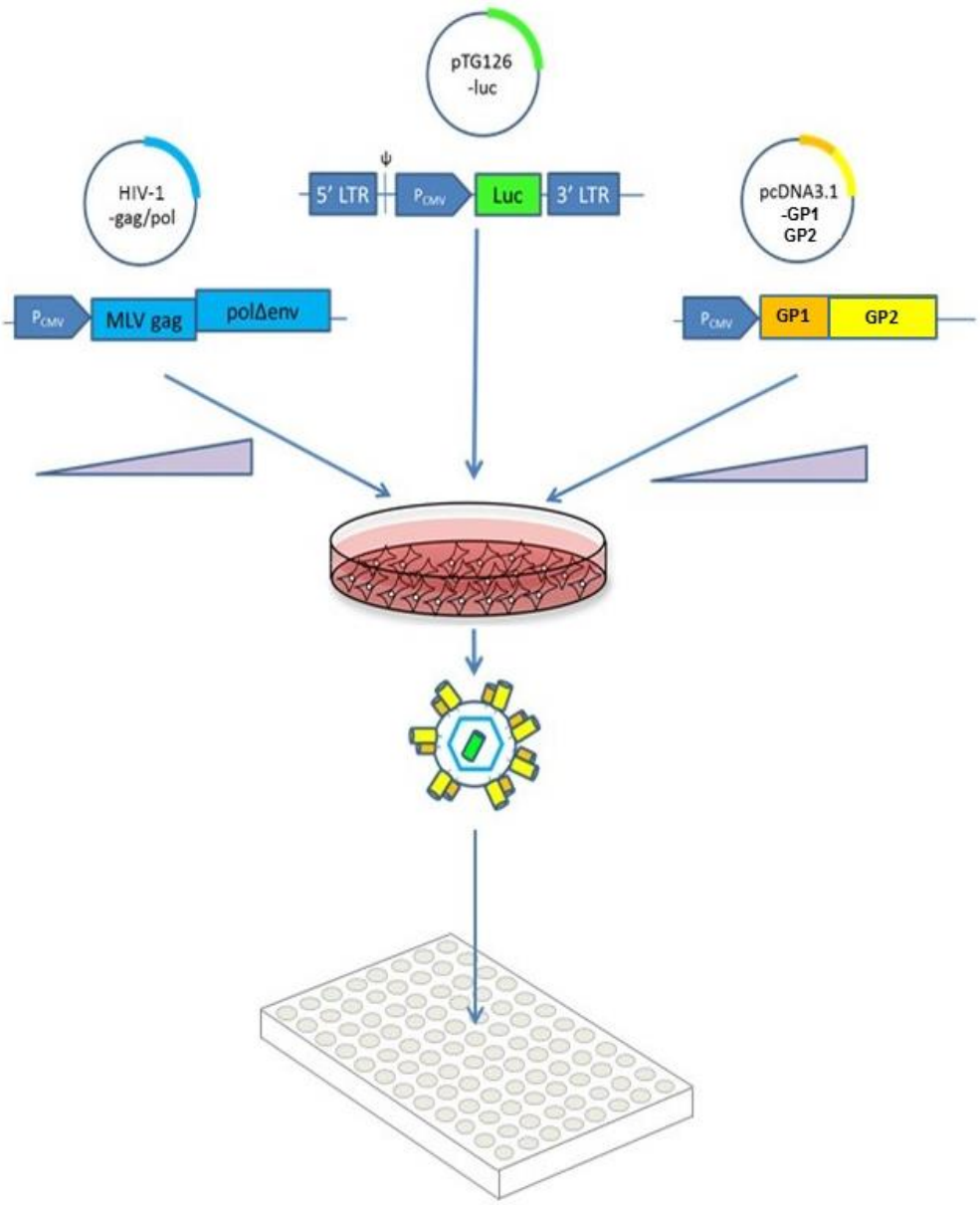


Figure 2.8: Experimental setup for LCMV pseudotype production in this study. Transfections were prepared with a fixed amount pnl4.3 *luc* R- E-. This plasmid was co-transfected with a fixed amount of the GP1/GP2-encoding plasmid (derived from pcDNA3.1) to create a matrix of transfections. HEK293T cells were transfected with the mixture of plasmids, and the pseudotypes generated were used to infect HuH7 cells in a 96-well format. Infectivity was determined as RLU from the expressed reporter gene in the HuH7 cells. Adapted from (Urbanowicz *et al.*, 2016)

3. Assessment of RNA Quality and Molecular Identification of Animal Species

3.1. Assessment of RNA Purity and Integrity

The quality of RNA used in molecular studies greatly determines the outcome of the investigation. RNA is subject to rapid deterioration and as such if in poor quality may lead to false-negative results in amplification assays (Fleige & Pfaffl, 2006). Deterioration arises through exposure of samples to RNases in the environment and, for *post mortem* samples this is facilitated by poor handling, processing and storage of organs and tissues (Catts *et al.*, 2005; Perez-Novo *et al.*, 2005). The presence of molecules including melanin, heparin and haemoglobin also affect RNA purity and may inhibit downstream processes. This calls for proper assessment of RNA purity and integrity. This can be achieved by a range of techniques including nanodrop spectrophotometric measurement, gel electrophoresis, fluorescent-based dye quantification and amplification of housekeeping genes (Fleige & Pfaffl, 2006; Wieczorek *et al.*, 2012). In this thesis, nanodrop and housekeeping genes were used to assess RNA purity and integrity.

Due to the method of sampling utilized (snap trap/ poison/ predator (cat) kill), the time period between the death and collection of animals for this thesis could not be determined. Animals may have undergone considerable decay before collection and storage was carried out.

3.1.1. Nanodrop spectrophotometry

Nanodrop readings were taken immediately after RNA extraction from tissues (Appendi). Nucleic acid purity is estimated by calculating the ratio of the absorbance contributed by the nucleic acid to the absorbance contributed by the contaminants (A260/A280). The specific ratio for ‘pure’

RNA is debatable but most authorities accept a ratio of about 2 (Thermo Scientific, 2017; Wieczorek *et al.*, 2012).

3.1.2. Amplification of Housekeeping Genes

Housekeeping genes encode proteins necessary for basic cellular maintenance and are essential for cell survival (Mahadevappa & Warrington, 2003). Housekeeping genes are used as reference genes in PCR assays with the assumption that they are always expressed and as such should be easily detected in samples (Kozera & Rapacz, 2013). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a reference gene in this thesis. Gene-specific primers for the amplification of GAPDH were designed based on an alignment of sequences obtained from NCBI database (Figure 3.1).

Following the successful design of primers, GAPDH gene was successfully amplified from samples obtained from fresh and frozen carcasses. The amplicon size of the target DNA was 571bp (Figure 3.2).

a)

Species	258 * *** *** *** *** *** *** 276
Mus musculus (House mouse)	CAT CAC CAT CTT CCA GGA GCG AGA CCC
Rattus rattus (Black rat)	CAT CAC CAT CTT CCA GGA GCG AGA TCC
Meriones unguiculatus (Mongolian Gerbil)	CAT CAC CAT CTT CCA GGA GCG AGA TCC
Homo sapiens (Man)	CAT CAC CAT CTT CCA GGA GCG AGA TCC
Jaculus jaculus (Lesser Egyptian Jerboa)	CAT CTC CAT CTT CCA GGA GCG AGA CCC
Cavia porcellus (Guinea pig)	CAT CAC CAT CTT CCA GGA GCG AGA TCC
Equus caballus (Horse)	CAT CAC CAT CTT CCA GGA GCG AGA TCC
Sus scrofa (Pig)	CAT CAC CAT CTT CCA GGA GCG AGA TCC
Canis lupus (Dog)	CAT CTC CAT CTT CCA GGA GCG AGA TCC
Myotis brandtii (Bat)	CAT CTC CAT CTT CCA GGA GCG AGA TCC
Oryctolagus cuniculus (Rabbit)	CAT CAC CAT CTT CCA GGA GCG AGA TCC
GAPDH F	... ..C CAT CTT CCA GGA GCG AGA ...

b)

Species	810 * *** *** *** *** *** *** 828
Mus musculus (House mouse)	CAT CAA GAA GGT GGT GAA GCA GGC ATC
Rattus rattus (Black rat)	CAT CAA GAA GGT GGT GAA GCA GGC GGC
Meriones unguiculatus (Mongolian Gerbil)	CAT CAA GAA GGT GGT GAA GCA GGC ATC
Homo sapiens (Man)	CAT CAA GAA GGT GGT GAA GCA GGC GTC
Jaculus jaculus (Lesser Egyptian Jerboa)	CAT CAA GAA GGT GGT GAA GCA GGC GTC
Cavia porcellus (Guinea pig)	CAT CAA GAA GGT GGT GAA GCA GGC ATC
Equus caballus (Horse)	GAT CAA GAA GGT GGT GAA GCA GGC ATC
Sus scrofa (Pig)	CAT CAA GAA GGT GGT GAA GCA GGC GTC
Canis lupus (Dog)	CAT CAA GAA GGT GGT GAA GCA GGC ATC
Myotis brandtii (Bat)	TAT CAA GAA GGT GGT GAA GCA GGC ATC
Oryctolagus cuniculus (Rabbit)	CAT CAA GAA GGT GGT GAA GCA GGC ATC
GAPDH R	... ..A GAA GGT GGT GAA GCA GGC ...

Figure 3.1: Alignment of GAPDH sequences of some mammals obtained from the NCBI database showing the binding site of (a) Forward (GAPDH_F) and (b) Reverse (GAPDH_R) primers and their respective positions.

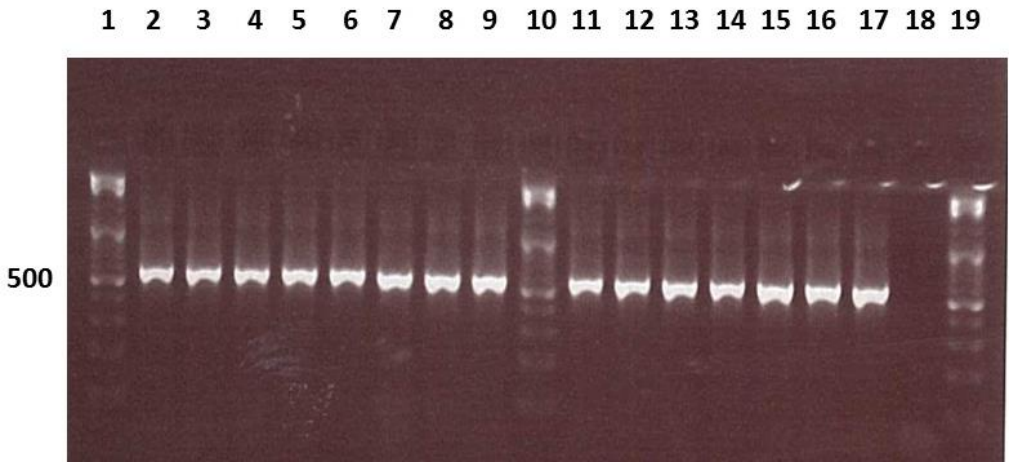


Figure 3.2: Gel electrophoresis of amplification products of RT-PCR assay for the detection of GAPDH in samples. Amplicon size of the target DNA was 571bp. Lane 1: DNA Ladder; Lanes 2-9: KD11 to KD18 (*M. musculus* rodents); Lane 10: DNA Ladder; Lanes 11 - 17: KD19 to KD25 (*M. musculus* rodents); Lane 18: Negative Control; Lane 19: DNA Ladder. GAPDH_F/GAPDH_R primers were used in the assay.

3.2. Species Identification

The animals used in this thesis were identified by trained veterinarians. This was further confirmed with the amplification and sequencing of a partial segment (419bp) of the cytochrome B gene (MT-CYB). MT-CYB is commonly used in phylogenetic studies due to its sequence variability in species of the same genus or family (Castresana, 2001; Clare *et al.*, 2011; Thinh *et al.*, 2010). Gene-specific primers for the amplification of a 419bp fragment of the *cytochrome b* gene were designed based on an alignment of the complete sequence of various mammals obtained from the NCBI database (Figure 3.3).

The fragment was then amplified (Figure 3.4) and analysed on a 2% gel. Samples with clear bands of the correct size were sent for sequencing. The sequences were confirmed after a BLAST search on the NCBI database. Thus *Cytochrome b* gene was amplified from samples obtained from fresh and frozen carcasses.

a)

Species	424 * *** * * * * * * *** *** * * ** 447
Homo sapiens (Human)	CAT TCT GAG GGG CCA CAG TAA TTA CAA ACT TAC T
Nomascus concolor (Black Crested Gibbon)	CTT TCT GAG GTG CCA CAG TAA TCA CAA ACC TAC T
Colobus guereza occidentalis (Black and White colobus)	CAT TTT GAG GGG CTA CAG TAA TTA CAA ACT TAC T
Mus musculus (House mouse)	CAT TCT GAG GCG CCA CAG TTA TTA CAA ACC TCC T
Sorex minutus (Pygmy shrew)	CAT TCT GAG GCG CAA CAG TTA TTA CCA ACC TGC T
Euphonia fulvicrissa (Fulvous-vented euphonia)	CAT TCT GAG GCG CCA CAG TAA TTA CAA ACC TCT T
Anas platyrhynchos (Wild duck)	CGT TCT GAG GAG CTA CCG TAA TTA CCA ACT TAT T
Panthera pardus orientalis (Amur leopard)	CCT TTT GAG GGG CAA CCG TAA TTA CCA ACC TCC T
NHPCyt_421F	... ... GAG GBC CYA CAG TWA TYA CAA AC. ... .

b)

Species	827 * *** *** ** * ** * *** 848
Homo sapiens (Human)	TTT CCT ATT CGC CTA CAC AAT TCT ACG ATC CG
Nomascus concolor (Black Crested Gibbon)	TTT TTT ATT CGC ATA CGC TAT CCT ACG ATC TG
Colobus guereza occidentalis (Black and White colobus)	TTT TCT ATT TGC ATA CGC AAT CTT ACG ATC CG
Mus musculus (House mouse)	TTT CCT ATT TGC ATA CGC CAT TCT ACG CTC AA
Sorex minutus (Pygmy shrew)	CTT TCT ATT CGC CTA CGC CAT TCT ACG ATC CA
Euphonia fulvicrissa (Fulvous-vented euphonia)	CTT CCT ATT TGC CTA CGC CAT CCT ACG ATC CA
Anas platyrhynchos (Wild duck)	CTT CCT ATT CGC CTA CGC CAT CCT GCG GTC AA
Panthera pardus orientalis (Amur leopard)	CTT CCT ATT TGC ATA CGC CAT CCT CCG ATC CA
NHPCyt_840R	... ..T ATT YGC ATA YGC CAT CCT RCG ... ..

Figure 3.3: Alignment of MT-CYB sequences of some animals obtained from the NCBI database showing the binding site of (a) Forward (Cyt_421F) and (b) Reverse (Cyt_840R) primers and their respective positions.

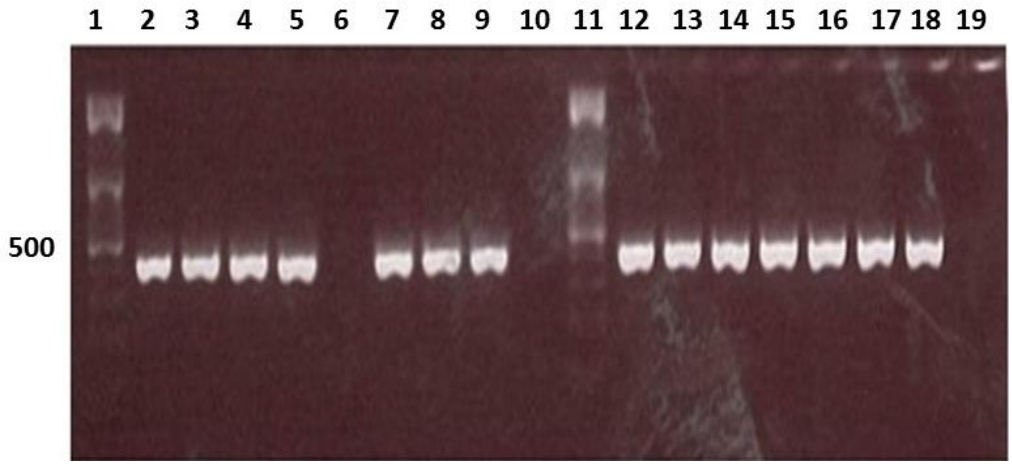


Figure 3.4: Gel electrophoresis of amplification products of RT-PCR assay screen for the *Cytochrome b* gene. Amplicon size of the target DNA was 419bp. Lane 1: DNA Ladder; Lanes 2-9: Samples KD 11 – 18 (*Mus musculus* rodents); Lane 10: Negative Control; Lane 11: DNA Ladder; Lanes 12 - 18: Samples KD 19 – 25 (*Mus musculus* rodents); Lane 19: Negative Control. Cyt_421F/ Cyt_840R primers were used in the assay.

4. Detection of Novel Filoviruses

4.1. Design and Construction of Plasmid Controls for Filoviruses.

To monitor RT-PCR efficiency, a positive control is required. A major problem with the screening of highly pathogenic microorganisms is the danger of infection associated with the use of viral genome as positive controls (Health and Safety Executive, 2016). Positive clinical samples may have inhibitors including haemoglobin and heparin which may affect the amplification process. Standardization of such controls is very important (Lee *et al.*, 2011b) (Minegishi *et al.*, 2014; Stocher *et al.*, 2003). Synthetic plasmids can be used as suitable controls in PCR assays. Such plasmids are designed to contain the DNA fragment of interest. Advantages of synthetic plasmids include ease of construction, stability and replication with *Escherichia coli*. There are successful reports on the use of plasmids as controls in a variety of PCR methods (Brod *et al.*, 2013; Corman *et al.*, 2013; Minegishi *et al.*, 2014; Niwa *et al.*, 2007). Synthetic plasmid controls were constructed for RT-PCR in this thesis.

The plasmid controls were constructed from the backbone of colicin E9, a bacterial nonspecific endonuclease (Wallis *et al.*, 1992). A 240bp fragment of colicin E9 was selected for amplification. Three sets of chimeric primers, were designed so their 3' ends were complementary to the E9 plasmid while the 5' end had sequences complementary to the filoviruses Lloviu cuevavirus (LIOSF/LIOSR), Reston ebolavirus (RSF/RSR) and Marburg Marburgvirus (LVSF/LVSR). The filovirus sequences were chosen based on their positions in a phylogenetic tree based on the polymerase gene (Figure 4.1). Amplification of the E9 plasmid would result in products containing a 240bp fragment of the E9 plasmid flanked between 40 – 43 bases of the respective filovirus primer sequences.

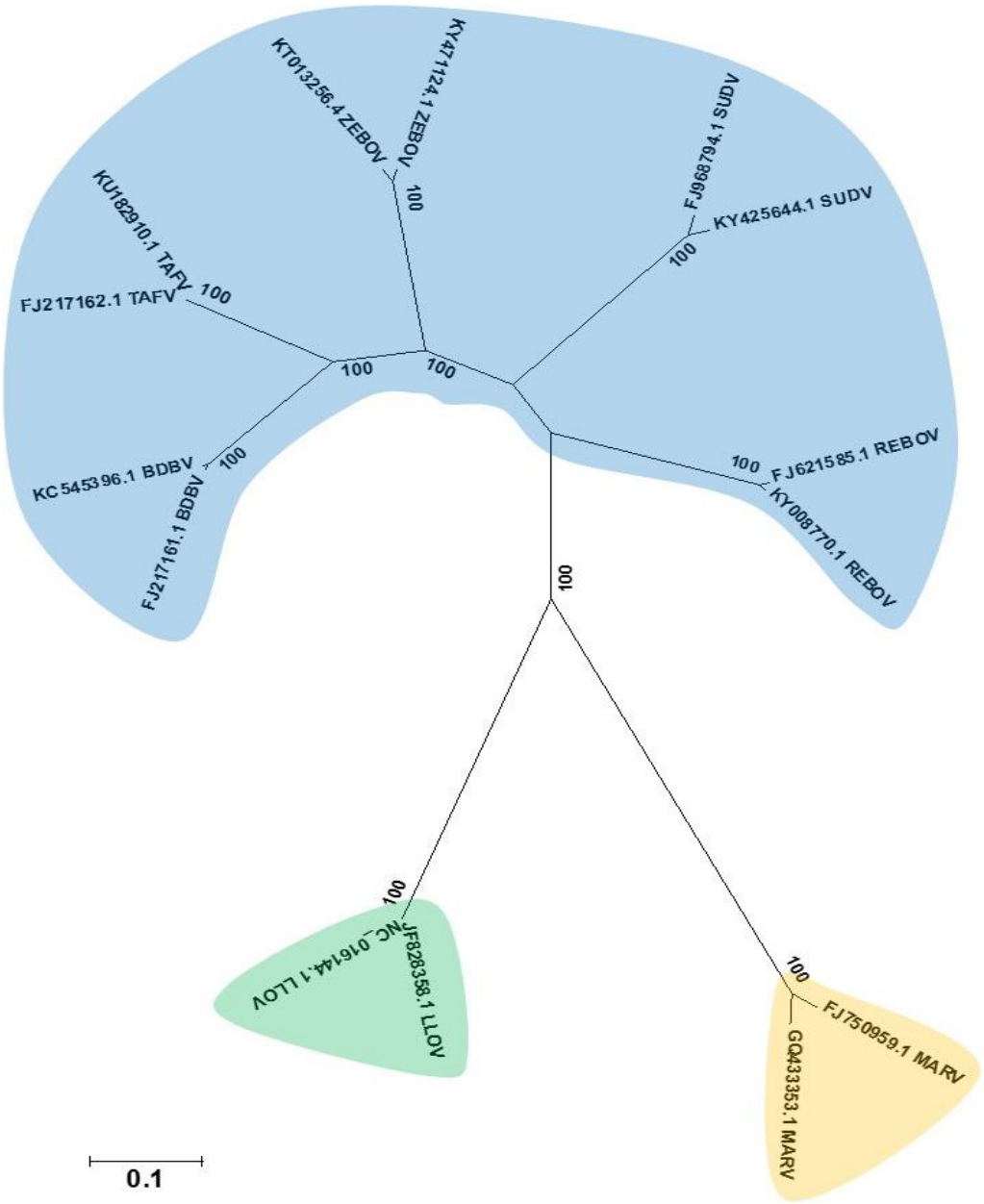


Figure 4.1: Unrooted phylogenetic tree showing relationships among filoviruses. The tree was computed with complete L gene fragment using the Maximum Likelihood method. Reference sequences are indicated by their Genbank Accession Numbers. Coloured regions represent the following groups: Blue – ebolaviruses (Bundibugyo ebolavirus (BDBV), Reston ebolavirus (REBOV), Sudan ebolavirus, Tai Forest ebolavirus (TAFV), Zaire ebolavirus (ZEBOV)); Green – Lloviu cuevaviruses (LLOV) and Yellow – Marburg marburgviruses (MARV). Branch lengths are drawn to scale: the bar indicates 0.1 nucleotide substitutions per site. Numbers above individual branches indicate the percentage that that branch was found in 1000 bootstrap replicates; only percentages >75% are shown

The 240bp fragment of the E9 plasmid was amplified using the chimeric primers (Figure 4.2). Amplicons were then ligated into pGEM®T Easy plasmid vector and used to transform competent *Escherichia coli* cells. Transformed cells were confirmed by colony RT-PCR. Positive control plasmids were extracted from cells and purified before storage at -20°C.

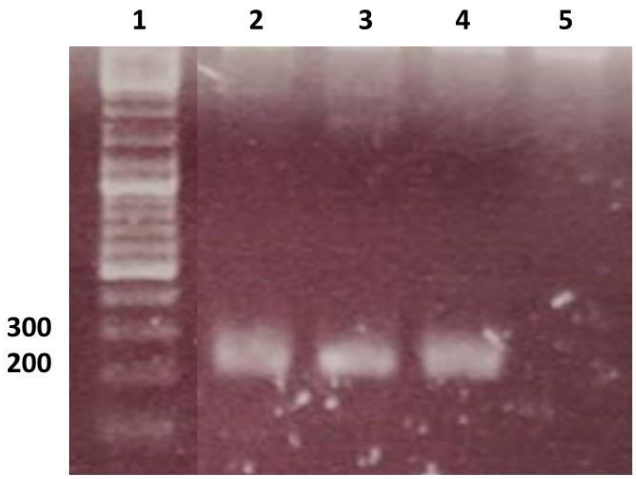


Figure 4.2: Gel electrophoresis of amplification products (240bp) of RT-PCR assay for E9 plasmid. Lane 1: DNA Ladder; Lanes 2-4: E9 plasmid using LIOSF/LIOSR, LVSF/LVSR, RSF/RSR primer sets respectively; Lane 5: Negative control.

4.2. Design of Gene-specific and Broadly-Reactive Filovirus Primers

With the construction of synthetic positive controls representing each cluster on the phylogenic tree, gene-specific primer sets representing Lloviu cuevavirus, Reston ebolavirus and Marburg Marburgvirus were designed. A set of pan-filovirus degenerate primers were also designed.

While gene-specific primers only have complementarity with the specific virus which they were designed for, an ideal broadly-reactive primer will bind to all the virus species at conserved sites shared across the group (Tsoleridis *et al.*, 2016; Vieth *et al.*, 2007). An alignment of L-gene of seven of the currently recognised filoviruses was carried out to determine conserved sites suitable for primer binding. The L-gene was chosen for

alignment and primer design due to the conserved amino acid motifs shared within virus families (Vieth *et al.*, 2007).

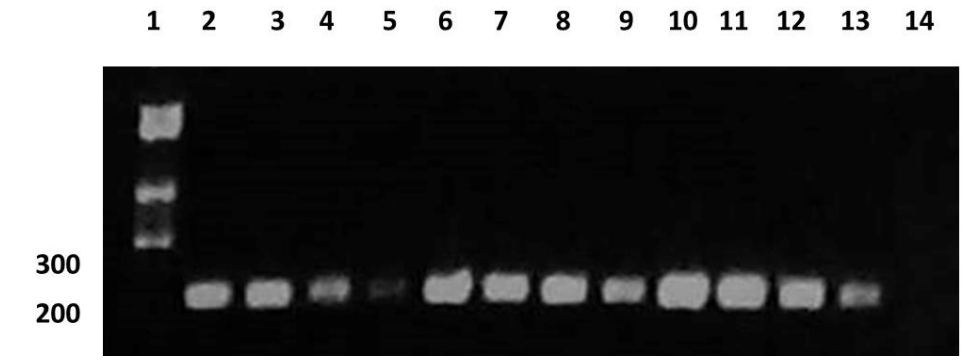
The sites chosen had sequences that were complementary to the sequences at the 5' end of the chimeric primers used in the construction of the plasmid controls. In this thesis, all sites of complete degeneracy were replaced with inosine. Three sets of gene-specific primers (LIO_F1/LIO_R1, LVM_F1/LVM_R1 and RES_F1/RES_R1) and one set of broadly-reactive primers (FILO_F1/FILO_R1) were designed. The gene-specific primers were designed with no inosines in them.

4.3. Amplification of Positive Controls

The sensitivity of the primer set was determined by the amplification of various concentrations of plasmid controls using the gene-specific and broadly reactive primers respectively. Concentrations used in the assay were 10^3, 10^2, 10^1 and 10^0 copies/µL. Amplification of positive controls at the four concentrations with gene-specific primers was first conducted using RT-PCR. The gene-specific primers successfully amplified the plasmid controls at all four concentrations (Figure 4.3).

The next step involved the amplification of the plasmid controls with the degenerate FILO_F1/FILO_R1 primer pair. The FILO_F1/FILO_R1 primer pair successfully amplified all concentrations of Lloviu cuevavirus plasmid control and Marburg Marburgvirus plasmid control. The FILO_F1/FILO_R1 primer pair did not amplify the Reston plasmid control at 10^0 copies/µL (Figure 4.3).

a)



b)

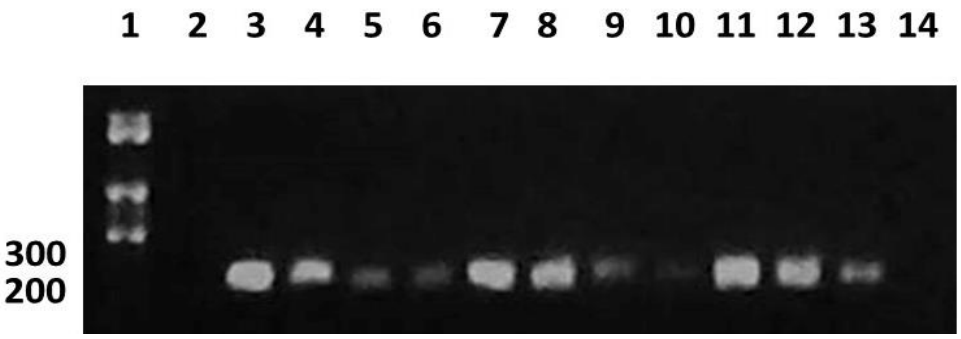


Figure 4.3: Gel electrophoresis of amplification products of RT-PCR assay for filovirus plasmid controls (a) Using the gene-specific primers. Lane 1: DNA Ladder; Lanes 2-5: Lloviu cuevavirus control (10^3, 10^2, 10^1, 10^0 copies/µL) using LIOSF/LIOSR; Lanes 6-9: Marburg Marburgvirus control (10^3, 10^2, 10^1, 10^0 copies/µL) using LVSF/LVSR; Lanes 10-13: Reston ebolavirus control (10^3, 10^2, 10^1, 10^0 copies/µL) using RSF/RSR. (b) Using broadly-reactive FILO_F1/FILO_R1 primer pair. Lane 1: DNA Ladder; Lane 2: Negative control; Lanes 3-6: Lloviu cuevavirus control (10^3, 10^2, 10^1, 10^0 copies/µL); Lanes 7-10: Marburg Marburgvirus control (10^3, 10^2, 10^1, 10^0 copies/µL); Lanes 11-14: Reston ebolavirus control (10^3, 10^2, 10^1, 10^0 copies/µL).

4.4. Screening of Samples for Novel Filoviruses

Following the successful amplification of plasmid controls at various concentrations with both gene-specific and degenerate primers, a RT-PCR assay was then carried out to screen cDNA obtained from the moles, shrews and rodents for the presence of filoviruses using FILO_F1/FILO_R1 primer pair.

The assay was carried out under the same conditions as with the amplification of the positive controls. Amplified products generated were shown not to have resulted from amplification of filovirus nucleic acids based on their band size and the result obtained from sequencing of PCR products. All cDNA screened were therefore negative for the presence of filoviruses. These included samples donated by Dr Rachael Tarlinton (sampled between 2006 and 2008) (Table 4.1), samples collected by the doctoral student between 2013 and 2015 (Table 4.2) and samples obtained from Poland and Egypt. The samples donated by Public Health England were not available at the time when the filovirus screening assay was conducted.

Table 4.1: Screening of Rodent samples Donated by Dr Rachael Tarlinton Obtained Between 2006 and 2008 for Filovirus Genome in the Present Study

Species	**Total**	**No Positive/ No Tested**	**Prevalence (%)**
M. musculus	21	0/21	0
R. norvegicus	7	0/7	0
M. agrestis	11	0/11	0
A. sylvaticus	6	0/6	0
S. minutus	3	0/3	0
Total	**48**	**0/48**	

Table 4.2: Screening of Animal samples Obtained Between 2013 and 2015 for Filovirus Genome in the Present Study

Species	**Total**	**No Positive/ No Tested**	**Prevalence (%)**
M. musculus	399	0/399	0
R. norvegicus	89	0/89	0
A. sylvaticus	3	0/3	0
S. minutus	1	0/1	0
Mole*	5	0/5	0
Duckling*	3	0/3	0
Finch*	1	0/1	0
Total	**501**	**0/501**	

* The information presented on the animal was insufficient to allow for proper identification.

5. Detection of Novel Arenaviruses

The samples obtained from moles, rodents and shrews were also screened for the presence of arenaviruses. To ensure an efficient RT-PCR assay was used, synthetic positive controls were constructed.

5.1. Design and Construction of Plasmid Controls for Arenaviruses.

The plasmid controls were constructed from the backbone of colicin E9. A 240bp fragment of colicin E9 was selected for amplification. Ten sets of chimeric primers were designed so their 3' ends were complementary to the E9 plasmid while the 5' end had sequences complementary to the arenaviruses LCMV (LCSF/LCSR), LUJV (LJSF/LJSR), IPPYV (IPSF/IPSR), LASV (LASVF/LASVR), OLVV (OLSF/OLSR), GTOV (GTSF/GTSR), PICV (PCSF/PCSR), CASV (CVSF/CVSR), GGV (GGSF/GGSR) and UHV (UHSF/UHSR).

The arenavirus sequences were selected based on their position in a polymerase gene phylogenetic tree (Figure 5.1). Amplification of the E9 plasmid would result in products containing 240bp fragment of the E9 plasmid flanked between 20 bases of the respective arenavirus sequences.

With the successful design of the primers, the 240bp fragment of the E9 plasmid was amplified (Figure 5.2). Successful amplicons were then ligated into pGEM®T Easy plasmid vector and used to transform competent *Escherichia coli* cells. Transformed cells were confirmed by colony RT-PCR. Positive control plasmids were extracted from cells and purified before storage at -20°C.

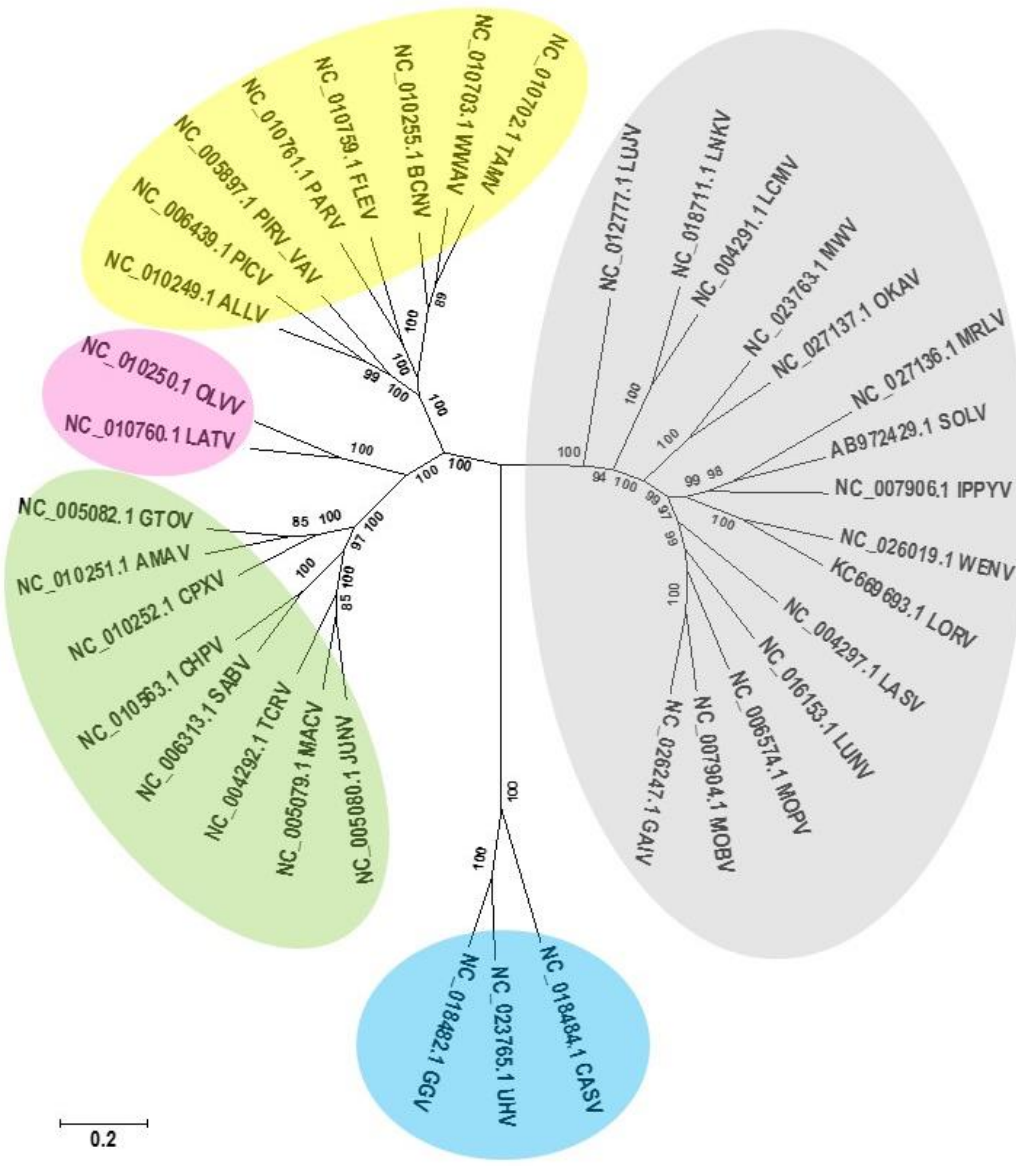


Figure 5.1: Unrooted phylogenetic tree showing relationships among arenaviruses. The tree was computed with complete L gene fragment using the Maximum Likelihood method. Reference sequences are indicated by their Genbank Accession Numbers. Individual arenaviruses are written as acronyms of full names in Table 1.3, Table 1.4 and Table 1.5. Coloured oval regions represent the following: Blue – Reptarenaviruses, Grey – Old World mammarenaviruses, Yellow – Clade A New World mammarenaviruses, Green – Clade B New World mammarenaviruses, Pink – Clade C New World mammarenaviruses. Branch lengths are drawn to scale: the bar indicates 0.1 nucleotide substitutions per site. Numbers above individual branches indicate the percentage that that branch was found in 1000 bootstrap replicates; only percentages >75% are shown.

5.2. Design of Gene-specific and Broadly-Reactive Arenavirus Primers

Having achieved the successful construction of synthetic positive controls representing each cluster on the phylogenic tree, ten sets of gene-specific primer sets representing Lassa mammarenavirus, Lymphocytic choriomeningitis mammarenavirus, Lujo mammarenavirus, Ippy mammarenavirus, Pichinde mammarenavirus, Guanarito mammarenavirus, Oliveros mammarenavirus, Alethinophid 1 reptarenavirus, Alethinophid 2 reptarenavirus and Alethinophid 3 reptarenavirus were designed.

The recently discovered reptarenaviruses are a highly divergent species of arenaviruses. As a result of this, two sets of broadly-reactive degenerate primers were made. The first set of primers (MAMF/MAMR) were constructed based on an alignment of L-gene sequences from twenty six of the currently recognised mammarenaviruses while the second set of primers (REPF/REPR) were constructed based on an alignment of L-gene sequences from three of the currently recognised reptarenaviruses. These alignments revealed conserved sites suitable for primer binding. The sites chosen had sequences that were complementary to the sequences at the 5’ end of the chimeric primers used in the construction of the plasmid controls (Figure 5.3, Figure 5.4 and Figure 5.5).

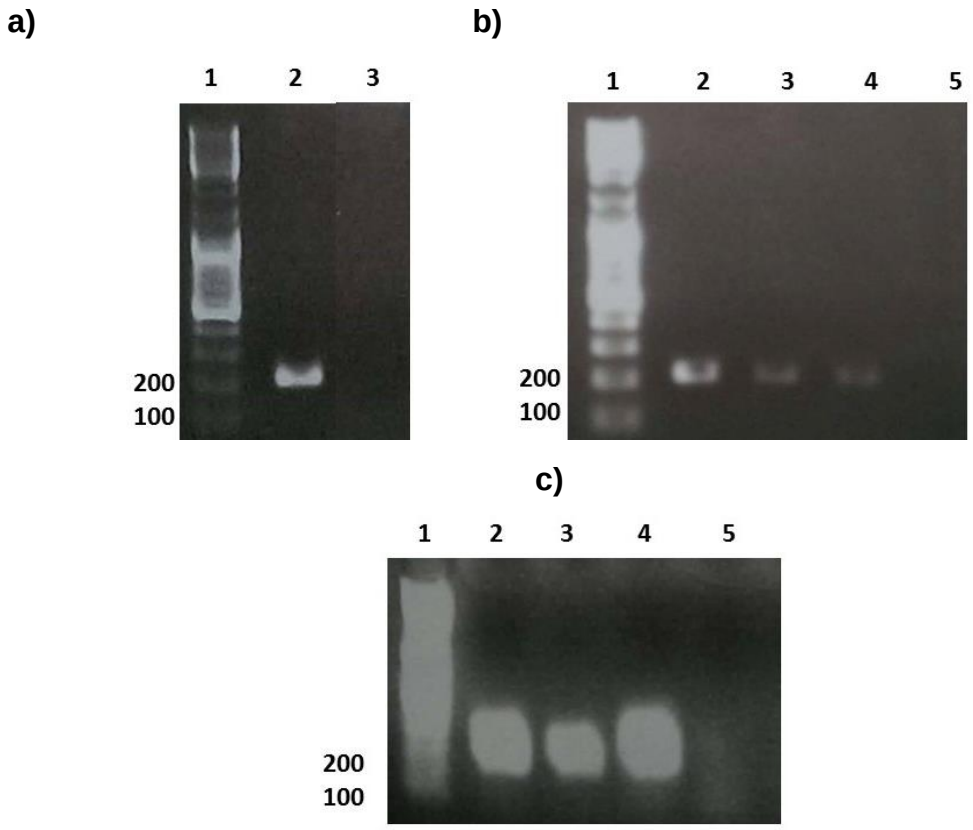


Figure 5.2: Gel electrophoresis of amplification products (240bp) of RT-PCR assay for E9 plasmid. a) Lane 1: DNA Ladder; Lane 2: E9 plasmid using LASVF1/LASVR1 primer set; Lane 3: Negative control. b) Lane 1: DNA Ladder; Lanes 2-4: E9 plasmid using IPSF/IPSR, LJSF/LJSR, LCSF/LCSR primer sets respectively; Lane 5: Negative Control. c) Lane 1: DNA Ladder; Lanes 2-4: E9 plasmid using CVSF/CVSR, GGSF/GGSR, UHSF/UHSR primer sets respectively; Lane 5: Negative Control.

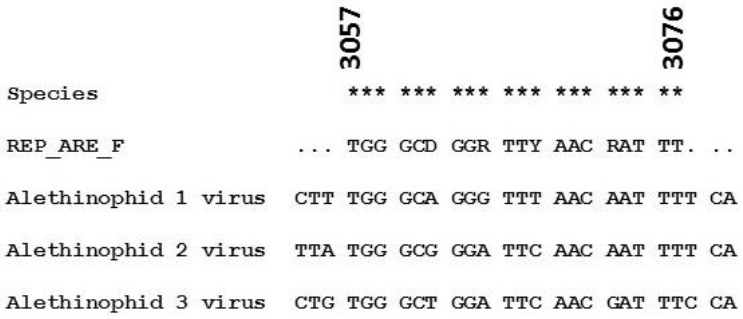
Species	4126 *** *** *** *** *** *** ** 4145
MAM_ARENA_F	... ... GGI CAR GGI ATH YTN CAH AA. ... .
Allpahuayo mammarenavirus	GAC ATG GGT CAA GGG ATT CTA CAC AAT TTG T
Amapari mammarenavirus	GAC ATG GGG CAG GGA ATA CTT CAC AAC CTC T
Bear Canyon mammarenavirus	GAC ATG GGA CAG GGA ATT TTG CAT AAT ACC T
Cupixi mammarenavirus	GAT ATG GGA CAA GGG ATA CTC CAT AAT TTA T
Guanarito mammarenavirus	GAT ATG GGA CAA GGC ATA CTT CAT AAT TCA T
Oliveros mammarenavirus	GAT ATG GGT CAA GGC ATT CTT CAC AAC ACT T
Pichinde mammarenavirus	GAT ATG GGT CAA GGC ATT TTG CAC AAT ACC T
Lymphocytic choriomeningitis mammarenavirus	GAC ATG GGA CAG GGA ATC TTA CAC AAT GCC T
Chapare mammarenavirus	GAT ATG GGA CAA GGA ATT TTA CAC AAC TTA T
Flexal mammarenavirus	GAT ATG GGG CAA GGA ATT TTG CAT AAT GTG T
Junin mammarenavirus	GAC ATG GGC CAG GGA ATC TTG CAC AAC ACA T
Latino mammarenavirus	GAC ATG GGC CAG GGG ATT CTT CAT AAT ATG T
Machupo mammarenavirus	GAC ATG GGG CAA GGG ATA CTA CAT AAC ACT T
Parana mammarenavirus	GAC ATG GGA CAA GGG ATT CTG CAT AAT GTC T
Pirital mammarenavirus	GAT ATG GGA CAA GGA ATA CTA CAC AAT ATG T
Sabia mammarenavirus	GAC ATG GGC CAA GGC ATC CTA CAC AAT TTG T
Tamiami mammarenavirus	GAT ATG GGG CAA GGA ATC CTA CAC AAT GCT T
Tacaribe mammarenavirus	GAT ATG GGC CAA GGC ATC CTG CAT AAC ATG T
Whitewater Arroyo mammarenavirus	GAT ATG GGT CAG GGA ATT TTA CAC AAC ACA T
Ippy mammarenavirus	GAT ATG GGG CAG GGA ATC CTG CAC AAC ACA T
Lassa mammarenavirus	GAC ATG GGT CAG GGA ATA TTA CAT AAC ACA T
Lujo mammarenavirus	GAC ATG GGT CAG GTA ATA TTG CAT AAC GCA T
Lunk mammarenavirus	GAT ATG GGT CAA GCA ATC CTA CAT AAT GCC T
Merino walk mammarenavirus	GAT ATG GGG CAA GTA ATC CTG CAC AAC ACA T
Mobala mammarenavirus	GAT ATG GGT CAA GAA ATT CTG CAA AAT ACA T
Mopeia mammarenavirus	GAC ATG GGG CAA GGA ATC TTG CAC AAT GCT T

Figure 5.3: Alignment of L-gene sequences of Old and New World mammarenaviruses showing the binding site of Forward MAMF primer and its position. The inosines (denoted by the letter 'I') compensate for the complete degeneracies in the 5' region.

Species	4363 * *** *** *** *** *** *** 4382 *
MAM_ARENA_R	... ..A ART TYR TIA GYC CIA ARA S.. ...
Allphahuayo mammarenavirus	TTA ATA AAT TTG TAA GTC CGA AGA CTG TTG
Amapari mammarenavirus	TAA ACA AAT TTG TAA GTC CCA AGA CTG TTT
Bear Canyon mammarenavirus	TCA ACA AAT TTG TAA GCC CCA AAA CAG TGG
Cupixi mammarenavirus	TCA ATA AGT TTG TAA GCC CCA AGA CTG TGT
Guanarito mammarenavirus	TCA ACA AAT TTG TGA GCC CCA AGA CTG TTT
Oliveros mammarenavirus	TCA ATA AAT TTG TAA GCC CTA AAA CAG TAA
Pichinde mammarenavirus	TCA ATA AAT TTG TGA GTC CTA AAA CAG TTG
Lymphocytic choriomeningitis mammarenavirus	TGA ATA AGT TCA TCA GTC CGA AAA GTG TGG
Chapare mammarenavirus	TGA ACA AAT TTG TCA GTC CCA AAA GTG TTG
Flexal mammarenavirus	TAA ACA AGT TTG TGA GTC CTA AGA CAG TGG
Junin mammarenavirus	TAA ACA AGT TTA TCA GCC CAA AGA GTG TGA
Latino mammarenavirus	TTA ACA AAT TTG TCA GTC CAA AAA CAG TTG
Machupo mammarenavirus	TGA ATA AGT TTG TCA GTC CCA AGA GTG TCA
Parana mammarenavirus	TCA ATA AGT TCA TTA GTC CAA AGA CTG TTG
Pirital mammarenavirus	TGA ATA AAT TCG TCA GTC CTA AAA CAG TAG
Sabia mammarenavirus	TGA ACA AAT TTG TAA GTC CAA AGA GTG TGG
Tamiami mammarenavirus	TTA ATA AAT TTA TCA GCC CCA AAA CAG TTG
Tacaribe mammarenavirus	TGA ACA AAT TTG TTA GCC CCA AGA GTG TAG
Whitewater Arroyo mammarenavirus	TGA ATA AGT TTG TTA GCC CCA AAA CTG TTG
Ippy mammarenavirus	TAA ACA AGT TTA TAA GCC CAA AAA GTG TTG
Lassa mammarenavirus	TCA ATA AAT TTG TGA GTC CTA AAA GTG TTA
Lujo mammarenavirus	TTA ATA AGT TTG TCA GTC CCA AAA GCT CGC
Lunk mammarenavirus	TGA ACA AAT TTG TGA GTC CAA AGA GTG TGG
Merino walk mammarenavirus	TCA ACA AAT TTG TTA GTC CTA AGA GTT CTA
Mobala mammarenavirus	TGA ACA AAT TTA TCA GCC CTA AAA GTG TGA
Mopeia mammarenavirus	TGA ATA AAT TCA TCA GTC CAA AGA GTG TTA

Figure 5.4: Alignment of L-gene sequences of Old and New World mammarenaviruses showing the binding site of Reverse MAMR primers and its positions. The inosines (denoted by the letter 'I') compensate for the complete degeneracies in the 5' region.

a)



b)

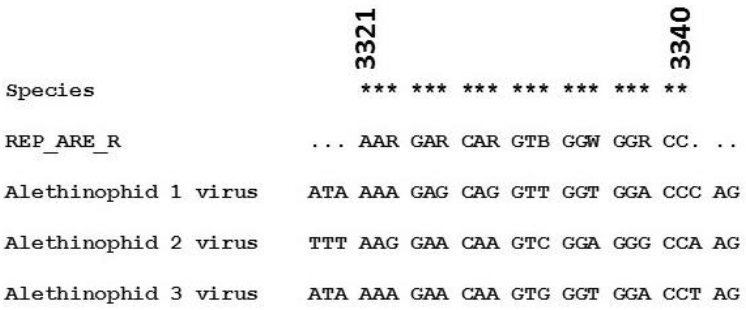


Figure 5.5: Alignment of L-gene sequences of reptarenaviruses showing the binding site of (a) Forward (REPF) and (b) Reverse (REPR) primers and their respective positions.

5.3. Amplification of Positive Controls

The broadly-reactive degenerate arenavirus primers were able to amplify the Lassa mammarenavirus, Lymphocytic choriomeningitis mammarenavirus, Lujo mammarenavirus, Ippy mammarenavirus, Alethinophid 1 reptarenavirus, Alethinophid 2 reptarenavirus and Alethinophid 3 reptarenavirus plasmid controls (Figure 5.6, Figure 5.7 and Figure 5.8).

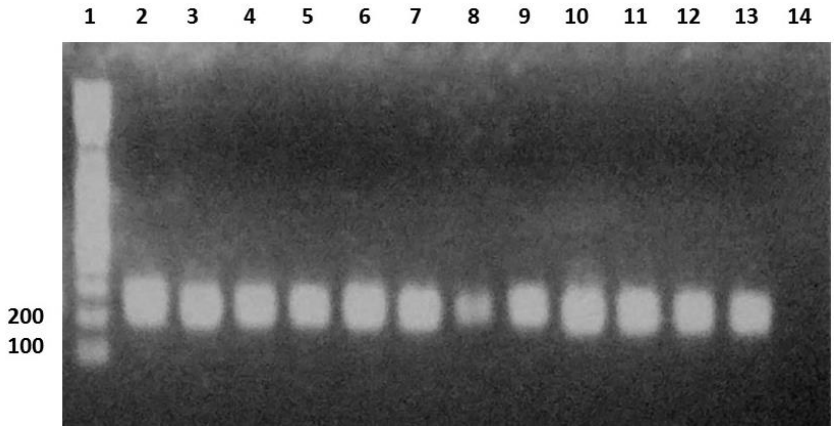


Figure 5.6: Gel electrophoresis of amplification products of RT-PCR assay for reptarenavirus plasmid controls using REPF/REPR primer set. a) Lane 1: DNA Ladder; Lanes 2-5: Alethinophid 1 reptarenavirus plasmid control (10^3, 10^2, 10^1, 10^0 copies/µL); Lane 6-9: Alethinophid 2 reptarenavirus plasmid control (10^3, 10^2, 10^1, 10^0 copies/µL); Lane 10-13: Alethinophid 3 reptarenavirus plasmid control (10^3, 10^2, 10^1, 10^0 copies/µL). Lane 14: DNA Ladder.

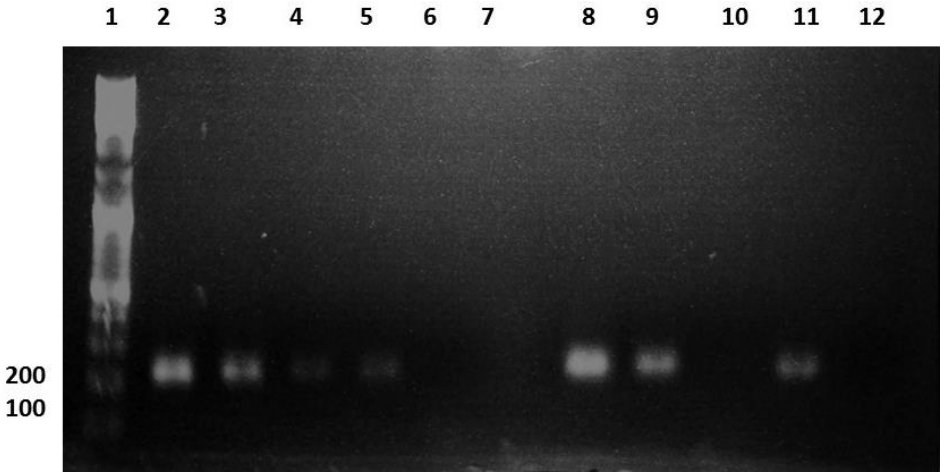


Figure 5.7: Gel electrophoresis of amplification products of RT-PCR assay for mammarenavirus plasmid controls using MAMF/MAMR primer set. Lane 1: DNA Ladder; Lanes 2-5: Ippy mammarenavirus plasmid control (10^3, 10^2, 10^1, 10^0 copies/µL); Lane 6: Negative Control; Lanes 7-10: Lassa mammarenavirus plasmid control (10^3, 10^2, 10^1, 10^0 copies/µL).

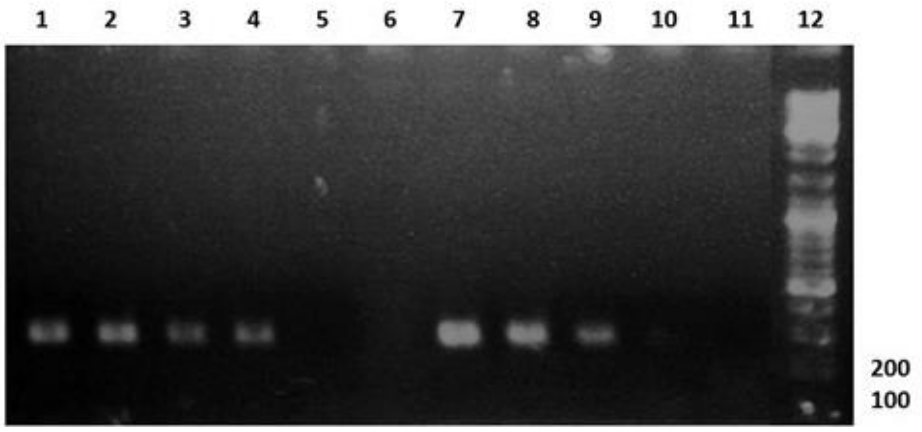


Figure 5.8: Gel electrophoresis of amplification products of RT-PCR assay for mammarenavirus plasmid controls using MAMF/MAMR primer set. Lane 1-4: Lymphocytic choriomeningitis mammarenavirus plasmid control (10^3, 10^2, 10^1, 10^0 copies/µL). Lane 5: Negative Control; Lanes 7-10: LUJV plasmid control; Lane 11: Negative Control; Lane 12: DNA Ladder

5.4. Screening of Samples for Novel Arenaviruses

The cDNA samples previously screened for the presence of filovirus nucleic acids were also screened for arenavirus RNA. The RT-PCR assay was carried out under the same conditions as with the amplification of the positive controls using the in-house broadly-reactive primers (MAMF/MAMR) and a set of four published primers designed for the detection of novel arenaviruses (Vieth *et al.*, 2007). The full sequences of the primers (LVL_3359D_Y+, LVL_3754A_R-, LVL_3359G_Y+ and LVL_3754D_R-) are listed in Appendix 6.

Four samples (SB15, SB16, SB18 and SB35) were positive for LCMV (Figure 5.9). These were from *M. musculus* rodents (Table 5.1) and were of the set of samples obtained from Twycross zoo by Dr Rachael Tarlinton (sampled between 2006 and 2008). These samples were

detected using the in-house broadly-reactive primers (MAMF/MAMR) and the published primers designed designed by Vieth and colleagues in 2007 (Vieth *et al.*, 2007)

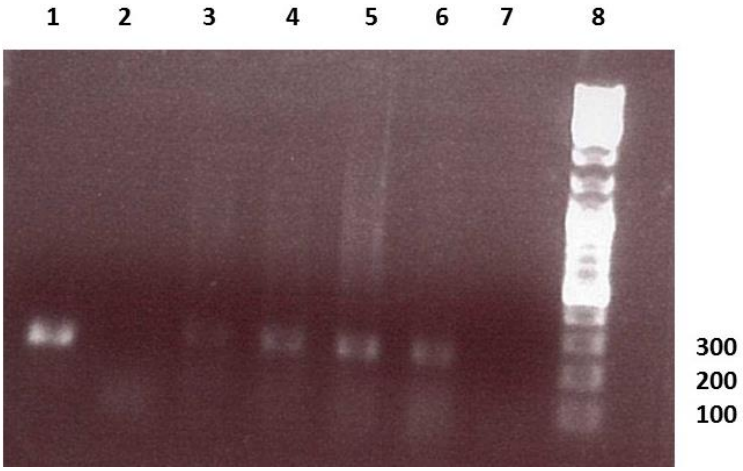


Figure 5.9: Gel electrophoresis of amplification products of RT-PCR assay for the detection of novel arenaviruses using the MAMF/MAMR primer pair. Lane 1: Positive Plasmid Control; Lane 2: Negative Control; Lane 3-6: SB15 (*M. musculus*), SB16 (*M. musculus*), SB18 (*M. musculus*), SB35 (*M. musculus*); Lane 8: DNA Ladder.

All other cDNA screened were negative for the presence of arenavirus genome. These included samples collected by the doctoral student between 2013 and 2015 (Table 5.2) and samples obtained from Poland and Egypt. The samples donated by Public Health England were not available at the time when the arenavirus screening assay was conducted.

Table 5.1: Screening of Rodent samples Donated by Dr Rachael Tarlinton Obtained Between 2006 and 2008 for Arenavirus Genome in the Present Study

Species	**Total**	**No Positive/ No Tested**	**Prevalence (%)**
M. musculus	21	4/21	19
R. norvegicus	7	0/7	0
M. agrestis	11	0/11	0
A. sylvaticus	6	0/6	0
S. minutus	3	0/3	0
Total	**48**	**0/48**	

Table 5.2: Screening of Animal samples Obtained Between 2013 and 2015 for Arenavirus Genome in the Present Study

Species	**Total**	**No Positive/ No Tested**	**Prevalence (%)**
M. musculus	399	0/399	0
R. norvegicus	89	0/89	0
A. sylvaticus	3	0/3	0
S. minutus	1	0/1	0
Mole*	5	0/5	0
Ducking*	3	0/3	0
Finch*	1	0/1	0
Total	**501**	**0/501**	

* The information presented on the animal was insufficient to allow for proper identification.

6. Molecular Epidemiology of an Outbreak of LCMV in the United Kingdom

6.1. Description of the Outbreak

The LCMV outbreak occurred between 2005 and 2006 in Twycross zoo, Warwickshire, United Kingdom. The actual number of deaths among NHPs from CH was not available but included at least two pygmy marmosets, one Northern white-cheeked gibbon, one black and white colobus and one purple-faced langur. Mice were not included in the diet fed to the NHPs in accordance with present zoo regulations and advice (Montali *et al.*, 1995; Montali *et al.*, 1993). To minimise contacts between captive animals with rodent material, traps were placed outside of the zoo enclosures.

Various animals were collected during and after the outbreak. Histopathology procedures were carried out by Twycross zoo on some of the animals. A group of animals were also screened for LCMV infection by Public Health England (PHE) using a combination of serology and polymerase chain reaction assays. Information on the precise techniques used by Public Health England were unavailable at the time of writing this report.

The animals collected after the outbreak were sampled between 2013 and 2015. Samples used for the present study were sourced either directly from the zoo or from Public Health England. Not all of the animals initially screened by Public Health England were available for re-screening in this thesis. The animals sampled in the present study were screened solely by polymerase reaction assays.

6.2. Histopathology

Histopathology was conducted on various animals (including reptiles and birds) by a separate group. No examination was carried out on *Mus musculus* rodents. The full necropsy reports for the animals examined are located in the Appendix 12.

6.3. Serology

Serological assays were carried out by Public Health England (PHE) on plasma, serum and post-mortem tissue (Table 6.1). All serological tests were conducted only on samples collected during the outbreak period and not on samples collected between 2013 and 2015. The results of the serological screening on rodents were not available at the time of writing this thesis. The overall seroprevalence of LCMV in NHPs was 19% (18/121). *C. didactylus* had the highest seroprevalence (100%) although the sample size for this species was very low (n = 2). Other NHPs that had detectable levels of LCMV antigen or antibody were *C. geoffroyi* (25%), *P. troglodytes* (83%), *G. gorilla gorilla* (12%), *C. guereza occidentalis* (14%), *A. niviridus* (33%) and *Ateles sp.* (25%). Screening results for one *C. geoffroyi* and *G. gorilla gorilla* were equivocal. These were not included in calculation of prevalence value. Similarly, unidentified bat species were negative for LCMV antigens or anti-LCMV antibodies while screening results for *M. memphitis*, *P. pardus orientalis* and *C. perspicillata* were unavailable. These were not screened for LCMV antigens or anti-LCMV antibodies. Serological assays were not conducted in the present study.

6.4. Amplification of LCMV Viral Genome

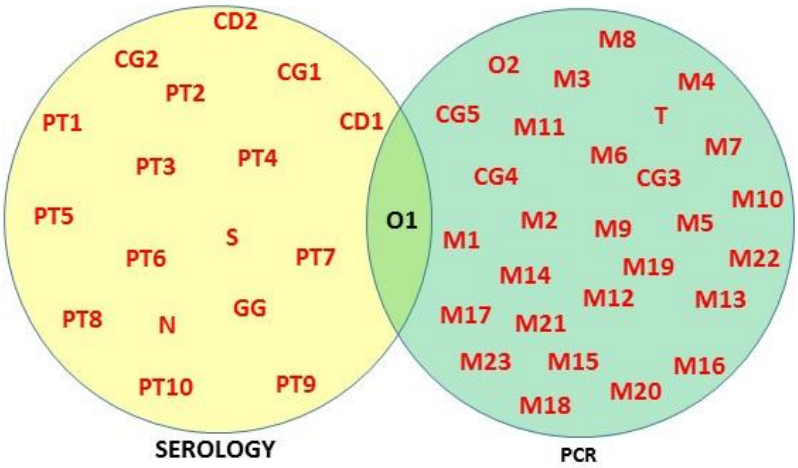
Two separate RT-PCR assays were conducted by separate groups to screen samples for the presence of LCMV genome. The first assays

were conducted by Public Health England. The second series assays were carried out independently in the present study.

6.4.1. LCMV Genome Screening by Public Health England

Specific information on the primers and protocol used by Public Health England was unavailable as at the time of writing this report. The results are shown in Table 6.1. LCMV genome was detected in 29 animals tested. Of the 47 rodents screened, 23 *M. musculus* alone were positive for LCMV infection. The overall LCMV prevalence in rodents was 49% (23/47). The prevalence of LCMV in *M. musculus* was 56% (23/41).

Not all results from the molecular screening of the 124 NHPs by PHE were available. Six NHPs were positive for LCMV infection (Overall prevalence of 10%). These were *C. geoffroyi* (38%), *C. guereza occidentalis* (29%) and *T. vetulus monticola* (33%). Four bats, 1 skunk and 1 amur leopard were also screened. These were all negative for LCMV infection. Not all samples screened with the serological assay were available for screening by PCR and vice versa. Of all animals screened, 1 *C. guereza occidentalis* was confirmed positive by both assays (Figure 6.1).



KEY

PHE ID Number	Designation	PHE ID Number	Designation
H06 276 0592 02	CG1	H06 266 0587 01	M1
H06 276 0593 01	CG2	H06 266 0588 02	M2
H06 094 0752	CG3	H06 266 0589 02	M3
H06 094 0753	CG4	H06 266 0590 03	M4
H06 336 0621 01	CG5	H07 196 0678 01	M5
H06 230 0544 01	CD1	H07 196 0676 01	M6
H06 230 0545 01	CD2	H07 196 0677 01	M7
H06 230 0551 01	PT1	H07 196 0674 01	M8
H06 230 0555 01	PT2	H07 196 0675 01	M9
H06 230 0556 01	PT3	H07 196 0637 01	M10
H07 196 0626 01	PT4	H07 196 0638 01	M11
H07 196 0628 01	PT5	H07 196 0641 01	M12
H07 196 0629 01	PT6	H07 196 0648 01	M13
H07 196 0630 01	PT7	H07 196 0649 01	M14
H07 196 0631 01	PT8	H07 196 0651 01	M15
H07 196 0632 01	PT9	H07 196 0652 01	M16
H07 196 0633 01	PT10	H07 196 0658 01	M17
H07 196 0592 01	GG	H07 196 0661 01	M18
H06 230 0553 01	O1	H07 196 0662 01	M19
H06 336 0625 01	O2	H07 196 0663 01	M20
H07 196 0608 01	N	H07 196 0664 01	M21
H07 196 0623 01	S	H07 196 0665 01	M22
H06 336 0622 01	T	H07 196 0679 01	M23

Figure 6.1: Venn diagram comparing positive results from serology and PCR by Public Health England. Specimen screened with serological assays are in yellow circle while specimen screened with PCR are in green circle. Specimen tested by PCR and not serology and vice versa are written in red. Specimen shown to be positive by both assays are written in black. Acronyms represent the following: CG – *C. geoffroyi*, CD – *C. didactylus*, O – C. *guereza occidentalis*, N – *A. niviridus*, S – *Ateles* sp., T – *T. vetulus monticola*, M – *M. musculus*.

Table 6.1: Prevalence of LCMV Infection in Animals Sampled During 2005/2006 Sampling Period by Public Health England

Organism		Serology		PCR	
Common Name	**Scientific Name**	**No positive/ No tested**	**Prevalence (%)**	**No positive/ No tested**	**Prevalence (%)**
Geoffroy's marmoset	*C. geoffroyi*	2/8	25	3/8	38
Linne's Two-toed Sloth	*C. didactylus*	2/2	100	NA	NA
Hamlyn's Guenon	*C. hamlyni*	0/3	0	NA	NA
Lesser Spot-nosed Guenon	*C. petaurista*	0/2	0	NA	NA
Roloway Monkey	*C. roloway*	0/1	0	NA	NA
Chimpanzee	*P. troglodytes*	10/12	83	0/12	0
Western Lowland Gorilla	*G. gorilla gorilla*	1/8	13	NA	NA
Black and White Colobus	*C. guereza occidentalis*	1/7	14	2/7	29
Unidentified primate*	-	0/10	0	NA	NA
Javan Leaf Monkey	*T. auratus auratus*	0/1	0	NA	NA
Red Howler Monkey	*A. sara*	0/6	0	0/6	0
Variegated Columbian Spider Monkey	*A. belzebuth hybridus*	0/1	0	NA	NA
Dusky Leaf Langur	*T. obscurus*	0/6	0	0/6	0
Kloss Gibbon	*H. klossii*	NA	NA	0/1	0
Northern White-cheeked Gibbon	*N. leucogenys*	NA	NA	0/1	0
Agile Gibbon	*H. agilis*	NA	NA	NA	NA
Black Howler Monkey	*A. caraya*	NA	NA	0/4	0
Allen's Swamp Monkey	*A. niviridus*	1/3	33	0/3	0
Golden Headed Lion Tamarin	*L. rosalia*	NA	NA	0/2	0
Purple-faced Langur	*T. vetulus monticola*	0/3	0	1/3	33
Common marmoset	*C. jacchus*	0/9	0	0/9	0

Organism		Serology		PCR	
Common Name	**Scientific Name**	**No positive/ No tested**	**Prevalence (%)**	**No positive/ No tested**	**Prevalence (%)**
Emperor tamarin	*S. imperator subgrisescens*	0/3	0	0/3	0
Cotton-top Tamarin	*C. Oedipus*	NA	NA	NA	NA
Red-handed Tamarin	*S. midas*	0/1	0	NA	NA
Langur*	-	0/4	0	NA	NA
Spectacled Langur	*T. obscurus*	0/2	0	NA	NA
Diana Monkey	*C. diana diana*	0/2	0	NA	NA
Woolly Monkey	*L. lagotricha*	0/1	0	NA	NA
Howler Monkey	-	0/7	0	NA	NA
Spider Monkey	*Ateles sp.*	¼	25%	NA	NA
Black-tailed Marmoset	*C. argentata melanura*	NA	NA	0/1	0
Pygmy Marmoset	*C. pygmaea*	NA	NA	NA	NA
Amur Leopard	*P. pardus orientalis*	NA	NA	NA	NA
Seba's Short-tailed Bat	*C. perspicillata*	NA	NA	0/2	0
Bat*	-	0/3	0	NA	NA
Striped Skunk	*M. mephitis*	NA	NA	0/1	0
Brown Rat	*R. norvegicus*	NA	NA	0/2	0
House Mouse	*M. musculus*	NA	NA	23/41	56
Guinea Pig	*C. porcellus*	0/4	0	0/4	0

6.4.2. LCMV Screening in Present Study

In the present study, RT-PCR assays were conducted to screen for both Small (S) and L (Large) segments of the LCMV genome in order to ensure an accurate prevalence value.

Screening for was carried out using 4 sets of primers. These included the in-house MAMF/MAMR primer set as well as the published primer set described by Vieth and colleagues (2007) (Vieth *et al.*, 2007). Additional primers used included an additional LCMV degenerate primer set (DLCMF/DLCMR), designed based on an alignment of L-gene sequences from NCBI database (Figure 6.2). This primer set was used to amplify a 247bp fragment of the LCMV L-gene. Screening was also carried out using a set of published primers Arena1+, LCMV-S 13^{+} and LCMV-S 322^{-}, designed by Asper and colleagues to amplify a 310bp fragment of the 5' end of the S-gene (Asper *et al.*, 2001).

The 4 primer sets used had different degrees of sensitivity in the detection of viral nucleic acid in the samples. All primer sets except the published set by Asper *et al.* (2001) successfully detected LCMV RNA in 4 of the 49 samples donated by Dr Rachael Tarlinton which were obtained between 2006 and 2008. These samples (SB15, SB16, SB18 and SB35) were the same samples detected during the screening assay for novel arenaviruses (19% prevalence) (Table 5.1).

The samples donated by Public Health England were also screened by RT-PCR using the 4 sets of primers. The published primer set described by Vieth and colleagues (Vieth *et al.*, 2007) detected LCMV genome in 20 samples of 76 samples screened.

The in-house DLCMF/DLCMR primer set were used to screen only 62 of the 76 samples obtained from Public Health England and detected LCMV RNA in 13 of 76 samples screened. Screening of the remaining samples

was hindered by the unavailability of animal tissue to carry out further nucleic acid extractions.

The published primer set described by Asper and colleagues (Asper *et al.*, 2001) detected LCMV RNA in 19 of the 76 samples screened.

The in-house MAMF/MAMR primer set were used to screen only 62 out of the 76 samples obtained from Public Health England out of which only 4 samples were positive for LCMV genome. Screening of the remaining samples was hindered by the unavailability of animal tissue to carry out further nucleic acid extractions.

All the 17 samples detected by the DLCMF/DLCMR primer set had already been detected by the published primer set described by Vieth and colleagues. All the samples detected using the MAMF/MAMR set were also detected using the published primer set described by Vieth and colleagues (Appendix 15).

Some LCMV-positive samples detected using the published primer sets by Asper and colleagues (Asper *et al.*, 2001) were not detected using the published primer sets by Vieth and colleagues (Vieth *et al.*, 2007) (Figure 15).

The results of all screening assays were combined to provide a more robust estimate of the prevalence value of the samples obtained from Public Health England (Table 6.2). A total of 24 samples had detectable virus RNA. LCMV RNA was detected in 4 out of 35 NHPs (Overall prevalence of 11%). The LCMV-positive species and the respective prevalence of infection are *C. geoffroyi* (67%), *C. guereza occidentalis* (33%) and *N. leucogenys* (100%) respectively.

a)

Species	3847 * *** *** *** *** *** *** *** *** 3869 *
DEGLCMV_F	... ..G GRC ARG GRA THY TDC AYA AYG C.. ...
KJ603309.1 LCMV Strain IGS31	ATA TGG GGC AGG GAA TCT TGC ATA ATG CTT CTG
KJ603307.1 LCMV Strain IGS32	ATA TGG GGC AGG GAA TCT TGC ATA ATG CTT CTG
JF912084.1 LCMV Strain HP65-2009/1	ACA TGG GAC AAG GAA TCT TGC ACA ATG CCT CTG
AB627956.1 LCMV Strain BRC	ACA TGG GAC AAG GGA TCT TGC ATA ATG CCT CTG
AB627955.1 LCMV Strain OQ28	ACA TGG GAC AAG GAA TTT TAC ATA ATG CCT CTG
AB627954.1 LCMV Strain WE Sub-strain Nagasaki	ACA TGG GAC AGG GGA TCC TAC ACA ATG CTT CTG
DQ286932.1 LCMV Strain Marseille 12	ACA TGG GAC AGG GAA TCT TGC ACA ATG CTT CTG
AB477530.1 LCMV Strain M1	ACA TGG GAC AAG GGA TCT TGC ATA ATG CCT CTG
GQ862981.1 LCMV Strain Bulgaria	ATA TGG GGC AAG GAA TTC TGC ACA ATG CTT CTG
FJ607027.1 LCMV Strain 810362	ATA TGG GGC AGG GAA TCT TAC ACA ATG CTT CTG
FJ607025.1 LCMV Strain WE Sub-strain UBC No. 57135	ATA TGG GAC AGG GAA TCT TGC ACA ATG CTT CTG
DQ361066.1 LCMV Clone 13	ATA TGG GGC AGG GAA TCT TGC ATA ATG CTT CTG
DQ868488.1 LCMV Strain Traub	ACA TGG GAC AGG GAA TCT TAC ACA ATG CCT CTG
DQ868486.1 LCMV Strain Pasteur	ATA TGG GAC AAG GGA TAC TGC ATA ACG CTT CGG
EU195889.1 LCMV Strain MX	ATA TGG GAC AAG GAA TAC TGC ATA ACG CTT CGG
AY847351.1 LCMV Strain Armstrong 53b	ATA TGG GGC AGG GAA TCT TGC ATA ATG CTT CTG

b)

Species	4069 * *** *** *** *** *** *** *** *** *** 4094 *
DEGLCMV_R	... ..A AYA ART TYA TYA GYC CIA ARA GYG T..
KJ603309.1 LCMV Strain IGS31	TGT TAA ACA AAT TTA TCA GCC CAA AAA GTG TGG
KJ603307.1 LCMV Strain IGS32	TGT TAA ACA AAT TTA TCA GCC CAA AAA GTG TGG
JF912084.1 LCMV Strain HP65-2009/1	TGT TAA ATA AGT TTA TCA GTC CAA AAA GCG TAG
AB627956.1 LCMV Strain BRC	TTC TGA ATA AGT TCA TTA GTC CAA AAA GTG TCA
AB627955.1 LCMV Strain OQ28	TGT TGA ACA AGT TCA TCA GTC CGA AGA GTG TGG
AB627954.1 LCMV Strain WE Sub-strain Nagasaki	TGT TGA ACA AGT TCA TCA GTC CAA AAA GTG TGG
DQ286932.1 LCMV Strain Marseille 12	TAT TGA ACA AAT TCA TCA GTC CCA AAA GTG TGG
AB477530.1 LCMV Strain M1	TTC TAA ATA AGT TCA TTA GTC CAA AAA GTG TCA
GQ862981.1 LCMV Strain Bulgaria	TTC TAA ACA AAT TTA TTA GTC CAA AAA GTG TAG
FJ607027.1 LCMV Strain 810362	TGT TAA ACA AGT TCA TCA GTC CAA AAA GTG TGG
FJ607025.1 LCMV Strain WE Sub-strain UBC No. 57135	TAT TAA ACA AAT TCA TCA GTC CAA AAA GTG TGG
DQ361066.1 LCMV Clone 13	TGT TAA ACA AAT TTA TCA GCC CAA AAA GTG TGG
DQ868488.1 LCMV Strain Traub	TGC TAA ACA AAT TCA TCA GTC CGA AAA GTG TGG
DQ868486.1 LCMV Strain Pasteur	TGT TAA ACA AAT TCA TCA GTC CCA AAA GTG TGG
EU195889.1 LCMV Strain MX	TGT TGA ACA AAT TCA TCA GTC CTA AAA GTG TGG
AY847351.1 LCMV Strain Armstrong 53b	TGT TAA ACA AAT TTA TCA GCC CAA AAA GTG TGG

Figure 6.2: Alignment of L-gene sequences of various LCMV strains obtained from the NCBI database showing the binding site of (a) Forward (DLCMF) and (b) Reverse (DLCMR) primers and their respective positions. The inosines (denoted by the letter 'I') in DLCMR compensate for the complete degeneracies.

Table 6.2: Prevalence of LCMV Infection among Samples Obtained from Public Health England during 2005/2006 Sampling Period in this Thesis

Organism		**No. Positive/ No Tested**	**Prevalence (%)**
Common Name	**Scientific Name**		
Geoffroy's marmoset	*C. geoffroyi*	1/3	67
Chimpanzee	*P. troglodytes*	0/1	0
Western Lowland Gorilla	*G. gorilla gorilla*	0/2	0
Black and White Colobus	*C. guereza occidentalis*	1/3	33
Red Howler Monkey	*A. sara*	0/6	0
Dusky Langur	*T. obscurus*	0/3	0
Kloss Gibbon	*H. klossii*	0/1	0
Northern White-cheeked Gibbon	*N. leucogenys*	1/1	100
Agile Gibbon	*H. agilis*	0/2	0
Black Howler Monkey	*A. caraya*	0/4	0
Allen's Swamp Monkey	*A. niviridus*	0/1	0
Golden-Headed Lion Tamarin	*L. rosalia*	0/1	0
Purple-faced Langur	*T. vetulus monticola*	0/1	0
Common marmoset	*C. jacchus*	0/2	0

Organism		**No. Positive/ No Tested**	**Prevalence (%)**
Common Name			
Emperor tamarin	*S. imperator subgrisescens*	0/2	0
Cotton-top Tamarin	*C. Oedipus*	0/1	0
Spectacled Langur	*T. obscurus*	0/1	0
Black-tailed Marmoset	*C. argentata melanura*	0/1	0
Pygmy Marmoset	*C. pygmaea*	0/1	0
Amur Leopard	*P. pardus orientalis*	0/1	0
Seba's Short-tailed Bat	*C. perspicillata*	0/1	0
Bat*	-	0/3	0
Striped Skunk	*M. mephitis*	0/1	0
Brown Rat	*R. norvegicus*	0/8	0
House Mouse	*M. musculus*	20/27	74
Guinea Pig	*C. porcellus*	0/3	0

* The information presented on the animal was insufficient to allow for proper identification.

Of the 38 rodents samples donated by Public Health England and collected during the outbreak, 20 were positive for LCMV. All LCMV-positive samples were *Mus musculus* (74% prevalence).

None of the shrews, moles or rodents obtained after the outbreak were positive for LCMV. Veterinary staff and zookeepers were not tested for LCMV infection after exposure to the infected animals. The additional samples of 4 bats, 1 skunk and 1 amur leopard were also tested. These were all negative for the presence of LCMV RNA.

A comparison of the results obtained from this screening with the results presented by PHE revealed that not all LCMV-positive samples detected in this study were detected in the screening conducted by PHE and vice versa (Appendix 15). This difference was partly because some of the samples confirmed to be LCMV RNA-positive by one group was unavailable for screening by the other group. However samples screened by both groups were used to assess primer performance which was based on the ability of the primers to detect LCMV RNA in the samples. Only samples confirmed to be positive and available for screening using all primers were selected (Table 6.3). The primers used by PHE detected LCMV RNA in 16 samples (41%). The MAMF/MAMR primer set detected LCMV RNA in 2 samples (5%). The published primer set by Asper and colleagues detected LCMV RNA in 5 samples (13%). The DLCMF/DLCMR primer set detected LCMV RNA in 6 samples (15%) and the published primer set by Vieth and colleagues detected LCMV RNA in 9 samples (23%). The primer set used by PHE had the greatest performance having detected LCMV in more samples than the other primer sets.

Table 6.3: Comparison of Different Primer Sets in RT-PCR Detection of LCMV RNA

SAMPLE	SPECIES	PRIMERS					RESULT SUMMARY
		Vieth *et al.*, (2007)	DLCMF/DLCMR	Asper *et al.* (2001)	MAMF/MAMR	PHE	
NA2	*M. musculus*	NEGATIVE	NEGATIVE	NEGATIVE	NEGATIVE	POSITIVE	POSITIVE
NA3	*M. musculus*	NEGATIVE	NEGATIVE	NEGATIVE	NEGATIVE	POSITIVE	POSITIVE
NA4	*M. musculus*	NEGATIVE	NEGATIVE	NEGATIVE	NEGATIVE	POSITIVE	POSITIVE
NA5	*M. musculus*	POSITIVE	POSITIVE	NEGATIVE	NEGATIVE	POSITIVE	POSITIVE
NA14	*C. geoffroyi*	POSITIVE	POSITIVE	POSITIVE	POSITIVE	POSITIVE	POSITIVE
NA15	*T. vetulus monticola*	NEGATIVE	NEGATIVE	NEGATIVE	NEGATIVE	POSITIVE	POSITIVE
NA18	*C. guereza occidentalis*	POSITIVE	POSITIVE	POSITIVE	POSITIVE	POSITIVE	POSITIVE
NA56	*M. musculus*	POSITIVE	POSITIVE	NEGATIVE	NEGATIVE	POSITIVE	POSITIVE
NA57	*M. musculus*	NEGATIVE	NEGATIVE	NEGATIVE	NEGATIVE	POSITIVE	POSITIVE
NA62	*M. musculus*	NEGATIVE	NEGATIVE	NEGATIVE	NEGATIVE	POSITIVE	POSITIVE
NA63	*M. musculus*	NEGATIVE	NEGATIVE	NEGATIVE	NEGATIVE	POSITIVE	POSITIVE
NA64	*M. musculus*	POSITIVE	NEGATIVE	POSITIVE	NEGATIVE	POSITIVE	POSITIVE
NA65	*M. musculus*	POSITIVE	POSITIVE	NEGATIVE	NEGATIVE	POSITIVE	POSITIVE
NA66	*M. musculus*	POSITIVE	POSITIVE	POSITIVE	NEGATIVE	POSITIVE	POSITIVE
NA70	*M. musculus*	POSITIVE	NEGATIVE	NEGATIVE	NEGATIVE	POSITIVE	POSITIVE
NA71	*M. musculus*	POSITIVE	NEGATIVE	POSITIVE	NEGATIVE	POSITIVE	POSITIVE

6.5. Molecular and phylogenetic characterization of the causative LCMV strains

Having successfully screened for the presence of LCMV S-gene, a phylogenetic analysis was required to assess the evolutionary relationship between the novel LCMV strains and other previously identified strains. This was carried out using MEGA and was based on the 213bp fragment of the SSP.

The phylogenetic tree generated (Figure 6.3) allowed for the comparison of the novel LCMV sequences with sequences obtained from a similar study carried out by Blasdell and colleagues to investigate arenavirus diversity in rodents of the United Kingdom (Blasdell *et al.*, 2008b). In the study by Blasdell *et al*, only 97 small nucleotide sequences of LCMV RNA were generated and these were the only contemporaneous UK strains available for comparison with the sequences generated in this thesis. The genetic distance within the newly sequenced LCMV strains based on amino acid and nucleotide sequences were 0% to 10.9% and 0% to 8.3% respectively. The genetic distance between the newly sequenced LCMV strains and the nearest UK strain (MM10) from the study by Blasdell and colleagues based on amino acid and nucleotide sequences were 9.9% to 11.7% and 7.1% to 9.4% respectively. The sequences generated in this thesis clustered with those obtained from Blasdell and colleagues suggesting the existence of a UK-specific LCMV lineage. Thus the UK strains are monophyletic.

A large number of branches had low bootstrap support thus larger fragments of the glycoprotein would be required for a more detailed analysis of LCMV phylogeny. Complete GPC sequences of eight of the novel LCMV strains were achieved following the amplification of the entire glycoprotein gene (~1480bp) from strains NA14, NA18, NA29, NA69, NA72, NA74, NA76 and SB18 (Table 6.4).

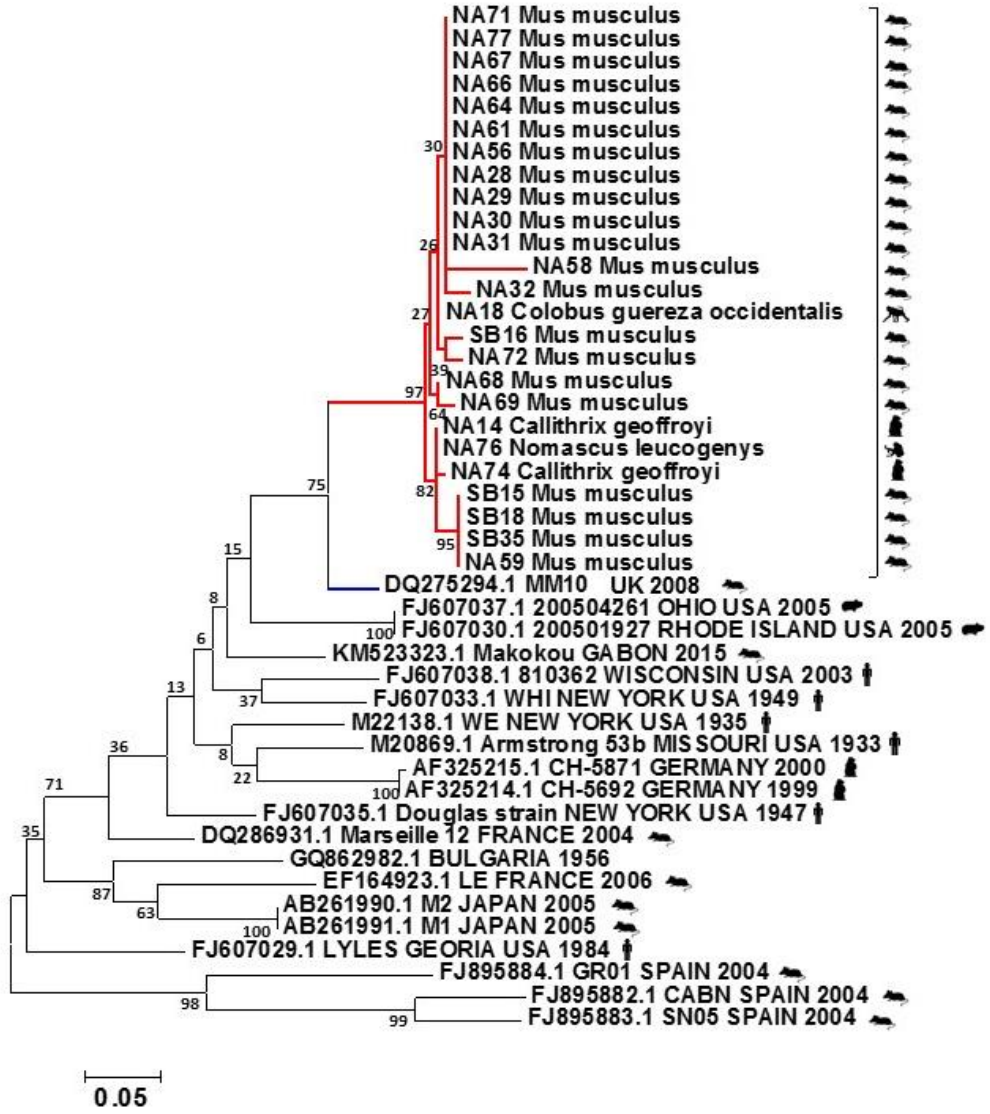


Figure 6.3: Maximum likelihood phylogenetic analysis of 213bp fragment of LCMV glycoprotein gene for novel sequences identified in this thesis (Red line) and other sequences from NCBI databases. The closest strain MM10 (Blue line), was obtained from the study by Blasdell and colleagues (Blasdell *et al.*, 2008b). Reference sequences are indicated by their Genbank Accession Number, country of origin and year of isolation. Branch lengths are drawn to scale: the bar indicates 0.05 nucleotide substitutions per site. Numbers above individual branches indicate the percentage that that branch was found in 1000 bootstrap replicates.

Table 6.4: LCMV Strains and Sources for Full-Length Glycoprotein

Identification Number	Source of Strain	Identification Number	Source of Strain
NA14	*C. geoffroyi*	NA72	*M. musculus*
NA18	*C. guereza occidentalis*	NA74	*C. geoffroyi*
NA29	*M. musculus*	NA76	*N. leucogenys*
NA69	*M. musculus*	SB18	*M. musculus*

Amplification was carried out either through a single run involving a set of primers (GPF1/GPF8) targeting both ends of the GPC or through primer walking using the following combinations of primers; GPF1, GPF2, GPF3, GPF4, GPF5, GPF6, GPF7, GPF7F, GPF7R, GPF8R, GPF8, GPF8R1, LCMV227 and LCMV1026 that amplified fragments of the GPC which were later joined together (Figure 6.4).

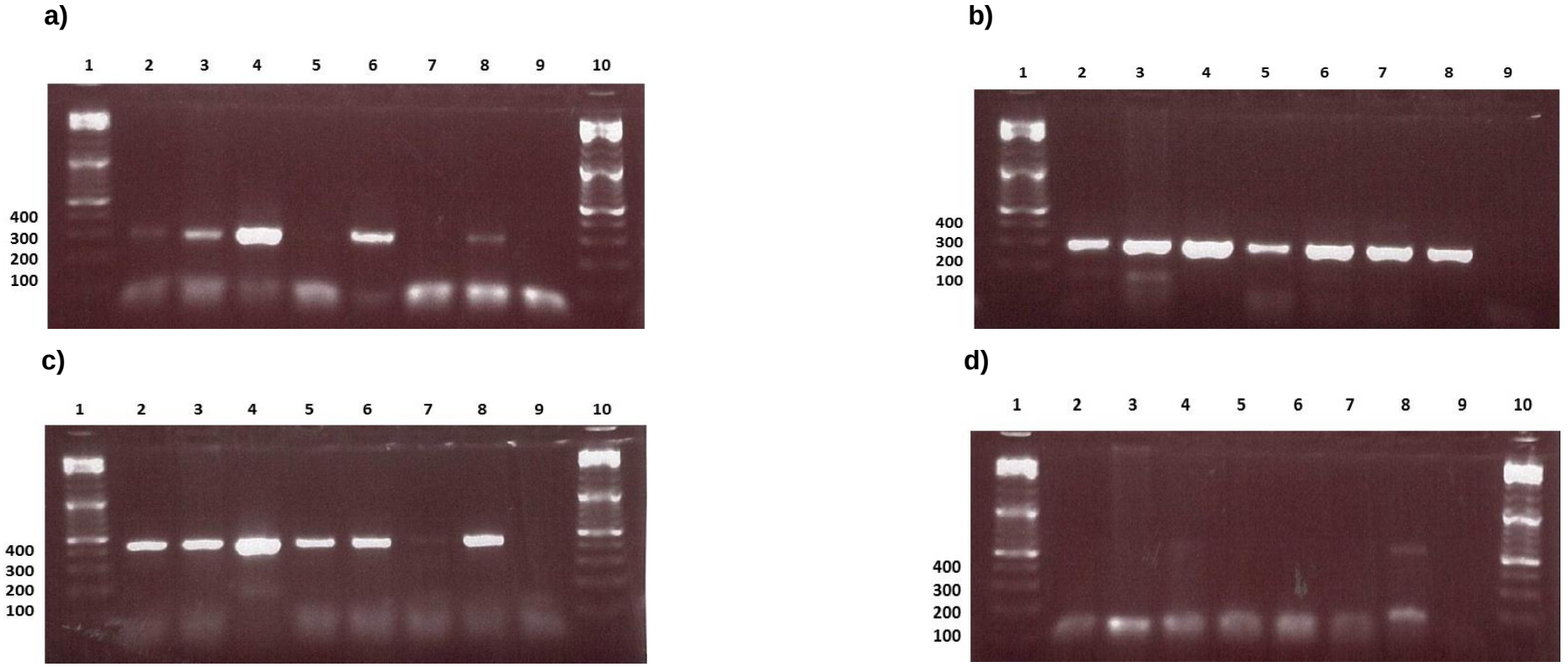


Figure 6.4: Gel electrophoresis of amplification products of RT-PCR assay for amplification of full length GPC. Lane 1: DNA Ladder; Lanes 2-8: SB18, NA14, NA18, NA29, NA69, NA74, NA76 Lane 9: Negative Control; Lane 10: DNA Ladder. Primers used are as follows; (a) GPF1/GPF2 (Region 1 – 278) (b) GPF3/GPF4 (Region 258 – 551) (c) GPF5/GPF6 (Region 530 – 982) (d) GPF7/GPF8R1 (Region 963 – 1497).

Direct sequence comparison was achieved using MEGA. An alignment of the full-length GPC sequences of all the novel strains revealed that they had high similarity but were not identical. The differences in amino acid and nucleotide sequences amongst the novel LCMV strains were 0% to 2.9% and 0.1% to 3% respectively. Amongst the rodent-derived strains, the differences in sequences were 2% to 3% for both amino acid and nucleotide respectively. Amongst the NHP-derived strains, the differences in amino acid and nucleotide sequence were 0% to 0.8% and 0.2% and 3% respectively.

A maximum likelihood tree was generated using the full sequence of the GPC of all the novel LCMV strains and other reference strains from the NCBI database (Figure 6.5). This revealed that the former belonged to lineage I together with the Armstrong and WE archetype strains. The differences in amino acid and nucleotide sequences between the novel LCMV strains and other lineage I strains were 5.3% to 14.0% and 15.6% to 30.4% respectively. Amino acid and nucleotide differences between the novel LCMV GPC sequences and those from lineages II, III and IV were 5.3% to 21.4% and 19.2% to 37.3% respectively.

A maximum clade credibility tree was generated from Bayesian coalescent analysis of LCMV based on the GPC gene segment (Figure 6.6). This enabled estimation of the rate of evolution of the sequences as well as the most recent common ancestor (TMRCA). The molecular evolutionary rate for the GPC segment was estimated to be 7.7×10^{-3} substitutions/site/year with 95% highest posterior density of 6.2×10^{-3} to 9.4×10^{-3}. The TMRCA for the European strains was 61.48. The analysis also revealed that the novel LCMV strains were within the clade of other strains isolated from France, Germany and Slovakia. The tree also showed that GR01, SN05 and CABN LCMV strains are the most divergent from the novel strains.

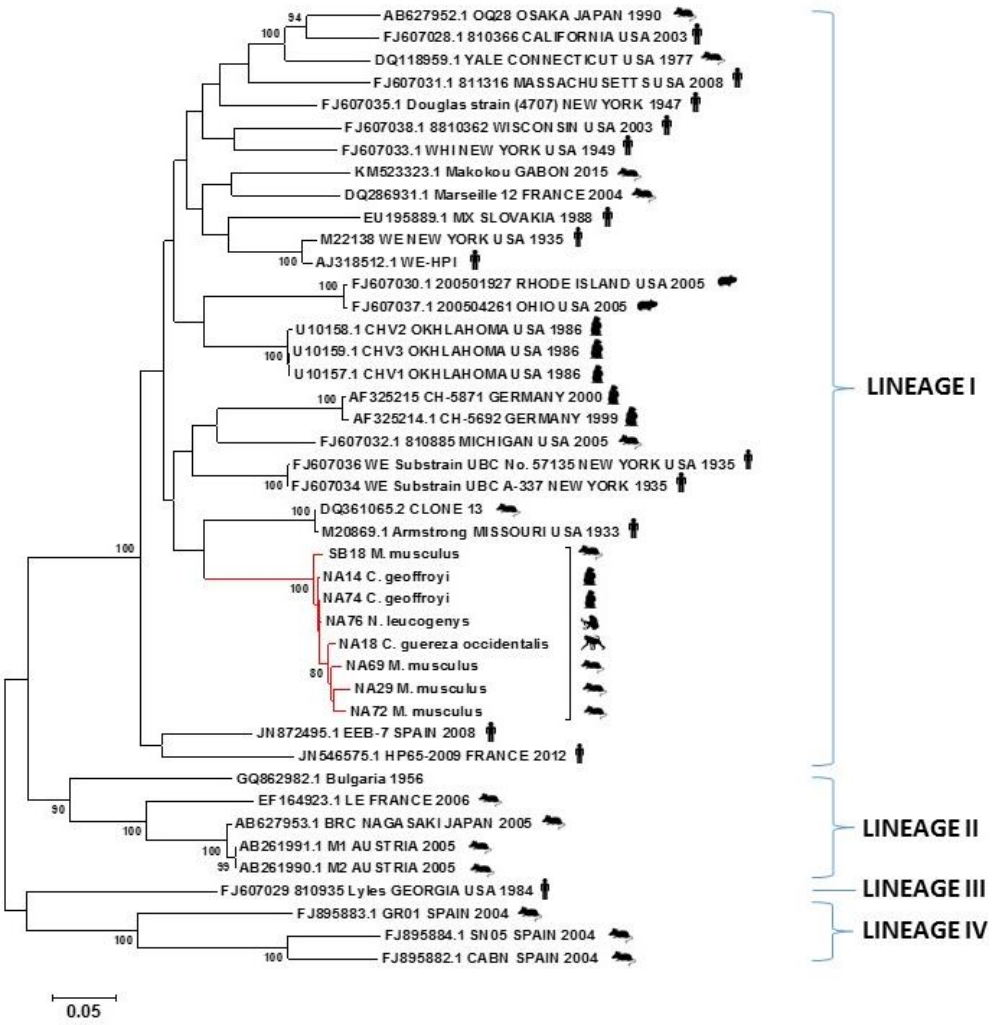


Figure 6.5: Maximum likelihood phylogenetic analysis of complete LCMV glycoprotein gene for all novel sequences identified in this thesis (red line) compared with reference sequences obtained from the NCBI database. Reference sequences are indicated by their Genbank Accession Number, country of origin and year of isolation. Branch lengths are drawn to scale: the bar indicates 0.05 nucleotide substitutions per site. Numbers above individual branches indicate the percentage that that branch was found in 1000 bootstrap replicates; only percentages >75 are shown.

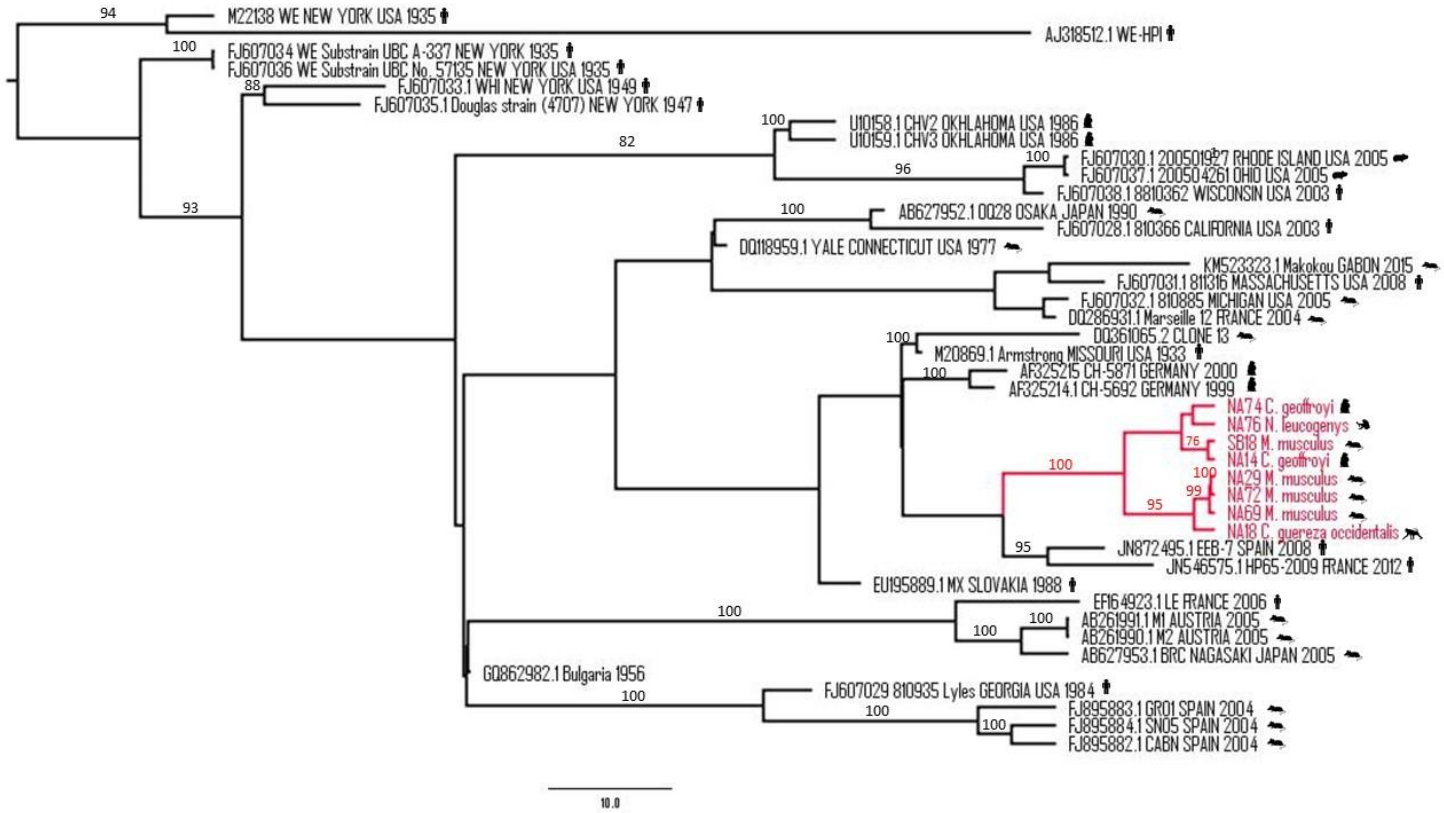


Figure 6.6: Maximum clade credibility tree generated from Bayesian coalescent analysis of lymphocytic choriomeningitis virus based on GPC segment. Novel LCMV sequences are highlighted in red. Branch lengths are proportional to the number of substitutions/site/year. Posterior probabilities are listed above the branches for supported nodes. Scale bar indicates nucleotide substitutions per site. Only percentages >75 are shown.

There were a total of 24 amino acid exchanges in GPC among the novel strains (Figure 6.7). A mutational 'hotspot' involving the substitutions at identical positions between amino acid residues 119 and 247 was observed. At position 211 the aminoacid residue was the threonine (T) in all the primates and lysine (K) in all the rodents. Also within the hotspot of NA18 and NA76 (strains obtained from *C. guereza occidentalis* and *N. leucogenys*) was the presence of two mutations that were not present in any of the other novel strains identified. At position 119 all the novel NHP-derived LCMV strains and majority of the mouse-derived LCMV strains had histidine whereas the LCMV strain from the black and white colobus (NA18) had glutamine (Q) instead. Also at position 438, all the mouse-derived and NHP-derived LCMV strains had Aspartic acid (D) whereas NA76 (Northern white-cheeked gibbon) which had glutamic acid (E) instead. The SSP/GP1/GP2 cleavage sites and N-linked glycosylation sites in the GPC were completely conserved.

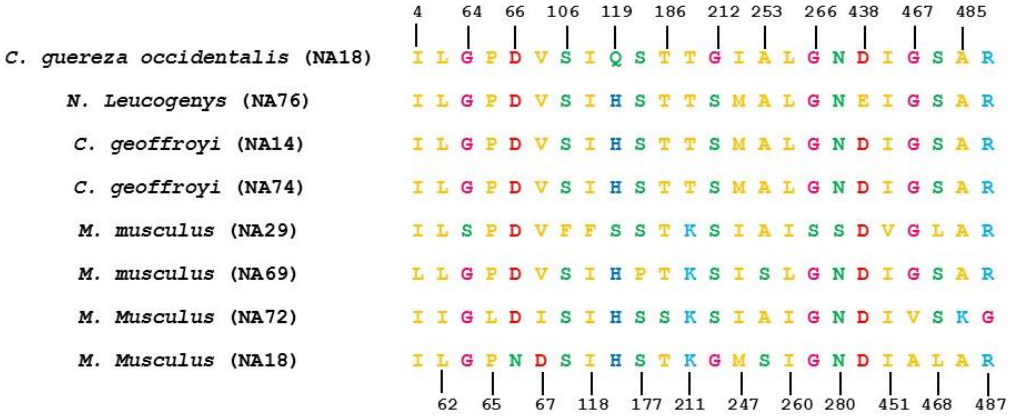


Figure 6.7: Amino acid differences amongst the Full-length GPC sequences of 8 of the novel LCMV strains identified in this thesis. Numbers indicate the position of the amino acids.

Due to the consistent presence of threonine and lysine at position 211 of the novel LCMV amplified from the primates and rodents respectively, GPC sequences in the NCBI database were analysed to determine if the same pattern would be observed in reference sequences. Strains isolated from cell lines which had undergone passaging were noted

(Table 6.5). The passage histories for most of the sequences were unavailable and most were sourced from the report by Albarino and colleagues (Albarino *et al.*, 2010a). The Armstrong and Douglas-4707 strains originated from humans but had been passaged in rodents and NHPs and had threonine at position 211. WE-UBC-57135 and WE-UBC-A337 originated from humans but had been passaged in mouse brain and had alanine at position 211. Marseille 12 and HP65-2009 originated from mice but had been passaged in Vero cells and had lysine at position 211. OQ28 originated from mice but had been passaged in Vero cells and mouse brain and had threonine at position 211. Therefore, although the observed differences in position 211 partitioned between primate and non-primate hosts in this study, the same trend was not observed with mouse and NHP-derived strains from other studies.

Table 6.5: Passage History of LCMV strains used in the Present Study. Strains originally sourced from primates but subsequently passaged using rodent cell lines are highlighted in yellow. Strains originally sourced from rodents but subsequently passaged using primate cell lines are highlighted in green.

Strain	Isolation/ Passage History	Amino acid at 211	NCBI Accession no.	Source
811316	Blood of transplant patient	Threonine	FJ607022, FJ607031	(Albarino *et al.*, 2010a)
810850	Hamster kidney	Threonine	FJ607021, FJ607030	(Albarino *et al.*, 2010a)
Makokou	House mouse liver and spleen	Threonine	KM523323, KM882857	(N'Dilimabaka *et al.,* 2015)
Douglas-4707	Intracranial inoculation of suckling mice with CSF from patient suffering from aseptic meningitis; One passage in VE6 in 2005	Threonine	FJ607024; FJ607035	(Albarino *et al.*, 2010a)
810885	Mouse spleen	Threonine	FJ607023, FJ607032	(Albarino *et al.*, 2010a)
810896	Hamster kidney	Threonine	FJ607026, FJ607037	(Albarino *et al.*, 2010a)
810366	CSF of congenitally-infected infant	Threonine	FJ607019, FJ607028	(Albarino *et al.*, 2010a)
810935/ Lyles Georgia	Human CSF; Passaged in Vero cells	Lysine	FJ607020, FJ607029	(Albarino *et al.*, 2010a)
EEB-7	CSF of patients with acute meningitis; passaged in Vero cells	Threonine	JN872494, JN872495	(Perez-Ruiz *et al.,* 2012)
810362	Human CSF	Threonine	FJ607027, FJ607038	(Albarino *et al.*, 2010a)
Armstrong	Patient suffering from encephalitis; passaged in mouse and monkey brains	Threonine	AY847351, M20869	(Armstrong & Lillie, 1934; Grande-Perez *et al.*, 2005; Salvato *et al.*, 1988)

Strain	Isolation/ Passage History	Amino acid at 211	NCBI Accession no.	Source
WE	Meningo-encephalitis patient	Alanine	M22138; AF004519	(Albarino *et al.*, 2010a)
WE-UBC-57135	Meningo-encephalitis patient; 7 passages in mouse brain; One passage in VE6 in 2005	Alanine	FJ607025, FJ607036	(Albarino *et al.*, 2010a)
WE-UBC-A337	Meningo-encephalitis patient; Seven passages in mouse brain	Alanine	FJ607034	(Albarino *et al.*, 2010a)
CHV1, CHV2, CHV3	Monkey liver. CHV1 and CHV3 were isolated from liver of experimentally infected marmoset; CHV2 was isolated from infected Vero cells.	Threonine	U10157, U10158, U10159	(Stephensen *et al.*, 1995)
WHI	Human CSF.	Threonine	FJ607033	(Albarino *et al.*, 2010a)
Marseille 12	House mouse kidney; Passaged in Vero cells	Lysine	DQ286932, DQ286931	(Emonet *et al.*, 2007)
CH-5692, CH-5871	Monkey spleen and serum.	Threonine	AY363903, AF325214, AF325215	(Asper *et al.*, 2001)
HP65-2009	Mouse; Passaged in Vero cells	Lysine	JN546573 – 6	(Yama *et al.*, 2012)
Yale	Mouse	Threonine	DQ118959	(Albarino *et al.*, 2010a)
MX	Persistently infected cell line	Alanine	EU195888, EU195889	(Tomaskova *et al.*, 2008)

Strain	Isolation/ Passage History	Amino acid at 211	NCBI Accession no.	Source
LE	Amniotic fluid in congenital infection	Lysine	EF164923	(Albarino *et al.*, 2010a)
Bulgaria	Patient with LCMV infection	Threonine	GQ862981, GQ862982	(Albarino *et al.*, 2010a)
OQ28	House mouse spleen; passaged in Vero cells and mouse brain	Threonine	AB627952, AB627955	(Takagi et al., 2012)
BRC	House mouse kidney; passaged in mouse brain	Lysine	AB627953, AB627956	(Takagi *et al.*, 2012)
M1, M2	House mouse spleen	Lysine	AB261990 – 1	(Ike *et al.*, 2007)
CABN, GR01, SN05	Wood mouse lung	Threonine	FJ895882, FJ895883, FJ895884	(Ledesma *et al.*, 2009)
BU1-8, MM9, MM10, MM16, MC1, MC24, M20, HM13, HM14, C1, C2, C6, C9, C8, C20, C21, BU12-87	House mouse and wood mouse serum	Threonine	DQ275199-DQ275295	(Blasdell *et al.*, 2008b)

6.6. Molecular Characterisation of Alphadystroglycan

Amino acid positions 169 to 408 on alpha-dystroglycan have been implicated to play an essential role in virus binding. The specific positions within this region are yet to be identified however (Kunz *et al.*, 2001). To help identify these, a partial segment of alpha-dystroglycan was amplified and sequenced. Amino acid variations in rodent and NHP alpha-dystroglycan were investigated. On the sequences from primates (including humans) it was observed that positions 303, 346, 410 were occupied by proline, arginine and alanine respectively while the rodents sequences had serine, glutamine, threonine (or valine) respectively at those positions (Table 6.6).

6.7. LCMV Pseudoparticle Production and Infection of Hepatocellular Carcinoma (Huh7) Cells

Having successfully amplified and sequenced the full GPC gene of the novel LCMV strains derived from rodents and NHPs an attempt was made to investigate virus entry into host cells and the role of the LCMV glycoprotein. This can be achieved using pseudotype infectivity assays. The method typically involves the use of chimeric virions composed of the structural and enzymatic core of one virus and the protein(s) or glycoprotein of another (King & Daly, 2014; Urbanowicz *et al.*, 2016). Once introduced to suitable cell cultures, the virions engage with the host cell via the surface proteins resulting in transduction and subsequent integration and expression of reporter gene. Levels of gene expression can then be measured quantitatively (Mather *et al.*, 2013). A variety of cell lines have been successfully transduced pseudoviruses bearing full-length LCMV glycoproteins (Beyer *et al.*, 2002).

Table 6.6: Amino Acid Substitutions at Positions 303, 346 and 410 on the Alphadystroglycan Receptor of rodents and NHPs from which the novel LCMV strains were derived compared with sequences of other rodents and NHPs obtained from the NCBI database

Organism		Amino Acid Position		
Common Name	Scietific Name	303	346	410
Syrian hamster	*Mesocricetus auratus*	S	G	T
Guinea pig	*Cavia porcellus*	S	G	T
Prairie vole	*Microtus ochrogaster*	S	G	T
House mouse	*Mus musculus*	S	G	T
House mouse	*Mus musculus* (SB18)	S	G	T
House mouse	*Mus musculus* (NA29)	S	G	T
House mouse	*Mus musculus* (NA69)	S	G	T
Northern white-cheeked gibbon	*Nomascus leucogenys (NA76)*	P	R	A
Black and white colobus	*Colobus guereza occidentalis (NA18)*	P	R	A
Geoffroy's marmoset	*Callithrix geoffroyi (NA14)*	P	R	A
Coquerel's sifaka	*Propithecus coquereli*	P	R	A
Western lowland gorilla	*Gorilla gorilla*	P	R	A
Bonobo	*Pan paniscus*	P	R	A
Nancy Ma's night monkey	*Aotus nancymaae*	P	R	A
Northern white-cheeked gibbon	*Nomascus leucogenys*	P	R	A
Northern greater galago	*Otolemur garnetti*	P	R	A
Gray mouse lemur	*Microcebus murinus*	P	R	A
Angolan colobus	*Colobus angolensis*	P	R	A
Common marmoset	*Callithrix jacchus*	P	R	A
Common chimpanzee	*Pan troglodytes*	P	R	A
Human	*Homo sapiens*	P	R	A

These include Huh7 and HEK 293T cell lines. Successful pseudoparticles were constructed using the viral envelopes obtained from strains SB18, NA29 and NA14. These were produced in HEK 293T cells and used to

infect Huh7 cells. The infectivity using the various glycoproteins was then measured and compared (Figure 6.8). Of the three pseudotypes, NA29 had the highest infectivity. The lowest infectivity was observed with the NA14 pseudotype.

Statistical difference in the infectivities of the pseudotypes were compared using a one-way ANOVA followed by a Bonferroni-adjusted multiple comparison using the negative control as the control group. The difference in infectivity between NA29 and the negative control was statistically significant while the infectivities of SB18 and NA14 pseudotypes were not significantly different from the negative control.

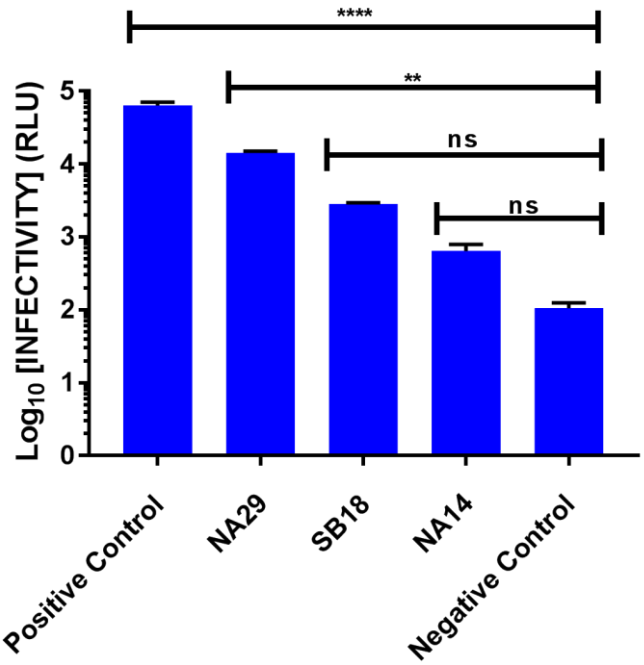


Figure 6.8: Pseudotype Infectivity Assay for using complete GP from novel LCMV strains. Bars from Left to Right: Positive Control (Pseudoparticle with VSV envelope), NA29, SB18, NA14 and Negative control (Pseudoparticles without envelopes). Error bars were calculated from the standard deviation of three assay replicates. One way ANOVA was performed with Bonferronis-adjusted multiple comparison test. Significance of infectivity of pseudoparticles compared with that of the negative control are shown; ** P = 0.003, **** P = <0.0001, ns – no significance.

7. Discussion

7.1. Screening of Animal Tissue for Novel Filoviruses and Arenaviruses

7.1.1. Design of Primers for RT-PCR Assays

RT-PCR assays for the detection of known and unidentified members of *Arenaviridae* and *Filoviridae* were developed. The L-gene (encoding the viral polymerase) is a highly conserved region even amongst members of different virus families when compared to the N gene (encoding for the virus nucleocapsid) (Lukashevich *et al.*, 1997; Vieth *et al.*, 2007). Family-wide degenerate and gene-specific primers were designed based on conserved sites on the L-gene (encoding viral RNA-dependent RNA polymerase) identified by the alignment of members of the viral families. To compensate for regions of complete degeneracy amongst members and increase the likelihood of identifying new members of each family, degenerate primers were designed with the inclusion of inosines. Assay reaction conditions such as primer and magnesium concentrations were optimized to increase sensitivity and specificity (van Boheemen *et al.*, 2012). Synthetic plasmid controls were also constructed to monitor RT-PCR efficiency (Brod *et al.*, 2013; Corman *et al.*, 2013; Minegishi *et al.*, 2014).

There are reports on the design of primers for the detection of Old World and New World mammarenaviruses (Charrel *et al.*, 2003; Vieth *et al.*, 2007) as well as individual members of the *Filoviridae* family available in literature (Leroy *et al.*, 2000; Sanchez *et al.*, 1999). These primers were placed in the alignments of L-gene sequences constructed for arenaviruses and filoviruses respectively and a number of mismatches were observed especially amongst the recently discovered reptarenaviruses and filoviruses (Bodewes *et al.*, 2014; Negredo *et al.*,

2011; Stenglein *et al.*, 2012). In this thesis, two sets of primers were designed for the detection of novel mammarenaviruses and reptarenaviruses. While a set of primers were designed for the detection of filoviruses.

As stated previously, all the primers used in this study had inosines in them. Prior to the selection of inosine-based primers, we used primers with 'N' representing a four-fold degeneracy. These were not effective in the RT-PCR assays (results not shown) and did not pick up positive results with the plasmid controls or test samples. This was not surprising as a drop in primer sensitivity is normally associated with increased degeneracy. In a study by Maki and colleagues, the sensitivity of universal primers for the detection of genital Human Papilloma virus (HPV) were compared to those of gene-specific primers. While the gene-specific primers were able to detect viral titres as low as 10 copies, the universal primers on the other hand were only able to detect 1000 copies of HPV in the sample (Maid *et al.*, 1991). In this thesis, it is was also a challenge to select the optimal concentration that would be effective in the RT-PCR assay using these primers. The use of inosine in primer design is well-documented and its presence in degenerate primers reduces the occurrence of mismatching during primer annealing, increases amplification efficiency and product yield as well as increases the likelihood of detecting novel DNA sequences (Tong *et al.*, 2008; van Boheemen *et al.*, 2012; Watanabe *et al.*, 2001). Inosines are used to replace nucleotides usually at positions of four-fold degeneracy thus reducing the need to use multiple primers (Knoth *et al.*, 1988). However, only a maximum of 3 inosines per primer were used as their presence in primers could also result in nonspecific amplification of host-cell nucleic acids which might consequently obscure the virus-specific product (Nix *et al.*, 2006; Rose *et al.*, 1998). The inosines were placed mostly at the 5′ end because annealing of the last three bases at the 3′ end is sufficient to cause undesirable amplification at the wrong sites on the target DNA

(Ben-Dov *et al.*, 2006). Optimization of RT-PCR assay for the detection of mammarenaviruses was achieved by increasing the magnesium (Mg^{2+}) and primer concentrations. Magnesium (Mg^{2+}) is a DNA polymerase cofactor and is essential for enzyme activity. It binds to the nucleic acid, primers, and nucleotides present in the amplification reaction. The chessboard approach reported by Gunson *et al.*, (2003) was used to determine the optimal Mg^{2+} concentration to be used in the assay as higher concentrations could cause the stability of nonspecific bonds resulting in decreased yields of specific Amplified products and the appearance smears in the background (Gunson *et al.*, 2003).

Positive controls for PCR assays used in virus detection are usually in the form of the whole viruses or viral nucleic acid(s) of interest (Faye *et al.*, 2013; Watzinger *et al.*, 2004). However, due to challenges associated with standardization, quantification and safety of viral RNA, synthetic DNA constructs are used as suitable alternatives (Brod *et al.*, 2013; Corman *et al.*, 2013; Minegishi *et al.*, 2014; Niwa *et al.*, 2007). The RT-PCR assays amplified various concentrations of the synthetic plasmids controls representing the representative members of the arenavirus and filovirus families.

7.1.2. Screening of Samples for Filovirus Genome

No filovirus RNA was detected in any of the animal tissues screened, 99% of which were rodents. This result may be due to a number of factors. The first is the geographical region where the specimen were collected from as the likelihood of detecting such pathogens in specimen may be higher in one region as compared to another (Blasdell *et al.*, 2008b; Lavergne *et al.*, 2016; Leroy *et al.*, 2005). This may raise the question of why there have been no reported cases of filovirus infections amongst humans in the countries from which the specimen were

collected if such viruses are indeed present. Firstly, not all filoviruses are associated with human disease (Barrette *et al.*, 2009; Negredo *et al.*, 2011) and if a yet undiscovered one were to cause human infection, there may still be a reduced likelihood of detection if the pathogen in question is not virulent, thus presenting only mild general symptoms in the infected host. Given that filoviruses are not routinely tested for in UK laboratories, it would be difficult to confirm a filovirus as the culprit behind such an infection. Health staff would most likely only suspect a filovirus infection if the patient was showing the symptoms unique to viral haemorrhagic fevers and had recently visited any of the countries where outbreaks had occurred or had contact with people who had done so (Karwowski *et al.*, 2014; NHS Choices, 2016a).

Other important factors are the small sample size and period of sampling. It is widely accepted that the chances of detecting the novel pathogens is increased significantly with higher sample numbers (Peterson *et al.*, 2004b; Pourhoseingholi *et al.*, 2013). However, as the virus being screened for is novel in the species examined, some of the parameters required to determine a suitable sample size for the study including the prevalence and precision were not available. Therefore the approach taken in this thesis was to screen all available samples. The period of sampling is also a very important factor as filoviruses exhibit seasonality in reservoirs and as such there exists a higher possibility of detecting these viruses in the wild at particular times of the year (Amman *et al.*, 2012; Leendertz *et al.*, 2016). Nonetheless, these two factors seem highly improbable particularly with the animals sampled from the East Midlands as they were obtained from the zoo surroundings where a high potential of these viruses to be transmitted to other animals in the zoo would have been noticed. Non-human primates in particular are susceptible to infections from Marburg and Ebola viruses (Bermejo *et al.*, 2006; Formenty *et al.*, 1999).

Finally, it may be that the animals examined in this thesis are not reservoirs for filoviruses. This thesis is the only reported study on the screening of rodents from Egypt, Poland and the United Kingdom for novel filoviruses. There is limited data on the screening of rodents for filovirus infections generally. The absence of filovirus genome in the rodent samples appears to support other reports which suggest that rodents are not suitable reservoirs for filoviruses, as immunocompetent mice and rats cannot be infected by human-derived strains of Ebola and Marburg viruses (Gonchar *et al.*, 1991; Swanepoel *et al.*, 1996). Furthermore, molecular screenings of rodents collected in regions where outbreaks have occurred have yielded negative results. The results from such studies are slightly skewed as only small populations of rodents per specie were screened (Swanepoel *et al.*, 2007). In studies involving larger sample numbers of rodents, the results are different. *Mus musculus* made up a majority of the rodents screened. Peterson and colleagues agree that rodents may be potential hosts for filoviruses but rule out peridomestic mammals especially those of the genus *Rattus* and *Mus* on the basis that the reservoir would not be peridomestic as most human infections have been around fieldworkers such as miners (Bausch *et al.*, 2003; Bertherat *et al.*, 1999; Leirs *et al.*, 1999; Peterson *et al.*, 2004b). However the detection of Ebolavirus genes in *Praomys sp* and *Mus setulosus* rodents does suggest that rodents (especially belonging to the genus *Mus*) play an important role in filovirus transmission (Morvan *et al.*, 1999). .

Studies involving the screening of potential reservoirs have involved the sampling of more bat specimen than any other animal groups. The suspicion of bats as natural hosts for filovirus is not without reason as experimentally inoculated bats support replication and circulation of high virus titres without becoming ill (Swanepoel *et al.*, 1996). Furthermore,

their tendency to live in large colonies and flight pattern over notable distances greatly enhances pathogen transmission and dissemination amongst various species (Luis *et al.*, 2013; Wynne & Wang, 2013). The discovery of novel filoviruses in European *M. schreibersii* bats (Negredo *et al.*, 2011) further strengthens this opinion. However, while filovirus RNA has been detected in bats, *R. aegyptiacus* remain the only species till date that live virus has been isolated from (Amman *et al.*, 2012; Towner *et al.*, 2009). With regards to LLOV particularly, the infection was fatal in *M. schreibersii* bats and as such it is unlikely that they are the reservoirs for the virus assuming that the ideal candidate would experience less severe effects of the infection which will ensure long-term stability and reduce probability of extinction of the pathogen (Peterson *et al.*, 2004b). Finally, there is minimal overlap with the Marburg haemorrhagic fever and Ebola haemorrhagic fever occurrence sites. This hints that the filoviruses may have reservoirs with different ecological needs (Peterson *et al.*, 2004a). The distant position of MARV in relation to LLOV and the other Ebolaviruses (Figure 1.4) is further evidence of this. A similar pattern is observed amongst the *Paramyxoviridae* virus family as the distinct positions occupied by the constituting genera on the phylogenetic tree is also reflected in their host species. The henipaviruses for instance have bats as their natural reservoirs while the avulaviruses have been detected only in birds (Gogoi *et al.*, 2017; Halpin *et al.*, 2011).

Alternative methods for filovirus screening have involved the detection of antibodies in specimen. Filovirus antibodies have been detected in bats and rodents (Leroy *et al.*, 2005; Olival *et al.*, 2013; Taniguchi *et al.*, 2011). Georges and Mathiot screened 826 rodents from the Central African Republic (inclusive of the genus *Mastomys*) for the presence of antibodies to filoviruses and 4.5% of them were seropositive (Georges & Mathiot, 1989). The presence of antibodies is not necessarily evidence of acute infection; however it is proof that the population has been

previously exposed to the virus. There is currently no information regarding the screening of animal populations in Europe for filovirus antibodies. Results obtained from these will be invaluable and will guide future screening programmes.

The results from molecular screening this thesis are therefore inconclusive on the potential of rodents to serve as reservoirs for filovirus transmission. Further investigations involving the screening of rodents from regions where the outbreaks have occurred with an emphasis on testing samples of each species large enough to give a reasonable probability of actual detection (Peterson *et al.*, 2004b), will provide insight into the roles they play in filovirus transmission. The lack of data in the screening of rodents for filovirus infection also calls for the publication of negative results if available, as this will provide much needed guidance on further investigations into rodent species that have not been studied previously (Dirnagl & Lauritzen, 2010; Leendertz *et al.*, 2016).

7.1.3. Screening of Samples for Arenavirus Genome

LCMV was the only arenavirus detected in the specimens screened. Four out of 989 animals had detectable LCMV viral genome. There is limited information on studies to detect and isolate known or potentially novel arenaviruses from rodent reservoirs in Egypt, Poland and the United Kingdom. In a recent study by Yama and colleagues generic and specific assays were used to screen for arenavirus viral genome. LCMV was the only arenavirus detected from a total 821 rodent tissue samples collected from Cameroon, France, Germany, Niger, Nigeria, Portugal, Slovakia and South Africa. Only two *M. musculus* rodents sampled from France were positive for LCMV (Yama *et al.*, 2012). This supports the results obtained from our study. A separate study to assess the host range and genetic diversity of Arenaviruses in rodents in the United Kingdom also

yielded similar results (Blasdell *et al.*, 2008b). Although no novel arenavirus genome was confirmed in the rodent populations, these two independent groups agree on the possible presence of arenaviruses in other rodent reservoirs within Europe.

7.1.4. Screening of Samples Otained from LCMV Outbreak

The outbreak of callitrichid hepatitis in the East Midlands Region of the United Kingdom took place between 2005 and 2006. The outbreak was caused by closely-related LCMV strains and resulted in the infection of NHPs and rodents. LCMV infection was confirmed using histopathological findings, serological tests and PCR assays.

Seroreactivity was observed in *C. didactylus*, *C. geoffroyi*, *P. troglodytes*, *G. gorilla gorilla*, *C. guereza occidentalis*, *A. niviridus* and *Ateles sp.* as reported by Public Health England. However, the unavailability of information on the actual assay that was used in the screening would not permit for a proper analysis on its sensitivity in screening for viral antigens or antibodies in this thesis. The specificity of such an assay may also have been questioned as there may have been cross-reactivity between antigens and antibodies making it difficult to determine whether the specimens tested actually had detectable levels of LCMV antigens or anti-LCMV antibodies or if the serological proteins detected were the result of the presence of a yet unconfirmed, potentially novel arenavirus. Most recent literature on the serological screening of LCMV involved the use of commercial IFA and ELISA kits (Becker *et al.*, 2007; Blasdell *et al.*, 2008b; Knust *et al.*, 2014). While these kits show some degree of effectiveness, it is challenging to determine how sensitive and specific they are. Furthermore, the PCR assays designed by the same research group were not able to amplify viral genome in some of those same

NHPs. Precise information on the exact primers used, the regions amplified and the steps to optimise the assay were also unavailable as at the time of writing this thesis. Assuming the primers were either LCMV-specific or pan-arenavirus and the assay was fully optimised, the negative test result may imply one of five things. Firstly, the quality of the viral genome may have been poor owing to extensive degradation making it challenging for the primers to effectively bind to the target regions (Fleige *et al.*, 2006). The second reason may have been as a result of a reduced viral titre concentration. Low viral load may either result in weakly-positive of false-negative PCR results depending on the sensitivity of the adopted assay (Blasdell *et al.*, 2008b; Debiasi & Tyler, 2004). Thirdly, the specimen may have had LCMV infection but the particular organ screened may not have had virus present within them and as such the screening of other organs of the same animal may have yielded positive results. LCMV has been detected from blood, kidney, spleen, liver and CSF (Blasdell *et al.*, 2008b; Ciurea *et al.*, 1999; N'Dilimabaka *et al.*, 2015; Schulte *et al.*, 2006). A significant challenge was that tissue samples obtained from the NHPs were not properly identified as some of the collection bottles were simply labelled as 'PM (Post-mortem) tissue'. A fourth reason is the possibility of a yet unconfirmed arenavirus circulating among the specimens. This reason is highly improbable because although none of the serologically-positive NHPs were available for screening by the follow-up RT-PCR assays conducted in this thesis using broadly reactive primers, the inability of the published primers (Hetzel *et al.*, 2013; Vieth *et al.*, 2007) to detect this 'novel' virus in any of the surrounding rodent species from which the infection may have originated makes the occurrence of a new arenavirus species in the NHPs unlikely. Furthermore, there is currently no report of natural arenavirus infection other than LCMV affecting NHPs various species although some have been successfully inoculated with highly pathogenic arenaviruses *in vivo* (Carrion *et al.*, 2007; Kastello *et al.*, 1976; Maiztegui *et al.*, 1998). The final reason for the seropositive yet genome-negative results in the NHPs screened is that the latter had

previous exposure to the virus which was later cleared (Becker *et al.*, 2007). This is the most likely explanation for the results. Either way, this is the first report of LCMV/Arenavirus infections in additional species of NHPs than previously known.

Of 124 NHPs screened by PCR assays conducted by PHE, 6 were positive for LCMV infection which included 3 Geoffroy's marmosets (*C. geoffroyi*), 2 black and white colobus (*C. guereza occidentalis*) and 1 purple-faced leaf monkey (*T. vetulus monticola*). Out of these, 2 *C. geoffroyi* and 1 *C. guereza occidentalis* were confirmed to be positive in this thesis. In addition, 1 Northern White-cheeked gibbon (*Nomascus leucogenys*) was also confirmed to be LCMV-positive. RT-PCR assays used were designed to detect fragments of the S-gene and L-gene. The unavailability of information on the primers and protocols used by PHE made it impossible for the comparison of assay conditions. Previous reports on LCMV infection in callitrichids include but are not restricted to *C. jacchus*, *S. fusciollis*, *S. imperator*, *L. rosalia*, *S. oedipus, C. pygmaea* and *Callimico goeldii (Asper et al., 2001; Montali et al., 1995; Scanga et al., 1993; Stephensen et al., 1992; Stephensen et al., 1991)*. This is however the first report of a natural LCMV infection in a different species of *Callitrichidae*, *C. geoffroyi* as confirmed by RT-PCR. More significantly, it is the first report of LCMV infection in non-callitrichid NHPs. Although successful experimental infection of Old World NHPs has been previously described in rhesus and crab-eating macaques (Beier *et al.*, 2015; Lukashevich *et al.*, 2002; Peters *et al.*, 1987; Zapata *et al.*, 2011), there have been no reports of natural LCMV infections in *N. leucogenys*, *T. vetulus monticola* or *C. guereza occidentalis*.

This is not the first LCMV outbreak in zoo in the United Kingdom. In 1982, Lucke and Bennet reported a similar outbreak at the Hollywood Tower estate of the Bristol, Clifton and West of England Zoological Society

which caused the death of a total of twelve marmosets (6 silvery, 5 goeldi and I cotton-top) (Lucke & Bennett, 1982). Since 1982, no other zoo outbreaks have been reported in the UK. The number of zoo outbreaks in the USA is particularly alarming. Montali and colleagues reported that an astonishing 11 outbreaks of callitrichid hepatitis had taken place between 1981 and 1989 (Montali *et al.*, 1993). The total number of callitrichid deaths from CH in North America alone was estimated to be 57 by the year 1991 (Beck *et al.*, 1991; Dittus *et al.*, 2008; Montali *et al.*, 1989; Montali *et al.*, 1993). The current situation has far reaching consequences and can potentially lead to the extinction of already endangered primates. The International Union for the Conservation of Nature (IUCN) Red List places *Callithrix geoffroyi* and *C. guereza occidentalis* as species of 'least concern' (Kingdon *et al.*, 2008; Rylands & Mendes, 2008) as there has been no noticeable decline in their relative numbers. The same however cannot be said of some other species. The purple-faced leaf monkey (*T. vetulus monticola*) is already endangered while the *N. leucogenys* are critically endangered. Various authorities have argued on the impact of infectious diseases on the conservation status of a population, suggesting most of the decline to be a consequence of habitat loss, hunting and the presence of invasive species (Wilcove *et al.*, 1998). Data sourced from the IUCN Red list and reports in the ISI Web of Science revealed that disease seemed to contribute only a small percentage (<4%) of species extinctions known to have occurred since 15th century (Smith *et al.*, 2006). But the uncertainty in threat assessment and the bias in the data available are two strong limtations of the evidence obtained from these sources. The IUCN for one is more focused on the correct categorization of species status than on the systematic ranking of threats. (Pedersen & Davies, 2009; Pedersen *et al.*, 2007; Smith *et al.*, 2009). The Hawaiian bird extinctions linked to avian pox is a good example of the devastating effect of cross-species transmission of virus disease on the population size of susceptible hosts. Van Piper and colleagues investigated the prevalence and altitudinal distribution of forest birds infected with avian pox in Hawaii

between 1977 and 1980 and concluded that the extinction of the birds was a direct result of the spread of avian pox (Van Riper *et al.*, 2002). There have also been various reports of a decline in non-human primate numbers owing to disease outbreaks (Kondgen *et al.*, 2008; Leroy *et al.*, 2004). Therefore the emergence of virulent pathogens amongst a species that had never encountered it previously presents a huge potential for extinction. The recent introduction of LCMV to rodent populations in Africa could have a negative impact on the survival of primate populations in the continent. We therefore suggest that LCMV also presents a significant threat to the survival of NHPs.

Twenty three *M. musculus* were LCMV-positive out of the 47 tested by PHE. Some of these were available for screening in this thesis. Out of 79 shrews and rodents captured between 2005 and 2006 in the present study, 24 *M. musculus* rodents had detectable viral RNA. These were obtained from two sample sets during the same period and the prevalence values were 19% and 74% respectively. Although the primary rodent reservoir for LCMV is *M. musculus*, other rodent species including *A. sylvaticus*, *C. porcellus* and *M. agrestis* are regarded as alternative hosts (Charbonnel *et al.*, 2008b; Kallio-Kokko *et al.*, 2006; Laakkonen *et al.*, 2007; Tagliapietra *et al.*, 2009). None of the other shrews or rodents obtained during the outbreak were positive for LCMV. The absence of viral genome in *A. sylvaticus*, *C. porcellus* and *M. agrestis* screened in this thesis may be a consequence of the small number of specimen tested (Goodman & Greenland, 2007). Ledesma and colleagues had similar results when screening for arenaviruses in the United Kingdom. While LCMV genome was detected in *A. sylvaticus* rodents, all *M. musculus* investigated tested negative for LCMV virus (Ledesma *et al.*, 2009). *C. porcellus* was also represented in the present study but all also tested negative for LCMV. The overall sample size used in this thesis was however limited to the diversity of rodents caught during and after the outbreak by the zoo workers.

Other species screened in this study were negative for LCMV although the sample sizes were small. Previous reports on LCMV prevalence studies in common shrew (*Sorex araneus*) and white-toothed shrew (*Crocidura russula*) have also yielded similar negative results (Laakkonen *et al.*, 2006; Ledesma *et al.*, 2009) which suggest that pygmy shrew (*S. minutus*) may not be susceptible to LCMV infection. The absence of anti-LCMV antibodies or antigens in serological assays will confirm this. *S. minutus* may however be a potential reservoir for other novel arenaviruses as observed with the Asian house shrews (*Suncus murinus*) in southeastern China which are likely reservoirs of recently discovered WENV. LCMV and WENV share a 59% and 47% similarity on the S-gene and L-gene respectively (Li *et al.*, 2015). The absence of viral RNA in *R. norvegicus* supports results from other molecular studies that provide no evidence of natural infection of rats with LCMV although seroprevalence studies seem to suggest otherwise (Blasdell & Read, 2004; Easterbrook *et al.*, 2007; Ledesma *et al.*, 2009). Experimental infection of rats through intracerebral inoculation have been successful and disease manifestation was dependent on the age and also appears to be dose-dependent (Bonthius, 2012; Bonthius & Perlman, 2007a).

There is a noticeable difference between the overall prevalence values obtained from the study group at PHE (42.86%) and the present study (19% and 74%). One clear reason is the difference in sample size. However some positive samples that were detected by the PHE group were not detected in the present study and vice versa. This could be owed to the sensitivity and specificity of the assays used. Broadly reactive and gene-specific primers were used in amplification by RT-PCR in the present study. The primers and protocols used by the PHE study group were not available however and as such PCR efficiency by both groups could not be compared. The LCMV prevalence in *Mus musculus* obtained in this study from screening of samples donated by PHE are also considerably higher than that of similar studies conducted globally

(Blasdell *et al.*, 2008b; Knust *et al.*, 2014; N'Dilimabaka *et al.*, 2015). The likelihood of detection is dependent on the method of screening employed as well as the sampling period. Morita and colleagues arrived at a prevalence of 25.9% in Japan using virus isolation (Morita *et al.*, 1991a; Takagi *et al.*, 2012). Serological surveys carried out by other groups in the United Kingdom however report a lower prevalence value in *M. musculus* rodents (4 to 17.5%) (Becker *et al.*, 2007; Blasdell *et al.*, 2008b). The huge variability between the values observed in the present study and others carried out globally may be due to a number of reasons. The first is the location where the samples were obtained from. The distribution of rodents across various locations has an effect on prevalence estimates (N' Dilimabaka *et al.*, 2015). Variability may also be a consequence of screening methods used. Most prevalence studies on LCMV infection utilized antibody screening methods predominantly (Becker *et al.*, 2007; Blasdell *et al.*, 2008b; Childs *et al.*, 1992; Lledo *et al.*, 2003; Morita *et al.*, 1991b; Morita *et al.*, 1996; Tagliapietra *et al.*, 2009). Limitations of results based on antibody screening include the generation of false-negative and false-positive results (Becker *et al.*, 2007; Morita *et al.*, 1991a). Furthermore LCMV strains such as clone 13 do not generate an effective humoral response making diagnoses by antibody detection impossible and the time taken for seroconversion in immunocompetent hosts may not allow for detection during outbreaks (Ahmed *et al.*, 1984; Besselsen *et al.*, 2003; Wilson & Brooks, 2010). The small sampling size of *M. musculus* rodents investigated within 2005 and 2006 (48) may also be a contributing factor to the high prevalence value obtained in the present study and may also account for the absence of LCMV infection in other rodents including *A. flavicolis* that were sampled during the outbreak making the results slightly skewed.

LCMV genome was not detected in any samples tested on a follow-up period 8 years after the outbreak (2013 and 2015). These results are not unusual. A study on the spatial and temporal dynamics of LCMV in wild

rodents in Northern Italy between 2000 and 2005 reported low relative prevalence of the virus in *Apodemus flavicolis* in 2000 in comparison to the other years of rodent trapping and screening (Tagliapietra *et al.*, 2009). There have been reports of the absence of detectable antibodies within a reservoir population at certain periods. Morita and colleagues conducted a serological survey of LCMV in wild house mice in Yokohama port, Japan between 1985 and 1989. While 6 out of 31 mice (19.4%) and 3 out of 23 mice (13.0%) captured in 1985 and 1986 respectively were positive for LCMV, mice captured between 1988 and 1989 were negative for the presence of LCMV antibody (Morita *et al.*, 1991b). The population of house mice used in the present study between 2013 and 2015 however was lower than the number required (372 out of 523) to detect any LCMV using Fischer's exact test with an expected prevalence of 17%.

The absence of LCMV genome in *M. musculus* may also be a consequence of external factors such as the surrounding environment. A complex relationship exists not only between a virus and its natural reservoir but also with climate patterns and the general ecosystem such that weather conditions, food supply and habitat quality generally affects mammalian reproductive rate. A general increase in number of reservoir species would also generally increase the frequency of interaction between the latter and other potential hosts within the same geographical region and hence increase the probability of infection (Mills, 2005). The two important weather conditions we considered in this thesis are rainfall and temperature. The 'tropic cascade' hypothesis put forward by Yates and colleagues explains how rainfall directly affects food production which in turn determines rodent population density (Yates *et al.*, 2002). This hypothesis explained the cause of the 1993 outbreak of Sin Nombre virus (SNV) which occurred in southwestern United States of America (Nichol *et al.*, 1993). It presented the El Niño–Southern Oscillation (ENSO) phenomenon of 1992 as a factor that led to increased

precipitation which in turn increased food availability for rodents and resulting in population expansion of the latter. This expansion caused increased transmission of hantavirus to human hosts and thus was responsible for the high prevalence of hantavirus pulmonary syndrome (HPS) that occurred in that region in 1993. The occurrence of ENSO between 1997 and 1998 also caused increased cases of hantavirus infection in 1998 (Yates *et al.*, 2002). The effect of rainfall on expansion of *M. musculus* and transmission of LCMV was not investigated in the present study to test this hypothesis as detailed information on the quantity of annual rainfall in parts of the United Kingdom was unavailable during the write up of this thesis.

Temperature is another environmental factor that may have accounted for the presence of detectable LCMV in the 2005/2006 cohort and its absence in the 2013/2015 cohort. Changes in temperature can have a favourable effect on the transmission of certain diseases as insect-borne diseases such as malaria, dengue fever and yellow fever thrive in warmer regions (Cannell *et al.*, 1989; Maantay & Becker, 2012). An indirect link between temperatures and outbreaks of rodent-borne diseases has been described. Results by Tersago *et al.*, 2009 revealed that high summer and autumn temperatures which occurred 2 years and 1 year before an outbreak of nephropathia epidemica (NE) would result in a high incidence of the latter (Tersago *et al.*, 2009). The study further revealed that the high summer temperature recorded in 2003 throughout Europe was responsible for the highest recorded incidence of NE which occurred in Belgium in 2005, two years afterwards. This heatwave of 2003 is described as the hottest summer in Europe for 500 years as temperatures reached as high as 40°C and 38.5°C in France and the United Kingdom respectively. Other Hantavirus infections were recorded at their highest peaks in 2005 all over Europe as well (Mailles *et al.*, 2005; Schneider & Mossong, 2005). Incidentally the outbreak of LCMV in the zoo in the current study also occurred between 2005 and 2006.

Therefore it is possible that the heatwave of 2003 was a major contributing factor to the LCMV outbreak that occurred between 2005 and 2006. The results obtained from screening of LCMV infection in rodents from Northwest England by Blasdell *et al.*, 2008 also support our results as a high percentage of the LCMV-positive rodents were sampled during the 2005 period. Through the data derived from the study by Tersago *et al.*, (2009), a successful prediction was made for another outbreak of NE to take place in 2008 based on the high summer and autumn temperatures of Europe in 2006. The highest report of 372 human cases of Hantavirus infection in Belgium was reported in 2005 and is closely followed by the report of 336 cases made in 2008 (Heyman *et al.*, 2011; Tersago *et al.*, 2009). We were not able to investigate the implications of the prediction on LCMV infection in the UK in 2008 due to the unavailability of rodent or primate samples from that period.

As stated earlier, the abundance of food in rodent communities is an important driver of population expansion. Mast trees produce heavy crops at sporadic intervals (Cannell *et al.*, 1989). Rodent population reach high densities during mast years through a number of ways including an improved chance of survival during winter as well as winter breeding (Tersago *et al.*, 2009). Reports on the effect of tree masting on the population densities of *A. flavicolis*, *M. musculus* and *M. glareolus* are well documented (Fitzgerald *et al.*, 2004; Pucek *et al.*, 1993; Tersago *et al.*, 2009). Tagliapietra *et al.*, 2009 reported a positive correlation between LCMV prevalence in *A. flavicolis* and rodent density, and this was related to tree masting (Tagliapietra *et al.*, 2009). The investigation of cases of nephropathia epidemica (NE) in Belgium yielded similar results. Hence the low population density and negative LCMV results in the rodent population sampled in this study may have been due low fruit production. The prediction of masting in trees is difficult as it varies with species and is dependent on a number of factors. The weather and time of flowering are of paramount importance. A direct relationship exists

between tree masting and temperature (Broome *et al.*, 2007; Hilton & Packham, 1997; Matthews, 1955). In a stusy of beeh not production in Swedish forests, hot summers directly preceded high mast production (Cannell *et al.*, 1989; Philipson, 1983). Periods of heat and drought can induce flowering in young spruce trees. Although the level of masting in trees in the East Midland regions before, during and after the outbreak could not be investigated, the results from the present study may have been caused by the irregular masting periods which were in turn influenced by the summer temperatures of the region. These results therefore contribute to the claims made by the various authorities on the effect of warmer temperatures and food abundance on the transmission rates of rodent-borne zoonoses (Tagliapietra *et al.*, 2009; Tersago *et al.*, 2009).

The spread of disease amongst wild populations can also be controlled by natural and human activities such as the presence of natural predators and the placement of traps and other control agents such as poisons. Seasonal outbreaks occur mainly as a result of human activities such as the elimination of natural predators and competitors or deforestation for the construction of buildings, roads and bridges which in turn cause disturbances that can encourage inter-community interactions (Howard & Fletcher, 2012; MacKay *et al.*, 2007; Patergnani *et al.*, 2010; Patterson *et al.*, 2014). The extensive use of traps in the outbreak and afterwards may have controlled further spread of the disease within the wild mice in that region, and contributed to the seeming eradication of LCMV from the population. Similar results have been reported with the elimination of the Calomys callosus, the reservoir of Machupo mammarenavirus as a measure to control disease spread of Bolivian Haemorrhagic fever. This resulted in the temporary absence of reported cases within the endemic region (Patterson *et al.*, 2014). Some predators also appear to selectively prey on a population and having preference for infected animals due to the lethargic movement and general ease of catch of the latter (Khalil *et*

al., 2016). There is no information available on the presence of rodent predators on the surroundings of the zoo and as such their effect on LCMV transmission could not be investigated further.

7.1.4.1. Comparison of Primer Performance in LCMV RNA Detection

A comparison of primer performance in LCMV screening by both groups was carried out. This revealed the primer set used by PHE had the greatest performance having detected LCMV in more samples than the other primer sets. However information on the primer sequence used by PHE was unavailable and prevented further analysis into reasons for the enhanced detection.

It is widely accepted that the parameters required for a successful primer design include primer length, GC content, melting temperature (T_m) and PCR product length (Dieffenbach *et al.*, 2003). The average primer length is normally between 15 and 30 nucleotides long (Koressaar & Remm, 2007). GC content should be between 40% and 60% with the 3' ending in G or C to facilitate binding to target template. T_m values of between 65°C and 75°C, and within 5°C of each other are generally sufficient (ThermoFisher Scientific, 2018). Such parameters were carefully considered when selecting the primer sets used in this thesis. One explanation for better detection rate in the assay by Public Health England may be the primer sequences. The primers used by Public Health England may have been gene-specific thus enhancing assay sensitivity. Degenerate primers may have less sensitivity in assays because of the lower effective concentration of each individual primer (Briese *et al.*, 2005; Jothikumar *et al.*, 2006). The inclusion of degenerate bases in the primer sequences used in this study may have increased the ability of the respective assays to detect RNA from a variety of arenaviruses at the cost of sensitivity.

As the PCR assay conducted by Public Health England detected LCMV RNA in more samples from the UK outbreak than the other assays, it would be most suitable for the molecular diagnosis of strain-specific LCMV infections. It may however be limited in its use for the detection of other LCMV strains.

The pathological findings in the LCMV-infected non-human primates revealed tubular necrosis of kidneys and acute encephalomalacia of brainstem in *T. vetulus monticola*. There was acute cell necrosis in various organs in *C. guereza occidentalis*. Disseminated intestinal adenocarcinoma was observed in *N. leucogenys*. Infections in the younger *C. geoffroyi* marmosets were marked by nephritis, hepatitis and vasculitis while the older *C. geoffroyi* marmoset developed Iron storage disease leading to possible hepatorenal syndrome.

The histopathology report revealed marked differences between primates of the same species as well as primates of different species. The summary of the findings within the 2 younger *C. geoffroyi* NHPs were similar, consisting of hepatitis, nephritis and vasculitis while infection in the older *C. geoffroyi* was only characterised by haemochromatosis, a disease caused by excess deposits of iron in the tissues which may be in turn, a result of parasitic infection in the host. *Capillaria hepatica* eggs were found in the liver of the older marmoset. The LCMV infection may however have exacerbated the condition (NHS Choices, 2016b; Pietrangelo, 2004; Uzoigwe, 2010). The necropsy findings in the older *C. geoffroyi* were insufficient to allow for suitable comparisons between it and the younger species. Age is an important determinant of disease outcome in LCMV-infected hosts. Asper and colleagues reported differences in histopathology for LCMV-infected *Cebuella pygmaea* of different ages. While infection in the liver of NHPs aged 4 years were marked by hepatocellular necrosis and degeneration, fatty change and

the presence of acidophilic (Councilman-like) bodies, infection in the liver of the 2-year old NHP was different and marked by the presence of multiple foci of inflammatory cell infiltrate with mild to moderate periportal mononuclear infiltrate and the diffused activation of Kupffer cells (Asper *et al.*, 2001). In humans, postnatal LCMV infection manifests typically as aseptic meningitis although most human cases are moderately asymptomatic and full recovery occurs after a few weeks. Prenatal LCMV infection in humans may be more severe as the virus exhibits strong tropism for the brain which can result in serious damage of the brain which is often marked by microencephaly, periventricular calcifications and hydrocephalus. Chorioretinitis is also a common outcome with congenital LCMV infection (Bonthius, 2012). Age-dependent pathogenesis of LCMV-infection has also been demonstrated in laboratory rats. These animal models replicate all of the pathology observed in affected humans. LCMV infection the rats displayed different targets of infection, viral titres, immune response to infection and location of neuropathology. Microcephaly in younger rat pups was much more severe especially when inoculation was made on day 1, day 4 and day 6. Inoculation after day 10 caused no changes in brain weight (Bonthius & Perlman, 2007b; Bonthius *et al.*, 2007).

Although the disease phenotypes observed in the non-callitrichids were different from those in the callitrichids in this thesis, similar findings have been reported in LCMV-infected NHPs from other studies (Montali *et al.*, 1989; Ramsay *et al.*, 1989). Acute cell necrosis in various organs was observed in *C. guereza occidentalis*; disseminated intestinal adecarcinoma was observed in *N. leucogenys* and tubular necrosis of kidneys and acute encephalomalacia of brainstem was observed in *T. vetulus monticola*. The necrosis observed in the kidneys of *T. vetulus monticola* have also been observed in LCMV-infected tamarins and marmosets (Montali *et al.*, 1995). The findings provided by the veterinarians in this thesis did not include precise information on the

necrotic foci observed. This also applies to the observation of acute cell necrosis of various organs in *C. guereza occidentalis* as the absence of specific details regarding the actual organs affected made comparisons of these observations with similar results in published literature challenging. The observations in *C. guereza occidentalis* however support the description of callitrichid hepatitis as a 'widely disseminated systemic' infection. Necrosis of various organs has not only been reported in LCMV-infected callitrichids but also in Lassa fever infections in both human and experimentally-infected NHPs (Callis *et al.*, 1982; Walker *et al.*, 1982a; Walker *et al.*, 1982b).

7.2. Phylogenetic Analyses of Novel LCMV strains

Previously identified LCMV strains have been grouped into 4 lineages (Albarino *et al.*, 2010a; Ledesma *et al.*, 2009; N' Dilimabaka *et al.*, 2015). Most of the strains fall within lineage I and were isolated from the United States of America. These include the archetype strains Armstrong and W.E (Armstrong & Lillie, 1934; Rivers & McNair Scott, 1935). Phylogenetic analyses of the novel LCMV detected in the present study revealed that the latter clustered together within lineage I. Interestingly, lineage I consists of strains most frequently associated with human disease. Furthermore all previously LCMV strains isolated from NHPs (including those from this study) all fall within this same lineage.

The most probable source of infection for the zoonotic outbreak was direct or indirect contact with LCMV-infected mice or mouse-contaminated products such as saliva, urine or faeces as *M. musculus* sampled from the zoo surroundings also tested positive for LCMV. Furthermore LCMV isolated from the rodents and NHPs were strongly linked in the phylogenetic trees. The novel LCMV sequences came from a common ancestor and that the NHPs were infected by repeated

introduction from the *M. musculus*. Infection of NHPs through contact with LCMV-infected house mice or their products is clearly reported in published literature as the source of previous zoo outbreaks (Barton *et al.*, 2002; National Research Council (US) Committee on Occupational Health and Safety in the Care and Use of Nonhuman Primates, 2003). Although there is strong regulation on the feeding of neonatal mice to NHPs, infected wild mice can easily find their way to feed otherwise prepared for the zoo animals, contaminate them thereby transferring the virus to unsuspecting callitrichids in the process (Rousseau *et al.*, 1997). Interspecies transmission of LCMV amongst primates has been hypothesised (Asper *et al.*, 2001; Montali *et al.*, 1989; Montali *et al.*, 1993) but this remains to be demonstrated. Nonetheless, it is unlikely that LCMV was transmitted amongst the NHPs in the present outbreak. Ramsay and colleagues reported cases of CH where healthy NHPs remained seronegative despite being placed in intimate contact with infected ones. There are reports of seroconversion of veterinarians exposed to infected NHPs but there are no reports of infection transfer from infected humans to NHPs (Montali *et al.*, 1989; Montali *et al.*, 1993; Ramsay *et al.*, 1989).

The novel strains also formed a unique cluster when analysed with other UK sequences obtained from the study by Blasdell and colleagues (Blasdell *et al.*, 2008b) therefore hinting that the novel strains are unique to the East Midland region of the United Kingdom. The small sequence size used in the analyses was taken into account as larger sequences from the strains identified by Blasdell et al., (2008) are unavailable on the NCBI database. Results from analysis of the small fragments had low bootstrap support which is an indication of its accuracy. Thus larger sequences would be required to provide a more accurate phylogenetic analyses between the strains the UK strains.

The placement of all discovered LCMV strains into 4 lineages is by no means exhaustive and can be further extended by the discovery of more diverse strains especially in new hosts (Ledesma *et al.*, 2009) or in parts of the world that have been under-investigated. LASV a close relative of LCMV, was initially thought to be comprised of 4 lineages (Bowen *et al.*, 2000; Ehichioya *et al.*, 2011). However increased availability of full-length sequences from Ivory Coast and Mali (Gunther *et al.*, 2000; Safronetz *et al.*, 2010; Safronetz *et al.*, 2013) have led to the discovery of a fifth lineage (Manning *et al.*, 2015). Also MACV although a new world arenavirus, comprises of eight distinct lineages (Aguilar *et al.*, 2009).

Phylogenetic analyses reveal a relationship between geography and genetic clustering in the Lassa virus strains and other clade B new world arenaviruses. This is not the case with LCMV as the Bayesian coalescent analysis of GPC sequences did not reveal any clear correlation of virus genetic lineages to particular geographic locations. This was also reported by Albarino and colleagues who analysed both S and L segments (Albarino *et al.*, 2010a). This observation is perhaps a consequence of the complex global distribution of *M. musculus* (Cucchi *et al.*, 2005; Suzuki *et al.*, 2004) and is further affected by the commercial transport of infected rodents to be used as pets and animal food (Amman *et al.*, 2007; Knust *et al.*, 2014).

7.3. Molecular Characterisation of the Novel LCMV Strains

In previous zoo outbreaks of LCMV involving NHPs, viral sequences from infected rodents were unavailable and as such, comparisons could not be made between LCMV strains obtained from the reservoir and those obtained from host species (Asper *et al.*, 2001; Stephensen *et al.*, 1991).

This is therefore the first report on the molecular characterisation of rodent-derived and NHP-derived strains from the same outbreak.

A comparison of the amino acid sequences of LCMV from published studies with those obtained from this thesis revealed various substitutions in the former. These substitutions were spread all over the three domains of the GPC. The amino acid sequences of strains NA14 and NA74 glycoprotein are identical. This was hardly surprising as both were amplified from *C. geoffroyi*. The nucleotide sequences were however different at various positions and therefore rules out any possibility of contamination. RNA was extracted from all tissues using separate sterile instruments. In addition, the assays were also repeated.

A mutational 'hotspot' was observed at positions 119 to 247 on GP1 domain. GP1 mediates receptor binding of arenaviruses to host cells. Substitutions in GP1 have been reported to play an important role in host receptor affinity, tissue tropism and disease outcome (Kunz *et al.*, 2001; Smelt *et al.*, 2001). As a whole, the mutational 'hotspot' present between positions 119 and 247 were interesting observations as substitutions within the GP1 will shed light on potential mutations required for a cross-species transmission. At position 119, most identified sequences have serine (S) but for a few others which contained glutamine (Q), histidine (H), alanine (A), arginine (R), threonine (T), asparagine (N) and aspartic acid (D) at this position. The presence of glutamine in position 119 of GP1 in the LCMV detected in *C. guereza occidentalis* may be indicative of its role in virus crossover from rodents to this primate species but this is also unlikely as other published strains including M1, M2 and 811316 also have glutamine at that position and have only been detected in rodent hosts. The M1 and M2 strains where isolated in Japan from the spleen of a mouse colony from Austria but belong to lineage II (Ike *et al.*,

2007) while strain 811316 a lineage I strain, was isolated from a human transplant recipient (Albarino *et al.*, 2010a).

Most of the aminoacid substitutions were common in both rodent and NHP LCMV sequences. Therefore the role of these in cross-species transmission is highly unlikely. An unexpected observation however was the amino acid substitutions at position 211. All sequenced LCMV strains obtained from *M. musculus* rodents had lysine (K) at this position while the strains obtained from the NHPs had threonine at the same position. This consistency across primate-derived and rodent-derived LCMV strains was truly surprising and may be a possible determinant for cross-species transmission. Hastie and colleagues analysed the effect of amino acid substitutions at position 211 on LCMV binding affinity to alphadystroglycan. WE54, a high-affinity strain has threonine at this position while low-affinity strains (WE2.2 and WE HPI) have alanine at this position. No effect was observed and binding affinity was reported as most likely a consequence of substitutions at other positions in the glycoprotein (Hastie *et al.*, 2016). Results from further investigation into amino acids at this position in other published LCMV strains derived from rodents and NHPs were however not consistent with the findings in this thesis as most human, NHP and rodent-derived LCMV strains had threonine at position 211, or occasionally alanine and lysine.

An extensive exploration of the passage histories of published LCMV strains was conducted to determine the possible effects of passaging on amino acid identity at position 211. This also did not reveal any noticeable trend. The impact of passaging on human-derived strains such as Armstrong and Douglas-4707 could not be established as both had been passaged in rodents and NHPs. This was also applicable to OQ28, a rodent-derived strain that had been passaged both in Vero cells and mouse brain.

A single amino acid change (phenylalanine to leucine or isoleucine) at position 260 on the GP1 has also been reported as an important determinant of cell tropism and the establishment of LCMV persistent infection in mice (Matloubian *et al.*, 1990; Salvato *et al.*, 1991). This is a consequence of a strong affinity to alpha-dystroglycan receptor. LCMV strains with weaker affinities to alpha-dystroglycan are easily cleared from the host after a short period (Sullivan *et al.*, 2011). A similar change at position 153 from serine-to-phenylalanine has also been implicated as a determinant for alpha-dystroglycan affinity. LCMV strains with serine at position 153 exhibited high affinity for alpha-dystroglycan (Hastie *et al.*, 2016; Smelt *et al.*, 2001). The complete sequence of novel LCMV strains in this thesis all had serine at position 153 and leucine and isoleucine at position 260.

GP2 facilitates fusion within the endosome (Igonet *et al.*, 2011). Amino acid substitutions within this domain have been linked to susceptibility of arenaviruses to various antiviral agents (Larson *et al.*, 2008; Ngo *et al.*, 2015). Position 438 of NA76 detected in *N. leucogenys* had glutamic acid (E) in the place of aspartic acid (D) on position 438. All LCMV strains detected in this thesis as well as other published strains from the NCBI database have aspartic acid at this position suggests that the occurrence of glutamic acid on position 438 in the NHP-derived LCMV strain may have been due to an error in the sequencing of the viral genome.

7.4. Infectivity Assay of Pseudoviruses Bearing Full-length GPC from Rodent-derived and NHP-derived LCMV Strains

Attempts were made to investigate virus entry into host cells using pseudoviruses bearing the full GPC gene of three of the novel LCMV strains derived from rodents and NHPs. Pseudoviruses made from the full GPC of the SB18 strain obtained from *M. musculus* had the highest infectivity in Huh7 cells while pseudoviruses made from the full GPC of the NA14 LCMV strain obtained from *C. geoffroyi* had the lowest infectivity in Huh7 cells. This result was surprising as the Huh7 cell is a human hepatocyte-derived carcinoma cell line (Nakabayashi *et al.*, 1982) and as such the pseudoviruses from the NHP were expected to have the highest infectivity.

One interpretation for the results is the different binding affinities of the glycoproteins to a suitable receptor on the Huh7 cells. Alpha-dystroglycan has been reported to be the host receptor for Old World arenaviruses (Cao *et al.*, 1998). This protein is one of two that constitute dystroglycan (DG) which is expressed by a broad range of primate and murine cells (Durbeej *et al.*, 1998). LCMV strains differ in their affinity to alphadystroglycan and thus the possibility of an alternative receptor has been put forward (Kunz *et al.*, 2004b). The identity of this receptor remains unknown. Shimojima and Kawaoka suggested that Axl and Tyro3 (from the TAM family) and DC-SIGN and LSECtin (from the C-type lectin family), which are also alternative receptors for LASV may be likely candidates (Shimojima & Kawaoka, 2012; Shimojima *et al.*, 2012). This may explain the unsuccessful infection of the NA14-derived pseudovirus clones of the hepatocellular carcinoma cells despite the presence of serine at position 153 and leucine at position 260 of their GPC sequences, which are reported as being responsible for high affinity of

LCMV GP to α-DG (Smelt *et al.*, 2001; Sullivan *et al.*, 2011). NA29- and SB18-derived pseudovirus clones also had serine at position 153 and leucine at position 260 of their respective GPC sequences and yet successfully infected hepatocellular carcinoma cells.

Amino acid variations in rodent and NHP alpha-dystroglycan gene were also investigated to determine if a similar trend existed in the known host receptor. Kunz and colleagues determined the receptor binding site of LCMV to alpha-dystroglycan to be between amino acid positions 169 to 408 (Kunz *et al.*, 2001). Positions 303, 346 and 410 on the primate-derived sequences (including humans) were occupied by proline, arginine and alanine respectively while the rodent-derived sequences had serine, glutamine, threonine (or valine) respectively at those positions. The specific amino acids within this region of the host receptor associated with virus binding have not yet been discovered. The results from this thesis put forward these three loci as having roles in virus GP and host receptor interaction. The outcome of infection of various LCMV strains with different binding affinities to alphadystroglycan on rodent and primate cell lines will have to be investigated to determine the implication of these substitutions on virus GP - host cell receptor interaction.

8. Conclusion and Future Work

The current global response to emerging infectious diseases is mostly reactive as the focus is on control and protection rather than prevention. Huge amounts of money are used caring for those affected by outbreaks. This has had severe economic impact especially on developing countries (Anthony *et al.*, 2013b; Morens & Fauci, 2013). The early detection and characterization of novel pathogens in potential animal reservoirs remains a viable strategy for the prevention of outbreaks (Jones *et al.*, 2008; Woolhouse & Gowtage-Sequeria, 2005).

Rodents serve as important reservoirs for the transmission of many zoonoses to humans and wildlife (Lõhmus *et al.*, 2013). Most discovered arenaviruses are associated with a rodent host (Charrel & de Lamballerie, 2010). The absence of a novel arenavirus or filovirus in any of the rodents screened does not infer their absence in UK rodent populations as a whole. Successful detection is dependent on various factors including sample number, study region and period of sampling as well as the reservoir species screened. Future studies will involve the expansion of the cDNA library to include rodents from other species sampled from various regions in the United Kingdom. Samples will also be obtained from rodent pets as these present a high risk for transmission of arenaviruses to pet owners (Amman *et al.*, 2007; Knust *et al.*, 2014). The role of rodents in arenavirus infection of mammals and reptiles (Asper *et al.*, 2001; Stenglein *et al.*, 2012) also hints at the possibility that other small animals that feed on rodents could also be infected. Thus tissue samples will also be obtained from cats and screened for the presence of arenavirus RNA. The detection of Lloviu cuevavirus RNA in Spanish bats would result in extensive screening tissues obtained from bats in the United Kingdom for filovirus RNA. Screening would not be restricted to the detection of filoviruses and arenaviruses alone and will include those belonging to other families (Anthony *et al.*, 2013b; Tong *et al.*, 2008; Tsoleridis *et al.*, 2016). Assays employed will include standard

RT-PCR assays as well as quantitative PCR assays. Viral RNA will be used as positive controls in such assays using gene-specific and broadly-reactive primers. Synthetic plasmids will only be used as alternative controls if suitable viral nucleic acids are unavailable as the results from the thesis show. Serological assays will also be developed to detect the presence of antigens or antibodies especially in samples that were negative for the presence of viral genome. This is will confirm the exposure of mammals to the virus. Samples will also be obtained from humans (especially veterinarians) that had contact with the infected animals and screened for antibodies. Other techniques will include virus isolation. Morita and colleagues successfully isolated virus particles from antibody-negative mice (Morita *et al.*, 1991a).

Molecular characterisation of LCMV glycoprotein and alphadystroglycan receptor from and NHPs revealed consistency in the amino acid identities at particular positions in the respective proteins. This trend was also observed in the rodent-derived LCMV GPC. Future investigations will include whole genome sequencing of all novel LCMV to allow comparisons of other gene segments such as the polymerase gene which appears to play a critical role in suppression of LCMV-specific CTL responses, causing to virus persistence in host tissue (Salvato *et al.*, 1988). The identification of virus and host proteins will also result in further analysis of the GPC-alphadystroglycan interactome using Mathematical and computational simulations. These have been employed as prediction tools for understanding receptor binding and host-type selectivity in virus variants including HIV and influenza (Behera *et al.*, 2015; Tastan *et al.*, 2009).

Pseudovirus infectivity assays were conducted to assess the impact of these substitutions on viral entry into hepatocellular carcinoma cells and the results argue the existence of alternative host receptors which is supported by other scientific groups (Kunz *et al.*, 2004a; Shimojima & Kawaoka, 2012). Assays involving the infection of other permissible

rodent and NHP-derived cell lines having reduced expression of α-DG, (Shimojima *et al.*, 2012) with pseudovirus clones generated using LCMV GPC (where position 211 is substituted with other amino acid residues) will help elucidate the role of position 211 in cross-species transmission. Virus neutralisation assays will also be developed to detect virus neutralising antibodies. These have been used for a number of viruses including Nipah virus and HIV (Kishishita *et al.*, 2013; Tamin *et al.*, 2009). These assays have the potential to be rapid and cost-effective in diagnoses and may prove valuable in basic research and vaccine development.

Appendices

Appendix 1: Filovirus Sequences Used in Phylogenetic Analyses

Strain	NCBI Accession Number	Source
Bundibugyo ebolavirus	FJ217161.1	(Towner *et al.*, 2008)
Cote d'Ivoire ebolavirus	FJ217162.1	(Towner *et al.*, 2008)
Reston strain Reston08-E	FJ621585.1	(Barrette *et al.*, 2009)
Lake Victoria marburgvirus strain Uganda 331Bat2007	FJ750959.1	(Towner *et al.*, 2009)
Sudan ebolavirus strain Boniface	FJ968794.1	Unpublished
Lake Victoria marburgvirus strain Ci67	GQ433353.1	(Lofts *et al.*, 2011)
Lloviu virus strain MS-Liver-86/2003	JF828358.1	(Negredo *et al.*, 2011)
Bundibugyo ebolavirus isolate EboBund-14 2012	KC545396.1	(Albarino *et al.*, 2013)
Zaire ebolavirus H. sapiens-tc/ZAI/1995/Zaire-199510621	KR824526.1	(Sozhamannan *et al.*, 2015)
Zaire ebolavirus isolate Ebola virus/H.sapiens-wt/GIN/2014/Makona-C07	KT013256.4	Unpublished
Sudan virus/C.porcellus-lab/SSD/1976/Nzara-Boneface	KT878488.1	(Wong *et al.*, 2016)
Tai Forest virus/H. sapiens-tc/CIV/1994/Tai Forest-CDC807212	KU182910.1	Direct submission
Reston virus/M.fascicularis-tc/USA/1989/Philippines89-AZ-1435, complete genome	KY008770.1	Direct submission
Sudan ebolavirus isolate IRF0154	KY425644.1	Direct submission

Strain	NCBI Accession Number	Source
Ebola virus/Hsapiens-tc/GAB/2011/inoculum strain	KY471124.1	(Piorkowski *et al.*, 2017)
Marburg marburgvirus isolate Marburg virus/H.sapiens-tc/KEN/1980/Mt. Elgon-Musoke	NC_001608.3	(Enterlein *et al.*, 2006)
Reston ebolavirus isolate Reston virus/M.fascicularis-tc/USA/1989/Philippines89-Pennsylvania	NC_004161.1	(Groseth *et al.*, 2002)
Tai Forest ebolavirus isolate Tai Forest virus/H.sapiens-tc/CIV/1994/Pauleoula-CI, complete genome	NC_014372.1	(Towner *et al.*, 2008)
Bundibugyo ebolavirus isolate Bundibugyo virus/H.sapiens-tc/UGA/2007/Butalya-811250	NC_014373.1	(Towner *et al.*, 2008)
Lloviu cuevavirus isolate Lloviu virus/M.schreibersii-wt/ESP/2003/Asturias-Bat86	NC_016144.1	(Negredo *et al.*, 2011)

Appendix 2: Arenavirus Sequences Used in Phylogenetic Analyses

Strain	NCBI Accession Number	Source
Latino virus	NC_010760.1	(Charrel *et al.*, 2008)
Oliverus virus	NC_010250.1	(Charrel *et al.*, 2003)
Allpahuayo virus	NC_010249.1	(Charrel *et al.*, 2003)
Pirital virus	NC_005897.1	(Cajimat & Fulhorst, 2004)
Pichinde virus	NC_006439.1	(Zhang *et al.*, 2001)
Parana virus	NC_010761.1	(Charrel *et al.*, 2008)
Flexal virus	NC_010759.1	(Charrel *et al.*, 2008)
Bear Canyon virus	NC_010255.1	(Cajimat *et al.*, 2007)
Whitewater Arroyo virus	NC_010703.1	(Cajimat *et al.*, 2007)
Tamiami virus	NC_010702.1	(Cajimat *et al.*, 2007)
Lunk virus NKS-1	NC_018711.1	(Ishii *et al.*, 2012)
Lymphocytic choriomeningitis virus	NC_004291.1	(Salvato *et al.*, 1989)
Merino Walk virus	NC_023763.1	(Palacios *et al.*, 2010)
Ippy virus	NC_007906.1	(Emonet *et al.*, 2006)
Lassa virus	NC_004297.1	(Djavani *et al.*, 1997)
Luna virus	NC_016153.1	(Ishii *et al.*, 2011)
Mobala virus	NC_007904.1	(Emonet *et al.*, 2006)
Mopeia virus AN20410	NC_006574.1	Unpublished
CAS Virus	NC_018484.1	(Stenglein *et al.*, 2012)
University of Helsinki virus strain UHV-1	NC_023765.1	(Hetzel *et al.*, 2013)
Golden Gate virus	NC_018482.1	(Stenglein *et al.*, 2012)
Cupixi virus	NC_010252.1	(Charrel *et al.*, 2003)
Amapari virus	NC_010251.1	(Charrel *et al.*, 2003)
Sabia virus	NC_006313.1	Direct Submission
Junin virus	NC_005080.1	Direct Submission
Machupo virus	NC_005079.1	Direct submission
Tacaribe virus	NC_004292.1	(Iapalucci *et al.*, 1989)
Guanarito virus	NC_005082.1	Direct submission

Strain	NCBI Accession Number	Source
Lujo virus	NC_012777.1	(Briese *et al.*, 2009)
Gairo virus isolate TZ-27421	NC_026247.1	(Gryseels *et al.*, 2015)
Mariental virus strain N27 MRMi.n9	NC_027136.1	Unpublished
Okahandja virus strain N73 OkhMi.n4	NC_027137.1	Unpublished
Wenzhou virus isolate Rn-242	NC_026019.1	(Li *et al.*, 2015)
Arenaviridae sp. 13ZR68	AB972429.1	Unpublished
Loie River virus isolate R5074	KC669693.1	(Blasdell *et al.*, 2016)

Appendix 3: Origins of LCMV Strains used in this Thesis

Strain	Collection date	Isolation/ Passage History	NCBI Accession no.	Source
811316	Massachusetts, USA, 2008	Blood of transplant patient	FJ607022, FJ607031	(Albarino *et al.*, 2010a)
810850	Rhode Island, USA, 2005	Hamster kidney	FJ607021, FJ607030	(Albarino *et al.*, 2010a)
Makokou	Gabon, 2015	House mouse liver and spleen	KM523323, KM 882857	(N'Dilimabaka *et al.,* 2015)
Douglas-4707	New York, USA, 1947	Intracranial inoculation of suckling mice with CSF from patient suffering from aseptic meningitis; One passage in VE6 in 2005	FJ607024; FJ607035	(Albarino *et al.*, 2010a)
810885	Michigan, USA, 2005	Mouse spleen	FJ607023, FJ607032	(Albarino *et al.*, 2010a)

Strain	Collection date	Isolation/ Passage History	NCBI Accession no.	Source
810896	Ohio, USA, 2005	Hamster kidney	FJ607026, FJ607037	(Albarino *et al.*, 2010a)
810366	California, USA, 2003	CSF of congenitally-infected infant	FJ607019, FJ607028	(Albarino *et al.*, 2010a)
810935	Georgia, USA, 1984	Human CSF; Passaged in Vero cells	FJ607020, FJ607029	(Albarino *et al.*, 2010a)
EEB-7	Spain, 2008	CSF of patients with acute meningitis; passaged in Vero cells	JN872494, JN872495	(Perez-Ruiz *et al.,* 2012)
810362	Wisconsin, USA, 2003	Human CSF	FJ607027, FJ607038	(Albarino *et al.*, 2010a)
Armstrong	Missouri, USA, 1933	Patient suffering from encephalitis; passaged in mouse and monkey brains	AY847351, M20869	(Armstrong & Lillie, 1934; Grande-Perez *et al.*, 2005; Salvato *et al.*, 1988)
WE	New York, USA, 1935	Meningo-encephalitis patient	M22138; AF004519	(Romanowski *et al.*, 1985)

Strain	Collection date	Isolation/ Passage History	NCBI Accession no.	Source
WE-UBC-57135	New York, USA, 1935	Meningo-encephalitis patient; 7 passages in mouse brain; One passage in VE6 in 2005	FJ607025, FJ607036	(Albarino *et al.*, 2010a)
WE-UBC-A337	New York, USA, 1935	Meningo-encephalitis patient; Seven passages in mouse brain	FJ607034	(Albarino *et al.*, 2010a)
CHV1, CHV2, CHV3	Okhlahoma, USA, 1986	Monkey liver. CHV1 and CHV3 were isolated from liver of experimentally infected marmoset; CHV3 was isolated from infected Vero cells.	U10157, U10158, U10159	(Stephensen *et al.*, 1995)
WHI	New York, USA, 1949	Human CSF.	FJ607033	(Albarino *et al.*, 2010a)

Strain	Collection date	Isolation/ Passage History	NCBI Accession no.	Source
Marseille 12	France, 2004	House mouse kidney; Passaged in Vero cells	DQ286932, DQ286931	(Emonet *et al.*, 2007)
CH-5692, CH-5871	Germany, 1999; Germany, 2000	Monkey spleen and serum.	AY363903, AF325214, AF325215	(Asper *et al.*, 2001)
HP65-2009	France, 2012	Mouse; Passaged in Vero cells	JN546573, JN546574, JN546575, JN546576	(Yama *et al.*, 2012)
Yale	Conneticut, USA, 1977	Mouse	DQ118959	(Albarino *et al.*, 2010a)
MX	Slovakia, 1998	Persistently infected cell line	EU195888, EU195889	(Tomaskova *et al.*, 2008)
LE	France, 2006	Amniotic fluid in congenital infection	EF164923	(Albarino *et al.*, 2010a)
Bulgaria	Bulgaria, 1956	Patient with LCMV infection	GQ862981, GQ862982	(Albarino *et al.*, 2010a)

Strain	Collection date	Isolation/ Passage History	NCBI Accession no.	Source
OQ28	Japan, 1990	House mouse spleen; passaged in Vero cells and mouse brain	AB627952, AB627955	(Takagi *et al.*, 2012)
BRC	Japan, 2005	House mouse kidney; passaged in mouse brain	AB627953, AB627956	(Takagi *et al.,* 2012)
M1, M2	Austria, 2005	House mouse spleen	AB261990, AB261991	(Ike *et al.*, 2007)
CABN, GR01, SN05	Spain, 2004	Wood mouse lung	FJ895882, FJ895883, FJ895884	(Ledesma *et al.*, 2009)
BU1-8, MM9,MM10, MM16, MC1, MC24, M20, HM13, HM14, C1, C2, C6, C9, C8, C20, C21, BU12-87	United Kingdom, 2008	House mouse and wood mouse serum	DQ275199-DQ275295	(Blasdell *et al.*, 2008b)

Appendix 4: Cytochrome B Sequences Used in Phylogenetic Analyses

Organism	NCBI Accession Number	Source
Mus musculus	LC062067.1	(Hayashi *et al.*, 2015)
Cavia porcellus	AY228361.1	Unpublished
Microtus agrestis	AF159402.1	(Martin *et al.*, 2000)
Mesocricetus auratus	KT626636.1	Unpublished
Myodes glareolus	KX815385.1	(Rosenfeld *et al.*, 2017)
Apodemus sylvaticus	AF159395.1	(Martin *et al.*, 2000)
Callithrix geoffroyi	KT626643.1	Unpublished
Nomascus leucogenys	GU594999.1	(Thinh *et al.*, 2010)
Colobus guereza occidentalis	U38264.1	(Collura & Stewart, 1995)
Callithrix jacchus	KM588314.1	(Wang *et al.*, 2016)
Leontopithecus rosalia	KR528404.1	(Muniz *et al.*, 2015)
Saguinus imperator	HM368019.1	(Matauschek *et al.*, 2011)
Saguinus Oedipus	KC757409.1	(Finstermeier *et al.*, 2013)

Appendix 5: Alphadystroglycan Sequences Used in Phylogenetic Analyses

Organism	NCBI Accession Number	Source
Homo sapiens	L19711.1	(Ibraghimov-Beskrovnaya *et al.*, 1993)
Propithecus coquereli	XM_012637972.1	Model
Pan troglodytes	XM_016941097.1	Model
Pan paniscus	XM_014344252.1	Model
Microcebus murinus	XM_012771288.2	Model
Aotus nancymaae	XM_012467733.1	Model
Nomascus leucogenys	XM_003257072.3	Model
Otolemur garnettii	XM_012811930.1	Model
Colobus angolensis	XM_011939083.1	Model
Callithrix jacchus	XM_017964905.1	Model
Mesocricetus auratus	XM_013116972.2	Model
Cavia porcellus	XM_003476671.3	Model
Microtus ochrogaster	XM_005347896.2	Model
Mus musculus	NM_001276493.1	(Fruh *et al.*, 2016)

Appendix 6: Oligonucleotides used in this study

Name	Nucleotide Sequence	Gene	Source
Cyt_421F	TGAGGBGCYACAGTWATYACAAAC	Cytochrome B	Present study
Cyt_840R	CGYAGGATDGCRTATGCRAATA		
ADG_460F	ATYGAGGTCTACCCYGAAGACCA	Dystroglycan	Present study
ADG_1297R	ACACCTGTYACTGCCATTGGRCC		
GAPDH_F	CCATCTTCCAGGAGCGAGA	Glyceraldehyde 3-phosphate dehydrogenase	(Tsoleridis *et al.*, 2016)
GAPDH_R	GCCTGCTTCACCACCTTCT		
LCSF	GGACAAGGGATCTTGGCATAAACGTCC AGGTGTTTTTACGG	Colicin E9 Plasmid	Present study
LCSR	CTTTTTGGACTAATGAACTTCGAATAACT GCATCCCTGGT		
LJSF	GGTCAGGGTATATTGCATAAACGTCCAG GTGTTTTTACGG		
LJSR	CTTTTGGGACTGACAAACTTCGAATAAC TGCATCCCTGGT		

Name	Nucleotide Sequence	Gene		Source
IPSF	GGGCAGGGAATCCTGCACAAACGTCCA GGTGTTTTTACGG	Colicin Plasmid	E9	Present study
IPSR	CTTTTTGGGCTTATAAACTTCGAATAACT GCATCCCTGGT			
LASVF	GGGCAAGGTATTCTACATAAACGTCCAG GTGTTTTTACGG			
LASVR	CTTTTGGGACTCACAAATTTCGAATAAC TGCATCCCTGGT			
OLSF	GGTCAAGGCATTCTTCACAAACGTCCAG GTGTTTTTACGG			
OLSR	GTTTTAGGGCTTACAAATTTCGAATAAC TGCATCCCTGGT			
GTSF	GGACAGGGCATACTTCACAAACGTCCA GGTGTTTTTACGG			
GTSR	CTCTTGGGGCTCACAAATTTCGAATAAC TGCATCCCTGGT			
PCSF	GGTCAAGGCATTTTGCACAAACGTCCA GGTGTTTTTACGG			

Name	Nucleotide Sequence	Gene		Source
PCSR	GTTTTAGGACTCACAAATTTCGAATAACTGCATCCCTGGT	Colicin Plasmid	E9	Present study
LCMV_F	GGACAAGGGATCTTGCATAA			
LCMV_R	CTTTTTGGACTAATGAACTT			
LJVF	GGTCAGGGTATATTGCATAA			
LJVR	CTTTTGGGACTGACAAACTT			
IPPYF	GGGCAGGGAATCCTGCACAA			
IPPYR	CTTTTTGGGCTTATAAACTT			
LASF	GGGCAAGGTATTCTACATAA			
LASR	CTTTTGGGACTCACAAATTT			
OLVF	GGTCAAGGCATTCTTCACAA			
OLVR	GTTTTAGGGCTTACAAATTT			
GNRF	GGACAGGGCATACTTCACAA			
GNRR	CTCTTGGGGCTCACAAATTT			

Name	Nucleotide Sequence	Gene	Source
PICF	GGTCAAGGCATTTTGCACAA	Colicin E9 Plasmid	Present study
PICR	GTTTTAGGACTCACAAATTT		
CVSF	TGGGCGGGATTCAACAATTTACGTCCA GGTGTTTTTACGG		
CVSR	GGCCCTCCGACTTGTTCCTTCGAATAAC TGCATCCCTGGT		
GGSF	TGGGCAGGGTTTAACAATTTACGTCCAG GTGTTTTTACGG		
GGSR	GGTCCACCAACCTGCTCTTTCGAATAAC TGCATCCCTGGT		
UHSF	TGGGCTGGATTCAACGATTTACGTCCAG GTGTTTTTACGG		
UHSR	GGTCCACCCACTTGTTCTTTCGAATAAC TGCATCCCTGGT		
CAS_F	TGGGCGGGATTCAACAATTT		
CAS_R	GGCCCTCCGACTTGTTCCTT		
GG_F	TGGGCAGGGTTTAACAATTT		

Name	Nucleotide Sequence	Gene	Source
GG_R	GGTCCACCAACCTGCTCTTT	Colicin E9 Plasmid	Present study
UH_F	TGGGCTGGATTCAACGATTT		
UH_R	GGTCCACCCACTTGTTCTTT		
LVSF	GCATTCCCTAGTAACATGATGGTACGTC CAGGTGTTTTTACGG		
LVSR	GCACATGATATGCATGTCCACGAATAAC TGCATCCCTGGT		
RSF	GCCTTCCCCAGTAACATGATGGTACGTC CAGGTGTTTTTACGG		
RSR	GCACAGGATATACTCGTCCACGAATAAC TGCATCCCTGGT		
LIOSF	GCATTTCCGAGTAATATGATGGTACGTC CAGGTGTTTTTACGG		
LIOSR	GCACAAGAAATACTTGTCCACGAATAAC TGCATCCCTGGT		
FILO_F1	GCMTTYCCZAGYAAYATGATGGT	Filovirus L gene	Present study
FILO_R1	GCACAZGAZATRGWZGTCCA		

Name	Nucleotide Sequence	Gene	Source
LVM_F1	GCATTCCCTAGTAACATGATGGT	Filovirus L gene	Present study
LVM_R1	GCACATGATATGCATGTCCA		
RES_F1	GCCTTCCCCAGTAACATGATGGT		
RES_R1	GCACAGGATATACTCGTCCA		
LIO_F1	GCATTTCCGAGTAATATGATGGT		
LIO_R1	GCACAAGAAATACCTGTCCA		
MAMF	GGICARGGIATHYTNCAHAA	Arenavirus L gene	
MAMR	STYTTIGGRCTIAYRAAYTT		
MAMF2	GGNCARGGNATHYTNCAHAA		
MAMR2	STYTTNGGRCTNAYRAAYTT		
REPF	TGGGCDGGRTTYAACRATTT		
REPR	GGYCCWCCVACYTGYTCYTT		
LVL_3359D_Y+	AGAATCAGTGAAAGGGAAAGCAAYTC		(Vieth *et al.*, 2007)

Name	Nucleotide Sequence	Gene	Source
LVL_3754A_R-	CACATCATTGGTCCCCATTTACTATGRTC	Arenavirus L gene	(Vieth *et al.*, 2007)
LVL_3359G_Y+	AGAATTAGTGAAAGGGAGAGTAAYTC		
LVL_3754D_R-	CACATCATTGGTCCCCATTTACTGTGRTC		
DLCMF	GGRCARGGRATHYTDCAYAAYGC	LCMV L gene	Present study
DLCMR	ACRCTYTTZGGRCTRATRAAYTTRTT		
LCMV_NP_1F	ATGCCVAGYYTRACHATGGC	Arenavirus S gene	(Perez-Ruiz *et al.*, 2012)
LCMV_NP_2R	CCHACYTGRTCNGADACRAACAT		
Arena-1^{+}	CGCACCGDGGATCCTAGGC		(Asper *et al.*, 2001)
LCMV-S 13^{+}	TatggcgcgcCTAGGCTTTTTGGATTGCGCTTT		
LCMV-S 322^{-}	GACATRTCAAACTCCACTGA		
LMCV_GP_0	GGYCARMTHRTSACRATGTT		Present study
LCMV_11R	GARCAHGCRTTGGGCATNGT		
LCMVGP111F	ACNATGCCCAAYGCDTGYTC		

Name	Nucleotide Sequence	Gene	Source
LCMVGP22R	AACATRTCYARVACYCTNCC		
LCMV_GPC_1F	TARTTRCARTANGGYACNCCCAT	Arenavirus S gene	(Perez-Ruiz *et al.*, 2012)
LCMV_GPC_2R	AGRGGNAGRGTBYTRGAYATGTT		
LCMVGP5	TRATWGAYTAYAAYAARGCHGC		Present study
LCMVGP6	AGCGTCTYTTCCARAYNGT		
GPF1	GgctagcgtttaaacttaagctcaccATGGGCCAGATAGTGACG		
GPF2	GAACACGCATTGGGCATCGTC		
GPF3	GACGATGCCCAATGCGTGTTC		
GPF4	CACTGGCTCAGAGCACTTTGTG		
GPF5	CACAAAGTGCTCTGAGCCAGTG		
GPF6	CAGCCTTATTATAATCAATC		
GPF7	GATTGATTATAATAAGGCTG		
GPF7F	GAGACTAGTACTCCCAAGTGTTG		
GPF7R	CCATTAAGGCTAAGGGTGTACTC		

Name	Nucleotide Sequence	Gene		Source
GPF8R	TCAGCGTCTTTTCCAGATGGTC			
GPF8	AccacactggactagtggatccTCAGCGTCTTTTCCAGATGG	Arenavirus gene	S	Present study
GPF8R1	AccacactggactagtggatccAGCGTCTYTTCCARAYNGT			
LCMV_227F	CAGTGGAATTTGATATGTCTCAC			
LCMV_1026R	CAGGTGCAGGGCAGATTC			

Appendix 7: Tissues Obtained During the LCMV Zoo Outbreak Donated by Dr. Rachael Tarlinton*

Rodent ID	Area Trapped/ Caught	Age	Sex	Species	Extracted Organs	Extracted Nucleic Acid
SB1	Sutton Bonington	Juvenile	-	*Microtus agrestis*	Liver	RNA
SB2	Sutton Bonington	Adult	-	*Microtus agrestis*	Liver	RNA
SB3	Sutton Bonington	Adult	Male	*Mus musculus*	Liver	RNA
SB4	Twycross Zoo	Adult	Female	*Mus musculus*	Liver	RNA
SB5	Sutton Bonington	Juvenile	Female	*Sorex minutus*	Liver	RNA
SB6	Sutton Bonington	Adult	Female	*Microtus agrestis*	Liver	RNA
SB7	Sutton Bonington	Juvenile	Male	*Microtus agrestis*	Liver	RNA
SB8	Sutton Bonington	Juvenile	Female	*Sorex minutus*	Liver	RNA
SB9	Sutton Bonington	Adult	-	*Apodemus sylvaticus*	Liver	RNA
SB10	Sutton Bonington	Adult	Female	*Microtus agrestis*	Liver	RNA
SB11	Sutton Bonington	Juvenile	Male	*Microtus agrestis*	Liver	RNA

Rodent ID	Area Trapped/ Caught	Age	Sex	Species	Extracted Organs	Extracted Nucleic Acid
SB12	Sutton Bonington	Adult	Female	*Microtus agrestis*	Liver	RNA
SB13	Twycross Zoo	Juvenile	Female	*Rattus norvegicus*	Liver	RNA
SB14	Twycross Zoo	Adult	Female	*Mus musculus*	Liver	RNA
SB15	Twycross Zoo	Adult	Male	*Mus musculus*	Liver	RNA
SB16	Twycross Zoo	Juvenile	Male	*Mus musculus*	Liver	RNA
SB17	Twycross Zoo	Adult	Female	*Mus musculus*	Liver	RNA
SB18	Twycross Zoo	Juvenile	Female	*Mus musculus*	Liver	RNA
SB19	Twycross Zoo	Adult	Female	*Rattus norvegicus*	Liver	RNA
SB20	Twycross Zoo	Adult	Male	*Rattus norvegicus*	Liver	RNA
SB21	Sutton Bonington	Adult	Female	*Apodemus sylvaticus*	Liver	RNA
SB22	Sutton Bonington	Adult	Male	*Apodemus sylvaticus*	Liver	RNA
SB23	Twycross Zoo	Adult	Male	*Rattus norvegicus*	Liver	RNA
SB24	Twycross Zoo	Adult	Male	*Mus musculus*	Liver	RNA
SB25	Belper	Adult	Female	*Microtus agrestis*	Liver	RNA

Rodent ID	Area Trapped/ Caught	Age	Sex	Species	Extracted Organs	Extracted Nucleic Acid
SB26	Belper	Adult	Male	*Apodemus sylvaticus*	Liver	RNA
SB27	Sutton Bonington	Adult	Female	*Microtus agrestis*	Liver	RNA
SB28	Sutton Bonington	Adult	Male	*Microtus agrestis*	Liver	RNA
SB29	Sutton Bonington	Juvenile	Male	*Microtus agrestis*	Liver	RNA
SB30	Sutton Bonington	Juvenile	Male	*Apodemus sylvaticus*	Liver	RNA
SB31	Belper	Adult	Male	*Apodemus sylvaticus*	Liver	RNA
SB32	Twycross Zoo	Adult	Male	*Rattus norvegicus*	Liver	RNA
SB33	Twycross Zoo	Adult	Female	*Rattus norvegicus*	Liver	RNA
SB34	Twycross Zoo	Adult	Female	*Sorex minutus*	Liver	RNA
SB35	Twycross Zoo	Adult	Female	*Mus musculus*	Liver	RNA
SB36	Twycross Zoo	Adult	Female	*Mus musculus*	Liver	RNA
SB37	Twycross Zoo	Adult	Female	*Mus musculus*	Liver	RNA
SB38	Twycross Zoo	Adult	Female	*Mus musculus*	Liver	RNA
SB39	Twycross Zoo	Adult	Male	*Mus musculus*	Liver	RNA

Rodent ID	Area Trapped/ Caught	Age	Sex	Species	Extracted Organs	Extracted Nucleic Acid
SB40	Twycross Zoo	Adult	Female	*Mus musculus*	Liver	RNA
SB41	Twycross Zoo	Adult	Female	*Mus musculus*	Liver	RNA
SB42	Twycross Zoo	Adult	Female	*Mus musculus*	Liver	RNA
SB43	Twycross Zoo	Adult	Female	*Mus musculus*	Liver	RNA
SB44	Twycross Zoo	Adult	Female	*Rattus norvegicus*	Liver	RNA
SB45	Twycross Zoo	Adult	Female	*Mus musculus*	Liver	RNA
SB46	Twycross Zoo	Adult	Male	*Mus musculus*	Liver	RNA
SB47	Twycross Zoo	Adult	-	*Mus musculus*	Liver	RNA
SB48	Twycross Zoo	Juvenile	Female	*Mus musculus*	Li6ver	RNA
SB49	Twycross Zoo	Adult	Female	*Mus musculus*	Liver	RNA

*The dates of collection were unavailable at the time of writing this thesis

Appendix 8: Tissues Obtained During the LCMV Zoo Outbreak Donated by Public Health England

THESIS ID	PHE ID	SPECIES	SAMPLING DATE
NA1	H06 230 0563 01	*R. norvegicus*	05/06/2006
NA2	H06 266 0587 01	*M. musculus*	26/06/2006
NA3	H06 266 0588 02	*M. musculus*	26/06/2006
NA4	H06 266 0589 02	*M. musculus*	26/06/2006
NA5	H06 266 0590 03	*M. musculus*	26/06/2006
NA6	H06 336 0611 01	*P. troglodytes*	23/08/2006
NA7	H06 336 0612 01	*H. klossi*	23/08/2006
NA8	H06 336 0614 03	*A. sara*	23/08/2006
NA9	H06 336 0615 02	*A. caraya*	23/08/2006
NA10	H06 336 0616 04	*A. sara*	23/08/2006
NA11	H06 336 0617 01	*A. niviridus*	23/08/2006
NA12	H06 336 0618 01	*A. sara*	23/08/2006
NA13	H06 336 0619 02	*T. obscurus*	23/08/2006
NA14	H06 336 0621 01	*C. geoffroyi*	23/08/2006
NA15	H06 336 0622 01	*T. vetulus monticola*	23/08/2006
NA16	H06 336 0621 01	*C. guereza occidentalis*	23/08/2006
NA17	H06 336 0624 01	*C. guereza occidentalis*	23/08/2006
NA18	H06 336 0625 01	*C. guereza occidentalis*	23/08/2006
NA19	H06 336 0626 01	*T. obscurus*	23/08/2006
NA20	H06 336 0627 01	*A. sara*	23/08/2006
NA21	H06 336 0628 01	*C. jacchus*	23/08/2006
NA22	H06 336 0629 01	*S. imperator subgrisescens*	23/08/2006
NA23	H07 196 0635 01	*C. porcellus*	20/08/2006
NA24	H07 196 0636 01	*M. mephitis*	10/10/2006
NA25	H07 196 0639 01	*A. caraya*	23/10/2006
NA26	H07 196 0640 01	*C. jacchus*	02/04/2007

THESIS ID	PHE ID	SPECIES	SAMPLING DATE
NA27	H07 196 0642 01	*M. musculus*	09/08/2006
NA28	H07 196 0643 01	*M. musculus*	08/08/2006
NA29	H07 196 0645 01	*M. musculus*	08/08/2006
NA30	H07 196 0647 01	*M. musculus*	08/08/2006
NA31	H07 196 0650 01	*M. musculus*	20/07/2006
NA32	H07 196 0656 01	*M. musculus*	19/07/2006
NA33	H07 196 0666 01	*C. porcellus*	23/04/2007
NA34	H07 196 0667 01	*C. porcellus*	23/04/2007
NA35	H07 196 0668 01	*C. perspicillata*	14/04/2007
NA36	H07 196 0669 01	*C. argentata melanura*	15/04/2007
NA37	H07 196 0670 01	*L. rosalia*	07/04/2007
NA38	H07 196 0671 01	Bat?	31/03/2007
NA39	H07 196 0672 01	Bat?	31/03/2007
NA40	H07 196 0673 01	Bat?	31/03/2007
NA41	H08 122 0595 01	*A. sara*	18/04/2007
NA42	H08 122 0596 01	*T. obscurus*	18/12/2007
NA43	H08 122 0597 01	*T. obscurus*	20/12/2007
NA44	H08 122 0603 01	*S. Oedipus*	23/08/2006
NA45	H08 122 0604 01	*G. gorilla*	20/09/2006
NA46	H08 122 0605 01	*H. agilis*	10/10/2006
NA47	H08 122 0606 01	*P. pardus orientalis*	19/01/2007
NA48	H08 122 0607 01	*C. pygmaea*	07/02/2007
NA49	H08 122 0608 03	*G. gorilla*	26/02/2007
NA50	H08 122 0609 01	*A. caraya*	02/04/2007
NA51	H08 122 0610 01	*A. caraya*	31/05/2007
NA52	H08 122 0611 01	*H. agilis*	08/08/2007
NA53	H08 122 0612 02	*S. imperator subgrisescens*	13/08/2007
NA55	H06 336 0614 03	*A. sara*	NO DATE
NA56	H07 196 0638 01	*M. musculus*	18/08/2006
NA57	H07 196 0648 01	*M. musculus*	19/07/2006
NA58	H07 196 0649 01	*M. musculus*	20/07/2006

THESIS ID	PHE ID	SPECIES	SAMPLING DATE
NA59	H07 196 0651 01	*M. musculus*	24/07/2006
NA60	H07 196 0652 01	*M. musculus*	19/07/2006
NA61	H07 196 0658 01	*M. musculus*	17/07/2006
NA62	H07 196 0661 01	*M. musculus*	08/08/2006
NA63	H07 196 0662 01	*M. musculus*	04/08/2006
NA64	H07 196 0663 01	*M. musculus*	02/08/2006
NA65	H07 196 0664 01	*M. musculus*	27/07/2006
NA66	H07 196 0665 01	*M. musculus*	24/07/2006
NA67	H07 196 0674 01	*M. musculus*	01/10/2006
NA68	H07 196 0675 01	*M. musculus*	06/10/2006
NA69	H07 196 0676 01	*M. musculus*	28/09/2006
NA70	H07 196 0677 01	*M. musculus*	28/09/2006
NA71	H07 196 0678 01	*M. musculus*	22/09/2006
NA72	H07 196 0679 01	*M. musculus*	21/09/2006
NA73	H06 094 0752	*C. geoffroyi*	08/01/2006
NA74	H06 094 0753	*C. geoffroyi*	27/01/2006
NA76	H06 336 0613 01	Northern White-cheeked Gibbon	23/08/2006
NA77	H07 196 0644 01	*M. musculus*	08/08/2006

* The information presented on the animal was insufficient to allow for proper identification.

Appendix 9: Rodents Obtained from Redmile, Leicestershire and Donated by Dr. Patrick McClure

S/No	THESIS ID	SAMPLING DATE	SPECIES	HARVESTED ORGAN	EXTRACTED NUCLEIC ACID
1.	KA1	11/03/2014	*M. musculus*	Liver	RNA
2.	KA2	11/03/2014	*M. musculus*	Liver	RNA
3.	KA3	11/03/2014	*M. musculus*	Liver	RNA

Appendix 10: Rodents Obtained from Tollerton, Nottinghamshire and Donated by Professor Jan Bradley

S/No	THESIS ID	SAMPLING DATE	SPECIES	HARVESTED ORGAN	EXTRACTED NUCLEIC ACID
1.	KA4	11/03/2014	*M. musculus*	Liver	RNA
2.	KA5	11/03/2014	*M. musculus*	Liver	RNA
3.	KA6	11/03/2014	*M. musculus*	Liver	RNA
4.	KA7	11/03/2014	*M. musculus*	Liver	RNA
5.	KA8	11/03/2014	*M. musculus*	Liver	RNA
6.	KA9	11/03/2014	*M. musculus*	Liver	RNA
7.	KA10	11/03/2014	*M. musculus*	Liver	RNA
8.	KA11	17/10/2013	*M. musculus*	Liver	RNA
9.	KA12	30/10/2013	*M. musculus*	Liver	RNA
10.	KA13	30/10/2013	*M. musculus*	Liver	RNA

Appendix 11: Animal Specimen Obtained from Twycross Zoo between 2013 and 2015

S/No	THESIS ID	SAMPLING DATE	SPECIES	HARVESTED ORGAN	EXTRACTED NUCLEIC ACID
1.	KA14	21/01/2014	*M. musculus*	Liver	RNA
2.	KA15	25/01/2014	*M. musculus*	Liver	RNA
3.	KA16	07/10/2013	*M. musculus*	Liver	RNA
4.	KA17	24/01/2014	*M. musculus*	Liver	RNA
5.	KA18	23/01/2014	*M. musculus*	Liver	RNA
6.	KA19	03/02/2014	*M. musculus*	Liver	RNA
7.	KA20	28/10/2013	*M. musculus*	Liver	RNA
8.	KA21	24/05/2013	*M. musculus*	Liver	RNA
9.	KA22	17/02/2014	*M. musculus*	Liver	RNA
10.	KA23	26/01/2014	*M. musculus*	Liver	RNA
11.	KA24	17/11/2013	*R. norvegicus*	Liver	RNA
12.	KA25	25/10/2013	*R. norvegicus*	Liver	RNA
13.	KA26	28/01/2014	*R. norvegicus*	Liver	RNA
14.	KA27	28/11/2013	*R. norvegicus*	Liver	RNA
15.	KA28	22/01/2014	*R. norvegicus*	Liver	RNA
16.	KA29	05/02/2014	*R. norvegicus*	Liver	RNA
17.	KA30	11/02/2013	*R. norvegicus*	Liver	RNA
18.	KA31	DATE UNAVAILABLE	*R. norvegicus*	Liver	RNA
19.	KA32	DATE UNAVAILABLE	*R. norvegicus*	Liver	RNA
20.	KA33	DATE UNAVAILABLE	*R. norvegicus*	Liver	RNA
21.	KA34	DATE UNAVAILABLE	*R. norvegicus*	Liver	RNA
22.	KA35	21/03/2014	*M. musculus*	Liver	RNA
23.	KA36	21/03/2014	*M. musculus*	Liver	RNA
24.	KA37	12/06/2013	*M. musculus*	Liver	RNA
25.	KA38	DATE UNAVAILABLE	*M. musculus*	Liver	RNA
26.	KA39	DATE UNAVAILABLE	*M. musculus*	Liver	RNA
27.	KA40	01/02/2014	*R. norvegicus*	Liver	RNA
28.	KA41	30/03/2014	*M. musculus*		

S/No	THESIS ID	SAMPLING DATE	SPECIES	HARVESTED ORGAN	EXTRACTED NUCLEIC ACID
29.	KA42	29/01/2014	*M. musculus*	Liver	RNA
30.	KA43	DATE UNAVAILABLE	*M. musculus*	Liver	RNA
31.	KA44	10/03/2014	*M. musculus*	Liver	RNA
32.	KA45	20/01/2014	*M. musculus*	Liver	RNA
33.	KA46	23/06/2013	*M. musculus*	Liver	RNA
34.	KA47	14/02/2014	*M. musculus*	Liver	RNA
35.	KA48	14/03/2014	*M. musculus*	Liver	RNA
36.	KA49	25/02/2014	*M. musculus*	Liver	RNA
37.	KA50	21/01/2014	*M. musculus*	Liver	RNA
38.	KA51	24/05/2013	*M. musculus*	Liver	RNA
39.	KA52	DATE UNAVAILABLE	*R. norvegicus*	Liver	RNA
40.	KA53	24/02/2014	*M. musculus*	Liver	RNA
41.	KA54	DATE UNAVAILABLE	*M. musculus*	Liver	RNA
42.	KA55	28/03/2014	*M. musculus*	Liver	RNA
43.	KA56	14/02/2014	*M. musculus*	Liver	RNA
44.	KA57	30/05/2013	*M. musculus*	Liver	RNA
45.	KA58	27/05/2013	*M. musculus*	Liver	RNA
46.	KA59	26/11/2013	*M. musculus*	Liver	RNA
47.	KA60	15/07/2013	*M. musculus*	Liver	RNA
48.	KA61	14/09/2013	*M. musculus*	Liver	RNA
49.	KA62	05/03/2014	*M. musculus*	Liver	RNA
50.	KA63	10/02/2014	*M. musculus*	Liver	RNA
51.	KA64	12/03/2014	*M. musculus*	Liver	RNA
52.	KA65	10/02/2014	*M. musculus*	Liver	RNA
53.	KA66	10/02/2014	*M. musculus*	Liver	RNA
54.	KA67	20/01/2014	*M. musculus*	Liver	RNA
55.	KA68	25/03/2014	*M. musculus*	Liver	RNA
56.	KA69	12/02/2014	*M. musculus*	Liver	RNA
57.	KA70	24/01/2014	*M. musculus*	Liver	RNA
58.	KA71	10/02/2014	*M. musculus*	Liver	RNA
59.	KA72	11/03/2014	*M. musculus*	Liver	RNA
60.	KA73	31/01/2014	*M. musculus*		

S/No	THESIS ID	SAMPLING DATE	SPECIES	HARVESTED ORGAN	EXTRACTED NUCLEIC ACID
61.	KA74	10/01/2014	*M. musculus*	Liver	RNA
62.	KA75	21/03/2014	*M. musculus*	Liver	RNA
63.	KB1	06/06/2014	*M. musculus*	Liver	RNA
64.	KB2	06/06/2014	*M. musculus*	Liver	RNA
65.	KB3	06/06/2014	*M. musculus*	Liver	RNA
66.	KB4	06/06/2014	*M. musculus*	Liver	RNA
67.	KB5	06/06/2014	*M. musculus*	Liver	RNA
68.	KB6	03/05/2014	*M. musculus*	Liver	RNA
69.	KB7	03/05/2014	*M. musculus*	Liver	RNA
70.	KB8	25/04/2014	*M. musculus*	Liver	RNA
71.	KB9	07/04/2014	*M. musculus*	Liver	RNA
72.	KB10	15/04/2014	*M. musculus*	Liver	RNA
73.	KB11	14/04/2014	*M. musculus*	Liver	RNA
74.	KB12	07/04/2014	*M. musculus*	Liver	RNA
75.	KB13	06/04/2014	*M. musculus*	Liver	RNA
76.	KB14	01/04/2014	*M. musculus*	Liver	RNA
77.	KB15	03/04/2014	*M. musculus*	Liver	RNA
78.	KB16	10/04/2014	*M. musculus*	Liver	RNA
79.	KB17	07/04/2014	*M. musculus*	Liver	RNA
80.	KB18	02/05/2014	*M. musculus*	Liver	RNA
81.	KB19	12/05/2014	*M. musculus*	Liver	RNA
82.	KB20	02/05/2014	*M. musculus*	Liver	RNA
83.	KB21	14/04/2014	*M. musculus*	Liver	RNA
84.	KB22	10/04/2014	*M. musculus*	Liver	RNA
85.	KB23	29/04/2014	*R. norvegicus*	Liver	RNA
86.	KB24	21/04/2014	*R. norvegicus*	Liver	RNA
87.	KB25	DATE UNAVAILABLE	*R. norvegicus*	Liver	RNA
88.	KB26	25/04/2014	*R. norvegicus*	Liver	RNA
89.	KB27	10/04/2014	*R. norvegicus*	Liver	RNA
90.	KB28	27/04/2014	*R. norvegicus*	Liver	RNA
91.	KB29	22/04/2014	*R. norvegicus*	Liver	RNA
92.	KB30	08/04/2014	*R. norvegicus*	Liver	RNA

S/No	THESIS ID	SAMPLING DATE	SPECIES	HARVESTED ORGAN	EXTRACTED NUCLEIC ACID
93.	KB31	18/04/2014	*R. norvegicus*	Liver	RNA
94.	KB32	30/04/2014	*R. norvegicus*	Liver	RNA
95.	KB33	17/04/2014	*Duckling*	Liver	RNA
96.	KB34	26/04/2014	*Duckling**	Liver	RNA
97.	KB35	26/04/2014	*Duckling**	Liver	RNA
98.	KC1	DATE UNAVAILABLE	*M. musculus*	Whole animal*	RNA
99.	KC2	DATE UNAVAILABLE	*M. musculus*	Whole animal*	RNA
100.	KC3	DATE UNAVAILABLE	*M. musculus*	Whole animal*	RNA
101.	KC4	DATE UNAVAILABLE	*M. musculus*	Whole animal*	RNA
102.	KC5	DATE UNAVAILABLE	*M. musculus*	Whole animal*	RNA
103.	KC6	DATE UNAVAILABLE	*M. musculus*	Whole animal*	RNA
104.	KC7	DATE UNAVAILABLE	*M. musculus*	Whole animal*	RNA
105.	KC8	DATE UNAVAILABLE	*M. musculus*	Whole animal*	RNA
106.	KC9	DATE UNAVAILABLE	*M. musculus*	Whole animal*	RNA
107.	KC10	DATE UNAVAILABLE	*M. musculus*	Whole animal*	RNA
108.	KC11	DATE UNAVAILABLE	*M. musculus*	Whole animal*	RNA
109.	KC12	DATE UNAVAILABLE	*M. musculus*	Whole animal*	RNA
110.	KC13	DATE UNAVAILABLE	*M. musculus*	Whole animal*	RNA
111.	KC14	DATE UNAVAILABLE	*M. musculus*	Whole animal*	RNA
112.	KC15	DATE UNAVAILABLE	*M. musculus*	Whole animal*	RNA
113.	KC16	DATE UNAVAILABLE	*M. musculus*	Whole animal*	RNA
114.	KC17	DATE UNAVAILABLE	*M. musculus*	Whole animal*	RNA
115.	KC18	DATE UNAVAILABLE	*M. musculus*	Whole animal*	RNA
116.	KC19	DATE UNAVAILABLE	*M. musculus*	Whole animal*	RNA
117.	KC20	DATE UNAVAILABLE	*M. musculus*	Whole animal*	RNA
118.	KC21	DATE UNAVAILABLE	*M. musculus*	Whole animal*	RNA
119.	KC22	DATE UNAVAILABLE	*M. musculus*	Whole animal*	RNA
120.	KC23	DATE UNAVAILABLE	*M. musculus*	Whole animal*	RNA
121.	KD1	10/06/2014	*M. musculus*	Liver	RNA
122.	KD2	24/04/2014	*M. musculus*	Liver	RNA
123.	KD3	20/06/2014	*M. musculus*	Liver	RNA
124.	KD4	24/06/2014	*M. musculus*	Liver	RNA

S/No	THESIS ID	SAMPLING DATE	SPECIES	HARVESTED ORGAN	EXTRACTED NUCLEIC ACID
125.	KD5	16/06/2014	*M. musculus*	Liver	RNA
126.	KD6	19/06/2014	*M. musculus*	Liver	RNA
127.	KD7	04/06/2014	*M. musculus*	Liver	RNA
128.	KD8	18/06/2014	*M. musculus*	Liver	RNA
129.	KD9	17/06/2014	*M. musculus*	Liver	RNA
130.	KD10	20/06/2014	*M. musculus*	Liver	RNA
131.	KD11	09/05/2014	*M. musculus*	Liver	RNA
132.	KD12	16/06/2014	*M. musculus*	Liver	RNA
133.	KD13	22/06/2014	*M. musculus*	Liver	RNA
134.	KD14	04/06/2014	*M. musculus*	Liver	RNA
135.	KD15	03/06/2014	*M. musculus*	Liver	RNA
136.	KD16	03/07/2014	*M. musculus*	Liver	RNA
137.	KD17	18/06/2014	*M. musculus*	Liver	RNA
138.	KD18	23/06/2014	*M. musculus*	Liver	RNA
139.	KD19	30/06/2014	*M. musculus*	Liver	RNA
140.	KD20	23/06/2014	*M. musculus*	Liver	RNA
141.	KD21	20/06/2014	*M. musculus*	Liver	RNA
142.	KD22	03/07/2014	*M. musculus*	Liver	RNA
143.	KD23	25/06/2014	*M. musculus*	Liver	RNA
144.	KD24	23/06/2014	*M. musculus*	Liver	RNA
145.	KD25	25/06/2014	*M. musculus*	Liver	RNA
146.	KD26	01/07/2014	*M. musculus*	Liver	RNA
147.	KD27	01/07/2014	*M. musculus*	Liver	RNA
148.	KD28	26/06/2014	*M. musculus*	Liver	RNA
149.	KD29	20/06/2014	*M. musculus*	Liver	RNA
150.	KD30	26/06/2014	*M. musculus*	Liver	RNA
151.	KD31	25/06/2014	*M. musculus*	Liver	RNA
152.	KD32	20/06/2014	*M. musculus*	Liver	RNA
153.	KD33	25/06/2014	*M. musculus*	Liver	RNA
154.	KD34	23/06/2014	*M. musculus*	Liver	RNA
155.	KD35	23/06/2014	*M. musculus*	Liver	RNA
156.	KD36	24/05/2014	*R. norvegicus*		

S/No	THESIS ID	SAMPLING DATE	SPECIES	HARVESTED ORGAN	EXTRACTED NUCLEIC ACID
157.	KD37	24/06/2014	*R. norvegicus*	Liver	RNA
158.	KD38	21/06/2014	*R. norvegicus*	Liver	RNA
159.	KD39	23/06/2014	*R. norvegicus*	Liver	RNA
160.	KD40	29/06/2014	*R. norvegicus*	Liver	RNA
161.	KD41	29/06/2014	*R. norvegicus*	Liver	RNA
162.	KD42	01/07/2014	*R. norvegicus*	Liver	RNA
163.	KD43	23/06/2014	*R. norvegicus*	Liver	RNA
164.	KD44	10/06/2014	*M. musculus*	Liver	RNA
165.	KD45	29/06/2014	*M. musculus*	Liver	RNA
166.	KD46	28/06/2014	*M. musculus*	Liver	RNA
167.	KD47	DATE UNAVAILABLE	*M. musculus*	Liver	RNA
168.	KD48	02/07/2014	*M. musculus*	Liver	RNA
169.	KD49	02/07/2014	*M. musculus*	Liver	RNA
170.	KD50	02/07/2014	*M. musculus*	Liver	RNA
171.	KD51	02/07/2014	*M. musculus*	Liver	RNA
172.	KD52	23/06/2014	*M. musculus*	Liver	RNA
173.	KD53	26/06/2014	*M. musculus*	Liver	RNA
174.	KD54	26/06/2014	*M. musculus*	Liver	RNA
175.	KD55	26/06/2014	*M. musculus*	Liver	RNA
176.	KD56	26/06/2014	*M. musculus*	Liver	RNA
177.	KE1	DATE UNAVAILABLE	*M. musculus*	Liver	RNA
178.	KE2	04/08/2014	*M. musculus*	Liver	RNA
179.	KE3	10/07/2014	*Vole*	Liver	RNA
180.	KE4	13/07/2014	*M. musculus*	Liver	RNA
181.	KE5	11/07/2014	*M. musculus*	Liver	RNA
182.	KE6	01/08/2014	*M. musculus*	Liver	RNA
183.	KE7	08/08/2014	*M. musculus*	Liver	RNA
184.	KE8	07/07/2014	*M. musculus*	Liver	RNA
185.	KE9	DATE UNAVAILABLE	*M. musculus*	Liver	RNA
186.	KE10	29/07/2014	*M. musculus*	Liver	RNA
187.	KE11	07/07/2014	*M. musculus*	Liver	RNA
188.	KE12	DATE UNAVAILABLE	*M. musculus*		

S/No	THESIS ID	SAMPLING DATE	SPECIES	HARVESTED ORGAN	EXTRACTED NUCLEIC ACID
189.	KE13	DATE UNAVAILABLE	*M. musculus*	Liver	RNA
190.	KE14	15/07/2014	*M. musculus*	Liver	RNA
191.	KE15	16/07/2014	*M. musculus*	Liver	RNA
192.	KE16	16/07/2014	*R. norvegicus*	Liver	RNA
193.	KE17	28/07/2014	*M. musculus*	Liver	RNA
194.	KE18	09/07/2014	*M. musculus*	Liver	RNA
195.	KE19	06/08/2014	*M. musculus*	Liver	RNA
196.	KE20	04/07/2014	*M. musculus*	Liver	RNA
197.	KE21	11/07/2014	*M. musculus*	Liver	RNA
198.	KE22	11/07/2014	*M. musculus*	Liver	RNA
199.	KE23	07/08/2014	*M. musculus*	Liver	RNA
200.	KE24	27/07/2014	*M. musculus*	Liver	RNA
201.	KE25	20/07/2014	*M. musculus*	Liver	RNA
202.	KE26	06/07/2014	*M. musculus*	Liver	RNA
203.	KE27	20/07/2014	*M. musculus*	Liver	RNA
204.	KE28	19/07/2014	*M. musculus*	Liver	RNA
205.	KE29	04/07/2014	*M. musculus*	Liver	RNA
206.	KE30	05/08/2014	*M. musculus*	Liver	RNA
207.	KE31	20/07/2014	*M. musculus*	Liver	RNA
208.	KE32	20/07/2014	*M. musculus*	Liver	RNA
209.	KE33	21/07/2014	*M. musculus*	Liver	RNA
210.	KE34	16/07/2014	*R. norvegicus*	Liver	RNA
211.	KE35	06/07/2014	*M. musculus*	Liver	RNA
212.	KE36	04/07/2014	*M. musculus*	Liver	RNA
213.	KE37	04/07/2014	*M. musculus*	Liver	RNA
214.	KE38	04/07/2014	*M. musculus*	Liver	RNA
215.	KE39	06/07/2014	*M. musculus*	Liver	RNA
216.	KE40	04/07/2014	*M. musculus*	Liver	RNA
217.	KE41	09/07/2014	*R. norvegicus*	Liver	RNA
218.	KE42	01/07/2014	*R. norvegicus*	Liver	RNA
219.	KE43	16/07/2014	*R. norvegicus*	Liver	RNA
220.	KE44	15/07/2014	*R. norvegicus*		

S/No	THESIS ID	SAMPLING DATE	SPECIES	HARVESTED ORGAN	EXTRACTED NUCLEIC ACID
221.	KE45	16/07/2014	*R. norvegicus*	Liver	RNA
222.	KE46	15/07/2014	*R. norvegicus*	Liver	RNA
223.	KE47	15/07/2014	*R. norvegicus*	Liver	RNA
224.	KE48	15/07/2014	*R. norvegicus*	Liver	RNA
225.	KE49	15/07/2014	*R. norvegicus*	Liver	RNA
226.	KE50	28/07/2014	*R. norvegicus*	Liver	RNA
227.	KE51	14/07/2014	*R. norvegicus*	Liver	RNA
228.	KE52	15/07/2014	*R. norvegicus*	Liver	RNA
229.	KE53	DATE UNAVAILABLE	*R. norvegicus*	Liver	RNA
230.	KE54	15/07/2014	*R. norvegicus*	Liver	RNA
231.	KE55	15/07/2014	*R. norvegicus*	Liver	RNA
232.	KE56	16/07/2014	*R. norvegicus*	Liver	RNA
233.	KE57	15/07/2014	*R. norvegicus*	Liver	RNA
234.	KE58	16/07/2014	*R. norvegicus*	Liver	RNA
235.	KE59	16/07/2014	*R. norvegicus*	Liver	RNA
236.	KE60	13/07/2014	*R. norvegicus*	Liver	RNA
237.	KE61	15/07/2014	*R. norvegicus*	Liver	RNA
238.	KE62	15/07/2014	*R. norvegicus*	Liver	RNA
239.	KE63	11/07/2014	*R. norvegicus*	Liver	RNA
240.	KE64	16/07/2014	*R. norvegicus*	Liver	RNA
241.	KE65	13/07/2014	*R. norvegicus*	Liver	RNA
242.	KE66	15/07/2014	*R. norvegicus*	Liver	RNA
243.	KE67	15/07/2014	*R. norvegicus*	Liver	RNA
244.	KE68	14/07/2014	*R. norvegicus*	Liver	RNA
245.	KE69	16/07/2014	*R. norvegicus*	Liver	RNA
246.	KR70	16/07/2014	*R. norvegicus*	Liver	RNA
247.	KE71	15/07/2014	*R. norvegicus*	Liver	RNA
248.	KE72	15/07/2014	*R. norvegicus*	Liver	RNA
249.	KE73	15/07/2014	*R. norvegicus*	Liver	RNA
250.	KF1	07/09/2014	*M. musculus*	Liver	RNA
251.	KF2	05/10/2014	*M. musculus*	Liver	RNA
252.	KF3	20/09/2014	*M. musculus*		

S/No	THESIS ID	SAMPLING DATE	SPECIES	HARVESTED ORGAN	EXTRACTED NUCLEIC ACID
253.	KF4	19/09/2014	*M. musculus*	Liver	RNA
254.	KF5	22/09/2014	*M. musculus*	Liver	RNA
255.	KF6	08/09/2014	*M. musculus*	Liver	RNA
256.	KF7	08/09/2014	*M. musculus*	Liver	RNA
257.	KF8	08/09/2014	*M. musculus*	Liver	RNA
258.	KF9	DATE UNAVAILABLE	*M. musculus*	Liver	RNA
259.	KF10	07/09/2014	*M. musculus*	Liver	RNA
260.	KF11	10/08/2014	*M. musculus*	Liver	RNA
261.	KF12	07/10/2014	*M. musculus*	Liver	RNA
262.	KF13	DATE UNAVAILABLE	*M. musculus*	Liver	RNA
263.	KF14	01/11/2014	*M. musculus*	Liver	RNA
264.	KF15	02/10/2014	*M. musculus*	Liver	RNA
265.	KF16	06/10/2014	*M. musculus*	Liver	RNA
266.	KF17	07/10/2014	*M. musculus*	Liver	RNA
267.	KF18	27/09/2014	*M. musculus*	Liver	RNA
268.	KF19	06/10/2014	*M. musculus*	Liver	RNA
269.	KF20	02/10/2014	*M. musculus*	Liver	RNA
270.	KF21	09/10/2014	*M. musculus*	Liver	RNA
271.	KF22	17/10/2014	*M. musculus*	Liver	RNA
272.	KF23	22/10/2014	*M. musculus*	Liver	RNA
273.	KF24	09/10/2014	*M. musculus*	Liver	RNA
274.	KF25	08/10/2014	*A. sylvaticus*	Liver	RNA
275.	KF26	19/09/2014	*M. musculus*	Liver	RNA
276.	KF27	22/09/2014	*M. musculus*	Liver	RNA
277.	KF28	23/10/2014	*M. musculus*	Liver	RNA
278.	KF29	07/09/2014	*M. musculus*	Liver	RNA
279.	KF30	04/10/2014	*M. musculus*	Liver	RNA
280.	KF31	11/08/2014	*M. musculus*	Liver	RNA
281.	KF32	05/09/2014	*M. musculus*	Liver	RNA
282.	KF33	30/10/2014	*M. musculus*	Liver	RNA
283.	KF34	12/08/2014	*M. musculus*	Liver	RNA
284.	KF35	18/08/2014	*M. musculus*		

S/No	THESIS ID	SAMPLING DATE	SPECIES	HARVESTED ORGAN	EXTRACTED NUCLEIC ACID
285.	KF36	31/10/2014	*M. musculus*	Liver	RNA
286.	KF37	05/09/2014	*M. musculus*	Liver	RNA
287.	KF38	17/08/2014	*M. musculus*	Liver	RNA
288.	KF39	22/10/2014	*M. musculus*	Liver	RNA
289.	KF40	12/08/2014	*M. musculus*	Liver	RNA
290.	KF41	15/08/2014	*M. musculus*	Liver	RNA
291.	KF42	27/10/2014	*M. musculus*	Liver	RNA
292.	KF43	27/10/2014	*M. musculus*	Liver	RNA
293.	KF44	17/10/2014	*M. musculus*	Liver	RNA
294.	KF45	15/08/2014	*M. musculus*	Liver	RNA
295.	KF46	08/09/2014	*M. musculus*	Liver	RNA
296.	KF47	31/10/2014	*M. musculus*	Liver	RNA
297.	KF48	01/10/2014	*M. musculus*	Liver	RNA
298.	KF49	30/10/2014	*M. musculus*	Liver	RNA
299.	KF50	02/11/2014	*M. musculus*	Liver	RNA
300.	KF51	05/09/2014	*Mole**	Liver	RNA
301.	KF52	02/09/2014	*Mole**	Liver	RNA
302.	KF53	07/08/2014	*Mole**	Liver	RNA
303.	KF54	26/09/2014	*Mole**	Liver	RNA
304.	KF55	26/09/2014	*Mole**	Liver	RNA
305.	KF56	06/08/2014	*R. norvegicus*	Liver	RNA
306.	KF57	21/10/2014	*R. norvegicus*	Liver	RNA
307.	KF58	29/10/2014	*R. norvegicus*	Liver	RNA
308.	KF59	30/08/2014	*R. norvegicus*	Liver	RNA
309.	KF60	17/08/2014	*R. norvegicus*	Liver	RNA
310.	KF61	07/10/2014	*R. norvegicus*	Liver	RNA
311.	KF62	26/10/2014	*M. musculus*	Liver	RNA
312.	KF63	31/08/2014	*M. musculus*	Liver	RNA
313.	KF64	28/10/2014	*M. musculus*	Liver	RNA
314.	KF65	01/09/2014	*M. musculus*	Liver	RNA
315.	KF66	10/08/2014	*M. musculus*	Liver	RNA
316.	M1	13/12/2014	*M. musculus*		

S/No	THESIS ID	SAMPLING DATE	SPECIES	HARVESTED ORGAN	EXTRACTED NUCLEIC ACID
317.	M2	04/12/2014	*M. musculus*	Liver	RNA
318.	M3	14/11/2014	*M. musculus*	Liver	RNA
319.	M4	12/12/2014	*M. musculus*	Liver	RNA
320.	M5	04/12/2014	*M. musculus*	Liver	RNA
321.	M6	16/11/2014	*M. musculus*	Liver	RNA
322.	M7	22/11/2014	*M. musculus*	Liver	RNA
323.	M8	11/12/2014	*M. musculus*	Liver	RNA
324.	M9	12/12/2014	*M. musculus*	Liver	RNA
325.	M10	23/11/2014	*M. musculus*	Liver	RNA
326.	M11	07/11/2014	*M. musculus*	Liver	RNA
327.	M12	11/11/2014	*M. musculus*	Liver	RNA
328.	M13	19/11/2014	*A. sylvaticus*	Liver	RNA
329.	M14	15/12/2014	*M. musculus*	Liver	RNA
330.	M15	14/12/2014	*M. musculus*	Liver	RNA
331.	M16	11/12/2014	*M. musculus*	Liver	RNA
332.	M17	12/11/2014	*M. musculus*	Liver	RNA
333.	M18	08/11/2014	*M. musculus*	Liver	RNA
334.	M19	06/11/2014	*M. musculus*	Liver	RNA
335.	M20	11/12/2014	*M. musculus*	Liver	RNA
336.	M21	14/12/2014	*M. musculus*	Liver	RNA
337.	M22	16/11/2014	*M. musculus*	Liver	RNA
338.	M23	23/11/2014	*M. musculus*	Liver	RNA
339.	M24	17/11/2014	*M. musculus*	Liver	RNA
340.	M25	19/11/2014	*M. musculus*	Liver	RNA
341.	M26	17/12/2014	*M. musculus*	Liver	RNA
342.	M27	12/12/2014	*M. musculus*	Liver	RNA
343.	M28	15/11/2014	*M. musculus*	Liver	RNA
344.	M29	18/11/2014	*M. musculus*	Liver	RNA
345.	M30	15/11/2014	*A. sylvaticus*	Liver	RNA
346.	M31	05/11/2014	*M. musculus*	Liver	RNA
347.	M32	27/12/2014	*M. musculus*	Liver	RNA
348.	M33	17/11/2014	*M. musculus*		

S/No	THESIS ID	SAMPLING DATE	SPECIES	HARVESTED ORGAN	EXTRACTED NUCLEIC ACID
349.	M34	11/11/2014	*M. musculus*	Liver	RNA
350.	M35	27/12/2014	*M. musculus*	Liver	RNA
351.	M36	03/01/2015	*M. musculus*	Liver	RNA
352.	M37	13/12/2014	*M. musculus*	Liver	RNA
353.	M38	14/12/2014	*M. musculus*	Liver	RNA
354.	M39	19/12/2014	*M. musculus*	Liver	RNA
355.	M40	18/12/2014	*M. musculus*	Liver	RNA
356.	M41	17/11/2014	*R. norvegicus*	Liver	RNA
357.	M42	16/11/2014	*R. norvegicus*	Liver	RNA
358.	M43	06/11/2014	*R. norvegicus*	Liver	RNA
359.	M44	10/12/2014	*R. norvegicus*	Liver	RNA
360.	M45	13/11/2014	*R. norvegicus*	Liver	RNA
361.	M46	13/12/2014	*R. norvegicus*	Liver	RNA
362.	M47	09/11/2014	*R. norvegicus*	Liver	RNA
363.	M48	10/11/2014	*R. norvegicus*	Liver	RNA
364.	M49	28/12/2014	*M. musculus*	Liver	RNA
365.	M50	04/12/2014	*M. musculus*	Liver	RNA
366.	M51	20/03/2015	*M. musculus*	Liver	RNA
367.	M52	17/03/2015	*M. musculus*	Liver	RNA
368.	M53	10/12/2015	*M. musculus*	Liver	RNA
369.	M54	09/01/2015	*M. musculus*	Liver	RNA
370.	M55	19/01/2015	*M. musculus*	Liver	RNA
371.	M56	11/11/2014	*M. musculus*	Liver	RNA
372.	M57	03/01/2015	*M. musculus*	Liver	RNA
373.	M58	06/01/2015	*M. musculus*	Liver	RNA
374.	M59	11/01/2015	*M. musculus*	Liver	RNA
375.	M60	17/03/2015	*M. musculus*	Liver	RNA
376.	M61	18/03/2015	*M. musculus*	Liver	RNA
377.	M62	16/01/2015	*M. musculus*	Liver	RNA
378.	M63	19/01/2015	*M. musculus*	Liver	RNA
379.	M64	18/01/2015	*M. musculus*	Liver	RNA
380.	M65	09/01/2015	*M. musculus*		

S/No	THESIS ID	SAMPLING DATE	SPECIES	HARVESTED ORGAN	EXTRACTED NUCLEIC ACID
381.	M66	10/11/2015	*M. musculus*	Liver	RNA
382.	M67	28/11/2015	*M. musculus*	Liver	RNA
383.	M68	17/03/2015	*M. musculus*	Liver	RNA
384.	M69	17/01/2015	*M. musculus*	Liver	RNA
385.	M70	26/02/2015	*M. musculus*	Liver	RNA
386.	M71	16/02/2015	*M. musculus*	Liver	RNA
387.	M72	12/02/2015	*M. musculus*	Liver	RNA
388.	M73	10/02/2015	*M. musculus*	Liver	RNA
389.	M74	13/02/2015	*M. musculus*	Liver	RNA
390.	M75	14/01/2015	*M. musculus*	Liver	RNA
391.	M76	03/02/2015	*M. musculus*	Liver	RNA
392.	M77	09/02/2015	*M. musculus*	Liver	RNA
393.	M78	19/02/2015	*M. musculus*	Liver	RNA
394.	M79	26/01/2015	*M. musculus*	Liver	RNA
395.	M80	01/02/2015	*M. musculus*	Liver	RNA
396.	M81	13/01/2015	*M. musculus*	Liver	RNA
397.	M82	12/01/2015	*M. musculus*	Liver	RNA
398.	M83	28/01/2015	*M. musculus*	Liver	RNA
399.	M84	17/01/2015	*M. musculus*	Liver	RNA
400.	M85	13/01/2015	*M. musculus*	Liver	RNA
401.	M86	09/01/2015	*M. musculus*	Liver	RNA
402.	M87	11/03/2015	*M. musculus*	Liver	RNA
403.	M88	15/01/2014	*M. musculus*	Liver	RNA
404.	M89	30/03/2015	*M. musculus*	Liver	RNA
405.	M90	08/02/2015	*M. musculus*	Liver	RNA
406.	M91	18/03/2015	*M. musculus*	Liver	RNA
407.	M92	09/02/2015	*M. musculus*	Liver	RNA
408.	M93	28/03/2015	*M. musculus*	Liver	RNA
409.	M94	11/01/2015	*M. musculus*	Liver	RNA
410.	M95	30/03/2015	*M. musculus*	Liver	RNA
411.	M96	01/04/2015	*M. musculus*	Liver	RNA
412.	M97	06/03/2015	*M. musculus*		

S/No	THESIS ID	SAMPLING DATE	SPECIES	HARVESTED ORGAN	EXTRACTED NUCLEIC ACID
413.	M98	28/03/2015	*M. musculus*	Liver	RNA
414.	M99	03/04/2015	*M. musculus*	Liver	RNA
415.	M100	05/04/2015	*M. musculus*	Liver	RNA
416.	M101	04/04/2015	*M. musculus*	Liver	RNA
417.	M102	18/03/2015	*M. musculus*	Liver	RNA
418.	M103	10/03/2015	*M. musculus*	Liver	RNA
419.	M104	11/03/2015	*M. musculus*	Liver	RNA
420.	M105	03/04/2015	*M. musculus*	Liver	RNA
421.	M106	12/04/2015	*M. musculus*	Liver	RNA
422.	M107	04/04/2015	*M. musculus*	Liver	RNA
423.	M108	31/03/2015	*M. musculus*	Liver	RNA
424.	M109	08/01/2015	*M. musculus*	Liver	RNA
425.	M110	08/01/2015	*M. musculus*	Liver	RNA
426.	M111	07/01/2015	*M. musculus*	Liver	RNA
427.	M112	31/01/2015	*M. musculus*	Liver	RNA
428.	M113	17/03/2015	*M. musculus*	Liver	RNA
429.	M114	16/01/2015	*M. musculus*	Liver	RNA
430.	M115	26/03/2015	*M. musculus*	Liver	RNA
431.	M116	27/03/2015	*M. musculus*	Liver	RNA
432.	M117	27/03/2015	*M. musculus*	Liver	RNA
433.	M118	07/01/2015	*M. musculus*	Liver	RNA
434.	M119	07/04/2015	*M. musculus*	Liver	RNA
435.	M120	29/03/2015	*M. musculus*	Liver	RNA
436.	M121	26/03/2015	*M. musculus*	Liver	RNA
437.	M122	10/04/2015	*M. musculus*	Liver	RNA
438.	M123	16/03/2015	*M. musculus*	Liver	RNA
439.	M124	09/02/2015	*M. musculus*	Liver	RNA
440.	M125	09/04/2015	*M. musculus*	Liver	RNA
441.	M126	10/04/2015	*M. musculus*	Liver	RNA
442.	M127	25/03/2015	*M. musculus*	Liver	RNA
443.	M128	25/03/2015	*M. musculus*	Liver	RNA
444.	M129	17/01/2015	*R. norvegicus*		

S/No	THESIS ID	SAMPLING DATE	SPECIES	HARVESTED ORGAN	EXTRACTED NUCLEIC ACID
445.	M130	13/01/2015	*R. norvegicus*	Liver	RNA
446.	M131	06/04/2015	*R. norvegicus*	Liver	RNA
447.	M132	06/03/2015	*R. norvegicus*	Liver	RNA
448.	M133	02/02/2015	*R. norvegicus*	Liver	RNA
449.	M134	11/01/2015	*R. norvegicus*	Liver	RNA
450.	M135	08/04/2015	*R. norvegicus*	Liver	RNA
451.	M136	07/04/2015	*R. norvegicus*	Liver	RNA
452.	M137	08/02/2015	*M. musculus*	Liver	RNA
453.	M138	14/04/2014	*M. musculus*	Liver	RNA
454.	M139	08/04/2015	*M. musculus*	Liver	RNA
455.	M140	10/01/2015	*M. musculus*	Liver	RNA
456.	M141	14/04/2015	*M. musculus*	Liver	RNA
457.	M142	11/04/2015	*M. musculus*	Liver	RNA
458.	M143	08/04/2015	*M. musculus*	Liver	RNA
459.	M144	26/03/2015	*M. musculus*	Liver	RNA

* The information presented on the animal was insufficient to allow for proper identification.

Appendix 12: Clinical and Histopathological Findings on Animals as Conducted by Public Health England

Organism		Age	Necropsy
Common Name	**Scientific Name**		
Cornsnake/red rat snake	*Elaphe guttata guttata*	N/A for Group	Disappeared. Presumed dead
Woolly monkey	*Lagothrix lagotricha*	0Y, 0M, 3D	Aborted foetus
Cornsnake/red rat snake	*Elaphe guttata guttata*	N/A for Group	Euthanased – Not grown to normal size. Very thin.
Cornsnake/red rat snake	*Elaphe guttata guttata*	N/A for Group	Euthanased – Very poor condition
Cornsnake/red rat snake	*Elaphe guttata guttata*	N/A for Group	Euthanased – Very thin
Northern pine snake	*Pituophis melanoleucus melanoleucus*	15Y, 0M, 14D	No post-mortem
L'Hoest's monkey	*Cercopithecus ihoesti*	6Y, 7M, 11D	Postpartum trauma
L'Hoest's monkey	*Cercopithecus ihoesti*	0Y, 0M, 3D	Peripartum dystocia
South American Red-footed Tortoise	*Geochelone carbonaria*	8Y, 2M, 12D	No post-mortem

Organism		Age	Necropsy
Common Name	**Scientific Name**		
Purple-faced leaf monkey	*Trachypithecus vetulus monticola*	0Y, 0M, 15D	Hypothermia
Red howler	*Alouatta sara*	0Y, 0M, 4D	Postpartum weakness. Rejected by mother. Died overnight after start of handrearing.
Addra gazelle	*Gazelle dama mhorr*	2Y, 10M, 24D	Pleuropneumonia and pleuritis consistent with arcanobacterium pyogenes
Axolotl	*Ambystoma mexicanum*	105Y, 2M, 11D*	No post-mortem
Cornsnake/red ratsnake	*Elaphe guttata guttata*	4Y, 1M, 16D	No post-mortem
Red howler	*Alouatta sara*	10Y, 8M, 14D	End stage nephritis and an ulcerative mucotaneous disease
Waldrapp ibis	*Geronticus eremita*	13Y, 8M, 22D	Euthanased – Tumour on left foot. Unable to fly. Difficulty walking.
Blue-and-yellow macaw	*Ara ararauna*	15Y, 7M, 30D	Euthanased – Serious balance and feather problems after falling on head.
Scarlet ibis	*Eudocimus ruber*	8Y, 11M, 2D	Starvation after break injury.
Variegated spider monkey	*Ateles belzebuth hybridus*	33Y, 2M, 14D	Herpesvirus Ateles infection
Long-tailed chinchilla	*Chinchilla lanigera*	1Y, 3M, 16D	No post-mortem. Killed by other group members.
Marcaroni penguin	*Eudyptes chrysolophus*	8Y, 6M, 8D	Died under anaesthetic – isoflourane and oxygen.

Organism		Age	Necropsy
Common Name	**Scientific Name**		
Bornean orangutan	*Pongo pygmaeus pygmaeus*	14Y, 5M, 9D	Died at Paignton – No cause of death determined.
Puna ibis	*Plegadis ridgwayi*	2Y, 11M, 15D	Injury
Red ruffed lemur	*Varecia variegata rubra*	0Y, 0M, 1D	No significant findings
Red ruffed lemur	*Varecia variegata rubra*	0Y, 0M, 1D	No significant findings
Red ruffed lemur	*Varecia variegata rubra*	0Y, 0M, 1D	No significant findings
Scheltopusik/glass lizard	*Ophisaurus apodus*	9Y, 6M, 25D	Bacterial hepatitis
Chestnut teal	*Anas castenea*	0Y, 0M, 4D	Disappeared – Presumed dead
Chestnut teal	*Anas castenea*	0Y, 0M, 4D	Disappeared – Presumed dead
North American wood duck	*Aix sponsa*	0Y, 0M, 2D	Eaten by kookaburra
North American wood duck	*Aix sponsa*	7Y, 1M, 11D	Found dead
North American wood duck	*Aix sponsa*	0Y, 0M, 3D	Missing. Presumed dead
North American wood duck	*Aix sponsa*	0Y, 0M, 1D	Missing. Presumed dead
Mandarin duck	*Aix galericulata*	0Y, 0M, 1D	Missing. Presumed dead.
Red ruffed lemur	*Varecia variegata rubra*	0Y, 0M, 20D	Post-mortem on site. No significant findings. No milk in stomach and some head trauma. Mismothering.
White-faced scops owl	*Otus leucotis*	0Y, 0M, 13D	Post-mortem not possible

Organism		Age	Necropsy
Common Name	**Scientific Name**		
Sheep/mouflon	Ovis aries	0Y, 0M, 1D	Euthanased – Joints in all 4 legs seized up
White-faced scops owl	*Otus leucotis*	0Y, 0M, 14D	No post-mortem
White-faced scops owl	*Otus leucotis*	0Y, 0M, 15D	Found dead on aviary floor.
White-faced scops owl	*Otus leucotis*	0Y, 0M, 15D	Post-mortem not possible.
Leopard tortoise	*Geochelone pardalis*	0Y, 6M, 13D	Substrate injestion and gut torsion
Addra gazelle	*Gazella dama mhorr*	5Y, 0M, 26D	Euthanased – chronic suppurating joint lesion and poor condition.
North American wood duck	*Aix sponsa*	0Y, 0M, 1D	Not caught. Presumed dead.
Crowned hornbill	*Tockus alboterminatus*	1Y, 10M, 17D	Disappeared from aviary.
Mandarin duck	*Aix galericulata*	0Y, 0M, 1D	Not caught. Presumed dead.
Mandarin duck	*Aix galericulata*	0Y, 0M, 1D	Not caught. Presumed dead.
Mandarin duck	*Aix galericulata*	0Y, 0M, 1D	Not caught. Presumed dead.
North American wood duck	*Aix sponsa*	0Y, 0M, 22D	Euthanased. Slipped tendon – No post-mortem.
North American wood duck	*Aix sponsa*	0Y, 0M, 6D	No post-mortem.
Asian lion	*Panthera leo persicus*	1Y, 3M, 17D	Died 3 weeks after being donated to Ostrava zoo. Renal disease with associated cerebral oedema.

Organism		Age	Necropsy
Common Name	**Scientific Name**		
Golden lion tamarin	*Leontopithecus rosalia*	0Y, 0M, 16D	Still born
Macaroni penguin	*Eudyptes chrysolophus*	0Y, 0M, 6D	Cause of death not established.
Chestnut teal	*Anas castenea*	0Y, 0M, 1D	Not caught – assumed dead
Chestnut teal	*Anas castenea*	0Y, 0M, 1D	Not caught – assumed dead
Chestnut teal	*Anas castenea*	0Y, 0M, 1D	Not caught – assumed dead
Chestnut teal	*Anas castenea*	0Y, 0M, 1D	Not caught – assumed dead
Chestnut teal	*Anas castenea*	0Y, 0M, 1D	Not caught – assumed dead
Chestnut teal	*Anas castenea*	0Y, 0M, 1D	Not caught – assumed dead
North American wood duck	*Aix sponsa*	0Y, 0M, 23D	No post-mortem
North American wood duck	*Aix sponsa*	0Y, 0M, 10D	No post-mortem
Brown pintail	*Anas georgica spinicauda*	0Y, 0M, 7D	No post-mortem
Seba's short-tailed bat	*Carollia perspicillata*	N/A for Group	Inanition
North American wood duck	*Aix sponsa*	0Y, 1M, 0D	No post-mortem
North American wood duck	*Aix sponsa*	0Y, 0M, 13D	No post-mortem
Black howler	*Alouatta caraya*	0Y, 8M, 1D	Euthanased – Very weak. Rejected by mother due to her being pregnant again.
North American wood duck	*Aix sponsa*	0Y, 0M, 30D	No post mortem
Red shoveler	*Anas platalea*	0Y, 0M, 9D	Unavailable
Long-tailed chinchilla	*Chinchilla lanigera*	1Y, 5M, 8D	Suspected nutritional hepatopathy

Organism		**Age**	**Necropsy**
Common Name	**Scientific Name**		
Chestnut teal	*Anas castanea*	0Y, 0M, 25D	No post-mortem
Seba's short-tailed bat	*Carollia perspicillata*	N/A for Group	Inanition
Chestnut teal	*Anas castanea*	0Y, 0M, 27D	No post-mortem
Ornate horned frog	*Ceratophrys ornata*	0Y, 7M, 7D	Compacted peat in gut
Cotton-top tamarin	*Sanguinus oedipus*	0Y, 0M, 2D	Euthanased – Not being reared.
Seba's short-tailed bat	*Carollia perspicillata*	N/A for Group	Third death – Deep abscess
North American wood duck	*Aix sponsa*	0Y, 1M, 14D	No post mortem
Cotton-top tamarin	*Sanguinus oedipus*	0Y, 0M, 5D	Tubulointerstitial nephritis. Bacterial aetiology suspected.
Central Asian tortoise	Testudo horsefieldii	10Y, 5M, 27D	Huge urolith and mineral deficiency
North American wood duck	*Aix sponsa*	0Y, 1M, 4D	No post-mortem
Red shoveler	*Anas platalea*	0Y, 0M, 29D	No post-mortem
Laysan teal	*Anas platyrhynchos laysanesis*	3Y, 1M, 1D	Disappeared – assumed dead.
Chestnut teal	*Anas castanea*	0Y, 1M, 18D	No post-mortem
Southern pudu	*Pudu puda*	9Y, 1M, 19D	Peracute mastitis and necrotic stomatitis
Chilean rose tarantula	*Grammostola rosea*	5Y, 6M, 5D	No post-mortem
North American wood duck	*Aix sponsa*	0Y, 1M, 25D	No post-mortem
North American wood duck	*Aix sponsa*	0Y, 1M, 14D	Euthanased

Organism		Age	Necropsy
Common Name	**Scientific Name**		
Sheep/mouflon	*Ovis aries*	0Y, 0M, 29D	Pneumonia
Leopard gecko	*Eublepharis macularius*	3Y, 0M, 14D	Chronic gastritis, moderate hepatic lipidosis, mild/minimal multifocal hepatitis and nephritis.
Cotton-top tamarin	*Saguinus oepidus*	0Y, 0M, 30D	Bacterial septicaemia
Eastern black and white colobus	*Colobus guereza occidentalis*	7Y, 3M, 23D	Acute cell necrosis in various organs, but not organism isolated.
Bush dog	*Speothos venaticus*	0Y, 1M, 2D	Not seen. Presumed dead
Slender-tailed meerkat	*Suricata suricatta*	0Y, 0M, 9D	Remain found. Probably eaten. No post-mortem
Slender-tailed meerkat	*Suricata suricatta*	0Y, 0M, 9D	No post-mortem
Black-tailed prairie dog	*Cynomys ludovicianus*	N/A for group	Three not seen since 9 June. Presumed dead.
Bush dog	*Speothos venaticus*	0Y, 1M, 2D	Not seen. Presumed dead
Slender-tailed meerkat	*Suricata suricatta*	0Y, 0M, 13D	Starvation
Giraffe	*Giraffa carmelopardalis*	2Y, 2M, 12D	Profound hind limb, ataxia and hip dysplasia.
Slender-tailed meerkat	*Suricata suricatta*	0Y, 0M, 14D	Body not found
Bactrian camel	*Camelus bactrianus*	0Y, 0M, 5D	Neonatal septicaemia
Bush dog	*Speothos venaticus*	0Y, 1M, 12D	Various traumatic lesions. Mild to moderate pneumonia.
Black-necked swan	*Cygnus melanocoryphus*	2Y, 2M, 17D	No significant findings. Severe bumblefoot of both feet may have caused long term deterioration.

Organism		**Age**	**Necropsy**
Common Name	**Scientific Name**		
West African dwarf crocodile	*Osteolaemus tetraspis tetraspis*	3Y, 10M, 14D	Severe renal gout
Boobook hawk owl	*Ninox novaeseelandie*	0Y, 3M, 2D	Unavailable
Iguana	*Iguana iguana rhinolopha*	12Y, 7M, 22D	Chronic-active hepatitis, fungal pneumonia, chronic-active splenitis.
Southern pudu	*Pudu puda*	0Y, 2M, 30D	Necrotic lesion obstructing the larynx (calf diphtheria)
Marcaroni penguin	*Eudyptes chrysolophus*	13Y, 7M, 15D	Malaria
Brazilian rainbow boa	*Epicrates cenchria cenchria*	7Y, 1M, 10D	Too decomposed for post-mortem
Red howler	*Alouatta sara*	0Y, 0M, 3D	Euthanased – Rejected by mother. Unsuccessful handrearing.
Seba's short-tailed bat	*Carollia persicillata*	N/A for group	Severeintraluminal haemorrhage of small intestine. Ishcaemia secondary to obstruction of venuous drainage by torsion possible.
Chestnut teal	*Anas castanea*	0Y, 1M, 25D	Euthanased. No post-mortem
Chestnut teal	*Anas castanea*	0Y, 1M, 28D	Euthanased. Bilateral sinusitis. No post-mortem.
Black-tailed prairie dog	*Cynomys ludovicianus*	0Y, 5M, 2D	Euthanased. Broken spine.

Organism		Age	Necropsy
Common Name	**Scientific Name**		
Patagonian mara	*Dolichotis patagonum*	6Y, 3M, 0D	Abscess of right lymphatic bulla causing compression of ventral brain. Specimen euthanased due to neurological signs.
Siberian chipmunk	*Tamias sibiricus*	N/A for group	Killed by exhibit mate
Siberian chipmunk	*Tamias sibiricus*	N/A for group	Killed by exhibit mate
Seba's short-tailed bat	*Carollia persicillata*	N/A for group	Previous members of group had died due to undiagnosed but presumed stress-related causes.
Siberian chipmunk	*Tamias sibiricus*	N/A for group	Euthanased
Black howler	*Alouatta caraya*	27Y, 0M, 19D	Euthanased. Chronic colitis with annular band of fibrosis (possible neoplasia) of caecum. Valvular endocardiosis of the left a-v valve and left atrial dilation. End-stage renal fibrosis.
Lesser sulphur-crested cockatoo	*Cacatua sulphurea*	28Y, 9M, 18D	Severe emaciation and arterioschleriosis.
White-cheeked turaco	*Tauraco leucotis*	5Y, 4M, 26D	Unavailable
White-naped crane	*Grus vipio*	0Y, 3M, 6D	Syngamus and scarid spp
Red-kneed tarantula	*Brachypelma smithi*	4Y, 10M, 7D	Unable to post-mortem – too decomposed.
Coscoroban swan	*Coscoroba coscoroba*	10Y, 4M 26M	Bumblefoot – showed nothing else abnormal.
Red ruffed lemur	*Varieca variegate rubra*	13Y, 7M, 4D	Died at Colchester

Organism		Age	Necropsy
Common Name	**Scientific Name**		
Oriental small-clawed otter	*Amblonyx cinereus*	0Y, 0M, 8D	Neonatal death - not reared
Red-fronted macaw	*Ara rubrogenys*	2Y, 4M, 2D	Unavailable
Dusky leaf monkey	*Trachypithecus obscurus*	0Y, 8M, 11D	Subacute suppurative bronchopneumonia (commensurate with previous aspiration of enteric contents)
Puna ibis	*Plegadis ridgwayi*	14Y, 1M, 29D	
Allen's swamp monkey	*Allenopithecus nigroviridis*	17Y, 9M, 1D	Aortic thromboembolism
Chilean teal	*Anas flavirostris flavirostris*	7Y, 8M, 4D	All deaths occurred after movement of these teal into an enclosure housing stork which were seen to be aggressive towards them. Although no lesions indicative of physical trauma were noted, the most likely cause of death would appear to be bullying and resultant stress.
Chilean teal	*Anas flavirostris flavirostris*	1Y, 5M, 21D	All deaths occurred after movement of these teal into an enclosure housing stork which were seen to be aggressive towards them. Although no lesions indicative of physical trauma were noted, the most likely cause of death would appear to be bullying and resultant stress.

Organism		Age	Necropsy
Common Name	**Scientific Name**		
Chilean teal	*Anas flavirostris flavirostris*	1Y, 5M, 21D	All deaths occurred after movement of these teal into an enclosure housing stork which were seen to be aggressive towards them. Although no lesions indicative of physical trauma were noted, the most likely cause of death would appear to be bullying and resultant stress.
Rufous-vented chachalaca	*Ortalis ruficauda*	15Y, 4M, 2D	Tracheal avulsion with coincidental, or possibly aetiological connected, iron storage disease.
Chilean teal	*Anas flavirostris flavirostris*	0Y, 4M, 4D	All deaths occurred after movement of these teal into an enclosure housing stork which were seen to be aggressive towards them. Although no lesions indicative of physical trauma were noted, the most likely cause of death would appear to be bullying and resultant stress.
Chilean teal	*Anas flavirostris flavirostris*	0Y, 4M, 4D	All deaths occurred after movement of these teal into an enclosure housing stork which were seen to be aggressive towards them. Although no lesions indicative of physical trauma were noted, the most

			likely cause of death would appear to be bullying and resultant stress.
Organism		**Age**	**Necropsy**
Common Name	**Scientific Name**		
Pygmy marmoset	*Callithrix pygmaea*	0Y, 1M, 21D	No diagnosis made at gross PM examination or subsequent histology.
Chilean teal	*Anas flavirostris flavirostris*	7Y, 2M, 23D	All deaths occurred after movement of these teal into an enclosure housing stork which were seen to be aggressive towards them. Although no lesions indicative of physical trauma were noted, the most likely cause of death would appear to be bullying and resultant stress.
Golden poison dart frog	*Phyllobates terribilis*	N/A for group	Unavailable
Golden poison dart frog	*Phyllobates terribilis*	N/A for group	No post-mortem planned
Yellow-banded poison dart frog	*Dendrobates leucomelas*	N/A for group	Unavailable
Yellow-banded poison dart frog	*Dendrobates leucomelas*	N/A for group	No post-mortem planned
Scarlet ibis	*Eudocimus ruber*	0Y, 2M, 18D	Unavailable
Whooper swan	*Cygnus cygnus cygnus*	8Y, 9M, 7D	Unavailable

Organism		Age	Necropsy
Common Name	**Scientific Name**		
Dusky leaf monkey	*Trachypithecus obscurus*	0Y, 0M, 18D	Stillborn. No breath taken but reason remains undiagnosed.
Rufous-vented chachalaca	*Ortalis ruficauda*	15Y, 5M, 21D	No diagnosis made on post-mortem. Histology shows iron storage disease also significant finding in this bird.
South American Tapir	*Tapirus terrestris*	0Y, 0M, 12D	Euthanased after attempt to resuscitate.
Pink-backed pelican	*Pelecanus rufescens*	6Y, 4M, 4D	Probably became hypothermis following water-logging of plumage during attempts to exit pond.
Palawan peacock peasant	*Polyplectron emphanum*	15Y, 6M, 11D	Abscessation and haemorrhage of the liver
Brush-tailed rock wallaby	*Petrogale penicillata penicillata*	5Y, 4M, 19D	Acute bacterial hepatitis of unknown etiology
White-fronted marmoset	*Callithrix geoffroyi*	11Y,9M, 28D	Iron storage disease leading to possible hepatorenal syndrome. Capillaria eggs found in liver.
White-cheeked gibbon	*Hylobates leucogenys*	31Y, 6M, 3D	Disseminated intestinal adenocarcinoma. Due to deteriorating health, age and the finding of a grossly apparent neoplasia under anaesthetic, the animal was enthanased.

Organism		Age	Necropsy
Common Name	**Scientific Name**		
Bell's hingeback tortoise	*Kinixys belliana*	10Y, 0M, 14D	Post-ovulatory egg stasis and weak plastron, presumably due to calcium deficiency. Histology of bone revealed severe diffuse osteopenia, whilst histology of the liver showed yolk coelomitis and severe lipidosis.
Pileated gibbon	*Hylobates pileatus*	22Y, 1M, 8D	Mediastinal ad intestinal lymphadenopathy, lyphatic enlargement of spleen, liver abd kidney and gastric ulceration.
White-fronted marmoset	*Callithrix geoffroyi*	3Y, 10M, 24D	Hepatits, nephritis and vasculitis suggestive of lymphocytic choriomenigitis virus (LCMV).
Oriental small-clawed otter	*Amblonyx cinereus*	3Y, 3M, 4D	Death presumed to be from acute respiratory infection, presumed bacterial in origin.
Black howler	*Alouatta caraya*	0Y, 5M, 28D	Colitis and lymphoid hyperplasia of intestine
Common marmoset	*Callithrix jacchus*	0Y, 0M, 24D	No diagnosis was made after gross post-mortem and histological examination.
Pygmy marmoset	*Callithrix pygmaea*	0Y, 0M, 6D	Found dead. No diagnosis made on post-mortem or histological diagnosis.
Giant African millipede	*Archispirostreptus*	N/A for group	This millipede apparently suffered an internal fungal infection. This may well have originated from the

			environment. Keeping the vivarium free of gross fungal growth in the future may be beneficial.
Organism		**Age**	**Necropsy**
Common Name	**Scientific Name**		
Siberian chipmunk	*Tamias sibiricus*	N/A for group	Euthanased on management and welfare grounds
White-fronted marmoset	*Callithrix geoffroyi*	4Y, 5M, 22D	Found dead am. Post-mortem and histology are very suggestive of LCMV – acute necrotising hepatitis, nephritis and vasculitis, subcuticular haemorrhage, pleural effusion and hepatosplenomegaly
Pygmy marmoset	*Callithrix pygmaea*	0Y, 0M, 14D	Found dead. Never breathed.
Praying mantis	*Polyspilota aeruginosa*	3Y, 1M, 29D	This animal became swollen and autolytic. No post-mortem was conducted but these females do apparently die from egg-binding, and this may have resulted from inadequate humidity.
Purple-faced leaf monkey	*Trachypithecus vetulus monticola*	0Y, 5M, 15D	Tubular necrosis of kidneys and acute encephalomacia of brainstem. Aetiology unknown but possibly due to vascular compromise following an enterotoxaemia.
Red howler	*Alouatta sara*	9Y, 2M, 17D	The chronic skin lesions caused immune complex production resulting in a chronic glomerulonephritis.

			This led to a proteinuria and resultant multicentric thromboses of the systemic and pulmonary circulations.
Organism		**Age**	**Necropsy**
Common Name	**Scientific Name**		
Chilean rose tarantula	*Grammostola rosea*	11Y, 2M, 29D	Found dead. It is assumed that this animal was ffected by hypothermia due to heating failure. A heavy growth of *Proteus* spp. Cateria isolated from the coelomic cavity may well have occurred as a secondary feature.
Domestic rabbit, breed unspecified	*Oryctolagus cuniculus domestic*	3Y, 2M, 28D	Treatment of a facial abscess had reduced it but not controlled the infection. At PME, the upper right teeth arcade was abnormal suggesting involvement. It is presumed that this animal became septicaemic. Although the abscess tracked deep into facial tissue it did not communicate with intracranial tissue.
Bornean orantugan	*Pongo pygmaeus*	31Y, 3M, 25D	The death may be due to a generalised infection, which probably started with the necrotic mass. The origin of this mass is still unclear.

Organism		Age	Necropsy
Common Name	**Scientific Name**		
Red howler	*Alouatta sara*	11Y, 9M, 5D	Euthanased after symptomatic treatment for perineal lesions was not successful.
Brush-tailed rock wallaby	*Petrogale penicillata penicillata*	3Y, 11M, 15D	No diagnosis made
Fiery-shouldered conure	*Pyrrhura egregia*	1Y, 9M, 9D	This bird carcass was predated, probably post-mortem by terrapin in the pond, making diagnosis more difficult.
Long-tailed chinchilla	*Chinchilla lanigera*	2Y, 3M, 18D	Suspected anteritis may have led to a bacteraemia and formation of cardiac thrombus.
Praying mantis	*Polyspilota aeruginosa*	3Y, 3M, 18D	This animal may have been female and been suffering from egg-binding, in which a lack of adequate humidity is implicated.
Black-winged ground dove	*Metropelia melanoptera*	1Y, 3M, 17D	Collision with roof of aviary suspected.
Scarlet ibis	*Eudocimus ruber*	8Y, 5M, 7D	This bird almost certainly collided with the aviary roof causing brain haemorrhage and resultant death.
Black-tailed prairie dog	*Cynomys ludovicianus*	N/A for group	This animal was seen to be subject to aggression from non-specifics. It had superficial and deeper wounds to the upper and lower left abdomen (with

			rupture of abdominal contents into the subcuticular space), a fractured last right rib and a deep puncture wound to the atlanto-ocipital region of the neck, with significant associated haemorrhage. It was found dead outside the enclosure (suggesting some of the injuries at least may have been inflicted by predators)
Organism		**Age**	**Necropsy**
Common Name	**Scientific Name**		
Red-eared slider	*Trachemys scripta elegans*	16Y, 3M, 22D	Hepatic lipidosis – Probably as a secondary result of post hibernation anorexia. Animal 2 also had a severe suppurative bronchopneumonia – probably the primary cause of death.
Red-eared slider	*Trachemys scripta elegans*	16Y, 3M, 22D	Hepatic lipidosis – Probably as a secondary result of post hibernation anorexia. Animal 2 also had a severe suppurative bronchopneumonia – probably the primary cause of death.
Patagonian mara	*Dolichotis patagonum*	2Y, 5M, 28D	Gross post-mortem and histology suggest that mineralisation of aorta and renal arteries may have played a part in the death of this animal.

Organism		Age	Necropsy
Common Name	**Scientific Name**		
Bali mynah	*Leucospar rothschildi*	9Y, 0M, 4D	Mostly eaten by predator – very few remains. No post-mortem.
Black-cheeked lovebird	*Agapornis nigrigenis*	N/A for group	Several nematodiasis of the small intestine resulting in severe emaciation.
Jan silvered leaf monkey	*Trachypithecus auratus auratus*	0Y, 0M, 10D	Histology of the placenta revealed intervillous fibrin plaques which may have resulted in foetal death prior to expulsion. The aetiology of these plaques is not clear.
Patagonian mara	*Dolichotis patagonum*	0Y, 0M, 16D	Failure to breathe at birth may have been related to birthing trauma.
Red-bellied lemur	*Eulemur rubriventer*	0Y, 0M, 16D	Abandoned. Post-mortem and histology showed no abnormalities.
Dwarf siamang	*Hylobates klossi*	38Y, 5M, 5D	Significant iron storage disease involving liver, kidney, spleen and intestine. Severity may have been adequate to produce significant hepatic impairment.
Chimpanzee	*Pan troglodytes*	1Y, 1M, 29D	Acquired atelectasis and overinflation of unknown aetiology but laryngeal spasm suspected.

Organism		Age	Necropsy
Common Name	**Scientific Name**		
Hissing cockroach	*Gromphadorhina oblongata*	N/A for group	Swabs were taken from the coelomic cavity and gastrointestinal tract for fungal and bacterial culture, but did not elucidate any infectious cause.
Red-bellied lemur	*Eulemur rubriventer*	0Y, 0M, 26D	No post-mortem – Body sent to museum of Scotland
Sacred ibis	*Threskiornis aethiopicus*	0Y, 0M, 30D	Plumage entirely waterlogged
Capybara	*Hydrochaeris hydrochaeris*	0Y, 0M, 16D	Attacked by babirusa in adjoining paddock.
Red howler	*Alouatta sara*	9Y, 9M, 9D	Severe end-stage kidney disease was responsible for the clinical signs seen prior to the decision to euthanase. This has been a historical feature of adult red howlers at Twycross zoo, but is of unknown aetiology and from this case at least, appears unconnected to the perineal lesions seen recently in all red howlers at the zoo.
Black-tailed marmoset	*Callithrix argentata melanura*	0Y, 0M, 23D	This neonate failed to suckle and showed partial to complete atelectasis on histology. Although unusual it is possible for lung inflation to only partially occur at birth, and presumably total collapse to follow –

			this neonate was definitely alive for 3 days, or severe autolysis wold have been a feature.
Organism		**Age**	**Necropsy**
Common Name	**Scientific Name**		
Orange-winged amazon	*Amazona amazonica*	0Y, 0M, 27D	No post-mortem. No body
Orange-winged amazon	*Amazona amazonica*	0Y, 0M, 27D	No post-mortem. No body
Domestic zebu	*Bos Taurus indicus*	0Y, 0M, 1D	Approximately two-thirds term foetus – lacking any hair, eyelids still fused, hooves not properly formed. No abnormalities detected. This abortion almost certainly relates to the anaesthesia of the dam some 5 days previously for TB test pre export. Every effort needs to be made in future to establish which livestock are likely to be pregnant in order to avoid a repeat of this.
Common marmoset	*Callithrix jacchus*	0Y, 0M, 7D	Found dead in enclosure. Suspect abandonment as came from group of quadruplets (even triplets are often not reared). Severe trauma to limbs, as pelvis (abiating sexual organs), abdomen, thorax and head, consistent with rodent scavenging. Not sent for histology.

Organism		Age	Necropsy
Common Name	**Scientific Name**		
Common marmoset	*Callithrix jacchus*	0Y, 0M, 10D	PME not performed since inanition was almost certainly the cause of death.
Green iguana	*Iguana iguana iguana*	7Y, 6M, 14D	A disseminated systemic mycobacterial infection. This has potential consequences for all inhabitants of the Tropical House. However, partial identification of the organism to blame suggests a mycobacterium of non-mammalian pathogenicity – possibly one of many spp seen surviving in soil as a saprophyte but occasionally causing disease in reptilian hosts.
Patagonian mara	*Dolichotis patagonum*	1Y, 1M, 13D	This gravid female died as a result of retained foetuses.
Javan silvered leaf monkey	*Trachypithecus auratus auratus*	13Y, 7M, 23D	2 month history of emesis. Suspected GIT obstruction then gestational hyperemesis. Non-responsive to therapy and became moribund. Euthanased. Most significant lesion appears to be a stenosis of the upper small intestine of unknown aetiology, although this may be normal species anatomy (pers. Comm. With D. Chivers). Histology

			suggests chronic cholelithiasis and a suppurative cholangitis are likely aetiology.
Organism		**Age**	**Necropsy**
Common Name	**Scientific Name**		
Domestic rat. Breed unspecified.	*Rattus norvegicus domestic*	3Y, 6M, 18D	Liz developed a bloody vulval discharge in April 2006. No abdominal mass was apparent. A vulval swab showed a profuse growth of mixed anaerobes and Liz was treated accordingly with metronidazole. The discharge improved but never stopped. Surgical exploration was deemed inappropriate. Bleeding suddenly became significant on 21st May, 2006 necessitating euthanasia. PME revealed both uterine horns to be distended with soft homogenous brown material (blood?) and several white plaques across the pleura, as well as a pericardial and pleural effusion. Histology suggests that Mycoplasma pulmonis may have been the organism responsible for both uterine and respiratory pathology.
Golden lion tamarin	*Leontopithecus rosalia*	0Y, 0M, 16D	Nothing abnormal found on histology.

Organism		Age	Necropsy
Common Name	**Scientific Name**		
Emperor tamarin	*Sanguinus imperator subgrisescens*	0Y, 0M, 3D	Oversized foetus appears to have resulted in dystocia and death in utero.
Patagonian mara	*Dolichotis patagonium*	0Y, 0M, 12D	Retained foetuses.
Patagonian mara	*Dolichotis patagonium*	0Y, 0M, 12D	Retained foetuses.
Laysian teal	*Anas platyrhynchos laysanensis*	0Y, 0M, 27D	Bird found dead. Carcass already decomposed beyond worth of a PME. Prbable predation.
Eastern black and white colobus	*Colobus guereza occidentalis*	0Y, 0M, 10D	This foetus was aborted whilst the dam was undergoing treatment. It is possible that the stress of anaesthesia of the dam, and associated care was a factor in the abortion, or that bacterial pyometra played a part.
Macaroni penguin	*Eudyptes chrysolophus*	20Y, 2M, 20D	Avian malaria
Macaroni penguin	*Eudyptes chrysolophus*	14Y, 7M, 13D	Avian malaria with positive identification of both Plasmodium and Leucocytozoon spp.
Golden-headed tamarin	*Leontopithecus chrysomelas*	4Y, 11M, 26D	Died post-anaesthesia and subsequent to traumatic wounds sustained from conspecifics.
Eastern black and white colobus	*Colobus guereza occidentalis*	12Y, 10M, 10D	Dolly was suffering from a suppurative and ongoing severe metritis. It is unclear whether this infection

			preceded (and potentially caused) the abortion, or resulted as a sequel to it.
Organism		**Age**	**Necropsy**
Common Name	**Scientific Name**		
Macaroni penguin	*Eudyptes chrysolophus*	14Y, 7M, 25D	Avian malaria
Cotton-top tamarin	*Sanguinus Oedipus*	0Y, 0M, 14D	Failure to suckle
Slender-tailed meerkat	*Suricata suricatta*	0Y, 1M, 11D	This neonate was almost certainly suffering from inanition and was abandoned and then partially consumed, probably by conspecifics.
Domestic guinea pig	*Cavia porcellus*	N/A for group	Chronic cholecystitis
Whiteknee tarantula	*Acanthoscurria geniculata*	3Y, 8M, 10D	No diagnosis made.
Macaroni penguin	*Eudyptes chrysolophus*	20Y, 3M, 16D	Bacterial pneumonia appears predominant cause of death. Although no exoerythrocytic protozoa were found, hepatitis and splenitis are characteristic of avian malaria as previously seen in the group.
Black-faced ibis	*Theristicus melanopis*	9Y, 4M, 16D	Sustained a fractured right tarsometatarsus. Inoperable and euthanased. Otherwise normal at PM.
Macaroni penguin	*Eudyptes chrysolophus*	14Y, 9M, 6D	Although there are no definite examples of protozoa found, the histological lesions are strongly suggestive that this bird died from avian malaria.

Organism		Age	Necropsy
Common Name	**Scientific Name**		
White-faced scops owl	*Otus leucotis*	1Y, 2M, 22D	Bird died of an ascending bacterial infection causing a severe nephritis.
Patagonian mara	*Dolichotis patagonum*	0Y, 0M, 20D	Foetal atelectasis with no inflammation.
Western lowland gorilla	*Gorilla gorilla gorilla*	33Y, 1M, 9D	Severe chronic fibrosing cardiomyopathy as previously described in captive population of Western Lowland Gorilla with associated histological findings. Probably a multi-factorial aetiology that may include various infectious agents, stress, obesity and arteriosclerosis.
Macaroni penguin	*Eudyptes chrysolophus*	9Y, 4M, 27D	This bird appeared to have died of a bacterial pneumonia and airsacculitis secondary to generalised plasmodium infection.
Macaroni penguin	*Eudyptes chrysolophus*	14Y, 9M, 18D	This bird died from a combination of gastro-intestinal obstruction and avian malaria.
Striped skunk	*Mephitis mephitis*	0Y, 5M, 28D	Although no obvious bacterial pathogens were isolated, the haemorrhagic colitis, renal tubular necrosis and lung oedema, are the most suggestive of a bacterial endotoxaemia

Organism		Age	Necropsy
Common Name	**Scientific Name**		
Silvery marmoset	*Callithrix argentata argentata*	0Y, 0M, 30D	Partial atelectasis and subsequent inflammation.
Silvery marmoset	*Callithrix argentata argentata*	0Y, 0M, 30D	No diagnosis made
Dusky leaf monkey	*Trachypithecus obscurus*	0Y, 0M, 28D	Animal euthanased after maternal rejection and policy of not attempting to hand-rear this species.
Black howler	*Alouatta caraya*	0Y, 0M, 9D	Atelectasis
Alpaca	*Lama pacos*	0Y, 2M, 12D	Although impossible to confirm definitely, the history, gross post-mortem and microbiology results suggest that this animal died of a clostridial infection. Vaccination of the adults had been overlooked during the summer and has been re-instigated. The histology is less convincing and may suggest a liver lobe torsion as the initial cuase.
Hylobates agilis	*Hylobates agilis*	1Y, 1M, 11D	It is probable that this gibbon died as a result of primary shigellosis, although a cryptosporidial infection may also have been significant. Terminally, it seems that an endotoxaemia and pneumonia were responsible for death.

Organism		Age	Necropsy
Common Name	**Scientific Name**		
Oriental small-clawed otter	*Amblonyx cinereus cinereus*	9Y, 1M, 28D	This animal had birthed two neonates before experiencing dystocia. All microbiology and histology points towards a mechanical problem, with changes ween almost certainly post-mortem.
Pygmy marmoset	*Callithrix pygmaea*	0Y, 0M, 17D	The animal was kidnapped by common marmosets and sustained severe head trama.
Neon tetra	*Paracheirodon innesi*	N/A for group	Found dead. No diagnosis made. Other fish in tank okay.
Peppered catfish	*Corydoras paleatus*	N/A for group	Acute septicaemic bacterial infection. No evidence of parasitism to be concerned about for tank mates.
Domestic guinea pig	*Cavia porcellus*	N/A for group	This animal was suffering from progressive loss of condition and had very large palpable cystic ovaries. Euthanased on welfare grounds.
Inland bearded dragon	*Pogona vitticeps*	6Y, 10M, 22D	Blood showed renal failure and a significant inflammatory process going on. Microbiology revealed a heavy *Pseudomonas aeruginosa* stomatitis. The animal was euthanased and histology diagnosed a hepatic carcinoma with metastases to heart and coelom.

Organism		Age	Necropsy
Common Name	**Scientific Name**		
Blue poison dart frog	*Dendrobates azureus*	0Y, 1M, 26D	Despite gross evidence of fungal infestation, there is none to suggest this tadpole died of it. No diagnosis made.
Red-crested turaco	*Tauraco erythrolophus*	0Y, 3M, 10D	This bird had a grossly enlarged liver with some subcapsular haemorrhage. Presumed aggression between parents and these juveniles was responsible, although subsequent events suggest an aetiology of yersiniosis is more likely.
Emperor tamarin	*Saguinus imperator subgrisescens*	4Y, 0M, 27D	This animal died from Marmoset Wasting Syndrome.
Laughing kookaburra	*Dacelo novaeguineae*	17Y, 0M, 23D	Impression at gross PME are that this bird died of a bacterial infection – probably Yersiniosis. Histology suggests that the infection is probably due to *E.coli* rather than Yersinia.
Puna ibis	*Plegadis ridgwayi*	6Y, 3M, 26D	Missing majority of beak and extensive head trauma. Otherwise normal externally and internally. Very good body condition.

Organism		Age	Necropsy
Common Name	**Scientific Name**		
Puna ibis	*Plegadis ridgwayi*	1Y, 7M, 14D	Head absent, neck de-gloved and thoracic inlet open. Otherwise normal externally and internally. Very good body condition.
Sacred ibis	*Threskiornis aethiopicus*	10Y, 8M, 29D	Missing digits of left foot (surgery). Some feather loss and abrasions over avulas. Poor body condition. Significant valvular disease and segmental myocardial depigmentation.

Y = Years; M = Months; D = Days

Appendix 13: Results of LCMV Screening by Public Health England and Doctoral Student in Present Study

Organism		PHE ID	THESIS ID	PHE Study		Present Study
Common Name	Scientific Name			Serology	PCR	RT-PCR
Geoffroy's Marmoset	*C. geoffroyi*	H06 094 0752	NA73	NA	Positive	Negative
Geoffroy's Marmoset	*C. geoffroyi*	H06 094 0753	NA74	NA	Positive	Positive
Geoffroy's Marmoset	*C. geoffroyi*	H06 336 0621 01	NA14	NA	Positive	Positive
Geoffroy's Marmoset	*C. geoffroyi*	H06 276 0592 02	-	Positive	Negative	NT
Geoffroy's Marmoset	*C. geoffroyi*	H06 276 0593 01	-	Positive	Negative	NT
Geoffroy's Marmoset	*C. geoffroyi*	H06 276 0594 02	-	Negative	Negative	NT
Geoffroy's Marmoset	*C. geoffroyi*	H07 196 0594 01	-	Equivocal	NA	NT
Geoffroy's Marmoset	*C. geoffroyi*	H07 196 0596 01	-	Negative	NA	NT

Organism		PHE ID	THESIS ID	PHE Study		Present Study
Common Name	**Scientific Name**			**Serology**	**PCR**	**RT-PCR**
Linne's Two-toed Sloth	*C. didactylus*	H06 230 0544 01	-	Positive	NA	NT
Linne's Two-toed Sloth	*C. didactylus*	H06 230 0545 01	-	Positive	NA	NT
Hamlyn's Guenon	*C. hamlyni*	H06 230 0549 01	-	Negative	NA	NT
Hamlyn's Guenon	*C. hamlyni*	H07 196 0609 01	-	Negative	NA	NT
Hamlyn's Guenon	*C. hamlyni*	H07 196 0610 01	-	Negative	NA	NT
Lesser Spot-nosed Guenon	*C. petaurista*	H06 230 0561 01	-	Negative	NA	NT
Lesser Spot-nosed Guenon	*C. petaurista*	H07 196 0613 01	-	Negative	NA	NT
Roloway Monkey	*C. roloway*	H06 230 0550 01	-	Negative	NA	NT
Chimpanzee	*P. troglodytes*	H06 230 0551 01	-	Positive	NA	NT
Chimpanzee	*P. troglodytes*	H06 230 0555 01	-	Positive	NA	NT
Chimpanzee	*P. troglodytes*	H06 230 0556 01	-	Positive	NA	NT

Organism		PHE ID	THESIS ID	PHE Study		Present Study
Common Name	**Scientific Name**			**Serology**	**PCR**	**RT-PCR**
Chimpanzee	*P. troglodytes*	H06 336 0611 01	NA6	NA	Negative	Negative
Chimpanzee	*P. troglodytes*	H07 196 0626 01	-	Positive	NA	NT
Chimpanzee	*P. troglodytes*	H07 196 0627 01	-	Negative	NA	NT
Chimpanzee	*P. troglodytes*	H07 196 0628 01	-	Positive	NA	NT
Chimpanzee	*P. troglodytes*	H07 196 0629 01	-	Positive	NA	NT
Chimpanzee	*P. troglodytes*	H07 196 0630 01	-	Positive	NA	NT
Chimpanzee	*P. troglodytes*	H07 196 0631 01	-	Positive	NA	NT
Chimpanzee	*P. troglodytes*	H07 196 0632 01	-	Positive	NA	NT
Chimpanzee	*P. troglodytes*	H07 196 0633 01	-	Positive	NA	NT
Chimpanzee	*P. troglodytes*	H08 140 0520	-	NT	NT	NT
Chimpanzee	*P. troglodytes*	H08 140 0521	-	NT	NT	NT
Western Lowland Gorilla	*G. gorilla*	H06 230 0552 01	-	Negative	NA	NT

Organism		PHE ID	THESIS ID	PHE Study		Present Study
Common Name	**Scientific Name**			**Serology**	**PCR**	**RT-PCR**
Western Lowland Gorilla	*G. gorilla*	H07 196 0589 01	-	Negative	NA	NT
Western Lowland Gorilla	*G. gorilla*	H07 196 0591 01	-	Equivocal	NA	NT
Western Lowland Gorilla	*G. gorilla*	H07 196 0592 01	-	Positive	NA	NT
Western Lowland Gorilla	*G. gorilla*	H08 122 0604 01	NA45	NA	NA	Negative
Western Lowland Gorilla	*G. gorilla*	H08 122 0608 03	NA49	NA	NA	Negative
Western Lowland Gorilla	*G. gorilla*	H08 122 0613 01	-	NA	NA	NT
Western Lowland Gorilla	*G. gorilla*	H08 122 0617 01	-	NA	NA	NT
Black and White Colobus	*C. guereza occidentalis*	H06 230 0553 01	-	Positive	Positive	NT
Black and White Colobus	*C. guereza occidentalis*	H06 336 0623 01	NA16	NA	Negative	Negative
Black and White Colobus	*C. guereza occidentalis*	H06 336 0624 01	NA17	NA	Negative	Negative
Black and White Colobus	*C. guereza occidentalis*	H06 336 0625 01	NA18	NA	Positive	Positive

Organism		PHE ID	THESIS ID	PHE Study		Present Study
Common Name	**Scientific Name**			**Serology**	**PCR**	**RT-PCR**
Black and White Colobus	*C. guereza occidentalis*	H06 276 0590 01	-	Negative	Negative	NT
Black and White Colobus	*C. guereza occidentalis*	H06 276 0591 01	-	Negative	Negative	NT
Black and White Colobus	*C. guereza occidentalis*	H08 122 0614 01	-	NA	NA	NT
Unidentified primate*	-	H06 266 0574 01	-	Negative	NA	NT
Unidentified primate*	-	H06 266 0575 01	-	Negative	NA	NT
Unidentified primate*	-	H06 266 0576 01	-	Negative	NA	NT
Unidentified primate*	-	H06 266 0577 01	-	Negative	NA	NT
Unidentified primate*	-	H06 266 0579 01	-	Negative	NA	NT
Unidentified primate*	-	H06 266 0580 01	-	Negative	NA	NT
Unidentified primate*	-	H06 266 0583 01	-	Negative	NA	NT
Unidentified primate*	-	H06 266 0584 01	-	Negative	NA	NT

Organism		PHE ID	THESIS ID	PHE Study		Present Study
Common Name	**Scientific Name**			**Serology**	**PCR**	**RT-PCR**
Unidentified primate*	-	H06 266 0585 01	-	Negative	NA	NT
Unidentified primate*	-	H06 266 0586 01	-	Negative	NA	NT
Javan Leaf Monkey	*T. auratus auratus*	H06 230 0554 01	-	Negative	NA	NT
Red Howler Monkey	*A. sara*	H06 230 0557 01	-	Negative	NA	NT
Red Howler Monkey	*A. sara*	H06 336 0616 04	NA10	NA	Negative	Negative
Red Howler Monkey	*A. sara*	H06 336 0618 01	NA12	NA	Negative	Negative
Red Howler Monkey	*A. sara*	H06 336 0627 01	NA20	NA	Negative	Negative
Red Howler Monkey	*A. sara*	H06 336 0614 03	NA8	NA	Negative	Negative
Red Howler Monkey	*A. sara*	H08 122 0595 01	NA41	NA	NA	Negative
Variegated Columbian Spider Monkey	*A. belzebuth hybridus*	H06 230 0558 01	-	Negative	NA	NT
Dusky Leaf Langur	*T. obscurus*	H06 230 0559 01	-	Negative	NA	NT
Dusky Leaf Langur	*T. obscurus*	H06 230 0560 01	-	Negative	NA	NT

Organism		PHE ID	THESIS ID	PHE Study		Present Study
Common Name	**Scientific Name**			**Serology**	**PCR**	**RT-PCR**
Dusky Leaf Langur	*T. obscurus*	H06 336 0619 02	NA13	NA	Negative	Negative
Dusky Leaf Langur	*T. obscurus*	H06 336 0626 01	NA19	NA	Negative	Negative
Dusky Leaf Langur	*T. obscurus*	H07 196 0606 01	-	Negative	NA	NT
Dusky Leaf Langur	*T. obscurus*	H08 122 0597 01	NA43	NA	NA	Negative
Kloss Gibbon	*H. klossii*	H06 336 0612 01	NA7	NA	Negative	Negative
Northern White-cheeked Gibbon	*N. leucogenys*	H06 336 0613 01	NA76	NA	Negative	Positive
Agile Gibbon	*H. agilis*	H08 122 0605 01	NA46	NA	NA	Negative
Agile Gibbon	*H. agilis*	H08 122 0611 01	NA52	NA	NA	Negative
Black Howler Monkey	*A. caraya*	H06 336 0615 02	NA9	NA	Negative	Negative
Black Howler Monkey	*A. caraya*	H07 196 0639 01	NA25	NA	Negative	Negative
Black Howler Monkey	*A. caraya*	H08 122 0609 01	-	NA	NA	NT
Black Howler Monkey	*A. caraya*	H08 122 0610 01	NA51	NA	NA	Negative
Allen’s Swamp Monkey	*A. niviridus*	H06 336 0617 01	NA11	NA	Negative	Negative

Organism		PHE ID	THESIS ID	PHE Study		Present Study
Common Name	**Scientific Name**			**Serology**	**PCR**	**RT-PCR**
Allen's Swamp Monkey	*A. niviridus*	H07 196 0608 01	-	Positive	NA	NT
Allen's Swamp Monkey	*A. niviridus*	H07 196 0612 01	-	NA	Negative	NT
Golden - Headed Lion Tamarin	*L. rosalia*	H06 336 0620 01	-	NA	Negative	NT
Golden - Headed Lion Tamarin	*L. rosalia*	H07 196 0670 01	NA37	NA	Negative	Negative
Purple-faced Langur	*T. vetulus monticola*	H06 336 0622 01	NA15	NA	Positive	Negative
Purple-faced Langur	*T. vetulus monticola*	H07 196 0601 01	-	Negative	NA	NT
Purple-faced Langur	*T. vetulus monticola*	H08 334 0684 01	-	NA	NA	NT
Common Marmoset	*C. jacchus*	H06 336 0628 01	NA21	NA	Negative	Negative
Common Marmoset	*C. jacchus*	H06 492 0472 02	-	Negative	Negative	NT
Common Marmoset	*C. jacchus*	H06 492 0473 02	-	Negative	Negative	NT

Organism		PHE ID	THESIS ID	PHE Study		Present Study
Common Name	**Scientific Name**			**Serology**	**PCR**	**RT-PCR**
Common Marmoset	*C. jacchus*	H06 492 0474 01	-	Negative	Negative	NT
Common Marmoset	*C. jacchus*	H06 492 0475 01	-	Negative	Negative	NT
Common Marmoset	*C. jacchus*	H07 196 0595 01	-	Negative	NA	NT
Common Marmoset	*C. jacchus*	H07 196 0640 01	NA26	NA	Negative	Negative
Common Marmoset	*C. jacchus*	H07 196 0599 01	-	Negative	NA	NT
Common Marmoset	*C. jacchus*	H08 122 0616 01	-	NA	NA	NT
Emperor tamarin	*S. imperator subgrisescens*	H06 336 0629 01	NA22	NA	Negative	Negative
Emperor tamarin	*S. imperator subgrisescens*	H07 196 0598 01	-	Negative	NA	NT
Emperor Tamarin	*S. imperator subgrisescens*	H08 122 0612 02	NA53	NA	NA	Negative
Cotton-top Tamarin	*S. Oedipus*	H08 122 0603 01	NA44	NA	NA	Negative
Red-handed Tamarin	*S. midas*	H07 196 0593 01	-	Negative	NA	NT

Organism		PHE ID	THESIS ID	PHE Study		Present Study
Common Name	**Scientific Name**			**Serology**	**PCR**	**RT-PCR**
Langur*	-	H07 196 0600 01	-	Negative	NA	NT
Langur*	-	H07 196 0603 01	-	Negative	NA	NT
Langur*	-	H07 196 0604 01	-	Negative	NA	NT
Langur*	-	H07 196 0605 01	-	Negative	NA	NT
Spectacled Langur	*T. obscurus*	H07 196 0602 01	-	Negative	NA	NT
Spectacled Langur	*T. obscurus*	H07386024101	-	NA	NA	NT
Spectacled Langur	*T. obscurus*	H08 122 0596 01	NA42	NT	NT	Negative
Diana Monkey	*C. diana diana*	H07 196 0607 01	-	Negative	NA	NT
Diana Monkey	*C. diana diana*	H07 196 0611 01	-	Negative	NA	NT
Woolly Monkey	*L. lagotricha*	H07 196 0614 01	-	Negative	NA	NT
Howler Monkey*	-	H07 196 0615 01	-	Negative	NA	NT
Howler Monkey*	-	H07 196 0616 01	-	Negative	NA	NT

Organism		PHE ID	THESIS ID	PHE Study		Present Study
Common Name	**Scientific Name**			**Serology**	**PCR**	**RT-PCR**
Howler Monkey*	-	H07 196 0617 01	-	Negative	NA	NT
Howler Monkey*	-	H07 196 0618 01	-	Negative	NA	NT
Howler Monkey*	-	H07 196 0619 01	-	Negative	NA	NT
Howler Monkey*	-	H07 196 0620 01	-	Negative	NA	NT
Howler Monkey*	-	H07 196 0621 01	-	Negative	NA	NT
Spider Monkey	*Ateles sp*	H07 196 0622 01	-	Negative	NA	NT
Spider Monkey	*Ateles sp*	H07 196 0623 01	-	Positive	NA	NT
Spider Monkey	*Ateles sp*	H07 196 0624 01	-	Negative	NA	NT
Spider Monkey	*Ateles sp*	No ID	-	NA	NA	NT
Black-tailed Marmoset	*C. argentata melanura*	H07 196 0669 01	NA36	NA	Negative	Negative
Pygmy Marmoset	*C. pygmaea*	H08 122 0607 01	NA48	NA	NA	Negative
Pygmy Marmoset	*C. pygmaea*	H08 122 0615 01	-	NA	NA	NT

Organism		PHE ID	THESIS ID	PHE Study		Present Study
Common Name	**Scientific Name**			**Serology**	**PCR**	**RT-PCR**
Pygmy Marmoset	*C. pygmaea*	H08 122 0619 01	-	NA	NA	NT
Amur Leopard	*P. pardus orientalis*	H08 122 0606 01	NA47	NA	NA	Negative
Seba's Short-tailed Bat	*C. perspicillata*	H06 330 0340 01	-	NT	NA	NT
Seba's Short-tailed Bat	*C. perspicillata*	H07 196 0668 01	NA35	NA	Negative	Negative
Bat*	-	H07 196 0671 01	NA38	Negative	NA	Negative
Bat*	-	H07 196 0672 01	NA39	Negative	NA	Negative
Bat*	-	H07 196 0673 01	NA40	Negative	NA	Negative
Striped Skunk	*M. mephitis*	H07 196 0636 01	NA24	NA	Negative	Negative
Brown Rat	*R. norvegicus*	H06 230 0562 02	-	NA	Negative	NT
Brown Rat	*R. norvegicus*	H06 230 0563 01	NA1	NA	Negative	Negative
House Mouse	*M. musculus*	H06 266 0587 01	NA2	NA	Positive	Negative
House Mouse	*M. musculus*	H06 266 0588 02	NA3	NA	Positive	Negative
House Mouse	*M. musculus*	H06 266 0589 02	NA4	NA	Positive	Negative

Organism		PHE ID	THESIS ID	PHE Study		Present Study
Common Name	**Scientific Name**			**Serology**	**PCR**	**RT-PCR**
House Mouse	*M. musculus*	H06 266 0590 03	NA5	NA	Positive	Positive
House Mouse	*M. musculus*	H07 196 0678 01	NA71	NA	Positive	Positive
House Mouse	*M. musculus*	H07 196 0676 01	NA69	NA	Positive	Positive
House Mouse	*M. musculus*	H07 196 0677 01	NA70	NA	Positive	Positive
House Mouse	*M. musculus*	H07 196 0674 01	NA67	NA	Positive	Positive
House Mouse	*M. musculus*	H07 196 0675 01	NA68	NA	Positive	Positive
House Mouse	*M. musculus*	H07 196 0637 01	-	NA	Positive	NT
House Mouse	*M. musculus*	H07 196 0638 01	NA56	NA	Positive	Positive
House Mouse	*M. musculus*	H07 196 0641 01	-	NA	Positive	NT
House Mouse	*M. musculus*	H07 196 0642 01	NA27	NA	Negative	Negative
House Mouse	*M. musculus*	H07 196 0643 01	NA28	NA	NA	Positive
House Mouse	*M. musculus*	H07 196 0644 01	NA77	NA	NA	Positive
House Mouse	*M. musculus*	H07 196 0645 01	NA29	NA	NA	Positive

Organism		PHE ID	THESIS ID	PHE Study		Present Study
Common Name	**Scientific Name**			**Serology**	**PCR**	**RT-PCR**
House Mouse	*M. musculus*	H07 196 0646 01	-	NA	NA	NT
House Mouse	*M. musculus*	H07 196 0647 01	NA30	NA	NA	Positive
House Mouse	*M. musculus*	H07 196 0648 01	NA57	NA	Positive	Negative
House Mouse	*M. musculus*	H07 196 0649 01	NA58	NA	Positive	Positive
House Mouse	*M. musculus*	H07 196 0650 01	NA31	NA	NA	Positive
House Mouse	*M. musculus*	H07 196 0651 01	NA59	NA	Positive	Positive
House Mouse	*M. musculus*	H07 196 0652 01	NA62	NA	Positive	Negative
House Mouse	*M. musculus*	H07 196 0653 01	-	NA	NA	NT
House Mouse	*M. musculus*	H07 196 0654 01	-	NA	NA	NT
House Mouse	*M. musculus*	H07 196 0655 01	-	NA	NA	NT
House Mouse	*M. musculus*	H07 196 0656 01	NA32	NA	NA	Positive
House Mouse	*M. musculus*	H07 196 0657 01	-	NA	NA	NT
House Mouse	*M. musculus*	H07 196 0658 01	NA61	NA	Positive	Positive

Organism		PHE ID	THESIS ID	PHE Study		Present Study
Common Name	**Scientific Name**			**Serology**	**PCR**	**RT-PCR**
House Mouse	*M. musculus*	H07 196 0659 01	-	NA	NA	NT
House Mouse	*M. musculus*	H07 196 0660 01	-	NA	NA	NT
House Mouse	*M. musculus*	H07 196 0661 01	NA62	NA	Positive	Negative
House Mouse	*M. musculus*	H07 196 0662 01	NA63	NA	Positive	Negative
House Mouse	*M. musculus*	H07 196 0663 01	NA64	NA	Positive	Positive
House Mouse	*M. musculus*	H07 196 0664 01	NA65	NA	Positive	Positive
House Mouse	*M. musculus*	H07 196 0665 01	NA66	NA	Positive	Positive
House Mouse	*M. musculus*	H07 196 0679 01	NA72	NA	Positive	Positive
House Mouse	*M. musculus*	H071960643-04	-	NA	NA	NT
House Mouse	*M. musculus*		-	NA	NA	NT
House Mouse	*M. musculus*	H071960645-6	-	NA	NA	NT
House Mouse	*M. musculus*	H071960647 No ID	-	NA	NA	NT
Guinea pig	*C. porcellus*	H07 196 0634 01	-	Negative	NA	NT

Organism		PHE ID	THESIS ID	PHE Study		Present Study
Common Name	**Scientific Name**			**Serology**	**PCR**	**RT-PCR**
Guinea pig	*C. porcellus*	H07 196 0635 01	NA23	NA	Negative	Negative
Guinea pig	*C. porcellus*	H07 196 0666 01	NA33	NA	Negative	Negative
Guinea pig	*C. porcellus*	H07 196 0667 01	NA34	NA	Negative	Negative
Field vole	*M. agrestis*	-	SB1	NT	NT	Negative
Field vole	*M. agrestis*	-	SB2	NT	NT	Negative
House Mouse	*M. musculus*	-	SB3	NT	NT	Negative
House Mouse	*M. musculus*	-	SB4	NT	NT	NT
Pygmy Shrew	*S. minutus*	-	SB5	NT	NT	Negative
Field vole	*M. agrestis*	-	SB6	NT	NT	Negative
Field vole	*M. agrestis*	-	SB7	NT	NT	Negative
Pygmy Shrew	*S. minutus*	-	SB8	NT	NT	Negative
Wood Mouse	*A. sylvaticus*	-	SB9	NT	NT	Negative

Organism		PHE ID	THESIS ID	PHE Study		Present Study
Common Name	**Scientific Name**			**Serology**	**PCR**	**RT-PCR**
Field vole	*M. agrestis*	-	SB10	NT	NT	Negative
Field vole	*M. agrestis*	-	SB11	NT	NT	Negative
Field vole	*M. agrestis*	-	SB12	NT	NT	Negative
Brown Rat	*R. norvegicus*	-	SB13	NT	NT	Negative
House Mouse	*M. musculus*	-	SB14	NT	NT	Negative
House Mouse	*M. musculus*	-	SB15	NT	NT	Positive
House Mouse	*M. musculus*	-	SB16	NT	NT	Positive
House Mouse	*M. musculus*	-	SB17	NT	NT	Negative
House Mouse	*M. musculus*	-	SB18	NT	NT	Positive
Brown Rat	*R. norvegicus*	-	SB19	NT	NT	Negative
Brown Rat	*R. norvegicus*	-	SB20	NT	NT	Negative
Wood Mouse	*A. sylvaticus*	-	SB21	NT	NT	Negative

Organism		PHE ID	THESIS ID	PHE Study		Present Study
Common Name	**Scientific Name**			**Serology**	**PCR**	**RT-PCR**
Wood Mouse	*A. sylvaticus*	-	SB22	NT	NT	Negative
Brown Rat	*R. norvegicus*	-	SB23	NT	NT	Negative
House Mouse	*M. musculus*	-	SB24	NT	NT	Negative
Field vole	*M. agrestis*	-	SB25	NT	NT	Negative
Wood Mouse	*A. sylvaticus*	-	SB26	NT	NT	Negative
Field vole	*M. agrestis*	-	SB27	NT	NT	Negative
Field vole	*M. agrestis*	-	SB28	NT	NT	Negative
Field vole	*M. agrestis*	-	SB29	NT	NT	Negative
Wood Mouse	*A. sylvaticus*	-	SB30	NT	NT	Negative
Wood Mouse	*A. sylvaticus*	-	SB31	NT	NT	Negative
Brown Rat	*R. norvegicus*	-	SB32	NT	NT	Negative
Brown Rat	*R. norvegicus*	-	SB33	NT	NT	Negative

Organism		PHE ID	THESIS ID	PHE Study		Present Study
Common Name	**Scientific Name**			**Serology**	**PCR**	**RT-PCR**
Pygmy Shrew	*S. minutus*	-	SB34	NT	NT	Negative
House Mouse	*M. musculus*	-	SB35	NT	NT	Positive
House Mouse	*M. musculus*	-	SB36	NT	NT	Negative
House Mouse	*M. musculus*	-	SB37	NT	NT	Negative
House Mouse	*M. musculus*	-	SB38	NT	NT	Negative
House Mouse	*M. musculus*	-	SB39	NT	NT	Negative
House Mouse	*M. musculus*	-	SB40	NT	NT	Negative
House Mouse	*M. musculus*	-	SB41	NT	NT	Negative
House Mouse	*M. musculus*	-	SB42	NT	NT	Negative
House Mouse	*M. musculus*	-	SB43	NT	NT	Negative
Brown Rat	*R. norvegicus*	-	SB44	NT	NT	Negative
House Mouse	*M. musculus*	-	SB45	NT	NT	Negative

Organism		PHE ID	THESIS ID	PHE Study		Present Study
Common Name	**Scientific Name**			**Serology**	**PCR**	**RT-PCR**
House Mouse	*M. musculus*	-	SB46	NT	NT	Negative
House Mouse	*M. musculus*	-	SB47	NT	NT	Negative
House Mouse	*M. musculus*	-	SB48	NT	NT	Negative
House Mouse	*M. musculus*	-	SB49	NT	NT	Negative

PM Tissue = Post-mortem tissue **NA** = Not available **NT** = Not tested

* The information presented on the animal was insufficient to allow for proper identification.

Appendix 14: CLUSTAL O(1.2.4) Multiple Sequence Alignment for Complete Glycoprotein Gene of Novel LCMV Detected in Thesis

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NA29      ATGGGCCAGATAGTGACGATGTTTGAGGCTTTGCCTCATATCATCGATGAGGTGATCAAC      60
NA72      ATGGGCCAGATAGTGACGATGTTTGAGGCTTTGCCTCATATCATCGATGAGGTGATTAAC      60
NA69      ATGGGCCAGCTTGTGACGATGTTTGAGGCTTTGCCTCATATCATCGATGAGGTGATTAAC      60
SB18      ATGGGCCAGATAGTGACGATGTTTGAGGCTTTGCCTCATATCATCGATGAGGTGATTAAC      60
NA18      ATGGGCCAGATAGTGACGATGTTTGAGGCTTTGCCTCATATCATCGATGAGGTGATTAAC      60
NA76      ATGGGCCAGATAGTGACGATGTTTGAGGCTTTGCCTCATATCATCGATGAGGTGATTAAC      60
NA74      ATGGGCCAGATTGTCACGATGTTTGAGGCTTTGCCTCATATCATCGATGAGGTGATTAAC      60
NA14      ATGGGCCAGATAGTGACGATGTTTGAGGCTTTGCCTCATATCATCGATGAGGTGATTAAC      60
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NA29      ATTGTGATTATTGTACTTATCATTATCACAAGCATCAAGGCAGTGTACAACTTTGCCACC      120
NA72      ATTGTGATTATTGTACTTATCATTATCACAAGCATCAAGGCAGTGTACAACTTTGCCACC      120
NA69      ATTGTGATTATTGTACTTATCATAATCACAAGCATCAAGGCAGTGTACAACTTTGCCACC      120
SB18      ATTGTGATTATTGTACTTATCATTATCACAAGCATCAAGGCAGTGTACAACTTTGCCACC      120
NA18      ATTGTGATTATTGTACTTATCATTATCACAAGCATCAAGGCAGTGTACAACTTTGCCACC      120
NA76      ATTGTGATTATTGTACTTATCATTATCACAAGCATCAAGGCAGTGTACAACTTTGCCACC      120
NA74      ATTGTGATTATTGTACTTATCATTATCACAAGCATCAAGGCAGTGTACAACTTTGCCACC      120
NA14      ATTGTGATTATTGTACTTATCATTATCACAAGCATCAAGGCAGTGTACAACTTTGCCACC      120
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NA29      TGCGGGATACTTGCACTGATCAGTTTCCTTTGTTTGGCTGGCAGATCCTGTGGGATGTAT      180
NA72      TGCGGGATACTTGCACTGATCAGTTTCCTTTGTTTGGCTGGCAGATCCTGTGGGATGTAT      180
NA69      TGCGGGATACTTGCTCTGATCAGTTTCCTTTGTTTGGCTGGCAGATCCTGTGGGATGTAT      180
SB18      TGCGGGATACTTGCACTGATCAGTTTCCTTTGTCTGGCCGGCAGATCTTGTGGGATGTAT      180
NA18      TGCGGGATACTTGCACTGATCAGTTTCCTTTGTTTGGCTGGCAGATCCTGTGGGATGTAT      180
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NA76      TGCGGGATACTTGCACTGATCAGTTTCCTTTGTCTGGCTGGCAGATCTTGTGGGATGTAT      180
NA74      TGCGGGATACTTGCACTGATCAGTTTCCTTTGTCTGGCTGGCAGATCTTGTGGGATGTAT      180
NA14      TGCGGGATACTTGCACTGATCAGTTTCCTTTGTCTGGCTGGCAGATCTTGTGGGATGTAT      180
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NA29      GGTCTTGATAGTCCCGATGTTTACAAAGGGGTCTATCAGTTTAAGTCAGTGGAATTTGAT      240
NA72      GGTATTGATGGTCTCGATATTTACAAAGGGGTCTATCAGTTTAAGTCAGTAGAATTTGAT      240
NA69      GGTCTTGATGGTCCTGATGTTTACAAAGGGGTCTATCAGTTTAAGTCAGTGGAATTTGAT      240
SB18      GGTCTTGATGGTCCTAACGTTTACAAAGGGGTCTATCAGTTCAAGTCAGTGGAATTTGAT      240
NA18      GGTCTTGATGGTCCCGATGTTTACAAAGGGGTCTATCAGTTTAAGTCAGTGGAATTTGAT      240
NA76      GGTCTTGATGGTCCTGATGTTTACAAAGGGGTCTATCAGTTTAAGTCAGTGGAATTTGAT      240
NA74      GGTCTTGATGGTCCTGATGTTTACAAAGGGGTCTATCAGTTTAAGTCAGTGGAATTTGAT      240
NA14      GGTCTTGATGGTCCTGATGTTTACAAAGGGGTCTATCAGTTTAAGTCAGTGGAATTTGAT      240
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NA29      ATGTCTCACTTAAACCTGACGATGCCCAATGCGTGTTCAGCCAACAACTCACACCATTAC      300
NA72      ATGTCTCACTTAAACCTGACGATGCCCAATGCGTGTTCAGCCAACAACTCACACCATTAC      300
NA69      ATGTCTCACTTAAACCTGACGATGCCCAATGCGTGTTCAGCCAACAACTCACACCATTAC      300
SB18      ATGTCTCACTTAAACCTGACGATGCCCAATGCATGCTCAGCCAACAACTCACACCATTAC      300
NA18      ATGTCTCACTTAAACCTGACGATGCCCAATGCGTGTTCAGCCAACAACTCACACCATTAC      300
NA76      ATGTCTCACTTAAACCTGACGATGCCCAATGCGTGTTCAGCCAACAACTCACACCATTAC      300
NA74      ATGTCTCACTTAAACCTGACGATGCCCAATGCGTGTTCAGCCAACAACTCACACCATTAC      300
NA14      ATGTCTCACCTAAACCTGACGATGCCCAATGCATGCTCAGCCAACAACTCACACCATTAC      300
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NA29      ATCAGTATGGGAACTTTTGGCTTGGAATTAACCTTCACCAATGATTCCATCTTTAGTCAC      360
NA72      ATCAGTATGGGAACTTCTGGCTTGGAATTAACCTTTACCAATGATTCCATCATTAGTCAC      360
NA69      ATCAGTATGGGAACTTCTGGCTTGGAATTAACCTTCACCAATGATTCCATCATTCATCAC      360
SB18      ATCAGTATGGGAACTTCTGGCTTGGAATTAACCTTTACCAATGATTCCATCATTCATCAC      360
NA18      ATCAGTATGGGAACTTCTGGCTTGGAATTAACCTTTACCAATGATTCCATCATTCAGCAC      360
NA76      ATCAGTATGGGAACTTCTGGCTTGGAATTAACCTTTACCAATGATTCCATCATTCATCAC      360
NA74      ATCAGTATGGGAACTTCTGGCTTGGAATTAACCTTTACCAATGATTCCATCATTCATCAC      360
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NA14      ATCAGTATGGGAACTTCTGGCTTGGAATTAACCTTTACCAATGATTCCATCATTCATCAC      360
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NA29      AACCATTGCAACCTTACCTCTTCCTTCAACAAGAAGACCTTTGACCACACGCTTATGAGC      420
NA72      AACCATTGCAACCTCACCTCTTCCTTCAACAAGAAGACCTTTGACCACACACTTATGAGC      420
NA69      AACCATTGCAACCTCACCTCTTCCTTCAACAAGAAGACTTTTGACCACACACTTATGAGC      420
SB18      AACCATTGCAACCTCACCTCTTCCTTCAACAAGAAGACCTTTGACCACACACTTATGAGC      420
NA18      AACCATTGCAACCTCACCTCTTCCTTCAACAAAAAGACCTTTGACCACACGCTTATGAGC      420
NA76      AACCATTGCAACCTCACCTCTTCCTTCAACAAGAAGACCTTTGACCACACACTTATGAGC      420
NA74      AACCATTGCAACCTCACCTCTTCCTTCAACAAGAAGACCTTTGACCACACACTTATGAGC      420
NA14      AACCATTGCAACCTCACCTCTTCCTTCAACAAGAAGACCTTTGACCACACACTTATGAGC      420
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NA29      ATAGTCTCAAGTCTACATCTCAGCATCAGAGGGAACTCCAACTATAAGGCAGTGTCTTGT      480
NA72      ATAGTCTCAAGTCTACATCTCAGCATCAGAGGGAACTCCAACTATAAGGCAGTGTCTTGT      480
NA69      ATAGTCTCAAGTCTACATCTCAGCATCAGAGGGAACTCCAACTATAAGGCAGTGTCTTGT      480
SB18      ATAGTCTCAAGTCTACATCTCAGCATCAGAGGGAACTCCAACTATAAGGCAGTGTCTTGT      480
NA18      ATAGTCTCAAGTCTACATCTCAGCATCAGAGGGAACTCCAACTATAAGGCAGTGTCTTGT      480
NA76      ATAGTCTCAAGTCTACATCTCAGCATCAGAGGGAACTCCAACTATAAGGCAGTGTCTTGT      480
NA74      ATAGTCTCAAGTCTACATCTCAGCATCAGAGGGAACTCCAACTATAAGGCAGTGTCTTGT      480
NA14      ATAGTCTCAAGTCTACATCTCAGCATCAGAGGGAACTCCAACTATAAGGCAGTGTCTTGT      480
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NA29      GATTTCAACAATGGCATCACCATTCAATACAACTTGACATTCTCAGATTCACAAAGTGCT      540
NA72      GATTTCAACAATGGCATCACCATTCAATACAACTTGACATTCTCAGATTCACAAAGTGCT      540
NA69      GATTTCAACAATGGCATCACCATTCAATACAACTTGACATTCTCAGATCCACAAAGTGCT      540
SB18      GATTTCAACAATGGCATCACCATTCAATACAACTTGACATTCTCAGATTCACAAAGTGCT      540
NA18      GATTTCAACAATGGCATCACCATTCAATACAACTTGACATTCTCAGATTCACAAAGTGCT      540
NA76      GATTTCAACAATGGCATCACCATTCAATACAACTTGACATTCTCAGATTCACAAAGTGCT      540
NA74      GATTTCAACAATGGCATCACCATTCAATACAACTTGACATTCTCAGATTCACAAAGTGCT      540
NA14      GATTTCAACAATGGCATCACCATTCAATACAACTTGACATTCTCAGATTCACAAAGTGCT      540
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NA29      CTGAGCCAGTGTAGGACCTTCAGGGGTAGGGTGTTGGATATGTTTAGAACTGCTTTTGGG      600
NA72      CTGAGCCAGTGTAGGTCCTTCAGGGGTAGGGTGTTGGATATGTTTAGAACTGCTTTTGGG      600
NA69      CTGAGCCAGTGTAGGACCTTCAGGGGTAGGGTGCTGGATATGTTTAGAACTGCTTTTGGG      600
SB18      CTGAGCCAGTGTAGGACCTTCAGGGGTAGGGTGTTGGATATGTTTAGAACTGCTTTTGGG      600
NA18      CTGAGCCAGTGTAGGACCTTCAGGGGTAGGGTGTTGGATATGTTTAGAACTGCTTTTGGG      600
NA76      CTGAGCCAGTGTAGGACCTTCAGGGGTAGGGTGTTGGATATGTTTAGAACTGCTTTTGGG      600
NA74      CTGAGCCAGTGTAGGACCTTCAGGGGTAGGGTGTTGGATATGTTTAGAACTGCTTTTGGG      600
NA14      CTGAGCCAGTGTAGGACCTTCAGGGGTAGGGTGTTGGATATGTTTAGAACTGCTTTTGGG      600
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NA29      GGAAAATACATGAGAAGTGGTTGGGGTTGGAAAAGTTCAGATGGCAAAACTACTTGGTGT      660
NA72      GGAAAATACATGAGAAGTGGTTGGGGTTGGAAAAGTTCAGATGGCAAGACTACTTGGTGT      660
NA69      GGAAAATATATGAGAAGTGGTTGGGGTTGGAAAAGTTCAGATGGCAAGACTACTTGGTGT      660
SB18      GGAAAATACATGAGAAGTGGTTGGGGTTGGAAAGGTTCGGATGGCAAGACTACTTGGTGT      660
NA18      GGAAAATACATGAGAAGTGGTTGGGGTTGGACAGGTTCAGATGGCAAGACTACTTGGTGT      660
NA76      GGAAAATACATGAGAAGTGGTTGGGGTTGGACAAGTTCAGATGGCAAGACTACTTGGTGT      660
NA74      GGAAAATACATGAGAAGTGGTTGGGGTTGGACAAGTTCAGATGGCAAGACTACTTGGTGT      660
NA14      GGAAAATACATGAGGAGTGGTTGGGGTTGGACAAGTTCAGATGGCAAGACTACTTGGTGT      660
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NA29      AGTCAAACAAATTACCAGTACCTAATTATACAAAATAGGACTTGGGAGAACCATTGCACA      720
NA72      AGTCAGACAAATTACCAGTACCTAATTATACAAAATAGGACTTGGGAGAACCATTGCACA      720
NA69      AGTCAGACAAATTACCAGTACCTAATTATACAAAATAGGACTTGGGAGAACCATTGCACA      720
SB18      AGTCAGACAAATTACCAGTACCTAATTATACAAAATAGGACTTGGGAAAACCATTGCACA      720
NA18      AGTCAAACAAATTACCAGTACCTAATTATACAAAACAGGACTTGGGAGAACCATTGCACA      720
NA76      AGTCAGACAAATTACCAGTACCTAATTATACAAAATAGGACTTGGGAGAACCATTGCACA      720
NA74      AGTCAGACAAATTACCAGTACCTAATTATACAAAATAGGACTTGGGAGAACCATTGCACA      720
NA14      AGTCAGACAAATTACCAGTACCTAATTATACAAAATAGGACTTGGGAGAACCATTGCACA      720
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NA29      TATGCAGGCCCTTTCGGAATATCTAGAATCCTTTTTGCCCAGGAAAAGACAAAATTTATC      780
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NA72      TATGCAGGCCCTTTCGGAATATCTAGAATTCTTTTTGCCCAGGAAAAGACAAAATTTATC      780
NA69      TATGCAGGCCCTTTCGGAATATCTAGAATTCTTTTTTCCCAGGAAAAGACAAAATTTCTT      780
SB18      TATGCAGGCCCTTTCGGAATGTCTAGAATTCTTTTCTCCCAGGAAAAGACAAAATTTATC      780
NA18      TATGCAGGCCCTTTCGGAATATCTAGAATTCTTTTTGCCCAGGAAAAGACAAAATTTCTC      780
NA76      TATGCAGGCCCTTTCGGAATGTCTAGAATTCTTTTTGCCCAGGAAAAGACAAAATTTCTC      780
NA74      TATGCAGGCCCTTTCGGAATGTCTAGAATTCTTTTTGCCCAGGAAAAGACAAAATTTCTC      780
NA14      TATGCAGGCCCTTTCGGAATGTCTAGAATTCTTTTTGCCCAGGAAAAGACAAAATTTCTC      780
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NA29      ACTAGAAGACTTGCAAGCACATTCACTTGGACTCTGTCAGACTCCTCAGGAGTGGAAAGC      840
NA72      ACTAGAAGACTTGCAGGCACATTCACTTGGACTCTGTCAGACTCCTCAGGAGTGGAAAAC      840
NA69      ACTAGAAGACTTGCAGGCACATTCACTTGGACTCTGTCAGACTCCTCAGGGGTGGAAAAC      840
SB18      ACTAGAAGACTTGCAGGCACATTCACTTGGACTCTGTCAGACTCCTCAGGAGTAGAAAAC      840
NA18      ACTAGAAGACTTGCAGGCACATTCACTTGGACTCTGTCAGACTCCTCAGGAGTGGAAAAC      840
NA76      ACTAGAAGACTTGCAGGCACATTCACTTGGACTCTGTCAGACTCCTCAGGAGTGGAAAAC      840
NA74      ACTAGAAGACTTGCAGGCACATTCACTTGGACTCTGTCAGACTCCTCAGGAGTGGAAAAC      840
NA14      ACTAGAAGACTTGCAGGCACATTCACTTGGACTCTGTCAGACTCCTCAGGAGTGGAAAAC      840
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NA29      CCAGGTGGTTATTGCCTAACCAAATGGATGATTCTTGCTGCAGAGCTTAAATGCTTTGGG      900
NA72      CCAGGTGGTTATTGCCTAACCAAATGGATGATTCTTGCCGCAGAGCTTAAATGCTTTGGG      900
NA69      CCAGGTGGTTATTGCCTAACCAAATGGATGATTCTTGCCGCAGAGCTTAAATGCTTTGGG      900
SB18      CCAGGTGGTTATTGCCTAACCAAATGGATGATTCTTGCCGCAGAGCTTAAATGCTTTGGA      900
NA18      CCAGGTGGTTATTGCCTAACCAAATGGATGATTCTTGCCGCAGAGCTTAAATGCTTTGGG      900
NA76      CCAGGTGGTTATTGCCTAACCAAATGGATGATTCTTGCCGCAGAGCTTAAATGCTTTGGA      900
NA74      CCAGGTGGTTATTGCCTAACCAAATGGATGATTCTTGCCGCAGAGCTTAAATGCTTTGGA      900
NA14      CCAGGTGGTTATTGCCTAACCAAATGGATGATTCTTGCCGCAGAGCTTAAATGCTTTGGA      900
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NA29      AACACAGCTGTTGCAAAGTGCAATGTCAATCATGATGAGGAGTTCTGTGATATGCTGCGA      960
NA72      AACACAGCTGTTGCAAAGTGCAATGTCAATCATGATGAGGAGTTCTGTGATATGCTGCGA      960
NA69      AACACAGCTGTTGCAAAGTGCAATGTCAATCATGATGAGGAGTTCTGTGATATGCTGCGA      960
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SB18      AACACAGCTGTTGCAAAGTGCAATGTCAATCATGATGAGGAGTTCTGTGATATGCTGCGA      960
NA18      AACACAGCTGTTGCAAAGTGCAATGTCAATCATGATGAGGAGTTCTGTGATATGCTGCGA      960
NA76      AACACAGCTGTTGCAAAGTGCAATGTCAATCATGATGAGGAGTTCTGTGATATGCTGCGA      960
NA74      AACACAGCTGTTGCAAAGTGCAATGTCAATCATGATGAGGAGTTCTGTGATATGCTGCGA      960
NA14      AACACAGCTGTTGCAAAGTGCAATGTCAATCATGATGAGGAGTTCTGTGATATGCTGCGA      960
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NA29      CTGATTGATTATAATAAGGCTGCTCTGAGCAAGTTCAAAGAAGATGTGGAATCTGCCCTG     1020
NA72      CTGATTGATTATAATAAGGCTGCTCTGAGCAAGTTCAAAGAAGATGTAGAATCTGCCCTG     1020
NA69      CTGATTGATTATAATAAGGCTGCTCTGAGCAAGTTCAAAGAAGATGTAGAATCTGCCCTG     1020
SB18      CTGATTGATTATAATAAGGCTGCTTTGAGTAAGTTCAAAGAAGATGTAGAATCTGCCCTG     1020
NA18      CTGATTGATTATAATAAGGCCGCTCTGAGCAAGTTCAAAGAAGATGTAGAATCTGCCCTG     1020
NA76      CTGATTGATTATAATAAGGCTGCTTTGAGTAAGTTCAAAGAAGATGTAGAATCTGCCTTG     1020
NA74      CTGATTGATTATAATAAGGCTGCTTTGAGTAAGTTCAAAGAAGATGTAGAATCTGCCCTG     1020
NA14      CTGATTGATTATAATAAGGCTGCTTTGAGTAAGTTCAAAGAAGATGTAGAATCTGCCCTG     1020
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NA29      CACCTCTTCAAAACAACAGTGAATTCTCTGATTTCTGACCAACTGCTGATGAGGAACCAC     1080
NA72      CACCTCTTTAAAACAACAGTGAATTCTCTGATTTCTGACCAGCTGCTGATGAGGAACCAC     1080
NA69      CACCTCTTCAAGACAACAGTGAATTCTCTGATTTCTGACCAGCTGCTGATGAGGAACCAC     1080
SB18      CACCTCTTCAAAACAACAGTGAATTCTCTGATTTCTGACCAGCTGTTGATGAGGAACCAC     1080
NA18      CACCTTTTCAAAACAACAGTGAATTCTCTGATTTCTGACCAGCTGCTGATGAGGAACCAC     1080
NA76      CACCTCTTCAAAACAACAGTGAATTCTCTGATTTCTGACCAGCTGTTGATGAGGAACCAC     1080
NA74      CACCTCTTCAAAACAACAGTGAATTCTCTGATTTCTGACCAGCTGTTGATGAGGAACCAC     1080
NA14      CACCTCTTCAAAACAACAGTGAATTCTCTGATTTCTGACCAGCTGTTGATGAGGAACCAC     1080
          ***** ** ** ***************************** *** **************

NA29      CTAAGGGATCTGATGGGGGTGCCATACTGTAATTACTCAAAATTCTGGTATTTGGAACAT     1140
NA72      CTAAGGGATCTGATGGGGGTGCCATACTGTAATTACTCAAAATTCTGGTATTTGGAACAT     1140
NA69      CTAAGGGATCTGATGGGGGTGCCATACTGTAATTACTCAAAATTCTGGTATTTGGAACAT     1140
SB18      CTAAGGGATCTGATGGGGGTGCCATACTGCAATTACTCAAAATTCTGGTATTTGGAACAT     1140
NA18      CTAAGGGATCTGATGGGGGTGCCATACTGTAATTACTCAAAATTCTGGTATTTGGAACAT     1140
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NA76      CTAAGGGATCTGATGGGGGTGCCATACTGCAATTACTCAAAATTCTGGTATTTGGAACAT      1140
NA74      CTAAGGGATCTGATGGGGGTGCCATACTGCAATTACTCAAAATTCTGGTATTTGGAACAT      1140
NA14      CTAAGGGATCTGATGGGGGTGCCATACTGCAATTACTCAAAATTCTGGTATTTGGAACAT      1140
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NA29      GCGAAGACTGGGGAGACTAGTACTCCCAAGTGTTGGTTAGTTACTAATGGTTCTTATCTG      1200
NA72      GCGAAGACTGGGGAGACTAGTACTCCCAAGTGTTGGTTAGTTACTAATGGTTCTTATCTG      1200
NA69      GCGAAGACTGGGGAGACTAGTACTCCCAAGTGTTGGTTAGTTACTAATGGTTCTTATCTG      1200
SB18      GCGAAGACTGGGGAGACCAGTACTCCCAAGTGTTGGTTAGTTACTAATGGTTCTTACCTG      1200
NA18      GCGAAGACTGGGGAGACTAGTACTCCCAAGTGTTGGTTAGTTACTAATGGTTCTTATCTG      1200
NA76      GCGAAGACTGGGGAGACTAGTACTCCCAAGTGTTGGTTAGTTACTAATGGTTCTTACCTG      1200
NA74      GCGAAGACTGGGGAGACTAGTACTCCCAAGTGTTGGTTAGTTACTAATGGTTCTTACCTG      1200
NA14      GCGAAGACTGGGGAGACTAGTACTCCCAAGTGTTGGTTAGTTACTAATGGTTCTTACCTG      1200
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NA29      AATGAGACCCATTTTAGTGATCAAATAGAGCAAGAAGCAGACAACATGATCACAGAAATG      1260
NA72      AATGAGACCCATTTTAGTGATCAAATAGAGCAAGAAGCAGACAACATGATCACAGAAATG      1260
NA69      AATGAGACCCATTTCAGTGATCAAATAGAGCAAGAAGCAGACAACATGATCACAGAAATG      1260
SB18      AATGAGACCCATTTTAGTGATCAAATAGAGCAAGAAGCAGACAACATGATCACAGAAATG      1260
NA18      AATGAGACCCATTTTAGTGATCAAATAGAGCAAGAAGCAGACAACATGATCACAGAAATG      1260
NA76      AATGAGACCCATTTTAGTGATCAAATAGAGCAAGAAGCAGACAACATGATCACAGAAATG      1260
NA74      AATGAGACCCATTTTAGTGATCAAATAGAGCAAGAAGCAGACAACATGATCACAGAAATG      1260
NA14      AATGAGACCCATTTTAGTGATCAAATAGAGCAAGAAGCAGACAACATGATCACAGAAATG      1260
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NA29      CTGAGAAAAGATTACATAAAAAGGCAAGGGAGTACACCCTTAGCCTTAATGGATCTTTTG      1320
NA72      CTGAGAAAAGATTACATAAAAAGGCAAGGGAGTACACCCTTAGCCTTAATGGATCTTTTG      1320
NA69      CTGAGAAAAGATTACATAAAAAGGCAAGGGAGTACACCCTTAGCCTTAATGGATCTTTTG      1320
SB18      CTGAGAAAAGATTACATAAAAAGGCAAGGGAGTACACCCTTAGCCTTAATGGATCTTTTG      1320
NA18      CTGAGAAAAGATTACATAAAAAGGCAAGGGAGTACACCCTTAGCCTTAATGGATCTTTTG      1320
NA76      CTGAGAAAAGATTACATAAAAAGGCAAGGGAGTACACCCTTAGCCTTAATGGAACTTTTG      1320
NA74      CTGAGAAAAGATTACATAAAAAGGCAAGGGAGTACACCCTTAGCCTTAATGGATCTTTTG      1320
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NA14      CTGAGAAAAGATTACATAAAAAGGCAAGGGAGTACACCCTTAGCCTTAATGGATCTTTTG      1320
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NA29      ATGTTCTCCACATCGGCATATTTAATCAGCGTATTTTTACATCTCATAAAGATACCAACA      1380
NA72      ATGTTCTCCACATCGGCATATTTAATCAGCATCTTTTTACATCTCATAAAGATACCGACA      1380
NA69      ATGTTCTCCACATCGGCATATTTAATCAGCATCTTTTTACATCTCATAAAGATACCAACA      1380
SB18      ATGTTCTCCACATCGGCATATTTAATCAGCATCTTTTTGCATCTCATAAAGATACCAACA      1380
NA18      ATGTTCTCCACATCGGCATATTTAATCAGCATCTTTTTGCATCTCATAAAGATACCAACA      1380
NA76      ATGTTCTCCACATCGGCATATTTAATCAGCATCTTTTTGCATCTCATAAAGATACCAACA      1380
NA74      ATGTTCTCCACATCGGCATATTTAATCAGCATCTTTTTGCATCTCATAAAGATACCAACA      1380
NA14      ATGTTCTCCACATCGGCATATTTAATCAGCATCTTTTTGCATCTCATAAAGATACCAACA      1380
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NA29      CATAGACATATAAAAGGTGGTTTATGCCCAAAGCCGCACCGCCTGACTAGCAAAGGGATC      1440
NA72      CATAGACATATAAAAGGTGTTTCATGCCCAAAGCCGCACCGCCTGACAAGCAAAGGGATC      1440
NA69      CATAGACATATAAAAGGTGGTTCATGCCCAAAGCCGCACCGCCTGACTAGCAAAGGGATC      1440
SB18      CACAGACATATAAAAGGTGCCTTATGCCCAAAGCCGCACCGCCTGACTAGCAAAGGGATC      1440
NA18      CACAGACATATAAAAGGTGGTTCATGCCCAAAGCCGCACCGCCTGACTAGCAAAGGGATC      1440
NA76      CACAGACATATAAAAGGTGGTTCATGCCCAAAGCCGCACCGCCTGACTAGCAAAGGGATC      1440
NA74      CACAGACATATAAAAGGTGGTTCATGCCCAAAGCCGCACCGCCTGACTAGCAAAGGGATC      1440
NA14      CACAGACATATAAAAGGTGGTTCATGCCCAAAGCCGCACCGCCTGACTAGCAAAGGGATC      1440
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NA29      TGTAGCTGTGGTGCATTTAGAGTGCCCGGTGTAA     1474
NA72      TGCAGCTGTGGCAAATTTGGAGTGCCCGGTGTAA     1474
NA69      TGCAGCTGTGGTGCATTTAGAGTGCCCGGTGTAA     1474
SB18      TGTAGCTGTGGTGCATTTAGAGTGCCCGGTGTAA     1474
NA18      TGCAGCTGTGGTGCATTTAGAGTGCCCGGTGTAA     1474
NA76      TGCAGCTGTGGTGCATTTAGAGTGCCCGGTGTAA     1474
NA74      TGCAGCTGTGGTGCATTTAGAGTGCCCGGTGTAA     1474
NA14      TGCAGCTGTGGTGCATTTAGAGTGCCCGGTGTAA     1474
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Appendix 15: Results of LCMV Screening Using In-house, Published and PHE Unknown primers

SAMPLE	SPECIES	PRIMER SETS				
		1	2	3	4	5
SB15	*M. musculus*	POSITIVE	POSITIVE	NEGATIVE	POSITIVE	NOT TESTED
SB16	*M. musculus*	POSITIVE	POSITIVE	POSITIVE	POSITIVE	NOT TESTED
SB18	*M. musculus*	POSITIVE	POSITIVE	POSITIVE	POSITIVE	NOT TESTED
SB35	*M. musculus*	POSITIVE	POSITIVE	NEGATIVE	POSITIVE	NOT TESTED
NA1	*R. norvegicus*	NEGATIVE	NEGATIVE	NEGATIVE	NEGATIVE	NEGATIVE
NA2	*M. musculus*	NEGATIVE	NEGATIVE	NEGATIVE	NEGATIVE	POSITIVE
NA3	*M. musculus*	NEGATIVE	NEGATIVE	NEGATIVE	NEGATIVE	POSITIVE
NA4	*M. musculus*	NEGATIVE	NEGATIVE	NEGATIVE	NEGATIVE	POSITIVE
NA5	*M. musculus*	POSITIVE	POSITIVE	NEGATIVE	NEGATIVE	POSITIVE
NA6	*P. troglodytes*	NEGATIVE	NEGATIVE	NEGATIVE	NEGATIVE	NEGATIVE
NA7	*H. klossi*	NEGATIVE	NEGATIVE	NEGATIVE	NEGATIVE	NEGATIVE
NA8	*A. sara*	NEGATIVE	NEGATIVE	NEGATIVE	NEGATIVE	NEGATIVE
NA9	*A. caraya*	NEGATIVE	NEGATIVE	NEGATIVE	NEGATIVE	NEGATIVE
NA10	*A. sara*	NEGATIVE	NEGATIVE	NEGATIVE	NEGATIVE	NEGATIVE
NA11	*A. niviridus*	NEGATIVE	NEGATIVE	NEGATIVE	NEGATIVE	NEGATIVE
NA12	*A. sara*	NEGATIVE	NEGATIVE	NEGATIVE	NEGATIVE	NEGATIVE
NA13	*T. obscurus*	NEGATIVE	NEGATIVE	NEGATIVE	NEGATIVE	NEGATIVE

SAMPLE	SPECIES	PRIMER SETS				
		1	2	3	4	5
NA14	*C. geoffroyi*	POSITIVE	POSITIVE	POSITIVE	POSITIVE	POSITIVE
NA15	*T. vetulus monticola*	NEGATIVE	NEGATIVE	NEGATIVE	NEGATIVE	POSITIVE
NA16	*C. guereza occidentalis*	NEGATIVE	NOT TESTED	NEGATIVE	NOT TESTED	NEGATIVE
NA17	*C. guereza occidentalis*	NEGATIVE	NEGATIVE	NEGATIVE	NEGATIVE	NEGATIVE
NA18	*C. guereza occidentalis*	POSITIVE	POSITIVE	POSITIVE	POSITIVE	POSITIVE
NA19	*T. obscurus*	NEGATIVE	NOT TESTED	NEGATIVE	NOT TESTED	NOT TESTED
NA20	*A. sara*	NEGATIVE	NEGATIVE	NEGATIVE	NEGATIVE	NOT TESTED
NA21	*C. jacchus*	NEGATIVE	NEGATIVE	NEGATIVE	NEGATIVE	NOT TESTED
NA22	*S. imperator subgrisescens*	NEGATIVE	NEGATIVE	NEGATIVE	NEGATIVE	NOT TESTED
NA23	*C. porcellus*	NEGATIVE	NEGATIVE	NEGATIVE	NEGATIVE	NEGATIVE
NA24	*M. mephitis*	NEGATIVE	NEGATIVE	NEGATIVE	NEGATIVE	NEGATIVE
NA25	*A. caraya*	NEGATIVE	NEGATIVE	NEGATIVE	NEGATIVE	NEGATIVE
NA26	*C. jacchus*	NEGATIVE	NOT TESTED	NEGATIVE	NOT TESTED	NEGATIVE
NA27	*M. musculus*	NEGATIVE	NEGATIVE	NEGATIVE	NEGATIVE	NEGATIVE
NA28	*M. musculus*	POSITIVE	POSITIVE	POSITIVE	NEGATIVE	NOT TESTED
NA29	*M. musculus*	POSITIVE	POSITIVE	POSITIVE	POSITIVE	NOT TESTED
NA30	*M. musculus*	POSITIVE	NEGATIVE	POSITIVE	NEGATIVE	NOT TESTED
NA31		POSITIVE	POSITIVE	NEGATIVE	POSITIVE	NOT TESTED

SAMPLE	SPECIES	PRIMER SETS				
		1	2	3	4	5
NA32	*M. musculus*	POSITIVE	POSITIVE	NEGATIVE	NEGATIVE	NOT TESTED
NA33	*C. porcellus*	NEGATIVE	NEGATIVE	NEGATIVE	NEGATIVE	NEGATIVE
NA34	*C. porcellus*	NEGATIVE	NEGATIVE	NEGATIVE	NEGATIVE	NEGATIVE
NA35	*C. perspicillata*	NEGATIVE	NEGATIVE	NEGATIVE	NEGATIVE	NEGATIVE
NA36	*C. argentata melanura*	NEGATIVE	NEGATIVE	NEGATIVE	NEGATIVE	NEGATIVE
NA37	*L. rosalia*	NEGATIVE	NEGATIVE	NEGATIVE	NEGATIVE	NEGATIVE
NA38	Bat?	NEGATIVE	NEGATIVE	NEGATIVE	NEGATIVE	NEGATIVE
NA39	Bat?	NEGATIVE	NEGATIVE	NEGATIVE	NEGATIVE	NEGATIVE
NA40	Bat?	NEGATIVE	NEGATIVE	NEGATIVE	NEGATIVE	NEGATIVE
NA41	*A. sara*	NEGATIVE	NEGATIVE	NEGATIVE	NEGATIVE	NOT TESTED
NA42	*T. obscurus*	NEGATIVE	NEGATIVE	NEGATIVE	NEGATIVE	NOT TESTED
NA43	*T. obscurus*	NEGATIVE	NEGATIVE	NEGATIVE	NEGATIVE	NOT TESTED
NA44	*S. Oedipus*	NEGATIVE	NEGATIVE	NEGATIVE	NEGATIVE	NOT TESTED
NA45	*G. gorilla*	NEGATIVE	NEGATIVE	NEGATIVE	NOT TESTED	NOT TESTED
NA46	*H. agilis*	NEGATIVE	NOT TESTED	NEGATIVE	NEGATIVE	NOT TESTED
NA47	*P. pardus orientalis*	NEGATIVE	NEGATIVE	NEGATIVE	NEGATIVE	NOT TESTED
NA48	*C. pygmaea*	NEGATIVE	NEGATIVE	NEGATIVE	NEGATIVE	NOT TESTED
NA49	*G. gorilla*	NEGATIVE	NEGATIVE	NEGATIVE	NOT TESTED	NOT TESTED
NA50	*A. caraya*	NEGATIVE	NOT TESTED	NEGATIVE	NEGATIVE	NOT TESTED
NA51	*A. caraya*	NEGATIVE	NEGATIVE	NEGATIVE	NEGATIVE	NOT TESTED
NA52	*H. agilis*	NEGATIVE	NEGATIVE	NEGATIVE	NEGATIVE	NOT TESTED

SAMPLE	SPECIES	PRIMER SETS				
		1	2	3	4	5
NA53	*S. imperator subgrisescens*	NEGATIVE	NEGATIVE	NEGATIVE	NEGATIVE	NOT TESTED
NA54	*A. sara*	NEGATIVE	NEGATIVE	NEGATIVE	NEGATIVE	NOT TESTED
NA55	*A. sara*	NEGATIVE	NEGATIVE	NEGATIVE	NEGATIVE	NEGATIVE
NA56	*M. musculus*	POSITIVE	POSITIVE	NEGATIVE	NEGATIVE	POSITIVE
NA57	*M. musculus*	NEGATIVE	NEGATIVE	NEGATIVE	NEGATIVE	POSITIVE
NA58	*M. musculus*	POSITIVE	POSITIVE	POSITIVE	NOT TESTED	POSITIVE
NA59	*M. musculus*	POSITIVE	NOT TESTED	POSITIVE	NEGATIVE	POSITIVE
NA60	*M. musculus*	NEGATIVE	NEGATIVE	NEGATIVE	NOT TESTED	POSITIVE
NA61	*M. musculus*	NEGATIVE	NOT TESTED	NEGATIVE	NEGATIVE	POSITIVE
NA62	*M. musculus*	NEGATIVE	NEGATIVE	NEGATIVE	NEGATIVE	POSITIVE
NA63	*M. musculus*	NEGATIVE	NEGATIVE	NEGATIVE	NEGATIVE	POSITIVE
NA64	*M. musculus*	POSITIVE	NEGATIVE	POSITIVE	NEGATIVE	POSITIVE
NA65	*M. musculus*	POSITIVE	POSITIVE	NEGATIVE	NEGATIVE	POSITIVE
NA66	*M. musculus*	POSITIVE	POSITIVE	POSITIVE	NEGATIVE	POSITIVE
NA67		POSITIVE	POSITIVE	POSITIVE	NOT TESTED	POSITIVE

SAMPLE	SPECIES	PRIMER SETS				
		1	2	3	4	5
NA68	*M. musculus*	POSITIVE	NEGATIVE	POSITIVE	NOT TESTED	POSITIVE
NA69	*M. musculus*	NEGATIVE	NOT TESTED	POSITIVE	NEGATIVE	POSITIVE
NA70	*M. musculus*	POSITIVE	NEGATIVE	NEGATIVE	NEGATIVE	POSITIVE
NA71	*M. musculus*	POSITIVE	NEGATIVE	POSITIVE	NEGATIVE	POSITIVE
NA72	*M. musculus*	POSITIVE	POSITIVE	POSITIVE	NOT TESTED	POSITIVE
NA73	*C. geoffroyi*	NEGATIVE	NOT TESTED	NEGATIVE	NOT TESTED	POSITIVE
NA74	*C. geoffroyi*	NEGATIVE	NOT TESTED	POSITIVE	NOT TESTED	POSITIVE
NA76	*N. leucogenys*	NEGATIVE	NOT TESTED	POSITIVE	NOT TESTED	NEGATIVE
NA77	*M. musculus*	POSITIVE	NOT TESTED	POSITIVE	NOT TESTED	POSITIVE

1 = LVL_3359D_Y+/ LVL_3754A_R-/ LVL_3359G_Y+/ LVL_3754D_R primer set (Vieth *et al.*, 2007)
2 = DLCMF/DLCMR primer set (In-house)
3 = LCMV-S 13^{+}/LCMV-S 322^{-} primer set (Asper *et al.*, 2001)
4 = MAMF/MAM_ARE_R Primer set (In-house)
5 = PHE Unknown Primer set

Appendix 16: Pseudotype Infectivity Assay Results for Pseudoviruses Derived from LCMV Glycoproteins of the Novel Strains

Pseudoparticle	Origin	Infectivity (RLU)		
Positive Control	NA	66678	68109	55193
SB18	House mouse	2814	2952	2768
NA14	Geoffroy's Marmoset	811	565	538
NA29	House mouse	14147	13310	14975
Negative Control	NA	106	86	125

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