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CHAPTER III

IDENTIFICATION AND CHARACTERIZATION OF NEW LAGOS BAT VIRUS ISOLATES FROM SOUTH AFRICA

3.1 Introduction

Lagos bat virus (LBV) is a member of the *Lyssavirus* genus in the *Rhabdoviridae* family. Rabies virus (RABV) was first isolated as a unique virus within this group. However, following the isolation of rabies-related lyssaviruses in Africa and Europe in the mid 1950's, the *Lyssavirus* genus was created and RABV (gt 1) was designated as the type species member of the genus. At least seven different lyssavirus genotypes are currently recognized by the International Committee of the Taxonomy of viruses (Tordo *et al*., 2005), but the genus will be expanded with the addition of new representatives, Irkut, Aravan, Khujand and WCBV, isolated from Eurasia in recent years currently added as putative species (Kuzmin *et al*., 2003; Kuzmin *et al*., 2005). All indications are that more lyssaviruses remain to be discovered. At present, four lyssavirus genotypes are recognized in Africa; LBV (gt 2), MOKV (gt 3) and DUVV (gt 4). Although RABV infection of bats is well known in the Americas (Belotto *et al*., 2005) this virus has only been associated with infections of terrestrial mammals on the African continent. MOKV has also never been isolated from bats but from various terrestrial species only. Of the remaining African genotypes, both LBV and DUVV are thought to be bat viruses, although LBV infections of terrestrial animals have been reported (Mebatsion *et al*., 1992b; Swanepoel, 2004). RABV is an important zoonotic agent throughout Africa. DUVV and MOKV, but not LBV, have also been responsible for rare zoonotic events (Swanepoel, 2004; Kemp *et al*., 1972; Paweska *et al*., 2006). Commercial vaccine strains belong to gt 1 (RABV) and the corresponding vaccines provide protection against RABV and probably some level of protection against gt 4-7, however, these vaccines (gt 1 based) do not protect against gt 2 and 3 viruses (Nel, 2005; Hanlon *et al*., 2005).

In southern Africa two biotypes of RABV are recognized (Nel *et al*., 1993; Von Teichman *et al*., 1995). They are the canid biotype that mainly circulates among dogs, jackals and bat-eared foxes, and the mongoose biotype that has been shown to be very well adapted and unique to mongooses in southern Africa (Nel *et al*., 2005). Prior to this study, RABV has been responsible for all mongoose rabies cases in Africa. In South Africa the principal vector of the mongoose biotype is the yellow mongoose (*Cynictis penicillata*) but there have also been reports of RABV in other

mongoose species such as the slender (*Galerella sanguinea*), water (*Atilax paludinosus*), small grey (*Galerella pulverulenta*), banded (*Mungos mungo*), selous (*Paracynictis selousi*), dwarf (*Helogale parvula*) and white tailed mongoose (*Ichneumia albicauda*). In South Africa mongoose rabies commonly occurs in the central highveld regions of the country (Nel *et al*., 2005; Swanepoel, 2004), whereas the KwaZulu Natal Province, located on the eastern seaboard of South Africa, is associated with epizootic canid rabies in domestic dogs and mongoose rabies is not reported from here.

LBV was first isolated from a fruit bat (*Eidolon helvum*) in 1956 at Lagos Island in Nigeria (Boulger and Porterfield, 1958), but it was not until 1970 that it was identified as LBV (Shope *et al*., 1970). Since then (and prior to this study), eleven more isolations of LBV were made throughout Africa (Chapter II:Table 2.4), including five isolates from South Africa (Swanepoel, 2004). All five isolates of LBV made from South Africa was from one area, the KwaZulu Natal Province. Four of these isolations were made from a frugivorous bat (*Epomophorus wahlbergi*) and one isolate was from a vaccinated cat. *E. wahlbergi* is of the suborder *Megachiroptera* and the *Pteropodidae* family and many observers noted that the head of this fruit bat resembles that of a dog. *E. wahlbergi* is common garden inhabitants in the coastal KwaZulu Natal area (Taylor, 2000) and known to roost in well-treed urban and suburban areas as well as under bridges and under the eaves of houses. They occur in colonies varying from a few individuals up to a hundred or more. These bats are named from the long white fur (or epaulets) that sprout from their shoulders. Due to their frugivorous diet, their jaws are strong, and their teeth are adapted to best process the fruit. They feed mainly on figs, mangoes, guavas, bananas, peaches, papayas, apples, and small berries and the smell of ripening fruit attracts them to their food source. This fruit bat is native to Africa, and is found anywhere south of the Sahara desert. When roosting, they do not pack themselves tightly next to one another and do not intrude on each others space.

Lyssaviruses are bullet shaped and have a single continuous negative stranded RNA genome coding for five proteins: nucleoprotein, glycoprotein, matrixprotein, phosphoprotein and the polymerase (Tordo and Poch, 1988). The N gene is well conserved making it an ideal target for diagnostic purposes and for genetic and antigenic characterization of lyssavirus genotypes (species). Lyssavirus infection can be identified from brain material using the gold standard for lyssavirus diagnostics, the fluorescent antibody test (FAT). In South Africa a polyclonal fluorescein

isothiocyanate conjugated immunoglobulin prepared by the Onderstepoort Veterinary Institute, Rabies Unit, is used. This conjugate is prepared by immunizing a goat with MOKV and RABV nucleocapsid and the produced conjugate has been validated to detect all lyssavirus genotypes, including LBV, by the Centre of Expertise for Rabies, Canadian Food Inspection Agency, Canada. A positive result with the FAT only indicates that it is a lyssavirus infection and cannot discriminate between lyssavirus genotypes. The mouse inoculation test is used as a confirmatory test and also to amplify isolates for further characterization.

Previously, identification of LBV isolates was performed using complement fixation and virus neutralization test e.g. identification of the 1956 Nigeria- (Shope *et al*., 1970), 1974 Central African Republic- (Sureau *et al*., 1977), 1985 Senegal- (Institute Pasteur, 1985), 1985 Guinea- (Institute Pasteur, 1985) and the 1980 South African LBV isolates (Meredith and Standing, 1981; Crick *et al*., 1982). The development of monoclonal antibodies provided a method to discriminate between lyssavirus genotypes and even virus variants that are part of the same genotype. Several monoclonal antibody panels have been described that can discriminate between the seven lyssavirus genotypes (King, 1993; Flamand *et al*., 1980; Dietzschold *et al*., 1988; Schneider *et al*., 1973). The LBV isolate from Ethiopia (Mebatsion *et al*., 1992b), Zimbabwe (Foggin, 1988) and South Africa (1990) (Swanepoel, 2004) have previously been characterized using monoclonal antibodies. A panel of 16 monoclonal antibodies provided by the Centre of Expertise for Rabies, Canadian Food Inspection Agency, Canada has been used in this study. This panel is able to differentiate antigenetically between lyssavirus genotypes circulating in Africa as well as between the canid and mongoose biotypes of gt 1. With the development of molecular techniques, monoclonal antibody typing has been largely replaced by genetic characterization using RT-PCR and DNA sequencing. These techniques have been used in previous studies to characterize LBV and determine its phylogenetic relationship in the lyssavirus genus (Bourhy *et al*., 1993a) as well as aiding in the identification of the 1999 LBV isolate from Africa (Aubert *et al*., 1999; Picard-Meyer *et al*., 2004). Several methods and primers sets claiming to be able to amplify all seven lyssavirus genotypes have been described in the literature (Black *et al*., 2002; Heaton *et al*., 1997; Heaton *et al*., 1999; Smith *et al*., 2000; Whitby *et al*., 1997; Vazquez-Moron, 2006) but these methods have only been analysed on limited representative samples of each genotype and may not be able to detect lyssaviruses in all field samples submitted to a diagnostic laboratory. Molecular methods should therefore be used in combination with other lyssavirus diagnostic methods.

When samples are submitted to a diagnostic laboratory it is usually only the removed brain material and not the complete animal carcass. The identification of the species is then dependent on the person who removed the brain. The correct identification can be difficult especially with wildlife species due to the inexperience of the examiner. It is very difficult to make informed decisions about lyssavirus epidemiology and control if the host species involved is unknown. The development of molecular techniques provides an opportunity to identify the host species from brain material using PCR and DNA sequencing techniques (Hsieh *et al*., 2001). These techniques target the cytochrome *b* gene of the mitochondrial DNA of the host (Kocher *et al*., 1989; Veron and Heard, 2000; Veron *et al*., 2004). This gene is 1140 bp in length and complete or partial sequence can be used to identify the host species. Genomic DNA is isolated from brain tissue and the cytochrome *b* region is amplified using PCR after which a DNA sequence is generated. This sequence can then be compared to other available DNA sequence information in the public domain, GenBank, to identify the host species.

Prior to this study LBV has not been identified in South Africa since 1990. The aim of this study was to instigate surveillance efforts that could identify and collect bats that display neurological disease signs that may be indicative of encephalitis due to lyssavirus infection but also to collect other apparently healthy wildlife species that are susceptible to lyssavirus infection. Samples were then analysed for the presence of lyssavirus antigens and positive results further characterized using antigenic and molecular methods. The unidentified host species was identified using genetic methods.

3.2 Materials and methods

3.2.1 Sample collection

During the period 2002 to 2006 a surveillance effort focusing on specific geographic regions in South Africa was instigated to identify the presence of rabies-related lyssaviruses. Surveillance included passive surveillance where wildlife species were submitted for lyssavirus testing that demonstrated signs of encephalitis or abnormal behaviour as well as samples from apparent healthy animals. The surveillance also included field trips where small amounts of wildlife samples were collected with the aim of identifying lyssavirus infection. Rodent samples were included since the reservoir species for MOKV is unknown and a rodent has previously been implicated

in a gt 3 lyssavirus infection (Saluzzo *et al.*, 1984). Table 3.1 lists samples tested during this study.

Table 3.1: Samples submitted for diagnostic testing for lyssavirus infection from 2002 to 2006.

DATE	SPECIES	GEOGRAPHIC LOCATION	SAMPLE NR	SUBMISSION INFORMATION
2002	Insectivorous bat (*Scotophilus dinganii*)	Durban, KwaZulu Natal Province	DM7848	b, f
2002	Insectivorous bat (*Pipistrellus nanus)*	Durban, KwaZulu Natal Province	DM7915	b, f
2002	Frugivorous bat (*Epomophorus wahlbergi*)	Durban, KwaZulu Natal Province	DM7998	b, f
2002/09/01	Insectivorous bat (*Chaerephon pumilus*)	Illovo, KwaZulu Natal Province	DM7913	b, f
2002/10/01	Insectivorous bat (*Rhinolophus sp*)	Swaziland	DM2894	b, f
2002/10/01	Insectivorous bat (*Rhinolophus sp*)	Swaziland	DM7893	b, f
2002/10/01	Insectivorous bat (*Rhinolophus sp*)	Swaziland	DM7897	b, f
2002/12/05	Frugivorous bat (*E. wahlbergi*)	Durban, KwaZulu Natal Province	DM7912	b, f
2002/12/17	Insectivorous bat (*Miniopterus schreibersii*)	Eston, KwaZulu Natal Province	DM7904	b, f
2003/02/07	Insectivorous bat (*C. pumilus)*	Durban, KwaZulu Natal Province	DM8001	b, f
2003/02/07	Insectivorous bat (*Nycteris thebaica*)	Durban, KwaZulu Natal Province	DM7908	b, f
2003/02/20	Insectivorous bat (*Otomops matiensseni)*	Durban, KwaZulu Natal Province	DM8002	b, f
2003/04/04	Frugivorous bat (*E. wahlbergi*)	Umdloti, KwaZulu Natal Province	W17	b, f
2003/04/22	Insectivorous bat (*C. pumilus*)	Pinetown, KwaZulu Natal Province	DM7910	b, f
2003/04/25	Insectivorous bat (*P. nanus)*	Umdloti, KwaZulu Natal Province	DM8000	b, f
2003/06/14	Frugivorous bat (*E. wahlbergi*)	Durban, KwaZulu Natal Province	LagSA2003	a, e
2003/07/14	Insectivorous bat	Durban, KwaZulu Natal Province	DM7851	b, f
2003/07/21	Canine (vaccinated)	Richards Bay, KwaZulu Natal Province	LagSA2003 canine	a, e
2003/11/11	Insectivorous bat (*N. thebaica*)	Westville, KwaZulu Natal Province	04-KW-2	b, f
2004/01/04	Insectivorous bat (*P. nanus*)	Durban, KwaZulu Natal Province	DM7911	b, f
2004/01/04	Insectivorous bat (*C. pumilus*)	KwaZulu Natal Province	DM7907	b, f
2004/02/27	Insectivorous bat (*C. pumilus*)	Amanzimtoti, KwaZulu Natal Province	DM8003	b, f
2004/04/14	Insectivorous bat (*M.schreibersii*)	Nkandla forest, Nkandla Town, KwaZulu Natal Province	04-KW-4	b, f
2004/04/15	Insectivorous bat (*P.hesperidares*)	Nkandla forest, Nkandla Town, KwaZulu Natal Province	04-KW-3	b, f
2004/04/15	Insectivorous bat (*P. hesperidares*)	Nkandla forest, Nkandla Town, KwaZulu Natal Province	04-KW-7	b, f
2004/05/02	Insectivorous bat (*N. thebaica*)	Shongweni valley, KwaZulu Natal Province	DM7842	b, f
2004/05/15	Insectivorous bat (*R.darlingi*)	Umzinto, KwaZulu Natal Province	04-KW-6	b, f
2004/05/17	Insectivorous bat (*O.matiensseni)*	Durban, KwaZulu Natal province	DM7909	b, f
2004/05/19	Insectivorous bat (*S.dinganii*)	Durban, KwaZulu Natal Province	DM8004	b, f

2004/05/27	Insectivorous bat (*O.matiensseni)*	Durban, KwaZulu Natal Province	DM7914	b, f
2004/06/17	Insectivorous bat (*P.kuhlii)*	Amanzimtoti, KwaZulu Natal Province	04-KW-1	b, f
2004/08/18	Frugivorous bat (*E. wahlbergi*)	Laughing waters farm, Nelspruit, Mpumalanga Province	04-NE-1	d, f
2004/08/20	Frugivorous bat (*E. wahlbergi*)	Laughing waters farm, Nelspruit, Mpumalanga Province	04-NE-2	d, f
2004/08/20	Frugivorous bat (*E. wahlbergi*)	Laughing waters farm, Nelspruit, Mpumalanga Province	04-NE-3	d, f
2004/08/20	Frugivorous bat (*E. wahlbergi*)	Laughing waters farm, Nelspruit, Mpumalanga Province	04-NE-4	d, f
2004/08/20	Frugivorous bat (*E. wahlbergi*)	Laughing waters farm, Nelspruit, Mpumalanga Province	04-NE-5	d, f
2004/08/20	Frugivorous bat (*E. wahlbergi*)	Laughing waters farm, Nelspruit, Mpumalanga Province	04-NE-6	d, f
2004/08/20	Frugivorous bat (*E. wahlbergi*)	Laughing waters farm, Nelspruit, Mpumalanga Province	04-NE-7	d, f
2004/08/20	Frugivorous bat (*E. wahlbergi*)	Laughing waters farm, Nelspruit, Mpumalanga Province	04-NE-8	d, f
2004/08/20	Insectivorous bat (*P. nanus*)	Laughing waters farm, Nelspruit, Mpumalanga Province	04-NE-9	d, f
2004/08/21	Insectivorous bat (*P.nanus*)	Laughing waters farm, Nelspruit, Mpumalanga Province	04-NE-10	d, f
2004/08/21	Insectivorous bat (*P.nanus*)	Laughing waters farm, Nelspruit, Mpumalanga Province	04-NE-11	d, f
2004/08/21	Frugivorous bat (*E. wahlbergi*)	Laughing waters farm, Nelspruit, Mpumalanga Province	04-NE-12	d, f
2004/08/22	Frugivorous bat (*E. wahlbergi*)	Durban, KwaZulu Natal Province	LagSA2004	c, e
2004/08/26	Rodent (*Aethomys namaquensis*)	Andries Vosloo Kudu Reserve, Grahamstown, Eastern Cape Province	04-PE-1	d, f
2004/08/26	Rodent (*Otomys unisulcatis*)	Andries Vosloo Kudu Reserve, Grahamstown, Eastern Cape Province	04-PE-2	d, f
2004/08/26	Rodent (*A.namaquensis*)	Andries Vosloo Kudu Reserve, Grahamstown, Eastern Cape Province	04-PE-3	d, f
2004/08/26	Rodent (*A.namaquensis*)	Andries Vosloo Kudu Reserve, Grahamstown, Eastern Cape Province	04-PE-4	d, f
2004/08/26	Rodent (*A. namaquensis*)	Andries Vosloo Kudu Reserve, Grahamstown, Eastern Cape Province	04-PE-5	d, f
2004/08/26	Insectivorous bat (*Rhinolophus sp*)	Andries Vosloo Kudu Reserve, Grahamstown, Eastern Cape Province	04-PE-6	d, f
2004/08/26	Insectivorous bat (*Rhinolophus sp*)	Andries Vosloo Kudu Reserve, Grahamstown, Eastern Cape Province	04-PE-7	d, f
2004/08/26	Insectivorous bat (*Rhinolophus sp*)	Andries Vosloo Kudu Reserve, Grahamstown, Eastern Cape Province	04-PE-8	d, f
2004/08/26	Insectivorous bat (*Rhinolophus sp*)	Andries Vosloo Kudu Reserve, Grahamstown, Eastern Cape Province	04-PE-9	d, f
2004/08/26	Insectivorous bat (*Rhinolophus sp*)	Andries Vosloo Kudu Reserve, Grahamstown, Eastern Cape Province	04-PE-10	d, f

2004/08/26	Insectivorous bat (*Rhinolophus sp*)	Andries Vosloo Kudu Reserve, Grahamstown, Eastern Cape Province	04-PE-11	d, f
2004/08/26	Insectivorous bat (*Rhinolophus sp*)	Andries Vosloo Kudu Reserve, Grahamstown, Eastern Cape Province	04-PE-12	d, f
2004/08/27	Rodent (*A.namaquensis*)	Andries Vosloo Kudu Reserve, Grahamstown, Eastern Cape Province	04-PE-13	d, f
2004/08/27	Rodent (*A.namaquensis*)	Andries Vosloo Kudu Reserve, Grahamstown, Eastern Cape Province	04-PE-14	d, f
2004/08/27	Rodent (*A.namaquensis*)	Andries Vosloo Kudu Reserve, Grahamstown, Eastern Cape Province	04-PE-15	d, f
2004/08/27	Rodent (*O.unisulcatis*)	Andries Vosloo Kudu Reserve, Grahamstown, Eastern Cape Province	04-PE-16	d, f
2004/08/27	Rodent (*A.namaquensis*)	Andries Vosloo Kudu Reserve, Grahamstown, Eastern Cape Province	04-PE-17	d, f
2004/08/27	Rodent (*A.namaquensis*)	Andries Vosloo Kudu Reserve, Grahamstown, Eastern Cape Province	04-PE-18	d, f
2004/08/27	Rodent (*A.namaquensis*)	Andries Vosloo Kudu Reserve, Grahamstown, Eastern Cape Province	04-PE-19	d, f
2004/08/29	Rodent (*A.namaquensis*)	Hogsback, Eastern Cape Province	04-PE-20	d, f
2004/08/29	Rodent (*A.namaquensis*)	Hogsback, Eastern Cape Province	04-PE-21	d, f
2004/08/29	Rodent	Hogsback, Eastern Cape Province	04-PE-22	d, f
2004/08/29	Shrew (*Crocidura sp*)	Hogsback, Eastern Cape Province	04-PE-23	d, f
2004/08/29	Shrew (*Crocidura sp*)	Hogsback, Eastern Cape Province	04-PE-24	d, f
2004/08/29	Shrew (*Crocidura sp*)	Hogsback, Eastern Cape Province	04-PE-25	d, f
2004/08/29	Shrew (*Crocidura sp*)	Hogsback, Eastern Cape Province	04-PE-26	d, f
2004/08/29	Shrew (*Crocidura sp*)	Hogsback, Eastern Cape Province	04-PE-27	d, f
2004/08/29	Shrew (*Crocidura sp*)	Hogsback, Eastern Cape Province	04-PE-28	d, f
2004/08/29	Rodent	Hogsback, Eastern Cape Province	04-PE-29	d, f
2004/08/29	Rodent	Hogsback, Eastern Cape Province	04-PE-30	d, f
2004/08/29	Rodent	Hogsback, Eastern Cape Province	04-PE-31	d, f
2004/08/29	Rodent	Hogsback, Eastern Cape Province	04-PE-32	d, f
2004/08/30	Rodent	Hogsback, Eastern Cape Province	04-PE-33	d, f
2004/08/30	Shrew (*Crocidura sp*)	Hogsback, Eastern Cape Province	04-PE-34	d, f
2004/08/30	Rodent	Hogsback, Eastern Cape Province	04-PE-35	d, f
2004/08/30	Rodent	Hogsback, Eastern Cape Province	04-PE-36	d, f
2004/08/30	Rodent	Hogsback, Eastern Cape Province	04-PE-37	d, f
2004/08/30	Rodent	Hogsback, Eastern Cape Province	04-PE-38	d, f
2004/08/31	Rodent	Hogsback, Eastern Cape Province	04-PE-39	d, f
2004/08/31	Rodent	Hogsback, Eastern Cape Province	04-PE-40	d, f
2004/08/31	Rodent	Hogsback, Eastern Cape Province	04-PE-41	d, f

2004/08/31	Rodent	Hogsback, Eastern Cape Province	04-PE-42	d, f
2004/08/31	Rodent	Hogsback, Eastern Cape Province	04-PE-43	d, f
2004/08/31	Rodent	Hogsback, Eastern Cape Province	04-PE-44	d, f
2004/09/04	Insectivorous bat (*Rhinolophus sp*)	Swaziland	DM7899	b, f
2004/09/04	Insectivorous bat (*Rhinolophus sp*)	Swaziland	DM7898	b, f
2004/09/24	Insectivorous bat (*M.schreibersii*)	Swaziland	DM7921	b, f
2004/09/24	Insectivorous bat (*Hipposideros caffer*)	Swaziland	DM7918	b, f
2004/09/24	Insectivorous bat (*M.schreibersii*)	Swaziland	DM7917	b, f
2004/09/25	Insectivorous bat (*H. caffer*)	Swaziland	DM7920	b, f
2004/09/28	Insectivorous bat (*R.hildebrandt*)	Mpumulanga Province	DM7886	b, f
2004/10/04	Mongoose (*Herpestidae sp*)	Westville, KwaZulu Natal Province	Mongoose2 004	a, e
2004/10/05	Insectivorous bat (*C .pumilus*)	Swaziland	DM7922	b, f
2004/10/05	Insectivorous bat (*S .dinganii*)	Swaziland	DM7900	b, f
2004/10/05	Insectivorous bat (*Neromicia Africana)*	Swaziland	DM7919	b, f
2004/10/06	Frugivorous bat (*E. wahlbergi*)	Swaziland	DM7901	b, f
2005	Mongoose (*Herpestidae sp*)	KwaZulu Natal Province	94/05	a, e
2005/02/23	Insectivorous bat (*P.hesperidares*)	Sudwana Bay, KwaZulu Natal Province	DM8013	b, f
2005/05/14	Insectivorous bat (*R. clivosus*)	Eshowe, KwaZulu Natal Province	DM8375	b, f
2005/05/14	Insectivorous bat (*R. clivosus*)	Eshowe, KwaZulu Natal Province	DM8374	b, f
2005/05/14	Insectivorous bat (*M.schreibersii*)	Entumeni, KwaZulu Natal Province	DM8369	b, f
2005/05/14	Insectivorous bat (*R. clivosus*)	Eshowe, KwaZulu Natal Province	DM8373	b, f
2005/05/15	Insectivorous bat (*R. clivosus*)	Melmoth, KwaZulu Natal Province	DM8376	b, f
2005/05/15	Insectivorous bat (*R.clivosus*)	Melmoth, KwaZulu Natal Province	DM8378	b, f
2005/05/15	Insectivorous bat (*R. clivosus*)	Melmoth, KwaZulu Natal Province	DM8377	b, f
2005/05/15	Insectivorous bat (*M.schreibersii*)	Melmoth, KwaZulu Natal Province	DM8380	b, f
2005/05/15	Insectivorous bat (*M.schreibersii*)	Melmoth, KwaZulu Natal Province	DM8384	b, f
2005/05/15	Insectivorous bat (*M.schreibersii*)	Melmoth, KwaZulu Natal Province	DM8382	b, f
2005/05/15	Insectivorous bat (*M.schreibersii*)	Melmoth, KwaZulu Natal Province	DM8383	b, f
2005/05/15	Insectivorous bat (*M.schreibersii*)	Melmoth, KwaZulu Natal Province	DM8381	b, f
2005/05/15	Insectivorous bat (*R .clivosus*)	Melmoth, KwaZulu Natal Province	DM8379	b, f
2005/06/17	Frugivorous bat (*E. wahlbergi*)	Durban, KwaZulu Natal Province	LagSA2005	c, e
2005/06/21	Frugivorous bat (*E. wahlbergi*)	Durban, KwaZulu Natal Province	2005pup	c, e
2005/07/28	Frugivorous bat (*E. wahlbergi*)	Amanzimtoti, KwaZulu Natal Province	W74	c, e
2005/11/23	Frugivorous bat (*E. wahlbergi*)	Durban, KwaZulu Natal Province	W68	c, e
2005/11/23	Frugivorous bat (*E. wahlbergi*)	Durban, KwaZulu Natal Province	W69	c, e
2005/11/23	Frugivorous bat (*E. wahlbergi*)	Durban, KwaZulu Natal Province	W70	c, e
2005/12/25	Frugivorous bat (*E. wahlbergi*)	Durban, KwaZulu Natal Province	W71	c, e
2005/12/25	Frugivorous bat (*E. wahlbergi*)	Durban, KwaZulu Natal Province	W72	c, e

2005/12/25	Frugivorous bat (*E. wahlbergi*)	Durban, KwaZulu Natal Province	W73	c, e
2006/02/26	Insectivorous bat (*N. capensis*)	Umkomaas, KwaZulu Natal Province	W79	c, e
2006/02/28	Insectivorous bat (*O.matiensseni)*	Umkomaas, KwaZulu Natal Province	W80	c, e
2006/03/17	Insectivorous bat (*N. capensis*)	Umkomaas, KwaZulu Natal Province	W81	c, e
2006/03/22	Frugivorous bat (*E. wahlbergi*)	Amanzimtoti, KwaZulu Natal Province	LBVSA2006	c, e
2006/03/25	Insectivorous bat	Umkomaas, KwaZulu Natal Province	W5	c, e
2006/03/25	Insectivorous bat (*P.kuhlii*)	Umkomaas, KwaZulu Natal Province	W82	c, e
2006/04/20	Insectivorous bat	Umkomaas, KwaZulu Natal Province	W3	c , e
2006/05/29	Insectivorous bat	Umkomaas, KwaZulu Natal Province	W1	c, e
2006/06/06	Insectivorous bat	Umkomaas, KwaZulu Natal Province	W4	c, e
2006/06/21	Frugivorous bat (*E. wahlbergi*)	Durban, KwaZulu Natal Province	481/06	c, e
2006/06/21	Frugivorous bat (*E. wahlbergi*)	Durban, KwaZulu Natal Province	482/06	c, e
2006/06/21	Frugivorous bat (*E. wahlbergi*)	Durban, KwaZulu Natal Province	483/06	c, e
2006/06/23	Insectivorous bat (*O.matiensseni)*	Umkomaas, KwaZulu Natal Province	W2	c, e
2006/06/23	Insectivorous bat	Umkomaas, KwaZulu Natal Province	W6	c, e
2006/07/17	Frugivorous bat (*E. wahlbergi*)	Durban, KwaZulu Natal Province	W7	b, e

Samples tested:	
Insectivorous bats	69
Frugivorous bat	29
Rodents	31
Shrews	7
Other	3
Total amount of samples tested	**138**

[a] Directorate of Veterinary Services, Allerton Provincial Veterinary Laboratory, Pietermaritzburg, KwaZulu Natal Province, South Africa
[b] EThekwini Heritage Department, Natural Science Museum, Durban, KwaZulu Natal Province, South Africa
[c] KwaZulu Natal Bat Interest Group, KwaZulu Natal Province, South Africa
[d] Captured during field surveillance effort
[e] Animal displaying abnormal behavior and subsequently died
[f] Apparent healthy animal

3.2.2 Analysis of samples

3.2.2.1 The fluorescent antibody test (FAT)

All samples indicated in Table 3.1 were tested for the presence of lyssavirus antigens using the FAT as described by Dean *et al*, (1996). Briefly, smears including all areas of the brain were prepared on microscope slides, air-dried and acetone fixed for 30 minutes. A polyclonal fluorescein isothiocyanate conjugated immunoglobulin (Onderstepoort Veterinary Institute, Rabies Unit, South Africa) that is capable of detecting all lyssavirus genotypes was used at a 1:20 dilution. Evans Blue counterstain (0.5% in PBS (0.01 M phospate buffer, pH 7.4; 0.138 M NaCl; 0.0027 M KCl, Sigma-Aldrich) was added to the working dilution conjugate. After adding the

conjugate, slides were incubated for 30 minutes at 37˚C in a humidity chamber and then washed in PBS three times for 5 minutes, air dried and mounted with 20% glycerol solution (0.05 M Tris-buffered saline pH 9.0 with 20% glycerol, Sigma-Aldrich). Slides were read using a fluorescent microscope and fluorescent staining intensity and distribution were graded from 4^{+} to 1^{+}. Samples demonstrating fluorescence were further characterized as described in Section 3.2.3. The necessary positive and negative controls were included.

3.2.3 Virus characterization

3.2.3.1 Mouse inoculation test

Virus isolation was attempted from samples demonstrating immunofluorescence in the FAT using the intracerebral mouse inoculation test (MIT) as described by Koprowski, (1996). Briefly, animal brains were weighed and pooled to prepare 10% (w/v) suspensions in PBS. Brain material was homogenized in a 2 ml glass Dounce homogenizer after which the suspensions were centrifuged at 200 *g* at 4℃ for 10 minutes. The supernatant (30 µl) was inoculated intracranially into two to three day old suckling mice with a 0.5cc tuberculin syringe and 8 mm, 31 gauge needle (Becton Dickinson and Company). Animals were monitored for up to 21 days after inoculation and either euthanized or collected upon death. Subsequently brain smears of brain removed aseptically from dead suckling mice were analysed with FAT (Section 3.2.2.1) to indicate the presence of lyssavirus antigen in the mouse brains. The brain material that tested positive by FAT was pooled and stored at -70˚C for further use.

3.2.3.2 Monoclonal antibody typing

Antigenic typing was performed using the FAT (Section 3.2.2.1) with a panel of sixteen antinucleocapsid monoclonal antibodies (N-MAbs) (Dr. Alex Wandeler, Centre of Expertise for Rabies, Canadian Food Inspection Agency, Nepean, Ontario, Canada) on suckling mice brain material of positive lyssavirus samples. This monoclonal antibody panel are able to distinguish between all seven lyssavirus genotypes and also the canid and mongoose biotypes (gt 1) present in South Africa.

3.2.3.3 Isolation of total RNA

Total RNA was extracted from originally infected animal brain material of samples positively identified for the presence of lyssavirus antigen in the FAT or

demonstrating non-specific fluorescence. TRIzol™ reagent (Invitrogen) was used for extraction of RNA based on the method originally developed by Chomzynski and Sacchi, (1987). Briefly, 50-100 mg of brain material was added to 1 ml TRIzol™ and homogenized using repeated pipetting. The mixture was incubated for 5 minutes at 22ºC in order to permit the dissociation of nucleoprotein complexes, after which 0.2 ml of chloroform was added. The mixture was mixed by shaking for 15 seconds and incubated for a further 3 minutes and then centrifuged at 12 000 g for 15 minutes at 4ºC. The aqueous phase containing the RNA was transferred to a new microcentrifuge tube. Isopropyl alcohol (500 µl) was added to precipitate the RNA for 10 minutes at 22˚C and RNA was recovered by centrifugation at 12 000 g for 30 minutes at 4ºC. The RNA precipitate was washed with 1 ml of 75% ethanol, allowed to dry, and dissolved in 50 µl nuclease free water (Promega). The RNA preparations were then stored at –70ºC until further use.

3.2.3.4 Primer design

Primers specific for the amplification of LBV were designed after alignment of LBV nucleoprotein sequences available from GenBank. The primers were 100% homologous to the LBV isolate from Ethiopia (GenBank accession number: AY3331110) available in the public domain. A forward and reverse primer was designed in a conserved region of the nucleoprotein (Table 3.2). Primers were ordered from Integrated DNA Technologies (IDT), diluted to a 100 pmol stock solution using nuclease free water (Promega) and stored at -70˚C.

Table 3.2: Sequence information of primers used for the genetic characterization of LBV isolates.

Primer	Primer sequence (5´– 3´)	Position on the LBV genome (Using GenBank accession number: AY333110 as reference)
LagNF	GGGCAGATATGACGCGAGA	374 – 392
LagNR	TTGACCGGGTTCAAACATC	813 – 831

3.2.3.5 Reverse transcription

Total extracted RNA (1 µg) was denatured together with 1 µl of 10 pmol of LagNF primer (IDT) and 1 µl of 10 mM dNTP mix (Invitrogen) at 65ºC for 5 minutes. The reaction was made up to a final volume of 13 µl with nuclease free water (Promega). The mixture was subsequently cooled on ice for 1 minute and briefly centrifuged after

which 4 µl of 5 X First strand buffer [0.25 M Tris-HCl, pH 8.3; 0.375 M KCl; 0.015 M $MgCl_2$ (Invitrogen)]; 1 µl of 0.1 M dithiothreitol (Invitrogen); 1 µl RNaseOUT™ recombinant RNase inhibitor (40U/µl, Invitrogen) and 1 µl of Superscript™ III reverse transcriptase (200U/µl, Invitrogen) was added. This was followed by reverse transcription at 55ºC for 60 minutes and inactivation at 70˚C for 15 minutes.

3.2.3.6 Polymerase chain reaction (PCR)

PCR was performed in a 50 µl reaction containing 5 µl of the cDNA prepared in Section 3.2.3.5; 2 µl of both the LagNF and LagNR primers (20 pmol); 5 µl 10 X Accuprime™ PCR buffer I (200 mM Tris-HCL, pH 8.4; 500 mM KCl; 15 mM $MgCl_2$; 2 mM dNTPs; thermostable Accuprime™ protein; 10% glycerol, Invitrogen) and 0.2 µl Accuprime™ Taq DNA polymerase High Fidelity (5U/µl, Invitrogen). The reaction was made up to a final volume of 50 µl with nuclease free water (Promega). The PCR was performed in a GeneAmp PCR 2700 thermocycler (Applied Biosystems) using a profile that involved an initial denaturation for 5 minutes at 94ºC, 30 cycles of 94ºC for 30 seconds; 55ºC for 1 minute; 72ºC for 1 minute and a final elongation step at 72ºC for 5 minutes. PCR products were visualized by agarose gel electrophoresis after adding loading buffer (0.25% bromophenol blue; 40% sucrose) in a 5:1 ratio to the PCR mixture. Samples were electrophoresed at 120V in a 1% ethidium bromide stained agarose gel in 1 X TAE buffer (40 mM Tris-acetate; 1 mM EDTA) using a Hoefer power station PS500X (Hoefer) and a HE33 Hoefer electrophoresis tank (Hoefer). DNA was visualized by UV fluorescence and the size of the amplified product was estimated using a 100 bp DNA weight marker (Promega).

3.2.3.7 Purification of PCR products

The correct size amplified band was excised from the agarose gel and purified using a Wizard® SV gel and PCR Clean-up system (Promega) according to the manufacturers instructions. In brief, 10 µl of membrane binding solution (4.5 M guanidine isothiocyanate; 0.5 M potassium acetate, pH 5.0) was added per 10 mg of excised gel, vortexed and incubated at 60ºC for 10 minutes until the gel slice completely dissolved. The gel mixture was transferred to a Wizard® SV minicolumn in a collection tube and incubated at room temperature for 1 minute after which the mixture was centrifuged through the column at 12 000 g for 1 minute. The DNA bound to the column was washed two times with membrane wash solution (10 mM potassium acetate; 80% ethanol; 16.7 µM EDTA, pH 8.0), and eluted into 30 µl of

nuclease free water (Promega). The purified DNA product was then quantified using agarose gel electrophoresis and a marker (100 bp DNA weight marker (Promega))of known concentration as comparison.

3.2.3.8 DNA nucleotide sequencing

Automated fluorescent DNA nucleotide sequencing was performed of the amplified PCR products. Briefly, 100 ng of purified PCR product (Section 3.2.3.7) was added to a reaction mixture containing 3.2 pmol LagNF or LagNR primer; 2 µl BigDye™ Terminator Ready reaction mix V 3.1 (Applied Biosystems); 1 µl BigDye™ sequencing buffer (5X) and nuclease free water (Promega) to a final volume of 10 µl. The reaction was processed in a thermocycler (GeneAmp PCR 2700, Applied Biosystems) using a profile that consisted of an initial denaturation step at 94ºC for 1 minute, 25 cycles of 94ºC for 10 seconds; 50ºC for 5 seconds and 60ºC for 4 minutes. After the reaction was completed, unincorporated ddNTPs were removed by adding 1 µl of 125 mM EDTA, 1 µl of 3 M Sodium acetate and 25 µl of 100% ethanol. The mixture was vortexed, incubated at room temperature for 15 minutes and centrifuged at 12 000 g for 30 minutes at 4˚C. The supernatant was carefully removed and the pellet washed with 100 µl 75% ethanol. After being allowed to dry on the bench for 15 minutes, the completed reaction was submitted to the Natural and Agricultural Science faculty's sequencing facility (University of Pretoria, South Africa) for resolution on an ABI 3100 DNA sequencer (Applied Biosystems). DNA sequencing were performed with both forward and reverse primes and repeated at least twice.

3.2.3.9 Phylogenetic analysis

DNA sequencing information obtained was compared with nucleoprotein sequence information for lyssavirus genotypes available in the public domain (GenBank). DNA sequences were trimmed using Bioedit (Hall, 1999). ClustalW (Thompson *et al*., 1994) was used to produce sequence alignments and phylogenetic tree construction was carried out using the MEGA3 software version 3.1 (Kumar *et al*., 2004). A neighbor-joining tree (NJ) was constructed using the Kimura's two-parameter method (Kimura, 1980). The branching order of the tree was evaluated by using bootstrap analysis of 1000 data replications.

3.2.4 Characterization of the host species

In cases where the animal carcass was destroyed, the animal species was accurately identified using DNA sequencing analyses of the mitochondrial cytochrome *b* region of genomic DNA obtained from the original brain sample (Veron *et al*., 2004).

3.2.4.1 DNA isolation

Genomic DNA was extracted from brain material using the DNeasy® tissue kit (Qiagen) as described by the manufacturers. Briefly, 25 mg of brain tissue was added to 180 µl ATL buffer (Qiagen) and 20 µl proteinase K (Qiagen) and incubated at 56˚C until the tissue was lysed. The sample was vortexed and 200 µl of buffer AL (Qiagen) and 200 µl 100% ethanol was added and mixed. The mixture was transferred to a DNeasy® Mini spin column (Qiagen) and centrifuged at 6000 g for 1 minute to discard the flow through into a collection tube. After placing the DNeasy® Mini spin column (Qiagen) in a new collection tube, 500 µl buffer AW1 (Qiagen) was added and centrifuged at 6000 g for 1 minute after which 500 µl of buffer AW2 (Qiagen) was added and centrifuged at 20 000 g for 3 minutes. The DNA was then eluted from the membrane in 200 µl AE buffer (Qiagen) after incubation for 1 minute at room temperature. The elute was collected by centrifugation for 1 minute at 6000 g.

3.2.4.2 PCR and DNA sequencing

PCR was performed according to the materials and methods of Veron *et al*, (2004). The complete cytochrome *b* region was amplified in three separate PCR reactions using the following primers: L14724 (GATATGAAAAACCATCGTTG) and H15149 (AAACTGCAGCCCCTCAGAATGATATTTGTCCTCA); L14841 (CATCCAACATCTC AGCATGATGAAAA) and H15553 (TAGGCAAATAGGAAATATCATTCTGGT) and L15146 (CATGAGGACAAATATCATTCTGAG) and H15915 (TTCATCTCTCCGGTT TACAAGAC). PCR was carried out in a 50 µl reaction containing 300 ng of the genomic DNA prepared in Section 3.2.4.1. The reaction mixture consisted of 1 µl each of the forward and the reverse primer (10 pmol); 5 µl 10 X Accuprime™ PCR buffer I (200 mM Tris-HCL, pH 8.4; 500 mM KCl; 15 mM $MgCl_2$; 2 mM dNTPs; thermostable Accuprime™ protein; 10% glycerol, Invitrogen) and 0.2 µl Accuprime™ Taq DNA polymerase (5 U/µl, Invitrogen) were added. The reaction was made up to

a final volume of 50 µl with nuclease free water (Promega). The PCR was performed in a GeneAmp PCR 2700 thermocycler (Applied Biosystems) using a profile that involved an initial denaturation for 4 minutes at 94ºC, 35 cycles of 93ºC for 30 seconds; 50ºC for 40 seconds, 72ºC for 40 seconds and a final elongation step at 72ºC for 7 minutes. PCR products were visualized by agarose gel electrophoresis and the size of the amplified product was estimated with a 100bp DNA weight marker (Promega). The PCR products were purified using the Wizard® SV PCR and Gel purification kit (Promega) (Section 3.2.3.7) and the DNA nucleotide sequence was determined by using the Big Dye™ Termination Cycle Sequencing Ready Reaction Kit 3.1 (Applied Biosystems), according to the manufacturer's protocol, with subsequent analysis on an Applied Biosystems 3100 DNA automated sequencer.

3.2.4.3 Phylogenetic analysis

DNA sequence of 893 bp of cytochrome *b* was compared to cytochrome *b* sequences for animal species available on GenBank, using the same methodology as described earlier for the analysis of LBV nucleoprotein gene sequences (Section 3.2.3.9).

3.3 Results

3.3.1 Results of epidemiological surveillance using the FAT

Five samples of 138 samples collected for lyssavirus diagnostic testing (Table 3.1), demonstrated a positive result for the presence of lyssavirus antigen using the FAT (Table 3.3). One sample (LagSA2005) demonstrated non-specific staining and was subjected to further testing using additional methods as indicated in Section 3.3.2.

Table 3.3: Information of samples that tested positive for the presence of lyssavirus antigens with the FAT or demonstrated non–specific fluorescence.

SAMPLE NUMBER	CASE REPORT	RESULT OF FAT
LagSA2003	On 14 June 2003, an *E. wahlbergi* carcass was recovered in Durban, KwaZulu Natal Province, after being caught by a domestic cat. The brain was submitted for rabies testing	3+
LagSA2003 canine	On 21 July 2003 an Australian Cattle dog attacked people on the beach unprovoked in Richards Bay, KwaZulu Natal Province. The owner requested euthanasia to avoid any future accidents. The dog was vaccinated against rabies but the date of vaccination was unknown. Rabies was not suspected but after consultation it was decided to send brain material for testing.	2+
LagSA2004	On 22 August 2004 a resident of Umbilo, Durban, KwaZulu Natal Province, found a dead *E. wahlbergi* fruit bat on her lawn in the morning after hearing squeaking noises around the house during the night. The complete bat carcass was submitted for rabies testing.	4+
Mongoose 2004	On 25 October 2004 a mongoose was captured by the Society for the Prevention of Cruelty to Animals (SPCA) from a marshy valley in a residential area in Westville near Durban, KwaZulu Natal Province, after the mongoose displayed abnormal behaviour. The animal was disorientated, attacking inanimate objects and its behavior alternated between being friendly and aggressive. Only the brain of the animal was submitted for testing and the carcass was not preserved. The mongoose species was not identified.	4+
LagSA2005	On 17 June 2005, a caretaker/gardener at a communal outdoor sports complex in the Bluff, Durban, KwaZulu Natal Province, found a bat on the lawn of the complex. At the time, birds were picking at it and on closer inspection, it was found to be an immobile adult animal with a pup attached to it. The caretaker collected the two animals and placed them in a nearby tree. Later on, the two bats, still attached to each other, were again found on the ground, where eyewitnesses also saw a cat toying with it. The animals were then presented to a local bat rehabilitator, part of the KwaZulu Natal Bat Interest Group. The adult animal was found to be dead and was submitted for diagnostic testing, but FAT carried out on brain smears were repeatedly found to be negative. The pup had at least one evident bite wound, presumed to be as result of the interaction with the cat, but otherwise appeared healthy and was taken care of by the rehabilitator. Although the pup was reported to be feeding and doing well, it suddenly died about 4 days after being found, on June 21, 2005. The bats were identified as *E. wahlbergi*. FAT on the pup brain was negative.	Negative (Non-specific staining)
LBVSA2006	On 22 March 2006 an *E. wahlbergi* fruit bat was submitted for testing by a bat rehabilitator, part of the KwaZulu Natal Bat Interest Group. The bat was very calm, unusual for a wild fruit bat and was having trouble breathing and was very thirsty. It was unable to eat solid fruit because it was unable to spit out the remains after sucking out the juice but was able to drink water and liquidised fruit. The throat was red and inflamed and almost totally closed by the swelling. After rehydration, the bat started producing large quantities of saliva and breathing rapidly deterioted and choked to death. The fluid in the eyes turned milky. The bat shooked it's head and sprayed saliva all over the bat rehabilitator.	4+

3.3.2 Characterization of new LBV isolates

Virus was isolated from four (LagSA2003; LagSA2004; Mongoose2004 and LBVSA2006) of the six cases indicated in Table 3.3. Suckling mice died 9-14 days following intracerebral inoculations with brain suspensions prepared from the original animal's brain material. The FAT performed on brain harvested from the inoculated suckling mice tested positive for lyssavirus antigens. Virus isolations could not be obtained from the LagSA2003canine and LagSA2005 cases.

To identify the specific lyssavirus genotype, the isolates were antigenically and genetically characterized. Antigenic typing was carried out using a panel of anti-lyssavirus nucleocapsid monoclonal antibodies prepared by the Centre of Expertise for Rabies, Canadian Food Inspection Agency, Nepean, Ontario, Canada. The smears were read by ultra-violet microscopy, and the fluorescence of each well scored as positive or negative. In the antigenic typing the LagSA2003; LagSA2004; Mongoose2004 and LBVSA2006 isolates reacted with N-MAb 38HF2, an antibody that reacts with all lyssavirus representatives tested, and with the antibody N-MAb M612, that is highly specific for LBV and does not react with any other lyssaviruses tested (Table 3.4). This suggested that these new isolates belong to gt 2 (LBV). Isolate LagSA2003canine and LagSA2005 could not be antigenically typed due to the low amount of fluorescence present in original brain samples and the inability to amplify these isolates in suckling mice.

Table 3.4: Immunoflourescence reaction of a panel of sixteen monoclonal antibodies (Centre of Expertise for Rabies, Ontario, Canada) against the nucleoprotein of LBV isolations made from South Africa. The typical immunoflourescence antibody pattern observed for all lyssavirus genotypes that are present on the African continent (Genotype 1, 2, 3 and 4) are also included as a reference.

	Canid Biotype (gt1)	Mongoose Biotype (gt1)	LBV (gt2)	MOKV (gt3)	DUVV (gt4)	Lag2003	Lag2004	Mongoose 2004	LBVSA2006
1C5	-	-	-	-	-	-	-	-	-
26AB7	+	var	-	-	-	-	-	-	-
26BE2	+	var	-	-	-	-	-	-	-
32GD12	var	var	-	-	-	-	-	-	-
38HF2	+	+	+	+	+	+	+	+	+
M612	-	-	+	-	-	+	+	+	+
M837	-	-	-	-	+	-	-	-	-
M850	-	var	-	-	+	-	-	-	-
M853	+	-	-	-	+	-	-	-	-
M1001	-	-	-	+	-	-	-	-	-
M1335	-	var	-	var	-	-	-	-	-
M1386	-	+	-	-	-	-	-	-	-
M1400	-	var	-	-	-	-	-	-	-
M1407	+	var	-	-	-	-	-	-	-
M1412	+	var	-	-	-	-	-	-	-
M1494	-	var	-	-	+	-	-	-	-

***The typical immunofluorescence antibody patterns observed for all lyssavirus genotypes present in Africa are included in the table. (-) no fluorescence, (+) fluorescence observed and (var) variable pattern observed.**

Confirmation of the diagnosis was done by RT-PCR and DNA sequencing of a 457 bp region of the nucleoprotein encoding gene that is known to be highly conserved within the *Lyssavirus* genus. For this purpose a novel set of PCR and sequencing primers specific for LBV were designed. All six isolates indicated in Table 3.3 yielded an amplicon of the correct size using the LBV specific primers. Subsequent DNA sequencing information of these amplicons was compared with cognate nucleoprotein sequence information for other lyssavirus genotypes available in the public domain (GenBank). A phylogenetic analysis indicated that the six new isolates cluster together with LBV isolates from Nigeria and Ethiopia (Figure 3.1) with a high

statistical bootstrap value support (86%). The genetic characterization therefore indicated that all six isolates belong to gt 2.

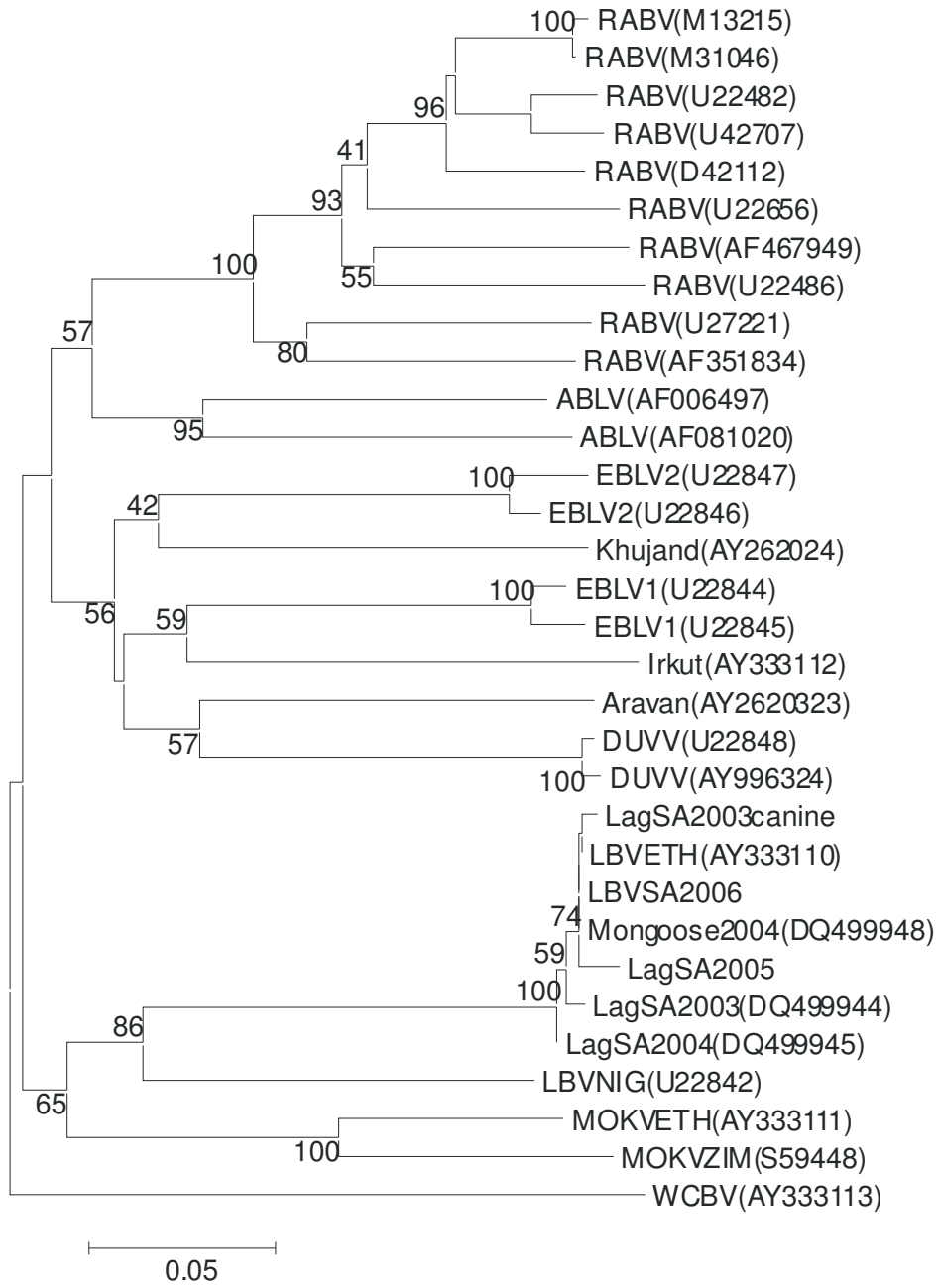


Figure 3.1: A neighbor-joining phylogenetic tree comparing 457 nucleotides of the nucleoprotein gene of six new Lagos bat virus (LBV) isolates from South Africa and representative sequences of all other genotypes of lyssaviruses. Branch lengths are drawn to scale, and bootstrap values for 1000 replicates are shown for the key nodes. The accession numbers for all sequences available from GenBank are included in the figure.

3.3.3 Species identification of the LBV-infected mongoose

The species of the LBV infected mongoose (Mongoose2004) was not identified because the animal carcass was not kept and only brain material was submitted for diagnostic testing. Analysis of 893 bp of the cytochrome *b* gene obtained from the mongoose brain material indicated that the infected animal shared a 98% DNA nucleotide sequence homology with the African water mongoose (*Atilax paludinosus*) (Figure 3.2). Water mongooses (also known as marsh mongooses) are solitary and mainly nocturnal mammals but can also be active during the day. These animals live closely to water in areas with sufficient bush cover and they have been reported to occur throughout sub-Saharan Africa.

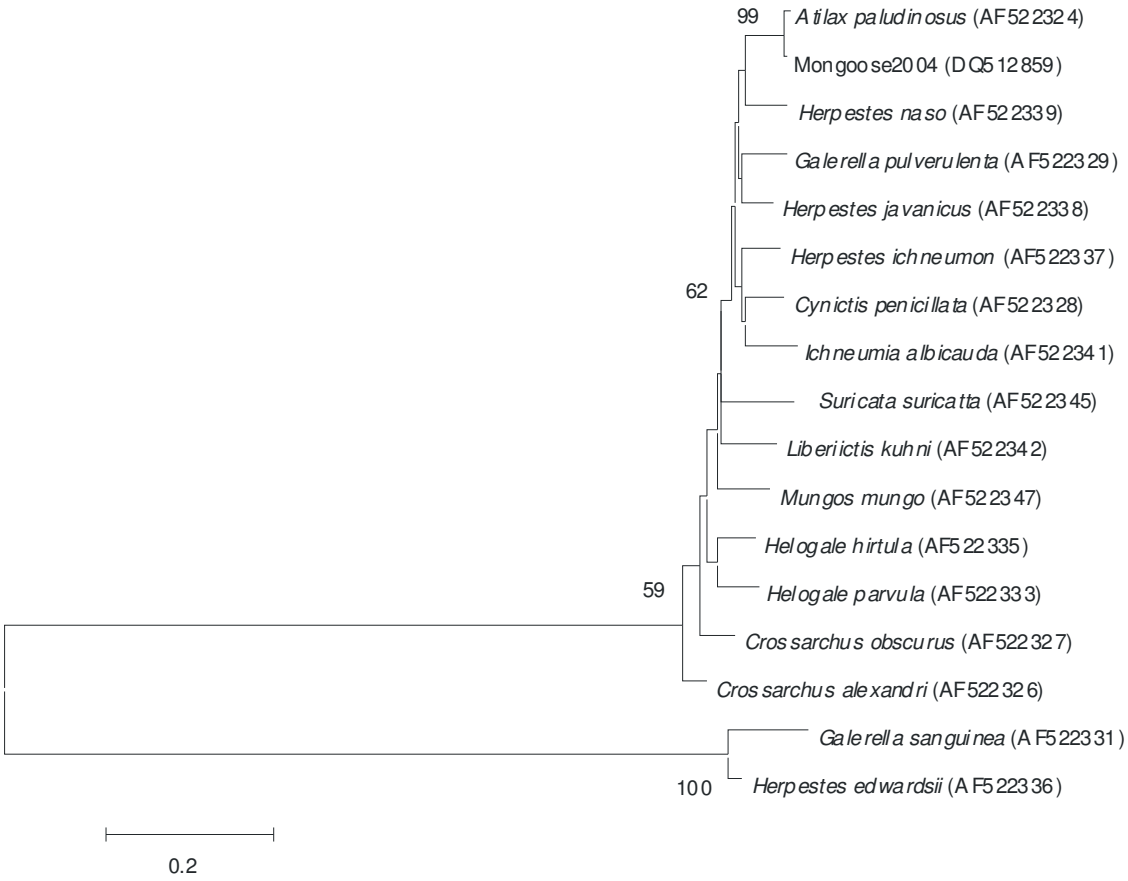


Figure 3.2: A neighbor-joining phylogenetic tree comparing 893 bp of the cytochrome *b* region of *Herpestidae* family sequences available in the public domain (GenBank). The sequence obtained from the LBV-infected mongoose (Mongoose2004) is 98% identical to the known cytochrome *b* sequences of *Atilax paludinosus* (water mongoose). The GenBank accession numbers are indicated on the phylogenetic tree and bootstrap values were determined with 1000 replicates.

3.4 Discussion

Although LBV appears to be rare and has not been identified in the previous 12 years before the start of this study in 2002, a small-scale surveillance effort enabled us to make six new isolations of LBV in a relatively short period of time. Poor surveillance of rabies-related lyssaviruses and poor diagnostic capability through most of Africa are large contributors to our lack of information and the obscurity of the African lyssaviruses. Even if more active surveillance programs were to be considered, very few laboratories in Africa would be capable of detecting rabies-related lyssaviruses and be able to differentiate between rabies and rabies-related lyssaviruses with any degree of certainty. The fluorescent antibody test used as a diagnostic test for rabies can only indicate the presence of lyssavirus antigens and cannot distinguish between lyssavirus genotypes. To identify a lyssavirus precisely, antigenic typing or genetic characterization is necessary, but beyond the capability of most laboratories responsible for rabies diagnostics on the African continent.

The difficulty of identifying rabies-related lyssaviruses with certainty has also been indicated by the LagSA2005 and Lag SA2003canine cases in this study. In the LagSA 2005 case a positive FAT test could not be established and virus isolation could not be obtained in suckling mice. This isolate was only positively identified as LBV with genetic characterization using RT-PCR and DNA sequencing. Diagnostic samples for rabies tesing are usually taken at the terminal stages of illness when the virus is present in high copy numbers in the brain. Lyssavirus infection can therefore be easily detected using the FAT. In the LagSA2005 case a cat was observed playing with the bat and it may therefore may have been killed by the cat in the early stages of disease when low amounts of virus are present in the brain. RT-PCR is more sensitive and can detect low copies of viral RNA that may not have been detected with the FAT (Centers for Disease Control and Prevention, 1997). Its been shown that RT-PCR can also detect viral RNA in samples diluted 100 to 1000 fold beyond the level of virus isolation in cell culture (Trimarchi and Smith, 2002). Successful virus isolation may also be dependent on the antibody status of the host (Trimarchi and Smith, 2002) and if neutralizing antibodies have developed, virus isolation is most often not successful. In the LagSA2003canine case the dog was euthanized without suspecting rabies and therefore was not demonstrating rabies signs at the time of death and may have been euthanized in early stages of the disease. Low amounts of virus present in the brain may explain the low intensity and distribution of the FAT result (2+ positive) and inability to isolate virus. The animal

was also vaccinated against rabies and therefore the presence of neutralizing antibodies may also have an influence on virus isolation. Cases like these must be treated with caution before making a positive diagnosis and extra care must be taken that no cross-contamination with other laboratory samples occurred that may led to a false positive result. In both these cases all the necessary controls were included and all techniques were performed aseptically. The FAT test must still be used as the classical technique for lyssavirus diagnostics before using molecular methods.

Phylogenetic analysis in this study indicated a close nucleoprotein sequence homology between LBV isolates from South Africa when comparing a partial region of the nucleoprotein. Geographical partitioning is a well known characteristic of RABV epidemiology worldwide and similarly; the close sequence homology found in this case is likely to be accounted for by virtue of the defined geographical location that all these LBV isolates were obtained from. The infected LBV bats, canine and mongoose identified in this study all demonstrated abnormal behaviour. The mongoose exhibited aggressive behavior and was captured in a populated residential area and the canine demonstrated aggressive behaviour and bit two people. Even though the incidence of the rabies-related lyssaviruses seems to be low there is a definitive potential risk of human exposure to these viruses. The virus has also been reported in domestic animals, cats and dogs (Mebatsion *et al*., 1992b; Swanepoel, 2004; Foggin, 1988). There has been close contact between humans and the LBV-infected bats and canine described in this study. The predator-prey relationship between cats and bats also leads to a seemingly inevitable interaction between these species and was clearly illustrated in the cases presented here. The extent and status of potential exposures of the cats involved in these cases are not known. It is clear from cross-neutralization data obtained in rodent models that rabies pre- and post-exposure prophylaxis is unlikely to be effective against LBV (Hanlon *et al*., 2005; Nel, 2005). It is not possible to provide informed advice on the control and prevention of any disease where basic epidemiological data are as scarce as for the African lyssaviruses. However, although this may be contrary to popular belief among some bat rehabilitators and handlers, there is clearly a possibility of LBV infection within these bat populations. Appropriate care when interacting with these animals is therefore strongly recommended. Even though the value of rabies vaccination is doubtful, it should also be considered in view of the possibility of some cross-reactivity and the lack of any alternative.

It should be noted that the amount of LBV infected bats in the more active surveillance effort of the 1980's amounted to almost 5% (Swanepoel, 2004), which is comparable to bat surveillance standards in the Americas. Employing a low key surveillance plan, it was demonstrated that LBV can still be readily identified and isolated from *Megachiroptera* in South Africa despite having not been reported from any species in this region for more than a decade. Cumulatively, all the presently available evidence indicates that LBV is highly likely to be persistently maintained in Megachiroptera populations in South Africa and probably elsewhere in Africa from where LBV had been reported in the past. The isolation of LBV from terrestrial wildlife serves as further confirmation of our lack of understanding of the incidence and host range of lyssaviruses in Africa. This underscores the need for surveillance of rabies-related lyssaviruses and also the need for accurate identification of lyssavirus genotypes even if the host involved is normally only associated with RABV. From these new cases identified it seems likely that LBV persist in pteropid bats in South Africa, implicating continual opportunity for spillover into terrestrial species. Towards determining the extent of risk to human and veterinary public health, it is therefore important to establish the prevalence of LBV not only in bats, but also in potential terrestrial animal vectors, to which mongoose species should be added.

In this study the value of the use of cytochrome *b* DNA sequencing to accurately identify the host in a positive rabies case has been demonstrated. Diagnostic laboratories do not routinely receive the complete carcass of suspected rabid animals and identification is dependent on the reports of the persons responsible for capturing the animal or for removal of the brain prior to submission to the diagnostic facility. Host identity is rarely a problem in the case of domestic animals, but wildlife species do present a potential uncertainty, such as demonstrated in the case reported here. Clearly, one important aspect of disease epidemiology is accurate information on the host species involved, enabling informed decisions on epidemiological patterns and potential threats to public and veterinary health.

CHAPTER IV

NON-NEURONAL VIRAL TISSUE DISTRIBUTION AND SEROLOGY OF NATURALLY INFECTED FRUGIVOROUS BATS WITH LAGOS BAT VIRUS

4.1 Introduction

Previous studies of lyssavirus infections in bats have mostly been performed under laboratory conditions and very little is known about non-neuronal tissue distribution and serology of lyssaviruses in naturally infected bats. Table 4.1 provides a summary of previous studies of the non-neuronal tissue distribution of lyssaviruses in experimentally and naturally infected bats that have succumbed to rabies.

Table 4.1: A summary of results of previous studies investigating non-neuronal tissue distribution of lyssavirus infection in experimentally (A) or naturally infected bats (B).

A

GT	REFERENCE	TISSUES TESTED*												
		Brain	Salivary gland	Brown fat	Heart	Esophagus	Lung	Kidney	Tongue	Bladder	Spleen	Stomach	Intestines	Liver
Aravan	Hughes *et al.*, 2006	+ +	+ -	+ -	NT	NT	+ -	+ -	+ -	NT	- -	NT	NT	NT
Irkut	Hughes *et al.*, 2006	+ +	+ +	+ -	NT	NT	+ +	+ -	NT	+ -	NT	NT	NT	NT
Khujand	Hughes *et al.*, 2006	+ +	+ +	+ +	NT	NT	+ +	+ -	+ +	NT	+ -	NT	NT	NT
5	Van der Poel *et al.*, 2000	+ +	+ +	NT	NT	NT	NT	NT	NT	- -	NT	NT	NT	NT
1	Baer and Bales, 1967	NT +	NT +	NT +	NT	NT	NT +	NT +	NT	NT	NT	NT	NT	NT
1	Shankar *et al.*, 2004	+ +	+ +	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT
1	Moreno and Baer, 1980	NT +	NT +	NT +	NT +	NT	NT +	NT +	NT	NT	NT +	NT	NT	NT +

B

GT	REFERENCE	TISSUES TESTED*												
		Brain	Salivary gland	Brown fat	Heart	Esophagus	Lung	Kidney	Tongue	Bladder	Spleen	Stomach	Intestines	Liver
6	Johnson *et al.*, 2003; Johnson *et al.*, 2006	+ +	NT	NT	+ -	NT	NT	+ -	+ -	+ -	NT	+ +	+ -	+ -
6	Bourhy *et al.*, 1992	+ +	- -	- -	- +	NT	+ -	- -	+ -	NT	- -	NT	NT	NT -

*** - (negative) or + (positive) with RT-PCR/FAT;** - (negative) or + (positive) with virus isolation;

NT (Not tested)

Experimental infection of bats with a lyssavirus results in fatal encephalitis and virus has always been detected in brains collected from these bats and frequently also in salivary glands. Low titres of virus could also be found in other non-neuronal tissue such as brown fat, lungs, kidney, tongue, bladder and spleen [Table 4.1(A)]. Viral distribution to non-neuronal tissues were found to differ between bats even if bats were infected with the same amount of virus and same virus isolate. Before the development of RT-PCR techniques, virus was detected in tissues collected using mouse inoculation or cell culture virus isolation. The development of RT-PCR provided a much more sensitive tool to detect viral RNA but virus isolation methods are still used to detect the presence of infectious virus. Studies of the non-neuronal distribution in tissue collected from naturally lyssavirus infected bats that succumbed to rabies were performed for EBLV-1 (Bourhy et al., 1992) and EBLV-2 (Johnson *et al*., 2003; Johnson *et al*., 2006). Viral RNA was detected by a very sensitive nested RT-PCR in various non-neuronal tissues such as heart, kidney, tongue, bladder, stomach, intestines and liver (Johnson *et al*., 2003; Johnson *et al*., 2006). However virus isolation was unsuccessful from most tissues that were positive by RT-PCR and was only successfully isolated from the brain and stomach. Another study indicated that EBLV-2 virus antigen can be detected in lung, tongue and brain tissue using FAT and virus was successfully isolated from brain, heart and lung (Bourhy *et al*., 1992). In these studies virus antigen was only detected in non-neuronal tissue if present in brain material. However the presence of EBLV-1 RNA in various non-neuronal bat organs (heart, esophagus, lung and tongue) has been indicated in another study after RT-PCR on brain material from the same bats was negative (Serra-Cobo *et al*., 2002). No virus could be isolated from any RT-PCR positive tissue. Previous studies also reported the presence of EBLV-1 RNA in apparently healthy bat's tissue samples using nested RT-PCR and FAT (Wellenberg *et al*., 2002). Salivary glands, bladder, lung, rectum and genital organs tested positive with RT-PCR but no virus could be isolated from any RT-PCR positive tissue including brain material. It was suggested that these results are indicative of a low or non-productive infection. This phenomenon has not been supported by any of the experimental studies performed in bats (Table 4.1).

It has been shown that viral RNA could be detected by RT-PCR in oral swabs collected from bats that were experimentally infected with Irkut, Aravan and Khujand virus (Hughes *et al*., 2006). Detectable amounts of viral RNA was not present earlier than 5 days before the onset of clinical symptoms or within a week of death and virus isolation was successful from only two oral swabs. Another study indicated that virus

was present in saliva of RABV experimentally infected bats 0 to 15 days before the onset of clinical signs (Baer and Bales, 1967). Other studies were also able to isolate virus from an oral swab taken from bats displaying signs of rabies (Shankar *et al*., 2004) and was able to isolate virus from saliva samples taken from vampire bats up to 8 days before signs of rabies appear (Moreno and Baer, 1980). In one particular animal, RABV was also found to be present in the saliva, but absent from the salivary glands (Moreno and Baer, 1980). There appeared to be no correlation between the dose of inoculum, presence of virus in saliva and presence of virus in salivary glands.

Baer and Bales, (1967) observed neutralizing antibodies only in bats that did not display any signs of clinical illness. However another study reported a high antibody titre in a rabid bat that subsequently died of rabies (Shankar *et al*., 2004). The complexity and many unanswered questions are demonstrated by a study performed of RABV in raccoons (*Procyon lotor*) (Niezgoda *et al*., 2002). A complex transmission pattern was exhibited in that animals may lack clinical signs, may or may not show RABV antibody titers, may become rabid with no detectable antibody titres, or may succumb to rabies with detectable antibody titres. If neutralizing antibodies were detected at all in animals with rabies infection, it usually was either at the onset of illness or, more commonly, in the terminal stages of the disease (Niezgoda *et al*., 2002).

Virus antigen in different tissues can be detected using standard lyssavirus diagnostic methods (Chapter II; Section 2.6). RT-PCR is the most sensitive method to detect viral RNA but may provide false negative results if the RNA is degraded or enzyme inhibitors are present. To improve the confidence of RT-PCR results an internal control can be included and it was therefore suggested to target ribosomal RNA (rRNA) with RT-PCR producing a 488 bp amplicon (Smith *et al*., 2000). rRNA is present in abundance and may out-compete the viral target RNA in a duplex RT-PCR and low levels of viral RNA may not be detected. An improved method was then suggested whereby competimers are added that compete with the internal control target for the 18S primers and therefore reducing the availability of primers (Smith *et al*., 2000).

LBV, a gt 2 lyssavirus, has recently been isolated in South Africa from *E. wahlbergii* fruit bats in 2003, 2004, 2005 and 2006 as described in Chapter III. In the 2004 (LagSA2004), 2005 (LagSA2005) and 2006 (LBVSA2006) positively identified LBV cases, the complete bat carcass was submitted for diagnostic testing. A positive

result for lyssavirus antigens with the FAT was obtained after testing brain material removed from these bats and either antigenic typing or genetic characterization identified the infectious agent as LBV. Since very little is known about the pathogenesis of LBV, the three positively identified LBV cases and the availability of the complete carcass of the infected animals allowed us with the unique opportunity to investigate the distribution of the virus in the tissues of naturally LBV infected bats as well as determine neutralizing antibody titres at the time of death.

4.2 Materials and methods

4.2.1 Collection of bat tissues

Bat organs were removed using sterilized instruments for each tissue sampled, in order to eliminate cross-contamination at necropsy. Special care was taken not to cross contaminate any tissues. Blood was collected into a serum separator tube from the heart cavity of the bat carcass with a pipette. Blood samples were centrifuged for 20 minutes at 5000 g and serum was extracted and stored at -20˚C. All tissues removed at necropsy were stored at -70ºC. Table 4.2 indicates the tissues collected.

Table 4.2: Tissues collected during necropsy of LagSA2004, LagSA2005 and LBVSA2006 LBV infected frugivorous bats.

TISSUE	LAGSA2004	LAGSA2005	LBVSA2006
Brain	Yes	Yes	Yes
Salivary Gland	Yes	Yes	Yes
Skin	No	Yes	Yes
Muscle	No	Yes	Yes
Tongue	Yes	Yes	Yes
Esophagus	No	Yes	Yes
Lung	Yes	Yes	Yes
Stomach	No	Yes	Yes
Intestine	No	Yes	Yes
Testes/Ovary	No	No	Yes
Heart	Yes	Yes	Yes
Diaphragm	No	Yes	Yes
Liver	Yes	Yes	Yes
Kidney	Yes	Yes	Yes
Adrenal	No	Yes	Yes
Bladder	No	Yes	Yes
Spleen	Yes	Yes	Yes
Oral swab	No	Yes	No
Blood	No	Yes	Yes

Collected (Yes); Not collected (No)

4.2.2 Total RNA isolation from bat tissues

Total RNA was extracted from all tissues collected (Table 4.2) using the TRIzol™ method (Invitrogen) as described by the manufacturers. Briefly, 50-100 mg of tissue

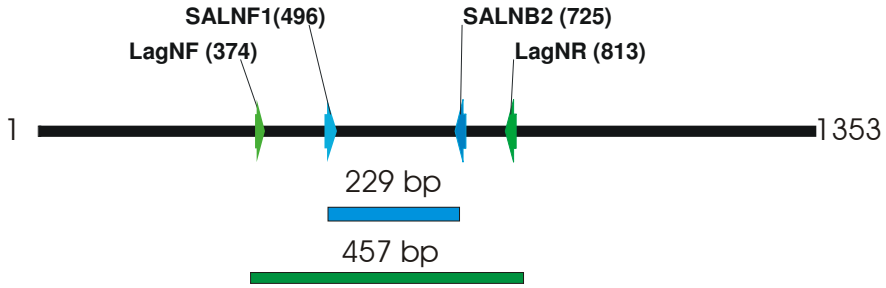
was added to 1 ml TRIzol™ and homogenized using a sterile 2 ml Dounce homogenizer for each tissue. The mixture was transferred to a microcentrifuge tube and incubated for 5 minutes at room temperature after which 0.2 ml of chloroform was added. The mixture was vortexed for 15 seconds and incubated for a further 3 minutes and then centrifuged at 12 000 g for 30 minutes at 4ºC, The aqueous phase containing the RNA was transferred to a new microcentrifuge tube. Isopropyl alcohol (500 µl) was added to precipitate the RNA for 10 minutes at room temperature and RNA was recovered by centrifugation at 12 000 g for 30 minutes at 4ºC. The RNA precipitate was washed with 1 ml of 75% ethanol, allowed to dry, and dissolved in 50 µl nuclease free water (GIBCO). The RNA preparations were then stored at –70ºC until further use. Extraction of RNA was performed in a level-2 biosafety laboratory in a separate room from where RT-PCR is performed using aerosol-resistant filter tips.

4.2.3 Assessment of RNA template quality using RT-PCR

The extracted RNA template quality was assessed by performing a ribosomal (rRNA) control RT-PCR on extracted RNA as described by Smith *et al*, (2000) with modifications. 5 µl (1-2 µg) of extracted RNA from the bat tissues was added to 1 µl of 18SD primer (5 pmol) (GGACATCTAAGGGCATCACA) and heated to 94˚C for 1 minute and then cooled on ice for 5 minutes. 14 µl of RT-RXN mix (4.5 µl 5 X Reverse transcriptase buffer (250 mM Tris-HCl; 40 mM $MgCl_2$; 150 mM KCl; 5 mM dithioerythritol; pH 8.5, Roche Diagnostics); 2.2 µl 10 mM dNTPs (Roche Diagnostics) and 7.3 µl DEPC H_2O (GIBCO); 0.4 µl AMV Reverse transcriptase (20U/µl, Roche Diagnostics) and 0.4 µl RNase inhibitor (40U/µl, Roche Diagnostics) were then added. The reaction mixture was centrifuged briefly and 80 µl of PCR mix; [66.75 µl DEPC H_2O (GIBCO); 10 µl 10 X Reverse transcriptase buffer (250 mM Tris-HCl; 40 mM $MgCl_2$; 150 mM KCl; 5 mM dithioerythritol; pH 8.5, Roche Diagnostics); 18SU (TCAAGAACGAAAGTCGGAGG) and 18SD (GGACATCTAAGGGCATCACA) primers (40 pmol) and 1 µl AmpliTaq® (2U/µl, Applied Biosystems)] were added. The reaction mixture was then subjected to the following cycling conditions on a GeneAmp 9700 thermocycler (Applied Biosystems); Denaturation of 94˚C for 1 minute followed by 40 cycles of 94˚C for 30 seconds, 37˚C for 30 seconds and 72˚C for 90 seconds. A final extension step of 72˚C for 7 minutes was performed and the reaction was kept at 4˚C until ready to be removed.

4.2.4 Primer design

For cDNA synthesis and the first round of PCR to detect LBV genomic RNA, primers already described in Chapter III, LagNF and LagNR (Section 3.2.3.4) were used. For the subsequent nested PCR two primers were designed using the Ethiopian LBV isolate's sequence available from GenBank (Accession number: AY333110). The SALNF1 (GCAGACAGRATGGAGCAGATT) and SALNB2 (TGTTTAATRAACCCTGTAA) primers were designed to amplify a 229 bp region as indicated in Figure 4.1.



LBVETH(GenBank accession: AY333110)

Figure 4.1: A diagrammatic representation indicating the position of primers targeting the nucleoprotein of LBV in RT-PCR and nested PCR performed on tissues collected from naturally infected bats.

4.2.5 RT-PCR and nested PCR to detect LBV RNA

5 µl (1-2 µg) of RNA extracted from the bat tissues was added to 1 µl of LagNF primer (5 pmol) and heated to 94˚C for 1 minute and then cooled on ice for 5 minutes. 14 µl of RT-RXN mix [4.5 µl 5 X Reverse transcriptase buffer (250 mM Tris-HCl; 40 mM $MgCl_2$; 150 mM KCl; 5 mM dithioerythritol; pH 8.5, Roche Diagnostics); 2.2 µl 10 mM dNTPs (Roche Diagnostics) and 7.3 µl DEPC H_2O (GIBCO); 0.4 µl AMV Reverse transcriptase (20U/µl, Roche Diagnostics) and 0.4 µl RNase inhibitor (40U/µl, Roche Diagnostics] were then added. The reaction mixture was centrifuged briefly and 80 µl of PCR mix [66.75 µl DEPC H_2O (GIBCO); 10 µl 10 X Reverse transcriptase buffer buffer (250 mM Tris-HCl; 40 mM $MgCl_2$; 150 mM KCl; 5 mM dithioerythritol; pH 8.5, Roche Diagnostics); 1.0 µl LagNF and LagNR primers (40 pmol) and 1 µl AmpliTaq® (2U/µl, Applied Biosystems)] were added. The reaction mixture was then subjected to the following cycling conditions on a GeneAmp 9700 thermocycler (Applied Biosystems): Denaturation of 94˚C for 1 minute followed by 40

cycles of 94˚C for 30 seconds, 37˚C for 30 seconds and 72˚C for 90 seconds. A final extension step of 72˚C for 7 minutes was performed and the reaction was kept at 4˚C until ready to be removed. For nested PCR, 10 µl of the first round PCR product was used as template. The second amplification was performed as described above with the following modifications: 40 pmol of primers SALNF1 and SALNB2 (described in Section 4.2.4) were used. Aliquots (10 µl) of first round amplified products and nested PCR were analysed by agarose gel electrophoresis (2%). Gels were stained with 1 µg/ml ethidium bromide and photographed under UV light. A DNA molecular weight marker was also loaded to identify the size of the amplicons (DNA molecular weight Marker XIIV (Roche Diagnostics)).

4.2.6 Determining the threshold of viral and rRNA detection

The threshold of detection of the RT-PCR method was determined by preparing 10-fold dilutions (10^{-1} to 10^{-14}) of a mouse brain tissue suspension in TRIzol™ (Invitrogen) of the 1982 LBV isolate made from South Africa (LBVSA1982). The titre of the brain suspension was 10^7 50% mouse lethal dose (LD_{50}) per 1 ml. Total RNA extraction, cDNA synthesis, and the RT-PCR procedures were performed targeting both rRNA and LBV viral genomic RNA as described in Sections 4.2.2, 4.2.3 and 4.2.5. The necessary positive and negative controls were included.

4.2.7 PCR product purification

PCR amplicons generated in Section 4.2.5 were purified using the Wizard® PCR Preps DNA Purification System (Promega). PCR products were separated by agarose gel electrophoresis in low melting temperature agarose (2%). The correct size band was excised from the agarose gel using a sterile razor blade and transferred to a 1.5 ml microcentrifuge tube and incubated at 70˚C until the agarose has melted. 1 ml resin (Promega) was added to the melted agarose and mixed for 20 seconds. For each PCR product a Wizard® minicolumn was prepared by attaching a Syringe Barrel to the Luer-Lok® extension of each minicolumn and inserting this assembly into a vacuum manifold. The mixture was then added to the Syringe Barrel and a vacuum was applied. The DNA bounded to the column was washed with 2 ml of 80% isopropanol and the resin was dried by applying a vacuum for a further 30 seconds. The minicolumn was transferred to a 1.5 ml centrifuge tube and residual isopropanol was removed by centrifugation at 10 000 g for 2 minutes. DNA was eluted in a separate microcentrifuge tube by adding 50 µl of nuclease free water

(GIBCO) and centrifugation for 20 seconds at 10 000 g. The purified PCR product was analysed by agarose gel electrophoresis to determine the concentration and quality of the product. A DNA molecular weight marker was also loaded to identify the size of the amplicons (DNA molecular weight Marker XIIV (Roche Diagnostics)).

4.2.8 DNA sequencing

The purified PCR products were sequenced with the BigDye™ Termination Cycle Sequencing Ready Reaction Kit 1.1 (Applied Biosystems), according to the manufacturer's protocol. Briefly, 100 ng of purified PCR product (Section 4.2.7) was added to a reaction mixture containing 3.2 pmol forward primer; 4 µl BigDye™ Terminator Ready reaction mix V 1.1 (Applied Biosystems) and nuclease free water (GIBCO) to a final volume of 10 µl. The reaction was processed in a thermocycler (GeneAmp PCR 9700, Perkin Elmer) using a profile that consisted of an initial denaturation step at 94ºC for 1 minute, 25 cycles of 94ºC for 10 seconds; 50ºC for 5 seconds and 60ºC for 4 minutes. After the reaction was completed unincorporated ddNTPs were removed using the CENTRI-SEP columns (Princeton Separations) as described by the manufacturers. Briefly, the column gel was hydrated with nuclease free water (GIBCO) for 30 minutes at room temperature. Air bubbles were removed by inverting and tapping the column. Excess column fluid was allowed to drain into a 2 ml wash tube and fluid was discarded. Remaining fluid was removed by centrifugation at 750 g for 2 minutes. The DNA sequencing reaction was transferred to the top of the gel and the column was placed in a sample collection tube. The elute was collected by centrifugation at 750 g for 2 minutes. The sample was dried in a vacuum centrifuge (Eppindorf). DNA sequencing samples was analysed on an ABI 377 DNA sequencer (Applied Biosystems) after preparing a DNA sequencing gel following the manufacturer's instructions.

4.2.9 The fluorescent antibody test (FAT)

The FAT was performed on thin frozen sections from all bat tissues indicating a positive RT-PCR result. The standard operational procedure as indicated at www.cdc.gov/ncidod/dvrd/Rabies/Professional/Publications/DFA_diagnosis) was followed. Thin frozen sections were prepared in Tissue freeze medium (Triangle Biomedical), frozen on dry ice and sectioned in a cryostat-microtome at a thickness of 8 µm. At least 15 sections of each tissue were prepared. Control slides were prepared from known positive or negative brain material in the same way as described above. The slides were fixed in ice-cold acetone for 60 minutes and

allowed to air dry at room temperature. The tissue sections were then flooded with anti-rabies fluorescein isothiocyanate conjugate (Fujirebio Diagnostics, Inc) through a syringe with a 0.45 µm low protein binding filter. The slides were then incubated at 37℃ in a high humidity chamber for 30 minutes. The conjugate was drained from the slides and the slides washed three times for 5 minutes in PBS. The slides were blotted to remove excess PBS and briefly air dried at room temperature. The slides were subsequently mounted in wet mounting fluid (20% glycerol, Tris buffered saline pH 9.0) and cover slipped. The slides were examined under a fluorescence microscope and photographs captured with a digital camera at a magnification of 200X.

4.2.10 Virus isolation from bat tissues

Virus isolation using the mouse inoculation test was attempted from bat tissues testing positive with RT-PCR or the FAT. Three-week-old ICR mice were obtained from Harlan Sprague Daly (USA). Animals were housed and handled according to protocols approved by the Institutional Animal Care and Use Committee (IACUC) of the Centers for Disease Control and Prevention. For inoculation, tissue suspensions were prepared in Minimal essential medium (MEM-10; GIBCO) supplemented with 10% fetal bovine serum (GIBCO). Tissue was homogenized in a 2 ml sterile Dounce homogenizer after which the mixture was transferred to a microcentrifuge tube. The mixture was clarified by centrifugation at 3 200 g for 15 minutes. The supernatant (30 µl) was inoculated intracranially into three week old ICR mice with a 0.5cc tuberculin syringe and 8 mm, 31 gauge needle (Becton Dickinson and Company). Animals were monitored for up to 28 days after inoculation and either euthanized or collected upon death. Brain smears of brain removed aseptically from dead mice were analysed with FAT (Section 3.2.2.1) to indicate the presence of lyssavirus antigen in the mouse brains. Brain material that tested positive with the FAT was also analysed using RNA extraction, RT-PCR and DNA sequencing (Section 4.2.2, 4.2.5, 4.2.7 and 4.2.8) to confirm that the infectious agent was the same as the original virus and not a contaminant.

4.2.11 Determination of the presence of neutralizing antibodies

Blood collected from the positively identified LBV bats (LagSA2005 and LBVSA2006) was tested for the presence of neutralizing antibodies using the Rapid Fluorescent Focus Inhibition Test (RFFIT) as described by Smith *et al*, (1996) with slight modifications.

4.2.11.1 Preparation of LBV challenge virus

Challenge virus to detect LBV neutralizing antibodies using the RFFIT was prepared. Briefly, a complete monolayer of Mouse neuroblastoma cells (MNA) in a 75 cm^2 culture flask (Nalge Nunc International) was resuspended in a 50 ml conical centrifuge tube (~30 X 10^6 cells) in 2.7 ml MEM-10 (GIBCO) medium supplemented with 10% fetal bovine serum (GIBCO). A LBV isolate, isolated in Senegal in 1985*, was added to the cells and then incubated at 37ºC for 15 minutes. The cells were mixed once during this incubation time. 10 ml of MEM-10 (GIBCO) was added and the cells were collected by centrifugation at 500 g for 10 minutes. The supernatant was discarded and the cells were resuspended in 30 ml MEM-10 (GIBCO) and transferred to a 75 cm^2 culture flask (Nalge Nunc International). At the same time three 8 well Lab-Tek® chamber slides (Nalge Nunc International) were prepared by pipetting 0.2 ml of the cell suspension into each well. The flasks and slides were incubated at 37ºC in a humidified incubator with 0.5% CO_2. A slide was acetone fixed at 20, 40 and 64 hours and stained using immunofluorescence methods (Section 4.2.9) to determine the virus infectivity. The supernatant was harvested 24 hours after the cells reached 100% infectivity. The supernatant was transferred to a 50 ml centrifuge tube and centrifuged at 4 000 g for 10 minutes. The supernatant was collected and distributed in 0.5 ml aliquots and stored at -70ºC.

One aliquot of the stock suspension was thawed and serial 10 fold dilutions were prepared (10^{-1} to 10^{-10}). 0.1 ml of each dilution was added to the well of an 8 well Lab-Tek® chamber slide (Nalge Nunc International) together with 0.2 ml MNA cells resuspended in MEM-10 (GIBCO). The cells and virus were mixed and incubated at 37ºC in a humidified incubator with 0.5% CO_2 for 20 hours. The slide was acetone fixed and stained using immunofluorescence methods (Section 4.2.9). The focus forming dose (FFD_{50}) of the challenge virus was determined by counting the infected cells in 20 microscopic fields. One unit of virus for the RFFIT is determined by the dilution at which 50% of the observed microscopic fields contain one or more foci of infected cells. Stock suspension of the virus was then diluted to contain $50FFD_{50}$ for use in the RFFIT.

4.2.11.2 The Rapid Fluorescent Focus Inhibition Test (RFFIT)

The test was prepared in a 8 well Lab-Tek® chamber slide (Nalge Nunc International). The collected sera (Section 4.2.1) were heat inactivated at 56℃ for 30 minutes. Briefly, 100 µl MEM-10 (GIBCO) supplemented with 10% fetal bovine serum

The lineage A strain (LBVSEN1985) was chosen as a challenge virus before sequencing data was available indicating the high sequence diversity compared to other gt 2 isolates. A representative number of serum samples were retested for the presence of antibodies using the LagSA2004 isolate as a challenge virus in the RFFIT and similar results were obtained.

(GIBCO) was transferred into each well followed by the addition of 50 µl of serum which was added to the first well of each slide. 50 µl was serially carried over from the first well to the last well on the slide creating a 5 fold dilution of the serum. 100 µl of challenge virus preparation (Section 4.2.11.1) was then added to each well of the test. A control slide was prepared with 75 µl MEM-10 (GIBCO) in the first well of the slide and 100 µl in the remaining wells. The controls were prepared by setting up a back titration of the challenge virus in a 10 fold serial dilution. One well was left uninfected to serve as a cell culture control in the test. The dilutions were incubated at 37℃ and 0.5% CO_2 for 90 minutes. After incubation about 5.0 X 10^5 MNA cells per ml were added to the reactions and incubated again at 37℃ and 0.5% CO_2 for 20 hours. After the incubation the cell culture supernatants were decanted and the slides dip-rinsed in phosphate buffered saline (PBS) (13.7 mM NaCl, 0.27 mM KCl, 0.43 mM $Na_2HPO_4.2H_2O$, 0.14 mM KH_2PO_4, pH 7.3) and transferred to ice-cold acetone for 30 minutes. Slides were then washed three times in PBS and air dried, stained with 100 µl of rabies conjugate (Fujirebio Diagnostics Inc) and incubated at 37℃ for 30 minutes. Following the incubation the conjugate was washed from the wells with PBS. The slides were dip-rinsed in distilled water and air dried before reading. The slides were read at 160 to 200 times magnification under a fluorescent microscope and 20 microscope fields per well were observed

4.3 Results

4.3.1 Determination of the sensitivity of RT-PCR and nested PCR to detect LBV RNA

The threshold of RT-PCR detection of LBV genomic RNA was determined using mouse brain material. A control RT-PCR targeting rRNA was also included to assess the template quality of extracted RNA. The control (targeting rRNA) and LBV specific RT-PCR was performed in two separate reaction tubes but under identical conditions. For the control rRT-PCR amplification was achieved from RNA extracted from all dilutions down to 10^{-14} (Figure 4.2). LBV RNA could still be detected from all dilutions down to 10^{-10} with RT-PCR and with subsequent nested PCR down to 10^{-14} dilution. This indicates the threshold of detection of the RT-PCR to be at least 10^{-3} LD_{50}/ml for RT-PCR (Figure 4.3) and 10^{-7} LD_{50}/1 ml for nested PCR (Figure 4.4). Although not indicated in Fig 4.2, no amplicon were detected in the negative control.

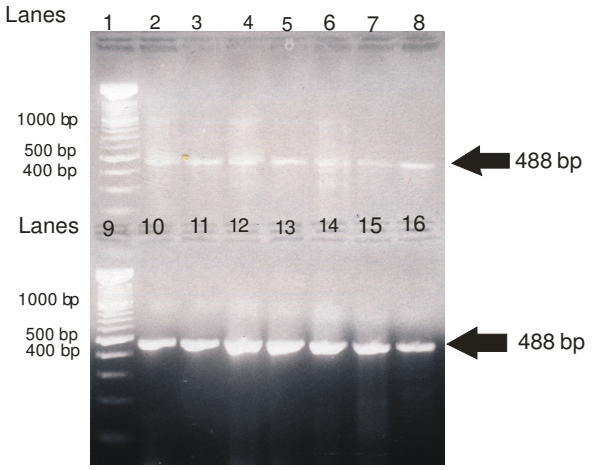


Figure 4.2: Result of agarose gel electrophoresis (2%) analysis of amplicons generated after performing rRT-PCR to assess the RNA template quality after RNA extraction from mouse brain material. Lane 1 and 9: DNA molecular weight Marker XIIV (Roche Diagnostics), Lane 2-8 and 10-16: rRT-PCR results performed using RNA extracted from 10^{-1} to 10^{-14} dilutions of mouse brain tissue. An amplicon of 488 bp was generated indicating that the RNA template quality was intact.

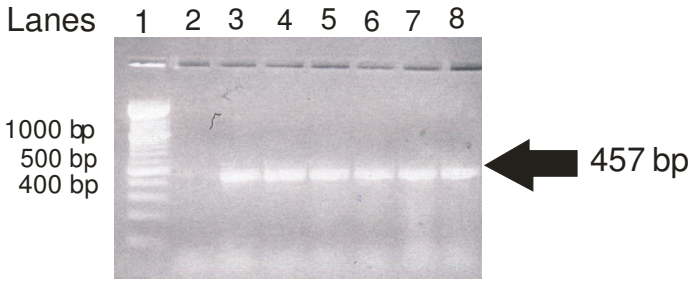


Figure 4.3: Result of agarose gel electrophoresis (2%) analysis of amplicons generated after performing RT-PCR specific for LBV RNA. Lane 1: DNA molecular weight Marker XIIV (Roche Diagnostics), Lane 2: Negative control, Lane 3-8: RT-PCR results performed using RNA extracted from 10^{-5} to 10^{-10} dilutions of brain tissue. An amplicon of 457 bp was generated in lane 3-8 indicating that the threshold for RNA detection is 10^{-3} LD_{50}/ml. No amplicon was observed in the negative control.

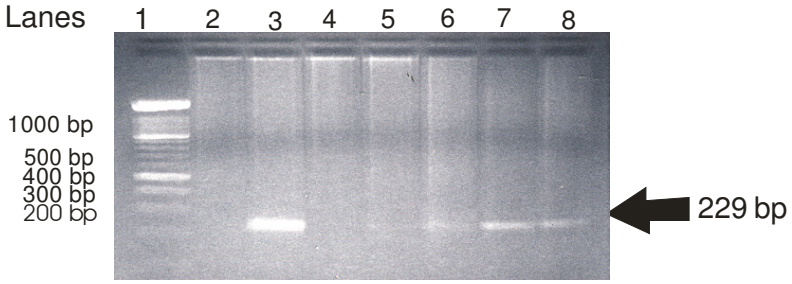


Figure 4.4: Result of agarose gel electrophoresis (2%) analysis of amplicons generated after performing a nested PCR targeting the LBV nucleoprotein. Lane 1: DNA molecular weight Marker XIIV (Roche Diagnostics), Lane 2: Negative control, Lane 3-8: Nested RT-PCR results performed using RNA extracted from 10^{-9} to 10^{-14} dilutions of brain tissue. An amplicon of 229 bp was generated in lane 3-8 indicating that the threshold for RNA detection is 10^{-7} LD_{50}/1 ml. No amplicon was observed in the negative control.

4.3.2 Detection of LBV RNA in naturally infected bat tissues

LBV RNA was detected (Figure 4.5 and 4.6) using RT-PCR and nested PCR in various tissues collected from frugivorous bats that were naturally infected with LBV (Table 4.3). A control PCR targeting the 18S rRNA to verify the integrity of the RNA template was also performed and an amplicon of 488 bp was obtained for all bat tissues analysed.

Table 4.3: A summary of results obtained with RT-PCR and nested PCR performed on various bat tissues collected from the LagSA2004, LagSA2005 and LBVSA2006 frugivorous bats.

	LAGSA2004		LAGSA2005		LBVSA2006	
	RT-PCR	Nested PCR	RT-PCR	Nested PCR	RT-PCR	Nested PCR
Brain	Yes	Yes	Yes	Yes	Yes	Yes
Salivary gland	No	Yes	No	No	No	Yes
Skin	NT	NT	No	No	No	Yes
Tongue	Yes	Yes	No	No	No	Yes
Esophagus	NT	NT	NT	NT	No	Yes
Lung	No	Yes	No	No	No	No
Stomach	NT	NT	No	No	No	No
Intestine	NT	NT	No	No	No	Yes
Heart	No	Yes	No	No	No	No
Diaphragm	NT	NT	No	No	No	No
Liver	No	No	No	no	No	No
Kidney	No	Yes	No	No	No	No
Adrenal	NT	NT	No	No	No	No
Bladder	NT	NT	No	No	No	Yes
Spleen	No	No	No	No	No	Yes

Yes (amplicon obtained); No (No amplicon obtained); NT (Not tested)

When analyzing the tissues collected from bat LagSA2004 for the presence of viral RNA, first round RT-PCR produced an amplicon from RNA extracted from brain and tongue whereas nested PCR also yielded a PCR amplicon from salivary gland, lung, heart and kidney (Result not indicated).

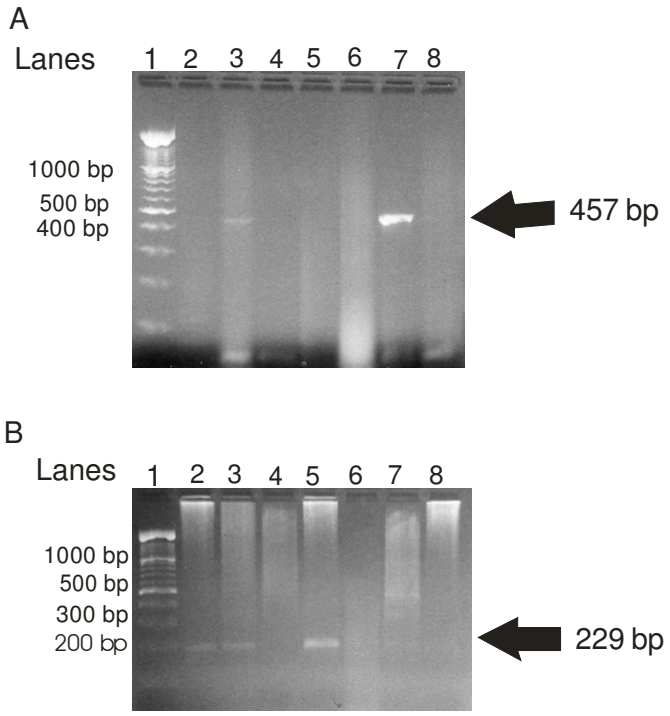


Figure 4.5: Agarose gel (2%) electrophoresis analysis of RT-PCR (A) and nested PCR (B) performed on tissue collected from bat LagSA2004.

A: Lane 1: DNA molecular weight Marker XIIV (Roche Diagnostics); Lane 2: Salivary gland; Lane 3: Tongue; Lane 4: Lung; Lane 5: Heart; Lane 6: Liver; Lane 7: Brain; Lane 8: Kidney. No band was observed in the negative control (data not shown)

B: Lane 1: DNA molecular weight Marker XIIV (Roche Diagnostics); Lane 2: Salivary gland; Lane 3: Tongue; Lane 4: Lung; Lane 5: Heart; Lane 6: Liver; Lane 7: Kidney; Lane 8: Spleen. No band was observed in the negative control (data not shown)

Bat LagSA2005 only yielded a PCR amplicon from brain material and none of the other tissues tested indicated the presence of viral RNA. Bat LBVSA2006 only yielded a first round RT-PCR product from RNA extracted from the brain but nested PCR was positive for salivary gland, skin, tongue, esophagus, intestine and bladder

(Figure 4.6). Heart, lung and kidney indicated no presence of viral RNA as were positively indicated for the bat LagSA2004. For bat LBVSA2006 saliva was also collected from the bat's oral cavity and nested PCR yielded an amplicon of the correct size (Data not shown).

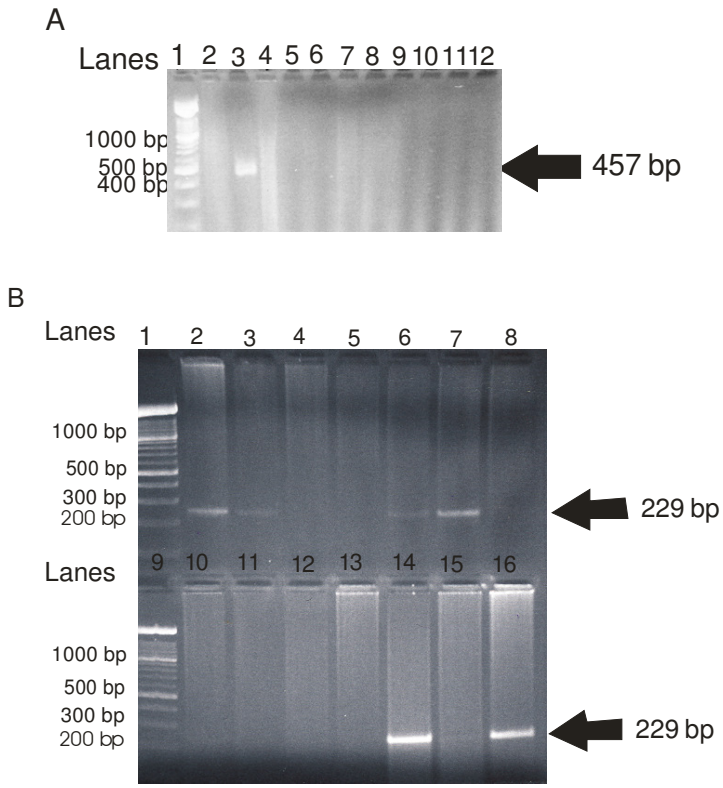


Figure 4.6: Agarose gel (2%) electrophoresis analysis of RT-PCR (A) and nested PCR (B) performed on tissue collected from bat LBVSA2006.

A: Lane 1: DNA molecular weight Marker XIIV (Roche Diagnostics); Lane 2: Salivary gland; Lane 3: Brain; Lane 4: Lung; Lane 5: Stomach; Lane 6: Skin Lane 7: Tongue Lane 8: Heart; Lane 10: Diaphragm; Lane 11: Liver; Lane 12: Kidney; Lane 13: Adrenal; Lane 14: Esophagus; Lane 15: Bladder; Lane 16: Spleen. No band was observed in the negative control (data not shown).

B: Lane 1 and 9: DNA molecular weight Marker XIIV (Roche Diagnostics); Lane 2: Brain; Lane 3: Salivary gland; Lane 4: Skin; Lane 5: Tongue; Lane 6: Esophagus; Lane 7: Lung; Lane 8: Stomach; Lane 9: Intestines; Lane 10: Heart; Lane 11: Diaphragm; Lane 12: Liver; Lane 13: Kidney; Lane 14: Adrenal; Lane 15: Bladder; Lane 16: Spleen. No band was observed in the negative control (data not shown).

DNA nucleotide sequences of the RT-PCR and nested PCR amplicons obtained from the bat tissues as indicated in Table 4.3 were determined. All amplicons obtained demonstrated a DNA sequence strictly similar to the sequence of the LagSA2004, LagSA2005 and LBVSA2006 respectively as described in Chapter III. This result was taken as confirmation of the the specificity of the products amplified from the various tissues collected from the bats.

4.3.3 Detection of LBV antigens and virus isolation from tissues collected from naturally LBV infected bats

The FAT was only performed on frozen sections prepared from bat tissues that were found to be positive by RT-PCR, the latter being more sensitive. Therefore only non-neuronal tissue samples of bat LagSA2004 and bat LBVSA2006 were analysed and none of the tissues of bat LagSA2005. All non-neuronal tissue analysed for bat LagSA2005 generated a negative result with RT-PCR. Virus isolation in 3-week-old ICR mice was only attempted on tissue samples indicating a positive FAT result. Table 4.4 provides a summary of results obtained with the FAT and subsequent virus isolation.

Table 4.4: A summary of results obtained with the FAT and virus isolation in 3-week-old ICR mice from selected tissue collected from bats, LagSA2004 and LBVSA2006.

	LAGSA2004		LBVSA2006	
	FAT	**Mouse inoculation***	**FAT**	**Mouse inoculation***
Brain	Yes	5/5	Yes	5/5
Salivary gland	Yes	0/5	Yes	1/5
Skin	NT	NT	Yes (Very faint)	0/5
Tongue	Yes	0/5	Yes	2/5
Esophagus	NT	NT	Yes	0/5
Lung	No	NT	No	NT
Intestine	NT	NT	No	NT
Heart	No	NT	No	NT
Liver	No	NT	No	NT
Kidney	No	NT	No	NT
Bladder	NT	NT	No	NT
Spleen	No	NT	No	NT

NT (Not tested)

*** Results are indicated as the amount of mice that died out of a total of 5 mice inoculated.**

The FAT results of the brain, salivary gland and tongue (Figure 4.7) collected from bat LagSA2004 were positive and brain, salivary gland, skin, tongue and esophagus (Figure 4.8) collected from bat LBVSA2006 were positive. Fluorescence observed in the skin of bat LBVSA2006 was very low and could not be clearly photographed.

Virus was only isolated from the brain of bat LagSA2004 and was successfully isolated from brain, salivary glands and an oral swab from bat LBVSA2006. Both FAT and subsequent RT-PCR sequencing performed on collected mouse brains confirmed the induction of rabies by the LagSA2004 and LBVSA2006 isolates in 3-week-old ICR mice.

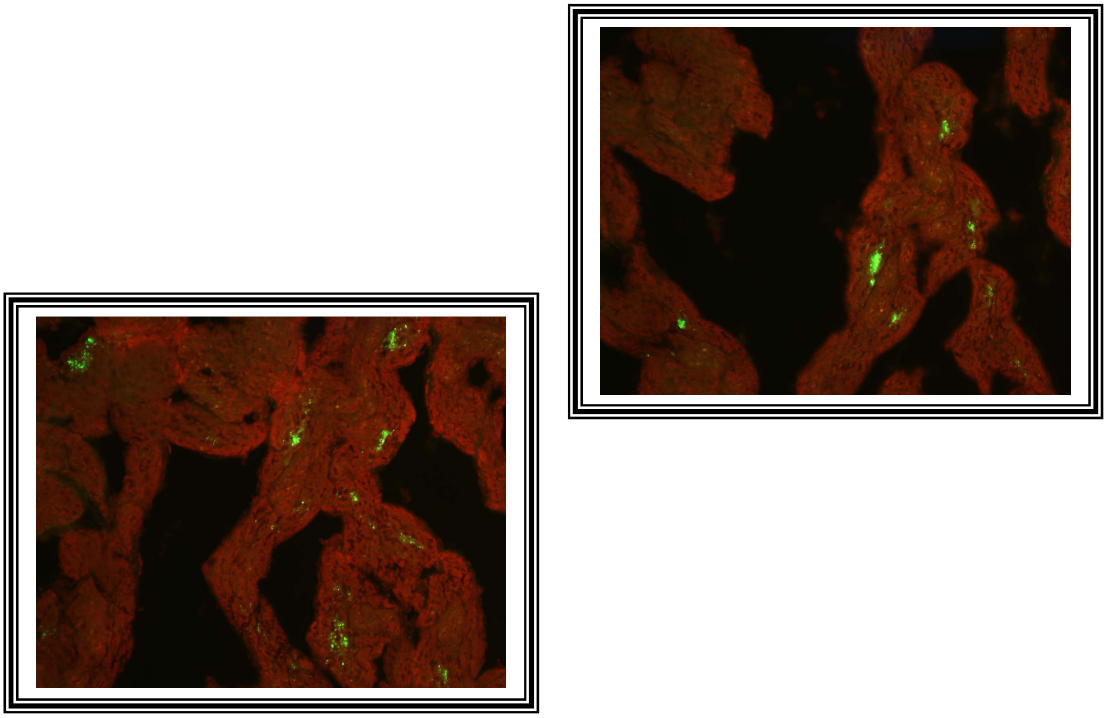
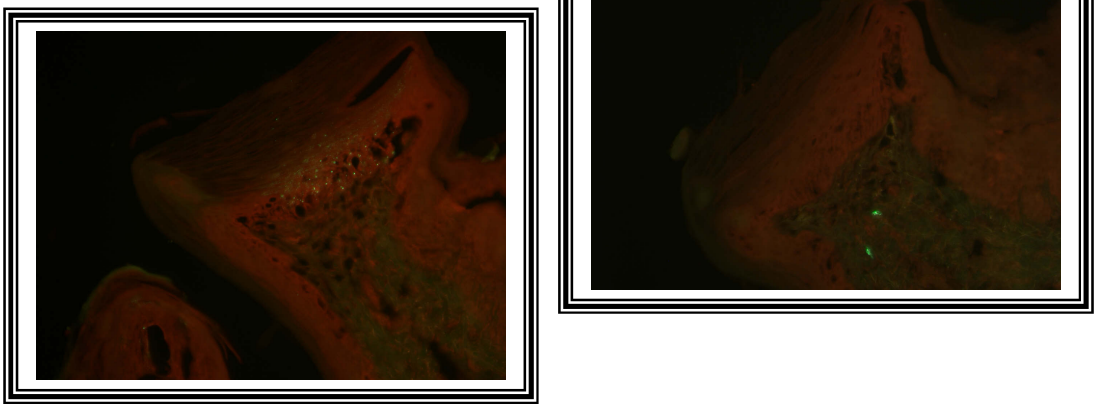
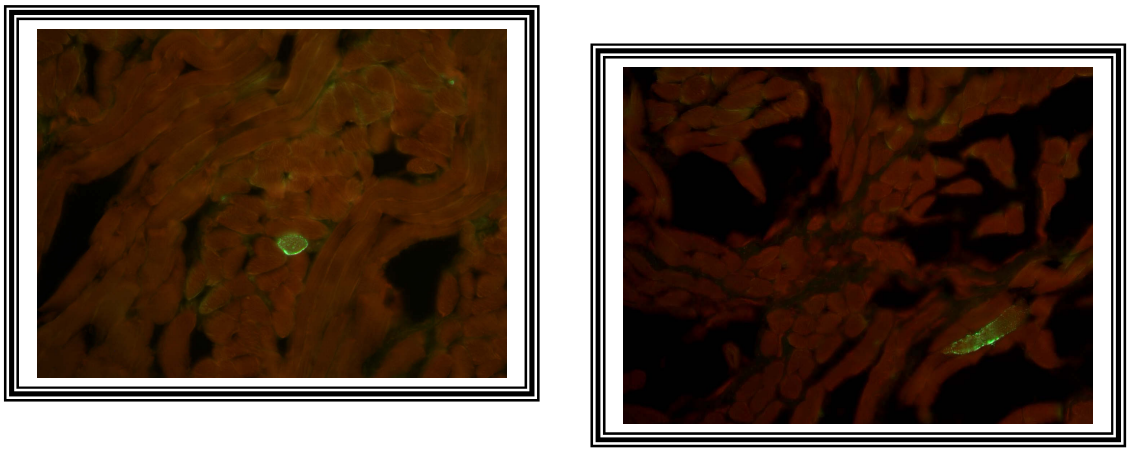


Figure 4.7: Thin frozen sections prepared of salivary glands collected from bat LagSA2004 after staining with FAT. [Photos taken with the assistance of Mike Niezgoda (CDC, Atlanta, USA)].

A



B



C

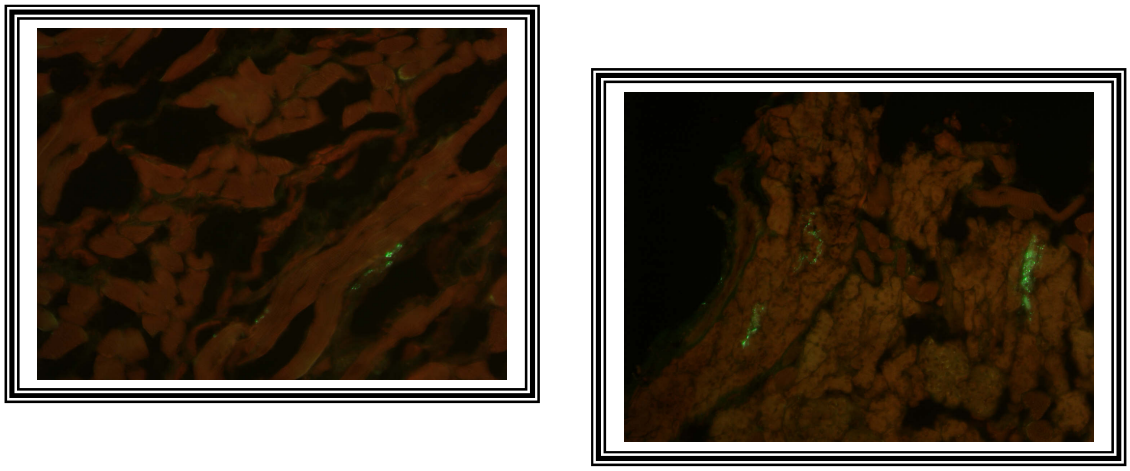
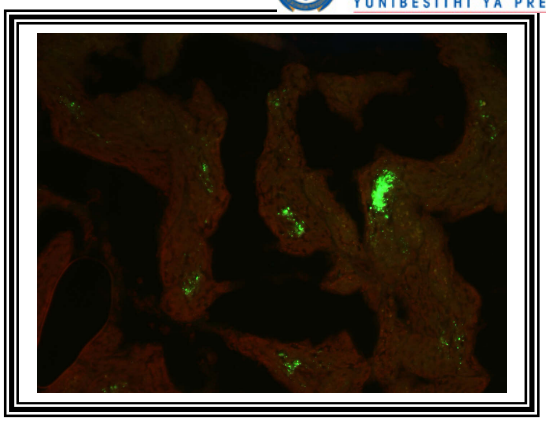
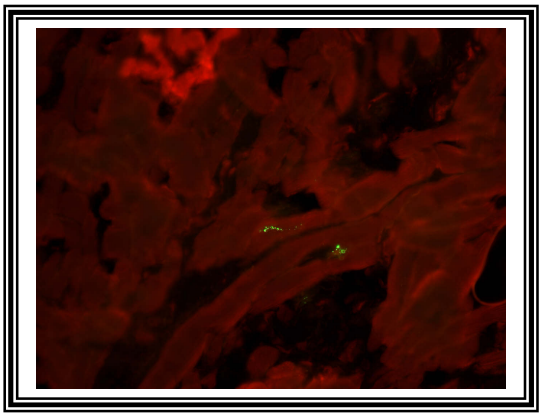


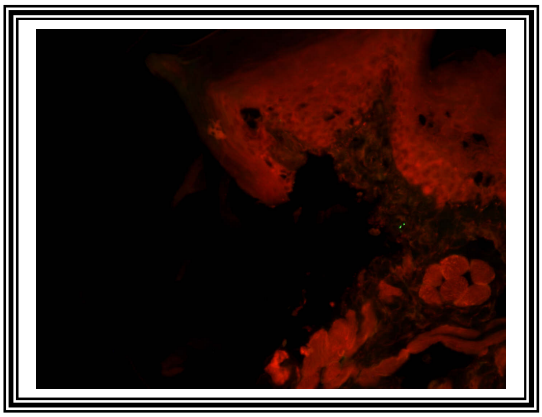
Figure 4.8: FAT results on different thin frozen sections prepared on tongue collected from bat LagSA2004. A: Fluorescence observed in the papilla; B Fluorescence observed in the epithelial cells and C: Fluorescence observed in the nerves [Photos taken with the assistance of Mike Niezgoda (CDC, Atlanta, USA)].

A



B





C

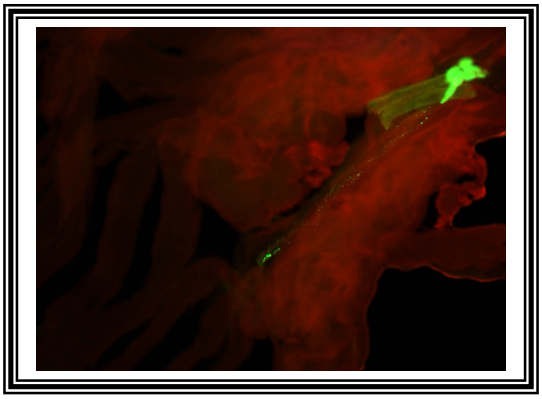


Figure 4.9: FAT results on thin frozen sections prepared on tissue collected from bat LBVSA2006. A: Salivary Gland; B: Tongue (Papilla and nerve tissue) and C: Esophagus. [Photos taken with the assistance of Mike Niezgoda (CDC, Atlanta, USA)].

4.3.4 The presence of neutralizing antibodies in naturally LBV-infected bats

Blood was collected from bats, LagSA2005 and LBVSA2006, and analysed for the presence of VNAs. RFFIT results indicated the presence of neutralizing antibodies only in the serum collected from bat LagSA2005. Neutralization was observed at a 1:10 dilution of the serum. Since no reference sera for LBV is available the virus neutralizing antibody titre could not be converted into international units. The current available international standard for rabies serology is based on gt 1.

4.4 Discussion

The only published study of the non-neuronal tissue distribution of LBV was performed by Murphy *et al*, (1973) in a hamster model (4 day old Syrian hamsters) with a LBV isolate from Nigeria (1956). These authors reported very similar results for gt 1 (RABV) and rabies-related lyssaviruses. Tissue of limb muscle, salivary glands, brown fat, heart, liver, kidney, spleen, thymus and pancreas were collected and titrated for the presence of virus by intracerebral inoculation of suckling mice. Detectable amounts of LBV were found in limb muscle, kidney, salivary glands and brain. In an experimental study (in shrews) of another African lyssavirus, MOKV, virus was recovered from brain and also from oral swabs, bladder, lung, adrenals, pancreas, salivary glands, kidney, heart, spleen and liver tissues (Kemp *et al.*, 1973). Virus titre was generally the highest in the brain although salivary glands also demonstrated high titres. The distribution of virus in tissues varied widely from shrew to shrew and no correlation between route of infection or virus dose was apparent. One shrew that succumbed to MOKV infection also demonstrated neutralizing antibodies. Presence of virus in salivary glands and tongue suggested that salivary glands and taste buds of the tongue could participate in producing infectious oral secretions.

In this study virus was only detected in brain material from bat LagSA2005 using RT-PCR and not in any other tissues. The FAT and virus isolation was negative even when performed on brain material. RT-PCR is a much more sensitive method than FAT and virus isolation and it is expected to detect very low amounts of viral RNA when other methods provided negative results. The RFFIT test performed on serum collected from this bat also indicated the presence of a low amount of neutralizing antibodies and can therefore explain why no virus could be isolated since it is very difficult to isolate virus in the presence of neutralizing antibodies due to neutralization

of the virus. In bats, LagSA2004 and LBVSA2006, virus was detected in several non-neuronal tissue and in both cases from tongue and salivary glands. Virus isolation was only obtained from brain material of bat LagSA2004 and not from any other tissue collected. This bat was repeatedly freezed and thawed, which may have resulted in inactivation of virus in various tissues. Viable virus was recovered from brain, salivary glands and tongue collected from bat LBVSA2006 and this bat was kept in a much better condition. Virus was also isolated from an oral swab indicating that virus was shed in the saliva at the time of death. This indicates the possibility of virus transmission through saliva. The low amount of viral RNA present in non-neuronal tissues underscores the need to use RT-PCR and nested PCR as a very sensitive technique for detection of viral RNA.

Little information on the pathogenesis of LBV exists and no previous studies in bats have been reported. Most LBV isolates in South Africa have been made from *E. wahlbergii* frugivorous bats and is seems that the virus persist in this species. There is no information about incubation period, non-neuronal tissue distribution, presence of virus in the saliva and immune responses in general upon LBV infection. There has been evidence that bats can tolerate EBLV infection (Ronsholt *et al*., 1998; Van der Poel *et al*., 2000) and clinically silent infections in bats with EBLV has been reported in *Rousettus aegyptiacus* (Ronsholt *et al*., 1998) and *Tadarida brasiliensis mexicana* (Steece *et al*., 1989; Baer and Smith, 1991). It has been shown that bats can be seropositive for lyssavirus neutralizing antibodies, indicating exposure to lyssaviruses and acquired herd immunity (Wellenberg *et al*., 2002; Niezgoda *et al*., 2002). The sensitivity of different species to lyssavirus infection probably varies and the risk of transmission to other species and the possibility that the virus can be maintained in the new species must also be assessed. Further studies are needed to determine the pathogenesis and infection dynamics of LBV in its reservoir host in South Africa (*E. wahlbergii*) and to enable us to make informed decisions about the risk of LBV infections for other species including humans, particularly in lieu of the absence of a vaccine for LBV.

Our study provides the first evidence for the distribution of LBV in non-neuronal tissues of naturally LBV infected frugivorous bats**.** It seems that LBV demonstrates a similar neurotropism than gt 1 where virus is disseminated from the brain to other non-neuronal sites. In all three *E. wahlbergii* frugivorous bats analysed in this study, virus was present in the highest amounts in the brain. No support for the presence of

low or non-productive infections was obtained where virus was present in other organs but not in the brain.