MOLECULAR STUDIES OF RABIES IN TRADE DOGS AND DETECTION OF SOME RNA VIRUSES IN BATS IN PLATEAU STATE, NIGERIA

BY

GRACE SABO NOK KIA,

DEPARTMENT OF VETERINARY PUBLIC HEALTH AND PREVENTIVE MEDICINE,
FACULTY OF VETERINARY MEDICINE,
AHMADU BELLO UNIVERSITY, ZARIA
NIGERIA

MARCH, 2014

MOLECULAR STUDIES OF RABIES IN TRADE DOGS AND DETECTION OF SOME RNA VIRUSES IN BATS IN PLATEAU STATE, NIGERIA

BY

Grace Sabo Nok KIA, DVM 2005; M.Sc. 2009 (ABU)

PhD/Vet-Med/01791/09-10

A DISSERTATION SUBMITTED TO THE SCHOOL OF POSTGRADUATE STUDIES, AHMADU BELLO UNIVERSITY, ZARIA
IN PARTIAL FULFILMENT FOR THE AWARD OFDOCTOR OF PHILOSOPHY IN VETERINARY PUBLIC HEALTH AND PREVENTIVE MEDICINE

DEPARTMENT OF VETERINARY PUBLIC HEALTH AND PREVENTIVE MEDICINE, AHMADU BELLO UNIVERSITY, ZARIA
NIGERIA

MARCH 2014

DECLARATION

I declare that the work in the dissertation entitled "Molecular Studies of Rabies in Trade Dogs and Detection of some RNA viruses in Bats in Plateau State, Nigeria"has been performed by me in the Department of Veterinary Public Health and Preventive Medicine, under the Supervision of Professor J. U.Umoh,ProfessorJ. K. P. Kwaga, and Professor H. M. Kazeem. The information derived from the literature has been duly acknowledged in the text and a list of references provided. No part of this dissertation was previously presented for another degree or diploma at this or any other institution.

Grace Sabo Nok KIA _____ _____

Name of student **Signature** **Date**

CERTIFICATION

This dissertation entitled "MOLECULAR STUDIES OF RABIES IN TRADE DOGS AND DETECTION OF SOME RNA VIRUSES IN BATS IN PLATEAU STATE, NIGERIA"by Grace Sabo NokKIA, meets the regulations governing the award of the degree of Doctor of Philosophy (Phd) of Ahmadu Bello University, and is approved for its contribution to knowledge and literary presentation.

Prof. J.U. Umoh	_____	
Chairman, Supervisory Committee	Signature	Date
Prof. J. K. P. Kwaga	_____	
Member, Supervisory Committee	Signature	Date
Prof. H. M. Kazeem	_____	_____
Member, Supervisory Committee	Signature	Date
Dr. JunaidKabir	_____	_____
Head of Department, Veterinary Public Health and Preventive Medicine	Signature	Date
Prof. A. A. Joshua	_____	_____
Dean, School of Postgraduate Studies	Signature	Date

ACKNOWLEDGEMENTS

To my awesome God who promised me that His grace is sufficient for me in all I have been or will ever pass through, and has never and will never fail to fulfill His promise, is my ultimate gratitude.

I acknowledge my supervisory team (Professors J. U. Umoh, J. K. P. Kwaga, and H. M. Kazeem). Specifically my gratitude goes to Professor J.U. Umoh, who connected me with the Professor in University of Georgia Athens (UGA), USA where the Dog rabies work was done, for his consistent fatherly advice, correction and guidance; Professor J. K. P. Kwagafor always being available to profer solutions to difficulties encountered, rebuke me when the need arises and also the encouragement you have been to me; Professor H. M. Kazeem for accepting to supervise me impromptu and listening, willingly enlightening, correcting and always giving me that asking query "look" which kept me working hard to finish the first draft of the thesis. My acknowledgement goes tomembers of the Department of Veterinary Public Health and Preventive Medicine for their assistance and encouragement and to every academic and non-academic staff of the Faculty of Veterinary Medicine ABU Zaria, who had been of great help to this study knowingly or unknowingly, directly or indirectly, I am grateful to you. I am grateful to Dr. JunaidKabir the HOD, for being very kind and considerate to me and all the advice and encouragement he provided. I acknowledge Prof. A.B. Ogunkoya who helped provide impromptureferee report when I applied for TETFUND grant and encouraged me at the initial stages of the research while my major supervisor was away on sabbatical leave. I appreciate the encouragement of my dear friends and "classmates" DVM, MSc, and PhD. The following people are appreciated;Prof. I. Ajogi, Drs. B. Adewumi, P.H. Mamman, B.V. and V. Maikai, S.M. Maidawa, P. Kobo, V. Sinkalu, N. Useh, E.C. Okolocha, L. Alam, J.K. Kadima For their encouragement. The "Bat team to Idanre", it was an experience

working with you. To Dr. B.M. Jahun who got me into the “Bat team”, I salue you. The Faculty staff Friday prayer group, I am grateful.I am grateful to Dr. A.A. Dzikwi, for the helpful input.

I acknowledge David Ehizibolo for his help and encouragement.My gratitude to Drs. Chika Nwosuh and Luka Pam for providing a storage space for me in their NVRI laboratories. I acknowledge with deep gratitude Professors A.J. Nok and IshayaNok for always being available when I need them. I am grateful to all the Staff at the Veterinary Clinic of the Jos Museum. The cooperation I received from the people in my study area, who gave me needed assistance and information are highly appreciated.

My gratitude goes to my parents, brothers and sisters in Jos who helped and made my transit from Zaria to the study areas bearable, FunmiEhindero and my dear children Dorcas and Abraham who have been a comfort, help and joy to me; may God grant you grace to be fulfilled in life. To my dearly beloved husband in whom I am well pleased, thank you for always believing in me and the enormous support you have been, may God cause you to reap more than you have given me. Amen! I acknowledge the Vineyard family worship Zaria for all the help and prayers and support you have been to me and my family especially in my absence.

I acknowledge the following for providing for me a “home away from home while away from home” my beloved country; Reverend Dr. and Mrs. C. Kia, Jessie Kia Orlando, Stephen and Anna Kia, Connor Kia, EmekaOkere, Hassana Moses, Aunt Susan, the Osinubis, the Wises, the Fletchers,Vineyard Athens, and the International fellowship at UGA to mention but a few.

I acknowledge Professor Zhen Fu (UGA), Jerry Saliki (Director of Veterinary Diagnostic Lab UGA), and Ming Zhou, Christiana Leyson, Clement Gnaduraiand all the staff of the Zhen Fu's Laboratory and College of Veterinary Medicine, Department of Pathology, UGA, USA. My gratitude also goes to the Staff of the Rabies Branch of the Division of High Consequence Pathogens and Pathology of the Centres for Disease Control and Prevention (CDC) especially C. Rupprecht, I. B. Kuzmin, M. O. V. Osinubi, M. Niezgoda, P.Yager, M. Douglas, O.Urazova, A.Vellasva-Villa just to mention a few. I appreciate Suxiang Tong and the staff of the Pathogen Discovery laboratory of the Division of Viral Diseases, CDC especially Christiana Conrardymy lovely teacher and friend, Ying, Yang and all the staff of the Rotavirus unit of the Division of Viral Diseases at CDC.

I acknowledge the divine provision for this work through grants in part by the Public Health Service grant A1-051560 from the National Institute of Allergy and Infectious Diseases (Z.F.F.), the Tertiary Education Trust Fund TETF/DESS/AST&D/UNIV/ABU/ZARIA/VOL.2, (K.G.S.N.) and Science and Technology Education Post Basic (STEP-B) Project, Innovators of Tomorrow Grants HME STEP-B/IOT/33/Vol.1/22 (K.G.S.N.).

DEDICATION

I dedicate this work to God Almighty whose awesome grace, mercy and faithfulness keeps me going.This work is also dedicated to my sweet loving, understanding and ever helpful husband John Abu Kia, my Mum and Dad, Mr. and Mrs. Sabo Turner ChiromaNok, for their love, compassion, forbearance, sacrifice and continuous encouragement.

TABLE OF CONTENTS

Title Page i
Declaration iii
Certification iv
Acknowledgements v
Dedication viii
Table of Contents ix
List of Tables xii
List of Figures xiii
List of Plates xv
List of Appendices xvi
List of Abbreviations and Symbols xvii
Abstract xviii
CHAPTER 1:INTRODUCTION 1
1.1 Background to the Study 1
1.2 Statement of Research Problem 2
1.3 Justification 4
1.4 Aim 6
1.5Objectives 6
1.6Research Questions 6
CHAPTER 2:LITERATURE REVIEW 8
2.1The Rabies Virus 8
2.2 Rabies in Nigeria 8
2.2.1 Rabies in dogs and other animals 9
2.2.2Sub-clinical rabies 12
2.2.3Dogmeat consumption in Nigeria 12
2.3 Lyssaviruses and Bats 13
2.3.1 Bat lyssaviruses in Africa 14
2.3.2 Insectivorous bat-associated rabies 17
2.3.3Bat rabies and host switching 19
2.4 Coronaviruses in Bats 20

2.5Paramyxoviruses in Bats **22**

2.6Reoviruses in Bats **24**

2.7 Rotaviruses in bats **25**

CHAPTER 3:MOLECULAR STUDIES OF RABIES IN TRADE DOGS IN PLATEAU STATE NIGERIA **27**

3.1 Introduction **27**

3.2 Materials and Methods **28**

3.2.1 Studyarea 28

3.2.2 Selection of dog markets 28

3.2.3 Dawaki and Amper markets. 29

3.2.4 Direct flourescent antibody test (DFAT) 32

3.2.5 Reverse transcription polymerase chain reaction (RT-PCR) 33

3.3 Results **39**

3.4 Discussion **48**

3.5 Conclusion **50**

CHAPTER 4:DETECTION OF ANTIGENS AND ANTIBODIES TO LYSSAVIRUSES IN BATS IN PLATEAU STATE. **52**

4.1Introduction **52**

4.2 Bat Samples **54**

4.2.1Study area 54

4.2.2 Bat survey and sample collection 54

4.2.3Bat identification 58

4.3 *Lyssavirus* Diagnosis **58**

4.3.*1Lyssavirus* antigen detection by DFA test on bat brain tissues. 58

4.3.2 Modified rapid fluorescent focus inhibition test (RFFIT) detection of virus neutralizing antibodies in batsera 59

4.3.3 Reading and interpretation of results 61

4.4 Results **63**

4.4.1Bats sampled 63

4.4.2 Lyssavirus neutralising antibody and antigen detection 63

4.5Discussion **68**

CHAPTER 5: SEARCH FOR SOME RNA VIRUSES IN BATS CAPTURED FROM PLATEAU STATE, NIGERIA 71

5.1Introduction 71

5.2 Materials and Methods 73

5.2.1 Study area 73

5.2.2 Bat survey, sampling and identification 73

5.2.3 Molecular techniques used for detection of selected RNA viruses at the CDC, GA. 73

5.2.4 TOPO® TA cloning with Invitrogen pCR 4.0 83

5.3Results 91

5.4. Discussion 108

CHAPTER 6: GENERAL DISCUSSION AND CONCLUSION……..…….…113

6.1General Discussion 113

6.2 Conclusion 115

6.3Recommendations 115

6.4Limitations of the Study 116

REFERENCES 117

APPENDICES……..…….135

LIST OF TABLES

Table	Title	Page
3.1:	Primers used for RT-PCR diagnosis of rabies in dog brain tissues	35
3.2:	Distribution of Dog study population by source, age, and sex and DFAT positive	42
4.1:	Bats sampled and geographic positions of their roost sites	56
4.2:	Biodata and serologic data on bats captured for detection of lyssaviruses	65
4.3:	Rapid flourescent focus inhibition test (RFFIT) for viral neutralizing antibody ToLBV and SHBV	66
4.4	Rapid flourescent focus inhibition test (RFFIT) for viral neutralizing antibody toLBV, MOKV and SHBV	67
5.1:	Consensus degenerate primers used for pan-viral RNA detection in this study	77
5.2:	Sequencing Reaction Protocol	81
5.3:	Components for adding Poly-A tail per reaction (using Invitrogen Kit)	84
5.4:	Colony PCR Protocol- using MasterAmp DNA polymerase	87
5.5:	Colony Sequencing Reaction Mix	88
5:6:	Frequency of some RNA viruses detected in faecal samples from different species of bats in Plateau State	99

LIST OF FIGURES

Figure	Title	Page
3.1:	A map of Plateau State showing Dawaki and Amper …….……..	30
3.2:	Sequence contigs of RD135, and RD138……..…….	43
3.3:	Sequence contigs of RD154, and RD187….…….……..…..…44	
3.4:	A radial Phylogenetic tree showing the four rabies viruses from this study…….	45
3.5:	Phylogenetic tree drawn using the entire genome of DRV-NG11/Nigeria(RD135)…….	46
3.6:	Phylogenetic tree of the completely sequenced DRV-NG11/Nigeria isolate……..	47
4.1.	Map showing the geographic location where specific bats were obtained……..…….....	57
5.1:	Phylogenetic tree of eight coronaviruses from Plateau State bats and 60 selected human and animal coronaviruses…….	100
5.2:	BLAST sequence of *Coronavirus* identified in Plateau State bats in this Study…….……..	101
5.3:	A Phylogenetic tree generated of a*Paramyxovirus* from Plateau State batand 49 selected human and animal paramyxoviruses from the NCBI database…….……..	102
5.4:	BLAST sequence of the *Paramyxovirus*obtained in a Plateau State bats 'NGR_1410_Paramyxovirus' with 16 nucleotide sequences from theGenBank…….……..	103
5.5:	Phylogenetic tree of *Reovirus*identified in bat in this study compared with 16 nucleotide sequences of human and animal reoviruses from the GenBank……...…….…	104
5.6:	BLAST sequence of *Reovirus*identified in Plateau State bats 'NGR_1354_orthoreovirus' with 16 nucleotide sequences from the	

GenBank…….…….……105
5.7: Phylogenetic tree of rotaviruses (16) identified in a Plateau State bats compared with 26 nucleotide sequences of human and animal rotaviruses fromthe GenBank……..……..…106
5.8: BLAST sequence of Rotavirusidentified in Plateau State bats and those obtained from NCBI for the Phylogenetic analysis……107

LIST OF PLATES

Plate	Title	Page
I:	An agarose gel under uv light of Ngene of Rabies virus……..…….41	
II:	Typical microscopic fields viewed after carrying out RFFIT…….....….62	
III:	Agarose gel showing amplicons following nested RT-PCR with *Coronavirus* positive amplicons……..……93	
IV:	An agarose gel under UV light following nested RT-PCR showing *Paramyxovirus* positive amplicon……..……..…….94	
V:	An agarose gel under UV light following nested RT-PCR showing *Rotavirus*……..95	
VI:	An agarose gel under UV light following amplificationshowing *Rotavirus* following nested RT-PCR…….……...96	
VII:	An agarose gel under UV light following nested RT-PCR *Rotavirus* specificamplicons obtained in lane 61(upper panel) and 77 (lower panel)…….....97	
VIII:	An agarose gel under UV light following nested RT-PCR of *Reovirus* (133bp) in 28 and *Rotavirus* ……..…….....…98	

LIST OF APPENDICES

Appendix	Title	Page
I:	"Genome announcement" A published work from this study…….....	135
II:	Unique wing features in identification of bats…….…….....	136
III:	Unique tail and tail membrane features in identification of bats…….…….	137
IV:	Unique facial features in identification of bats……..……	138
V:	Unique characteristics of palatal ridges in different bats……..…..……....	139
VI:	Some unique bats sampled in Plateau State…… ……..…....	140
VII:	Preparation of cell culture medium (MEM) and Mouse neuroblastoma cells (MNA) Preparation, Propagation and maintenance for RFFIT…….…….....	141
VIII:	An example of RFFIT end-point titration result…….……	144
IX:	Determining the 50% end-point titers of serum:……..……...…	145
X:	Pathologies observed in some bats that were sampled…….…….....	147
XI:	RT-PCR protocol for arenaviruses…….…….....	148
XII:	RT-PCR protocol for coronaviruses…….....	149
XIII:	RT-PCR protocol for influenza viruses…...…….....	150
XIV:	RT-PCR protocol for paramyxoviruses …….....	151
XV:	RT-PCR protocol for pneumoviruses …….…….....	152
XVI:	RT-PCR protocol for *Rhabdivirus*…….…….……...…….....	153
XVII:	RT-PCR protocol for *Rhabdivirus* (VSV-1)…….……	154
XVIII:	RT-PCR protocol for reoviruses ……...…….…….....	155
XIX:	RT-PCR protocol for rotaviruses….……..…….....	156

LIST OF ABBREVIATIONS AND SYMBOLS

MEGA	Molecular Evolutionary Genetics Analysis version
NVRI	National Veterinary Research Institute
RNA	Ribonucleic acid
DFAT	Direct fluorescent antibody test
RT-PCR	Reverse transcription polymerase chain reaction
RFFIT	Rapid fluorescent focus inhibition test
RABV	Rabies Virus
LBV	Lagos bat virus
MOKV	Mokola virus
DUVV	Duvenhage virus
WCBV	West Caucasian bat virus
SHBV	Shimoni bat virus
CVS	Classical rabies virus strain
SARS	Severe acute respiratory syndrome
MERS	Middle East respiratory syndrome
CoV	*Coronavirus*
NJ	Neighbour joining
NCBI	National Center for Biotechnology Information
RdRp	RNA dependent RNA polymerase
CVS	Rabies challenge virus standard
TOPO	Topoisomerase
TA	Adenine and thymine
WHO	World Health Organization
CDC	Centres for Disease Control and Prevention
FFD	Fluorescent focus dose
MEM	Minimum Essential Medium
SOP	Standard operating procedure
UV	Ultraviolet light
BSL	Biological safety laboratory
Exo-SAP	Exonuclease and shrimp alkaline phosphatase
ORF	Open reading frame
PBS	Phosphate-buffered saline hosphate-buffered saline
MNA	Mouse neuroblastoma cells
TBE	Tris-borate-Ethylenediaminetetraacetic acid
TAE	Tris-Acetate-Ethylenediaminetetraacetic acid
dNTPs	Deoxynucleotide triphosphates
bp	Base pairs
pH	Potential Hydrogen
VSV	Vesicular stomatitis virus

ABSTRACT

Rabies, the cause of an almost invariably fatal encephalomyelitis, remains a public health threat in Nigeria where the dog is the main reservoir. Ribonucleic acid (RNA) viruses are most commonly implicated in emerging and reemerging diseases. Bats are established natural reservoir hosts for many emerging viral zoonoses of enormous human and animal health impacts. Bat and dog meats are consumed by some ethnic groups in Nigeria.This study determinedthe characteristics of rabies virus in slaughtered dogs, detected some RNA viruses in bats and serologically detected some lyssaviruses in bats from Plateau State. Brain tissues were obtained from 532 slaughtered dogs and the direct fluorescent antibody test (DFAT) was used to screen for rabies antigen**.** TheDFATpositive brain tissues were subjected to the reverse transcription polymerase chain reaction (RT-PCR). Bats (356) consisting of *Eidolon helvum*(244),*Epomophorusfranqueti*(12),*Epomophorusgambianus*(5),*Chaerophonpumila*(33),*La via frons* (3),*Nycterismacrotis*(18), *Rhinolophuslanderi*(37), *and Rhinopomamicrophylum* (4) were collected from 8 locations during 2010- 2011.Bat brain samples (356) were tested for Lyssavirus antigen by DFAT. Modified rapid fluorescent focus inhibition test (RFFIT) was used to test serum samples from 76 bats for antibodies to *Lagos bat* (LBV), *Mokola* (MOKV), *Duvenhage* (DUVV), *West Caucasian bat* (WCBV), *Shimoni bat* (SHBV) and the classical rabies virus strain (CVS-11). Using pan-viral RT-PCR with consensus degenerate primers, rectal swabs from bats (n=95) were screened for some RNA viruses. Neighbour joining (NJ) was used to construct phylogenetic trees for sequences obtained with 1000 bootstrap values. DFAT detected rabies antigen in 92 (17.29%) dog brain tissues, of which 4(4.35%) were positive by RT-PCR. The N gene of all 4 were amplified and sequenced and all had a 99% homology to other previously documented rabies viruses identified from Nigeria. Among these 4 samples, one

was further isolated and its entire genome was characterized and phylogenetic analysis showed it belongs to the Africa 2 lineage. No *Lyssavirus*was detected in the 356 bat brains. Of 76 bat sera tested, 28.94% (22/76) had neutralizing antibodies to LBV, MOKV, and SHBV. Twenty one (27.6%) neutralized LBV, 6(8%) neutralized MOKV and 18(24%) neutralized *Shimoni* bat virus. All the positive sera originated from the straw-coloured fruit bat (*Eidolon helvum*) with the exception of one *Chaerophonpumila* serum which neutralized both LBV and SHBV. Four samples neutralized both MOKV and LBV. No serum neutralized CVS-11, WCBV and DUVV. Among the 95 bat faecal swabs screened, eight (8.42%) were positive for *Coronavirus*(CoV). Seven of the identified CoVs had an average of 95% homology to the Ghana bat CoV and one had 97% identity to the Kenya bat CoV (BtKy56). All eight sequences clustered with the BetaCoV group of the SARS-CoV. One bat faecal sample was positive for *Paramyxovirus* with 70% homology to the J-virus from Kenya and one specimen was also positive for *Reovirus* with sequence relating closely (93%) to the mammalian *Orthoreovirus.* Sixteen specimens (16.84%) were positive for *Rotavirus* (RV) RNA of which phylogenetically, 13 sequences were related to RV A, 1 to RV B and 2 to RV C. None of the faecal swabs was positive for *Arenavirus*, *Influenza* virus, and *Rhabdoviruses*. The continued detection of rabies virus in trade dogs and other lyssaviruses in bats show their importance in the epidemiology of lyssaviruses in Nigeria. The detection of *Coronavirus, Paramyxovirus, Reovirus* and *Rotavirus* in feacal samples of the bats is an indication of the role that bats may play in the emergence of viral diseases.

CHAPTER ONE

INTRODUCTION

1.1 Background to the Study

Rabies virus (RABV), genus *Lyssavirus* and family *Rhabdoviridae*, is enzootic throughout most of the world. The domestic dog (*Canisfamiliaris*) acts as the principal vector in Africa and Asia (Knobel *et al.,* 2005; 2007). However, a range of mammalian carnivores also can act as hosts (Nel *et al*.,1993; Swanepoel *et al.,* 1993; Rupprecht *et al*.,1995; Real *et al.,* 2005; Velasco-Villa *et al.,* 2005; Hass and Dragoo, 2006; Childs *et al*., 2007; Davis *et al.,* 2007).

Bats are established as the natural reservoir hosts for many emerging viral zoonoses and more than 60 viral species have been detected in various regions of the world with enormous human and animal health impacts. Some of these viruses include: Lyssaviruses (Calisher *et al.,* 2006), paramyxoviruses (Murray *et al.,* 1995; Chua *et al.,* 2000; Drexler *et al.,* 2012), filoviruses (Leroy *et al.,* 2005; Calisher *et al.,* 2006), coronaviruses (Li *et al.,* 2005; Tong *et al.,* 2009; Quan *et al.,* 2010), *influenza* virus (Tong *et al.,* 2012)and reoviruses (Esona *et al*., 2010; Wong *et al*., 2012). Viruses from bats could be transmitted to humans via several means such as: bites, exposure to infected saliva, faecal aerosols and contact with infected tissue materials or infected host (Wong *et al.,* 2012).

Recently, a severe acute respiratory syndrome *Coronavirus* (SARS-CoV) like virus was identified and characterized in a leaf-nosed bat (*Hipposideros* spp) in Zaria, Nigeria (Quan *et al.,* 2010). The outbreak of SARS caused by SARS-CoV in 2003 with subsequent implication of the Chinese horseshoe bats (*Rhinolophus*) as possible reservoir of the SARS-CoVs-like viruses

(Baric, 2008; Ksiazek *et al.,* 2003; Marra *et al.,* 2003; Peiris *et al.,* 2003; Rota *et al.,* 2003) has renewed surveillance interest.

In some parts of Nigeria, bats are eaten by the local people, while in other parts, tradition ensures the protection of colonies and consequently, undisturbed colonies may exist in villages and towns. Occasionally, the skulls and smoke-dried bodies of straw coloured fruit bats *Eidolon helvum*, Gambian Epaulet bats and Franquets bats are found in "juju" stalls because they are thought to cure bareness and promote fertility in women (Happold, 1987).

1.2 Statement of Research Problem

Rabies history in Nigeria dates back to 1912 and 1925 in man and animals respectively (Boulger and Hardy, (1960). Aghomo *et al.*(1987), reported the presence of rabies virus antibodies in over 15.9% of unvaccinated, apparently healthy dogs in south western part of Nigeria.However, it has been reported that a few dogs infected with rabies do not die but may remain as carriers (Bell, 1975).Conditions conducive to such nonfatal outcomes after exposure to rabies virus may include the infectious dose, route of exposure, and particular virus variant, among other variables. For example, the intraperitoneal and mucosal routes appear to be associated with less severe outcomes than do typical intracerebral or intramuscular exposures (Bell and Moore, 1971; Correa-Giron *et al*., 1970).

Rabies is a deadly infection having almost 100% case fatality and kills between 35,000 to 55,000 people every year worldwide (Warrell and Warrell, 2004). It has greater impact in the developing world and transmission from dogs represents more than 99% of all human cases reported

worldwide (WHO, 1992). The bulk of the estimated 55,000 annual global human mortalities due to rabies occur in developing countries, with 56% occurring in Asia and 44% in Africa. These two continents alone bear an estimated cost of US$583.5 million (WHO, 2005) related to rabies prevention.

The domestic dog is the main reservoir for RABV in Nigeria (WHO, 1992).Bats play an important or exclusive role in the epidemiological cycles of many Lyssaviruses (Badrane and Tordo, 2001). Human consumption of bats occurs in many parts of Nigeria and the public health implication of this practice is yet to be assessed. Commercially available antirabies biologicals do not provide adequate immunity against LBV, MOKV, SHBV and West Caucasian bat virus (WCBV) (Hanlon *et al.,* 2005). The need to know if these lyssaviruses circulate in this country will shed more light to the understanding of *Lyssavirus* epidemiology and its subsequent prevention and control.

The association of bats with emerging infectious agents, the frequency and diversity of virus detection in bats, now in many continents, demonstrate that bats are likely an important source for many viral introductions into other species globally. The biodiversity of bats second only to rodents, high population densities, wide distribution and ability to fly over long distances allow them to harbour and easily spread multiple infectious agents. Bats have long been known as natural hosts for lyssaviruses (Childs *et al*., 2007) and more recently have been recognized as potential reservoirs for emerging human pathogens, including *Influenza, Ebola, Marburg, Nipah, and Hendra* viruses in addition to SARS-CoV (Wong *et al.,* 2009; Leroy *et al.,* 2005).

Monitoring of certain viral infections in bats in Nigeria provides a foundation for detecting new disease introductions that may present a public health threat.

1.3 Justification

Bat and dog meats are commonly consumed by varied ethnic groups in Plateau State. Rabies has been a neglected disease and of low public health priority in many countries. This has resulted in rabies being the disease with the highest financial expenditure in most countries with regards to cost of post exposure prophylaxis (WHO, 2005). This monetary loss in addition to the tens of thousands of annual human deaths is potentially preventable. Accurate data are non-existent in many countries making it difficult for national policies on rabies control to be instituted.

Rabies virus has been detected in some apparently healthy Nigerian dogs with no vaccination history (Aghomo *et al*., 1987; Garba *et al.*, 2008; Akombo, 2009). In a previous study Sabo (2009), established a high prevalence of rabies (42.6%) in apparently healthy, non vaccinated dogs slaughtered for human consumption in two of Plateau State's major dog markets. Inapparent rabies in dogs may be more widespread than previously envisaged.

Studies conducted in Ibadan in 1990 (Aghomo *et al*., 1990a) and small settlements around Zaria and Mubi in 2006 (Dzikwi *et al*., 2010) detected rabies virus (RABV) and LBV neutralizing antibodies in the sera of fruit bats.

Majority of *Lyssavirus* isolates are not identified, and the actual significance of lyssaviruses for public and veterinary health is unknown (Kitala *et al*., 2000). A recent study in Malawi

demonstrated that 11.5% of human cases of cerebral malaria were actually misdiagnosed rabies cases (Mallewa *et al*., 2007). The presence of numerous lyssaviruses in bat species has led to increasing research efforts towards search for lyssaviruses in bat populations globally. In Nigeria like many other African countries, rabies surveillance is lacking. There is the need to establish an understanding of the epidemiology of rabies in Nigeria.

Bats represent approximately one fifth of the 5676 recognized species of mammals and in recent years they have been identified as hosts for viruses of human and veterinary importance (Calisher *et al*., 2006). Identification of reservoirs for such viruses, surveillance for host-jumping events, and elucidation of viral and host factors that may facilitate such events are warranted **(**Dominguez *et al*., 2007). Bats are numerous and found in distant as well as close proximity to humans in Nigeria. The handling, processing and consumption of bats of various species by children and adults, may pose some danger to the public.

This study was undertaken to detectrabiesvirus antigens in dogs slaughtered for human consumption, lyssavirus antigen and antibody and some other RNA viruses (*Arenavirus, Coronavirus, Influenza*virus, *Paramyxovirus, Reovirus, Rhabdovirus* and *Rotavirus*) of zoonotic potential in bats from Plateau State, Nigeria. Presently very few studies on zoonotic viral pathogens in bats have been documented in Nigeria; among these are the lyssaviruses (Aghomo *et al*., 1990a; Dzikwi *et al*., 2010) and coronaviruses (Quan *et al*., 2010).

1.4 Aim

To conduct a survey on *Lyssavirus* in slaughtered dogs and wild bats and determine the occurrence and molecular phylogeny of some RNA viruses of public health significance in bats from Plateau State, Nigeria.

1.5Objectives

The specific objectives of the study are;

1. To identify of rabies virus antigen in the brains of slaughtered dogs and to do phylogenetic analysis of one identified (representative) virus.
2. To investigate evidence of Lyssavirus antigens in the brain tissues of the trapped bats.
3. To determine the presence of neutralizing antibodies to Lyssaviruses in the serum samples of bats from Plateau State.
4. To search for and characterize some RNA viruses in bats from Plateau State.

1.6Research Questions

1. Are rabies antigenspresent in the brain tissues of dogs slaughtered for human consumption in Plateau State?

*2.*Do brain tissues of the bats captured in Plateau State have *Lyssavirus* antigens?

3. Are antibodies to Lyssaviruses (*Lagos bat virus* (LBV), *Mokola virus* (MOKV), *Duvenhage virus* (DUVV), *West Caucasian bat virus* (WCBV), *Shimoni bat virus* (SHBV) and the Rabies challenge virus standard (CVS) detectable in the sera of wild bats caught in Plateau State?

4. Are *Arenavirus, Coronavirus, Influenza virus, Paramyxovirus, Reovirus, Rhabdovirus* and *Rotavirus* absent in bats from Plateau State?

CHAPTER TWO

LITERATURE REVIEW

2.1**The Rabies Virus**

Rabies virus belongs to the order Mononegavirales, viruses with non-segmented, negative-stranded RNA genomes. Within this group, viruses with a distinct "bullet" shape are classified in the Rhabdoviridae family, which includes at least three genera of animal viruses, *Lyssavirus, Ephemerovirus,* and *Vesiculovirus* (Radostits *et al*., 1995).

Rhabdoviruses are approximately 180 nm long and 75 nm wide. The rabies genome encodes five proteins: nucleoprotein (N), phosphoprotein (P), matrix protein (M), glycoprotein (G) and polymerase (L). All rhabdoviruses have two major structural components: a helical ribonucleoprotein core (RNP) and a surrounding envelope. In the RNP, genomic RNA is tightly encased by the nucleoprotein. Two other viral proteins, the phosphoprotein and the large protein (L-protein or polymerase) are associated with the RNP. The glycoprotein forms approximately 400 trimeric spikes which are tightly arranged on the surface of the virus. The M protein is associated both with the envelope and the RNP and may be the central protein of rhabdovirus assembly(Panleuk, 2007).

2. 2 Rabies in Nigeria

In Nigeria, almost all the dialects have a name for rabies such as: “Chiwon hauka” (Hausa), “digbolugi” (Yoruba), “ginnaji” (Fulani), “idat ebua” (Efik/Ibibio)(Nuru,1973), “mballaha/chihwu kila” (Bhurra), “mbige dzive” (Higgi),” ara nkita” (Igbo) (Nottidge,1994), “Oga Abia” (Ighalal), “Iwa hundu” (Tiv), “Ewo-jele” (Idoma) (Ogunkoya, 1986), “vow lolog”

(Berom) and “ass titar” (Ngas) (Sabo *et al*., 2008), all which essentially translate to “mad dog disease”. This is an evidence of the long history of the disease in Nigeria.

The first documented report of rabies in Africa was in a dog imported from England at Port Elizabeth, South Africa in 1893 (Rollingson,1956). Since then outbreaks have been reported in all African countries (Umoh and Belino, 1979).

Rabies history in Nigeria dates back to 1912 and 1925 in man and animals respectively (Boulger and Hardy, 1960). Despite efforts to control rabies, it continues to be a major health problem in Plateau State and many parts of Nigeria (Umoh and Belino, 1979; Fagbami *et al.,* 1981; Okoh, 1986). Okoh (1982) reported the prevalence of rabies to be higher in Plateau State compared to other states in Nigeria.

2.2.1 Rabies in dogs and other animals

The disease in dogs in Nigeria was first investigated by Smith (1928) who described the clinical signs of five of the six cases reported. The laboratory in Yaba confirmed 1,046 cases of rabies in dogs between 1928 and 1959 which represented 95.1% of the total confirmed cases of rabies. In 1942, outbreaks were reported in Kano, Zaria, Katsina, Borno, Ilorin and the Cameroons (Umoh and Belino, 1979). In 1943, outbreaks were reported in Katsina and Kano and in 1946 in Kano, Plateau, Makurdi and Enugu (Anonymous, 1946).The disease was also reported in Sokoto involving 16 dogs (Umoh and Belino, 1979). In Vom, Plateau State, the National Veterinary Research Institute reported 64 canine rabies cases in 1964; 48 cases in 1965 and 35 cases in dogs and cats in 1966 (Bisseru,1972). A ten year survey (1963-1972) in Kano State revealed 59

confirmed cases of rabies and 56 unconfirmed suspects (Nuru, 1973), while a survey conducted in 1975 reported rabies to be on the increase in most states except Rivers (Umoh and Belino, 1979). In Accra, Ghana (which is not too far from Nigeria) canine rabies has been observed to peak in September and October. Interestingly, Nuru (1973) observed a similar peak for canine rabies in Kano in 1963 to 1972 and a similar peak also between January and February in two studies. In Kaduna, rabies cases seemed to cluster around April and September (Ezeokoli and Umoh, 1987).

The next in importance to the dog in frequency of rabies in Nigeria are cats. The laboratory at Yaba, reported 48 confirmed cases between 1928 and 1959 (Boulger and Hardy, 1960). They also reported 8 cases of rabies from Lagos between March, 1956, and December, 1959. Many people still associate rabies with dogs only and tend to neglect the fact that cats and other domestic animals can be rabid. For a four year period from 1965 to 1968, there were only 4 confirmed feline cases and all of them were from the Plateau Province (Owolodun, 1969).

Rabies in farm animals in Nigeria causes some concern especially as Nigeria has a great need for animal protein. An outbreak occurred in Butu, near Minna, which affected a herd of 55 cattle and a flock of 15 sheep (Elegbe and Banerjee, 1970). A rabid dog bit 45 cattle and 10 adult sheep. Four of these animals showed signs of rabies and the rest were slaughtered. In another outbreak, Friesian cows imported from England by the Kano State Government for livestock improvements were involved (Nuru and Barnes, 1974), rabies was confirmed in four of the animals. Rabies has been reported in Nigerian dwarf goat (Ojo and Adeoye, 1967), donkey (Owolodun, 1969) and pig (Boulger and Hardy, 1960). In a case study of 20 Local Government

Areas (LGA) of Bauchi State for fifteen years (1987-2001), 62 cases of rabies were recorded out of which 93.6% were dogs, 4.8% sheep, and 1.6% cow (Bello et al., 2007).

Four rabies related viruses have been isolated from Nigeria.These are Lagos bat virus isolated from pooled brains of frugivorous bat, *Eidolon helvum*, on Lagos Island by Boulger and Porterfield in 1958, the Nigerian horse virus isolated from the brain of a horse which had died from "staggers"(Porterfield *et al*.,1958), the Mokola virus isolated from viscera of shrews (Crocidura sp.) in Ibadan in 1968 (Shope *et al*.,1970) and from patients with neurologic diseases on 2 occasions (Familusi *et al*.,1972), and the Kotonkan virus isolated in 1967 from culicoides in Ibadan (Kemp *et al*.,1973). A suggestion was made that the Lagos bat, Nigerian horse and Mokola viruses were new serotypes of rabies (Shope, 1982; Kemp *et al.*, 1973).

Members of the Lyssaviruses are all morphologically, morphogenetically and pathologically indistinguishable except by the use of monoclonal antibodies. Mokola virus, for example, is capable of producing a rabies-like disease in humans and offers an explanation for the difficulties in rabies diagnosis and intermittent failure of canine rabies vaccination. Mokola virus was isolated from a dog and six cats suspected to have died of rabies in Zimbabwe (Shope, 1982; Foggin, 1983; Wiktor *et al*., 1984). Shope(1982) proposed the possibility of Mokola, Lagos bat or Duvenhage viruses as the cause of deaths in rabies post vaccinated dogs. Aghomo and Rupprecht (1990) reported that five hundred healthy privately owned dogs were tested serologically for detection of antibodies to lyssaviruses using a modification for the rapid fluorescent focus inhibition technique. Three serum samples were seropositive to Mokola virus while negative to Lagos bat and Duvenhage viruses. The three Mokola seropositive samples

were negative to rabies virus neutralizing antibodies. This study suggests that Mokola virus may not be uncommon in dogs in Nigeria.

2.2.2Sub-clinical rabies

In recent times, latent infection and inapparent carriers of rabies have assumed some importance in rabies epidemiology. Carrier status occurs in bats (Radostits *et al.,* 2000). Many authors reported cases in Africa and elsewhere which show that infected dogs may not die but continue to live as carriers (Arko *et al.,* 1973; Bell, 1975; Aghomo *et al*., 1987; 1990b). An immunohistochemical survey for rabies virus infection among apparently healthy dogs in Maiduguri, Nigeria, reported 31% prevalence (Ajayi *et al*., 2006). Also 463 serum samples from non-vaccinated dogs in Nigeria were examined and neutralizing antibodies to rabies was detected in 142 dogs indicating rabies exposure in canine population from Kaduna, Oyo and Lagos states of Nigeria (Ogunkoya, 1986). A study of four south western Nigerian states of Oyo, Ogun, Ondo and Lagos, obtained 15.0% sero-prevalence of rabies antibody (Aghomo *et al.,* 1990b). Recently, Okoh (2007) in an augural lecture estimated that about 10,000 people are exposed to rabies each year in Nigeria. Reports from studies conducted on brain tissues of dogs slaughtered for human consumption showed alarming prevalence of 31% in Maiduguri, Borno State (Ajayi *et al*., 2006), 31% in Sokoto (Garba *et al*., 2008), 15.8% in Benue State (Akombo, 2009), and 42.9% in Plateau State (Sabo *et al*., 2009).

2.2.3Dogmeat consumption in Nigeria

In Nigeria, dogs are not slaughtered in approved abattoirs and slaughter slabs as other livestock, because they are nationally not considered as food animals. In spite of this, dogs are still

slaughtered and sold locally for human consumption in Army Barrack community markets. Dog meat is considered a delicacy in some states of Nigeria particularly in Cross River, Plateau, Kaduna, and Gombe and the Federal Capital Territory (Senan, 2007). Some people consume it as an alternative source of meat or for specific medicinal benefits attributed to various parts of the dog. There exist three major dog meat pepper soup restaurants (commonly referred to as 'joints') in Kubwa, a satellite town in Abuja, the Nigerian Federal Capital territory. "South Africa" is the name given to the most popular of the three having twenty employees (Humphrey, 2007). The dog meat eating population in Cross River and FCT refer to the meat commonly as "404" (for the local breed of dogs), and "J-5" (refers to the exotic breed) a tribute to a dog's ability to run fast.

2.3 Lyssaviruses and Bats

The *Lyssavirus* infection of bats occurs across much of the globe, although different virus species are present in different regions and tend to infect particular bat species (Streicker *et al.,* 2010). In the Americas, only RABV is associated with bats, whereas across Europe, Africa, Asia, and Australia, otherbat lyssaviruses are present but with the complete absence of bat associated RABV in these countries. Phylogenetic analyses and virus–host relationships suggest that all lyssaviruses, including the RABV, likely originated in bats. With the exception of Mokola virus (MOKV), all lyssaviruses have been isolated from bats (Badrane and Tordo, 2001; Nel and Rupprecht, 2007).

2.3.1 Bats lyssaviruses in Africa

2.3.1.1*Lagos bat virus (LBV)*

There have been more isolations of LBV than any of the other African bat lyssaviruses recognized, with the virus apparently circulating among bats in sub-Saharan Africa (Calisher *et al*., 2006). Several bat species have been associated with LBV infection, and these include Wahlberg's epauletted fruit bat (*Epomorphorus wahlbergi*), the straw-coloured fruit bat (*Eidolon helvum*), the Egyptian fruit bat (*Rousettus aegyptiacus*), and an insectivorous bat, the Gambian slit-faced bat (*Nycteris gambiensis*). Spill-over events of LBV from bats into water mongooses have been reported (Markotter *et al.,* 2006a, 2006b) with infection of humans never having been demonstrated (Markotter *et al.,* 2008). LBV was first isolated from pooled *E. helvum* brain material in Nigeria in 1956 (Boulger and Porterfield, 1958).

Studies have revealed a high seroprevalence of antibodies against LBV in two fruit bat species, *E. helvum* and *R. aegyptiacus* (Kuzmin *et al.,* 2008a; Hayman *et al*., 2008; Dzikwi, *et al.,* 2010). Seroprevalence ranged from 14% to 67% in *E. helvum* and 29% to 46% in *R. aegyptiacus,* with adult *R. aegyptiacus* having a higher seroprevalence (60%) than subadults (31%). As previously reported, a substantial proportion (38%) of sera that neutralized LBV also neutralized MOKV (Badrane and Tordo,2001; Hanlon *et al.,* 2005; Kuzmin *et al.,* 2008b; Wright *et al.,* 2010). Kuzmin *et al.* (2008b) reported an absence of detectable LBV from the oral swabs of 931 healthy bats by nested RT-PCR, but from the brain material of a bat found dead they isolated LBV andsequenced the entire genome.

The discovery of further LBV isolates and their genome analysis have suggested that LBV phylogeny is more complex than originally thought (Kuzmin *et al.,* 2008b, 2010; Nadin-Davis *et al.,* 2002). A Senegalese (1985), a Kenyan (2007), and a French isolate (either Togolese or Egyptian origin, 1999) are highly similar (>99% nucleotide identity across the N gene) and constitute lineage A. The original isolate (Nigeria, 1956) is genetically distant from lineage A isolates and constitutes lineage B, whereas a third lineage (C) is made up of isolates from the Central African Republic, Zimbabwe, and South Africa (Markotter *et al.,* 2006a). Also a fourth lineage (D) has been characterized in Kenya from an Egyptian fruit bat having only 79.5–80.9% similarity to lineages A- C (Kuzmin *et al.*, 2010). These four distinct groups are geographically clustered with the eight isolates from South Africa showing very little sequence variation, despite isolations occurring over a 25-year period (Markotter *et al.,* 2008). Divergence between lineages is high with lineage A sharing <80% identity with the other lineages across a fragment of the N gene, a percentage cutoff value previously suggested as suitable for *Lyssavirus* genotype division (Bourhy *et al.,*1993; Kissi *et al*.,1995).

2.3.1.2 Mokola *virus (MOKV)*

Although MOKV has not been isolated from bats, isolates are available from other mammalian species and the host reservoir(s) remain unknown (Meredith *et al.,* 1996; Sabeta *et al.,* 2007). Cross-neutralization occurs between lysaviruses of same phylogroups for LBV and MOKV as observed by Kuzmin *et al.* (2008b).

2.3.1.3 *Duvenhage virus (DUVV)*

DUVV was first isolated in South Africa in 1970 from a human who died following a bat bite (Meredith *et al.,* 1971). Further isolations from insectivorous bats occurred from *M. schreibersii* in South Africa in 1981 and *Nycteris thebaica* in Zimbabwe in 1986 (King and Crick, 1988; Paweska *et al.,* 2006). Interestingly, the *N. thebaica* was trapped in a survey, with no clinical signs of rabies reported (Foggin, 1983). Two further cases of DUVV in humans have been reported; one from South Africa (Paweska *et al.,* 2006) and the other from the Netherlands, the virus being of Kenyan origin (van Thiel *et al.,* 2008).

2.3.1.4 *West Caucasian bat virus (WCBV)*

WCBV appears to have a wide geographical range, including both Eurasia and some parts of Africa. A recent study reported neutralizing antibodies against WCBV in *Miniopterus* bats collected in Kenya with prevalence ranging from 17%to 26% in sampled bats (Kuzmin *et al.,* 2008c). WCBV seropositive bats were detected in four of five locations sampled across Kenya. This finding provides evidence that WCBV, originally isolated in Europe, may emerge in other continents.

2.3.1.5*Shimoni bat virus (SHBV)*

SHBV was first isolated in 2009, and represents the most recently detected bat lyssavirus, potentially a new species, isolated from the brain of a dead Commerson's leaf-nosed bat (*Hipposideroscommersoni*) in Kenya. The virus lies antigenically within phylogroup II, being classified phylogenetically between MOKV and WCBV (Kuzmin *et al.,* 2010). The

Hipposideros subfamily has a broad distribution in the Old World from tropical Africa to China. Interestingly, *M. schreibersii,* a species from which EBLV-1, DUVV, and WCBV have been isolated or RNA detected, has a distribution from the Middle East and Caucasus, across southern Europe and down through Africa (Banyard *et al*., 2011).

2.3.2 Insectivorous bat-associated rabies

Initial detection of RABV in insectivorous and frugivorous bats was made during surveys into haematophagous bat populations and rabies transmission in South America (Carini, 1911; Pawan, 1936). It was not until the first detection of rabies in an insectivorous bat in the early 1950s that insectivorous bats were also found to harbour the virus in North America (Baer and Smith, 1991; Brass, 1994; Sulkin and Greve, 1954). A recent study in Brazil documented that 41 species of bats have historically been associated with transmission of bat rabies, including 25 genera and three bat families: the *Phyllostomidae*, the *Vespertilionidae,* and the *Molossidae* (Sodre *et al.,* 2010). Across the Americas, 16% of wildlife-associated rabies cases were reported from bat species, mostly nonhaematophagous bats, between 1993 and 2002 (Belotto *et al.*, 2005). With the elimination of terrestrial RABV in companion animals in North America, the transmission of RABV to humans from bats has become of increasing importance as a public health risk (Messenger *et al.,* 2002). Due to increased surveillance and general scientific interest in RABV as a viral pathogen and the ecology and biology of bat species there has been a dramatic surge in the number of bat rabies cases being reported in the United States (Blanton *et al.,* 2010). Despite the large number of different bat species associated with RABV transmission in Central and Southern America, association of RABV infection in North America has highlighted a key role in infection of certain bat species. The big brown bat (*E. fuscus*), the little

brown bat (*Myotislucifigus*), and the Brazilian (Mexican) free-tailed bat (*Tadaria brasiliensis*) are most commonly submitted for rabies testing, although only 23.2% of those submitted are found to be positive for RABV infection (Blanton *et al.,* 2010). Interestingly, the species that are most frequently associated with transmission to humans are silver-haired bats (*Lasionycteris noctivagans*), eastern pipistrelle bats (*Perimyotis subflavus*) and Brazilian (Mexican) free-tailed bats (*T. brasiliensis*) in the United States between 2000 and 2009 (Blanton *et al.,* 2010; Brass, 2009).

Recent studies have also highlighted an apparent host restriction seen within circulating bat RABV variants in North America with a suggestion that phylogenetic barriers exist to cross-species transmission at the level of both initial infection and sustained transmission within the newly infected species (Streicker *et al.,* 2010). A series of experiments in the late 1950s highlighted potential mechanisms for virus persistence by virtue of body temperature as well as suggesting a possible role for the adipose tissue of hibernating bats in the maintenance of RABV (Sulkin, 1962; Sulkin *et al.,* 1954, 1960), although later studies were unsuccessful in corroborating an involvement of brown fat in virus maintenance through periods of torpor (Kuzmin *et al.,* 1994). Throughout the 1960s, extensive studies were undertaken in caves where naturally infected insectivorous bats were present to attempt to elucidate potential transmission mechanisms. Caged coyotes and foxes were positioned under large roosts and monitored, and the caged animals invariably developed clinical rabies (Constantine, 1967) highlighting the potential for aerosol transmission of RABV (Constantine, 1966). However, it is not clear that biting can be ruled out as a potential transmission mechanism in this study. Although natural exposure via bats and aerosol route has been reported (Winkler, 1968; Winkler *et al.,* 1973), experimental attempts

to infect mice by this route have had success with RABVs but not with other lyssaviruses (Johnson *et al.,* 2006).

Genetic analysis of glycoprotein sequences from viruses circulating in both bat and terrestrial carnivore species suggests that host switching of lyssaviruses from bats to other mammals had occurred repeatedly and successfully in history (Badrane and Tordo, 2001). Clearly, the role of antibody development following infection differs between individuals, and ultimately, other immunological components such as cell-mediated mechanisms play an important role in host response to infection (Turmelle *et al*., 2010).

Other studies have addressed the susceptibility of different bat species to infection and there appears to be a virus–host dependence on the outcome of infection. Numerous observations suggest that both haematophagous and nonhaematophagous bats that form highly social roosts appear to be less susceptible to disease with indigenous RABV isolates (Banyard *et al*., 2011). Where infection occurs, clinical disease is observed as a nonaggressive, paralytic form of rabies. In direct contrast, less social bats appear to have a heightened susceptibility to RABV variants and appear more likely to develop a furious form of the disease following infection (Banyard *et al*., 2011).

2.3.3Bat rabies and host switching

Successful species-to-species transmission of viral pathogens is an important factor in the emergence and evolution of microorganisms (Banyard *et al*., 2011). For viruses, such transmission can lead to host switching whereby a virus can spill-over into a novel host and

theoretically alter the virus genome to enable efficient replication and maintenance within the recipient species. This fact is of particular importance for zoonotic pathogens where host switching may lead to establishment of novel human pathogens with altered virulence. Host switching has been proposed as the evolutionary mechanism by which lyssaviruses have evolved through spill-over from bats to infect terrestrial mammals (Badrane and Tordo, 2001).

In 1993, a small outbreak of rabies occurred in foxes on Prince Edward Island, Canada. The genetic detection of a bat RABV variant within this fox population was confirmed, and from the detection of low levels of virus in salivary gland material, it was assumed that some degree of intraspecific transmission among the foxes had occurred (Daoust *et al.,* 1996). A second report from Arizona, USA in 2001, highlighted the infection of a number of skunks with a bat RABV variant. This virus was phylogenetically most similar to that known to be present in *Eptesicus* and *Myotis* spp. populations from the same region (Leslie *et al.,* 2006). Sustained intraspecies transmission of this bat variant of RABV in the skunk population was the most likely explanation as the skunk cases occurred over a period of only 7 months (Leslie *et al.,* 2006).

2.4 Coronaviruses in Bats

Coronaviruses (CoVs) are enveloped viruses with a positive-sense, single-stranded RNA genome (Woo et al., 2009). CoVs are classified into 4 genera: Alphacoronavirus (α), Betacoronavirus (β) (grouped further into clades 2a–2d), Gammacoronavirus(ɣ) and Deltacoronavirus (б). Horseshoe-nosed bats of several species (suborder Microchiroptera, family Rhinolophidae, genus *Rhinolophus*) from different locations in southern People's Republic of China and the Hong Kong Special Administrative Region were found to be infected with SARS-like CoVs, and

some of the bats had antibodies to these newly recognized coronaviruses (Li *et al*., 2005; Woo *et al*., 2006). Phylogenetic analysis of the complete genome sequences of the bat SARS-like CoVs showed that they form a large and diverse clade within phylogenetic group 2b (also called group 4), which includes SARS-CoVs from palm civets and humans obtained during the 2002–2003 outbreak (Lau *et al*., 2005). Thus, the virus responsible for the SARS pandemic may have originated from bats, perhaps with the palm civet as an intermediate host. In addition to SARS-like CoVs, RNAs of many other viruses belonging to coronavirus groups 1 and 2a, and proposed new group 5, were detected in several species of Asian bats (Poon *et al*., 2001; Tang *et al*., 2006; Woo *et al*., 2007).

Two human coronaviruses (hCoVs), termed hCoV-OC43 and −229E, have been known since the 1960s and cause chiefly mild respiratory disease (Saif, 2004). In the aftermath of the SARS pandemic, 2 hCoVs, termed hCoV-NL63 and -HKU1, and numerous novel bat CoVs were described (Annan *et al*., 2013). In September 2012, health authorities worldwide were notified of 2 cases of severe respiratory disease caused by a novel hCoV (Corman *et al*., 2012; Zaki *et al*., 2012). This virus, termed EMC/2012, was related to the 2c betacoronavirus clade, which had only been known to contain *Tylonycteris bat coronavirus* HKU4 and *Pipistrellus bat coronavirus* HKU5 (Woo*et al*., 2006). A novel coronavirus, now called Middle East respiratory syndrome coronavirus (MERS-CoV), has been identified in several countries across the Middle East and Europe, with primary infections found in Saudi Arabia, Qatar, Jordan, and The United Arab Emirates (UAE) (WHO, 2014). MERS-CoV is more closely related to the bat coronaviruses HKU4 and HKU5 (lineage 2C) than it is to SARS-CoV (lineage 2B) sharing more than 90% sequence identity with their closest relatives, bat coronaviruses HKU4 and HKU5 and

therefore considered to belong to the same species(de Groot *et al*., 2013). But there is no definitive evidence that MERS-CoV originates in bats (de Groot *et al*., 2012).

In a recent study, an extended sample of 4,758 bats from Ghana and 272 bats from 4 European countries were analysed and a novel clade 2c betacoronaviruses was detected in *Nycteris* bats in Ghana and *Pipistrellus* bats in Europe that were phylogenetically related to the novel hCoV EMC/2012. All previously known 2c bat CoVs originated from vespertilionid bats: VM314 originated from a *P. pipistrellus* bat from the Netherlands and HKU4 and HKU5 originated from *Tylonycteris pachypus* and *P. abramus* bats, respectively, from the People's Republic of China. The *Nycteris* bat virus in Africa extends this bat CoV clade over 2 different host families, (*Nycteridae* and *Vespertilionidae*) (Annan *et al*., 2013).

Genetic analysis revealed that bat coronaviruses mainly clustered into three different groups: group 1, a group including all SARS and SARS-like coronaviruses from different hosts (putative group 4), and an independent bat coronavirus group (putative group 5). Further characterization of bat coronaviruses revealed high genetic diversity across a large geographic distribution and revealed that different species of bats maintain coronaviruses from different groups and that the same species of bat from different geographic locations can also contain the same type of coronavirus (Drexler *et al*., 2010).

2.5Paramyxoviruses in Bats

Many bat-associated paramyxoviruses have emerged and some of them have caused diseases in animals and humans (Wang *et al.,* 2001). A large and diverse host species are infected by

paramyxoviruses, including avian, porcine, canine, bovine, equine, ovine, reptiles, aquatic species and humans (Horwood *et al.,* 2008; Lamb and Parks, 2007; Nollens *et al.,* 2008; Nylund *et al.,* 2008).

Mapuera virus (MPRV) was isolated in 1979 from an apparently healthy tropical fruit bat (Sturnira lilium) in Brazil (Karabatsos, 1985). Genomic studies showed that MPRV was closely related to La Piedad Michoacán paramyxovirus (LPMV) (Wang *et al.,* 2007), also known as porcine rubulavirus (PoRV), the only well studied neurotropic paramyxovirus isolated from pigs prior to the 1990s (Moreno-Lopez *et al.,* 1986). The host range and disease-causing potential of MPRV is still unknown. In late 1994 in Australia, Hendra virus (HeV) caused an outbreak of severe respiratory disease resulting in the death of 13 horses and their trainer (Murray *et al.,* 1995), followed by sporadic HeV outbreaks in horses and humans (Field *et al.,* 2007a). Subsequent serosurveillance of wild-caught wildlife showed evidence that flying foxes (genus *Pteropus* and order *Chiroptera*) were the natural hosts of HeV (Field *et al.,* 2007b). Hendra and Nipah virus have been identified in various *Pteropus* spp. (flying foxes) fruit bats (Halpin *et al*., 2000; Chua *et al*., 2002; Drexler et a., 2009; Wibbelt *et al*., 2010; Lau *et al*., 2010). A closely related virus, Nipah virus (NiV) from Malaysia caused severe febrile encephalitis and death in pigs and humans (Chua *et al.,* 2000), which spread to Bangladesh and India (Chadha *et al.,* 2006; Hsu *et al.,* 2004). HeV and NiV were assigned as a genus, *Henipavirus*, in the sub-family Paramyxovirinae (Eaton and Broder,2006). For Menangle virus, another paramyxovirus isolated from fruit bats, less pathogenic courses of disease in humans have been described (Halpin *et al*., 1999). In 1997, Menangle virus (MenV) was isolated in Australia from still-born pigs with deformities (Philbey *et al.,* 1998), and associated human illness (Chant *et al.,* 1998). Neutralizing

antibodies against this virus was also detected in several fruit bat species (Philbey *et al.*, 1998). In 2000, Tioman virus (TioV) was isolated from urine collected beneath a fruit bat colony on Tioman Island, Malaysia (Chua *et al.*, 2001).

For other bat paramyxoviruses isolated, infections in humans have yet to be associated, for example, Tioman virus from flying fox (Chua*et al*., 2001), bat parainfluenza virus from flying fox (Pavri *et al*., 1971), Tuhoko virus from flying fox and Mapuera virus from non-pteropid fruit bat (Lau *et al*., 2010). All viruses of the family *Paramyxoviridae* so far detected in bat species have been identified in fruit bats across Africa, Australia, South America, Asia, and Madagascar (Halpin *et al*., 2000; Chua *et al*., 2002; Wong *et al*., 2009; Drexler *et al*., 2009; Lau *et al*., 2010). Only a few studies attempting the isolation of paramyxoviruses in bats concerned insectivorous bat species, and all of them turned out with negative results (Young *et al*., 1996; Yob *et al*., 2001). The only indication of paramyxoviruses in this group of bat species was the detection of Nipah virus antibodies in lesser Asiatic yellow bats (*Scotophilus kuhlii*) (Yob *et al*., 2001).

2.6Reoviruses in Bats

The first reovirus of bat origin, Nelson Bay virus (NBV), was isolated in 1968 from the blood of a flying fox (*Pteropus poliocephalus*) in New South Wales, Australia (Gard and Marshall, 1973). NBV was the first mammalian orthoreovirus to display the fusogenic properties characteristic of avian orthoreoviruses (Gard and Compans, 1970). In 1999, during a search for NiV in pteropid bats on Tioman Island, a novel orthoreovirus, Pulau virus (PulV), was isolated from*Pteropus hypomelanus* (Chua, 2003; Pritchard *et al*., 2006). Serological and sequence characterization revealed that PulV was closely related to NBV. It is not known whether these bat orthoreoviruses

are capable of infecting and causing disease in animals or humans (Chua *et al*., 2007). Since 2006, two fusogenic orthoreoviruses, Melaka virus and Kampar virus, have been independently isolated in Malaysia from respiratory samples of patients with acute respiratory illness though no death has been reported (Du *et al.,* 2010; Chua *et al.,* 2008). All of these newly isolated orthoreoviruses are genetically closely related to the Nelson Bay virus and Pulau virus, both of bat origin (Gard and Marshall, 1973; Pritchard *et al*., 2006). Due to the high genetic similarity of genome sequences and strong cross reactivity at the serological level (Chua *et al.,* 2008), it has been proposed, after recent consultation with the International Committee on Virus Taxonomy, that all of these viruses be placed under a new species, *Pteropine orthoreovirus* (PRV), replacing the existing species, *Nelson Bay orthoreovirus*. The new name has a better fit with other existing species names in the genus such as *Avian orthoreovirus* (ARV) and *Mammalian orthoreovirus* (MRV).

2.7 Rotaviruses in bats

Until 2010, *Rotavirus* was not detected in bats, although other viruses in the family *Reoviridae* have been detected in bats (Calisher *et al.,* 2006). Esona *et al*. (2010) in a study reported the first detection and genetic characterization of a bat rotavirus with a VP4 gene that was nearly identical to human P [6] strains suggesting introduction as a result of a ressortment event between human and bat rotaviruses. The NSP4 gene of this bat *Rotavirus* also suggests a reassortment with human or animal strains. The possibility that the VP4 and NSP4 gene segments were originally bat rotavirus genes, which have evolved and become ubiquitous among human rotaviruses for several years, cannot be excluded. However, this possibility is less

likely because 7 of the Bat/KE4852/07 genes were genetically divergent from any previously reported rotavirus genotype for humans or animals (Esona *et al*., 2010).

CHAPTER THREE

MOLECULAR STUDIES OF RABIES IN TRADE DOGS IN PLATEAU STATE NIGERIA

3.1 Introduction

Rabies remains a public health threat around the world with more than 55,000 human deaths each year, mostly in the developing countries where canine rabies is endemic (Wunner, 2007). In Nigeria, rabies was reported as far back as 1912 and 1925 in man and animals, respectively (Boulger and Hardy, 1960). Plateau State has flourishing dog markets where dogs from different states of the country and from Niger and Chad Republics are brought in and sold to traders from within the State and other parts of the country (Sabo *et al.,* 2008).

Sabo (2009) established a high prevalence of rabies (42.6%) in apparently healthy, non-vaccinated dogs slaughtered for human consumption at two major dog markets in Plateau State. Based on the findings in that study, dogs sold in these markets were sourced by hunters from ten northern states (Kaduna, Katsina, Jigawa, Kano, Yobe, Bauchi, Sokoto, Borno, Zamfara and Plateau) in Nigeria and two border countries (Niger and Chad Republics). The occurrence of apparently healthy dogs with rabies virus in the brain tissues, yet not showing clinical signs of rabies may be more widespread than previously envisaged as rabies antigens were also found in the brain tissues of apparently healthy dogs slaughtered for human consumption in Borno, Sokoto and Benue state (Ajayi et al., 2006; Garba et al., 2008; Akombo, 2009). The need to safe guard the health of the people by reducing the risk of exposure warrants continued surveillance. Thus, this study aims to determine the status and characteristics of rabies in the dogs slaughtered at the Dawaki and Ampere markets in in Kanke local government area (LGA) of Plateau State.

3.2 Materials and Methods

3.2.1 Studyarea

Kanke Local Government area (LGA) of Plateau State has two major dog markets. The markets are, Dawaki (commonly called 'Calabar' market) and Amper. Plateau state is located on a plateau landform. The land formation is categorized into two: upper plateau (rocky and a lot of hills) and lower plateau (sandy). It has striking physical features with high lands rising from 1,200 meters above sea level at the low lands to a peak of 1,829 meters above sea level. The state is located in the middle-belt zone of Nigeria (coordinates *9°10'N 9°45'E*) with a population of about 3,178,712 people. Plateau State has a near temperate climate with an approximate mean high temperature of 22°C and mean minimum low temperature of 18°C. The mean annual rainfall varies from 131.75 cm in the southern part to 146 cm on the Plateau, and highest rainfall is usually recorded in the months of July and August. The state has about 20 different ethnic groups and 17 Local Government Areas.The ethnic groups are Birom, Angas, Mwaghavul, Tarokh, Geomai, Tal, Fier, Afizere, Miango, Youm Bogghom, Rukuba, Piapung, Kwalla, Montol, Challa, Ron-Kulere, Pyem, Miship, Mupum, and Buji (http://en.wikipedia.org/wiki/Plateau_Stateaccessed 12/1/14 4:41 pm).

3.2.2 Selection of dog markets

The major dog markets in Plateau State (Bukuru (aka 'kugia'), Dawaki and Ampere) were visited, with the assistance of the then Local Government chairman of Kanke LGApermission for the study was obtained (via oral communication) from village heads and market officials.

Thus the criteria used to finally select the dog markets in this study included:

Consent of dog slaughter slab owners, consent of owners of dogs slaughtered, and participation of butchers.

The markets where more than 10 dogs are available for slaughter on a market day.

The markets that met these criteria were Dawaki (aka 'Calabar" Market) and Amper in Kanke Local Government Areas (LGA) of Plateau state.

3.2.3 Dawaki and Amper markets.

Dawaki (Latitude $9^{o}27'$N; Longitude 9^{o} 30'E) and Amper (Latitude 9^{o} 20'N; Longitude 9^{o} 45'E) which are the two major markets where dogs are sold, slaughtered and processed for human consumption in Kanke Local Government Area of Plateau State were used for the study (Fig. 3.1). The slaughter of dogs in these markets occurs at the backyards of many houses which are within the markets. Four of these backyard slaughter points identified to have consistent availability of dogs brought for slaughter consented to the study.

The market days are Wednesdays for Dawaki and Saturdays for Amper. A minimum of 40 dogs were slaughtered on each of these market days. Dawaki is known all over Nigeria by the dog eating population as a major point for mass purchase of dogs for resale elsewhere (Sabo *et al.,* 2008).

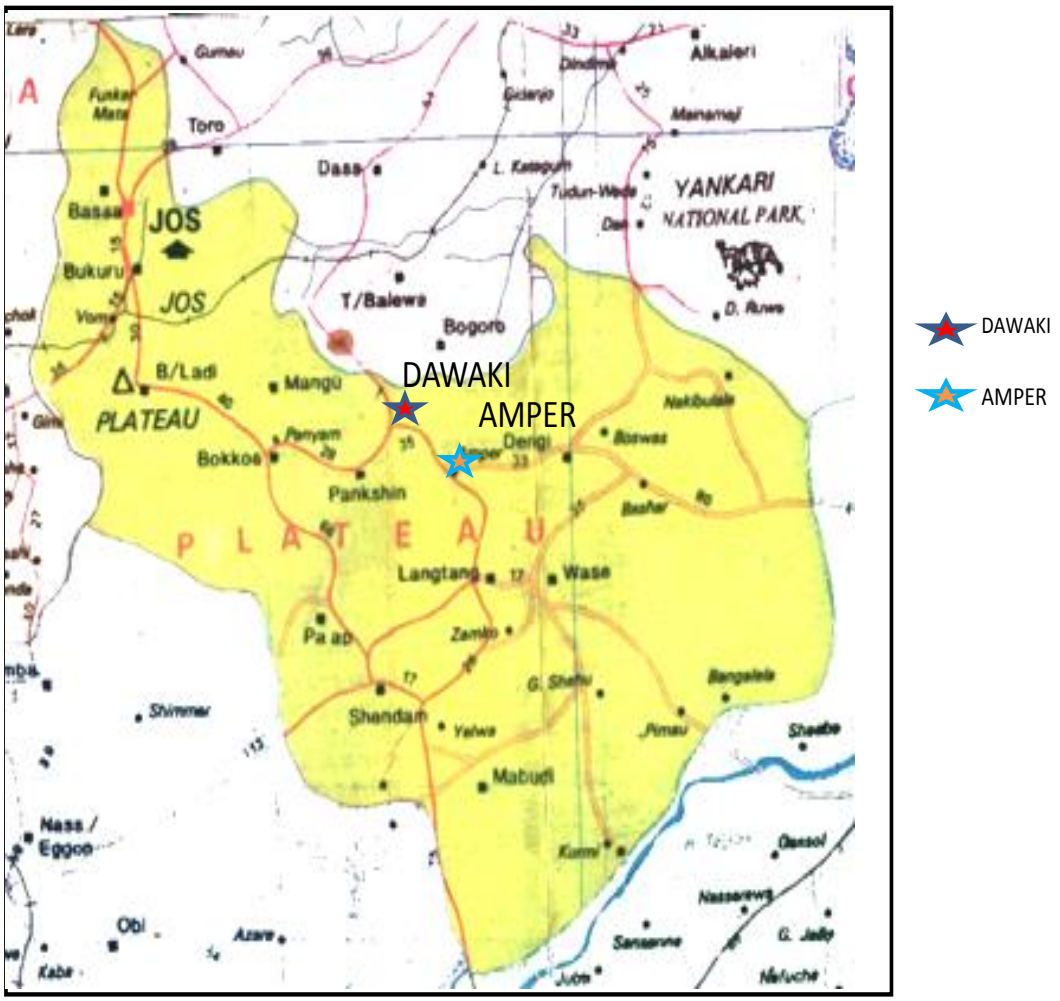


Figure 3.1: A map of Plateau State showing Dawaki and Amper in Kanke LGA

Based on visual observations and information obtained from dog owners, dogs sampled were categorized as young adult (if the dog is between 6 months to one year) or adult (for any dog one year and above). The sex of dogs, gross pathologies and abnormal behavior were observed. Additional information as regard the source, bitehistory or health condition of each dog sampled,was obtained by interviewing the dog traders and butchers.

Brain tissue samples from slaughtered dogs (532) were collected (according to the method of Barrat, 1996) between 15th December, 2010 and 18th June, 2011. Collection of brain tissues was carried out by inserting a drinking-straw (5mm in diameter) through the occipital foramen (foramen magnum) which is exposed after the dog head is cut off the body at the atlanto-occipital joints. This is a technique for collecting brain samples without opening the skull.The technique allows samples to be taken from the medulla oblongata, the base of the cerebellum and the hippocampus (Ammons horn and the cerebral cortex). Usually the process of singeing off of the hair coat occurs prior to the cutting off of the dog heads for sample collection. Brain tissues collected were put into individual sterile labeled tubes and immediately stored at -20 °C in a small Thermocool® freezerwhile on the field and later transferred to -80°C freezers in the National Veterinary Research Institute (NVRI) Vom, Plateau State Nigeria. Later, the samples were shipped on dry ice to the College of Veterinary Medicine, University of Georgia, Athens (UGA) USA, where they were stored at -80°C until use.

Of all the dogs sampled, 10 dogs from Plateau State had history of having bitten someone prior to sample collection and 2 dogs sourced from Kano were brought to slaughter having some form of limb paralysis and were not able to walk.

3.2.4 Direct flourescent antibody test (DFAT)

The samples were subjected to the direct fluorescent antibody (DFA) test as described by Dean *et al.,* (1996), to test for the presence of lyssavirus antigens. Brain impressions were made on 4-well, Teflon-coated slides. The slides were fixed in cold acetone; air dried and stained with a fluorescent isothiocyanate-labelled anti-rabies virus monoclonal antibodies (Fujirebio Diagnostics, Inc., Malvern, Pennsylvania, USA). RABV-infected and normal mouse brains were used to prepare positive and negative-control slides respectively.

After staining (of the smears with the conjugate), the slides were incubated for 30 minutes in a humid chamber. Using a gentle stream of 0.01M phosphate buffered saline (PBS) pH 7.4 from a wash bottle; excess conjugate was washed off the slides. Thereafter, they were removed, rinsed twice by immersion in PBS for 5minutes in separate containers. The slides were then removed from PBS, air dried and observed under Flourescent Microscope (Zeiss, Axioskop with 20x magnification) for the presence of *Lyssavirus* antigen. The procedure consists of antigen reacting with antibody tagged with flourescein isothiocyanate, the dye most frequently used for diagnosis of rabies. If it appears under ultraviolet light as brightly coloured, apple-green or greenish yellow objects against a dark background it was interpreted as a positive result. Absence of such bodies signifies a negative result.

3.2.5 Reverse transcription polymerase chain reaction (RT-PCR)

3.2.5.1*Ribonucleic acid extraction*

From the DFAT positive brain tissues (n=92), total RNA was extracted from about 100mg of the dog brain samples with QIAGEN BioSprint One-for-all Vet Kit (Catalog no. 947055) according to manufacturer's instructions. Briefly,disruption and homogenization of 100 mg brain tissue placed in 1.5ml microcentrifuge tube was carried out in 300 µl Trizol using a small sterile pestle.Then the homogenate was allowed to incubate at room temperature for 5 minutes on the bench. A mixture of 300µl RLT (RNA lysis buffer containing guanidine thiocyanate) combined with 1 µl of *β*-mercaptoethanol (ß-ME) was added to the lysate and the mixture shaken vigorously for 15 seconds and again incubated at room temperature for 2–3 minutes. The mixture was then centrifuged at 12,000 rpm for 3 minutes at 4°C. The upper aqueous phase was then transfered to a new tube, to which one volume of 70% ethanol was added, and vortexed. Then it was transfered to RNeasy column in 2 ml tube and centrifuged at 10,000 rpm for 15 seconds, and then the flow-through was discarded. Buffer RW1 (700 µl) (contains ethanoland guanidine thiocyanate) was added to RNeasy column and centrifuged at 10,000 rpm for 15 seconds and again the flow-through was discarded. Buffer RPE 500 µl was then added to RNeasy column and centrifuged at 10,000 rpm for 15 seconds and again the flow-through was discarded. RPE (500 µl) was again added and centrifugation repeated as before but for 2 minutes. RNeasy column was then placed in a new 2 ml tube, and centrifuged at 12,000 rpm for 1 minute. RNeasy column was then placed in new 1.5 ml tube and 30µl RNase-free water was added, and centrifuged for 1 minute at 10,000 rpm to elute the RNA.

3.2.5.2 *Reverse Transcription*

The extracted RNA from DFAT positive brain tissues (n=92) were later subjected to the reverse transcription –polymerase chain reaction (RT-PCR). The SuperScript® lll reverse transcriptase (Invitrogen, San Diego, CA, USA Cat.18080-093) was used to perform RT-PCR according to manufacturer's instructions. Primers used were the same as that used by Davis *et al.* (2005) (Table 3.1) to target the Ngene with a product size between 1.3-1.5kb.

The procedure was done according to the manufacturer's instructions briefly as follows; To a nuclease-free microcentrifuge tube, 1 µl of forward primer (50mM) was added, 1 µl 10 mM dNTP Mix (10 mM each dATP, dGTP, dCTP and dTTP at pH 7.0) and 11 µl of extracted RNA was also added. The mixture was briefly centrifuged and was heated to 65°C for 5 minutes and incubated on ice for at least 1 minute. The contents of the tube were collected by brief centrifugation and 4 µl 5X first-strand buffer [250 mM Tris-HCl (pH 8.3 at room temperature), 375 mM KCl, 15 mM MgCl2], 1 µl 0.1 M DTT (Dithiothreitol), 1 µl RNaseOUT™, 1 µl of SuperScript™ III RT (200 units/µl) were added, mixed by pipetting gently up and down and incubated at 50°C for 45 minutes. The reaction was inactivated by heating at 70°C for 15 minutes. This produced cDNA which was used as a template for amplification in PCR.

Table 3.1. Primers used for RT-PCR diagnosis of rabies in dog brain tissues.

Primer Name	Sequence (5'-3')	G-C%	Tm	Coverage
N127F	ATGTAACACCTCTACAATGG	40	49.3	Nigeria (100%) Africa (100%)
N8mR	CAGTCTCYTCNGCCATCTC	57.9	54.7	Nigeria (100%) Africa (100%)

Tm – The melting temperature of a primer

Y-represents C T nucleotides

N-represents AGCT nucleotides

3.2.5.3*PCR amplification reaction*

The following were added to a PCR reaction tube: nuclease free water 10µl, 10 x PCR buffer [200 mM Tris-HCl (pH 8.4)], 2.5 µl, forward primer (10 µM) 1 µl, reverse primer (10 µM) 1 µl, Taq DNA polymerase (5 U/µl) 0.5 µl, cDNA (from first-strand reaction) 10 µl and mixed gently (total reaction volume is 25 µl). This mix was then short spinned and heated at 95°C for 2 minutes and amplified for 35 cycles at 95°C for 15seconds, 56°C for 30 seconds, 68°C for 1minute and 40 seconds, was carried out and finally extension at 68°C for 10 minutes was done in a thermal cycler (Applied Biosystems GeneAmp® PCR system 9700).

3.2.5.4*Preparation of 0.7% agarose gel*

Agarose gel preparation was done as described (Green, 2012). Briefly, about 0.7g of agarose (Bio-rad laboratories) wasweighed. The agarose was mixed with 100ml 1X TAE buffer in an Ehlenmeyer flask and microwaved for 3 minutes till it melted completely. It was then allowed to cool on the bench top for 10 minutes. Ethidium bromide (EtBr) was added to the gel (final concentration 0.5 ug/ml) at this point to facilitate visualization of DNA after electrophoresis.

Approximately 5µl of each sample were mixed with 1 µl gel loading dye (bromophenol blue, glycerol and xylene cyanol) before they were run on 0.7% agarose gels. Molecular weight marker (1 Kb Plus DNA Ladders, Invitrogen, San Diego, CA, USA) was included in each run, as well as positive (cDNA of RABV infected mouse brain) and negative controls (product of RT-PCR of a non RABV infected mouse brain). The gel was run at 120V for 25minutes depending on the expected size of PCR product. The gel was placed on the UV transilluminator and the Kodak EDAS 290 system for gel documentation.

3.2.5.5*Purification of deoxyribonucleic acid (DNA) fragments*

PCR amplicons were extracted and purified using QIAquick gel extraction kit according to the manufacturer's instructions (Qiagen, Germantown, MD, USA). Briefly, the DNA fragments of expected sizes were excised from the agarose gels with clean, scalpel blade and the gel slices were placed in their corresponding ready labelled colourless 1.5ml tubes and weighed. Three volumes of QG buffer (5.5 M guanidine thiocyanate 20 mM Tris HCl pH 6.6) was added to 1 volume of each gel piece (100 mg~100 ul) and incubated at 50ºC for 10 min (or until the gel slice completely dissolved). After the gel slices had dissolved completely, they were visualized to ensure that the colour of the mixture is yellow. Buffer QG contains a pH indicator which is yellow at pH less than or equal to 7.5 (This pH is required for the efficient adsorption of DNA to the QIAquick membrane). Isopropanol (1 gel volume) was then added to the sample and mixed in order to increase the yield of DNA fragments. A QIAquick spin column was placed in a provided 1.5 ml collection tube. The samples were applied to already labeled tubes corresponding QIAquick column to bind DNA and centrifuged for 1 min. The flow-through was then discarded and the QIAquick column was placed back in the same collection tube. Buffer QG 500 ul was added to QIAquick column and centrifuged for 1 min. They were washed by adding 750 ul of Buffer PE (10 mM Tris-HCl pH 7.5 and 80% ethanol) to QIAquick column and centrifuged for 1min. The flow through was again discarded and the QIAquick column was centrifuged for an additional 1 min at ≥ 10,000 x g (13,000 rpm). QIAquick column was placed into a clean 1.5ml microcentrifuge tube. To elute DNA, 10 ul of Buffer EB (10 mN Tris-Cl, pH 8.5) or H2O was applied to the center of the QIAquick membrane and allowed to stand for only a minute and then centrifuged at 13,000 rpm for 1 min.

3.2.5.6*PCR pre-sequencing reaction*

For the sequencing reaction, two sets of master-mixes were prepared in a clean 1.5ml microcentrifuge tube, one for the forward primer N127 and the other for the reverse primer N8m. The contents of the sequencing master-mixes were as follows; Nuclease free water (7.5µl), PCR product (6 µl) and primer (1.5µl). For each positive sample,the N gene was sequenced at the Georgia Genomic Facility, UGA Athens GA., USA**,** using the Big Dye® Terminator cycle sequencing method.

About 4µl BigDye® Terminator which contains buffer, 4dNTPs, Taq polymerase, pyrophosphatase 4 BigDye dideoxyterminators each labeled with a different fluorescent dye (each nucleotide having different dye colour). The tubes were vortexed and spunned down briefly in the centrifuge. The thermocycler was loaded and 25cycles of 96ºC for 10 seconds, 50ºC for 5 seconds, and 60ºC for 4 minutes, were completed followed by a hold at 4ºC until used.

3.2.5.7Sequencing *reaction clean-up and sequencing*

On completion of the cycling, sequencing reactions were cleaned using the ABI procedure so as to remove excess salts, unincorporated dNTPs and primers. In brief, 80µl of 70% isopropanol was added to all the wells, then sealed, vortexed and left at room temperature for 15minutes. This was followed by centrifugation at 3,000rpm for not less than 30minutes and the supernatant

was tipped off gently. The plate was then inverted over a paper towel, spunned for 15seconds at 300rpm and air dried.

The Capillary (Sanger) DNA sequencing method was used on ABI Prism 3730 Automated Sequencer (Applied Biosystems, Foster City, CA, USA) using an ABI Prism Big Dye Terminator Cycle ready reaction kit (Applied Biosystems) to sequence the contents of the plate.

3.2.5.8*Sequence editing and analysis*

The sequencing result was obtained on excel spread sheet as raw data on ABIF file format. These were trimmed using the Sequence Navigator software (Applied Biosystems). The forward and reverse sequences were assembled to resolve all differences by comparing the chromatogram profiles. Homology searches and comparisons of all the sequences obtained were carried out with the aid of the Lasergene package (DNASTAR Inc.). Sequences of encoded proteins were aligned using MEGA version 5 (Tamura, 2007). Neighbor-joining phylogenetic trees were constructed from DRV-NG11 isolate (originally 138) and 36 full-length nucleoprotein gene sequences from Africa. Bootstrap values are indicated at the nodes (only those with values more than 90% are indicated), and the scale bar indicates nucleoprotein substitutions per site.

3.3 Results

The DFAT of the 532 dog brain tissue samples screened showed 92 (17.3%) to be positive (Table 3.2). Of the 92 DFAT positive samples, the specific rates obtained based on reported

origin of the dogs from various states were 7(10.4%) from Jigawa, 4(15.4%) from Kaduna, 38(18.5%) from Kano, 8(9.5%) from Katsina, 17(25.0%) from Borno, 3(60.0%) from Niger Republic, 10(35.7%) from Plateau, 1(10.0%) from Sokoto and 4(10.3%) from Yobe State. The highest number of dogs in this study originated from Kano 205(38.5%), followed by Borno 68(12.8%) and then Jigawa 67(12.6%).

Fifty (9.4%) of the 532 dog brains collected were from dogs between the ages of 6months and one year while the remaining 482 (90.6%) were above one year of age. Of the 532 dog brain tissues screened, 375 (70.5%) were obtained from female dogs. Ten of the dogs had history of dog bite while 2 dogs sourced from Kano were brought to slaughter with some form of limb paralysis and were not able to walk. None of these 2 was positive by DFAT. Eight of the 10(80%) slaughtered dogs positive for rabies antigen sourced within Plateau State had histories of dog bite. These positive specimens came from dogs with some form of dog bite history although the situation surrounding the bite is unclear weither they were provoked or not.

Out of the 92 samples that were positive by DFAT, only 4 (Plate 1) yielded rabies-specific bands of expected amplicon size (1.3kb). Phylogenetic analysis of the sequenced N gene amplicons revealed they all clustered with the Genotype 1 RABV (Figure 3.1; 3.2; 3.3). The whole genome of one of these four rabies viruseswas further completely sequenced and termed asDRV-NG11 (Figure 3.4; 3.5) and is available in the GenBank under accessionnumber KC196743 (Appendix 1).

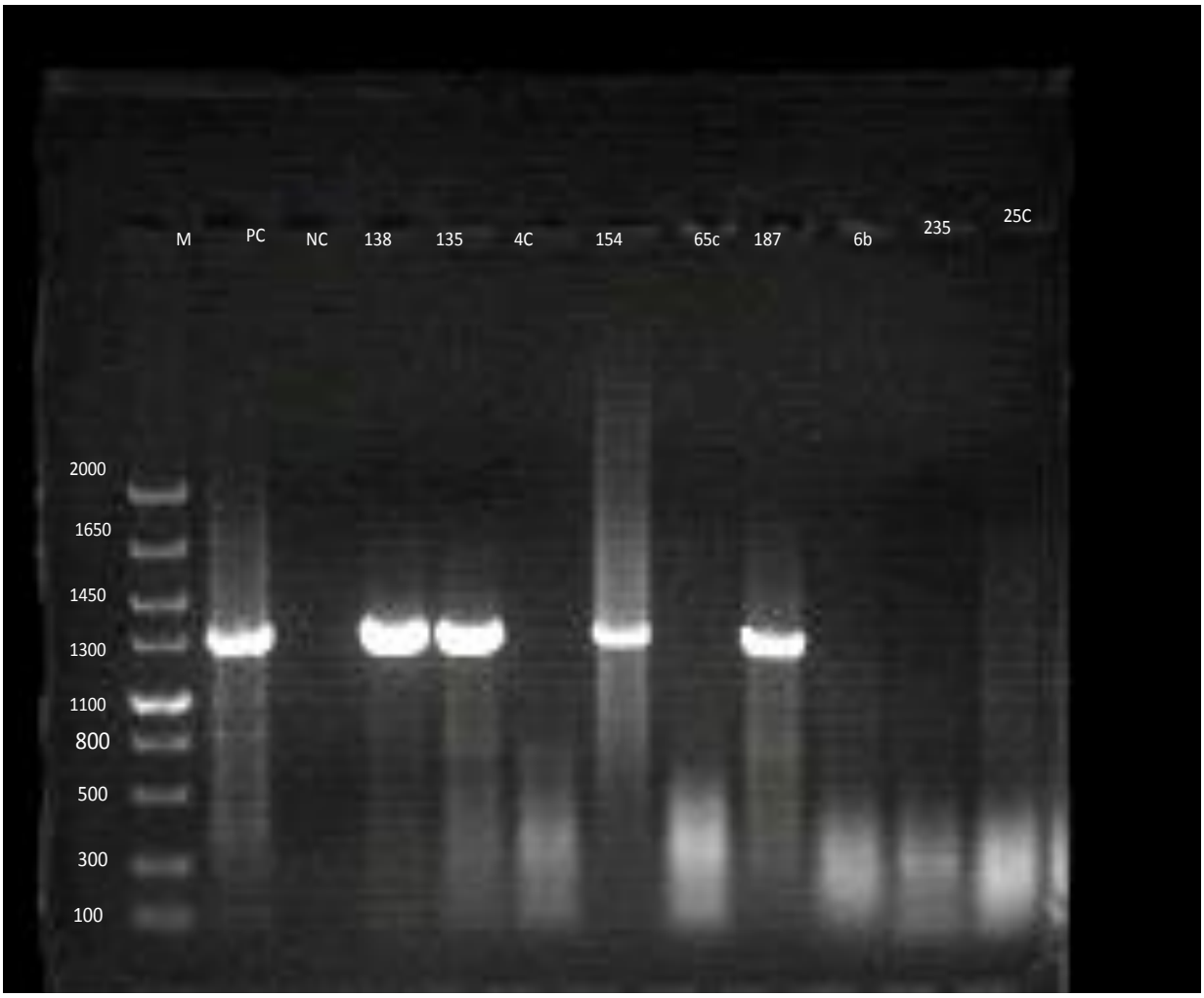


Plate I: An agarose gel under UV light following amplification of N gene from DFAT positive control (CVS-11) and NC the negative control. Subsequent wells were loaded with test samples 138,135, 4C, 154, 65c, 187, 66, 235, and 25C.*Rabies* specific amplicons were obtained from lanes 138, 135, 154 and 187.

Table 3.2: Distribution of Dog study population by source, age, and sex and DFAT positive rates.

Source (State/Country)	Number Examined	Age Adult/ Young (%)	Sex Male/female (%)	DFAT positive rate (DFAT +/No tested (%)
Jigawa	67	55/12	11/56	7/67 (10.4)
Kaduna	26	26/0	7/19	4/26 (15.4)
Kano	205	192/13	73/132	38/205 (18.5)
Katsina	84	81/3	21/63	8/84 (9.5)
Borno	68	64/4	23/45	17/68 (25.0)
Plateau	28	17/11	7/21	10/28 (35.7)
Sokoto	10	10/0	3/7	1/10 (10.0)
Yobe	39	32/7	11/28	4/39 (10.3)
Niger Republic	5	5/0	1/4	3/5 (60.0)
Total	532	482/50(90.6/9.4)	157/375(29.5/70.5)	92/532 (17.3)

>UNC:>RD135

CTCATATTTGGGATGGTTCGCAAGGAGAGGTGTTAGTTTTTTTCATGAAGGATGTACACAAGTTGTCGTTTCCTAG
AATCCTGTGATTCAACTCTTTATGAGTCACTAGAATATGTTTTGTTTAGAAACTCAGCAAATGAGTTTGGGCGAGC
TTGATGATTGGAACTGACTGAAACATATCTCCTTATGTGAGATCTCTTTAGTCGCCCTCCGTTCATCATGATCCGA
GTATAAACGGCTTCAGGACTCCTGGTCTCACCGGAAAAGTAATCCTCATCATCAGAGTTGACGGTTCCGTCATCTG
CCAGTGCCACATCAGTCTTCGTCAATTCAGCTGCCTCATATTCTTGGAGTTCTTTTTCGTCTCTAAAGAATCTTCT
CTACTGCATTTGACGAATACGGGGACTTCCCGCTCAGACCCAACGAGCGGAAGTGAATGAAATAGGAGTGAGGAAC
CGCAGTCTCCTGCCCTGGCTCGAACATTCTTCGTATCTCTTCTTCAAAGTTCTTGTGGAAGAAATATAGTATTGCT
TCTCTCGCAGTGAGATTTATCTGTTTTATGAACCCTGTGAACGACACCAGCCCAGAGCAATCTTCATAAGCAGTGAAGA
AGGAAACTGGGCTCTAACAGGAGGTATGGAATTGACGAGAGACCCCACGGTTTCTGAACATGCATCCTTAGTC
GGTCTGCTCTTGAGTCTGTATAGGTTGAGCAAAATATCAGGACAAAACACAGGCAACTACAAGACAAACATTGCAG
CTACTGTGCCCACTCTGATAGCTGAATATAGATGCTCAATCCGGGAGAAATACATGTCGTATGTTCAGTTTTATCA
GGCATGAATGCTGCCAAGCTTGATCCTGACGATGTATGTTCCTATTTGGCAGCTGCGATGCATCTCTTTGAAGGGA
CATGTCCTGAAGACTGGACCAGCTATGGAATCTTGATTGCAAGAAAGGGAGACAAGATCACCCCAGATTCTCTTGT
AGAGATCAAGCGTACAGATATAGAAGGGAACTGGGCTCTAACAGGAGGTATGGAACTGACGAGAGACCCCACGGTT
TCTGAACATGCATCCTTAGTCGGTCTGCTCTTGAGTCTGTATAGGTTGAGCAAAATATCAGGACAAAACACAGGCA
ACTACAAGACAAACATTGCAGATCGGATAGAGCAGATTTTTGAGACAGCCCCTTTCGTTAAAATTGTGGAACATCA
TACCC

>UNC:RD138

GTGGTTTCCTTGAAGCCAGAGATTATTGTAGATCAATATGAGTACAAATACCCTGCTATCAAAGACTTGAAGAAGC
CCAGTATAACCTTAGGGAAAGCCCCCGATTTAAACAAAGCATACAAGTCAGTTTTATCAGGCATGAATGCTGCCAA
GCTTGATCCTGACGATGTATGTTCCTATTTGGCAGCTGCGATGCAGCTCTTTGAAGGGACATGTCCTGAAGACTGG
ACCAGCTATGGAATCTTGATTGCAAGAAAGGGAGACAAGATCACCCCAGATTCTCTTGTGGAGATCAAGCGTACAG
ATATAGAAGGAAACTGGGCTCTAACAGGAGGTATGGAACTGACGAGAGACCCCACGGTTTCTGAACATGCATCCTT
AGTCGGTCTGCTCTTGAGTCTGTATAGGTTGAGCAAAATATCAGGACAAAACACAGGCAACTACAAGACAAACATT
GCAGATCGGATAGAGCAGATTTTTGAGACAGCCCCTTTCGTTAAAATTGTGGAACATCATACCCTGATGACAACTC
AAAAATGTGCGCTAATTGGAGTACCATACCGAACTTCCGATTCTTGGCGGGAACATACGACATGTATTTCTCCCGG
ATTGAGCATCTATATTCAGCTATCAGAGTGGGCACAGTAGTCACTGCTTATGAAGATTGCTCTGGGCTGGTGTCGT
TCACAGGGTTCATAAAACAGATAAATCTCACTGCAAGAGAAGCAATACTATATTTCTTCCACAAGAACTTTGAAGA
AGAGATACGAAGAATGTTCGAGCCAGGGCAAGAGACTGCGGTTCCTCACTCCTATTTCATTCACTTCCGCTCGTTG
GGTCTGAGCGGGAAGTCCCCGTATTCGTCAAACGCAGTAGGTCATGTATTCAATCTCATTCACTTTGTTGGATGTT
ATATGGGTCAGGTCAGGTCCTTAAATGCGACGGTCATTGCTGCATGTGCTCCTCATGAGATGTCTGTCCTAGGGGG
TTATCTAGGGGAAGAGTTTTTTGGAAAGGGCACATTTGAAAGAAGATTCTTTAGAGACGAAAAAGAACTCCAAGAA
TATGAGGCAGCTGAATTGACGAAGACTGATGTGGCACTGGCAGATGACGGAACCGTCAACTCTGATGATGAGGATT
ACTTTTCCGGTGAGACCAGGAGTCCTGAAGCCGTTTATACTCGGATCATGATGAACGGAGGGCGACTAAAGAGATC
TCACATAAGGAGATATGTTTCAGTCAGTTCCAATCATCAAGCTCGCCCAAACTCATTTGCTGAGTTTCTAAACAAA
ACATATTCTAGTGACTCATAAAGAGTTGAATCACAGGATTCTAGGAAACGACAACTTGTGTACATCCTTCATGAAA
AAAACTAACACCTCTCCTTGCGAACCATCCCAAATATGAGCAAGATCTTTGTCAATCCGA

Figure 3.2: Sequence contigs obtained of the N gene from sample RD135 and RD138

>UNC:RD154

GTGGTTTCCTTGAGCCAGAGATTATTGTGGATCAATATGAGTACAAATACCCTGCTATCAAAGACTTGAAGAAGCC
CAGTATAACCTTAGGGAAAGCCCCCGATTTAAACAAAGCATACAAGTCAGTTTTATCAGGCATGAATGCTGCCAAG
CTTGATCCTGACGATGTATGTTCCTATTTGGCAGCTGCGATGCAGCTCTTTGAAGGGACATGTCCTGAAGACTGGA
CCAGCTATGGAATCTTGATTGCAAGAAAGGGAGACAAGATTACCCCAGATTCTCTTGTGGAGATCAAGCGTACAGA
TATAGAAGGAAACTGGGCTCTAACAGGAGGTATGGAATTGACGAGAGACCCCACGGTTTCTGAACATGCATCCTTA
GTCGGTCTGCTCTTGAGTCTGTATAGGTTGAGCAAAATATCAGGACAAAACACAGGCAACTACAAGACAAACATTG
CAGATCGGATAGAGCAGATTTTTGAGACAGCCCCTTTCGTTAAAATTGTGGAACATCATACCCTGATGACAACTCA
AAAATGTGCGCTAATTGGAGTACCATACCGAACTTCCGATTCTTGGCCGGAACATACGACATGTATTTCTCCCGGA
TTGAGCATCTATATACAGCTATCAGAGTGGGCACAGTAGTCACTGCTTATGAAGATTGCTCTGGGCTGGTGTCGTT
CACAGGGTTCATAAAACAGATAAATCTCACTGCATTTCTTCCCCAGAACTTTGAAGAGAGATACGAAGAATGTTCG
AGCCAGGGCAAGAGACTGCGGTTCCTCACTCCTATTTCATTCACTTCCGCTCGTTGGGTCTGAGCGGGAAGTCCCC
GATTCGTCAAATGCAGTAGGTCATGTATTCAATCTCATTCACTTTGTTGGATGTCTATATGGGTCAGGTCAGGTCC
TTAAATGCGACGGTCATTGCTGCATGTGCTCCTCATGAGATGTCTGTCCTAGGGGGTTATCTAGGGGAAGAGTTTT
TTGGAAAGGGCACATTTGAAAGAAGATTCTTTAGAGACGAAAAAGAACTCCAAGAATATGAGGCAGCTGAATTGAC
GAAGACTGATGTGGCACTGGCAGATGACGGAACCGTCAACTCTGATGATGAGGATTACTTTTCCGGTGAGACCAGG
AGTCCTGAAGCCGTTTATACTCGGATCATGATGAACGGAGGGCGACTAAAGAGATCTCACATAAGGAGATATGTTT
CAGTCAGTTCCAATCATCAAGCTCGCCCAAACTCATTTGCTGAGTTTCTAAACAAAACATATTCTAGTGACTCATA
AAGAGTTGAATCACAGGATTCTAGGAAACGACAACTTGTGTACATCCTTCATGAAAAAAACTAACACCTCTCCATG
CGAACCATCCCAAATATGAGC

>UNC:RD187

TTTCCTTGAAGCCAGAGATTATTGTGGATCAATATGAGTACAAATACCCTGCTATCAAAGACTTGAAGAAGCCCAG
TATAACCTTAGGGAAAGCCCCCGATTTAAACAAAGCATACAAGTCAGTTTTATCAGGCATGAATGCTGCCAAGCTT
GATCCTGACGATGTATGTTCCTATTTGGCAGCTGCGATGCAGCTCTTTGAAGGGACATGTCCTGAAGACTGGACCA
GCTATGGAATCTTGATTGCAAGAAAGGGAGACAAGATTACCCCAGATTCTCTTGTGGAGATCAAGCGTACAGATAT
AGAAGGAAACTGGGCTCTAACAGGAGGTATGGAATTGACGAGAGACCCCACGGTTTCTGAACATGCATCCTTAGTC
GGTCTGCTCTTGAGTCTGTATAGGTTGAGCAAAATATCAGGACAAAACACAGGCAACTACAAGACAAACATTGCAG
ATCGGATAGAGCAGATTTTTGAGACAGCCCCTTTCGTTAAAATTGTGGAACATCATACCCTGATGACAACTCATAA
AATGTGCGCTAATTGGAGTACCATACCGAACTTCCGATTCTTGGCCGGAACATACGACATGTATTTCTCCCGGATT
GAGCATCTATATTCAGCTATCAGAGTGGGCACAGTAGTCACTGCTTATGAAGATTGCTCTGGGCTGGTGTCGTTCA
CAGGGTTCATAAAACAGATAAATCTCACTGCAAGAGAAGCAATACTATATTTCTTCCACAAGAACTTTGAAGAAGA
GATACGAAGAATGTTCGAGCCAGGGCAAGAGACTGCGGTTCCTCACTCCTATTTCATTCACTTCCGCTCGTTGGGT
CTGAGCGGGAAGTCCCCGTATTCGTCAAATGCAGTAGGTCATGTATTCAATCTCATTCACTTTGTTGGATGTTATA
TGGGTCAGGTCAGGTCCTTAAATGCGACGGTCATTGCTGCATGTGCTCCTCTGAGATGTCTGTCCTAGGGGGTTAT
CTAGGGGAAGAGTTTTTTGGAAAGGGCACATTTGAAAGAAGATTCTTTAGAGACGAAAAAGAACTCCAAGAATATG
AGGCAGCTGAATTGACGAAGACTGATGTGGCACTGGCAGATGACGGAACCGTCAACTCTGATGATGAGGATTACTT
TTCCGGTGAGACCAGGAGTCCTGAAGCCGTTTATACTCGGATCATGATGAACGGAGGGCGACTAAAGAGATCTCAC
ATAAGGAGATATGTTTCAGTCAGTTCCAATCATCAAGCTCGCCCAAACTCATTTGCTGAGTTTCTAAACAAAACAT
ATTCTAGTGACTCATAAAGAGTTGAATCACAGGATTCTAGGAAACGACAACTTGTGTACATCCTTCATGAAAAAAA
CTAACACCTCTCCTTGCGAACCATCCCAAATATGAGCAAG

Figure3.3: Sequence contigs obtained of the N gene from sample RD154 and RD187

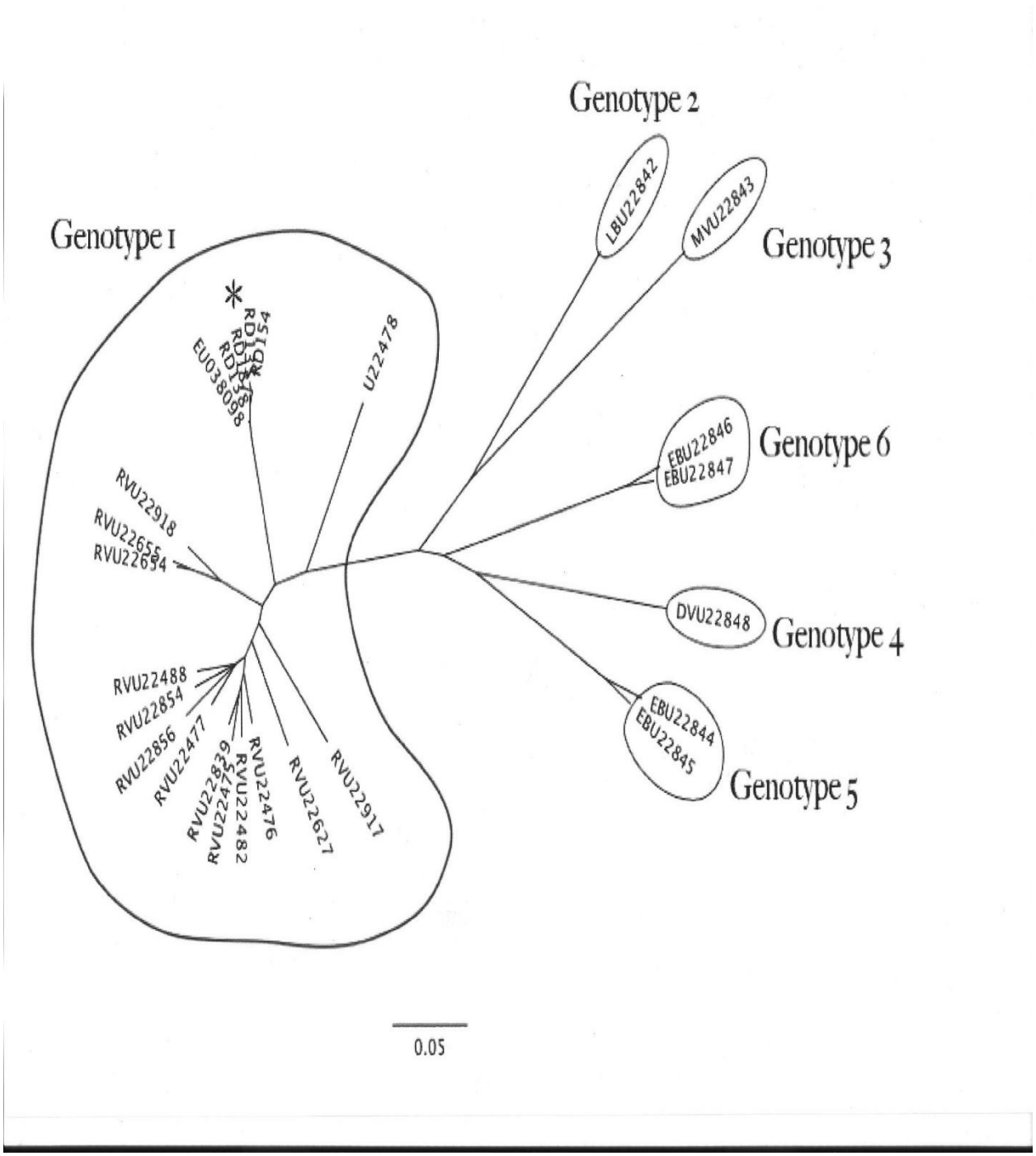


Figure 3.4: A radial phylogenetic tree of the four rabies viruses from Plateau State dogs in this study (* RD154, RD135, RD187 and RD138) and their genotypic relationship to other lyssaviruses based on N gene sequence alignment.

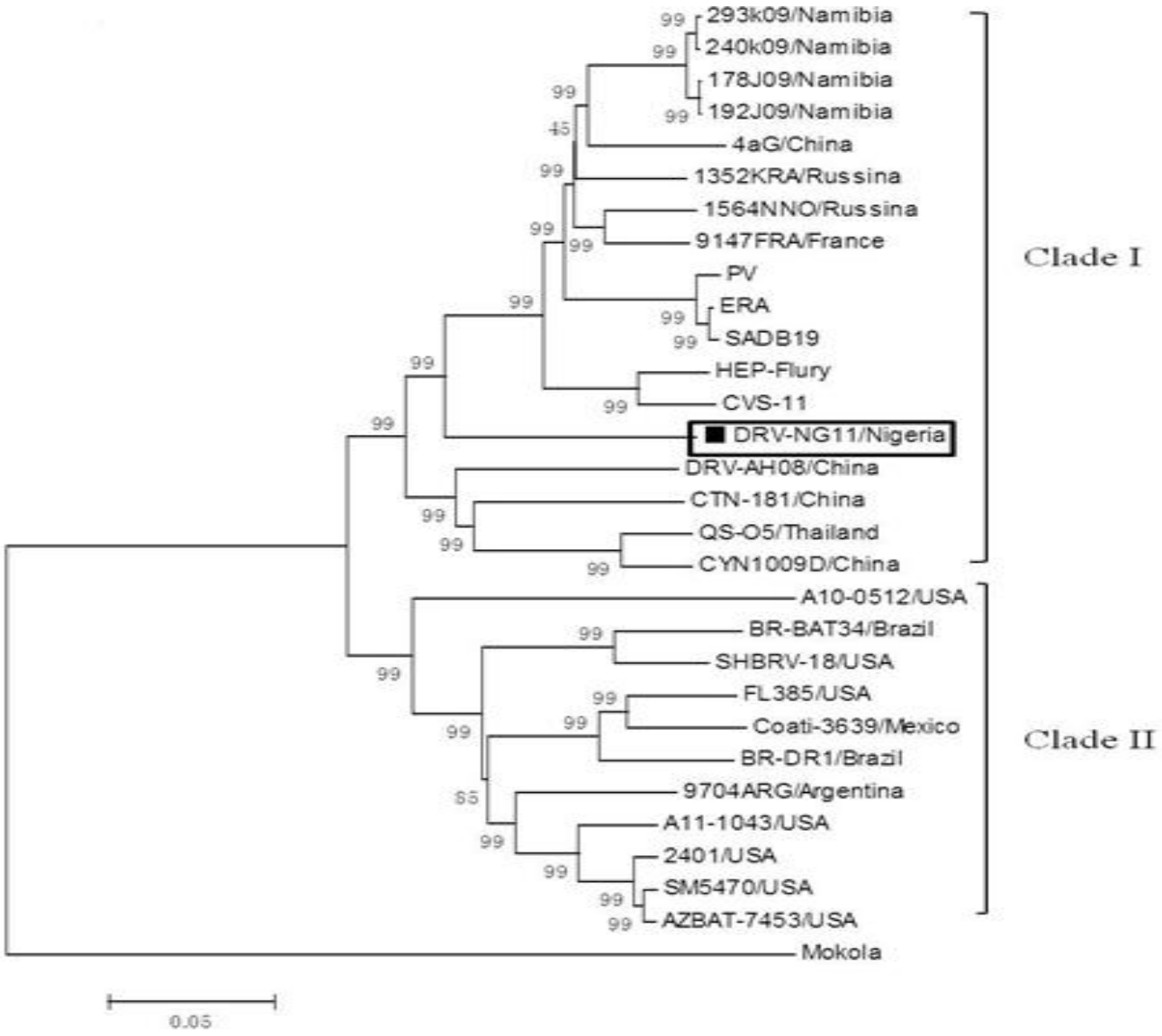


Figure 3.5: Phylogenetic tree drawn using the entire genome of DRV-NG11/Nigeria (RD135) isolate from Plateau State compared to other sequences found in the GenBank showing its location within Clade 1.

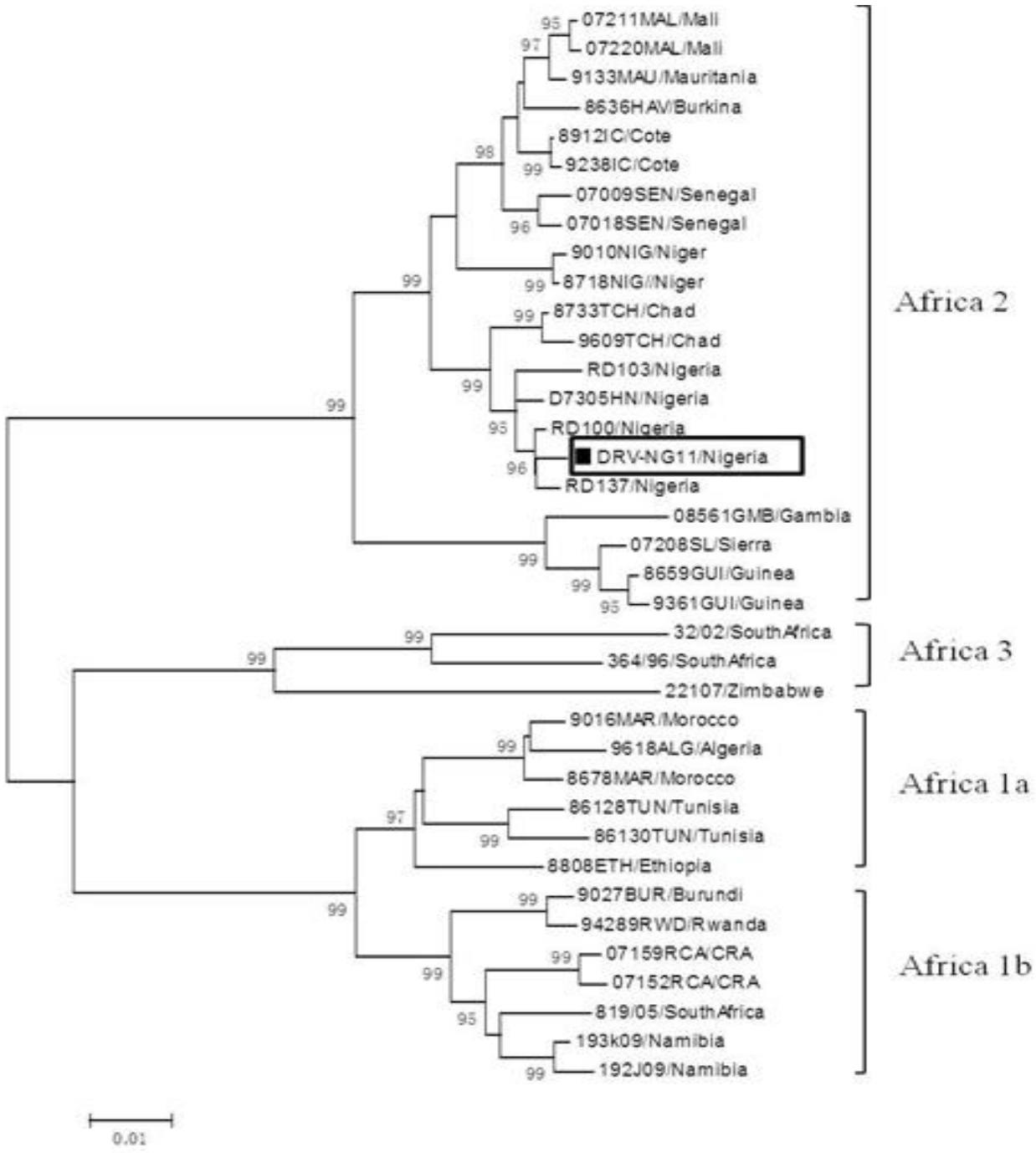


Figure 3.6: Phylogenetic tree of the completely sequenced DRV-NG11/Nigeria isolate Plateau State (Africa 2) compared with 36 different rabies isolates from across Africa.

3.4 Discussion

Brain tissues were collected from dogs slaughtered for human consumption in two dog markets in Kanke Local Government Area of Plateau State. Of the 532 dogs, 527 originated from eight Northern States of the country and five from Niger Republic.About 70.5 % (375/532) of the dogs sampled were females. Dog ownership favours male dogs more than female dogs for varied reasons (Okoh, 2007). This may result in more female dogs constituting the majority of the strays in communities. These may subsequently be captured and traded at the dog markets (Sabo *et al*., 2008). Adult dogs made up majority, (482/532; 90.6%) of the dogs sampled. This is probably due to the economics of production and sales. From visual observation, it was noted in this study that majority of the young adult dogs were slaughtered to be prepared as roasted meat, commonly known as "suya". Five of the dogs were from Niger Republic and none was from Chad Republic. Sabo (2009) documented 10 states in Nigeria (Sokoto, Zamfara, Katsina, Kano, Kaduna, Plateau, Jigawa, Borno, Bauchi and Yobe) and Niger and Chad Republics that serve as sources of dogs sold at the Dawaki and Amper markets. The disparities in number of dogs sampled based on sex, age and source are due to the dogs available in the markets at the time of sampling.

All 532 dog brain tissues were examined by DFAT and 92(17.3%) were positive for RABV antigen. The 17.3% dog brain tissues positive for rabies antigen by DFA, is lower when compared to previous studies: Ajayi *et al*. (2006) in a study of dogs slaughtered in Maiduguri (Borno State), reported a 31% prevalence of rabies; Garba *et al.,* (2008), reported a 28% prevalence of rabies in dogs slaughtered for meat in Sokoto, while Sabo (2009), reported a prevalence of 43%, from dogs slaughtered in Plateau State. These findings imply that dogs sold

at the dog markets may harbour the virus and even serve as means of transmission of rabies to man and other in contact animals with subsequent economic losses. After subjecting these 92 DFAT positive brain samples to RT-PCR, RABV RNA was detected in only four samples. This result was contrary to what was expected considering the high sensitivity of PCR. This may be due to some technical problems. Probably optimizing conditions such as the use of other short sequence primers targeting the N gene in a nested RT-PCR may have increased the sensitivity of the PCR (Dzikwi, 2008). Also optimizing reaction conditions such as magnesium, primer and/or template RNA concentration and the thermal cycling temperatures especially the annealing temperature maximizes sensitivity of PCR (Davis et al., 2005).

The genetic relatedness of the rabies viruses detected in this study to those from Republics of Niger and Chad (Table 3.5) of the Africa 2, can be easily understood in light of the transboundary movement of dogs as a result of dog trade. Similar observations were previously made in which Nigerian RABV isolates obtained clustered with the Africa 2 lineage with isolates from Chad, Cameroun, Niger, Cote d'Voire Central African Republic, Burkina Faso, and Mauritania (Dzikwi, 2008; Ogo *et al.,* 2011). These observations imply that, the West African isolates may have originated from a common ancestral lineage which evolved and is expanding rabies epidemiology in these regions.

The N genes of all four identified rabies viruses were sequenced. Comparison of the N gene sequence indicated that this virus is related to another RABV identified in Nigeria (EU038098) (Figure 3.3) with high homology (99.4%). Viral isolation of one of the positive specimens was done and the entire genome was completely sequenced and published (Appendix 1). The whole

genome sequence also clustered with isolates from Chad, Niger, Cameroon, Mali, Senegal, Burkina Faso Mauritania, Gambia, Guinea, and Sierra Leone. The homology between this RABV and those in the "cosmopolitan" group ranges from 86.5% to 87.8%. Kissi*et al*. (1995) and Talbi *et al*., (2009) also studied the molecular epidemiology of rabies in Africa and described three phylogenetic lineages of RABV: Africa 1, 2 and 3. All the RABV identified in this study however are of Genotype 1. The RABV completely sequenced fell within Clade 1 and the Africa 2 lineage with other West African strains. This further authenticates the origin of West African rabies to be of a common ancestry since Nigeria is located on the geographical coordinates 10^{o}00'N, 8^{o}00'E and shares land boundaries with Benin Republic, Niger Republic, Chad and Cameroon.

The variants found in Nigeria are distinguishable from those of Central, North Africa (Africa 1a) and those of East (Africa 1b) and South Africa (Africa 3). Our findings are supported by evidence from past studies which showed a homogenous and close relatedness of dog rabies virus (99% sequence homology) belonging to the Africa 2 rabies lineage (Dzikwi, 2008, Ogo *et al.,* 2011). The finding of rabies virus among slaughtered dogs confirms dog trade as one of the risk factors for rabies transmission.

3.5 Conclusion

The rabies viruses detected in this study belong to the Genotype 1 and Africa 2 lineage.The movements of dogs for trade from various states within the country and from neighbouring countries may be enhancing rabies spread within the dog and humanpopulations and

subsequently constituting a danger to the public. There is need to educate communities at risk about the dangers associated with dog trade.

CHAPTER FOUR

DETECTION OF ANTIGENS AND ANTIBODIES TO LYSSAVIRUSES IN BATS IN PLATEAU STATE.

4.1Introduction

One of the most significant zoonotic pathogens of bat origin is rabies virus (RABV) (Calisher *et al*., 2006). This virus and other lyssaviruses cause fatal encephalitis for which there is no effective treatment (Rupprecht *et al*., 1995). The *Lyssavirus* genus can be differentiated into 11 genetically divergent species based on genetic analyses of the viral genome (ICTV, 2009; Kuzmin *et al.*, 2005). The species are: *Rabies virus* (RABV; *Lagos bat virus* (LBV); *Mokola virus* (MOKV); *Duvenhage virus* (DUVV); *European bat Lyssav*irus-1 (EBLV-1); *European bat Lyssavirus*-2 (EBLV-2), *Australian bat Lyssavirus* (ABLV), *Irkut virus* (IRKV), *Aravan virus* (ARAV), *Khujand virus* (KHUV) and *West Caucasian bat virus* (WCBV) (ICTV, 2009). Two newly identified lyssaviruses, *Shimoni* bat virus (SHBV) (Kuzmin *et al., 2010*) and *Bokeloh* bat *lyssavirus* (Freuling *et al,* 2011), both detected in bats, have not yet been classified.

Approximately 20% of mammalian species are bats with more than 1100 species recognized worldwide (Teeling *et al.*, 2005). They have many characteristics that differentiate them from other mammalian species, and at the same time, they exhibit an enormous degree of intra-species diversity. Bats are hypothesized to have evolved between 50 and 70 million years ago and have undergone a rapid diversification during this period (Teeling *et al.,* 2005). All bat species belong to the Order Chiroptera, that is subdivided into two suborders, Yangochiroptera and Yinpterochiroptera, the later including the superfamily Pteropodidae, the old world fruit bats, and Rhinolophoidea (Giannini and Simmons, 2003). Scientific interest in bats has increased

substantially following the identification of bats as important reservoirs of pathogens of both zoonotic and veterinary importance (Calisher *et al.,* 2006; Dominguez *et al.,* 2007; Field, 2009; Towner *et al.,* 2009; Wang and Eaton, 2007).

Four *Lyssavirus* species have been documented in Africa. Of these, RABV occurs worldwide, but LBV, MOKV, and DUVV have not been naturally encountered outside of Africa (WHO, 2005). Nigeria is particularly important for *Lyssavirus* ssurveillance because the initial isolations of LBV and MOKV occurred in the country. Historically, LBV was isolated over four decades ago from the brains of the straw-coloured fruit bat, *E.helvum,* collected on Lagos island in 1956 (Boulger and Porterfield, 1958), MOKV was isolated from the viscera of shrews (*Crocidura* sp.) in 1968 in Mokola (Shope *et al*., 1970). A study carried out in Ibadan (western Nigeria) in 1990 suggested the presence of RABV-neutralizing antibodies in the sera of fruit bats (Aghomo *et al*., 1990a). In another study Dzikwi (2010), demonstrated the presence of lyssavirus-neutralizing antibodies to Lagos bat virus in sera from the straw-colored fruit bat (*E. helvum*) and the Gambian epaulet bat (*E.gambianus*).

Human consumption of bat meat is practised in many parts of Nigeria, and the public health implication of this practice remains to be assessed. Host switching has been proposed as the evolutionary mechanism by which lyssaviruses have evolved through spill-over events from bats to infect terrestrial mammals (Badrane and Tordo, 2001). Thus, the need for continued surveillance of all lyssaviruses in bats wherever they may be found is needful. This study seeks to investigate evidence of *Lyssavirus* antigens in the brain tissues of the bats and the presence of antibodies to lyssaviruses (*Lagos bat virus* (LBV), *Mokola virus* (MOKV), *Duvenhage virus*

(DUVV), *West Caucasian bat virus* (WCBV), *Shimoni bat virus* (SHBV) and classical rabies virus (RABV) in the sera of bats from Plateau State, Nigeria.

4.2 Bat Samples

4.2.1Study area

Plateau State (9°10'N, 9°45'E) is located in Nigeria's middle belt. The State covers an area of 26,899 square kilometers, with an estimated population of 3,178,712 people. The state is bounded on the northeast by Bauchi State, Northwest by Kaduna State, Southwest by Nasarawa State and Southeast by Taraba.

Although Plateau State is situated in the tropical zone, it has a temperate climate. Harmattan winds cause the coldest weather between December and February. The warmest temperatures usually occur in the dry season months of March and April. The mean annual rainfall varies from 131.75 cm (52 in) in the southern part to 146 cm (57 in) on the Plateau. The highest rainfall is recorded during the months of July and August (SCRD, 2011).

4.2.2 Bat survey and sample collection

In December 2009, in preparation for a *Lyssavirus* surveillance study of bats in Plateau State, north-central Nigeria, bat roost location site survey was carried out and information on the seasonal migration times and locations where bats roost, were obtained. Based on this survey, consents from relevant community leaders and officers of government as applicable were obtained.

Collection of bats was based on the availability of bat roost, consent from relevant authorities, and the cooperation of local hunters and community inhabitants who were knowledgeable of bat

roosts and available to assist in bat capture. Bats were collected from 8 different locations within 4 Local Government Areas (LGA) in Plateau State (Jos South, Kanke, Lantang South and Shendam)(Table 4.1; Figure 4.1). The megachiroptera were captured by the use of catapults, and traps made of bamboo branches. The microchiroptera were captured using improvised mist nets (made from mosquito net) and manually from a cave, old deserted well, culvert, hospital and human dwellings from March 2010 through June 2011 and sent to the laboratory in plastic cages. Bats were handled using protective, hard rubber gloves. Sampling and handling techniques for bats were described in detail by Wimsatt *et al*.(2005) and Ellison *et al*. (2006).These bats were anaesthetized by an intramuscular injection of ketamine hydrochloride (0.05-0.1 mg per body weight) and euthanized while on sedation. Blood samples were collected via cardiac puncture. For bats intended for consumption, blood collection was from the jugular vein where possible. The blood was allowed to clot and serum was collected and put into clean tubes. After euthanasia, complete necropsy was performed on each bat. Brain samples were collected immediately into sterile tubes and stored in liquid nitrogen in the field and subsequently transferred to -80°C in the National Veterinary Research Institute (NVRI) Vom, Plateau State Nigeria. Later, the samples were shipped on dry ice to Centers for Disease Control and Prevention (CDC) Laboratory, Atlanta, GA where they were stored at -80°C until use.

Table 4.1: Bats sampled and Geographic Positions of their Roost sites

S/No	Bat species (Family)	Number of bat/ species	Bat common name	Name of location bat roost sites	Geopositioning of bat roost
1.	*Eidolon helvum* (*Pteropodidae)*	244	African straw coloured bats or African fruit bat	National Commission for Museums and Monuments, Jos, roosting on trees.	9º54'48N; 8º53'10E Altitude 1223m
2.	*Rhinopoma microphylum* (*Rhinopomatidae*)	4	Mouse-tailed bat	Leptur, roosting in a cave	9º26´3N, 9º32´10E Altitude 966m
3.	*Chaerophon pumila*(*Molossidae*)	33	Little free-tailed bats	Dawaki, roostingin hospital roof and house wall.	9º25´41N 9º34´12E Altitude 759m
4	*Rhinolophus landeri*(*Rhinolophidae*)	37	Lander's horseshoe bat.	Pandam Game Reserve, roosting in an ancient well.	8 º38'12N; 8 º 57'52E Altitude 162m
5.	*Nycteris macrotis* (*Nycteridae*)	18	Large-eared slit-faced bat.	Pandam, roosting in a culvert	8º32´49N, 8º39´9E Altitude 145m
6.	*Epomophorus franquetti* (*Pteropodidae*)	12	Franquet Epaulet	Sabon layi, roosting on trees in the Chief's compound	8 º34'59N ; 9 º37'45E Altitude 173m
7.	*Epomorphorus Gambianus* (*Pteropodidae*).	5	Gambian Epaulet	Sabon layi, roosting on trees beside the bridge	8º33´59N, 9º38´29E Altitude 158
8.	*Lavia frons* (*Megadermatidae*)	3	Yellow winged bat	Lakushi,roostingon thorny trees in a swampy forest-like area	8º34´24N, 9º39´13E Altitude 152m

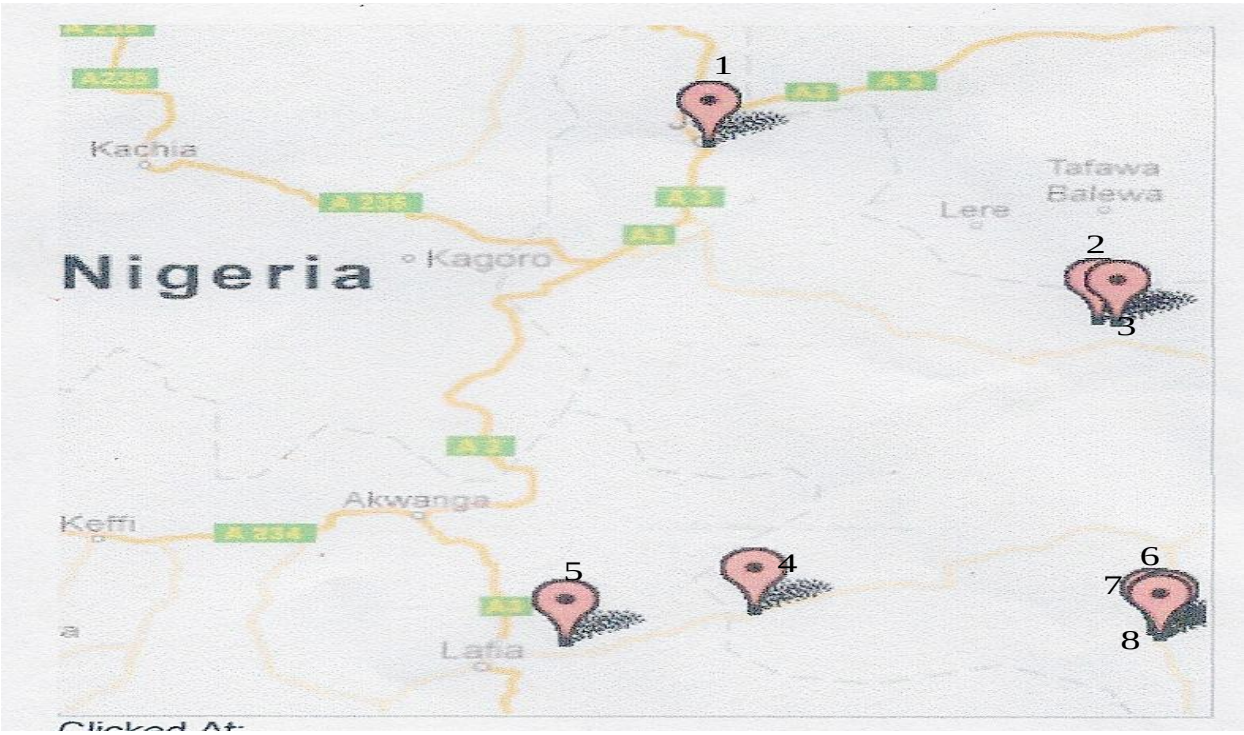


Figure 4.1. Map showing the geographic location where specific bats were obtained.

1.*E. helvum*(on trees inJos Zoo in Jos North LGA);**2.***R. microphylum*(in a cave in leptur, Kanke LGA); **3**. C.*pumila*(from hospital roof and house wall in Dawaki, Kanke LGA); **4.***R. landeri*a (in a well in PGR in Shendam LGA); **5.***N. macrotis*, (in a culvert in Pandam,Shendam LGA); **6.***E. franquetti* (from trees, Sabo layi at the chief's compound in Shendam LGA); **7.***E. gambianus*(from Lakushi bridge inShendam LGA); **8.***L. frons* (from lakushi swampy forestShendam LGA).

4.2.3 Bat identification

Bats are classified in the phylum Chordata, sub-phylum Vertebrata, class Mammalia, order Chiroptera. The order is divided on anatomical grounds into two major divisions, or suborders: the Megachiroptera, or fruit bats, found only in the Old World tropics, and the Microchiroptera, or insect-eating bats, with a worldwide distribution (Infoplease, 2012). Identification was done based on documented unique characteristic features (Happold, 1987) of the wings (Appendix II), the tail and tail membrane (Appendix III), the face, tragus, nose leaf (Appendix VI) the palatal ridges (Appendix VII). Their specific habitat in which they were found roosting and the colour of their pelage all clearly described by Happold (1987) aided the bats identification. Bats identification was further confirmed at the CDC Atlanta Georgia by Ivan Kuzmin an experienced researcher who is very good with bat identification.

4.3 *Lyssavirus* Diagnosis

4.3.*1Lyssavirus* antigen detection by DFA test on bat brain tissues.

All bat brain tissues (n=356) were subjected to the direct fluorescent antibody (DFA) test as described by Dean *et al*.(1996). Touch-brain impressions were made on 4-well, Teflon-coated slides and air-dried. The slides were fixed in ice-cold acetone; air dried and stained with an anti-rabies monoclonal (Fujirebio Diagnostics, Inc., Malvern, Pennsylvania, USA) and polyclonal (Chemicon Int., Temecula, CA) fluorescein isothiocyanate-labelled anti-rabies virus antibodies. RABV-infected and normal mouse brains were used to prepare positive and negative-control slides respectively.

After staining of the smears with the conjugate, the slides were incubated at 37ºC for 30 minutes and then washed off by tipping drops of the monoclonal antibodies off the wells into 1000ml beakers containing four mls of concentrated lysol (QAC). The slides were removed, washed properly with a wash bottle using PBS (pH 7.4-7.8). This was followed by two rounds of 3-5minutes washing of slides in PBS in individual holders.The slides were air dried and observed under flourescent microscope (Zeiss, Axioskop) at X 20 magnification under UV or blue light and read as the emitted light of 525nm. The reaction with the FITC results in apple green flourescence. The labeled antibodies recognize specific epitopes of RNP and antigen-antibody complex are formed.

4.3.2 Modified rapid fluorescent focus inhibition test (RFFIT) detection of virus neutralizing antibodies in batsera

This test was done as described in the standard operating procedure (SOP) for the rapid fluorescent focus inhibition test for determining rabies virus neutralizing antibody (CDC, 2006). The RFFIT was carried out under a biological safety cabinet (Baker, Sterilgard III) because of the live viruses used.

4.3.2.1. *Challenge virus and back titration*

Six different challenge live viruses were used in RFFIT including RABV (strain CVS – 11), LBV (isolate from Nigeria, 1956), DUVV (isolate from South Africa, 1970), MOKV (isolate form South Africa, 1998), WCBV (isolate from Russia, 2002) and SHBV (isolate from Kenya, 2009)**.** A dilution of 4µl of each of the virus stock per ml medium which had been determined beforehand in this laboratory was used. This gives a concentration of 50 FFD_{50}/0.1ml (neat

virus). To make a 2-fold serial dilution (1:10 and 1:100), 0.9ml (900µ) MEM was added to 2 cryovials (Sarstedt, Corning) tubes using a micropipette. About 100µl rabies virus from the 50_{50}/0.1 tubes was added to the first tube and mixed by gently swirling around to make a 5.0 FFD_{50}. Approximately 100µl was then transferred from the 5.0 FFD_{50} dilution tube to the second cryovial to give a 0.5 FFD_{50}/0.1ml.

About 12.5µl of the 50 FFD_{50}/0.1 dilution of the challenge virus was added to each well and mixed properly with the tip. To the control slide, the first received the 50 FFD_{50}/0.1 dilutions, while an equal volume of the 5.0 FFD_{50} and 0.5 FFD_{50}/0.1ml dilutions were added into the 2^{nd} and 3^{rd} were respectively. The slides were incubated at 37^{o}C for about 90 minutes simultaneously with the modified RFFIT of the bat serum samples to be tested as explained below.

4.3.2.2*Modified RFFIT on bats serum samples*

Of the 356 bats collected, only 76 serum samples were available for testing. In brief, the modified RFFIT was performed using four – well (6mm) glass slides (Cel – Line, Eric Scientific, Portsmouth, New Hampshire, USA). Serum samples were heat inactivated at a stable temperature of 56ºC for 30minutes. The heat inactivated sera were then tested initially in a 1:10 and 1:25 dilutions. About 14µl Eagle's Minimum Essential Medium (MEM) -10 was added to the first and third wells of 4-well Teflon-coated slides. Approximately 7.5µl of the same medium was dispensed into the second and forth well. Approximately 3.6µl of two separate test sera were added to the first and third wells respectively, and mixed properly using the pipette tip. About 5.0µl of the mixture was then transferred to corresponding second and fourth well and mixed

properly. The slides were incubated in humidity chamber at 37^{o}C for about 90 minutes. Thereafter, mouse neuroblastoma cells (MNA) were added unto these slides, the control and back titration reactions and the volume of 25µl (concentration, approximately 2 x 10^{6} cells/ml), and the slides were incubated at 37^{0}C, 0.5% CO_2 during 20 hours for RABV or 44 hours for LBV, MOKV, DUVV, WCBV and SHBV.

Slides were then fixed in ice-cold acetone for 30 minutes, air-dried and subjected to DFA test as described above. At microscopy, 10 separate fields were counted for each well. For all positive or inconclusive RFFIT results, additional titration of the serum was done in dilutions 1:10, 1:50, 1:250 and 1:1250. Therefore, each positive result was confirmed at least twice.

4.3.3 Reading and interpretation of results

Ten microscopic fields were examined per well for the modified RFFIT. Fields inhibited indicate antibody presence in the test sera since immunoflourescense is an indicator of viral growth (Plate II A and B). The cell control well should have no fields with fluorescing cells and of the ten fields inspected in each well of the virus back titration, the 50FFD_{50}/0.1ml should have at least 9 to ten positive fields and the 5.0 FFD_{50}/0.1ml should have 5 to ten positive fields. The totalnumbers of fluorescing fields were recorded. Fields inhibited indicate antibody presence in the test sera since immunoflourescence is an indicator of viral growth. The titers were calculated by Reed and Muench method (1938) and expressed as log dilution of test serum (Appendix VIII). Only the samples that had a 50% end-point neutralizing titer greater than 1 $\log_{10}$ (e.g less than five fields contained infected cells at serum dilution 1:10) were considered as positive.

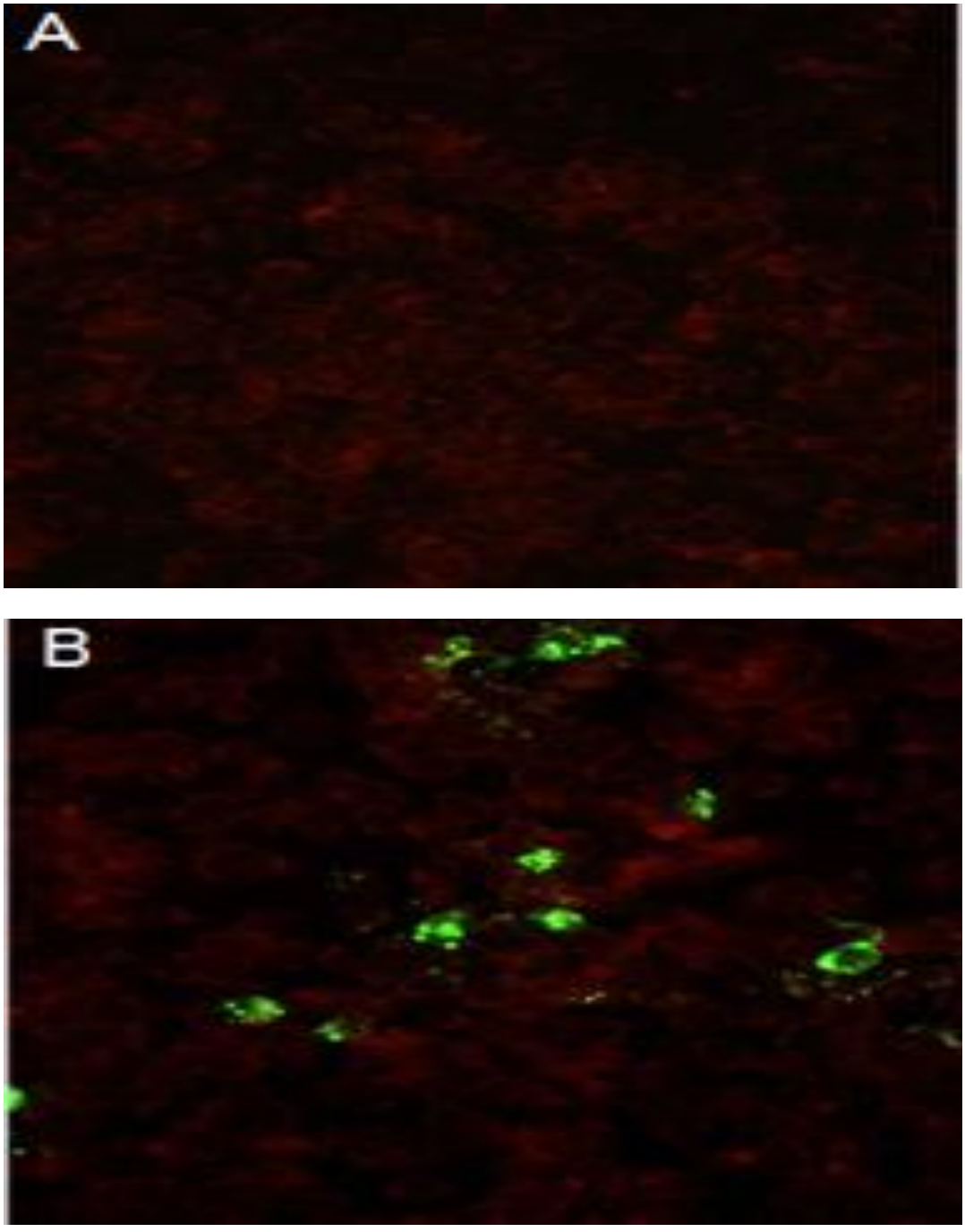


Plate II: Typical Microscopic fields viewed after carrying out RFFIT
A: An example of a microscopic field where virus has been neutralized.
B: An example of a microscopic field where virus was not neutralized

4.4 Results

4.4.1Bats sampled

The bats sampled were identified to be of seven genera, totaling 356 bats consisting of *Eidolon helvum, Epomops franqueti, Epomops gambianus, Chaerophon pumila, Lavia frons, Nycteris macrotis, Rhinolophus landeri and Rhinopoma microphylum* (two frugivorous and five insectivorous bats)(Table 4.1).

4.4.2 Lyssavirus neutralising antibody and antigen detection

No *lyssavirus* antigen was detected in the 356 bats brains tested by DFA. Most of the bat sera 92% (70/76) tested by RFFIT originated from *E.helvum.* Of the remainder, *Chaerephon Pumila* constituted 5% (4/76),whereas*Epomophorus gambianus* and*Nycteris macrotis* were 1% (1/76),each (Table 4.2).

Of the 76 bat sera tested 22 (29%), 18 (24%), 6 (8%) had neutralizing activity against LBV, SHBV and MOKV respectively. Four (5%) of the 76 sera tested neutralized LBV, SHBV and MOKV; 12 (16%) neutralised LBV and SHBV, whereas only 2 (3%) of the sera neutralized both LBV and MOKV (Table 4.2). Four (5%) bat sera neutralized only LBV while 2(3%) neutralised SHBV. None of the sera neutralized CVS-11, WCBV, and DUVV. Eight of the serum samples from *E. helvum* bats were cytotoxic to MNA cells. Cytotoxic seum samples might have resulted probably due to contamination or poor storage.

The distribution of the number and species tested by DFAT and RFFIT is as indicated on Table 4.2. Different titers of neutralizing antibodies against different lyssaviruses were found in some bats (Table 4.3: 4.4).The positive samples came from only two locations and two species. Of these positive samples all except one came from the common fruit bat,*E. helvum.*

Table 4.2:Biodata and serologic data on bats captured for detection oflyssaviruses in Plateau State Nigeria (2010 to 2011)

S/no	Species and Location	Number tested	Positive bat sera tested by RIFFT		
			LBV (%)	MOKV (%)	SHBV (%)
1.	***Chaerephon pumila*** Kanke	4	1	0	1
2.	***Eidolon helvum*** Jos Zoo	70	21	6	17
3.	***Epomophorus gambianus*** Shendam	1	0	0	0
4.	***Nycteris macrotis*** Pandam culvert	1	0	0	0
	Total	76	22(29.0)	6(7.9)	18(23.7)

% = Number of sera positive with neutralizing antibodies to the *Lyssavirus*/ total bat sera tested multiplied by one hundred.
All serum samples were negative for CVS-11, DUVV and WCBV
All bat brains tested by DFAT were negative for *Lyssavirus* antigen

Table 4.3:Rapid flourescent focus inhibition test (RFFIT) for viral neutralizing antibody titres at 50% endpoint dilution of bat sera from Plateau state, to both Lagos bat virus (LBV) and Shimoni bat virus (SHBV)

S/no	Bat ID	Bat Species	Antibody titres to LBV	Antibody titres to SHBV
1.	37	*Eidolon helvum*	1:16	1:112
2.	53	*Eidolon helvum*	1:36	1:955
3.	54	*Eidolon helvum*	1:16	1:22
4.	106	*Eidolon helvum*	1:50	1:562
5.	111	*Eidolon helvum*	1:251	1:16
6.	115	*Eidolon helvum*	1:955	1:132
7.	122	*Eidolon helvum*	1:32	1:50
8.	135	*Eidolon helvum*	1:22	1:562
9.	176	*Eidolon helvum*	1:91	1:4786
10.	178	*Eidolon helvum*	1:33	1:50
11.	183	*Eidolon helvum*	1:32	1:145
12.	B24	*Chaerophon pumila*	1:38	1:195

Table 4.4: Rapid flourescent focus inhibition test (RFFIT) for viral neutralizing antibody titres at 50% endpoint dilution of bat sera from Plateau state, Nigeria to Lagos bat virus (LBV), Mokola virus (MOKV) and Shimoni bat virus (SHBV)

S/no	Bat ID	Bat Species	Antibody titres to LBV	Antibody titres to MOKV	Antibody titres to SHBV
1.	3	*Eidolon helvum*	1:50	1:251	1:79
2.	12	*Eidolon helvum*	1:91	N	N
3.	13	*Eidolon helvum*	N	N	1:32
4.	22	*Eidolon helvum*	1:91	N	N
5.	45	*Eidolon helvum*	N	N	1:549
6.	48	*Eidolon helvum*	1:83	N	N
7.	89	*Eidolon helvum*	1:246	1:1995	N
8.	90	*Eidolon helvum*	1:125	1:251	1:16
9.	92	*Eidolon helvum*	1:32	1:50	1:138
10.	114	*Eidolon helvum*	1:50	1:229	1:18
11.	131	*Eidolon helvum*	1:22	N	N
12.	186	*Eidolon helvum*	1:79	1:501	N

6 bat sera seropositive for LBV, MOKV or SHBV

4 bat sera were seropositive for LBV, MOKV, and SHBV

2 bat sera were seropositive for LBV and MOKV.

N= negative

4.5Discussion

This study provides evidence of bat exposure to MOKV, LBV and SHBV, co-infection and/or possible interference betweenthem. MOKV may be circulating in bats in Nigeria. Cross-neutralization to MOKV has been shown by LBV seropositive bat sera (Dzikwi *et al*., 2010; Kuzmin *et al*., 2008a). Although no viral antigen was detected in the brain tissues of bats the study suggest the the occurrence of LBV, MOKV and SHBV in the wild in Nigeria.

There have been more isolations of LBV than any of the other African bat lyssaviruses recognized, with the virus apparently circulating among bats in sub-Saharan Africa (Kuzmin *et al*., 2008c). Several bat species have been associated with LBV infection, including Wahlberg's epauletted fruit bat (*Epomorphorus wahlbergi*), the straw-colored fruit bat (*Eidolon helvum*) the Egyptian fruit bat (*R. aegyptiacus*) and an insectivorous bat, the Gambian slit-faced bat (*Nycterisgambiensis*) (Banyard *et al.,* 2011). In this study, only *E. helvum* and *C. pumila* were seropositive for LBV. This may be due to the limited number of samples (76 out of 356 bats sera samples) and bat genera from which sera were obtained. Mokola virus has been isolated from terrestrial mammals in Africa (Swanepoel, 2004), but never from bats therefore there is a need for continued surveillance among animals in the wild for efficient *Lyssavirus*prevention and control.

Shimoni bat virus (SHBV) neutralizing activity occurred in 24% (18/76) of the bats (*E. helvum* and *C.pumila*). SHBV is a recently detected bat *lyssavirus*, isolated in 2009, from the brain of a dead Commerson's leaf-nosed bat (*Hipposideros commersoni*) in Kenya (Kuzmin *et al.*, 2010). The Hipposideros subfamily has a broad distribution in the Old World from tropical Africa to

China (Happold, 1987; Banyard *et al*., 2011), but it was not collected in this study. The evidence of SHBV circulating in the common African fruit bat and an insectivorous bat other than *Hipposideros* sp. Suggests that the virus may have no host species restriction.

Despite the serological evidence obtained in this study for the presence of LBV, MOKV and SHBV, none of the brain samples showed evidence of *Lyssavirus* antigen by DFA test. Several previous studies did not succeed in detecting *Lyssavirus* antigen or RNA in bats (Amengual *et al.,* 1997;Van der Poel *et al*., 2000; Arguin*et al.,* 2002; Wellenberg *et al.,* 2002; Dzikwi *et al.,* 2010). Thismay be attributed to a lengthy co-evolution period between bats and lyssaviruses. Due to limited susceptibility (Warrilow *et al.,* 2003), a majority of exposures may lead to the development of immunity, caused by peripheral virus activity and abortive clearance rather than by central nervous system infection (Shankar *et al.,* 2004).

Several hypotheses have been advanced to explain the observation of rabies virus neutralizing antibody (RVNA)in apparently healthy bats. As with any virus, there is a minimum infectious dose required to initiate a progressive infection and that dose is influenced by the genetics of the host and the pathogen. It is likely that colonial bats in particular are frequently exposed to low doses of rabies virus. Although such exposures may not lead to disease, but induces immune response, as has been demonstrated following experimental exposure of bats to aerosols containing rabies virus (Davis *et al*., 2007). Experimental exposure of big brown bats to an *E. fuscus* rabies variant isolated from a naturally infected big brown bat suggests some protective effect of past exposures tovery low doses(Turmelle *et al*., 2010). Another mechanism that may commonly result in the development of antibodies without disease is exposure of young bats to the virus while they still enjoy passive protection from maternal antibody (Steece and Altenbach,

1989). This situation may result in development of acquired protective immunity that would be boosted periodically by re-exposure to the virus giving a general pattern of having a significant fraction of healthy adults with RVNA. The absence of reports does not mean absence of disease or infection. In the meantime, post exposure treatment should be considered in the event of a bat bite. The public, especially persons in close contact with bats, should be educated about the risk of lyssaviruses transmission from bats.

CHAPTER FIVE

SEARCH FOR SOME RNA VIRUSES IN BATS CAPTURED FROM PLATEAU STATE, NIGERIA

5.1Introduction

Bats are the natural reservoir hosts for many emerging viral zoonoses of enormous human and animal health impact. These viruses among many others include: Filoviruses (Leroy *et al.*, 2005), Lyssaviruses (Calisher *et al.*, 2006), Reoviruses (Esona *et al*., 2010), Coronaviruses (Quan *et al.*, 2010) and Paramyxoviruses (Drexler *et al.*, 2012). Viruses from bats could be transmitted to humans via several means such as; bites, exposure to infected saliva, faecal aerosols, contact with infected material, infected host and/or vector (Calisher *et al*., 2006; Wong *et al*., 2012).

Quan *et al.*, (2010) identified and characterized a severe acute respiratory syndrome coronavirus (SARS-CoV) -like virus in a leaf-nosed bat (*Hipposideros*) in Nigeria. CoVs cause respiratory, enteric and seldom neurologic diseases in a wide variety of vertebrates (Lai *et al.*, 2007; Woo *et al.*, 2009). They are classified into 3 genera: alpha-, beta- and gamma-coronaviruses formerly referred to as groups 1, 2, and 3 respectively (Carsten, 2009). The alpha- and beta- CoVs are associated with diseases of mammals including humans while the gamma- CoVs are chiefly implicated in avian diseases (Lane *et al.*, 1997). The outbreak of severe acute respiratory syndrome (SARS) was caused by the SARS-CoV in 2003 with subsequent implication of the Chinese horseshoe bats (*Rhinolophus*) as possible reservoir of the SARS-CoVs-like viruses (Baric, 2008; Ksiazek *et al.*, 2003; Marra *et al.*, 2003; Peiris *et al.*, 2003; Rota *et al.*, 2003). Among the most urgent concerns prompted by the SARS epidemic is the likelihood of similar future events. Thus, it seems highly relevant to study the ecology of bat CoVs.

Some of the most significant emerging infectious diseases of humans and animals are associated with paramyxoviruses. Recent discoveries suggest bats as the major mammalian host of paramyxoviruses(Drexler *et al.,* 2012). Bat associated orthoreoviruses have recently been identified from humans with respiratory infections (Chua *et al.,* 2007; 2008) thus raising serious concerns. The family *Reoviridae* consist of numerous diversified viruses of segmented, double stranded RNA (dsRNA) genome. Reoviruses have a wide host range which includes mammals, birds, fishes, insects, reptiles and plants (Du *et al.,* 2010). Rotaviruses (RV) are very important aetiologic agents of severe diarrhoeal disease in humans and animals. Of the 7 known RV serogroups, the serogroups A, B, and C have been identified in humans and animals while D, E, F and G have only been identified in animals. Although host species barriers may limit RV adaptation and circulation, gene segments from animal-origin RVs have been frequently identified in human RVs. Therefore bats may serve as reservoir host of RV to human or other animals, as annual reemergence of RV occurs due to viral genomic reassortments among already circulating RVs (Estes and Kapikan, 2007; Esona *et al.,* 2010).

Oral reports of some flu-like symptoms experienced for many years by some zoo staff which they had claimed coincided with the seasonal arrival of the fruit bats were obtained during bat roost survey in 2009 prior to the *Lyssavirus* study documented in this dissertation (Chapter 4). Also, a few gross lesions including necrotic lesions on liver, splenomegaly, whitish hard nodular lesions on spleen, pale and fibrotic lungs (Appendix X) were seen during necropsy of some of the bats. These therefore served as the empirical bases for the search of viruses. This study therefore, was designed to detect the presence of*Arenavirus, Coronavirus, Influenza virus,*

Paramyxovirus, Pneumovirus, Reovirus, Rhabdovirus (both snakehead and vesicular stomatitis virus) and *Rotavirus* in bats from Plateau State Nigeria by nested reverse transcription-polymerase chain reaction (RT-PCR) and to characterize them by molecular methods.

5.2 Materials and Methods

5.2.1 Study area

Specimens obtained for this study were collected from the same bats examined for lyssaviruses as shown in Table 4.1 and figure 4.1.

5.2.2 Bat survey, sampling and identification

Bat survey, collection, identification, processing and storage are as explained in sections 4.2.2 and 4.2.3.

5.2.3 Molecular techniques used for detection of selected RNA viruses at the CDC, GA.

The molecular techniques used to screen for viral RNA in the 95 bat faecal swabs incuded, nucleic acid extraction and purification, DNA amplification by Nested RT-PCR, agarose gel electrophoresis, Exo- SAP, nucleotide sequencing and sequence analysis, TOPO TA- cloning (where necessary).

5.2.3.1 *Total nucleic acid (TNA) extraction*

Faecal swabs (n=95) obtained from seven bat genera were individually suspended and vortexed in 700 µL phosphate-buffered saline (PBS). Buffer AL (guanidine thiocyanate)with magnetic beads and isopropanol was added to specimen already in the sample plate 1. All plates prior to

use were properly and carefully labelled according to the bat identification number and each well loaded as follows: Plate 1-lysis contained different specimen in each separate well, with 400µlBuffer AL as the lysis buffer; Plate 2-AW1 contained 700µl buffer AW1 (consist of ethanoland guanidine thiocyanate) a wash buffer; Plate 3-AW1 contained 500µl buffer AW1 for a second wash; Plate 4-RPE contained 500 µl buffer RPE (ethanol); Plate 5-RPE also contained 500 µl buffer RPE; Plate 6 contained nuclease-free water as elution buffer and plate 7 (the rod cover plate). After these steps automated extraction using Thermo Scientific KingFisher96® instrument and the QIAGEN BioSprint One-for-all Vet Kit Catalog no. 947057 was carried out.

Before loading KingFisher 96® with the actual specimen, it was first test run with test run plates specially kept in a ziplock bag for that purpose. The volume of the reagents used for the 96 wells (95 samples and one blank) prepared was 120% of the required volume so as to compensate for pipetting error and possible evaporation.

Following the protocol after loading, the KingFisher 96 machine was turned on for automatic extraction and when finished the 'Start' key was pressed to remove plate 6 (elution plate). The total extracted using this automated machine last for about 35minutes.The elution plate containing the extracted RNA from the bat faecal swabs was then sealed for storage in -80ºC until when required for RT-PCR, while the other plates were disposed of.

5.2.3.2*Broadly reactive oligonucleotide primer selection*

Conserved amino acid sequences for the families, subfamilies, and genera were selected from alignment of deduced L-protein-coding sequences recorded in GenBank (National Institutes of

Health, Bethesda, MD). Highly conserved domains were selected and back translated into degenerate nucleotide sequences to represent all possible codons for the corresponding amino acids. To minimize the number of primers, primers were designed with restricted number of mixed degenerate. To minimize the potential for nonspecific cross-reactivity, a Blastn search analysis of GenBank was performed for similarities with known sequences. The primers were synthesized at the Biotechnology Core Facility, Division of Scientific Resources of the Centers for Disease Control and Prevention (CDC). The list of primers used is given in Table 5.1.

5.2.3.3*Reverse transcription polymerase chain reaction (RT-PCR) and nested RT-PCRamplification*

To maximize sensitivity and specificity, nested or seminested PCR assays were used. Optimized reaction conditions, including primer concentration, magnesium (Mg2) concentration, and thermal cycling temperatures and profiles were used. Total nucleic acids from each rectal swab were screened for selective viral enteric and respiratory pathogens (*Arenavirus, Coronavirus, Influenza Virus, Paramyxovirus, Pneumovirus, Reovirus, Rhabdovirus* (snakehead and vesicular stomatitis virus) and *Rotavirus*). The PCR assays were done using the Superscript III One-step RT-PCR kit and Platinum Taq Kit (Invitrogen, San Diego, CA, USA) and analysed as previously described (Tong *et al.,* 2009) and as shown in the pan-viral RT-PCR protocols (Appendix III-X1).

Prior to the RT-PCR on the bat rectal swab samples, the master mixes were made and tested with on the relevant corresponding protocols (Appendix XI-XX). The PCR products were visualized by UV light after electrophoresis on a 2% agarose gel. Expected amplicons were obtained in all

viral family/genera by use of the tested master mixes except *Reovirus* and *Rotavirus*. After troubleshooting we discovered that *Reovirus* and *Rotavirus* did not produce any amplicon because of an initial step of denaturation unique for Reoviruses due to their double bonds, which was mistakenly skipped resulting in absence of amplicons. The test run was repeated after error correction, and amplicons of expected sizes were obtained for the two genera.

To avoid PCR contamination, sample extraction, PCR reagent preparation, addition of reference controls, PCR, and sequencing were carried out in separate BSL 2 laboratory rooms. Negative controls (water) were included in each step. The final nested or seminested PCR products were visualized after electrophoresis on a 2% agarose gel.

Table 5.1: Consensus degenerate primers used for pan-viral RNA detection

Viral Family/ Genus	Function of Primer in PCR	Primer sequence/primer name if unpublished	Target gene	Product Size (bp)
Arenaviridae	1st round forward 1st and 2nd round reverse 2nd round forward	Arena- F915 (Unpublished) Arena – R1440 (Unpublished) Arena – F990 (Unpublished)	Arena-NP	450
Pan-coronavirus	1st and 2nd round forward 1st round reverse; 2nd round reverse	5′-ATGGGITGGGAYTATCCWAARTGTG3′ 5′-AATTATARCAIACAACISYRTCRTCA-3′ 5′-CTAGTICCACCIGGYTTWANRTA-3′	Orf1AB	199
Pan-bat coronavirus	1st and 2nd round forward 1st round reverse 2nd round reverse	5′-ATGGGITGGGAYTATCCWAARTGTG3′ 5′-TATTATARCAIACIACRCCATCRTC-3′ 5′-CTGGTICCACCI GGYTTNACRTA-3′	Orf1AB	199
Pan-influenza	Forward round Reverse round	FLU-PAN-F3 (Unpublished) FLU-PAN-R4-2 (Unpublished)	FLU-PB1	324
Paramyxovirinae	1st round forward 2nd round forward 1st and 2nd round reverse	5′-GAAGGITATTGTCAIAARNTNTGG AC3′ 5′-GTTGCTTCAATGGTTCARGGNGAYAA-3′ 5′-GCTGAAGTTACIGGITCICCDATRTTNC-3′	L gene	563
Pneumovirinae	1st round forward 2nd round forward 1st and 2nd round reverse	5′-GTGTAGGTAGIATGTTYGCNATGCARCC3′ 5′- ACTGATCTIAGYAARTTYAAYCARGC-3′ 5′- GTCCCACAAITTTTGRCACCANCCYTC-3′	L gene	108
Rhabdoviridae – snake head -1	1st round forward 2nd round forward 1st and 2nd round reverse	Rha-S-F808 (Unpublished) Rha-S-F958 (Unpublished) Rha-S-R1277 (Unpublished)	L gene	319
Rhabdoviridae – (VSV-1)	1st round forward 2nd round forward 2nd round reverse	Rha-F1926 (Unpublished) Rha-F2014 (Unpublished) Rha-R2250 (Unpublished)	L gene	236
Reoviridae (ReoAM)	1st round forward 1st and 2nd round reverse 2nd round forward	2073F (Unpublished) 2222R (Unpublished) 2090F-3 (Unpublished)	VP2 gene	133
Reoviridae-Rotavirus 24-1	1st round forward 1st round reverse 2nd round reverse 2nd round forward 2nd round forward	1738F (Unpublished) 2100R (Unpublished) 1985R (Unpublished) 1756F-1A (Unpublished) 175s6F-1B (Unpublished)	VP1 gene	230

5.2.3.4 *Preparation of agarose gel*

A 2% agarose gel was prepared by using electrophoresis buffer, 100ml Tris-borate EDTA (TBE) pH 8.0, and 2g agarose (Invitrogen, UltraPure ® Cat.No 16500-500) and melted in a microwave oven. The molten gel mix was allowed to cool and 5 µl of Ethidium Bromide solution (0.5µg/ml) was added and poured into mould. Combs were inserted to create wells and the agarose was allowed to stand and set for 30 minutes. The electrophoresis chambers were filled with 1X TBE buffer and the gels were submerged on the trays.

Approximately 5µl loading dye (bromophenol blue, glycerol and xylene cyanol) were placed on clean parafilm or in a 96 well plate to which about 20µl cDNA was added and mixed, then loaded into the wells.A DNA VIII marker (Roche, Indianapolis, IN) was run in the gels alongside the sample wells in the first well to estimate the amplicon size. Electrophoresis was achieved at 125-150V for 20-30 minutes depending on the expected size of PCR product. Gels were placed on trays and read on Ultraviolet (UV) transluminator of a molecular imager and gel pictures were taken using the Quantity One software pacage (Lasergene, UK, London).

5.2.3.5*Several options to aid identifying possible positives in the specimens*

If a specimen presents a single or multiple bands on the gel including the desired band around ± 100bpcompared to positive control on the gel, there are several procedures to use in purifying the band of interest, these includes;

If the band appears strong it was purified using the QIAquick PCR purification kit.

If the band appears to have adequate intensity after extraction and purification it was directly sequenced. The remaining dNTP's and primers were removed by an enzymatic reaction using Exonuclease and Shrimp Alkaline Phosphatase(ExoSAP).

If the band appears faint, after extraction and purification, a reamplification step was carried out using 2nd run PCR primers.

If the band was faint and re-amplifications gave a mixed sequence, then TA cloning was carried out to clarify the true sequence.

5.2.3.6*Purification of DNA fragments*

Positive PCR products were purified by gel extraction using the QIAquick Gel Extraction kit (QIAGEN USA) according to the manufacturer's instructions exactly as describedearlier in section 3.2.4.toobtain DNA in 10µl of elution buffer.

5.2.3.7*Exo-SAP protocol*

To remove excess unincorporated primersanddNTPs, the Exo-SAP (NEB Exonuclease 1 (Mo293L - OU/ µl) and Roche shrimp alkaline phosphatase Cat. N. 11758250001) method was used. Briefly, after performing PCR amplification, the product was checked on an agarose gel with a size standard for the desired bright and dominant band. If the band was too faint for direct sequencing, the extracted DNA was reamplified using the same PCR primer set and then the product of the second amplificationwas rechecked on a gel to make sure there was a single bright dominant band. For every 45-50 µl PCR product, 1 µl Exo I and 2µl SAP (a total of 3 µl) was

used per template reaction**.** PCR Buffer is compatible with enzyme buffers, so a working stock solution of Exo-SAP was prepared 1:2 and stored ready for use at -20 ºC. Incubation at 37º for 15 minutes to degrade the remaining primers and dephosphorylate excess dNTPs was then conducted, followed by an inactivation of the Exo-SAP enzymes at 80º for 15 minutes. This was all programmed into the thermal cycler for ease of operation. The products were held at 4ºC until ready for downstream application.

5.2.3.8*Preparation of materials for sequencing*

For the sequencing reaction the template used are the products of the Exo-SAP reaction. Two sets of tubes were obtained and labeled for the forward and reverse primers used for amplification (Table 5.2). BigDye® Terminator version 3.1(Applied Biosystems, Fosta city, CA) is a premix containing buffer, 4 dNTPs, Taq polymerase, heat stable pyrophosphatase, 4 BigDye dideoxyterminators each labeled with a different energy transfer fluorescent dye. The tubes were vortexed and spun down briefly in the centrifuge. They were then placed in the thermal cycler to complete 25cycles as was programmed (Table 5.2). The products were held at 4ºC until ready to be removed from the machine.

Table 5.2: Sequencing Reaction Protocol

		Cycling Parameters		
Content	**Volume µl**	**Temp(ºC)**	**Length**	
H_2O	8 ~ 4ul*	96	2min	**Initial denaturation**
BigDye (version 3.1)	2ul**	96	30	25 cycles
5 µM Primer (Forward or Reverse)	1ul	50	15	
Template	1~ 5ul***	60	3min	
Total	12 µl	4	Until required	

*Varies based on volume of template. (The total volume of water and template equals 9ul)
** 1ul may also be adequate for easy-to-sequence template
*** for DS DNA, 0.5 ug DNA in MAX volume 5 µl
for SS DNA, 0.25 ug DNA in MAX volume 5µl
for PCR DNA, 0.07-0.2 ug DNA in MAX volume 5 µl

5.2.3.9 *Clean-up of sequencing reaction*

After the cycling was completed the sequencing products were cleaned up using ABI procedure. Cleaning of sequencing reaction was achieved using Centri-Sep columns (Princeton separations, Inc. Adelphia, NJ) as recommended by ABI for fast, efficient removal of excess dye terminators from completed DNA sequencing reactions. Briefly, formamide was removed from -20ºC freezer and allowed to thaw on bench while clean up plate was prepared [350 ul per run (1run= 16 samples)]. The required Centri-Sep plate/strips was taken out of - 4ºC refridgerator and allowed to equilibrate to room temperature before use. Centri-sep strips to be used were separated using exactor knife, then the bottoms were cut off with scissors and the top adhesive were removed. The strips were placed in a balance and 2 balances were weighed to ensure they were within 0.1g of each other and they were centrifuged for 3 min at 3,000 rpm.During the spinning of the plates 20ul of formamide was added to the relevant rows in the 96 well sequencing plate. The plate holder was gently removed and sequencing plate with formamide was added. Silica strips were then placed over the rows to be used. Sequencing reaction was spun down and 12ul of sequencing reaction (template) was added to the silica strips taking care to place the template in the center of the gel bed without touching it with the pipette tips and centrifuged as before.

5.2.3.10*Nucleotide sequencing and sequence analysis*

After the sequence reaction clean-up, purified DNAs were sequenced on ABI Prism 3130 DNA Automated Sequencer (Applied Biosystems, Foster City, CA, USA) using an ABI Prism Big Dye Terminator Cycle Sequencing Kit, version 3.1 (Applied Biosystems) according to the manufacturer's instructions. Sequencing reaction set-up is as shown in Table 5.2. Each of the four fluorescent dyes emits a peak emission wavelength spectrally resolved from other dyes. The A emits green, G emits black, T emits red and C emits blue. None specific nucleotides (N) were purple in colour. Editing of sequence chromatogram files was done and sequence contigs were assembled using Sequencher 4.8 software (Gene Codes Corporation, Inc., Ann Arbor, MI, USA). Nucleotide sequence alignments were conducted. To characterize the diversity of the viruses screened, all genetic trees were constructed using the Molecular Evolutionary Genetics Analysis version 5 (Mega5) software package (Tamura *et al*., 2011) with the neighbour joining method based on other existing fragments, selections, for the positive virus from the NCBI database based on Bayesian-Monte Carlo Markov Chain (Tong *et al.,* 2009) with 1000 bootstraps number**.**

5.2.4 TOPO® TA cloning with Invitrogen pCR 4.0

Faint PCR amplicons or those with mixed sequence results were cloned for further sequencing. This was achieved using the TOPO® TA Cloning Kits (Invitrogen) according to the manufacturer's instructions as briefly described in the following procedures;

5.2.4.1 *Addition of a poly-A tail to products of gel extraction for TA- cloning*

Fresh PCR products generally contain dA 3´ overhang due to the activity of Taq polymerase. If the PCR products are more than one day old or if they are gel purified, 3'dA will be absent and will not be ligated efficiently into a TA-cloning vectors such as pCR2.1 or pCR4-TOPO supplied by Invitrogen. This protocol adds dAs to the 3´ end of gel extract preparations.

The template, a product of gel extraction (2-3 µl) was added to 3-4ul Master Mix (Table 5.3) for each sample to be cloned. Each reaction was incubated for 10-15 minutes at 72ºC after heat starting the thermal cycler at 94ºC for 2 minutes, followed by 4ºC storage or transferred to -20 ºC until when required.

Table 5.3: Components for adding Poly-A tail per reaction (using Invitrogen Kit)

Poly-A tail Master mix	**Volume µl/reaction**
Taq Polymerase	0.1
10X Buffer	0.5
dNTPs	0.5
Mg^{2+}	0.5
dH_2O	1.5
Gel extraction product	2-3
Total Volume	**5.1 (or 6.1)**

5.2.4.2 *Ligation with TOPO TA cloning® kit*

Ligation was conducted according to the manufacturer's instructions (Invitrogen pCR 4.0). Briefly, for each reaction of about 3-4 µl of PCR product was added TOPO salt solution (1µl) and Topo vector pCR 4.0 (1µl). This reaction was mixed by gently tapping at the bottom of the tube. Then it was incubated at room temperature (RT) of 22-23ºC for 20 minutes and placed on ice ready for transformation.

5.2.4.3*Transformation of TOP10® competent cells*

Before commencing transformation, the water bath was equilibrated to 42ºC. The S.O.C. medium (2% Tryptone, 0.5% Yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM MgCl2,10 mM MgSO4, 20 mM glucose) supplied with the kit was warmed to room temperature and selective agar plates were put in 37ºC incubator for 30 minutes (one plate was used for each transformation) for optimal growth of *E. coli* cells.

Transformation was done according to the manufacturer's instructions (Invitrogen One Shot® TOP 10) into chemically competent *E. coli* (Cat. No. C4040-06) cells (50 µl cells per transformation), equivalent to the number of samples/templates were taken out of -80ºC freezer and thawed on ice.

The ligated plasmids (3 µl) were added to the correspondingly labeled vials of One Shot® cells and gently tapped at the bottom of the tube about 10-20 times and incubated on ice for 15-30 mins. Thereafter, it was heat shocked at 42ºC in water bath for 30 seconds and immediatelyplaced on ice for 2-3 minutes. Thereafter, 250ul of Super Optimal broth with Catabolite repression (S.O.C.) medium was added for higher transformation efficiencies of plasmids and put in a horizontally shaking incubator at 225rpm for 1hr at 37ºC. A 200ul transformation product was spread onto a pre-warmed 15cm-diameter LB (Luria Berani agar) plate and allowed to absorb for 5-10minutes. The plates were incubated at 37ºC overnight and inspected for colonies the next day.

5.2.4.4 *Colony PCR*

For the colony PCR, master mix was made (Table 5.4) and aliquoted into labeled PCR tubes. Using micropipette tip, a single putative colony was picked off a plate (8 single colonies per sample) and each tip was inserted into the labeled PCR mixture and gently mixed. After colony PCR , the samples were run on gel and the expected amplicons were subsequently purified

according to the Exo-SAP protocol as previously elaborated (5.2.3.7.) before proceeding to sequencing of the purified PCR products using colony sequencing master mix using universal primer such as M13 was prepared as shown below (Table 5.5). Sequence cycling parameter used was same as in Table 5.2.

Table 5.4: Colony PCR Protocol- using MasterAmp Tfi DNA polymerase

Reaction Mix content	**Volume µl**
H2O	15.9
20x MasterAmp TFl buffer	1.0
25mM MgCl2 (TFl kit)	1.2
10mMdNTPs (RocheLifesciences)	0.4
20 µM M13 Primer F	0.5
20 µM M13 Primer R	0.5
MasterAmp TFl DNA polymerase (1U/µl)	0.5
Total	20.0

Table 5.5: Colony Sequencing Reaction Mix

Colony Sequencing Reaction Mix	Volume µl
Big Dye 3.1	1
5x buffer	2
5 µM of M13primer	1
Water	5
Sub-total volume	9
Template (purified products)	3
Total	12

5.2.3.11*Sequence editing, aligning and analysis*

After the completion of ABI 3130 sequencer run, the data from sequencer was transferred to the computer and analyzed using a simplified program known as the Sequencher Program. This program was used to sort out, verify sequences and edit as needed. In brief, the files were sorted out in the folder of sequences by file type. All ABI files were then selected and dragged into the sequencer window then saved. Chromatograms were viewed and trimmed.The window was closed and automatically saving the edits. This was done for all the sequences and saved intermittently. Once this was finished 30 -40 sequences were selected to File and exported as FASTA files.

Exported sequences were uploaded to http://1.usa.gov/kCt9cW and BLAST using http://blast.ncbi.nlm.nih.gov/ . Each time after obtaining the BLAST the sequence was checked to make sure it's in the right orientation. MEGA program(Tamura *et al*., 2011) was used to reverse any that was found not to align. For each of the sequence BLAST the closest hit and the closest known species were chosen. All sequences were checked for the possibility of Chimeras using the software; http://35.8.164.52/html/analyses.html. It was noted if sequences were in the right orientation (5′- to 3′), if not, corrections were made by clicking the sequence in Bio-edit 5.0 package and changed. Inserts and deletions of the sequences obtained versus the closest hits were also checked.

The evolutionary history was inferred by using the Maximum Likelihood method based on the Kimura 2-parameter model (Kimura, 1980). The tree with the highest log likelihood

(-9733.3341, -9415.1048, -1388.6187 and -3597.2729 for CoV, *Paramyxovirus*, *Reovirus* and *Rotavirus* respectively) were shown. Initial tree(s) for the heuristic search were obtained automatically as follows. When the number of common sites was less 100 or less than one fourth of the total number of sites, the maximum parsimony method was used; otherwise BIONJ method with MCL distance matrix was used. A discrete Gamma distribution was used to model evolutionary rate differences among sites. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The branching orders of the trees were evaluated by the bootstrap analysis of a 1000 replicates. The sequences used for comparison and the phylogenetic analysis were obtained from the GenBank.Evolutionary analyses were conducted in MEGA5 (Tamura *et al.,* 2011).

5.3Results

Among the 95 bat faecal swabs tested by semi-nested RT-PCR, a positive PCR result was observed as bright visible amplicon of expected size for each virus tested (Plate 2 to 7). Eight (8.42%) *Coronavirus* RNA were identified, and sequenced. Of these, the BLAST search demonstrated seven of the CoVs (6 from *Rhinolophus* sp. and 1 *Chaerophon* sp.) had between 94% - 96% homology to the Ghanabat CoV (BtCoVGhana) and another from an *Epomop gambianus* had a 97% homology to the Kenya batCoV (BtKy56). When compared with 60 other CoVs of animals and humans obtained from the GenBankusing the NJ tree method to construct a phylogenetic tree (Figure 5.1; 5.2) the eight newly detected Nigeria CoVsall of which clustered withthe β-group CoV as the SARS-COV, their numbers and the name of the specific bat host written next to each (Figure 5.1; 5.2).

Sequence corresponding to*Paramyxovirus* was obtained in one bat(*Rhinolophus landeri)* and this had 70% homology to the J-virus cluster found in Kenya when compared with 48 sequences (belonging to *Morbillivirus, Respirovirus, Henipavirus, Avulavirus, Rubula* virus all of man and animals) obtained from the GenBank . A phylogenetic tree was constructed using NJ tree method and the virus "Nigeria *ParamyxovirusChaerophon pumila*" is marked by a bold blue circle (Figure 5.3; 5.4).

None of the 95 fecal swabs were positive for *Arenavirus, Influenza virus* and Rhabdoviruses (Table 5.6).Faint amplicons were obtained approximately of the expected size for *Reovirus* (133bp) in lanes 23, 24 and 28 (Plate 7) but the amplicon in lane 28 was brighter and of exact

size. After sequencing only sample 28had reovirus sequence (Figure 5.6)**.** A *Reovirus* was detected from an *Epomop franqueti* with sequence relating closely (93%) to the mammalian *Orthoreovirus.* The sequence was compared with 16 other reoviruses of animals and humans obtained from the GenBank (Figure 5.5; 5.6).

Sixteen (17%) faecal samples were positive for *Rotavirus*(Figure 5.8) and these includedsamples from *E. helvum* (8), *R. landeri* (2), *N. macrotis* (1), *C. pumila* (3), *E. franqueti* (1), and *L. frons* (1). Initial 17 samples were positive and sequenced but one was not reproducible**(**sample from lane 17, Plate 5**)** after repeated RT-PCR and sequencing**.**The partial VP1 gene sequence identified in a *Nycterismacrotis*, and a horse shoe bat, *R. landeri*(both insectivorous bats), shared 90% and 89% nucleotide identity with human RV A strain B10 from Kenya respectively. A *rotavirus*with 96% identical sequence to RVA02V0002G3 from chickenswas also detected from*R. landeri*. There were five other sequences obtained from *E. helvum*which were also closely related to the RVA02V0002G3 strain with a mean maximum identity of 79%.

Sequences similar to RVA human/Vanderbilt/VU08-09-39 were obtained from two *C. pumila* with an average maximum identity of 81%. A rotavirus sequence from a *C. pumila* had 84% identity to another human RVA (Hu/Cl-81/2011/KOR). One*E.helvum* was also found having RV sequence that closely relates to another different RVA strain (RVA/Human-wt/NPL/KTM368/2004/G11P). The*Rotavirus* sequence obtained from *E. franquetti* alsoclustered with the serogroup A.

From *Laviafrons* (an arboreal insect bat) was found a RV sequence clustering in the group B (Figure 5.7) with close homology to Human RVB, while from two *E. helvums* were two sequences with an average mean identity of 89% to *Porcine* RVC. A phylogenetic tree (Figure 5.7) based on a 200 bp fragment of the VP1 gene demonstrated sequences related to RVA (13), RVB (1), and RVC (2).

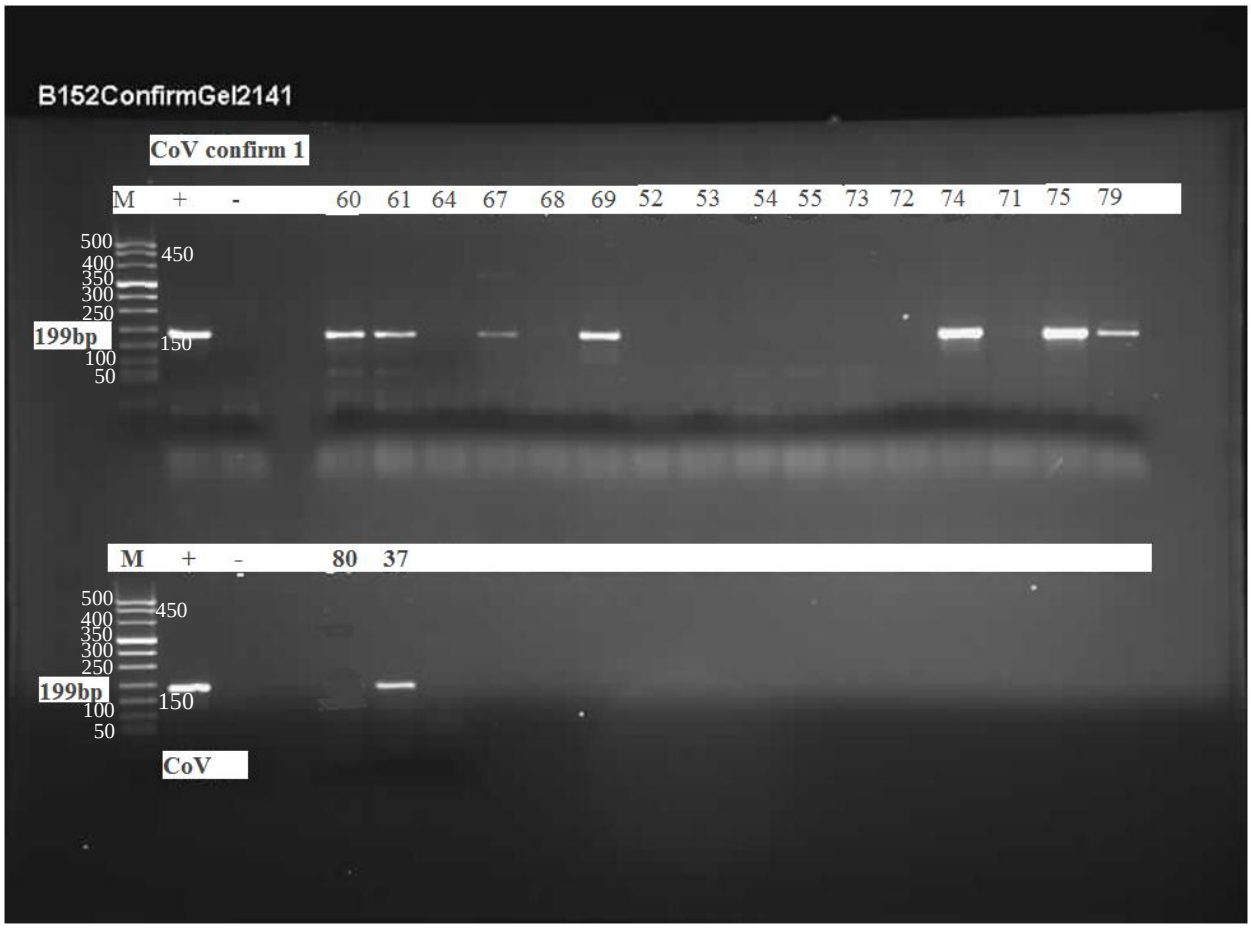


Plate 2: Agarose gel showing amplicons following nested RT-PCR with CoVprimers. Lane M: Molecular marker. Lanes + and - : signify positive and negative controls respectively. Expected positive amplicons of approximately 199bp were seen in lanes with sample identification numbers 60, 61, 67, 69, 74, 75, 79 in upper panel and 37 on the bottom panel.

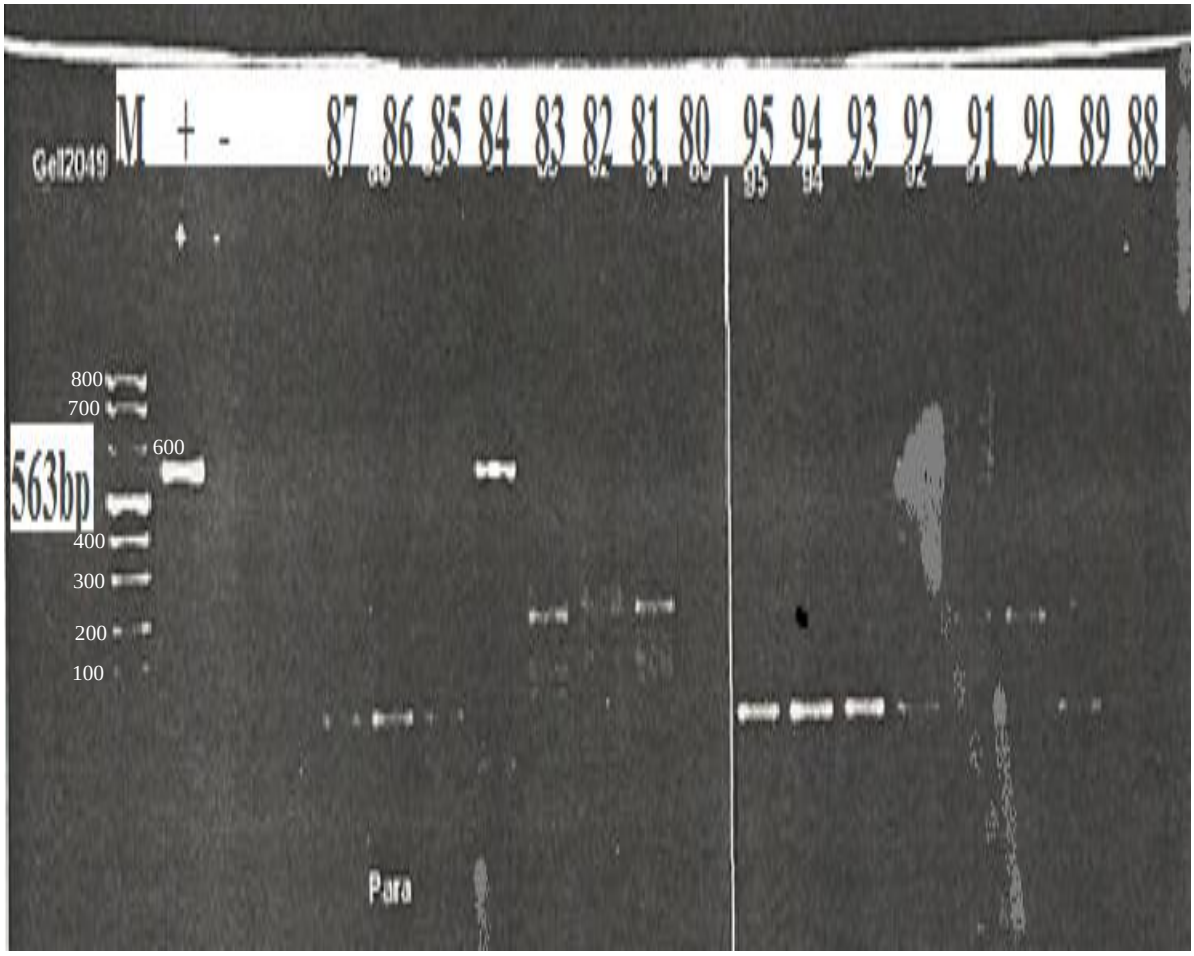


Plate 3: An agarose gel under UV light following nested RT-PCR for *paramyxovirus*
Molecular marker (lane M), positive (+) and the negative (-) controls are as indicated. Sample number 84 showed band of expected sizeapproximately 563bp.

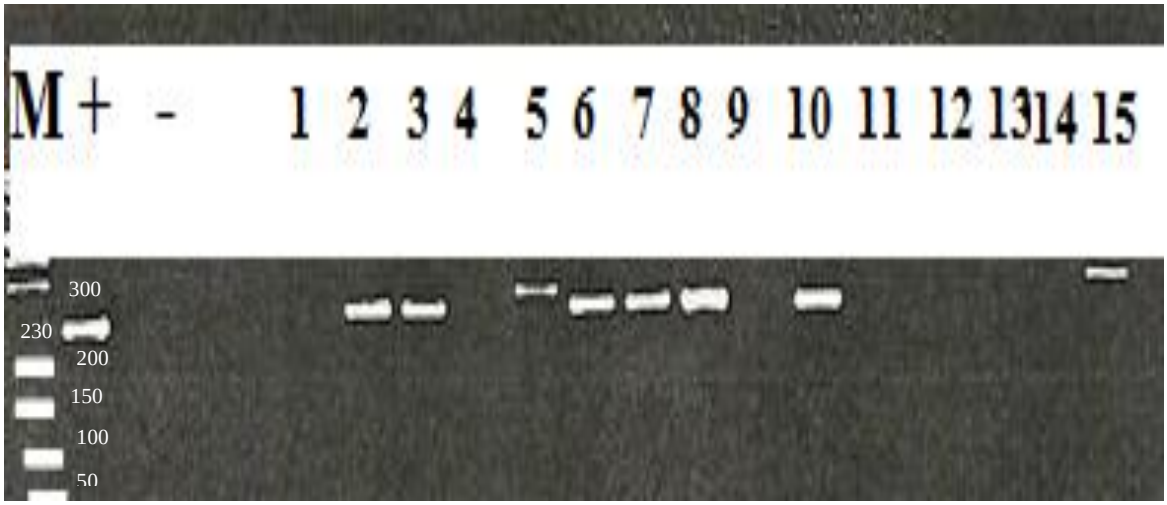


Plate 4: An agarose gel under UV light following nested RT-PCR for *Rotavirus*.
Molecular marker (lane M), positive (+) and the negative (-) controls are as indicated. Rotavirus specific amplicons of expected product size of approximately 230bp following nested RT-PCR, were obtained in wells with sample Id; 2, 3, 5, 6, 7, 8, 10, and 15.

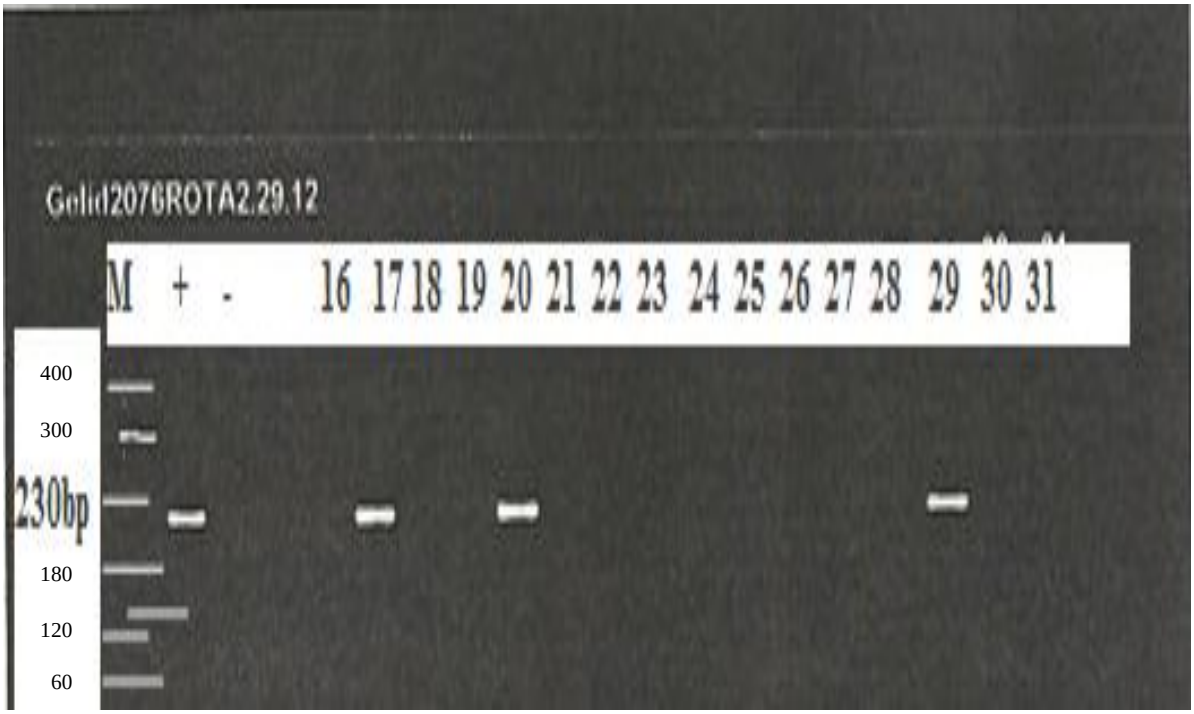


Plate 5: An agarose gel under UV light following nested RT-PCR for Rotavirus.
Specific amplicons of expected product size approximately 230bp following nested RT-PCR, were obtained in wells with samples 17, 20 and 29.

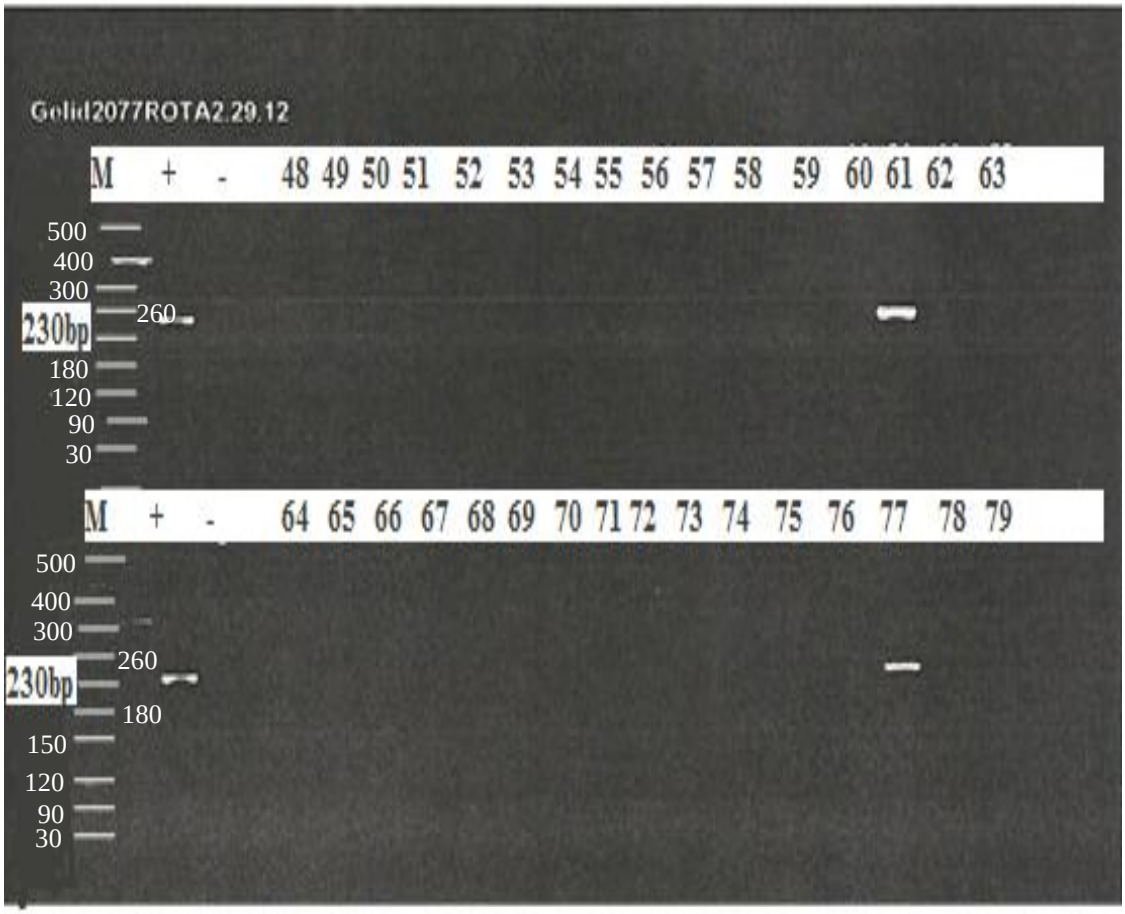


Plate 6: An agarose gel under UV light following nested RT-PCR for *Rotavirus*.
Moleccular marker (lane M), positive (+) and the negative (-) controls are as indicated showing *Rotavirus* specific amplicons of expected product size approximately 230bp were obtained in lane with samples 61(upper panel) and 77 (lower panel).

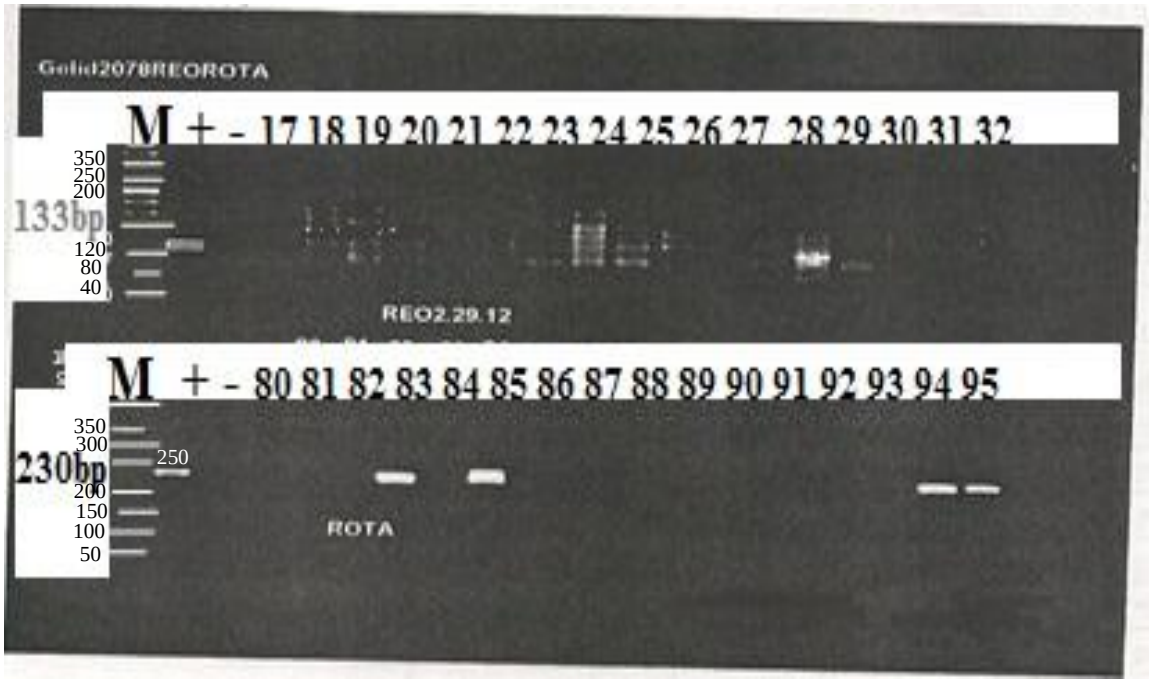


Plate 7: An agarose gel under UV light following nested RT-PCR for *Rotavirus*.
Moleccular marker (lane M), positive (+) and the negative (-) controls are as indicated loaded with test samples for both *Reovirus* (upper panel)and *Rotavirus* (lower panel). Faint amplicons were obtained approximately of expected size for *Reovirus* (133bp) in lanes with samples 23, 24 and 28 (the latter was brighter and of exact size). *Rotavirus* specific amplicons of expected product size approximately 230bp were obtained in lanes with samples 82, 84, 94 and 95.

Table 5:6: Frequency of some RNA viruses detected in faecal samples from different species of bats in Plateau State

Species (location)	**Total number tested**	***Coronavirus* positive (Specific rate %)**	***Paramyxovirus* positive (Specific rate %)**	**Rotavirus positive (specific rate %)**	**Reovirus positive (specific rate %)**
Chaerophon pumila (Kanke)	18	1 (5.6)	1 (5.6)	3 (18.3)	0 (0.0)
Eidolon helvum (Jos Zoo)	23	0 (0.0)	0 (0.0)	8 (34.8)	0 (0.0)
Epomop Franquetti (Shendam)	9	0 (0.0)	0 (0.0)	1 (11.1)	1 (11.1)
Epomop gambianus (Lakushi bridge)	5	1 (20.0)	0 (0.0)	0 (0.0)	0 (0.0)
Lavia frons (Lakushi forest)	3	0 (0.0)	0 (0.0)	1 (33.3)	0 (0.0)
Nycteris macrotis (Pamdam culvet)	16	0 (0.0)	0 (0.0)	1(6.3)	0 (0.0)
Rhinolophus landeri Pandam GR well	18	6 (33.3)	0 (0.0)	0 (0.0)	2 (11.1)
Rhinopoma microphylum Leptur cave	4	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Grand Total	95	8 (8.4)	1 (1.1)	16 (16.8)	1 (1.1)

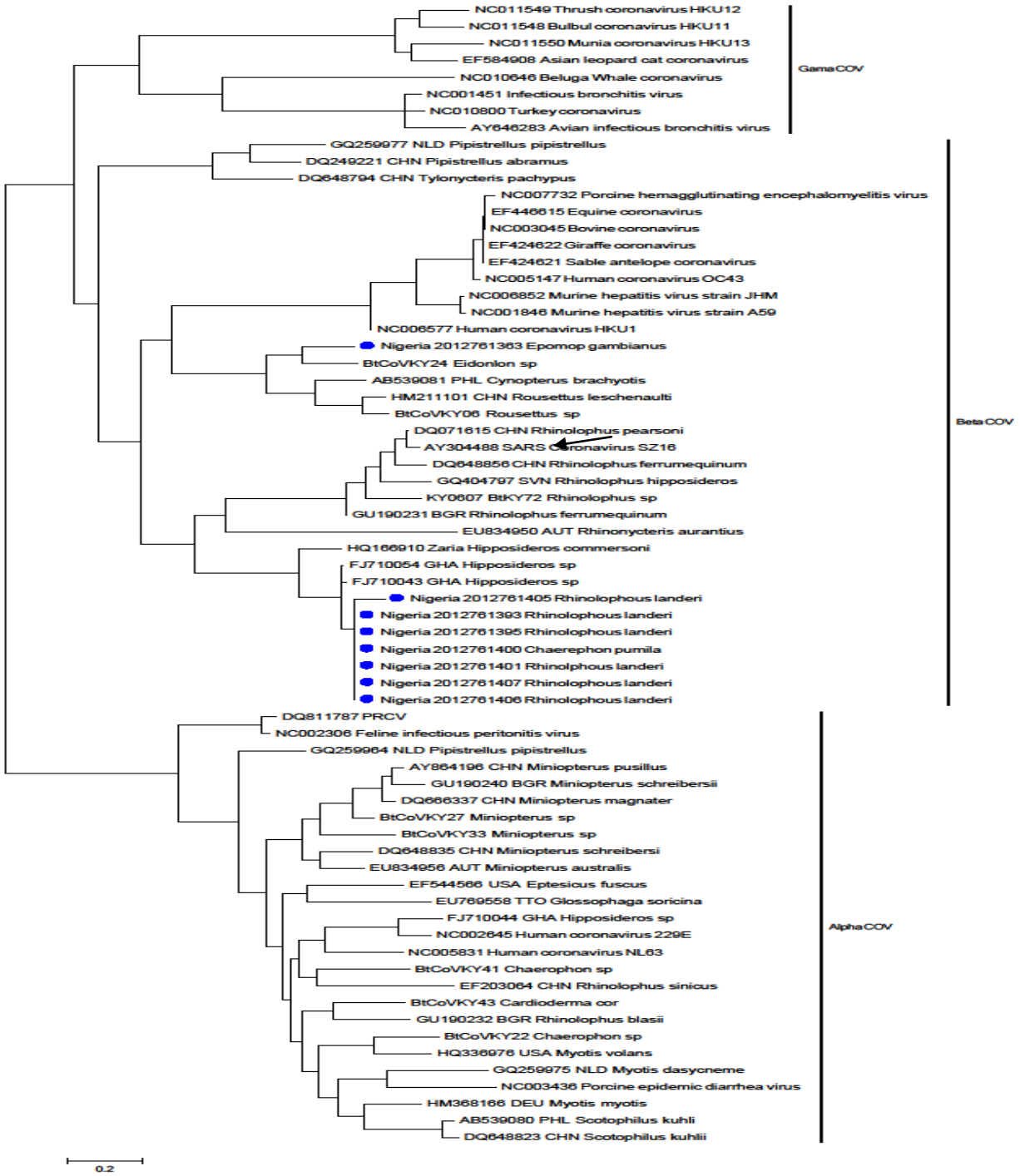


Figure 5.1: Phylogenetic tree of the 121-bp fragment of RNA dependent RNA polymerase (RdRp) was generated from eight coronaviruses from Plateau State bats in this study (blue circles) and 60 selected human and animal coronaviruses from the National Center for Biotechnology Information (NCBI) database. Note the arrow showing the position of SARS CoV.

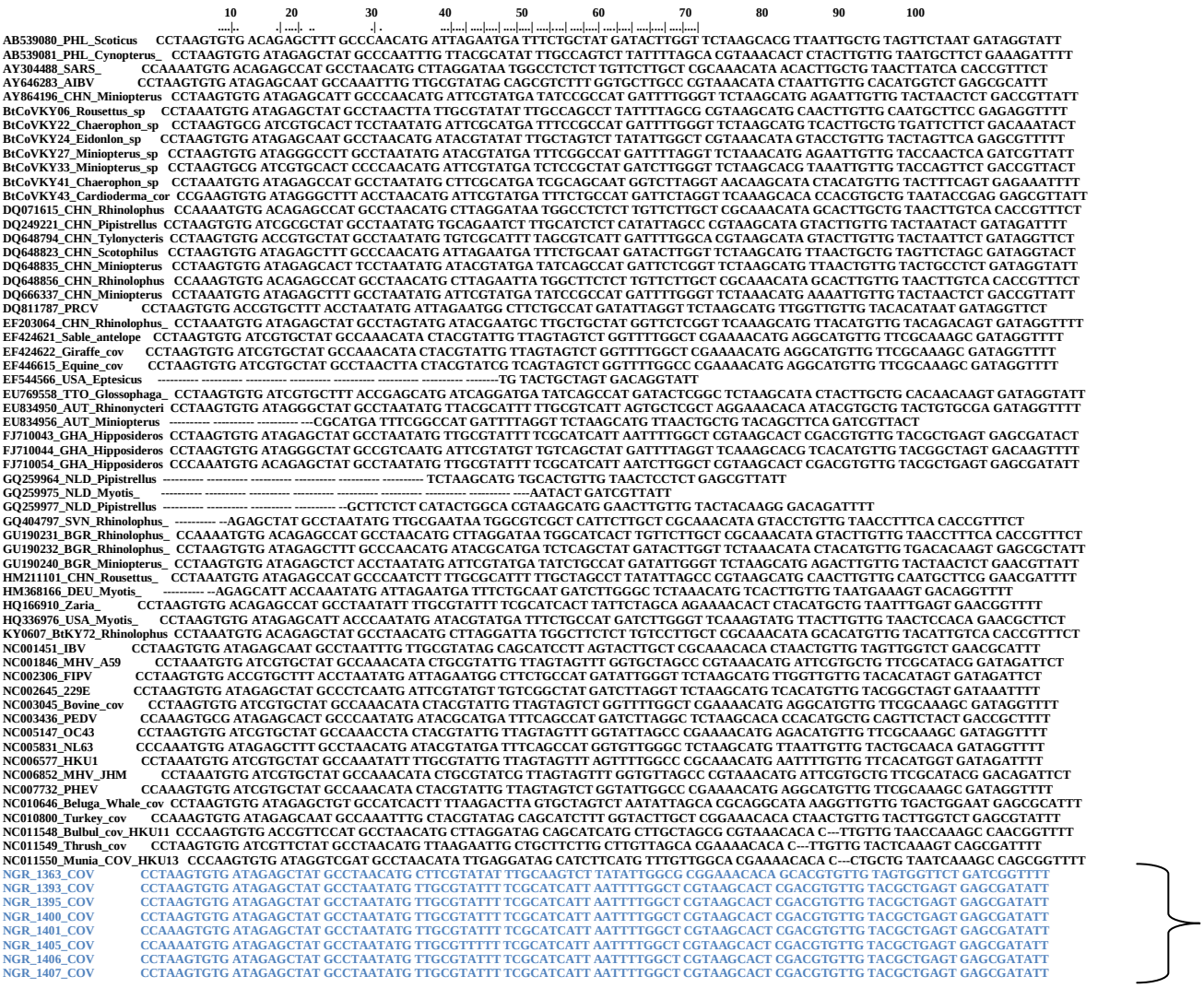


Figure 5.2: BLAST sequence of 121-bp fragment *Coronavirus*amplicon identified in Plateau State bats in this study (in blue font colour indicated by a bracket on the right side) and those obtained from NCBI for the Phylogenetic analysis.



Figure 5.3:A Phylogenetic tree generated of one*Paramyxovirus* from a bat inPlateau State in this study (in blue circle) and 49 selected human and animal paramyxoviruses from the NCBI database**.**

```
                    10        20        30        40        50        60        70        80        90        100
                    ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
NGR_1410_Paramyxovirus    GGACACAATC TCAAAGCAAA TGAAACAATA GTTAGCAGTC ATTTTTTTGT CTACTCCAAG AGAGTGTATT ATGATGGATT GGTACTCTCA    CAAGCTCTCA
Goose_paramyxovirus_SF02  GGCCATAACC TGAAGGATCG TGAAACCATT AGGTCAGACA CATTCTTCAT ATACAGCAAA CGAATATTCA AAGATGGAGC AATACTCAGT CAGGTCCTCA
Sendai                    GGACATGAGC TAAAATTGAA CGAGACCATC ATTAGTAGTA AAATGTTTGT CTATAGTAAA AGGATATATT ATGATGGAAA GATCCTACCG CAATGTCTGA
HPIV3                     GGTCATGAAC TTAAATTAAA TGAAACGATT ATAAGTAGCA AGATGTTCAT ATATAGCAAA AGAATCTATT ATGATGGGAG AATTCTTCCT CAAGCTCTGA
Hendra                    GGACTCAACT TAAAAGCAAC AGAGACCATT ATAAGTACAC ACTTATTTGT CTACTCTAAG AAAATACATT ATGATGGTGC AGTATTATCA CAAGCCCTCA
CDV                       GGACATCATT TGAAAGCAAA TGAAACAATA ATCTCTTCCC ACTTTTTTGT ATACTCAAAA GGAATCTATT ATGATGGAAT GTTAATTTCA CAATCCCTGA
BPIV3                     GGTCATGAGC TCAAACTAAA TGAAACTATA ATAAGTAGTA AAATGTTTAT ATATAGCAAA AGGATATACT ATGACGGAAG AATCCTTCCT CAGGCATTAA
Tupaia                    GGACACAACT TAAAAGCTAG CGAAACTATA ATTAGCTCTC ACTTTTTCGT CTATTCTAAA AGGATTTACT ACGATGGTGT TTGTATGACA CAAGGTCTGA
Mumps                     GGACACCACT TAAAAGAACA AGAAACAATC CTTAGTTCTG ATTTCTTCAT ATACAGTAAG AGGGTGTTTT ACAAAGGTCG AATTTTGACT CAAGCGTTAA
NDV                       GGCCATAATT TGAAGGATCG TGAAACCATC AGGTCAGACA CATTCTTCAT ATACAGCAAA CGAATCTTCA AAGATGGAGC AATCCTCAGT CAAGTCCTCA
Nipah                     GGCCACAATC TTAAAGCTAC AGAAACTATC ATCAGTACAC ATCTTTTTAT TTATTCGAAG AAAATTCATT ATGATGGTGC TGTGCTGTCT CAGGCACTCA
Avian_paramyxovirus_6     GGGCACAACT TAAAAGTCCA AGAAACAATT CATAGTGAGT CATTCTTCGT GTACTCGAAA CGGATCTTCT TTGAGGGGAA GATCCTCAGT CAACTACTAA
HPIV2                     GGTCATCAGC TAAAGGCTCA AGAAACTATA ATAAGTTCCA CGTTCTTCAT ATATAGTAAA CGGGTATTTT ATCAAGGACG TATACTAACA CAGGCACTCA
HPIV1                     GGACATGAAT TAAAATTAAA TGAGACCATT ATAAGTAGCA AAATGTTTGT ATACAGCAAA CGGATATATT ATGATGGGAA AATCCTCCCA CAGTGCCTCA
Tioman                    GGACATCATT TAAAAGAACA AGAAACTATT GTAAGTTCAG AATTCCTAAT CTATAGCAAG AGGATTATTT ACAACGGAAG AATACTAAAT CAATCCCTCA
Measles                   GGCCATCACC TCAAGGCAAA TGAGACAATT GTTTCATCAC ATTTTTTTGT CTATTCAAAA GGAATATATT ATGATGGGCT ACTTGTGTCC CAATCACTCA
Fer-de-lance              GGACATAACT TGAAACTGAA CGAGACTATC ATATCCAATC AGTTCTTTAT CTACTCGAAG CGAATATACT TTGATGGTTC TATCTTACCC CAAGGTCTTA
Dorphin_morbillivirus     GGGCATCATT TGAAAGCAAA TGAAACAATT ATATCCTCAC ATTTCTTTGT TTACTCAAAA GGTATATATT ATGATGGAAT GCTGATCTCT CAATCACTTA
Mossman                   GGGCATAACC TAAAGGCAAA TGAGACTCTA ATTTCAAGTC ATTTCTTTAT TTATTCAAAG CAGATTTACT ATGACGGAGT TGTTCTATCT CAAGCTCTCA
Rinderpest                GGCCATCACC TTAAGGCAAA CGAAACTATC GTGTCATCAC ACTTCTTTGT TTACTCCAAG GGGATATATT ACGACGGACT ACTAATCTCA CAGTCTCTCA
Peste-des-petits-ruminant GGTCATCACT TAAAGGCTAA TGAAACCATA GTATCTTCTC ACTTTTTTGT ATATTCCAAG GGGATTTATT ATGACGGCCT GCTAGTCTCA CAATCACTAA
J_virus                   GGTCACAATC TTAAGGCAAA TGAGACAATA GTCAGTTCAC ACTTTTTTAT ATACTCCAAG AGGATCTATT ATGATGGTCA AGTCTTGTCC CAAGCATTGA
Menangle                  GGTCATCATT TGAAAGAACA GGAGACTATC ATTAGTTCTG AGTTTATTGT GTACAGCAAA AGAATCATAT ACAATGGTAG AATCCTAAAT CAAGCCCTCA
Beilong_virus             GGGCACAATC TGAAAGCGAA TGAAACCATT GTGAGTTCGC ACTTTTTCAT TTATTCAAAG AGGATATATT ACGATGGACA AGTGTTGTCA CAAGCTCTAA
Atlantic_salmon           GGTCATGAAC TTAAACTTCA AGAGACAGTA ATCAGCAGTA AGTTCTTTGT ATATAGCAAA CGTATATACT ATGATGGACA AATTCTGCCT CAGTGTCTTA
Mapuera                   GGGCATCATC TAAAGGCACA AGAAACCATC CTCAGCTCTG ATTTCTTCAT ATATAGCAAA CGGGTATTCT TTAGAGGCCG TATCTTGACA CAGGCCTTAA
SV_41                     GGTCATCAGC TTAAAGCACA GGAAACAATT ATCAGTTCTC AATTCTTCAT CTACAGTAAA AGGGTTTTTT ATCAGGGTAG GATCCTAACT CAGGCTCTCA
SV_5                      GGTCACCATT TGAAAGAACA AGAGACTATC ATTAGTTCTC ACTTCTTTGT TTATAGCAAG AGAATATTCT ATCAGGGGAG GATTCTAACG CAAGCCTTAA
PDPRV                     GGTCATCACT TAAAGGCTAA TGAGACCATA GTATCTTCTC ACTTTTTTGT ATACTCTAAA GGGATTTATT ATGACGGCCT GTTAGTCTCA CAATCACTAA
Pigeon_paramyxovirus      GGCCACAACC TGAAAGATCG TGAAACCATC AGATCAGACA CATTCTTCAT ATACAGCAAG CGGATATTCA AAGACGGAGC AATACTCAGT CAGGTCCTCA
Porcine_rubulavirus       GGACACCACT TAAAGGCGCA AGAGACCATC ATTAGTTCGG ACTTCTTTGT TTATAGCAAG AGGGTCTTTT TTAGAGGAAG GATTCTCAAC CAAGCTTTGA
```

Figure 5.4: BLAST sequence of the *Paramyxovirus* obtained in a Plateau State bat in this study (in blue font colour in the first line) 'NGR_1410_Paramyxovirus' with 16 nucleotide sequences from the GenBank.

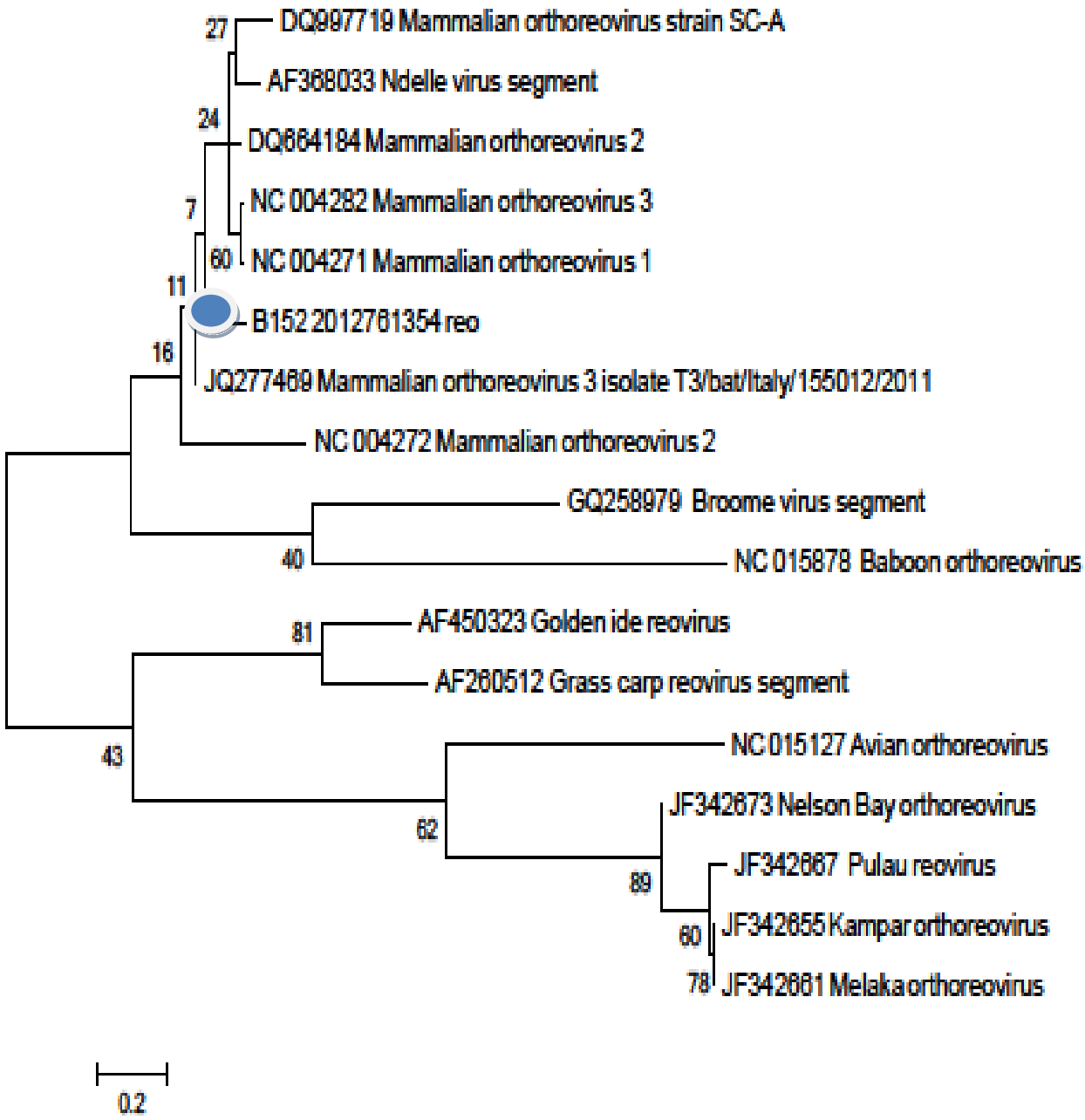


Figure 5.5: Phylogenetic tree of *Reovirus* identified in bat in this study (in blue circle) compared with 16 nucleotide sequences of human and animal reoviruses from the GenBank.



Figure 5.6: BLAST sequence of *Reovirus* identified in Plateau State bats in this study (in blue font colour shown also by bracket on the right side), 'NGR_1354_orthoreovirus' with 16 nucleotide sequences from the GenBank.

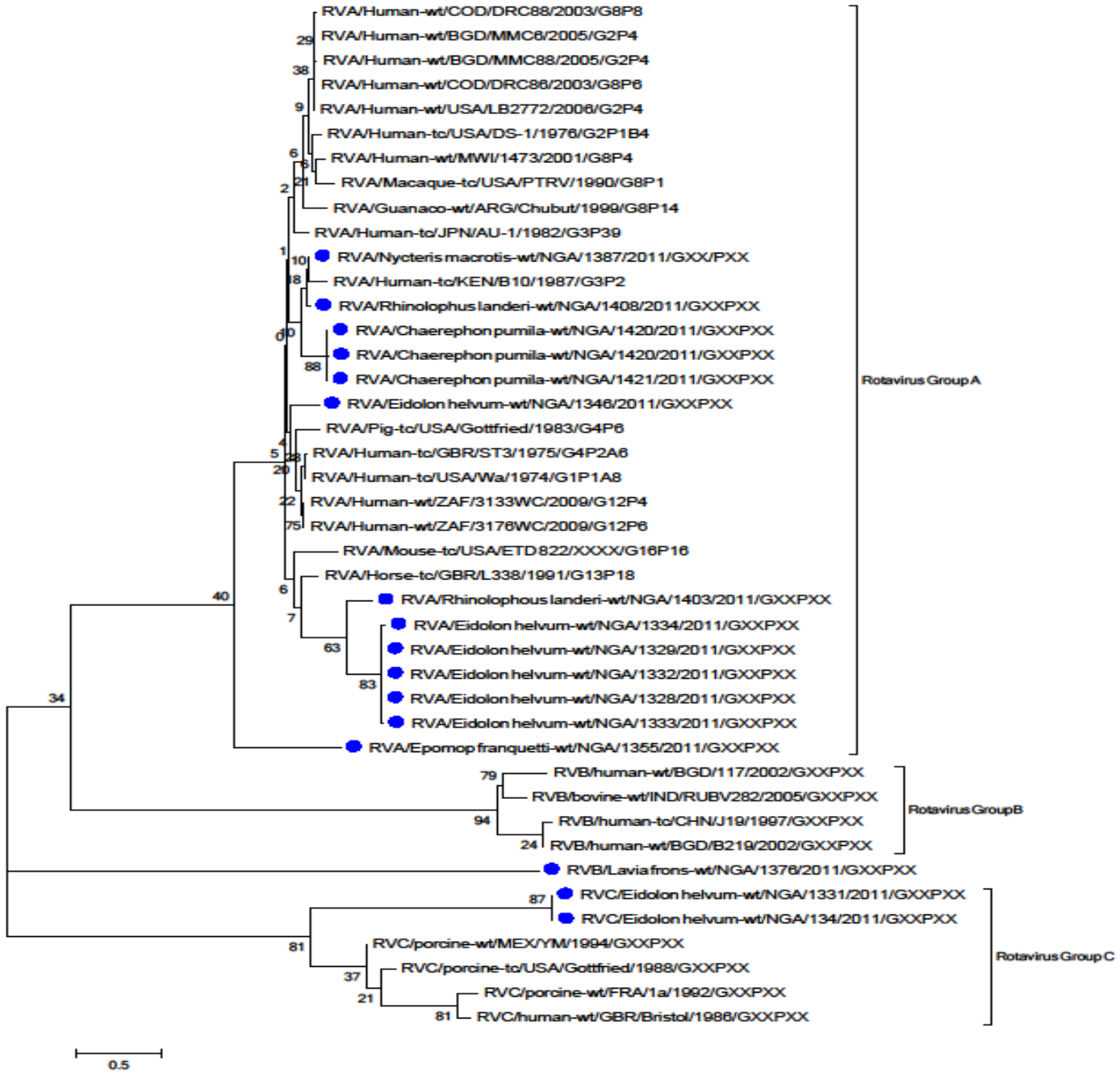


Figure 5.7: Phylogenetic tree of rotaviruses (16) identified in a Plateau State bat in this study (in blue circles) compared with 26 nucleotide sequences of human and animal rotaviruses from the GenBank.



Figure 5.8: BLAST sequence of Rotavirusidentified in Plateau State bats in this study (in blue ink and indicated also by bracket on the right side) and those obtained from NCBI for the Phylogenetic analysis.

5.4. Discussion

The use of PCR primers that collectively target highly conserved amino acid sequences within each family or genus, identified viruses belonging to the genera *Coronavirus*, *Paramyxovirus*, *Reovirus* and *Rotavirus* in bats tested in this study. This implies that bats in Nigeria, not just in Plateau State, may be harbouring these viruses considering the migratory nature of bats and their ability to fly long distances (Calisher *et al*., 2006). No *Arenavirus*, influenza virus, and *Rhabdovirus* RNA was identified. This finding may not be sufficient to conclude on the absence of these three viral genera, because the number of samples (95) bat species (8) tested, the limited study location and season may have affected the outcome.

Nigerian bat CoVs identified clustered among the beta-CoV all belonging to the group 2b SARS-CoV-like viruses. Furthermore, in addition to earlier finding of Zaria bat CoVs in a *Hipposideros commersoni* bat (Quan *et al*., 2010), this study reports the detection of eight CoV all in the same group with the Kenya bat CoV (Tong *et al*., 2009), Ghana bat CoV (Pfefferle *et al.*, 2009), Zaria bat CoV as well as SARS CoV. This findings are not surprising considering the long distances that bats travel and possibility of bats translocation due to global travel via planes and ships,thus resulting viral introductions and spread to naïve population. In the same bat genera (*Rhinolophus*) earlier implicated as the major reservoir of SARS CoV in the 2003 outbreak (Lau *et al*., 2005), was found six genetically similar but different CoVs in individuals bats inhabiting the same roost site (a well at Pandam game reserve). A similar CoV with 98% homology to the six CoVs in the *R. landeri*was also found in a *Chaerophon pumila*,a different insectivorous bat from a different location (Dawaki). Tong *et al*., (2009) also detected genetically similar CoVs in several different bat species. For example, CoVs from the BtHKU7-like cluster detected in both *Chaerophon* spp. and *Otomops martinsseni* bats; CoVs from the BtCoVA970-like cluster were

detected in *Cardioderma cor* and *Rousettus aegyptiacus* bats; CoVs from the BtKY18-like cluster were detected in *Chaerophon* spp., *Eidolon helvum*, and *R. aegyptiacus* bats; and CoVs from the BtHKU9-like cluster were detected in *Hipposidereos commersoni* and *R. aegyptiacus* bats. In this study a CoV was detected from a fruit bat *Epomop gambianus,* whichclustered withBtCoVKY24 (a Kenya bat CoV) from an *Eidolon spp*.

The findings in the present study however are not in agreement with the claim by Quan *et al*., (2010) of the absence of *Rhinolophus* bats in Nigeria. They attributed this to their inability to obtain more CoVs from bats collected from Idanre and Zaria in Nigeria. Five species of *Rhinolophus* bats have been documented to be present in Nigeria (Happold, 1987); *R. alcyone, R. alticolus, R. fumigates, R. landeri* and *R. maclaudi*. *Rhinolophus* have been the most commonly implicated reservoirs of SARS CoV and SARS CoV-like viruses (Calisher *et al.*, 2006), as also found in this study. From this and many previous studies, it is also obvious that CoVs may be harboured by other species of bats. Fruit bats have reportedly been found harboring CoV, though not as frequent as from insectivorous bats (Calisher *et al*., 2006).

Drexler *et al*. (2012), have gathered evidence of bats being in close evolutionary and ecological relationship with several genera of mammalian paramyxoviruses (PVs). Their major findings included evidence of an origin of Hendra- and Nipah virus in Africa, identification of a bat virus conspecific with the human mumps virus, detection of close relatives of respiratory syncytial virus, mouse pneumonia and canine distemper virus in bats. In this study, only one paramyxovirus was identified which is genetically closely related to a Kenyan bat paramyxovirus (BtKY248) and the virus was from the *Chaerophon* spp. of bat. These paramyxoviruses fall within the unclassified cluster. The limited sample size tested could explain why only one

Paramyxovirus was obtained in this study or it could be that the pan viral family PCRs failed to detect the virus because it was below the limit of detection by the assay. Although it has been documented that, for the isolation of henipaviruses or other paramyxoviruses, it is best to take urine samples (Chua *et al.*, 2002) while faecal swabs are a better specimen when coronaviruses are the target (Lau *et al.*, 2005; Li *et al.*, 2005). This may explain the high number of CoVs obtained as compared to paramyxoviruses which are found to be abundant in bats of different species (Drexler *et al*., 2012).

Only one *Reovirus*clustering to a mammalian orthoreovirus was identified from an *E. franqueti* (a pteropine bat). The finding of this virus is very significant in light of their segmented nature which facilitates virus reassortment, thereby contributing to virus diversity, a determinant of evolution of reoviruses (Wong *et al.*, 2012). Among the new bat zoonotic viruses discovered in recent years, several reoviruses were found to be able to cause acute respiratory infections in humans, and these include Melaka and Kampar viruses discovered in Malaysia, all of them belonging to the genus *Orthoreovirus* (Chua *et al*., 2007). A similar virus was discovered in Hong Kong from a patient who visited Bali a few days before the onset of the severe respiratory illness (Cheng *et al*., 2009). All of these newly isolated orthoreoviruses are genetically closely related to the Nelson Bay virus and Pulau virus, both of bat origin (Gard and Marshall, 1973; Pritchard *et al*., 2006). Recent studies demonstrated that there are a large number of genetically diverse reoviruses circulating among different bats in geographically separated locations. Many, if not all of them, have the potential to infect human and cause respiratory disease outbreaks (Chua *et al*., 2007; Chua *et al*., 2008; Cheng *et al*., 2009).

Rotavirus (RV) was first reported in bats (*E. helvum*) from Kenya(Esona *et al.,* 2010), with evidence of reassortment as have also been documented in other animal species in the recent past (Gentsch *et al.,* 2005; Fiztergald *et al.,* 1995; Gouvea *et al.,* 1999). In this study 16 sequences of rotavirus belonging to *Rotavirus* A, B, and C were identified, some of which are unique (novel). The majority of the RVs in this study came from the common fruit bat *Eidolon helvum* and clustered in the RV group A and C. The *E.helvum* bats were captured from the Jos zoo where many animals are kept and many people including tourists often visit. Indiscriminate defaecation by humans and animals, poor hygiene and sanitary practices may result in faecal runoffs into water bodies. Bats may drink from these waterbodies contaminated with RVs of both human and animal origin resulting in possible reassortment to new strains (Gentsch *et al*., 2005). Similar report of RV in *E. helvum* from Kenya was made (Esona *et al*., 2010). Human rotaviruses have been detected in surface water, reservoirs, and sewage; and viable viruses have been isolated from drinking water (Ferreira *et al.*, 2009; Rutjes *et al.*, 2009).

In addition, three *Chaerophon* bats harboured genetically distinct but similar RV all within group A. These bats were found in roofs and walls of human habitations. Human and bat activities provide ample opportunities for bats and humans to contact each other resultingin the sharing of their respective viruses. Fruit bats and some insect bats often live near human habitats (Calisher *et al.,* 2006) as a result of deforestation and expanded settlement of human populations.

The identification of *Coronavirus*, *Paramyxovirinae*, *Reovirus* and rotaviruses from bats in Nigeria further show the role of bats as reservoir hosts for these viruses. Active animal disease surveillance is very needful to aid in the prediction and subsequent prevention of disease outbreaks. The findings in the present study, to the best of our knowledge, may be the first report

on the detection of *Rotavirus* in bats from West Africa and the second successful bat RV detection worldwide.

CHAPTER SIX

GENERAL DISCUSSION AND CONCLUSION

6.1General Discussion

Dog "hunters" travel through 9 Northern States in Nigeria as well as Niger and Chad republics to source for dogs (strays, wild and owned) to be sold at the dog markets in Plateau State (Sabo *et al.,* 2008). Rabies antigen in the brains of slaughtered dogs presents risks for those involved in the dog trade: hunters, handlers, butchers, as well as consumers**.**Phylogenetic analysis shows that DRV-NG11 from a slaughtered dog in this study belongs to the Africa 2 lineage.

RFFIT was used to measure the neutralizing activity of some *Lyssavirus* specific antibodies present in the serum. This study alongside others (Dzikwi *et al.,* 2010) has established the circulation of some lyssaviruses other than rabies in Nigeria. With the exception of Mokola virus (MOKV), all lyssaviruses have been isolated from bats (Badrane and Tordo, 2001; Nel and Rupprecht, 2007). Thus there is need for more cross-reactive vaccine formulations that is protective against all circulating lyssaviruses due to the close contact between humans and bats in Nigeria generally. These data (Table 4.2), suggest that bats in Nigeria may be commonly exposed to rabies-related viruses and develop a humoral immune response suggesting some degree of viral replication, but many infections fail to progress to clinical disease or else may go unnoticed.

Many studies have documented the association of bats with emerging infectious agents globally of which RNA viruses have posed the greatest danger due to their potential for reassortment. This study assayed for some RNA viruses in bat population in Plateau State and identified some bat SARS CoV-like viruses and others (*Paramyxovirus*, *Reovirus* and some rotaviruses).

Corornaviruses are known to cause respiratory and enteric diseases in animals and humans, some of which are mild while others are fatal (de Groot *et al*., 2011). The two major CoVs that have been implicated with epidemics that have claimed lives are the SARS and MERS-CoVs which all belong to the β-CoV group same as the CoVs from this study (Figure 5.1). Although MERS-COV differs from the SARS-CoV and the CoVs from this study (of β-CoV sub-group 2a) because it belongs to the sub-group 2c. Outbreaks such as the SARS CoV and the ongoing Middle East Respiratory Syndrome CoV (MERS CoV) or novel CoV (nCoV) (Zaki *et al*., 2012; Corman *et al*., 2012), calls for establishing efficient surveillance systems for continuous and sustained monitoring in order to prevent similar future outbreaks in the nation.

The paramyxovirus detected in this study was closely related to the J virus from mice. The J virus is unclassified and has the largest paramyxovirus genome characterized to date. The J virus genome has a unique structure containing eight genes, a significant divergence to date from the common six-gene genome structure of *Paramyxovirinae* (Jack *et al*., 2005). Nevertheless, the disease-causing potential of J virus remains somewhat unclear (Jun *et al*., 1977).

The family *Reoviridae* includes the *Reovirus* and *Rotavirus* found in bats in this study. The segmented nature of these viruses results in evolution of new variants as consequence of reassortment. This may possibly explain the detection of 16 RV sequences in this study, which was mainly from *E.helvum*, the common straw-coloured fruit bat. Other recent studies have also identified new *Rotavirus* genotypes in many captive and free ranging wildlife (Matthijnssens *et al.*, 2009). The close association between bats, other animals (domestic and feral) and humans warrants close monitoring to avoid future epidemics.

6.2 Conclusion

In conclusion this studies;

- Reports the identification of RABV from Plateau State Nigeria as belonging to Genotype 1, Africa 2 lineage and Clade 1.
- The first complete genome sequence of RABV isolate from a dog in Nigeria.
- Provides serologic evidence of viral neutralizing antibodies to LBV (29.0%) and MOKV (7.9%) in Plateau State bats and the first evidence of SHBV (23.7%) in Nigerian bats.
- Documents the detection of Coronavirus (8.42%), Paramyxovirus (1.05%), Reovirus (1.05%) and Rotavirus (16.84%) in bats in Plateau State, Nigeria.

In summary, the identification of classical rabies virus in trade dogs from Plateau State, and RNA viruses in bats provide insight into the occurrence of these viruses. The findings reinforce the potential role of bats as reservoirs of viral pathogens that may threaten human health. Global surveillance for viruses in bats and other wildlife is needed to corroborate, elaborate, and more fully understand the origin, adaptation, and evolution of bat virus strains.

6.3Recommendations

Based on the findings from this study the following are recommended:

- Dog trade and movement both into and within the country should be strictly monitoredtoensure no rabied dog is allowed into the country and control of strays to prevent rabies virus introduction and spread.
- Public education of high risk individuals on the likelihood of contacting rabies from infected trade dogs and how to prevent it.

- People in locations where such contacts are possible should be informed on the risk associated with keeping close contact with bats and consumption of under cooked bats and other wild life.
- There is need for continued surveillance nationwide to have a better understanding of the epidemiology of *Lyssavirus, Rotavirus, Coronavirus, Paramyxovirus, Reovirus* and other viral agents of economic and public health importance in dogs, bats and other wild animals so as to aid in their prevention, control and eradication.
- Equipping of existing laboratories in Research institutes and Universities in the nation for modern, speedy and quality diagnosis.
- There is need to sequence more strains or isolates of rabies from clinical cases to further understand the epidemiology of the virus. Further genomic characterization of the viral isolates obtained is needful to give more information about their origin and evolution.

6.4Limitations of the Study

- Due to the then on-going political and religious unrest it was not possible to get access to some certain communities in Plateau state to adequately sample available bat species.
- Sample storage problems resulted in the loss of some dog brain samples thus reducing the samples available for testing as well as the quality of some samples.
- Inability to amplify rabies viral RNA from most DFAT positive dog brain tissues (only 4 out of 92).
- Non-availability of facilities for proper capture of live bats resulting in fewer sera samples.
- Limited time resultingin search for few RNA viruses in 95 out of the 356 bat faecal swabs.

REFERENCES

Aghomo, H. O. and Rupprecht, C. E. (1990). Antigenic characterization of virus isolates from vaccinated dogs dying of rabies. *Tropical Animal Health Production,* 22:275-280.

Aghomo, H. O., Ako-nai, A. K., Oduye, O. O., Tomorin, O. and Rupprecht, C. E. (1990a). Detection of rabies virus antibodies in fruit bats (Eidolon helvum) from Nigeria. *Journal of Wildlife Diseases*, 26: 258–261.

Aghomo, H. O., Ako-nai, A. K., Oduye, O. O. and Tomori, O. (1990b). Further studies on rabies virus isolated from healthy dogs in Nigeria. *Veterinary Microbiology*, 212: 17-22.

Ajayi, B. B., Rabo, J. S., and Baba, S. S. (2006). Rabies in apparently healthy dogs: Histological and Immunohistochemcal studies. *The Nigerian Postgraduate Medical Journal, 12:128-134.*

Akombo, P. M. (2009). Dog Ecology and epidemiological studies of canine Rabies in Benue State, Nigeria. MSc. thesis submitted to the Department of Veterinary Public Health and Preventive Medicine, Faculty of Veterinary Medicine, Ahmadu Bello University, Zaria

Amengual, B., Whitby, J. E., King, A., Serra Cobo, J. and Bourhy, H. (1997). Evolution of European bat lyssaviruses. *Journal of General Virology,* 78:2319–28.

Annan, A., Baldwin, H. J., Corman, V. M., Klose, S. M., Owusu, M. and Nkrumah, E. E. (2013). Human betacoronavirus 2c EMC/2012–related viruses in bats, Ghana and Europe. *Emerging Infectious Diseases*, 14(9) Retrieved 6/5/2012, from http://wwwnc.cdc.gov/eid/article/19/3/12-1503_article.htm.

Anonymous, (1946). Rabies in Nigeria. Annual Report of Veterinary Division, Northern Nigeria, pp. 10-12.

Arguin, P. M., Murray-Lillibridge, K., Miranda, M. E. G., Smith, J. S., Calaor, A. B. and Rupprecht, C.E. (2002). Serologic evidence of lyssavirus infections among bats, the Philippines. *Emerging Infectious Diseases,* 8:258–62.

Arko, R. J., Schneider, L. G. and Baer, G. M. (1973). Non-fatal canine rabies. *American Journal of Veterinary Research,* 34:937-938.

Badrane, H. and Tordo, N. (2001). Host switching in *Lyssavirus* history from the *Chiroptera* to the *Carnivora* orders. *Journal of Virology,* 75:8096–8104.

Baer, G. M. and Smith, J. S. (1991). Rabies in non-hematophagous bats. In: *The Natural History of Rabies* (G. M. Baer, ed.) CRC Press, Boca Raton, FL, USA. pp. 341–366.

Banyard, A., Hayman, D.T.S., Johnson, N., McElhinney, L. and Fooks, A.R. (2011). Bats and Lyssaviruses. *Advance Virus Research,* 79:239–289.

Baric, R. S. (2008). SARS-CoV: lessons for global health. *Virus Research,* 133: 1-3.

Barrat, J. (1996). Simple technique for the collection and shipment of brain specimens for rabies diagnosis. In: Meslin, F-X, Kaplan., MM, Koprowski H (editors). *Laboratory Techniques in Rabies*. *4th*Edition. Geneva: World Health Organization. pp. 1-16.

Bell, J. F. (1975). Latency and abortive rabies. In: Baer,G.M (editor). *The Natural History of Rabies.* Academic Press, New York. pp. *331-354.*

Bell, J. F. and Moore, G. J. (1971). Susceptibility of carnivora to rabies virus administered orally. *American Journal of Epidemiology*,93:176–182.

Bello, M., Lukshi, B. M., and Usman, B. (2007). A fifteen-year retrospective study of prevalence of rabies in Bauchi State. *The Nigerian Veterinary journal,* 28(2): 18-23.

Belotto, A., Leanes, L. F., Schneider, M. C., Tamayo, H. and Correa, E. (2005). Overview of rabies in the Americas. *Virus Research,* 111:5–12.

Bisseru, B. (1972). Human rabies. In: B. Bisseru (Ed.), *Rabies*. London: William Heinemann Medical Books Ltd. Black, D., & Wiktor, T. I. pp. 385-453. Rabies. William Heinemann Medical Books, Ltd., London, pp. 66-70.

Blanton, J. D., Palmer, D. and Rupprecht, C. E. (2010). Rabies surveillance in the United States during 2009. *Journal of American Veterinary Medicine Association,* 237:646–657.

Boulger, L. R. and Porterfield, J. S. (1958). Isolation of a virus from Nigerian fruit bats. *Transactions of the Royal Society of Tropical Medicine and Hygiene,* 52:421–424.

Boulger, L. R. and Hardy, J. (1960). Rabies in Nigeria. *West African Medical Journal,* 9:223-234.

Bourhy, H., Kissi, B. and Tordo, N. (1993). Molecular diversity of the Lyssavirus genus. *Virology,* 194:70–81.

Brass, D. A. (1994). Epizootiology of vampire-bat rabies. In: *Rabies in bats: natural history and public health implications*. Livia Press, Ridgefield, Connecticut), pp. 151–162.

Brass, D. A. (2009). Rabies vaccine strategies: Concepts of rabies prophylaxis for the caving community. *In: Proceedings for Rabies Symposium*, 109:6–16.

Calisher, C. H., Childs, J. E., Field, H. E., Holmes, K. V. and Schountz, T. (2006). Bats: Important reservoir hosts of emerging viruses. *Clinical Microbiology Review*, 19: 531 – 545.

Carini, A. (1911). About one large epizootie of rabies. *Annals of Pasteur Institute (Paris)*, 25:843–846.

Carstens, E. B. (2009). Ratification vote on taxonomic proposals to the International committee on Taxonomy of Viruses. *Archives of Virology,* 155:133-146.

CDC (2006). Standard Operating procedure for the rapid fluorescent focus inhibition test (RFFIT) for determining rabies virus neutralizing antibody, pp. 1-192.

Chadha, M. S., Comer, J. A., Lowe,L., Rota, P. A., Rollin, P. E., Bellini, W. J., Ksiazek, T. G. and Mishra, A. C. (2006). Nipah virus-associated encephalitis outbreak, Siliguri, India. *Emerging Infectious Diseases,*12(2): 235-240.

Cheng, P., and Lau, C. S., Lai, A, Ho, E. and Leung, P. (2009). A novel *Reovirus* isolated from a patient with acute respiratory disease. *Journal of Clinical Virology,* 45: 79–80.

Childs, J., Real, A. L., Jackson, A. C. and Wunner, W. H. (2007). Epidemiology. In:*Rabies (eds* Jackson,A C, Wunner,W H*),*New York, NY: Elsevier. pp.123–199.

Chua, K. B., Bellini, W. J., Rota, P. A., Harcourt, B. H., Tamin, A., Lam, S. K., Ksiazek, P. E. Rollin, S. R. Zaki, W.-J. Shieh, C. S. Goldsmith, D. J. Gubler, J. T. Roehrig, B. Eaton, A. R. Gould, J. Olson, H. Field, P. Daniels, A. E. Ling, C. J. Peters, L. J. Anderson, B. and Mahy, W. J. **(**2000). Nipah virus: a recently emergent deadly *Paramyxovirus*. *Science*, 288:1432-1435.

Chua, K. B., Wang, L. F., Lam, S. K., Crameri, G., Yu, M., Wise, T., Boyle, D., Hyatt, A. D. and B. T. Eaton. (2001). Tioman virus, a novel *Paramyxovirus* isolated from fruit bats in Malaysia. *Virology,* 283:215-229.

Chua, K. B., Wang, L. F., Lam, S. K. and Eaton, B. T. (2002). Full length genome sequence of Tioman virus, a novel *Paramyxovirus* in the genus, *Rubulavirus* isolated from fruit bats in Malaysia. *Archives of Virology,* 147:1323-1348.

Chua, K. B. (2003). A novel approach for collecting samples from fruit bats for isolation of infectious agents. *Microbes and Infection,* 5: 487–490.

Chua, K. B., Crameria, G., Hyatt, A., Yu, M., Tompang, M., Rosli, K., McEachern, J., Crameri, S., Kumarasamy, V., Eaton, B.T. and Wang, L.F. (2007). A previously unknown *Reovirus* of bat origin is associated with an acute respiratory disease in humans. *Proceedings of National Academy of Science USA*, 104: 11424 – 11429.

Chua, K. B., Voon, K., Crameria, G., Tan, H. S., Rosli, J., McEachern, J. A., Suluraju, S., Yu, M., and Wang, F. L. (2008). Identification and characterization of a new *Orthoreovirus* from patients with acute respiratory infections. *PLOS One,* 3 (11): e3803.

Constantine, D. G. (1967). Bat rabies in the southwestern United States. Public Health service Publication, Washington DC.,82:867-888.

Constantine, D. G. (1966). Transmission experiments with bat rabies isolates: responses of certain *Carnivora* to rabies virus isolated from animals infected by nonbite route. *American Journal of Medical Research,* 27(116):13–15.

Corman, V. M., Eckerle, I. and Bleicker, T. (2012). Detection of a novel human *Coronavirus* by real-time reverse-transcription polymerase chain reaction. *European Surveillance,* 17:20285.

Correa-Giron, E. P., Allen, R. and Sulkin, S. E. (1970). The infectivity and pathogenesis of rabies virus administered orally. *American Journal of Epidemiology,*91:203–215.

Daoust, P. Y., Wandeler, A. I. and Casey, G. A. (1996). Cluster of rabies cases of probable bat origin among red foxes in Prince Edward Island, Canada. *Journal Wildlife Diseases,* 32:403–406.

Davis, P. L., Holmes, E. C., Larrous, F., Van der Poel, W. H. M., Tjornehoj, K., Alonso. W. J. and Bourhy, H. (2005). Phylography, population dynamics and Molecular evolution of European bat lyssaviruses. *Journal of Virology,* 799(16):10487-10497.

Davis, P.L. Rambaut, A. Bourhy, H., Holmes, E.C., (2007). The evolutionary dynamics of canid and mongoose rabies virus in Southern Africa. *Archives of Virology,* 152:1251–1258.

de Groot, R. J., Baker, S. C., Baric, R., Enjuanes, L., Gorbalenya, A. E., Holmes, K. V., Perlman, S., Poon, L., Rottier, P .J. M., Talbot, P. J., Woo, P. C. Y., Ziebuhr, J.(2012). *Coronaviridae*. In: King, A. M. Q., Adams, M. J., Carstens, E. B., Lefkowitz, E. J. (Eds.), *Virus Taxonomy*: Ninth Report of the International Committee on Taxonomy of Viruses. Elsevier Academic Press, San Diego, pp. 774–796.

de Groot, R. J., Baker, S. C., Baric, R., Enjuanes, L., Gorbalenya, A. E., Holmes, K. V., Perlman, S., Poon, L., Rottier, P. J. M., Talbot, P. J., Woo, P. C. Y. and Ziebuhr, J. (2013). *Coronaviridae.* In: King, A.M.Q., Adams, M. J., Carstens, E. B. and Lefkowitz, E.J. (Eds.), Middle East Respiratory Syndrome *Coronavirus* (MERS-CoV): Announcement of the *Coronavirus* Study Group. *Journal of Virology*; 87(14): 7790–7792.

Dean, D. J., Abelseth, M. K. and Atanasiu, P. (1996). The fluorescent antibody test. In F.-X. Meslin, M. M. Kaplan, and H. Koprowski (ed.), *Laboratory techniques in rabies,* 4th ed. WHO, Geneva, Switzerland, pp. 88–93.

Dominguez, S. R., O'Shea, T. J., Oko, L. M., and Holmes, K.V. (2007). Detection of group 1 coronaviruses in bats in North America. *Emerging Infectious Diseases,* 13: 1295–1300.

Drexler, J. F. M., Pfefferle, S., Yordanov, S., Zhelyazkov, L., Hermanns, U., Vallo, P., Lukashev, A., Muller, M. A., Deng, H., Herrler, G. and Drosten, C. (2009). Henipavirus RNA in African bats. *PLoS ONE* ,4, e6367.

Drexler, J. F., Corman, V. M., Muller, M. A., Maganga, G. D., Vallo, P. (2012). Bats host major mammalian paramyxoviruses. *Nature Communication,* 3:796.

Drexler, J. F., Gloza-Rausch, F., Glende, J., Corman, V. M., Muth, D., Goettsche, M., Seebens, A., Niedrig, M., Pfefferle, S., Yordanov, S., Zhelyazkov, L., Hermanns, U., Vallo, P., Lukashev, A., Muller, M. A., Deng, H., Herrler, G. and Drosten, C. (2010). Genomic characterization of severe acute respiratory syndrome-related coronavirus in European bats and classification of coronaviruses based on partial RNA-dependent RNA polymerase gene sequences. *Journal of Virology*, 84:11336-11349.

Du, L., Lu, Z., Fan, L., Meng, K., Jiang, Y., Zhu, Y., Wang, S., Gu, W., Zou, X. and Tu C. (2010). Xi river virus, a new bat *Reovirus* isolated in Southern China. *Archives of Virology*, 155:1295-99.

Dzikwi, A. A., Kuzmin, I. I., Umoh, J. U., Kwaga, J. K., Ahmad, A. A., and Rupprecht, C. E. (2010). Evidence of Lagos bat virus circulation among Nigerian fruit bats. *Journal of Wildlife Diseases,* 46:267–271.

Dzikwi, A. A. (2008). Role of Dogs and Bats in the epidemiology of Lyssavirus infection in Northern Nigeria PhD. thesis submitted to the Department of Veterinary Public Health and Preventive Medicine, Faculty of Veterinary Medicine, Ahmadu Bello University, Zaria.

Eaton, B. T. and Broder, C. C. (2006). Hendra and Nipah viruses: different and dangerous. *Nature Review of Microbiology,* 4(1): 23-35.

Elegbe, S. O. and Banerjee, A. K. (1970). A case Report of Rabies in Bovine in the North Western State of Nigeria. *Bulletin of Epizootic Diseases Africa*, 18: 57-62.

Ellison, L. E., O'Shea, T. J., Wimsatt, J., Pearce, R. D., Neubaum, D. J., Neubaum, M. A. and Bowen, R. A. (2006). Sampling blood from big brown bats (*Eptesicus fuscus*) in the field with and without anesthesia: Impacts on survival. *Journal of Wildlife,* 42 (4): 849–852.

Esona, M. D., Mijatovic-Rustempasic, S., Conrardy, C., Tong, S., Kuzmin, I. V. and Agwanda B. (2010). Reassortant group A rotavirus from straw-colored fruit bat (Eidolon helvum). *Emerging Infectious Diseases,* 16:1844-52.

Estes, M. K., and Kapikan, A. (2007). Rotaviruses In: Knipe, D. M., Howley, P. M, Griffin, D. E, Lamb, R. A., Martin, M, A. and Roizman B, editors. *Fields virology,* 5th ed. Philadelphia: Kluwer/Lippincott, Williams and Wilkins, pp. 1917–1974.

Ezeokoli, C. D. and Umoh, J. U. (1987). Epidemiology of Rabies in Northern Nigeria. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, 81:268-272.

Fagbami, A. H., Anosa, V. O. and Ezebuiro, E. O. (1981). Hospital records of human rabies and antirabies prophylaxis in Nigeria 1969-78. *Transactions of the Royal Society of Tropical Medicine Hygiene, 95:872-876.*

Familusi, J. B., Osunkoya, B. O., Moore, D. L., Kemp, G. E. and Fabiyi, A. (1972). A fatal human infection with Mokola virus. *American Journal of Tropical Medicine and Hygiene,* 21:959–963.

Ferreira, F. F., Guimaraes, F. R., Fumian, T. M., Victoria, M., Vieira, C. B. and Luz, S. (2009). Environmental dissemination of group A *Rotavirus*: P-type, G-type and subgroup characterization. *Water ScienceTechnology,* 60:633–642.

Field, H. E., Breed, A. C., Shield, J., Hedlefs, R. M., Pittard, K., Pott, B. (2007a). Epidemiological perspectives on Hendra virus infection in horses and flying foxes. *Australian Veterinary Journal*, 85:268–269.

Field, H. E., Mackenzie, J. S. and Daszak, P. (2007b). Henipaviruses: Emerging paramyxoviruses associated with fruit bats. *Curriculum of Tropical Microbiology and Immunology*, 315:133–159.

Field, H. E. (2009). Bats and emerging zoonoses: Henipaviruses and SARS. *Zoonoses Public Health*, 56:278–284.

Fitzgerald, T. A., Munoz, M., Wood, A. R. and Snodgrass, D. R. (1995). Serological and genomic characterisation of group A rotaviruses from lambs. *Archives of Virology*, 140:1541–8.

Foggin, C. M. (1983). Atypical rabies virus in cats and a dog in Zimbabwe. *Veterinary Record*, 110:338.

Freuling, C. M., Beer, M., Conraths, F. J., Finke, S., Hoffmann, B. and Keller, B. (2011). Novel lyssavirus in Natterer's bat, Germany. *Emerging Infectious Diseases*, 17:1519–1522.

Garba, S. I., Oboegbulem, A. T., Elsa, A. U., Junaidu, A. W., Magaji, A. A., Umoh, J. U., Yahaya, K., Danbirni, S., Habu, A. K., and Masdooq, A. A. (2008). A comparative rabies laboratory diagnosis: peculiar features of samples from apparently healthy dogs in Nigeria. *Sokoto Journal of Veterinary Sciences,* 7: 30-33.

Gard, G. and Compans, R. W. (1970). Structure and cytopathic effects of Nelson Bay virus. *Journal of Virology,* 6: 100–106.

Gard, G. P. and Marshall, I. D. (1973). Nelson Bay virus. A novel *Reovirus*. *Achives of Virology*, 43: 34–42.

Gentsch, J. R., Laird, A. R, Bielfelt, B., Griffin, D. D., Banyai, K. and Ramachandran, M. (2005). Serotype diversity and reassortment between human and animal rotavirus strains: implications for rotavirus vaccine programs. *Journal of Infectious Diseases*, 192:S146–59.

Giannini, N. P. and Simmons, N. B. (2003). A phylogeny of megachiropteran bats (Mammalia: Chiroptera: *Pteropodidae*) based on direct optimization analysis of one nuclear and four mitochondrial genes. *Cladistics*, 19:496–511.

Gouvea, V., Lima, R. C., Linhares R. E., Clark H. F., Nosawa C. M. and Santos, N. (1999). Identification of two lineages (WA-like and F45-like) within the major rotavirus genotype P [8]. *Virus Research*, 59: 141 – 7.

Green, S. A. (2012). How to make an agarose gel for electrophoresis Retrieved 7/3/2012, from http://www.mcdb.lsa.umich.edu/labs/maddock/protocols/DNA/agarosegels.html.

Halpin, K., Young, P. L., Field, H. E. and Mackenzie, J. S. (2000). Isolation of Hendra virus from pteropid bats: a natural reservoir of Hendra virus. *Journal of General Virology*, 81: 1927–1932.

Halpin, K., Young, P. L., Field, H. E. and Mackenzie, J. S. (1999). Newly discovered viruses of flying foxes. *Veterinary Microbiology*, 68: 83–87.

Hanlon, C. A., Kuzmin, I. I., Blanton, J. D., Weldon, W. C., Manangan, J. S. and Rupprecht C. E. (2005). Efficacy of rabies biologics against new lyssaviruses from Eurasia. *VirusResearch*, 111: 44–45.

Hass, C. C. and Dragoo, J. W. (2006). Rabies in hooded and striped skunks in Arizona. *Journal of Wildlife Dis*eases, 42:825– 829.

Happold, D. C. D. (1987). Bats. In: *The mammals of Nigeria.* Clarendon, Oxford University Press, Oxford. ISBN 0-19-85756 5-3. pp 34-82.

Hayman, D. T., Fooks, A. R., Horton, D., Suu-Ire, R., Breed, A. C., Cunningham, A. A. and Wood, J. L. (2008). Antibodies against Lagos bat virus in megachiroptera from West Africa. *Emerging Infectious Diseases*, 14:926–928.

Horwood, P. F., Gravel, J. L. and Bresee, J. (2008). Identification of two distinct bovine parainfluenza virus type 3 genotypes. *Journal of General Virology*, 89(Pt 7): 1643-1648.

Hsu, V. P., Hossain,M. J. U. D. Parashar, M. M. Ali, T. G. Ksiazek, I. Kuzmin, M. Niezgoda, M., Rupprecht, Bresee, J. and Breiman, R. F. (2004). Nipah virus encephalitis reemergence, Bangladesh. *Emerging Infectious Diseases,* 10:2082-2087.

Humphrey, C. (2007). Abuja dog pepper soup joint. The Daily Sun. Retieved 6/5/2008, from mhtml:file://E:\The%20sun%20news%20On-line.

International Committee on Taxonomy of Viruses (ICTV). (2009). Taxonomy: Updates Since the 8th Report, 2009. Retieved 6/5/2013, from http://talk.ictvonline.org/files/ictv official taxonomy updates since the 8th report/m/vertebrate-2008/1208.aspx.

Infoplease, (2012). Bats classification. The Columbia Electronic Encyclopedia, 6th ed. Copyright ©. Columbia University Press. All rights reserved. Retieved 1/8/2012, from http://www.infoplease.com/encyclopedia/science/bat-classification.html.

Jack, P. J., Boyle, D. B., Eaton B. T. and Wang L. F.(2005). The complete genome sequence of J virus reveals a unique genome structure in the family *Paramyxoviridae*. *Journal of Virology*,79(16): 10690-10700.

Johnson, N., McKimmie, C. S., Mansfield, K. L., Wakeley, P. R., Brookes, S. M., Fazakerley, J. K., and Fooks, A. R. (2006). *Lyssavirus* infection activates interferon gene expression in the brain. *Journal of General Virology*, 87:2663–2667.

Jun, M. H., Karabatsos, N. and Johnson R. H. (1977). A new mouse paramyxovirus (J virus). *Australian Journal of Exploratory Biology of Medicine and Science,* 55:645-647.

Karabatsos, N. (1985). International Catalogue of Arboviruses. San Antonio, Texas, *American Society of Tropical Medicine and Hygiene,* 45:266–26.

Kemp, G, E., Lee, V. H., Moore, D. L., Shope, R. E., Causey, O. R. and Murphy, F. A. (1973). Kotonkan Virus, a New Rhabdovirus Related to Mokola Virus of the Rabies Subgroup. *American Journal of Epidemiology,* 98: 43-49.

Kimura, M. (1980). A simple method for estimating evolutionary rate of base substitutions through comparative studies of nucleotide sequences. *Journal of Molecular Evolution,*16:111-120.

King, A., and Crick, J. (1988). Rabies-related viruses. In: *Rabies*, (K. M. Charlton andJ. B. Campbell, eds.). Kluwer Academic Publishers, Boston, pp. 177–200.

Kissi, B., Tordo, N., and Bourhy, H. (1995). Genetic polymorphism in the rabies virus nucleoprotein gene. *Virology,* 209:526-37.

Kitala, P. M., J. J. McDermott, M. N. Kyule, and J. M. Gathuma. (2000)**.** Community-based active surveillance for rabies in Machakos District, Kenya. *Preventive Veterinary Medicine,* 44:73–85.

Knobel, D. L., Cleaveland, S., Coleman, P. G., Fevre, E. M., Meltzer, M. I., Miranda, M. E., Shaw, A., Zinsstag, J. and Meslin, F. X. (2005). Re-evaluating the burden of rabies in Africa and Asia. *Bulletin of World Health Organization,* 83:360–368.

Knobel, D. L., Kaare, M.; Fevre, E. M. and Cleaveland, S. (2007). In: *Rabies*. Jackson, A.C.and Wunner, W. H., editors. Elsevier, pp. 573-594.

Ksiazek, T. G., Erdman, D. C. S., Goldsmith, S. R. Zaki., Peret, T., Emery, S., Tong, S., Urbani, C., Comer, J. A., Lim, W., Rollin, P. E., Dowell, S. F., Ling, A. E., Humphrey, C. D., Shieh, W. J., Guarner, C., Paddock, C. D., Rota, P., Fields, B., DeRisi, J., Yang, J. Y., Cox, N., Hughes, J. M. LeDue, J. W., Bellini, W. J. and Anderson, J. J.(2003). A novel coronavirus associated with severe acute respiratory syndrome. *New England Journal of Medicine,* 348:1953-1966.

Kuzmin, I. V., Botvinkin, A. D., and Shaimardanov, R. T. (1994). Experimental *Lyssavirus* infection in chiropters. *Voprosk Virusol*, 39:17–21.

Kuzmin, I. V., Hughes, G. J., Botvinkin, A. D., Orciari, L. A. and Rupprecht, C. E. (2005). Phylogenetic relationships of Irkut and West Caucasian bat viruses within the *Lyssavirus* genus and suggested quantitative criteria based on the N gene sequence for *Lyssavirus* genotype definition. *Virus Research,* 111:28–43.

Kuzmin, I. V., Wu, X., Tordo, N. and Rupprecht, C. E. (2008a). Complete genomes of Aravan, Khujand, Irkut and West Caucasian bat viruses, with special attention to the polymerase gene and non-coding regions. *Virus Research,* 136:81–90.

Kuzmin, I. V., Niezgoda, M., Franka, R., Agwanda, B., Markotter, W. Beagley, J. C., Urazova, O.Y., Breiman, R.F. and Rupprecht, C.E. (2008b). Possible emergence of West Caucasian bat virus in Africa. *Emerging Infectious Diseases,* 14:1887–1889.

Kuzmin, I. V., Niezgoda, M., Franka, R., Agwanda, B., Markotter, W., Beagley, J. C., Urazova, O. Y., Breiman, R. F., and Rupprecht, C. E. (2008c). Lagos bat virus in Kenya. *Journal of Clinical Microbiology*, 14:1887–1889

Kuzmin, I. V., Mayer, A. E., Niezgoda, M., Markotter, W., Agwanda, B., Breiman, R.F., Rupprecht, C. E. (2010). Shimoni bat virus, a new representative of the *Lyssavirus* genus. *Virus Research,* 149:197–210. .

Lamb, R. A. and Parks, G. D. (2007). *Paramyxoviridae*: the viruses and their replication. In: Knipe, D. M., Howley, P. M., Griffin, D. E., Lamb, R. A., Straus, S. E., Martin, M. A. and Roizman, B., editors. *Fields Virology*. 5th Ed. Vol. 1. Wolters Kluwer Lippincott Williams and Wilkins; Philadelphia, PA, USA. pp. 1449–1496.

Lane, T. E. and Buchmeier, M. J. (1997). Murine coronaviruses infection: a paradigm for virus-induced demyelinating disease. *Trends Microbiology,* 5: 9-14.

Lai, M., Perlman, S. and Anderson, L. (2007*). Coronaviridae.* In: Knipe, D. M., Howley, P. M., Griffin, D. E., Lamb, R. A., Straus, S. E., Martin, M. A. and Roizman, B., editors. *Fields Virology*. 5th Ed. Vol. 1. Wolters Kluwer Lippincott Williams and Wilkins; Philadelphia, PA, USA. pp. 1306–1335.

Lau, S. K., Woo, P. C., Li, K. S., Huang, Y., Tsoi, H.W., Wong, B.H., Wong, S.S.,Leung, S.Y., Chan, K.H. and Yuen, K.Y. (2005). Severe acute respiratory syndrome coronavirus-like virus in Chinese horseshoe bats. *Proceedings of the National Academy of Science, U S A*, 102(39):14040-14045.

Lau, S. K. Woo, P.C., Li, K.S., Huang, Y., Tsoi, H. W., Wong, B. H., Wong, S. S., Leung, S.Y., Chan, K. H. and Yuen, K.Y. (2010). Identification and complete genome analysis of three novel paramyxoviruses, Tuhoko virus 1, 2 and 3, in fruit bats from China. *Virology*, 404:106–116

Leroy, E.M., Kumulungui, B., Pourrut, X., Rouquet, P., Hassanin, A., Yaba, P. (2005). Fruit bats as reservoirs of Ebola virus. *Nature*, 438: 575 – 576.

Leslie, M. J., Messenger, S., Rohde, R. E., Smith, J., Cheshier, R., Hanlon, C. and Rupprecht, C. E. (2006). Bat-associated rabies virus in skunks. *Emerging Infectious Diseases*, 12:1274–1277.

Li, W., Shi, Z., Yu, M., Ren, W., Smith, C., Epstein, J. H., Wang, H., Crameri, G., Hu, Z., Zhang, H., Zhang, J., Mceachern, J., Field, H., Daszak, P., Eaton, B. T., Zhang, S. and Wang, L. F. (2005). Bats are natural reservoirs of SARS-like coronaviruses. *Science*,310(5748): 676-679.

Mallewa, M., Fooks, A. R., Banda, D., Chikungwa, P., Mankhambo, L., Molyneux, E., Molyneux, M. E. and Solomon, T. (2007). Rabies encephalitis in malaria-endemic area, Malawi, Africa. *Emerging Infectious Diseases*, 13:136–139.

Markotter, W., Kuzmin, I., Rupprecht, C. E., Randles, J., Sabeta, C. T., Wandeler, A. I. and Nel, L. H. (2006a). Isolation of Lagos bat virus from water mongoose. *Emerging Infectious Diseases*, 12:1913–1918.

Markotter, W., Randles, J., Rupprecht, C. E., Sabeta, C. T., Taylor, P. J., Wandeler, A. I. and Nel, L. H. (2006b). Lagos bat virus, South Africa. *Emerging Infectious Diseases*, 12:504–506.

Markotter, W., Kuzmin, I., Rupprecht, C. E., and Nel, L. H. (2008). Phylogeny of Lagos bat virus: Challenges for *Lyssavirus* taxonomy. *Virus Research*, 135:10–21.

Marra, M. A., Jones, S. J., Astell, C. R., Holt, R. A., Brooks-Wilson, A. (2003). The genome sequence of the SARS-associated *Coronavirus*. *Science*, 300: 1399 – 1404.

Matthijnssens, J., Bilcke, J., Ciarlet, M., Martella, V., Banyai, K. and Rahman, M. (2009). Rotavirus disease and vaccination: impact on genotype diversity. *Future Microbiology,* 4:1303–1316.

Meredith, C. D., Prossouw, A. P., and Koch, H. P. (1971). An unusual case of human rabies thought to be of chiropteran origin. *South African Medical Journal,* 45:767–769.

Meredith, C. D., Nel, L. H., and von Teichman, B. F. (1996). Further isolation of Mokola virus in South Africa. *Veterinary Record,* 138:119–120.

Messenger, S. L., Smith, J. S., and Rupprecht, C. E. (2002). Emerging epidemiology of bat associated cryptic cases of rabies in humans in the United States. *Clinical Infection and Disease,* 35:738–747.

Moreno-Lopez, J., Correa-Girón, P., Martinez, A. and Ericsson, A. (1986). Characterization of a *Paramyxovirus* isolated from the brain of a piglet in Mexico. *Archives of Virol*ogy, 91(3-4): 221-231.

Murray, K., Selleck, P., Hooper, P., Hyatt, A., Gould, A. and Gleeson, L. (1995). A morbillivirus that caused fatal disease in horses and humans. *Science*, 268: 94 – 97.

Nadin-Davis, S. A., Abdel-Malik, M., Armstrong, J., and Wandeler, A. I. (2002). *Lyssavirus* P gene characterisation provides insights into the phylogeny of the genus and identifies structural similarities and diversity within the encoded phosphoprotein. *Virology,* 298:286–305.

Nel, L. H., Thomson, G. R. and Von Teichman, B. F. (1993). Molecular epidemiology of rabies virus in South Africa. *Onderstepoort Journal of Veterinary Research,* 60:301–306.

Nel, L. H. and Rupprecht, C. E. (2007). Emergence of lyssaviruses in the old world: The case of Africa. *Curriculum of Tropical Microbiology and Immunology,* 315:161–193.

Nollens, H. H., Wellehan, J. F., Saliki, J. T., Caseltine, S. L., Jensen, E. D., Van Bonn, W. and Venn-Watson, S. (2008). Characterization of a parainfluenza virus isolated from a bottlenose dolphin (Tursiops truncatus). *Veteterinary Microbiology,*128: 231–242.

Nottidge, H.O. (1994). Seroevaluation of the immunogenicity of Flurry LEP strain Rabies vaccine purchased from Local Vendors. *Tropical Veterinarian, 12:179-184.*

Nuru, S. (1973). Epizootiology of Canine Rabies and its Public Health Significance in Kano State, Nigeria. *Nigerian Veterinary Journal,* 2(2): 498-542.

Nuru, S. and Barnes, H. J. (1974). Bovine Rabies in Kano State, Nigeria. *Bulletin of Epizootic Diseases in Africa,* 22 (3): 211-215.

Nylund, S., Karlsen,M.and Nylund, N. *(*2008). The complete genome sequence of the Atlantic salmon *Paramyxovirus* (ASPV). *Virology*, 373:137-148..

Ogo, M. F., Nel, L. H., and Sabeta, C. T. (2011). Phylogenetic evidence and Public and Veterinary Health threat of dog rabies in Nigeria. *Nigerian Veterinary Journal*, 32(1): 40-44.

Ogunkoya, A.B. (1986). Epidemiology of rabies in Kaduna, Oyo and Lagos states of Nigeria. *MSc*. *Thesis in Ahmadu Bello University, Zaria.*

Ojo, M. O. and Adeoye, A. (1967). Rabies in a Nigerian Dwarf Goat. *Bulletin of Epizootic Diseases in Africa*, 15: 409-410.

Okoh, A. E. J. (1982). Canine rabies in Nigeria: Reported cases in vaccinated dogs. *InternationalJournal of Zoonoses*, 9:118-125.

Okoh, A. E. J. (1986). Dog ecology with reference to surveillance of rabies and characterization of rabies in Plateau State, Nigeria. Ph.D. Thesis, Ahmadu Bello University, Zaria, Nigeria virus isolates in Plateau State, Nigeria.

Okoh, A. E. J. (2007). Rabies in Nigeria: Issues and Challenges, 6thInaugural Lecture of the University of Agriculture Makurdi. pp. 61- 62.

Owolodun, Y. (1969). Rabies: Present Situation in Nigeria. *West African Medical Journal*, 18: 143-144.

Panleuk, (2007). The rabies virus: Rabies virus in dogs and cats. Retieved 9/13/2012, from http://www.vendian.org/howbig/UnstableURLs/virus_rabiesorig.jpg.

Pavri, K. M., Singh, K. R. and Hollinger, F. B. (1971). Isolation of a new parainfluenza virus from a frugivorous bat, *Rousettus leschenaulti*, collected at Poona, India. *American Journal of Tropical Medicine and Hygiene*, 20:125–130.

Pawan, J. L. (1936). Rabies in the vampire bat of Trinidad, with special reference to the clinical course and the latency of infection. *Annals of Tropical Medical Parasitol*ogy, 30:410–422.

Paweska, J. T., Blumberg, L. H., Liebenberg, C., Hewlett, R. H., Grobbelaar, A. A.,Leman, P. A., Croft, J. E., Nel, L. H., Nutt, L. and Swanepoel, R. (2006). Fatal human infection with rabies-related Duvenhage virus, South Africa. *Emerging Infectious Diseases*, 12:1965–1967.

Peiris, J. S., Chu, C. M., Cheng, V. C., Chan, K. S., Hung, I. F., Poon, L. L., Law, K. I.,Tang, B. S., Hon, T. Y., Chan, C. S., Chan, K. H., Nge, J. S., Zheng, B. J., Nge, W. L., Lai, R. W., Guan, Y. and Yuen, K. Y. (2003). Clinical progression and viral load in a community

outbreak of coronavirus-associated SARS pneumonia: a prospective study. *Lancet,*361(9371):1767-1772.

Pfefferle, S., Oppong, S., Drexler, J. F., Rausch, F. G. and Ipsen, A. (2009). Distant relatives of severe acute respiratory syndrome *coronavirus* and close relatives of human *Coronavirus* 229E in bats,Ghana 15(9). Retrieved 6/5/2012, from http://www.cdc.gov/EID/content/15/9/1377.htm.

Philbey, A. W., Kirkland, P. D., Ross, A. D., Davis, R. J., Gleeson, A. B. Love, R. J., Daniels,P. W., Gould, A. R. and Hyatt, A. D. (1998). An apparently new virus (family, *Paramyxoviridae*) infectious for pigs, humans, and fruit bats. *Emerging Infectious Diseases*, 4:269-271.

Poon, L. L., Chan, K. H., Wong, O.K., Yam, W.C., Yuen, K.Y., Guan, Y., Lo, Y. M. and Peiris, J. S. (2001). Early diagnosis of SARS coronavirus infection by real time RT-PCR. *Journal Clinical Virology*, 28(3) 233-238.

Porterfield, J. S., Hill, D. H. and Morris, A. D. (1958). Isolation of a Virus from the Brain of a Horse with "Staggers" in Nigeria. *British Veterinary Journal,* 114:425-433.

Pritchard, L. I., Chua, K. B., Cummins, D., Hyatt, A. and Crameri, G. (2006). Pulau virus; a new member of the Nelson Bay orthoreovirus species isolated from fruit bats in Malaysia. *Archives of Virology,* 151: 229–239.

Quan, P. L., Firth, C., Street, C., Hendriquez, J. A., and Petrosov A. (2010). Identification of a severe acute respiratory syndrome coronavirus-like virus in a leaf nosed bat in Nigeria. mBio(4):e00208-e00210.

Radostits, O. M., Blood, D. C. and Gay, C. C. (1995). Rabies. In: *Veterinary medicine*. 8th ed. ELBS Bailliere Tindall, London, pp. 1087-1094.

Radostitts, O. M., Gay, C. C. Blood, D.C. and Hinchcliff, K. W. (2000). Rabies. In: *Veterinary Medicine,* 9th edition. W.B. Saunders Co.Ltd., pp. 1100-1123.

Real L. A., Russell C., Waller L., Smith D. and Childs J. (2005). Spatial dynamics and molecular ecology of North American rabies. *Journal of Hereditary*, 96:253–260.

Reed, L. J. and Muench, H. (1938). A simple method of estimating fifty percent endpoints. *The American Journal of Hygiene*, 27: 493–497.

Rollinson, D. H. L. (1956). Problems of Rabies Control in Africa. *East African Medical Journal*, 33:425

Rota, P. A., Oberste, M. S., Monroe, S. S., Nix, W. A. and Campagnoli, R. (2003). Characterization of a novel *coronavirus* associated with severe acute respiratory syndrome, *Science,* 300: 1394 – 1399.

Rupprecht, C. E., Smith, J. S., Fekadu, M. and Childs J. E. (1995). The ascension of wildlife rabies: a cause for public health concern or intervention? *Emerging Infectious Diseases,* 1:107–114.

Rutjes, S. A., Lodder, W. J, van Leeuwen, A. D. and de Roda Husman, A. M. (2009). Detection of infectious rotavirus in naturally contaminated source waters for drinking water production. *Journal of Applied Microbiology,* 107:97–105.

Sabeta, C. T., Markotter, W., Mohale, D. K., Shumba, W., Wandeler, A. I. and Nel, L. H. (2007). Mokola virus in domestic mammals, South Africa. *Emerging Infectious Diseases,* 13:1371–1373.

Sabo, G. (2009). The role of dog trade in the epidemiology of rabies in Plateau State Nigeria. MSc. thesis submitted to the Department of Veterinary Public Health and Preventive Medicine, Faculty of Veterinary Medicine, Ahmadu Bello University, Zaria.

Sabo, G., Umoh, J.U., Sackey, A.K.B., Ahmad, A. and Okolocha E.C. (2008). The role of dog trade in the epidemiology of rabies. *A Review Veterinary Clinical Practice Bulletin,* 1 (1): 63-72.

Saif, L. J. (2004). Animal coronaviruses: what can they teach us about the severe acute respiratory syndrome? *Review of Scientific Technics*, 23: 643–660.

Senan, M. (2007). Dogs' dinners prove popular in Nigeria. Retieved 15/6/2008, from http//BBCnews/world/Africa.com.

Shankar, V., Bowen, R. A., Davis, A. D., Rupprecht, C. E. and O'Shea, T. J. (2004). Rabies in a captive colony of Big Brown bats (Eptesicus fuscus). *Journal of Wildlife Diseases,* 40:403–413.

Shope, R. E., Murphy, F. A., Harrison, A. K., Causey, O. R., Kemp, G. E., Simpson, D. I. and Moore, D. L. (1970). Two African viruses serologically and morphologically related to rabies virus. *Journal of Virology,* 6:690–692.

Shope, R. E. (1982). Rabies-related viruses.*The Yale Journal of Biology and Medicine*, 55:271–275

Sodre, M. M., da Gama, A. R. and de Almeida, M. F. (2010). Updated list of bat species positive for rabies in Brazil. *Review of Institute of Medicine in Tropical Sa˜o Paulo,* 52:75–81.

State Creation for Rapid Development (SCRD). (2011). History of Plateau State. Retrieved 1/6/2014, from www.plateaustate, Nigeria History Geographic Information.htm.

Steece, R. and Altenbach, J. S. (1989). Prevalence of rabies specific antibodies in the Mexican free tailed bat (*Tadarida brasiliensis mexicana*) at Lava Cave, New Mexico. *Journal of Wildlife Diseases,* 25: 490–496.

Streicker, D. G., Turmelle, A. S., Vonhof, M. J., Kuzmin, I. V., McCracken, G. F. and Rupprecht, C. E. (2010). Host phylogeny constrains cross -species emergence and establishment of rabies virus in bats. *Science,* 329:676–679.

Sulkin, S. E. (1962). Bat rabies: Experimental demonstration of the ‘‘reservoiring mechanism’’. *American Journal of Public Health,* 52:489–498.

Sulkin, S. E., Allen, R., Sims, R., Krutzsch, P. H. and Kim, C. H. (1960). Studies on the pathogenesis of rabies in insectivorous bats II. Influence of environmental temperature. *Journal of Experimental Medicine,* 112:595–617.

Sulkin, S. E. and Greve, M. J. (1954). Human rabies caused by bat bite. *Texas State Journal of Medicine,* 50:620–621.

Swanepoel, R. (2004). Rabies. In: *Infectious diseases of livestock: With special reference to southern Africa*. 2nd Edition, J. A. W. Coetzer and R. C. Tustin (eds.). Oxford University Press, Cape Town, South Africa, pp. 1123–1182.

Swanepoel, R., Barnard, B. J., Meredith, C.D., Bishop, G.C., Bruckner G.K., Foggin C.M. and Hubschle O.J. (1993). Rabies in Southern Africa. Onderstepoort *Journal Veterinary Research,* 60:325–346.

Talbi, C., Holmes, E. C., De Benedictis, P., O. Faye, E. Nakoune, D. Gamatie, A. Diarra, B. O., Elmamy, A., Sow, E. V., Adjogoua, O., Sangare, W. G., Dundon, I., Capua, A. A. and Bourhy, H. (2009). Evolutionary history and dynamics of dog rabies virus in western and central Africa. *Journal of General Virology,* 90:783-791.

Tamura, K., Dudley,J., Nei, M. and Kumar. S. (2007). MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. *Molecular Biology of Evolution,* 24:1596-1599.

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M. and Kumar S. (2011). MEGA5: Molecular Evolutionary Genetics Analysis using Maximum Likelihood, Evolutionary Distance, and Maximum Parsimony Methods. *Molecular Biology and Evolution*(In Press).

Tang, X.C., Zhang, J.X., Zhang, S.Y.,Wang, P., Fan, X.H., Li, L.F., Li, G., Dong, B.Q., Liu, W., Cheung, C.L., Xu, K.M., Song, W.J., Vijaykrishna, D., Poon, L.L., Peiris, J.S., Smith, G. J., Chen, H. and Guan, Y. (2006). Prevalence and genetic diversity of Coronaviruses in bats from China. *Journal of Virology,* 80: 7481.

Teeling, E. C., Springer, M. S., Madsen, O., Bates, P., O'Brien, S. J. and Murphy, W. J. (2005). A molecular phylogeny for bats illuminates biogeography and the fossil record. *Science,* 307:580–584.

Tong, S., Conrardy, C., Ruone, S., Kuzmin, I. V. and Guo, X. (2009). Detection of novel SARS-like and other coronaviruses in bats from Kenya. *Emerging Infectious Diseases,*15:482-485.

Tong, S., Li Y., Rivailler, P., Conrardy, C., Castillo A. A. D., Chen, L., Recuenco, S., Cox, N. J., Anderson, J. L., Rupprecht, C. and Donis, R.O. (2012). A distinct lineage of influenza A virus from bats. Publication of the National Academy of Science, early edition. Retrieved 20/2/2012, from www.pnas.org/cgi/doi/10.1073/pnas.1116200109.

Towner, J. S., Amman, B. R., Sealy, T. K., Carroll, S. A., Comer, J. A., Kemp, A., Swanepoel, R., Paddock, C. D., Balinandi, S., Khristova, M. L., Formenty, P. B. and Albarino, C. G.(2009). Isolation of genetically diverse Marburg viruses from Egyptian fruit bats. *PLoS Pathogen.* 5:e1000536.

Turmelle, A. S., Jackson, F. R., Green, D., McCracken, G. F. and Rupprecht, C. E. (2010). Host immunity to repeated rabies virus infection in big brown bats. *Journal of General Virology,* 91:2360–2366.

Umoh, J. U. and Belino, E. D. (1979). Rabies in Nigeria. A historical review. *International Journal of Zoonoses, 6:* 41-48.

Van der Poel, W. H., Van der Heide, R., Van Amerongen, G., Van Keulen, L. J.,Wellenberg, G. J., Bourhy, H., Schaftenaar, W., Groen, J. and Osterhaus, A. D. (2000).Characterisation of a recently isolated lyssavirus in frugivorous zoo bats. *Archives of Virology*, 145:1919–1931.

van Thiel, P. P., van den Hoek, J. A., Eftimov, F., Tepaske, R., Zaaijer, H. J., Spanjaard, L., deBoer, H. E., van Doornum, G. J., Schutten, M., Osterhaus, A. and Kager, P. A. (2008). Fatal case of human rabies (Duvenhage virus) from a bat in Kenya: The Netherlands, December 2007. *European Surveillance,* 13:8007.

Velasco-Villa, A., Orciari, L. A., Souza, V., Juarez-Islas, V., Gomez-Sierra, M., Castillo, A., Flisser, A. and Rupprecht C. E. (2005). Molecular epizootiology of rabies associated with terrestrial carnivores in Mexico. *Virus Research,* 111:13–27.

Wang, L. F., Yu, M., Hansson, E., Pritchard, L. I., Shiell, B., Michalski, W. P. and Eaton, B. T. (2007). Full-length genome sequence and genetic relationship of two paramyxoviruses isolated from bat and pigs in the Americas. *Archives of Virology*, 152(7):1259-1271.

Wang, L. F. and Eaton, B. T. (2007). Bats, civets and the emergence of SARS. *Curriculum of Tropical Microbiology and Immunology*, 315:325–344.

Wang, L. F., Harcourt,B. H., Yu, M., Tamin, A., Rota,P. A., Bellini W. J. and Eaton, B. T. (2001). Molecular biology of Hendra and Nipah viruses. *Microbes and Infection*, 3:279-287.

Warrell, M. J. and Warrel, D. A.(2004). Rabies and other *Lyssavirus* diseases. *The Lancet*, 363:959-969.

Warrilow, D., Harrower, B., Smith, I. L., Field, H., Taylor, R., Walker, C. and Smith G. A. (2003). Public health surveillance for Australian bat Lyssavirus, in Queensland, Australia, 2000– 2001. *Emerging Infectious Diseases*, 9: 263–264.

Wellenberg, G. J., Aubry, L., Ronsholt, L., Van der Poel, W. H., Bruschke, C. J. and Bourhy, H. (2002). Presence of European bat lyssavirus RNAs in apparently healthy *Roussetus aegyptiacus* bats. *Archives of Virology*, 349–61.

Wibbelt, G., Moore, M. S., Schountz, T. and Voigt, C. C. (2010). Emerging diseases in *Chiroptera*: why bats? *Biology Letters*, 6: 438–440.

Wiktor, T. J., Macfarlan, R. I., Reagan, K. J., Dietzschold, B., Curtis, P. J., Wunner, W. H., Kieny, M. P., Lathe, R., Lecocq, J. P., Macketi', M., Moss, B. and Koprowski, H. (1984). Protection from rabies by a vaccinia virus recombinant containing the rabies virus glycoprotein gene. *Proceedings of the National Academy of Sciences*, U.S.A., 81: 7194-7198.

Wimsatt, J., O'Shea, T. J., Ellison, L. E., Pearce, R. D. and Price, V. R. (2005). Anesthesia and blood sampling of wild big brown bats (Eptesicus fuscus) with an assessment of impacts on survival. *Journal of Wildlife Diseases*, 41 (1): 87–95.

Winkler, W. G. (1968). Airborne rabies virus isolation. *Journalof Wildlife Diseases*, 4:37–40.

Winkler, W. G., Fashinell, T. R., Leffingwell, L., Howard, P. and Conomy, P. (1973). Airborne rabies transmission in a laboratory worker. *Journal of American Medical Association*, 226:1219–1221.

Wong, A. H., Cheng, P. K. C, Lai, M. Y. Y., Leung, P. C. K., Wong, K. Y. and Lee, W.Y. (2012). Virulence potential of fusogenic orthoreoviruses. *Emerging Infectious Diseases*. Retieved 2/2/2013, from http://www.cdc.gov/Other?disclaimer.html.

Wong, K. T., Robertson, T., Ong, B. B., Chong, J. W., Yaiw, K. C., Wang, L. F., Ansford, A. J. and Tannenberg, A. (2009). Human Hendra virus infection causes acute and relapsing encephalitis. *Neuropathology and Applied Neurobiology*, 35: 296–305.

Woo, P. C., Lau, S. K., Li, K. S., Poon, R. W., Wong, B. H., Tsoi, H. W.,Yip, B. C., Huang, Y., Chan, K. H. and Yuen, K. Y. (2006). Moleculardiversity of coronaviruses in bats. *Virology*, 350:180.

Woo, P. C., Wang, M. and Lau, S. K. (2007). Comparative analysis of twelve genomes of three novel group 2c and group 2d coronaviruses reveals unique group and subgroup features. *Journal of Virology*, 81:1574-1574.

Woo, P. C, Lau, S. K., Huang, Y. and Yuen, K.Y. (2009). Coronavirus diversity, phylogeny, and interspecies jumping. *Experimental Biology of Medicine*, 234: 1117-1127.

World Health Organization (WHO), (1992).Geneva, Switzerland.World Health Organization. Report of the World Health Organization Expert Committee and Rabies. *World Health Organization Technical Report Series*, No. 824.

World Health Organization (WHO), (2005). World Health Organization expert consultation on rabies: first report. Geneva, Switzerland: *World Health OrganizationTechnical Report*,931: 1–88.

World Health Organization (WHO), (2014). *Coronavirus* infections. Global alert and response. Retrieved 7/1/2014, from http://www.who.int/csr/disease/coronavirus_infections/en/.

Wright, E., Hayman, D. T., Vaughan, A., Temperton, N. J., Wood, J. L., Cunningham, A. A., Suu-Ire, R., Wright, E., Hayman, D. T., Vaughan, A., Temperton, N. J., Wood, J. L., Weiss, R. A. and Fooks, A. R. (2010). Virus neutralising activity of African fruit bat (*Eidolon helvum*) sera against emerging lyssaviruses. *Virology*, 408:183–189.

Wunner, W. H. (2007). Rabies virus. In: Rabies. Jackson, A. C. and Wunner, W. H. (eds), 2nd edn. Elsevier, Academic Press, London, United Kingdom, pp. 23-68.

Yob, J. M., Field, H., Rashdi, A. M., Morrissy, C. and van der Heide, B. (2001). Nipah virus infection in bats (order *Chiroptera*) in peninsular Malaysia. *Emerging Infectious Diseases, 7*: 439–441.

Young, P. L., Halpin, K., Selleck, P. W., Field, H., Gravel, J. L., Kelly, M. A. and Mackenzie, J. S. (1996). Serologic evidence for the presence in pteropus bats of a paramyxovirus related to equine morbillivirus. *Emerging Infectious Diseases,* 2: 239-240.

Zaki, A. M., van Boheemen, S., Bestebroer, T. M., Osterhaus, A. E. and Fouchier, R. A. M. (2012). Isolation of a Novel Coronavirus from a Man with Pneumonia in Saudi Arabia. *The New England Journal of Medicine,* 367 (19):1814 -1820.

Appendix I

A Published Work from this study



genomeA
Journals.ASM.org

Complete Genome Sequence of a Street Rabies Virus Isolated from a Dog in Nigeria

Ming Zhou,[a] Zutao Zhou,[a,b] Grace S. N. Kia,[a,c] Clement W. Gnanadurai,[a] Christina M. Leyson,[a] Jarlath U. Umoh,[c] Jacob P. Kwaga,[c] Haruna M. Kazeem,[d] Zhen F. Fu[a,b]

Department of Pathology, College of Veterinary Medicine, University of Georgia, Athens, Georgia, USA[a]; State Key Laboratory of Agricultural Microbiology, College of Veterinary Medicine, Huazhong Agricultural University, Wuhan, China[b]; Department of Veterinary Public Health and Preventive Medicine, Ahmadu Bello University, Zaria, Nigeria[c]; Department of Veterinary Microbiology, Faculty of Veterinary Medicine, Ahmadu Bello University, Zaria, Nigeria[d]

M.Z., Z.Z., G.S.N.K., and Z.F.F. contributed equally to this work.

A canine rabies virus (RABV) was isolated from a trade dog in Nigeria. Its entire genome was sequenced and found to be closely related to canine RABVs circulating in Africa. Sequence comparison indicates that the virus is closely related to the Africa 2 RABV lineage. The virus is now termed DRV-NG11.

Received 18 December 2012 **Accepted** 31 December 2012 **Published** 21 February 2013

Citation Zhou M, Zhou Z, Kia GSN, Gnanadurai CW, Leyson CM, Umoh JU, Kwaga JP, Kazeem HM, Fu ZF. 2013. Complete genome sequence of a street rabies virus isolated from a dog in Nigeria. Genome Announc. 1(1):e00214-12. doi:10.1128/genomeA.00214-12.

Copyright © 2013 Zhou et al. This is an open-access article distributed under the terms of the Creative Commons Attribution 3.0 Unported license.

Address correspondence to Zhen F. Fu, zhenfu@uga.edu.

Rabies, which can cause an almost invariably fatal encephalomyelitis, remains a public health threat around the world. Although effective vaccines are available, more than 55,000 people still die from rabies each year throughout the world, with most of them in developing countries of Asia and Africa (1). Rabies virus (RABV) belongs to the genus *Lyssavirus* in the *Rhabdoviridae* family. Its genome is a single-stranded negative-sense RNA of approximately 12 kb in length (2, 3). In previous studies, on the basis of phylogenetic analysis of the nucleoprotein (N) and/or the glycoprotein (G) gene sequences, all RABVs (genotype 1 of lyssaviruses) have been divided into two major clades, one comprising those isolated from terrestrial animals around the world and the other containing viruses isolated from bats and raccoons in the Americas (4–7). A strain of RABV was isolated from a dog brain originating in Nigeria and was designated DRV-NG11. To obtain the whole genomic sequence of this virus, total RNA was extracted from dog brain using TRIzol LS reagent (Invitrogen, Carlsbad, CA). Ten pairs of oligonucleotide primers for amplifying regions of the RABV genomes were designed as previously described (8–11). All PCR products were purified using a QIAquick gel extraction kit (Qiagen, Germantown, MD) and then cloned into the pCR-Blunt II vector (Invitrogen). Cloned DNA was sequenced using a BigDye Terminator cycle sequencing ready reaction kit and an ABI Prism 3730 sequencer. The genomic sequence was assembled by using SeqMan software (DNASTAR Inc.). Homology searches and comparisons of all the sequences obtained were carried out with the aid of the Lasergene package (DNASTAR Inc.). Sequences of encoded proteins were aligned using MEGA version 5 (12). The complete genome of DRV-NG11 is 11,923 nucleotides (nt) in length, similar to genomes of other street RABVs published to date (10, 11, 13). The lengths of the coding sequences are as follows: 1,353 nt for the nucleoprotein (N), 894 nt for the phosphoprotein (P), 609 nt for the matrix protein (M), 1,575 nt for the glycoprotein (G), and 6,384 nt for the RNA-dependent RNA polymerase (L) genes. Comparison of N gene sequences indicates that this virus is related to another RABV identified in Nigeria (EU038098) with high homology (99.4%) and thus it belongs to the Africa 2 lineage (14, 15). The homology between this RABV and those in the "cosmopolitan" group ranges from 86.5% to 87.8% (14, 15). To our knowledge, this is the first report of a complete genome sequence of RABV isolated from Nigeria. The complete genome analysis of DRV-NG11 will advance further studies in rabies epidemiology and pathogenesis.

Nucleotide sequence accession number. The complete genome sequence of DRV-NG11 is available in GenBank under accession number KC196743.

ACKNOWLEDGMENTS

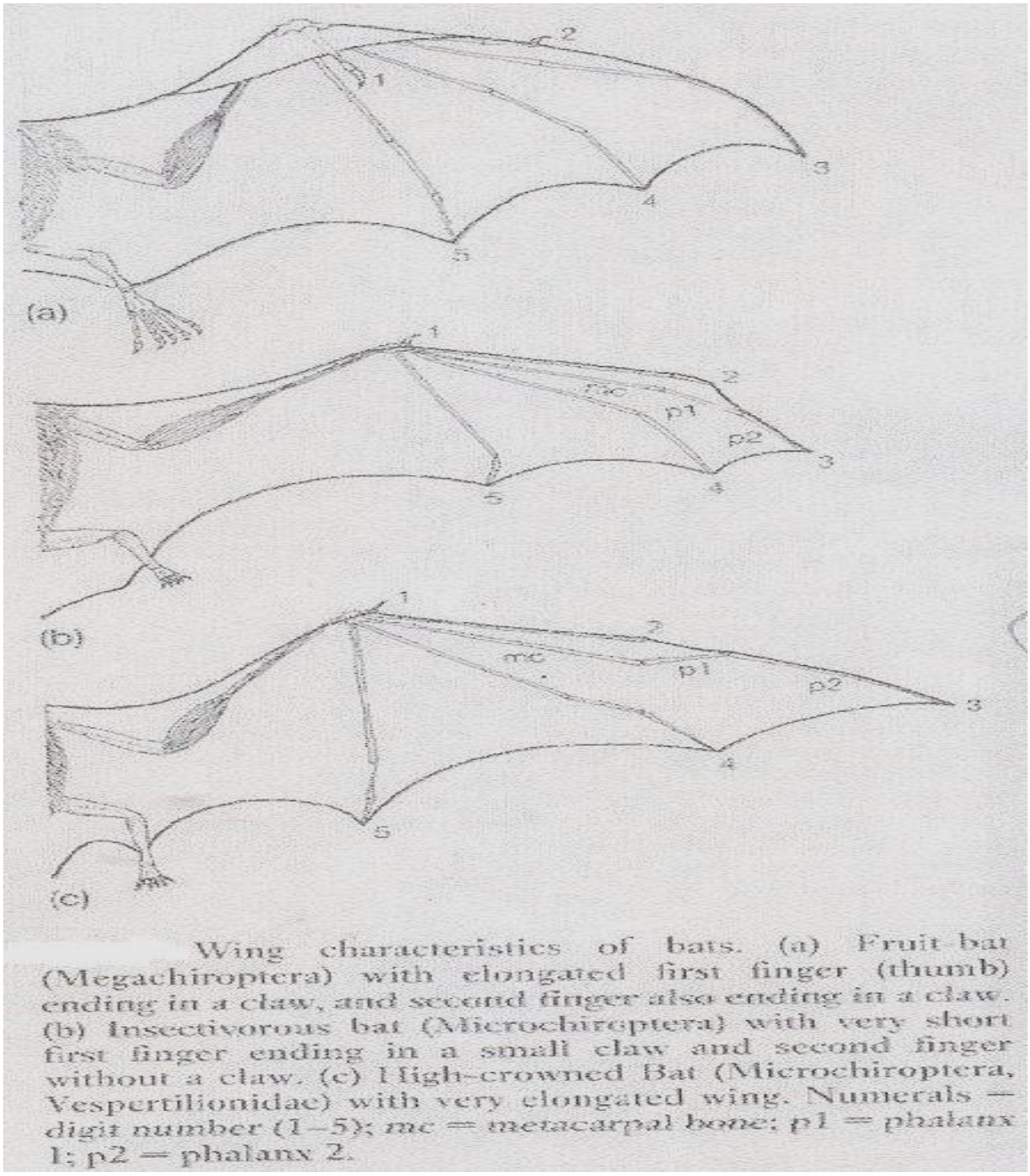
This work was partially supported by Public Health Service grant AI-051560 from the National Institute of Allergy and Infectious Diseases (Z.F.F.) and by the Education Trust Fund ETF/DESS/AST&D/UNIV/ABU/ZARIA/VOL.2 and the Science and Technology Education Post Basic (S. T. E. P.-B.) Project, Innovators of Tomorrow Grants HME STEP-B/IOT/33/Vol.1/22 (G.S.N.K.).

REFERENCES

1. Dietzschold B, Rupprecht CE, Fu ZF, Koprowski H. 1996. Rhabdoviruses. p 1137–1159. *In* Fields B, Knipe D, Howley PM (ed), Fields virology, 3rd ed. Lippincott Raven, Philadelphia, PA.
2. Tordo N, Poch O, Ermine A, Keith G, Rougeon F. 1986. Walking along the rabies genome: is the large G-L intergenic region a remnant gene? Proc. Natl. Acad. Sci. U. S. A. 83:3914–3918.
3. World Health Organization 2005. WHO Expert Committee on Rabies. 2004. First report. WHO technical report series 931. World Health Organization, Geneva, Switzerland.
4. Bourhy H, Kissi B, Audry L, Smreczak M, Sadkowska-Todys M, Kulonen K, Tordo N, Zmudzinski JF, Holmes EC. 1999. Ecology and evolution of rabies virus in Europe. J. Gen. Virol. 80:2545–2557.
5. Davis PL, Bourhy H, Holmes EC. 2006. The evolutionary history and dynamics of bat rabies virus. Infect. Genet. Evol. 6:464–473.
6. Nadin-Davis SA, Sampath MI, Casey GA, Tinline RR, Wandeler AI.

Appendix II

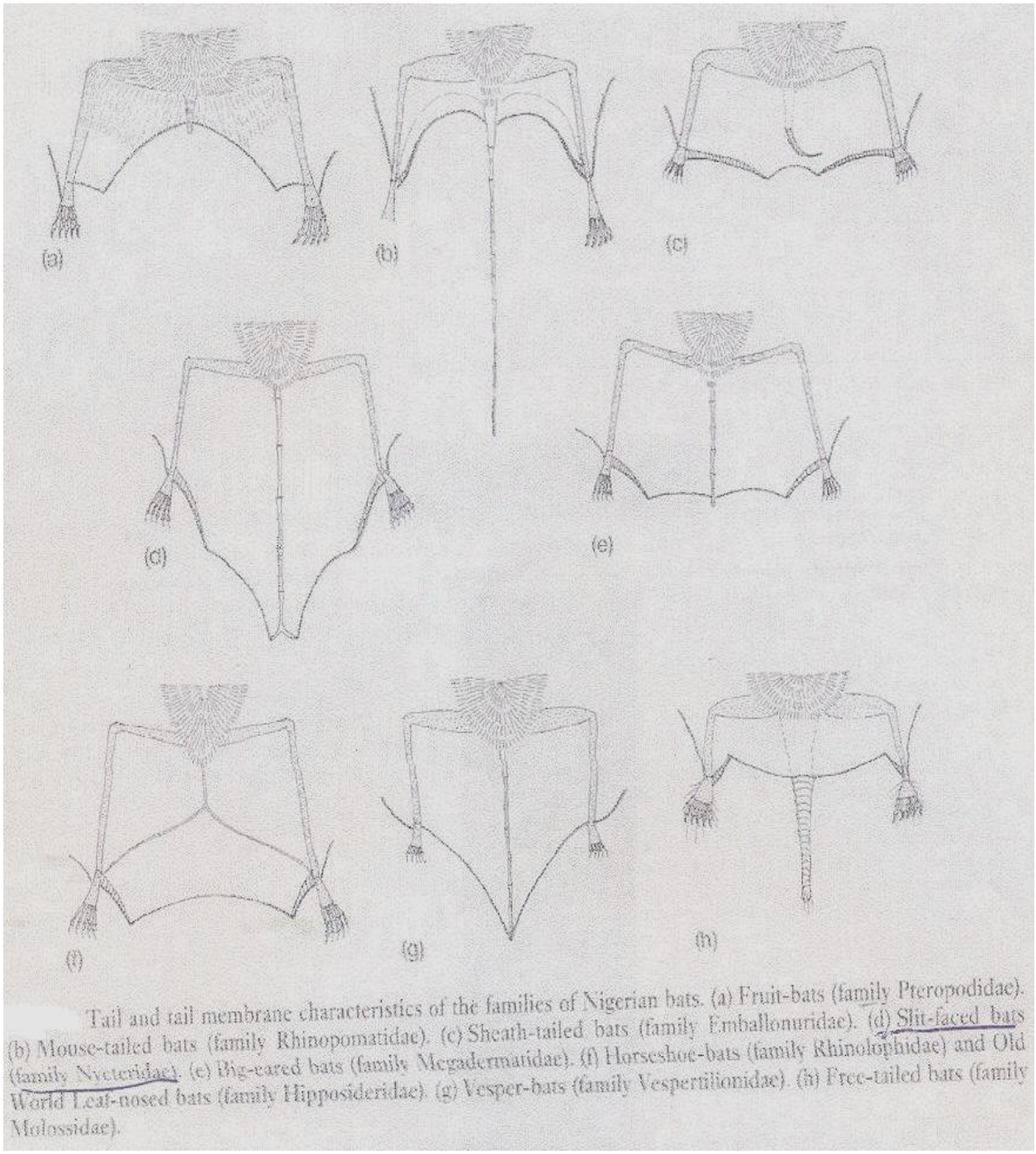
Unique wing features in identification of bats



Wing characteristics of bats. (a) Fruit bat (Megachiroptera) with elongated first finger (thumb) ending in a claw, and second finger also ending in a claw. (b) Insectivorous bat (Microchiroptera) with very short first finger ending in a small claw and second finger without a claw. (c) High-crowned Bat (Microchiroptera, Vespertilionidae) with very elongated wing. Numerals — *digit number* (1–5); *mc* = *metacarpal bone*; *p1* = phalanx 1; p2 = phalanx 2.

Appendix III

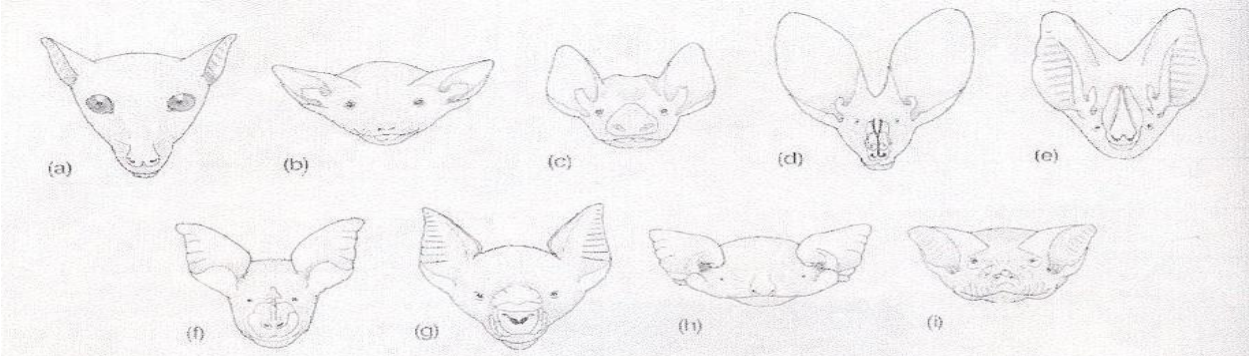
Unique tail and tail membrane features in identification of bats



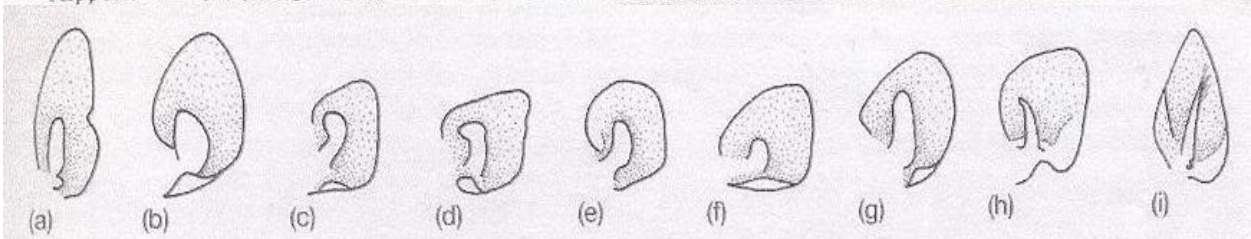
Tail and tail membrane characteristics of the families of Nigerian bats. (a) Fruit-bats (family Pteropodidae). (b) Mouse-tailed bats (family Rhinopomatidae). (c) Sheath-tailed bats (family Emballonuridae). (d) Slit-faced bats (family Nycteridae). (e) Big-eared bats (family Megadermatidae). (f) Horseshoe-bats (family Rhinolophidae) and Old World Leaf-nosed bats (family Hipposideridae). (g) Vesper-bats (family Vespertilionidae). (h) Free-tailed bats (family Molossidae).

Appendix IV

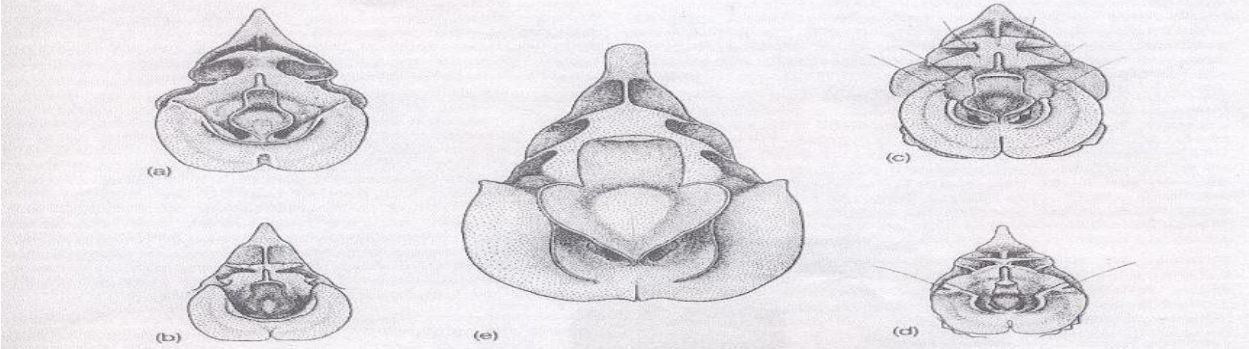
Unique facial features in identification of bats



Facial characteristics of the families of Nigerian bats. (a) Fruit-bats (family Pteropodidae). (b) Sheath-tailed bats (family Emballonuridae). (c) Mouse-tailed bats (family Rhinopomatidae). (d) Slit-faced bats (family Nycteridae). (e) Big-eared bats (family Megadermatidae). (f) Horseshoe-bats (family Rhinolophidae). (g) Leaf-nosed bats (family Hipposideridae). (h) Vesper-bats (family Vespertilionidae). (i) Free-tailed bats (family Molossidae).



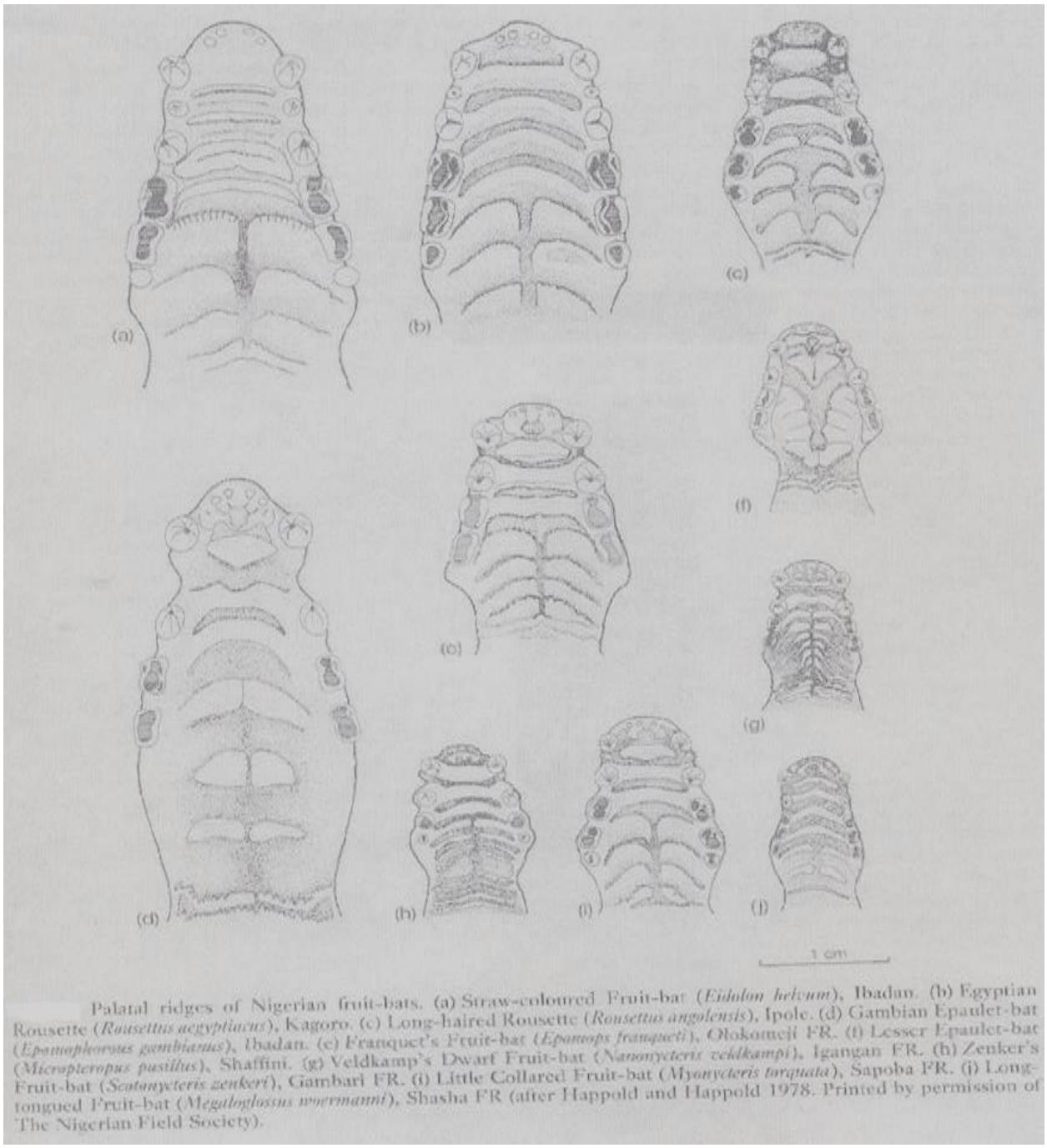
Tragus characteristics of some vesper-bats (family Vespertilionidae). (a) Rufous Mouse-eared Bat (*Myotis bocagei*). (b) Schlieffen's Bat (*Nycteiceus schlieffeni*). (c) *Scotoecus* sp. (d) *Pipistrellus* sp. (e) *Glauconycteris* sp. (f) Moloney's Flat-headed Bat (*Mimetillus moloneyi*). (g) *Eptesicus* sp. (h) *Scotophilus* sp. (i) Smith's Woolly-bat (*Kerivoula smithi*). Not drawn to scale.



Nose-leaves of horseshoe-bats (family Rhinolophidae); frontal views. (a) Halcyon Horseshoe-bat (*Rhinolophus alcyone*). (b) Cameroun Horseshoe-bat (*Rhinolophus alticolus*). (c) Abyssinian Horseshoe-bat (*Rhinolophus fumigatus*). (d) Lander's Horseshoe-bat (*Rhinolophus landeri*). (e) Maclaud's Horseshoe-bat (*Rhinolophus maclaudi*) (adapted from Rosevear 1965).

Appendix V

Unique characteristics ofpalatal ridges in different bats



Palatal ridges of Nigerian fruit-bats. (a) Straw-coloured Fruit-bat (*Eidolon helvum*), Ibadan. (b) Egyptian Rousette (*Rousettus aegyptiacus*), Kagoro. (c) Long-haired Rousette (*Rousettus angolensis*), Ipole. (d) Gambian Epaulet-bat (*Epomapleorsus gambianus*), Ibadan. (e) Franquet's Fruit-bat (*Epomops franqueti*), Olokomeji FR. (f) Lesser Epaulet-bat (*Micropteropus pusillus*), Shaffini. (g) Veldkamp's Dwarf Fruit-bat (*Nanonycteris veldkampi*), Igangan FR. (h) Zenker's Fruit-bat (*Scotonycteris zenkeri*), Gambari FR. (i) Little Collared Fruit-bat (*Myonycteris torquata*), Sapoba FR. (j) Long-tongued Fruit-bat (*Megaloglossus woermanni*), Shasha FR (after Happold and Happold 1978. Printed by permission of The Nigerian Field Society).

Appendix VI

Some uniquebats sampled in Plateau State

Lavia frons

Unique elongated nose leaves, big ears, yellow wings. Pelage smoky grey, and long soft fur.





Rhinolophus spp.

They are known for their horseshoe shaped nose with orange brown pelage.



Nycteris spp.

Slit faced bats with long ears.

Has a long tail which terminates in a Y-shaped cartilage enclosed in the interfemoral membrane



Rhinopoma spp.

mouse-tailed bats. Nasal region thickened to give wide flattened snout. Tail thin and very long.



Appendix VII

Preparation of cell culture medium (MEM) and Mouse neuroblastoma cells (MNA) Preparation, Propagation and maintenance for RFFIT

About 500mls cell culture medium was prepared at a time. A 500 ml disposable filter of 0.22μm pore (Nalgene) was connected to the Vacuum pump under the safety cabinet and the underlisted reagents stored as aliquots for ease of use were added. About 370ml distilled deionized sterile water (Cellgro®) was dispensed into the filter to which about 50ml of 10x Eagle's Minimum Essential Medium with 10% calf fetal serum (MEM -10) was added. The MEM was stored at 4°C and wrapped in aluminum foil to keep out light. This was followed by addition of 10mls MEM vitamin solution liquid (Invitrogen) stored at -20°C, then 10mls L-glutamine (Invitrogen) previously stored at -20ºC. The glutamine was mixed properly by shaking vigorously to mix the precipitate and achieve a homogenous suspension. About 5mls antibiotic-antimycotic (Invitrogen) containing penicillin, streptomycin and amphotericin B stored at -20°C was added. About 50mls fetal bovine serum (Hyclone, Utah, USA) which was previously stored a -20°C and heat inactivated at 56°C for 30 minutes in a water bath just before use was added. Lastly, 5mls Sodium bicarbonate (Invitrogen) stored at 4°C was added. The vacuum was turned on and the filterate collected in the receptacle. The red coloured resulting mixture was shaken to mix and dispensed into 50 ml centrifuge tubes and kept at 4°C until used.

The cell culture line MNA used a CDC was originally obtained from the Wistar Institute, Rabies section, Philadelphia, PA, USA. A flask of stock MNA culture of about 3 to 4 days ie near confluency was taken out of CO_2 incubator at 37°C (Forma Scientific Inc., Model 3110, series II) and checked for viability using an inverted microscope (Zeiss, Axiovert 25). The MEM was

taken out using a 25ml plastic pipette and a pipette aid (Fisher Scientific) taking care not to disturb the monolayer. The medium was washed off using two changes of 5mls PBS 4550 (pH 7.4, Ca^{2+} and Mg^{2+} free, Cellgrow ®) using a new pipette each time to maintain sterility. The flask was rocked back and forth each time to wash off residual MEM which otherwise can inactivate the next step. About two mls trypsin-EDTA was dispensed into the flask, rocked gently and kept for 5 minutes at room temperature or incubated at 37°C for 3 minutes. Once the cells started to detach, the bottom of the flask was gently tapped against the palm of the hand to facilitate the dislodgement of the cells from the wall of the flask. About 8mls of fresh MEM was dispensed into the flask immediately to stop enzymatic activity of trypsin. The cells were dispersed by gently sucking in and out of the pipettes to break clumps and make an even suspension. The re-suspended cells were seeded by transferring approximately 2mls into new culture flasks to which about 23 mls MEM was added using a 25ml pipette to bring the total volume to about 25ml per flask. This gives a 1:5 split ratio. The cells were dispersed by gently swirling. The flask lid were completely tightened and were kept gently flat under the hood, labeled with cell type, new passage number, date of subculture, and the cell concentration. The flasks were placed in an incubator at 37°C. This process was repeated every three to four days to keep the cell line since the cells have potentially infinite subpassages.

Preparation of MNA cells

This was done just prior to use. About 30 minutes before end of the 90 minute incubation step following virus challenge, a flask with monolayer cells was taken from incubator at 37°C and the medium taken out and processed as described above using PBS 4588 (pH 7.4 - 7.6). After dislodging the cells and addition of eight mls MEM, the cells were transferred into 50mls

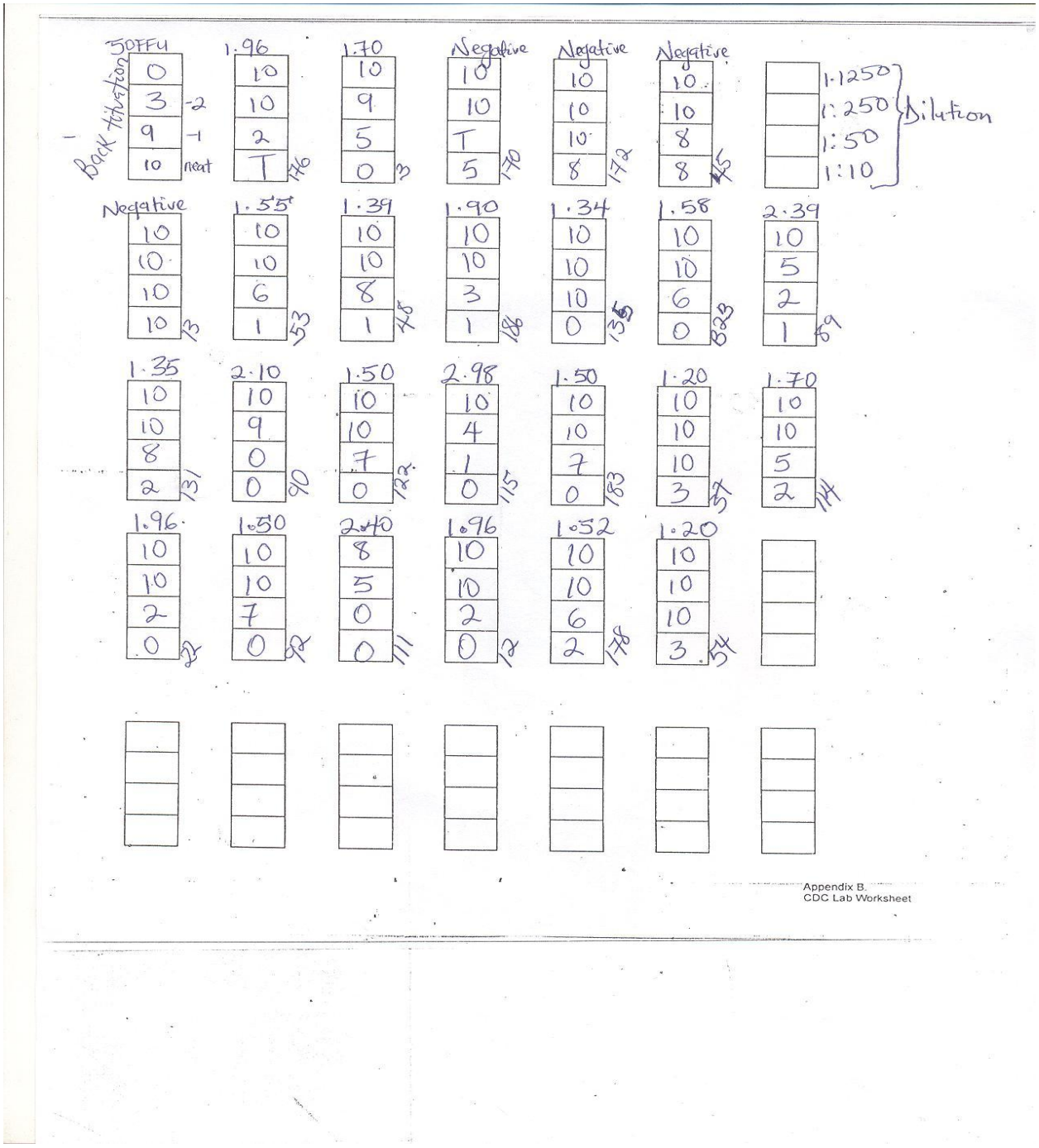
centrifuge tubes and spun at 500rpm for 10 minutes at 4°C. The medium was taken out using pipette, taking care to leave the cells at the bottom of the tube. About 16mls MEM was added to the cells to make a 5.0 X 10^5 cell/ml.

Reference serum standard

The U.S standard rabies immunoglobulin (National Institute for Biological Standards and Control, Herts, EN6 3QH, UK) with a pre-determined unit value was used to obtain the dilution necessary for a laboratory stock containing 2IU/ml.

Appendix VIII

An example of RFFIT end-point titration result.



Appendix IX

Determining the 50% end-point titers of serum:

The Reed-Muench formula is applied to calculate the difference between the logarithm of the starting dilution and the logarithm of the 50% end-point dilution.An example of calculated Reed-Muench Method 50% serum end-point titers corresponding to the number of infected fields observed for different serum dilutions of forinstance the first test sera ID 22 in the last row (Appendix VII) is sown as follows;

Serum dilutions	Number of fields showing fluorescing cells out of ten fields
1:1250	10/10
1:250	10/10
1:50	2/10
1:10	0/10

The Reed-Meunch formula:

$$\frac{50\% - (infectivity\ next\ below\ 50\%)}{(infectivity\ next\ above\ 50\%) - (infectivity\ next\ below\ 50\%)} \times logarithm\ of dilution\ factor$$

In this example the starting point dilution (the dilution showing infectivity next below 50%) is 50 (with only 2 infected fields out of 10) The infectivity next above 50% is 10 at a dilution point of 250. The dilution factor is 5. Hence, the difference in logarithms is:

$\frac{50 - (2x10)}{(10x10) - (2x10)}$ X log 5 (where log 5 = 0.69897)

= 0.26211

Since the infectivity is increasing as the dilution increases, the 50% end point dilution is higher than the starting point dilution and is calculated by adding the difference of logarithms as follows:

Log (reciprocal of the starting point dilution) + difference of logarithms

Log 50% next lowest to end point = log 50 + 0.26211

= 1.6989 + 0.26211

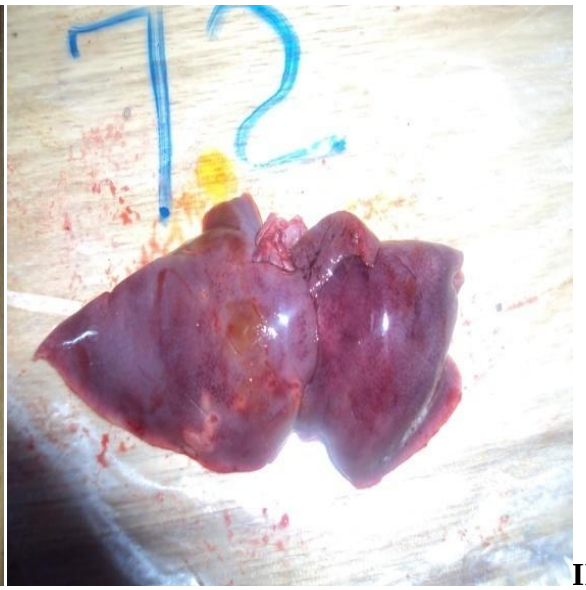
= 1.96 approximately

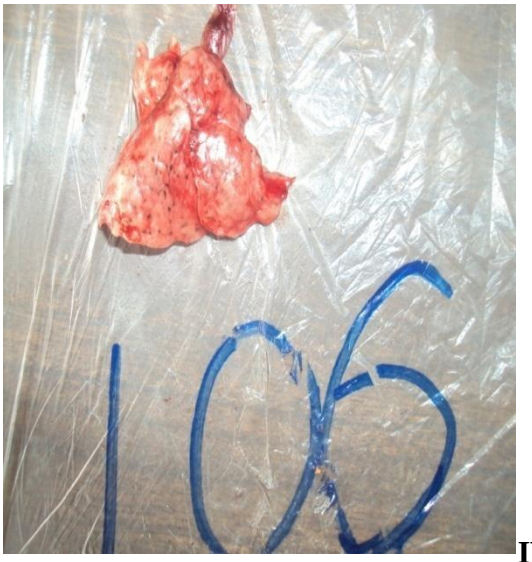
Hence, log (50% end point dilution) = 1.96

Antilog 50% endpoint = $10^{-1.96}$ = 91.2

Therefore the antibody titer at 50% end point dilution = 1: 91

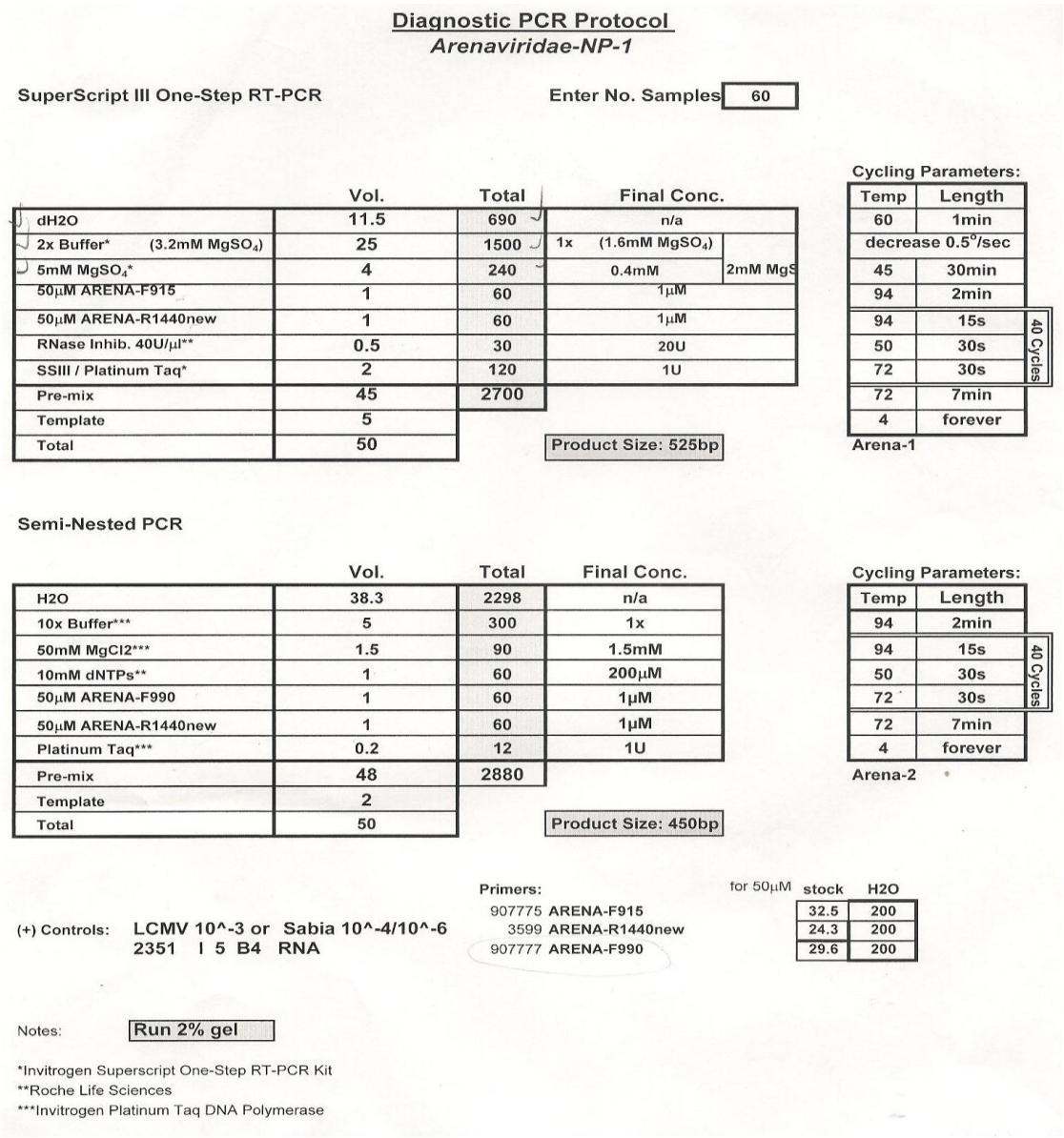
Appendix X

I   **II**

III   **IV**

Plates I-IV showing pathologies observed in some bats that were collected: An *E.helvum* bat with intact internal organs (Plate I) from which the liver with necrotic lesion (Plate II) was obtained. Splenomegaly with nodular lesions on spleen ((Plate III) and lungs with multiple areas of fibrosis (Plate IV).

Appendix XI



Diagnostic PCR Protocol
Arenaviridae-NP-1

SuperScript III One-Step RT-PCR — Enter No. Samples: 60

	Vol.	Total	Final Conc.
dH2O	11.5	690	n/a
2x Buffer* (3.2mM $MgSO_4$)	25	1500	1x (1.6mM $MgSO_4$)
5mM $MgSO_4$*	4	240	0.4mM 2mM MgS
50µM ARENA-F915	1	60	1µM
50µM ARENA-R1440new	1	60	1µM
RNase Inhib. 40U/µl**	0.5	30	20U
SSIII / Platinum Taq*	2	120	1U
Pre-mix	45	2700	
Template	5		
Total	50		Product Size: 525bp

Cycling Parameters:

Temp	Length	
60	1min	
decrease 0.5°/sec		
45	30min	
94	2min	
94	15s	40 Cycles
50	30s	
72	30s	
72	7min	
4	forever	

Arena-1

Semi-Nested PCR

	Vol.	Total	Final Conc.
H2O	38.3	2298	n/a
10x Buffer***	5	300	1x
50mM MgCl2***	1.5	90	1.5mM
10mM dNTPs**	1	60	200µM
50µM ARENA-F990	1	60	1µM
50µM ARENA-R1440new	1	60	1µM
Platinum Taq***	0.2	12	1U
Pre-mix	48	2880	
Template	2		
Total	50		Product Size: 450bp

Cycling Parameters:

Temp	Length	
94	2min	
94	15s	40 Cycles
50	30s	
72	30s	
72	7min	
4	forever	

Arena-2

(+) Controls: LCMV 10^-3 or Sabia 10^-4/10^-6
2351 I 5 B4 RNA

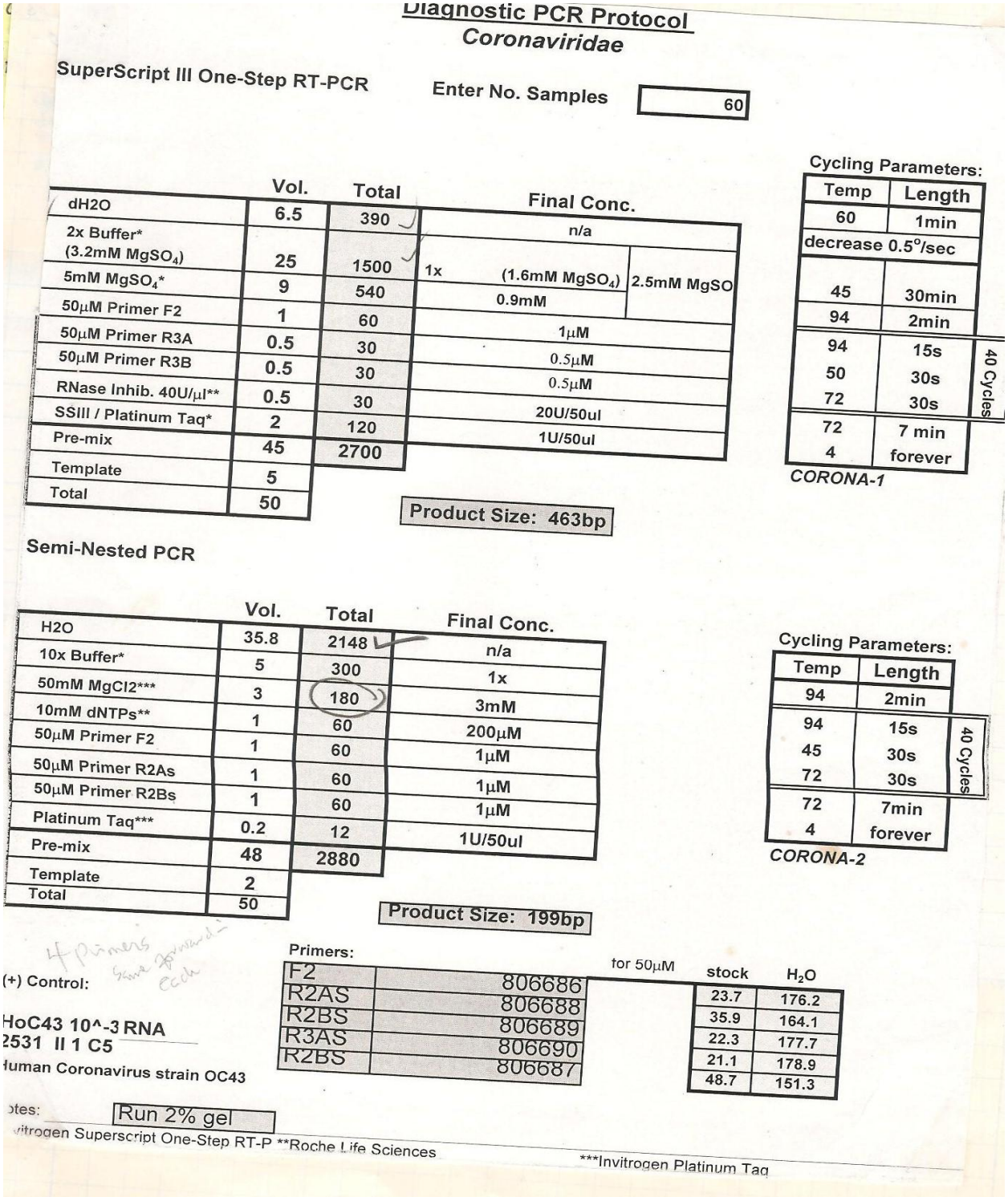
Primers:
907775 ARENA-F915
3599 ARENA-R1440new
907777 ARENA-F990

for 50µM	stock	H2O
	32.5	200
	24.3	200
	29.6	200

Notes: Run 2% gel

*Invitrogen Superscript One-Step RT-PCR Kit
**Roche Life Sciences
***Invitrogen Platinum Taq DNA Polymerase

Appendix XII



Diagnostic PCR Protocol
Coronaviridae

SuperScript III One-Step RT-PCR

Enter No. Samples: 60

	Vol.	Total	Final Conc.		
dH2O	6.5	390	n/a		
2x Buffer* (3.2mM $MgSO_4$)	25	1500	1x	(1.6mM $MgSO_4$)	2.5mM MgSO
5mM $MgSO_4$*	9	540	0.9mM		
50µM Primer F2	1	60	1µM		
50µM Primer R3A	0.5	30	0.5µM		
50µM Primer R3B	0.5	30	0.5µM		
RNase Inhib. 40U/µl**	0.5	30	20U/50ul		
SSIII / Platinum Taq*	2	120	1U/50ul		
Pre-mix	45	2700			
Template	5				
Total	50				

Cycling Parameters:

Temp	Length	
60	1min	
decrease 0.5°/sec		
45	30min	
94	2min	
94	15s	40 Cycles
50	30s	
72	30s	
72	7 min	
4	forever	

CORONA-1

Product Size: 463bp

Semi-Nested PCR

	Vol.	Total	Final Conc.
H2O	35.8	2148	n/a
10x Buffer*	5	300	1x
50mM MgCl2***	3	180	3mM
10mM dNTPs**	1	60	200µM
50µM Primer F2	1	60	1µM
50µM Primer R2As	1	60	1µM
50µM Primer R2Bs	1	60	1µM
Platinum Taq***	0.2	12	1U/50ul
Pre-mix	48	2880	
Template	2		
Total	50		

Cycling Parameters:

Temp	Length	
94	2min	
94	15s	40 Cycles
45	30s	
72	30s	
72	7min	
4	forever	

CORONA-2

Product Size: 199bp

4 Primers same dilution each

(+) Control:

HoC43 10^-3 RNA
2531 II 1 C5
Human Coronavirus strain OC43

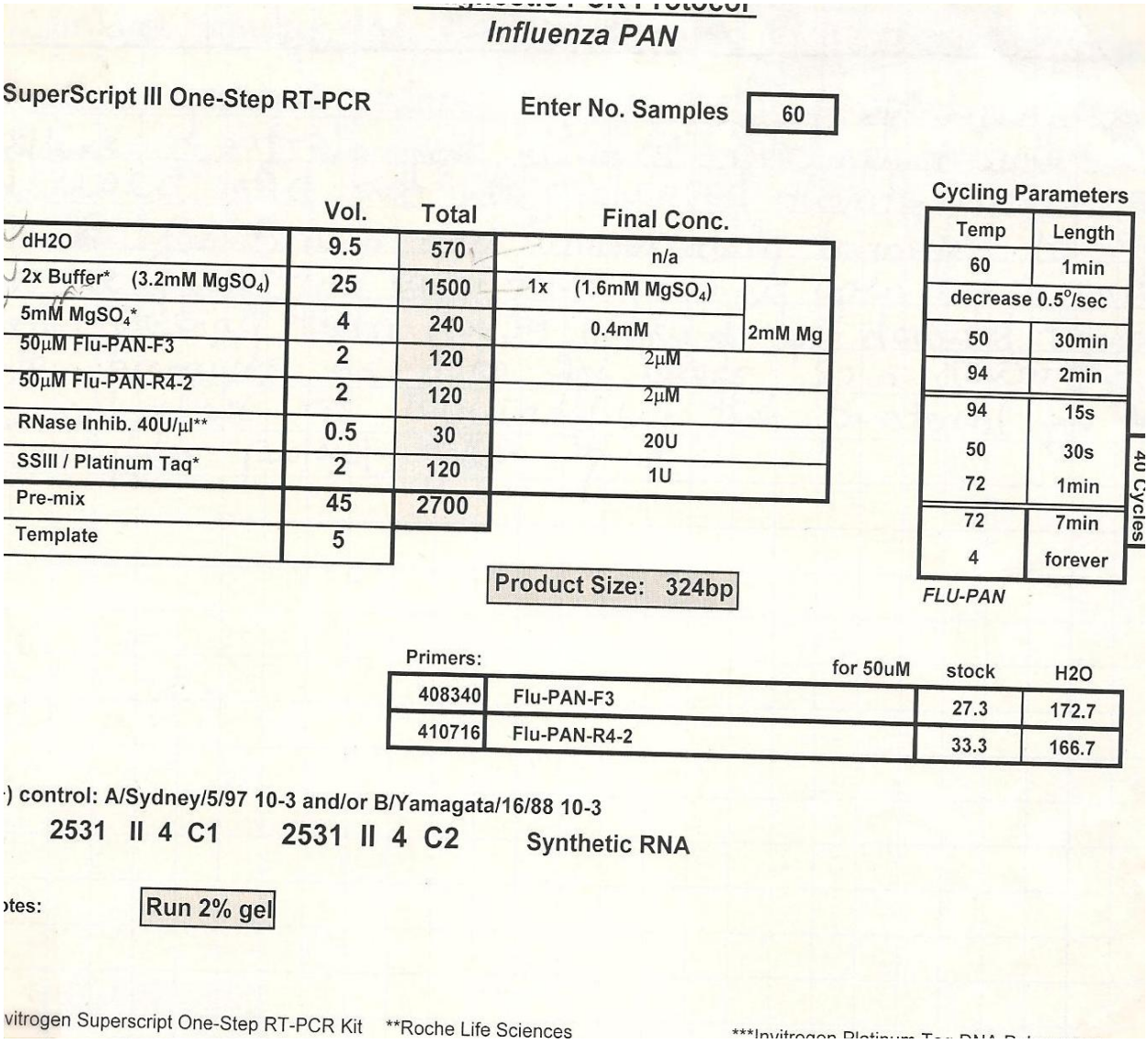
Primers:

		for 50µM stock	H_2O
F2	806686	23.7	176.2
R2AS	806688	35.9	164.1
R2BS	806689	22.3	177.7
R3AS	806690	21.1	178.9
R2BS	806687	48.7	151.3

Notes: Run 2% gel

Invitrogen Superscript One-Step RT-P **Roche Life Sciences ***Invitrogen Platinum Taq

Appendix XIII



Influenza PAN

SuperScript III One-Step RT-PCR

Enter No. Samples: 60

	Vol.	Total	Final Conc.	
dH2O	9.5	570	n/a	
2x Buffer* (3.2mM $MgSO_4$)	25	1500	1x (1.6mM $MgSO_4$)	2mM Mg
5mM $MgSO_4$*	4	240	0.4mM	
50µM Flu-PAN-F3	2	120	2µM	
50µM Flu-PAN-R4-2	2	120	2µM	
RNase Inhib. 40U/µl**	0.5	30	20U	
SSIII / Platinum Taq*	2	120	1U	
Pre-mix	45	2700		
Template	5			

Product Size: 324bp

Cycling Parameters

Temp	Length	
60	1min	
decrease 0.5°/sec		
50	30min	
94	2min	
94	15s	40 Cycles
50	30s	
72	1min	
72	7min	
4	forever	

FLU-PAN

Primers:

		for 50uM	stock	H2O
408340	Flu-PAN-F3		27.3	172.7
410716	Flu-PAN-R4-2		33.3	166.7

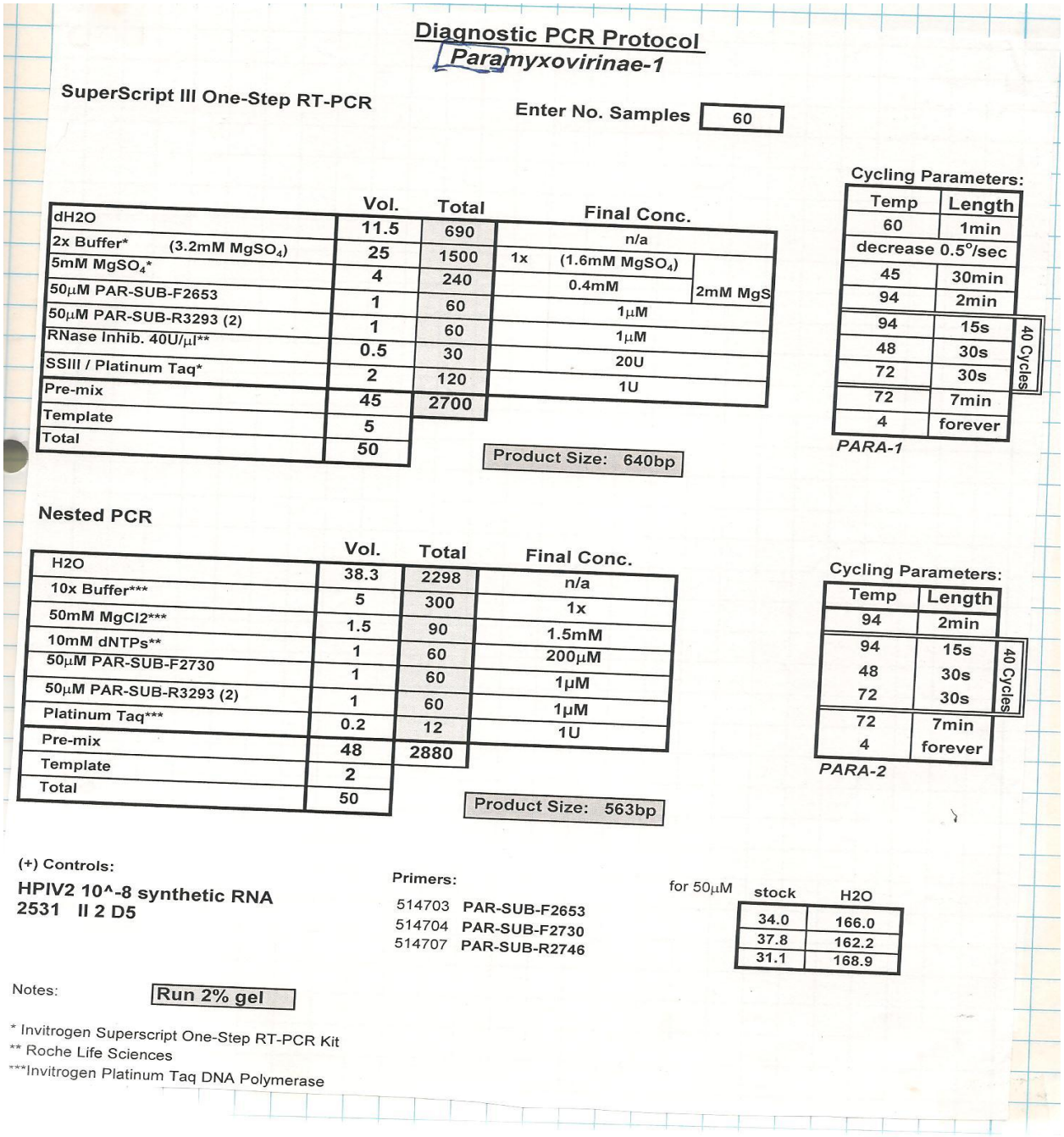
') control: A/Sydney/5/97 10-3 and/or B/Yamagata/16/88 10-3

2531 II 4 C1 2531 II 4 C2 Synthetic RNA

otes: Run 2% gel

vitrogen Superscript One-Step RT-PCR Kit **Roche Life Sciences ***Invitrogen Platinum Taq DNA P

Appendix XIV



Diagnostic PCR Protocol
Paramyxovirinae-1

SuperScript III One-Step RT-PCR

Enter No. Samples: 60

	Vol.	Total	Final Conc.	
dH2O	11.5	690	n/a	
2x Buffer* (3.2mM $MgSO_4$)	25	1500	1x (1.6mM $MgSO_4$)	
5mM $MgSO_4$*	4	240	0.4mM	2mM MgS
50µM PAR-SUB-F2653	1	60	1µM	
50µM PAR-SUB-R3293 (2)	1	60	1µM	
RNase Inhib. 40U/µl**	0.5	30	20U	
SSIII / Platinum Taq*	2	120	1U	
Pre-mix	45	2700		
Template	5			
Total	50			

Product Size: 640bp

Cycling Parameters:

Temp	Length	
60	1min	
decrease 0.5°/sec		
45	30min	
94	2min	
94	15s	40 Cycles
48	30s	
72	30s	
72	7min	
4	forever	

PARA-1

Nested PCR

	Vol.	Total	Final Conc.
H2O	38.3	2298	n/a
10x Buffer***	5	300	1x
50mM MgCl2***	1.5	90	1.5mM
10mM dNTPs**	1	60	200µM
50µM PAR-SUB-F2730	1	60	1µM
50µM PAR-SUB-R3293 (2)	1	60	1µM
Platinum Taq***	0.2	12	1U
Pre-mix	48	2880	
Template	2		
Total	50		

Product Size: 563bp

Cycling Parameters:

Temp	Length	
94	2min	
94	15s	40 Cycles
48	30s	
72	30s	
72	7min	
4	forever	

PARA-2

(+) Controls:
HPIV2 10^-8 synthetic RNA
2531 II 2 D5

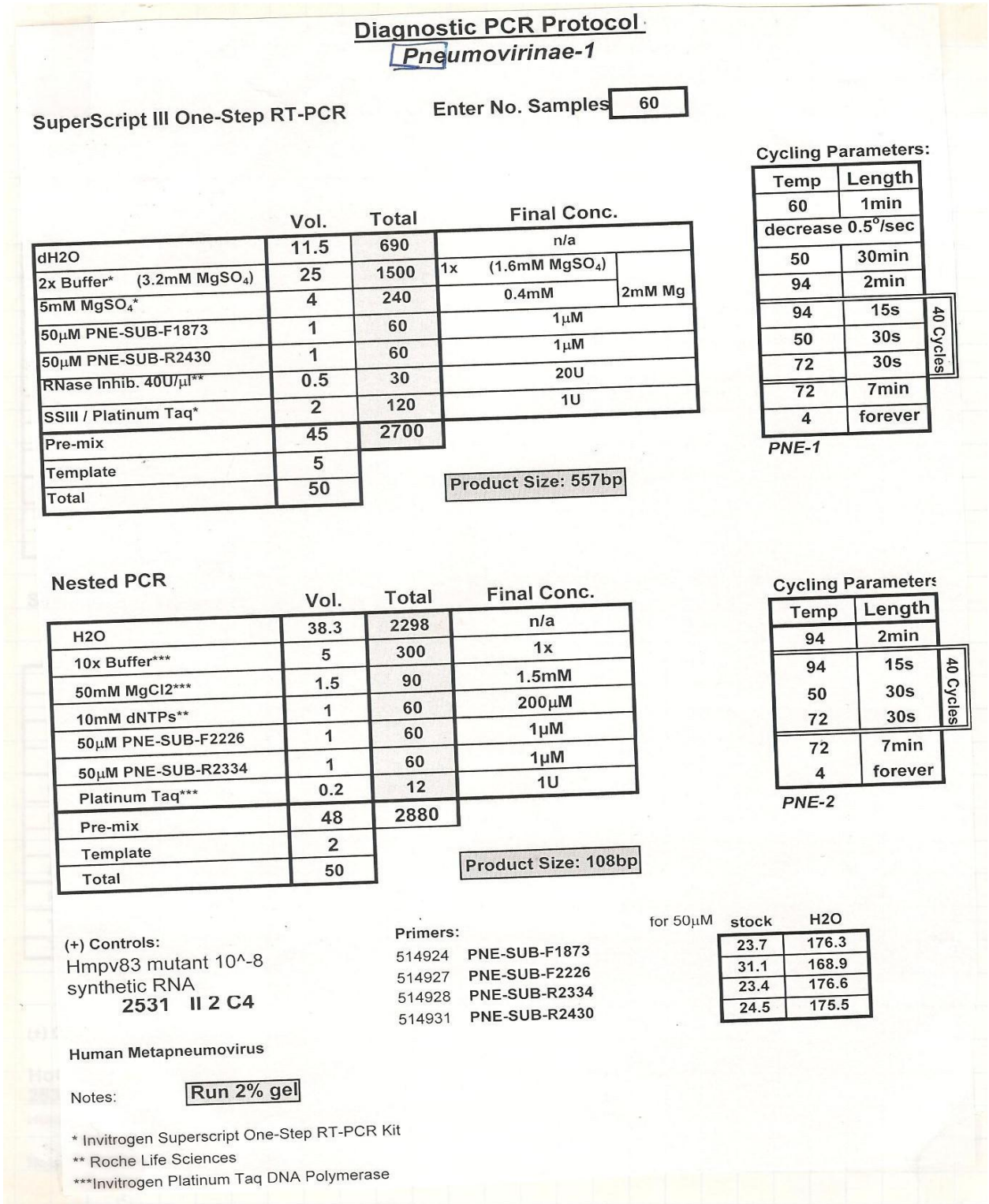
Primers:
514703 PAR-SUB-F2653
514704 PAR-SUB-F2730
514707 PAR-SUB-R2746

for 50µM	stock	H2O
	34.0	166.0
	37.8	162.2
	31.1	168.9

Notes: Run 2% gel

* Invitrogen Superscript One-Step RT-PCR Kit
** Roche Life Sciences
***Invitrogen Platinum Taq DNA Polymerase

AppendixXV



Diagnostic PCR Protocol
Pneumovirinae-1

SuperScript III One-Step RT-PCR Enter No. Samples: 60

	Vol.	Total	Final Conc.	
dH2O	11.5	690	n/a	
2x Buffer* (3.2mM $MgSO_4$)	25	1500	1x (1.6mM $MgSO_4$)	
5mM $MgSO_4$*	4	240	0.4mM	2mM Mg
50µM PNE-SUB-F1873	1	60	1µM	
50µM PNE-SUB-R2430	1	60	1µM	
RNase Inhib. 40U/µl**	0.5	30	20U	
SSIII / Platinum Taq*	2	120	1U	
Pre-mix	45	2700		
Template	5			
Total	50			

Product Size: 557bp

Cycling Parameters:

Temp	Length	
60	1min	
decrease 0.5°/sec		
50	30min	
94	2min	
94	15s	40 Cycles
50	30s	
72	30s	
72	7min	
4	forever	

PNE-1

Nested PCR

	Vol.	Total	Final Conc.
H2O	38.3	2298	n/a
10x Buffer***	5	300	1x
50mM MgCl2***	1.5	90	1.5mM
10mM dNTPs**	1	60	200µM
50µM PNE-SUB-F2226	1	60	1µM
50µM PNE-SUB-R2334	1	60	1µM
Platinum Taq***	0.2	12	1U
Pre-mix	48	2880	
Template	2		
Total	50		

Product Size: 108bp

Cycling Parameters

Temp	Length	
94	2min	
94	15s	40 Cycles
50	30s	
72	30s	
72	7min	
4	forever	

PNE-2

(+) Controls:
Hmpv83 mutant 10^-8
synthetic RNA
2531 II 2 C4

Human Metapneumovirus

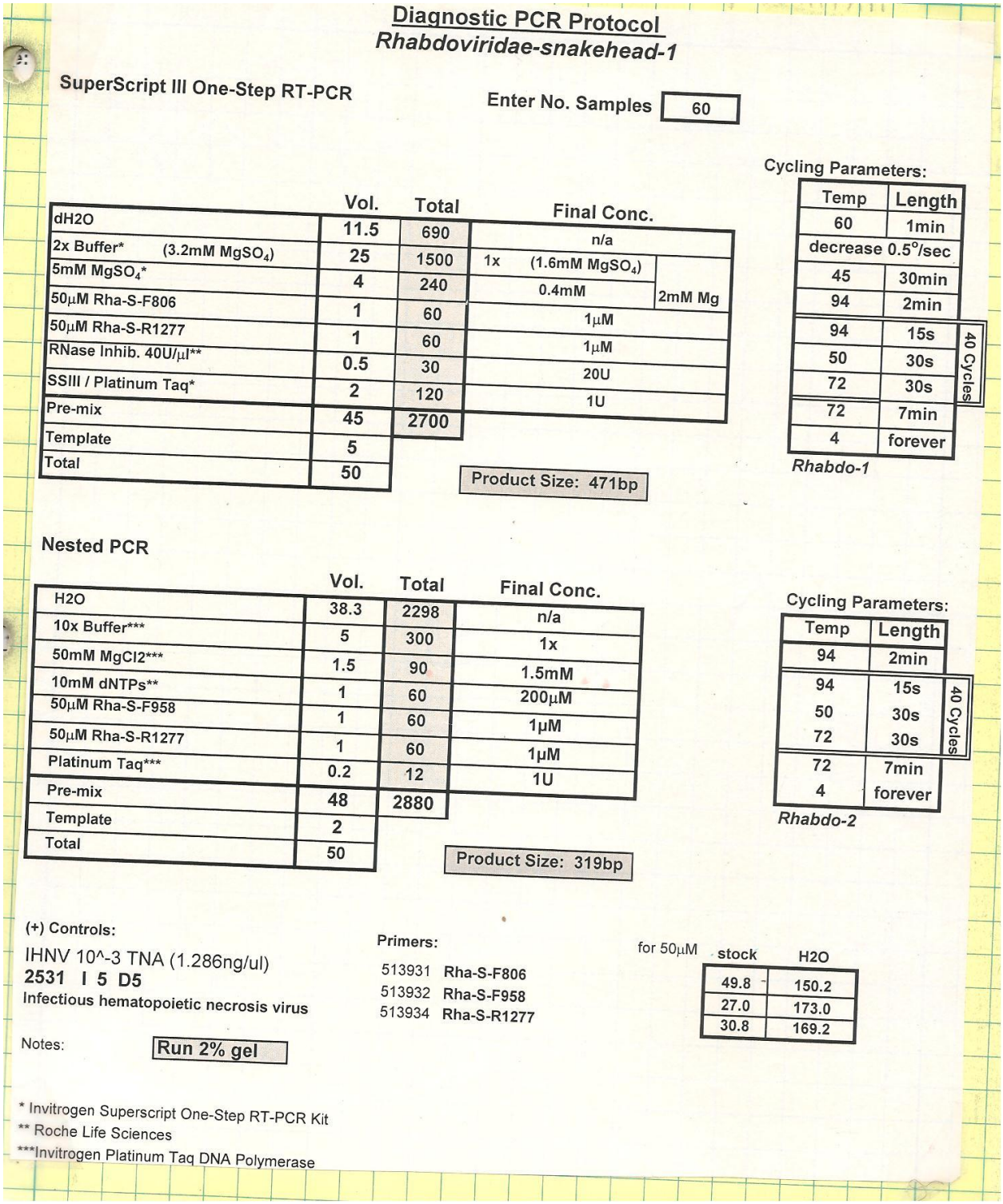
Primers:
514924 PNE-SUB-F1873
514927 PNE-SUB-F2226
514928 PNE-SUB-R2334
514931 PNE-SUB-R2430

for 50µM	stock	H2O
	23.7	176.3
	31.1	168.9
	23.4	176.6
	24.5	175.5

Notes: Run 2% gel

* Invitrogen Superscript One-Step RT-PCR Kit
** Roche Life Sciences
***Invitrogen Platinum Taq DNA Polymerase

Appendix XVI



Diagnostic PCR Protocol
Rhabdoviridae-snakehead-1

SuperScript III One-Step RT-PCR

Enter No. Samples: 60

	Vol.	Total	Final Conc.
dH2O	11.5	690	n/a
2x Buffer* (3.2mM $MgSO_4$)	25	1500	1x (1.6mM $MgSO_4$)
5mM $MgSO_4$*	4	240	0.4mM (2mM Mg)
50µM Rha-S-F806	1	60	1µM
50µM Rha-S-R1277	1	60	1µM
RNase Inhib. 40U/µl**	0.5	30	20U
SSIII / Platinum Taq*	2	120	1U
Pre-mix	45	2700	
Template	5		
Total	50		

Product Size: 471bp

Cycling Parameters:

Temp	Length	
60	1min	
decrease 0.5°/sec		
45	30min	
94	2min	
94	15s	40 Cycles
50	30s	
72	30s	
72	7min	
4	forever	

Rhabdo-1

Nested PCR

	Vol.	Total	Final Conc.
H2O	38.3	2298	n/a
10x Buffer***	5	300	1x
50mM MgCl2***	1.5	90	1.5mM
10mM dNTPs**	1	60	200µM
50µM Rha-S-F958	1	60	1µM
50µM Rha-S-R1277	1	60	1µM
Platinum Taq***	0.2	12	1U
Pre-mix	48	2880	
Template	2		
Total	50		

Product Size: 319bp

Cycling Parameters:

Temp	Length	
94	2min	
94	15s	40 Cycles
50	30s	
72	30s	
72	7min	
4	forever	

Rhabdo-2

(+) Controls:

IHNV 10^-3 TNA (1.286ng/ul)
2531 I 5 D5
Infectious hematopoietic necrosis virus

Primers:

513931 Rha-S-F806
513932 Rha-S-F958
513934 Rha-S-R1277

for 50µM	stock	H2O
	49.8	150.2
	27.0	173.0
	30.8	169.2

Notes: **Run 2% gel**

* Invitrogen Superscript One-Step RT-PCR Kit
** Roche Life Sciences
***Invitrogen Platinum Taq DNA Polymerase

Appendix XVII

Diagnostic PCR Protocol
Rhabdoviridae-VSV-1

SuperScript III One-Step RT-PCR **Enter No. Samples** 60

	Vol.	Total	Final Conc.	
dH2O	11.5	690	n/a	
2x Buffer* (3.2mM $MgSO_4$)	25	1500	1x (1.6mM $MgSO_4$)	
5mM $MgSO_4$*	4	240	0.4mM	2mM Mg
50µM Rha-F1926	1	60	1µM	
50µM Rha-R2250	1	60	1µM	
RNase Inhib. 40U/µl**	0.5	30	20U	
SSIII / Platinum Taq*	2	120	1U	
Pre-mix	45	2700		
Template	5			
Total	50		Product Size: 324bp	

Cycling Parameters:

Temp	Length	
60	1min	
decrease 0.5°/sec		
45	30min	
94	2min	
94	15s	40 Cycles
50	30s	
72	30s	
72	7min	
4	forever	

Rhabdo-1

Nested PCR

	Vol.	Total	Final Conc.
H2O	38.3	2298	n/a
10x Buffer***	5	300	1x
50mM $MgCl_2$***	1.5	90	1.5mM
10mM dNTPs**	1	60	200µM
50µM Rha-F2014	1	60	1µM
50µM Rha-R2250	1	60	1µM
Platinum Taq***	0.2	12	1U
Pre-mix	48	2880	
Template	2		
Total	50		Product Size: 236bp

766 x 3

1200 Aliquot

Cycling Parameters:

Temp	Length	
94	2min	
94	15s	40 Cycles
50	30s	
72	30s	
72	7min	
4	forever	

Rhabdo-2

(+) Controls:

VSV 10^-3 RNA
2531 I 5 D2
Vesicular Stomatitis Virus

Primers:

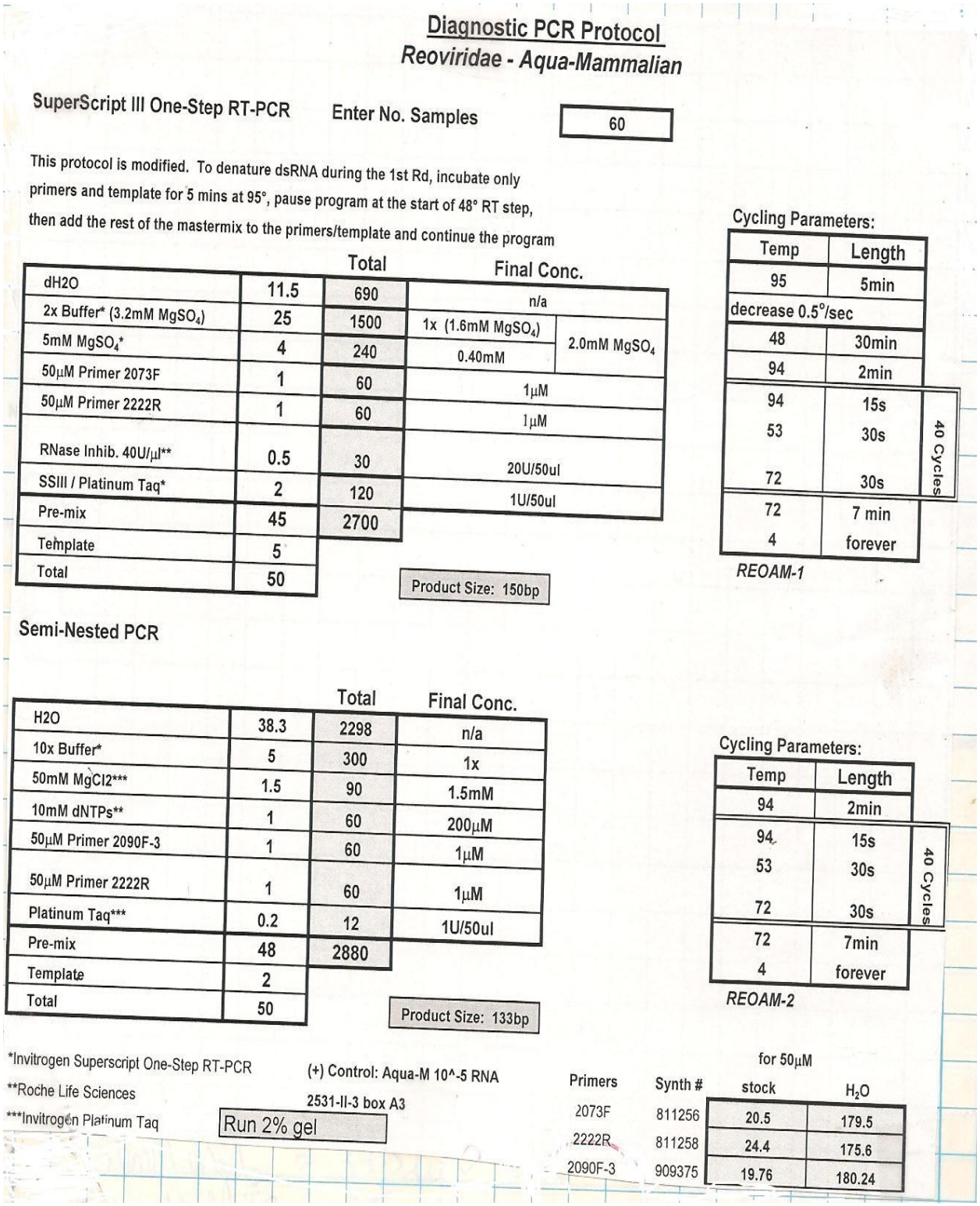
513344 Rha-F1926
513345 Rha-F2014
513349 Rha-R2250

for 50µM	stock	H2O
	31.8	168.2
	15.4	184.6
	24.4	175.6

Notes: Run 2% gel

* Invitrogen Superscript One-Step RT-PCR Kit
** Roche Life Sciences
***Invitrogen Platinum Taq DNA Polymerase

Appendix XVIII



Diagnostic PCR Protocol
Reoviridae - Aqua-Mammalian

SuperScript III One-Step RT-PCR Enter No. Samples: 60

This protocol is modified. To denature dsRNA during the 1st Rd, incubate only primers and template for 5 mins at 95°, pause program at the start of 48° RT step, then add the rest of the mastermix to the primers/template and continue the program

		Total	Final Conc.	
dH2O	11.5	690	n/a	
2x Buffer* (3.2mM $MgSO_4$)	25	1500	1x (1.6mM $MgSO_4$)	2.0mM $MgSO_4$
5mM $MgSO_4$*	4	240	0.40mM	
50µM Primer 2073F	1	60	1µM	
50µM Primer 2222R	1	60	1µM	
RNase Inhib. 40U/µl**	0.5	30	20U/50ul	
SSIII / Platinum Taq*	2	120	1U/50ul	
Pre-mix	45	2700		
Template	5			
Total	50			

Product Size: 150bp

Cycling Parameters: *REOAM-1*

Temp	Length	
95	5min	
decrease 0.5°/sec		
48	30min	
94	2min	
94	15s	40 Cycles
53	30s	
72	30s	
72	7 min	
4	forever	

Semi-Nested PCR

		Total	Final Conc.
H2O	38.3	2298	n/a
10x Buffer*	5	300	1x
50mM $MgCl2$***	1.5	90	1.5mM
10mM dNTPs**	1	60	200µM
50µM Primer 2090F-3	1	60	1µM
50µM Primer 2222R	1	60	1µM
Platinum Taq***	0.2	12	1U/50ul
Pre-mix	48	2880	
Template	2		
Total	50		

Product Size: 133bp

Cycling Parameters: *REOAM-2*

Temp	Length	
94	2min	
94	15s	40 Cycles
53	30s	
72	30s	
72	7min	
4	forever	

*Invitrogen Superscript One-Step RT-PCR
**Roche Life Sciences
***Invitrogen Platinum Taq

(+) Control: Aqua-M 10^-5 RNA
2531-II-3 box A3
Run 2% gel

Primers	Synth #	for 50µM stock	H_2O
2073F	811256	20.5	179.5
2222R	811258	24.4	175.6
2090F-3	909375	19.76	180.24

Appendix XIX

Diagnostic PCR Protocol
Reoviridae - Rotavirus 24 - 1

SuperScript III One-Step RT-PCR Enter No. Samples: 60

This protocol is modified. To denature dsRNA during the 1st Rd, incubate only primers and template for 5 mins at 95°, pause program at the start of 48° RT step, then add the rest of the mastermix to the primers/template and continue the program

		Total	Final Conc.	
dH2O	11.5	690	n/a	
2x Buffer* (3.2mM $MgSO_4$)	25	1500	1x (1.6mM $MgSO_4$)	2.0mM $MgSO_4$
5mM $MgSO_4$*	4	240	0.40mM	
50µM Primer 1738F	1	60	1µM	
50µM Primer 2100R	1	60	1µM	
RNase Inhib. 40U/µl**	0.5	30	20U/50ul	
SSIII / Platinum Taq*	2	120	1U/50ul	
Pre-mix	45	2700		
Template	5			
Total	50			

Product Size: 363 bp

Cycling Parameters:

Temp	Length	
95	5min	
decrease 0.5°/sec		
48	30min	
94	2min	
94	15s	40 Cycles
50	30s	
72	30s	
72	7 min	
4	forever	

REO-1

Semi-Nested PCR

		Total	Final Conc.
H2O	37.3	2238	n/a
10x Buffer*	5	300	1x
50mM MgCl2***	1.5	90	1.5mM
10mM dNTPs**	1	60	200µM
50µM Primer 1756F-1A	1	30	0.5µM
50µM Primer 1756F-1B	1	30	0.5µM
50µM Primer 1985R	1	60	1µM
Platinum Taq***	0.2	12	1U/50ul
Pre-mix	48	2880	
Template	2		
Total	50		

Product Size: 230 bp

Cycling Parameters:

Temp	Length	
94	2min	
94	15s	40 Cycles
50	30s	
72	30s	
72	7min	
4	forever	

REO-2

*Invitrogen Superscript One-Step RT-PCR
**Roche Life Sciences
***Invitrogen Platinum Taq

(+) Control: Rotavirus A DS-1
10^-5 RNA
2531 I 5 B3

Run 2% gel

Primers	Synth #	for 50µM stock	H_2O
1738F	811226	25	175
2100R	811231	20.3	179.7
1985R	811229	19.6	180.4
1756F-1A	910200	28.16	171.84
1756F-1B	910201	28.92	171.08