Public health awareness and seroprevalence of rabies in dogs in Limpopo National Park, and the phylogeny of rabies virus in Mozambique

By

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A thesis submitted to the Faculty of Veterinary Science, University of Pretoria, South Africa, in fulfilment of the requirements for the degree

Doctor of Philosophy Veterinary Science Veterinary Tropical Diseases

August 2021

DEDICATION

I dedicate this thesis to my wife (Eva Dora da Cruz João) and my little daughter (Maggui Mapatse), to my parents and siblings, by divine inspiration and encouraging words uttered during this time that I had to devote myself to the Doctoral program.

DECLARATION

I hereby declare that this thesis is a result of my own work. It is submitted in fulfilment of the degree, Doctor of Philosophy Veterinary Science Veterinary Tropical Diseases, in the University of Pretoria, South Africa. It has not been submitted before for any degree or examination in this or any other University.

Milton Francisco Mapatse

ACKNOWLEDGEMENTS

I would like to sincerely thank my supervisors, Professor José Manuel Fafetine, Dr Claude Taurai Sabeta and Professor Darrell Abernethy for their guidance, support, indispensable advices, teachings and positive criticism throughout this study. I will be eternally grateful to you all for this.

I am grateful to the directive board of the Biotechnology Centre, University Eduardo Mondlane, for having accepted me to be part of the project and Belgian Directorate General for Development Co-operation, for making it possible to carry out this project under the agreement (FA3-III DGD/ITM 2014-2016) benefiting the Department of Veterinary Tropical Diseases, Faculty of Veterinary Science, University of Pretoria.

I could not fail to thank the Directorate of Animal Sciences (DCA), Maputo, especially Dr Sara Achá for helping me to send samples to South Africa. My thanks are extended to the staff of the Virology Section, DCA, in particular to Dr Iolanda Anahory, to Dr Lourenço Mapaco, to Dr Virgínia and to Dr Lourenço for their help in the processing of samples and friendship.

I thank the laboratory staff of Rabies OIE-Rabies Reference Laboratory (Agricultural Research Centre-Onderstepoort Veterinary Institute), Pretoria, RSA, especially to Debra Mohale, Baby Phahladira and Dr Ernest Ngoepe, for the laboratory techniques' teaching, support and friendship during my stay in Pretoria. This thesis would not exist without you.

I am so grateful to Dr Guilhermina Sitoe (former Massingir District Health director) for allowing me to work at health post/centres of Massingir District and also to all the administrative staff at the Limpopo National Park, especially Dr Sérgio Bié and Ludovico Salinha for their support in the field and the friendship since the beginning of this research.

I can't fail to thank Dr Gracinda Carlos, director of the District Services for Economic Activities (SDAE) in Massingir and her staff, especially Dr Remígio Jojó, for having created all necessary conditions for the study to be carried out smoothly within the communities residing in the park.

I thank all the academic staff of University of Pretoria, especially the DVTD for the great support during the program. I really appreciate them for having welcomed me at UP campus and especially for the good times during my stay in Pretoria.

I would also like to thank the Directive board of the Veterinary Faculty of Eduardo Mondlane University, for allowing me to be part of the project.

Special thanks go to the communities of all the villages and to the health workers of all post and health centers in Massingir.

To my colleagues and friends, Abel Chilundo, Alberto Pondja, Iara Gomes, Vlademiro Magaia, Ofélia Nhambirre, Leonid Guiliche and Jimis Deve.

I thank all the people and institutions who directly or indirectly contributed for the success of this thesis. May God bless you all!

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LIST OF ABBREVIATIONS

ABC Animal Birth Control

ABLV *Australian bat lyssavirus*

AEC Streptavidin peroxidase-amino ethyl carbazole

ARAV *Aravan lyssavirus*

ARC Alliance for Rabies Control

ARES ASEAN Rabies Elimination Strategy

ASEAN Association of Southeast Asian Nations

B.C. before Christ

BBLV *Bokeloh bat lyssavirus*

BEI Binary ethyleneimine

BHK Baby Hamster Kidney

BPL β-propiolactone

CCEEV Cell culture or embryonated egg–based vaccine

CCV Cell Culture Vaccine

CDC Centers for Disease Control and Prevention

CME Clathrin-mediated endocytosis

CNS Central Nervous System

CRU Cellular Receptor Unit

CSF Cerebral spinal fluid

CVL Central Veterinary Laboratory

CVS Challenge virus standard

DFAT Direct fluorescent antibody test

DNA Deoxyribonucleic acid

DRC Democratic Republic of Congo

DUVV *Duvenhage lyssavirus*

EBVL *European bat lyssavirus*

ELISA Enzyme-linked immunosorbent assay

FAT Fluorescent antibody test

FITC Fluorescein isothiocyanate

FAVNT Fluorescent antibody virus neutralization test

GARC Global Alliance for Rabies Control

GBLV *Gannoruwa bat lyssavirus*

IFN Interferon

IKOV *Ikoma Lyssavirus*

IRKV *Irkut lyssavirus*

IU International Unit

KBLV *Kotalahti bat lyssavirus*

KHUV *Khujand lyssavirus*

LAC Latin America and the Caribbean

LBV *Lagos bat virus*

LLEBV *Lleida bat Lyssavirus*

LNP Limpopo National Park

MADER Ministry of Agriculture and Rural Development

MASA Ministry of Agriculture and Food Safety

MCMC Markov chain Monte Carlo

MEGA Molecular Evolutionary Genetics Analysis

MIT Mouse Inoculation test

MLV Modified Live Virus

MOKV *Mokola virus*

MRCA Most Recent Common Ancestor

MRI Magnetic Ressonance Imaging

NCAM Neural cell adhesion molecule

OIE World Animal Health Organization

ORF Open Reading Frame

PARACON Pan-African Rabies Control Network

PCECV Purified chick embryo cell vaccine

PEP Post-exposure prophylaxis

PHE Public Health England

PNISA National Agricultural Investment Plan

PrEP Pre-Exposure Prophylaxis

PrP Partners for Rabies Prevention

PVRV Purified Vero Cell Vaccine

RABV *Rabies lyssavirus*

RFFIT Rapid Fluorescent Focus Inhibition Test

RIG Rabies Immunoglobulin

RIT Rapid Immunohistochemical Test

RIWA Rabies in West Africa Group

RNA Ribonucleic acid

RNP Ribonucleoprotein

RSA Republic of South Africa

RT-PCR Reverse transcription polymerase chain reaction

RT-qPCR quantitative Reverse transcription polymerase chain reaction

SAD Street-Alabama-Dufferin

SARE Stepwise Approach to Rabies Elimination

SEARG Southern and Eastern African Rabies Group

SHIBV *Shimoni bat Lyssavirus*

STAT Signal transducers and activator of transcription

TWBLV *Taiwan bat lyssavirus*

UK United Kingdom

UPGMA Unweighted Pair Group Method with Arithmetic Means

USA The United States of America

VNA Virus-neutralizing antibodies

WCBV *West Caucasian bat Lyssavirus*

WHO World Health Organization

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THESIS SUMMARY

Public health awareness and seroprevalence of rabies in dogs in

Limpopo National Park, and the phylogeny of rabies virus in Mozambique

by

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Degree: **Doctor of Philosophy Veterinary Science Veterinary Tropical Diseases**

Rabies is a fatal and neglected viral disease representing one of the major public and veterinary health concerns in developing countries, particularly in Africa. The prototype causative agent (*Rabies lyssavirus* (RABV)) belongs to the genus *Lyssavirus*, family *Rhabdoviridae*. RABV cycles are sustained mainly by domestic dogs, responsible for most animal and human rabies cases. In Mozambique, dog-transmitted rabies has been acknowledged as a significant public health threat since 1908. This project sought to assess the level of knowledge, attitudes and practices with regard to rabies in remote areas of Limpopo National Park (LNP) in Massingir district and to understand the molecular epidemiology of the disease not only in that region, but in Mozambique in general. A cross-sectional study was conducted between 2016 and 2018 in 233 households residing in eight LNP villages and among 42 health practitioners from eight health facilities who were selected

using snowballing and purposive procedures, respectively, in Massingir district. In summary, 18.9% (44/233) and 13.3% (31/233) of the households had good knowledge and practices of rabies, respectively. For health practitioners, only 16.7% (7/42) had good knowledge, whilst 33.3% (14/42) adopted adequate attitudes/practices towards the disease. In conclusion, both households and health practitioners displayed poor levels of knowledge and adopted bad attitudes and practices towards rabies. A lack of community-based education and professional retraining courses were determining factors contributing for the poor awareness of rabies. This study therefore provides an opportunity to enhance public health knowledge and reduce dog-mediated human rabies deaths. To assess the level of circulating rabies antibodies in dogs from LNP, canine sera samples were tested using a BioPro® Rabies ELISA Antibody kit. Among the 418 surveyed dogs, (89.2%; n=373) were negative for rabies antibodies (PB < 40%, 95% CI: 85.77 - 91.96) and only 6.5% (n=27; PB ≥ 70%, 95% CI: 4.4 - 9.4) had positively adequate circulating rabies antibodies levels. These findings confirm the low coverage of parenteral mass vaccination campaigns indicating high susceptibility of these dog populations to rabies virus infection. Molecular tools including real-time reverse transcription polymerase chain reaction testing and nucleotide sequencing are useful for lyssavirus identification. To enhance our understanding of rabies molecular epidemiology in Mozambique, eight rabies viruses (RABV) collected in domestic dogs (*n*=7) and a cat (*n*=1) in Maputo, Gaza, Nampula and Sofala provinces of Mozambique between 2017 and 2018 were characterized by both reverse transcription polymerase chain reaction (RT-PCR) and quantitative real-time PCR (RT-qPCR). Five (62.5%) were positive by RT-PCR. However, lyssavirus RNA was detected in all eight (100%) samples using the most sensitive RT-qPCR. The amplicons representing partial regions of nucleoprotein and glycoprotein genes were sequenced using Sanger dideoxy chain termination. Phylogenetic trees generated from nucleotide sequences of both genes and constructed using Molecular Evolutionary Genetics Analysis (MEGA X) software, had similar topologies. The viruses clustered with other canid viruses of the Africa 1b lineage of rabies viruses originating from dogs from neighbouring South Africa, Tanzania and Zimbabwe. Further analysis demonstrated that the RABVs from Mozambique clustered with other dog rabies viruses from Zimbabwe and South Africa. It can be concluded that rabies virus circulating in Mozambique has a common progenitor, highlighting the important role of the domestic dog in rabies cycles and the transboundary nature of rabies. Rabies surveillance, laboratory diagnosis capacity and sample submission are still inadequate. The implementation of rabies control and prevention strategies that take

into consideration low level of community awareness to rabies and the inadequate level of rabies antibodies in dogs in villages in and around the LNP should be considered.

Key words: Rabies, Awareness, Lyssavirus, Epidemiology, Dogs, KAPs, Canine, Mozambique, Limpopo National Park, Serology

Chapter 1 : General Introduction

1.1 Background

Rabies is a neglected tropical disease affecting all warm-blooded animals (Woldehiwet 2002). It causes and acute myelo-encephalitis which is fatal to most mammalian species including humans (Lafon 2008; Crowcroft and Thampi 2015). The causative agent of the disease {Latin: *rabere* (to rage)} is classical rabies virus (RABV) and is responsible for the vast majority of human rabies cases (Knobel et al. 2005; Nel and Markotter 2007; Hampson et al. 2015). The bullet-shaped RABV belongs to the family *Rhabdoviridae*, order *Mononegavirales*, genus *Lyssavirus* (Greek: lyssa, meaning "frenzy"), (Leung et al. 2007; Nel and Markotter 2007; Walker et al. 2018). RABV has a non-segmented, single-strand and negative-sense RNA genome of approximately 12 kb in length (Tordo et al. 1986; Zhou et al. 2013), comprising five genes that encode the following proteins: nucleoprotein (N), phosphoprotein (P), matrix protein (M), glycoprotein (G), and RNA-dependent RNA polymerase (L) (Luo et al. 2012).

Seventeen distinct *lyssavirus* species (previously referred to as genotypes) can be distinguished within the genus (ICTV 2019). These species are segregated into three phylogroups, based on their genetic relatedness (Rupprecht et al. 2017). Phylogroup I consists of classical *Rabies lyssavirus* (RABV), *Duvenhage lyssavirus* (DUVV), *European bat type 1 lyssavirus* (EBLV-1), *European bat type 2 lyssavirus* (EBLV-2), *Australian bat lyssavirus*(ABLV)*, Khujand lyssavirus* (KHUV), *Aravan lyssavirus* (ARAV), *Irkut lyssavirus* (IRKV), *Bokeloh bat lyssavirus* (BBLV), *Gannoruwa bat lyssavirus* (GBLV), *Taiwan bat lyssavirus* (TWBLV) (Rupprecht et al. 2017). Phylogroup II consists of *Lagos bat virus* (LBV), *Mokola virus* (MOKV) and *Shimoni bat lyssavirus* SHIBV) (Rupprecht et al. 2017; Shipley et al. 2019). Phylogroup III includes *West Caucasian bat lyssavirus* (WCBV), *Ikoma lyssavirus* (IKOV) and *Lleida bat lyssavirus* (LLEBV) (Aréchiga et al. 2013; Nokireki et al. 2013; Fooks et al. 2019). Recently, one related and recently discovered lyssavirus *Kotalahti bat lyssavirus* (KBLV) (tentatively Phylogroup I), was isolated from bats (Nokireki et al. 2018; Hu et al. 2018; ICTV 2019).

Of all the lyssaviruses known to date, RABV is the most important one from a public and veterinary health perspective, and it has the highest case fatality rate (99.9%) of all infectious diseases known worldwide (Nel et al. 2009). Rabies virus is capable of infecting a wide variety of warm-blooded wild and domestic animals, including humans

(Von Teichman et al. 1995; Banyard et al. 2013). Rabies is present in more than 150 countries and territories of all continents except the Antarctica (Woldehiwet 2002). Although the correct figures on human deaths due to rabies are unknown, the studies have estimated 59,000 deaths annually (Hampson et al. 2015; Wilde et al. 2016; WHO 2018). In developing countries RABV accounts for almost all reported humans deaths (>97%), with the tropical and subtropical regions in Africa and Asia being the most affected (Swanepoel et al. 1993; Leung et al. 2007; Banyard et al. 2013; Chiou et al. 2014; Singh et al. 2014).

In Southern African countries including Mozambique, rabies virus infects many animal host species, but the canid rabies cycles are maintained mainly by domestic dogs (Bingham et al. 1999), the carnivore host responsible for most human cases (Dias et al. 1985). However, there have also been documented cases of humans bitten by the wildlife hosts such as the bat-eared foxes (Swanepoel et al. 1993; Bingham et al. 1999; Banyard et al. 2013; WHO and Global Alliance for Rabies Control 2014).

Despite implementation of the National Strategy for the Control of Rabies initiative (Government of Mozambique 2010), in Mozambique, in a retrospective study of rabies over a 29-year period (1989 to 2017), 1,001 human rabies cases were documented, with the Province of Maputo contributing more than 25% of the total cases (n=274; Bilaide 2019). It is recognized that reported animal rabies cases are highly underestimated, especially in rural areas where the animal health rabies surveillance is almost non-existent (Nel 2013; WHO 2013; Salomao et al. 2017). Most healthcare workers seem to have poor knowledge of rabies and its symptoms (WHO and Global Alliance for Rabies Control 2014), leading to under-reporting, both in animals and humans, in part because it may easily be misdiagnosed with other fatal encephalitis, such as cerebral malaria (Mallewa et al. 2007).

In a 2003 country report, it was revealed that 73.8% (n=240) of all dog bites of people in Maputo, were by owned dogs, suggesting that at some point during the day dogs are allowed to roam freely by most of their owners (Dias and Rodrigues 2003). Although rabies is a preventable disease, throughout Mozambique, the number of humans exposed to suspected rabid animals is high (Dias and Rodrigues 2003). Constraints for an effective rabies control in the country include the very low parenteral dog (and cat) vaccination coverage, due to limited availability of resources and funds for the implementation of parenteral vaccination in the field (Ministry of Agriculture and Food Security 2014), insufficient veterinary field staff and inefficient or non-existent disease notification procedures (Dias and Rodrigues 2003; WHO 2013). Although the total population of dogs and cats is unknown, these species are the most affected and play a very important role in maintaining rabies in urban areas and therefore it is very difficult to assess vaccination coverage.

The spread of rabies virus from infected dogs to wildlife is reported to occur in canine rabies-endemic areas (Stuchin et al. 2018). The opposite has also been suggested to occur, although further studies on the genetic and antigenic characterisation of the rabies viruses are needed to prove inter-species transmission, mainly between clusters of different species of bats, and clusters of foxes and domestic carnivores (Hemachuda et al. 2007; Yung et al. 2012; Wallace et al. 2014; Singh et al. 2017; Polupan et al. 2019). In rural areas, there is evidence and documented reports of people being attacked by rabid wild mammals such as African wild dogs, bat-eared fox, jackals, mongoose and hyenas (Government of Mozambique 2010; WHO 2013).

However, in Mozambique, effective surveillance of rabies in wildlife does not exist and consequently, there have been only speculation of the occurrence of this disease in those animal populations, with no confirmed cases (WHO and Global Alliance for Rabies Control 2014). There is a big gap in national rabies reports, especially on those referring to rural areas, which has prompted concerns that the epidemiology of the disease may be more complex, with a possible involvement of wildlife carnivores that may also sustain rabies infection cycles (Dias 1992; Stuchin et al. 2018).

Domestic dogs are the primary source of the disease in many African countries and therefore vaccination of this host species is the starting point towards primary rabies prevention in humans (Crowcroft and Thampi 2015), even in the remote and poorest regions in developing countries (Lembo et al. 2010). In Mozambique and according to national policy, rabies outbreaks should be followed by vaccination of the adjacent canine populations (WHO 2013), since the reporting of animal rabies cases is mainly assigned on the dog. However, as in other African countries (Lembo et al. 2010), in rural areas of Mozambique there is not only uncertainty about exact demographic data on the dog population, but also about the level of vaccination coverage required for rabies control in this species. Furthermore, according to Perry (1992) in southern and eastern African countries the different status of dog owners or keepers has also diversified, making the task of dog populations' vaccination a much more complicated one.

Social change, such as urbanization of Mozambican conservation areas, resulted in an uncontrolled movement and violation of national parks boundaries by illegal entry of domestic animals, including dogs and cats, with the likelihood of introduction of diseases such as rabies. An increase in dog and human movement as well as conflict between humans and wildlife (Massé 2016) is believed to enhance rabies persistence in such a dog metapopulation, especially if there are reports of circulation of infected dogs (Bingham 2005).

In fact, dogs whether feral, owned or residing in the neighbourhood, are considered here because they can to some extent interact with people for food and shelter, and also could be pivotal in hunting and be house guards (Wandeler et al. 1993; Jackman and Rowan 2007). In this context, one cannot rule out also the possibility of rabid animals wandering in these protected areas, a suggestion put forward by Thomson and Meredith (1993).

Intrusions of rabid dogs from Mozambique into the Kruger National Park is a situation that has been reported (Bishop et al. 2003), and this phenomenon can threaten the animal and human lives of the Greater Limpopo Transfrontier Park. During a meeting of the Southern and East Africa Rabies Group (SEARG), held in Maputo (Mozambique) in 2011, the protection of conservation areas through vaccination of dogs in neighbouring communities was highlighted.

Although the domestic dogs are considered as the main species responsible for sustaining the animal rabies cycle, in Mozambique, little is known about their ecology. Better understanding of dog structure, dynamics, confinement status and its resources (food, water and shelter) especially in the context of Limpopo National Park's Wildlife-Livestock-Human interface environment and culture, is of extreme importance for predicting the spread of rabies infections and designing the strategies for its control (Perry 1993; Bourhy et al. 2010).

Rabies control in Mozambique or regions where it is ineffective has traditionally been driven by the government veterinary authorities, but with limited collaboration of a one-health approach. This has been due to a lack of communication between workers responsible for human and animal health, including physicians and veterinarians, albeit knowingly that success relies on concerted multi-disciplinary partnerships and a national reference laboratory to support diagnosis and surveillance (Lembo et al. 2010; Crowcroft and Thampi 2015).

Such partnerships should include awareness programs such as educating the general public about the importance of immediate wound cleansing after a bite contact, rapid consultation at the dog-bite centre, if there is any, and compliance with vaccination schedules (Dodet 2007).

Children who are the main victims of rabies should be educated on how and where to report and receive treatment after potential exposure to the virus. There is a need for proper and continuous training of personnel and for a sustained availability of good quality biological for post-exposure prophylaxis. For instance, human rabies immunoglobulin (HRIG) is often not administered because of its perceived cost or unavailability, resulting in many human deaths (Hemachuda et al. 2007; Lembo et al. 2010).

In areas where HRIG is not available, equine rabies immunoglobulin (ERIG) has equally provided the necessary protection albeit that there have been some adverse reaction recorded (Wilde et al. 1989; Wilde and Chutivongse 1990).

In Africa, the cost of post-exposure prophylaxis (PEP) is extremely expensive and amounts to more than 10% of annual per capital gross national income (Nel et al. 2009). For example, in the Republic of South Africa, full PEP treatment with vaccine and immunoglobulin G exceeds USD 152 per individual (Nel et al. 2009). In Mozambique, human rabies vaccine for PEP is reported to be available at least in Maputo city and in 2010, 40.4% of the people bitten by dogs received PEP (WHO and Global Alliance for Rabies Control 2014).

On the other hand, pre-exposure prophylaxis (PrEP) is recommended by the World Health Organization for people at high risk of exposure such as those working in rabies diagnostic or research laboratories, veterinarians, animal handlers, animal rehabilitators and wildlife officers and veterinary students (WHO 2005). With good, organized multidisciplinary teams against rabies, coupled with an accurate database on the burden of rabies in Mozambique, it will be easier for the country to receive financial and other type of support from various entities such as the Global Alliance for Rabies Control (GARC), Partners for Rabies Prevention (PrP), the Bill and Melinda Gates Foundation and WHO, organizations which promote systematic PrEP and also PEP of children living in regions where canine rabies is highly endemic, since they have the highest risk of severe rabies exposure.

1.2 Thesis rationale

The motivation for the work described in this thesis is driven by the scarcity of data on the epidemiological situation of rabies in conservation areas in Mozambique. This project seeks to fill that gap. Hence, this project will add value for being the first of its kind to be carried out in a protected area such as the Limpopo National Park (LNP), it may contribute to help the key stakeholders in the design and implementation of the best rabies control and prevention strategies, based on the results obtained in respect of the levels of knowledge, attitudes and practices regarding rabies among the rural communities of the LNP, and the virus variants circulating in the park and in Mozambique in general.

The gaps such as lack data on awareness, on community perceptions and practices, on dog serological status, on molecular epidemiology of rabies variants are expected to be filled by assessing the dynamics of the canine population, its ecology, responsible pet ownership, including knowledge of rabies prevention methods within the population yet to be resettled, and health practitioners working in remote area, and finally by laboratory sequencing of rabies positive samples.

Vaccination campaigns against rabies do not take place regularly in the Massingir District in general, let alone in LNP in particular. However, priority is generally being given to cattle vaccination against Foot-and-Mouth-Disease and chickens against Newcastle disease. With this in mind, this study is intended to demonstrate that the data on the anti-rabies vaccination and immunization coverage rates are non-existent or grossly inadequate, which may call for a review of the control measures and epidemiological surveillance of rabies in dogs.

The constant movement of people and their animals into and out of that conservation area could be a factor affecting these actions. The district veterinary services of

Massingir do not have a laboratory for the diagnosis of rabies, therefore all suspected cases are sent for rabies diagnosis to the Central Veterinary Laboratory (CVL) in Maputo. Given the distance between Massingir and the CVL, specimens are usually delayed and the delay in receipt of results interferes with the proper actions and good decision-makings for rabies control in animals and management of human bite cases.

The results obtained here will be of added value to sensitize the government on the importance of installing a local laboratory for the diagnosis of this important zoonotic disease. The lack of decentralized rabies diagnostic laboratories is a major obstacle to the quick response required for suspected cases of rabies. For instance, Mozambique, a country with 784,090 square kilometres has only one central veterinary laboratory and only three regional laboratories in the provinces of Gaza, Manica, and Nampula. These last three laboratories can perform the Seller's stain diagnostic technique, a test demonstrated to lack adequate sensitivity (CDC 2018).

The correct definition of rabies cases by health professionals is very important, especially if we take into account that this disease is often misdiagnosed with other tropical diseases presenting with encephalitis as observed in Malawi (Mallewa et al. 2007; Mudiyanselage et al. 2016). Through surveys administered to healthcare practitioners, it is also possible to raise their awareness for the need for collection and systematization of data on cases of animal bites and human rabies cases, procedures that currently do not exist in LNP health centres.

Moreover, it is common practice in many African countries (Dodet et al. 2008), that people always rely on local healers when they get sick or when bitten by (potentially rabid) animals, which can further complicate the work of district health practitioners in regard to the management of human rabies cases. People then visit the medical centres when the disease has progressed to a level where it is difficult to manage. Thus, it is envisaged that work of this thesis will raise the awareness of the local population about the attitudes and good practices regarding the bite wounds management especially at home level which may help to minimize the possible negative impacts of rabies in the park.

1.2.1 Overarching research question

Compared to what is observed in the bordering and neighbouring Kruger National Park, rabies research in LNP is poor and challenging due to poor infrastructure, dispersed communities and uncertainty around local commitment. The lack of rabies information in communities in the park has prompted the following research question: What do the population and health professionals in the LNP know about rabies and how can this baseline knowledge be used to support the design and implementation of disease control strategies in the human population, domestic animals and wildlife in the region?

1.3 Study objectives

The main objectives of this research were:

1.3.1 General objective

- To better understand the public health awareness and canine seroprevalence of rabies in Limpopo National Park (LNP) and, molecular epidemiology of rabies virus in Mozambique

1.3.2 Specific objectives

- To assess the Knowledge, Attitudes and Practices regarding rabies among LNP households,
- To assess the Knowledge, Attitudes and Practices towards rabies among general health practitioners at Health Centres and Posts in Massingir District,
- To detect and evaluate the levels of rabies antibodies in the dog population in LNP,
- To detect and characterize molecularly rabies viruses from domestic dogs and cat in Mozambique.

Chapter 2 : Literature review

2.1 Historical perspective of rabies

Rabies is considered to be one of the oldest zoonotic and most-feared diseases known since ancient times. The word *"Rabies"* is derived from the Latin word "rabere" ("to rave") or "rabhas" ("to do violence") according to Sanskrit etymology (Nel and Markotter 2007). It was first recorded in the 23rd century before Christ (B.C.) (Table 2.1) when Mosaic Eshnunna code of Babylon demanded a financial penalty from the owner of any vicious animal that bit a person: "…if a dog is mad and the authorities have brought the fact to the knowledge of its owner; if he does not keep it in, and it bites a man and causes his death, then the owner shall pay two thirds of a mina (40 shekels) of silver…" (Baer 2007; Kumar 2009). It was also described in the sixth century B.C. and in the Avesta (Persia), 4th century B.C. by Aristotle (ancient Greece; Baer 2007).

There were two main ways of interpreting the causes of the disease. The Egyptians, Hebrews and other people of different regions thought it to be religious in nature and as early as 500 B.C., there were even special rabies gods: one to prevent rabies (Aristaeus, son of Apollo and Cyrene) and one to heal rabies (Artemis; (Schneider and Santos-Burgoa 1994). The other interpretation was developed by the Chinese and Hindus in which the disease was conceived as an imbalance of the constituents of the human organism (Babboni and Modolo 2011).

The Greek word for rabies, "*lyssa"*, "*lussa*", "*lutta*" or "*lytta*" derives from the root "lud" which means "madness". Thus, the genus of viruses to which rabies belongs is lyssa. Since the first century it was believed that dogs were more likely to become rabid when a lunar eclipse occurred at year's end (Yuhong 2001). Since ancient times (4th Century B.C.), some attempts to describe the disease have been reported (Blancou 1994; King et al. 2004), but in the 1st Century B.C., Asclepiades was reputed to have given the first adequate description of rabies (Adamson 1977; Tarantola 2017). Another widespread myth of the cause of rabies described in ancient times (1st century B.C.) by Pliny and Ovid was that the disease was transmitted by a worm lodged under the tongue of dogs (Huang et al. 2015). Accordingly, the fraenulum of the tongue of the dogs was cut and the piece where it was thought to be present removed (King et al. 2004; Babboni and Modolo 2011).

In 1804, Zinke, a Germany scientist recognised the infectious nature of saliva from infected dogs (Pearce 2002; Rupprecht et al. 2002). No effective preventive or curative treatment in animals was available before Pasteur's discovery in 1885 (Pearce 2002). Before that time, various remedies were attempted, often with increasingly arcane methods including herbal remedies and cauterization of wounds caused by rabid animals (Neville 2004).

Other revealed that the bite from a rabid dog, when untreated, ordinarily produced the horror of water, which the Greeks called "hydrophobia". It was terrible in which the patient was both plagued by thirst and fear of water (Debbie 1988; Babboni and Modolo 2011). The only remedy one could try was to throw the rabid person into a pool. If he did not know how to swim one should let him sink so that he swallowed some water, then he was taken out in order to be plunged in again. This saturation of water was to suppress the thirst (Baer 2007).

Louis Pasteur, a Parisian chemist, successfully used a crude rabies vaccine for the very first time, in 1885. He infected rabbits with a virulent fixed strain of the virus, harvested their spinal cords and then attenuated the virus in these by desiccation (Berche 2012). He finally decided to test his vaccine on a human, to save the life of young Joseph Meister, presenting multiple dog bite wounds (Bourhy et al. 2010). Paul Remlinger demonstrated in 1903 the filterability of the rabies virus and Negri described inclusion corpuscles in nerve cells of rabid dogs (later called Negri corpuscles) (Cortez 2006).

Table 2.2.9.1.1:1: Chronological history of rabies virus

Year	Scientist	Discovery (reference)
Twenty-third century B.C.	–	Vicious dogs referenced in the Laws (A iv 20-23) of Eshnunna in Mesopotamia (Goetze 1951)
Seventh century B.C.	–	In the Persian Avesta, the sacred book of Zoroastrianism, canine rabies was described (Iran) (Rosner 1974)
Fourth century B.C.	Aristotles	Canine rabies was described in Greece by Aristotles as a transmissible disease to other animals, with the exception of man (Wilkinson 1977)
18th-19th Century	–	Animal and human rabies in southern Africa documented by colonialist powers (Tarantola 2017)
1804	Zinke	Demonstrated the infectivity of the saliva inoculating it in healthy dogs (Wilkinson 1977)
1881	Louis Pasteur	The neurotropic nature of rabies virus has been demonstrated (Baer et al. 1965)
1885	Louis Pasteur	First live, attenuated rabies vaccine developed from infected rabbits' spinal cord was inoculated to a schoolboy Joseph Meister (Tarantola 2017)
1903	Remlinger	The nature of rabies virus has been suggested by demonstrating its filterability (Remlinger 1903; Wilkinson 1977)
1903	Adelchi Negri	Intracytoplasmic inclusion bodies called "Negri bodies" have been detected (Frothingham 1906)
1908	-	Animal rabies referenced as an endemic disease in Mozambique (Dias et al. 1985)
1940s	–	Red foxes (*Vulpes vulpes*) implicated in the epidemiology of fox-mediated rabies across the Kaliningrad region and its spread to Central and Western Europe (King et al. 2004)

1946	–	First mass rabies vaccination campaign of dogs in Japan (Shimada 1971)
1950	Francisco Valadão	First laboratory confirmation of rabies in Tete Province, Mozambique (Dias et al. 1985)
1978	–	Field oral rabies vaccination campaign in foxes was conducted for the first time in Switzerland and afterwards in Germany, France and other European countries (Stöhr and Meslin 1996)
1994	–	First rabies virus infection generated from cloned cDNA (Schnell et al. 1994)
2004	–	Report of first human survivor of rabies without immunisation in the USA (Willoughby Jr et al. 2005)
2007	–	USA declared free of terrestrial canine rabies (CDC 2007)
2015	WHO, OIE, GARC, FAO	Endorsed global campaign to end human deaths due to dog-mediated rabies by 2030 (Abela-Ridder et al. 2016)

2.2 Rabies virus and other lyssaviruses genome organization

2.2.1 Structure and composition

The aetiological agents of rabies are enveloped, bullet-shaped viruses of 180 nm long and 75 nm wide in size, containing a 12 kilobases of single-strand, negative sense, non-segmented ribonucleic acid (RNA) genome (Figure 2.1) (Chenik et al. 1998). Like other *Rhabdoviruses*, lyssaviruses consists of 2-3% of RNA, 67-74% of protein, 20-26% of lipid and 3% of carbohydrate as integral components of their structure (Wunner and Conzelmann 2013).

Rabies virus genome comprises five different structural genes in the order 3'-5'and codes for five different proteins (Table 2.2), namely: a large RNA-dependent RNA polymerase or large protein (L), nucleoprotein (N), phosphoprotein (P), matrix protein (M), and a surface glycoprotein (G) (Singh et al. 2017) in the order 3'-N-P-M-G-L-5' (Singathia et al. 2012). In general, with the exception of some isolates of *Irkut lyssavirus*, the order of conservation level of the lyssaviruses' genes is as follows: N>L>M>G>P (Murphy et al. 1999; Liu et al. 2013). The antigenic glycoprotein G is responsible for activation of helper T and cytotoxic T lymphocytes and for the production of viral-neutralizing antibodies (VNAs) induced by the B cells (Dietzschold et al. 2008; Wunner and Conzelmann 2013), during infection process with lyssaviruses, while the N, P, L proteins and negative-strand genomic RNA (ribonucleoprotein complex) are involved in the induction of cellular immunity required to augment VNA production as well as to establish immunologic memory and long-lasting immunity (Dietzschold et al. 2008; WHO 2017).

The structure of the ribonucleoprotein (RNP) complex, which are structural proteins of the virion, are essential for mRNA synthesis, viral replication virus and spread inside the cells (Marschalek 2010; WHO 2017; Guo et al. 2019).

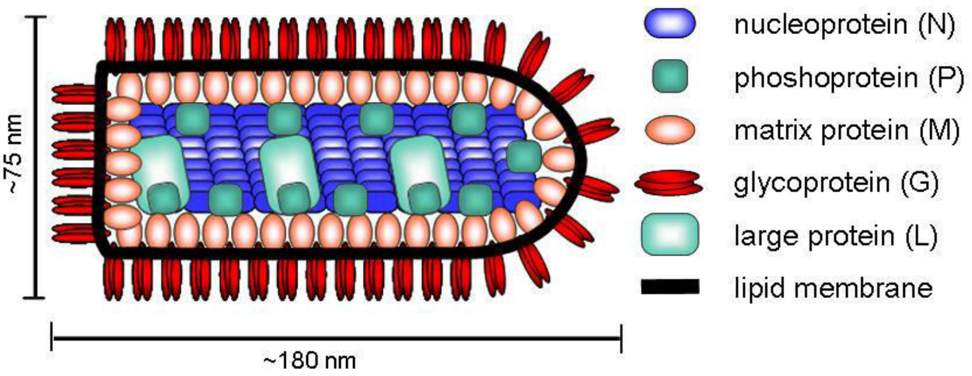

Figure 2.1: Virus structure. The Matrix protein (M) covers the inside of viral membrane while the glycoprotein (G) forms trimeric spikes which are embedded into the membrane Adapted from Marschalek (2010).

The five genes are separated by four non-coding intergenic sequences (from the 5′ end of one gene to the 3′ start of the next gene), and are made up of one dinucleotide (N-P gene border), two pentanucleotide (P-M and M-G gene borders) and one long 423-

nucleotide sequence (G-L), which are unique to lyssaviruses (de Novaes Oliveira et al. 2010; Wunner and Conzelmann 2013).

From the *lyssavirus* genome, short non-coding sequences (58 and 70 nucleotides) at the 3′ and 5′ ends called the "leader" (Le) and "trailer" (Tr) sequences, respectively, flank the structural genes in the strictly conserved order 3′-N-P-M—G-L-5′ (Figure 2.2) (Wu et al. 2007; Wunner and Conzelmann 2013).

Leader RNA is the first viral product made in rabies virus infected cells during infection (Yang et al. 1999) and is also observed in several members of the *Rhabdoviridae* family including Indiana vesiculovirus (Zhang et al. 2017). It is believed that during transcription the LeRNA (57-58 ribonucleotides) carries the promoter of encapsidation near 5′-end and cleaves it from the distal messenger RNA (mRNA) transcripts (Meslin et al. 1996; Tordo 1996). LeRNAs of RABV are also capable of activating dendritic cells (DCs) and its level determines the status of DCs activation (Yang et al. 2015; Zhang et al. 2017). The long intergenic region between the G and L genes (Figure 2.2) is called a remnant gene or pseudogene (Ψ) in recognition of a sequence of suitable length but lacking an open reading frame (ORF) to code for a detectable protein (Tordo et al. 1986).

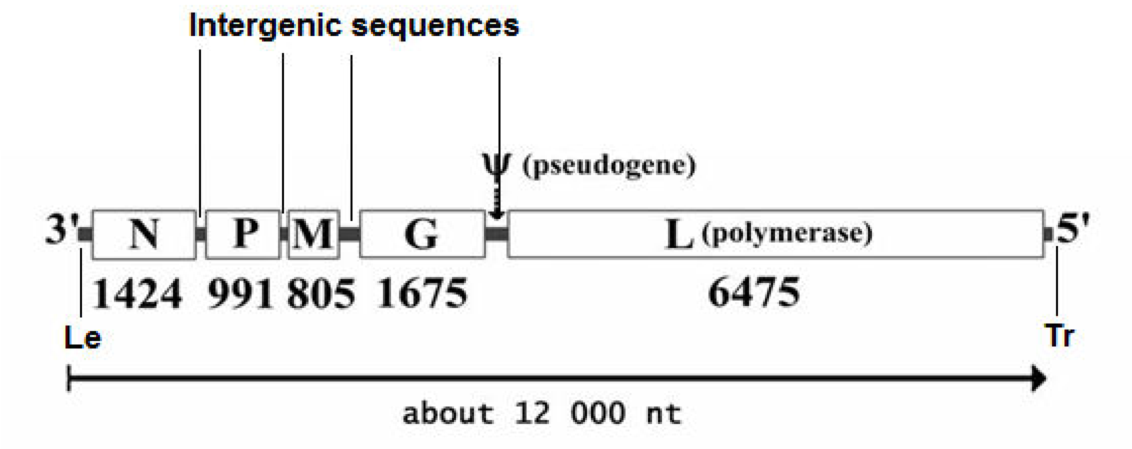

Figure 2.2: Schematic illustration of the structure of the rabies virus genome. The length of the respective genes (nucleotides) is indicated in each gene**.** Adapted from Metlin (2008).

Table 2.2.9.1.1:1: Characteristics and biological functions of the rabies virus constituent genes. Adapted from (Tordo et al. 1986; Wunner et al. 1988; Metlin 2008; Wunner and Conzelmann 2013; Singh et al. 2017)

Gene	Characteristics	Functions
Nucleoprotein (N)	The major structural component of the internal viral nucleocapsid (NC); 450 amino acids in length; Contains several antigenic and immunodominant sites with one of the immunodominant sites located at position 404-418.	Acts as a template for replication and transcription by the viral polymerase complex. It encapsidates and protects the positive-strand leader RNA by forming the nucleocapsid (NC).; Prevents further transcription of the genomic RNA
Phosphoprotein (P)	297 amino acids in length; Forms NC that wraps around the viral RNA along with the N and L proteins; Encodes more than one protein i.e., P1, P2, P3, P4, and P5; Has two antigenic sites located at positions 75-90; The domain 83-172 contain the major antigenic determinants.	Participates in the process of transcription and replication along with the L protein and the nucleoprotein to support viral RNA encapsidation; Important determinant in retrograde transport of the virus within axons; Antagonize the type I interferon (IFN)-mediated antiviral responses and suppresses activation of interferon regulatory factor 3; Binds to the transcriptional factors signal transducers and activator of transcription 1 (STAT1) and STAT2.
The matrix	The smallest virion protein;	Interacts with the transmembrane

protein (M)	202 amino acid polypeptide; It covers the ribonucleoprotein (RNP) coil to keep it in a condensed form; Interacts specifically with the glycoprotein; The major antigenic site is located between the amino acid residues 1 and 72.	spikes of G protein; Viral assembly, maturation and budding; Stimulating factor for viral replication and transcription; An inhibiting factor of cellular RNA transcription.
Glycoprotein (G)	524 amino acid polypeptide; Protein with a trimeric structure; Contains at least 8 antigenic sites and epitopes.	Acts as immune-protective and neutralising antigen; Binds the target cell receptors; Enhances the entry and trans-synaptic spread of virus from the peripheral.
Large RNA-dependent RNA polymerase (L)	The largest protein of the rabies virus; 2142 amino acids in length.	Plays role in pathogenesis along with G gene by means of transcription, making 5 individual mRNAs, one for each viral protein.
G-L intergenic sequence	Large non-coding region of 423 nucleotides; most variable region of the genome;	Useful in phylogenetic studies to discriminate between closely related RABVs;

2.3 Classification of *Lyssavirus* genus

The aetiological microorganism that cause rabies belong to the Order *Mononegavirales*, Family *Rhabdoviridae*, Genus *Lyssavirus*, a group of bullet shaped viruses consisting of 18 genera and 134 species genetically and immunopathologically distinct, based on nucleotide sequence and phylogenetic analyses (Rupprecht et al. 2017). The family is ecologically diverse with members infecting animals (mammals, birds, reptiles or fish) and plants (Walker et al. 2018). The *Lyssavirus* genus is distributed worldwide, except the Antarctica and several isolated islands (Table 2.3).

According to The International Committee on Taxonomy of Viruses (ICTV), the genus *Lyssavirus* is composed of 17 species of lyssaviruses, plus one unclassified related virus (*Kotalahti bat lyssavirus*) isolated from bats (Hu et al. 2018; Nokireki et al. 2018; Leopardi et al. 2019; ICTV 2019) (Table 2.3).

Rabies virus (RABV) the causative agent of classical rabies in animals and humans is the most important member of the genus as it has a global distribution. Besides RABV, viruses belonging to all other known lyssavirus species can cause acute and progressive fatal encephalitis in humans (Dacheux et al. 2011; Rupprecht et al. 2017).

2.3.1 *Lyssavirus* phylogroups

According to genetic distances, phylogenetic topology and consistency obtained with various evolutionary models, serologic cross-reactivity and other additional characteristics (ecological properties, host and geographic range, pathological features) the genus *Lyssavirus* can be subdivided into phylogroup 1 (RABV, DUVV, EBLV-1, EBLV-2, ABLV, ARAV, KHUV, IRKV, BBLV, and GBLV) and phylogroup 2 (LBV, MOKV, and SHIBV) (Fooks et al. 2019). The remaining species, WCBV, IKOV, and LLEBV, fall outside of these two defined phylogroups (Amarasinghe et al. 2018).

2.4 RABV clades

RABVs can be grouped into seven major clades (Figure 2.3) designated according to their geographical distribution and they are estimated to have emerged within the last 1,500 years (Bourhy et al. 2008).

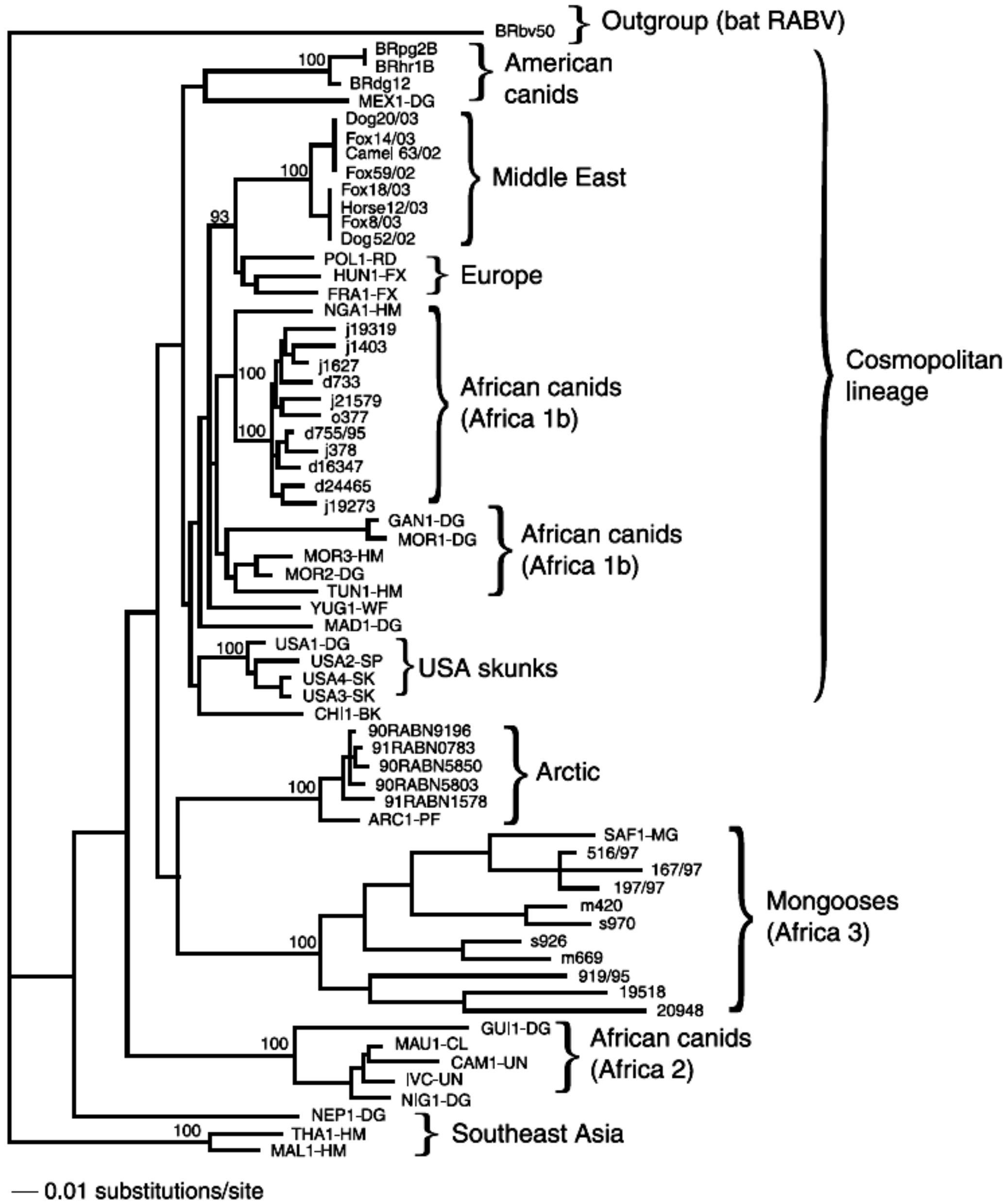

Figure 2.3: **Phylogenetic tree, illustrating some RABVs clades (Davis et al. 2007).**

2.4.1 Indian clade

The highly divergent Indian clade (Nanayakkara et al. 2003) are found in southern India and Sri Lanka (Meng et al. 2011). In the phylogenetic tree of all rabies viruses associated with terrestrial mammals, it has the most basal position, suggesting that it may have been the progenitor for all RABV lineages harboured by terrestrial mammals (Bourhy et al. 2008). Dogs are considered as main reservoirs of RABV in nature (Nanayakkara et al. 2003).

2.4.2 Asian clade

The large heterogeneous Asia clade, estimated variously to have emerged from a most recent common ancestor (MRCA) around 1412 or 1654 (Gong et al. 2010; Ming et al. 2010). It is widely distributed across the greater parts of Asia. The clade can be subdivided into several regional subgroups, identified in various regions of China (Zhang et al. 2006; Meng et al. 2007; Zhang et al. 2009b). The dog is the principal reservoir host across the range of this lineage. Phylogenetic studies are consistent with past spread of RABV variants from China to other Asian countries and island nations such as Indonesia and the Philippines during periods of extensive human and animals migration from China (Bourhy et al. 2008; Gong et al. 2010; Nadin-Davis and Real 2011).

2.4.3 Cosmopolitan clade

This clade has been distributed worldwide, mainly throughout human-assisted movement of pets from Europe during colonialism around 1870 (Bourhy et al. 2008; Nadin-Davis and Real 2011). It includes viruses circulating among terrestrial animals not only in Europe, but also in the Arabic Peninsula, Iran, Kazakhstan, Russia, America and the genetic group previously referred to as Africa 1 clade (Troupin et al. 2016). The latter has a wide geographical distribution and consists of subclade 1a and 1b (Bourhy et al. 1993; Kissi et al. 1995), with the former circulating mainly in Northern and Western Africa, while the latter circulates mainly in Southern and Eastern Africa (Kissi et al. 1995; Sadeuh-Mba et al. 2017).

2.4.4 Africa 2 clade

Viruses from the Africa 2 clade are distributed in West and Central Africa, with the dog being the reservoir host (Kissi et al. 1995; Bourhy et al. 2008).

2.4.5 Africa 3 clade

The Africa 3 clade comprises viruses well adapted to the wild carnivores of the family *Herpestidae* (mainly the yellow mongoose), and although there is confirmation of their circulation in herpestes in South Africa, Botswana and Namibia, groups of this lineage (mongoose biotype) can also be found in other wild species such as the slender mongoose (*Galerella sanguinea*) and the African civet (*Civettictis civetta*) in Zimbabwe in an independent epidemiological cycle (Johnson et al. 2004; Nel et al. 2005; Mansfield et al. 2006b; Sabeta et al. 2008; Van Zyl 2010; Sabeta et al. 2020).

2.4.6 Artic-related clade

Arctic-related or Arctic-like lyssaviruses include viruses circulating across the Northern hemisphere, ranging from central to eastern Asia as well as Greenland and North America (Mansfield et al. 2006a; Nadin-Davis and Real 2011). Phylogenetic analysis suggests that Indian isolates fall into a basal position, thus suggesting that the entire clade evolved from Indian dog viruses, with subsequent differentiation into genetic sub-clades according to the geographical location (Kuzmin et al. 2008a; Nadin-Davis and Real 2011).

•

Table 2.2.9.1.1:1: Lyssaviruses species and geographic distribution of their important reservoir species (WHO 2017; Hu et al. 2018; Nokireki et al. 2018; ICTV 2019)

Virus	**Abbreviation**	**Potential vectors/reservoirs**	**Distribution**
Aravan Lyssavirus	ARAV	Insectivorous bats *(Myotis blythi)*	Central Asia
Australian bat lyssavirus	ABLV	Frugivorous/insectivorous (?) bats *(Megachiroptera/Microchiroptera)*	Australia
Bokeloh bat lyssavirus	BBLV	Insectivorous bats	Europe
Duvenhage Lyssavirus	DUVV	Insectivorous bats	S. Africa
European bat lyssavirus 1	EBLV-1	Insectivorous bats *(Eptesicus serotinus)*	Europe
European bat lyssavirus 2	EBLV-2	Insectivorous bats *(Myotis daubentonii, M. dasycneme)*	Europe
Ikoma Lyssavirus	IKOV	*Civettictis civetta*	Africa
Khujand Lyssavirus	KHUV	Insectivorous bats	Central Asia
Lagos bat lyssavirus	LBV	Frugivorous bats (*Megachiroptera)*	Africa
Mokola Lyssavirus	MOKV	Unknown	Africa
Rabies Lyssavirus	RABV	Carnivores worldwide, and bats (Americas)	Worldwide
Shimoni bat Lyssavirus	SHIBV	Insectivorous bats *(Microchiroptera; Hipposideros commersoni)*	East Africa
West Caucasian bat Lyssavirus	WCBV	Insectivorous bats	Caucasian region
Irkut lyssavirus	IRKV	Insectivorous bats *(Murina leucogaster)*	East Siberia
Gannoruwa bat lyssavirus	GBLV	Frugivorous bats *(Pteropus giganteus)*	Asia
Lleida bat Lyssavirus	LLEBV	Insectivorous bats *(Miniopterus schreibersi)*	Europe (Spain)
Taiwan bat lyssavirus	TWBLV	Insectivorous bats *(Pipistrellus abramus)*	Asia (Taiwan)
Kotalahti bat lyssavirus	KBLV	Brandt's bat *(Myotis brandtii)*	Europe (Finland)

2.5 RABV life cycle

The sequence of events in the rabies virus replication cycle *in vivo* and *in vitro*, can be divided into three phases as shown in figure 2.4.

The first phase starts with the virus attaching to surface receptors on susceptible host cells, entry into the cell by direct fusion of the virus externally to either the plasma or internally to endosomal membrane, uncoating of the virus particles, and subsequently release of the helical RNP into the cell cytoplasm (Wunner and Conzelmann 2013). The second phase involves the transcription of mRNA and viral protein synthesis, the replication of the viral genome (Wunner and Conzelmann 2013). Finally, after the assembly of progeny virions, release (budding) of the completed particles from the surface of the infected cell into the surrounding cellular environment takes places at the plasma cell of the host cell (Wunner and Conzelmann 2013), typical of many virus infection cycles.

2.5.1 RABV transcription, translation and replication mechanisms

The steps for RABV replication begin with a series of events from the moment the virion particles binds to a susceptible host cell surface receptor molecules, a process that is mediated by interactions of glycoprotein G with cellular receptors that to date include the nicotinic acetylcholine receptor (nAChR) found at neuromuscular junctions (Tuffereau et al. 1998; Lafon 2005). Other receptors which the RABV interacts with include the neural cell adhesion molecule (NCAM or CD56), the low-affinity nerve growth factor receptor (p75NTR), metabotropic glutamate 2 receptor (mGluR2), carbohydrates, and gangliosides (Thoulouze et al. 1998; Wunner and Conzelmann 2013). After receptor binding, the virus enters the cell via the low pH (pH 6.3–6.5) dependent endocytic pathway (Albertini et al. 2011; Davis et al. 2015; Maclachlan and Dubovi 2017).

The endocytic pathway that mediates fusion of the viral envelope with the cellular membrane includes clathrin-mediated endocytosis (CME) and caveolae-dependent endocytosis (CavME) leading to the entry of the virion into the cell (Albertini et al. 2011; Xu et al. 2015). The transcription process is initiated immediately after the release of the helical negative-sense viral RNP into the cytoplasm of an infected cell (uncoating) (Wunner and Conzelmann 2013; CDC 2019). The vRNA transcription and replication is initiated at the 3′ end of the genomic ribonucleoprotein (leader sequence) and involves sequential production of monocistronic mRNAs by the antigenic RNA polymerase (the L–P polymerase complex) (Finke and Conzelmann 2005).

During transcription phase, the L-P polymerase complex enters the vRNA by recognizing a specific promoter at the 3′-end of the genome and progress toward the 5′ -end by a stop–start mechanism, producing six consecutive transcripts: first, the uncapped and non-polyadenylated positive leader RNA and then the five successive capped and polyadenylated mRNAs coding for the N, P, M, G, and L proteins in a gradient manner (Albertini et al. 2008; Albertini et al. 2012).

Translation occurs on free ribosomes in the cytoplasm (CDC 2019). Later, the viral L polymerase switches from transcription to replication of the RNA genome and starts producing a full-length positive-sense antigenome that subsequently serves as a template for the synthesis of new viral RNA genomes (Leyrat et al. 2011; Albertini et al. 2012). Replication requires the continuous production of soluble N and M proteins necessary to encapsidate both antigenomes and the newly synthesized RNA by N protein (Finke and Conzelmann 2005; Albertini et al. 2012).

These positive-stranded RNAs are also encapsidated by the N protein, bind the L–P complex, and serve as templates for synthesis or amplification of full-length negative strands of the viral genome (Albertini et al. 2012; CDC 2019). In the late phase and once sufficient pool of viral N, L and NS (P) proteins and RNA genomes have accumulated in the infected cell, ribonucleoprotein complexes (i.e. the core nucleocapsids) form and virus particles are assembled near the inner surface of the plasma membrane through the action of the matrix protein (M) protein, from which mature virions are released after budding (Wunner and Conzelmann 2013; Davis et al. 2015).

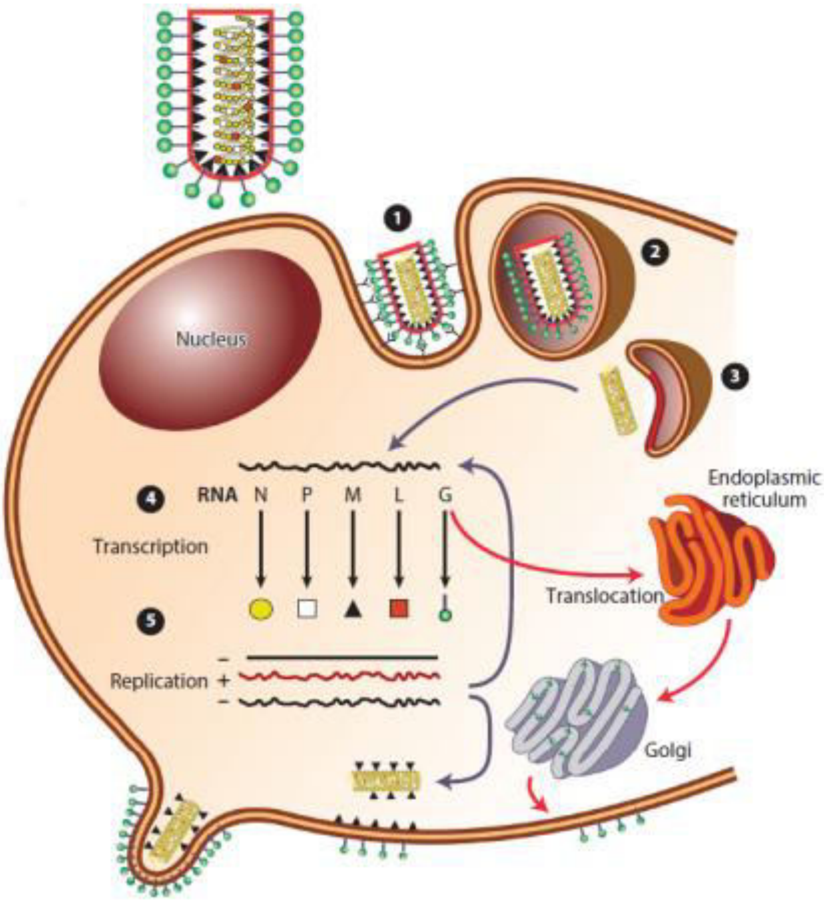

Figure 2.4: Rabies virus life cycle.

RABV particle attaches to a host cell receptor and its entry is via endocytosis (step 1) and the enveloped viral particles are engulfed by the host cell membrane (step 2). After pH-mediated fusion of the virion membrane with the endosomal membrane (step 3), the capsid is released from vesicles into cytoplasm, the individual genes transcribed (step 4), and the genome replicated (step 5). Adapted from Davis et al. (2015).

2.6 Mode of transmission

2.6.1 Animal contact

Rabies virus has a broad host range and can infect almost all mammals, including humans (Rao 2019). Although several routes of transmission have been described for RABV, the most common and natural one is through inoculation of the agent present in the saliva of an infected animal into another host tissue by biting, scratching or licking (Hanlon and Childs 2013; Maclachlan and Dubovi 2017; Markotter et al. 2018). Contamination of fresh, open bleeding wounds, cuts or direct contact with mucosal membranes, by fresh saliva of a rabid animal may result in infection, but not via intact skin (Gongal et al. 2012; Hanlon and Childs 2013; Singh et al. 2017). The risk of rabies infection is 5%–80% following a bite, which is about 50 times more effective than by licks or scratches (0.1%-1%) (Hemachudha et al. 2013).

2.6.2 Ingestion

In addition to bites, oral exposure to rabies virus as a result of slaughtering, processing, or consumption of RABV-infected tissue is possible, although there are few cases of human infection resulting from these practices (Bishop et al. 2003; Garg 2013; WHO 2018). Therefore, in rabies-endemic countries (e.g. in some Asian and African countries) the entire process, from the slaughter of unvaccinated dog and cats to the end-consumer, should be considered a risk factor for rabies virus infection (Wertheim et al. 2009; Nguyen et al. 2011; Ekanem et al. 2013).

2.6.3 Aerosol transmission

Inhalation of aerosolised rabies virus is another potential non-bite route of exposure, and transmission has been documented in research laboratory workers as well as in cave explorers from bat caves containing highly concentrated live rabies virus (Winkler et al. 1973; World Health Organization 1977; Kumar 2009).

2.6.4 Human-to-human transmission

Although rare, iatrogenic human-to-human transmission of rabies has been well documented for recipients of transplanted human tissues (Hanlon and Childs 2013). In the USA, four recipients of kidneys, a liver, and an iliac artery graft from a common organ donor died of rabies in 2004 (Srinivasan et al. 2005). In Germany, in 2005, two recipients of kidney and lung transplants developed rabies and died within days of symptomatic disease. Another kidney and pancreas recipient died of rabies 95 days after the transplantation (Maier et al. 2010).

In February 2013, in the USA, a kidney recipient with no reported exposures to potentially rabid animals died from rabies 18 months after transplantation (Vora et al. 2013). In China, from 2015 to 2017, four solid organ recipients were diagnosed with rabies that was considered to have been transmitted from two donors (kidney and liver) who died due to viral encephalitis of unknown cause and acute disseminated encephalomyelitis (Zhang et al. 2018).

Rabies virus has been isolated from many tissues and body fluids of infected patients (Helmick et al. 1987; Stantic-Pavlinic 2002; Chosewood and Wilson 2009) but transmission through sexual intercourse has not yet been documented (Tenzin 2012; Warrell 2014). In scientific literature though are reports of two cases of human-to-human rabies transmission in Ethiopia: the first one is of a mother bitten on her finger by her child. The child who later died of rabies had previously been bitten by a rabid dog (Kolars 2003). The second case is that of a 5-year-old child who showed signs consistent with those of rabies. It was reported that the mother repeatedly kissed the child on his mouth during her illness, preceded by a bite from a rabid dog (Fekadu et al. 1996; Kolars 2003; Jackson 2013).

2.6.5 Transplacental and transmammary transmission

Transplacental transmission of rabies virus in humans was reported in Turkey (Sipahioglu and Alpaut 1985). A Turkish woman and her 2-day-old infant died of rabies in 1981 (Sipahioglu and Alpaut 1985; Warrell 2014). Transmammary transmission of rabies virus has also been documented in some countries such as Vietnam and elsewhere in Latin America (Lemos-Filho and Fries 2006; Nguyen et al. 2018). As early as this century in Paraguay, a child who was breastfed from a mother who died of rabies also received a vaccine and anti-rabies serum regimen and did not develop the disease (Kotait et al. 2009). In Brazil there were two cases of pregnant women who had rabies, but their babies subsequently survived the disease after PEP in the State of São Paulo (1960s) and in Pernambuco, in the 1990s (Kotait et al. 2009).

2.6.6 Resistance to physical and chemical agents

The enveloped RABV is not viable outside the host and is very sensitive to some environmental factors. It can be inactivated by direct sunlight, ultraviolet and X-ray exposure and heat (Okonko et al. 2010; Tenzin 2012). At 56°C the half-life is less than one minute, but at 37°C it is prolonged to several hours in moist conditions, while at 4°C, there is little loss after two weeks (Warrell 2014). Desiccation and lipid solvent (chloroform, acetone, 45-70% alcohol, 10% formalin, ether), 5% phenol, halogens, mercurial, mineral acids, sodium deoxycholate, trypsin and 1% soap solution and other common detergents are used to disrupt the lipid coat of RABV (Okonko et al. 2010; Tenzin 2012).

2.6.7 Pathogenesis

The highly neurotropic rabies virus gains entry into a new host by introduction of virus-containing saliva into a bite wound (Warrell 2014). The virus remains latent at the point of inoculation and it does not usually stimulate an immune response at this time, but is susceptible to neutralization if antibodies are present (Bishop et al. 2003; Markotter et al. 2018; Belay 2019). Immediately after its entry, the virus may replicate in the striated muscle (myocytes) or in the connective tissue (Warrell 2014) until it reaches sufficient concentration before invading the peripheral nerves through the neuromuscular junction of motor nerves (Castro et al. 2014; Maclachlan and Dubovi 2017). It may then persist there up to 18 days (Singh et al. 2017).

Currently, three distinct cellular proteins for lyssavirus binding are speculated as necessary for the completion of the life cycle of RABV: the nicotinic acetylcholine receptor (nAChR), the neuronal cell adhesion molecule (NCAM or CD56) and the p75 neurotrophin receptor (p75NTR). The virus is capable of binding to p75NTR from mammalian cells, but not to bird cells (Tuffereau et al. 2007; Kotait et al. 2009). Apart from causing abnormalities in the neurotransmitters (serotonin, gamma-aminobutyric acid and muscarinic acetylcholine), neuronal infections also causes dysfunction of sodium-potassium ion channels and increases nitric oxide production (Fu and Jackson 2005; Jackson and Fu 2013; Singh et al. 2017). The virus then spreads to the CNS through the endoneurium of the Schwann cells in a fast centripetal retrograde movement within the motor axons, with no uptake by sensory or sympathetic endings, typically to the spinal cord with resultant neuron-to-neuron spread (Dacheux et al. 2011; Hemachudha et al. 2013; Barecha et al. 2017; WHO 2018b).

The viral genome is estimated to have a travel speed up to 100 mm per day, depending on virus concentration, viral strain and the density of motor endplates at the wound site until it reaches the CNS (Tsiang et al. 1991; Kelly and Strick 2000; Jackson and Fu 2013). From intense CNS replication, the virus moves in a centrifugal direction, via slow anterograde axoplasmic flow in motor axons to the ventral roots and nerves and peripheral sensory axons of the infected dorsal root ganglia, leading to infection of a variety of extraneural organs (lungs, heart, kidneys, bladder, uterus, testes, hair follicle, adrenal glands, tongue, etc.) and salivary glands (Ugolini 2011; WHO 2018b).

Centrifugal spread is slow, as it is probably mediated by passive diffusion rather than active transport. This viral spread ends in the sensory nerve endings in the cutaneous tissue of the head and neck, where the lyssaviral antigen can be demonstrated by fluorescent antibody test (FAT) or RT-PCR (Davis et al. 2015; Mahadevan et al. 2016; Markotter et al. 2018). Usually skin and subcutaneous tissues are rich in sensory and autonomic innervations, which are involved in infection due to deeper biting of vectors. Bats however, generally inflict more superficial bites than terrestrial vectors with the host usually unaware of the bite (Singh et al. 2017).

By the time of clinical onset, the virus is widely disseminated throughout the central nervous system leading to behavioural changes due to lesions in the neurons of limbic system. Death follows, usually through respiratory paralysis or through secondary circulatory, metabolic or intense inflammation of the spinal nerve roots (Bishop et al. 2003; Ghosh et al. 2009, Jackson 2010). In animal experiments, it could be demonstrated that rabies virus is excreted in the saliva of infected dogs, cats and ferrets for 4 to 5 days before developing the signs of disease (Fekadu et al. 1982; Zhang et al. 2008; Maclachlan and Dubovi 2017).

2.7 Immunity

Interferons are extremely important at the onset of infection and they act by directly inhibiting viral replication and consequently its spread or inducing immune cell reactions (Kotait et al. 2009; Warrell 2014). The rabies virus is capable of inducing interferon production prior to its migration to the central nervous system (Kotait et al. 2009; Davis et al. 2015). Antigen-presenting cells (macrophages, dendritic cells, Langherans cells etc.), when they come in contact with the rabies virus, phagocyte and process them for presentation to immune cells (Kotait et al. 2009). This presentation is fundamental for the activation of helper T lymphocytes, which will produce different cytokines; they activate different cells implicated in the direct elimination of the virus or infected cells, and assist in the production of antibodies by B lymphocytes (Kotait et al. 2009; Warrell 2014). B lymphocyte stimulation for antibody production in natural infection occurs only after the onset of clinical symptoms (Kotait et al. 2009).

The possibility of neutralizing the viral infectious capacity occurs, therefore, only after the invasion of the CNS and, at this moment, the disease has acquired an irreversible form (Warrell 2014). The neutralizing antibody titre remains low until the terminal stage of the disease, and peaks near death. The main activity of antibodies is to block the extracellular virus before it encounters the muscle cell receptor, preventing its spread at the site of infection and its progression to the central nervous system. The cellular immune response is perhaps the most important mechanism of rabies virus immune response. T lymphocytes participate in protection in different ways: by stimulating, through helper T lymphocytes, B cells to produce antibodies; as immune effectors in the form of cytotoxic T cells by lysing infected cells (Bishop 2003); inducing synthesis of stimulating mediators of different cells; and as immune memory cells (Bishop et al. 2003; Kotait et al. 2009; Davis et al. 2015).

During apoptosis (host mechanism to limit viral spread) the cell shrinks, stands out from the others, with no evident changes in the cytoplasm, but in the nucleus, with chromatin clustering and DNA cleavage. If the cell does not die, the cell fragments and the materials are phagocytized, with no signs of inflammation (Kotait et al. 2009). This phenomenon plays an important role in pathogenicity and is induced by phylogroup II lyssaviruses and non-pathogenic phylogroup I mutants (Sugamata et al. 1992; Consales and Bolzan 2007). As an example, RABV, EBLV type-1 and type-2, DUVV and ABLV were found to be more pathogenic for mice when injected by the intracerebral or the intramuscular route, whereas MOKV and LBV were only pathogenic by the intracerebral route (Badrane et al. 2001; Kgaladi et al. 2013; Sabeta 2015).

Even so, to fight viral infection of the nervous system, neuronal apoptosis more than the death of “protective” T cells, seems to be more important in transient infections by less pathogenic RABV strains such as Evelyn-Rotnycki-Abelseth (ERA), Pasteur virus (PV) and others, while apoptosis resulting from T cells (CD3 +) that migrate and consequently killed into the infected nervous system is more important in successful invasion of nervous system by pathogenic strains of RABV such as the challenge virus standard (CVS) as a result of the overexpression of immune-subversive molecules such as Fas ligand (FasL), human leukocyte antigen G (HLA-G) or immunoglobulin-like immune suppressive molecule (B7-H1) (Thoulouze et al. 1997; Lafon 2004; Lafon 2008; Lafon et al. 2008).

2.8 Epidemiology of rabies

Rabies is prevalent throughout the world except in Antarctica and most of Pacific Oceania (Malerczyk et al. 2010; Singh et al. 2017; Rupprecht et al. 2018a). Every year, rabies kills nearly 60,000 people, mostly children from Africa and Asia (Hampson et al. 2015; Cleaveland and Hampson 2017). It is estimated that every nine minutes one person in the world dies from rabies with over 99% of human cases being caused by the bite of a rabies-infected dog (WHO 2018d).

2.8.1 Rabies in Asia

More than 30,000 people die annually of rabies in Asia (WHO 2010; WHO 2018b). For instance, in 2015, of the estimated 37,045 deaths due to rabies across the Asian continent , 56% occurred in India (Hampson et al. 2015). In 2005, about 50% of all rabies deaths in India occurred in children under 15 years old (Suraweera et al. 2012). This disease poses a potential risk of infection to around 1.4 billion people in the Southeast Asia alone (Gongal and Wright 2011). This region, where rabies is maintained in two epidemiological cycles involving respectively dog populations as the most important reservoirs rabies and wildlife (Gongal and Wright 2011), accounts for about 71% of all mortality cases in the continent (Hampson et al. 2011; Tenzin and Ward 2012). Cats and wildlife such as the small Indian mongoose (*Herpestes auropunctatus*), Golden jackal (*Canis aureus*), Bengal fox (*Vulpes bengalensis*) and wolf (*Canis lupus*) have also been reported as the other secondary source of rabies (Kuzmin et al. 2004; Gongal and Wright 2011; Yousaf et al. 2012).

Rabies is still endemic in most rural regions of Asia where poverty, political instability, insufficient and deficient health and veterinary services, and cultural beliefs contribute to its persistence (Yousaf et al. 2012; Singh et al. 2017). In India, an estimated 17 million animal bites are reported per year and 97% of humans report dogs as the main source, followed by cats (2%) and wildlife - mongoose (*Herpestes spp*.) and jackals (*Canis aureus*) one per cent (Tenzin and Ward 2012). Despite the Association of Southeast Asian Nations (ASEAN) regional elimination strategy (ARES) to eliminate human rabies in the Region by 2020, India still accounts for 59.9% of rabies deaths in Asia and 35% (approximately 20,800 deaths) globally. The cost of Post-Exposure Prophylaxis (PEP) is estimated to be US$ 1.5 billion per year (Wunner and Briggs 2010; Yousaf et al. 2012; Hampson et al. 2015; WHO 2018b).

In East Asia, the biggest concern about the epidemiological situation of rabies lies in China, where it is currently considered a re-emerging disease (Tian et al. 2018). From 1950 to 2015, about 130,494 human rabies cases were reported in China, an average of

1,977 cases per year (Song et al. 2014; Ruan 2017). Over a 6-year period (2007–2011) at least 15,715 human rabies deaths were reported in China (Tenzin and Ward 2012). Dogs were associated with 88.53% of rabies in rural China (Yin et al. 2012). Evidences shows that rabies viruses can also be found in wildlife such as ferret, badgers and wolves, foxes (*Vulpes bengalensis*), deer, but plays a very minimal role in the epidemiology of rabies in China (Hu et al. 2009; Tenzin and Ward 2012; Tan et al. 2017).

2.8.2 Rabies in Europe

In Europe, animal and human rabies has been controlled or eradicated from most countries through the control of stray dog populations (Yousaf et al. 2012; Hanlon and Childs 2013; Singh et al. 2017) and most importantly by the oral immunization of Raccoon dogs (*Nycterentes procyonoides*) and especially Red foxes (*Vulpes vulpes*) in Western, Central and Eastern Europe (Bourhy et al. 1999; Cliquet et al. 2014; Maclachlan and Dubovi 2017). About 10 cases per year were reported in Europe from 1990 to 2012, with a total of 210 human fatalities (Cliquet et al. 2014).

The arctic fox (*Vulpes lagopus*), which has a circumpolar distribution (northern Europe) and raccoon dogs (*Nyctereutes procyonoides*) act as rabies transmitters in large parts of northern, eastern and central Europe (King and Turner 1993; Wandeler 2008; Sihvonen 2015; Hanke et al. 2016). *Microcheroptera* bats such *as Eptisecus serotinus* and *Myotis sp.* are responsible for maintaining European bat lyssaviruses type-1 and type-2 in Europe (McColl et al. 2000; Banyard et al. 2013).

From 2000 to 2013, a total of twenty-one dogs and one cat were diagnosed positive for rabies in Western Europe following importation from rabies-enzootic countries (Cliquet et al. 2014; Ribadeau-Dumas et al. 2016). The first rabies in a bat was reported from Germany in 1954 and to date 1064 cases have been confirmed from 16 European countries in 11 of 45 indigenous species (Hammarin et al. 2016; Singh et al. 2017).

2.8.3 Rabies in Central, North America and Australia

Since 2006, no cases of dog-transmitted human rabies were reported from the USA or Mexico (Yousaf et al. 2012; Secretaría de Salud 2014; Ma et al. 2018a). Mass pet vaccination campaigns, along with animal birth control, have dramatically reduced the number of reported cases of canine rabies in humans in Mexico (Secretaría de Salud

2014). As a result of this effort, Mexico was declared by WHO, in November 2019, as a country free of human rabies transmitted by dogs (PAHO 2019a).

In the USA, wild animals accounted for the majority of rabies cases: 91% and 91.4% in 2017 and in 2016 respectively (Ma et al. 2018a; Ma et al. 2018b). The major reservoir hosts responsible for sylvatic cycles of rabies are raccoons (*Procyon lotor*), striped skunks (*Mephitis mephitis*), bat species (*Eptesicus fuscus*, *Tadarida brasiliensis*, *Myotis lucifugus*, *Lasiurus cinereus*, *Lasionycteris noctivagans*, *L. borealis* and *Pipistrellus hesperus*), foxes and coyotes (Tordo et al. 1993; Marks and Bloomfield 1999; Finnegan et al. 2002; Ma et al. 2018b).

In Canada, the red fox (*Vulpes vulpes*) is implicated in the maintenance and spread of a sylvatic cycle of the disease, but no human cases of rabies had been reported in the last three years, i.e. 2016-2018 (Finnegan et al. 2002; Ma et al. 2020). From January to October 2018, two human rabies cases were reported in the United States, with one of the cases being bat- associated (*Tadarida brasiliensis*) (Ma et al. 2018a). Australia is historically free of canine rabies, but *Megachiroptera* and *Michrochiroptera* bats are reservoirs for *Australian bat lyssavirus* (Johnstone-Robertson et al. 2017), a lyssavirus associated with human deaths.

2.8.4 Rabies in Latin America and the Caribbean

In Latin America and the Caribbean (LAC) countries cases of human and canine rabies have been reduced by nearly 90% over the last three decades following implementation of a regional programme for rabies control introduced in 1983 (Vigilato et al. 2013; Freire de Carvalho et al. 2018). From 1998 to 2014, 778 human rabies cases were reported in the 21 LAC countries (Argentina, Belize, Bolivia, Brazil, Chile, Colombia, Costa Rica, Cuba, Ecuador, El Salvador, Guatemala, Haiti, Honduras, Mexico, Nicaragua, Panama, Paraguay, Peru, the Dominican Republic, Uruguay, and Venezuela) of which 49% (382) were dog-mediated; 298 cases (38%) were bat-mediated rabies and unknown aggressor species 44 (7%) were the third most reported (Freire de Carvalho et al. 2018). Many factors, including national disasters and social, cultural, and economic factors have interfered with canine rabies control programs in these countries (Vigilato et al. 2013).

Currently and according to PAHO (Pan American Health Organization), from 1983 to 2018, human rabies cases fell from 258 to 13 and in dogs, fell from 11,276 to 163 in the same period. If the surveillance system and canine vaccination coverage are improved, particularly in high-risk countries such as Haiti and Dominican Republic it is possible to eliminate cases of human rabies by 2022 (PAHO 2019b).

However, throughout most of continental Latin America (from Mexico, Brazil, Peru, Venezuela to north-eastern Argentina) and, Trinidad and Tobago and Grenada in the Caribbean, the principal vector of sylvatic rabies, vampire bats (*Desmodus rotundus*) poses a serious threat to humans as well as to livestock (De Mattos et al. 1999; Animal and Plant Health Inspection Service 2008; Rosatte 2013; Taylor et al. 2013; Johnson et al. 2014). In the Caribbean Islands such as Puerto Rico, Grenada, West Indies and Cuba, the small Indian mongoose (*Herpestes auropunctatus*), is recognized as an important rabies vector and a growing public health problem since the beginning of the 20th century (Zieger et al. 2014; CDC 2016; Seetahal et al. 2018).

2.8.5 Rabies in the Middle East

The epidemiological situation of rabies in Middle Eastern countries is not well understood (Bannazadeh Baghi et al. 2018). The endemicity of the disease is evident in Lebanon, Syria, Jordan, Iran, Israel, Saudi Arabia and Iraq where dog-mediated rabies, remains a significant public health threat (Horton et al. 2015; Memish et al. 2015; Bannazadeh Baghi et al. 2018). Annually, approximately 300 human rabies cases are reported (Aylan et al. 2011; Bizri et al. 2014) with the annual incidence varying from 0.02 to 1.3 per million (Horton et al. 2015). The sylvatic cycle occurs involving jackals, coyotes, mongooses, foxes and wolves are the main or intermediary viral reservoirs (Tordo et al. 1993; Seimenis 2008; Bannazadeh Baghi et al. 2018).

2.9 Imported rabies cases

An estimated 1.06 million dogs are imported annually into the US (Pieracci et al. 2019) The global movement of animals and the illegal importation of dogs and cats, has negatively impacted the efforts of governments in European and North American countries in preventing the reintroduction of canine rabies cases. In the USA, since 2015, three canine rabies cases have been reported from imported dogs adopted from countries with a high risk for rabies (Pieracci et al. 2019). In Europe, from 2012 to

2020, five illegally imported dogs infected with rabies have been reported in France, Spain and the Netherlands from north Africa (ECDC 2019). In 2019, four imported human rabies case were reported in Latvia, Spain, Italy, and Norway after having contracted the disease through a dog or cat bite while travelling in India, Morocco, Tanzania, and Philippines, respectively (ECDC 2019; Vega et al. 2021).

2.9.1 Rabies in Africa

Although the history of rabies in Africa is poorly understood, on this continent it has probably been present since ancient times (Rupprecht et al. 2020) and is thought to have been introduced into sub-Saharan Africa from North Africa and Eurasia following European colonization (Bourhy et al. 2008; Talbi et al. 2009). In recent decades, numerous initiatives and organizations such as the African Rabies Expert Bureau (AfroREB), the Southern and Eastern African Rabies Group (SEARG), the Rabies in West Africa group (RIWA), the Global Alliance for Rabies Control (GARC), and the Pan-African Rabies Control Network (PARACON) and Stepwise Approach to Rabies Elimination (SARE) have been set up to strengthen surveillance, control and elimination strategies in Africa (Dodet 2009; Broban et al. 2018).

The cosmopolitan lineage, Africa 1, is the most widespread with Africa 2 and 3 having more restricted geographical distributions (Markotter et al. 2018). Domestic dogs (*Canis familiaris*) are hosts of rabies virus in most of Africa; they cause most human rabies cases and bites that require medical intervention (Bingham 2005). Since 1912 when rabies was first confirmed in Kenya, approximately 23,000 people die annually from rabies, with maximum mortality rates reported in children and underprivileged agrarian people (Barecha et al. 2017; Singh et al. 2017).

The high proportion of unvaccinated and free-roaming dogs makes this population the most important reservoir host and the primary source of infection to humans and other domestic carnivores (Lembo et al. 2010; Morters et al. 2014). Overall estimates of the annual incidence of bites received from suspect rabid dogs are 100 per 100,000 persons in urban and rural settings in Africa (Hanlon and Childs 2013). In addition to domestic dogs, wild dogs (*Lycaon pictus*), jackals (*Canis mesomelas* and *Canis adustus*), Ethiopian wolves (*Canis simensis*), yellow mongooses, bat-eared foxes (*Octocyon megalotis*) and some bats are involved in rabies and rabies-related epidemics (Adeiga and Audu 1996; Bingham 2005). Lagos bat virus was isolated from straw-coloured fruit bats (*Eidolon helvum*) in Nigeria, Ghana, Senegal and Kenya; Wahlberg's epauletted fruit bat (*Epomophorus wahlbergi*) in South Africa; Egyptian fruit bat (*Rousettus aegyptiacus*) in Egypt or Togo and Kenya; insectivorous Gambian slit-faced bat (*Nycteris gambiensis)* in Guinea and from dwarf epaulet fruit bats (*Micropteropus pussilus*) in Central African Republic (Markotter et al. 2008; Kuzmin et al. 2008b; Banyard et al. 2011; Binger et al. 2015; Freuling et al. 2015).

Duvenhage virus was isolated from *Miniopterus natalensis* in South Africa in 1970 (Meredith et al. 1971) and *Nycteris thebaica* bats in Zimbabwe in 1986 (Paweska et al. 2006). SHIBV was isolated from a Commerson's leaf-nosed bat (*Hipposideros commersoni*) in Kenya (Banyard et al. 2011; Kuzmin et al. 2011). Bat-related human rabies cases remain rare but may be underreported because of limited surveillance and characterization of viruses (WHO 2018b). On the other hand, black-backed jackal is a host that maintain rabies in different regions of Africa, ranging from South Africa to Sudan (Zulu et al. 2009; Markotter et al. 2018), while the side-striped jackal are responsible for the transmission of rabies in Zimbabwe (Bingham and Foggin 1993).

2.9.1.1 Rabies in Southern Africa

Rabies was first diagnosed in the Eastern Cape (South Africa) in 1893 during an outbreak in dogs following an importation of an rabid Airedale terrier from England (Swanepoel et al. 1993). Since then, the disease has assumed epidemic proportions in some geographic areas of the country (Bingham 2005; Markotter et al. 2018). In addition to the domestic dog, the side-striped jackal (*C. adustus*), the black-backed jackal (*C. mesomelas*), the bat-eared fox (*Otocyon megalotis*), the wild dog (*Lycaon pictus*) and the Ethiopian wolf (*C. simensis*) have also been implicated as independent maintenance hosts (Bingham 2005).

The disease is endemic in the coastal provinces of KwaZulu-Natal (KZN) and Eastern Cape (EC) of South Africa (Swanepoel 2004). In Mpumalanga the initial emergence of canid rabies resulted from an epizootic that originated in Angola in 1947 and spread through Namibia, Botswana, and northern South Africa in 1950 (Swanepoel 2004). The re-emergence of canid rabies in 2008 in Mpumalanga province occurred in districts where it had never been reported or where it had been under control (Mkhize et al. 2010; Sabeta et al. 2013). Wildlife species such as the black-backed jackal and bat-eared fox also contribute to the epidemiological cycle of canine rabies in the northern and south-western of the country, respectively, while whereas yellow mongoose *(Cynictis penicillata)* and other herpestids maintain the “mongoose” biotype across the central plateau of South Africa (Swanepoel 2004; Bingham 2005; Sabeta et al. 2013). Cases of rabies have also been reported in suricates (*Suricata suricata*) and ground squirrels (*Xerus inauris*) in the mongoose rabies endemic regions of South Africa (Blancou 1988; Swanepoel et al. 1993; Bishop et al. 2003). Human rabies cases are confirmed annually in South Africa and the majority of cases are linked to domestic dog exposures, this emphasises the importance of controlling the disease in dogs in order to prevent human cases (Weyer et al. 2011).

Rabies is endemic throughout Botswana with two interrelated cycles (urban and sylvatic) with the principal reservoir hosts not only being stray dogs and cats, but also wildlife including the black-backed jackal and other carnivores such as the genet (*Genetta genetta*) and yellow mongoose (*Cynictis penicillata*) (Letshwenyo 2003; Moagabo et al. 2009; Ditsele 2016). These wildlife-associated biotype (Mongoose subtype) and the canine-associated biotype dominate the southern and northern parts of the country, respectively (Johnson et al. 2004). Over 70% of all rabies cases in Botswana are in domestic ruminants, with cattle being the most commonly affected animal (Johnson et al. 2004). Cattle, sheep, goats, dogs and cats are the main potential risk species for rabies transmission to men when handling or eating dead carcasses or due to their close proximity with them (Letshwenyo 2003).

Other wildlife species, such as the black-backed and the side-striped jackal are pivotal in specific areas of the western and northern parts of South Africa, as well as in Namibia, Zambia and Zimbabwe (Perry 1992; Bishop et al. 2003; Bingham 2005; Mansfield et al. 2006).

Rabies has been recorded in Namibia since the early 19th century and the domestic dog is the principal reservoir, particularly in the northern communal areas adjacent to Angola (Hikufe et al. 2019). Rabies in wildlife species is predominantly reported from farmlands in central Namibia, mostly affecting kudu (*Tragelaphus strepsiceros)* and canids such as jackals or bat-eared foxes causing spill-over infection in domestic and wildlife ruminants (Mettler 2003; Hikufe et al. 2019). Between 2001 and 2017, dog-mediated rabies contributed to 242 human rabies deaths in the country, with the majority of these victims being children (Athingo et al. 2020).

In 1902 the first case of rabies was recorded in Bulawayo province in Zimbabwe after an outbreak of the disease in the neighbouring country of Zambia (Swanepoel et al. 1993). After a period of about 30 years of apparent control of the disease, in the 1950s it was reintroduced from either Botswana or South Africa (Swanepoel et al. 1993) and became established throughout Zimbabwe in both the domestic dog populations and terrestrial wild carnivores (primarily side-striped jackal and black-backed jackals) (Swanepoel et al. 1993; Sabeta et al. 2003; Coetzer et al. 2019). Over 40% of all confirmed rabies cases reported between 1950 and 2000, appear to be maintained in rural areas by rabid dogs where 71.3% of its country's population is to be found (Foggin 1988; Brooks 1990; Bingham et al. 1999; Sabeta et al. 2003; Sabeta and Nel 2003).

In a retrospective study of rabies in wild animals, it was actually revealed that jackals (*Canis mesomelas* and *Canis adustus*), were the major maintenance wild hosts, representing approximately 91% of the total rabies cases confirmed, with the majority of the positive cases (83.7%) recorded in commercial farming areas in the northeast parts of the country (Pfukenyi et al. 2009). Between 1992 and 2003, domestic dogs were responsible for 42 human cases (90.5%) and other animals acting as sources were two jackals species and two honey badgers (*Mellivora capensis*) (Pfukenyi et al. 2007).

Rabies was first recorded in Lesotho in the 1980s when the disease was introduced into the north-eastern part of the country from the KwaZulu-Natal (KZN) province of South Africa (Swanepoel et al. 1993). Since then, it has remained endemic throughout the country and has been especially recorded in dogs, with 58 and 20 positive rabies cases, in 2011 and 2012, respectively (WHO and Global Alliance for Rabies Control 2014b). There are also cases reported in other domestic animals such as cattle, sheep goats, horses and donkeys (Swanepoel et al. 1993; WHO and Global Alliance for Rabies Control 2014b). The fact that Lesotho is bordered by three rabies-endemic South African provinces (Free State (FS), Eastern Cape (EC) and KZN) may contribute to the cross-border transmission of rabies between the two countries (Ngoepe 2008; Coetzer et al. 2017b). In fact, molecular epidemiological analyses suggested that in Lesotho there are at least three independent rabies cycles implicated in instances of cross-border transmission (Coetzer et al. 2017b). Rabies in humans in Lesotho is mostly transmitted through the bite of the domestic dog, but inconsistent data submitted to the OIE in 2012, recorded 15 human rabies cases with two deaths (WHO and Global Alliance for Rabies Control 2014b).

In eSwatini, Zambia, Angola and Malawi, dogs act as the main vector for transmission and sustenance of the RABV in the domestic environment since the early 1900s (Swanepoel et al. 1993; Dlamini et al. 2020). The majority of human cases reported in these southern African countries are due to bites by domestic dogs (Swanepoel et al. 1993; Babaniyi et al. 2016). Cattle are affected in the south-central part of Zambia, particularly in locations where jackal rabies is diagnosed close to nature reserves (Swanepoel et al. 1993; WHO and Global Alliance for Rabies Control 2014e). Rabies in cattle has also been reported in eSwatini (WHO and Global Alliance for Rabies Control 2014d). Despite the lack of rabies surveillance in the most of these countries, the disease has been documented in wildlife including the African civet (*Civettictis civetta*) (Angola), wild foxes (Zambia), jackals and hyenas (Malawi) (Edelsten 1995; de Fontes-Pereira et al. 2012; WHO and Global Alliance for Rabies Control 2014a; WHO and Global Alliance for Rabies Control 2014e).

2.9.1.1.1 Rabies in Mozambique

In Mozambique, rabies has been endemic since 1908 with domestic dogs contributing at least 88% of confirmed cases (Swanepoel et al. 1993; Dias and Rodrigues 2003; WHO 2013). The epidemiological data on rabies that has been disclosed in Mozambique, is inconsistent and probably underreported, meaning that they do not reflect the reality of the disease in the country (Dias and Rodrigues 2003; Barreto et al. 2004; Nel 2013). As an example of the discrepancy in the data provided, is illustrated in a study carried out to assess the occurrence of animal rabies between 2001 and 2017 and, in humans (1989 to 2017) which controversially revealed less rabies cases (898) in animals than in humans (1,001) (Bilaide 2019). Maputo province had the highest occurrence, with 274 animal rabies cases, followed by the provinces of Nampula (134) and Gaza (122), respectively. The lowest occurrence of was recorded in Zambézia and Sofala provinces, with 26 and 30 cases, respectively.

The domestic dog is the species that is most affected by rabies with 714 (79.5%) cases during 2001 and 2017. Other affected species during this period (2001-2017) included goats (16 cases; 1.8%), cattle (71 cases; 7.9%), felines (44 cases; 4.9%), pigs (50 cases; 5.6%) and unspecified wild animals (3 cases; 0.3%). In humans, the provinces of Nampula and Zambézia recorded most cases of human rabies, that is, 216 (21.5%) and 160 (16%), respectively. In evolutionary terms, few cases occurred between 1989 and

2001. Despite some numerical fluctuation, the situation of rabies is still unreliable in Mozambique and more effective actions are necessary for the reversal of this neglected problem (Bilaide 2019).

Dog rabies control programmes are carried out through regular rabies vaccination campaigns. In 2010, the Rabies Control Strategy was approved by the government of Mozambique for a period of four years (2010-2014), but so far there is still no information on the goals achieved during the period covered. Among other measures, the strategy aimed to educate people about the different ways of rabies prevention in dogs, cats and humans. The approved plan also sought to provide better care to people attacked by animals suspected to be infected. The strategy also aimed to reduce the incidence of animal rabies to less than 10 cases per year. However, until then, vaccination coverage rates are around 9.2%, much lower than the rate recommended by the World Organisation for Animal Health (OIE). Poor revitalization of stray dog capture services also seems to contribute to the weak control of rabies in this species.

2.10 Incubation period and clinical signs of rabies

2.10.1 Clinical manifestations of rabies in domestic animals

Rabies affects the CNS and hence it is usually associated with behavioural changes. However, these changes are not necessarily species specific and can manifest in different ways. Special epidemiological surveillance and clinical attention should be paid to the symptoms that can occur when rabies in dogs and cats is transmitted by bats, a fact that has been occurring in some countries such as France and Brazil (Kotait et al. 2009). Experimentally, dogs were reported to be susceptible to bat rabies-related viruses such as LBV, MOKV and DUVV (Fekadu 1993). In Brazil, passive epidemiological surveillance activities for rabies-related virus infections have been developed, particularly in some south-eastern states (Kotait et al. 2009).

2.10.1.1 Canine rabies

The prodromal phase, a phase preceding the onset of active symptoms, clinical signs are often nonspecific and frequently overlooked, which is why international standards set by the World Health Organization (WHO) recommend the observation of dogs for ten days (Bishop et al. 2003; Maclachlan and Dubovi 2017). It usually persists for one to three days and the dogs may have fever, vomiting, diarrhoea and a change in behaviour, such as photophobia, unusual agitation, lethargy, abnormal appetite, dysphagia and mydriasis (Fekadu 1993; Beeler and Ehnert 2020). After one to three days, the symptoms of excitation are accentuated (excitatory or encephalitic phase) and may last up to a week, although there are cases of rabies that progress directly from the prodromal phase to the paralytic stage (Lackay et al. 2008; Kotait et al. 2009).

During the excitatory (mad or furious) phase, the dog becomes vicious and aggressive, exhibiting violent mania, confusion, a tendency to bite objects, animals or man, including its owner, and self-biting of limbs and hindquarters (Kumar 2009; Zhu and Liang 2012). During this phase, dogs have a tendency to leave their homes and travel great distances, during which they can attack other animals, thus spreading rabies especially if the ecological through which they pass include water channels, which in turn, in the dry season serve as accumulation points for food waste and garbage (Graham and Dunlap 1937; Knobel et al. 2014; Castillo-Neyra et al. 2017; Raynor et al. 2020). There is also a development of a high pitched bark, which becomes hoarse or bitonal, due to partial paralysis of the vocal cords (Kotait et al. 2009; Kumar 2009; Castro et al. 2014).

In the last period (paralytic or dumb) phase, salivation becomes abundant due to paralysis of swallowing muscles. Generalized seizures are common, followed by motor incoordination and paralysis of the trunk and limbs, and tongue prolapse. Paralysis begins with the musculature of the head and neck, including jaw and tongue. Paralysis of the hind quarters becomes obvious and, finally respiratory paralysis or failure which leads to death occurs within three to eight days after the initial symptoms (Zhu and Liang 2012; Belay 2019; Beeler and Ehnert 2020).

2.10.1.2 Feline rabies

Furious and paralytic are identified in cats (Frymus et al. 2009; Shimshony 2009). The furious form is observed more often than in dogs and the symptoms are similar, with cats showing behaviour abnormalities (Fogelman et al. 1993; Barecha et al. 2017). During the prodromal phase (one to two days) of both forms, a wide range of non-specific clinical signs (including fever, anorexia, vomiting, and diarrhoea) may occur, sometimes accompanied by neurological signs (Shimshony 2009).

As mentioned previously, marked behavioural changes such as unusual friendliness or shyness, aggressive behaviour or increased frequency of vocalization may be noticed at first (Fogelman et al. 1993; Shimshony 2009). The tendency to bite or to strike at the air with its forepaws may be the consequence of the loss of inhibitory control by cortical neurons over the subcortical bite reflex; if this is the case, the animal snaps without warning or showing any emotion when doing it (Bishop et al. 2003; Shimshony 2009; Garg 2013).

The paralytic phase which is characterized by generalized paralysis/paraparesis, incoordination, coma and death, are usually evident within two to four days after the first signs are observed. Nerves affecting the head and throat are the first involved tissues and cats may begin to salivate as a result of their inability to swallow (Nilakanth et al. 2013). The animal will get weaker with deep laboured breathing and a dropped jaw due to convulsions and respiratory, facial and diaphragm muscles paralysis and will die after a clinical course of one to ten days (Frymus et al. 2009).

2.10.2 Clinical manifestations of rabies in wild animals

In wild animals, rabies must be suspected when they exhibit behavioural changes such as loss of fear and wariness towards humans or domestic species and/ or unusual friendliness (Garg 2013). Other signs include changes in their activity cycles, where some seek solitude or become more gregarious (Rupprecht et al. 2001). For example, nocturnal animals such as bats, raccoons, coyotes, foxes and skunks may show abnormal activity during daytime and may attack humans (Foggin 1985; Oertli et al. 2009; Garg 2013). In the furious form of rabies, there is unprovoked aggression and some animals may attack anything that moves or even inanimate objects. The affected animal may appear disoriented, confused or uncoordinated, or wander aimlessly (Garg 2013). There may be additional CNS excitation, photophobia, pica, and convulsive seizures (Rupprecht et al. 2001). Labile hypertension, hyperventilation, muscle tremors, priapism, altered libido, hypothermia, or hyperthermia may be noticed as a result of autonomic systemic excitation (Rupprecht et al. 2001).

A paralytic phase (dumb rabies) may follow the agitated or furious phase, or the animal may progress directly to the paralytic phase from the prodromal stage (Rupprecht et al. 2001). Ascending, flaccid, symmetrical or asymmetrical paralysis often begins in the hind legs or throat and eventually leads to respiratory and cardiac failure (Rupprecht et al. 2001; Garg 2013). Paralysis of the throat muscles can cause the animal to bark, whine, drool, choke, or froth at the mouth (Garg 2013).

Skunks, raccoons, and foxes usually display furious rabies (Garg 2013). Bats often display dumb rabies but an excitability phase can occur followed by paralysis mainly of the wings, which causes these animals to stop flying and consequently dropping to the ground (Kotait et al. 2009). Yellow mongooses generally demonstrate tame behaviour, but some are very aggressive (Bishop et al. 2003). Jackals are usually aggressive and lose fear of humans (Bishop et al. 2003). Wild cats display similar behaviour to domestic cats (Bishop et al. 2003). Badgers are usually vicious and fierce. Kudu salivate profusely, may be paralysed, docile or tame, even entering houses (Garg 2013). Duikers (*Sylvicapra grimmia*) are sometimes very aggressive (Bishop et al. 2003). Death due to generalized multi-organ failure occurs one to ten days (or more) after the onset of primary signs or may result in acute death with no premonitory signs (Rupprecht et al. 2001).

2.11 Diagnosis of rabies

Current diagnostic tools are not suitable for detecting rabies infection before the onset of clinical disease (WHO 2019). In rabies-endemic countries the diagnosis of rabies is routinely based on clinical and epidemiological information, especially when exposures are reported (Fooks et al. 2009). With no history of viral exposure or typical symptoms, a diagnosis of rabies on clinical grounds alone may be difficult (WHO 2018b), often unreliable and generally contributes to misdiagnosis and under-reporting of cases (WHO 2018b). On the other hand, laboratory confirmation of positive cases is also beneficial for disease surveillance in defining the current epidemiological patterns of rabies as well as for development of rabies control programmes in that country (Garg 2013).

2.11.1 Laboratory diagnosis

Laboratory diagnosis of rabies is performed in most countries only in level 2 and/ or in level 3 biosafety approved laboratories (BSL-2 and BSL-3), by qualified and experienced personnel (Meslin et al. 1996; Duong et al. 2016). Brain tissues (thalamus, pons and medulla) are the preferred samples for *post-mortem* diagnosis in both humans and other animals (Meslin et al. 1996; WHO 2018b). If it is not possible to keep brain tissues refrigerated or frozen until testing, samples can be preserved at ambient temperature in a 50% glycerine–saline solution (Aguilar-Setién et al. 2003; WHO 2018b). Samples should never be preserved in 10% formalin as the fixation process takes about seven days, and if this is the case, the specimens should be transferred rapidly to absolute ethanol for subsequent molecular testing (WHO 2018b).

2.11.1.1 Direct fluorescent antibody test (dFAT)

The direct fluorescent antibody test (dFAT or FAT) is a rapid, cheap, highly sensitive and specific method for diagnosing rabies in animals and humans and it gives reliable results on fresh specimens in less than two hours (Meslin et al. 1996; OIE 2018). It is the most widely used *post-mortem* test recommended by both WHO and OIE (McElhinney et al. 2020) and is also used to confirm the presence of rabies virus antigen in cell culture or in brain tissue of mice that have been inoculated for diagnosis (Robardet et al. 2011; OIE 2018; Rupprecht et al. 2018b; WHO 2018b). The test detects all rabies lyssavirus variants examined to date and all representative lyssaviruses (Lembo et al. 2006).

The test is based on microscopic examination, under ultraviolet light, of impressions, smears, or frozen sections of brain or nervous tissue after staining with anti-rabies serum or globulin conjugated with fluorescein isothiocyanate (FITC) (Garg 2013; OIE 2018). Smears prepared from a composite sample of brain tissues are fixed in 100% high- grade cold acetone (-20°C) for at least 20 minutes or heat fixed by passing the slide two to three times through a flame (OIE 2018). They are subsequently air dried, and then stained with a drop of FITC or labelled monoclonal anti-rabies antibody conjugate for 30 minutes at 37°C in a humid chamber (Garg 2013; OIE 2018; Rupprecht et al. 2018b).

Commercially available anti-rabies fluorescent conjugates include polyclonal or monoclonal antibodies (mAbs), specific to the entire virus or to the rabies nucleocapsid protein, conjugated to a fluorophore such as FITC (Garg 2013; OIE 2018; WHO 2018b). Upon incubation with rabies suspect brain tissue, labelled antibody will bind to rabies antigen. Unbound antibody can be washed away and areas where antigen is present can be visualised as fluorescent- apple-green areas using an inverted fluorescence microscope (Garg 2013; OIE 2018; Rupprecht et al. 2018b; WHO 2018b). The main limitations of this method and the factors that reduce the sensitivity and specificity of the test are the degree of autolysis, the requirement for a high quality fluorescence microscope, the quality of the reagents (conjugates) and the need for trained and experienced personnel (Fooks et al. 2009; Hanlon and Nadin-Davis 2013).

2.11.1.2 Direct Rapid Immunohistochemical Test (dRIT)

The direct rapid immunohistochemical test (dRIT), was developed by Niezgoda & Rupprecht in the early 2000's (Niezgoda and Rupprecht 2006). The test is used for diagnosis of rabies using fresh, frozen, glycerol-preserved or formalin-fixed brain tissue (Lembo et al. 2006; Coetzer et al. 2014) and was validated for use in Africa, including Mozambique (Coetzer et al. 2017a). As it is equally sensitive and specific to the FAT, dRIT can be used in routine rabies diagnosis as an alternative to FAT and as a field application (Lembo et al. 2006; Coetzer et al. 2014).

The test is based on capture of rabies nucleoprotein (N) antigen in brain smears using a cocktail of highly concentrated and purified biotinylated rabies virus-monoclonal or polyclonal antibodies and colour development by streptavidin peroxidase enzyme such as acetyl 3-amino-9-ethylcarbazole (AEC) and counterstaining with haematoxylin (Lembo et al. 2006; Fooks et al. 2009; Duong et al. 2016; OIE 2018). The viral nucleoprotein appears under light microscope as reddish brown (magenta) inclusions against a light blue background (Madhusudana et al. 2012; Coetzer et al. 2014; Rupprecht et al. 2014; OIE 2018). The biotinylated antibodies are available from the South African OIE rabies laboratory (Pretoria-RSA).

Interpretation of results by dRIT is easier and requires a simple light microscope thereby reducing the cost of this test (Dürr et al. 2008; McElhinney et al. 2020). In addition, dRIT technique is simple, recognizes all rabies lyssavirus examined to date and all representative lyssaviruses, is cost effective and can be performed in a shorter time (< 1 hour) than required routinely for the FAT. In addition, dRIT has been shown to have field applicability particularly in low-resource settings of developing countries (Lembo et al. 2006; Madhusudana et al. 2012; Coetzer et al. 2014).

2.11.1.3 Virus isolation

2.11.1.3.1 *Mouse inoculation test (MIT) and cell culture*

Specimen containing small amounts of rabies virus may be difficult to confirm as rabies positive by routine methods (Garg 2013). Wherever possible, virus isolation in cell culture should replace the mouse inoculation test via intracranial as recommended by the OIE and WHO (OIE 2018; WHO 2018b). However, in developing countries, the use of mice will continue because cell culture requires expensive equipment and reagents, specialized facilities, and highly trained personnel (Kienzle 2007; Rupprecht et al. 2018b).

For MIT, the suckling mice are observed daily for 28 days, and every dead mouse is examined for rabies using FAT starting four days post-intracranial inoculation of small piece of the brain tissue homogenized by a mortar and pestle in a buffered saline diluent containing protein stabilizer and antibiotics (Webster et al. 1976; Meslin et al. 1996; Rupprecht et al. 2018b; WHO 2018b). Cell culture tests are as sensitive as MIT (Rudd and Trimarchi 1989; Robardet et al. 2011; Corona et al. 2018).

A homogenized sample is added to the mouse neuron cells (C-1300) (murine neuroblastoma cells) in a sterile multi-well plastic plates, on multi-chambered glass slides, or on glass cover slips (Rudd and Trimarchi 1989; Garg 2013) and within five days, the presence or absence of rabies can be determined by examining the cells using FAT (Kumar 2009; Rupprecht et al. 2018b).

2.11.1.4 Serological Tests

Rapid fluorescent focus inhibition test (RFFIT) and fluorescent antibody virus neutralization test (FAVNT) are currently recommended by the WHO and OIE for detecting and titration of the rabies virus neutralizing antibodies (Smith et al. 1973; Cliquet et al. 1998; Moore et al. 2013). Serological tests have been developed to measure the level of virus neutralizing antibody in vaccinated humans and animals and to detect host response to rabies infection by measuring antibodies in CSF/serum (Lackay et al. 2008; Fooks et al. 2009). RFFIT and FAVN are the most commonly used serum neutralisation tests but requires a costly fluorescent microscope and fluorochrome antibody conjugate against rabies may limit its use (Meslin et al. 1996; OIE 2018). FAVN test involves *in vitro* neutralization of a constant amount of rabies virus (challenge virus standard strain adapted to cell culture) and then inoculating into the cell culture (Singh et al. 2017).

In several countries around the world, numerous enzyme-linked immunosorbent assays (ELISA) based methods using either the whole virus or purified G glycoprotein as the detection antigen are available to effectively detect and quantify rabies antibody in large numbers of field sera of vaccinated animals or humans (Grassi et al. 1989; Sugiyama et al. 1997; Cliquet et al. 2000; Servat et al. 2007; Zhang et al. 2009a). Numerous commercial kits are already available for the determination of anti-rabies antibodies in sera, but some are expensive and require highly trained technicians as well as special laboratory facilities to handle tissue culture and the virulent rabies virus (Wasniewski and Cliquet 2012; Singh et al. 2017).

Nevertheless, in general ELISA methods are rapid, require little expertise, do not need high-level biohazard facilities to be performed and several steps of the procedure can be automated (i.e., serial dilution of the sera, addition of reagents, and optical density reading) (Moore et al. 2013). However, the main concern when using assays that employ whole virus as the target antigen is the possibility of cross-reactivity with other antigens that may lead to false positives or inaccurate estimations of the levels of neutralizing-related antibodies present in the sample (Servat et al. 2007; Zhang et al. 2009a; Moore et al. 2013). In accordance with the WHO and OIE recommendations, 0.5 IU per mL of rabies antibodies is the minimum measurable antibody titre considered to represent a level of immunity in individuals that correlates with the ability to protect against rabies infection (Moore and Hanlon 2010; Garg 2013; OIE 2018).

2.11.1.5 Molecular based methods

Molecular techniques play an increasingly important role in many countries, as they can be used for confirmatory testing and epidemiological surveys in laboratories with strict quality control procedures (Fooks et al. 2009; WHO 2018b).

2.11.1.5.1 *Polymerase Chain Reaction (PCR)*

Various molecular methods such as the reverse transcription polymerase chain reaction (RT-PCR), a gel-based hemi-nested RT-PCR assay (hnRT-PCR), a real-time RT-PCR assay (RT-qPCR) (based on a fluorescent DNA stain) and PCR-enzyme linked immunosorbent assay (PCR-ELISA) are available for rapid detection of viral RNA (WHO 2018b).

As described elsewhere, these methodologies were developed to target different genes with different primers (WHO 2018b). In their favour, such molecular-based methods are reported to be highly sensitive (Hanlon and Nadin-Davis 2013). PCR techniques may be applied to fresh saliva, skin samples (often collected for *ante-mortem* diagnosis in humans), salivary glands, and, indeed, virtually to any other tissue or sample including brain material from a suspect animal or human (Madhusudana and Sukumaran 2008; WHO 2018b).

2.11.1.5.2 *Nucleotide sequencing*

Since the PCR fragments generated are usually very short, rabies, or rabies-related viruses from different vector species or geographical areas, can be differentiated by genetic sequence analysis (Warrell 2014; Duong et al. 2016) through direct comparison of the genome obtained with other reference genomes available in databases (Nadin-Davis 2013).

Sanger method

Sanger dideoxy terminator sequencing has become the most widely used sequencing method during the past three decades (Sanger et al. 1977). The method is based on the use of fluorescently labelled ddNTPs (2′,3′-dideoxynucleotides) in addition to the deoxynucleosidetriphosphates (dNTPs) found in DNA. Each of the chain terminator ddNTPs is labelled with a different fluorescent dye, each of which emit light at different wavelengths (Rupprecht et al. 2019). The four labelled ddNTPs (ddATP, ddGTP, ddCTP, or ddTTP) are included in an *in vivo* reaction with the four normal dNTPs and the DNA polymerase.

Sanger sequencing enables the rapid, reliable and relatively inexpensive virus typing of lyssaviruses PCR products or amplicons. The resulting DNA fragments are terminated by the incorporation of the ddNTP at different sites along the template sequence, thus generating fragments of different sizes that can be separated by gel electrophoresis (Rupprecht et al. 2019). Automated DNA sequencers perform capillary electrophoresis for size separation and detection (Shendure and Ji 2008) thereby recording the incorporation of the labelled ddNTPs as fluorescent peak trace chromatograms (Smith et al. 1987).

Using sequence data on several genomic targets, especially N gene for taxonomic classification and immunogenetic studies and the noncoding G-L region for evolutionary events of the lyssaviruses from hundreds of specimens from around the world, a clear picture of the extent of RABV diversity and phylogeny has emerged (Nadin-Davis and Real 2011).

Pyrosequencing

Pyrosequencing is an alternative to the conventional Sanger method of sequencing, which relies on the luminometric detection of a released pyrophosphate molecule during the DNA synthesis (Ronaghi and Elahi 2002). The visible light is released during primer-directed DNA polymerase catalysed nucleotide incorporation. The pyrophosphate released is then converted to ATP. The resulting ATP provides the energy and drives the luciferase enzyme mediated luciferin to oxyluciferin conversion, and visible light is detected. Because the added nucleotide is known, the sequence of the template can be determined (Ronaghi and Elahi 2002). This technique is a widely applicable as an alternative approach for the detailed characterization of nucleic acids, since it is accurate, flexible, enables parallel processing and can be easily automated. Furthermore, the technique avoids the need for labeled primers, labeled nucleotides, and gel-electrophoresis (Fakruddin et al. 2012).

2.11.1.6 Phylogenetic technique

Today, more than ever the study of molecular phylogenetics has become an essential tool to define genetic evolutionary relationships of groups of organisms and for understanding their molecular sequence variation (Nei and Kumar 2000; Drummond et al. 2006). In the past decade, huge progress has been made in the study of molecular epidemiology methods for inferring phylogenies and estimating divergence dates in terms of infectious agents' origin, their geographical distribution, the onset of a newly emergent epidemic and the order of its transmission events (Nei and Kumar 2000). This development has been characterized by increases both in the complexity of the models used to describe molecular sequence evolution, and in the sophistication of the methods for analysing these new models. The objective of most phylogenetic studies is to construct the tree-like pattern of organisms using aligned nucleotide or protein sequence data (Brown 2002). Phylogenetic trees are generated from sequence data by computer programs that employ a variety of different algorithms and methods for tree reconstruction (Nadin-Davis 2013). There are many methods for constructing trees according to the kind of genetic data they use, discrete character states or a distance matrix and according to the algorithm, clustering algorithm and optimality criteria (Jobling et al. 2013) (Table 2.4).

Table 2.2.11.1.6:1: Classification of phylogenetic methods

	Type of data	
Methodology	Characters	Distances (Algorithmic)
Clustering	-	Neighbour joining, UPGMA
Searching	Maximum Parsimony, Maximum Likelihood, Bayesian inference	Minimum evolution
Networks	Maximum spanning, Median Network	Split decomposition

2.11.1.6.1 *Distance methods*

In distance method or matrix methods, evolutionary distances are computed for all pairs of taxa or operational taxonomic units (OUTs), and a phylogenetic tree is constructed by considering the relationships among these determined distance values (Nei and Kumar 2000). They are rapid in terms of execution and show to be efficient as other more computationally intensive methods to identify groups or clades (Nadin-Davis 2013). The distances values are expressed as the fraction of sites that differ between the two sequences. The phylogenetic relationship inferred from pairwise distance matrix can be built by a variety of methods including:

UPGMA (Unweighted Pair Group Method with Arithmetic Means)

The UPGMA is the simplest method of tree construction. It was originally developed for constructing taxonomic phenograms, but it can also be used to construct phylogenetic trees if the rates of evolution are approximately constant among the different lineages (Sneath and Sokal 1973). For this purpose the number of observed nucleotide or amino-acid substitutions can be used. UPGMA employs a sequential clustering algorithm, in which local topological relationships are identified in order of similarity, and the phylogenetic tree is built in a stepwise manner. Although this method is guaranteed to find the correct tree if the distance matrix obeys the ultrameric property, it turns out to be an inaccurate algorithm in practice. Apart from lack of robustness, it suffers from the molecular clock assumption that the mutation rate over time is constant for all species (Horiike 2016).

The neighbour-joining (NJ)

The NJ method (Saitou and Nei 1987) is a commonly used distance-based method for inferring phylogenetic trees, which overcomes the disadvantage of UPGMA. It requires an evolutionary distance matrix calculated from multiple sequence alignment but it does not require a molecular clock (Horiike 2016). The principle of NJ method is to find pairs of operational taxonomic units (OTUs) that minimize the total branch length at each stage of clustering of OTUs starting with a star-like tree. It is, therefore, a special case of the star decomposition method. The merit of the NJ method is that it is fast and can be used to analyse a large dataset (Tamura et al. 2004). PHYLIP (Felsenstein 1993), Clustalw, Clustal X and MEGA X are widely used for inferring phylogenetic tree by this method.

2.11.1.6.2 *Discrete or character-based method*

The main generic advantages of character-based methods are that they enable the computation of ancestral sequences (i.e. the sequence at interior nodes of the tree) and there is no loss of information (they operate directly on the multiple sequences alignment data).

Maximum parsimony (MP)

Maximum parsimony is based on the assumption that the most likely tree is the one that requires the fewest number of changes to explain the data in the alignment. It finds the tree topology for a set of aligned sequences that are subject to the constraint of invoking the fewest possible evolutionary changes (Horiike 2016). Parsimony, or minimum change, is the criterion for choosing the best tree. An algorithm is used to determine the minimum number of steps necessary for any given tree to be consistent with the data. That number is the score for the tree, and the tree or trees with the lowest scores are the most parsimonious trees (Nei and Kumar 2000).

Maximum likelihoods (ML)

ML tries to infer an evolutionary tree by finding the tree that maximises the probability of observing the data. For sequences, the data is the alignment of nucleotides or amino acids. Starting from one nucleotide position, the evolutionary model that gives the instantaneous rates at which each of the four nucleotides changes to each of the other three possible nucleotides, and a hypothetical tree of some topology and with branches of some length. Several scenarios exist, thus the probabilities of each of the scenarios must be determined and added together to obtain the probability of the tree (Nei and Kumar 2000).

Bayesian analysis

The Bayesian method is a character-state method for inferring phylogenetic trees and is based on posterior probabilities under the estimated best model. It employs the concept of likelihood and searches for a set of plausible trees. The method requires the information of a prior distribution on the model parameters, such as substitution model parameters, branch lengths, and tree topology. Posterior probabilities are obtained by exploring tree space using a sampling technique, called Markov chain Monte Carlo (MCMC) algorithms (Ronquist and Huelsenbeck 2003). The Bayesian approach has become popular due to advances in computing speeds and is one of the most widely used methods now because it seeks the tree that maximizes the probability of the tree given the data and the model of evolution (Horiike 2016).

2.12 Rabies diagnosis in Mozambique

In Mozambique, in the veterinary sector, there are few trained and experienced personnel to perform rabies diagnostics. The largest centre for diagnosis of animal diseases in the country is the Central Veterinary Laboratory (CVL) located in Maputo. The CVL is able to diagnose rabies using the following methods: FAT, dRIT, Seller's stain (Negri bodies) and RT-PCR. In addition to the CVL, Mozambique has regional laboratories in the provinces of Gaza, Manica and Nampula. These laboratories can perform the Seller's stain diagnostic test. The human sector does not routinely perform rabies diagnosis, except in cases of requisition during investigations of suspected cases of rabies. The technique used is the Seller's stain test (CDC 2018).

2.13 Rabies differential diagnosis

The clinical signs and length of the incubation period can help in the diagnosis of rabies in animals but since there is no definitive clinical presentation for rabies, all cases of behavioural change and/ or signs of abnormal mental state or paralysis, or a combination of both, should be included as a differential diagnosis of rabies (Constable et al. 2017). Among the various conditions affecting the nervous system and that must be differentiated from rabies, are: a) diseases: canine distemper, ehrlichiosis, toxoplasmosis (cats); pseudorabies, African swine fever (pigs), listeriosis (ruminants), tetanus b) toxicity: equine and bovine encephalitis poisoning due to lead and organochloride compounds, benzoic acid and strychnine poisoning (dogs and cats); c) metabolic: thiamine deficiency, hepatic encephalopathy, polioencephalomacia spongiform encephalopathy, lactation tetany and pregnancy toxaemia (ruminants) (Fekadu 1993; Bishop et al. 2003; Kotait et al. 2009; Constable et al. 2017; Singh et al. 2017).

2.14 Rabies prevention and control

2.14.1 Rabies prevention in humans

Rabies is almost always fatal, but can be effectively prevented by vaccination before or after suspected or proven exposure to the virus. The World Health Organization (WHO) and the Centers for Disease Control and Prevention (CDC) have regular updated detailed guidelines on the prevention of rabies in humans (Jackson 2014; WHO 2018a; WHO 2018b). Eliminating the possibility of exposure through education and awareness campaigns; administering pre-exposure prophylaxis (PrEP) to those at increased risk of exposure and administering post-exposure prophylaxis (PEP) through appropriate wound care, administration of rabies immune globulin (RIG) or a cell culture-based rabies vaccine (CCV) to patients that have been exposed are the strategies involved in the prevention of rabies in humans (Taylor et al. 2013).

2.14.1.1 Prevention of human rabies in Mozambique

As in most of African nations, human rabies cases in Mozambique are also managed at least initially, in peripheral or even village health centres, where human and material resources for basic wound care and rabies prevention are often extremely limited or absent (WHO 2018b). Furthermore, a study by Simone (2016) revealed that traditional healers are an alternative for treating cases of animal bites in Mozambique. The Ministry of Health (MISAU) recognized that some health centres are not in a position to administer treatment to people who have been bitten by dogs, since not all have the capacity to manage and conserve rabies vaccines.

In the first instance, the rabies vaccine and the rabies immunoglobulin are available at the Medical Examination Centres (CEMs) (Guilengue 2018). As a way of ensuring greater action and efficiency for rabies control, a manual was published in 2004 on the prophylaxis and epidemiological surveillance of the disease in Mozambique (Barreto et al. 2004). In the 2000s, the most used human rabies vaccine in Mozambique was RABIVAC® (prepared from the inactivated rabies virus and grown in Human diploid cells - HDCV) (Barreto et al. 2004), but currently and according to information from the Mozambique National Directorate of Pharmacy (DNF), VERORAB® (inactivated and prepared from purified Vero cell rabies vaccine) is the vaccine commonly imported for use in humans in the country.

2.14.2 Control of rabies in domestic animals

Rabies control, especially in stray (free-roaming) dogs is one of the most important and priority areas for the prevention of human rabies, since they are the main vectors for the disease and responsible for more than 99% of rabies cases that occur in humans (Knobel et al. 2005; Meslin and Briggs 2013; Fooks et al. 2014). Control involves the mass vaccination of dogs and cats alongside with (1) eliminating stray animals, (2) public health education and awareness on rabies, (3) responsible pet (dogs and cats) ownership, (4) movement restriction through registration and legislation, (5) national and international collaboration and cooperation, (6) epidemiological surveillance, (7) dog population control and management and (8) laboratory diagnosis.

2.14.2.1 Mass dog vaccination campaigns and vaccine coverage

Mass vaccination of dog populations is considered to be the most cost-effective measure for rabies control and elimination of Dog-Mediated Human Rabies (Wandeler et al. 1993; Cleaveland et al. 2006). Furthermore, compared to the actual cost of emergency PEP treatments for people who have been bitten (US$ 108.07 average costs), the cost of vaccinating dogs remains minimal (US$ 4.03 average), that is, 10% of the overall cost of these treatments would be sufficient to considerably reduce or even eliminate canine rabies (WHO 2018c; OIE 2019).

In rabies endemic countries, the WHO recommends vaccination coverage of at least 70% of the susceptible canine population to ensure the disease is gradually eradicated (Coleman and Dye 1996; WHO 2018b). This goal would be sufficient to maintain the required level of herd immunity in the period between mass vaccination campaigns despite dog population turnover (Hampson et al. 2009). As demonstrated in North

America, western Europe, Japan and some countries of South America (Sabeta and Ngoepe 2018), in certain regions of Africa, there has also been a significant decrease in the incidence of canine rabies and human exposures through parenteral vaccination campaigns of reservoir hosts (free or charged), which have achieved effective vaccine coverage similar to that recommended by the WHO (≥70%) (Davlin and VonVille 2012; Jibat et al. 2015). Among them are the cases of Iringa, in Tanzania with 78% (Gsell et al. 2012); Two cases in Chad with 74% and 71%, respectively (Kayali et al. 2003; Durr et al. 2009); Serengeti ecological region in Tanzania (80%) (Kaare et al. 2009); Tunisia (70%) (Touihri et al. 2011); Zambia with 80% of coverage (De Balogh et al. 1993). In Namibia, there have been successes in the control of dog-mediated rabies, through the development and implementation of a strategy which started in 2015. Among others, the project included training of vaccination teams, health practitioners, laboratory staff and improvement of awareness-raising methods. Surveillance and the implementation of good rabies diagnosis practices were key points for the success of this project, through the involvement of veterinarians, para-professionals and farmers. As a result of mass parenteral vaccination of dogs, human rabies deaths also showed a declining trend, from 23 cases in 2015, 13 cases in 2016, six cases in 2017, to only one case in 2018 (Athingo et al. 2020).

2.14.3 Control of rabies in wildlife

Controlling rabies in wildlife includes reduction or elimination of the reservoir species and elimination of rabies in the reservoir species (Garg 2013). Another option includes protection of the victim species from the reservoir species, especially those behaving abnormally (CFSPH - The Center for Food Security and Public Health 2009; Garg 2013). It involves reducing the opportunities of interaction or contact of wildlife with humans, pets, or livestock by certain measures, such as garbage management, modification or elimination of habitat, and proper storage or removal of human and pet foods (Hanlon et al. 1999; Rupprecht et al. 2001; Rosatte 2011).

These methods may be applied individually or in combination. Initially, depopulation and destruction of reservoir species was the primary and the only feasible measure for controlling rabies in wildlife, but during the past few decades, there have been significant changes in the tactics of wildlife rabies control (Aubert et al. 1994; Garg 2013). The advances in the research and development of oral rabies vaccines and delivery systems for wildlife have now made it feasible to apply the control measures to substantially large areas (Garg 2013).

2.14.3.1 Rabies control in bats

So far there is no practical means of controlling rabies in some inaccessible vector species such as insectivorous bats, despite their potential for infecting people (Warrell and Warrell 2004). Controlling bats by numerically reducing their population in a given region is a short-term strategy (McColl et al. 2000) and not recommended from a conservation perspective. This procedure, which has been carried out through the destruction of their shelters (by means of explosives and/ or fire) or by direct trapping and killing has often resulted in the simultaneous loss of beneficial insectivorous and frugivorous bats (McColl et al. 2000; Mayen 2003; Banyard et al. 2013). On the other hand, the availability of food in the treated area quickly attracts bats from adjacent regions, resulting in rapid re-colonization (McColl et al. 2000; Banyard et al. 2013).

Haematophagous bats have been vaccinated orally with a recombinant vaccinia rabies glycoprotein, and this has been found to confer adequate protection if administered between eighteen and thirty days before challenge (Setién et al. 1998; McColl et al. 2000). However, some studies demonstrated that anti-rabies vaccination in the common vampire bat was effective but is costly and unlikely to reach the widespread populations found throughout Latin America (Setién et al. 1998; Aguilar-Setién et al. 2002; Almeida et al. 2008).

2.15 Vaccines for use in animals

2.15.1 Parenteral Rabies vaccine

Rabies vaccines for animals should be approved by the competent State authorities and comply with national requirements for vaccine (WHO 2018c).

- **Modified Live Virus (MLV) Vaccines:** were widely used for injection in domestic animals, especially in pet dogs and cats. These vaccines have also been used widely for oral immunization of wildlife (e.g. foxes in Canada and Europe, raccoon dogs in Finland) (Day et al. 2016). MLV are all safe derivatives of the SAD (Street-Alabama-Dufferin) virus strain which was developed using hamster kidney cells (Fenje 1960; Yang et al. 2013). However, several of these products have been documented to cause vaccine-induced rabies in target and non-target species, and therefore parenteral use should be discontinued (Fehlner-Gardiner et al. 2008; Müller et al. 2009; Yang et al. 2013; Hostnik et al. 2014; OIE 2018; Pfaff et al. 2019). MLV are also more sensitive to changes in temperature. In addition, accidents of self-inoculation with these vaccines pose a high risk to the vaccinator, hence, in 2004, WHO stopped recommending MLV rabies vaccines for parenteral inoculation in animals (Yang et al. 2013).

- **Vectored Recombinant Rabies Vaccines:** Recombinant vaccine viruses are prepared by inserting non-infectious rabies virus nucleic acid coding for rabies virus glycoprotein into a vector such as avipox for injectable vaccine (Meslin et al. 1996; OIE 2018). They are particularly safe because they do not contain live rabies virus; instead, they contain only the rabies virus glycoprotein G gene that is relevant for protection. Poxvirus (vaccinia and canary pox) and adenovirus vectors expressing the rabies virus glycoprotein are used routinely in North America for the control of rabies in wildlife by the oral route, and in cats (canary pox vector) by the parenteral route. These vaccines are avirulent in all avian and mammalian species tested (Day et al. 2016).

- **Inactivated (Killed) Vaccines**: Worldwide, the following RABV strains have been used for inactivated rabies vaccine: CVS 11, Pittman-Moore–NIL2, RC-HL derived from the Nishigahara strain, and Pasteur virus (Roumiantzeff 1988; Yang et al. 2013). These inactivated rabies vaccine strains are grown in culture systems on baby hamster kidney, hamster lung, guinea pig brain, chick embryo, murine neuroblastoma, or Vero cells. The use of inactivated vaccines is indicated for individual dog and cat protection and mass canine vaccination campaigns (Yang et al. 2013). The virus is inactivated by addition of an inactivant of the first order, usually β-propiolactone (BPL) or ethyleneimine (EI) in the form of binary ethyleneimine (BEI) or ultraviolet light (Yang et al. 2013; OIE 2018). Inactivated rabies vaccines are usually formulated as liquid or freeze dried (Singh et al. 2017). The liquid vaccine is prepared by adsorbing the antigen onto an adjuvant, for example aluminium hydroxide gel, aluminium phosphate and saponin (Yang et al. 2013; OIE 2018). The killed vaccines are easier to manage than live preparations because of their stability at ambient temperatures, and accidents of self-inoculation do not represent a risk (Day et al.

2016). WHO and OIE strongly recommend the use of inactivated vaccines (with or without adjuvant) (OIE 2018; WHO 2018b). Until August 2019, 22,2 million anti-rabies vaccines have been disseminated by the OIE through the vaccine banks. Of these, 6 million have been directly delivered by the OIE to 22 countries to aid their national vaccination programmes (OIE 2019). In the USA, multiple monovalent inactivated rabies vaccines are licensed and marketed for use in dogs and cats, while recombinant rabies vaccine including monovalent (rabies glycoprotein; live canary pox vector) and combination (rabies glycoprotein; live canary pox vector) are available for use in cats and horses, not for dogs (Brown et al. 2016).

2.15.2 Animal rabies vaccines for oral use

Oral rabies vaccines have been produced for free-ranging animals and wildlife species that serve as vectors (Yang et al. 2013). Several types of modified-live, attenuated or recombinant vaccines has been evaluated for the oral rabies vaccination of dogs (Cliquet et al. 2018). The first generation oral vaccines were attenuated rabies viruses developed by conventional *in vivo* and/or *in vitro* serial passaging of virulent field virus isolates resulting in SAD (Street-Alabama-Dufferin) Bern, SAD B19, SAD P5/88, Vnukovo-32 and RV-97 (Metlin 2008; Müller et al. 2015; Pfaff et al. 2019). The original SAD strain was recovered from the salivary glands of a dog infected with RABV during 1935 in the USA (Singh et al. 2017).

The second generation was developed by selection of monoclonal antibody escape mutants, e.g. SAD VA1, SAG1, or SAG2 (Le Blois et al. 1990; Müller et al. 2015). Later, site-directed mutagenesis (reverse genetics) led to the development of a third generation of oral rabies vaccines, e.g. ERA G333 (Bankovskiy et al. 2008; Müller et al. 2015). Finally, a recombinant vaccinia virus expressing the rabies virus glycoprotein from the ERA strain (V-RG) has been used (Kieny et al. 1984; Müller et al. 2015).

In conclusion, SAD B19, SAD Bern and SAD P5/88 strains resulted in mild pathogenic lesions in rodents and in domestic and wild carnivores after the oral administration in these species (Le Blois et al. 1990; Mähl et al. 2014; Singh et al. 2017). Therefore, using all three strains in the field during oral vaccination programmes results in potential risk of disease outbreak and spread in target and non-target host species (Hostnik et al. 2014). However, currently in Europe, RABV strains like SAG2, SAD

Bern, SAD B19 and V-RG strain are used for oral immunization in wildlife for rabies control programmes (Cliquet and Aubert 2004; Animal Health and Welfare - AHAW 2015).

A more recent study suggests that if all the infrastructural, fiscal, and socio-demographic issues surrounding oral vaccination in dogs are taken into account, this method may well be used in dogs as a way to reinforce parenteral vaccination campaigns (Wallace et al. 2020).

2.16 Rabies control in Mozambique

2.16.1 Dog vaccination campaigns

In Mozambique, Central point vaccination strategy is used in urban areas, whereas in rural areas, dip tanks and crush pens constitute vaccination points (Dias and Rodrigues 2003). The Ministry of Agriculture and Rural Development (MADER) is responsible for the vaccination of dogs and cats aged three months or more and for general public awareness of prevention and control of the disease in Mozambique (MASA-DINAV 2016). The Mozambican government, through the National Strategy for the Control of Rabies (2010-2014) initially planned to allocate more funds (60 million meticais, approximately US$ 2 million) for the purchase of vaccines as well as education programs in the country (Government of Mozambique 2010). Over 50% of the estimated total budget for the MADER action plans was intended for the purchase of vaccine. The National Strategy for Rabies Control (2010-2014) also included the use of collars and implementation of Registration and Identification Services for dogs in the municipalities, as a way of recognizing vaccinated animals (Government of Mozambique 2010).

Raising public health awareness consisted of disseminating information on the most popular television and radio channels. Approximately 80% of dogs were targeted for annual vaccination (Ministry of Agriculture and Food Security 2014). The estimated number of dogs in Mozambique is around 800 thousand. Regarding rabies control, the number of vaccinated dogs increased from 163 900 in 2013 to 226 500 in 2016 (an increase of about 38.2%). In 2014 and 2015, the vaccination covered 203.6 thousand and 224 300 dogs, respectively. According to the estimates of the canine population in the country, it is estimated that the vaccination coverage achieved from 2013 to 2016 was about 26%. This vaccination coverage is still far short of the target set by the National Agricultural Investment Plan (PNISA) and by the National Strategy for the Control of Rabies, which was to achieve in dogs, vaccination coverage of 80% (Government of Mozambique 2010; Ministry of Agriculture and Food Security 2017).

2.16.2 Surgical sterilization

Mozambique, since 2001, has included in its rabies control programs, not only rabies vaccination, but also the sterilization of dogs and cats, in cities like Maputo and Matola, Maputo Province, Ibo Island among other places (Felgate 2011; Protect Ponta 2016; Arm 2018). In the country the costs estimated by a team of volunteers for the sterilization of dogs and cats ranged from 20 to 90 US dollars per surgery (Protect Ponta 2016; Arm 2018).

In developing countries, the cost for medicines and consumables per surgical sterilization may vary from approximately US$ 3 to US$ 15 (US$ 7.50 average) depending upon the country (Partners for Rabies Prevention 2010; Garg 2013). The full costs (including veterinarians and veterinary support staff, clinic running costs, all medicines, and consumables) may range from US$10 to US$ 52, with an average of US$30 per sterilisation (Partners for Rabies Prevention 2010).

2.16.3 Current status of dog vaccination coverage and prospects of oral vaccination in Mozambique

According to the Mandatory Sanitation Programs of the National Directorate of Veterinary, rabies vaccination campaigns must occur throughout the year while free vaccination campaigns must be carried out between April-July and September-November of each year (MASA-DINAV 2016).

Despite several parenteral dog rabies-mass vaccination campaigns that are carried out annually in different regions of the country, vaccination coverage for the control of this fatal disease in Mozambique is still not satisfactory, that is, about 9.2%, well below the 70% recommended by WHO (Government of Mozambique 2010). Several factors described in several studies have also been pointed out as the same ones that compromise the achievement of the goals set for vaccination coverage of dogs in Mozambique. Among the various factors, the following stand out: (a) lack of funding, infrastructure and political will; (b) poorly organized campaigns; (c) owners' inability to control their dogs; (d) veterinarians in the field (e) vaccinators' inability to reach dogs without extraordinary effort (f) large proportions of free-roaming dogs; (g) inefficient or non-existent disease notification procedures (Ministry of Agriculture and Food Security 2014; Smith et al. 2019).

As an example, in 2010 the government of Mozambique planned to purchase around 177,557 doses of the canine rabies vaccine, but at the end of the same year, only 121,200 doses were acquired, which makes up only 68% of the forecast (MEF 2011). Despite all these constraints, in recent years there seems to be some progress in controlling canine rabies. However, even that the proliferation of free-roaming dogs in the streets, with incidence in the garbage dumps is recognized by the Mozambican veterinary authorities (MADER 2018). As probable consequence, in 2017 alone, there were about 89 cases of dog-mediated human rabies, out of a universe of over 10,000 animal bite cases across the country (MADER 2018; Bilaide 2019). However, to date, there are no reports of oral dog vaccination programs in the country, an action which could be associated with other forms of rabies control. The same is no longer the case in some African countries such as Tunisia (Matter et al. 1998; Youssef et al. 1998); the Republic of South Africa (Bishop 2001) and Morocco (Darkaoui et al. 2014) where field trials have had some success in controlling rabies through oral vaccination of dogs that are difficult to capture or handle.

2.17 Rabies surveillance in Mozambique

In Mozambique, there is a weekly epidemiological report (Bulletin epidemiological surveillance or BES) that provides updates on eleven selected epidemic-prone diseases, including rabies (WHO 2006). Both animal bites and rabies deaths are notified weekly through a routine surveillance system, which covers all public health facilities in the country (Salomao et al. 2017).

In 2006, a consultancy was requested by the Ministry of Health of Mozambique to the World Health Organization aiming, among others, to assess the country's surveillance system (with regard to detection, reporting, confirmation, analysis, preparedness, response and feedback on selected priority diseases). Although the current epidemiological surveillance system was functioning well at the district, provincial and central levels it was found that in some areas the quality of information generated needed improvement in terms of case detection and reporting; data management and communication; epidemic preparedness and response; feedback and supervision; human resource and training; and coordination of laboratory services (WHO 2006). Other aspects found on the ground also revealed that:

- Laboratory confirmed epidemic pathogens were not reported timely.
- Epidemiological data was not being collected, analysed or reported as planned.
- Inadequate coordination and supervision of the surveillance activities could have contributed to some of these shortcomings.

However, Barreto and co-workers (Barreto et al. 2004) described the measures to be implemented for epidemiological surveillance for the country. Clearly, the following surveillance measures are addressed:

- The case definition;
- Disease control guidelines whose applications depend not only on the health sector, but also involve Municipalities and Veterinary services. These include:
    - Registration and licensing of all dogs;
    - Capture and culling of stray or unowned dogs;
    - Maintenance of vaccination coverage (≥ 75%) of animals through routine vaccination or mass campaign;
    - Capture and clinical observation for 10 days of domestic animals that have bitten someone, or are suspected of rabies;;
    - Immediate shipment of the dog's intact head, preserved on ice (do not freeze) to the laboratory in Maputo;
    - Promotion of cooperation with entities responsible for animals;
    - Treatment of bite wounds including immediate washing with plenty of water and soap;
    - Prophylaxis to all exposed individuals, with vaccines and/ or immunoglobulin;
    - Mandatory notification of the case to the competent health unit;
    - Fill in the registration form and send the copy to the local veterinary services;
    - Inform local veterinary services.

Chapter 3 : Dog Demography, Ecology and Awareness Towards Rabies Among Community Households (HHs) and Health Practitioners (HPs) at the Human-wildlife interface in Limpopo National Park (LNP), Massingir District, Mozambique

3.1 Introduction

Rabies is one of the oldest and neglected infectious diseases known in medical history, and affects all warm-blooded animals, including humans. Today, rabies remains a major public and veterinary health problem in developing countries, especially in the tropical and subtropical regions of Africa and Asia (Banyard et al. 2013; Chiou et al. 2014), where it is considered a re-emerging disease in some areas. The causative agent is a classical rabies virus (RABV) that belongs to the Genus *Lyssavirus*, family *Rhabdoviridae* and responsible for more than 95% of human rabies deaths (Horton et al. 2010; Weyer et al. 2011; Wallace et al. 2017).

Globally, human mortality due to rabies is estimated to be at least 60,000 deaths per year, with 56% of these occurring in Asia and the rest (44%) in Africa, especially in rural areas where stray dogs are responsible for 99% of cases and parenteral dog vaccination is not generally performed (Jackman and Rowan 2007; Digafe et al. 2015; Singh et al. 2017). Poverty and lack of awareness of the disease and its associated hazards have led to increased vulnerability to rabies and are, consequently, major obstacles in its prevention, especially in rural areas (Dodet et al. 2008; Felgate 2011).

Rural communities' understanding of dog behaviour and rabies prevention, as well as responsible pet ownership and appropriate responses to dog bites are essential elements of public awareness programmes (Nilsson 2014; Hasanov et al. 2018). In Mozambique, RABV affects many host species, but cycles are sustained mainly by domestic dogs (*Canis familiaris*) (Bingham et al. 1999), with humans being the dead-end hosts (Dias et al. 1985). However, there have also been reported cases of people being bitten by wild bat-eared foxes and wild dogs albeit with far less frequencies than those attributed to dogs (Swanepoel et al. 1993; Bingham 2005; Banyard et al. 2013; Coetzer et al. 2017).

The National Strategy for the Control of Rabies in Mozambique, was approved by the Council of Ministers in 2010 (Government of Mozambique 2010), with a view to control rabies in animal populations, but rabies cases are reported every year from all ten provinces of the country (Ministry of Agriculture and Food Security 2014). During the last four decades, the human population of Mozambique has grown rapidly, from approximately 12 million in 1980 to about 30 million in 2017 (INE 2017), with consequent occupation of conservation areas (Cuco and Liesegang 2012). These remote communities rely on their dogs for hunting and guarding. The occurrence of rabies reservoirs (mongooses, African wild dogs, genets) in the neighbouring Kruger National Park (Chaparro and Esterhuysen 1993; Marnewick et al. 2014; Grover et al. 2018) may increase the risk of exposure and the spread of rabies in the adjacent Limpopo National Park. The risk to domestic dogs and other wild animals may be increased at the interface resulting from the opening of borders between these two conservation areas, the expansion or introduction of new hosts, and movement of animals (Vial et al. 2006; Newmark 2008; Brown 2011; Bekker et al. 2012).

In Mozambique, there is a general lack of studies addressing the knowledge of, and attitudes to, rabies (Bragança 2005; Guimarães and Moreira 2012). Children under 15 years, who are the main victims of this highly preventable disease and are unaware on where to report bite contact incidents or receive proper treatment according to WHO protocols (Lembo et al. 2010; Dzikwi et al. 2012). In these cases, the majority of bite contact victims seek help from traditional healers rather than their nearest health centres. Furthermore, the suspected rabid dogs are destroyed without first being quarantined (Ghosh et al. 2016; Salomao et al. 2017; WHO 2019).

Despite implementation of the Mozambique National Health Plan for strengthening primary health care, the lack of adequate health facilities, poor infrastructure and inadequate training of medical staff, particularly on the management of dog bites and rabies cases, remains a great concern (Schwitters et al. 2015). Enhancing the level of awareness of public health rabies, particularly in the complex socio-ecological systems that encompass wildlife protected areas, will address identified gaps, cultural beliefs and behaviour patterns. Furthermore, these studies are important in designing relevant public health educational campaigns, and provision of baseline data for planning, implementation and evaluation of national control programmes (Sambo et al. 2014; Hudson et al. 2016). This may assist towards the elimination of dog-mediated human rabies on the continent.

In Massingir District, the ratio of medical doctors to the population ratio in 2018 was 0.1 physicians per 1000 population, similar to the rest of the country (0.07 physicians/1000 population), meaning that healthcare services are mostly provided by technicians with elementary to intermediate skills in medical care (INE 2017; INE 2018). It is crucial therefore that these have the correct level of knowledge on the management of animal bites and prevention of rabies. Some healthcare practitioners do not have an in depth-knowledge of rabies case definitions or are unfamiliar with its clinical signs and symptoms, misdiagnosing it with other fatal encephalitis and thereby contributing to underreporting of rabies cases (Mallewa et al. 2007; Taylor and Knopf 2015). This lack of knowledge is further complicated by the lack of reliable surveillance or epidemiological data (Lembo et al. 2010; Ameh et al. 2014; Ministry of Agriculture and Food Security 2014).

3.2 Objectives

3.2.1 General

This work was carried out with the following objective:

- To assess the level of knowledge, attitudes, practices and awareness of rabies and rabies control measures among households and health practitioners in Limpopo National Park (LNP), in Massingir district, Mozambique.

3.2.2 Specific objectives within the LNP households

The specific objectives of this study aimed:

- To understand the demography and ecology of the LNP canine population
- To assess the knowledge, attitudes and practices of the households related to rabies and its control
- To assess the knowledge, attitudes and practices of the households related to animal bites
- To assess the general practices of the households related to veterinary care seeking behaviour

3.2.3 Specific objectives within the health practitioners

- To assess the knowledge about rabies and guidelines for its prevention
- To assess the knowledge, attitudes and practice related to bite wounds and its management
- To assess the knowledge the WHO rabies vaccination guidelines including the sites and routes

3.3 Materials and Methods

3.3.1 Characteristics of the study area and population

The study was carried out in Limpopo National Park (LNP), Mozambique (Figure 3.1) between September 2016 and April 2018 (Households) and in September 2016 and in April 2018 (health practitioners). The LNP is located to the west of Gaza Province and is delimited by 190 km of fenced international border with South Africa (Kruger National Park) further west, and by both Limpopo (about 260 km) and Elefantes (about 85 km) rivers in the east and south, respectively. It covers an area of approximately 11,000 km^2 (Stalmans et al. 2004; Cambule and Smaling 2013).

Figure 3.1: Limpopo National Park Map showing the study area and study villages indicated in the rectangles (Courtesy of the LNP administration).

The LNP contains 13 villages, situated along the three major rivers, namely the Limpopo River (Macaringue, Munhamane, Cunze, Chipanzo and Maconguele villages); the Shingwedzi River (Bingo, Machamba, Chimangue and Mavoze villages) and Elefantes River (Madingane, Mahlaúle, Chibotane and Macuachane villages). Machamba, Bingo and Mavoze are located inside the park, while the remaining villages are located along the Limpopo River (Buffer Zone), an area still considered to be within the boundaries of the park (Figure 3.1). This buffer zone includes settlements, agricultural lands (crop) and 70% of the livestock population of the park (Massé 2013).

The Buffer Zone extends westwards from the Limpopo River for a distance of 8 - 10 km and northwards from the Elefantes River in the area between the latter's confluence with the Limpopo and Massingir Dam (Le Bel et al. 2011).

The human population of LNP was estimated to about 27,000 in 2001 (Witter 2013), with most inhabitants concentrated in areas with arable alluvial soils (Le Bel et al. 2011). Game hunting and bush meat constitute an important source of protein. Fishing is also an important economic activity for the communities living close to the rivers and along the Massingir Dam (Ministry of Tourism 2003). According to the data provided by the District Services of Economic Activities of Massingir, the census carried out in 2015 in the study area revealed that the population of domestic animals was as follows: 38,147 cattle; 19,338 goats; 6,838 sheep; 2,337 pigs; 1,551 dogs and 51 donkeys.

In Massingir District, there are eight health facilities, namely: Zulo Health Post (Buffer zone), Machamba Health Post (Inside the park), Macaringue Health Post (Buffer zone), Chibotane Health Post (Buffer zone), Mavoze Health Post (Inside the park), Cubo Health Post (Outside the park), Massingir Health Center (Outside the park) and Mucatine Health Center (Outside the park) (Figure 3.2).

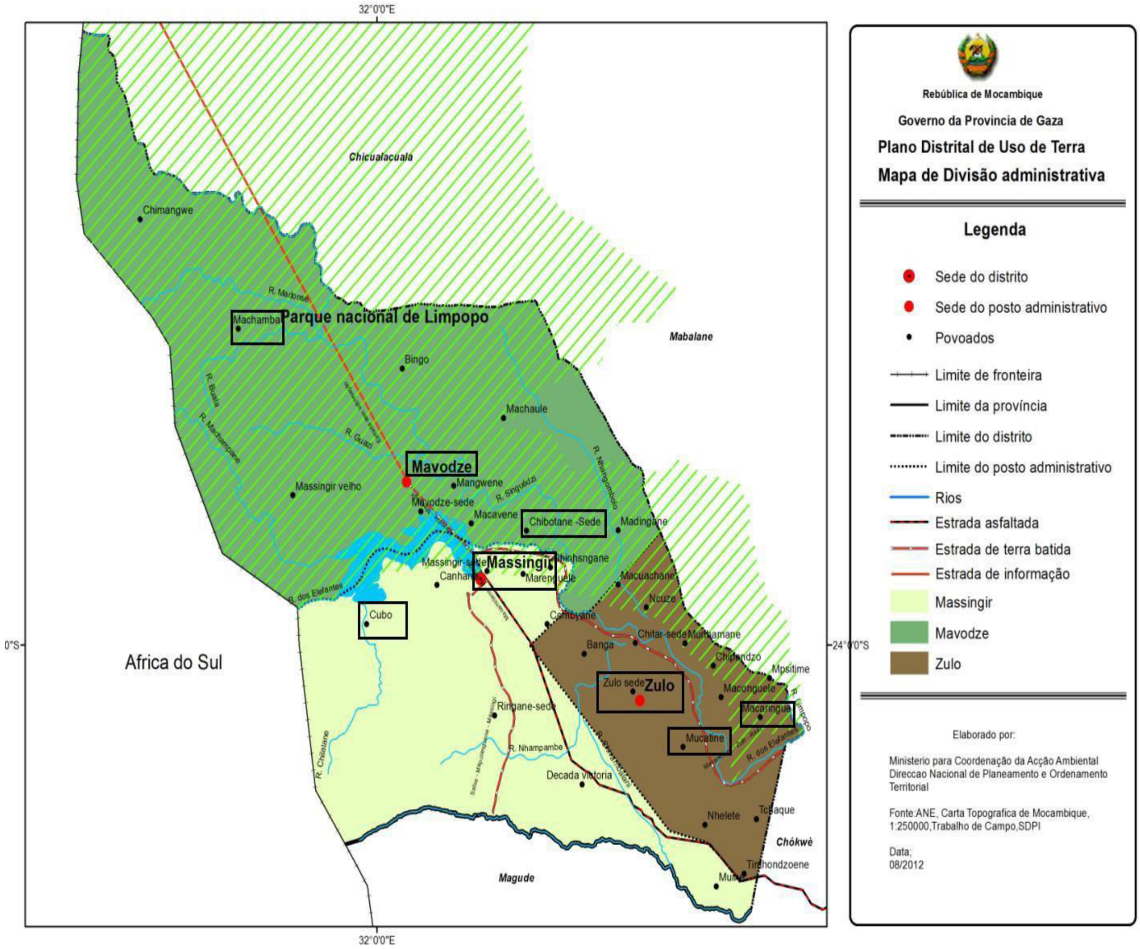

Figure 3.2: Location of all eight Health Facilities of Massingir District (Courtesy of District Services of Economic Activities of Massingir).

3.3.2 Study design and sampling framework

3.3.2.1 Household (HHs) Survey

A cross-sectional study was undertaken between 2016 and 2018 among 233 households selected within eight of the 13 LNP villages. The villages were selected by stratified multi-stage random sampling. A sampling frame of all villages was constructed and the "RAND" function in Microsoft Excel 2010 used to generate random entries. The randomly selected villages were Machamba, Bingo and Mavoze (inside the park) and Macaringue, Mahlaúle, Madingane, Cunze and Munhamane (in the buffer zone) each representing the three main LNP riverine domains (Figure 3.1).

3.3.2.1.1 Sample size

The sample size was calculated using the formula given by Dohoo et al. (2003) taking into account the only two surveys conducted in the country. Due to the difference of absolute number of households within the eight randomly selected villages, an estimated weighted measure (87%) of respondents having knowledge of rabies based on two previous community-based cross-sectional studies of rabies' awareness in central and northern Mozambique (Bragança 2005; Simone 2016) was used to calculate the target sample size, considering 95.0% confidence interval, 80% power, 9% of non-responses.

$$n=Z^2 * (pq/L^2)$$

Where: n = required sample size; Z=1.96 is the value required for confidence of 95%; p = a prior estimate of the proportion; q = 1-p the complementary of prior estimate; L = Margin of error.

With 1968 households in the study area (Parque Nacional do Limpopo 2017), the sample size representative of the Limpopo National Park region was estimated to be 174. However, the sample size was extended to 233 to include another 59 interviewees who voluntarily agreed to participate in the study. In addition, we had resources to administer the questionnaires to 250 respondents.

$$w = n*nh/tnh$$

Where: w = weighted sample per village; n = required sample size (n = 174); nh -total of households per village; tnh – total number of households

The stratification of the population by villages according to the formula is presented in Annex II (Number of households per study villages).

3.3.2.1.2 Household selection

Households aged ≥18 years were selected using the snowballing technique as long as they owned at least a dog, including those that a month prior to the surveys were left without a dog due to death. Existing study subjects helped us recruit future subjects from among their acquaintances.

3.3.2.1.3 Questionnaire preparation, recruitment and interviews

Pre-survey visits were undertaken to explain the general objectives of the project to the village leaders and to request their assistance in encouraging the selected households to participate in the study. The selected participants orally agreed to complete the questionnaire after also being informed of the study objectives. Thereafter, face-to-face interviews were conducted in Changana, the local language spoken in the region of Massingir for appropriateness and ease among study participants, but answers were recorded in Portuguese. The questionnaire provided written response options for each of the questions presented in it. The questions were read out to the respondents, and before the response options were provided, participants were allowed to answer openly.

The semi-structured questionnaire included three main sections: (i) Socio-demographic characteristics (sex, group age, marital status, education, occupation, number of people in the household and type of house); (ii) dog demography, ownership and care practices (numbers of dogs, sex, age, source of dog, purpose of dog, source of food/water, vaccination records, housing and control of dog movement, history and management of dog bites (iii) rabies related questions, consisting of knowledge of the existence of rabies, source of the information, the range of species affected, rabies mode of transmission, symptoms and the outcome of disease and the methods of prevention and other related dog demographic characteristics.

3.3.2.1.4 Healthcare practitioners (HPs) survey

To assess the knowledge, attitudes and practices (KAP) and awareness regarding the transmission and preventive measures of rabies among healthcare practitioners, a cross-sectional study was carried out in all eight health facilities, in Massingir District, Gaza Province (2016 to 2017). The study included 42 health practitioners (two doctors, eight-physician's assistant, 11 caregivers and two technicians) from these allopathic government health and post centres. Participants were purposively selected (Thrusfield 2007) among those who deal directly with patients on a daily basis (clinical, nursing and laboratory personnel). The investigator personally visited the HPs at their workplaces, explained the purpose of the study, assured them of confidentiality with regard to any information they might provide and obtained their oral consent. The medical personnel then completed the self-administered questionnaire during the investigator's visit. The document included, among other variables, those related to age, gender, professional qualifications, place of work, knowledge of rabies, management of bite wounds, vaccination sites and schedules of pre- and post-exposure prophylaxis.

3.4 Data entry and analysis

Two databases were created in Epi-Info, version 7 (CDC, USA) for the two study populations. Data were exported and analysed using SPSS Statistics for Windows, version 18.0 (SPSS Inc., Chicago, Ill., USA). The analysis focused on descriptive statistics to calculate the frequency, proportions, medians and means of the answers regarding both target groups (households and health practitioners).

To assess the dimension of responses (knowledge, attitudes and practices), all potential variables were dichotomized and scored following the scoring systems used in similar previous studies (Sambo et al. 2014; Kishore et al. 2015; da Costa and Fernandes 2016; Kapoor et al. 2019), with some modifications. Details of the scoring criteria for the final assessment of the level of household knowledge, attitudes and practices in the Limpopo National Park are illustrated in Annex IV.

Depending on the type of question, the scores assigned ranged from 0 to 3 according to the degree of clarity and accuracy of the answer. For example, for the questions considered for households knowledge assessment, the maximum overall score stipulated was 13, for attitudes and practices, were 10 and 8 respectively. Knowledge scores were categorized into “Poor” (0-6) and “Good” (7-13); attitudes into “Negative” (0-5) and “Positive” (6-10) and practice scores were categorized into “Poor” (0-3) and “Good” (4-8).

For health practitioners, the maximum overall score was 27 for “knowledge” and five for attitudes/practices. Knowledge scores regarding health practitioners were categorized into “Poor” (0-13) and “Good” (14-27); attitudes/practices into “Inadequate” (0-2) and “Adequate” (3-5).

The questionnaire was tested for internal consistency using the Cronbach alpha index. In general, the previous validation of its contents was done with the help of supervisors' expertise and judgement, and also by means of guidelines published in peer-reviewed scientific journals studies (Sambo et al. 2014; Kishore et al. 2015; da Costa and Fernandes 2016; Kapoor et al. 2019). The KAP on rabies (dependent variables) were compared against HH respondents’ village, gender, educational level, and occupation; health practitioners’ health post, years of service and occupation (independent variables).

Inferential analysis was done using Chi-square test. All analyses were performed considering 95% confidence interval (CI) and *P*-values <0.05 were considered statistically significant.

3.5 Results

3.5.1 Households (HHs) characteristics

3.5.1.1.1 Socio-demographic characteristics

The results of socio-demographic characteristics of HHs are shown in Table 3.1. The majority of interviewees were male (63.1%; 147/233). Most of the participants were aged between 50 and 60 years, comprising 40.3% of study population. Over 60% (141/233) of respondents had at least attended primary schooling and 82.8% (193/233) were farmers.

Table 3.1: Socio-demographic characteristics of households in LNP, Mozambique (n=233)

Variables		Frequency	%
Gender	Male	147	63.1
	Female	86	36.9
Age group (years)	18-21	29	12.4
	22-28	30	12.9
	29-35	27	11.6
	36-42	23	9.9
	43-49	26	11.2
	50-56	42	18.0
	57-60	52	22.3
	>60	4	1.7
Education	None	85	36.5
	Primary	141	60.5
	Secondary	7	3.0
Occupation	Unemployed	21	9.0
	Farmer	193	82.8
	Public servant	4	1.7
	Merchant	1	0.4
	Student	4	1.7
	Bricklayer	4	1.7
	Self-employment	2	0.9
	Fisherman	1	0.4
	Driver	1	0.4
	Miner	1	0.4
	Security guard	1	0.4

Household size

Households comprised 2185 people, with an average of 9.4 people per family. Most household members were under 5 years old (507 people) while 166 people were older than 50 years old (Figure 3.3).

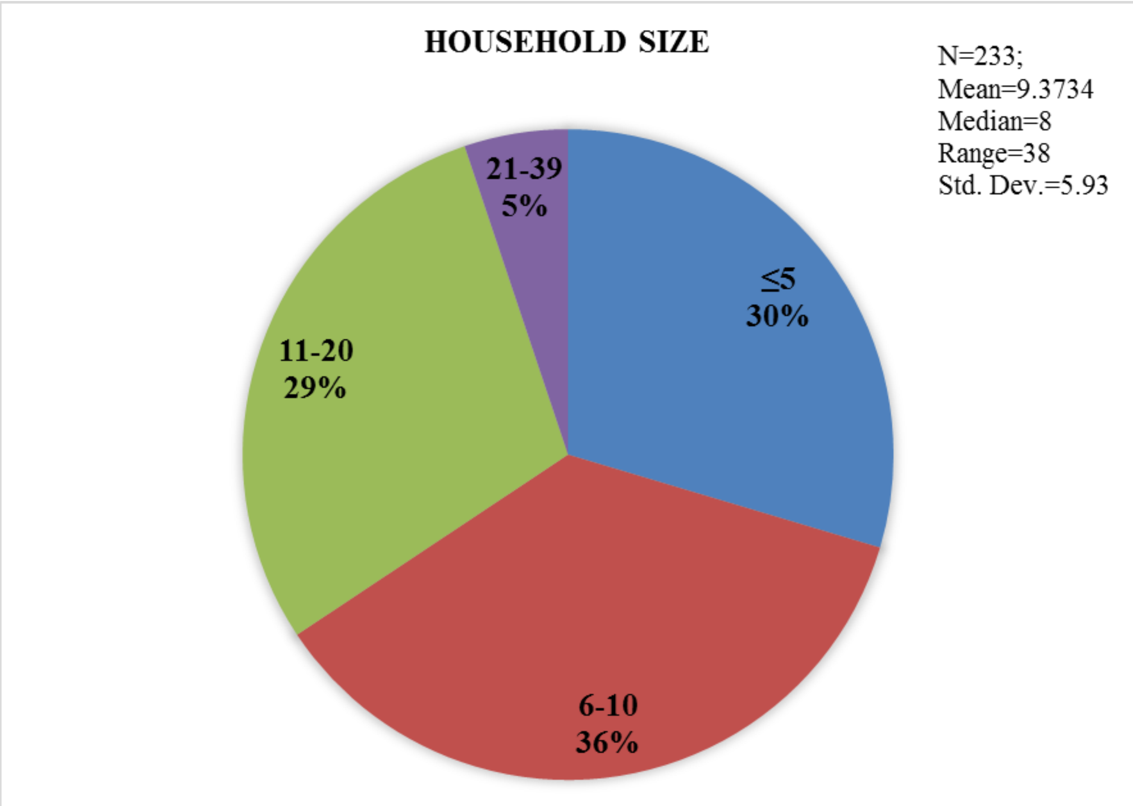

Figure 3.3: Pie chart illustrating households' composition according to the number of people in them

***Livestock* possession**

The majority (75.1%; 173/233) of households owned cattle with a median of five animals. Over 60% (141/233) of households owned poultry with a median of two birds, range 0-37 (Table 3.2).

Table 3.2: Distribution of livestock by household (HH>=1 means households with at least one animal)

Species	HH >=1	% HH>=1	Mean	Median	Range
Cattle	173	75.1	10.5	5	0 – 100
Sheep	25	10.8	0.7	0	0 – 28
Goats	141	60.8	5.8	2	0 – 100
Pigs	32	13.8	0.8	0	0 – 36
Donkeys	3	1.3	0.1	0	0 –3
Poultry	141	60.8	4.4	2	0 – 37

Type of houses and enclosure

Most homesteads were traditional single family houses (Figure 3.4) with 4.7% (11/233) having a fence or wall (Table 3.3).

Table 3.3: Type of households' homesteads and enclosure in LNP, Mozambique (n=233)

Variables		Frequency	%
Households' type of house	Traditional single family house	133	57.1
	Traditional multi-family house	62	26.6
	Modern single family house	33	14.2
	Modern multi-family house	5	2.1
	Total	233	100.0
Type of enclosure	No fence or wall	222	95.3
	Fence or wall, but does not restrain dogs	11	4.7
	Total	233	100.0

Figure 3.4: Traditional single family houses in Madingane Village – LNP

(Author's collection, 2016)

3.5.1.2 Dog ownership and structure

Almost 90% (208/233) households, across all the villages (except Bingo) owned at least one dog with an average of 3.1 dogs per household or 1 dog to 3 person, considering the 2185 persons belonging to the 233 households and a total of 649 recorded in this survey (Table 3.4).

Most of the households (78.5%) owned male dogs and of those, 20.2% (42/208) had their dog sterilised (Table 3.5). The ratio of male to female was (1:0.66). Using logistic regression, there was no association between household size (P=0.832) or the total number of livestock owned (P=0.119) and owning a dog.

Table 3.4: Dog demographics per household size

Variables		
HH size	HH that own a dog	**% of all HHs**
<=5	62	89.9
6-10	72	85.7
>10	74	92.5
Total	208	89.2

Table 3.5: Frequency of households according to their dogs' demographic information

Variables		Frequency	%
Dog gender	Male	183	78.5
	Female	120	51.5
Dog age category	Puppies (0-4 months)	36	15.5
	Juvenile dogs (5-12 months)	46	19.7
	Adult dogs (>12 months)	184	79.0
Neuter/spay dog status	Sterilized dogs	42	20.2
	Intact	166	79.8

Number of owned dogs according to household size

For households with a single dog (n = 64), there was a significant difference in the proportion with male compared to female dogs (73.4% v 26.4% respectively; $P<0.05$). This was also observed in households with multiple dogs (n = 144 households; number of dogs: 2 – 28), where the mean number of male to female dogs was 2.6 versus 1.5 respectively ($P<0.05$). Of the 144 households, 60% (n=86) contained more male than female dogs (Table 3.6).

Table 3.6: Number of owned dogs according to household number (n=233)

Num owned Dogs	Num HHs	1 dog		2 dogs		3 dogs		>3 dogs		Tot al
		M	F	M	F	M	F	M	F	
0	25	0	0	0	0	0	0	0	0	0
1	64	47	17	0	0	0	0	0	0	64
2	53	23	23	26	4	0	0	0	0	76
3	30	7	11	11	7	9	3	0	0	48
4	18	2	7	4	4	7	2	4	0	30
5	15	0	3	6	5	5	6	4	0	29
6	9	0	1	2	2	4	4	3	0	16
7	4	0	2	0	0	0	1	4	0	7
8	7	0	0	1	2	1	2	5	2	13
10	5	0	1	1	2	0	1	4	1	10
11	1	0	0	0	0	0	1	1	0	2
15	1	0	0	0	0	0	0	1	1	2
28	1	0	0	0	0	0	0	1	1	2
Total	**233**	79	65	51	26	26	20	27	5	

M=male; F=Female, Num=Number, HHs=Households, Num=Number

Dog's dynamic indicators (2015-2016)

Table 3.7 summarizes information on the dog dynamics for 12 months (2015-2016), where, among those owning a dog, 213 puppies were whelped (mean of 4.3 puppies per dog- owning household, range 1-10). Puppies' mortality rate was 72.3 deaths per 100 live births.

Table 3.7: Dynamics indicators of female dogs among dog-owning households, according to number of puppies born, number of dead dogs, number of live dogs, offered and acquired in Limpopo National Park, Massingir District, Mozambique (2015-2016) (n=233)

Variables	#HH	%	# Dogs	Mean	Range
Number of puppies born per dog-owning household in the last 12 months	49	21.0	213	4.3	1 - 10
Dogs died from diseases	36	15.5	154	2.7	1 - 9
Dogs alive and in the household's possession	7	3.0	19	4.6	1 - 5
Dogs offered to other individuals	18	7.7	56	3.1	1 - 6
Dogs acquired	9	3.9	41	4.3	1 - 11

Source of the dog and reasons for keeping them

Most (70.2%) dogs were received as a gift. The need to protect crop fields against monkeys' invasions (68.8%; 143/208) followed by security (21.6%; 45/208) were the reasons advanced to keep dogs (Figure 3.5).

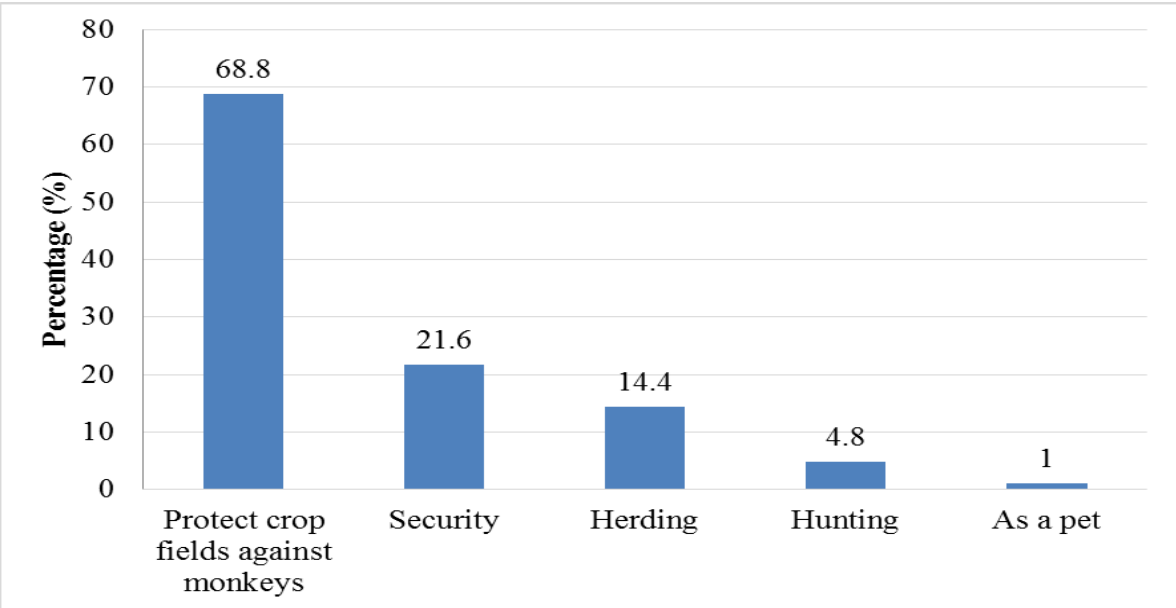

Figure 3.5: Frequency of households' responses regarding the reasons to keep dogs

Type of dog confinement

Less than 20% (41/208) of owners confined their dogs, and those who do, 47.4%, they confine them all the time, followed by 44.7% during daylight and 7.9% at night (Table 3.8). There was no difference in the proportion of households with a single dog that confined their dog compared to those with multiple dogs (22.2% versus 16.5% resp.; P=0.605).

Table 3.8: Source of dog and confinement practices in LNP, Mozambique

Variables		Frequency*	%
Source of owned dog	Breeder	18	8.7
	Found as stray	1	0.5
	Offered/received as gift	146	70.2
	Born in the homestead	37	17.8
	Bought from a neighbour	9	4.3
Dog confinement	Dog is never confined	167	80.3
	Dog confined	41	19.7
Type of dog confinement	Fenced yard	5	2.4
	Leashing (chain or rope)	34	16.3
	Kennel or other type of enclosure	2	1.0

* Multiple responses allowed, totals may not add up to 100%

Dog's source of food/drinking water

In terms of dogs' source of food, the majority of respondents (79.3%; 165/208) fed their dogs with meal leftovers while rivers were the main source of drinking water (81.7%; 170/208) (Table 3.9).

Table 3.9: Dog's source of food and water in LNP, Mozambique

Variables		Frequency *	%
Source of food	Scavenging	17	8.2
	Home leftovers	165	79.3
	Home-cooked food	29	13.9
Source of drinking water	River	170	81.7
	Lake	1	0.5
	Home	35	16.8
	Standpipes	12	5.8
	Other source	1	0.4

Information on unowned dogs

Regarding the possible existence of unowned dogs, most households (79.4%; 185/233) did not confirm their existence, while 16.7% affirmed that they exist. The rest (3.9%), were not sure of their existence in the villages of the park. Of those who pointed out the existence of unowned dogs, 59.5% said that their number had been increasing. The factors mentioned by them as being behind this increase were uncontrolled breeding (96.2%; 25/26) and abandonment and/ or neglect by owners (3.8%; 1/26).

3.5.2 General practices related to healthcare seeking behaviour

Approximately one-third (30.8%; 64/208) of households owning dogs visited an animal care health centre at least once a year (Table 3.10). There was no statistical difference in the proportion of households who visited an animal health officer, based on whether they had one or multiple dogs (29.7% v 31.3% resp. *P*=0.829). The most common reason for visiting an animal health officer was for vaccination (82.8% of visits) while suspicion of rabies or aggressive behaviour accounted for 9.4% (6/64).

Table 3.10: Respondents' general practices related to the veterinary care seeking behaviour in LNP, Mozambique (n=233)

Variables		Frequency	%
Visit to an animal health officer	No	144	69.2
	Yes	64	30.8
	Total	208	100
Reasons for visiting a vet	Suspicion of rabies infection	5	7.8
	Abnormal aggression/barking	1	1.6
	Vaccination	53	82.8
	Prevention of other diseases	4	6.3
	Cropping ears	1	1.6
Reasons for not visiting the veterinarian	There is no veterinary clinic	103	60.9
	Not aware of need to visit a vet	32	18.9
	Do not have a dog	25	14.8
	Unable to handle the dog	3	1.8
	The dog never got sick	3	1.8
	It is far	2	1.2
	It is costly	1	0.6

Attitudes, practices towards bite wound and its management

The majority of respondents (91%; 212/233) would first seek help at the health centres for the treatment of bite wounds, while 4.7% (11/233) would consult traditional healers. At the home level, 64.8% (151/233) of households indicated they would wash their wounds with water (Table 3.11).

The results on bite cases experienced in the previous 12 months within households, and the respective measures taken by victims, as well as the attitudes of the study participants would adopt towards future bite wounds and rabies prevention, are illustrated in Table 3.11.

Among all study respondents, 17.6% (41/233) had experienced a member being bitten by a dog in the previous 12 months. The majority of which (65.9%; 27/41) were bitten by a neighbour's dog(s) and only one (1/41) by a stray dog (Table 3.11). Few of such victims (12.2%; 5/41) sought post-exposure vaccination following a dog bite. In most cases (56.1%; 23/41), a nurse provided the treatment, while a traditional healer assisted in four cases (9.8%; 4/41). Where the respondent knew the status of the dog after the incident (90.2%; 37/41), in 35.1% of cases, the dog had died while the remaining (64.9%; 24/37) had stayed healthy.

Table 3.11: Households' health-seeking behaviour, dog bite experience and treatment applied in case of bites in LNP, Mozambique (n=233)

Variables		Frequency (%)
First place to look for first aid in case of dog bites	None	3 (1.3)
	Health centre	212 (91.0)
	Home level	3 (1.3)
	Home level + health centre	4 (1.7)
	Traditional healer	11 (4.7)
First aid actions at home level if bitten	Nothing	12 (5.2)
	Wash wound with water	151 (64.8)
	Wash wound with soapy water	57 (24.5)
	Traditional/spiritual medicine	13 (5.6)
Household with at least one person bitten in the past 12 months	Yes	41 (17.6)
	No	192 (82.4)
Source of biting dog in previous 12 months	Household's dogs	13 (31.7)
	Neighbours' dogs	27 (65.9)
	Stray dog	1 (2.4)
Treatment applied after bite	Local bite area treatment	28 (68.3)
	Rabies PEP	5 (12.2)
	Traditional treatment	4 (9.8)
	Medical wound treatment	3 (7.3)
	Other	3 (7.3)
	Nothing	1 (2.4)
	Not known	1 (2.4)

Knowledge of rabies: causes, mode of transmission and host range

The results concerning the knowledge of rabies, its transmission, clinical signs, and disease outcome are presented in the supporting information (Table 3.12). In summary, nearly all participants (97.9%; 228/233) had heard of rabies and "Mulunguisse" or "Munyuwisse" (meaning "Madness") are the two local names by which the disease was best known.

A large number of respondents (78.9%) had heard about rabies through a single source of information. When asked about the source of rabies, 96.5% (220/228) respondents correctly identified dogs, while 4.8% (11/228) identified cats, but significantly fewer correctly identified other species to be susceptible to virus infection. More than eighty per cent (82.9%; 189/228) of households were aware of the zoonotic nature of rabies and bites were highlighted by most (88.4%; 167/189) as the most frequent form of rabies transmission to humans.

Table 3.12: Households' knowledge of rabies in LNP, Mozambique (n=233)

Variables		Frequency	%
Ever heard of rabies	Yes	228	97.9
	No	5	2.1
Source of information	Family elders	118	51.8
	Close relatives	23	10.1
	Community neighbours	19	8.3
	Friends	11	4.8
	Teacher	4	1.8
	School	2	0.9
	Radio	2	0.9
	Health professionals	1	0.4
	Combined sources (including traditional healers)	48	21.1
Cause of rabies	Psychological problem	71	31.1
	Worm	1	0.4
	Virus	23	10.1
	Lack of food/water	6	2.6
	Poisoning	2	0.9
	Ears not cropped	1	0.4
	Don't know	124	54.4
Source of rabies	Dogs	220	96.5
	Goats	27	11.8
	Sheep	27	11.8
	Cats	11	4.8
	Other responses	4	1.8
Animals affected by rabies	Dogs	224	98.2
	Goats	26	11.4
	Sheep	26	11.4
	Cats	18	7.9
	Cattle	17	7.5
	Wild animals	4	1.8
Mode of transmission among animals	Bite from rabid animal	199	87.3
	Don't know	29	12.7
Rabies potential zoonotic	Yes	189	82.9
	No	22	9.6
	Unsure	17	7.5
Mode of transmission to humans	Bite from infected animal	167	88.4
	Contact infected saliva	5	2.6
	Don't know	21	11.1

Knowledge of the clinical signs and fatal nature of rabies

In this study, 89.9% (205/228) of interviewees stated they had seen a case of rabies; Rabid dogs were mentioned by all participants (100%; 205/205) as the species they observed with the signs of the disease. Only 7.9% (18/228) observed rabies cases in other species, including three in humans and ten in livestock. Aggression and aimless wandering were the most commonly reported symptoms, accounting for 50% of all observed symptoms. Of the study respondents, 82% (187/228) were aware that rabies is a fatal disease following the onset of clinical signs (Table 3.13).

Table 3.13: Respondents' knowledge of clinical signs of rabies in LNP, Mozambique (n=228)

Variables		**Frequency**	**%**
If ever seen a rabid animal	Yes	205	89.9
	No	23	10.1
Animal seen with rabies	Dogs	205	100
	Cats	5	2.4
	Cattle	2	1.0
	Sheep	4	2.0
	Goats	4	2.0
Humans seen with rabies	Yes	3	1.5
	No	202	98.5
Clinical signs in animals and humans	Aggression	103	50.2
	Aimless wandering	102	49.8
	Salivation	41	20.0
	Loss of appetite	28	13.7
	Open mouth	23	11.2
	Behavioural changes	6	2.9
	Hydrophobia	5	2.4
	Tongue stuck out	4	2.0
	Abnormal vocalization	2	1.0
	Depression	1	0.5
	Restlessness	1	0.5
	Reddish eyes	1	0.5
	Don't know	3	1.5
Recognition of rabies as a deadly disease	No	13	5.7
	Yes	187	82.0
	Unsure	28	12.3

Knowledge of rabies as preventable or curable disease

Concerning the prevention of rabies, 54.8% (125/228) of respondents stated that it could be prevented, usually by parenteral dog vaccination (84.8%; 106/228). Half (50%; 114/228) of participants were aware that, after the onset of symptoms, rabies can no longer be cured, while 25.9% (59/228) incorrectly thought that it was curable through unspecified oral or injectable drugs (45.8%; 21/59) (Table 3.14). Only 1.6% (2/125) were aware of population control (confining dogs) as a preventive measure. When asked about the practices taken in case of an animal with behavioural signs or changes resembling those of rabies, 83.8% (191/228) of respondents said they would kill it, while eight (3.5%; 8/228) would do nothing (Table 3.14).

Table 3.14: Households knowledge and attitudes of rabies as a preventable or curable disease in LNP, Mozambique (n=228)

Variables		Frequency *	%
Perception of rabies as a preventable disease	No	45	19.7
	Yes	125	54.8
	Uncertain	58	25.4
Methods of prevention	Vaccination of dogs	106	84.8
	Cropping ears	5	4.0
	Drug administration	4	3.2
	Killing all suspicious dogs	3	2.4
	Controlling the population of stray dogs	2	1.6
	Cropping the dog ears, cooking and let them eat	2	1.6
	Seeking traditional healers	1	0.8
	Seeking health care	1	0.8
	Don't know	5	4.0
Rabies is curable after onset of clinical signs	No	114	50.0
	Unsure	55	24.1
	Yes	59	25.9
Method of cure (if yes) *	Through administration of anti-rabies vaccine	9	15.3
	Wound washing (soapy water)	2	3.4
	Through traditional/spiritual medicine	2	3.4
	Through oral or injectable drugs	27	45.8
	By cropping the dog ears and add to their food	3	5.1
	By killing the animal	2	3.4
	Curable, but don't know how	14	25.5
Attitudes towards an animal suspected of rabies	Kill the animal	191	83.8
	Expel from home/neighbourhood	30	13.2
	Do nothing	8	3.5

* Multiple responses allowed, total may not add up to 100%.

Dogs' vaccination records

Concerning the vaccination status of their dogs, few (25.8%; 60/233) respondents indicated that their dogs were vaccinated against rabies while, the majority (63.5%) who assumed they did not have their dogs vaccinated, advised this was due to the lack of rabies vaccination campaigns in their villages. The extent of dog vaccination, as proven by anti-rabies vaccination certificate or later by laboratory results of rabies antibody levels, differed significantly among villages (*P*=0.001) as shown in Table 3.15, being Mavoze the one with the highest proportion of vaccinated dogs (6%; 14/27).

Table 3.15: HHs history of dogs' vaccination by village in LNP, Mozambique (n=208) (# = number of households and % = percentage)

Village	**Vaccinated (# and %)**		**No Dogs (# and %)**
	No	Yes	
Macaringue	27 (11.6)	7 (3.0)	7 (3.0)
Machamba	18 (7.7)	0 (0.0)	5 (2.1)
Munhamane	13 (5.6)	12 (5.2)	4 (1.7)
Mavoze	27 (11.6)	14 (6.0)	2 (0.9)
Madingane	12 (5.2)	13 (5.6)	1 (0.4)
Mahlaúle	9 (3.9)	4 (1.7)	4 (1.7)
Cunze	16 (6.9)	7 (3.0)	2 (0.9)
Bingo	26 (11.2)	3 (1.3)	0 (0.0)
Total	148 (63.5)	60 (25.8)	25 (10.0)
		#	%
Reasons for not having the dog vaccinated	Never had information on any vaccination campaign	105	62.1
	Absent at time of vaccination campaign	17	10.1
	Not aware of need to vaccinate	16	9.5
	Was still a puppy	5	3.0
	Was aggressive	3	1.8
	Don't know where to get the vaccine	2	1.2
	Lack of time	1	0.6

Attitudes towards rabies control programme

Almost all respondents who have dogs expressed their willingness to have their dogs registered (96.2%; 200/208). Nearly all participants (99.1%; 231/233) gladly showed their openness to participate in future rabies vaccination campaigns and 95.3% (222/233) to notify the health or veterinary authorities if bitten by a suspect rabid dog (Table 3.16).

Table 3.16: Households attitudes and willingness towards rabies control programme

Variables		Frequency	%
Willingness to have their dogs registered	Yes	200	95.7
	No	2	1.0
	Unsure	6	2.9
Willingness to participate in vaccination campaigns	Yes	231	99.1
	No	0	0.0
	Unsure	2	0.9
Notification to authorities if bitten by dog	Yes	222	95.3
	No	8	3.4
	Unsure	3	1.3
Euthanasia if it is a rabid dog?	Yes	212	93.0
	No	2	0.9
	Uncertain	14	6.1

3.5.3 Results regarding the level of knowledge, attitudes and practices within households

To assess the level of knowledge, attitudes and practices towards rabies among households, the following variables were considered: (a) Villages; (b) Gender; (c) Age; (d) Education; and (e); Occupation. In general, the respondents had good knowledge (18.9%; n=44) and practices (13.3%; n=31) on rabies, and a positive attitude (87.1%; n=203) towards the disease (Table 3.17).

Table 3.17: Knowledge, attitudes and practices towards rabies among households in LNP, Mozambique (n = 233)

Measured variables	Level	Frequency	%
Knowledge	Poor	189	81.1
	Good	44	18.9
Attitudes	Negative	30	12.9
	Positive	203	87.1
Practices	Poor	202	86.7
	Good	31	13.3

Associations between variables under study within households

Table 3.18 shows statistically significant differences (P <0.05) between knowledge of rabies, location of villages and educational level of the respondents.

Respondents living in Mavoze village, for example, demonstrated a good knowledge (27.9%), adopted positive attitudes (90.7%) and most (25.6%) implemented good practices towards rabies. No households in Madingane and only 3.4% in Bingo had good knowledge and good practices towards rabies, respectively. More male respondents had good knowledge (19%), positive attitudes (88.4%) and good practices (13.6%) than females (18.6%, 84.9% and 12.8%, respectively). The 30 to 39 years age group had the most respondents with 22% demonstrating good knowledge on rabies. For attitudes and good practices, the best results were observed in individuals between 20-29 years (91.2%) and 50-59 years (19.1%). It appeared that those with secondary education, showed good knowledge (28.6%) and positive attitudes (100%) towards rabies. Only 10.6% of the respondents without education adopted good practices in relation to rabies. Eight non-farmers (20%) had the best level of knowledge of rabies, while only 10% displayed good practices.

Table 3.18: Test of (χ^2) associations of households' knowledge, attitudes and practices towards rabies and some independent variables in LNP, Mozambique (n = 233)

Factors	Knowledge			Attitudes			Practices		
	Good (%)	Poor (%)	*P* value	Positive (%)	Negative (%)	*P* value	Good (%)	Poor (%)	*P* value
Villages									
Macaringue	12 (29.3)	29 (70.7)	0.032	35 (85.4)	6 (14.6)	0.910	5 (12.2)	36 (87.8)	0.133
Machamba	6 (26.1)	17 (73.9)		20 (87.0)	3 (13.0)		1 (4.3)	22 (95.7)	
Munhamane	5 (17.2)	24 (82.8)		25 (86.2)	4 (13.8)		5 (17.2)	24 (82.8)	
Mavoze	12 (27.9)	31 (72.1)		39 (90.7)	4 (9.3)		11 (25.6)	32 (74.4)	
Madingane	0 (0)	26 (100)		21 (80.8)	5 (19.2)		3 (11.5)	23 (88.5)	
Mahlaúle	1 (5.9)	16 (94.1)		15 (88.2	2 (11.8)		1 (5.9)	16 (94.1)	
Cunze	5 (20.0)	20 (80.0)		21 (84.0)	4 (16.0)		4 (16.0)	21 (84.0)	
Bingo	3 (10.3)	26 (89.7)		27 (93.1)	2 (6.9)		1 (3.4)	28 (96.6)	
Gender									
Male	28 (19.0)	119 (81.0)	0.934	130 (88.4)	17 (11.6)	0.435	20 (13.6)	127 (86.4)	0.860
Female	16 (18.6)	70 (81.4)		73 (84.9)	13 (15.1)		11 (12.8)	75 (87.2)	
Age in years									
≤ 19	4 (14.8)	35 (85.2)	0.847	24 (88.9)	3 (11.1)	0.931	0 (0)	27 (100.0)	0.142
20-29	6 (17.6)	28 (82.4)		31 (91.2)	3 (8.8)		3 (8.8)	31 (91.2)	
30-39	9 (22.0)	32 (78.0)		36 (87.8)	5 (12.2)		5 (12.2)	36 (87.8)	
40-49	4 (12.1)	29 (87.9)		28 (84.8)	5 (15.2)		5 (15.2)	28 (84.8)	
50-59	20 (21.3)	74 (78.7)		81 (86.2)	13 (13.8)		18 (19.1)	76 (80.9)	
≥ 60	1 (25.0)	3 (75.0)		3 (75.0)	1 (25.0)		0 (0)	4 (100)	
Education									
Primary	35 (24.8)	106 (75.2)	0.007	123 (87.2)	18 (12.8)	0.562	22 (15.6)	119 (84.4)	0.322
Secondary	2 (28.6)	5 (71.4)		7 (100)	0 (0)		0 (0)	7 (100)	
None	7 (8.2)	78 (91.8)		73 (85.9)	12 (14.1)		9 (10.6)	76 (89.4)	
Occupation									

Farmer	36 (18.7)	157 (81.3)	0.843	170 (88.1)	23 (11.9)	0.337	27 (14.0)	166 (86.0)	0.499
Non-farmer	8 (20.0)	32 (80.0)		33 (82.5)	7 (17.5)		4 (10.0)	36 (90.0)	

3.5.4 HEALTH PRACTITIONERS (HPs)

3.5.4.1 HPs Demographic characteristics

Fourty-two medical staff from eight health centres completed the questionnaire. Females represented the majority of participants (57.1%; n=24). In terms of professional qualifications, nurses in general medicine were the majority (45.2%; n=19) and only two (4.8%; n=2) were physicians (Table 3.19). The average length of service was 4.57 years (median: 2 years; range: one to 30 years) (Figure 3.6).

Table 3.19: Demographic characteristics of Massingir health practitioners in Massingir District, Mozambique (n=42)

Variables		Frequency	%
Gender of respondents	Female	24	57.1
	Male	18	42.9
Age group	18-24	11	26.2
	25-30	24	57.1
	31-35	3	7.1
	36-40	2	4.8
	> 40	2	4.8
Professional qualifications	Nurse (general medicine)	16	38.1
	Care assistant	11	26.2
	Physician assistant	8	19.0
	Nurse (psychiatric)	2	4.8
	Physician (general practice)	2	4.8
	Nurse (preventive medicine)	1	2.4
	Medical Laboratory Assistant	1	2.4
	Pharmacy technician	1	2.4
Years of service	1-5	31	73.8
	6-10	7	16.7
	11-15	1	2.4
	>15	3	7.1

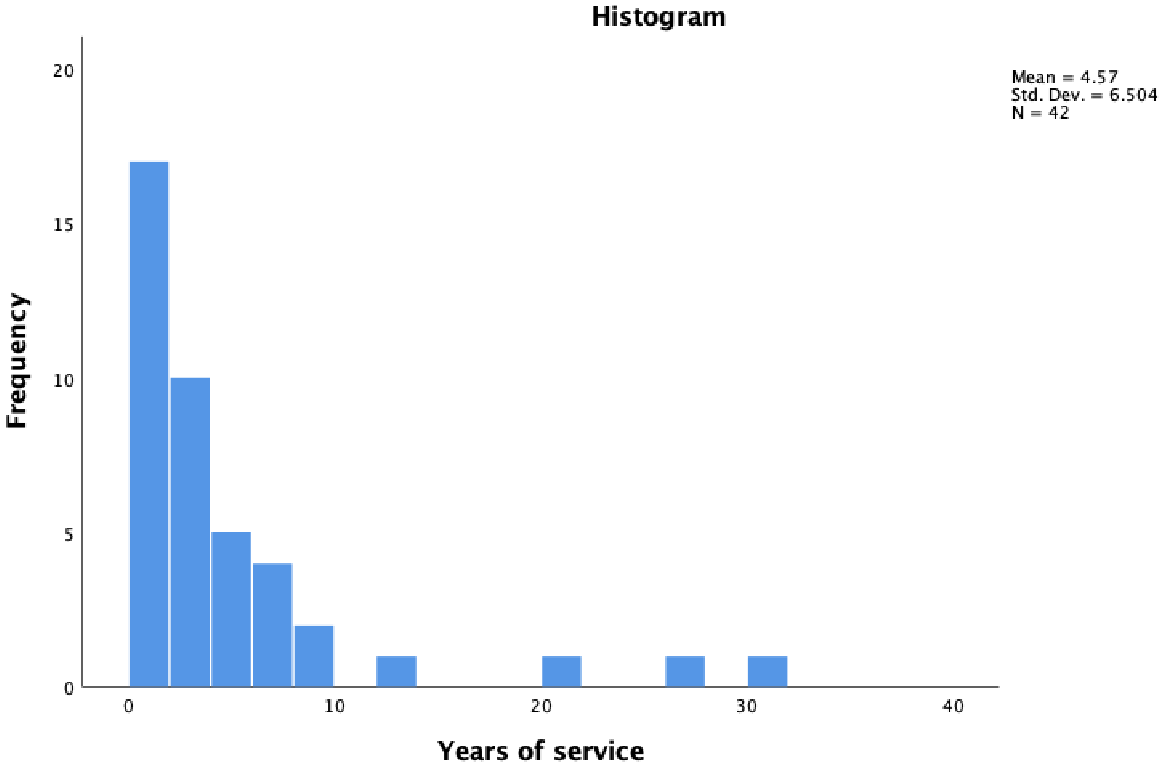

Figure 3.6: Unimodal histogram representing the years of service of health professionals, in Massingir district

Table 3.20: The distribution of health practitioners by gender and profession

Gender	Nurse	Physician	Care Assistant	Physician Assistant	Technician	Total
Female	14	1	8	1	0	24
Male	5	1	3	7	2	18
Total	19	2	11	8	2	42

Experience of handling animal bites and rabies cases

More than half the respondents (n = 24; 57.1%) had some prior experience of handling dog bite cases while 22 (47.6%) reported they had previous experienced of handling rabid cases.

Knowledge of rabies causative agent, transmission, incubation period and clinical features

A virus was correctly mentioned by 76.2% (32/42) of HPs as being the causative agent of rabies, while 85.7% (n=36) stated that dog was the most important source of rabies in Mozambique. Of those who incorrectly identified the source of rabies, five were in their first year of service and one in her second year. Bites were correctly reported as a mode of transmission by the majority of respondents (88.1%; n = 37). However, only 26.2% (n=11) correctly identified saliva and 2.4% (n=1) identified raw meat as a threat. Almost all (95.2%; n = 40) respondents believed they knew symptoms associated with rabies and most (87.5%; n = 35) knew the disease was fatal. However, only 40% (n=16) correctly identified the early, flu-like symptoms of rabies in humans. Similarly, 50% (n=21) of interviewees correctly indicated hydrophobia as a symptom but only 14.3% (n=6) identified paralysis. In all these cases, there was no significant difference between those with more advanced training (e.g. nurses) and the assistants (*P*>0.05) (Table 3.21).

Table 3.21: Respondents' knowledge of rabies transmission and clinical features in Massingir District, Mozambique (n=42)

Variables		Frequency	%
Causative agent of rabies	Virus	32	76.2
	Bacteria	3	7.1
	Don't know	7	16.7
Most important source of rabies in Mozambique*	Dog	36	85.7
	Cat	2	4.8
	Monkeys	1	2.4
	Wild animals	2	4.8
	Other animal species	4	9.5
	Don't know	3	7.1
Mode of rabies transmission to humans*	Bites	37	88.1
	Scratches by claws (nails)	11	26.2
	Contact with infected saliva	11	26.2
	Eating raw meat	1	2.4
	Blood contact	1	2.4
	Don't know	1	2.4
Incubation period of rabies virus in humans*	< 1Week	16	38.1
	1-5 Weeks	7	16.7
	< 3 Months	1	2.4
	Don't know	18	42.9
Clinical signs of rabies in humans*	Fever	27	64.3
	Headache	19	45.2
	Nausea	8	19.0
	Insomnia	7	16.7
	Anxiety	16	38.1
	Paralysis	6	14.3
	Restlessness	14	33.3
	Hallucinations	18	42.9
	Hypersalivation	28	66.7
	Dysphagia	5	11.9
	Hydrophobia	21	50.0
	Death	35	83.3
	Don't know	2	4.8

* Multiple responses allowed, totals may not add up to 100%.

Knowledge of wound categorization and management of bite wounds

Twenty nine (69%) of the 42 interviewees confirmed they knew about the WHO classification of rabies exposure (Figure 3.7) and, of these, most (65.5%; n=19) were correct in identifying mild (Category I) exposure. However, only 10.3% (n=3) health practitioners (10.3%) correctly identified all type of exposure categories (Table 3.22).

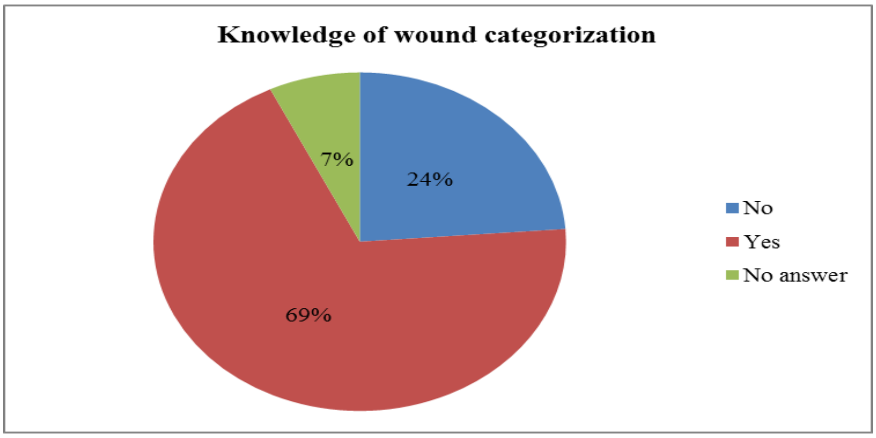

Figure 3.7: Health Practitioners' knowledge of wound categorization (n=42)

Table 3.22: HPs' knowledge of type of exposure in Massingir District, Mozambique (n=42)

Variables		**Frequency (%)**			**Don't know**	**Guidelines**
		CAT I	**CAT II**	**CAT III**		
Rabies exposure categories	Touching or feeding animals, licks on intact skin	19 (65.5)	0 (0)	0 (0)	23 (35.5)	CAT I
	Nibbling of uncovered skin, minor scratches/abrasions without bleeding	0 (0)	9 (27.6)	0 (0)	33 (73.4)	CAT II
	Single transdermal bites or scratches, licks on broken skin	0 (0)	0 (0)	11 (34.5)	31 (66.5)	CAT III
	Multiple transdermal bites or scratches, licks on broken skin	0 (0)	0 (0)	17 (58.6)	25 (42.4)	
	Contamination of mucous membranes or broken skin with saliva	0 (0)	0 (0)	8 (27.6)	34 (73.4)	

Knowledge of the relationship between the site/ severity of the bite wound and the onset of clinical signs

Concerning the knowledge of the relationship between the site/ severity of the bite wound and the period of RABV incubation, over half of HPs (57.1%; n=24) were not aware about this relationship (Table 3.23).

Table 3.23: Knowledge of the relationship between the bite wound severity or location and the onset of the clinical signs (n=42)

Variables		Frequency	%
Awareness of severity/location of the bite and onset of clinical signs	No	24	57.1
	Yes	7	16.7
	Don't know	9	21.4
	Unsure	2	4.8

Practices towards bite wound

Thirty-eight (90.5%) respondents confirmed they knew about wound management. Thirty-seven (97.4%) advised bite wounds should be washed with soapy water, while significantly fewer (18.4%; n=7) felt that antiseptic was required. Interviewees with more than two years' experience (33.3%) were more likely to know this than their less experienced colleagues (12.5%) (P = 0.136) (Figure 3.8).

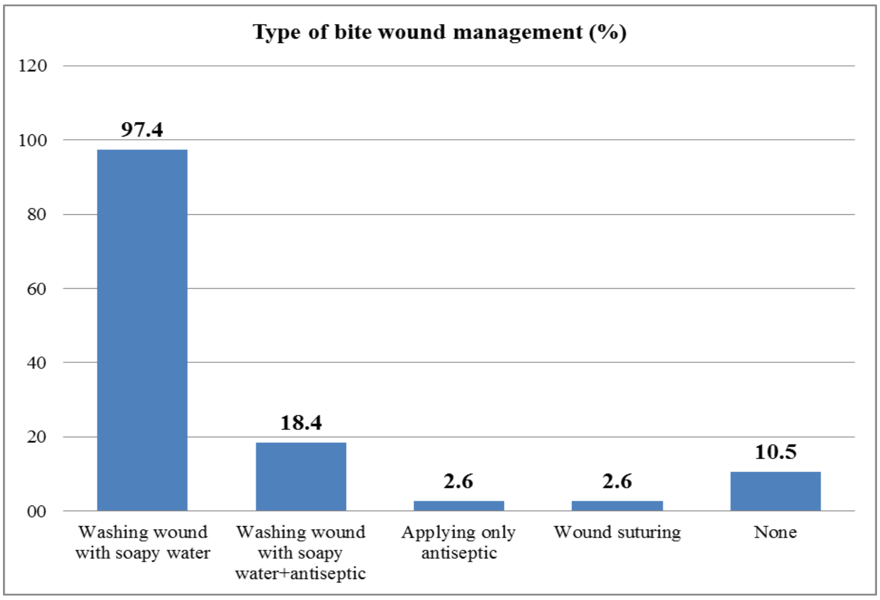

Figure 3.8: Frequency of health practitioners' responses regarding bite wound practices (n=42)

Thirty four (89.4%) indicated they were aware of advice to be given about a suspect rabid dog. However, only thirteen (30.9%) correctly identified the necessity for isolating suspect dogs up to ten days. A significantly higher proportion of interviewees with more than two years' experience (56.3%) were more likely to know this than their less experienced colleagues (22.2%) (*P*= 0.044) (Table 3.24).

Table 3.24: HPs frequency of responses towards advice given to the bitten victim

Variables	Frequency	%
Any advice given to the bitten patient *		
To confine and monitor the biting animal for 1-5 days	10	23.8
To confine and monitor the biting animal for 5-10 days	13	30.9
To confine and monitor the biting animal for 10-20 days	2	4.8
To confine and monitor the biting animal for 20-30 day	8	19.0
Find the owner of the biting animal and ask of vaccination status	4	9.5
None	8	19.0

* Multiple responses allowed, totals may not add up to 100%.

Knowledge of rabies prevention methods and WHO guidelines for the recommended route(s) and site(s) for the anti-rabies vaccination

Regarding rabies prevention, most respondents (83.3%; n=35) correctly reported that parenteral dog vaccination was a valuable preventive measure. Fewer study participants (33.3%; n=14) identified other useful measures such as destruction of suspect animals, public education (26.2%; n=11) or pre-exposure prophylaxis (16.7%; n=7) (Table 3.25). Based on the World Health Organization (WHO) recommendations for the route and sites for rabies vaccine administration, 59.5% (n=25) of HPs stated properly that rabies vaccine could be administered via the intramuscular (IM) route, while 21.4% (n=9) correctly reported intradermal (ID) route as another method of application. On the other hand, 57.1% (n=24) of HPs, correctly answered that the site of application of the anti-rabies vaccine was in the deltoid muscle and the minority of HPs (21.4%; n=9) also mentioned the thigh as the other region for the vaccine application.

Twelve (28.6%) respondents - two nurses (with one and three years of service) and ten assistants - did not know the route of vaccination in people.

Table 3.25: HPs' knowledge of rabies prevention methods, WHO guidelines, route(s) and site(s) for vaccine administration in at-risk population

Variables		Frequency	%	Guidelines
Prevention/control methods *	Parenteral dog vaccination	35	83.3	
	Destruction of suspected rabid animals	14	33.3	
	Pre-Exposure Prophylaxis (PrEP)	7	16.7	
	Educating people on PrEP/Post-Exposure prophylaxis (PEP)	11	26.2	
	Confining all suspected animals	1	2.4	
	Don't know	2	4.8	
Routes of rabies vaccine administration	Subcutaneous	8	19.0	IM/ID
	Intramuscular	25	59.5	
	Intradermal	9	21.4	
	Intravenous	3	7.1	
	Don't know	12	28.6	
Site of rabies vaccine administration	Deltoid	24	57.1	Deltoid/Thigh/Suprascapular
	Gluteus	11	26.2	
	Thigh	9	21.4	
	Don't know	11	26.2	

* Multiple responses allowed, totals may not add up to 100%.

Knowledge on PrEP and PEP regimen and guidelines

Results shown in Table 3.26 below indicate that when asked if they had knowledge about the prophylaxis schemes for PrEP and PEP, more than 80% (n≥34) of HPs were unaware of the schemes. Similarly, only 19% (n=8) and 11.9% (n=5) reported were aware of the regimen to be followed for post-exposure and pre-exposure vaccination respectively.

Table 3.26: Respondents' knowledge of PEP and PrEP schedule in Massingir District, LNP (n=42)

	Prophylaxis			
	PEP		PrEP	
Schedule	Yes	Don't know	Yes	Don't know
Frequency (%)	8 (19.0)	34 (81.0)	5 (11.9)	37 (88.1)

When asked about the best strategy to handle Category I, II and III patients, relatively few respondents identified the correct measures: for Category I – three (7.1%), for Category II - seven (16.7%) and for Category III – 12 (28.6%) (Table 3.27).

Table 3.27: Health practitioners who answered correctly about WHO recommended rabies treatment

Variables*	CAT I	CAT II	CAT III	Guidelines
N	3 (7.1%)	-	-	CAT I (N)
WM+V	-	7 (16.7%)	-	CAT II (WM+V)
WM+V+RIG	-	-	12 (28.6%)	CAT III (WM+V+RIG)

*Variables (N-Nothing; WM-Wound management; V-Vaccine; RIG-Rabies immunoglobulin)

3.5.5 Level of knowledge, attitudes/practices within health practitioners

The results of the level of knowledge and attitudes/practices of rabies are shown in table 3.28. In short, 16.7% (n=7) had good knowledge, whilst 33.3% (n=14) adopted adequate attitudes/practices towards the disease.

Table 3.28: Knowledge, attitudes/practices towards rabies among HPs in LNP, Mozambique (n = 42)

Measured variables	Level	Frequency	%
Knowledge	Poor	35	83.3
	Good	7	16.7
Attitudes/Practices	Inadequate	28	66.7
	Adequate	14	33.3

Associations between variables under study within health practitioners

The results in Table 3.29 show statistically significant differences between knowledge of rabies, gender and occupation. One respondent (100%) of Machamba and Cubo Health posts demonstrated a good knowledge and adopted adequate attitudes/practices of rabies, respectively. There were more male respondents who had good knowledge (33.3%) and adequate attitudes/practices (50%) than females. The 36-40 year age group was the one with the most respondents (50%) and demonstrated good knowledge of rabies, while for attitudes/practices, best results were observed in the 31-35 year old individuals (66.7%), respectively. With regard to years of service, those with 11-15 years of practice (100%) had good knowledge and 100% HPs with 11 years onwards adopted adequate attitudes/practices towards rabies. All physicians (100%) and 62.5% physician assistant were knowledgeable and had adequate attitudes/practices of rabies.

Table 3.29: HPs' level of knowledge, attitudes and practices towards rabies, according to location, gender, age, years of service and occupation, in Massingir district, Mozambique

Factors	**Knowledge**			**Attitudes/Practices**		
	Good (%)	Poor (%)	*P* value	Adequate (%)	Inadequate (%)	*P* value
Villages						
Macaringue	0 (0)	4 (100)	0.226	2 (50.0)	2 (50.0)	0.283
Machamba	1 (100)	0 (0)		0 (0)	1 (100)	
Massingir-Sede	5 (22.7)	17 (77.3)		8 (36.4)	14 (63.6)	
Mavoze	0 (0)	2 (100)		0 (0)	2 (100)	
Cubo	0 (0)	1 (100)		1 (100)	0 (0)	
Chibotane	0 (0)	3 (100)		2 (66.7)	1 (33.3)	
Mucatine	0 (0)	6 (100)		0 (0)	6 (100)	
Zulo	1 (33.3)	2 (66.7)		1 (33.3)	2 (66.7)	
Gender						
Male	6 (33.3)	12 (66.7)	0.031	9 (50.0)	9 (50.0)	0.096
Female	1 (4.2)	23 (95.8)		5 (20.8)	19 (79.2)	
Age						
18-24	1 (9.1)	10 (90.9)	0.549	2 (18.2)	9 (81.8)	0.396
25-30	4 (16.4)	20 (83.3)		9 (37.5)	15 (62.5)	
31-35	1 (33.3)	2 (66.7)		2 (66.7)	1 (33.3)	
36-40	1 (50.0)	1 (50.0)		1 (50.0)	1 (50.0)	
>40	0 (0)	2 (100)		0 (0)	2 (100)	
Years of service						
1-5	4 (12.9)	27(87.1)	0.085	9 (29.0)	22 (71.0)	0.078
6-10	2 (28.6)	5 (71.4)		5 (71.4)	2 (28.6)	
11-15	1 (100)	0 (0)		0 (0)	1 (100)	
> 15	0 (0)	3 (100)		0 (0)	3 (100)	

Occupation						
Physician	2 (100)	0 (0)	0.019	1 (50.0)	1 (50.0)	0.241
Physician assistant	2 (25.0)	6 (75.0)		5 (62.5)	3 (37.5)	
Nurse	2 (10.5)	17 (89.5)		6 (31.6)	13 (68.4)	
Care assistant	1 (9.1)	10 (90.9)		2 (18.2)	9 (81.8)	
Technician	0 (0)	2 (100)		0 (0)	2 (100)	

3.6 Discussion

Rabies is a public and veterinary health problem that affects many nations around the world, including Mozambique. Assessment of knowledge, attitudes and practices concerning the disease, especially among rural communities and health workers residing or working in such a remote region of Limpopo National Park is very important as it can help identify gaps for appropriate intervention plans.

Households (HHs)

Dog demographic characteristics

Establishing effective rabies prevention and control programs involves not only the assessment of levels of knowledge, attitudes and practices of the disease within local communities, but also the understanding of dog demography, ecology and dynamics (Morters et al. 2014; Kisiel et al. 2016). Lack of knowledge about rabies, especially concerning risk factors and prevention, increases the risk of exposure among the Limpopo National Park community and harm for all dogs, livestock and humans – with severe implications for health and livelihoods. As long as the population of the LNP does not know or has doubts that rabies is a preventable disease, it may mean that not only its community, but also their dogs and their livestock, which are their sources of wealth, are at serious risk of exposure to the disease.

The majority of households include children under 5 years old, an age group that is considered to be at high risk of exposure to dog bites, including those suspected of rabies (WHO 2018b). This, again, means that education and awareness programmes really would be an asset to minimize such risks. On the other hand, in LNP, the numerical variation of the canine population increases proportionally to the size of the households. If a vaccination and/ or awareness campaign on rabies or responsible dog ownership education is to be implemented, these findings must be carefully considered. If we consider the percentage of LNP households owning dogs and compare them with other countries such as Tanzania (13%) (Gsell et al. 2012; Mauti et al. 2017), Zambia (42%) (De Balogh et al. 1993), Zimbabwe (62%) (Butler and Bingham 2000) and RSA (10%) (Morters et al. 2014) it can be concluded that in LNP, the human-dog bond is a very important socio-cultural aspect and it must always be taken into account in all circumstances.

The national ratio of dogs to inhabitants in Mozambique was approximately 1:20 in 2009 (Government of Mozambique 2010) and was significantly higher in the LNP (1:3). By contrast, it was 1:14 in some areas of Tanzania (Gsell et al. 2012; Sambo et al. 2014), 1:45 in Zambia (De Balogh et al. 1993) and 1:4.5 in Madagascar (Ratsitorahina et al. 2009).

Taking into account the 1:3 dog to human ratio, the total population of owned dogs in the park would rise from 649 to 6555. Therefore, for parenteral dog vaccination campaigns, there would also be a need to safeguard sufficient doses of rabies vaccine in order to achieve the target vaccine coverage recommended by WHO, which is 70% (Cleaveland et al. 2003; WHO 2018c). In fact, in 2009, the average vaccination coverage achieved in the country was very low, that is, 12.1% (Government of Mozambique 2010) and in 2018, only 8.2% of about 3,011,656 animals at risk (National Livestock Development Directorate 2019).

If the objective of this work was to implement a rabies vaccination campaign in the park, it should take into account the number of unowned dogs since some (16.7%) of respondents acknowledged their existence, while 3.9% were uncertain of their presence in the park.

Other studies also reported a low proportion of ownerless dogs in African communities. Examples include those that were carried out in Zambia with 17% of unowned dogs (De Balogh et al. 1993), 11% in Madagascar (Ratsitorahina et al. 2009), 7.8% in Mali (Muthiani et al. 2015) and 17% in the Democratic Republic of Congo (Mbilo et al. 2019). The apparent low proportion of ownerless dogs should however, be viewed critically, especially since their actual existence is not categorical, as it was based only on the statement of the interviewees. More transect studies, including those based on capture and recapture (Morters et al. 2014) although they are very resource intensive, should be conducted to prove the existence of unowned dogs in the LNP. Thus, in the design of dog population and rabies control programs in the region, until proven otherwise, it should encompass every dog found roaming freely, be it owned or not. In general, the low level of knowledge and the bad practices of rabies within the communities residing in the LNP are a matter of great concern and should be taken into account in awareness campaigns and education about the disease.

Mavoze, which is a village close to the main village of Massingir, with the largest number of inhabitants and the most socio-economically developed compared to other villages, had the best results in terms of knowledge, attitudes and practices about rabies. However, the general gaps observed in the knowledge of rabies, in turn, are advocated, among others, by the lack of knowledge about the causative agent and the different methods of preventing the disease.

Dog ownership

Most (68.8%) respondents owned dogs to protect crop fields against monkeys. These results differ from those obtained in studies conducted and reported in other African countries including Zimbabwe, Chad and Nigeria where nearly 60% of dogs were kept as house guards (security purposes) (Butler and Bingham 2000; Mbilo et al. 2017; Kolawole et al. 2018). Dogs are of greater value for safety and protection purposes in urban and peri-urban areas in some African countries (Butler and Bingham 2000; Bouli et al. 2019) in response to high crime rates (invasion of property) as a result of increased population density and heterogeneity, including differences in socio-economic opportunities (Kunnuji 2016).

For households with a single dog, there was a significant difference in the proportion of male to female dogs. Most of the surveyed dogs were adults and males with few being sterilized. These results are similar to those of Butler and Bingham (2000), Muriuki (2016) with nearly 60% of male and adult dogs as the majority versus only 20% of them sterilized, and Bouli et al. (2019) with 74% male and 62% adult dogs.

In a study carried out in Debark Woreda, in Ethiopia, only 6.9% of respondents had their dogs sterilized (Yalemebrat et al. 2016). Perhaps the predominance of males may be due to the fact that owners want to avoid the birth of undesirable puppies and the nuisance of a bitch in oestrus that attracts intact males and results in fighting. The other possibility may also have to do with security issues, which is the perception of such communities and others such as that of the Federal Capital Territory, in Nigeria (Mshelbwala et al. 2018). Kitala et al. (2001) reported that only males had been castrated as spaying of bitches is a specialized procedure and conceivably out of reach of most of the rural population. In addition to a slow turnover in the population, adults have a completely competent immune system (Day 2007), which allows them to be more resistant to possible debilitating and fatal diseases compared to puppies.

In reference to litter size, the average number of puppies per dog-owning households was 4.3 (range 1-10), consistent with the figures reported in Zimbabwe (4.6) (Butler and Bingham 2000) and Tanzania (4.9) (Czupryna et al. 2016). The high mortality rate in the first year of life (72.3%, i.e., 72.3 deaths per 100 live births) observed in this study has also been reported in poor communities in some countries such as Zimbabwe (71.8%) (Butler and Bingham 2000), Tanzania (72%) (Gsell et al. 2012) and Mali (73%) (Mauti et al. 2017).

Though the causes behind the high mortality rate have not been assessed, it was noticed that puppies do not receive any veterinary care or supervision and in most cases the diet (porridge) is poor. These and other factors that can induce diseases have also been recognized as causing mortality in puppies from South Africa and the Zimbabwean communal land (Rautenbach et al. 1991; Butler and Bingham 2000).

Although the fertility and mortality rates for each age group had not been established, perhaps the population's survival is offset by births and the number of puppies that are acquired annually by households. However, more research is needed for the perception of turnover that occurs in the canine population of LNP.

The LNP surveyed dogs were mostly acquired as a gift from friends and family and 17.8% were offspring of household bitches. The results are similar to those from a study on dog ecology and demography done in Machakos District in Kenya (Kitala et al. 2001) and in Bamako, Mali (Mauti et al. 2017), although in these studies, few owned dogs (9%) had been bought. Thus, we can conclude that the limited economic power of such communities residing in rural areas prevents this practice of buying dogs.

Less than 20% of respondents restricted their dog movements at all times through fencing, leashing, and confinement in a kennel or other type of enclosure. This was similar to those reported elsewhere in Kenya (69%) (Kitala et al. 2001; Muriuki 2016), 79% in Madagascar (Ratsitorahina et al. 2009) and 55% in Mali (Mauti et al. 2017). The same authors reported that such dogs are likely to spend their time roaming freely, foraging for food, but they always returned to their household of origin.

What hinders the practice of confinement among LNP communities are the socio-economic and cultural characteristics of the population. Most LNP residents are poor and the houses do not have fences or barriers that can restrict the movement of their dogs (Figure 3.4). Confinement, rather than a measure of responsible dog ownership, can also minimize the risk of contracting or spreading of rabies, in addition to helping to plan more effective parenteral vaccination campaigns (Tiwari et al. 2019). The consequence of non-confinement can lead to low rates of vaccine coverage. This situation was verified in the study where, the majority of dogs fled from the study researchers, or the owners did not know their whereabouts or even, the animals did not accept to be subjected to any type of restraint.

On the other hand, most households in LNP depend on home leftovers (maize-meal porridge) to feed their dogs. This result is similar to the observation that had previously been reported in Zimbabwe (Butler and Bingham 2000), in Siaya county (Kenya) (Muriuki 2016) and also in Machakos (Kitala et al. 2001) where 86.44% and 95% of the dogs are fed with household leftovers.

According to Léchenne (2015) and since the leftovers are insufficient in terms of quantities, dogs are left to scavenge. From these findings, we concluded that cultural practices and low income lead to poor supervision of dogs in developing countries, since the majority of people do not allocate resources for their dogs. In general, the demographic and ecological questionnaires presented here can serve among others as a baseline to assist the district government authorities and other relevant stakeholders in designing strategies for improving awareness of responsible dog ownership and, implementation of effective dog population and rabies control through an effective association of both sterilization and mass vaccination campaigns (Felgate 2011; Protect Ponta 2016; Hiby and Hiby 2017).

Households' rabies general knowledge and awareness

Poor knowledge and practices in regards to rabies within the LNP communities are matters of a great concern, especially concerning the causative agent, clinical signs and methods of prevention, so awareness and education campaigns should be carried out more routinely in the region. However, Mavoze, a village close to the main village in the district of Massingir (Tihovene) was the one that showed the best results in terms of knowledge, attitudes and practices of rabies. This apparent improvement is the result of it being the most populous and the most socio-economically developed compared to the other seven villages recruited in this study. Our results are in agreement with those of

Awuni et al. (2019) in Ghana, highlighting the influence of the location of households (village) on variables related to knowledge of rabies.

In addition to the influence of villages' location, the probability of those with some level of education to have some knowledge of rabies was more significant than those who never attended school. Results statistically in agreement with our findings regarding the influence of education on the knowledge of rabies and prevention methods were obtained in other studies conducted in Nigeria (Ameh et al. 2014), in Brazil (da Costa and Fernandes 2016), in Morocco (Bouaddi et al. 2020) and in Ethiopia (Alie et al. 2015).

The World Rabies Day could be one of the most important events and vehicle to achieve these objectives. In LNP most of respondents had heard of rabies basically from the elders of the family. Among several studies on rabies KAP, including some carried out in communities in Ethiopia by Alie et al. (2015), Digafe et al. (2015) and Yalemebrat et al. (2016), revealed similar results, although in South Gondar (Ethiopia), mass media had been mentioned by 52.6% of respondents as one of the most important sources of information on rabies (Alie et al. 2015).

In terms of causative agent of rabies, our results are different from those obtained in Ethiopia where 39.0-86% of respondents were aware that a virus was responsible for causing the disease (Jemberu et al. 2013; Guadu et al. 2014; Gebeyaw and Teshome 2016). The differences observed in the misperception of the rabies-causing agent may be due to dissimilarities in sample size, habits and local knowledge/awareness. Some misconceptions about the incorrect causes of disease tend to be easily widespread between the communities investigated.

Most respondents correctly mentioned the mode of transmission of the disease to humans. These positive findings, are in agreement with those of Yalemebrat et al. (2016), Jemberu et al. (2013) and Kabeta et al. (2015) in studies conducted in Ethiopia where interviewees correctly mentioned bites as the main form of transmission, respectively. The higher level of perception observed may due to the fact that most of our respondents had a minimum of primary school education.

One of the points that may facilitate the implementation of rabies control measures is focusing on its main reservoir and most respondents were aware that the domestic dog was indeed the major source of rabies. These findings are similar to other studies conducted in Bahir Dar Town with 71.3% and in Haiti (85.1%) (Guadu et al. 2014; Fenelon et al. 2017). Dogs are in fact responsible for more than 90% of all of human rabies source of exposures in developing countries (Ceballos et al. 2014). The role of cats, bats and wild animals as rabies vectors was little known among residents of Limpopo National Park. Cats and wild mammals (including bats) were also infrequently recognized as possible vectors of rabies in Guatemala as well among French travellers (Altmann et al. 2009; Moran et al. 2015). This low level of awareness expressed by LNP communities is extremely worrying given the location of these villages at the human-wildlife interface.

The lack of positive general awareness of rabies clinical signs observed in the present study is supported by the fact that only half of respondents described correctly at least one of the most common clinical signs of the disease. Most respondents did note the fatal nature of rabies, consistent with reports elsewhere, where knowledge levels ranged from 63%-98.7% (Dhand et al. (2012) in Bhutan, Sambo et al. (2014) in Tanzania and Matibag et al. (2007) in Sri Lanka). When compared with results from the study carried out by Ntampaka et al. (2019) in Rwanda (26%), they are almost similar, considering that the situation in terms of awareness of rabies fatality is not at all lost for the case of LNP households (82%). However, because death is the final outcome after the onset of rabies symptoms, more awareness and community education actions are needed to raise the hazards of the disease.

The majority of respondents mentioned that prevention could be achieved through vaccination of dogs, which is a positive outcome, regardless of the source where this knowledge or information was acquired. The finding is consistent with studies published in Kenya (Mucheru et al. 2014), Sri Lanka (Matibag et al. 2007) and Rwanda (Ntampaka et al. 2019) where a range of 73.9% to 88.1% respondents were aware of rabies prevention through dog vaccination. Unfortunately, we did not assess whether households would be willing to pay for their dogs' vaccination.

Only 25.8% of respondents indicated that they had their dogs vaccinated, slightly lower than those reported in Cameroon (34%) (Bouli et al. 2019), Haiti (35.9%) (Fenelon et al. 2017) and in Democratic Republic of Congo (DRC) (24%) (Mbilo et al. 2019). These may be over-estimates as only 19.7% of respondents were able to show us certificate of rabies vaccination for the previous year, as occurred in the DRC (11%; Mbilo et al. 2019).

Usually, it has been suggested that those who have their dogs vaccinated are likely to have experienced bites from dogs at home or from neighbours' (Mucheru et al. 2014). Nevertheless, it is also known that in Mozambique there are less than 500 veterinarians registered for a vast territory of around 30 million inhabitants (0.17 per 1000 inhabitants), fewer than the median set by the OIE (Bonnet et al. 2011), i.e., 5.4 per million inhabitants.

In addition, there is poor availability of the rabies vaccine in the rural areas. Furthermore, one of the justifications put forward by the DRC (Mbilo et al. 2017) and Ethiopian communities (Jemberu et al. 2013) for the non-vaccination of dogs, is the lack of information about any vaccination campaign, was also mentioned by population of the LNP.

To increase vaccination coverage, regular, joint actions between the local government and various partners regarding community education (for example, radio, television and community meetings) on the importance of vaccinating dogs and the provision of vaccines are required. The World Rabies Day, an event combining awareness campaigns, education and free vaccination of dogs and cats should be extended to rural areas, where the income is often seen as reduced for the purchase of rabies vaccine.

The frequent practice of killing animals suspected of being rabid by the LNP community shows not only a certain unawareness about the measures to be taken in such situations, but also reveals negative attitudes, as it endangers the canine and human populations. It is important to educate the population in Massingir not to destroy them, but to engage a veterinary officer, or to confine the animal for observation for up to 10 days, especially after a history of contact with a human. Such attitudes and practices are not unique to Mozambique but have also been reported in Tanzania, Kenya and in DRC (Cleaveland et al. 2003; Bragança 2005; Mucheru et al. 2014; Sambo et al. 2014; Muriuki 2016; Mbilo et al. 2019). Destruction of an animal suspected of being rabid, contributes to the reduction of reported cases of rabies, since the cadaver will not be examined by an expert, and samples will not be taken for laboratory confirmation.

Cleaveland et al. (2003) also pointed out that the attitude of elimination of suspected rabies dogs reduces the number of people who should receive the PEP.

In the period of this study (two years), a few people bitten by a dog went to the hospital for subsequent procedures. All measures taken in relation to the people bitten were at home level. These health-seeking behaviours are different from the ones documented by Dhand et al. (2012) (91.8%), Sambo (2012) in Tanzania (83%) and in RSA with 80% of respondents reporting that they treat bite wounds in hospitals (Hergert et al. 2016).

However, even with the long distances to reach the few existing health posts and bite management centres, the general willingness shown by most respondents to a case of bite or rabies (observed in the family or not) was that they would seek a health centre and that the home measures taken would be washing the wound with water. Therefore, this demonstrated will is already a good starting point for mitigating cases of rabies. However, in poor rural communities these actions must be reinforced and improved, mainly in relation to the time that must be spent for washing the wounds (15 minutes) (WHO 2018d) using soap and water or other alternative antiseptics (Bloomfield and Nath 2009; WEDC and WHO 2011).

The study disclosed a lack of awareness of the WHO-recommended first aid measure of washing the bite wound with soapy water when compared to respondents from the New Delhi community based study (32%) and respondents from Thailand (70%) (Agarwal and Reddaiah 2003; Matibag et al. 2009). The high response on these preferred first aid measure recommended by the WHO is possibly due to the fact that the studies were conducted in regions where respondents had received some awareness campaigns prior to the studies.

On the other hand, in LNP, as reported in some rural areas of Kwazulu-Natal) (Hergert et al. 2016), local communities do not have access to piped water or a conventional toilet. In LNP communities, water for their daily needs is obtained from rivers, which in turn are very distant from their homesteads. Water scarcity must be taken into account in public awareness programs, including on PEP, taking into account that a simple gesture of immediate washing of wounds with soap and water can substantially reduce (for a five-fold decrease) the risk of contracting rabies (Wilde 2007; Hampson et al. 2008; Sambo et al. 2014).

The low number of households that have their dogs vaccinated is also worrying. Once again, parenteral dog vaccination campaigns and education programs on the correct procedures in cases of bite wounds and the importance of PEP must be implemented regularly. Epidemiological surveillance also needs the same attention. Rural veterinary extension services and the community itself must play an active role in this regard. It is expected that this work alone and taking advantage of the fact that more than 95% of the population is willing to register and vaccinate their dog, is as an important starting point for promoting a discussion and reflection on the best actions and strategies that can be implemented on the ground by government entities and other national and international partners to reduce the risks and impact.

In LNP, the points of great gathering of the population and their livestock, including dogs, are limited to points of vaccination, acaricidal baths as well as crop fields. These can be considered, alongside churches and schools, as strategic places for the implementation of community-based awareness and education campaigns of rabies.

Health Practitioners (HPs) rabies knowledge and awareness

In 2004, MISAU (Ministry of Health) launched a rabies handbook edited by Barreto et al. (2004), in which all the basic information about the disease was described in a simple way. Surprisingly, the health practitioners still have gaps on knowledge of rabies and its prevention, as well as in the management of bites.

Therefore, one purpose of this work is to alert and guide the political actors and managers of the health sectors about the need for training and strengthening the skills of the HPs in matters of rabies prevention and PrEP/PEP guidelines. There is also a need to encourage the implementation of One-Health approaches, which may in turn improve the capacity for epidemiological surveillance and the control and prevention of cases of bites and rabies within the LNP communities.

An example of success with regard to improvements in knowledge about rabies was observed in Chad (Mbaipago et al. 2020) through training and assessments sessions aimed at getting insight into the basic knowledge, attitudes and practices of rabies. The other successful example of implementing a training course was that of Bourhy et al. (2015), held in Senegal, which resulted in the improvement of skills of several African experts (physicians, veterinarians, public health officers and specialists in infectious diseases, virology and / or epidemiology on rabies), in relation to the implementation of control strategies and rabies diagnosis capacity. This type of online and onsite training can also be very well suited and implemented for Massingir HPs and veterinarians.

As a way of showing the existing gaps, a virus as the causative agent of rabies, was not correctly mentioned by all respondents as expected, but by only 76.2%. Other studies have shown similar results (62%-95.23%) in Pakistan (Shah et al. 2009) and in India (Bhalla et al. 2005; Nayak et al. 2013). For a health professional, the correct knowledge of the causal agent of rabies is of paramount importance for making correct decisions about the best therapeutic or prophylactic approaches to the disease.

Rabies can be well mitigated with the correct hospital management of post-bite wounds. Unfortunately, there is no culture for LNP communities to seek the very few existing health facilities following an animal bites exposure. This is demonstrated by the fact that a considerable proportion of Massingir HPs never handled cases of bite (42.9%) or rabies cases (52.4%). A similar and more aggravating scenario was reported in a study conducted in Sokone Health District, in Senegal (Niang et al. 2020), in which only 28.6% of health practitioners had ever handled bite cases.

On the other hand, in LNP there are no specialized care sectors and professionals responsible for bite wound management. This lack of both animal bite clinics and community healthcare-seeking behaviour was also reported by Chowdhury et al. (2013), in a survey among interns of a government medical college in Kolkata, India and in Haiti (Fenelon et al. 2017), respectively. It must always be taken into account that an incorrect approach (e.g. wound suturing) and incorrect identification of patients who experienced different categories of exposure, can represent a risk of rabies for HPs (Jeanpetit et al. 2014). Hence, the need to raise their awareness and that of communities about the importance of both wound management at hospital and home-level.

In the present study there is an evident gap concerning the knowledge of major animal source of rabies in Mozambique, in which the dog was correctly mentioned by only 85.7% of respondents as the primary responsible for human rabies, lower than the reported in other studies carried out in India where at least 98% of respondents (Amrita et al. 2013; Dasgupta et al. 2014), mentioned dogs. Again, it shows that training and capacity building actions are actually necessary.

One-health approaches are important for the health and veterinary sectors particularly in relation to rabies and control of stray and unvaccinated dogs, including reciprocal teaching on proper management of bite wounds, which can decrease the risk of rabies transmission. One observation from the study was that, in addition to the lack of knowledge of the main vector of rabies, the primary mode of transmission of rabies virus was not yet known by all HPs in Massingir District.

Our findings are in agreement with those reported by Amrita et al. (2013) in Gujarat, India, where 98% of interns identified bites, licks, scratches of the infected animal as the modes of rabies transmission. In another study conducted in Delhi (Garg et al. 2013), also the majority (95.6%) knew that the main route of rabies transmission were through animal bites. However, licks on cuts and scratching were respectively listed only by 31.6% and 8.7% of medical doctors (Garg et al. 2013). Although contact with the blood of an rabid animal was wrongly mentioned as another form of rabies transmission by few interviewees, this misconception was also reported in the studies conducted by Singh et al. (2013), Holla et al. (2017) and Mbaipago et al. (2020).

Since contact with blood is not considered a potential means for rabies transmission, there is a need to clarify such issues to professionals. Although we did not assess the level of awareness and the importance of non-intact skin or mucosa for perpetuation of infection, it is pertinent to include these aspects in the training processes of health practitioners. Only then, they will be able to take the correct measures according to the type of exposure.

Knowledge of the incubation period, especially if associated with advanced surveillance (i.e. integrated bite case management) is very important for making a decision on the type of treatment to be instituted in the exposed patient. The existing gaps regarding the knowledge of the incubation period of the disease were evidenced by the majority of respondents and in addition, 23.8% of them declined to answer the question about the incubation period of rabies. Our results were similar to studies in Pakistan and India where the average of correct answers was around 50% in both (Shah et al. 2009; Holla et al. 2017). Usually, on average, the incubation period varies between 3-8 weeks, but may also vary from 5 days to several years (Shah et al. 2009; Hemachudha et al. 2013; Dutta 2014; WHO 2018c). According to some reports, there are recorded cases in which the rabies incubation period lasted for about 7 years (Smith et al. 1991; Grattan-Smith et al. 1992).

More than recognizing clinical signs of rabies in humans, it is important to take precautions in patients with history of wound bites, scratches, or other contact with potentially infectious vector. Clinical features of human rabies were not well-known by many HPs respondents and apart of death, a few have mentioned other clinical signs indicative of encephalitis due to rabies. Our findings are in complete disagreement with those found out in a study carried out by Kishore et al. (2015), where hydrophobia was one of the clinical signs most recognized by the health workers of Uttarakhand, in India (95.7%).

These gaps can be justified by the negligence attributed to the disease compared to others of an encephalitic nature such as malaria and also due to the rare admission of suspected cases of rabies to local health centers. A study conducted by Mallewa et al. (2007) in Malawi, pointed out that a central nervous system infection should not only be attributed to cerebral malaria, although most of the clinical signs are similar to those of rabies. Around ten per cent (10.5%) of the 133 fatal cases attended at the Queen Elizabeth Central Hospital, in Blantyre were due to rabies.

Since wound management and rabies PEP depends on an appropriate pre-assessment and wound classification according to the World Health Organization guidelines (WHO 2018a), the lack of general awareness among HPs in this regard may tarnish the actions aiming at the prevention and/ or induction of an adequate prophylaxis that will prevent rabies from reaching the stage where nothing or little can be done. Another study in coastal south India showed more worrying results, with only 13.6% of participants correctly knowing how to classify wounds in exposure category III (Holla et al. 2017).

Wound washing practices were well-known by the majority of respondents, contrasting with studies developed among Haitians (34%) (Fenelon et al. 2017) and Karachiites (19%) (Shah et al. 2009) health professionals. Unfortunately, it was not possible to collect information on the minimum time spent on wound washing, which according to WHO (2018c) should be performed for at least 15 minutes.

The observation of monitoring biting animals for ten days is important for the determination of the treatment to be instituted, that is, in modifying the treatment regimen from post-exposure prophylaxis to pre-exposure prophylaxis (Singh et al. 2013). One factor that may be behind the low proportion of HPs who would correctly advise this may be due to the fact that most LNP households do not have a fenced back yard, let alone kennels. LNP dogs are never confined and are allowed to roam freely in the neighbourhoods of the district villages.

In addition, the apparent lack of communication and coordination between HPs and animal health officers seems to complicate rabies prevention actions, as it is up to the veterinary sector's staff to confine and closely monitor animals suspected of rabies or those responsible for human bites. The measures or advice given unilaterally by the HPs to patients or victims without addressing the one-health approach were also mentioned and observed by Malhotra et al. (2017), Malhotra et al. (2018) in India, and by Niang et al. (2020) in Senegal.

An important finding is the fact that few respondents mentioned the importance of educating the communities on the importance of PrEP/PEP as actions to prevent rabies in humans. Fenelon et al. (2017) also found the same problem in HPs in Haiti, where only 2.8% mentioned rabies vaccination as a post-bite action. Physicians practicing in rural India are also not adequately informed about the importance of administering vaccinations and immunoglobulins in cases of any dog-bite injury, regardless of its severity (Shankaraiah et al. 2013; Tiwari et al. 2018).

The association between location of the bite and the wound severity (scratch versus deep skin penetration) is likely to affect the outcome into clinical rabies (Tschopp et al. 2016). In the present study, there was a lack of perception of that association and onset of clinical signs. These gaps were also observed in other developing countries such as Haiti (Fenelon et al. 2017), Chad (Mbaipago et al. 2020) where the majority (87.7% and 60%, respectively) of health centre practitioners are not aware of such association. This is very worrying scenario because it can result in death of many patients due to the lack of immediate administration of PEP according to the observed situation. Unfortunately, we did not have access to the records of bites and how they are handled, hence the impossibility of evaluating the risks associated with such conditions.

According to new WHO recommendations launched in April 2018, for both PEP and PrEP, vaccines can be administered by either the intradermal (ID) or intramuscular (IM) route (WHO 2018c). Concerning both site and route of rabies vaccinations, in general, a considerable percentage of our study participants were unaware of their guidelines. Our findings are consistent with those observed in other surveys (66%-73.8%) in Gujarat and in Kolkata in India (Amrita et al. 2013; Chowdhury et al. 2013) and in Karachi, Pakistan (43.7%) (Shah et al. 2009). The lack of refresher and updating courses on neglected diseases including rabies may be behind the concerned gaps.

Knowledge of the categories of exposure is very important for decision making on an effective preventive intervention after an exposure to the rabies virus (RABV) or before exposure to RABV (WHO 2018c). The results of awareness and actions normally taken by HPs according to the exposure category are quite worrying. This shows that for HPs, only big or multiple bite wounds are what matters for the compliance with the universally recommended treatment (Shankaraiah et al. 2013).

Regarding the pre- and post-exposure prophylaxis scheme, the fact that the majority did not know the WHO guidelines reflects the level of training within the HPs. Those who were aware of the pre and post-exposure guidelines were those who had a higher education (doctors), including the only professional in Preventive Medicine. These results are far below those observed by Shankaraiah et al. (2013) and Tiwari et al. (2018) in studies conducted in India, where they reported respectively that 92.6% and 87% of HPs were aware of the schedule of the ARV (antiretroviral) administration.

In Massingir District, less than half of HPs had specific background in the nursing area, and they do not always have access to practical training on anti-viral prophylaxis. The weak and slow level of development of the health sector, including the scarcity of rabies vaccine and RIGs, may also influence the lack of awareness of its use within the health staff. According to Tiwari et al. (2018), the same scenario is also worrying in India, where it was reported that Indian physicians are not adequately informed about the importance of administering vaccinations and immunoglobulins.

3.7 Conclusions, limitations and future steps

The conclusions of this study take into account the low number of health professionals interviewed, an aspect derived from the shortage of human resources in the Massingir district health sector. Based on this study, one can conclude that due to low level of knowledge, lack of training or educational courses, rabies is it a neglected disease in Limpopo National Park, in particular, and in Massingir District in general. The rural communities of LNP have a poor level of knowledge and adopt bad practices towards rabies and this may increase the risk of rabies in the community. The health practitioners have poor knowledge, attitudes and practices on the control and prevention of rabies. Lack of community-based education and medical retraining courses were determinant factors contributing for the poor awareness of rabies. Local education and public awareness raising actions towards rabies and the management of bite wounds should be implemented in the short term.

The Ministry of Health should promote more training and re-training courses on rabies among Massingir health professionals, especially those with a basic academic level, because they constitute the majority of the workers. It is also imperative that the Rabies Handbook published by Barreto et al. (2004), and the updated WHO rabies guidelines are available in all health facilities, health training centres throughout the country.

A major limitation of this study was the fact that there is still a tension between the government and the residents of LNP as regards the resettlement process started in 2006. The resettlement process has created a certain animosity and lack of openness to anyone who wants to work in such communities. In order to better understand the difficulties in accessing the villages and the recruitment of participants for this study, it is advised to read the research work carried out by Milgroom and Spierenburg (2008).

Chapter 4 : Prevalence of rabies antibodies in dogs in Limpopo National Park, Massingir District, Mozambique

4.1 Introduction

Rabies is an acute and fatal encephalitis caused by RNA viruses belonging to the genus *Lyssavirus* and family *Rhabdoviridae* (Singh et al. 2011; ICTV 2019). Currently, the disease poses a veterinary and public health threat especially in the developing countries of Asia and Africa (Zhu and Guo 2016). Annually, it is estimated that at least 59,000 human deaths occur worldwide due to dog-transmitted rabies (Hampson et al. 2015; Wilde et al. 2016; WHO 2018), with the vast majority of deaths (84%) occurring in rural areas (Adebiyi et al. 2014). In fact, more than 95% of human deaths due to the disease occur in Africa (36.4% of global human deaths) and Asia (59.6% of global deaths) (Hampson et al. 2015; WHO 2018). Although rabies is a preventable zoonosis, it is can be still considered as a neglected endemic disease in Mozambique as the true number of cases in humans is also considered to be much higher than recorded throughout the country (Dias and Rodrigues 2003; Salomao et al. 2017).

Despite huge efforts by the government to control the disease, every year the occurrence of animal rabies is reported from all provinces of Mozambique, with an average of 25 cases per year and about 287 human deaths between 2003 and 2010. The domestic dog contributes to at least 88% of the confirmed human rabies cases (Dias and Rodrigues 2003; Ministry of Agriculture and Food Security 2014).

As observed in many African countries, Mozambique also faces a huge problem of under-reporting due to an inefficient or almost non-existent routine epidemiological surveillance system for both animal and human rabies (Nel 2013; Coetzer et al. 2017). Despite the implementation of the Mozambique National Health Plan and the development of the Health Information System for strengthening the health sector and improvement of the quality of surveillance system, in many rural areas the progresses remained subdued.

For example, at the district level in particular, few health workers consider rabies as a priority disease in Mozambique, and it has also been noticed that health information is not translated into action. In addition, individuals do not have their own clinical records, implying that epidemiological data is not being collected, analysed and/or reported on time (WHO 2006; Schwitters et al. 2015; Baltazar et al. 2017; Percival et al. 2018).

In some countries such as the United States of America, where wildlife rabies virus circulates and is predominant, the cost of rabies prevention is substantial and an estimated US$ 300 million are spent annually (Mani and Madhusudana 2013). In Kwazulu-Natal (South Africa), approximately 640,000 dogs were vaccinated in 2012 at a cost of roughly US $3.8 million (Shwiff et al. 2014).

In Mozambique, there is no official data on the involvement of wildlife in the transmission of the disease, especially in rural areas (World Health Organization 1977; WHO 2013). In these areas, rabies is focused only on dogs, notwithstanding the role that wildlife plays in the epidemiology of rabies, since the side-striped jackal (*Canis adustus*), the black-backed jackal (*Canis mesomelas*), mongoose (*Cynictis penicillata*), the bat-eared fox (*Otocyon megalotis*) and the wild dogs (*Lycaon pictus*) are all considered as maintenance hosts of the disease, especially in Southern Africa (Hofmeyr et al. 2004; Nel et al. 2005; Sabeta et al. 2008; WHO and Global Alliance for Rabies Control 2014).

The World Health Organization recommends that for rabies-endemic countries mass parenteral vaccination programmes for dogs and enforcement of responsible dog ownership should be the main measures for rabies control (Banyard et al. 2013; WHO 2018). This is not the case in Mozambique, where in 2010, 2018 and 2019, the vaccination of the dog population at risk was estimated to cover 12 3910 animals (9.2% of all dogs in the country), illustrating the low vaccination coverage against the initial targets (Ministry of Agriculture and Food Security 2014). To highlight the low vaccination coverage in the country, data disclosed in the annual report of the National Livestock Development Directorate showed that in 2018, only 8.2% of about 3,011,656 animals at risk were vaccinated against rabies (National Livestock Development Directorate 2019).

Actually, the low vaccination coverage of dogs, the lack of prophylactic treatment and deficient diagnostic capacity contribute to the poor epidemiological surveillance and control of the disease in rural areas including national parks and reserves where the human population and their domestic animals are in constant conflict with wildlife (Milgroom and Spierenburg 2008; Le Bel et al. 2011; Guimarães and Moreira 2012).

The National Strategy for the Control of Rabies, approved by the Mozambican Government in 2010, with responsibilities shared between the Ministries of Agriculture and Food Safety, Health and State Administration aimed among others to reduce the incidence of animal rabies to less than 10 cases per year, but so far there is little information on the progress of the implemented strategy to the control of rabies in the country. In addition, vaccination campaigns are mainly undertaken in urban areas, forgetting that the re-emergence of rabies has also been associated with rapid urbanization, hence the particular attention should also be paid to both the suburban areas and conservation areas which represent a rapidly growing population (Salomao et al. 2017). However, notwithstanding these difficulties it is encouraging that an animal welfare group in Mozambique supported by the Bill and Melinda Gates Foundation has also begun vaccinating unrestricted dogs along the southern beaches of Mozambique along the border of Northern KwaZulu-Natal in South Africa (World Health Organization 2015).

The impact of these non-profit initiatives in conjunction with veterinary services has led to a reduction in the number of bite cases, increasing access to post-exposure prophylaxis (PEP) (from 9% in 2008 and 2009 to over 40% in 2010) as well as raising awareness of rabies and its control (Felgate 2011).

For the Massingir district in general, and the touristic Limpopo National Park (LNP) in particular, there is little information on the existence of routine rabies vaccination of dogs and cats. The same can be said of the human rabies vaccine for both pre-exposure (PrEP) and post-exposure prophylaxis (PEP), which due to the financial constraints, as well as limitations to its conservation, are not available not only in Massingir, but also in almost all district posts and health centres of the country. These together with the existence of the human population residing inside the park with their dogs and other domestic animals have been reported as being typical barriers of a developing country to conduct successful vaccination campaigns (Shwiff et al. 2014).

According to Limpopo National Park management and development plan, all dogs and cats resident in the park should be vaccinated and sterilized (ANAC - National Administration for Conservation Areas 2003). However, this established measure was considered a medium priority activity in terms of importance and until then, little or almost no information exists on what was actually carried out on the ground and if so, the extent of the long term implications of these vaccinations on the local dog population.

Dogs in LNP appear to be healthy and local communities continue to rely on them for game hunting, protection against predators and herding. However, if activities are limited due to poor vaccination campaigns in terms of dissemination, regularity, accessibility of dogs, knowledge of dog population sizes, sustained infection cycles in wildlife, operational handicaps, obviously the LNP population and their animals will remain at risk. This should be a reason for the Government to upscale its efforts to control rabies.

Even though there is no one and exclusive effective strategy to control the disease in rural areas such as Massingir, it is important to take some steps in this direction. Therefore, to our knowledge this is the first work undertaken and with a view to contribute to the control of rabies by evaluating the levels of antibodies in the dog population within a national park in Mozambique.

4.2 Objectives

4.2.1 General

The main objective of this work was to assess the level of RABV antibodies in the canine population of the Limpopo National Park, Massingir District.

4.2.2 Specifics

The specific objectives of this work were:

- To assess the level of RABV antibodies according to the age of the dogs
- To assess the level of RABV antibodies according to the sex of the dogs
- To evaluate the level of RABV antibodies according to the vaccination status of the dogs
- To determine the proportion of dogs with circulating levels of RABV antibodies capable of inducing an adequate immune response.

4.3 Material and methods

4.3.1 Study areas and population characteristics

The LNP is part of the Great Limpopo Transfrontier Park, which also includes the Kruger National Park (KNP) in South Africa and Gonarezhou National Park (GNP) in Zimbabwe (see Figure 4.1). It covers a vast area of 1,123,316 ha and is located adjacent to the international border with South Africa and to the south of the international border with Zimbabwe, in the west of Gaza Province (Figure 4.1) (ANAC - National Administration for Conservation Areas 2003).

The western perimeter of the Park is defined by the border with South Africa and stretches in a north-south direction for a distance of nearly 200 km. The Zimbabwean border is on the most northern tip of the area and extends in a north-eastern direction. The Limpopo River forms the eastern boundary, whilst the Olifants (Elefantes) River forms the southern boundary (ANAC - National Administration for Conservation Areas 2003).

Of the 27,000 people who reside within the borders of the LNP, 7,000 live in villages along the Shingwedzi River which transects the southern part of the park (Milgroom and Spierenburg 2008). These villages were designated for resettlement outside the park's boundaries in 2003, after the establishment of the park.

The human population within the central parts of LNP was estimated at 6,500 in 2003 with an additional 20,000 living in the eastern and southern boundary settlements (buffer zone) (Milgroom and Spierenburg 2008). Most of the human population is concentrated in areas of arable alluvial soils along the Limpopo, Shingwedzi and Elefantes Rivers. Subsistence hunting and bush meat constitutes an important source of protein (Milgroom and Spierenburg 2008).

Fishing is also an important economic activity for those communities living close to the rivers and along the Massingir Dam. According to the census carried out in 2015 the population of domestic animals was as follows: 38 147 cattle, 19 338 goats, 6 838 sheep, 2 337 pigs, 1 551 dogs and 51 donkeys.

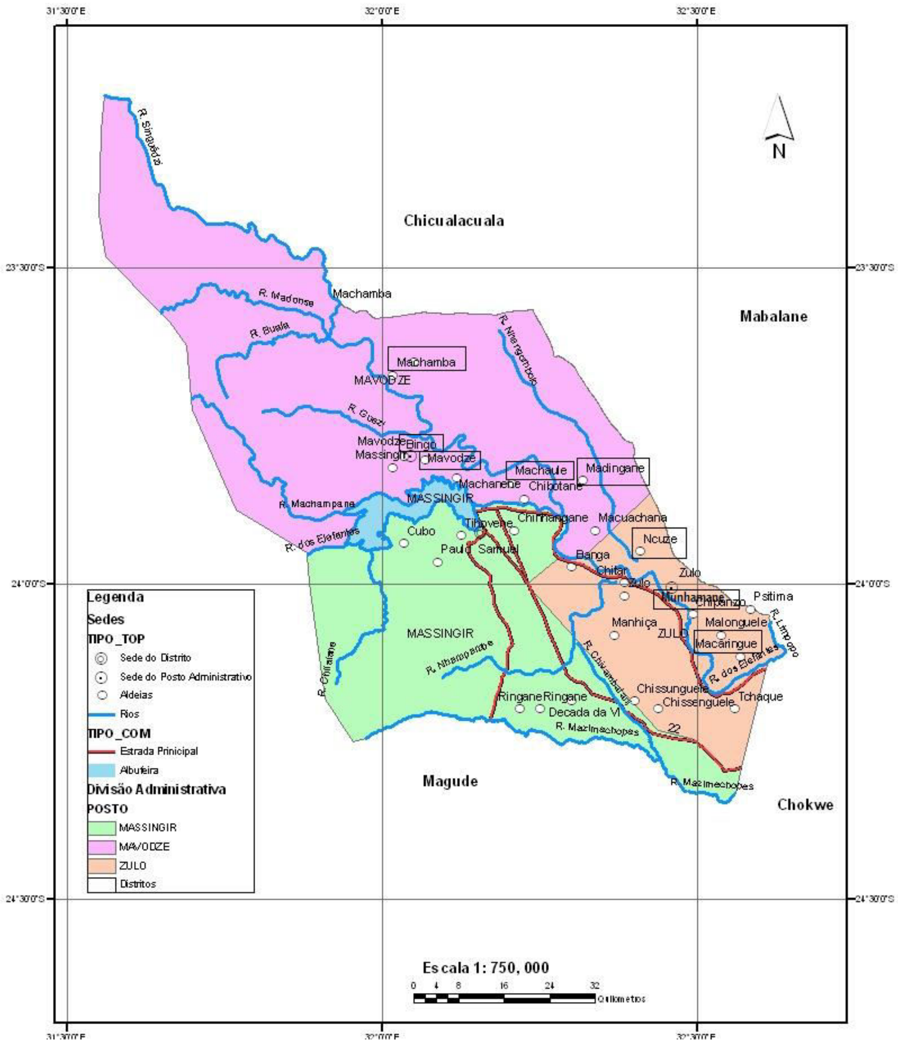

Figure 4.1: Massingir district map showing the study areas

4.4 Study design

4.4.1 Sampling methods and specimen collection

Some dogs included in the present study were vaccinated in the April 2015 vaccination campaign. Blood samples were collected in November and December 2016 and in April 2017, i.e. more than 1 year after the last vaccination. A total of four hundred and eighteen (n=418) blood samples were collected between November 2016 and April 2017 from apparently healthy dogs belonging to the households of eight (n=8) out of 13 LNP villages selected by stratified multi-stage random sampling. In 2015, there was a vaccination campaign in the park. These villages (Machamba, Bingo and Mavoze, Macaringue, Mahlaúle, Madingane, Cunze and Munhamane) were randomly selected, following a previous research study that assessed the level of knowledge, attitudes and practices of rabies within the local communities (Figure 4.1). Households with dog(s) were selected using the "chain referral" sampling method.

4.4.1.1 Selection of the dog population

All apparently healthy dogs from the selected households were targeted for blood collection irrespective of their age, sex, vaccination history or status and type of confinement. Owners were advised to ensure that the dogs were not roaming freely at the agreed time for the collection process. For this purpose, leftover food was used as bait. To prevent others from escaping, researchers resorted to the use of catch nets and dog-catchers.

As most of the dogs in the LNP do not have an identification document, their ages were determined based on oral information provided by the owners and confirmed based on size and/ or dentition. For all selected dogs, their owners were asked whether or not their animals had ever been vaccinated. If so, they had to show any proof (card, certificate, etc). If not, the district veterinary technicians who are usually in charge of the rare mass vaccination campaigns in the park, helped to confirm the veracity of the related information.

4.4.2 Sample size

The total sample size was estimated according to the formula given by Thrusfield (2007) assuming that 50% of the dogs would have circulating antibodies against rabies; with a 5%, margin of error and 95%:

$n = 1.96^2 \times P_{exp} (1-P_{exp}) / d^2$

Where: n = required sample size; P_{exp} = Expected prevalence; d = desired absolute precision.

According to the Thrusfield formula, a study sample of 384 was calculated. However, to increase statistical power, a total of 418 blood samples were collected. Using proportionate stratified random sampling the calculation for each stratum (dogs/per village) the following formula was used (Cochran 2007):

$n_h = (N_h / N) \times n$

where n_h is the sample size for stratum h, N_h is the dog population size for stratum h, N is total population size, and n is total sample size. Details of the dogs sampled per village are illustrated in Table 4.1.

Table 4.1: Details of the sample size of the canine population covered in this study

Village	**Total number of dogs estimated per selected village**	**Intended sample size per stratum**	**Sample size retrieved per stratum**
Bingo	63	37	64
Macaringue	126	74	51
Munhamane	56	33	44
Madingane	81	47	51
Malhaule	57	33	33
Machamba	64	37	75
Cunze	63	37	52
Mavoze	147	86	48
Total	657	384	418

a) Inclusion criteria and term of consent

Dogs were included taking into account the number of household members owning at least one dog until the intended sample size was reached. Pre-survey visits were performed to obtain an oral consent from the leaders of the villages and also to request them to mobilize their community members to participate in the study. The purpose of the study had already been explained to them in the interviews conducted on previous visits. All owned dogs that were present in the owners' homes or in the vicinity of the target village neighbourhood streets at the moment of the visit were included in this study, as long as the owners agreed orally that blood samples could be collected.

4.5 Blood collection and processing

Dogs were manually restrained and muzzled to prevent dog biting. For those difficult to handle, to restraint or to catch, dog-catchers poles and nets were used. Four millilitres of blood was collected from the cephalic or saphenous vein, using plain vacutainer blood tubes. The blood samples were allowed to clot at 5°C and the serum decanted into a sterile Eppendorf tube. To ensure that higher volume of serum was obtained, the remaining blood samples in plain vacutainer blood tubes were centrifuged at 2500 RPM for 15 minutes (Centrifuge 5430, Eppendorf, Hamburg, Germany), then transferred into 2 mL sterile Eppendorf tubes (Eppendorf, Hamburg, Germany) and stored at -40°C until required for analyses. Both vacutainer and Eppendorf tubes were immediately coded with the date of collection, sex of the dog, name of the owner and village. The serum samples were sent to the Rabies Unit laboratory at ARC-OVI (Agricultural Research Council-Onderstepoort Veterinary Institute, Pretoria, South Africa) for testing.

4.5.1 ELISA test

The rabies virus antibodies were assessed using the BioPro® Rabies ELISA kit (BioPro Rabies ELISA, BioPro, Prague, Czech Republic) according to the manufacturer's instructions. The BioPro ELISA, is a commercial blocking ELISA used to detect rabies virus binding antibodies in canine serum or plasma. In comparison with "Gold standard" methods like fluorescent antibody virus neutralisation (FAVN), or rapid fluorescent focus inhibition test (RFFIT), this is rapid, simple and more convenient especially when the serum is derived from foxes, which in turn, easily loses quality both through autolysis and bacterial contamination. The wells of microplates are coated with rabies antigen.

The test serum samples together with control sera included in the kit, were diluted two-fold as per the manufacturer's instructions. After washing, biotinylated anti-rabies antibody was added to wells. In the case of positive samples specific antibodies blocked the binding of biotinylated anti-rabies antibodies with coated rabies antigen. In the case of negative samples, biotinylated anti-Rabies antibodies form antigen-biotinylated antibody complex. Briefly, 60 µL of diluent buffer was distributed into dummy microplate wells, followed by 60 µL of serum samples as well as the control sera that included positive and negative controls. One hundred microliters of diluted serum samples were dispensed into the designated wells of rabies glycoprotein-coated microplates included in the kit after mixing with the aid of a pipette. The microplates were covered with adhesive foil and incubated overnight at 4°C, with gentle shaking on an orbital shaker at 125 rpm. Six washes were performed after incubation to remove unbound antibodies and other proteins contained in the sera. Then, 100 µL of the biotinylated anti-rabies antibodies were distributed into each well using a 12-channel micropipette. The microplates were incubated for 30 minutes at 37 °C with gentle shaking.

Four washes were performed to remove the unbound biotinylated anti-rabies antibodies. Then, 100 µL of the streptavidin peroxidase conjugate were distributed to each well. The microplates were incubated for further 30 min at 37 °C with gentle shaking. Four washes were performed after incubation to remove unbound conjugate. The presence of the antigen-biotinylated antibody complexes was revealed by adding 100 µL of 3,3', 5,5;-tetramethylbenzidine (TMB) chromogen solution to each well. The microplates were incubated in the dark for 20 minutes at room temperature with gentle shaking. The enzymatic reaction was stopped by adding 50 µL of a solution of 0.5 M H_2SO_4 per well and absorbance read at 450 nm using a microplate reader.

4.5.2 Test validation

As described in the manufacturer's guidelines, the optical density (OD) of a negative control was above 1.0. Differences between means of OD of negative and positive controls sera were also considered, as long as the values were ≥ 0.8. The percentage of blocking (%PB) was calculated for each sample according to the manufacturer's specifications (i.e. %PB = ((ODNC − ODsample)/ (ODNC − ODPC)) ×100) where ODNC is the optical density of the negative control, ODPC the optical density of the positive control and OD sample is the optical density of the sample. The conditions and specifications of validation set by the manufacturer were implemented to meet the results obtained for the different dog samples. To ensure that the test was working in optimal conditions, the panel of positive control sera (CS1, CS2 and CS3) were included in the test.

4.5.2.1 Interpretation of ELISA results

Dog sera with PB less than 40% were considered negative for rabies antibodies, whereas PB equal to or higher than 40% but less than 70% was regarded as sub-optimally positive to rabies antibodies and serum samples with Percentage Blocking (PB) equal to or higher than 70% were considered optimally positive, that is, with antibody levels equal to or higher than 0.5 IU/mL, a cut-off value based on the Fluorescent Antibody Virus Neutralization (FAVN) test (OIE 2019).

4.6 Data analysis

The MS-Excel spread sheet was used for entering the data and SPSS (Statistical Package for Social Sciences) version 18 for statistical analysis. The generated data were subjected to descriptive statistics analysis, where the frequencies were calculated for categorical variables. The association between variables were assessed using logistic binary regression analysis at 95% confidence interval; a *P*-value less than 0.05 was considered significant. The correlation between the level of seroconversion and age and sex of dog was determined using binary correlation test.

4.7 Results

4.7.1 Study population and vaccination status

All dogs included in this study were locally bred. Details of the study population and vaccination status are illustrated in the Table 4.2. According to the age of the dogs, six categories were created to facilitate the analysis of the data. The group with the lowest frequency were dogs younger than three months old (4.8%) and 119 dogs with ages ranging from 2 to 4 years were the most frequent (28.5%). Most of the dogs (80.1%) involved in the present study did not have history of vaccination.

Table 4.2: Frequency of studied dogs according to their age group**,** sex and history of vaccination

Variables	Frequency (%)
Dogs' age groups	
< 3 Months	20 (4.8)
3-6 Months	44 (10.5)
6-12 Months	100 (23.9)
1-2 Years	65 (15.6)
2-4 Years	119 (28.5)
> 4 Years	70 (16.7)
Total	418 (100)
Sex of the dogs	
Male	280 (66.9)
Female	138 (33.1)
History of dog vaccination	
Unvaccinated	335 (80.1)
Vaccinated	83 (19.9)

4.7.2 Level of rabies antibody

The results of the antibody titers are shown in Table 4.3. Most dogs (89.2%; n=373; 95% CI: 85.77% to 91.96%) were negative for rabies antibodies (according to the manufacturer's interpretation. Eighteen dogs (4.3%) (95% CI: 2.65% to 6.85%) showed the presence of rabies-specific antibodies. With only 6.5% (n=27; 95% CI: 4.4% to 9.4%) demonstrating adequate seroconversion.

Table 4.3: Antibody titres in both vaccinated and unvaccinated dogs in LNP

Variables	Frequency (%)
Antibody Level	
PB < 40%	373 (89.2)
40% ≤ PB <70%	18 (4.3)
PB ≥ 70%	27 (6.5)
Total	418 (100)

4.7.3 Vaccination status *versus* adequate level of antibody's titres

Table 4.4 shows the level of circulating antibodies in dogs according to the history of vaccination. Of the total number of unvaccinated dogs (n=335), only 4.5% had optimal levels of rabies antibodies titres, while only 14.5% (n=12) of the vaccinated dogs successfully seroconverted and presented with adequate levels of antibodies above the cut-off value of 0.5 IU/mL.

Table 4.4: Prevalence of circulating rabies antibodies level in dogs of Limpopo National Park according to the vaccination status

Variables	Frequency (%)			
		Percentage of Blocking (PB)		
	Negative	Sub-optimally positive	Optimally positive	Total
Vaccination status				
Unvaccinated	306 (91.3)	14 (4.2)	15 (4.5)	335 (100)
Vaccinated	67 (80.7)	4 (4.8)	12 (14.5)	83 (100)
Total	373 (89.2)	18 (4.3)	27 (6.5)	418 (100)

4.7.4 Frequency of dog vaccination status according to age and sex of dogs

Table 4.5 shows the frequency of vaccinated and non-vaccinated dogs according to age groups. Younger animals were in the lower percentage for both the vaccinated and non-vaccinated dogs. The trend was crescent for young animals. However, as age increased, the proportion of vaccinated and unvaccinated adults and elderly declined (dogs over 4 years old).

Table 4.5: Proportion of vaccinated and unvaccinated dogs according to age group

Variables	Frequency (%)		
Age of dogs	Unvaccinated	Vaccinated	Total
< 3 Months	20 (6)	0 (0)	20 (4.8)
3-6 Months	41 (12.2)	3 (3.6)	44 (10.5)
6-12 Months	81 (24.2)	19 (22.9)	100 (23.9)
1-2 Years	53 (15.8)	12 (14.5)	65 (15.6)
2-4 Years	82 (24.5)	37 (44.6)	119 (28.5)
> 4 Years	58 (17.3)	12 (14.5)	70 (16.7)
Total	335 (100)	83 (100)	418 (100)

Table 4.6 shows the proportion of vaccinated and non-vaccinated dogs according to sex. Males had higher proportions for both states (*P*=0.029) (Table 4.8).

Table 4.6: Proportion of vaccinated and unvaccinated dogs according to sex

Variables	Frequency (%)		
	Unvaccinated	Vaccinated	Total
Male	216 (77.1)	64 (22.9)	280 (100)
Female	119 (86.2)	19 (13.8)	138 (100)
Total	335 (80.1)	83 (19.9)	418 (100)

4.7.5 Level of antibodies titers according to age and sex of dogs

The results on the relationship between the antibody titers and the ages of dogs are illustrated in Table 4.7. Dogs younger than three months of age did not have antibodies to rabies. The highest levels of circulating antibodies were observed in dogs aged two to four years with 11.8% (n=14). Binary logistic regression of the combined results revealed a significant positive correlation ($P < 0.05$) between age and the level of circulating antibodies, especially for dogs that were in the 6-12 months and 1-2 years age groups. Compared with females, males appeared less likely to have circulating rabies antibodies, with 88.9% versus 89.9%. Regarding the sex of the dogs and the level of rabies antibodies capable of conferring an adequate immuno-response against rabies virus infection (equal or more than 0.5 IU/mL based on the FAVN test), the difference in proportions was not statistically significant ($P > 0.283$).

Table 4.7: Antibody titers according to the age and sex of dogs

Variables		Frequency (%)			**Odds Ratio (95% CI)**	***P*-Value**
Group age	Negative	Sub-optimally positive	Optimally positive	Total		
< 3 Months	20 (100)	0 (0)	0 (0)	20 (100)	Reference	Reference
3-6 Months	42 (95.5)	1 (2.3)	1 (2.3)	44 (100)	0.000	0.998
6-12 Months	95 (95)	3 (3)	2 (2)	100 (100)	0.129 (0.016; 1.040)	0.054
1-2 Years	60 (92.3)	2 (3.1)	3 (4.6)	65 (100)	0.219 (0.066; 0.721)	0.012
2-4 Years	99 (83.2)	6 (5)	14 (11.8)	119 (100)	0.455 (0.148; 1.397)	0.169
> 4 Years	57 (81.4)	6 (8.6)	7 (10)	70 (100)	1.057 (0.466; 2.397)	0.895
Total	373 (89.2)	18 (4.3)	27 (6.5)	418 (100)		
Sex						
Male	249 (88.9)	13 (4.6)	18 (6.4)	280 (100)	Reference	Reference
Female	124 (89.9)	5 (3.6)	9 (6.5)	138 (100)	1.506 (0.713; 3.179)	0.283
Total	373 (89.2)	18 (4.3)	27 (6.5)	418 (100)		

4.8 Discussion and conclusions

The present research will not only help to raise public health awareness about the attitudes, practices of rabies vaccination among the local residing communities, but it will also provide information to policy makers and other stakeholders if the little work done so far in LNP is bringing public health benefits of the elimination of canine rabies. With the knowledge of the level of circulating rabies antibodies, we also intend to contribute to the design of appropriate control measures for the disease and savings of funds that would be applied in programs of acquisition of vaccines for PEP for the population at risk.

It is still a rational premise that the parenteral vaccination of dogs remains one of the most cost-effective measures for the prevention of dog-mediated human rabies as it targets the source of the disease (Zinsstag et al. 2009). This rabies control strategy takes into account the high costs that would be or are assigned to pre and post-exposure prophylaxis, especially for high risk people such as those that reside in Limpopo National Park (Shwiff et al. 2014).

Irregular free mass vaccination campaigns may be contributory to the low levels of anti-rabies antibodies considered capable of conferring protection against rabies virus infection in the canine population in Mozambique. For example, all the vaccinated dogs (85/335) recorded in this study, regardless of their age, were only immunised two years prior to the study (in 2015). However, according to the information obtained in the District Services for Economic Activities of Massingir, which is the sector responsible for programs for the prophylaxis of Massingir's domestic animals, including those in the park, have revealed that in recent years there have been mass dog anti-rabies vaccination campaigns for free in dogs in LNP.

Nonetheless, some sectoral and socio-cultural and economic factors such as the accessibility of the dog population impaired by the lack of means of capturing or handling dogs, most of which are free-roaming, the lack of means for issuing rabies vaccination certificates as a proof of vaccination undermine attempts to control the disease. On the other hand, poor knowledge is evident by the myth patent in the LNP residing communities that rabies vaccine decreases the power of aggression and the hunting instinct of their dogs, aspects which have also contributed to low or no vaccination of dogs in specific localities. These and other factors were considered in a work carried out between 2016-2018 in the park (Mapatse et al. 2019) where there were owners who never heard of any vaccination campaign taking place in the LNP villages. Some stated that they were absent at the time of vaccination, and were also those who were not aware of need to vaccinate their dogs.

The same reasons for non-attendance at vaccination points were also reported in the Mozambican Province of Manica (Simone 2016) and in a study conducted in Kwazulu-Natal of South Africa (Hergert et al. 2016). Based on the results presented here, it is possible to alert the district and provincial veterinary services about the need to strengthen community education and to raise public awareness so that they participate massively in immunization campaigns.

The lack of time and information and scepticism in relation to the efficacy of the vaccine were also the main reasons mentioned by the aforementioned population of Manica Province for not having their dogs vaccinated. Studies conducted elsewhere in Tanzania (Bardosh et al. 2014), and in Indonesia (Widyastuti et al. 2015) have also revealed that residents were informed of the impending parenteral vaccination campaigns late, and that the majority of the population was pre-occupied with other tasks. In addition, the existence of free-roaming dogs and therefore difficult to handle, was another obstacle to the poor achievement of the targets. This is corroborated by the results of the results of the current study where 80.3% of LNP dogs are never confined.

This is the first study to evaluate the levels of rabies antibodies in the canine population in a conservation area (LNP) in Mozambique. The BioPro ELISA Ab kit, an internationally validated tool for the assessment of rabies antibodies in domestic dogs (Wasniewski and Cliquet 2012; Wasniewski et al. 2013; Wasniewski et al. 2016), has been assessed on different panels of serum samples by several independent research groups (Hikufe 2016; Moore et al. 2017; Eze et al. 2018; Smith et al. 2019), and shown to have good specificity and sensitivity characteristics.

Of all dogs covered in the present study, less than ten cent were positive for an adequate level of circulating specific rabies antibodies with an equivalent titre of 0.5 IU/mL, a minimum capable of conferring an adequate immune response against rabies virus infection. This result is much lower than that observed in the Province of Manica by Simone (2016) where 28.1% of dogs had levels of specific rabies antibodies optimally considered as adequate to confer a immunological response against rabies virus infection, regardless of their vaccination status. Other investigations carried out in Nigeria in neighbourhood dogs (Adebiyi et al. 2014) and also in hunting and roaming dogs in some peri-urban and rural areas of Ogun and Oyo States (Oluwayelu et al. 2014), and in South‐Eastern Nigeria (Eze et al. 2018) showed that less than two per cent of dogs had rabies antibodies of less than 0.5 IU/mL.

These results may reflect to a certain degree the level of neglect by the dog owners in preventing their pets receiving rabies vaccines. However, irregular vaccination campaigns, lack of education campaigns and awareness of local communities about the dangers of rabies in Limpopo National Park (Mapatse et al. 2019) are some aspects that may have contributed to the lack of adequate antibody titers capable of providing adequate immunological response against rabies infection to the park's dogs. These barriers are not only common to the LNP area but have also been reported in dogs from Ogun and Oyo states and other regions of Nigeria (Olugasa et al. 2011; Oluwayelu et al. 2014; Eze et al. 2018).

Encouraging results were reported in Federal Capital Territory (FCT) in Nigeria, where indigenous breed had the seroprevalence (antibody titres > 0.6 IU/mL) of 22.9%, that is, values above those observed for positively adequate antibody titres in LNP dogs. The data obtained in the FCT are not surprising since there was a significant association ($P < 0.05$) between the area of residence of dog owners and the antibody titers of the sampled dogs. Unlike the Limpopo National Park, a remote area, Abuja, the Federal Capital of Nigeria is a planned city with the upper social class residing mainly in the city center and with higher standards in terms of the notion of responsible dog ownership.

Seroconversion was observed in less than five per cent of the population with no history of prior vaccination, and this deserves special attention from local veterinary structures because they contrast with what would be the expected in this category of non-immunized animals, that is, zero per cent of seroconversion. Other similar studies also detected rabies antibodies in unvaccinated domestic dog populations in Kenya (Kitala et al. 2001). Several hypotheses may warrant the presence of rabies antibodies in non-vaccinated dogs of the present study, including false positive reactions (Kitala et al. 2001). Development of antibodies in apparently healthy dogs is unusual (Sabeta et al. 2018) and the controversial role of exposure to other closely related viruses and other pathogens such as canine distemper (Aubert 1992; Gold et al. 2020) that are suspected of causing cross-reactivity with anti-rabies antibodies, demonstrates that further studies are needed to prove their influence on the results obtained in this study.

In some of these cases, the history of the vaccination of the dogs may not have been accurate as the data was provided by children rather than by the head of the household. On the other hand, the previously mentioned study, carried out between 2016 and 2017 by Mapatse et al. (2019) in the park, revealed that most of dogs were received as gifts from a relative, friends or neighbours, which does not preclude the possibility that seropositive dogs might actually have been vaccinated by the previous owner. Other relevant factors could be that some local people in the community or neighbours would have captured and taken the owned dogs for vaccination, in the absence of their owner, the vaccination team would have also captured the dogs that were roaming freely nearby the vaccination points and vaccinated them (Gold et al. 2020).

Therefore, it is necessary to ensure that throughout the national territory, the issuance of an official certificate of (re) vaccination is complied with. Furthermore, the lack of accurate information on the vaccination status of some dogs in the LNP leads us to state that possibly positive cases of circulating rabies virus antibodies, may have been derived from maternal antibodies or as an anamnestic immune response to a previous natural non-lethal rabies exposure (Gold et al. 2020). Despite a decline in rabies vaccine antibody titres over time, cases have been reported in which a protective immune memory to rabies virus occurs in previously vaccinated dogs and these may last for up to six years (Dodds et al. 2020). Therefore, this factor should be taken into account for dogs in the park that have circulating rabies antibodies even two or three years after the history of the last date of vaccination.

As mentioned previously, rabies antibodies detected in unvaccinated dogs may suggest natural exposure of these animals to RABV and/ or abortive, subclinical, or latent infections (Carey and McLean 1983), as was reported in previous studies carried out in Nigeria, Kenya and in India (Wosu and Anyanwu 1990; Kitala et al. 2001; Ludrik et al. 2009; Eze et al. 2018). However, there is a need for more complementary research to ascertain whether there was any possibility that some seropositive dogs were in an extended latent period or were in fact in the non-lethal clinical stage (Gold et al. 2020) or not. This recommendation will also be a way of raising awareness of the need to implement more effective control actions for rabies in Limpopo National Park. So far, a few studies have investigated the serological response of vaccinated dogs to rabies vaccines in Mozambique. The low proportion of vaccinated dogs with rabies antibody levels capable of conferring adequate immune response was also observed in dogs from the Province of Manica (Simone 2016) with a prevalence of 37.7% of dogs with threshold of positivity to ≥70%. Although the latter results were slightly higher than those observed in our study, probably due to differences in the methodologies used and different animal populations targeted, it does not preclude the risk through the lack of adequate level of circulating rabies antibodies in Mozambique.

Although we do not have information on the specifications of the vaccines used in LNP in 2015, or recommendations for timing of the vaccinations, aspects such as the formulation and the production differences between vaccines together with the concentration and integrity of antigen content and the adjuvant used, were considered by other research groups (Kennedy et al. 2007; Wallace et al. 2017), and may have influenced the low seroconversion in vaccinated dogs. However, it is easy to realize that vaccinated dogs have probably never received vaccine boosters since the Veterinary Services in Mozambique recommend a single vaccine dose and a booster 12 months later. These protocols disagree with most protocols for inactivated, adjuvanted rabies virus vaccines, which usually recommend an initial vaccination and a booster 30 or 90 days later to complete the immune stimulation (Loza et al. 2009; Fernandes et al. 2017).

Even so, as reported by Cliquet et al. (2003) it should be noted that the fact that some vaccinated animals are not adequately positive for rabies specific antibodies can mean that they may have presented a seroconversion before the date of the blood test. The possible interruption of the cold chain and storage time that may have exceeded the maximum tolerance limits for its use, as defined in the work carried out by Lankester et al. (2016), including the improper storage and re-use of vaccine leftovers, after the vials are opened, are some factors quite often affecting the efficacy of the vaccine and should also be considered here in order for most dogs to have failed to reach the threshold level of 0.5 IU/mL.

This finding is in agreement with those of other researchers who have also stressed that preparation, transporting, storing and application of the vaccines play an important role effectiveness of the biological product (Gazi and Seyyal 2011). Studies carried out in Dakar by Akpapo et al. (1993) and by Ludrick et al. (2009) suggest that malnutrition, infections, external and internal parasites could also cause rapid decrease of antibodies after vaccination. In Massingir, 79.3% of dogs eat low-quality home-made leftovers, mostly maize meal porridge, hence these and other host-specific factors, such as genetics and immune status, should also be considered for antibody decline after initial effectiveness.

As in the present study several others also showed a tendency for a larger sample size in adult animals (i.e., over one year) and lower proportion in dogs less than three months and older than four years of age. These inclusion data are in agreement with those observed in other similar studies (Kitala et al. 2001; Kasempimolporn et al. 2007; Singh et al. 2011; Hikufe 2016). The younger ones are difficult to collect blood samples from and the fact that they are still under the care and protection of the mother prevents many researchers from being able to capture them for blood collection.

The older ones are more experienced and memorize the various capture attempts (Bardosh et al. 2014) and even calling them using voice commands or grooming using baits (food) did not always result. In addition, the difficulties reported by Bardosh et al. (2014) for the non-presentation of animals to anti-rabies vaccination points, were also observed in LNP dogs for blood sampling, since most dogs were free roaming, which made it difficult to capture or restrain them. However, it is noteworthy that mass immunization campaigns, including boosters, in dogs younger than one year old or from three months of age, is very important in Mozambique, since these have been reported as the group that besides being the one that presents a higher incidence of rabies, are the group age more active and always in closer contacts with humans (Kitala et al. 2001; Loza et al. 2009).

Although The World Small Animal Veterinary Association (WSAVA) Vaccination Guidelines Group (VGG) (Day et al. 2016) clearly specifies the conditions (age) under which dogs and cats should be vaccinated against rabies, it is necessary for the veterinary sector in Massingir district to take into account the new guidelines proposed by Arega et al. (2020), in which suggest parenteral vaccination of puppies at six weeks of age, so that from 21 days onwards they begin to show adequate antibody titres. In addition, dogs at this new-born age are easier to capture and handle for parenteral vaccination than most free-roaming adults (which is the case in the LNP).

Other studies showed that since 2009, 19 human deaths occurred due to rabies in the previous 7-year period, where five cases were caused by “scratches/abrasions without any bleeding” by unvaccinated pups below three months of age (Bharti et al. 2017). The same was also observed in Thailand, where human rabies cases were derived from bites by puppies under three months of age (Pimburage et al. 2017).

The same analysis can also be established between the age and the seroconversion level observed on the dogs of the present study where titers of puppies’ neutralizing antibodies were lower than in older dogs (newborn). Age has been one of the factors that influence the seroconversion of both newborn (less than one year old) and older dogs, irrespective of previous immunization or specific immunosuppressive conditions. There is no doubt that the newborn, with a few months of life, have the immune system still in development, being unable to respond positively to an antigenic stimulation, therefore, it is imperative to go for revaccination (Gazi and Seyyal 2011; Wallace et al. 2017). Studies carried out in India (Bharti et al. 2014) have shown that humans are susceptible to infection from puppies less than three months old, so it is recommended that puppies be vaccinated even from two weeks of age, since the maternal antibodies from a vaccinated dam do not interfere with seroconversion.

According to Mansfield et al. (2004), it is suggested that when titers of maternal antibodies have declined or if the dam has not been vaccinated, younger dogs can produce rabies antibodies from four weeks of age, since that there are no maternal antibodies which would be responsible for the activation of putative regulatory T cells and increased corticosteroid levels. The low seroprevalence observed in older dogs also suggests the need to assure their revaccination boosts, at specific intervals, in order to counteract the regulation of their immune system that gradually declines with the advancing age (Mastroeni et al. 1994; Scott and Nel 2016; WHO 2017).

In the present study, there was no statistically significant association between the sex of dogs and their rabies antibody titre ($P > 0.05$). These results are in agreement with various other studies, including the ones carried out by Gazi and Seyyal (2011) and Mansfield et al. (2004) and van Sittert et al. (2010) which revealed that gender did not have any effect on the neutralizing antibody titers formed against rabies virus.

This study reveals that the work that still has to be done towards the control of rabies in Massingir and especially in the conservation areas. The human population and domestic animals residing here provide a perfect system for disease transmission between man and dogs. The veterinary and health services sectors in the Massingir District should ensure that epidemiological surveillance systems are more effective by reviewing policies and strategies for disease control, as the level of protection of dogs against rabies virus infection is quite low. The herd immunity in the dog population is very low and demonstrates how susceptible it is to rabies infections/outbreaks.

Vaccination and revaccination campaigns are not consistent. Vaccination coverage levels and responsible dog ownership should be improved, so educating local communities about public health hazards of rabies should be enhanced. These present potential areas for intervention.

Hence, further studies need to be carried out to ascertain the status of animals that seroconvert in the absence of apparent vaccination. With good, organized multidisciplinary (one-health) teams against rabies, in Limpopo National park, one can even get support from various entities such as the Alliance for Rabies Control (GARC), Partners for Rabies Prevention (PrP), the Bill and Melinda Gates Foundation and WHO, which also promotes rabies control programs in regions where canine rabies is highly endemic.

Chapter 5 : Detection and Molecular Characterization of Rabies viruses from Limpopo National Park and Other regions of Mozambique

5.1 Introduction

Rabies is still considered one of the most deadly and viral zoonotic disease, with at least an estimated 59,000 global human deaths registered annually, with the majority of cases resulting from bites by infected dogs (Hampson et al. 2015; Wilde et al. 2016; WHO 2018). The viral aetiologic agent of rabies, is responsible for an acute infectious disease that compromises the central nervous system (CNS) resulting in a neurological disorder characterized by horrific clinical signs and symptoms in a wide range of warm-blooded animals, including humans (Kotait et al. 2009).

The bullet-shaped rabies virus, the causative agent of the disease has a ~12 Kb, single-stranded, non-segmented negative sense RNA genome and belongs to the *Mononegavirales* order, *Rhabdoviridae* family and *Lyssavirus* genus. The rabies virus genome encodes five proteins: the nucleoprotein (N), phosphoprotein (P), matrix protein (M), glycoprotein (G) and (L) RNA polymerase protein (Ortiz-Prado et al. 2016; Kgaladi et al. 2017).

In Africa, human rabies deaths are highly underestimated. It is estimated that about 21,000 people die annually due to the disease, representing approximately 36% of global rabies cases, and that these deaths are mainly dog-mediated (Hampson et al. 2015). In addition, even with the various awareness actions carried out at all levels worldwide on the importance of mass parenteral vaccination programs of dogs and the provision of biological for post-exposure prophylaxis (PEP) for humans exposed to the virus, it is still inconceivable that even to date, rabies continues to be under-reported in many developing countries of Asia and Africa, including Mozambique (Fooks 2005; Fooks et al. 2014).

Unlike the long-term success measures undertaken in South America to reduce human rabies deaths, in Africa and Asia, the factors that compromise disease control and actions for its elimination, have been mentioned elsewhere. For about three decades the lack of surveillance and laboratory infrastructure, cultural or social taboos (Bingham et al. 1999; Lembo et al. 2010; Banyard et al. 2013; Fooks et al. 2014; Rupprecht et al. 2017) have led to underreporting of the disease. Unfortunately, other economic and political aspects such as insufficient financial resources, inadequate health care infrastructure, inadequate reporting systems, imprecise public disclosure, unfortunately all contribute to this disastrous scenario of human rabies cases and its global burden induced by canine-mediated rabies in endemic areas (Banyard et al. 2013; Taylor et al. 2017; Coetzer et al. 2017a).

The absence of accurate data on disease incidence and an apparent neglect in terms of integrated government health policy actions, in which public and private human and animal health professionals should work together, has also compromised the eventual elimination of canine-mediated rabies on the African and Asian continents (Shwiff et al. 2008; Taylor and Partners for Rabies Prevention 2013).

As an example of different disease control approaches during the past two decades is shown in some comparative models in which for each fatal case of human rabies, Asia and Africa perform around 200 and eight PEPs respectively, while for Latin America the figure is over 41,000 PEPs (Shwiff et al. 2013; Regea 2017; Shwiff et al. 2018). Still in Latin America, over 2.8 million dogs are vaccinated per human life lost, whereas only some 1000 are vaccinated in Asia and 200 in Africa (Mani and Madhusudana 2013; Shwiff et al. 2013; Vigilato et al. 2013).

On the other hand, rabies is generally considered to be a fast-moving transboundary disease that does not respect borders (Banyard et al. 2013). It has been endemic in Mozambique since 1908 and constitutes a serious public health problem, with dog rabies accounting for over 88% of confirmed cases in the 1990s (Dias and Rodrigues 2003; King et al. 2004).

There is little existing information on the molecular epidemiology of rabies in Mozambique, with a greater focus to the prototype *Rabies lyssavirus* (RABV) (Salomao et al. 2017; Coetzer et al. 2017b), despite the fact that other 17 species within the genus *Lyssavirus,* namely *Lagos bat lyssavirus* (LBV), *Mokola lyssavirus* (MOKV), *Duvenhage lyssavirus* (DUVV), *European bat 1 lyssavirus* (EBLV-1), *European bat 2 lyssavirus* (EBLV-2), *Australian bat lyssavirus* (ABLV), *Aravan lyssavirus* (ARAV), *Khujand lyssavirus* (KHUV), *Irkut lyssavirus* (IRKV), *West Caucasian bat lyssavirus* (WCBV), *Shimoni bat lyssavirus* (SHIBV), *Bokeloh bat lyssavirus* (BBLV), *Ikoma lyssavirus* (IKOV), *Lleida bat lyssavirus* (LLEBV), *Gannoruwa bat lyssavirus* (GBLV) and *Taiwan bat lyssavirus* (TWBLV) are recognized worldwide (ICTV 2019). In fact, the Central Veterinary Laboratory located in Maputo is the only one of the four existing laboratories at the national level, capable of diagnosing rabies using molecular techniques (CDC 2018). This weak laboratory diagnostic capacity, together with the almost non-existent rabies surveillance and poor notification system in the country (Government of Mozambique 2010; World Health Organization 2013; Salomao et al. 2017; Coetzer et al. 2017b), are as mentioned previously, very worrying factors that have compromised the actions designed to effectively combat this devastating disease.

This is particularly true, especially if one takes into account that in other African countries, there are at least six lyssaviruses species that have been isolated, namely LBV, MOKV, DUVV, SHIBV, IKOV and RABV (Coertse et al. 2010; Ngoepe et al. 2014).

In this perspective, Mozambique must be alert to and improve its epidemiological surveillance system, as only RABV circulates in terrestrial mammals of neighbouring Republic of South Africa (RSA) (Ngoepe et al. 2014). We are not aware how many species actually circulate in host species in Mozambique. It is therefore possible that if surveillance was enhanced in Mozambique, more lyssavirus species could be identified and confirmed in the diagnostic centres of the country. Enormous efforts and resources should be made available for the control of the most important source of animal and human rabies, the dog-mediated RABV in the country.

The problem is worsened when it comes to rural areas, particularly in regions of human-animal conflict, such as in Mozambique's Limpopo National Park (LNP), located in the Gaza Province, where rabies is a serious public health problem (Osofsky et al. 2008). In such a conservation zones and in other regions of the country, although rabies is also linked to wild mammals, such as jackals, mongoose, wild dogs and hyenas (Government of Mozambique 2010), RABV cycles are in fact sustained mainly by domestic dogs (*Canis familiaris*) (Bingham et al. 1999), simply because the vaccination and stray dog control is inadequate (Osofsky et al. 2008).

The transboundary movement of the disease has been reported between Mozambique, South Africa and Zimbabwe (Zulu et al. 2008; Coetzer et al. 2017b). This phenomenon is not new. Since the early 1950s, it has been reported that epizootic rabies spread across Eastern and Central Africa and Southern countries to involve whole of Mozambique (Dias et al. 1985). In 1952, canine RABV spread from the extreme north-eastern corner of South Africa (Mpumalanga province), including the Kruger National Park into Southern Mozambique, from where in 1954 it spread into the Kingdom of eSwatini and into Kwazulu-Natal in mid-1961 and again in 1976 (Swanepoel et al. 1993; Mollentze et al. 2013; Coetzer et al. 2017b).

However, currently throughout Mozambique, the epidemiology of rabies is still not well defined (Coetzer et al. 2017b), but if attention is paid to enhance surveillance and phylogenetic analyses of lyssaviruses, it can lead to better control of incursions of rabies also in transfrontier conservation areas, through permeable borders within South Africa, Zimbabwe and Mozambique (Cohen et al. 2007; Zulu et al. 2008). The dog owners entering into Mozambique are required to be in possession of rabies vaccination certificates of their dogs, but translocation of infected dogs across national borders, is a regional issue (Zulu et al. 2008), mainly due to an increased illegal cross-border and migration of people and their animals between Mozambique and neighbouring countries and vice-versa, especially after the ceasefire in 1992. Most importantly, the rabies cycles across borders of neighbouring countries in southern African, are inter-connected. For instance, the removal of the boundary fence along the transboundary conservation regions shared with neighbouring countries, can promote the circulation not only of RABV, but also of other species of lyssavirus (Spierenburg et al. 2008; Khan 2009).

The lack of epidemiological surveillance and underreporting of rabies cases in Mozambique is a worrying situation, especially if one takes into account that RABV variants may undergo genetic adaptation to each particular host, resulting in new clades or biotypes relating to the local or neighbouring fauna. Fortunately, the inactivated vaccines in use cover most of lyssavirus variants encountered here. However, to the best of our knowledge, studies reporting evolutionary diversity of the rabies virus within Mozambique and the association with the strains in neighbouring countries are scarce. This makes it difficult to understand the epidemiology of rabies and to design measures for its control. Therefore, this research aims, among others, to understand the epidemiology of the disease through the detection, molecular characterization and comparison of the phylogenetic relationships of the rabies virus in Limpopo National Park, in particular, and of viruses from other regions of the country, in general.

5.2 Material and methods

5.2.1 Rabies viruses

Eight rabies viruses (n=8) were obtained from original brain-infected tissues of dogs (*Canis familiaris*) and a domestic cat (*Felis catus*) recovered from clinical reported cases from Maputo, Gaza, Sofala and Nampula provinces of Mozambique. They were submitted for routine diagnosis (fluorescent antibody test) performed between 2017 and 2018 at the Virology Section of the Central Veterinary Laboratory (CVL), in Maputo, Mozambique. Part of the same brain samples were also shipped frozen in 2018 to the OIE Rabies Reference

Laboratory (ARC-OVI, Pretoria, South Africa), and retested by the fluorescent antibody test (FAT) and subsequently subjected to genetic characterisation. The information of the viruses included in this research is shown in Table 5.1.

Table 5.1: Rabies specimens collected in four Mozambican provinces for the FAT, molecular and phylogenetic analysis

Virus #	Lab Ref. #[b]	Year of collection	Host species	Province of origin
1	496/18	2018	Canine	Maputo city
2	597/18	2018	Canine	Sofala, Beira
3	501/18	2018	Canine	Gaza, Xai-Xai
4[a]	343/18	2018	Canine	Gaza, Massingir
5	124/18	2018	Canine	Maputo, Marracuene
6	393/18	2018	Cat (feline)	Sofala, Beira
7	468/17	2017	Canine	Gaza, Xai-Xai
8	368/18	2018	Canine	Nampula city

[a]Exact origin: Limpopo National Park

[b]Reference # assigned by the CVL

5.2.2 Active surveillance and management of exposure

Within the scope of active surveillance for dogs suspected of rabies, implemented in Massingir in general and in LNP in particular, a task carried out by the researchers' group members and also by the District Veterinary Services, the virus #4 was obtained from the brain of an *Africanis* male dog breed, approximately six months old, from LNP, in Massingir district, Gaza province (Figures 5.1 and 5.2).

The dog was euthanized on May 15, 2018, after showing suspicious signs of rabies, which started four days earlier (May 10, 2018). Among the clinical signs, the lack of appetite, excessive salivation, aggressiveness and the attack on objects stood out. It was the only dog in the house, and usually roamed the neighbourhood freely. There were no reports that the dog had ever been vaccinated. During the morbid period of the rabid dog, it was documented that a 13-year-old girl suffered a category II exposure (nibbling of uncovered skin with minor scratches or abrasions without bleeding. The case was not immediately reported to the local health centre, but at home level abrasions and scratches were just washed with soap and water. The dog's frozen head was sent to Maputo on May 19, 2018. On the same day, after arriving in Maputo, the brain tissues were collected at the *post-mortem* room of the CVL for a subsequent rabies virus screening, using the FAT protocol as recommended by the WHO with minor modifications (Dean et al. 1996; Ronald et al. 2003).

The remaining seven brain samples used in this study were provided by Virology Section of the CVL. The provided samples were the only ones available in the diagnostic archives and that until then, were awaiting genetic characterization.

5.2.2.1.1 Fluorescent Antibody (FA) Test for Detection of RABV

The same eight brain specimens from Mozambique (Table 5.1), which were submitted to the ARC-OVI Laboratory, at the Rabies Unit in Pretoria, South Africa, for the identification of the rabies virus through the FAT, were also tested in the same facilities using RT-PCR, for the molecular detection of RABV.

Figure 5.1: Head (left) and brain tissue (right) of a LNP *Africanis* dog suspected of rabies (sample #4)

A thin impression smear from the brain specimens was prepared on a clean glass slide using wooden sticks. Two slides were prepared for each brain sample. Impression smears were air dried for 30 minutes and then fixed in cold acetone (-20°C) for 30 minutes. The fixed slides were air-dried for approximately 5 minutes in the Biological Safety cabinet until all traces of acetone and moisture had evaporated. The slides were then placed onto a moist chamber and stained with drops of anti-rabies nucleocapsid conjugate with fluorescein isothiocyanate (FITC) (BIORAD®; CA, USA) prior to 30 minutes incubation at 37°C. Slides were then washed three times in phosphate buffered saline (pH 7.26), air dried and then counterstained with Evans blue for 1 minute, and then the excess dye was decanted. One drop of 50% glycerol-phosphate buffered saline was added to smear slides, then covered with coverslips and examined under fluorescent microscope with using 40 X objectives, for the presence of clear cut apple green fluorescing Negri bodies indicating the presence of lyssavirus antigen (Figure 5.2). The smears were graded quantitatively with a score of +1 (weak positive), +2 (moderate positive) to +3 (strong positive), depending on the abundance of viral antigens according to the intensity of fluorescence.

5.2.2.1.2 Molecular Identification

Viral RNA extractions and Detection of Nucleoprotein (N) Gene and G gene of RABV using Reverse Transcription Polymerase Chain Reaction (RT-PCR)

Total viral RNAs were extracted from approximately 200 ng of the original brain-infected samples using TRI reagent (Sigma–Aldrich, St. Louis, MO, USA) as described in the manufacturer's protocol. Briefly, 200 ng of each of the brain samples (n=8) were placed in pre-labelled and sterile Eppendorf tubes. To each of the tubes containing the brain-infected samples, 1 mL of TRI Reagent was added. Then the tubes were briefly vortexed. Subsequently, 200 µL of chloroform was added to the homogenate, vortex mixed and kept on the bench for a further two minutes. The mixture was then centrifuged at 10000 rpm for 10 minutes at 4°C. Five-hundred microliters (500 µL) of the eight homogenates were transferred to pre-labelled tubes containing an equal volume of isopropanol (Sigma, USA), gently mixed and kept on the bench for a further 5 minutes. The mixtures were subsequently centrifuged at 12000 rpm for 10 minutes at 4°C, the supernatant decanted and the clear RNA pellet washed with 1 mL of 75% ethanol. Again, the tubes were centrifuged at 12000 rpm for 5 minutes at 4 °C and the supernatant decanted. The resulting RNA pellet was air dried (not completely) for 5 minutes, and solubilised in 40 µL of DEPC (diethyl pyrocarbonate) water at 55°C for 15 minutes. The purity and concentration of each viral RNA was evaluated using a spectrophotometer (NanoDrop™ 1000, Thermo Fisher Scientific) prior to long term storage at -80 °C until required.

5.2.2.1.3 Complementary deoxyribonucleic acid (cDNA) synthesis

For cDNA reaction, two Eppendorf tubes (0.6 mL each) were used for the two supermix (A and B) for the eight RNA samples, including a positive control from a South African black-backed jackal (laboratory reference #460/17) from the ARC-OVI, and previously sequenced and antigenically typed as a typical dog strain. Supermix "A" consisted of 50 µL PCR-water (Top-Bio™, Czech Republic), 10 µM dNTPs (10 mM) and oligonucleotides positive sense primer 001lys(+) (N-gene) and G(+) (20 picomoles per microliter). The mixture containing 5 µL (1µg/µL) of total viral RNA templates were denatured at 65℃ for 5 minutes then cooled on ice for one minute. The annealing and numbering positions of the oligonucleotides were based on that of Pasteur virus (PV) genome (Table 5.2) (Tordo et al. 1986; Tordo et al. 1988; Sacramento et al. 1991; Markotter et al. 2006). The contents of the tube were collected and supermix "B" consisting of 40 µL 5X first strand buffer (250 mM Tris-HCl, 375 mM KCl, 15 mM $MgCl_2$), 10 µL of 0.1 M DTT (Dithiothreitol), 40 U of RNaseOUT™ (Recombinant Ribonuclease Inhibitor) and 15 µL SuperScript III reverse transcriptase (200 U) (Invitrogen, Renfrew, UK) were added and then incubated for 50 minutes at 50°C.

PCR reaction

Two mastermixes were prepared for the 10 reactions. Mastermix 1 consisted of 10X PCR buffer composed of 200 mM Tris-HCl, ultra-pure water, 40 picomoles each of G (+) and L (-) (Sacramento et al. 1991; Markotter et al. 2006), 1.5 mM $MgCl_2$, 10 mM dNTP mix and 1.25 U Takara *Taq* DNA polymerase (Takara Taq DNA polymerase) (Takara Biotechnology, Shiga, Japan), making up a total volume of 480 µL. The second mastermix differed from the first only in the primers, that is, 40 picomoles each of 001lys and 550B primers targeting the partial N gene of the RABV genome (Table 5.2) (Sacramento et al. 1991; Markotter et al. 2006).

Two microliters (2 µL) of aliquot of the cDNA was added to 48 µL of each of Mastermix 1 and 2 to give a total final volume of 50 µL. The amplification reactions were performed in a Geneamp 2400 thermocycler (Perkin-Elmer, USA). For the G gene PCR reaction, the thermal cycling parameters included a denaturation at 94°C for 2 minutes and then followed by 30 cycles of 94°C for 30 seconds, 42°C for 30 seconds, 72°C for 90 seconds were used. Final extension was carried out at 72°C for 7 minutes. For the N-gene PCR reaction, the samples were denatured at 94°C for 2 minutes and then subjected to 35 cycles of: 94°C for 30 seconds, 50°C for 30 seconds, 72°C for 120 seconds, with an extension at 72°C for 10 minutes. For each PCR reaction for G and N gene, a negative control was included by substituting the cDNA for nuclease free water. To visualize the amplicons, five microlitres of each reaction mixture was stained with an equal volume loading dye 6X (New England Biolabs®, UK) and added to the wells of 1% agarose gel immersed in TE buffer. Five microlitres of the GeneRuler 100bp DNA ladders were also loaded on the last wells of every gel. The gel ran in an electrophoresis unit for 45 minutes at 100 Volts. After this, the amplicons were separated in 1%-ethidium bromide stained agarose gel for 30 minutes and viewd on a UV-transilluminator.**Resolution of diagnostic inconsistencies**

Quantitative Real Time-PCR (RT-qPCR)

The viral RNA samples extracted using Trizol at ARC-OVI (n=8) that produced diagnostic incongruities observed between the FAT and conventional PCR assays were sent and re-tested at the University of Pretoria to confirm the relevant diagnostic results (Table 5.2). This was performed by an amplification of viral nucleic acid from the total RNA extracted from each of the brain samples (Table 5.1) using TaqMan® platform, targeting the partial nucleoprotein gene in an established “one-step” quantification real-time PCR (RT-qPCR) on a QuantStudio™ 5 real-time PCR system (Thermo Fisher Scientific, USA) as described previously (Coertse et al. 2010; Coertse et al. 2015). Two NTCs (No template Control) and a standard RNA (STD) were included in the reactions. Viral RNA amplifications curves analysis were performed using external standard curves as described previously (Coertse et al. 2010; Coertse et al. 2015). For that purpose were used QuantStudio™ Design and Analysis software, version 1.5.0 (Thermo Fisher Scientific, USA).

5.2.2.1.4 PCR products Purification and nucleotide sequencing

The PCR amplicons of approximately 660-bp size (N gene) and 850-bp G gene were purified using spin columns (Qiagen™, Hilden, Germany), using the Big Dye™ v3.1 sequencing kit (Applied Biosystems) according to instructions of the manufacturer. The PCR products analysis was performed on an ABI3700 (Inqaba Biotech, Pretoria) sequencer machine. Nucleotide sequences of the purified PCR products were determined in both forward and reverse directions. For this, the N gene was sequenced with 001lys (+) and 550B (-) primers (Markotter et al. 2006) and the G was sequenced with the G (+) and (L-) primers, respectively (Table 5.2).

Table 5.2: Oligonucleotide primers used for cDNA, PCR and sequencing showing annealing positions and nucleotides sequences based on that of Pasteur virus (PV) genome

Oligonucleotide	Position on genome	Sequence (5'-3')	Application	Reference
001lys (+)	1-15	ACGCTTAACGAMAAA	cDNA, PCR, sequencing	Markotter et al. 2006
550B (-)	647-666	GTRCTCCARTTAGCRCACAT	PCR, sequencing	Markotter et al. 2006
G (+)	4665-4687	GACTTGGGTCTCCCGAACTGGGG	cDNA, PCR, sequencing	Sacramento et al. 1991
L (-)	5543-5520	CAAAGGAGAGTTGAGATTGTAGTC	PCR, sequencing	Sacramento et al. 1991

The symbols (+) and (-) refer to genomic and messenger sense respectively

Table 5.3: Rabies virus sequences used for partial N gene phylogenetic analysis

Virus#	Host Species of origin	Year of Collection	Country of origin	Lineage	Genbank Accession Number
343/18	Canine	2018	Mozambique (Gaza)	Africa 1b	MW248383
368/18	Canine	2018	Mozambique (Nampula)	Africa 1b	MW248384
393/18	Feline	2018	Mozambique (Sofala)	Africa 1b	MW248385
468/17	Canine	2017	Mozambique (Gaza)	Africa 1b	MW248386
124/18	Canine	2018	Mozambique (Maputo)	Africa 1b	MW248387
UPV130	Canine	2008	RSA	Africa 1b	JF747614
10_509	Canine	2010	RSA	Africa 1b	KJ744304
UPV150	Canine	2007	RSA	Africa 1b	JF747615
10_274	Canine	2010	RSA	Africa 1b	KJ744308
10_387	Canine	2010	RSA	Africa 1b	KJ744303
11_300	Canine	2011	RSA	Africa 1b	KJ744307
10_268	Canine	2010	RSA	Africa 1b	KJ744302
10_458	Caprine	2010	RSA	Africa 1b	KJ744309
UPV153	Canine	2007	RSA	Africa 1b	JF747616
11_217	Canine	2011	RSA	Africa 1b	KJ744310
8721AFS	Human	2008	RSA	Africa 1b	U22633
UPV128	Canine	2008	RSA	Africa 1b	JF747613
UPV167	Canine	2007	RSA	Africa 1b	JF747617

11_185	Canine	2011	RSA	Africa 1b	KJ744305
9137ALG	Canine	1982	Algeria	Africa 1a	U22643
87012MAR	Canine	1986	Morocco	Africa 1a	U22631
9107MAR	Human	1990	Morocco	Africa 1a	U22852
8693GAB	Canine	1986	Gabon	Africa 1a	U22629
8698GAB	Canine	1986	Gabon	Africa 1a	U22630
86031MOZ	Mouse	1986	Mozambique	Africa 1b	KX148203
8631MOZ	Canine	1986	Mozambique	Africa 1b	U22484
RV2775.1	Canine	2010	Tanzania	Africa 1b	KR906747
RV2780.1	Canine	2011	Tanzania	Africa 1b	KR906751
34312	Canine	2012	RSA	Africa 1b	KT336437
21467	Canine	1993	Zimbabwe	Africa 1b	KT336435
8697BEN	Canine	1995	Benin	Africa 2	U22485
8660GUI	Canine	1986	Guinea	Africa 2	U22487
9218TCH	Canine	1992	Chad	Africa 2	U22644
8801CAM	Canine	1987	Cameroon	Africa 2	U22634
8692EGY	Human	1979	Egypt	Africa 4	U22627
32/02	Yellow Mongoose	2002	RSA	Africa 3	FJ392371
57-06	Yellow Mongoose	2006	RSA	Africa 3	JQ692990
dog/S2/1999	Canine	1999	Egypt	Africa 4	DQ837462
dog/S3/1999	Canine	1999	Egypt	Africa 4	DQ837463
9126MEX	Canine	1991	Mexico	America 1	U22477
DgNYKprwsky1950	Canine	1950	USA	America	FJ228535
8738THA	Human	1983	Thailand	Asia	U22653
SPU94.06	Human	2006	RSA	OUTGROUP	DQ676932
Aravan	Myotis blythii	2002	Kyrgyzstan	OUTGROUP	AB094438

*Canine=domestic dog;

Table 5.4: Rabies virus sequences used for G gene phylogenetic analysis

Virus#	Host species	Year of Collection	Country/province	Genbank Accession Number
124/18	Canine	2018	Mozambique (Maputo)	MW349549
368/18	Canine	2018	Mozambique (Nampula)	MW349550
343/18	Canine	2018	Mozambique (Gaza)	MW377781
393/18	Feline	2018	Mozambique (Sofala)	MW377782
468/17	Canine	2017	Mozambique (Gaza)	MW377783
d22547	Canine	1994	Zimbabwe	AF177070
d332	Canine	1997	RSA	AF303069

d29103	-	-	Zimbabwe	AY604993
fe29406	-	-	Zimbabwe	AY604997
hum29175	-	-	Zimbabwe	AY605013
d28460	-	-	Zimbabwe	AY605034
ZW204/14	Canine	2014	Zimbabwe	MF425797
d35/00	Canine		RSA	EF686077
d333/06	Canine		RSA	EU123929
d652/08	Canine	2008	RSA	FJ842726
d206/07	Canine	2007	RSA	FJ842744
108.00	Canine	2000	RSA	GQ918285
132.92	Canine	1992	RSA	GQ918300
151.86	Canine	1986	RSA	GQ918318
326.86	Canine	1986	RSA	GQ983472
341.88	Canine	1988	RSA	GQ983477
366.84	Canine	1984	RSA	GQ983482
MOZdg298.93	Canine	1993	Mozambique	KM262037
MOZdg572.99	Canine	1999	Mozambique	KM262039
MOZdg633.00	Canine	2000	Mozambique	KM262040
MOZdg315.04	Canine	2004	Mozambique	KM262041
MOZdg558.05	Canine	2004	Mozambique	KM262043
MOZdg659.05	Canine	2005	Mozambique	KM262044
MOZdg482.12	Canine	2012	Mozambique	KM262047
MOZbov1018.12	Bovine	2012	Mozambique	KM262048
MOZdg233.13	Canine	2013	Mozambique	KM262049
RV131	Bat	2010	Zimbabwe	GU936870

5.2.2.2 Phylogenetic analysis

Phylogenetic analysis of the nucleotide sequences of the RABVs was performed using Molecular Evolutionary Genetics Analysis (MEGA X) software following the procedures outlined by (Kumar et al. 2018) with some modifications, based on an alignment of a partial region of the highly conserved nucleoprotein (N) gene and the G gene. Sequences were trimmed using algorithms in BioEdit Sequence Alignment Editor (Version 7.2.5) (Hall 1999) followed by the alignment of the forward and reverse sequences in order to obtain the consensus sequences also using the Bioedit software. Sequences were trimmed to about 600 nucleotides for partial N gene and for approximately 800 bp for partial G gene. Alignments of multiple sequences were carried out with the use of ClustalW software incorporated on MEGA X.

Based on the genetic distances generated in the alignments, phylogenetic trees were reconstructed using the neighbour-joining algorithm in MEGA X software (Kumar et al.

2018). The branching order of the trees was evaluated by bootstrap analysis of a 2000 replicates for both N gene and G gene. Bootstrap values of more than 70% on the nodes are generally regarded as providing evidence for phylogenetic grouping (Hillis and Bull 1993). In addition, nucleotide sequences of partial regions of the N and G gene for the lyssaviruses from this study, representative rabies viruses from other part of Mozambique, neighbouring countries, Africa, and other continents (GenBank) were included in the analysis (Table 5.3). The phylogenetic tree was rooted using *Aravan lyssavirus*. For G gene, the representatives' sequences of rabies virus from Southern Africa region (GenBank) were incorporated for phylogenetic analysis (Table 5.4) and the tree was rooted using *Duvenhage lyssavirus*.

5.3 Results

5.3.1 Fluorescent Antibody Test and PCR products

The results presented in Table 5.3, reveal that all (100%; n=8) brain samples submitted to FAT were positive for RABV. Samples (496/18, 597/18 and 501/18) were weak positive. All the eight samples (100%) were positive on RT-qPCR (Table 5.3) but only 62.5% (n=5) were positive on conventional PCR (Table 5.5 and Figure 5.4).

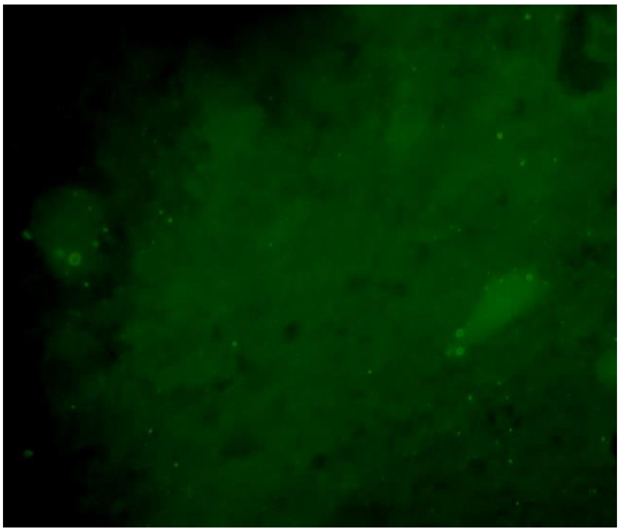

Figure 5.2: Rabies virus presented as clear rounded dots of apple green fluorescence. Objective 40X.

Table 5.5: Results of brain tissues submitted for the detection of rabies virus using FAT and PCR

Virus #	Lab Ref. #	Animal	FAT (in Mozambique)	FAT (in RSA)	RT-PCR results (RSA)	RT-qPCR result (in RSA)	Gene copies /µL
1	496/18	Canine	Positive	Positive	**Negative**	Positive	640
2	597/18	Canine	Positive	Positive[c]	**Negative**	Positive	789
3	501/18	Canine	Positive	Positive[c]	**Negative**	Positive	708
4	343/18	Canine	Positive	Positive	Positive	Positive	6.4 x 106
5	124/18	Canine	Positive	Positive	Positive	Positive	1 x 107
6	393/18	Feline	Positive	Positive	Positive	Positive	2.5 x 106
7	468/17	Canine	Positive	Positive	Positive	Positive	8.8 x 106
8	368/18	Canine	Positive	Positive	Positive	Positive	1 x 107

[c]Samples #1, #2 and #3 were weak (score: +1) and moderately positive (score: +2), respectively on FAT.

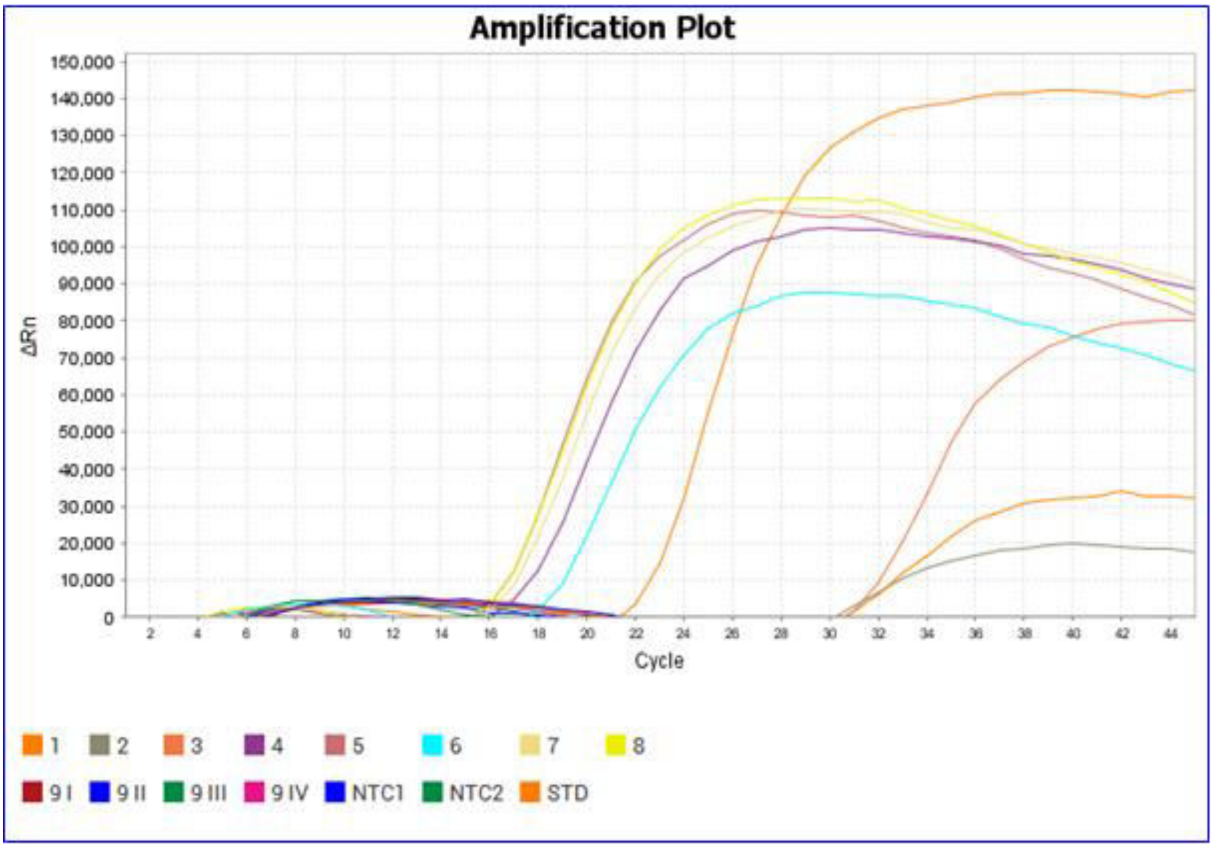

Figure 5.3: Real-time quantitative PCR amplification plot of eight RABV genomic RNA from eight Mozambican brain samples (1-496/18, 2-597/18; 3-501/18; 4-343/18; 5-124/18; 6-393/18; 7-468/17; 8-368/18); NTC-Non template control; STD-Standard; 9 I to 9 IV-Undetermined.

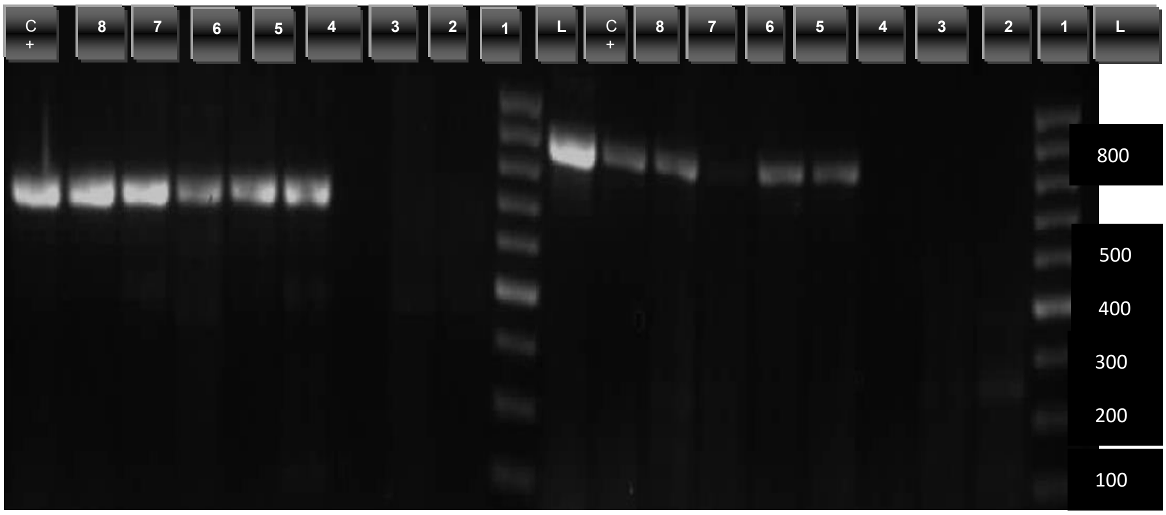

Figure 5.4: Conventional PCR results of the samples processed at ARC-OVI.

On the left are the N products and on the right are the G products. Columns 1 to 8 show the expected band of 660bp and 850bp, respectively. L-Ladder of 100 bp; C+ - Control Positive (South African jackal sample# 460/17).

5.3.2 Phylogenetic analysis

For the N gene, the phylogenetic analysis showed that all lyssaviruses recovered from animals from Mozambique belonged to Africa 1b and were closely related with other canid rabies viruses from dogs from South Africa (99% nucleotide sequence identity), Tanzania and Zimbabwe with 96% of identity (Figure 5.5). For G gene, three RABVs (MW349549, MW377781 and MW377781) clustered with viruses from Mozambique and RSA (Clade A), while MW349550 clustered with other Mozambican rabies viruses (Clade B). Other rabies virus (MW377782) clustered with viruses from Mozambique and Zimbabwe (Clade C) (Figure 5.6) highlighting the influence of geographic determinants in lyssavirus phylogeny.

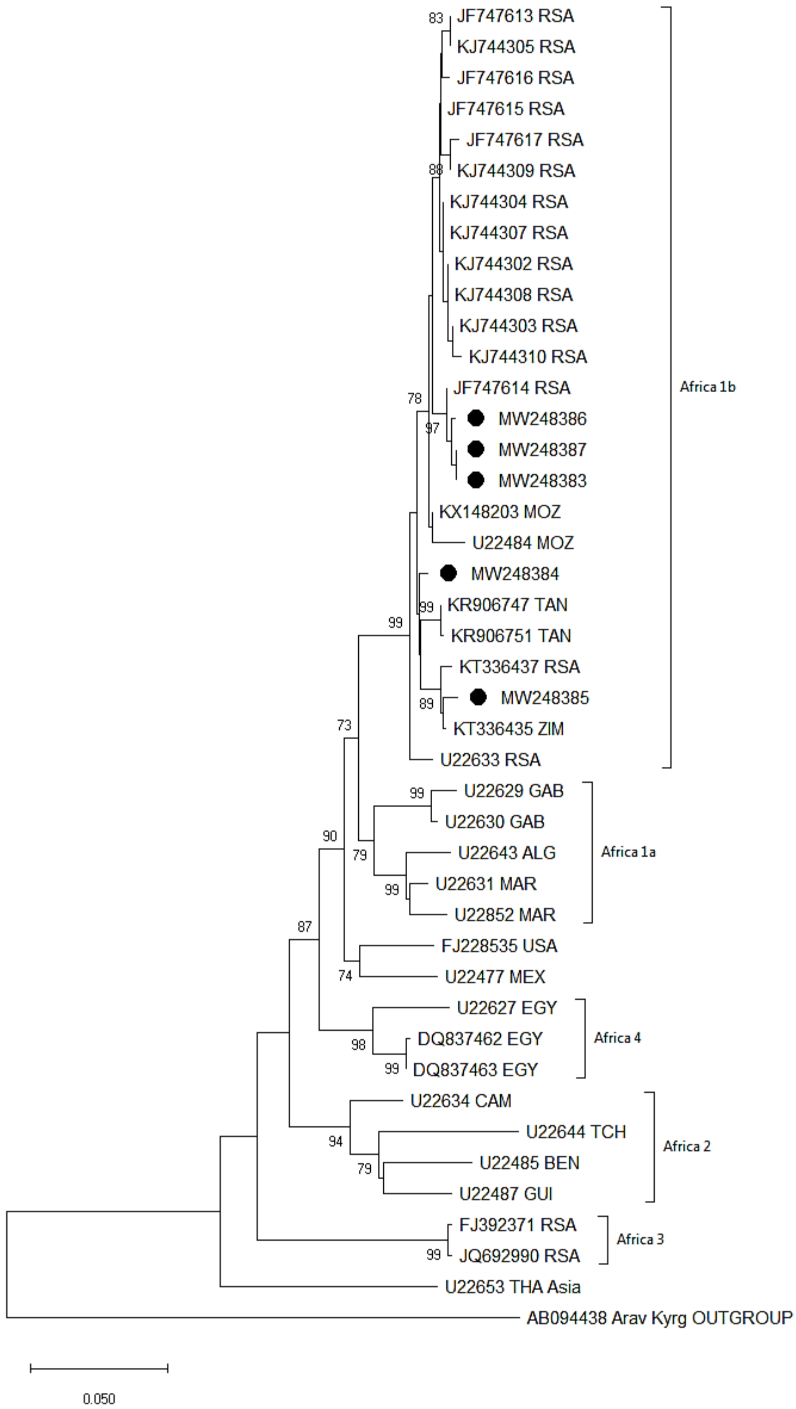

Figure 5.5: Neighbour-joining (NJ) tree derived from the five partial Mozambican RABV nucleoprotein gene generated in the bootstrap test (2000 replicates). The phylogenetic analysis was conducted using MEGA X software (Kumar et al. 2018). The bootstrap values (%) are shown on the nodes supporting the branches. The evolutionary distances were computed using the Kimura 2-parameter method and are in the units of the number of base substitutions per site (Kimura 1980). This analysis involved 43 rabies nucleotide sequences and the ones used in the study are represented by a dark circle.

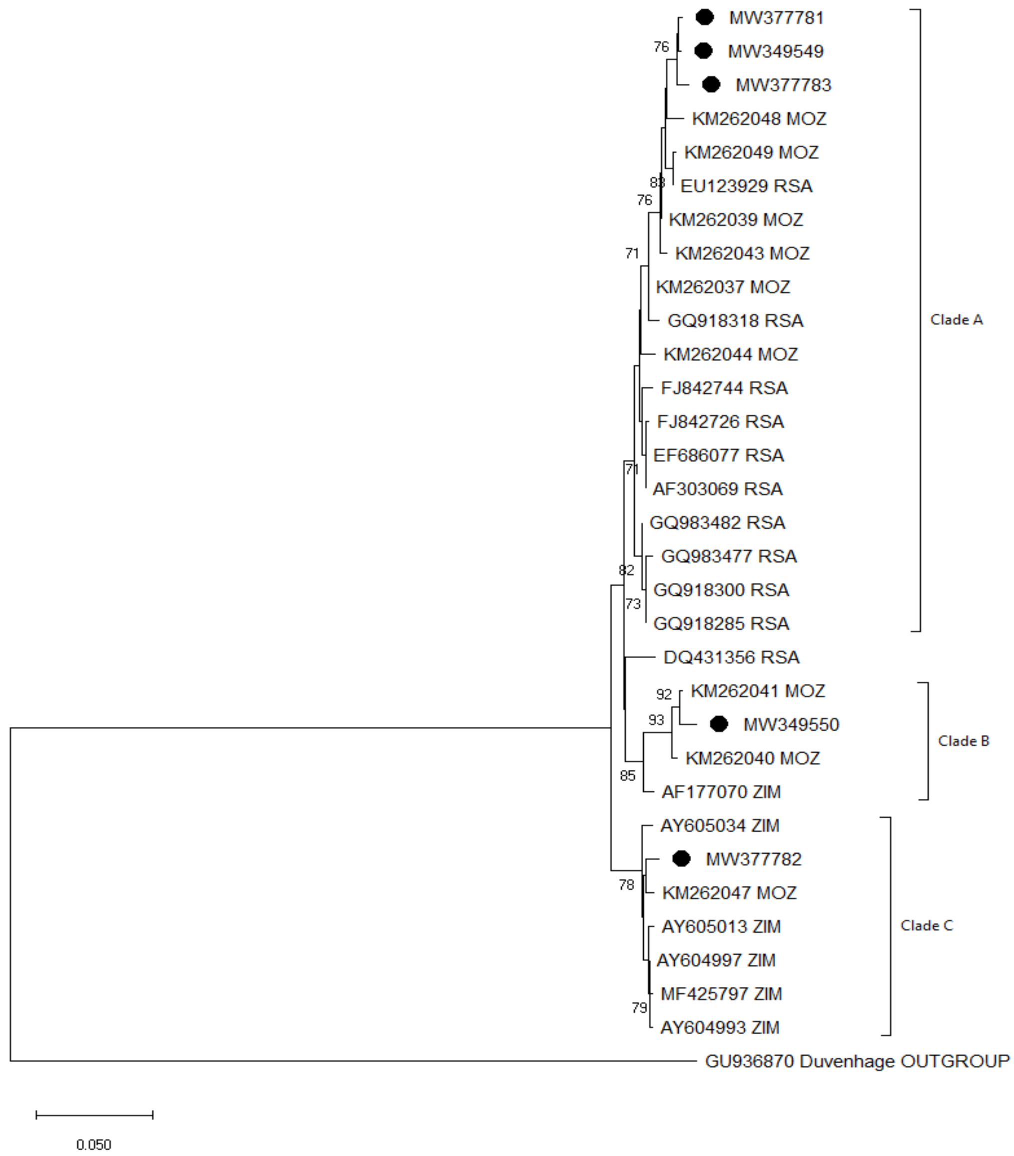

Figure 5.6: Neighbour-joining (NJ) tree showing the genetic relationship of the five Mozambican RABVs partial G gene sequences generated (2000 bootstrap replicates). Phylogenetic analysis were conducted using MEGA X (Kumar et al. 2018). The bootstrap values (%) are shown on the nodes supporting the branches of associated taxa (Felsenstein 1985). The evolutionary distances represented by horizontal scale were computed using the Kimura 2-parameter method and are in the units of the number of base substitutions per site (Kimura 1980). This analysis involved 32 nucleotide sequences and the ones used in the study are represented by a dark circle. The Zimbabwean *Duvenhage lyssavirus* (GU936870) was used to root the tree.

5.4 Discussion and conclusion

Rabies diagnosis using the primary method of the FAT at both the CVL Laboratory (Maputo, Mozambique) and the OIE Rabies Reference Laboratory at Onderstepoort highlight the technical competence in southern Africa. The technical competence in the Southern African Development Community (SADC) could be attributable to the diagnostic training provided by the OIE Reference Laboratory and the Global Alliance for Rabies Control (GARC). In particular, GARC has worked and trained personnel at the CVL (Mozambique) on the use of alternative methods including the direct rapid immunohistochemical test (dRIT). Rabies diagnosis can therefore be decentralised in Mozambique, and dRIT applied in those areas of the country where rabies cases in both animals and humans are still diagnosed based on clinical signs and a history of bites rather than laboratory confirmation by conventional methods, such as the direct fluorescent antibody test (FAT), direct rapid immunohistochemical test (dRIT) and molecular techniques such as the polymerase chain reaction (PCR) and Real time PCR (OIE 2018). In the provincial laboratories of Gaza, Nampula, Manica and the CVL, the Seller's stain (Negri bodies) technique (CDC 2018) is still used. Due to its relatively low sensitivity and linked with the fact this technique no longer being recommended by international entities such as The World Organization for Animal Health (OIE) (OIE 2018). It is therefore encouraged to adopt the dRIT in these settings that are distant from the main laboratory in Maputo.

The CVL is the only facility in Mozambique that uses FAT for rabies diagnosis, and it is important that continued validation with other methods that are recommended by the OIE be performed (Babu et al. 2012; Rupprecht et al. 2018). In a few and rare cases specimens sent from distant provinces, reach the CVL partly decomposed and hence the specimen condition may affect the sensitivity and specificity of the FAT (Duong et al. 2016). For the present study, all samples submitted were positive for FAT. Molecular detection of the viral antigen is becomes important particularly real-time PCR, but in many instances costs are prohibitive.

The use of molecular methods for epidemiological surveillance purposes is unquestionable and should be always performed either at the facility in Maputo or in collaboration with other centres.

The results obtained in the present study, indicate, by the difference in the number of positive samples that, the conventional RT-PCR did not pick the weak positive samples as detected by

FAT. This was rather surprising. It is possibly that the parts of the sample shipped to the OIE Rabies Reference Laboratory had very little antigen. The small amounts of antigen were observed in the three false-negatives samples (496/18, 597/18 and 501/18). In fact, false negatives due to low RABV antigen may occur as reported by Duong et al. (2016). In Brazil, Macedo et al. (2006) also identified samples that were negative by RT-PCR but positive to the MIT. In cases where results are inconclusive retesting the sample, is recommended. The final objective is to avoid discarding samples found negative using traditional techniques (Seller's staining, FAT or conventional RT-PCR), as would have happened in the present study. In Mozambique, and in many southern African laboratories, the OIE, WHO, GARC and FAO, have been improving capacity for rabies diagnosis, DFA should ideally be complemented with isolation of virus in cell culture and then RT-PCR performed on the isolate (at the CVL, Maputo) for continual validation of the diagnostic tests. Diagnostic facilities are being discouraged from using laboratory animals such as mice as these pose ethical challenges but in the absence of other confirmatory methods in use, MIT is still a good method to use. For instance, the CVL previously performed the MIT, suggesting that the laboratory adhered to specific techniques approved by both OIE & WHO. Furthermore, MIT enables propagation of virus for characterization albeit the long turnaround time, requirement for specialist and operator expertise and ethical issues owing to the use of *in vivo* models. Virus isolation (VI) in laboratory animals is an appropriate and relatively good confirmatory method for rabies diagnosis. The phylogenetic analysis using nucleotide sequences of the partial region of the N gene, showed that the Mozambican RABVs variants recovered from dogs and cats, all belong to sub-clade 1b (Africa 1 lineage). These viruses, in addition to being closely related and grouping with RABV variants from Tanzania, Zimbabwe, RSA and Mozambique (Kissi et al. 1995), suggest a common progenitor (Swanepoel et al. 1993), thus denoting, the nature of their possible cross-border transmissions.

In conclusion, the Africa 1b variants, which represents the canid variant of Southern Africa (Sabeta et al. 2003; Nel et al. 2005), were clearly grouped together, with genetic relationship to the RABVs canid variants included in the present study.

If we link our data with the history of the first laboratory confirmed cases in the epizootic of the 1950s in Mozambique, it leads us to consider that the viruses actually circulating in the country are still the same, as they were grouped (clades A and B) with those from the neighbouring regions (along the borders with Zimbabwe and South Africa). As the rabies virus is maintained in other hosts including the bat-eared fox (*Otocyon megalotis*), in South Africa (Sabeta et al. 2013), so it is important that further studies are carried out in Mozambique, in order to establish the definitive RABV hosts and other geographic, temporal and host factors that can help in understanding the dynamics of spread of rabies in the sub-region. LNP is prone to illegal immigration to South Africa across the border with the Kruger National Park (KNP) (Pool 2006; Spierenburg et al. 2009). The epidemiological situation in the north of the country is still largely unknown. Nampula province has one of the highest reported rabies prevalence in dogs (19.2%; 134/714; 2001 - 2017) and people (21.6%; (216/1001; 1989 - 2017) (Bilaide 2019). Due to cross-border migration between Zimbabwe, Zambia, Malawi, Tanzania and RSA, it can once again result in the cross-border transmission of the virus (Mkhize et al. 2010; Coetzer et al. 2017b), into Mozambique as demonstrated by genetic clustering shown in the figure 5.6. With this study, it can be conclude that rabies surveillance and laboratory diagnosis are still inadequate and clearly that more submissions must be sent to the diagnostic laboratories for surveillance. Another option would be to decentralise rabies diagnostics so as improve coverage, and in particular replace the Sellers test with dRIT. Decentralisation of rabies testing would overcome one of the limitations for surveillance in Mozambique and increase the overall numbers of samples submitted and tested. Rabies viruses identified in dogs all belong to the canine variant underscoring the role of the domestic dog (*Canis familiaris*) in rabies cycle in Mozambique. Furthermore, while in neighbouring RSA and eSwatini the importance of continuous control measures, including mass vaccination of dogs, and public awareness in regions where the disease had previously been controlled is underlined (Sabeta et al. 2013), it is necessary that these actions are also implemented in neighbouring Mozambique.

Chapter 6 : General discussion and conclusion

6.1 Introduction

The main objectives of the present study were: (i) to assess the level of Knowledge, Attitudes and Practices of rabies among LNP households, (ii) to assess the level of Knowledge, Attitudes and Practices regarding rabies among Massingir health practitioners (iii) to detect rabies antibodies in the dog population in LNP, and (iv) to characterize rabies viruses strains of domestic dogs in LNP and compare these to viruses from dogs from other parts of the country and the region. The methodologies and tools used to achieve such objectives included among others the administration of questionnaires, use of enzyme-linked immunosorbent assay, conventional PCR, RT-qPCR and genetic sequencing.

6.1.1 Dog demography, ecology and rabies awareness among households and health practitioners

In Mozambique dog-mediated rabies has been recorded since 1908 (Dias 1992). Despite being a disease with a negative impact on public health and taking into account that deaths can be prevented through regular campaigns of parenteral vaccination of dogs and the provision of post-exposure prophylaxis, rabies is still neglected in Mozambique. The neglect is also reflected in the lack of studies published in peer-reviewed journals and other scientific repositories aimed at understanding the situation of animal and human rabies in the country, its epidemiology, the diagnostic capacity and especially the prevention and control strategies that are more applicable to the real situation of the country.

The present information on the ecology and demography of the canine population, combined with the level of community KAP of rabies, will serve as a basis for the design of effective rabies control and prevention strategies in the Limpopo National Park region. Usually, official figures used for planning of vaccination campaigns frequently underestimate true dog population sizes (Lembo et al. 2010). Therefore, with the information presented here, coupled with other related strategies, will contribute to helping improve vaccination coverage rates in future dog vaccination campaigns (Matter and Daniel 2000; Kitala et al. 2002; Ratsitorahina et al. 2009; Chidumayo 2018).

The rural communities of LNP displayed a poor level of knowledge and adopted bad practices towards rabies and this may increase the risk of rabies in the community. Just to substantiate our conclusions, in regarding to the villages, Mavoze had a statistically significant influence for the (poor) knowledge. The majority of the LNP population does not confine their dogs nor take them to the veterinary services, nor to anti-rabies vaccination. Although the health centres have been correctly mentioned by most households as the places where they should seek for medical help in cases of dog bites, at the home level, it is still notorious the issue of poor knowledge demonstrated by most of the interviewees, in which they would guide by a wrong practice of washing the wounds by bites only with water.

Health practitioners had poor knowledge, attitudes and practices on the control and prevention of rabies.

Case definition of rabies was not well-known by many health practitioners. The majority also did not know the WHO guidelines regarding the pre- and post-exposure prophylaxis scheme. A considerable percentage of HPs were unaware of the guidelines related to the sites and routes of rabies vaccinations.

Poor levels of knowledge, attitudes and practices about rabies, both within rural communities, including health practitioners, have been an "Achilles' heel" not only in Mozambique, but in many countries in Africa (Dodet et al. 2008; Wallace et al. 2017; Mbilo et al. 2019). Despite the fact that poverty is considered here as one of the causes, another factor that aggravates actions to combat the disease, is the lack of training for health professionals. Therefore, the integration of the One-health approach and initiatives such as World Rabies Day, are excellent ways to minimize the gaps in knowledge of rabies that was observed in this study (Cleaveland et al. 2014; Balaram et al. 2016).

Some training actions are being carried out in other countries like Cameroon and in The Ivory Coast, especially with regard to the animal bite management of dog bites, human rabies case diagnosis and management, as well as diagnostics skills and competences (Broban et al. 2018). Regardless of some limited-scale efforts undertaken by the Mozambican government (Government of Mozambique 2010; Ministry of Agriculture and Food Security 2017), it is important that the percentage of households with vaccinated dogs rise from 25.8% to 70%, an recommended threshold that is capable of ensuring herd protection and/or eliminating or preventing rabies outbreaks. Although these actions do not yet cover conservation areas (LNP), they have already been timidly implemented in some regions of southern Mozambique in coordination with the local municipalities; private veterinary services and non-governmental animal welfare organizations (Felgate 2011; Protect Ponta 2016; Salomao et al. 2017).

6.1.2 Detection of rabies virus antibodies in canine population within the remote human-wildlife interface zone of limpopo national park, southern mozambique

A total of four hundred and eighteen (n=418) blood samples were collected from apparently healthy LNP households dogs in order to detect rabies antibodies using the commercial blocking ELISA test. The results obtained allow one to state that regardless of the dogs vaccination status, in general, the proportions of dogs with adequate level of antibodies titres (PB> = 70%) were quite low, that is, only 6.5% (n=27). This emphasizes the extent to which the government should invest more and on a regular basis in mass vaccination campaigns. It is necessary to consider that the community of the present study resides in an area where not only the human-animal conflict is a reality, but also the risk of rabies, which, according to Osofsky et al. (2008) is due to the lack of control and vaccination of dogs. The lack of accurate statistical data has had a negative impact on the irregular vaccination campaigns schedules carried out in rural areas. However, there is one positive aspect reported in this study, which is that young dogs (> = 2 years old) were the ones with the highest levels of adequately positive level of rabies antibodies (Tepsumethanon et al. 1991; Aubert 1992). However, according to Morters et al. (2015), it is important not only to embrace puppies from 3 months, but if the objective is to reduce the impact of rabies (Sabeta and Ngoepe 2018) in the park, it is also necessary to reflect on the possibility of immunizing puppies from 6-weeks old (Arega et al. 2020).

The great doubts and lack of information about whether the antibodies detected in the present study were of maternal origin or were derived from active immunisation or rabies infection, clearly demonstrates that seroepidemiological studies and surveillance programs, along with the routine immunization campaigns, are still a utopia in LNP.

Furthermore, some other aspects must be taken into account when assessing the level of antibodies considered as adequate to confer an adequte immuno-response, namely the amount and quality of vaccine, route of administration, the expression and involvement of major histocompatibility complex (MHC) genes, and the health status of the dogs, among others (Moore and Hanlon 2010). In LNP there is no evidence that such aspects are or have been safeguarded in the few campaigns carried out in the region. It is also not surprising that in other regions of Mozambique, as was the case in the study conducted in Manica province (Simone 2016), only 28.1% (100/356) of dogs vaccinated in the years 2012, 2013 and 2014, had adequate levels of rabies antibodies. At that time, the justification for the low proportion of adequately positive dogs (PB ≥70%) was attributed to non-compliance with vaccination boosters and also to interruptions in the cold chain beyond the limits and time tolerated, and other host-related factors, could have affected the efficacy of the vaccine and consequently the immune response. In general, community involvement and awareness campaigns on the importance of dog vaccination should be on the agenda of LNP managers.

6.1.3 Detection and molecular characterization of rabies virus strains in Limpopo National Park, Massingir district, Gaza province, south-western Mozambique

In order to contribute to the perception of virus strains circulating in Massingir district and to compare them with those of other regions of Mozambique and neighbouring countries, brain samples of dogs and a cat were analysed using direct immunofluorescence technique and confirmed by Real-time PCR technique. One of the constraints for an effective rabies control and prevention in LNP, and in Massingir district in general, is the lack of laboratory capacity and trained personnel for accurate rabies diagnosis. These limitations, including the ineffective surveillance system, a poor exchange of information across veterinary and medical authorities and under-reporting of rabies (Cleaveland et al. 2014; Salomao et al. 2017(Cleaveland et al. 2014; Salomao et al. 2017), have also been reported in other developing countries in sub-Saharan Africa (Banyard et al. 2013).

During the active surveillance instituted over the period covered by the present study, a case of rabies in a dog was reported in LNP but, due to the reasons mentioned above, the diagnosis of rabies had to be established in the Central Veterinary Laboratory in Maputo. Many brain samples are not submitted to rabies testing due to lack of logistical conditions and the long distance between where the case is reported and the central or provincial laboratory.

With this, not only the opportunity to start PEP is lost, but also the chance to adopt other measures inherent to the prevention and monitoring of possible contacts with the suspect rabid animal. The differences in sensitivity between the different techniques used for the detection of the rabies virus should be considered in future studies, since all samples were positive for the quantitative Real-time PCR and for the gold standard FA test. It is important to remember that the latter is recommended for routine veterinary and human laboratory diagnosis of rabies (Meslin et al. 1996; Rupprecht et al. 2019). Thus, it can be concluded that in Mozambique there are two clades (A and B) grouped with those from the neighbouring Zimbabwe and South Africa. RABVs variants circulating in Mozambique have the same origin in canid variants from Southern Africa.

Therefore, it is important that the transboundary surveillance system is up-scaled so that new strains are not introduced in the country.

6.2 General conclusions, recommendations and future prospects

Based on this study, it can be concluded that rabies is a neglected disease in Limpopo National Park, in particular, and in Massingir District in general. The rural communities of the LNP have a poor level of knowledge and adopt bad practices towards rabies. The similar findings were observed among the HPs. Local education and public awareness raising actions towards rabies and the management of bite wounds should be implemented in the short term. The proportion of positive canine population with an equivalent level of rabies antibodies of ≥ 0.5 IU/mL is low in the LNP. All partners, including governmental and non-governmental organizations, are invited to embrace vaccination campaigns through the provision of vaccines and cold storage facilities.

According to prior information, a well-established national laboratory capacity for rabies diagnosis is almost non-existent in LNP. Therefore, collaboration is required between the LNP administration and the district veterinary services, through the establishment of memoranda of understanding, for use of the park's laboratory. The Real-time PCR and the FAT techniques were both equally sensitive in the detection of LNP RABV. Molecular studies suggested that rabies virus strains had a common origin with the circulating canid variants of southern Africa (1b), thus denoting their transboundary nature. Better epidemiological surveillance is required on the ground through the implementation of a mobile phone network. Further studies are required on serological detection and evaluation of the levels of rabies antibodies in LNP dogs, before and after the rabies vaccination as a way to contribute to the improvement of epidemiological surveillance.

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ANNEX I. Eight (n=8) villages randomly selected in LNP according to the riverine system

Village	Rivers	Nr. of Cattle	Nr. of Goat	Nr. of Sheep	Nr. of Dogs	Random	Dogs'proportional stratified random sample size
Bingo	Shingwedzi	2402	1219	358	63	0,958845	37
Macaringue	Limpopo	1994	263	199	126	0,949527	74
Munhamane	Limpopo	1041	728	187	56	0,874357	33
Madingane	Elefantes	1564	349	416	81	0,84278	47
Malhaúle	Elefantes	807	291	147	57	0,837639	33
Chibotana	Elefantes	1686	755	138	0	0,8108	0
Machamba	Shingwedzi	1627	993	262	64	0,719319	37
Macuachane	Elefantes	1495	884	197	0	0,707354	0
Cunze	Limpopo	1488	582	180	63	0,574552	37
Chipandzo	Limpopo	731	171	128	0	0,535738	0
Maconguele	Limpopo	2088	773	243	0	0,420525	0
Chimangue	Shingwedzi	1790	1447	587	0	0,172832	0
Mavoze	Shingwedzi	3641	2627	1911	147	0,072928	86
Total					**657**		**384**

ANNEX II. Number of households per study villages

Selected Villages	Nº households	Intended sample size per/village	Final sample size/village
Mavoze	720	64	43
Bingo	175	15	29
Machamba	150	13	23
Madingane	137	12	26
Mahlaule	110	10	17
Munhamane	148	13	29
Macaringue	427	38	41
Cunze	101	9	25
Total	1968	174	233

ANNEX III: Questionnaire applied to the households and health practitioners in LNP

DOG DEMOGRAPHY, ECOLOGY AND HOUSEHOLDS KNOWLEDGE, ATTITUDES AND PRACTICES TOWARDS RABIES

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

UNIVERSIDADE EDUARDO MONDLANE

SECTION 1: QUESTIONNAIRE IDENTIFICATION

101. Questionnaire reference number: |__| __| __| __| __| __|

102. Date of interview (DD/MM/YY): |__| __| __| __| __| __|

103. Time of interview (Hr:Mn): |__| __| : |__| __|

104. Interviewer name: ____

105. Interviewee name:____

106. Village name:____

107. Neighbourhood name or Number: __________________________ |__| __|

108. House number: |__| __|

109. GPS latitude coordinates (North/South): |__| __| |__| __| __| __| __| __|

110. GPS longitude coordinates (East/West): |__| __| |__| __| __| __| __| __|

111. Respondent cell phone number: |__| __| __| __| __| __| __| __| __| __| __| __|

SECTION 2: HOUSEHOLD INFORMATION

201. Respondent gender: Male |__| Female |__|

202. Respondent age group: 18-21 |__| 22-28 |__| 29-35 |__| 36-42 |__|

43-49 |__| 50-56 |__| 57-64 |__| > 60 |__| Years

203. Marital status: Single |__| Married |__| Divorced |__| Widowed |__|

204. Respondent place of birth: ____

205. Educational level: None |__| Primary |__| Secondary |__| Higher |__|

Vocational |__| Other ____

206. Occupation: Unemployed |__| Civil servant |__| Farmer |__| Merchant |__|

Self-employed |__| Student |__| Other (specify) ______________________

207. Respondent religion: Christian |__| Muslim |__| Jewish |__| Atheist |__|

Other |__| ____

208. Number of people in the household < 5 years old |__| __| 5-10 years

old |__| __| 11-17 years old |__| __| 18-50 years old |__| __| > 50 years old |__| __|

209. If no, who is the household head? __________________________________

210. Type of home: traditional single family house |__|

traditional single family house |__| modern single family house |__|

modern multi-family house |__| apartment |__|

home in multi-apartment building |__| other (identify) |__| ________________

211. What type of enclosure does the house have? no fence or wall |__|

fence or wall, but does not restrain dogs |__|

fence or wall, completely restrains dogs |__|

212. Household Livestock details

ANIMAL SPECIES OWNED	NUMBER

Cattle	
Goats	
Sheep	
Pig	
Poultry	
Donkey	
Other (specify) ______________ ___ ___	

SECTION 3: DEMOGRAPHY OF DOG POPULATION

301. How many dogs do you own? 1 │__│2 │__│3 │__│4 │__│5 │__│ >5 │__│

302. How many are puppies (0–4 months)? │__│ __│

303. How many dogs are juveniles (5–12 months)? │__│ __│

304. How many dogs are adults (>12 months)? │__│ __│

305. If the household doesn't have any dog, explain why not?

Don't like dogs as pets │__│

To prevent dog bite │__│ To avoid dog ticks/lice │__│ Noise│__│

Against religious belief │__│ Prevent embarrassing guests │__│

To avoid contamination with dog faeces │__│ Superstitious belief │__│

Lack of resources to care for dogs │__│ Other reasons_____________________

306. What is the breed of the dog(s)? Crossbreed │__│ Native breed(Africanis) │__│

Exotic breed │__│ Other (specify)____________________________________

307. What is(are) number of owned dogs according to their sex?

Male |__| __| Female |__| __|

Pregnant female |__| __| Lactating female |__| __| Unsure |__| __|

308. Is (are) the dog(s) neutered/spayed? Yes |__| No |__| Unsure |__|

309. If yes, how many? Neutered/spayes |__| __|

310. How long have you owned the dog(s)? Months |__| __| Years |__| __|

311. Where did you acquire the dog(s)? Pet Store |__| Breeder |__|

Rescue Group |__| Found as a stray |__| Newspaper Ad |__|

Received from a friend/relative |__| Born at home |__|

Bought or traded from neighbour |__| Received as gift from outside

neighbourhood |__| Other ______________________________________

312. What was the age of the dog when you acquired it? |__| __| Months |__| __| Years

313. If it is a bitch, give the date of the last litter (dd-mm-yy) |__| __| - |__| __| - |__| __|

How many puppies were born? |__| __|

How many are still alive and with the household? |__| __|

How many died from diseases? |__| __|

How many were killed by the bitch? |__| __|

How many were killed by people? |__| __|

How many were given away, were sold or disappeared?

How many were abandoned? |__| __|

How many were killed intentionally? |__| __|

314. Why do you own a dog(s) for? Security | __| Hunting | __| Sale | __| Pet | __|

Breeding | __| Herding | __| Other reasons____________________________

SECTION 4: DOG HOUSING, CARE PRACTICES AND ECOLOGY

401. Explain how is the dog(s) confined to your property?

Fenced yard | __| Tied out, chain or runner | __|

Kennel or other type of enclosure | __| The dog is never confined | __|

Other (please explain) ____

402. If confined, at what period of day is the dog confined to house or garden?

During the day | __| At night | __| Day and night | __| Allowed to roam | __|

403. How many hours a day is the dog kept outside?

None | __| Less than an hour | __| 1-2 hours | __| 3-4 hours | __|

More than 5 hours | __| Live outdoors leashed | __| Live outdoors unleashed

Allowed inside only at night | __| Other (please explain)_________________

404. Who normally manage, handle and take care of the dog?

Father/owner | __| Mother | __| Children of household | __|

Everybody | __| Relatives | __| Strangers | __| Friends and neighbours

Nobody | __| Other (specify) _______________________________________

405. Who usually feeds the dog?

Household members │ __│ Neighbours │ __│ Dog finds its own food │ __│

Other (please explain)____

406. What is the source of food?

Commercial dog food │ __│ Family garbage and waste │ __│ Small rodents │ __│

Butchers' waste │ __│ Garbage on roads and dumps │ __│ Family left over │ __│

Especial cooked food │ __│ Other source (specify) ______________________

407. What is the source of water?

Home │ __│ River │ __│ Lake │ __│ Other (specify)_________________________

SECTION 5: INFORMATION ABOUT UNOWNED/STRAY DOGS

501. Are there unowned dogs in your neighbourhood? Yes │ __│ No │ __│ Unsure │ __│

502. If yes, are they known in the neighbourhood? Yes │ __│ No │ __│ Unsure │ __│

503. Are the number of unowned dogs increasing in your neighbourhood?

Yes │ __│ No │ __│ Unsure │ __│

504. If yes, what are (according to you own opinion) the reasons that are behind this increase?

Uncontrolled breeding │ __│ Lack of financial resources to cater for dog │ __│

Inadequate feeding by owners │ __│ Abandonment and neglect by owners │ __│

Non enforcement of dog control policies │ __│ Escape from home │ __│

Dogs searching for mating partners │ __│ Dog battering by owners │ __│

Dont know | __| Other reason (specify)_________________________________

SECTION 6: HOUSEHOLD INFORMATION ABOUT DOG BITE

601. Have members of your family been bitten by dog(s) in the previous12 months?

Yes | __| No | __| Unsure | __|

602. If yes, specify?

by household dog(s) | __| by neighbours' dog(s) | __|

by unowned dog(s) always in the community | __|

by unidentified strange dog(s) | __|

603. Number of people bitten by dog(s) in the household in the past 12 months | __| __|

604. Which treatment have the bitten person received afterwards?

Local wound treatment | __| Traditional bite treatment | __| Rabies post-exposure vaccination | __| None | __| Don't know/remember | __| Other(specify) ________

605. Who administered the treatment? Nurse | __| Physician | __|

Traditional healer | __| Her/himself | __|

Community neighbourhood member | __| Other (specify) _______________

606. What was/is the status of the dog after the bite?

Healthy | __| Died | __| Sick | __| Don't know/remember | __|

SECTION 7: VETERINARY CARE SEEKING BEHAVIOUR

701. Does the dog see a veterinarian/animal health care at least once a year?

Yes | __| No | __| Doesn't have a dog | __|

702. If yes, give the reasons for taking the dog to veterinarian/animal health centre.

Suspicion of rabies infection |__| Skin infection and loss of hair |__|

Abnormal aggression and barking |__| Vaccination |__|

Preventing them from other diseases |__|

Other reason (specify)____

703. If no, give the reasons for not taking the dog to veterinarian/animal care centre.

There is no Veterinary clinic |__| I am was/unable to handle the dog |__|

The dog never gets/got sick |__| I don't know where the clinic is? |__|

I don't think it is important |__| Not aware of need to go to vet clinic |__|

It is expensive |__| It is far |__| Don't want to |__| Other reasons(specify)______

704. Do you know any disease that affects a dog health and that represents a risk to your pet and for the people? Yes |__| No |__|

705. If yes, mention which one? Rabies |__| Tick infestation |__|

Ringworm |__| Worm infection |__| Don't know |__|

Other (specify) ____

SECTION 8: HOUSEHOLD LEVEL OF KNOWLEDGE, ATTITUDES AND PRACTICES (KAP) TOWARDS RABIES

801. Have you ever heard about rabies? Yes |__| *(If yes, continue with the following questions)* No |__|

802. How did you hear about rabies? *(Check all that apply)*

From the informal/non mass media |__| Please specify:

Family elders |__| Teacher/school |__| Traditional healer |__| Professionals |__|

Friends |__| Community neighbours |__| Colleagues |__| Radio |__|

Close relatives |__| Health professionals |__| Other informal source(specify)___

803. What is the name of rabies in your language? ____

804. What causes rabies? Virus |__| Psychological problem |__|

Evil spirit |__| Shortage of food and water |__| Do not know |__|

Other cause(s)____

805. In your opinion, what are the main animals' sources of rabies?

(Check all that apply)

Dogs |__| Cats |__| Donkeys |__| Cattle |__| Sheep |__| Goats |__|

Wild animals |__| Bats |__| Other (specify) ______________________

806. Which animal(s) can be affected by rabies? *(Check all that apply)*

Dogs |__| Cats |__| Donkeys |__| Cattle |__| Sheep |__| Goats |__|

Wild animals |__| Bats |__| Humans |__| Other (specify) ____________

807. Do you know that rabid animals can transmit rabies to humans?

Yes |__| No |__| Uncertain |__|

808. What are the means through which rabies is transmitted?

Bite |__| Ingestion of contaminated water or food |__| Scratch with teeth |__|

Eating raw meat |__| Drinking raw milk |__| Contact with infected saliva |__|

Blood contact |__| Don't know |__| Other means (specify)_____________

809. Have you ever seen a rabid animal(s)? Yes |__| No |__| Uncertain |__|

810. If yes, which animal(s)?

Dogs │ __│ Cats │ __│ Donkeys │ __│ Cattle │ __│ Sheep │ __│ Goat │ __│

Wild animals │ __│ Bats │ __│ Humans │ __│ Monkeys │ __│

Other (specify) ____

811. What was/were the major clinical sign(s) observed in such rabid humans or animal(s)?

Aggression │ __│ Loss of appetite │ __│ Depression │ __│ Restlessness │ __│

Tail paralysis │ __│ Mouth paralysis │ __│ Aimlessly wandering │ __│

Salivation │ __│ Vocal change │ __│ Open mouth │ __│ Hydrophobia │ __│

Loss of fear │ __│ Fainting │ __│ Don't know │ __│ Other (specify)__________

812. Considering rabies as a disease, do you know that it is a deadly disease?

Yes │ __│ No │ __│ Uncertain │ __│

813. Does dog rabies can be prevented? Yes │ __│ No │ __│ Uncertain │ __│

814. If yes, please tell us how can it be prevented?

Through dog vaccination │ __│ Leashing/tying the dog │ __│

Keeping the dog in a secure cage/confinement │ __│

Stray dog population control │ __│ By other means (specify)_______________

815. Does rabies has a cure after onset of clinical signs?

Yes │ __│ No │ __│ Uncertain │ __│

816. If yes, please tell us how is it cured?

By means of anti-rabies administration │ __│ Tetanus injection │ __│

Antiseptics applied to the bite wound │ __│

Wound washing with soap and water │ __│

Look for traditional/spiritual medicine │ __│

Don't know │ __│ Other means of treatment (specify)____________________

SECTION 9: DOG VACCINATION RECORDS

901. Is the dog vaccinated against rabies? Yes |__| No |__| Unsure |__|

902. If the dog is vaccinated against rabies, please indicate:

Date of last injection (dd-mm-yy) |__|__|-|__|__|-|__|__|

Type of vaccine used ____

Place/Post of vaccination ____

903. Who vaccinated the dog? Veterinarian |__| Animal Health Assistants |__|

Self |__| Self and Veterinarian |__| Other people (specify) _________________

904. At what frequency do you get the dog vaccinated? Yearly |__|

As advised by Veterinarian |__| At convenience |__| Other (specify)_________

905. If the dog isn't vaccinated against rabies, give the reason.

I was absent from the household at the time of the vaccination campaign |__|

I have never had information that there is/was a vaccination campaign against rabies in the community |__| I am was/unable to handle the dog |__|

The dog is/was sick |__| The dog is/was aggressive |__|

The dog is/was pregnant |__| I don't know where to get the vaccine |__|

I don't think it is important |__| Not aware of need to vaccinate |__|

It is expensive |__| Other reasons (specify) ____________________________

SECTION 10: ATTITUDES TOWARDS RABIES AND DOG BITE MANAGEMENT

1001. Are you willing to register your dog(s) Yes |__| No |__| Undecided |__|

1002. Would inform authorities if bitten by dog? Yes |__| No |__| Unsure |__|

1003. Would allow to euthanize dog if rabid? Yes |__| No |__| Unsure |__|

1004. Would seek treatment first if bitten by dogs? Where if yes?

Doctor/hospital |__| Native/traditional |__| None/Uncertain |__|

Other (specify)____

1005. What would be the first aid action at home level if a person is bitten?

Wash wound with water |__| Wash wound with water and soap |__|

Traditional/spiritual medicine |__|

Nothing |__| Other measure |__| Specify___________________________

1006. Are you willing to participate in vaccination campaigns against rabies?

Yes |__| No |__| Unsure |__|

KNOWLEDGE, ATTITUDES AND PRACTICES TOWARDS RABIES AMONG GENERAL HEALTH PRACTITIONERS

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UNIVERSIDADE
EDUARDO
MONDLANE

Self-administered questionnaire (please return it after answering)

SECTION 1: QUESTIONNAIRE IDENTIFICATION

Questionnaire reference number: |__| __| __| __| __| __|

Date of interview (DD/MM/YY): |__| __| __| __| __| __|

Time of interview (Hr:Mn): |__| __| : |__| __|

Interviewer name: ____

Interviewee name:____

Village name:____

Neighbourhood name or Number: __________________________ │__│ __│

Health Centre name: ____

GPS latitude coordinates (North/South): │__│ __│ │__│ __│ __│ __│ __│ __│

GPS longitude coordinates (East/West): │__│ __│ │__│ __│ __│ __│ __│ __│

Respondent cell phone number: │__│ __│ __│ __│ __│ __│ __│ __│ __│ __│ __│ __│

SECTION 2: INTERVIEWEES SOCIO-DEMOGRAPHIC INFORMATION

201. Respondent sex: Male │__│ Female │__│

202. Respondent age: │__│ __│ Years

203. Professional qualifications: Physician │__│ Nurse │__│ Other(specify)_____

204. Educational qualifications (degree/Diploma):

Doctor of Philosophy (Ph.D.) │__│ Master of Science (MSc.)

Doctor of Medicine (M.D.) │__│ Bachelor of Science (BSc.) │__│

Nurse Practitioner │__│ Advanced Practice Nurse │__│ Other ___________

205. Years of service: │__│ __│ Years │__│ __│ Months

206. Previous experience of managing dog bite case: Yes │__│ No │__│

207. Previous experience of seeing rabies case: Yes │__│ No │__│

SECTION 3: QUESTIONNAIRE ON RABIES AGENT AND MODE OF TRANSMISSION

301. What is the causative agent of rabies? Virus |__| Bacteria |__| Parasites |__|

Fungus |__| Others |__| Don't know |__|

302. What is the most important animal source of rabies in Mozambique?

Only dog |__| Cats |__| Monkeys |__| Livestock |__| Wild animals |__|

Other animals |__| Don't know |__|

303. Do you know that rabies it is a fatal disease? Yes |__| No |__| Uncertain |__|

304. How is rabies transmitted? Bite |__| Scratch with teeth |__| Inhalation |__|

Eating raw meat |__| Drinking raw milk |__| Contact saliva |__|

Blood contact |__| Don't know |__| Other means (specify) ______________

305. . What is the incubation period for rabies in humans?

1-7 days |__| 1 - 5 weeks |__| 6 -10 weeks |__| 6 -12 months |__|

1-2 years |__| > 2 years |__| Don't know |__| Other (specify) ___________

306. What are the main signs and symptoms of rabies in humans?

(Check all that apply)

Fever |__| Headache |__| Nausea |__| Insomnia |__| Anxiety |__|

Slight or partial paralysis |__| Restlessness |__| Hallucinations |__|

Salivation |__| Dysphagia |__| Hydrophobia |__| Death |__| Don't know |__|

SECTION 4: QUESTIONNAIRE ON DIFFERENT CATEGORY OF BITE AND THEIR MANAGEMENT

401. Do you know that wound can be categorized? Yes |__| No |__|

402. If yes, please categorize, by telling which statement according to wound type is Cat I, Cat II and Cat III *(one category per sentence)*:

Touching or feeding animals, licks on intact skin ______________________

Nibbling of uncovered skin, minor scratches or abrasions without bleeding ____

Single transdermal bites or scratches, licks on broken skin ________________

Multiple transdermal bites or scratches________________________________

Contamination of mucous membrane with saliva _______________________

403. What are the first aid and care management do you normally implement in case of animal wound bite among your patients?

Washing using soap and water |__| Washing using soap and water and antiseptic (ethanol/tincture of iodine) |__|

Apply antiseptic (ethanol/tincture of iodine) only |__| Wound suturing |__|

Wound bandaging |__| Wound cauterization |__|

Don't do anything |__| Other (specify) ________________________________

404. Do you usually give an advice to your patients regarding the observation of biting animal? Yes |__| No |__|

405. If yes, please tell which advice(s)?

To confine and observe the animal for 1-5 days |__|

To confine and observe the animal for 5-10 days |__|

To confine and observe the animal for 10-20 days |__|

To confine and observe the animal for 20-30 days |__|

Other advice (specify) ____

406. Does the severity and location of the bite have an implication on rabies incubation period and the onset of clinical signs?

Yes |__| No |__| Don't know |__| Unsure |__|

SECTION 5: KNOWLEDGE REGARDING SITE, ROUTE AND EXPOSURE PROPHYLAXIS

501. What are the preventive measures recommended for rabies in case of high risk population?

Vaccination of dogs |__|

Elimination of suspected animals |__|

Pre-exposure vaccination |__|

Educating people about the pre & post-exposure vaccination |__|

Don't know |__| Others (specify)_________________________________

502. What is/are according to the WHO guidelines the recommended route(s) for anti-rabies vaccination? *(Check all that apply)*

Subcutaneous (Sc) |__| Intramuscular (IM) |__| Intradermal (ID) |__|

Intravenous (IV) |__| Intraperitoneal (IP) |__| Do not know |__|

503. What is/are according to the WHO guidelines the recommended site(s) for anti-rabies vaccination? *(Check all that apply)*

Deltoid |__| Abdomen |__| Gluteus |__| Thigh |__| Others |__| Don't know |__|

504. What are the guidelines of rabies post-exposure prophylaxis?

Categories	DK	N	WM	V	RIG	WM+V	WM+RIG	V+RIG	WM+V+RIG
Cat I	|__|	|__|	|__|	|__|	|__|	|__|	|__|	|__|	|__|
at II	|__|	|__|	|__|	|__|	|__|	|__|	|__|	|__|	|__|
Cat III	|__|	|__|	|__|	|__|	|__|	|__|	|__|	|__|	|__|

DK = Don't know, N = nothing, WM = wound management, V = vaccination, RIG= rabies immunoglobulin

505. Do you know the prophylaxys scheme for PrEP?

Yes |__| No |__|

506. and for PEP?

Yes |__| No |__|

ANNEX IV. Score criteria for assessing the level of household knowledge, attitudes and practices in the Limpopo National Park, Massingir District, Gaza Province.

Question asked	**Criteria**	**Score**	**Binary outcome**
Causative agent of rabies			
Virus	Correct answer	1	NA
	Unknown/wrong route	0	NA
Transmission routes to humans			
Bite, Scratch with teeth, Contact with infected saliva	1 correct route transmission	1	NA
	2 correct route transmission	2	NA
	3 correct route transmission	3	NA
	Unknown/wrong route	0	NA
			NA
Transmission routes among animals			NA
Bite, Scratch with teeth, Contact with infected saliva	1 correct route transmission	1	NA
	2 correct route transmission	2	NA
	3 correct route transmission	3	NA
	Unknown/wrong response	0	NA
Clinical signs Animals and humans			NA
Aggression, Loss of appetite, Depression, Restlessness, Tail paralysis, Mouth paralysis, Salivation, Vocal change, Open mouth, Aimless wandering, Loss of fear, Fainting, Hydrophobia	1-2 correct symptoms	1	NA
	3-4 correct symptoms	2	NA
	5 or more correct symptoms	3	NA
	Unknown/wrong response	0	NA
Knowledge of rabies as fatal disease	Yes	1	NA
	No	0	NA
Animal source of rabies in Mozambique			
Only dog, Cats, Monkeys, Livestock, Wild animals	1 correct source	1	NA
	2 correct source	2	NA
	3 correct source	3	NA
	Unknown/wrong response	0	NA
Methods of rabies prevention			
Through dog vaccination, Leashing/tying the dog, Keeping the dog in a secure cage/confinement, Stray dog population	1 correct prevention measure	1	NA

control			
	2 correct prevention measures	2	NA
	3 or more correct measures	3	NA
	Unknown/wrong response	0	NA
MAXIMUM SCORE KNOWLEDGE		**13**	
KNOWLEDGEABLE OF RABIES PARTICIPANTS WHO SCORES WAS ≥ 7/13	**Yes**		**1**
UNKNOWLEDGEABLE OF RABIES PARTICIPANTS WHO SCORES WAS ≤ 6/13	**No**		**0**
Attitudes toward an animal suspected of rabies			
Euthanasia	1 correct attitude	3	1
	Unknown/wrong attitude	0	0
Attitude first place seek treatment bite cases			
Seek treatment at the Health Centre	1 correct attitude	3	1
	Unknown/wrong response	0	0
Attitudes if bitten by dog			
Notify to the authorities	Yes	1	1
	No/Uncertain	0	0
Attitudes towards willingness participate vaccination campaigns			
	Yes	1	1
	No/Uncertain	0	0
Attitudes willingness for euthanasia if rabid dog			
	Yes	1	1
	No/Uncertain	0	0
Attitudes towards willigness to have their dog registered			
	Yes	1	1
	No/Uncertain	0	0
MAXIMUM SCORE ATTITUDES		**10**	
Practices regarding vaccination status their dogs			
	Yes	1	1
	No	0	0
Practices in case of animal bites			
Washing wound Water Washing wound Water + Soap Applying antiseptic	1 correct response	1	1
	2 or more correct response	2	1
	Unknown/wrong response	0	0
Practices regarding visits to the veterinarian			
Suspicion of rabies infection, Skin infection and loss of hair,	One correct good	1	1

Abnormal aggression and barking, Vaccination, Preventing them from other diseases	practice		
	Two correct good practice	2	1
	Three or more correct good practice	3	1
	Unknown/wrong practices	0	0
Practices regarding dog confinement	Yes	1	1
	No	0	0
Practices regarding neutering status			
	Yes	1	1
	No	0	0
MAXIMUM SCORE PRACTICES		**8**	

ANNEX V. Score criteria for assessing the level of health practitioners' knowledge, attitudes and practices, in the Limpopo National Park, Massingir District, Gaza Province.

	Criteria Knowledge	**Score**	**Binary outcome**
Knowledge			
Causative agent of rabies			
Virus	Correct answer	1	1
	Unknown/wrong answer	0	0
Mode transmission to humans			
Bites Scratches by claws (nails) Contact with infected saliva Eating raw meat	1 correct mode transmission	1	1
	2 correct mode transmission	2	1
	3 correct mode transmission	3	1
	Unknown/wrong answer	0	0
Incubation period			1
1-5 weeks, < 3 weeks	1 correct answer	1	1
	2 correct answer	2	1
	Unknown answer	0	1
Clinical signs humans			
Fever, Headache, Nausea, Insomnia, Anxiety, Paralysis, Restlessness, Hallucinations, Hypersalivation, Dysphagia, Hydrophobia, Death	1 correct symptoms	2	1
	2 and more correct symptoms	2	1
	3 or more symptoms	3	1
Rabies as fatal disease	Yes	1	1
	No	0	0
Main source of rabies in Mozambique			
Dog	Correct source	2	1
	Unknown/ wrong answer	0	0
Methods of rabies prevention			
Parenteral dog vaccination, Destruction of suspected rabid animals, Pre-Exposure Prophylaxis (PrEP), Confining all suspected animals	1 correct prevention measure	1	1
	2 correct prevention measures	2	1
	3 or more correct measures	3	1
	Unknown/ wrong measure	0	0
Wound categorization			
Touching or feeding animals, licks on intact skin (CAT I); Nibbling of uncovered skin, minor scratches/abrasions without bleeding, Single transdermal bites or scratches,	Correct answer	3	1
	Two correct wound categorization	2	1
	One correct wound categorization	1	1

licks on broken skin (CAT II), Multiple transdermal bites or scratches, licks on broken skin, Multiple transdermal bites or scratches, licks on broken skin (CAT III)			
	Don't know/Incorrect	0	0
Knowledge severity bite wound onset clinical signs			
	Yes	1	1
	No	0	0
Knowledge route of vaccine administration			
	1 correct route	1	1
	2 correct routes	2	1
	Unknown/wrong route	0	**0**
Knowledge site of vaccine administration			
Deltoid, Thigh, suprascapular	1 correct site	1	1
	2 correct sites	2	1
	3 correct sites	3	1
	Unknown/wrong site	0	0
Knowlegde of Regimen PEP			
	Yes	1	1
	No	0	0
Knowledge of Regimen PrEP			
	Yes	1	1
	No	0	0
Post-exposure prophylaxis (PEP) management according category exposure			
CAT I (Nothing) CAT II (Wound Management + Vaccine) CAT III (Wound Management + Vaccine + Rabies Immunoglogulin)	One correct management	1	1
	Two correct management	2	1
	Three correct management	3	1
	Unknown/wrong answer	0	0
		27	

Attitudes/Practices	**Score Criteria Attitudes/Practices**	**Score**	
Bite wound management			
Soapy water, Soapy water and anti-septic, Anti-septic	1 correct attitude/practices	1	1
	2 correct attitude/practices	2	1
	3 correct attitude/practices	2	1
	Unknown/incorrect practice	0	0
Advice given to the bitten victim			
To confine and monitor the biting animal for 5-10 days, Find the owner of the biting animal and ask of vaccination status	One correct advice	1	1
	Two correct advice	2	1
	Three advice or more	3	1
	Unknown/Wrong answer	0	0
		11	

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Animal Ethics Committee

PROJECT TITLE	**Epidemiology of Rabies in Limpopo National Park**
PROJECT NUMBER	**V133-16**
RESEARCHER/PRINCIPAL INVESTIGATOR	**MF Mapatse**

STUDENT NUMBER (where applicable)	**UP_154 129 63**
DISSERTATION/THESIS SUBMITTED FOR	**PhD**

ANIMAL SPECIES	**Bats**	**Dogs**
NUMBER OF ANIMALS	**To be reported**	**260**
Approval period to use animals for research/testing purposes		**November 2016-November 2017**
SUPERVISOR	**Dr. JM Fafetine**	

Conditions: The AEC has noted that this project will be completed in a facility outside of South Africa. Since the AEC has not inspected the facility, please note that we cannot comment on the quality of the facility other than what was provided in the study questionnaire

KINDLY NOTE:

Should there be a change in the species or number of animal/s required, or the experimental procedure/s - please submit an amendment form to the UP Animal Ethics Committee for approval before commencing with the experiment

APPROVED	Date	28 November 2016
CHAIRMAN: UP Animal Ethics Committee	Signature	

S4285-15

Research Protocol Approval Letter

To whom it my concern

This document is aimed to inform that the research protocol titled "Epidemiology of Rabies in Limpopo National Park" has been reviewed and approved by Scientific Board of the Biotechnology Centre, Eduardo Mondlane University, Maputo, Mozambique. It does not conflict with World Organization for Animal Health. It complies with the International Epizooties Organization, national and local requirements concerning animal experimentation, care and welfare. The handling of animals will be carried by trained and approved staff, namely animal health technicians and veterinary doctors. The experimental procedures will based on blood sample collection in about 384 dogs and bats as well as wing tissues biopsy for bats identification in Limpopo National Park, located in Gaza Province. The field work for this purpose will be carried out from October 2016 to December 2017.

Date 21.10.16

Signature (Head of the Scientific Board)