MOLECULAR STUDY ON THE N GENE OF RABIES VIRUS ISOLATED IN DOGS FROM TWO SELECTED LOCAL GOVERNMENT AREAS OF BENUE STATE, NIGERIA

BY

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DEDICATION

I dedicate this Dissertation to God Almighty, the very essence of my being, and to my lovely parents, Mr. and Mrs.AlhajiAdaji, the ultimate source of my inspiration.

DECLARATION

I declare that the work of this dissertation entitled "Molecular Studies of the N Gene of Rabies Virus Isolated in Dogs from Benue State", has been carried out by me in the Department of Biochemistry, Ahmadu Bello University, Zaria. The information obtained 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.

_____ _____

Joseph Otafu ADAJIDate

CERTIFICATION

This dissertation entitled "MOLECULAR STUDY ON THE N GENE OF RABIES VIRUS ISOLATED IN DOGS FROM TWO SELECTED LOCAL GOVERNMENT AREAS OF BENUE STATE, NIGERIA" by JosephOtafu ADAJI meets the regulation governing the award of the degree of M.Sc Biotechnology of the Ahmadu Bello University, Zaria, and is approved for its contribution to knowledge and literary presentation.

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ABSTRACT

Rabies is a viral zoonosis that causes encephalomyelitis. The rabies virus is a negative sense non-segmented RNA virus that codes for 5 proteins: N protein, P protein, M protein, G protein, and L protein. This research was aimed at carrying out molecular studies on a segment of N gene of rabies virus isolated from dogs in Benue State. The study revealed the presence of Nucleo protein antigen using the Direct Fluorescent Antibody Test (DFAT) in 2% of the 50 dog brain samples collected for the study. Sixty eight percent (68%) of the samples were from male dogs while 32% were from females. The 1 DFAT positive sample was from a male dog. The positive Sample (J50) with DFAT fluorescence score of (++++) was positive for Polymerase Chain Reaction (PCR) amplification of N-gene segment using N-gene specific forward primer; RabN-533F; 5'-CATTGCAGATAGGATAGAGC-3' and reverse primer; RabN-888R; 5'-GAGGAACGGCGGTCTCCTG-3' primer set. The amplified N gene segment of the isolate was sequenced and compared with 31 other rabies virus N gene sequences from the Gene bank. There was a maximum homology of 99% (KR080522 Dog from Bida 2012) and a minimum of 93% () . The group variation occurred at 32 different Non-serial positions of the nucleic acid of the gene, with a group variation rate of 8.3%. The phylogenic analysis gave an evolutionary pattern, showing the rabies virus is of West African origin belonging to the African 2 lineage and a possible history of Cameroonian origin considering that in an earlier year a similar rabies virus was isolated in that country and also might be associated with the fact that Benue state share border with the country.

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CHAPTER ONE

1.0 INTRODUCTION

1.1 Background of Study

Rabies comes from the Latin word *rabere*, which means to rage or rave. Rabid dogs sometimes appear to be angry or in a rage. About 3000 BC, this Latin word *rabere* may have roots in a Sanskrit word *rabhas* meaning "violence" (where the victim becomes violent) (Nottidge, 2005). The Greeks called rabies *lyssa* or *lytta*, which means frenzy or madness. It has many synonyms worldwide such as delirious, ferocious, feverish, fierce, furious, violent and wild etc (Hossein, 2012).

Rabies is a viral zoonosis that causes encephalomyelitis (Dacheux *et al*., 2012). It affects all warm-blooded animals (Warrell and Warrell, 2004; Adedeji *et al*., 2010), and having almost 100% case-fatality rate (WHO, 2000). Human mortality due to rabies is estimated to be 50,000 deaths per year worldwide, mostly reported from Asia and Africa (Dacheux *et al*., 2012; Yousaf *et al*., 2012). The disease is of worldwide public health importance (Streicker *et al*., 2012). And have been found to be endemic in Nigeria (Ogunkoya, 2007; Ehimiyein *et al*., 2010; Okpe *et al*., 2011). In Nigeria, the first human rabies case was diagnosed in 1912 (Boulger and Hardy, 1960), while canine rabies was first diagnosed in 1925 at Yaba Rabies Laboratory, Lagos, Nigeria and officially reported in 1927 (Boulger and Hardy, 1960).

Dogs have been confirmed as the reservoirs and vectors of human rabies, accounting for 94% of the cases (Nel and Rupprecht, 2007). At least, 10,000 persons have been estimated to be exposed to rabies annually in Nigeria, which is apparently underestimated (Ehizibolo *et al*., 2011) because there is no efficient monitoring and reporting system of the disease. In Nigeria and many

other developing countries in the tropics, at least 10% of brain samples received at laboratories for rabies diagnosis are decomposed because of inadequacy of transportation and storage facilities (Barrat, 1996; Elsa & Ogunkoya, 1996; Durr *et al.*, 2008), leading to the misdiagnosis of most of the cases. For efficient transportation, various methods of preservation and transportation have been experimented (Elsa and Ogunkoya, 1996; Durr *et al.*, 2008). The use of 50% glycerol/phosphate-buffered sulphate for preservation is commonly practiced in Nigeria, and it is assumed that the virus remains viable in the medium for over six months (Elsa and Ogunkoya, 1996).

The Nucleoprotein gene which is the first gene after the leader sequence (LDR) of approximately 50bp, has a size of 1424bp and codes for the N protein, which is the most abundant protein in the Ribo Nucleo Protein (RNP) complex and is involved in the encapsidation of viral genome (Wunner, 2007). Besides, the N gene is also the most conserved gene in the viral genome, making it an ideal candidate for phylogenetic analysis (Wunner *et al.*, 1989). It was found that the N protein was phosphorylated in Rabies Virus possibly by a cellular casein kinase II and different phosphorylation states of N protein played an important role in viral transcription and replication (Wu *et al.*, 2009). Furthermore, previous studies have mapped several functional regions to the N protein. It has been shown that in addition to the G protein, the N protein also possessed antigenic and immunogenic properties. A number of antigenic sites have been identified in the N protein, including antigenic site I (aa 358-367), antigenic site II (aa 373-383) and antigenic site IV (aa 359=366 and aa 375-383) (Minamoto *et al.*, 1994, Goto *et al.*, 2000), and a phosphorylation site, Ser389, was also involved in the N antigenicity (Kouznetzoff, *et al.*, 1994; Kawai, *et al.*, 1999). In addition, several RABV-specific Th cell epitopes were identified in the N protein, comparing aa 21-35 and the so-called 31D (aa 404-418), the latter of which was

shown to be an immunodominant epitope to stimulate RABV-specific T bcell production in vitro. Finally, the RNA-binding site on N gene for interaction with viral RNA has been localized to region aa 298-352 of the N protein (Kouznetzoff *et al.*, 1998).

Rabies virus is very sensitive to environmental factors. It is highly sensitive to sunlight, ultraviolet irradiation and heat at 60^0C (for five minutes), lipid solvents (70% alcohol and ether), sodium deoxycholate, trypsin, and common detergents. However, it is preserved at sub-zero temperature and in glycerol (Awoyomi *et al.*, 2007; Adedeji *et al.*, 2010).

Dogs have been the major reservoirs of rabies to humans in Africa (Fitzpatrick *et al.*, 2012). Approximately 24,000 deaths occur per year in Africa, although most of the incidents are under-reported. The sites of exposures are usually in the rural areas where prompt diagnosis and post exposure prophylaxis are not readily available thereby leading to increase in human deaths. Some factors militate against the control and elimination of rabies in Africa; socio-cultural beliefs, lack of prompt field-based diagnostic tests, funding, illiteracy, etc. (Yousaf *et al.*, 2012).

1.2 Statement of Research Problem

The domestic dog is the main source of exposure and vector for human rabies in Africa (Zinsstag *et al.*, 2007). The domestic dog (*Canis familiaris*) plays a pivotal role in rabies transmission, with 85-95% of human rabies cases being associated with dog bites due to their close association with man (Mckenzie, 1993). Rabies remains a major public health threat and this is attributable to the low priority given to the disease (Ogo, 2009). In Benue and Nasarawa States there are no religious or cultural restrictions on the domestic keeping of dogs; hence dogs are freely kept by families in the city. Presence of dump sites in some locations in the city encourage dog

movement as they scavenge for food. Dog meat btchers also ply their trade in specific regions in the States and majority of these individuals are unaware of the risk they are exposed to in the course of their activities (Akombo, 2009)

Despite the availability of effective human and animal vaccines against rabies, and other measures for its control, rabies continues to account for at least 55,000 human deaths each year, mainly in the developing countries of Africa and Asia (Meslin *et al.*, 1994; Knobel *et al.*,2005). In these countries, most human rabies infections resulted from exposure to infected dogs, through bites, scratches, and mucosal exposures (Warrell, 1988). Vaccination of animals against rabies and postexposure prophylaxis (PEP) for humans is prohibitively expensive for most African governments, and it has long been contended that the effects of rabies are underestimated in Africa Knobel *et al.*,2005.

In Nigeria where dog bites continue to be the main mode of transmission of the disease to man, it remains a serious public health hazard (Awoyomi *et al.*, 2007). Since dogs have been established as the predominant vectors of rabies in Nigeria, the most logical and cost–effective approach to rabies control is elimination of stray and un-owned dogs combined with a programme of single mass immunization in the shortest possible time, covering at least 80% of the entire dog population (Awoyomi *et al.*, 2007). It appears many Nigerians still see rabies as insignificant and do not take serious measures to prevent or control its occurrence. Many people are still ignorant about the disease and do not vaccinate their dogs because they claimed it changes the taste of dog meat and affects the development of canine teeth for hunting and protection (Idachaba *et al.*, 2009).

However, rabies as a fatal viral zoonosis remains an important public health problem in Nigeria due to uncontrolled enzootic rabies, lack of vaccination and poor information. Questions are raised why rabies has not evolved more rapidly in the New World, given the frequency and ease with which antigenic changes can be induced in the laboratory, and how the virus became so extensively established in New World bats (Shope, 1982). However, the situation reported in Nigeria, only reaffirms that rabies is more of a neglected tropical disease than thought and which deserves attention (Durosinloun, 2009; Fagbo, 2009; Woolf, 2009).

Ajayi *et al*. (2006) in a histological and immunohistochemical studies reported the presence of rabies in apparently healthy dogs. Also, in 2006, Opaleye *et al*. (2006) reported the knowledge, attitude and practices among some Nigerians as the key factors affecting the control of rabies in Nigeria. In their study among 679 individuals, only 33.4% of the respondents knew rabies could be prevented by vaccination, while 38.7% believed that the infection could be treated with herbs. Of the 387 victims of dog bite, 240 (62%) never sought prophylactic postexposure treatment. Of the 10 people who received postexposure treatment, only one received the appropriate treatment consisting of washing, disinfection of wounds, tetanus toxoid and complete antirabies immunization.

Rabies is a disease of major public health importance and is widespread in Nigeria (Ehimiyein *et al*., 2010; Abubakar and Bakari, 2012). In Nigeria, rabies was recognized by local people prior to 1912 in various dialectic names such as *digbolugi* (Yoruba), *ciwon haukan kare* (Hausa), *ginnaji* (Fulani), e*bua idat* (Efik/Ibibio) and *ara nkita* (Igbo) (Nottidge, 2005). Official disease reporting system in Nigeria is rudimentary, hence information is lacking on the precise prevalence and distribution of rabies. No wildlife host has been clearly identified in Nigeria, although sporadic cases in wildlife reservoir have been reported (Adedeji *et al*., 2010). The domestic dog seems to

be the major source of human exposure to rabies and perhaps also the reservoir host in Nigeria (Ezeokoli and Umoh, 1989). Ehizibolo *et al.* (2011) reported that records from the National Veterinary Research Institute, Vom, Nigeria also showed that 95% of suspected rabies cases submitted for diagnosis between 1990-2000 were dogs and only one was a bat. The relative roles of stray and domestic dogs in the epidemiology of rabies in Nigeria follow the classical pattern in which the stray dog seems to be the disseminator among dogs (bringing the virus from one community to the other), while the domestic dog is the most important source of human exposure (Awoyomi *et al.*, 2007; Okpe *et al.*, 2011). Most domestic dogs in Nigeria are free-roaming, providing ample opportunity for contact with rabid strays (Awoyomi *et al.*, 2007).

1.3 Justification

Understanding of the molecular properties of rabies virus in dog populations is crucial in controlling the disease (Widdowson *et al.*, 2002). Dogs are the most widely spread and abundant animal amongst the carnivores (Wandeler *et al.*, 1993), therefore it is important to study the role of dogs in human cultures and the impact of their interactions. This can be achieved by having a good knowledge of the molecular epidemiology of canine rabies through studying the immunologic and genetic biology of dog rabies (Wandeler *et al.*, 1993). Even though some studies have shown prevalence of rabies antigens in the brain tissues of apparently healthy dogs slaughtered for human consumption in some parts of the country (Sabo *et al.*, 2008; Akombo, 2009; Idachaba *et al.*, 2009; Aliyu *et al.*,2010), confirming the presence of the viral strain molecularly, phylogeny analysis of the virus, disease and its health risk to the general public, there is a dearth of this information in many other parts of the country, including Benue State.

The application of genomic methods of analysis revealed that apart from antigenic differences, rabies virus isolates adapted to different species have conserved gene alterations which can also be used as epidemiological markers (Arai *et al.*, 1997; Ito *et al.*, 2001; 2003; Pa´ez *et al.*, 2003).

One important aspect of disease control is accurate information about the host species involved, which enables informed decisions to be made with regard to the epidemiologic patterns and potential threats to public, veterinary health and designing a suitable vaccine strain (Markotter *et al.*, 2006b).

Surveillance strategies for rabies virus and other rabies-ralated viruses in Nigeria must be improved to better understand the molecular epidemiology of thbis virus and to make informed decisions on future vaccine strategies because evidence is insufficient that current rabies vaccines provide protection against these rabies-related viruses (Markotter *et al.*, 2006).

The key for controlling zoonoses such as rabies, echinococcosis, and brucellosis is to focus on the animal reservoir (Zinsstag *et al.*, 2007). In this respect, ministries of health should question whether the public health sector really benefits from interventions for livestock (Zinsstag *et al.*, 2007). Effective, practicable and acceptable control strategy can only be put in place after the prevalence and molecular diversity of rabies virus associated with dog in each community in Nigeria have been studied.

Canine ecology studies can generate information that public health workers and veterinarians can use to characterize owned-dog populations and plan vaccination campaigns, hence reducing the risk of rabies in domestic animals and humans (Flores- Ibara and Estrella- Valenzuela, 2004). Knowledge of the epidemiology of rabies in dogs and the ecology of dogs including their population density and structure in Benue State will equip public health workers with relevant data that will help in the planning of rabies control programmes in this area.

1.4 Aim and Objectives of the Study

1.4.1 Aim: To carry out molecular studies on a segment of the N gene of rabies virus isolated in dogs from Benue State**.**

1.4.2 Objectives

The Objectives of the Study are:

1. To detect the presence of rabies virus nucleocapasid antigen in brain tissues of dogs using Direct Fluorescent antibody technique (DFAT).
2. To detect fragment of the N gene of the rabies virus genome by PCR amplification
3. To study the genetic relatedness of the amplified fragment of the N gene with other rabies virus isolates.
4. To study the evolutionary relationship of the amplified fragment of the N gene with other rabies virus isolates..

CHAPTER TWO

2.0 LITERATERATURE REVIEW

2.1 Rabies virus characteristics

2.1.1. Historical

Rabies has existed for thousands of years (Blancou, 2003). The first references to animal rabies seem to date back to the *laws of Eshunna* in Mesopotamina, in the 23rd century BC. Canine rabies was also described in the 6th century BC, in the Avesta (Persia), 4th century BC by Aristotle (Greece), and 1st century BC in the *Susrutasamhita* (India). Information on rabies in animals other than dogs or wild carnivores is much rarer. The methods for preventing rabies were also implausible and had no valid rationale for success. No effective preventive or curative treatment in animals was available before Pasteur's discovery in 1885.

2.1.2. Classification

The rabies virus belongs to the order *Mononegavirales,* family *Rhabdoviridae*, which includes at least three genera: *Lyssavirus, Ephemerovirus,* and *Vesiculovirus* (Virus Taxonomy, 2005). The *Lyssavirus* genus is further divided into seven genotypes. The genotype 1 is known to be the most widespread and comprises the classical field rabies viruses, and the laboratory and vaccine strains. The rabies-related viruses isolated in the African continent belong to genotypes 2, 3, and 4, with prototypes of the Lagos bat virus, Mokola virus, and Duvenhage virus, respectively. The viruses isolated from bats in Europe represent genotypes 5 and 6 (EBLV1 and EBLV2). The Australian bat Lyssavirus (ABLV) represents the seventh genotype (Gould *et al.,* 1998; Guyatt *et al.,* 2003). It has been found that some new rabies virus genotypes exist among viruses isolated

from the territory of the former Soviet Union. It was proposed that the Aravan virus isolated from a bat in Kyrgyzstan should be classified as the eighth genotype (Arai *et al.,* 1997). The Khujand virus isolated in Tajikistan can also be classified as a separate genotype of *Lyssavirus* (Kuzmin *et al.*, 2003). The existence of two additional *Lyssavirus* genotypes among strains isolated from bats (Irkut virus and West Caucasian Bat Virus) in the Russian Federation was also recently proposed (Botvinkin *et al.*, 2003; Kuzmin *et al.,* 2005). Other genotypes are Aravan virus (ARVA), Irkut virus (IRKV), Khujand virus (KHUV) and the West Caucasian bat virus (WCBV). Two newly identified lyssavireuses, the Shimoni bat virus (SHIBV) and the Bokeloh bat lyssavirus (Freuling *et al.*, 2011) have been detected in bats, but not yet classified.

2.2 Structure of Virion, Genome, and Proteins of Rabies Virus

Rhabdoviruses are approximately 180 nm long and 75 nm wide. They are bullet shape (Figure 2.1**)**. The rabies genome encodes five proteins: nucleoprotein (N), phosphoprotein (P), matrix protein (M), glycoprotein (G) and polymerase (L) (Figure**.** 2.2). 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 (CDC, 2016). Two other viral proteins, the phospoprotein 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 (CDC, 2016).

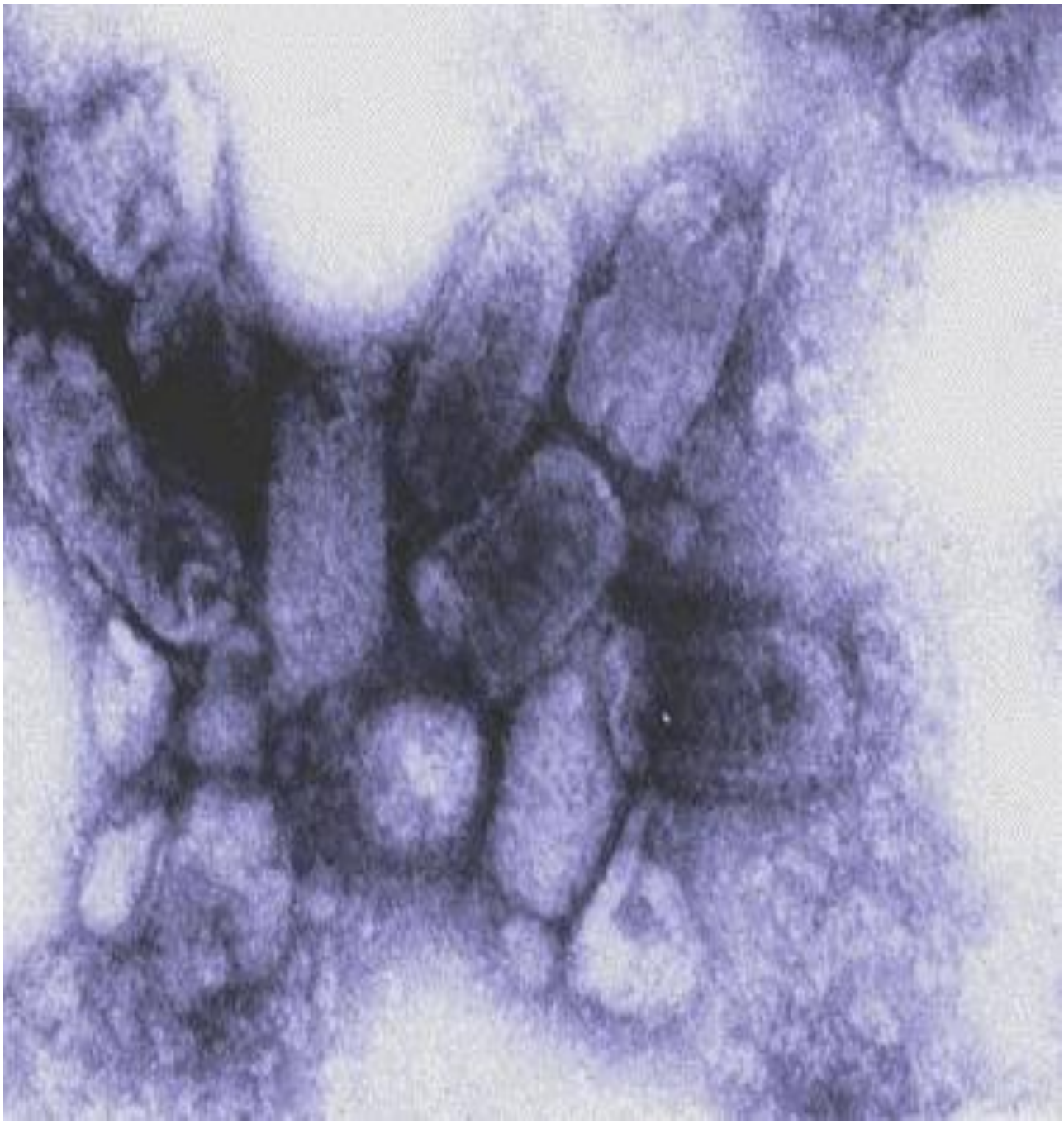

Figure 2.1 Rabies virions. (http://www.wadsworth.org/databank/rabies.html).

The genome of the rabies virus is a non-segmented negative-sense singlestranded RNA of approximately 12000 nucleotides (nt) in length (Fig. 2.1). The virus RNA codes 5 major proteins: the nucleoprotein (N protein), phosphoprotein (P protein), matrix protein (M protein), glycoprotein (G protein), and the RNA-dependent RNA-polymerase (L protein). The RNP complex is formed by the N, P, and L proteins associated with the viral RNA. The RNP is surrounded by a lipid bilayer associated with the G and M proteins (Goto *et al.*, 2000). The genome also contains several non-coding regions including a GL intergenic region called the "pseudogene" or Ψ-region, which became suppressed and degenerated during the evolution of the virus (Goto *et al.*, 2000).

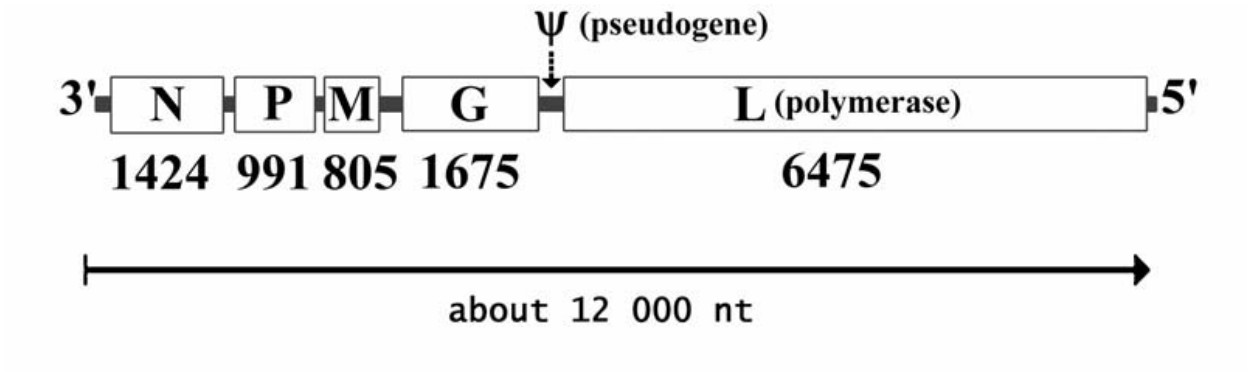

Figure. 2.2 Schematic structure of the rabies virus genome (adapted from *http://www.cdc.gov/ncidod/dvrd/rabies/the_virus/virus.htm*).

The N nucleoprotein consists of 450 amino acids and participates in the transition from RNA transcription to replication. It encapsidates the positive-strand leader RNA and prevents further transcription of the genomic RNA (Yang, 1998) and contains several antigenic and immunodominant sites. The antigenic sites I and III include stretches of amino acids at positions 374-383 and 313-337, respectively (Tordo, 1996). One of the immunodominant sites is known to be located at position 404-418 (Artem *et al.*, 2008). Recently, the crystal structure of the rabies virus nucleoprotein-RNA complex was determined (Albertini *et al.*, 2006).

The P protein is a phosphorylated protein of 297 amino acids, associated with the L protein to function as a noncatalytic cofactor for the RNA polymerization, and with the N protein to support adequate RNA encapsidation (Jacob *et al.*, 2001). Four additional proteins derived from the phosphoprotein gene of the rabies virus were also found to be present in infected cells, cells transfected with a plasmid encoding the wild-type P protein, and in the purified virus. It was shown that these proteins are initiated from secondary downstream in-frame AUG initiation codons. The P-gene is the only gene shown to encode more than one protein. Two antigenic sites were found to be located at positions 75-90 (Tordo, 1996). The domain 83-172 was shown to contain the major antigenic determinants (Artem *et al.*, 2008). An immunodominant site was also mapped in phosphoprotein at positions 191-206 and was identified as the responsible alpha/beta interferon antagonist (Brozka *et al.*, 2005).

The matrix protein is a 202 amino acid polypeptide which plays a key role in virus assembly and binding. It covers the ribonucleoprotein (RNP) coil to keep it in a condensed form and was found to interact specifically with the glycoprotein. The major antigenic site is located between the amino acid residues 1 and 72 (Artem *et al.*, 2008).

The glycoprotein is a 524 amino acid protein and is the most studied protein of the rabies virus. It has a trimeric structure, with the first 19 amino acids representing the signal domain which is found only in nascent protein. The glycoprotein contains several antigenic sites and epitopes. The antigenic epitope I is represented by a single amino acid at position 231 of the mature glycoprotein. The site II is known to be discontinuous and involves two separate stretches between positions 34-42 and 198-200. The antigenic site III is located at position 330-338, epitope VI at position 264, "a" – 342- 343 (Artem *et al.*, 2008).

The major immunodominant site of the glycoprotein is located between amino acids 222 and 332 (Johnson *et al.*, 2002b). The amino acid at position 333 of the mature glycoprotein was found to be associated with viral pathogenicity (Badrane *et al.*, 2001). It was shown that either arginine or lysine at position 333 of the ERA and CVS fixed rabies virus strains is necessary for rabies virulence in adult mice (Tuffereau *et al.*, 1989).

The L protein is the largest protein of the rabies virus and L gene occupies more than half of the virus genome. It has been the least studied rabies protein at biochemical and immunological levels, but the best analyzed theoretically (Tordo, 1996). The Lprotein is associated with the RNP, RNA-dependent RNA polymerase. It participates in transcription by making five individual mRNAs, one for each viral protein.

2.3 Replication

The fusion of the rabies virus envelope to the host cell membrane (adsorption) initiates the infection process. The interaction of the G protein and specific cell surface receptors may be involved. After adsorption, the virus penetrates the host cell and enters the cytoplasm by pinocytosis (via clathrin-coated pits). The virions aggregate in the large endosomes (cytoplasmic

vesicles). The viral membranes fuse to the endosomal membranes, causing the release of viral RNP into the cytoplasm (uncoating). Because lyssaviruses have a linear single-negative-stranded ribonucleic acid (RNA) genome, messenger RNAs (mRNAs) must be transcribed to permit virus replication (CDC, 2016).

A viral-encoded polymerase (L gene) transcribes the genomic strand of rabies RNA into leader RNA and five capped and polyadenylated mRNAs, which are translated into proteins. Translation, which involves the synthesis of the N, P, M, G and L proteins, occurs on free ribosomes in the cytoplasm. Although G protein synthesis is initiated on free ribosomes, completion of synthesis and glycosylation (processing of the glycoprotein), occurs in the endoplasmic reticulum (ER) and Golgi apparatus. The intracellular ratio of leader RNA to N protein regulates the switch from transcription to replication. When this switch is activated, replication of the viral genome begins. The first step in viral replication is synthesis of full-length copies (postive strands) of the viral genome. When the switch to replication occurs, RNA transcription becomes "non-stop" and stop codons are ignored. The viral polymerase enters a single site on the 3’ end of the genome, and proceeds to synthesize full-length copies of the genome. These positive strands of rabies RNA serve as templates for synthesis of full-length negative strands of the viral genome (CDC, 2016).

During the assembly process, the N-P-L complex encapsulates negative-stranded genomic RNA to form the RNP core, and the M protein forms a capsule, or matrix, around the RNP. The RNP-M complex migrates to an area of the plasma membrane containing glycoprotein inserts, and the M-protein initiates coiling. The M-RNP complex binds with the glycoprotein, and the completed

virus buds from the plasma membrane. Within the central nervous system (CNS), there is preferential viral budding from plasma membranes. Conversely, virus in the salivary glands buds primarily from the cell membrane into the acinar lumen. Viral budding into the salivary gland and virus-induced aggressive behavior in the host animal maximize chances of viral infection of a new host (CDC, 2016).

2.4 Pathogenesis

Bites by rabid animals generally inoculate virus-laden saliva through the skin into muscle and subcutaneous tissues. Other routes of infection are rare (Warrell, 2003). During the incubation period the virus can replicate locally in muscle cells or attach directly to nerve endings. Having gained access to peripheral nerves, it travels in a retrograde direction within the axoplasm. When the virus reaches the central nervous system, there is massive replication on membranes within neurons. Direct transmission of virus occurs from cell to cell across synaptic junctions. At the onset of illness when evidence of neuronal dysfunction appears, there is little or no apparent histopathological change. Centrifugal spread of virus from the central nervous system in somatic and autonomic nerves deposits virus in many tissues, including skeletal and cardiac muscle, adrenal glands, kidney, retina, cornea, pancreas, and nerves around hair follicles (Alan, 2005). Productive viral replication with budding from plasma membranes takes place predominantly in the salivary glands, excreting virus that is transmissible to other mammals (Alan, 2005).

Local replication of virus in striated muscle at the bite site, before any contact with nervous tissue, could account for long incubation periods. In skunks, antigen was detectable in muscle for 2 months after inoculation. Virus is soon detectable experimentally at local motor or sensory

nerve endings and, after superficial inoculation, in epithelial layers. Rabies virus can infect a great variety of neuronal and non-neuronal cells in vitro. Non-specific viral attachment to several types of cell-surface receptors including carbohydrates, phospholipids, and sialylated gangliosides has been demonstrated. Specific binding occurs at neuromuscular junctions, where virus colocalises with the nicotinic acetylcholine receptor (Lewis *et al.,* 2000). Binding at this postsynaptic site is competitive with cholinergic ligands, including the snake venom neurotoxin-bungarotoxin, which shows sequence homology with the envelope glycoprotein of rabies virus. Concentration of virus at this site increases its chances of entering the axon terminal across the synaptic cleft. Rabies virus attaches specifically to two other receptors on neuronal cell membranes: the neural cell adhesion molecule and the p75 neurotropin receptor (p75NTR) (Langevin *et al.,* 2002) Two neurotransmitter receptors in the central nervous system, for N-methyl-D-aspartate subtype R1 and gamma-Aminobutyric acid (GABA), have been suggested as possible receptors for rabies virus (Gosztonyi and Ludwig, 2001).

Rabies virus migrates along peripheral nerves towards the central nervous system via the fast axonal transport system at about 50–100 mm per day (Tsiang, 1993). Because this movement is strictly retrograde, it is used experimentally to track neural pathways (Graf *et al.*, 2002). Infection is thought to be via sensory as well as motor nerves, because antigen was detected in sensory nerve endings and dorsal root ganglia soon after peripheral inoculation in several studies. Viral replication is intraneuronal, but the mechanism of interneuronal spread is unknown. The fact that budding of virus is very rarely seen at synapses by electron microscopy suggests that infectious naked nucleocapsids are transferred across synapses. However, interneuronal infection is dependent on the presence of viral glycoprotein (Etessami *et al.,* 2000), which suggests that intact virus can cross the synapse. The mechanism of uptake is unknown, but one possibility is thought

to be through the synaptic vesicle recycling system (Lewis, 2000). Among street viruses, the dynamics and effects of infection can be influenced by the particular strain, since dog rabies viruses are specific for neurons, whereas bat strains can also infect astrocytes (Prosniak *et al.*, 2003).

In humans, the symptoms of encephalitis and even death can occur with only minor histopathological changes. Rabies virus must have some profound effects on the functions of infected and some uninfected neurons. Few abnormalities of organelle structure are seen on electron microscopy in neurons infected with street virus. Minor electroencephalographic changes during animal infection indicate neuronal dysfunction (Tsiang, 1993). Although MRI shows a range of abnormalities as the human encephalitis progresses, no consistent pattern has yet emerged.

Abnormalities of neurotransmitter functions affecting serotonin, opioid, gamma-Aminobutyric acid (GABA), and muscarinic acetylcholine transmission have been found experimentally, in some cases in specific brain areas, and not always associated with the presence of virus (Jackson, 2002; Tsiang, 1993; Prosniak *et al.*, 2003). Results show no clear explanation of the limbic-system dysfunction suggested by the classic clinical features. Changes in neurotransmitter functions could lead to failure of brain networking and regulation of responses (Jackson, 2002; Tsiang, 1993; Prosniak *et al.*, 2003).

The involvement of excitatory aminoacids in neuronal toxicity is a possibility. Many non-competitive antagonists of N-methyl-D-aspartate have an antiviral effect on viral replication (Tsiang, 1993). The effect of infection on the function of neuronal membrane ion channels could be reduction of normal inhibitory events (Iwata *et al.*, 2000). Apoptosis could contribute to

pathogenesis since fatal infection of mice is associated with apoptosis of T cells invading the brain and neuronal preservation. By contrast, neuronal apoptosis occurs in non-lethal infection, with development of an immune response (Jackson, 2002; Baloul and Lafon, 2003). The role of nitric oxide toxicity in neuronal dysfunction in rabies is not clear, (Jackson, 2002), but it could be related to the Dynein light chain (DLC8) inhibition of neuronal nitric oxide synthase, through interaction with the viral phosphoprotein (Jacob *et al.,* 2000). Although rabies infection progressively decreases host gene expression overall, a few genes are upregulated, some associated with the interferon response, host-cell protein synthesis, synaptic vesicle function, and neuron growth and spread, even in some uninfected or nonneuronal cells (Prosniak *et al.*, 2003). One hypothesis on the cause of death is therefore that short-circuiting of normal neural pathways results from the formation of new interneuronal connections (Prosniak *et al.,* 2001). Another hypothesis is that disruption of neuronal metabolism ends in the exhaustion of metabolic pools (Dietzschold *et al.*, 2001).

2.5 Clinical Signs

The clinical signs of rabies have been known since the ancient times (Blancou, 2004). The duration from bite to the appearance of clinical signs varies significantly, ranging from a few days to a several months. The clinical signs may appear only after the involvement of numerous neurons, and death may occur as a result of the involvement of vital nerve centers (Blancou, 2004).

There are three phases described in the clinical course of rabies:

Prodromal period – the first 1 to 3 days after the rabies virus reaches the brain. Vague neurological signs that progress rapidly - some animals may appear tamer, some will demonstrate intense drooling. Death usually follows within 10 days, due to paralysis (Blancou, 2004),

Excitative stage – the next 2 to 3 days. This is the "furious rabies" stage – tame animals suddenly become vicious, attacking humans and other animals as they roam and wander. Some animals will chew and eat odd objects (rocks, sticks, etc.). Paralysis then begins, and loss of the ability to swallow will cause frothing at the mouth of the affected animal (Blancou, 2004).

Paralytic stage - follows the excitative stage, or is the main clinical presentation for some animals. The throat and chewing muscles are paralyzed, and the animal is unable to swallow, causing excessive drool, the lower jaw is often dropped. This is a dangerous period for human contact with domestic animals such as cows and horses; "choke" (foreign body within the throat) can be a misdiagnosis of rabies, causing humans to be potentially exposed as they investigate the problem. Similar situations occur in dogs that appear to be choking (drooling and dropped jaw). This is also the period when wild animals may seem tame to humans and nocturnal animals are seen in the daytime. The paralysis progresses from the neck and jaw to all areas of the body, the animal falls into a coma, and dies within a few hours (Blancou, 2004).

In bats, the clinical signs of a Lyssavirus-infection include loss of body mass, lack of coordination, muscular spasms, agitation, increased vocalization, and overt aggression (Whitby *et al.*, 2000; Shankar *et al.*, 2004), but in many cases, rabies in bats can be clinically silent and left unnoticed before dead animals are found and laboratory tests are performed (Ronsholt *et al.*, 1998). When bats are found alive, the clinical signs are generally described as paralysis,

unprovoked vocalization, and aggression (biting) during handling (Rabies Bulletin Europe, 1989). However, almost all bats will bite when handled (Vos *et al.*, 2007).

Because dogs are often responsible for the transmission of rabies to humans, clinical signs in this species are more elaborately described, studied, understood, and include: drooping jaw, abnormal sounds when barking, dry drooping tongue, licking of its own urine, abnormal aversion from water, regurgitation, altered behavior, the biting and eating of abnormal objects, aggression, biting without provocation, running without no apparent reason, stiffness upon running or walking, restlessness, biting during quarantine, sleepy appearance, gait imbalance, and frequent demonstration of the "Dog sitting" position (Tepsumethanon *et al.*, 2005).

2.6 Diagnosis

To date, there are no tests available to diagnose human rabies infection ante-mortem, or before the onset of clinical disease. However, rabies should be included in the differential diagnosis of all patients who present with unexplained, acute, progressive viral encephalitis, even in areas where the disease is not endemic (WHO, 2016). Rabies diagnosis does not involve any clinical or gross pathogenesis. A laboratory technique is the only way of confirmation of infection (Anon, 2004a). The laboratory test for rabies is meant to be standardized, rapid, specific, economical, sensitive and reliable, due to the nature of rabies. The decision whether or not to introduce control strategies or to carry on with a treatment course is greatly influenced by the results generated from tests. The absence of infection is not basically confirmed by the failure to notice or identify the presence of the Rabies Virus or viral antigen or antibodies in a specimen by a single test (Anon, 2004a). To increase the chance of making a correct or right diagnosis, more than one test should be carried out simultaneously as a routine. The reason is that some tests are more sensitive and specific than others, depending on the type of specimen available, stage of

infection and genotype of the Rabies Virus (Anon, 2004b). Although the thalamus has been reported to be positive for the virus, a pool of brain tissue including the brain stem should be used for laboratory examinations (Bingham and Merwe, 2002). Salivary gland examination can also provide epidemiological information on the virus excretion by different animal species (Bingham and Merwe, 2002).

Timely administration of post exposure prophylaxis requires accurate and rapid laboratory diagnosis of rabies in human and other animals. The diagnostic laboratory can determine whether or not an animal is rabid and inform the responsible medical personnel, within a few hours. The laboratory result may save a patient from unnecessary physical and psychological trauma and financial burdens, if the animal is not rabid. Furthermore, the laboratory determination or identification of positive rabies cases may aid in defining current epidemiologic patterns of disease and make available the right information for the advancement of the rabies control programs.

The laboratory diagnosis of rabies is rarely attempted in less developed countries, but confirmation of infection during life will guide management of the patient, relatives, and staff; prevent unnecessary investigations; and allow characterisation of the virus. Routine tests might show plasma neutrophil leucocytosis. Mild pleiocytosis is seen in only 60% of patients in the first week. The diagnosis can be made by early identification of antigen or viral RNA or by virus isolation, and in unvaccinated people, antibody detection (Warrell, 2003).

At Ante-mortem (before death) several tests are required to diagnose rabies in humans; no single test is sufficient. Tests are performed on samples of spinal fluid, saliva, hair follicles at the nape of the neck and skin biopsies. Saliva can be tested by virus isolation or reverse transcription

followed by polymerase chain reaction (RT-PCR). Serum and spinal fluid are tested for antibodies to rabies virus. Skin biopsy specimens are examined for rabies antigen in the cutaneous nerves at the base of hair follicles. In the case of rabies, diagnosis often begins with assessing the risk for rabies exposure based on information such as the location where the incident occurred, the type of animal involved, the vaccination status of the animal, and whether or not the animal can be safely captured and tested for rabies. Several tests are used in the diagnosis of rabies, but the direct fluorescent antibody test (DFA) is the most commonly used. Other tests that can be used in diagnosis are: electron microscopy (EM), histologic examination using Sellers staining, immunohistochemistry (IHC), RT-PCR and viral isolation in cell culture.

2.6.1 Clinical History and Signs

Prior to suspecting the disease, there should be history of bite, scratch and/or contact with a rabid animal and clinical presentations of furious and/or paralytic form of the disease. History and clinical signs give only indication of disease. However definite diagnosis of rabies relies on the laboratory demonstration and identification of virus or its specific component(s).

2.6.2 Detection of the Antigen

A variety of methods are in practice to detect rabies virus and its specific component(s), briefly presented as follows.

2.6.3 Collection and transportation of the samples

The rabies virus can be demonstrated in the saliva, CSF, swabs from the throat, nasal mucosa or eye, eyeball impression smears and nuchal or facial skin biopsies in the living individuals or in the tissues from the brain (stem plus either cerebellum and/or hippocampus) in dead animals. Therefore either whole body (small animals *e.g.* bats), or head, brain/ a complete cross section of the brain of large animal are collected at necropsy for detection of virus using *Intra-vitam* test,-

the first reliable diagnostic test for rabies, applicable to either dead or living individuals. The test was first described by Schneider, (2010) in animals. The material collected must be securely packed in a leak-proof rigid container ensuring the least risk to human being handling the material and dispatched while maintaining cold chain to avoid inactivation of virus in transport. The material should reach the laboratory within 48 hours. Repeated freeze and thawing cycles must be avoided. Alternatively, approved preservation technique can be used in the event that the dispatch of the material under cold chain is not feasible. The Choice of preservative is closely linked with the test to be employed (histology/IFT/ cell culture). Isolation of virus is not possible from formalin preserved samples or those transported at room temperature. Vitality of virus could be extended for several days by storing the materials in a mixture of 50% glycerol in phosphate buffered saline (PBS), however this may not protect the virus/fragment against declining titer, due to thermal fluctuations. The viral genome integrity can also be secured from activities of RNAse by storing the sample in RNA later. Therefore, as far as possible low temperature should be maintained while transporting glycerol/saline preserved samples. As the virus is not inactivated by glycerol/PBS, all laboratory tests can be used on these samples (Shankar, 2009). Serum samples are also collected, stored and frozen in deep freezer until application of test to demonstrate anti rabies antibodies.

2.6.4 Light Microscopy (Demonstration of Negri Bodies)

Negri bodies corresponding to aggregates of viral proteins, are specific for rabies virus infection, the staining techniques are not specific since they merely detect affinity for acidophilic stains. Histological examination of brain sections or impression smears by light microscopy, in detection of characteristic Negri bodies, has been considered standard method of rabies diagnosis

for many years. Under light microscopy these Negri bodies are round or oval, acidophilic and strongly refringent, staining pink with haematoxylin-eosin and red with Mann's stain. Staining of an unfixed tissue smear by Seller's method allows diagnosis in less than one hour; whereas Mann's test generally involves fixing the tissue and embedding it in paraffin, with test results being available within three days. (Singathia *et al.,* 2012*).*

These techniques have the advantage that the laboratory equipment needed to perform them is inexpensive and any need to keep specimens cold after fixation is avoided. Whichever staining method is used, the evidence of infection is provided by intra cytoplasmic acidophilic bodies. These histological methods, especially the Seller's method, can no longer be recommended because they have very low sensitivity and should be abandoned (Singathia *et al.,* 2012*).* Additionally, false negatives results may occur because not all individuals develop Negri bodies and false positive results may occur if nonspecific inclusion bodies are present (Singathia *et al.,* 2012*).* Presence of Negri bodies in positive cases varies from 10 to 65% (Sellers, 1927).This is why this technique has gradually been replaced by fluorescent antibody test. The OIE no longer recommends histopathology for diagnosis of rabies. Canine distemper and canine hepatitis virus forms inclusion bodies, showing that other viruses can produce inclusion bodies, making diagnosis of rabies using this procedures difficult and lacks integrity (Lumtertdacher, 2005).

2.6.5 Electron microscopy

Examination by electron microscopy can be used to detect viral inclusions (corresponding to Negri bodies) and virus particles. Electron microscopy can be used on fresh specimens and formalin-fixed or paraffin-embedded specimens (Singathia *et al.,* 2012*).*

2.6.6 Fluorescent microscopy or direct Fluorescent Antibody Test (FAT)

Direct immunofluorescence for diagnosis of rabies was first developed by Goldwasser and kissling in 1958 and it became a routine diagnostic method in the 1970s (Dean *et al.*, 1996). FAT is presently the standard test for rabies virus detection, which is recommended by both WHO and OIE (Shankar, 2009). The FAT provides a reliable diagnosis in 98-100% cases (Dean *et al.*, 1996). This test is now the most widely used test for the detection of rabies antigens, and compared to the histological demonstration of Negri bodies, it has a high degree of sensitivity. The test is particularly sensitive with fresh specimens, but it can also be used in fixed tissues after treatment with trypsin or other enzymes (Whitfield *et al.*, 2001). The direct immunofluorescent antibody test on thin touch impressions of brain tissue (medulla, cerebellum and hippocampus) is preferred for rabies diagnosis. Thin touch impression smears are fixed in cold acetone for one to four hours, air dried then stained using fluorescein isothiocyanate (FITC)-labelled anti-rabies antibody. Examination at 400x to 1,000x magnification reveals dust-like orange green particles less than 1 µm diameter or intra-cytoplasmic large round/oval masses and strings (2.0 - 10 µm diameter), smooth, with bright edges. The main advantage of this test is that results can be obtained within 2 hr., but the requirement of specialist laboratories, properly immunized and well trained personnel, fluorescence microscope and rabies conjugate makes this technique relatively expensive. However, it is reported to be less sensitive and less specific than the mouse inoculation test (Woldehiwet, 2005).

2.7 Virus Isolation Techniques

Virus isolation can be performed in the laboratory animal as well as in the tissue culture.

2. 7.1 Virus Isolation in Mouse or Mouse Inoculation Test (MIT)

Mouse inoculation test involves intracerebral inoculation of (preferably) weanling mice (six per specimen), with test suspensions (*e.g.* pooled bilateral samples of hippocampus, cerebral cortex,

cerebellum and pons-medulla) and observation of the mice for development of neurological signs. Mice should be observed for at least 21 days, preferably 28 days. Death within 48 hr is considered non-specific. Paralysis occurs within 5-10 days in newborn mice, 8-15 days in adult mice. Diagnosis is confirmed using the FAT if available, otherwise by detection of Negri bodies and by virus neutralization index test. For a faster detection, individual mice in a group are sacrificed every day or every second day, starting from three days (for newborn) or five days (for adult) mice, the brain being tested using the FAT (Singathia *et al.*, 2012*)*. For many years, mouse inoculation was considered the most sensitive way to detect rabies virus because it can be easily and practicably applied in situations where skills and facilities for other tests (e.g. cell culture) are not available. But this *in vivo* test is expensive, particularly if specific pathogen free (SPF) mice are used and several days are required for a positive diagnosis compared with *in-vitro* inoculation test and this test is slightly less sensitive than immunofluorescence microscopy, due to these limitations, it has now been superseded by modern methods for routine diagnosis. This test should be avoided when possible on animal welfare grounds (Singathia *et al.*, 2012*)*.

The disadvantage of mouse inoculation test is the long interval before a diagnosis can be made since the inoculated mice need to be kept under observation for 28 days as some wild viruses may have a very long incubation period. If cell culture facilities exist in the laboratory, consideration should be given to replacing the mouse inoculation test with cell culture whenever possible as it avoids the use of live animals, is less expensive, and gives more rapid results. However, advantages of MIT are that when the test is positive, a large amount of virus can be isolated from a single mouse brain for strain identification purposes and that it can be easily and practicably applied in situations where skills and facilities for other tests (e.g., cell culture) are not available (Anon, 2012).

2.7.2 Virus isolation in Rapid Tissue Culture Infection Test (RTCIT)

Virus isolation by *in-vitro* technique (RTCIT) is done by using highly susceptible cell line murine neuroblastoma (NA-C 1300), neuroblastoma cell line *e.g* CCL-131. It can be used for testing saliva and cerebrospinal fluid samples from living individuals as well as for testing brain and salivary gland tissues from *post mortem* specimens. Virus isolation in cell culture can be used in cases where immunofluorescence is inconclusive. After overnight incubation (18hr.) at $37^{o}C$ in 5% CO_2, the cells are fixed, stained with an anti-nucleocapsid antibody conjugated to FITC and observed under fluorescent microscope (Singh, 2010). RTCIT has progressively replaced the MIT because it is less expensive as it avoids the use of live animals, relatively sensitive, easy to undertake and can substantially reduce the time required to obtain results from 30 days in MIT to 4 days (Webster and Casey, 1996). Several comparative studies between the RTCIT and both MIT and FAT have shown that the RTCIT is at least as sensitive as the MIT in demonstrating rabies virus in human and animal tissues, but others have found it to be less sensitive than MIT (Zanoni, 1990). Several laboratories have now replaced the MIT with RTCIT as the routine test for rabies diagnosis. But problem with this test is that a negative result in samples from a live animal is not conclusive, many animals have been found to be rabies virus positive in the brain but negative in saliva samples; even clinical patients with rabies may be negative for virus in biopsy, saliva or CSF. It is often advisable to carry out more than one type of test on each sample, particularly when there has been human exposure.

2.8. Immunoperoxidase Test (IPT)

Immunoperoxidase staining techniques have also been used, when good morphology is important in addition to requirement for the sensitive and specific detection of rabies antigen.

Immunoperoxidase test may be used instead of FAT. Both techniques have the same sensitivity, but direct FAT is only possible when fluorescent microscope is available. Its disadvantage is its impermanency and nonspecific reactions are also seen. These disadvantages can largely be avoided by employing direct IPT since the method does not require a special kind of microscope and it can keep for a long time. The sensitivity of the immunoperoxidase test can be improved by the addition of peroxidase and antiperoxidase or avidin biotin horseradish peroxidase conjugates (Jayakumar *et al.*, 1994).

2.9 Direct Rapid Immunohistochemical Test (DRIT)

The direct rapid immunohistochemical test (DRIT) was developed recently and validated as a field test for rabies surveillance. This test can be used on brain touch impressions and the reaction product is visible under an ordinary light microscope (magenta inclusions, while the neuronal background is blue). The test allows diagnosis within one hour. It can be used on frozen samples or samples preserved in glycerol, and have sensitivity similar to that of the direct FAT. Compared to the DFAT, the traditional standard in rabies diagnosis, the DRIT was 100% sensitive and specific (Singathia *et al.,* 2012*).*

2.10 Rapid Latex Agglutination Test

Latex beads sensitized with rabies immunoglobulins quickly agglutinate in the presence of rabies virus antigens. In the same manner latex beads sensitized with purified rabies antigen quickly agglutinates in the presence of rabies specific antibodies. This test could also be used as a quantitative test. This test requires less than 15 minutes to perform on clean microscopic slide of plate or VIDAL Test plates (Kasempimolporn *et al.*, 2000).

2.11 Rapid Rabies Enzyme Immuno Diagnosis (RREID)

The RREID test is an Enzyme Linked Immunosorbent Assay (ELISA) test performed on the supernatant of brain or salivary gland suspensions. This test is based on the immunocapture of the rabies nucleocapsid antigen by an antinucleocapsid polyclonal globulin coated to the ELISA plates, followed by the addition of the same globulin conjugated to horse radish peroxidase enzyme. The results are obtained within three hours. The correlation between FAT and RREID is 96-99% (Jayakumar *et al.*, 1997).

2.12 Dipstick / Dot ELISA

In the Dipstick / Dot ELISA nitrocellulose strips as solid support replaces the micro titer plates. This test is rapid, simple, reagent conservative, does not require sophisticated equipment and trained person and is ideal for rabies antigen detection in postmortem brain samples in geographically remote areas where FAT facility is not available. The results of this test can be preserved for longer time and this test can be used in retrospective epidemiological survey of rabies (Jayakumar *et al.*, 1997).

2.13 Serological Demonstration of Antibodies

2.13.1 Indirect Fluorescent Antibody Test (IFA)

The indirect fluorescent antibody test can be used for detection of rabies antibodies in serum samples. The indirect fluorescent antibody test has been accepted as a "rapid, sensitive and reproducible method for measuring rabies antibody" (Singathia *et al.*, 2012).

2.13.2 Mouse Neutralization Test (MNT)/ Mice protection test

Webster and Casey (1996) developed MNT which is a widely used and WHO recommended test. The mouse protection test was the first serological test developed for rabies antibody detection but is now outdated. It involves mixing dilutions of serum and virus before inoculating the mixture into mice and noting whether or not the mice die from rabies.

2.13.3 Rapid Fluorescent Focus Inhibition Test (RFFIT)

RFFIT is one of the most widely used substitutes of MNT. The principle of RFFIT is same with MNT except that the indicator system for virus infectivity in this case being a susceptible cell culture system usually BHK-21 or neuroblastoma cells. It is precisely a combination of cell culture and immunofluorescence. It is an advanced approach using international standards and thus provides international units of quantitative antibody. It is a rapid test and requires only 20 hr for completion. It involves the observation of foci of virus infected cells by fluorescent antibody staining. The sera are serially diluted and followed by the addition of predetermined FFD50 (focus forming dose) of virus (one FFD50 is the dilution of virus at which 50% of the observed fields contain one or more infected cells). The serum-virus mixture is incubated at 37^{o}C. Subsequently, suspension of cell line is added. After a 20hr incubation period at 37^{o}C in 5% CO2 incubator, the cells are stained with an anti-rabies antibody conjugated to FITC. The antibody titer is defined as the reciprocal of the serum dilution, which reduces the challenge virus to 1

FFD50. The rapidity with which the test can be carried out and the ease with which several samples may be tested at a time makes it suitable for routine diagnosis. The RFFIT is more sensitive than MNT for detecting virus neutralizing antibodies (Singh, 2010).

2.13.4 Fluorescence Inhibition Micro test (FIMT)

FIMT is a micro modification of the RFFIT which can be used for determination of rabies in sera of various species (Singathia *et al.,* 2012*).*

2.13.5 Fluorescent Antibody Virus Neutralization (FAVN)/Virus neutralization test in cell culture

Fluorescent Antibody Virus Neutralization (FAVN)/Virus neutralization test in cell culture is an adaptation of RFFIT which was developed by Cliquet *et al* (1998). In early phase of disease, when there is low titer of antibody, distinguishing negative sera from the positive sera is easier with the FAVN as compared to RFFIT. The principle of the FAVN test is the neutralization*in vitro* of a constant amount of rabies virus before inoculating cells susceptible to rabies virus. The serum titre is the dilution at which 100% of the virus is neutralized in 50% of the wells. Results obtained with this FAVN test show good agreement with MNT and RFFIT (Singh, 2010).

2.13.6 Enzyme Linked Immunosorbent Assay (ELISA)

ELISA has been developed for the detection of antibodies to either whole rabies virus or the envelope glycoprotein (G) of rabies virus. An indirect ELISA, using anti-canine immunoglobulin, has been shown to be useful for the measurement of rabies-specific antibody in sera of a variety of carnivores. ELISA is advantageous because it does not require cell culture techniques (as RFFIT does) or facilities for keeping live mice. ELISA is acceptable for international movement of dogs and cats provided kits used have been validated and adopted on the OIE register.

2.13.7 Counter Immune Electrophoresis (CIE)

Rani *et al. (*2002) reported a relatively simple, rapid, inexpensive and reliable technique *i.e.* CIE for detection of anti-rabies antibodies. The principle of Counter Immunoelectrophoresis method is that at pH 8.4 the immunoglobulins will migrate to the negative end of a capillary system if an electric field has been applied to the capillary system. In the same capillary system, many antigens will migrate towards the positive end at that pH. As the antibody and antigen move towards each other in an electric field, they will soon meet in optimal proportion and visible precipitate is formed.

2.14 Molecular detection of Rabies RNA

2.14.1 Reverse Transcriptase Polymerse Chain Reaction (RT-PCR)

RT-PCR to detect *Lyssavirus* nucleic acid is not used in routine rabies diagnosis, but is useful for confirmation of direct immunofluorescence test results in formalin-fixed or decomposed material unsuitable for virus isolation, and has been used in *antemortem* diagnosis. One advantage is that RT-PCR may give result in just a few hours. But there are some constraints like false-positive result from contamination or technical error and false-negatives may result, due to the diversity of lyssaviruses, if the initial primer cannot allow for sequence heterogeneity and there are not yet universal primers for all known lyssaviruses. This method is up to 100 fold more sensitive than southern blot and FAT. The complete detection methodology from RT-PCR to PCR ELISA detection could be completed within 10 hours. The RT–PCR method is likely to reduce the time required to establish diagnosis to a few hours, but its use remains limited to a few laboratories. This technique is reported to be particularly useful for the intravitam diagnosis of rabies because the other methods are not suitable or sensitive.

2.14.2 Dot and slot blot hybridization's

Viral RNAs are extracted from brain or salivary gland specimens after filtration on nylon membranes, hybridization is performed with 32p DNA probes or non-radioactive probes complementary to rabies genome RNA and mRNAs. Autoradiography or ELISA detects labeled probes. The results are obtained in 48 hr. (Reddy *et al.,* (2002).

2.14.3 In situ Hybridization (ISH)

This procedure allows the reproducible in situ detection of rabies virus antigen and both genome and messenger RNAs in formalin fixed tissues (Praveena, *et al*., 2007).These procedures can be used on sequential tissue sections and thereby permit comparison of results from tests detecting both antigen and RNA in the some tissue. This antigen detecting procedures has also been used to identify both the phylogenetically distinct rabies viruses like rabies and rabies related viruses (Warner *et al*., 1997).

2.14.4 Nucleic-Acid Sequence Based Amplification (NASBA)

NASBA is able to detect very small amounts of rabies virus RNA and has been used to test the saliva and CSF in humans with clinical illness. It was noted that rabies RNA was detected in the saliva and/or CSF of all patients from as early as two days after the onset of clinical signs, but that not all samples were positive. Repeated sampling and testing of saliva was recommended. Results could be obtained in about four hours. NASBA allowed detection of rabies RNA at about 100th of the amount required for detection by RT-PCR.

2.15 Fixed Strains of Rabies Virus

2.15.1 Laboratory Strains

Two laboratory strains, the CVS (Challenge/Control Virus Strain) and PMPV (Pitman-Moore Pasteur Virus), are widely used. Both strains are considered to be derived from the original Pasteur virus strain (Smith *et al.*, 1993). The CVS strain has two stable variants: CVS-B2c and CVS-N2c, these differ in pathogenicity for healthy mice and in the capacity to affect neurons (Morimoto *et al.*, 1999). These laboratory strains are used in different diagnostic tests such as the virus neutralization and focus inhibition tests, as well as for the potency testing of anti-rabies vaccines in laboratory animals.

2.15.2 Vaccine Strains and Anti-Rabies Vaccines

Since the first rabies vaccination in 1885 by Louis Pasteur (Pasteur, 1885), significant progress has been made in improving the pre- and post-exposure treatment of human rabies (Dietzschold *et al.*, 2003). There are several types of vaccines: live attenuated, inactivated (killed), DNA-based, and vector vaccines. For the production of anti-rabies vaccines, a number of attenuated vaccine strains are employed: the Pasteur Virus (PV), Evelyn Rokitniki Abelseth (ERA), Street-Alabama-Dufferin (SAD), 3aG, Fuenzalida S-51 and S-91, Ni-Ce, SRV9, PM, Nishigahara, RC-HL, Kelev, Flury, "Shelkovo-51", "O-73 Uz-VGNKI", "RV-71", "Krasnopresnenskii-85", and the RV-97 strain (Gruzdev and Nedosekov, 2001; Ito *et al.*, 2001; Borisov *et al.*, 2002). The PV is one of the first vaccine strains; it was isolated from a rabid cow in 1882 and attenuated by multiple passages in rabbits. The SAD strain was isolated from a rabid dog in Alabama (USA) in 1935 and adapted for cultivation in the mouse brain and in the baby hamster kidney cell culture. It has two main derivates: ERA and Vnukovo-32. Several variants of the SAD strain exist: SAD-

Berne, SAD B19, SADP5/ 88 etc., and also non-virulent mutants SAG-1 and SAG-2. The vaccine strains belonging to the SAD group are widely used throughout the world. One of the most widely used oral anti-rabies vaccines is prepared from the SAD B19 strain, the high immunogenicity and relative safety of this strain has been demonstrated experimentally (Vos *et al.,* 2000; Neubert *et al.,* 2001).

Live attenuated vaccines are still in use in some developing countries for parenteral vaccination of animals and humans. These contain live attenuated rabies virus which has been developed in cell cultures or in live animals such as sheep. In the developed world, live attenuated vaccines are only used for the oral immunization of wild animals. Oral vaccines are widely used and several vaccine strains are used for the production of such vaccines: the SAD B19 and other SAD-strains, SAG1 and SAG2 – apathogenic deletion mutants, Vnukovo-32, and the VRG strain (Vos *et al.*, 2000). The vaccine strain RV-97 is used in Russia for producing the oral anti-rabies vaccine "Sinrab". This strain was obtained in the FGI "Federal Centre for Animal Health" (Vladimir, Russia) from strain RB-71. The ancestor to these two strains is the strain "Sheep", derived from the PV strain. The strain "Moscow" is also believed to be a derivate of the PV strain (Gruzdev and Nedosekov, 2001), and was used in the former USSR for producing anti-rabies vaccine. The strain RV-97 is adapted for cultivation in cell culture BHK-21 (Borisov *et al.*, 2002).

Inactivated vaccines. Complete inactivated rabies virus particles are highly immunogenic. The vaccines based on this principle are used for the pre- and postexposure immunization of humans and domestic animals (Dietzschold *et al.,* 2003). The inactivated chicken embryo vaccines and vaccines based on virus cultivated in cell cultures are used for veterinary and medical purposes. Modern medical vaccines can be administered by the intradermal route (WHO, 1997).

DNA vaccines are based on plasmid vectors expressing rabies virus glycoprotein. These vaccines have been tested for their efficacy in several animal species (mice, dogs and nonhuman primates), and it has been found that the DNA vaccine develops virus neutralizing antibody (VNA)gaba levels and offers protection comparable with those obtained with the inactivated vaccines (Artem *et al.*, 2008; Lodmell *et al.*, 2002). On the basis of the results of the study conducted in mice, a single administration of the rabies DNA vaccine may be as effective as at least five injections of the cell-culture-derived vaccine (Bahloul *et al.*, 2003).

Vector vaccines are based on recombinant viruses, and several viruses have been tested for these purposes. The VRG vaccine was designed on the basis of poxvirus (vaccinia virus) expressing SAD strain glycoprotein and used for oral immunization of wildlife (Artem *et al.*, 2008). The Adrab.gp - vaccine is based on the adenovirus expressing the ERA strain glycoprotein and was found capable of inducing an immune response in dogs (Tims *et al.*, 2000). The canine herpesvirus (CHV) expressing the glycoprotein of rabies virus has also been used successfully as an anti-rabies vaccine. A raccoon poxvirus (RCNV) recombinant vaccine for the mmunization against feline panleukopenia and rabies has been developed and tested in cats. A recombinant rabies virus vaccine carrying two identical glycoprotein (G) genes (SPBNGA-GA) has also been constructed (Faber *et al.*, 2002).

The rabies virus vaccine strain based on vectors have shown great promise as vaccines against other viral diseases such as human immunodeficiency virus type 1 (HIV-1) infection and Hepatitis C, but a low residual pathogenicity remains a concern for their usage (McGettigan *et al.*, 2003).

Plant-derived antigens can also be used for the immunization against rabies. The coat protein of alfalfa mosaic virus has been used as a carrier molecule to express the antigenic peptides from

rabies virus. The *in vitro* transcripts of the recombinant virus with sequences encoding the antigenic peptides have been synthesized from DNA constructs and used to inoculate tobacco plants. The plant-produced protein (virus particles) has been purified and used for the immunization of mice, and specific anti-rabies virus neutralizing antibodies in immunized mice have been found; spinach has also been used for this purpose (Koprowski, 2002). The transgenic maize expressing the G protein of the Vnukovo strain has also been obtained and tested in mice. It was shown that the mice developed virus neutralizing antibodies which were able to provide protection of 100% against the challenge of a vampire bat strain (Loza-Rubio *et al.*, 2007).

2.16 Genetic Characterization of rabies virus

According to the WHO Expert Consultation on Rabies (2005) it is important to conduct molecular characterization of the new field isolates of the rabies virus. Several phylogenetic and molecular-epidemiological studies on rabies have been performed during the past 15 years (Holmes *et al.*, 2002; Kuzmin *et al.*, 2004; Real *et al.*, 2005). These studies have shown that the rabies viruses can be divided into two major groups, one comprising viruses isolated from terrestrial mammals and the other containing viruses isolated from bats or spillover infections from bats. Additionally, there is a viral lineage that is closely related to the bat rabies virus but which circulates independently in raccoons and skunks, suggesting that it might represent a secondary transmission from bats. It was also found that among terrestrial mammals, rabies viruses cluster more by geographical origin than by host species, and in this case, closely related viruses infect a variety of species (Davis *et al.*, 2006). The phylogenetic reconstruction strongly supports the hypothesis that host switching has occurred in the history of the *Lyssaviruses*. It has been proven that the *Lyssaviruses* have evolved in chiropters long before the emergence of the carnivoran rabies, probably due to spillovers from bats (Badrane and Tordo, 2001). The rabies

virus is an ancient virus but it has been suggested that the current diversities in the genotype 1 of the *Lyssaviruses* from diverse geographical locations and different species may have started only within the last 500 years (Holmes *et al.*, 2002).

The RT-PCR amplification, nucleotide sequencing of the different genome regions, and the subsequent genetic and phylogenetic analysis allows the determination of the genetic groups and the differentiation of the field and vaccine strains of the rabies virus. Different genome regions can be and have been used in molecular-epidemiological studies of the rabies virus. The G gene and the G-L intergenic region (pseudogene, ψ-n region) are much more variable than the N gene and evolutionary pressure on individual protein coding genes within the genome varies considerably (Johnson *et al.*, 2002a). The pseudogene region has been used for the genetic characterization of rabies viruses in several studies (Hyun *et al.*, 2005; Nel *et al.*, 2005). The analysis of the sequence of the nucleoprotein is considered to be adequate for reliable phylogenetic study of the rabies virus, and the additional glycoprotein gene sequence analysis is important for the characterization of antigenic and immunogenic properties of the virus (Kasempimolporn *et al.*, 2004; Kuzmin *et al.*, 2004).

During the last century, rabies virus was spreading to the West and South of Europe but natural barriers, such as the Vistula River in Poland, were able to limit its dissemination (Bourhy *et al.*, 1999). Bourhy *et al.*, (1999) have classified rabies viruses of European origin into four main groups: the NEE-group (North-East Europe), the EE-group (East Europe), the WE-group (Western Europe), and the CE-group (Central Europe). Recently, Kuzmin *et al.* (2004) studied a wide range of rabies viruses isolated from the territory of the former Soviet Union and classified these into five genogroups (A, B, C, D, and E). The data from that study have shown that the viruses with the same geographical origin often group together during phylogenetic analyses.

The number of rabies virus variants is co-circulating in Europe, and are often associated with the red fox; also there are dog-associated rabies and the role of raccoon dogs in maintaining rabies in the Baltic countries is increasing (McElhinney *et al.*, 2006). Mansfield *et al.* (2006) have demonstrated the existence of two groups within the general Arctic group: "Arctic 1" and "Arctic 2", the latter having two subgroups and a separate "Arctic-like" group. The study of isolates from countries in the Middle East has shown the existence of few closely related groups which are different from the viruses of European and African origin; no host dependent relations were found in that study (David *et al.*, 2000). A molecular study of Brazilian rabies viruses has shown the presence of three main host-specific groups, especially among bat viruses and victims of vampire bats (Bernardi *et al.*, 2005). Some authors have combined the use of mAbs and molecular methods to study the characteristics of rabies viruses (Nadin-Davis *et al.*, 2001; 2003; Favoretto *et al.*, 2002).

Most of the rabies cases in terrestrial animals and human beings are due to the genotype 1 viruses, but some cases are caused by viruses from other genotypes. Spillover of the EBLV1 virus into the stone marten (*Martes foina*) under natural conditions has been recorded in Germany (Muller *et al.*, 2004). Also in 1998 cases of rabies in sheep that were shown to have been infected with the EBLV-1a possibly derived from insectivorous bats were observed in Denmark, and in 2002, a second occurrence of the EBLV-type 1 in sheep was reported (Ronsholt, 2002). Also, several human cases caused by EBLVs and ABLV strains have been reported in some European countries and in Australia (Fooks *et al.*, 2003; Spooner, 2003). One of the most important issues in the field of rabies research is the identification and characterization of new genotypes. As a result of PCR and nucleotide sequencing during the last ten years, one new genotype has been identified and four additional ones were proposed. Fraser

et al. (1996) isolated a new *Lyssavirus* from bats in Australia, which was later identified by nucleotide sequencing as the new seventh genotype: Australian bat Lyssavirus or ABLV (Gould *et al*., 1998). The phylogenetic analysis of the ABLV viruses has shown that they form a monophyletic group distinct from the other *Lyssaviruses*, and two antigenic variants of ABLV were described (Guyatt *et al*., 2003). Arai *et al*., (2003) studied rabies virus isolated from the Lesser Mouse-eared Bat (*Myotis blythi*) in Kyrgyzstan and suggested that it belonged to a new genotype of the rabies virus (Aravan virus). Furthermore, the Khujand virus isolated from northern Tajikistan in 2001 can be classified as a separate genotype of *Lyssavirus* (Kuzmin *et al*., 2003). Two unique viruses (Irkut and West Caucasian) have been isolated from bats in Russia and based on genetic studies it has been suggested that they belong to new genotypes (Botvinkin *et al*., 2003; Kuzmin *et al*., 2005).

2.17 Rabies Epidemiology

2.17.1 Rabies Situation World-Wide

Rabies is widely distributed throughout the world and is present in all continents except Australia, where only bat Lyssavirus has been found, and in Antarctica. Worldwide, it has been estimated that approximately 55,000 persons die annually due to rabies; 99% of human rabies deaths have occurred in the developing countries. Customarily, the level of international resources committed to the control of an infectious disease is a response to the associated human morbidity and mortality. For most infectious diseases, these data adequately reflect the deserved public health attention. It is difficult, however, to estimate the global impact of rabies by using only human mortality data. Because vaccines to prevent human rabies have been available for more than 100 years, most deaths from rabies occur in countries with inadequate public health resources and limited access to preventive treatment. These countries also have few diagnostic facilities and almost no rabies surveillance (CDC, 2011). The total global expenditure on rabies prevention is well over US$ 1 billion annually (WHO Expert Consultation on Rabies, 2005). Different animal species can be responsible for viral circulation and rabies transmission in different continents and countries. Canids have been determined to be the main hosts of the rabies virus in Africa; in most cases they are also responsible for the transmission of the virus to humans. In addition to canids (domestic and wild dogs, jackals, and wolves), mongooses, and bats are involved in rabies epidemics, as occurs in Africa (Bingham, 2005). Dogs are also the primary reservoir for rabies in Thailand (Tepsumethanon *et al.*, 2005). In the USA, several species are involved in rabies epidemics but the main reservoirs are raccoons and skunks (Krebs *et al.*, 2003). An epizootic of raccoon rabies, begun in the USA in the late 1970s, and developed into one of the largest and most extensive in the history of wildlife rabies (Childs *et al.*, 2000).

Rabies has been detected in rodents and lagomorphs, mostly in woodchucks and also in arctic foxes (Ballard *et al.,* 2001). In addition, bats are sometimes responsible for the transmission of rabies to humans (Miah, 2005).

2.17.2 Rabies in Nigeria

In Nigeria, the dog is responsible for over 96% of rabies cases in animals (WHO, 2005). Rabies remains an endemic and neglected tropical disease in Nigeria and is often misdiagnosed, under-diagnosed and underreported (Adedeji *et al.,* 2010; Ehizibolo *et al.,* 2011). In some cases, despite vaccination, the disease has been reported (Adedeji *et al.,* 2010; Kujul *et al.,* 2010). Some of the factors responsible for the endemicity of rabies in Nigeria lack of knowledge and information about the disease and poor public awareness, socio-economic factors and increasing human activities involving dogs e.g. hunting. Dog trade is also gaining popularity in Nigeria. Although available records on rabies in Nigeria are far from complete, reports of human deaths from rabies in the country are a matter of great concern. Knowledge, attitude and practice (KAP) towards rabies among dog meat processors and dog meat consumers have been shown to be an important factor in the control of rabies in different part of the country (Opaleye *et al.,* 2006; Isek, 2013; Odeh *et al.,* 2014).

2.18 Vaccination

Vaccination against rabies has been shown to be an efficient way for its control and statistics have shown that successful immunization of 70% - 80% of dog population in a country places rabies under control. Mass vaccination has been used successfully in Western Europe and North America (Wandeler, 2000; WHO, 2004), showing that the disease can be controlled and eliminated by vaccination of reservoir animal populations. Japan was the first country to

implement mass vaccination of dogs, successfully eliminating the disease in 1956 (WHO 1996). In Nigeria however, it has not been possible to successfully control and eradicate rabies, instead evidences show that the disease is on the increase (Ogunkoya, 1997). Furthermore there have been several reports of rabies occurring in dogs vaccinated with low egg passage (LEP) – flurry strain anti-rabies vaccine, which is the official vaccine produced by National Veterinary Research Institute (NVRI) Vom – Nigeria and used in Nigeria.

Despite the availability of safe, effective and relatively cheap vaccines for dogs, rabies remains uncontrolled in most African and Asian countries (WHO, 2001). Insufficient funding compounded by lack of awareness about the true burden of the disease may be responsible for failure of current control programmes. It appears many Nigerians still see rabies as insignificant and do not take serious measures to prevent or control its occurrence. Many are still ignorant about the disease and do not vaccinate their dogs because they claim it changes the taste of dog meat and affects the development of canine teeth for hunting and protection (Idachaba *et al*., 2009).

2.19 Control and Prevention

All human cases of rabies were fatal until a vaccine was developed in 1885 by Louis Pasteur and Émile Roux. Their original vaccine was harvested from infected rabbits, from which the virus in the nerve tissue was weakened by allowing it to dry for five to 10 days. Similar nerve tissue-derived vaccines are still used in some countries, as they are much cheaper than modern cell culture vaccines (Srivastava *et al*., 2004).

Animal rabies control consists of vaccination of dogs and cats, elimination of stray animals, health education for public, etc (Artem *et al.*, 2008). Mass vaccination of dogs is not

implemented in some countries and effective coverage rate is not known; this and the elimination of stray dogs by shooting, has a minimal effect on transmission of rabies (Knobel *et al.*, 2005). For traveling purposes, vaccination of domestic carnivores is obligatory. Some countries require testing for neutralizing antibodies against rabies. The minimum threshold level accepted by OIE/WHO is 0.5IU/ml (Jackel *et al.*, 2008).

In Nigeria, where dogs serve as the main reservoirs of rabies virus and dog bites continue to be the main mode of transmission, the disease remains a serious public health problem (Awoyomi *et al.*, 2007). The most effective approach to control of rabies in Nigeria is by mass vaccination of at least 80% of dog population in country, within the shortest possible time, combined with elimination of stray and ownerless dogs (Awoyomi *et al.*, 2007). But this high level of immunity is rarely achieved in most African countries, due to socio-economic barriers.

Post-Exposure treatment in humans usually involves combined active and passive immunization immediately after exposure, which helps in elimination of the virus before it enters the nervous system (Wandeler, 2000). This treatment in humans should be done as soon as the exposure occurs. The treatment for rabies prevention in man must consist of thorough wound cleaning for about 15minutes with soap and clean water; a virucidal antiseptic such as povidone iodine or ethanol should be applied on the wound surface. This is then followed immediately by the administration of rabies passive immunization and cell culture or purified embryonated egg rabies vaccine of proven efficacy (WHO, 2004).

In dogs, vaccination after infection does not significantly alter the clinical picture or prevent the development of the disease (Fooks and McElihnney, 2000). This is in contrasts to post-exposure treatment in humans which is effective after infection. It is possible that an animal infected

before vaccination may continue to incubate the disease even after developing a high antibody titre (Fooks and McElihnney, 2000).

CHAPTER THREE

3.0 METHODOLOGY

3.1 Study Area

The study was carried out at dog slaughter points in Benue state. Benue state is in the mid-belt region of Nigeria, having a population of about 4,253,641 as estimated in 2006 census. Benue State lies within the lower river Benue through in the middle belt region of Nigeria. Its geographic coordinates are longitude 7° 47' and 10° 0' East. Latitude 6° 25' and 8° 8' North; and shares boundaries with five other states namely: Nasarawa State to the north, Taraba State to the east, Cross-River State to the south, Enugu State to the south-west and Kogi State to the west. The state also shares a common boundary with the Republic of Cameroon on the south-east. Idoma and Tiv, are the most predominantly tribes in Benue state but there are other ethnic groups, including Igede, Etulo and Abakwa, Jukun, Hausa, Akweya and Nyifon. With its capital at Makurdi, Benue is a rich agricultural region. Benue occupies a landmass of 34,059 km^2. Dog slaughter houses are located within some Local Government Areas in the State such as Makurdi and Agatu where dog brain samples were collected (Seibert, 2007).

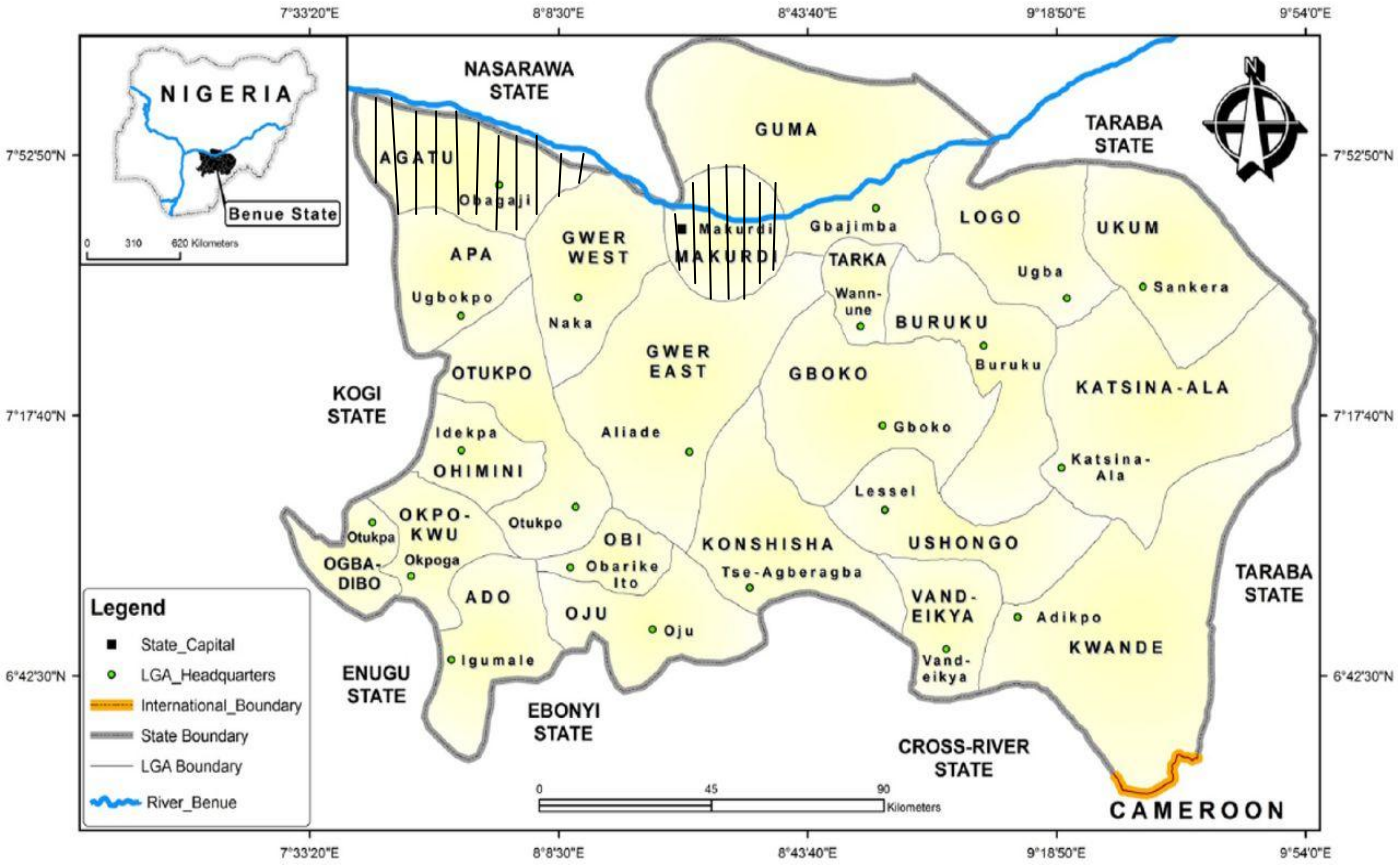

Figure. 3.1: Map of Benue State showing the Study Area
Source: Nigeria Spatial Infrastructure Data Base

3.3 Study Design

The study involved a purposive cross sectional study of trade dogs at two LOCAL Government Areas of Benue State and an experimental research involving the detection of rabies antigens in brain of slaughtered dogs and PCR identification of the virus and phylogenic analysis of sequenced PCR amplicons of the N gene.

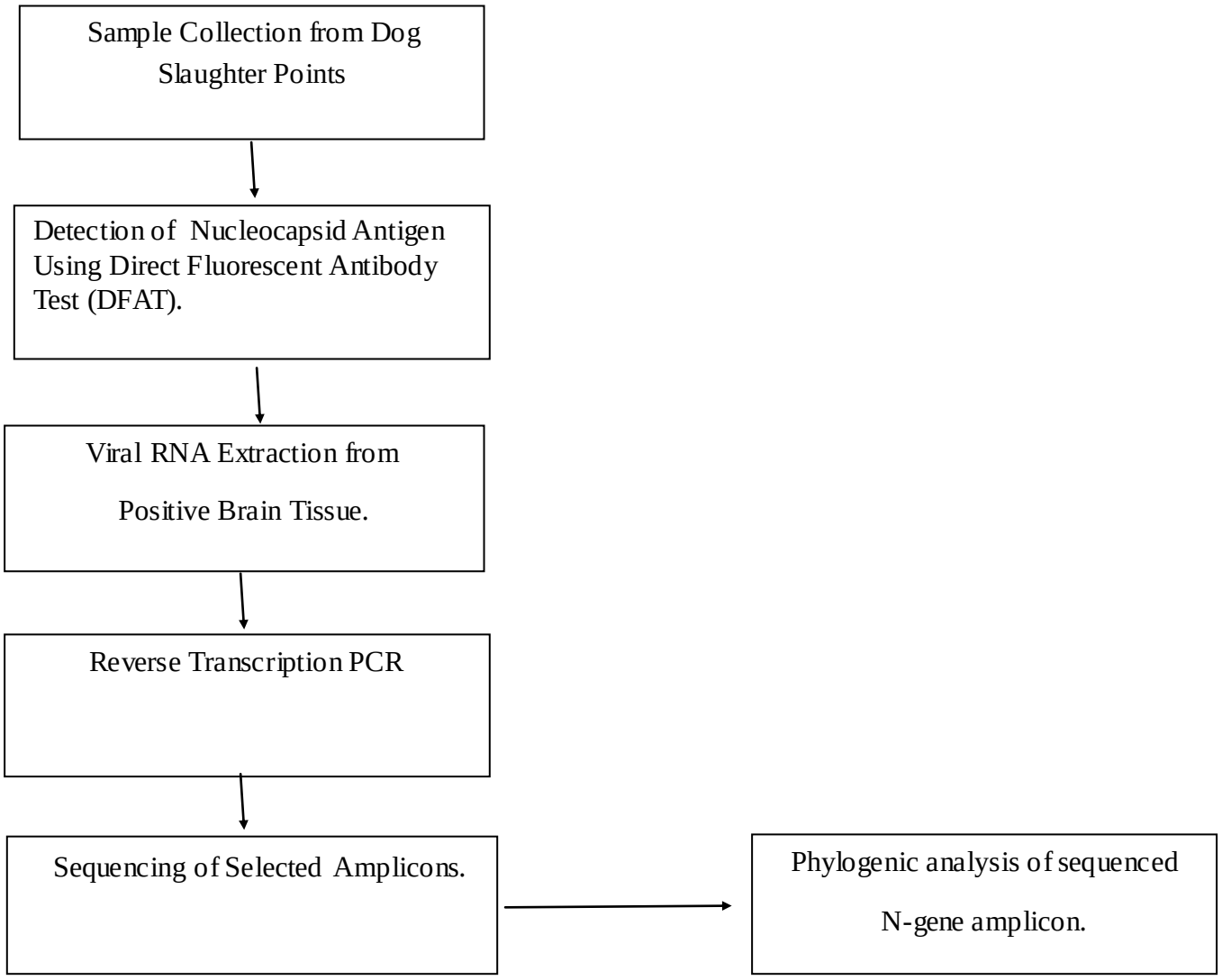

Figure 3.2 Research Design Outline

3.4 Sample Collection

A total of 50 brain samples were collected from slaughtered dogs at various slaughter points within the study area. Information on age of the dogs, sex, breed health status and source of dogs slaughtered were also collected. Samples were placed in sterile plastic bottles, preserved at -20^{0}C and transported in a cold box to the Viral Zoonoses Laboratory of the Department of Veterinary Public Health and Preventive Medicine, Ahmadu Bello University Zaria, for fluorescent antibody testing. Each slaughter point was visited and samples were collected based on availability. Fifty dog brain samples were collected from six (6) slaughter points within the Benue metropolis namely; North Bank, Wadata, Mobile Barracks, Railway market, High level, all in Markudi and Osugudu in Agatu Local Government Area. The samples were labeled or name by the alphabet J and the number of sequence of collection, that is, J1 to J50. Out of the 50 samples, 10 were collected from North Bank among which, 7 were males and 3 females, 8 samples from Wadata among which, 6 were males and 2 females, 5 samples from Mobile Barracks which 2 were males and 3 females, 14 from Osugudu among which 9 males and 5 females, 12 samples from Railway market which both male and female were 6 each, and 9 samples from where 4 were males and 5 females. In total there were 34 males and 16 females with 68.0% and 32.0% respectively.

3.5 Direct Fluorescent Antibody Test

Direct Fluorescent Antibody test (DFA) was performed on the brain samples collected from slaughtered dogs as described by Dean *et al.* (1996). Rabies direct fluorescent antibody assay (DFA) monoclonal antibody .reagent (Fujirebio Diagnostic Inc. Malvern, P.A 19355, USA) was used, working according to manufacturer's recommendations. An impression smear of part of the brain sample was made on a clean glass slide, air dried and fixed in cold acetone for 30 minutes

at -20^{0}C. The slide was then air dried for 5 minutes and a drop of the rabies conjugate was applied on the smear using a micro pipette and this was incubated for 30 minutes at 37^{0}C in a humid chamber. After the incubation step, excess conjugate was removed by washing with PBS (pH 7.4) for about 5 minutes, repeated three times, and allowed to air dry. The slides was then examined using the fluorescent microscope at X 40 magnification objective lens. The presence of brilliant apple green fluorescence or greenish-yellow objects against a dark background was regarded as positive and the intensity was noted and scored as +, ++, +++ or ++++ based on the number of fluorescents per unit area of the field, while the absence of specific apple green fluorescence was regarded as negative for rabies (Woldehiwet, 2005).

3.6 Molecular Analysis

3.6.1 RNA Extraction

Total RNA was extracted from all the DFAT positive samples using Trizol reagents according to the manufacturer's instruction (Life Technologies, Carlsbad, California, USA). Briefly about 0.1 g brain tissue was homogenized with 1 ml Trizol and then 200 ml chloroform (Sigma Chemical Co., St Louis, MO, USA) was added and mixed. After centrifugation of the sample at 10 000 g for 15 min, the top aqueous layer was recovered and RNA was precipitated by adding 0.5 ml isopropanol. The sample was spun at 10 000 g for 10 min, the liquid was removed and the pellet washed with 1 ml of 75% ethanol. The RNA was dissolved in RNase free buffer provided with the kit. RNA was quantified with a spectrophotometer (Gene Quant II Pharmacia, Piscataway, NJ, USA) and stored at -70 ^{o}C, prior to use.

3.6.2 Polymerase Chain Reaction (PCR)

PCR was performed using one-step RT-PCR kit following manufacturer's protocol. For the amplification, the N gene specific primers targeting the 355 bp conserved region based upon the previous submissions in the Genbank were used (Singh *et al.*, 2010).

Briefly, 25µl reaction mix was prepared consisting of 5µl purified RNA template, 5µl 5x QIAGEN one step RT-PCR buffer, 3µl 5x QIAGEN Q solution, 1.5µl dNTP mix (10mM), 3µl (30pmol) each of N specific forward primer; RabN-533F; 5'-CATTGCAGATAGGATAGAGC-3' and reverse primer; RabN-888R; 5'-GAGGAACGGCGGTCTCCTG-3', 1µl of QIAGEN RT-PCR enzyme mix, 0.5µl of RNase inhibitor (20U/µl), and 3µl of dH_2O. The reaction was incubated at 60°C for 1minute, 42°C for 10minutes, 50°C for 30minutes for the cDNA synthesis (step 1) followed by denaturation at 94°C for 5minutes (step 2). After the initial denaturation, the amplification was carried out for 30 cycles in three steps, 94°C for 30seconds, 55°C 30seconds and 72°C for 30seconds (step 3), with a final extension at 72°C for 5minutes.

PCR aliquots were electrophoresed through a 1.5% agarose gel, stained with ethidium bromide (5mg/ml) (Gibco-BRL, Grand Island, NY, USA), evaluated under UV light and photographed.

3.7 DNA Sequencing

The DNA fragment matching the size of expected amplicon was excised from the agarose gel and weighed in a 1.5ml micro tube (A gel of 100mg is approximately equal to 100ul.). 3 gel volumes of buffer GB was added and the mixture was incubated at 50°-60° for 8 minutes with mixing of the tube by tapping the tube bottom every 2-3 minutes till gel was completely melted. 2 gel volumes of isopropanol was added and mixed. 750ul was transferred to a DNA mini column with a collection tube and was centrifuged at 11000 rpm for 30 seconds before the flow through was discarded and the column placed back in the collection tube. This was repeated until there wass no remaining mixture. 750ul of wash buffer was added to the column and centrifuged

at 11000 rpm for 1 minute at room temperature, and the flow through was discarded. The empty column was further centrifuged at 11000 rpm for 1 minute to remove the residual ethanol.

The PCR fragments was sequenced using the ABI PRISM Big Dye terminator cycle sequencing kit (Perkin Elmer, USA) with primers RabN-533F and RabN-888R on an ABI 310 genetic analyzer. A labeled sterile 0.5ml tube was prepared for the sample. Fresh stop solution/glycogen mixture was prepared as follows per sequencing reaction: 2ul of 3M Sodium acetate, 2ul of 100mM Na2-EDTA and 1ul of 20mg/ml of glycogen (provided in the kit).To the labeled tube 5ul of the stop solution/glycogen mixture was added. Sequencing reaction was transferred to each of the appropriately labeled tube and mixed thoroughly. Sixty microlitres (60ul) cold 95% (v/v) ethanol from -20 freezer was added and mixed thoroughly. This was immediately centrifuged at 14,000rpm at 4^{o}C for 15min and carefully the supernatant was removed with a micropipette (the pellet being visible). The pellet was rinsed with 200ul 70% (v/v) ethanol from -20 freezer, centrifuged at 14,000rpm at 4C for a minimum of 2minutes and carefully all the supernatant was removed with a micropipette. The constituent was vacuum dried for 10min or until dry and resuspended in 40ul of the sample loading solution. The resuspended sample was transferred to the appropriate wells of the sample plate (PN 609801) and overlaid with one drop of mineral oil from the kit. The sample plate was loaded into the instrument and the desired method started.

3.8 Sequence Analyses and Phylogenetic Reconstructions

Sequence analyses and phylogenetic reconstructions was performed using Bio Edit (Hall, 1999) and MEGA 6 software platforms (Tamura *et al*., 2013). Phylogenetic analysis was conducted by

comparing the partial rabies sequences with other rabies isolates selected from the Genbank representing rabies virus isolates that circulate in Africa.

3.10 Statistical Analyses

Data obtained were subjected to descriptive statistics and categorical evaluation of gender, source or location and test outcome. Results were presented in Tables and charts.

CHAPTER FOUR

4.0 RESULTS

4.1. Detection of Rabies Virus Nucleocapasid Antigen in Brain Tissues of the Dogs using

Out of 50 samples tested for nucleocapasid antigen in brain tissues of the dogs using Direct Fluorescent antibody technique (DFAT) only 1 sample from Osugudu tested positive, that is, sample No.J50 (Table 4.1). The dog from which this sample was collected was showing the signs of full blown rabies, such as hydrophobia, ferociousness and secluction. The DFAT positive rate is 7.1% for Oshugudu and 0.0% for the rest locations. The DFAT positive rate among all the samples was 2.0% (Table 4.1).

Table 4.1 The Occurrence of Nucleo-capsid Antigen Detection among The Dog Brain Tissues based on Sex and Sampling Location in Benue State

Location	No. of Samples	Sex		No. Positive	Percentage
		Male	Female		
North Bank	10	7	3	0	(0.0)
Wadata	8	6	2	0	(0.0)
Mobile barracks	5	2	3	0	(0.0)
Osugudu	14	9	5	1	(7.1)
Railway market	12	6	6	0	(0.0)
High level	9	4	5	0	(0.0)
Total	50	34	16	1	(2.0)

4.2 PCR Amplification of DFAT Positive Sample

Out of the 50 samples tested only one was positive by DFAT, this DFAT positive sample was obtained from a male dog in Oshugudu. The DFAT Florescent score of the sample was estimated to be ++++ and the sample was also positive for N gene fragment amplification by PCR (Table 4.2). The electrophoregram slide shows the molecular weight marker on Lane M, the positive sample J50 on Lane 1 and the Negative control on Lane 2. The amplicon size as indicated by the arrow shows that the band as within the 355bp as to be amplified by the primer (Plate I).

Table 4.2 Positivity Score of DFAT Positive and PCR Test Outcome.

Sample ID	location	sex	No. Positive	positivity Score	PCR Amplification
J50	Osugudu	Male	**1**	**++++**	**1**
TOTAL			(1)		(1)

Key

+: positive and -: Negative

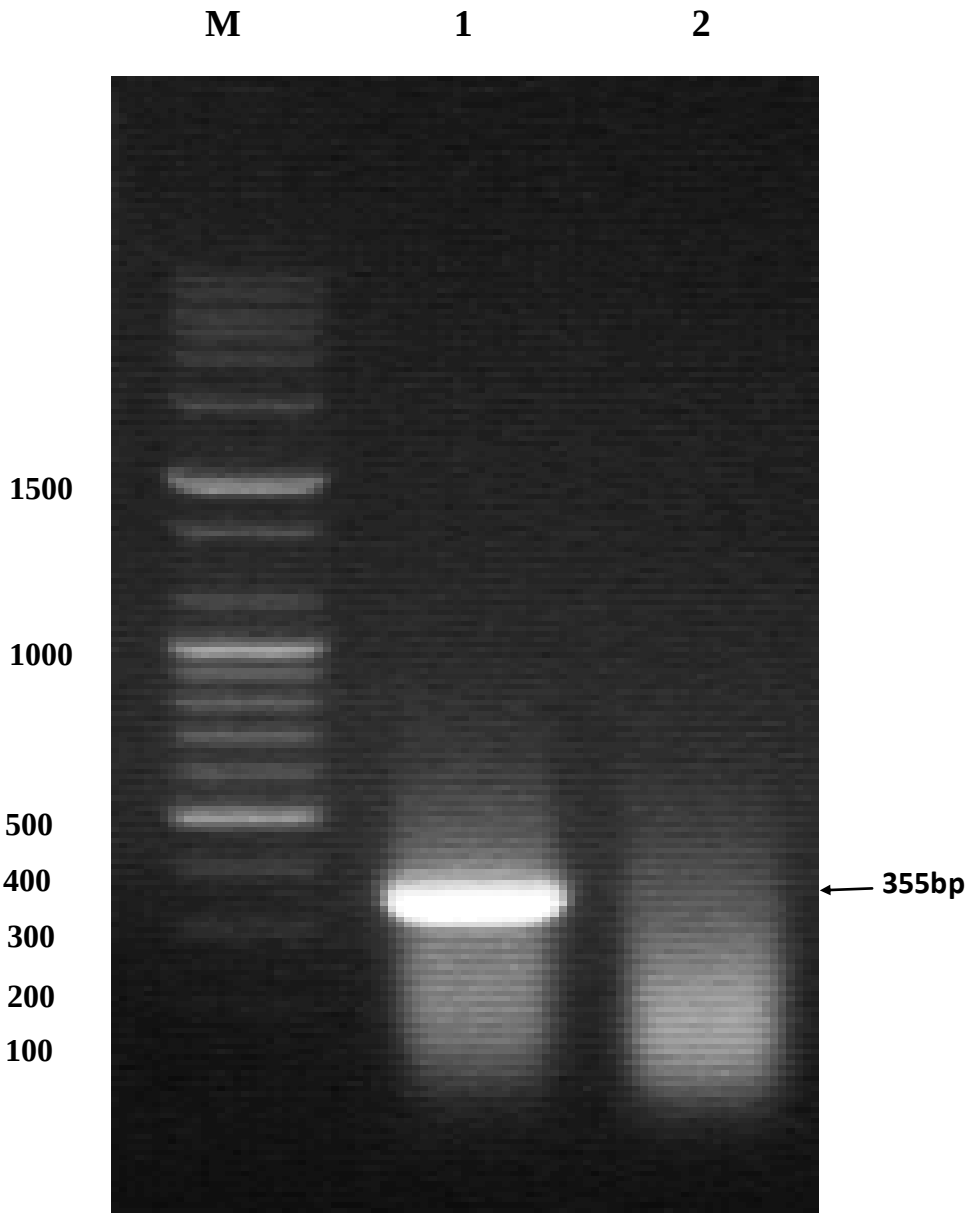

Plate I: An Agarose Gel under UV light following Amplification of N gene Fragment from DFAT Positive Samples.

KEY

Lane M: 100bp Molecular Weight Marker, Lane 1 is Sample J50, Lane 2: Negative Control

4.3 Sequence Reads of the N Gene Segment amplified from Sample J50

The amplified N gene segment was sequenced and 267 Nucleic acid was obtained.

‘’CTCTTGCAGTGAGATTTATCTGTTTTATGAACCCGGTGAATGACACCAACCCAGAG
CAATCTTCATAAGCTGTGACCACTGTGCCCACTCTGATAGCTGAATATAAATGCTCA
ATCCGGGAGAAATACATGTCGTATGTTCCGGCCAAGAATCGGAAGTTCGGTATGGT
ACTCCATTAGCGCACATTTTATGAGTTGTCATCAGGGTATGATGTTCCACAATTTTAA
CGAAGGGGCTGTCTCGAAAATCTGCTCTATCCTATCTGCA’’. The sequence was aligned with 31 other Rabies Virus N gene sequences from the Gen bank. Among these aligned sequences RBV J50 had a maximum similarity of 97% with isolate KR080522 (Dog 2012 Nigeria Bida)and a minimum similarity of 93% (Appendix II). There was a variation at 32 different non serial positions, with a variation rate of 8.3% among the whole group (Appendix IV).

4.4 Phylogenic Analysis of the Aligned Rabies Virus Sequences

The analysis involved 32 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 267 positions in the final dataset. Evolutionary analyses were conducted in MEGA6 software program. The evolutionary history was inferred using the Maximum Parsimony method. From the tree the possible lineage of Rabies Virus is the Africa 2 lineage and isolate KR080522 (Dog 2012 Nigeria Bida) is the most closely related to the RBV J50, in the west African region isolate KT119774 (Dog 1987 Cameroon) is the most closely related to the RBV J50 (Figure 4.1).

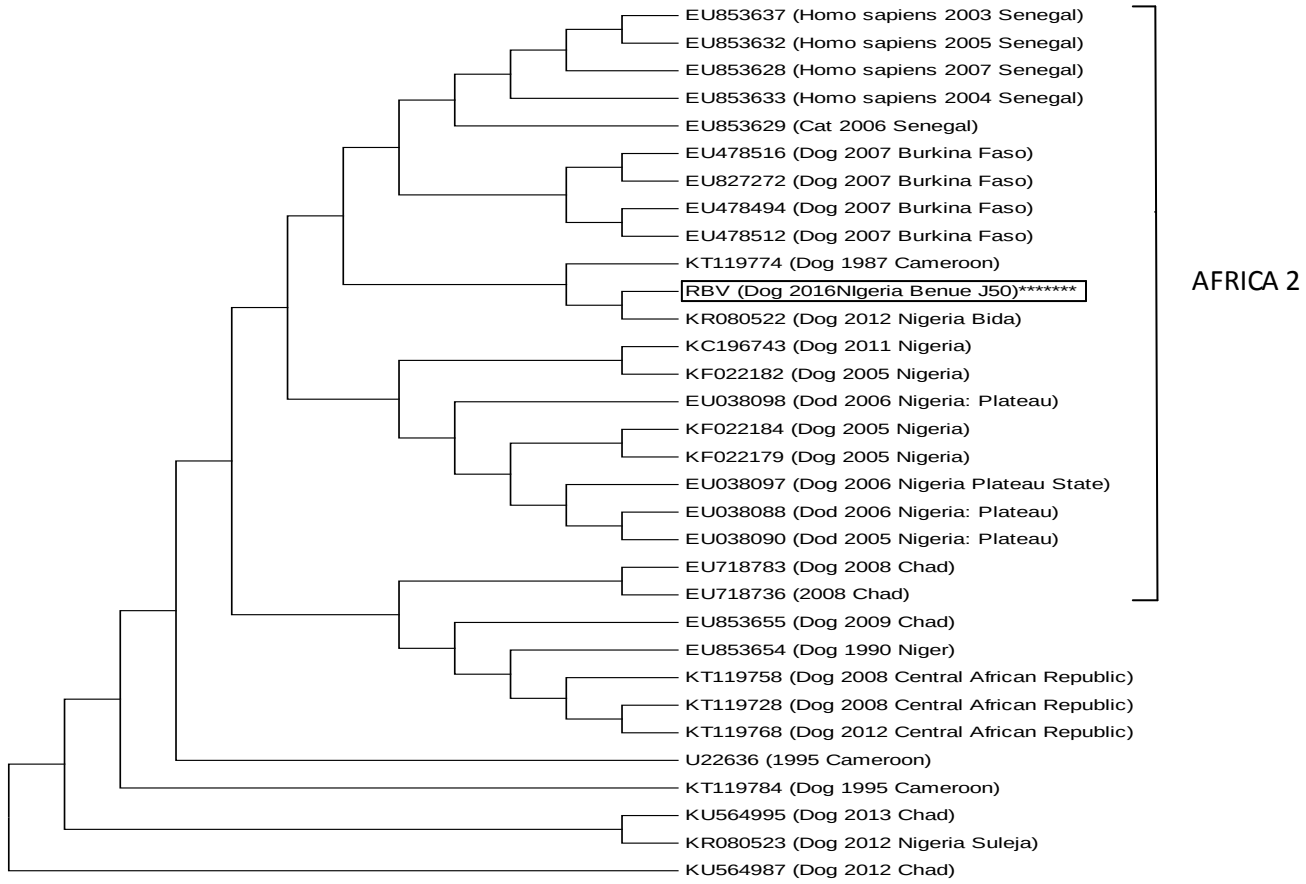

Figure 4.1 Phylogenic Analysis of the Rabies Virus RBV J50 with 31 other Sequences from the Gen Bank

CHAPTER FIVE

5.0 DISCUSSION

Rabies antigen was detected in 1 (2%) of the 50 dog brain samples tested. This indicates that dogs slaughtered for human consumption may be a source of infection to dog handlers and butchers. This rate is lower than those reported previously by (Akumbo *et al.*, 2009; Ameh *et al.*, 2014; Otolorin *et al.*, 2014). Most of the individuals involved in dog meat processing have been found not to utilize personal protective wears and also the method of slaughter predisposes these dog meat processors to bites from dogs during restraint. About 68.0% of the brain tissue samples tested was obtained from male dogs as more male dogs were slaughtered in the study area during the period of sample collection.

The intensity of fluorescence of the DFAT positive sample was estimated to be ++++ and the sample was also positive for N gene fragment amplification by PCR. This intensity of fluorescence of a sample may be a factor affecting the PCR Amplification of the sample, owing to the fact that the intensity represents the concentration of the rabies virus in the sample. Nevertheless PCR amplification can be affected by other factors that may cause RNA degradation. RNA degradation can affect the RNA content of a sample which can lead to total loss of RNA and unavailability of Nucleic acid to be amplified in the sample. The concentration of fluorescein entities, shows that viral load in sample J50 is high. This was also shown by the display of signs by the dog indicating that it was at advance state of the infection that is, full blown rabies. Often, people mistake the presence of artifacts as being fluorescein entities by Rabies Nucleoprotein antigen but with PCR better inference can be made.

The sequenced segment of the N gene of the Rabies virus was compared with 31 other sequences from the Gen bank. Among these aligned sequences RBV J50 had a maximum similarity of 97%

with isolate KR080522, Dog 2012 Bida and a minimum similarity of 93%. There was variation at 32 positions which was non serial with a nucleic acid variation rate of 8.3% among the whole group. The amnino acid variation occurred in 28 positions, showing that the nucleic variations were significant, resulting in different codons for amino acid synthesis. The variation in Nucleic acid composition affects the amino acid sequence, because amino acid changes can potentially affect the folding of proteins which can have an influence on the conformational antigenic sites (epitopes) (Moore *et al.,* 2005). The differences determined can theoretically influence the immunological characteristics of the Rabies virus. Further studies may be needed to support that, because immunogenicity and pathogenicity of rabies virus have complex nature and is not yet fully understood (Moore *et al.,* 2005).

A cladogram of the N gene fragment was constructed to infer phylogenetic and it has shown that the rabies virus (RBV J50) is of the African 2 lineage and has the highest homology to the KR080522, Dog 2012 Bida, Nigeria and KT119774 1987 Cameroun, which suggests that this has its evolutionary history from Cameroun considering the date (1987) a similar virus was isolated in Cameroun and the homology between the viral N gene sequences. The history of possible Cameroun origin can be justified by the fact that some parts of Benue state share boundary with Cameroun, thus can aid the virus movement through trade or through wild life movement. The Rabies virus (RBV J50) detected in this study is of a west African nativity, considering the evolutionary relatedness with sequence of the N gene of west African isolates from Gen bank. The relationship with Rabies viruses isolated in humans and cat in Senegal indicates that mutual infection and transmission of rabies virus might exist between animal hosts and humans. The construction of phylogenic cladogram of N gene from rabies viruses is the

process of evaluation and simulation for the topology of evolutionary relationship among the sequences, which can display the possible source and transmission routes of rabies as well as the cycling transmission and historic migration among the hosts in a certain degree. It can be used for laboratory diagnosis, genotyping and molecular epidemiological investigation for rabies virus infection (Tao *et al.*, 2009).

Practices of good vaccination of dogs handlers, advising dog handlers to wear protective clothing (such as hand gloves, coveralls and protective boots) and receiving human anti-rabies vaccine and washing of dog bite wounds with soap and water are also indicators that the community is involved in the control of Rabies disease (Otolorin *et al.*, 2014). Poor practices of seeking traditional medicine and non-washing of dog bite wounds go against the WHO recommendation of instituting medical treatment on victims of dog bite. These negative practices may be as a result of inadequate awareness of the possible dangers of rabies. Individuals involved in the handling and processing of dog meat are constantly being exposed to rabies virus through bites from dogs, cuts or wounds that are not well protected from infective tissue or saliva of these dogs. Presence of antigens to rabies in brain of dogs slaughtered for human consumption is very significant in the epidemiology of the disease. This means that dog meat processors and handlers are at risk of being exposed to rabies either from bites from dogs before slaughter or by coming in contact with infective tissue or saliva (Sabo *et al.*, 2008; Garba *et al.*, 2008; Akombo, 2009; Aliyu *et al.*, 2010, Isek, 2013). Sequencing data of biological genomes have increased exponentially now and the sequenced data have become hotspots for genomic and proteomic investigations (Bansal, 2005). The present study has carried out the sequencing of a segment of the N gene of the amplicon of the PCR positive sample and deduced the composition of nucleic acids of the N gene, its variation from others from the Gen bank which may provide the

molecular basis for further investigations on genomic and proteomic structures, biological characteristics, pathogenic mechanisms and other data for dog rabies.

CHAPTER SIX

6.0 CONCLUSION AND RECOMMENDATIONS

6.1 Conclusion

1. This study revealed the presence of Nucleo protein antigen using the Direct Flourescent Antibody Test (DFAT) in 2% of the 50 dog brain samples tested. The 1 DFAT positive sample was from a male dog.
2. The positive sample (J50) with DFAT fluorescence score of (++++) was positive by Polymerase Chain Reaction (PCR) for Rabies virus N-gene segment.
3. The amplified N-gene segment after sequencing was compared to 31 other Rabies virus N-gene segment sequences from the Gene bank and there was a maximum homology of 97% and a minimum of 93%. The group variation occurred at 32 different non-serial positions of the nucleic acid of the gene, with a group variation rate of 8.3%.
4. The phylogenic analysis gave an evolutionary pattern, showing the Rabies virus is of west African origin belonging to the African 2 lineage and a possible history from Cameroun. .

6.2 Recommendations

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

1. There is need for continued surveillance nationwide to have a better understanding of the epidemiology of rabies virus so as to aid in prevention, control and eradication.
2. There is need to sequence strains or isolates of rabies from clinical cases to further understand the molecular epidemiology of the virus. Further genomic characterization of the viral isolates obtained is needful to give more information about their origin and evolution of circulating strains.
3. Dog trade and movement both into and within the country should be strictly monitored to ensure rabid dogs are not allowed into the country and control of strays to prevent rabies virus introduction and spread.
4. Public education of high risk individuals on the likelihood of contracting rabies from infected trade dogs and how to prevent it.

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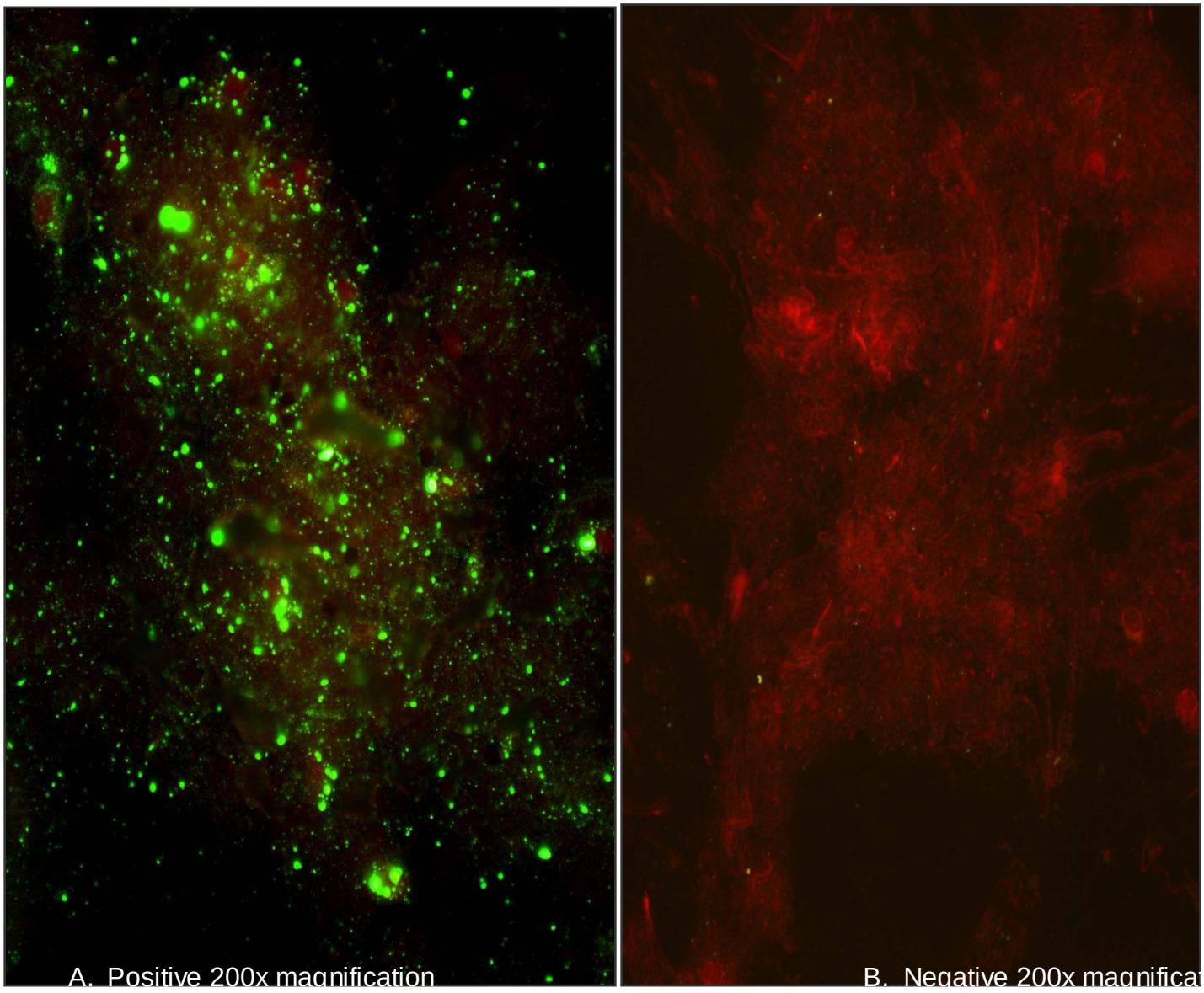
APPENDIX I

Control Slides for the Direct Fluorescent Antibody Test

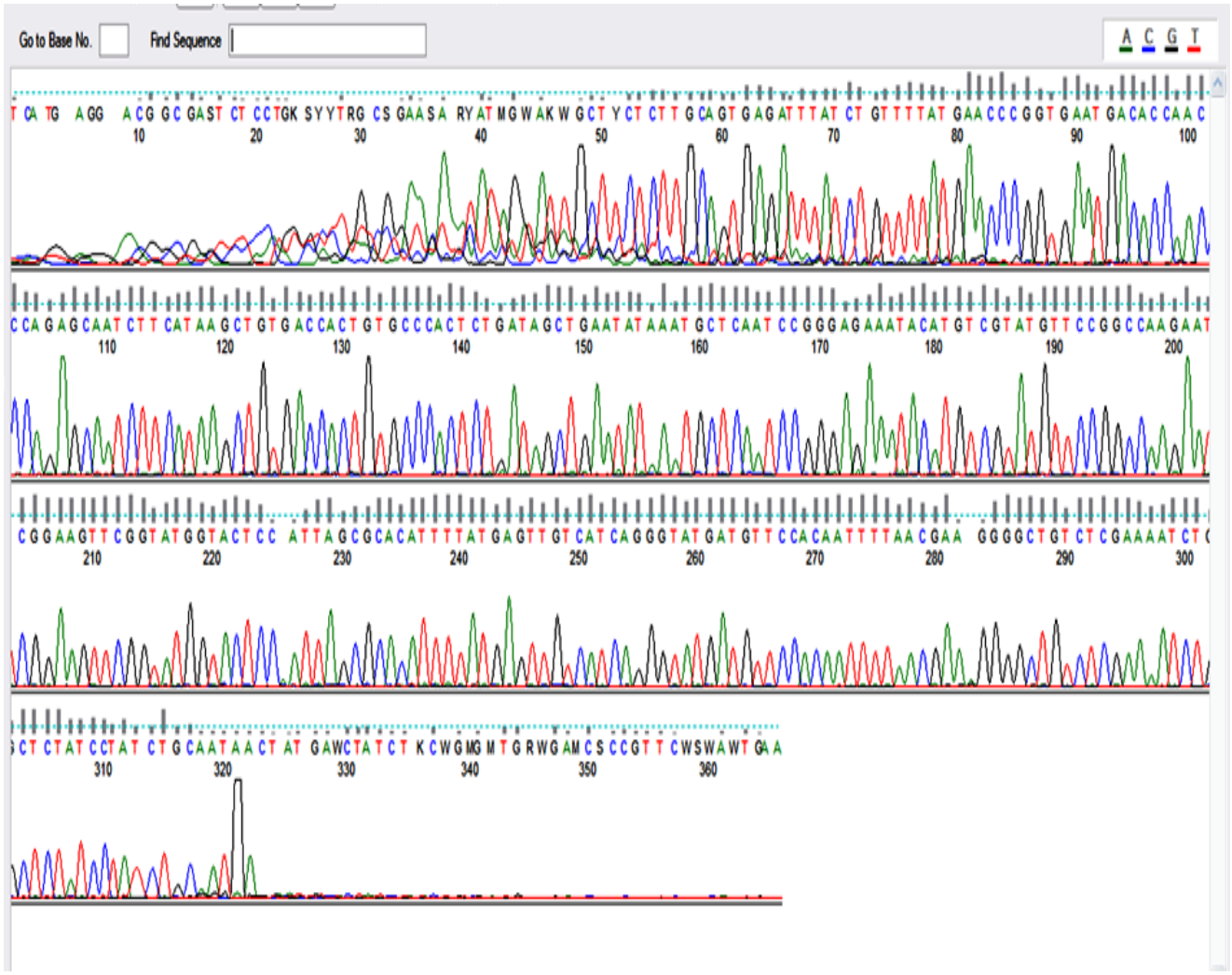


Slide A: Positive Control

Slide B: Negative Control

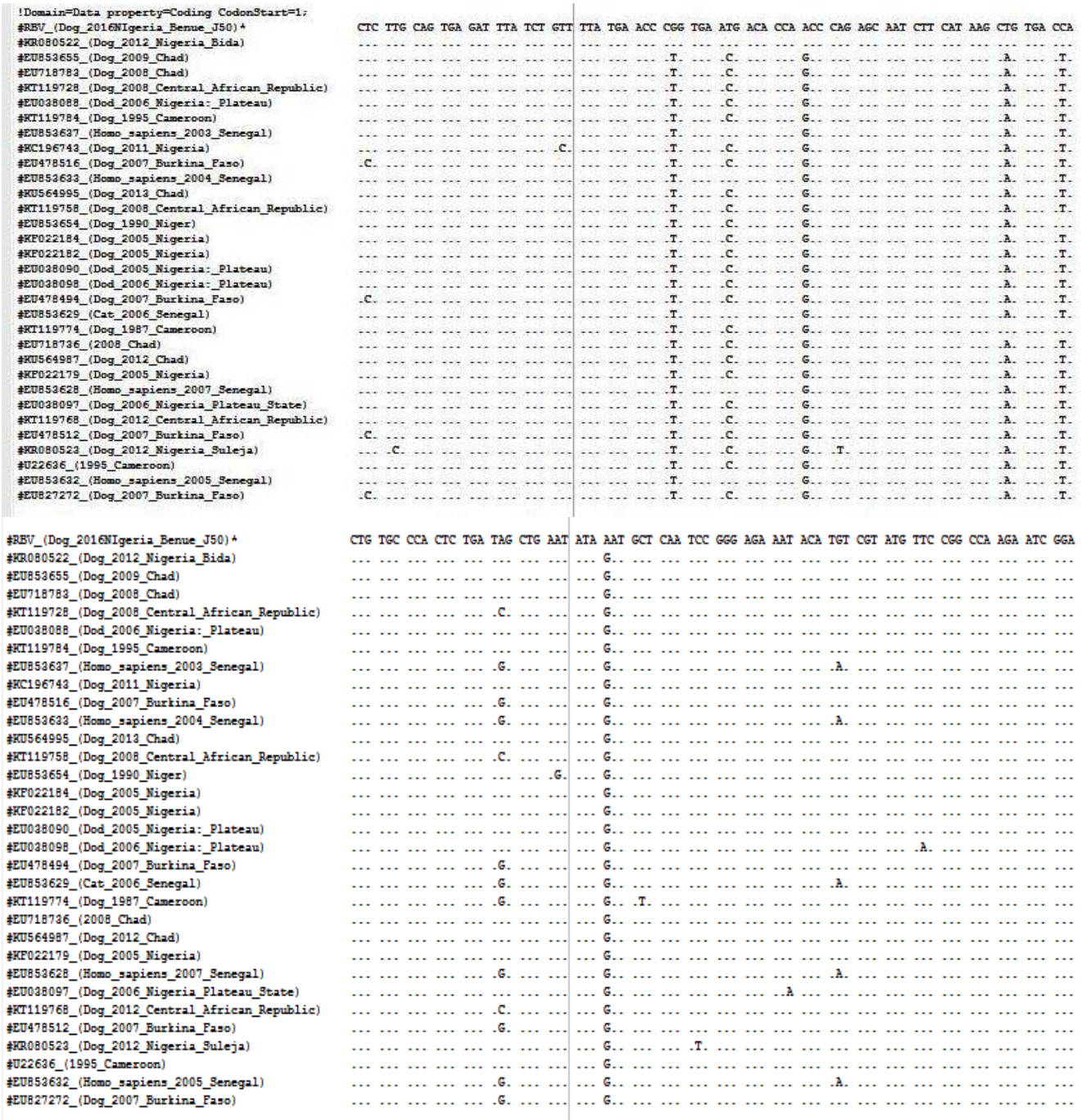
APPENDIX II

Chromatogram for the Sequencing of the Amplified N-Gene Segment



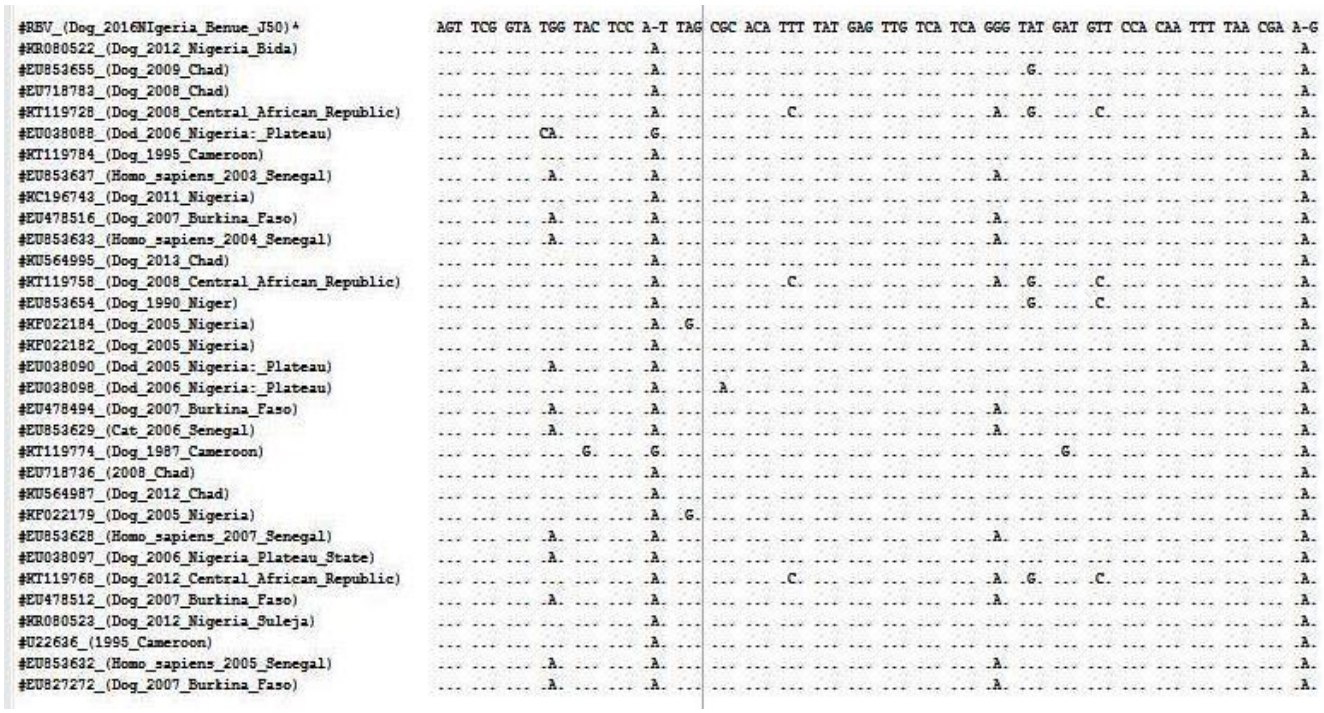
APPENDIX III

Sequence variability of the sequenced sample No 50 compared to related sequences from the Gene bank.

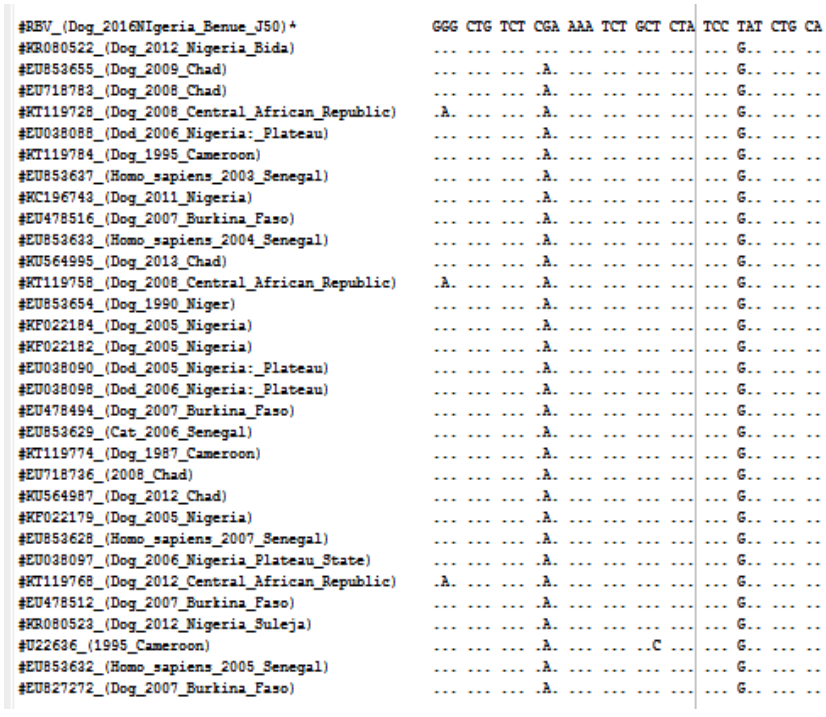


APPENDIX IV

Sequence variability of the sequenced sample No 50 compared to related sequences from the Gene bank cont'd



	AGT	TCG	GTA	TGG	TAC	TCC	A-T	TAG	CGC	ACA	TTT	TAT	GAG	TTG	TCA	TCA	GGG	TAT	GAT	GTT	CCA	CAA	TTT	TAA	CGA	A-G
#RBV_(Dog_2016NIgeria_Benue_J50)*																										
#KR080522_(Dog_2012_Nigeria_Bida)	...	...	...	...	...	...	.A.	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	.A.
#EU853655_(Dog_2009_Chad)	...	...	...	...	...	...	.A.	...	...	...	...	...	...	...	...	...	...	.G.	...	...	...	...	...	...	...	.A.
#EU718783_(Dog_2008_Chad)	...	...	...	...	...	...	.A.	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	.A.
#KT119728_(Dog_2008_Central_African_Republic)	...	...	...	...	...	...	.A.	...	...	...	.C.	...	...	...	...	...	.A.	.G.	...	.C.	...	...	...	...	...	.A.
#EU038088_(Dod_2006_Nigeria:_Plateau)	...	...	...	CA.	...	...	.G.	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	.A.
#KT119784_(Dog_1995_Cameroon)	...	...	...	...	...	...	.A.	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	.A.
#EU853637_(Homo_sapiens_2003_Senegal)	...	...	...	.A.	...	...	.A.	...	...	...	...	...	...	...	...	...	.A.	...	...	...	...	...	...	...	...	.A.
#KC196743_(Dog_2011_Nigeria)	...	...	...	...	...	...	.A.	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	.A.
#EU478516_(Dog_2007_Burkina_Faso)	...	...	...	.A.	...	...	.A.	...	...	...	...	...	...	...	...	...	.A.	...	...	...	...	...	...	...	...	.A.
#EU853633_(Homo_sapiens_2004_Senegal)	...	...	...	.A.	...	...	.A.	...	...	...	...	...	...	...	...	...	.A.	...	...	...	...	...	...	...	...	.A.
#KU564995_(Dog_2013_Chad)	...	...	...	...	...	...	.A.	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	.A.
#KT119758_(Dog_2008_Central_African_Republic)	...	...	...	...	...	...	.A.	...	...	...	.C.	...	...	...	...	...	.A.	.G.	...	.C.	...	...	...	...	...	.A.
#EU853654_(Dog_1990_Niger)	...	...	...	...	...	...	.A.	...	...	...	...	...	...	...	...	...	...	.G.	...	.C.	...	...	...	...	...	.A.
#KF022184_(Dog_2005_Nigeria)	...	...	...	...	...	...	.A.	.G.	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	.A.
#KF022182_(Dog_2005_Nigeria)	...	...	...	...	...	...	.A.	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	.A.
#EU038090_(Dod_2005_Nigeria:_Plateau)	...	...	...	.A.	...	...	.A.	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	.A.
#EU038098_(Dod_2006_Nigeria:_Plateau)	...	...	...	...	...	...	.A.	...	.A.	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	.A.
#EU478494_(Dog_2007_Burkina_Faso)	...	...	...	.A.	...	...	.A.	...	...	...	...	...	...	...	...	...	.A.	...	...	...	...	...	...	...	...	.A.
#EU853629_(Cat_2006_Senegal)	...	...	...	.A.	...	...	.A.	...	...	...	...	...	...	...	...	...	.A.	...	...	...	...	...	...	...	...	.A.
#KT119774_(Dog_1987_Cameroon)	...	...	...	...	.G.	...	.G.	...	...	...	...	...	...	...	...	...	...	...	.G.	...	...	...	...	...	...	.A.
#EU718736_(2008_Chad)	...	...	...	...	...	...	.A.	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	.A.
#KU564987_(Dog_2012_Chad)	...	...	...	...	...	...	.A.	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	.A.
#KF022179_(Dog_2005_Nigeria)	...	...	...	...	...	...	.A.	.G.	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	.A.
#EU853628_(Homo_sapiens_2007_Senegal)	...	...	...	.A.	...	...	.A.	...	...	...	...	...	...	...	...	...	.A.	...	...	...	...	...	...	...	...	.A.
#EU038097_(Dog_2006_Nigeria_Plateau_State)	...	...	...	.A.	...	...	.A.	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	.A.
#KT119768_(Dog_2012_Central_African_Republic)	...	...	...	...	...	...	.A.	...	...	...	.C.	...	...	...	...	...	.A.	.G.	...	.C.	...	...	...	...	...	.A.
#EU478512_(Dog_2007_Burkina_Faso)	...	...	...	.A.	...	...	.A.	...	...	...	...	...	...	...	...	...	.A.	...	...	...	...	...	...	...	...	.A.
#KR080523_(Dog_2012_Nigeria_Suleja)	...	...	...	...	...	...	.A.	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	.A.
#U22636_(1995_Cameroon)	...	...	...	...	...	...	.A.	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	.A.
#EU853632_(Homo_sapiens_2005_Senegal)	...	...	...	.A.	...	...	.A.	...	...	...	...	...	...	...	...	...	.A.	...	...	...	...	...	...	...	...	.A.
#EU827272_(Dog_2007_Burkina_Faso)	...	...	...	.A.	...	...	.A.	...	...	...	...	...	...	...	...	...	.A.	...	...	...	...	...	...	...	...	.A.



	GGG	CTG	TCT	CGA	AAA	TCT	GCT	CTA	TCC	TAT	CTG	CA
#RBV_(Dog_2016NIgeria_Benue_J50)*												
#KR080522_(Dog_2012_Nigeria_Bida)	...	...	...	...	...	...	...	...	...	G..	...	..
#EU853655_(Dog_2009_Chad)	...	...	...	.A.	...	...	...	...	...	G..	...	..
#EU718783_(Dog_2008_Chad)	...	...	...	.A.	...	...	...	...	...	G..	...	..
#KT119728_(Dog_2008_Central_African_Republic)	.A.	...	...	.A.	...	...	...	...	...	G..	...	..
#EU038088_(Dod_2006_Nigeria:_Plateau)	...	...	...	.A.	...	...	...	...	...	G..	...	..
#KT119784_(Dog_1995_Cameroon)	...	...	...	.A.	...	...	...	...	...	G..	...	..
#EU853637_(Homo_sapiens_2003_Senegal)	...	...	...	.A.	...	...	...	...	...	G..	...	..
#KC196743_(Dog_2011_Nigeria)	...	...	...	.A.	...	...	...	...	...	G..	...	..
#EU478516_(Dog_2007_Burkina_Faso)	...	...	...	.A.	...	...	...	...	...	G..	...	..
#EU853633_(Homo_sapiens_2004_Senegal)	...	...	...	.A.	...	...	...	...	...	G..	...	..
#KU564995_(Dog_2013_Chad)	...	...	...	.A.	...	...	...	...	...	G..	...	..
#KT119758_(Dog_2008_Central_African_Republic)	.A.	...	...	.A.	...	...	...	...	...	G..	...	..
#EU853654_(Dog_1990_Niger)	...	...	...	.A.	...	...	...	...	...	G..	...	..
#KF022184_(Dog_2005_Nigeria)	...	...	...	.A.	...	...	...	...	...	G..	...	..
#KF022182_(Dog_2005_Nigeria)	...	...	...	.A.	...	...	...	...	...	G..	...	..
#EU038090_(Dod_2005_Nigeria:_Plateau)	...	...	...	.A.	...	...	...	...	...	G..	...	..
#EU038098_(Dod_2006_Nigeria:_Plateau)	...	...	...	.A.	...	...	...	...	...	G..	...	..
#EU478494_(Dog_2007_Burkina_Faso)	...	...	...	.A.	...	...	...	...	...	G..	...	..
#EU853629_(Cat_2006_Senegal)	...	...	...	.A.	...	...	...	...	...	G..	...	..
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#KF022179_(Dog_2005_Nigeria)	...	...	...	.A.	...	...	...	...	...	G..	...	..
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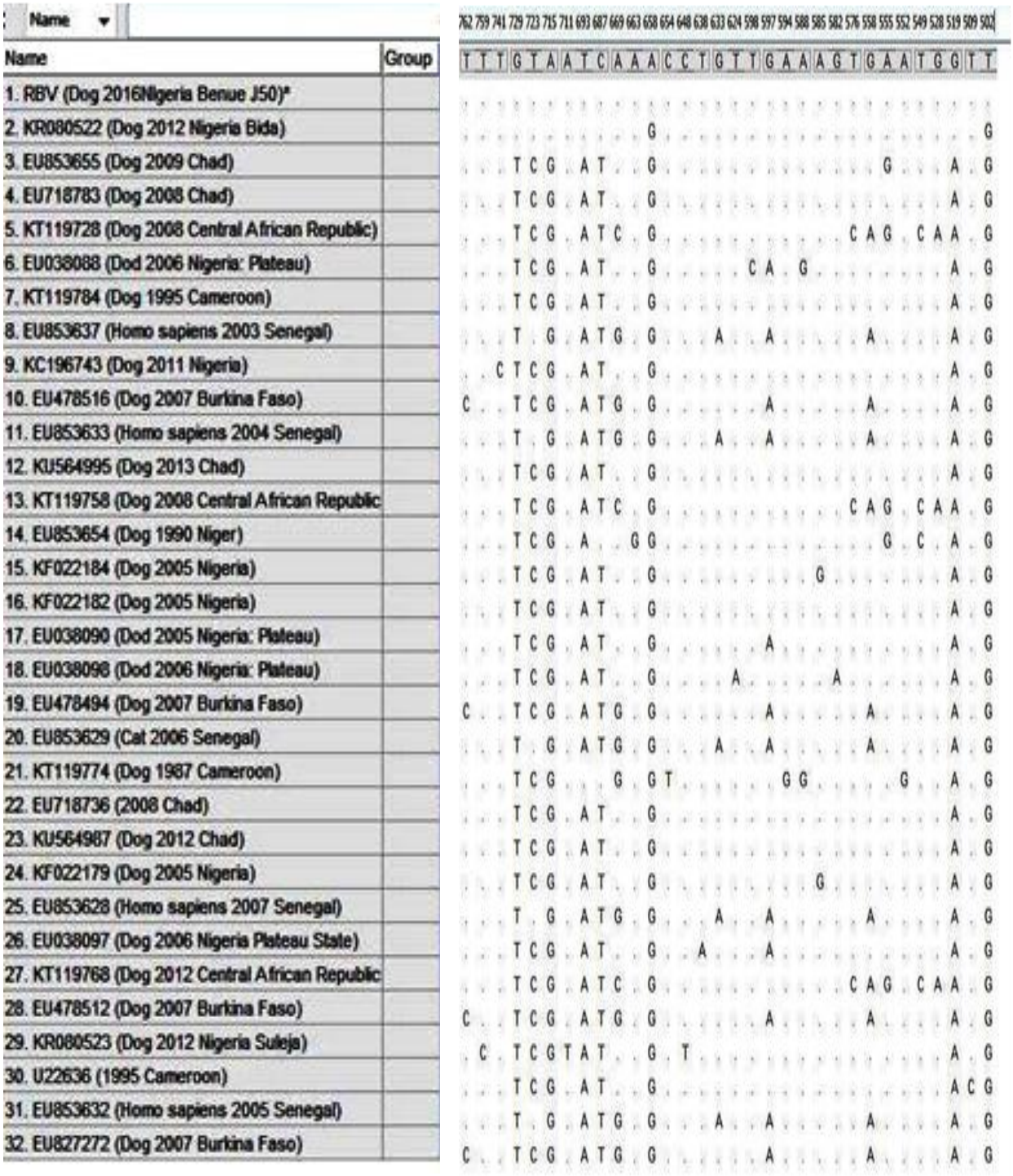
#EU478512_(Dog_2007_Burkina_Faso)	...	...	...	.A.	...	...	...	...	...	G..	...	..
#KR080523_(Dog_2012_Nigeria_Suleja)	...	...	...	.A.	...	...	...	...	...	G..	...	..
#U22636_(1995_Cameroon)	...	...	...	.A.	...	...	..C	...	...	G..	...	..
#EU853632_(Homo_sapiens_2005_Senegal)	...	...	...	.A.	...	...	...	...	...	G..	...	..
#EU827272_(Dog_2007_Burkina_Faso)	...	...	...	.A.	...	...	...	...	...	G..	...	..

APPENDIX V

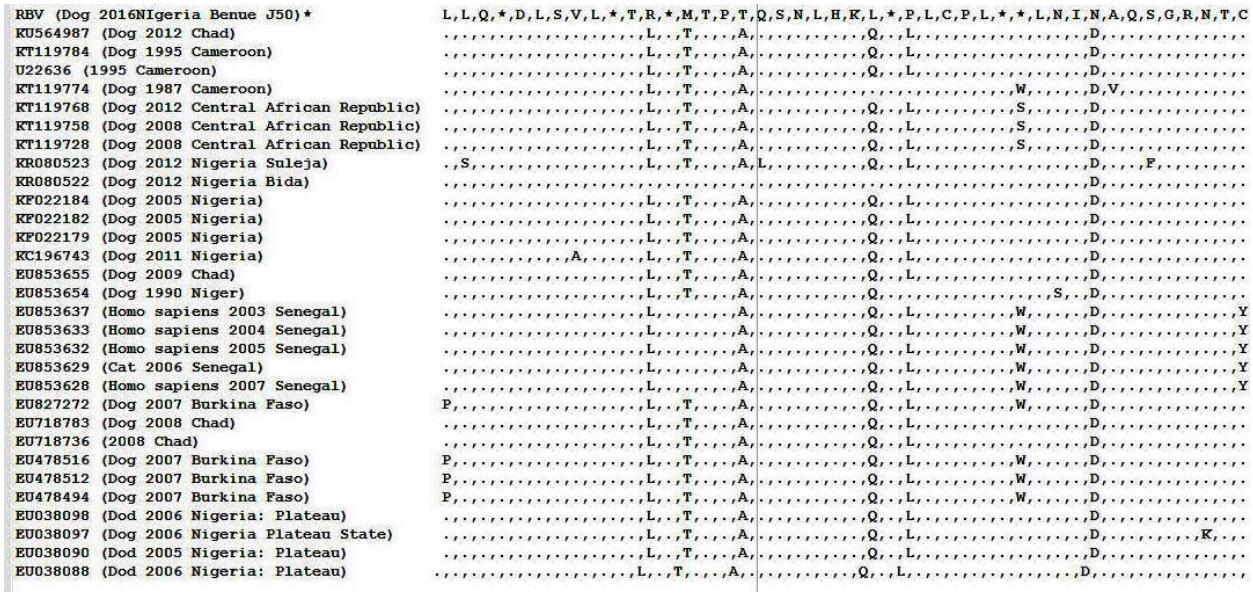
The Variable 32 different Non-serial Position and their Variability to the Sequenced RBV J50 sample.

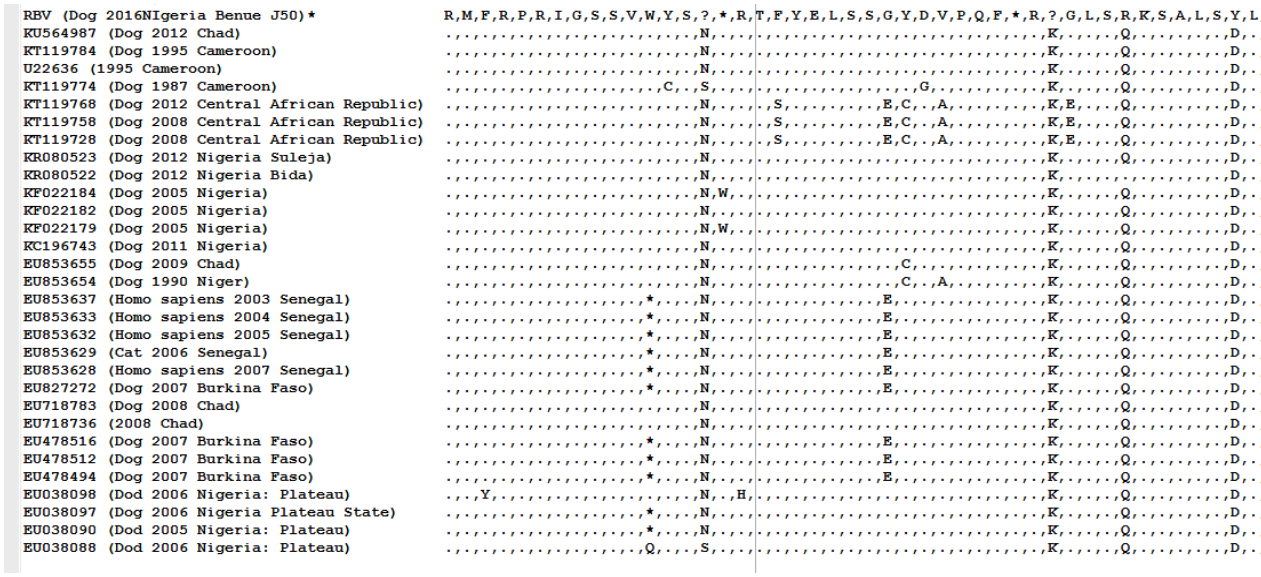


Name	Group
1. RBV (Dog 2016Nigeria Benue J50)*	
2. KR080522 (Dog 2012 Nigeria Bida)	
3. EU853655 (Dog 2009 Chad)	
4. EU718783 (Dog 2008 Chad)	
5. KT119728 (Dog 2008 Central African Republic)	
6. EU038088 (Dod 2006 Nigeria: Plateau)	
7. KT119784 (Dog 1995 Cameroon)	
8. EU853637 (Homo sapiens 2003 Senegal)	
9. KC196743 (Dog 2011 Nigeria)	
10. EU478516 (Dog 2007 Burkina Faso)	
11. EU853633 (Homo sapiens 2004 Senegal)	
12. KU564995 (Dog 2013 Chad)	
13. KT119758 (Dog 2008 Central African Republic	
14. EU853654 (Dog 1990 Niger)	
15. KF022184 (Dog 2005 Nigeria)	
16. KF022182 (Dog 2005 Nigeria)	
17. EU038090 (Dod 2005 Nigeria: Plateau)	
18. EU038098 (Dod 2006 Nigeria: Plateau)	
19. EU478494 (Dog 2007 Burkina Faso)	
20. EU853629 (Cat 2006 Senegal)	
21. KT119774 (Dog 1987 Cameroon)	
22. EU718736 (2008 Chad)	
23. KU564987 (Dog 2012 Chad)	
24. KF022179 (Dog 2005 Nigeria)	
25. EU853628 (Homo sapiens 2007 Senegal)	
26. EU038097 (Dog 2006 Nigeria Plateau State)	
27. KT119768 (Dog 2012 Central African Republic	
28. EU478512 (Dog 2007 Burkina Faso)	
29. KR080523 (Dog 2012 Nigeria Suleja)	
30. U22636 (1995 Cameroon)	
31. EU853632 (Homo sapiens 2005 Senegal)	
32. EU827272 (Dog 2007 Burkina Faso)	

APPENDIX VI

Amino Acid variability of the sequenced sample No 50 compared to related sequences from the Gene bank





APPENDIX VII

Contributions to Knowledge

- The study has provided information on the occurrence of Rabies Virus in Oshugudu in Agatu Local Government Area of Benue State.
- The first characterization of rabies virus of African lineage 2 in Benue State.