

EPIDEMIOLOGY OF RABIES IN NIGER STATE, NIGERIA

A PhD Dissertation (2015)

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ABSTRACT

Dog bite cases have been unofficially reported by residents of Niger State and slaughter of dogs for human consumption is occurring in the open. However, there are no official or researched documents on rabies in the state, despite published reports of rabies in neighboring states of Nigerian. This study sought to examine dog ecology and management, bite cases and rationale for dog meat consumption. The study also examined the presence of rabies virus antigen in the brain of dogs and bats slaughtered for human consumption. Duvenhage virus (DUVV) antibodies in bats and rabies antibodies in the sera of humans at risk in Niger State of Nigeria were also investigated. Out of 300 structured questionnaires administered to adult participants between the ages of 18-70 year old, only 237 questionnaires were returned. Hospital records of human dog bite cases across the study area and animal samples submitted to the National Veterinary Research Institute (NVRI), Vom for rabies Laboratory diagnosis were also examined. Descriptive statistics were used to analyze the data from the questionnaire survey, and the results recorded in tables and charts. A total of 471 dog brains from apparently healthy slaughtered dogs and 267 brain samples from captured fruit eating bats were collected. Direct fluorescent assay (DFA), direct rapid immunohistochemistry test (DRIT) and reverse-transcription-polymerase chain reaction (RT-PCR) were employed to detect rabies antigen in the brains. Sequencing and

phylogenetic analyses of the positive amplicons (252, 276 and 471) were carried-out using BioEdit, ClustalX, and MEGA Program software for blast comparison and to depict the phylogenetic tree of the rabies positive samples. A total of 162 sera from bats and 185 human sera were collected and subjected to a modified and standard rapid fluorescent focus inhibition test (RFFIT) to detect the presence of DUVV and rabies virus antibodies in bats and human sera respectively. Another set of 185 questionnaires were equally distributed to human volunteers who gave their blood were all returned. Descriptive statistics were employed to analyse the data. Results indicated that there is a population ratio of 1:5.4 dogs to humans and 1:1.9 female dogs to male ratio with an estimated 732,476 dog population in Niger State. Most of the dogs (58.6%) are kept for security reasons, but 52% of dogs are not housed/confined and majority of the dogs stray in the night (52.4%) and evenings (23.8%) from homes. Responsibility for dogs in terms of welfare, mostly (61.5%) rest on everybody in the family with 61% of dogs being fed on family left over. About 30.4% of dogs were never vaccinated and 31% of the respondents (or their family members) reported being inflicted with a dog bite. Hospital records of dog bite cases showed about 47% of cases were in children below the age of 15 years and that 81.2% of bites were on the legs with the highest cases (40 out of 223) seen in the year 2012. No animal sample was submitted for rabies confirmatory diagnosis at NVRI, Vom throughout the study period. Of the 471 dog brains analyzed, only 3 (0.63%) were positive for rabies antigen and all belonged to Africa 2 subgroup. However, one lineage found in Niger State of Nigeria is believed to have emanated from the variants seen in Niger Republic. The other variant also has never been reported in Nigeria but has close relatedness with those found in Chad republic and forms a new lineage. About 16.4% of humans at dog bite or rabies risk in Niger State had detectable rabies antibodies in their sera. About 3.9% of these volunteers were shown to have been vaccinated

over the previous ten years before this study. While no rabies antigen was detected in the brains of bats in Niger State, serological evidence of Duvenhage virus was seen in 3 (1.95%) out of 154 sera of bats. This study concludes that the pattern of dog ecology and management in Niger State is about the same as have been obtained in other parts of Nigeria and much of Africa with a dog to human ratio of 1:5.4 and an estimated 732,476 dog population in Niger State. The rabies virus circulating in Niger State is of Africa 2 subgroup. One isolate did not form cluster with the 3 known Nigerian lineages and thus a new lineage (possibly Nigeria 4) is proposed. There is serological evidence of Duvenhage virus circulating in fruit eating bats and of rabies virus infection in some unvaccinated persons. Measures should be instituted to reduce human exposure to rabies.

Keywords: Epidemiology of rabies, Dogs, Bats, Humans, Niger State, Nigeria

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BY

GARBA AHMED

**DEPARTMENT OF VETERINARY PUBLIC HEALTH AND PREVENTIVE
MEDICINE**

FACULTY OF VETERINARY MEDICINE

**AHMADU BELLO UNIVERSITY, ZARIA
NIGERIA**

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(PhD/ VET-MED/04908/2009-2010)**

**A DISSERTATION SUBMITTED TO THE SCHOOL OF POST GRADUATE
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**DEPARTMENT OF VETERINARY PUBLIC HEALTH AND PREVENTIVE
MEDICINE
AHMADU BELLO UNIVERSITY, ZARIA, NIGERIA**

MARCH, 2015

DECLARATION

I hereby declare that the work in this Dissertation titled “Epidemiology of Rabies in Niger State, Nigeria” was performed by me in the Department of Veterinary Public Health and Preventive Medicine, under the supervision of Prof. J. U. Umoh., Prof. H. M. Kazeem and Dr. A. A. Dzikwi. The information derived from literature has been duly acknowledged in the text and a list of references provided. No part of this work has been presented for another degree or diploma at any institution.

Garba AHMED

Signature.....

Date.....

CERTIFICATION

This dissertation entitled EPIDEMIOLOGY OF RABIES IN NIGER STATE, NIGERIA by GARBA AHMED meets the regulations governing the award of the degree of Doctor of Philosophy in Veterinary Public Health and Preventive Medicine of the Ahmadu Bello University, and is approved for its contribution to knowledge and literary presentation.

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I came to Ahmadu Bello University, Zaria having worked in the research environment, the National Veterinary Research Institute, Vom. Little did I know that a few months of rabies survey in dogs and humans I conducted during my national youth service corps in 1997/98 at Funtua Zone of Katsina State would become the topic of my Master's thesis and PhD dissertation. First, I give honour to God who is the head of my life, with Him, nothing is impossible. Secondly, I thank my late father, Alh. Ahmadu Dubagari, the Danburam of Kontagora and my Mother, Haj. Hafsat both of whom instilled in me the belief that I could accomplish anything I set my mind on right from my childhood. They encouraged me to use my God-given gifts to become a productive member of the society.

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Dog bite cases have been unofficially reported by residents of Niger State and slaughter of dogs for human consumption is occurring in the open. However, there are no official or researched documents on rabies in the state, despite published reports of rabies in neighboring states of Nigerian. This study sought to examine dog ecology and management, bite cases and rationale for dog meat consumption. The study also examined the presence of rabies virus antigen in the brain of dogs and bats slaughtered for human consumption. Duvenhage virus (DUVV) antibodies in bats and rabies antibodies in the sera of humans at risk in Niger State of Nigeria were also investigated. Out of 300 structured questionnaires administered to adult participants between the ages of 18-70 year old, only 237 questionnaires were returned. Hospital records of human dog bite cases across the study area and animal samples submitted to the National Veterinary Research Institute (NVRI), Vom for rabies Laboratory diagnosis were also examined. Descriptive statistics were used to analyze the data from the questionnaire survey, and the results recorded in tables and charts. A total of 471 dog brains from apparently healthy slaughtered dogs and 267 brain samples from captured fruit eating bats were collected. Direct fluorescent assay (DFA), direct rapid immunohistochemistry test (DRIT) and reverse-transcription-polymerase chain reaction (RT-PCR) were employed to detect rabies antigen in the brains. Sequencing and phylogenetic analyses of the positive amplicons (252, 276 and 471) were carried-out using BioEdit, ClustalX, and MEGA Program software for blast comparison and to depict the phylogenetic tree of the rabies positive samples. A total of 162 sera from bats and 185 human sera were collected and subjected to a modified and standard rapid fluorescent focus inhibition test (RFFIT) to detect the presence of DUVV and rabies

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ABBREVIATIONS, DEFINATIONS, GLOSSARY AND SYMBOLS

⁰ C	Degree centigrade
μl	Microlitre
ml	Millilitre
IU	International Unit
Ab	Antibody
ABL	Australian bat <i>Lyssa</i> virus
ABU	Ahmadu Bello University
CDC	Centers for Disease Control and Prevention
cDNA	Complimentary Deoxyribo nucleic acid
Centrifugal	Movement of rabies virus from site of bite to the central nervous system
Centripetal	Movement of the rabies virus from the CNS to affected organs like salivary glands, lungs etc
CNS	Central Nervous System
dRIT	Direct rapid immunohistochemistry test
DUVV	Duvenhage virus
Eg	For example
etc	and the rest
ELISA	Enzyme linked immunosorbent assay

EBLV	European Bat <i>Lyssa</i> virus
End point titre	Refers to the point when the antibodies could no longer neutralized the rabies virus
FAT	Flourescent antibody test
FAVN	Flourescent antibody virus neutralization test
FCT	Federal Capital Territory
ICTV	International Committee on Taxonomy of Virus
LBV	Lagos bat virus, LBV
MEGA	Molecular evolution genetic analysis
MEM	Eeagles Minimum Essential Medium
MIT	Mouse inoculation test
MNA	Mouse Neuroblastoma cell (for the development of the virus)
MOKV	Mokola Virus
N-gene	Nucleoprotein gene
NPC	National Population Commission
NVRI	National Veterinary Research Institute
OIE	Office international des epizooties (World organization for animal health)
RABV	Classical Rabies virus

RFFIT	Rapid fluorescent focus inhibition test
RFLP	Restriction fragment length polymorphism
RNA	Ribonucleic acid
RT-PCR	Reverse-transcriptase polymerase chain reaction
RTCIT	Rabies tissue culture inoculation test
rVNA	Rabies virus neutralizing antibodies
Titre	level of antibodies in the serum
WHO	World health organization

CHAPTER ONE

INTRODUCTION

1.1 Background of the Study

Rabies is a viral zoonotic disease of warm blooded animals and indeed the disease ever known to man that causes the most agonizing death the world over. Rabies with a timeline history of 2300 BC is preventable. Yet over 55,000 people die annually due to rabies (WHO, 2010). Most of the deaths (99%) are in the developing countries, Asia reports 30,000 annual deaths and Africa 24,500 deaths annually (WHO, 1998). More than 95% of human cases occurred in unvaccinated or improperly treated individuals (Pakamatz *et al.*, 2002). This is at least partly due to the fact that modern post-exposure rabies prophylaxis is expensive and therefore not readily available in the endemic regions where they are most needed (Wilde *et al.*, 1999). The rabies virus (RABV) belongs to the genus *Lyssavirus* of the family *Rhabdoviridae*, order *Mononegavirales*. (Gould *et al.*, 1998; Ramiz *et al.*, 2008).

The genome of rabies virus is a non-segmented, anti-sense, single-stranded RNA, which is about 12,000 nucleotides (nt) long (Linzhu, 2010). The viral RNA encodes five major proteins: nucleoprotein (N-protein), phosphoprotein (P-protein), matrix protein (M-protein), glycoprotein (G-protein), and RNA-dependent RNA-polymerase (L-protein) (Metlin *et al.*, 2008). These viral proteins, form the basis for diagnostic identification and antigenic relatedness of the family *Rhabdoviridae* in general and the genus *Lyssavirus* in particular (Kissi *et al.*, 1995).

Over the years 7 distinct members of *lyssavirus* genus have been identified, but recently additional members have been discovered making them up to 14 members (ICTV,

2013). The early seven members include: Genotype 1 (classical RABV), Genotype 2 (Lagos bat virus, LBV), Genotype 3 (Mokola Virus, MOKV), Genotype 4 (Duvenhage Virus, DUVV), Genotype 5 (European Bat *Lyssavirus* 1, EBLV 1), Genotype 6 (European Bat *Lyssa* virus 2, EBLV 2) and Genotype 7 (Australian bat *Lyssa* virus, ABL) are the earlier recognized *lyssaviruses* (Bourhy, *et al.*, 1992; 1993; Fraser, *et al.*, 1996; Black *et al.*, 2002). The classical rabies virus (Genotype 1) has a worldwide distribution. Lagos bat, Duvenhage and Mokola viruses appear to have distribution limited to Africa, while the EBLs have only been found in Europe and ABL in Australia (Black *et al.*, 2000). All genotypes, except LBV, are known to cause human disease, virtually indistinguishable from classical rabies (Smith, 1996).

Currently there are 14 members of *the Lyssavirus* genus some (LBV, MOKV, DUV, Shimoli Bat Virus, SHIBV) were first recognized in Africa (WHO, 2012). Rabies is a major public health problem in most parts of the developing world, where the domestic dog plays a principal role as a reservoir and transmitter of the disease in human (WHO, 1992). Developing countries account for almost all the reported human death (99.9%) and most cases of human post-exposure treatment (PETs) (WHO, 2002a). While bats are the reservoirs of rabies in some parts of the western world, dogs are the major reservoirs of rabies in most developing world particularly Asia and Africa

In Nigeria, the first laboratory confirmed human and canine rabies were at Rabies Laboratory Yaba in 1912 and 1925 respectively (Boulger and Hardy, 1960). The National Veterinary Research Institute, (NVRI) Vom is the officially recognized institution responsible for diagnosing animal rabies in Nigeria, with Faculties of Veterinary Medicine across the country providing support and teaching services in rabies diagnosis. Reports indicated that between 1928 and 1990 NVRI, Vom has

diagnosed (confirmed) a total of 3770 cases of animal rabies of which 3555(93.4%) were in dogs (Oboegbulem, 1994). In a similar report, between 1991 and 2005, NVRI Vom has diagnosed (confirmed) 1039 cases of animal rabies, 1024 (99.1%) being of dogs with only one sample submitted from Niger State and was negative (Garba *et al.*, 2007, 2008a). It is, therefore, obvious that the dog is the principal reservoir and transmitter host for rabies in Nigeria.

1.2 Statement of the Research Problem

For an effective control or eradication of rabies in the human and animal populations in an area, the ecology of the reservoir and transmitter host has to be known, the variant/ strain of the circulating virus has to be established and the ecosystem/ interaction between the virus and the hosts have to be studied. In Nigeria, all published reports and surveys have incriminated the dog as both the reservoir and transmitter of rabies (Umoh and Belino, 1979; Ezebuiro *et al.*, 1980; Fagbami *et al.*, 1981; Ezeokoli *et al.*, 1984; Oboegbulem, 1994; Ogunkoya, 1997; Garba *et al.*, 2007; 2008a).

Studies on dog ecology in Nigeria have been attempted by some workers (Okoh, 1986; Oboegbulem and Nwakonobi, 1989; El-Yuguda *et al.*, 2007; Hambolu *et al.*, 2014). In Niger State with an estimated dog population of over 199,812 (RIM, 1992), there are no published articles on dog ecology, rabies or dog bite cases.

Lagos bat virus was first isolated from the brain of frugivorous bat (*Eidolon helvum*) on Lagos Island in 1956 (Boulger and Porterfield, 1958). The rabies virus has been reported in many places in Nigeria, suggesting Nigeria as a hotspot location for *lyssavirus* research particularly, Niger State where no report of rabies documented. Clinical rabies, which manifest in either furious or paralytic form has historically been

regarded as uniformly fatal, because diagnosis could only be done at post mortem when brain tissue is available for test (Umoh and Blendin, 1980).

However, reports in Africa and elsewhere in the world have shown that dogs may not die, but live as the carrier, disseminating the virus in their saliva (Doego and Northrop, 1973; Bell, 1975; Fekadu, 1975; Fekadu and Baer, 1980; Fekadu, 1988).

Despite this long period of documentation and the fact that rabies is of great public health significance, there is little sustained surveillance of the etiologic agent across the states (particularly Niger State) in Nigeria. However, two decades ago, Aghomo *et al.* (1987) reported the presence of rabies virus antibodies in over 15.93% of unvaccinated dogs in the southwestern part of Nigeria and later the presence of rabies antigen in healthy dogs in Nigeria (Aghomo and Rupprecht, 1990).

Similarly, Ogunkoya *et al.* (1990) reported the presence of rabies antibodies in 30.7% and 28.6% of unvaccinated dogs and humans respectively in Nigeria. Ogunkoya *et al.* (2003) also reported the occurrence of nine rabies cases in an unnoticed circumstance in dogs brought to the Veterinary Teaching Hospital in Zaria. There was serological evidence of rabies antibodies in 7% of camels slaughtered in Maiduguri municipal abattoir (Baba *et al.*, 2005).

Also, Ajayi *et al.* (2006) reported the presence of rabies virus antigen in the brains of 31% apparently healthy dogs examined from northeastern Nigeria. Most recently Garba *et al.* (2010) reported the presence of rabies virus antigen in the brain of apparently healthy slaughtered dogs in 28% of dogs examined from Sokoto and Katsina in north western Nigeria.

In a similar report Dzikwi *et al.* (2010a) reported the presence of Lagos bat virus neutralizing antibodies among 19% of the fruit eating bats examined in Northern

Nigeria. There is also serological evidence of Mokola virus (Genotype 3 rabies related virus) in apparently healthy dogs in Zaria, Nigeria (Dzikwi *et al.*, 2010b). These findings could pose a public health threat, especially that *Lyssa* viruses (except Lagos bat virus) cause illness indistinguishable from classical rabies ((Smith, 1996).

Reports indicated that between 1991 and 2005, up to 2,143 animal samples were received by NVRI, Vom from across Nigeria for rabies confirmatory diagnosis (Garba *et al.*, 2008a). Of this number, 1,039 (48.5%) were positive for rabies, but only one sample was submitted from Niger State during that period and was found to be negative. Does this suggest there was no rabies in Niger State?

1.3 Justification for the Study

The endemic nature of rabies in Nigeria can be attributed to the prevalence of the disease in dogs; therefore, transmission of rabies to humans by rabid dogs might happen due to various favourable ecological and societal factors which need to be addressed. The effective control of rabies among dogs in Nigeria would be possible with the proper disposal of garbage, reductions of stray dogs, and the proper induction of "herd immunity" by mass vaccination. In Nigeria, the first nationally coordinated anti-rabies campaign held was in May 1982, but only about 44,627 (1.0%) of the total 4,543,003 dog population were vaccinated (Oboegbulem, 1994). Many dogs lost their immune status due to lack of annual booster (re-vaccination).

Similarly, a large population of dogs were not vaccinated and un-apprehended, a phenomenon that ensured the continuous spread of rabies cycle in Nigeria (Oboegbulem, 1994). More recent attempts on national anti-rabies campaign was in November, 2006 which kicked-off at Jos. During that campaign, only 40,000 doses of anti-rabies vaccines were purchased for use across the states in the country (Privilege

information from Federal Department of Livestock). Consequent to low vaccination coverage and lack of annual boosters, the presence of rabies virus antigens has been reported even in apparently healthy dogs in Nigeria (Ajayi *et al.*, 2006; Garba *et al.*, 2010). Dogs are the principal reservoirs of the rabies virus (RABV) in Nigeria (Oboegbulem, 1994; Garba *et al.*, 2008a). RABV isolates tend to form genetic clusters based on the geographical region (Nagarajan *et al.*, 2007).

Analysis of more samples is essential to identify the existence of other genetic clusters and also to identify the dominant gene clusters in Nigeria. The amino acid sequence may serve as epidemiological markers for RABV isolates in Niger State and could be used to trace the origins of travel-related rabies cases in humans as well as in animals. Studies of different genome targets (like the P gene and the N gene) should also be carried out to further characterize the RABV isolates of Nigerian origin. In a recent report the Nigeria isolates of rabies belong to Africa-group 2 (David *et al.*, 2007; Dzikwi, 2008; Ogo, 2009).

Considering the above stated problems of rabies and rabies related viruses circulating among apparently healthy dogs and bats; the presence of Lagos bat virus and Mokola virus neutralizing antibodies amongst bats and dogs in Nigeria; the need for understanding the dog ecology; and the absence of research document/information on the incidence of rabies in Niger State, this study became necessary.

1.4 Aim of the Study

To carry out the dog ecology and survey for the presence of rabies virus antigens in the brain of slaughtered dogs and captured bats and molecular characterization of the isolates as well as to check for DUVV and RABV antibodies in bats and human sera respectively in Niger State, Nigeria.

1.5 Objectives of the Study

1. To determine the dog population density, dog population structure, and pattern of dog ownership and management in Niger State.
2. To determine the patterns of dog bite and rabies cases in human hospitals across Niger State.
3. To determine the rationale for dog meat consumption in Niger State.
4. To determine the presence of rabies virus antigen in the brain of apparently healthy slaughtered dogs in Niger State.
5. To characterize the rabies virus isolates from dogs in Niger State.
6. To determine the presence of rabies virus antigen in the brain of bats and serological evidence of Duvenhage virus in bats in Niger State.
7. To determine the presence of antibody against rabies in human occupational risk groups (dog butchers, dog meat consumers, pet owners, hunters with dogs and veterinarians/animal health assistants) in Niger State.

1.6 Research Questions

1. What is the population density and management of dogs in Niger State?
2. Are there cases of dog bite and rabies in humans in Niger State?
3. Is there any rationale for dog meat consumption in Niger State.?
4. Is there rabies virus antigen in the brains of slaughtered dogs in Niger State?
5. Are there any previously reported or new rabies virus isolates in dogs from Niger State?

6. Is there serological evidence of DUVV in bats in Niger State?

7. Is there serological evidence of rabies in the blood of rabies occupational risk groups (dog butchers, dog meat consumers, pet owners, hunters with dogs and Veterinarians/animal health assistants) in Niger State.

CHAPTER TWO

LITERATURE REVIEW

2.1 Definition of Rabies

Rabies is a viral, zoonotic and fatal disease of warm blooded animals that causes severe encephalitis in the affected subjects. Rabies is caused by a *lyssavirus* of various genotypes. Rabies virus (genotype 1), a single-stranded RNA virus, which was the first of the fourteen *lyssavirus* genotypes so far identified is the cause of classical rabies. Of the other early six rabies-related viruses, all but Lagos bat virus have caused fatal encephalitis in humans, clinically indistinguishable from classic rabies (Smith, 1996; Warrell and Warrell, 2004).

2.2 Rabies Virus

Rabies virus, a single-stranded RNA virus, was the first of the many lyssavirus genotypes to be identified. Rabies virion measure 180×75 nm. The genome, a single non-segmented strand of negative-sense RNA of 11.9 kb, a nucleoprotein, a phosphoprotein, and an RNA-dependent RNA polymerase form the helical coil of the ribonucleoprotein complex core. A layer of matrix protein covers this cylindrical structure. The lipoprotein envelope is a host-derived lipid bilayer studded with rabies glycoprotein bearing trimeric spikes (Appendix XV) (Warrell and Warrell, 2004).

2.3 Rabies Related Viruses

In the current classification the genus *Lyssavirus* contains fourteen genotypes (Denise *et al.*, 2012.; ICTV. 2013). Eleven *Lyssavirus* species have been classified: *Rabies virus* (RABV), *Lagos bat virus* (LBV), *Mokola virus* (MOKV), *Duvenhage virus* (DUVV), *European bat lyssavirus* types -1 and -2, *Australian bat lyssavirus*, *Aravan virus*, *Khujand virus*, *Irkut virus*, and *West Caucasian bat virus* (WCBV), (Dietzgen *et al.*, 2011). All these viruses except MOKV have been detected in bats. Two newly identified lyssaviruses, *Shimoni bat virus* (SHIBV) (Kuzmin *et al.*, 2010) and *Bokeloh bat lyssavirus* (Freuling *et al.*, 2011), both detected in bats, have now been classified (ICTV, 2013).

The presence of numerous lyssaviruses in bat species has led to increasing research efforts toward lyssavirus discovery in bat populations globally. Additional *lyssavirus* (Ikoma virus) from an African Civet has recently been discovered in Tanzania (Denise *et al.*, 2012) making a total of 14 members of the genus *lyssavirus* (ICTV, 2013). However, lyssavirus surveillance in terrestrial mammals remains limited across most of Africa.

Of the 14 lyssaviruses, 5 (RABV, LBV, MOKV, DUVV, and SHIBV) circulate in Africa. LBV, MOKV, DUVV, and SHIBV are detected exclusively in Africa, whereas RABV is detected worldwide. The predominant RABV variants circulating in Africa are the mongoose and canine biotypes (Denise *et al.*, 2012).

2.4 Host Range

All warm-blooded animals are susceptible to rabies; but, according to the World Health Organization's Expert Committee on the disease, not all animals are equally susceptible

(Kaplan, 1977). The most highly susceptible animals are foxes, coyotes, jackals and wolves, kangaroo rats, cotton rats and common field voles (Kaplan, 1977). The group next most susceptible contain many species which are important in maintaining reservoirs of infection in wildlife in many different parts of the world such as raccoons, skunks and vampire bats. Some of the recent discoveries of lyssaviruses are from the bat species and particularly in Africa (Kuzmin *et al.*, 2010; Freuling *et al.*, 2011; Denise *et al.*, 2012).

Dogs, are listed as only moderately susceptible, but dogs are, however, without any doubt the animals most likely to spread the infection to human especially in Africa (Oboegbulem, 1994). Others mammals are cats, cattle, sheep, horses, pigs, monkeys, mice, civet, etc. Oboegbulem (1994). Generally, there are reservoir hosts that maintain the virus e.g. dogs, foxes, cat, etc. And there are dead-end hosts that can be infected develop the diseases but do not maintain the virus e.g. cattle, sheep, goats etc.

2.5 Mode of Transmission

Rabies is spread through the virus-laden saliva of an infected animal by bite, scratch, or contact with mucous membranes or a fresh break in the skin (WHO, 1973). Bites of some animals, such as bats, can inflict injury so minor (and may transmit rabies) that it goes undetected (Kuzmin *et al.*, 2010).

Indirect exposure to the saliva of a rabid animal can also occur through contact with a rabid animal (Fekadu, 1988), skin break or mucous membrane exposure to nervous tissue (brain, spinal cord) of an infected animal may also pose a risk of transmission (WHO, 1973). No direct person to person transmission has been documented (Oboegbulem, 1994). However, there have been cases documented after corneal

transplants from infected individuals (Lopez *et al.*, 1970; Beneson 1980). Airborne spread (for example, in a cave with a multitude of bats or in a laboratory with rabies virus or specimens) has occurred (Kaplan, 1977). Rabies is not transmitted through contact with blood, urine, skunk spray, or faeces of an infected animal (Oboegbulem, 1994)

However, transmission by ingestion has also been demonstrated (WHO, 1973), but it is an opinion, that, it is doubted if the rabies virus can survive for long in the highly acid environment of the stomach (Oboegbulem, 1994). Therefore, oral infection is not considered important in maintaining the disease in nature or transmitting the virus to animals and man (Schrurrenberger, 1975). There is no evidence of transmission by eating the meat or brain of rabid animals, although the possibility cannot be ruled out (Oboegbulem, 1994).

2.6 Pathogenesis

In most people exposed to rabies virus, the exact time and location of viral entrance are known. The treatment given thereafter must be based on a precise knowledge of the subsequent movement of the virus (Baer, 1975a). Baer (1975a) cited that the first to demonstrate the role of the central nervous system (CNS) in rabies (Pasteur *et al.*, 1881), wrote that “the central nervous system, and especially the bulb which joins the spinal cord to the brain, are particularly concerned and active in the development of the disease” later (1882) he found a virus in the spinal cord but not in the brain stem of dogs inoculated intravenously with virus and sacrificed at the first sign of paralysis.

However, before the virus moves toward the CNS via the nerves, a complicated and little understood series of events, including entrance, absorption and eclipse, occurs at the site of introduction (Baer, 1975a). Rabies virus injected into the footpad or muscle

tissue of experimental animals can be recovered from the site of inoculation for relatively short period of time (Baer, 1975a). Dean *et al.* (1963a) amputated the feet of mice inoculated with fixed virus and found that the procedure of removing the inoculated area was a life saving procedure for 4 hours or less after inoculation (cited by Baer, 1975a).

This result is similar to those of Fermi (1928) in which rat could be saved by amputation of the inoculated tail up to five hours after inoculation with fixed virus but no longer. It appears that development of disease may depend on the area of the body exposed. Dean *et al.* (1963b) reported that guinea pigs died after a drop of the fixed rabies virus was placed on a small hole cut in the skin of the neck, but none succumbed when the same dose was applied on the back leg. Similarly, statistics on human disease also indicate that the wound on the head and necks are generally more lethal than those on the hands and feet (Veeraraghavan, 1964).

After the short initial period of obvious viability of the virus in the bite or entrance site, it goes into eclipse, during which time it cannot be recovered from any tissue what so ever (Johnson, 1965; Bear *et al.*, 1968) and during this eclipse period, the virus may remain locally (at the site) or already proceeded onto its pathway to or within the CNS. It appears more probable however, that virus remains locally during most of the eclipse period (Baer and Cleary, 1972; Baer, 1975a). "Street virus isolates may persist locally for up to 96 hrs after intramuscular inoculation (Habel, 1941)

It has been found that following infection with the rabies virus, there is an initial replication at the myocytes before detection of the virus antigen at peripheral and central nervous system (Murphy *et al.*, 1973). This finding is remarkable in the study of post exposure rabies prophylaxis, since the efficacy of treatment procedures most

probably depends on the accessibility of the virus before it becomes entrapped in the central nervous system, which appears to be considerably longer than 72 hours (Baer, 1975a). The speed at which rabies virus travels in nerve has been reported by many workers. For instance, Dean *et al.* (1963b) showed that fixed rabies virus travels along nerve pathways at a rate of approximately 3 mm/hour. Similarly, Allen *et al.* (1995) demonstrated using polymerase chain reaction (PCR) that the speed of axonal transport of rabies virus in mice infected intramuscularly is 1mm/hour and that the replication cycle of the virus is approximately 12 hours. Warrell and Warrell (2004) in their finding; found that the speed of the rabies virus was 0.5 mm/hour.

Based on this data currently available, it indicates that rabies virus apparently replicates in the muscle, connective tissue, or nerves at the site of exposure. It may remain undetectable in that initial infected tissue for weeks or months before moving up the associated nerve. It is not known what form the virus assumes during these long periods, or what its relation is to the adjacent tissue (Baer, 1975a).

At a given time the virus appears to move passively up. It either follows the tissue spaces between the schwann cells and the perineural structures, or in the interstitial nerve spaces of the fasciculus. It then begins to replicate in the spinal ganglia and dorsal root ganglion cell corresponding to the involved peripheral nerves. Finally, it multiplies in the central nervous system (Baer, 1975a). In a general term the virus may enter the peripheral nervous system directly, or may replicate in muscle tissue after entering the host, remaining at or near the site of introduction for most of the incubation period. However, the precise sites of viral sequestration remain unknown, since neither antigen nor virus can usually be found in any organ during this phase (Baer, 1975a). The entrance of the virus into peripheral nervous system may be via the

Neuro-muscular junctions, and the virus moves rapidly centripetally to the central nervous system for replication and symptoms may develop shortly thereafter. The viruses then begin to pass centrifugally to many tissues and organs, such as the salivary glands (Baer, 1991, Charlton. 1994). It has been found that some dogs inoculated with American (Southern Texas) or Ethiopia canine street virus excreted virus in their saliva up to 14 days before signs appeared (Fekadu, 1988).

2.7 Epidemiology of Rabies

Rabies has a worldwide distribution except for a few areas of the world reported to be rabies-free. Such areas include Australia (before 1997), Antarctica, New Zealand, Japan, Hawaii, Pacific Islands, British Isles (United Kingdom and Ireland), Sweden and some of the West Indian Island (Black *et al.*, 2002). Nearly all the rabies-free countries are surrounded by a large body of water which to some extent provides natural protection. Some 87 countries of the world with a combined population of over 2.8 billion people are still afflicted by rabies, these countries account for more than 99% of all reported human death from rabies (Oboegbulem, 1994).

Current trends in rabies epidemiology indicates that the disease is spreading in many regions, mainly owing to increasing density and mobility of human and animal hosts' population. In the United States of America (US), Hawaii is the only state that has never reported an indigenously acquired rabies case in humans and animals. Wild animals accounted for nearly 92% of reported cases of animal rabies in the US in 1998 (Krebs *et al.*, 1999). In the US, raccoons represent 44% of animal rabies reported followed by skunks (28.5%), bats (12.5%), foxes (5.5%) then others (0.9%) (Krebs *et al.*, 1999).

In Europe, rabies are maintained in two cycles, urban and sylvatic, with the reservoir of infection in the wildlife (Pastoret and Brochier, 1999). Where all susceptible species, both wild and domestic, are involved, the red fox (*Vulpes Vulpes*) is involved in more than 75% of cases in Europe. Rabies was a common disease in Great Britain for many centuries (King and Turner, 1993). The prevalence of rabies increased from 1735 and by 1776 rabies was widespread in dogs throughout Britain (Pastoret and Brochier, 1999). Mad dogs were reported in London from 1752 to 1862. Despite a significant fox population, the disease was not observed in wild life except for two outbreaks in deers.

The implementation of the orders which includes powers for the muzzling of stray dogs, the elimination of stray dogs and the regulation and control of importation of dogs, led to the eradication of the disease for the first time in 1902 in Britain, Rabies was later re-introduced in 1918 (Pastoret and Brochier, 1999).

Rabies is a major public health problem in most parts of the developing world, where dog plays a principal role as reservoirs and transmitter of the disease to humans (WHO, 1992). Developing countries account for almost all the reported human death (99.9%) and most cases of human post-exposure treatments (PETs) (WHO, 1996). India has a large population of stray dogs which together with the lack of effective control strategies, might have led to the persistence of rabies virus in the canine population (Nagarajan *et al.*, 2007).

In Africa, the first report of confirmed rabies in a local dog was made in Ethiopia in 1893, similarly a disease of dogs locally known as '*Oulon fato*' was identified as rabies in French-speaking West African countries (Boulford, 1912).

However, there was a report of rabies in an exotic dog imported into the African Continent at Port Elizabeth, South Africa in 1884. Subsequently, rabies became

endemic in Africa in both animal and human population, cases being reported each year in all the ecological zones (Oboegbulem, 1994; Ogunkoya, 1997). In Nigeria, the first laboratory confirmed human and canine rabies were at Rabies Laboratory Yaba in 1912 and 1925 respectively (Boulger and Hardy, 1960).

On the other hand, bat rabies are causing a serious public health and economic problem across the western world. The involvement of vampire bats in transmitting the rabies virus was suggested at the beginning of last century (Carini, 1911). The hypothesis was confirmed by Haupt and Rehaag (1925) who identified the presence of Negri bodies in the central nervous system of a vampire bat that was feeding on a cow. Vampire bats, and especially *Desmodus rotundus*, are the reservoirs for the rabies virus in Latin American countries (Torres *et al.*, 1935). They are endemic in the region stretching from northern Chile and northern Argentina to northern Mexico and parts of the Caribbean (Koopman, 1988).

In 1953, in Florida (US), a boy was attacked by the insectivorous bat *Lastirus intermedius*, and this made the American researchers turn their attention to the question of rabies in non-vampire bats (Bigler *et al.*, 1974). Shortly afterwards, rabies was confirmed in different bat species of distinct feeding habits, including insectivores, frugivores, omnivores and piscivores (Baer, 1975b). In Brazil, in 2005, out of the 45 recorded cases of human rabies, 42 were transmitted by vampire bats (Karin *et al.*, 2007). In the US, all bat-related cases during 1985-2007, were domestic (not imported into the country), constituting 91% (43 out of 47) of the rabies cases reported. In addition, bats were the source of all 4 human rabies cases, documented during the 1985-2007 in Canada (Kuzmin, 2010).

In Nigeria, the first Lagos bat virus was isolated from the brain of frugivorous bat (*Eidolon helvan*) on Lagos Island in 1956 (Boulger and Porterfield, 1958). Recent investigation supports the evidence of Lagos Bat Virus (LBV) circulating amongst Nigerian Fruit eating bats with neutralizing antibodies in 19% of bats (sera) screened (Dzikwi *et al.*, 2010a).

2.7.1 Molecular epidemiology of rabies virus in Northern Nigeria

In Nigeria, the first laboratory confirmed human and canine rabies were at Rabies Laboratory Yaba in 1912 and 1925 respectively (Boulger and Hardy, 1960). National Veterinary Research Institute, (NVRI) Vom is the officially recognized institution responsible for diagnosing animal rabies in Nigeria, with the Faculties of Veterinary Medicine across the country providing support and teaching services in rabies diagnosis. Reports indicate that between 1928 and 1990 NVRI, Vom has diagnosed (confirmed) a total of 3,770 cases of animal rabies of which 3,555 (93.4%) were in dogs (Oboegbulem, 1994). In a similar report, between 1991 and 2005, NVRI Vom have diagnosed (confirmed) 1,039 cases of animal rabies, 1,024 (99.1%) being of dogs with only one sample submitted from Niger State and was negative (Garba *et al.*, 2007; 2008a). It is, therefore, obvious that the dog is the principal reservoir and transmitter host for rabies in Nigeria.

Viral sequencing and phylogenetic analyses helps in expressing the intrinsic and extrinsic relatedness of rabies virus (genotype 1) variants and other lyssaviruses (Kissi *et al.*, 1995). Using molecular techniques, the rabies virus variants circulating in Africa were classified into Africa 1a (circulating in Ethiopia, Gabon, Morocco and Algeria), Africa 1b (in Tanzania, Kenya, S/Africa and Zaire), Africa 2 (seen in Niger, Chad,

Cameroun, Senegal, Ivory Coast and Nigeria), Africa 3 (only in South Africa) and Africa 4 (in Egypt) (Kissi *et al.*, 1995).

In Nigeria some isolates were characterized from Jos using monoclonal antibodies and were linked to those found in Cameroun (Okoh, 1986). Using the RT-PCR and sequencing technologies, 3 clusters were reported, these included Nigeria 1 (from Bokkos, Barikin Ladi, Jos, Mangu, Vom, Kuru, Trade centre etc (all in plateau state) the Kaduna). Nigeria 2 (were isolated from Jos, Vom, Kaduna and Bauchi). Nigeria 3 (also in Jos and Kaduna). All of these clusters belong to Africa 2 subgroup and have some links with those reported in other west African countries (David *et al.*, 2007; Dzikwi 2008; Ogo, 2009; Nwosuh, 2010). It is therefore suggestive that the molecular epidemiology of rabies in northern Nigeria has gone far.

2.8 Rabies Diagnosis

Rabies is a disease of public health importance for which diagnostic techniques have been standardized internationally (OIE, 2000). Validated diagnostic tests that confirm the presence of rabies virus or a *lyssavirus* variant have been the foundation of rabies control strategies in many countries. Historically, histopathological techniques such as the Sellers Stain technique (Tierkel *et al.*, 1996) were used to determine the presence of Negri bodies as rabies virus-specific antigen, however, due to poor sensitivity and specificity, this technique is no longer recommended by the World Health Organization (WHO). The Fluorescent Antibody test (FAT) (Dean and Abelseth, 1973) relies on the ability of a detector molecule (usually fluorescein isothiocyanate) coupled with a rabies specific antibody forming a conjugate to bind to and allow the visualization of rabies antigen using fluorescent microscopy techniques.

Microscopic analysis of samples is the only direct method that allows for the identification of rabies virus-specific antigen in a short time and at a reduced cost, irrespective of geographical origin and status of the host (Fooks *et al.*, 2009). It has to be regarded as the first step in diagnostic procedures for all laboratories. Autolysed samples can, however, reduce the sensitivity and specificity of the FAT. The Rabies Tissue Culture Infection Test (RTCIT) (Sureau, 1986) and the Mouse Inoculation Test (MIT) (Webster and Dawson, 1935) are based on the propagation and isolation of the virus.

These diagnostic tests are used to detect virus particles either directly in tissue samples (FAT) or indirectly in animals and in tissue culture (MIT and RTCIT, respectively). The rationale for the use of virus isolation (RTCIT/MIT) from a sample where there is a suspicion of infection with rabies virus is always recommended (in order to propagate the virus), especially when Koch's postulates are likely to be met (Fooks *et al.*, 2009). Such amplification of the viral pathogen facilitates additional molecular analysis to be undertaken, including sequencing of the viral isolate and subsequent phylogenetic analysis. Conventional diagnostic tests for rabies (FAT, RTCIT, MIT) are not labour intensive and rely upon low throughput. The FAT can be completed in less than two hours. In contrast, both the RTCIT and MIT require longer turnaround times (4 days and 28 days, respectively). The fluorescent antibody virus neutralization (FAVN) test (Cliquet *et al.*, 1998) and the Rapid Fluorescent Focus Inhibition Test (RFFIT) (Smith *et al.*, 1973) utilize a similar principle, to measure the level of virus neutralizing antibody in vaccinated or exposed individuals.

'Indirect' serological methods, including the FAVN and RFFIT measure the host responses to infection/vaccination only and do not detect the presence of infectious

virus/antigen directly. However, host antibody detection (through FAVN/RFFIT) is an indirect tool to measure the presence of rabies virus in a non-immunized individual by evaluating the host response to infection (Smith *et al.*, 1973; Fooks *et al.*, 2009). The test may lack sensitivity and specificity, and the interpretation of the test results may be difficult as the host response to infection varies substantially between individuals. As such, the negative predictive value of serological tests for rabies diagnosis is considered poor (Fooks *et al.*, 2009). Therefore, serological assays are not suitable as diagnostic tools for routine rabies testing.

In numerous laboratories in rabies-endemic regions in the developing world, cost and simplicity are critical factors in the delivery of disease diagnosis and cannot be neglected, even when the principal consideration is for rapid diagnosis (Fooks *et al.*, 2009). To combat the use of prohibitive fluorescent microscopy (due to its high cost) in FAT; a direct Rapid Immunohistochemical Test (dRIT) for the postmortem detection of rabies virus antigen in brain smears has been developed (Niezgoda and Rupprecht, 2006). Using a cocktail of highly concentrated and purified biotinylated monoclonal antibodies, rabies antigen can be detected by direct staining of fresh brain impressions within 1 hour. This test employs anti-rabies monoclonal antibodies specific for the nucleoprotein, a viral protein produced in abundance during productive infection. In contrast to FAT antibody used, the new dRIT antibody cocktail is biotinylated such that following a short incubation with a Streptavidin-peroxidase complex, antibody-antigen binding complexes can be visualized through the addition of the substrate, 3-amino-9-ethylcarbazol.

Performed on brain tissues, the dRIT has been proven to be as sensitive as the FAT for fresh specimens (Durr *et al.*, 2008; Tao *et al.*, 2008). Brain impressions stained using

the dRIT technique can be read within one hour and the antibody cocktail used has been shown to detect classical rabies virus strains (genotype 1) that have been assessed (Lembo *et al.*, 2006). Using the dRIT in field studies in Tanzania, viral antigen could be detected in samples after considerable time periods post collection regardless of the regimen of glycerol preservative used (Lembo *et al.*, 2006). Applications of the dRIT to analyze field samples in other rabies endemic regions have also proven highly successful (Madhusudana *et al.*, 2012).

Field trials in Chad sought to study the dRIT in direct comparison to the FAT to attempt to confirm previous studies as to the incidence of rabies within a district known to be endemic. In this study, results between the two tests were 100% in agreement (Durr *et al.*, 2008) and the only issue regarding use of the dRIT over the FAT was the need for the dRIT kit to be stored refrigerated prior to use. In a similar study in India involving animal and human samples, dRIT and FAT showed 100% agreement (Madhusudana *et al.*, 2012).

2.8.1 Reverse-transcriptase polymerase chain reaction

Polymerase Chain Reaction (PCR) is a technique used to study the molecular pathogenesis and diagnosis of a variety of viral diseases, including rabies. Unlike the conventional method, however, this technology makes ante-mortem testing both practical and feasible (Allen *et al.*, 1995). With PCR a specific segment of a viral genome is amplified. Since primers were selected from conserved regions of the genome, most assays amplify parts of the nucleoprotein (N-) gene as earlier proposed (Tordo *et al.*, 1996). In generic approaches intended to detect all lyssaviruses either hemi-nested or fully nested amplifications, are used and have applications for both ante-mortem (saliva, CSF, brain) and postmortem samples (principally brain tissue)

(Fooks *et al.*, 2009). Some of these diagnostic procedures are also applied for further virus characterization, including sequencing reactions (Johnson *et al.*, 2002) or restriction fragment length polymorphism (RFLP) (Nadin-Davis *et al.*, 1993).

Subsequently, strain-specific RT-PCRs have been developed to distinguish the various rabies virus (RABV) strains circulating in a particular region (Kissi *et al.*, 1995; Nadin-Davis *et al.*, 1993). With the incorporation of TaqMan probe with RT-PCR, all the previous six rabies and rabies-related viruses were differentiated (Black *et al.*, 2002; Wakeley *et al.*, 2005). The use of molecular techniques, mainly Polymerase Chain Reaction (PCR), are useful tools in rabies diagnosis (Belák and Ballag, 1993). Many researches are being done with the PCR for rabies diagnosis, which showed that this technique is sensitive and specific in rabies diagnosis (Ermine *et al.*, 1989; Kamolvarin *et al.*, 1993).

RT-PCR and heminested RT-PCR (hnRT-PCR) can detect the rabies virus genome in highly decomposed samples, even when DFA and MIT present negative results, a common situation in countries with tropical weather like Brazil (Heaton *et al.*, 1997; Favoretto *et al.*, 2005). In a report, subjecting previously DFA and MIT positive brain samples to decomposition at room temperature for 72 hours, followed by RT-PCR and hnRT-PCR; analysis revealed that 34.3% and 75% of the decomposed samples were positive by RT-PCR and hnRT-PCR respectively (Araújo *et al.*, 2008). Suggesting that hnRT-PCR is more sensitive than RT-PCR on decomposed samples.

The hnRT-PCR technique allows a sensitive, specific and fast diagnosis of rabies virus, even when samples are in a decomposed state. Additionally, the amplified products can be used in techniques such as sequencing, RFLP, SS-PCR and Multiplex PCR for epidemiological characterization of the virus (Lopes *et al.*, 2006; Favoretto, *et al.*,

2005). However, the recommendation for RNA (after extraction process) is the storage on -80°C until the moment of its amplification (Azevedo *et al.*, 2003), but samples of cerebral tissue are routinely stored at -20°C, in this condition, viral RNA can be degraded disabling retrospective studies.

2.8.2 Detection of anti-glycoprotein antibodies

A quantitative indirect Enzyme Linked Immunosobent Assay (I-ELISA) is an indirect ELISA technique used in detecting the presence of anti-glycoprotein antibodies in vaccinated or unvaccinated individuals (Stantic-Pavlinic *et al.*, 2006; Feysseguet *et al.*, 2007). Based on the principle ELISA, a Platelia™ Rabies II Kit was developed (Bio-Rad, Marnes-la-coquette) to detect rabies virus anti-glycoprotein antibodies as described (Stantic-Pavlinic *et al.*, 2006; Feysseguet *et al.*, 2007; Babasola *et al.*, 2010). Briefly, the kit includes a microplate that was pre-coated with rabies glycoprotein extracted from the inactivated and purified virus membrane. The optical density (OD) values of the test specimens are usually compared with the OD of the positive controls and the antibody titres expressed as equivalence units per ml (EU/ml), obtained from the standard OD antibody titre curve.

All steps are conducted according to manufacturer's instruction as previously described (Stantic-Pavlinic *et al.*, 2006; Feysseguet *et al.*, 2007; Babasola *et al.*, 2010). The results are read using an ELISA reader (IRE 96™ Saint Jean d'Ilac) at a wavelength of 450-620 nm. Subjects are usually considered to be exposed to the virus or immune against rabies virus infection if they produced ELISA titres of ≥ 0.5 eu/ml (Stantic-Pavlinic *et al.*, 2006; Feysseguet *et al.*, 2007; Babasola *et al.*, 2010).

2.8.3 Immunochromatographic Techniques

Another method for the detection of rabies virus antigen from postmortem samples is a recently developed rapid immunodiagnostic test (RIDT) based on the principles of immunochromatography (Kang *et al.*, 2007). The immunochromatographic lateral flow strip test is a one-step test that facilitates low-cost, rapid identification of various analytes, including viruses (Zhang *et al.*, 2009).

2.8.4 Rapid fluorescence focus inhibition test

The fluorescent antibody virus neutralization (FAVN) test (Cliquet *et al.*, 1998) and the Rapid Fluorescent Focus Inhibition Test (RFFIT) (Smith *et al.*, 1973) utilize a similar principle, to measure the level of virus neutralizing antibody in vaccinated or exposed individuals.

‘Indirect’ serological methods, including the FAVN and RFFIT measure the host responses to infection/vaccination only and do not detect the presence of infectious virus/antigen directly. However, host antibody detection (through FAVN/RFFIT) is an indirect tool to measure the presence of rabies virus in a non-immunized individual by evaluating the host response to infection (Smith *et al.*, 1973; Fooks *et al.*, 2009). The test may lack sensitivity and specificity, and the interpretation of the test results may be difficult as the host response to infection varies substantially between individuals. As such, the negative predictive value of serological tests for rabies diagnosis is considered poor (Fooks *et al.*, 2009). Therefore, serological assays are not suitable as diagnostic tools for routine rabies testing.

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However, rabies virus infection almost invariably results in a fatal encephalomyelitis. Persons working in positions where they may be exposed to rabies virus should receive pre-exposure vaccination. Successful immunization is demonstrated by detecting adequate levels of virus neutralizing antibody in the serum of a vaccinated person. Several serologic tests have been evaluated for measuring antibodies against rabies in serum (Smith *et al.*, 1996). Serologic assays for the detection of rabies virus antibodies may vary depending upon the different types of antibodies in the sera of immunized, or naturally infected animals or humans.

The serum may contain mixtures of immunoglobulins induced by internal ribonucleoprotein antigens or surface glycoprotein antigens. Neutralization tests, such as the Rapid Fluorescent Focus Inhibition Test (RFFIT) measure antibody induced by exposure to rabies virus antigens (Smith *et al.*, 1996). This neutralizing antibody is

induced by the glycoprotein in the envelope on the virus. The Centers for Disease Control and Prevention (CDC) has adopted the RFFIT for rabies virus antibody determinations because it is rapid, economical, sensitive, and reproducible (CDC, 2006).

2.9 Latency and Abortive or Non Fatal Rabies

As suggested by Bell (1975) the term “infection” may be used in the broad sense of the mere presence of the virus while “Latent infection is reserved for situations in which the presence of the microbes cannot be demonstrated by any method, then available, and the fact infection is present can only be demonstrated in retrospect by the emergence of overt disease. “Abortion” on the other hand will refer to the cessation of adverse effects of virus upon the host (Bell, 1975). Latency and abortion are not always clearly distinguishable from masked, in-apparent, chronic, recrudescence infection, eclipse, long incubation periods, and carrier state (Walker *et al.*, 1958).

In 1762, Layard remonstrated against the practices of euthanasia as a reaction to the assumed hopelessness of rabies infections in man and went to some pains to try to prove that clinical cases need not necessarily prove fatal (Bell, 1975).

The incubation period of rabies, especially the occasional very long incubation period could be considered as Latency. Mc Dermott (1959) defined characteristically, that incubation periods of rabies are extremely variable with reliable records of more than 7 months in naturally infected bats (Moore and Raymond, 1970), 611 days in an experimentally infected cow (Albelseth and Lawson, 1972) minimum periods of more than 4 months in 7 of 30 quarantined dogs (Hole, 1969) and as long as 120 days in experimentally infected mice (Baer and Cleary, 1972) cited by Bell (1975). In the

infected mice, amputation of the inoculated Limb even up to the eighteen day after inoculation resulted significantly to low mortality, and it seems fair to infer from these data that virus may remain infective at or near the site of inoculation during long incubation period (Bell, 1975).

Nonfatal, abortive or recovery from rabies is a well documented phenomenon. However, to document recovery from rabies, it is necessary to establish that the active rabies infection has occurred. Circumstantial evidence can be convincing when large numbers of animals are inoculated and some become ill and die, whereas others with simultaneous and identical signs of illness, recover and survive (Bell, 1975). Pasteur (1882) on the other hand considered resistance to re-inoculate as a strong indication of previous abortive infection. Perhaps the most commonly used criterion of non fatal infection is the isolation of virus from saliva, brain or other tissue of animals that appear normal (Bell, 1975), yet it should be emphasized that the virus may be present in the CNS and salivary glands well before onset of illness (Wright; 1956, Schneider, 1969) and isolation of virus from saliva of normal appearing animals is significant only when the animal can be held for observation to see whether they will die, which have been done repeatedly (Bell, 1975).

Non fatal rabies was successfully reproduced in rabbits infected intracerebrally with a highly pathogenic strain of street virus isolated from a man who died of hydrophobia after a dog bite (Gribencha, 1975), as well in white rats infected intracerebrally with the CVS strain of fixed virus (Gribencha, 1975). Similarly, Fekadu (1988) indicated that up to 18% of dogs experimentally infected with the street rabies virus died without showing detectable signs of illness. He further reported that up to 20% of the dogs, showing signs of rabies later recovered without any supportive treatment; he concluded

that rabies is not invariably fatal. One dog that recovered from rabies intermittently excreted rabies virus in its saliva for a long time, while some dogs excreted virus in their saliva 14 days before signs appeared (Fekadu, 1988).

2.8.1 Rabies virus antigen in the brain of apparently healthy slaughtered dogs in Nigeria

In Nigeria, a report has indicated the presence of detectable rabies virus neutralizing antibodies (rVNA) of 15.93% in unvaccinated dogs (Aghomo *et al.*, 1987) and rabies virus antigen in apparently healthy dogs (Aghomo and Rupprecht, 1990). Furthermore Ogunkoya *et al.* (1990) reported the presence of 30.3% rVNA of the total unvaccinated dogs screened, all from southwestern Nigeria (Ogunkoya *et al.*, 1990). Recently, there are reports of the detection of rabies virus antigen in the brains of between 6 and 42% of apparently healthy slaughtered dogs destined for human consumption in Northern parts of Nigeria (Ajayi *et al.*, 2006; Garba, 2007; Sabo, 2009; Dzikwi *et al.*, 2010b; Garba *et al.*, 2010; Audu, 2011).

It is, therefore, a well known phenomenon that non fatal, abortive, recovered, carrier state of rabies in animals occur. The carrier state in rabies may play a significant role in the perpetuation and survival of the virus and may become a source of rabies out break whenever a new generation of rabies susceptible animals/host reaches a critical density.

2.10 Dog Ecological Studies in Nigeria

Dog population dynamics have a major impact upon the effectiveness of rabies control strategies. For this reason, understanding domestic dog ecology especially in Nigeria where dog is the principal reservoir host for rabies, has been recognized as central to the design of effective rabies control programmes. The population dynamics of dogs is an important factor in the epidemiology of endemic canine rabies. Lack of reliable

estimates of dog populations hampers rabies control campaigns in developing countries, as cost benefit analysis of control strategies cannot be made accurately. The WHO "Guidelines for dog rabies control" has stressed the need for research on dog populations and ecology in urban and rural areas (WHO, 1980). Determination of dog population density (dogs/km²) from established indicators of dog abundance (dog to human ratio and dogs per household) is one recommended procedure (WHO, 1980; Oboegbulem and Nwakanobi, 1989).

Unlike livestock kept in herds on farms or housed in flocks, dogs are owned in small numbers in widely and randomly scattered households (Oboegbulem and Nwakanobi, 1989). Until recently there appears to be no standardised field technique available for general application in dog population studies. One parameter is an estimation of dog population density, calculated from the dog to man ratio or dog per household and expressed as dog per unit area (WHO, 1980). Various workers have applied different techniques for estimating dog population densities. These techniques include total street-dog count (WHO, 1980), estimates from the rate of capture (Davis and Winstead, 1980) and estimates from rate of re-capture of the same dog (Beck, 1973). Most of these methods are adapted from techniques developed for estimating the density of wild animals in their natural habitat (WHO, 1980).

In Nigeria, ecological studies have been conducted using questionnaires and street dog counts in order to estimate the dog population of some states. For instance, Okoh (1986) reported a dog to human ratio of 1:4.2 in Jos. The dog to man ratios for urban and rural areas were 1:21 and 1:45 or 47 per 1,000 persons and 21.7 per 1,000 persons, respectively from Southeastern Nigeria (Oboegbulem and Nwakanobi, 1989). A 1:4.10 and 1:3.20 ratios were reported from urban and rural Borno State respectively (El-

Yuguda *et al.*, 2007). More recently, a dog to human ratio of 1:4 was reported in residential areas of Makurdi, Benue State of Nigeria (Omudu *et al.*, 2010) a dog: human ratio of 1:5.6 was reported from Lagos State (Hambolu *et al.*, 2014). Similarly, a dog to human ratio of 1: 7.8 was reported in Aba to of Abia State (Otolorin *et al.*, 2014).

CHAPTER THREE

MATERIALS AND METHODS

3.1 Study Area

The study was conducted in Niger State, Nigeria, a state in the north central region of Nigeria. The state is located between Latitudes 8° 30' N and 11° 30' N and between Longitudes 3° 30' and 7°20'E, comprising of three senatorial zones (Bida, Kontagora and Minna) with 26 Local Government Areas. The state has a total human population of over 3.9 million (NPC, 2006) with abundant livestock and a dog population of 199,812 (with 98.5% in the villages and 1.5% in the township) of the state (RIM, 1992).

The state falls within Guinea (southern) Savanna Belt of Nigeria, with about 90% of its population living in the rural areas practicing subsistence farming. Niger State is the largest state in the country in terms of land area, which is about 76,000sq Km (or nearly 9 per cent of Nigeria's total land area) (Anon, 2003). Figure 3.1 shows a map of Nigeria with Niger State bounded with arrows. Figure 3.2 is map of Niger State showing locations where samples and data were collected (Bida, Kontagora, Suleja and Minna accordingly).

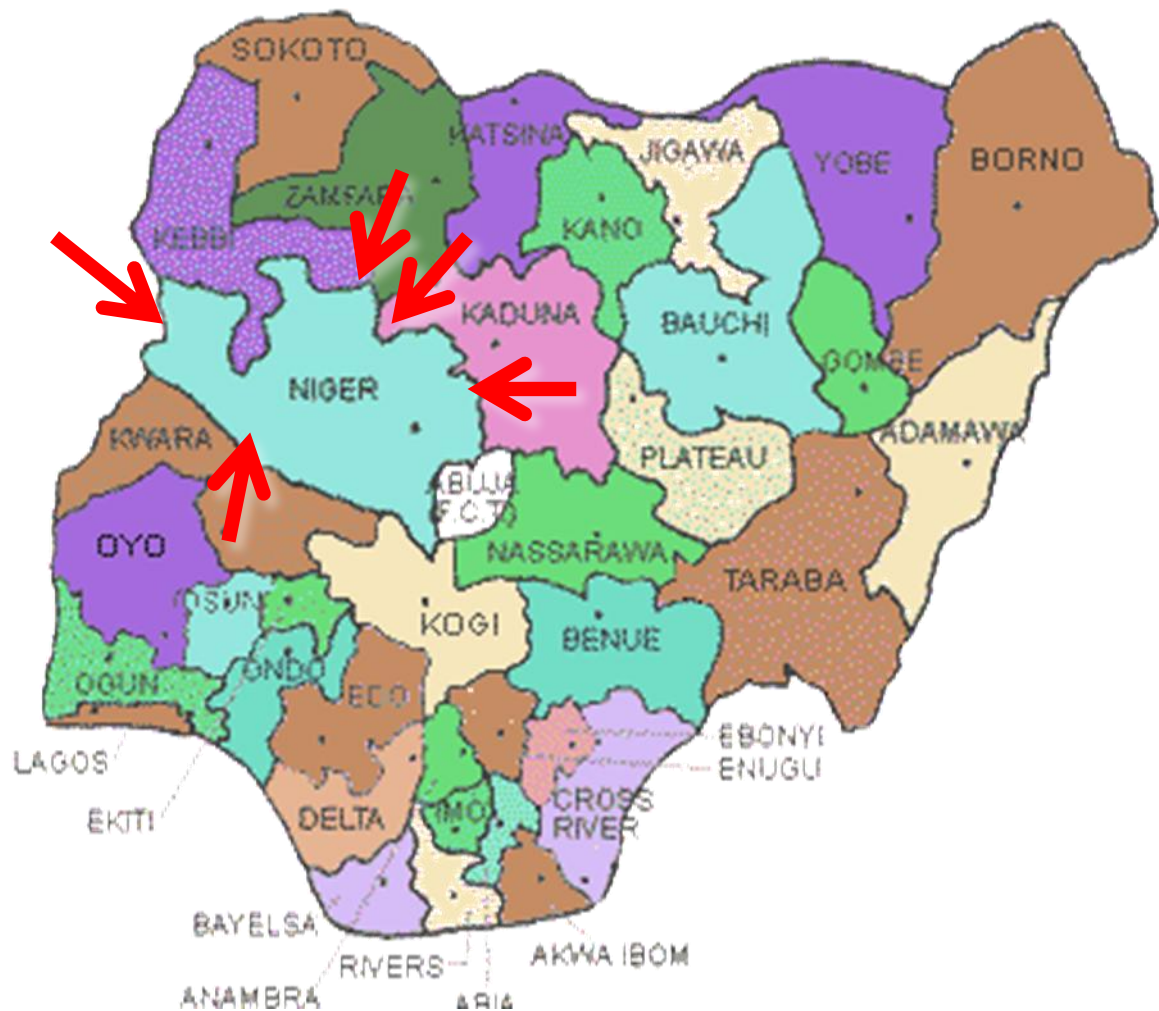


Figure 3.1: Administrative map of Nigeria showing 36 states and Abuja (Niger State bounded with arrows) (Source: Anon, 2003)

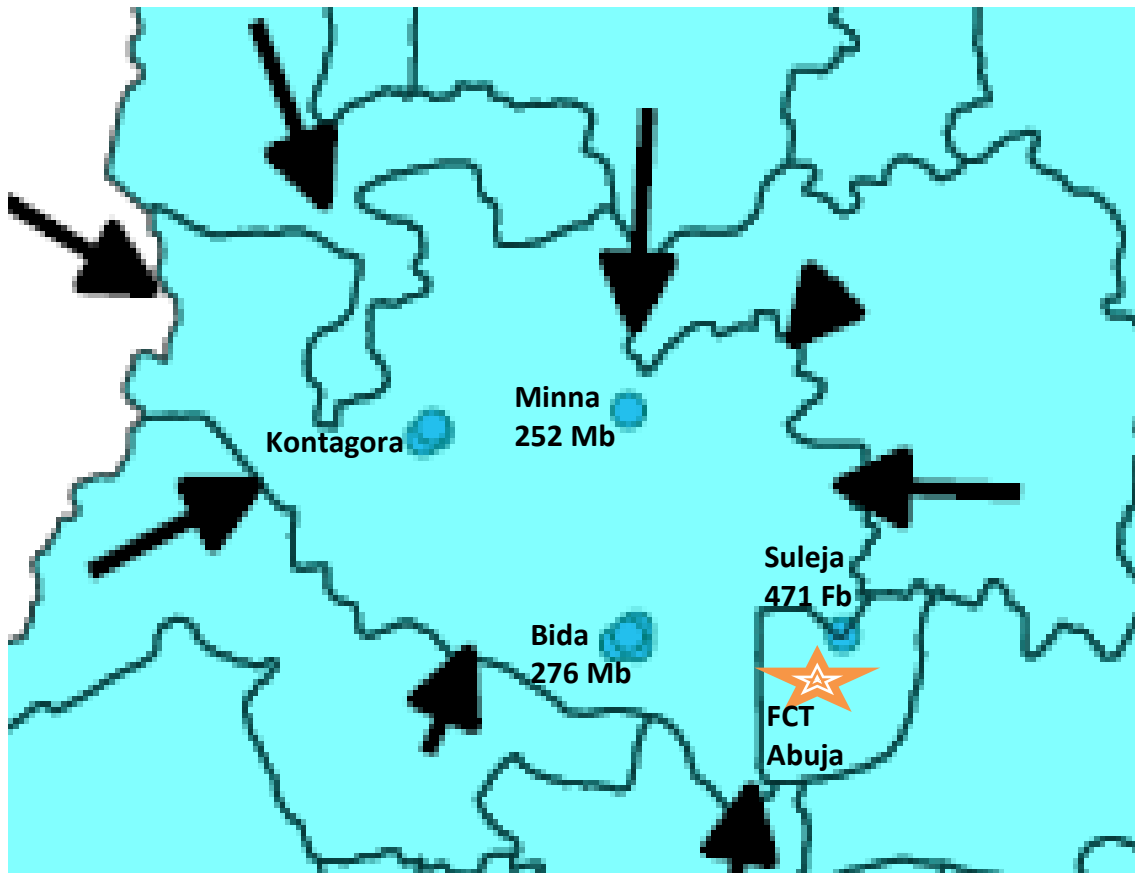


Figure 3.2: Study locations where samples and data were collected in Niger State, Nigeria. (Source: Anon, 2003)

3.2 Sample Size:

Sample sizes were determined based on previous reports on rabies/ related viruses and using the mathematical expression as described by Mahajan (1997).

Using the expression as described by Mahajan (1997), $n = \frac{Z^2 pq}{d^2}$

Where n = sample size

d = allowable error

P = percentage prevalence

Z = Standard Normal Deviate

q = (1-p)

1. For dogs, Garba *et al.* (2010) reported a rabies virus antigen prevalence of 28% in the brain of apparently healthy slaughtered dogs from North western Nigeria.

∴ n = ?

p = 28% (0.28)

q = 1-p (1-0.28) = 0.72

z = 1.96

d = 5% CI = 0.05

$n = \frac{3.841 \times 0.28 \times 0.72}{0.0025} = 309.74 \approx 310$ samples

A minimum of 310 dog brain samples were to be collected, but 471 dog brain samples from slaughtered dogs were collected and used in this study.

2. For bats, Dzikwi *et al.* (2010a) reported the prevalence of neutralizing antibodies of Lagos bat virus in 19% of the bats examined.

$$n = ?$$

$$p = 19\% (0.19)$$

$$q = (1-0.19) = 0.81$$

$$z = 1.96 \quad n = \frac{3.841 \times 0.19 \times 0.81}{0.0025} = 236.45 \approx 237 \text{ samples}$$
$$d = 5\% (0.05)$$

A minimum of 237 bats were to be captured and used for this study, but only 267 bats were captured in which brains (267), and sera (162) were collected and used in this study.

3. For human samples, based on the prevalence of 28.6% of rabies antibody in humans by Ogunkoya *et al.* (1990); the following samples were to be collected in Niger State.

$$n = \frac{3.841 \times 0.286 \times 0.714}{0.0025} = 314 \text{ human sera}$$

However, only 185 human volunteers participated and gave their blood for rabies antibody testing and same 185 volunteers filled the structured questionnaires on dog bite and vaccination. However, samples were collect from 5 categories of volunteers dog butchers, dog meat consumers, pet owners, hunters with dogs and veterinarians/animal health assistants, totaling 185 human samples volunteered.

This implies that a sample of 471 dog brains, 267 bat brains, 162 bats sera and 185 human sera were collected totaling 1085 samples and used in this research, for all the species. In addition, three questionnaire surveys via: 300 for general public on dog ecology and management (Appendix V), 155 for dog butchers/ consumers and hunters/pet owners (Appendix III), 30 for Veterinarians/animal health assistants (Appendix IV) were used.

3.3 Questionnaire Survey on Dog Ecology and Data Collection on Dog bite Cases in Hospital Records

3.3.1 Questionnaire survey on dog ecology and management

A total of 300 structured questionnaires were distributed in the three senatorial districts of Niger State. The questionnaire comprises of 4 sections with a total of 40 questions (Appendix V). Section (a) was on the demography of respondents, including age, location, household size etc., section (b) on dog population and structure, including keeping of dogs, number of dogs, sex, breed, age, the purpose of keeping dogs, acquisition, depopulation, confinement, etc. Section (c) on dog feeding and health management, including responsibility for dogs, feeding of dogs, vaccination, health care offered and section (d) was on dog bite cases, management and sequela, including household member (s) ever been bitten by a dog, fate of the offending dog, fate of the victim, ownership of the dog, health status of dogs, the causative agent of rabies etc.

Four major towns Bida, Kontagora, Suleja including the state capital Minna were conveniently selected. In every selected town, a major road that crosses the town was first identified and from one end towards the other, every third street radiating from the road was selected from the left and right hand sides of the road using the systematic randomization method. In the same way every third house/compound (on the left and right) in the selected streets were used to administer the questionnaires to the most senior and enlightened member of the family in the households. Of the 300 questionnaires distributed only 237 were returned. Of the 237 returned, 30 were discarded due to lack of merit, 207 were actually used. Data were entered into Microsoft excel 2010 spread sheet, cleaned and coded then Imported into SAS statistical program version 9.3 to generate the frequencies of variables. Information generated was summarized in tables and chart using Microsoft office tools.

3.3.2 Data collection on hospital records of dog bite and laboratory confirmation of dog rabies

A total of nine General Hospitals and two tertiary health providers (all government owned) were randomly selected in the state using simple randomization. The selected hospitals were visited across the State. Data from the records of dog bite cases between January 2006 and July 2013 in these hospitals were retrieved. All records of cases reported to the clinic with dog bite or suspected rabies were considered as dog bite cases and included in the research. Information sought in the cases included date of bite, sites of bite, sex and age of victims. Similarly, the records of rabies confirmatory diagnosis at NVRI, Vom-Nigeria between January 2006 and July, 2013 were retrieved.

3.4 Sample Collection

Brain Samples were collected from the three senatorial zones (SZ) of the state where dogs are slaughtered for human consumption (precisely army barrack Bida, Sachi Bida, Unguwan Kaje Minna, dog meat market Suleja and army barrack Kontagora) were used. Similarly, places were identified and visited across the State, in which bat colonies on trees were found in the three SZ. Hunters with local rifles loaded with low density bullets were used to shoot the bats, captured and samples collected. Furthermore, human samples from rabies occupational risk groups were collected at dog slaughter and consumption points and veterinary clinics in the study area.

3.4.1 Dog brain sample collection

Dogs were examined visually before slaughter to see if there was a rabies-like syndrome, they were all looking apparently normal. Following slaughter and decapitation of dog heads, brain samples were collected via the foramen magnum. The skin and the muscles at the base of the skull were removed, and hacksaw and bone

cutters were used to remove a portion of the skull wide enough to obtain the brain stem. The sample was then transferred to a pre-labelled (plastic) sample bottle. Samples were then shipped on ice to Virology Laboratory, Department of Veterinary Public Health and Preventive Medicine, Ahmadu Bello University Zaria, Nigeria and stored at -20°C until analysed in July, 2012. Immediately after slaughter of each dog, age, sex and breed were recorded.

3.4.2 Collection of brain and sera samples from bats

Following capture, about 1.5 to 2 ml of blood was collected from each bat using a 2 ml syringe and needles directly from the heart. The blood samples were transferred into vacutainers into a coleman box for serum separation by centrifugation at 3,000 rpm. Then the bat was slaughtered and whole brain was removed following the opening of the skull with scissors, placed into a pre-labeled bijou bottle and stored at -20°C until analyzed. Some representative samples of different carcasses were stored in 10% formalin for species identification.

3.4.3 Human blood serum collection

Following ethical permission from Ahmadu Bello University and the State Government (Appendices X and XI respectively), participant information leaflet was given (Appendix I) and the acceptance/ filling of the consent form (Appendix II) a pair by each volunteer (dog butchers, dog meat consumers, hunters with dogs, pet owners and veterinarians/animal health assistants). About 2 ml of blood was collected from the cephalic vein of each volunteer by a registered nurse. The blood was allowed to clot; centrifuged (3,000 rpm) and serum samples aspirated using a plastic pipette, then placed into a pre-labeled vacutainers and were stored at 4°C until analyzed. Prior to

blood collection, a structured questionnaire was filled by every donor. A total of 155 human sera and questionnaires (see Appendix III) was collected from dog butchers, dog meat consumers, hunters with dogs and pet owners, while a total of 30 human sera and questionnaires (see Appendix 4) was collected from veterinarians/animal health workers and used for statistical analysis.

3.5 Geographic Information System Mapping

All the areas where samples/specimens or data were collected or generated [the location co-ordinates] were captured using GPS navigator Etrex 10 Gamin. This was to facilitate mapping of all locations of brain samples and those positive on the map of Niger State, Nigeria.

3.6 Transportation of Samples

All samples collected in the field at intervals between January and July, 2012 were transported to the Viral Zoonosis Laboratory, Faculty of Veterinary Medicine, A. B U-Zaria in ice packed coleman boxes and stored at -20°C until used. All samples were further shipped in September, 2012 on dry ice to Rabies Program, Centers for Disease Control and Prevention (CDC), Atlanta, United States of America (USA) for further analyses.

3.7 Laboratory Analyses

3.7.1 Fluorescent antibody test procedure

All the 471 brain samples from dogs and 267 brain samples from bats were subjected to the FAT procedure at Virology laboratory, Department of Veterinary Public Health, Faculty of Veterinary Medicine, A. B. U-Zaria as previously described by Dean *et al.*

(1996) and later at the Centers for Disease Control and Prevention (CDC) Atlanta, USA following the CDC protocol.

Briefly, the brain impressions were made on 3 well Teflon-coated glass slides, air-dried for 15-30 minutes, then fixed in cold acetone for a minimum of 1 hour. After fixation the impressions were reacted with two anti-rabies nucleocapsid monoclonal antibody conjugates, EMD Millipore Cat# 5100 (Temecula, CA) and Fujirebio Diagnostics, Inc. (Seguin, TX, USA) and specificity negative control conjugate EMD Millipore Cat #5102 for 30 minutes in a humid chamber at 37⁰C. The slides were then removed, and the impressions washed in PBS, pH 7.5 twice for 5 minutes each. Cover slips were placed onto slides using 20% glycerol Tris-buffered mounting medium pH 9.0 and observed for the presence of typical rabies-like inclusions demonstrating 4+ sparkling apple- green fluorescence under fluorescence microscope using a 20x aplanachromat objective with > 0.75 aperture. Rabies virus infected and normal mice brain specimens were used as positive and negative control-slides respectively.

Presence of rabies typical apple green fluorescing inclusions in the examination fields of the impressions tested with anti-rabies conjugates and absence in the impression tested with the specificity control, non-rabies FITC conjugate was considered positive (presence) of rabies viral antigen. The absence of apple green fluorescing particle on the stained slides was considered negative for rabies. All slides were compared with the positive and negative controls. Results were recorded according to the intensity and distribution of viral particles graded from +1 (low) to +4 (high) intensity/distribution. Typical rabies virus inclusions demonstrated 4+ intensity fluorescence.

Fluorescence seen in each well of the test smear was compared with the positive control interpreted as follows:

++++, a massive infiltration of large and small brilliant apple green shining inclusions/ particles at varying size and shape in almost every area of the impression.

+++ , a brilliant apple green shining inclusion/ particle of varying size and shape are found in almost every microscopic field, the number of inclusions per field varies, but the inclusions are numerous most fields.

++ , a brilliant apple green shining inclusion /particles of varying size and shape are present in 10% to 50% of the microscopic fields and most field contain only a few inclusions.

+ , a brilliant apple green shining inclusion/particles of varying size and shape are present in < 10% of the microscopic field and only few inclusions are found per field (usually only one or two inclusions per field). This category 1+ was not considered true positive in this study, it was assumed doubtful due to human and experimental errors.

- , when there is no brilliant apple green shining particle/ inclusion in the microscopic fields.

All the DFA processed samples were equally re-tested using Direct rapid immunohistochemistry test (DRIT) procedure to compare results. Similarly, all the positive and questionable/non-specific stained specimens were further subjected to Reverse transcription polymerase chain reaction (RT- PCR) to further identify and sequence the viral amplicons.

3.7.2 Direct rapid immunohistochemistry test procedure

All the brain samples from dogs and bat were equally subjected to DRIT procedure at the Centers for Disease Control and Prevention (CDC), Atlanta, USA as described by Neizgoda and Rupprecht (2006) in accordance with the standard operating procedure (SOP) for DRIT CDC (CDC, SOP manual for DRIT 2006). The procedure was carried out as highlighted in Appendix VI in a step – by – step wise using 10 dishes containing various reagents/solution.

3.7.3 Reverse transcription - polymerase chain reaction

FAT and DRIT positive and questionable samples (totaling 13) from dog brain were used for the RT-PCR and Sequencing. Extraction of the viral RNA, RT-PCR, and sequencing was performed as previously described by David *et al.* (2007) with some modification as recommended in the RT-PCR protocol designed by CDC, USA (CDC, 2012a). Initially, total RNA was extracted, followed by reverse transcription (RT) to produce the cDNA, then polymerase chain reaction (PCR), gel electrophoresis, purification, sequencing PCR and phylogenetic analysis (CDC, 2012a). Complete protocol carried is presented in Appendix VII.

Briefly, total RNA was extracted using the TRIzol method (Invitrogen, Life Technologies, USA) following the manufacturer's recommendation by the use of (0.1 g of brain sample), 0.1ml of lysis buffer, 0.1ml TRIzol, exactly 0.2 ml of chloroform, 0.5 ml Isopropanol and 1.0 ml 75% ethanol.

This was followed by Reverse transcription (RT) to convert (transcribe) the extracted RNA into complimentary DNA (cDNA). This was briefly carried out by preparing three RT reaction tubes and two of these tubes received 2 µl 001degFw 10 µM and the remaining one received 2 µl of 921degFw 10 µM. Then 10 µl RNA was added to each reaction tube containing the RT primer and were further centrifuged. The sample and forward primer were denatured by heating the tube to 94°C for 1 min, and then cooled on ice for approximately 3 minutes. Then 14 µl RTRX cocktail (71.6 µl of RTRX mix, plus 2.0 µl reverse transcriptase and 2.0 µl RNase inhibitor) was added to each reaction tube briefly vortexed and centrifuged. The thermocycler block was pre-heated to 42°C before placing the tubes which were incubated at 42°C for 90 min with 4°C hold. At the end of the 90 minutes incubation, complimentary DNAs (cDNA) were produced.

Polymerase Chain Reaction premix/cocktail buffer was then prepared (as previously described for every 5.4 reactions) by adding 372.6 μ l molecular grade water, 43.2 μ l of Tris buffer pH 8.3, 2.7 μ l (5 U/ μ l) Taq polymerase (Amplitaq™ PE Biosystems# N880153), 5.4 μ l of 20uM now degenerated or 40uM degenerated forward Primers (three separated PCR cocktails 1) 001-921degB; 2) 001-550degB and 3) 1066degFw-304B). 80 μ l PCR cocktail was added to each of sample reaction tubes accordingly and centrifuged briefly. The tubes were then placed into the thermocycler preheated at 94°C and ran 40 cycles of PCR (1 minute at 94°C followed by 40 cycles of 94 °C for 30 seconds, 37°C for 30 seconds, and 72°C for 90 seconds; with the hold at 4°C following a 7 minute extension at 72°C).

A Hemi-nested PCR (Re-amplification of the primary PCR reactions) was carried out with primers, one only internal primer and one of the primers used for the primary RT-PCR). It was run with the same cycle parameters that were used for the primary RT-PCR. This was followed by gel electrophoresis using 2% agarose gel loadig dye with the RT-PCR products were subjected electrophosis at 150 V and amplicons were seen after UV light exposure and photography.

This was followed by purification of the primary and Heminested RT-PCR product using EXOSP-IT enzyme at 37⁰ C (destroy all double stranded DNA from the unused primers and proteins in the initial RNA to cDNA) and at 80⁰ C (to destroy the EXOSP-IT enzyme after its work) all set at 15 minutes periods. This was followed by sequencing-PCR using Big dye and 25 PCR cycles set at denaturalization temperature at 96°C for 10 seconds, the annealing temperature at 50°C for 5 seconds and the polymerization temperature at 60°C for 4 minutes. The sequencing-PCR product were

then re-purified with the use of 'Sepharose' centrifuged at 4000 rpm for 4 minutes, then formamide was added and mixed.

The mixture were then removed and transferred into a 96 well plate and placed into the PCR machine and set at denaturalization temperature of 94°C for 2 minutes to allow the single strand DNAs to be linear. The plate was then transferred to sequencing machine after putting fresh buffers required for sequencing analysis in the machine. An automatic sequencer 3700 DNA analyzer (Applied Biosystems) connected to a computer program was used according to manufacturer's recommendations

Phylogenetic analysis: Basically after the sequencing all sequences were selected and saved by exporting as ABI files. BioEdit software package was used to assemble and edit the complete N gene using as a template the complete nucleoprotein gene of the reference RABV SADB19. Correction or deletion of the sequences were made at appropriate points where sequences are not in the correct orientation using the BioEdit. The assembled nucleoprotein gene was then sent for a BLAST search to retrieve recent and historical sequences that have the higher identity values to be incorporated into a phylogenetic tree. The nucleotide sequences retrieved both from GenBank and the CDC Nigerian data base were then aligned using ClustalX software.

A phylogenetic tree encompassing complete N genes from recent and historical RABV circulating in Africa was constructed by the maximum likelihood method using the Molecular Evolutionary Genetic Analysis (MEGA version 9.1) software as described (Kumar *et al*, 2004). The reliability of the phylogenetic grouping was calculated using bootstrapping with 1000 replicates. Results and phylogenetic tree were recorded.

Table 3.1: Broadly reactive or degenerate primers used for RT-PCR for rabies diagnosis

Designation ID	Orientation	Genome position	Sequence	Reference
Lys001	Forward	1-16	ACGCTTAACGAMAAA	Markotter <i>et al.</i> , 2006
550F	Forward	647-666	ATGTGYGCTAAYTGGAGYAC	Markotter <i>et al.</i> , 2006
550B	Reverse	647-666	GTRCTCCARTTAGCRCACAT	Markotter <i>et al.</i> , 2006
1066degF	Forward	1136-1155	GARAGAAGATTCTTCAGRGA	Orciari Rupprecht, 2007
1066degB	Reverse	1136-1155	TCYCTGAAGAATCTTCTYTC	Orciari & Rupprecht <i>al.</i> , 2007
†304	Reverse	1514-1533	TTGACGAAGATCTTGCTCAT	Smith, 1995
921F	Forward	991-1011	YGTGTTCAAYCTHATYCACTT	CDC, 2012a
921B	Reverse	991-1011	RAGTGRADTAGRTTGAACAC	CDC, 2012a

.Key: † = Non-degenerate primers

3.7.4 Modified rapid fluorescence focus inhibition test for Duvenhage Virus antibodies on bat sera

This was carried out following the principle of Smith *et al* (1996) as described in the CDC protocol (Appendix VIII). Briefly there was an initial heat inactivation of the bat sera at 56°C for 30 minutes, followed with a four step 5-fold serial titration of the sera. A 12µl of Eagles minimum essential medium (MEM - 10) in each well was added, followed with the addition of the Duvenhage virus and incubated at 37°C in humid chamber for 90 minutes. This was followed with the addition of the cell line (Mouse Neuroblastoma, MNA cell), incubated at 37°C at humid condition for 44 hours, followed by staining as described by Dean *et al.* (1996) and examination of fields under fluorescent microscope at x20 and x40 magnification.

Ten examination fields were viewed for every well and presence of fluorescing apple green particles suggest the sample is negative. But absence of fluorescing apple green particles particularly in less than 5 of the 10 examination fields indicate presence of neutralizing antibodies and the sample was considered positive for Duvenhage virus antibodies. Results were recorded. Detailed procedure is shown in Appendix VIII.

3.7.5 Standard rapid fluorescence focus inhibition test on human sera

This was done as described by Smith *et al.* (1996) in accordance with the Standard Operating Procedure for RFFIT for determining rabies virus neutralizing antibody at the Centers for Disease Control and Prevention (CDC) as revised in 2006 (CDC, 2006). Complete standard RFFIT procedure is shown in Appendix IX.

Briefly, as in the modified RFFIT, the human serum was initially heat inactivated in water bath at 56°C but for 45 minutes, followed by a four folds, 5 serial dilution of the sera with Eagles minimum essential medium (MEM - 10) in a 8 chamber Tissue-tek

slide. This was followed with the addition of the virus and incubation at 37°C in humid chamber for 90 minutes.

The cell line (Mouse Neuroblastoma, MNA cell) was then added and incubated at 37°C in humid condition for 20 hours. The chamber was then removed, slides washed in PBS then in acetone followed by fixation in cold acetone for 30 minutes, stained with rabies monoclonal antibody (fujirebio) as described by Dean *et al.* (1996). The slides were then washed and the fields were examined under fluorescent microscope at x20 to scan the field and then at x40 magnification to view.

Examination of slides and Interpretation of results: Each of the 8-well Tissue-Tek slide chambers contains 25 to 50 distinct microscopic fields when observed at 160 to 200 times magnification. Twenty examination fields were viewed for each chamber under a fluorescent microscope (Zeiss, Axioskop with up to 200X magnification was used). The numbers of fields which contain fluorescing cells (even if it was one particle/chamber) were counted starting in one of the corners of the chamber. The number of positive fields out of 20 fields/ chamber examined was recorded on a RFFIT results data sheet. Presence of less than 10 infected fields/chamber per test serum in 1:5 dilution (suggesting presence of neutralizing antibodies), was considered positive for rabies antibody.

Determination of the potency of test serum in international units (IU) per ml: The potency (titre) of the test serum antibody was calculated based on the Reed-Muench Method (Reed and Muench, 1938) as described in the antibody titre table (Appendix XII) contained in the CDC Standard Operating Procedure for RFFIT for determining rabies virus neutralizing antibody (CDC, 2006). Using the expression below:

3.8 Data Analyses

3.8.1 Data analyses on questionnaire survey

The 207 completed and returned questionnaires on dog ecology were subjected to descriptive statistics following entry of cleaned and coded data into Microsoft excel 2010 spreadsheets. Statistical Analysis System (SAS) program version 9.3 was used to get the frequency of variables, the results being summarized in figures and tables. The data collected from the hospital records on dog bites in Niger State and laboratory confirmation of dog rabies at NVRI, Vom were also processed using descriptive statistics and results summarized in tables. The moving average ratio was used to calculate the seasonal index of dog bite cases. Some data collected on dog slaughters were equally entered into Microsoft excel spreadsheets and analyzed using an analysis tool for the Microsoft excel 2010, results being summarized in figures and tables.

3.8.2 Statistical analysis on laboratory specimens

Results of the FAT, DRIT and RT-PCR were summarized using descriptive statistics, while *Kappa coefficient* was used to measure agreement between FAT and DRIT on results of dog brain samples. While the maximum likelihood method was used for RT-PCR sequence and Phylogenetic analysis using BioEdit, ClustalX and MEGA version 9.1 software to construct the phylogenetic tree.. Data from questionnaire studies on human volunteers and the RFFIT test results on bat and human sera were subjected to descriptive statistics using proportions and percentages. The end point titre titre of human sera in intercalated using the expression below (based on Reed and Maunch, 1938 techniques)

$$\text{Number of IU/ml} = \frac{\text{End-point titre of the test serum}}{\text{End-point titre of the reference}} \times 2 \text{ IU / ml in the reference serum}$$

CHAPTER FOUR

RESULTS

4.1 Dog Ecology and Management in Niger State

It was shown that there was no wide difference in percentage of the returned valid questionnaires across the four representative towns used in studying the dog ecology in Niger State (Figure 4.1).

Results showed that there is a ratio of 1:5.4 dogs to human population in Niger State are bringing the current estimate of the dog population to 732,476 and compared with the actual human population of 3,955,372 in Niger State as at 2006 (NPC, 2006) (Table 4.1). Furthermore, for every two male dogs there is one female dog and over 75% of dogs were below the age of 5 years (Table 4.1.). Similarly, over 60% of dogs in Niger State are a native breed with about 25% being exotic breeds (Table 4.1).

The majority (58.6%) of the dogs in the study area was kept for security reason with a lower proportion (8.6%) kept as pets (Figure 4.2). Most of the populace (66.7%) in Niger State like keeping dogs and mostly acquire dogs through purchase (60.4%) or given by their friends or relatives (29.6%) and only few (3.1%) acquired dogs when they strayed into their houses (Table 4.2). Furthermore, only 5.8% (of puppies) and 1.5% (of adult dogs) disappeared or strayed away and over 66.2% of the respondents do not allow dogs stray into their compounds (Table 4.2). Most of the dogs (55.1%) stray into homes at night with the lowest proportion (8.2%) straying in the afternoons (Figure 4.3). It was also shown that 79.1% of dogs that strayed into homes, engaged in mating with other dogs resident in the houses and to some extent (10%) involved in eating left over foods (Figure 4.. 4).

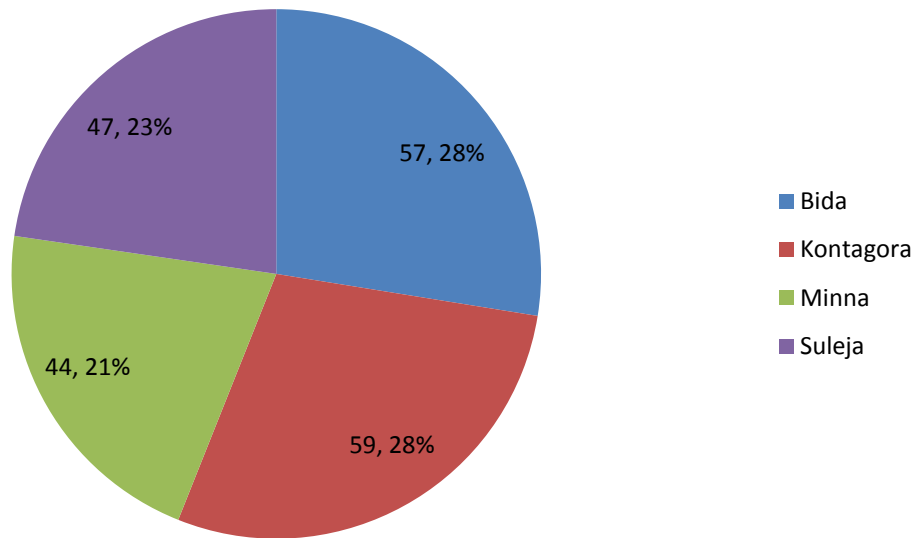


Figure 4.1: Distribution of respondents according to four towns surveyed.

Table 4.1: Dog population and structure in Niger State, Nigeria.

Variables	<u>Frequency</u>	
	No.	%
a. No. of compounds visited	207	
No. of persons	1965	
No. of dogs	366	
Human to Dog ratio	5.4 : 1	
Estimated Human*: Dog population	3,955,372* : 732,476	
b. Sex distribution of dogs		
Male	238	65
Female	128	35
Male to Female ratio	1.9 : 1	
c. Age distribution of dogs		
< 1 year old	120	32.8
1 – 5 year old	155	42.3
> 5 year old	91	24.9
d. Breed of dog		
Native	88	60.3
Exotic	37	25.3
Mixed	21	14.4

Key: No. = number, % = percentage, * = estimate based on NPC census, 2006

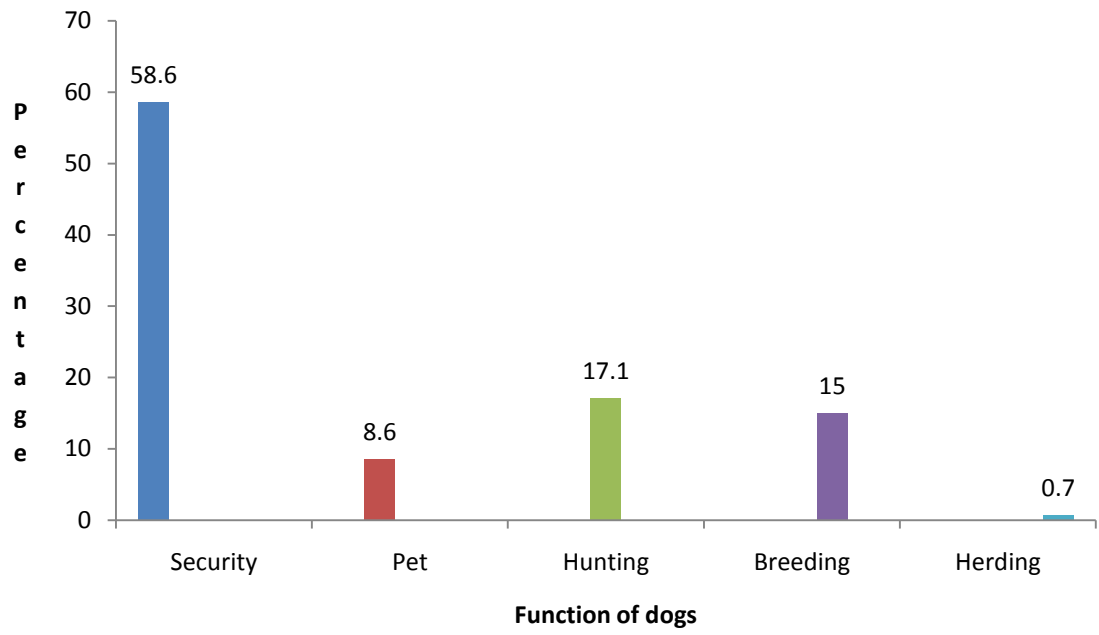


Figure 4.2: Purpose of keeping dogs in Niger State, Nigeria.

Table 4.2: Dog acquisition, depopulation and straying in Niger State, Nigeria.

Parameters	Frequency	
	%	
a. Do you like keeping dogs?		
No	33.3	
Yes	66.7	
b. How do you acquire dog?		
Friends and relatives	29.4	
Neighbours	6.9	
Purchase	60.6	
Roam into my house	3.1	
c. How do you depopulate your dogs?		
	Puppies	Adult
Give out	51.5	46.7
Sale	42.6	30.6
Disappear/stray away	5.9	1.5
Die	0	20.4
Automobile accident	0	0.7
d. Do you allow dogs stray into your compound?		
No	66.2	
Yes	33.8	

Key: % = percentage

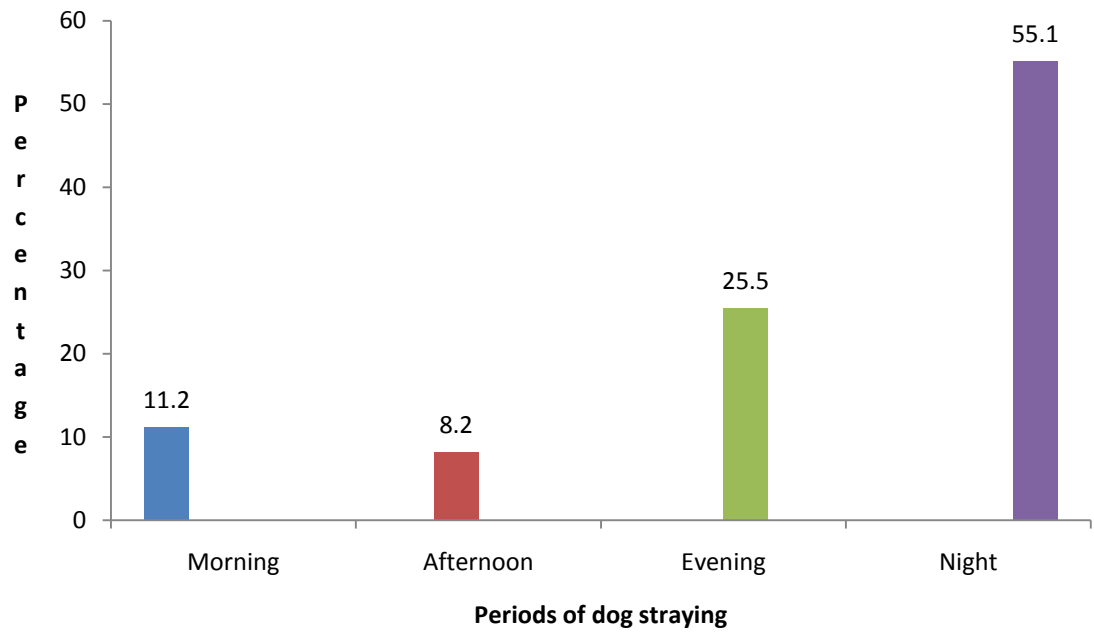


Figure 4.3: Periods in which dogs strayed into houses in Niger State, Nigeria.

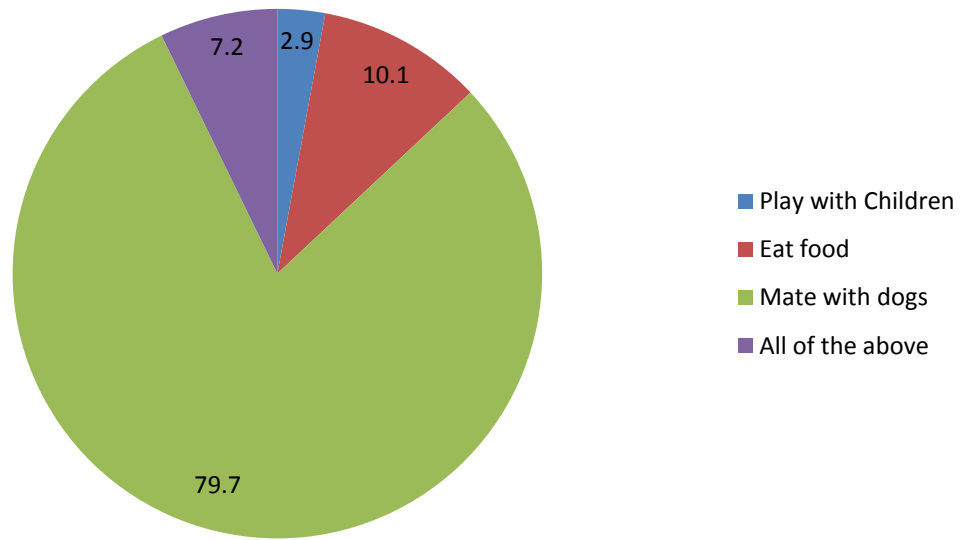


Figure 4.4: Activities of dogs when strayed in to houses in Niger State, Nigeria.

It was equally observed that some of the dogs (26.9%) were confined or are partially (25%) confined (Table 4.3). Furthermore, responsibility for dogs, mostly (61.5%) laid with everybody in the house/compound and 60.1% of the dog feed were from the family left over (Table 4.3). Although about 50% of the dogs received anti-rabies vaccine regularly; yet over 42% of the dogs received vaccines only once in their life time (Table 4.3).

Similarly, the preferred vaccine/regimen by respondents is injectable vaccine (69.7%) than the oral (30.3%) even if available. For those that do not vaccinate their dogs against rabies, majority (38.9%) claimed the vaccine was expensive, yet over 34% have no idea that anti-rabies vaccine was available or exist (Figure 4.5). It was noted that treatment of dogs when sick are mostly taken to the hospital (59%) for attention, however, about 30.4% and 10.6% are treated at home with herbs or left on their own respectively in the study area (Figure 4.6).

It was shown that 31% of the respondents or their family members have been inflicted by dog bite; which were mostly caused by households dogs (42.3%) or dogs from neighborhood (29.6%) (Table 4.4). However, 24.2% proportion of biting dogs seemed to be mad/sick at the time of bite, but majority (59%) of biting dogs, were assumed to be healthy by the respondents (Table 4.4). Unfortunately, about 40% of dog bite victims received only local wound dressing and 28.1% received post exposure prophylaxis (PEP), others were only treated in a traditional ways (Table 4.4). Consequently, 13.8% of the dog bite victims were reported to have died but 78.8% were reported to remain healthy (Table 4.4).

Table 4.3: Management and care of dogs in Niger State, Nigeria.

Parameters		Scores
		%
a.	Confinement of dogs	
	Never	26.9
	Partial	25
	Always	48.1
b.	Who takes care of dog?	
	Father	18.6
	Mother	6.4
	Children	13.5
	Everybody	61.5
c.	How are dogs fed?	
	Family left over	60.1
	Cook special pot	38.5
	Allow to scavenge	1.4
d.	Do you vaccinate dogs?	
	No	30.4
	Yes	69.6
e.	How often do you vaccinate?	
	Once in life time	42.9
	Twice in life time	8
	Regular annually	49.1

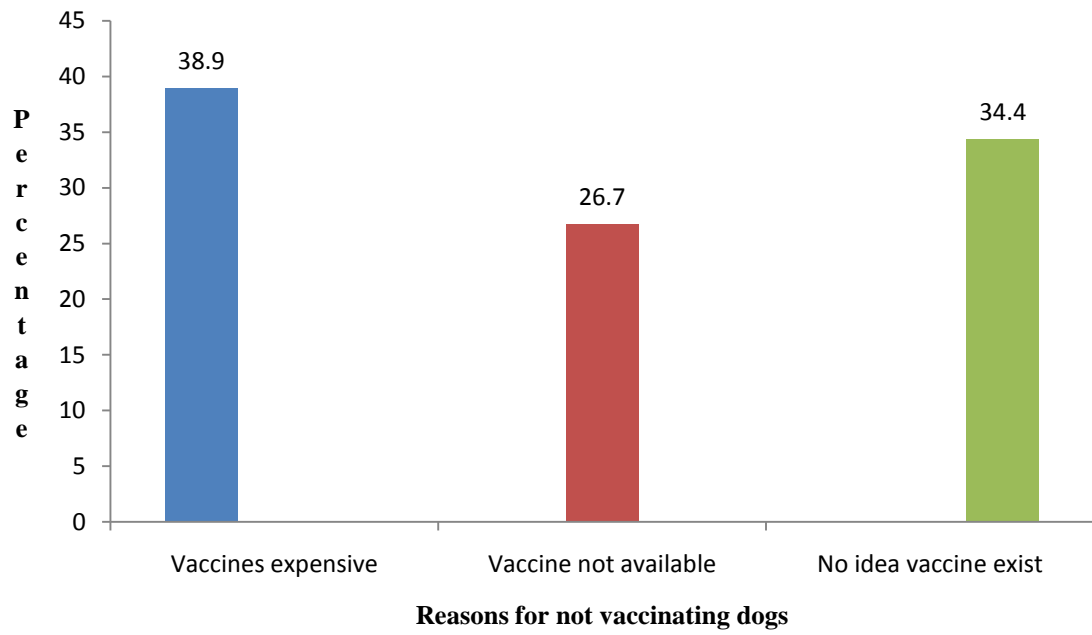


Figure 4.5: Some reasons amongst those who do not vaccinate their dogs against rabies in Niger State, Nigeria.

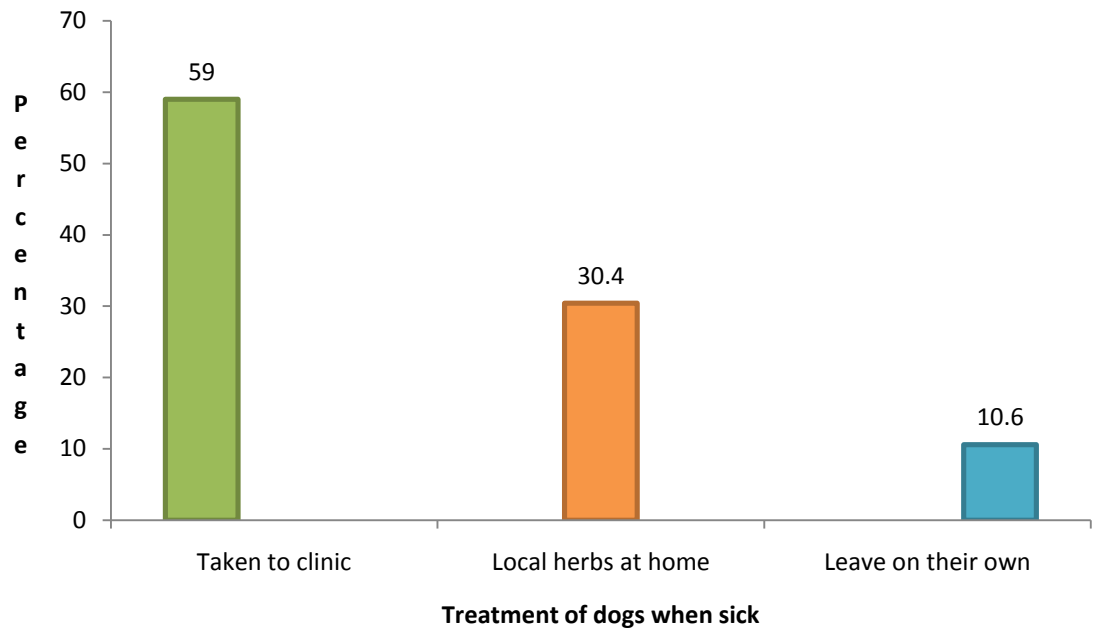


Figure 4.6: Mode of treatment for sick dogs in Niger State, Nigeria.

Table 4.4: Dog bite cases, management and sequela, in Niger State, Nigeria.

Parameter	Score	
	%	
A	Has any family member been bitten by dog?	
	No	69
	Yes	31
b.	If yes, who own the dog?	
	Households	42.3
	Neighbors	29.6
	Stray dog	28.1
c.	Health status of the dog?	
	Healthy	56.8
	Mad/sick	24.2
	Nursing bitch	19
d.	Treatment to the victim	
	Local wound dressing	39.3
	Post – exposure prophylaxis	28.1
	Traditional methods	32.6
e.	What happens to the victim?	
	Died later	13.8
	Remain healthy	78.8
	I do not know	7.4

Key: % = percentage

Furthermore, about 61% of biting dogs remained healthy, but about 33.7% of the offending dogs got killed instantly following the bite (Figure 4.7). Also, the most used traditional method in treating dog bite victim is cooking and feeding the victim with cooked part (viscerals or flesh) of the dog (in 50%) of the cases, then to some extent concoctions or incantations (Table 4.5). About 23.1% of respondents in the study area still believed that rabies is caused by evil spirit (Table 4.5).

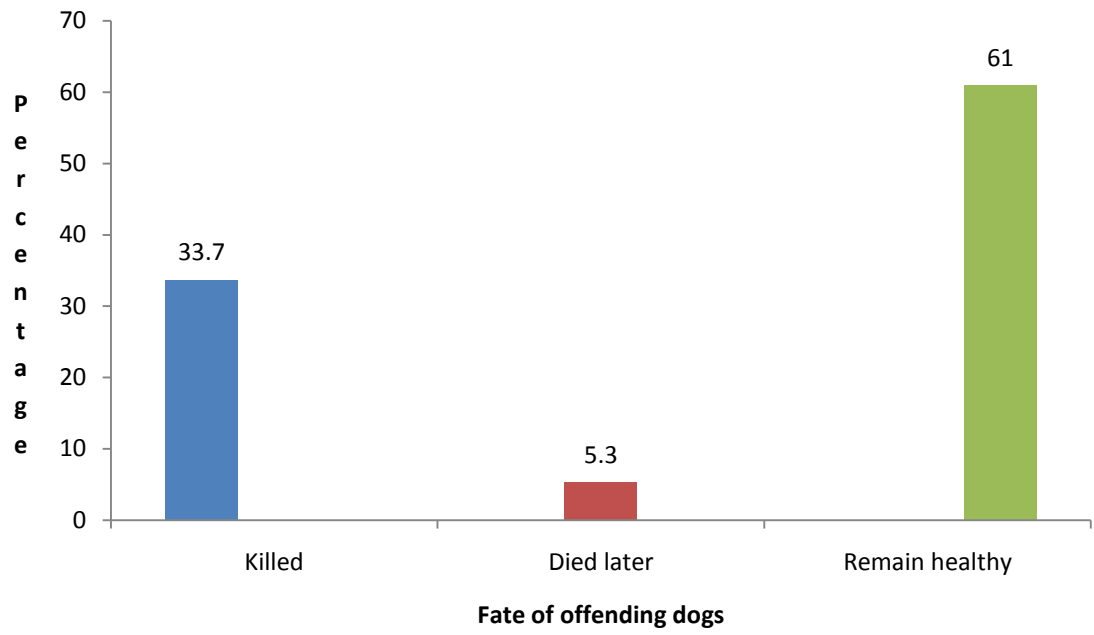


Figure 4.7: Fate of a biting dog in Niger State, Nigeria.

Table 4.5: Traditional concepts on rabies in Niger State, Nigeria.

Variable	Frequency (%)
a. Types of traditional treatments	
Concoction	31.3
Cooking and consuming of dog parts	50
Incantations	18.7
b. Do you believe in Traditional treatments	
No	65.4
Yes	34.6
c. What causes rabies?	
Infectious agent	69.6
Evil spirit	23.2
Hunger	7.7

4.2 Hospital Records of Dog Bite Cases and Rabies Diagnosis from Niger State at National Veterinary Research Institute (NVRI), Vom

Available hospital records of human dog bite cases shows that a total of 223 cases were recorded amongst the human hospitals visited in Niger State during the period January, 2006 and July, 2013. The highest number (40 cases) was recorded in the year 2012 and the least (10 cases) in 2013 (Table 4.6). General Hospital Tungan Magajiya had the highest total cases of 114 followed by General Hospital Minna with 62 cases and the least cases of 2 each were recorded in Bida and Wushishi General Hospitals. No record of dog bite cases were seen in Ibrahim Badamasi Babangida (IBB) specialist hospital and Federal Medical Center (FMC) assumed to be the tertiary hospitals in the State (Table 4.6).

Furthermore, the monthly distribution of cases suggested that more dog bite victims were received in the hospitals in the months of January and April during the reported period with 22 and 24 cases each. This was followed by 21 cases in the months of October, while the least cases were seen in the months of November and December with 14 and 11 cases each respectively (Table 4.7). The seasonal variation (trend) shows that dog bite cases occurred throughout the year but with the highest indices seen in the months of May, March and January of the year and the lowest rates seen in December and November in Niger State (Figure 4.8).

Sex distribution amongst victims of dog bite in Niger State indicated that majority of victims (66.8%) were males during the reporting period (Table 4.8). Similarly, the age distribution of victims suggests that about 46.3% of dog bite victims were children below the age of fifteen years (Table 4.8).

It was also observed that majority (81.2%) of the victims of dog bite were bitten on the legs, followed by hand (14.3%) while the least sites of bite was face and multiple site of the body seen in 2.25% each of the victims (Figure 4.9).

On the record of animal brain samples submitted to NVRI, Vom for rabies diagnosis over the same period of dog bite cases (January 2006 – July, 2013) from Niger State. This study showed that a total of 1976 animal samples were submitted for rabies diagnosis from all over Nigeria. A total of 1098 (55.6%) were confirmed positive for rabies but no sample was submitted from Niger State during the period (Table 4.9).

Table 4.6: Annual distribution of dog bite cases in Niger State, Nigeria January, 2006 – July, 2013.

Year	2006	2007	2008	2009	2010	2011	2012	2013	Total
Hospital									
T/Magajiya	15	16	14	16	15	19	17	2	114
Kontagora	0	2	0	1	2	3	5	1	14
Minna	5	11	7	9	9	6	10	5	62
Bida	0	0	0	2	0	0	0	0	2
Wushishi	0	0	0	0	0	0	1	1	2
Suleja	2	3	1	2	1	1	1	0	11
Kagara	0	1	3	1	2	0	5	1	13
Makwa	0	1	1	0	1	1	1	0	5
IBB	NR	NR	NR	NR	NR	NR	NR	NR	NR
FMC	NR	NR	NR	NR	NR	NR	NR	NR	NR
Zungeru	NR	NR	NR	NR	NR	NR	NR	NR	NR
Total	22	34	26	31	30	30	40	10	223

Key; IBB = Ibrahim Badamasi Babangida Specialist, FMC = Federal Medical Center, NR = No record

**Table 4.7: Monthly distribution of dog bite cases in Niger State, Nigeria
January, 2006 to July, 2013**

Year	2006	2007	2008	2009	2010	2011	2012	2013	Total
Month									
January	1	5	1	4	5	1	5	0	22
February	2	3	3	3	2	1	2	1	17
March	0	1	3	2	3	3	5	3	20
April	4	4	3	3	4	2	4	0	24
May	1	1	4	3	2	5	5	2	21
June	1	3	0	3	2	4	5	0	18
July	3	0	5	0	2	1	4	3	19
August	2	3	2	4	3	3	2	0	19
September	2	2	1	2	3	3	4	0	17
October	3	4	2	2	2	6	2	0	21
November	1	4	1	2	2	2	2	0	14
December	1	4	1	3	0	1	1	0	11
Total	22	34	26	31	30	30	41	9	223

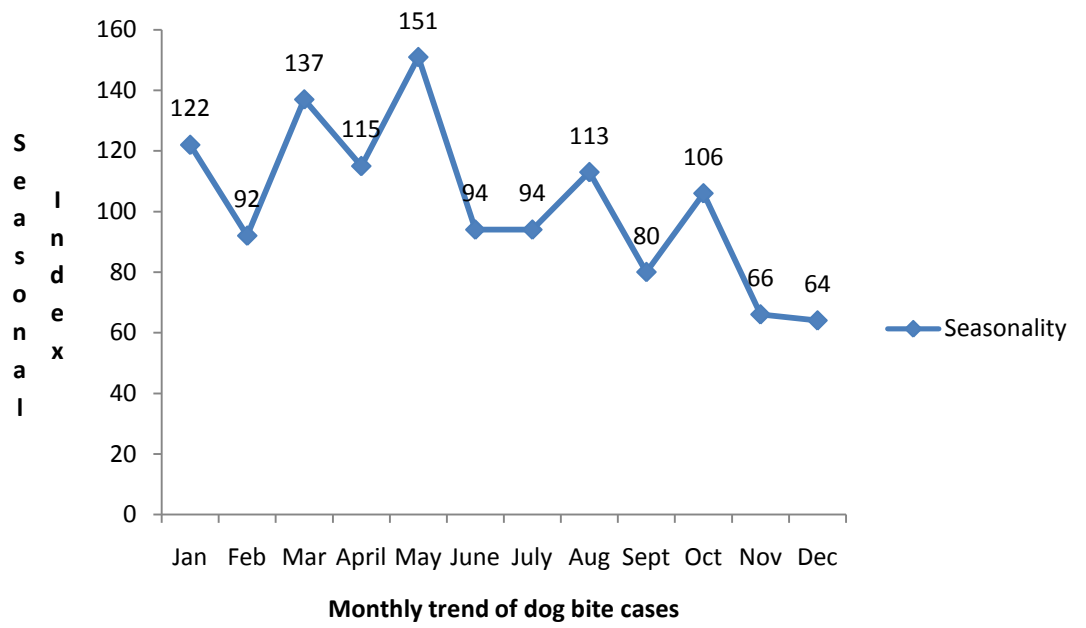


Figure 4.8: Seasonal trend of dog bite cases on humans (2006- 2013) in Niger State, Nigeria.

Table 4.8: Sex and age distribution of dog bite victims in Niger State, Nigeria, January, 2006 – July, 2013

Variable	Frequency	Specific rate
<u>Sex</u>		
Male	149	66.8%
Female	74	33.2%
Total	223	100%
<u>Age</u>		
< 15 year old	103	46.2%
> 15 year old	120	53.8%
Total	223	100%

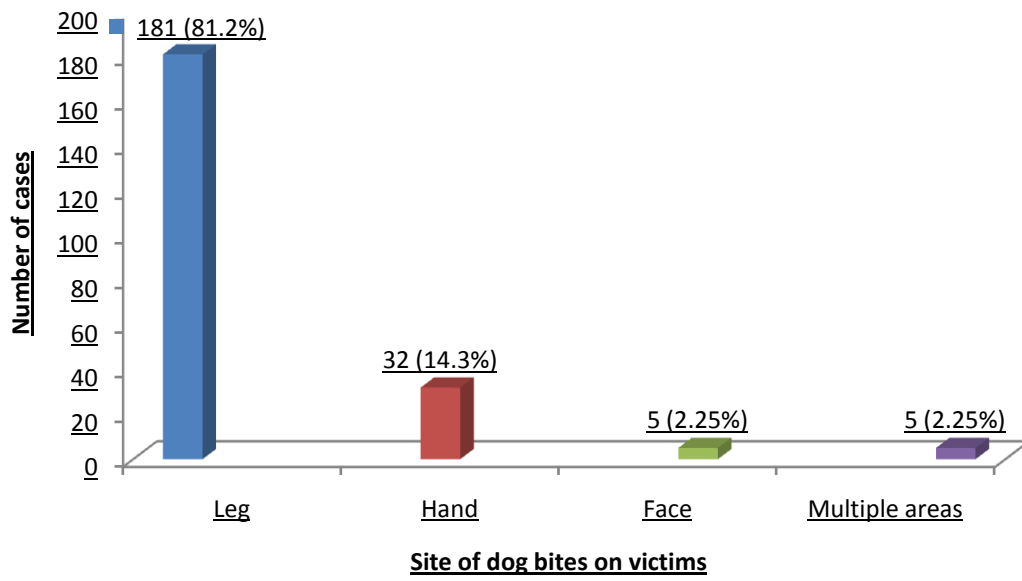


Figure 4.9: Site of bites on dog bite victims in Niger State, Nigeria.

Table 4.9: Animal samples submitted to NVRI, Vom across Nigeria for rabies confirmatory diagnosis January, 2006 – July, 2013

Samples	Samples submitted	Samples positive	Samples submitted
Year	across Nigeria	(%)	from Niger State
2006	200	93 (46.5)	None
2007	133	55 (41.4)	None
2008	242	169 (69.8)	None
2009	427	300 (70.3)	None
2010	302	179 (59.3)	None
2011	344	150 (43.6)	None
2012	226	116 (57.3)	None
2013	102	36 (35.3)	None
Total	1976	1098 (55.6)	None

4.3 Assessment of Dog Slaughter and Rational for Dog Meat Consumption in Niger State

Available records of dogs slaughtered between the months of January and July 2012 across five slaughter points in Niger State indicated that a total of 471 dogs were slaughtered. Of this number, 53.4% were male dogs and about 99.6% (469 dogs) are native breed. Also, 261 (55.4%) of dog slaughtered were under the age of one year, while only 13.4% were above the age of five years (Table 4.10).

Assessing the demographic information of dog meat consumers, it appears that the consumption of dog meat cut across all or many occupational groups. Of the one hundred and twenty five respondents that consume dog meat, 51 (40.8%) were civil servants, while 9.6% (12 out of 125) engaged in the actual dog meat butchering sourcing their dogs for slaughter from households within and outside their territories (Table 4.11).

Available record indicates that 91% (11 out of 12) of the dog butchers have secondary school education only one had primary education and that 66.7% of the butchers were married.

A maximum of 6 dogs and a minimum of 1 dog were found to be slaughtered daily per butcher. However, majority of butchers (41.7%) reported the slaughter of 2 dogs per day in Niger State during the reporting period (Figure 4.10).

Dogs were being slaughtered and consumed by individuals for different reasons. The enquiry for the rational for dog meat consumption in Niger State revealed that majority (64%) consumed the meat because it was delicious. Others consumed the meat for medicinal purposes and few felt it was a cheap source of protein, yet 0.8% believed it protects against the witches (Figure 4.11).

Table 4.10: Sex, breed and age distribution of dogs slaughtered in Niger State, Nigeria January – July, 2012.

Variable	Frequency	Specific rate
<u>Sex</u>		
Male	252	53.5%
Female	219	46.5%
Total	471	100%
<u>Breed</u>		
Local	469	99.6%
Exotic	2	0.4%
Total	471	100%
<u>Age</u>		
<1 year old	261	55.4%
1 – 5 year old	147	31.2%
> 5 year old	63	13.4%
Total	471	100%

Table 4.11: Summary of demographic information of dog meat consumers in Niger State, Nigeria.

Occupation	Number of dog meat consumers (%)	Vaccination against Rabies	Source of dog for slaughter
Civil Servants	51(40.8)	^{φφ} Never	*Only consume
Business	24(19.2)	^φ Never	*Only consume
Students	22(17.6)	Never	*Only consume
Farmers	7(5.6)	Never	*Only consume
Other Professionals	6(4.8)	Never	*Only consume
Hunters	3(2.4)	Never	*Only consume
Dog butchers	12 (9.6)	Never	**Households
Total	125(100)	Never	

Key: ^{φφ} = one respondent bitten by dog and vaccinated 21 years earlier, the other bitten but not vaccinated 8 year earlier

^φ = one respondent bitten by dog and vaccinated 19 years earlier

* = do not slaughter dogs, ** = slaughter and consume dog meat

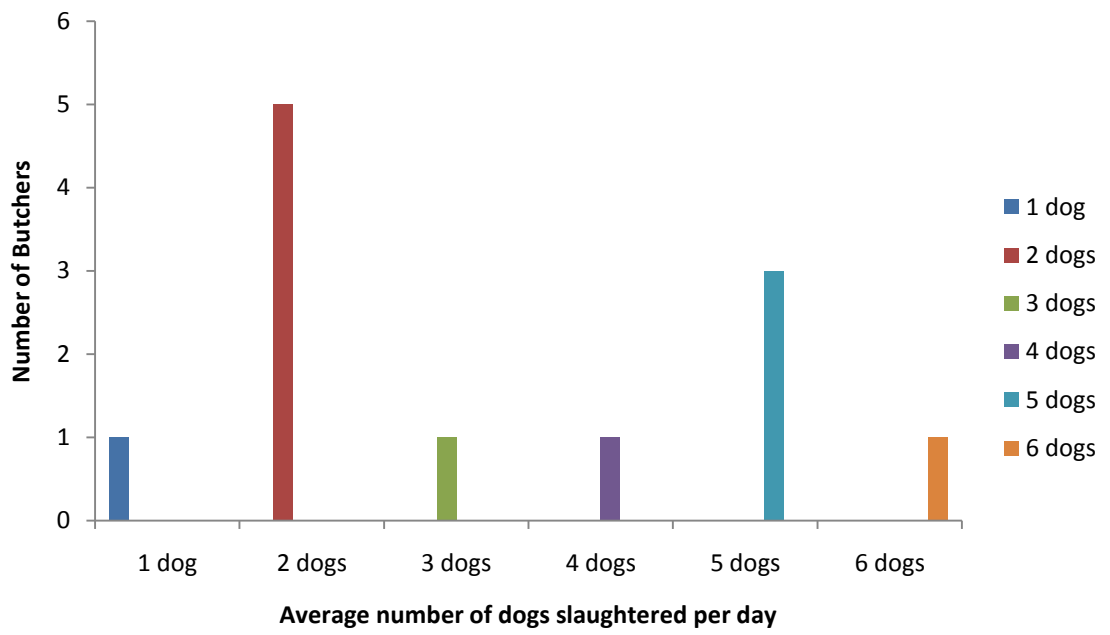


Figure 4.10: Average number of dogs slaughtered by dog butchers per day in Niger State, Nigeria.

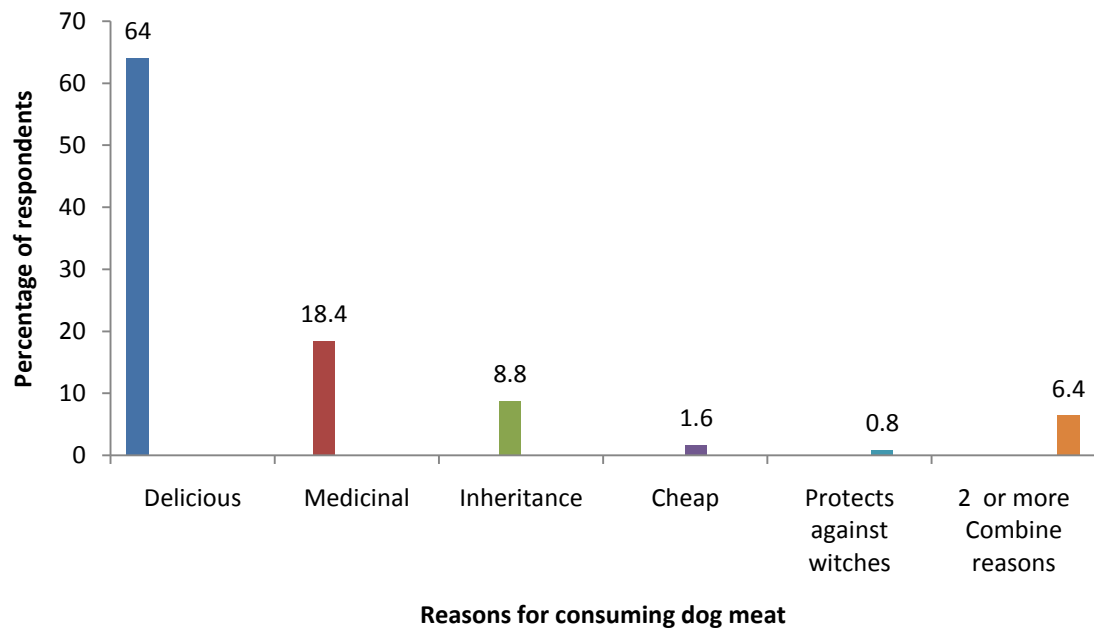


Figure 4.11: Reasons for dog meat consumption in Niger State, Nigeria.

4.4 Laboratory Confirmation of Rabies Antigen in Dog Brains by FAT, DRIT and RT –PCR

Of the 471 dog brain samples collected from slaughtered dogs in Niger State, only 3 (0.64%) were positive for rabies antigen using the DFA, DRIT and RT – PCR (Table 4.12). with high intensity (4+) and distribution (4+) of viral load in the samples (Plate I). High intensity (4+) and distribution (4+) of rabies viral antigen were observed by the used of direct fluorescent antibody test (FAT) and the DRIT in the study.

Of the 13 dog brain samples that undergone RT-PCR then electrophoresis only 3 samples (252, 276 & 471) that were DFA and DRIT positive that also showed positive bands of rabies viral amplicons at 921F- 304B region (Plate II). Following sequencing of the viral amplicons, Pair wise comparison of those three rabies positive samples indicated that the 3 samples are 96 – 97% correlated to each other (Table 4.13). Similarly, when these 3 positive dog brain samples were subjected to blast best matches with other isolates from Africa, all of them had 98 – 99% identity with those found in Niger, Chad and Burkina Faso republics (Table 4.14).

Phylogenetic tree constructed, shows that sample D471 (collected from Suleja town of Niger State, denoted ‘471 Nigeria, 2012’) falls within Nigeria 1 cluster. Sample D252 (collected from Minna town of Niger State, denoted ‘252 Nigeria, 2012’) falls within clusters previously reported from Niger Republic since 1990. While sample D276 (collected from Bida town of Niger State, denoted ‘276 Nigeria, 2012’) falls closely to Nigeria 2 cluster previously reported between 2005 – 2007 and Chad 2 cluster reported in 2006, but this sample (D276) forms a new lineage (Figure 4.12) Considering five slaughter points where samples were collected from 4 major towns of Niger State; only in 3 locations were positive samples seen/confirmed and all the samples were from local dogs (Table 4.15).

Table 4.12: Brain Samples from slaughtered dogs diagnosed positive for rabies antigen from Niger State, Nigeria

Diagnostic Methods	Total No. of Samples	No. Positive	%age Positive	Samples Positive	Intensity/distribution
DFA	471	3	0.64%	D252, D276, D471	4+/4+
DRIT	471	3	0.64%	D252, D276, D471	4+/4+ “
RT-PCR	13	3	23.2 %	D252, D276, D471	4+/4+ “

Kappa coefficient between tests = 1 (absolute agreement)

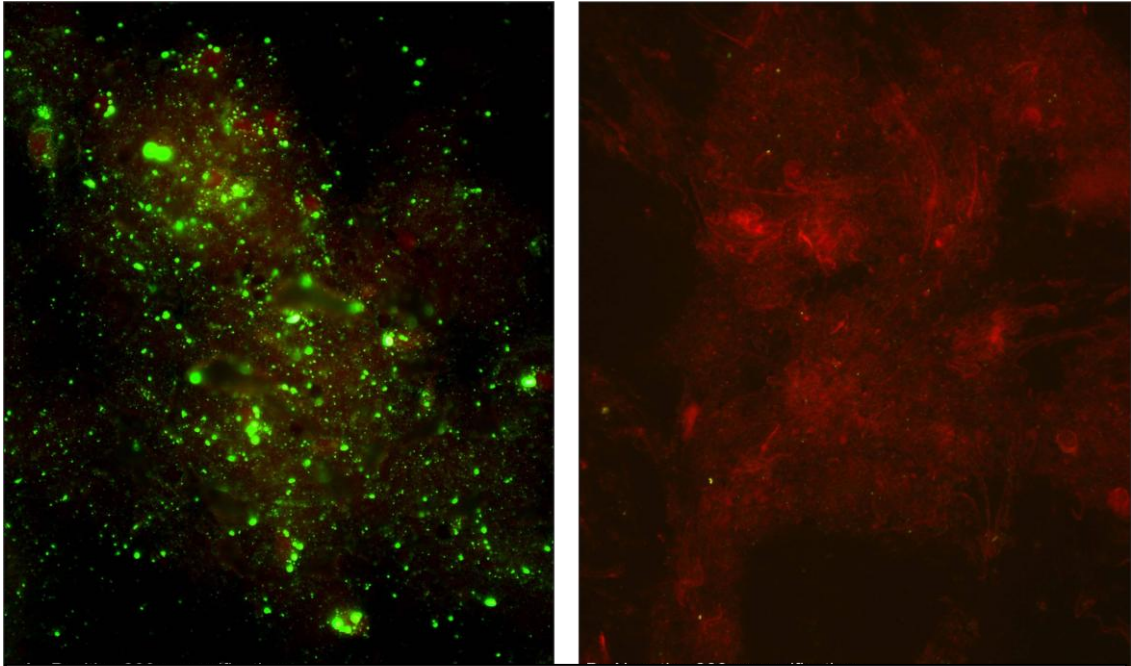


PLATE I: The direct fluorescent antibody (DFA) test slide on brain sample no. 276 touch impression A= Positive, B= Negative

A. Negative
Red background: even with illumination

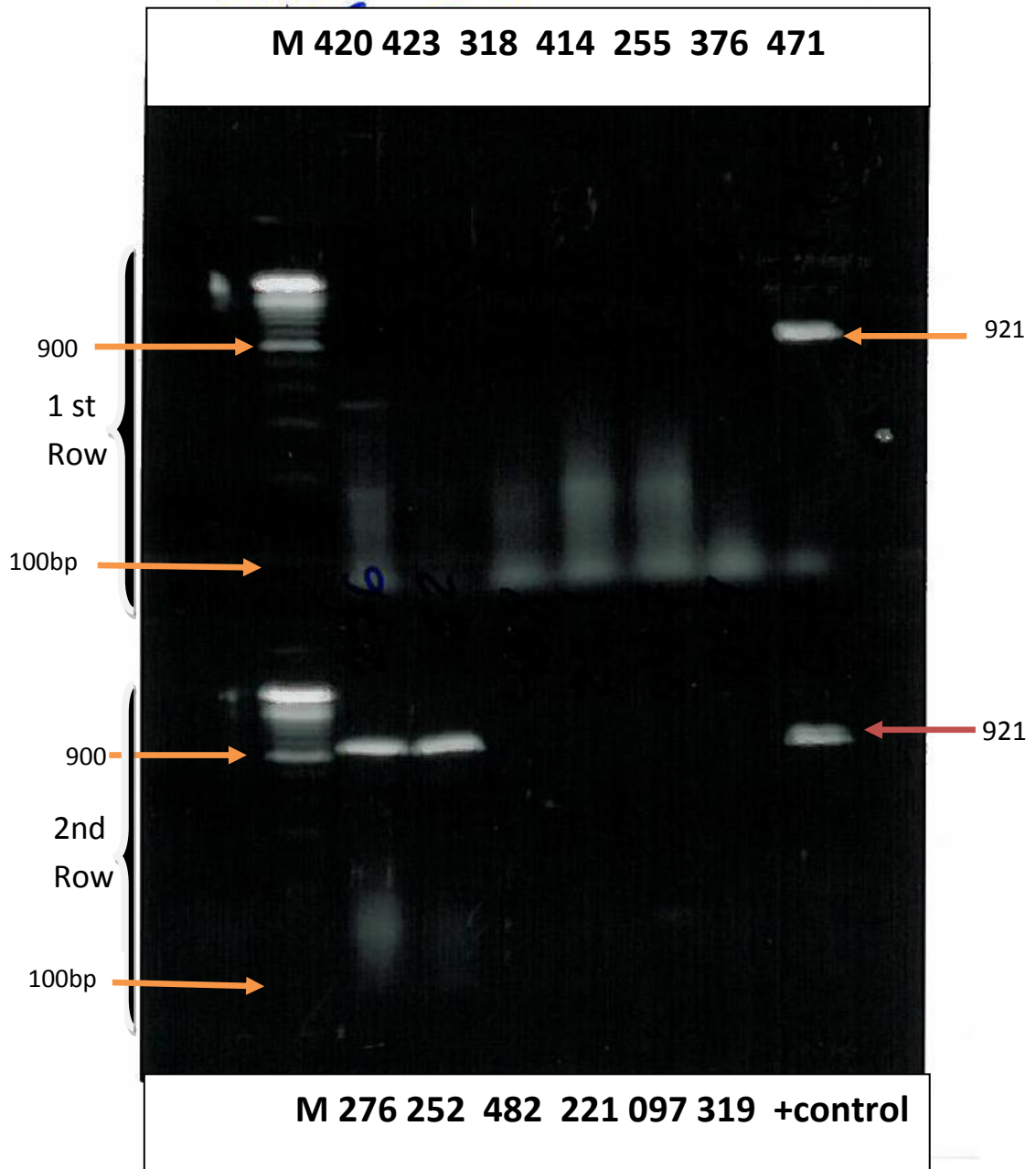


PLATE II: Bands of positivity (seen in samples lane 471 on 1st row and 276, 252, & +control on 2nd row) of RT-PCR products following agar gel electrophoresis, lane M= Molecular weight maker 100 bp ladder, other lanes are negative samples.

Table 4.13: Pair wise comparisons of positive samples within Niger State.

Sample comparison	Pair wise identity values
252 with 471	96.6%
276 with 252	96.2%
471 with 276	97.2%

Table 4.14: BLAST best matches of positive samples with the rest of Africa.

Sample ID	GenBank Accession no.	Blast comparison with Africa
252 NigeriaMinna 2012	KR080521	99% with Niger and Burkina Faso
276 NigeriaBida 2012	KR080522	98% with Chad, Niger republic
471 NigeriaSuleja 2012	KR080523	99% with Chad

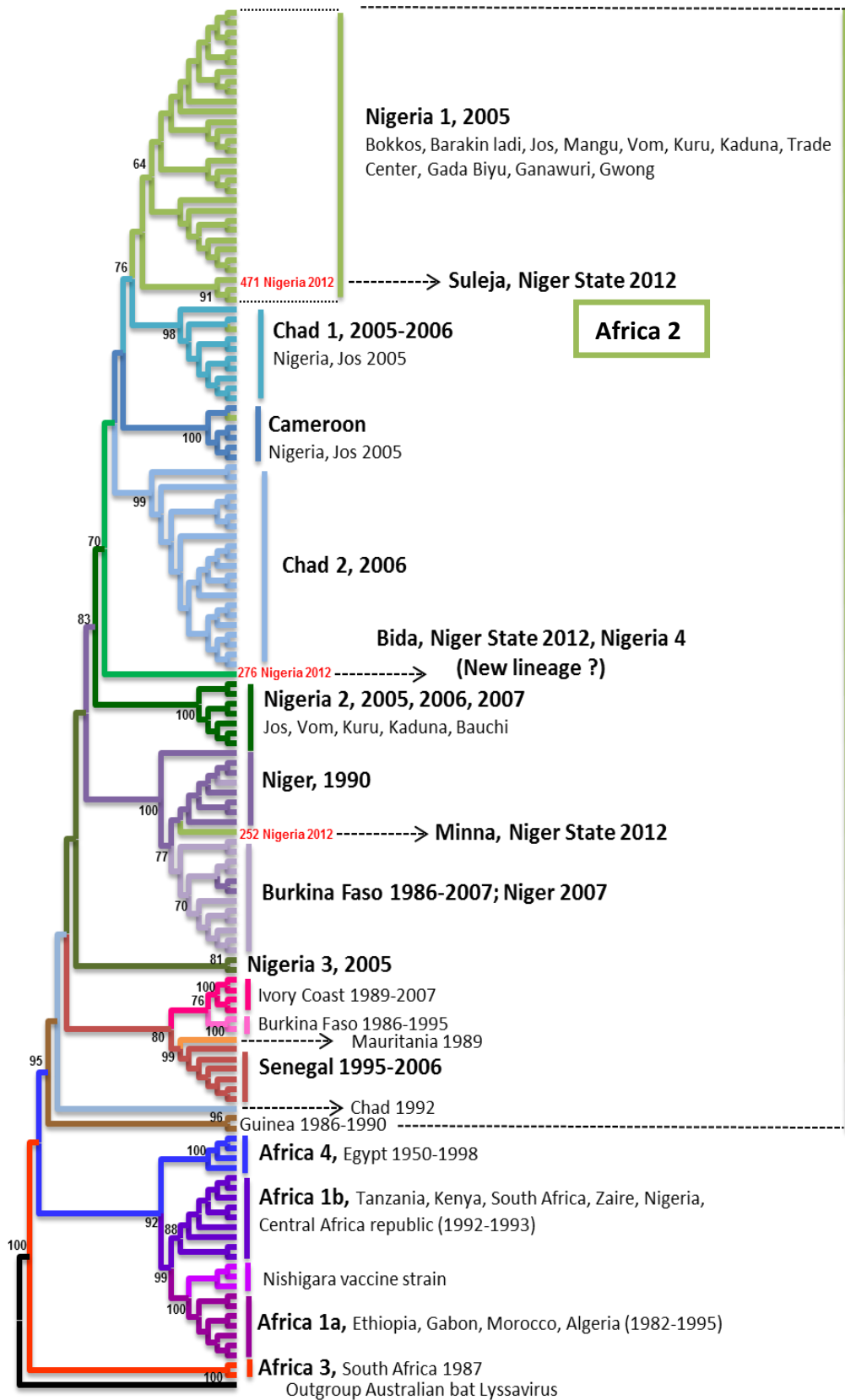


Figure 4.12: Phylogenetic tree of 3 positive samples with the rest of Africa

Table 4.15: Geographic locations of dog brain samples from Niger State, Nigeria

Town	Latitude	Longitude	Total dog samples collected	Dog samples positive (ID)
Bida A (Sachi)	9.066667	6.138611	85	0
Bida(Army barracks)	9.066944	6.042222	68	1 (252)Ma
Kontagora	10.07111	5.138611	26	0
Minna	9.133889	6.138611	158	1 (276)Fb
Suleja	9.108611	7.17	134	1 (471)Mb
Total	Niger	State	471	3

Key: ID = identification, M = male, F = female, a = < 1 year old, b = > 1< 5 year old

4.5 Analysis of Bat Brain and Serum Samples

Of the 267 brain samples from bats, none was positive for rabies antigen by the FAT employed.

However, of the 162 bat sera collected, 154 were checked for the presence of antibody to Duvenhage virus and only 3 (1.95%) showed detectable antibody (Table 4.5.1). All the 3 positive samples were *Epomorphorus gambiense* (fruit eating) bats which constituted over 95% of the total bat species captured/collected.(Table 4.16)

Geographic distribution of samples indicated that most bats 126 (47.3%) were collected at Rijiyang Nagwamatse and the least number 5 at Madangen (Table 4.17).

Table 4.16: Bat species with detectable antibodies against Duvnhage virus by Modified RFFIT test.

Species	No. Bats captured	Serum collected	Serum Not tested	No. Cyto-toxic	No. tested	No. Positive (%)
<i>E. gambiense</i>	254	151	11	2	148	3
<i>M. pusali</i>	13	11	5	0	6	0
Total	267	162	17	2	154	3 (1.95)

Table 4.17: Geographic distribution of bat's samples collected and analyzed for rabies antigen (FAT) and Duvenhage virus (modified RFFIT) in Niger State, Nigeria.

Location	Latitude	Longitude	Bats captured (No.)	spp	FAT +ve	RFFIT DUV +ve	ID^{SEX}
Kutigi	9.105833	6.100278	<i>E. gambiense</i>		0	1	B095 ^M
R/Nagwam atse	10.0427777	5.071667	<i>E. gambiense</i> (113) <i>M. pusali</i> (13)		0	1	B072 ^M
Samata	10.0941667	5.071667	<i>E. gambiense</i> (58)		0	0	0
Madangen	10.1730556	6.100278	<i>E. gambiense</i> (5)		0	0	0
Kontagora	10.0711111	5.138611	<i>E. gambiense</i> (33)		0	1	B244 ^M
Total	Niger	State	267		0	3	3

Key: M = male, ID = sample identification number

4.6 Rabies Virus Neutralizing Antibodies in Human Sera

Analysis of the human sera with standard RFFIT indicated that of the 155 sera from dog butchers, dog meat consumers, hunters and pet owners, only 111 were good for analysis and 19 (17.1%) samples had detectable rabies virus neutralizing antibody (Table 4.18). All the 155 participants were not previously vaccinated against rabies except two pet-owners who reported that they were bitten by dogs and vaccinated about 20 and 8 years prior to sampling; the later was amongst the positives. Between, 14.8 to 25% of each volunteer group had detectable rabies antibodies (Table 4.18).

Of the 30 serum samples collected from thirty veterinarians/animal health assistants, only 4 (13.4%) had detectable rabies antibodies (rVNA). One Veterinarian and one animal health worker claimed to have received human anti-rabies vaccines ten years earlier (Table 4.19). Feed back and follow up questioning, however, revealed that the second veterinarian was equally vaccinated since 1995 during clinical year of training, while the later animal health worker confessed to have been bitten by a dog and was vaccinated in 2011 following the bite.

On the overall, 16.4% (23 out of 140) of the human serum samples screened had detectable rabies virus neutralizing antibodies (Table 4.20). Going by the antibody titres of the 23 rabies positive human volunteers, it appears only 6 members had minimum protective titre, between 0.5 to 5.4 IU/ml and 3 of the 6 were previously vaccinated while 3 were never vaccinated. Other 17 members had titres below the protective level (Table 4.21).

Table 4.18: RFFIT rabies antibody detection on dog butchers and others in Niger State, Nigeria.

Volunteers	Total number collected	Cyto- toxic	Negative	Positive (%)
Butchers	12	4	6	2 (25)
Dog meat consumers	113	32	69	12 (14.8)
Pet owners	24	8	12	4 (25)
Hunters	6	0	5	1 (17)
Total	155	44	92	19 (17.1)

Table 4.19 RFFIT rabies antibody detection on Veterinarians & Animal health assistants in Niger State, Nigeria.

Volunteers	Total no. collected	Cyto-toxic	Negative	Positive (%)
Veterinarians	6	0	4	2 (33.3)
Animal Health Assistants	24	1	21	2 (8.7)
Total	30	1	25	4 (13.8)

Table 4.20: RFFIT rabies antibody detection for all human volunteers in Niger State, Nigeria.

Volunteers	Total	Cyto-toxic	Number	Negative	Positive
	number		Tested		
	collected				
Vet/Anim. Hlth	30	1	29	25	4 (13.8)
Assistants					
Dog	155	44	111	92	19 (17.1)
butchers/Others					
Total	185	45	140	117	23 (16.4)

Table 4.21: Rabies antibody titre for all the RFFIT positive human volunteers in Niger State, Nigeria.

S/no.	Sample ID.	Group	Sample date	Titre	IU/ml	Location of volunteer
1.	001H	DB	26 May 2012	5	0.05	Bida
2.	007H	DC	26 May 2012	7	0.07	Bida
3.	019H	DC	26 May 2012	11	0.11	Bida
4	020H	DC	26 May 2012	11	0.11	Bida
5.	031H	DC	27 May 2012	65	0.65	Minna
6.	032H	DB	27 May 2012	65	0.65	Minna
7.	040H	PO	28 April 2012	8	0.08	Minna
8.	045H	DC	28 April 2012	13	0.13	Minna
9.	048H	DC	24 May 2012	42	0.42	Bida
10.	049H	PO	24 May 2012	11	0.11	Bida
11.	073H	DC	24 May 2012	29	0.29	Bida
12.	076H	DC	24 May 2012	11	0.11	Bida
13.	092H	DC	31 May 2012	13	0.13	Minna
14.	096H	PO**	1 June 2012	7	0.07	Minna
15.	108H	DC	1 June 2012	11	0.11	Bida
16	111H	HT	2 June 2012	13	0.13	Bida
17.	114H	DC	2 June 2012	7	0.07	Bida
18.	127H	PO	5 June 2012	13	0.13	Minna
19.	139H	DC	22 June 2012	70	0.7	Bida
20.	167H	AH**	20 July 2012	50	0.5	Minna
21.	177H	VET*	20 July 2012	50	0.5	Minna
22.	181H	AH*	20 July 2012	17	0.17	Minna
23.	182H	VET*	20 July 2012	540	5.4	Suleja

Key: DB = Dog butcher, DC = Dog consumer, PO = Pet owner, HT = hunter, AH = Animal health personnel, VET = Veterinarian, * = Vaccinated, ** = Bitten & Vaccinated, H = Human sera. Reference serum titre used was 200.

CHAPTER FIVE

DISCUSSION

It was shown both from the questionnaire survey and laboratory results that dog bite and rabies are present in Niger State of Nigeria.. It has been observed from the distribution of respondents on the questionnaire survey on dog ecology and management that there was an approximately equal number of forms returned from the selected towns (Bida, Kontagora, Minna and Suleja towns) strategically located and representing the three agricultural zones of the state. This goes to suggest that the outcomes of this survey may represent the view from cultural set ups in the state.

It has been shown by the results of this study on dog ecology that there is a dog to human ratio of 1:5.4 in Niger State. This suggests that there is an increase in the activity of dog keeping in the recent years compared to the 1990s, when the dog population was estimated at 199,812 (RIM, 1992) and the human population was 2,482,367 (NPC, 1991) bringing the dog to human ratio at 1:12.4 then. This suggests that there is increased density of the dog population in Niger State. The implication of this finding is that there is, or will be close interactions between dogs and humans and thereby increased risk of rabies virus antigen spread in an event of rabies outbreak in the population. The increase of dog keeping and hence closer interaction in the recent years may be as a result of increased insecurity in Nigeria in terms of theft and arm robbery necessitating keeping of dogs.

Our observed dog to human ratio of 1:5.4 in the present report is similar to that reported in Lagos State with 1:5.6 ratio (Hambolu *et al.*, 2014) but differs significantly with that reported in Abia State having a dog to human ratio of 1 : 7.8 (Otolorin *et al.*, 2014).

These differences may be due to cultural, social and insecurity situation of each state or human set up which varies in some ways. On the average, a dog to human ratio was reported to be 1:21.2 in urban and 1:7.4 in rural Africa (Knobel *et al.*, 2005). A ratio as high as a 1:45 dog to human has been reported in urban Zambia (De Balogh *et al.*, 1993) and as low as 1:3.2 in a rural area of Borno State, Nigeria (El-Yuguda *et al.*, 2007) suggesting a Geo-cultural differences or influences related to dog ecology.

It has also been observed that for every two male dogs there is a female dog and over 75% of dogs are below the age of 5 years. This male to female dog ratio of 2:1 in Niger State is contrary to that reported in Lagos State which was 1:1.5 male: females (Hambolu *et al.*, 2014). The implication of this finding is that the humans in Niger State may stand a higher risk of contracting rabies or dog bite due to the higher number of male dogs. This is because male dogs have been implicated of having more chances of contracting rabies than the females in a study in Plateau State (Garba *et al.*, 2005). Furthermore, the ratio that for every one house there is 1.8 dogs may suggest a close interaction of dogs to humans in the study area and may enhance dog bite or even rabies spread. Having over 58% of dogs being kept for security reasons with only 8.6% kept as pets may suggest an increasing insecurity in the study area.

It has also been shown from the results of this study that about 67% of the respondents like keeping dogs, mostly (90%) acquired through purchase or given by friends. Despite that, some dogs are owned by straying of the dogs or puppies into homes. This suggests that dog straying is a phenomenon in the state, exhibited by the presence of dogs straying in to homes/houses, mostly in the night (seen in 55.1%) and evenings (25.5%) with a lower period of straying seen in the afternoon (8.2%). The possible reasons for higher dog stray in the periods of nights and evenings may be due to

perceived safety on quietness of the periods by the dogs in which they are not seen in the night when it is dark or quietness of the evening period when populace leave homes for outdoor activities (games, roadside viewing). This is further supported by responses that when dogs, stray into houses they mostly (79.1%) mate with other dogs and to some extents search for food (10.4%) remnant. This suggests that these two factors (mating and looking for food) are the main ingredients for straying, thus confinement by the provision of food and shelter are the major remedies to dog straying. However, despite the low percentage (2.9%) of dogs that play with the children when stray into the house, it is a serious public health threat especially when the straying dog is rabid. Hence, this factor (straying or free-roaming of dogs) therefore necessitates a public health awareness campaign. These findings suggest that most of the dogs are owned dogs but free-roaming and permanent straying of ownerless dog is very limited.

Furthermore, about half the respondents (48%) claimed to always confine their dogs, and only 26.7% never cared to confine their dogs. This goes to show that a lot of the dogs, though owned are free-roaming in the community. The consequence of this finding is the continuous interaction of dogs with humans (and other animals) which could disseminate rabies virus amongst the populace (human and animal) after a primary exposure. Another practical issue is that the responsibility for dogs in terms of care and welfare issues at homes (61.5%) is on the shoulder of everybody in the household. What this finding suggests is that no one is dedicated to cater for the dog, hence everyone assumes that the other members will take care of the dog thus they decline. Consequently, dogs are left un-catered for and may roam freely in search of food. In addition, 59% of respondents indicated that they fed their dogs on the family left over. This suggests that in the absence of family leftover, probably due to starvation

or shortfall; dogs are liable to see to their subsistence which favours free-roaming and its consequences.

On anti-rabies vaccination of dogs, it was shown that about 70% of respondents claimed to have been vaccinating their dogs. But it appears that only about 50% do vaccinate their dogs regularly, yet 43% vaccinated only once. One can infer that 43% of dogs (or their fraction) in the study area, may come down with rabies if exposed to rabid dog bite. Principally due to vaccination failure as a result of absence of annual booster as they are being vaccinated only once (in a lifetime) and never again. It was equally shown that amongst those who do not vaccinate their dogs, 38.9% said because the vaccine was expensive. This suggests that if anti-rabies vaccine is free or cheaper than currently is (N500); dog owners will probably have vaccinated their dogs. However, 34% claimed they have no idea that anti-rabies vaccine exists. This goes to show that public health officers and veterinarians have a lot of input to make in public awareness campaigns on rabies and its preventive measures.

Worth discussing is the way sick dogs are being treated in the study area which shows that about 60% of sick dogs are taken to the clinics when sick. Yet a substantial proportion (30.4%) is treated at home with herbs or left on their own (10.6%) to survive/heal. This practice suggests that dogs are not medically catered for as expected of a responsible ownership.

It has also been shown in the present study that 31% of respondents (or their family members) have been bitten by a suspected rabid dog. This finding suggests that one of the indicators of rabies (i.e dog bite) and likely rabies is present in the state. However, 17% of such respondents believed that the dogs were mad (rabid) but 57% believed that the dog was apparently healthy. It should however be noted that research has indicated

that infected dogs may not die, but survive as carriers shedding the virus intermittently in their saliva (Fekadu, 1988). Similarly, in some reports in Nigeria, 6–42% of dogs slaughtered for human consumption had rabies antigen in their brains (Ajayi *et al.*, 2006; Sabo, 2009; Garba *et al.*, 2010; Audu, 2012). This suggests that some of the 57% of the dogs that inflicted bites, but were thought to be apparently healthy in this report may be harbouring the rabies virus.

About 34% of the respondents reported that they killed the offending dogs instantly after they inflicted bite on humans while 5.3% died later on their own (probably due to rabies). This indicates that capture and quarantine of dogs for observation period is hardly/rarely carried out on the offending dogs. The implications of which are that dogs are pre-maturely killed and that the fate of the victims for vaccination lies in the skill and decision of a physician and not on clinical history or confirmatory diagnosis of rabies in the dog.

On the other hand, it has been shown that about 14% of the dog bite victims from the questionnaire responses died later. This could be a pointer that some of the offending dogs (though not laboratory confirmed) may be rabid dogs expressed by the death of about 14% of victims. However, about 78.8% of the victims from the responses were shown to remain healthy. This probably may be due to the nature of the virus which has invariably long incubation periods ranging from as low as 10 days to as high as 19 years in a patient in China (HESIS, 1998). Rabid dog bite victims with a long incubation period may be assumed by respondents as being healthy after a long period of bite due to long incubation period. However, the consequence of this is that, the victim may be harbouring the virus unnoticeable, hence may come down with rabies subsequently which may not be attributed to the previous dog bite by the respondents.

It has also been observed from the responses that 28.1% of victims received rabies post – exposure prophylaxis (PEP), however, a good proportion (39.3%) of victims were reported to rely only on local wound dressing and a good number (32.6%) treated in a traditional way. The low level of PEP may be due to non availability and expensive nature of the vaccine in the developing countries (WHO, 2002b). This further shows the over reliance of some local people onto traditional methods of treating rabies which needs to be checked.

It was shown that the major (50%) traditional method of treating dog bite victims was through cooking a part of the offending (killed) dog and giving to the victim. Administration of concoctions (33%) and reciting incantations (18%) are some of the other traditional treatment methods employed. Though 69.2% of respondents believe that rabies is caused by infectious agents, yet some (23.1%) had the belief that rabies is caused by an evil spirit and few (7.7%) felt that it was because the dog was hungry. These were some of the reasons why some people believed that incantations, eating of parts (flesh or visceral) of offending dogs could cleanse against the disease (Oboegbulem, 1994; Garba, 2011). Most sick dogs were taken to the clinic (59%), but about 14.3% are treated with herbs and 10.6% left alone. This suggests that dogs are not given the expected treatment when sick. However, because some of the dogs in the study area as shown earlier are not confined; inability to restrain them to the clinic may be one reason why they are not taken to the clinic for medications and other treatment. But lack of financial resources and public awareness may also contribute.

Relating to this study that summarizes the presence of dog bite cases in Niger State with over 223 different occurrences, it is a strong pointer to the possibilities of rabies in the state. Especially with the assumption that only moderate and severe cases of dog

bites are reported to the hospitals when there is perceived fear of infection with the rabies virus. It was observed, however, that there has been a steady annual increase in dog bite cases during the reporting period with the highest number of 40 cases recorded in 2012. This may either be due to increased awareness and reporting of dog bite cases to the hospitals, or there was a true increase of rabies in the dog population, hence increase in dog bite cases in the state.

Considering the hospital distribution of cases, it was observed that over 51% (114) of the total 223 dog bite cases were seen at the Tungan Magajiya General Hospital, followed by 27% (62) of the cases seen in Minna General Hospital. The most probable reason could be that Tungan Magajiya has been one of the oldest hospitals set up by the colonial masters providing primary and secondary health care in Nigeria. Additionally, it is an institutional (School of Health Technology, T/Magajiya) hospital surrounded by villages and rural settlements.

It is the large population and traffic of dogs in the rural settlements (which could inflict bite) coupled with the tract records of the hospital that made Tungan Magajiya hospital record the highest number of dog bite cases. Minna is the capital city of Niger State which houses civil servants, educated and enlightened citizens. These attributes may be the reason why Minna General Hospital recorded second highest dog bite cases.

For monthly distribution and seasonal trend of dog bite victims, more cases were recorded in the months of May, March, January and October. This observation may be due to National and Islamic festivities around these periods in the study area especially that majority of residents of Niger State are Muslims. However, it was observed that the least cases were in December during the reporting period. This is however, contrary to the report of Garba *et al.* (2005) in Plateau State.

It was observed that about 67% of dog bite victims were males. This may be due to the lifestyle of the residents of Niger State. More males are engaged in official and day to day street activities than the females (who mostly are restricted house wives), coupled with the fact that the majority of dogs, though owned are free-roaming (unleashed/unchained). This lifestyle could be responsible in exposing more males to dog bite than the females. Over 46% (103) of dog bite victims were children below the age of 15 years. This finding is in agreement with the report of the World Health Organization (WHO) that children below the age of 15 years are more vulnerable to rabies world over (WHO, 2010). The vulnerability of these children below the age of 15 years to dog bite incidences in the present study may not be unconnected to the following facts. The majority of these children (unprivileged) go to school, apprentice points and other places sent by their parents/guardians on foot (pedestrians). In the process, they could get bitten by dogs.

As shown from the results that over 81% of dog bite victims in this study were bitten on the legs and over 14% on the hands. This may be due to the close proximity of the extremities (legs and hands) to the offending dog during running and defense by the victim than other parts of the body. Reports have indicated that rabies virus migrates along peripheral nerves towards the central nervous system at about 0.5—1 mm per hour via the fast axonal transport system (Tsiang, 1993; Allen *et al.*, 1995; Warrell and Warrell, 2004). In this scenario where the majority of the bites being on the legs, it may suggest a prolonged incubation period (IP), which could lead to misdiagnosis of rabies in such victims. Particularly when the relatives of dog bite victims who is now expressing neurologic signs, might have forgotten about the previous bite and ascribe the current sickness to evil spirit or the witches leading to misdiagnosis of rabies.

During the same period (January 2006 and July 2013) when dog bite cases occurred in Niger State, no single sample was submitted from Niger State of Nigeria to NVRI for rabies diagnosis. The implication of this finding is that there is poor surveillance of rabies in Niger State of Nigeria, and a lot of dog bite victims could die due to undiagnosed rabies. This none submission of samples from Niger State is similar to the previous report that between 1991 and 2005, about 2143 animal samples were submitted to NVRI across Nigeria for rabies confirmation. But only one sample was submitted from Niger State which was diagnosed negative (Garba *et al.*, 2008b).

Results from data generated on dog slaughter and consumption practices in the study area, have made some revelations on rationales for dog meat consumption. The 471 dogs recorded to have been slaughtered in this study were based on those slaughtered at the time of our visits. This was accepted with the consent of the butchers who gave us details of each dog slaughtered. It was shown that, between 1 and 6 dogs are being slaughtered by an individual butcher daily. It was also shown that over 55% of dogs slaughtered were below the age of one year and only 13.4% were above the age of 5 years. These suggest that more dogs at their reproductive age (5 years and below) are being slaughtered in the study area. The implication of this finding is that the practice (of dog slaughter) may help in reducing the population of dogs (depopulation), or it will help in the spread and distribution of rabies virus variants across the country when a rabid dog was source from elsewhere and transported to another location for slaughter (Ajayi *et al.*, 2006; Garba *et al.*, 2010; Audu, 2011).

It was shown that the consumption of dog meat spread across almost all the occupational/professional groups with the highest percentage observed with the civil servants. This outcome suggests the continued and wide acceptance of dog meat

amongst the occupational groups in Nigeria. From this study, it shows that about two thirds (64%) or majority of dog meat consumers ate the meat because it was delicious. This claim has been made by some tribes in some parts of India (Anon, 2004; Mao, 2010). Some consumers (18.4%) ate due to its medicinal properties which tally with what was reported earlier for Nigerian dog meat consumers (Murray, 2007; Willy, 2007).

All the high risk groups (though aware of rabies) were not vaccinated against rabies pre-exposure prophylaxis (Pre-EP). Ordinarily consumption of well cooked dog meat will not cause exposure to the virus because of the inactivation of the virus at cooking temperature and acidic nature of the stomach (Oboegbulem, 1994). However, the source of infection could come from the contaminated utensils and sales tables used in the slaughter and sales points. Individual consumer (even the butcher) could get infected if he has a cut or open wound on his body. It was shown from the results that all the butchers sourced/purchased their dogs from households within and outside their vicinity and not by personal breeding. Hence, there is every assumption that those dogs could be unwanted by the owners who have seen illnesses or abnormalities in the dogs hence selling or giving them out for slaughter. Such abnormalities may include rabies (dumb or furious) and the dogs may be looking apparently normal on purchase and slaughter.

In a report from Vietnam, 5 human rabies patients did not have any history of dog or cat bites, but had an experience of butchering dogs or cats, or consuming their meat (Anh *et al.*, 2011). Rabies virus was also detected in 2 of the 100 sick dogs from slaughterhouses (Anh *et al.*, 2011). Similarly, in the following reports, all the rabies virus isolates found in Nigeria (David *et al.*, 2008), Burkino-Faso (De Benedictis *et al.*,

2010) and Vietnam (Anh *et al.*, 2011) were associated with the transboundary spread from Cameroon, Mauritania and China respectively. These above reports further elucidate the risks associated to dog trade/slaughter. Caging of dogs destined for human consumption is another means of disseminating the virus and torture of dogs, which are serious problems that need government intervention at all levels (WSPA, 2013). The low level of educational status of the butchers as shown, may affect the strategic interventions for the control of rabies, particularly, with the family burden of 1:6 ratio (butcher: household size) as shown in the results. This could drive the butchers to slaughter any dog (even sick or rabid) under the quest to solve family financial needs.

The sourcing of dogs for slaughter from households as shown in the results and the shipment to slaughter points could aid in the spread of the virus in particular and the disease across the sub-region. Particularly that reports of rabies virus antigens were seen in the brains of slaughtered dogs in Nigeria (Aghomo and Rupprecht, 1990; Ajayi *et al.*, 2006; Garba *et al.*, 2010). The practice of purchase, shipment and slaughter/consumption of dogs from within and outside their territory is a pointer to an evolving epidemiological situation that could aid in the spread of rabies.

The laboratory investigation aspect of this study, has confirmed the presence of dog rabies in Niger State. The distribution and intensity of the viral load of the 3 positive samples in the present study, however, were shown to be 4+/4+ each. These samples may be assumed to be full blown rabies antigen found in the brains of slaughtered dogs at the slaughter points. The public health implication of this finding is that the dog butchers (and indeed dog consumers) could contract rabies through contact with the contaminated materials during and after the processing period. In the present study, the dogs were visually examined by the researcher in their cages before slaughter and were

looking rabies free hence were assumed apparently healthy dogs. However, serological evidence exists were the presence of rabies antibodies in 15.93% of unvaccinated dogs examined from southern Nigeria was reported (Aghomo *et al.*, 1987). This suggests that such dogs were previously exposed to the virus, but remaining apparently healthy expressing the presence of such antibodies in that report. Indicating that the apparently healthy dogs could harbor rabies antigen as is the 3 rabies positive dog samples in the present study.

One positive sample each came from three locations (Bida, Minna and Suleja), no positive sample was seen in Kontagora. This may probably be due to the low number of samples (26 brain samples) collected in Kontagora compared with between 134, 153 and 158 samples collected from Suleja, Bida and Minna locations respectively. It was also noted that, of the 3 positive samples 2 (66%) were males. It suggests that male dogs are likely prone to contracting rabies than female dogs, especially when about equal number of samples (53.5% males and 46.5% females) were collected. Although our data are insufficient to make this conclusion, however, a similar observation was made by Garba *et al.* (2005) who attributed this to the level of male dogs in courtship especially during their breeding season. More male dogs were seen per bitch during courtship which exposed most of the male dogs to bite by a rabid dog.

Furthermore, one of the 3 positive samples was from a dog below the age of 1 year, probably a puppy. The implication of this finding is the continuous circulation of rabies antigen in unvaccinated puppies without suspicion of rabies at that age. Previous reports have, however, indicated the presence of rabies in an eight week old puppy (Adeyanju and Addo, 1977) and even two week old puppies reported (Garba *et al.*, 2008b) in Nigeria. The remaining two positive samples were from dogs between

the ages of 1 – 5 years. In other words, all the 3 dogs were below the age of 5 years. This suggests that rabies virus is circulating more amongst the age bracket of 1 – 5 year old dogs. However, it was shown that only about 13.3% of dogs slaughtered were above the age of 5 years. This low number of this age category (above 5 years old) of dogs may be responsible for non – positive viral antigen in this group.

As was shown, the BLAST best match of these three positive samples had 98 to 99% identical sequences with those variants found in Burkina Faso, Chad, Nigeria and Niger Republics. This indicates that the samples harboured rabies virus variant that are Africa 2 subgroups, which circulate in West Africa (Kissi *et al.*, 1995; David *et al.*, 2008; Dzikwi, 2008). Isolate 471 Nigeria 2012 has been shown to form cluster with Nigeria 1 lineage of Africa 2 subgroup (Dzikwi, 2008). This lineage (Nigeria 1); were samples collected and isolated from Kaduna and Plateau States of Nigeria (David *et al.*, 2008; Dzikwi, 2008). Kaduna State forms boundary with Niger State on North-eastern portion, while Plateau State forms boundary with Kaduna State on its western portion. This suggests that there may be flow of rabies virus from Plateau State of Kaduna then to Niger State or from Kaduna to Niger and Plateau States, especially as dogs are sourced for slaughter by butchers from within and outside their territories. There are reports that dogs are being exchanged for food commodities across Nigeria (Plateau State) and Cameroun, Chad and Niger republic borders (Ogunkoya, 2008; Sabo, 2009). In such exchanges, there are chances of importation of rabies variants in dogs (if they are infected) across our borders.

It is evident that the sample 252 Nigeria 2012 in the present study has never previously been reported in Nigeria but Niger Republic. The occurrence of this variant is suggestive of transboundary movement of rabies infected dogs. The most probable

means could be due to dog trading across our borders as mentioned earlier probably from Niger Republic. Another possibility could be the continuous flow of nomadic cattle rearers ‘Fulanis’ from Niger Republic into Nigeria for greener pasture during drought periods. These cattle rearers are accompanied by their donkeys, sheep, goats and dogs. Such dogs are used for guard, hunting and rearing or control of animal movement. In the event of illness by the dogs to the extent of biting their animals, such dogs are killed or sold out to dog butchers by the Fulanis.

Niger State covers 9% of Nigeria’s total land space and is a major stock (cattle) route from Cameroun and Niger Republic through Northern parts of Nigeria to the Southern part where greener pasture exists. This could be the probable reason for the presence of this rabies variant in Niger State due to the stock transit.

Another probable reason could be that a rabid dog from Niger Republic may have moved a long distance into the border towns of Sokoto and Katsina States of Nigeria and inflicted bites (transmitting the rabies variant) on other Nigerian dogs. These other Nigerian dogs may come down with rabies and keep inflicting bites and disseminating this rabies variant from Niger Republic to other Nigerian dogs but only detectable in Niger State in the present study. The exchange of dogs between hunters in Nigeria and Niger Republic should not be underscored and could be another source of transboundary movement of rabies variants.

The 3rd positive sample ‘276 Nigeria 2012’ was also never previously reported in Nigeria, but falls closely related to clusters previously reported in Chad and Niger Republic. Its emergence in Nigeria in the present study may be explained as to the earlier reasons given for the variant in Niger Republic. However, this variant is also closely related to Nigeria 2 clusters, but it forms a distinct lineage different from all

other lineages (Nigeria 1, 2 & 3) previously reported (David *et al.*, 2008; Dzikwi, 2008). This new lineage (though single sample) may be proposed as Nigeria 4 cluster.

Of the 267 samples of bat brain investigated in the present study for rabies antigen, none were positive for rabies antigen. This further augments the findings of other previous researchers who did not detect rabies antigen in the brains of bats in Nigeria but found other lyssaviruses (Aghomo *et al.*, 1990; Dzikwi *et al.*, 2010a). However, the absence of rabies antigen in these bats does not necessarily preclude the presence of rabies antibodies in their sera as reported by previous workers (Aghomo *et al.*, 1990). Aghomo *et al.* (1990) has detected rabies virus neutralizing antibodies in the sera of two out of the fifty bats whose brains were negative for rabies antigen by FAT.

It was evident that certain limitations in the present study could not warrant serological assay against rabies, Mokola and Lagos bat viruses. However, 1.95% (3) out of the total 154 bat serum samples screened against Duvenhage virus (DUVV) showed a detectable antibody titre. This is another milestone in the epidemiology of non-rabies lyssaviruses in Nigeria. Because since the first isolation of Lagos bat virus from fruit bats (*Eidolon helvum*) on Lagos Island in Nigeria in 1956 (Boulger and Porterfield, 1958), researchers have been reporting LBV antibodies in Nigeria (Aghomo *et al.*, 1990). Recently, there was serological evidence of Lagos bat virus in fruit eating bat (*Eidolon helvum*) in Nigeria (Dzikwi *et al.*, 2010a). In this present report fruit eating bat (*Epomorphorus gambiense*) is the culprit harbouring DUVV antibodies in their sera. Based on a literature search, it appears from the first report of Duvenhage virus in South Africa in 1970 in insectivorous bats (Kaplan and Koprowski, 1980), this is the first report of the DUV virus antibodies in fruit eating species (*Epomorphorus gambiense*) of bats in Nigeria and elsewhere in the world.

Most of the bats in the present study were captured in the public dwellings; if those bats detected with DUVV antibodies happens to harbour the virus; the consequences will be the possible contamination and infection of public with Duvenhage virus. It is known that all lyssaviruses (except LBV) causes illness indistinguishable from classical rabies in humans (Smith, 1996). This will further affect the problems of diagnosing classical rabies from DUVV infection in individual's dwelling and possibly eating of Duvenhage virus infected bats in Nigeria.

It was evident that despite the fact that only two species of bats were screened (*Epomorphorus gambiense* and *Micropteropus pusali*), only *E. gambiense* were seen with detectable antibodies against DUVV. Probable reason could be due to the number of samples screened. Particularly that out of total 154 bat sera screened, only 6 were *Micropteropus pusali*. Bats were captured from the trees at those selected locations in the study area by shooting giving equal chances of any species to be captured. Observing this high number of *Epomorphorus gambiense* in the samples, may suggest that it is the predominant species of bats in Niger State of Nigeria. This is in contrast to the findings of Aghomo *et al.* (1990) in the South west and Dzikwi *et al.* (2010a) in the Northeastern Nigeria where in their sample collection *Eidolon helvum* was the predominant species of bats captured. However, this does not necessarily inferred that these bats species are the confirmed predominant spp of bats in the study locations because *Epomorphorus gambiense* was equally captured in Northeastern Nigeria in addition to *Eidolon helvum* captured (Dzikwi *et al.*, 2010a).

The serological detection of DUVV antibodies in *Epomorphorus gambiense* in the present study area leaves us yet with another epidemiological niche for DUVV. Reports have indicated that there have been only five reported cases of DUVV infection, and

three of these have been fatal human cases (1970, 2006, and 2007), each case followed contact with a small unidentified bat (Van Eeden *et al.*, 2011). It was indicated that none of the bat has been found in these three human fatalities, thus no identification of such bats was made. The remaining two cases were believed to be from small bats, one from South Africa in 1981 (though no bat identification was made) and the other from *Nycteris thebaica* (insectivorous bat) in Zimbabwe in 1986 (Foggin, 1988). Recent reports (the 6th DUVV report) revealed the presence of DUVV antibodies in 30% of the insectivorous bats (*N. thebaica*) screened in Swaziland a border country to South Africa (Markotter *et al.*, 2013). Thus *N. thebaica* has been hypothesized as the only bat species linked with DUVV infection (Markotter *et al.*, 2013). But in this study a frugivorous (fruit eating) bat *Epomorphorus gambiense* is the implicating species with DUVV antibodies. This present report is therefore contrary to the hypothesis by Markotter *et al.* (2013) that *N. thebaica* (an insectivorous bat) is the only reservoir host for DUVV.

The classical rabies virus (RABV) is in the same phylogroup 1 with DUVV in the *lyssavirus* species segregation (WHO, 2012; ICTV, 2013). It was established that there is a significant serological neutralization within phylogroups but very limited cross-neutralization has been detected between phylogroups (WHO, 2012). This significant serological neutralization within phylogroup will help in the control and management of humans exposed to DUVV and clinically misdiagnosed as classical RABV infection. This holds most especially in rural Africa where laboratory confirmatory facilities are scanty. This present finding also opens more facets of research and understanding of the epidemiology of DUVV bats reservoirs in Nigeria and Africa at large. In the present findings, it was noted that all the 3 positive samples were from male *Epomorphorus*

gambiense despite about equal number of males (134) to female (133) were captured in this study. There is no epidemiological explanation for this observation, but, probably male bats of *Epomorphorus gambiense* are more vulnerable to DUVV than the females. However, this observation may open another facet of research in challenging different sexes with DUVV or other Lyssaviruses in order to test this hypothesis. Conclusively on this aspect, serological evidence of DUVV is established in the study area and probably the first report of DUVV antibodies in fruit eating bats in Nigeria.

On the human sera, results for detection of rabies neutralizing antibodies using RFFIT has revealed that about 16.4% of rabies occupational risk humans had some reasonable titre of rabies virus neutralizing antibodies (rVNA) in their body. This suggests prior exposure of these individuals to rabies viral antigen either at once or repeatedly in smaller doses due to their occupation or hobby. The detection of rabies antibodies in unvaccinated dogs and humans has since been reported by some workers in Nigeria. Aghomo *et al.* (1987) had reported the presence of rabies virus antibodies in over 15.93% of unvaccinated dogs in the southwestern part of Nigeria. Similarly, Ogunkoya *et al.* (1990) reported the presence of rabies antibodies in 30.7% and 28.6% of unvaccinated dogs and humans respectively in Nigeria. This present finding has therefore augmented these previous reports. Furthermore, recently Gilbert and her colleagues (2012) reported the presence of rVNA in 11% (7 out of 63) unvaccinated villagers in two villages where there was depredation of vampire bats in Amazonia areas of Peru (Gilbert *et al.*, 2012).

It should be noted, however, that some of these positives (5 out of 23 positives) in the present study had a history of vaccination over 10 years earlier based on the questionnaire results. This may suggest that the presence of rVNA in these individuals

may be due to previous vaccinations received. Unfortunately, however, for a pre- or – post-exposure anti-rabies prophylaxis to be efficient, it was recommended to be having an annual booster, especially when the titre falls below 0.5 IU/ml (WHO, 2002b). It is expected that for this long period (>10 years) of vaccination in these individuals, there is supposed to be no response to rVNA or should fall below the minimum titre (0.5 IU/ml). However, it was shown that 3 of the 5 previously vaccinated individuals had relatively high titre each (between 0.5 to 5.4 IU/ml) in their sera. This may suggest that the anti-rabies prophylaxis when instituted as recommended (WHO, 2002b) may last and remain protective in the human subjects for a longer period (about 10 years) before it wears down.

However, this claim is not absolute as it is experimentally not proven except these ones in the present study and one sero-positive individual who received PEP earlier as reported by Gilbert *et al.* (2012). More so 2 of the 5 sero-positives who were previously vaccinated had titres below 0.5 IU/ml the minimum protective titre recommended by WHO (WHO, 2002b).

Accordingly, it has been shown that 25% each of dog butchers and pet owners, then 20% hunters and 14.8% dog meat consumers tested had detectable rabies virus neutralizing antibodies (rVNA). This shows that dog butchers and pet owners have greater tendencies of being infected with rabies in their occupations if the dogs they process or keep as pets had rabies antigen. For instance, as previously mentioned earlier in this work that 3 out of 471 dog brains analysed in this study area from slaughtered dogs had rabies virus antigen in their brains. This could be a source of infection for these individuals (dog butchers, consumers, hunters and even pet owners).

Similarly, in a report from Vietnam, 5 human rabies patients did not have any history of dog or cat bites, but they had an experience of butchering dogs or cats, or consuming their meat (Anh *et al.*, 2011). Contrary to the above report, however, the human subjects in this study at the point of collecting their sera (in year 2012) up to this present time of writing these findings (year 2014), none was showing any sign of ailment suggestive of rabies. Yet they have a detectable rVNA titre ranging between 0.05 to 0.65 IU/ml amongst the (17 out of 23) previously unvaccinated sero-positive individuals. This may suggest they had a previous exposure and natural immune response was present hence the detectable titres.

It was seen that amongst the previously vaccinated (5 out of 23) sero-positive individuals, their titre was shown to be between 0.5 to 5.4 IU/ml. This may suggest that there was no demarcation whether the individual was vaccinated or not with respect to high titre response in rVNA detectable. Even amongst the 7 individuals reported by Gilbert *et al.* (2012) had titre range between 0.1 to 2.8 IU/ml and even then the only one person who was previously vaccinated had a lower titre of 0.1 IU/ml.

Similarly, the hunters and dog meat consumers have 20% and 14.8%, respectively tendencies of being infected with rabies antigen if they come in contact with a rabid animal (dog) as in the present study and those reported with bats by other workers (Gilbert *et al.*, 2012). For the hunters, the likely possibility of being infected lies in the ability of the infected dog contaminating the hunted (shot) animals or birds with their contaminated saliva. Or most likely during struggle between the hunter and the dog in collecting the hunted animal (bird) from the mouth of the apparently healthy looking infected dogs, transmission may occur especially in broken skin.

The likely mode of infection in the consumers may not be due to the consumption of the meat, but through touching, rubbing, and contact with contaminated utensils/materials during the purchase of the prepared dog meat. This is because the killing, processing and sales occurred in the same premises using the same utensils or materials (knives, plates, tables, etc.) in the dog meat sales points in Niger State, Nigeria. In addition to infection through contaminated utensils as in dog meat consumers; dog butchers could get infected through scratches or even bite from infected dogs (WHO, 2010) during the purchase, restrain and shipment of dogs in cages to slaughter points. For pet owners most likely source of infection may be through licking of broken skin/mucous membrane by an infected pet dog.

It has been shown that of the 5 previously vaccinated volunteers who were rabies antibody positive, 4 were from the veterinarians/animal health category. All other members of the group (26 out of 30 veterinarians/animal health personals) were neither vaccinated nor had detectable rVNA in their sera. This emphasizes the lack of rabies vaccination amongst the professionals (veterinarians/animal health personals) in the study area. This lack of vaccination amongst the professionals, particularly the veterinarians, animal health personals, staff of zoological garden and veterinary students have been reported from the southwestern region (Ibadan) of Nigeria (Babasola *et al.*, 2010). None detection of rVNA in any of the unvaccinated volunteers in this professional group may signify that the risk of contracting rabies through their activities may be minimal; probably they used personal protective equipments efficiently.

Unfortunately, one dog butcher and one dog meat consumer though previously unvaccinated yet had detectable rVNA which all were above the minimum protective

titre (0.65 IU/ml each) in the present study. These two were equally from the same dog slaughter point (Minna) where and when their sera were collected. This observation may target an epidemiological scenario depicting that these two individuals may have gotten the infection and subsequent/equal responses to the titre of rVNA from the same infected dog.

It should be remembered that 1 out of the 3 rabies positive dog brain samples (from the total 471 samples) analysed in the other aspect of the study was from Minna. This may implicate the chances of these two volunteers to have gotten infected from same particular dog (not necessarily from this present rabies positive dogs reported), probably through contact with contaminated utensils/materials. Consequently, they developed this protective rVNA 0.65 IU/ml which is a bit greater than the minimum recommended protective titre (0.5 IU/ml) by WHO (WHO, 2002b). This outcome suggests that these two and the remaining unvaccinated volunteers who had detectable rVNA may be resistance to rabies virus infection.

Willoughby (2012) challenges the orthodoxy that rabies is untreatable and universally fatal, especially with the recent report of rVNA in villagers in Peru who were previously unvaccinated (Gilbert *et al.*, 2012). However, he emphasizes that for a complete or relative resistance to rabies to occur in man, it should be detected sporadically, such as in the recent well-characterized reports of rabies survivors in Texas and California (CDC, 2010; 2012b). Our present report, though not well characterized (as it is not cases of abortive or recovery from clinical rabies) but presence of rVNA in these subjects; may constitute yet another sporadic report of rabies resistance in unvaccinated humans in Nigeria.

Based on demographic information about the volunteers, it has been shown that the majority of the rVNA positives were from Bida (52.2%) and Minna (43.5%) locations. Only 4.3% were from Suleja and none from Kontagora study location. This does not preclude the presence of rVNA in these two locations, but probably the very low number of volunteers from these locations, may have limited our findings from these locations

Furthermore, it was noticed that about 45 (24.35%) of samples processed were cytotoxic to the cell line used. What is responsible for this finding is not known, but it can be seen that about 44 out the 45 samples were those collected from other occupational groups at the dog slaughter points. It appears that the environment is not as controlled as those samples obtained from veterinarians/animal health personnels in the clinics. These samples were immediately clotted and centrifuged hence only one sample from this group (veterinarians/animal health personnels) was cytotoxic. It seems, therefore, that these 45 samples were not fully separated and possibly have some traces of red blood cells which might have interfered with the process and render the sera toxic to the cell line.

However, based on the principle of immunology, T-helper cells actually contribute to the development of immunity, whereas cytotoxic T cells do not appear to play a role in protection and may actually be detrimental to the host (Johnson *et al.*, 2010). This could be another possible reason why cytotoxicity was observed in these 45 samples in this study. On a general consideration, the rabies viruses spread from peripheral sites of entry to the central nervous system (CNS) tissues via axonal transport, thereby bypassing the specialized features of the neurovasculature, the blood-brain barrier (BBB) (Hooper *et al.*, 2009). Once the virus reaches CNS tissues three alternative

outcomes are likely to occur as hypothesized (Hooper *et al.*, 2009): (1) the BBB remains intact and the infection is lethal due to the absence of an antiviral CNS immune response (2) immune effectors cross the BBB and mediate a CNS antiviral immune response with extensive immunopathology that contributes to the disease, or (3) immune effectors cross the BBB and clear the virus from the CNS without significant pathological consequences. It is well known that in humans naturally infected with rabies virus the latter outcome is exceedingly rare (Hooper *et al.*, 2009). In our present study, whether the virus is in the CNS or not, whether the immune effectors (the T-helper cells, rabies specific B-cells) have crossed the BBB or not are not known. One fact is that peripheral immune response was confirmed by the presence of rVNAs as a response to natural infection due to occupational exposure by unvaccinated volunteers.

CHAPTER SIX

CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

Despite the lack of published reports on the presence of rabies in Niger state, it is evident from this study that rabies is present in the study area. The pattern of dog ecology and management in Niger State is about the same as seen in other parts of Nigeria and much of African countries. Dog to human ratio was found to be 1:5.4 with an estimated 732,476 dog population in Niger State. The majority of dogs in Niger State though owned are allowed to see to their subsistence except for a few who are catered for. There were at least 31% victims of dog bite cases amongst the respondents or their relatives.

In addition, 223 recorded cases of dog bites occurred in Niger State with 81% of bites being on the legs but no laboratory confirmation of dog rabies between year 2006 and 2013 in the state. It was also evident that the majority of dog meat consumers do eat dog meat because the meat was delicious while others ate for medicinal and spiritual purposes.

The rabies virus antigen present in the brains of slaughtered dogs and circulating in Niger State are Africa 2 subgroup with some of the rabies virus variants never previously reported in Nigeria but Chad and Niger Republics. The presence of these variants have been attributed to dog trade and slaughter as well the movement of Nomadic cattle rearers with dogs from neighbouring countries into Nigeria for greener pasture. New lineage of rabies virus variant (Nigeria 4) has been proposed based on the

present finding (that sample '276 Nigeria 2012' has never previously reported anywhere).

Serological evidence of Duvenhage virus was seen in some fruit eating bats (*Epomorphorus gambiense*) in Niger State without rabies virus antigen in their brains. This presence of DUVV antibodies in the study area appears to be the first serological evidence of the virus in West Africa.

Serological evidence of rabies virus neutralizing antibodies (rVNA) in the sera of rabies occupational risk groups, particularly, unvaccinated dog butchers, consumers, hunters and pet owners with a titre range of 0.5 to 0.65 IU/ml represents another epidemiological problem in the pathogenesis and control of rabies. It is equally evident that some of the vaccinated volunteers had a high rVNA titre (titre range 0.5 to 5.4 IU/ml) despite the fact that they were vaccinated over 10 years prior.

6.1 Recommendations

Based on the above conclusions, the following recommendations are hereby proffered:

- 1) Further dog ecological studies involving both urban and rural areas of Niger State be carried out.
- 2) Further researches including surveys, laboratory and molecular analyses of rabies in both animals and human subjects be carried out in Niger State, Nigeria.
- 3) Transboundary surveillance of rabies virus, particularly, amongst the dogs of nomadic cattle rearers, hunters and dog butchers in the West African sub-region should be employed.

- 4) The Niger State government as a matter of urgency through its ministries of Health and Animal health should make it a law that the heads of all killed dogs should be collected and transported to NVRI, Vom for rabies diagnosis with responsibility on logistics shouldered by the state government.
- 5) Rabies high risk individuals particularly the Veterinary personnel, surveillance officers, dog butchers in the state and Nigeria at large should be vaccinated and sero-monitored for rabies and other Lyssaviruses.
- 6) Further sero-monitoring of bats against Duvenhage virus and other lyssaviruses should be carried out in the state and Nigeria at large.
- 7) It is finally recommended that the New lineage '276 Nigeria 2012' be ascribed as Nigeria 4 cluster especially if more of such isolates are found in the field in future.

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APPENDICES

APPENDIX I: PARTICIPANT INFORMATION LEAFLET

Rabies is a zoonotic viral disease of great public health significance. It is caused by a rabies virus which is transmitted through bite and the saliva laden of an infected animal principally dogs. Cats, bats, cattle, sheep, goats amongst other mammals can also get the disease. Some dogs show real furious rabies by their mental expression of madness, while others become dumb without noticing rabies in them. At risk occupational groups like the dog butchers, hunters with dogs, pet owners, and Veterinary doctors / animal health workers, sometimes get contacted with the virus and become infected without knowing. It is on this note that you are invited to participate in this research as volunteer to donate 2 – 4 ml of your blood, also to give us small quantity of dog brain if you are a dog butcher as well to assist us in showing us caves and trees to get some representative bats in your area.

Thanking you in anticipation of your honest participation

Dr. Garba Ahmed 08036471758, 08095086862

APPENDIX II: BLOOD COLLECTION CONSENT FORM [No.-----]

I..... (volunteer) of Town do voluntarily and willingly agreed and accepted that my blood sample be collected and be use for rabies research purposes by Dr. Garba Ahmed of the above named address through his nurse/health officer.

This day.....of.....20....

Sign.....

Sign.....

(Volunteer)

(Nurse/Health officer)

Phone no.....

Phone no.....

Sign.....

(Dr. Garba Ahmed)

08036471758, 08027418282

APPENDIX III: QUESTIONNAIRE SURVEY ON DOG BUTCHERS, CONSUMERS, PET OWNERS AND HUNTERS IN NIGER STATE, NIGERIA [_ _ _ _]

INSTRUCTION: Kindly read and answer the following questions. The obtained information will be used strictly for academic purpose only and absolute confidentiality will be ensured. Thank you for the much needed cooperation and time spared. Fill or tick the space or box provided accordingly.

1. Age _____years
2. Sex a. Male [] b. Female []
3. Ethnic group a. Hausa [], b. Nupe [], c. Gwari [], d. Others (specify)

4. Marital status: a. single [], b. married [], c. divorced [], d. widow []
5. What is your religion: a. Islam [], b. Christianity [], c. Others (specify)

6. What is your occupation? a. civil servant [], b. private practitioner [],
c. businessman/woman [], d. farmer [], e. others (specify) _____
7. Duration of service/practice/business/farming/others? _____years
8. What is the size/number of your households'? _____ people
9. What is your education status? a. Primary [], b. Secondary [], c. Tertiary [],
d. Religious [], e. None []
10. How many dogs do you slaughter per day a. [1], b. [2], c. [3], d. others []

11. Have you been bitten by a dog a. Yes [], b. No []
12. Have you been vaccinated against rabies a. Yes [], b. No []
13. Where do you source your dogs for slaughter? a. From dogs market [], b. From households [], c. Stray dogs [], d. All of the above []
14. Do you eat dog meat? a. Yes [], b. No []
15. Why do you eat dog meat a. Because it is cheap [], b. Because it is sweet/delicious [], c. For medicinal purpose [], other reason [specify]_____

APPENDIX IV: QUESTIONNAIRE ON VETERINARIANS AND ANIMAL HEALTH ASSISTANTS IN NIGER STATE, NIGERIA [_ _ _ _]

INSTRUCTION: Kindly read and answer the following questions. The obtained information will be used strictly for academic purpose only and absolute confidentiality will be ensured. Thank you for the much needed cooperation and time spared. Fill or tick the space or box provided accordingly.

1. Age _____years
2. Sex a. Male [] b. Female []
3. Ethnic group a. Hausa [], b. Nupe [], c. Gwari [], d. Others (specify)

4. Marital status: a. single [], b. married [], c. divorced [], d. widow []
5. What is your religion: a. Islam [], b. Christianity [], c. Others (specify)

6. What is your occupation? a. civil servant [], b. Youth corps member [],
c. student on attachment[], d. others (specify) _____
7. Duration of service/practice/others? _____years
8. What is the size/number of your households'? _____ people
9. What is your highest education status? a. Primary [], b. Secondary [], c. OND [],
d. HND [], e. DVM [], f. others (specify)_____
10. What is your discipline a. Veterinarian [], b. Animal health personnel [], c.
Youth corps member [], Student on attachment [], Others
(specify).....

11. Have you ever been bitten by a dog a. Yes [] b. No []
12. Have you ever been vaccinated against rabies a. Yes [], b. No []
13. If yes in 12 above, how many years ago a. 1 year [], b. 2 years [], c. 3 years []
d. 4 years []. e. others (specify)_____
14. If yes in 12 above, who sponsor the cost? a. Self [-], b. School [], c.
Government [], d. Free by voluntary organization []
15. If yes to 12 above how many doses of the vaccine did you received at a time
a. 1 dose [], b. 2 doses [], c. 3 doses [], d. Others (specify)_____

APPENDIX V: QUESTIONNAIRE SURVEY ON DOG ECOLOGY AND MANAGEMENT IN NIGER STATE, NIGERIA. [_ _ _ _]

INSTRUCTION: Kindly read and answer the following questions. The obtained information will be used strictly for academic purpose only and absolute confidentiality will be ensured. Thank you for the much needed cooperation and time spared. Fill or tick the space or box provided accordingly.

SECTION A: BIODEMOGRAPHIC INFORMATION OF RESPONDENT

1. Age _____years
2. Sex a. Male [] b. Female []
3. Ethnic group a. Hausa [], b. Nupe [], c. Gwari [], d. Others (specify)

4. Marital status: a. single [], b. married [], c. divorced [], d. widow []
5. What is your religion: a. Islam [], b. Christianity [], c. Others (specify)

6. What is your occupation? a. civil servant [], b. private practitioner [],
c. businessman/woman [], d. farmer [], e. others (specify) _____
7. Duration of service/practice/business/farming/others? _____years
8. What is the size/number of your households'? _____ people

SECTION B: DOG POPULATION STRUCTURE

9. Do you or members of your house holds keep dog(s)? a. Yes [], b. No []

10. How many dog(s) do you or your households keep now? a. one [], b. two [],
c. three [], d. others (specify) _____ dogs

11. What is the sex and number of your dog(s)? a. Male [1], [2], [others specify____]
b. Female [1], [2], [others specify____]

12. What is the age of your dog(s)?

a. less than 1 year [1], [2], [3], [others, specify__]

b. 1-5 years [1], [2], [3], [others, specify__]

c. more than 5 years [1], [2], [3], [others, specify__]

13. What is the breed of your dog(s)? a. local [], b. exotic [], c. mixed []

14. Do you like keeping dogs? a. Yes [], b. No []

15. If No to 14 above, why No?

a. because I dislike dogs []

b. because my religion discourage keeping of dogs []

c. because dog bite people causing madness (rabies) []

d. two of the above specify, [] and []

e. all of the above []

16. If Yes to 14 above, why Yes?

a. for security [], b. as pets [], c. for hunting [], d. for herding animals [],

e. for breeding and selling []

17. How do you acquire your dog(s)? a. through purchase [], b. gift from friends [], c. gift from relatives [], d. gift from neighbors [], e. as free roaming into my house []
18. Do you allow stray dogs or dogs from neighbors roam freely into your compound? A. Yes [], b. No []
19. If yes, to 18 above, what do you do with them?
- a. allow them access to my food []
 - b. allow them play and mate with my dogs []
 - c. allow them play with my children []
 - d. all of the above []
 - e. none of the above []
20. How often do dogs stray into/ around your house?
- a. in the mornings [], b. afternoons [], c. evenings [], d. at night []
21. How do you dispose-off your puppy dogs?
- a. sale them off []
 - b. give to friends and relatives []
 - c. allow them to stray away []
 - d. two of the above, specify [] and []
 - e. all of the above []

SECTION C: DOG FEEDING AND HEALTH MANAGEMENT

22. Do you house or confine your dog(s) ?
- a. never confine [], b. partial confinement [], c. always confine []
23. Who take care of the dog(s)? a. father [], b. mother [], c. children []
- d. everybody []
24. How are the dog(s) fed? a. family left over [], b. cook special pot for the dog [], c. roam freely anywhere to feed themselves
25. Do you vaccinate your dog(s) against rabies a. Yes [], b. No []
26. If Yes to 25, how often do you vaccinate your dog(s) a. once [], b. twice [], c. regularly []
27. If No, why? a. vaccines are expensive [], b. vaccines not available [], c. I have no idea that dogs are vaccinated against rabies []
28. What type of vaccine regimen do you prefer if available?
- a. oral vaccination for dogs [], b. injectable vaccination []
29. How do you treat your dog(s) when they are sick?
- a. taken to the veterinary clinics b. give the medication at home by self []
- c. treat with traditional herbals [], d. leave the dogs on their own []
30. How are your dogs being depopulated?
- a. giving out to people [], b. selling to people [], c. disappear []

d. automobile accident [], e. died due to diseases []

SECTION D: DOG BITE CASES, MANAGEMENT AND SEQUELA

31. Has any member of your house holds been bitten by a dog?

a. Yes [], b. No []

32. If Yes, who owns the dog? a. house hold [], b. neighbors' dog [],

c. stray dog [].

33. What was the health status of the dog? A. sick [], b. healthy [],

c. nursing bitch [], d. mad (rabid) dog []

34. What happened to the dog later?

a. died [], b. killed [], c. remained healthy []

35. What treatment was given to the victim?

a. post-exposure prophylaxis treatment given []

b. local wound dressing and antibiotics []

c. traditional dog bite treatment given []

36. What happened to the victim? a. died later [], b. healthy [], c. I don't know []

37. Do you believe in the traditional way of treating dog bite injuries/ rabies?

a. Yes [], b. No []

38. How is dog bite injuries/rabies treated in your tradition?

a. placing pull out hairs on the wound & cooking parts of the dogs' body for the victim to eat []

b. reciting incantations to the victim [], c. giving concoctions to the victim[]

d. others (specify)_____

39. What do you call mad dog or rabies in your language _____

40. What do you think causes rabies? a. evil spirit [], b. infectious agent [],

c. hunger [], d. others specify_____

APPENDIX VI: PROTOCOL FOR DIRECT RAPID IMMUNOHISTOCHEMISTRY TEST PROCEEDURE



Plate III: A direct rapid immunohistochemical test diagnostic kit

1. A routine touch impressions of each sampled brain tissue were made on labeled glass microscope slides (including the standard positive and negative controls).
2. Slides were arranged in slides holder (24 slides at a time i.e 22 test slides and 1 positive and 1 negative control slides), air-dry for 5 minutes at room temperature in an examination/laboratory hood.
3. Slides were then immersed in 10% buffered formalin at room temperature for 10 minutes. **Dish I**
4. Slides were then removed and dip-rinsed several (10 -15) times to wash off any excess fixative in wash buffer Tween phosphate buffer saline TPBS (PBS with 1% tween 80). **Dish II.**
5. Slides were then immersed in 3% hydrogen peroxide (H_2O_2) for 10 minutes. **Dish III.**

6. Excess hydrogen peroxide was removed by dip-rinsing slides in TPBS, **Dish IV**. Slides were then transferred into the next rinse **Dish V containing TPBS** (after dip-rinsing, excess buffer were shake off from slides edges using wipe papers surrounding the impression). A slide at a time was removed wiped; leaving the remaining slides immersed with TPBS rinse until 8 slides were arranged on a wet paper towel on laboratory flat table top.

7. Slides were then incubated in a humidity chamber (e.g. we used the plastic top to a 96-well plate, on a moistened paper towel, on laboratory bench top) at room temperature after adding primary antibody – (biotinylated anti-rabies mAb) for 10 minutes (enough of primary antibody usually 2-3 drops using plastic pipettes to cover the impression were added).

8. After incubation with primary antibody, excess conjugate were shake off. Slides dip-rinsed in TPBS, **Dish V** (excess TPBS were shake off and blot buffer from slides edges surrounding the impression using wipe paper). This same wash buffer was used through step 10.

9. Slides were placed back on the wetted paper towel then incubated with Streptavidin – peroxidase complex (enough of this complex was added to the slide by drop to cover the impression) in humidity chamber as described in step 7 above at room temperature for 10 minutes. After incubation, slides were shake- off.

10. Slides were then dip-rinsed with TPBS, in **Dish V** (excess buffer shake off and blotted from slide edges surrounding the impression).

11. Slides were then incubated with peroxidase substrate, amino-ethylcarbizole (AEC) – the working dilution was prepared just prior to use. Enough of this substrate was

added to the slide by drop to cover the impression in a humidity chamber (as in step 7 above) at room temperature for 10 minutes. After incubation, shake off excess substrate.

12. Slides were then dip – rinsed in de-ionized/distilled water, **Dish VI**.

13. Then counter stain with Gills Hematoxylin (diluted 1:2 with de-ionized/distilled water) for 2 minutes **Dish VII**.

14. Immediately after the 2 minutes counter staining, the slides were dip-rinsed (10 -15 times) with de-ionized/distilled water in **Dish VIII**. A second dip-rinse of slides with fresh deionized/distilled water (**Dish IX**) was made to ensure removal of excess stain.

15. Slides were then transferred to fresh distilled water **Dish X**. Slides were mounted with cover slips using water-soluble mounting medium. Slides were not allowed to air-dry prior to cover-slipping.

16. Slides were viewed by light microscopy, using a x20 objective to scan the field, and a x40 objective for higher power magnification.

17. Rabies viral antigen appeared as red coloured particles/inclusions under a bluish neuronal background indicated the samples was positive. A complete bluish background suggested that the sample was negative. Results were recorded according to intensity and distribution of viral particles graded from +1 (low) to +4 (high) intensities.

APPENDIX VII: COMPLETE PROTOCOL FOR RT-PCR AND SEQUENCING FOR RABIES DIAGNOSIS

FAT and DRIT positive and questionable samples (totaling 13) from dog brain were used for RT-PCR and Sequencing: Extraction and purification of viral RNA, RT-PCR, and sequencing was performed as previously described by David *et al* (2007) with some modification as recommended in the RT-PCR protocol designed by CDC, USA (CDC, 2012a). Briefly, total RNA was extracted, followed by reverse transcription (RT) to produce the cDNA, then polymerase chain reaction (PCR) to amplify the product, gel electrophoresis, purification, sequencing and phylogenetic analysis (CDC, 2012a). The procedures were carried out as shown below:

RNA extraction: RNA was extracted using the TRIzol method (Invitrogen, Life Technologies, USA) following the manufacturer's recommendations.

1. Initially the biological safety cabinet (BSC) and bench surfaces were cleaned with 3 % hydrogen peroxide prior to starting. Only equipment for the current sample was kept in the BSC during an extraction. Frozen brain samples were thawed just prior to testing, while reagents and samples were always kept cold on ice.
2. In a clean area sterile micro-centrifuge tubes were labeled, and aliquot in the tubes the following reagents: lysis buffer 0.1 ml, TRIzol 1.0 ml, chloroform 0.2 ml, isopropanol 0.5 ml were made just before starting the procedure.
3. About 0.1 grams of brain tissue was transferred into the screw cap micro-centrifuge tube containing 100 µl lysis buffer and homogenize using mini bead beater. Same was done for each of the 13 brain samples and the positive control.
4. 1.0 ml each of TRIzol reagent was added into each tube containing the specimen and lysis buffer suspension, homogenized thoroughly using mini bead beater again, the

tubes were cap tightly and mix approximately 30 seconds by shaking vigorously and allowed to sit for 5 minutes at room temperature.

5. Exactly 0.2 ml of chloroform was added to each tube. Which were vigorously shake approximately 30 seconds by hand, and allowed to settle 2-3 minutes. Upon mixing, the suspension became opaque as chloroform emulsifies the cellular lipids.

6. The tubes were centrifuged at 12,000 x G at 4°C for 10 minutes to separate phases (aqueous phase in the clear upper layer contains RNA, middle white layer contains protein, and the bottom red layer contains phenol and DNA).

7. By careful pipetting, the upper aqueous phase was removed and added into the tube containing 0.5 ml Isopropanol. Then mixed by vortex briefly and we allowed RNA to precipitate for 10-15 minutes at room temperature.

8. The RNA/Isopropanol tubes were then centrifuged for 10 minutes at 12,000 x G at 4°C. Carefully the Isopropanol was poured off and discarded into chemical waste.

9. Then 1.0 ml 75% ethanol, was added into the tube (RNA tube) and centrifuged at 7,500 x G for 5 minutes.

10. The ethanol was then carefully poured off from the sample tube, discarded into the chemical waste, and the sample tube was blotted on a sterile gauze pad. The RNA was rehydrated by adding 100 µl molecular grade DEPC water to the tube.

11. The tube was then vortexed on low to medium speed for 2 minutes and spin down during 5 seconds, incubated at 56°C for 10 minutes in a water bath to dissolve the RNA pellet. The tube was never kept in water bath longer than 10 minutes as this can lead to

RNA degradation. The RNA was used immediately for RT or sometime freeze at -70°C until used. RNA prepared in this way is stable for several years.

Reverse transcription (RT): Initially the reverse transcription reaction (RTRX) working buffer was prepared: For each set of five samples, one tube RTRX mix (71.6 µl) was thawed. Followed by addition of 2.0 µl (approximately 50 units) reverse transcriptase and 2.0 µl (approximately 80 units) protector RNase inhibitor to make the working RTRX buffer. All reagents and samples were kept on ice while setting up the RTRX reactions. RNA and RT enzyme are heat labile. Briefly all RNA sample tubes in a micro centrifuge tube were spinned to avoid cross-contamination.

Working dilutions of the RT primers were prepared using molecular grade water with DEPC. RT primers: 10 µM (921F or forward primer) from 40 µM stock and 10 µM (001F primer or forward) from 40 µM stock. Both of these primers were degenerated to increase their spectrum of annealing with most of RABV and some non- rabies lyssaviruses. Three separated RT reactions were set up to further generate three independent overlapping amplicons covering the complete nucleoprotein gene (001-921B, 001-550B, 921F-304).

1. A 2 µl of the RT primers (10 µM degenerate forward primers) were added to each of the three labeled PCR reaction tubes (snap-cap size 0.5 ml) set up for each sample. Two of these tubes received 2 µl 001degFw 10 µM and the remaining one 2 µl of 921degFw 10 µM. At the end three tubes were set up for each sample with different primer sets which were labelled with sample identification and primer information on the tube as follows 001-921B, 001-550B and 921F-304.

2. Then the RNA sample tubes (from RNA extraction) were briefly vortex and centrifuged (2 minutes), and 10 µl RNA were added to each reaction tube containing the RT primer and were further centrifuged.

3. The sample and forward primer were denatured by heating the tube to 94°C for 1 min, and then cool on ice for approximately 3 minutes.

4. This was followed with the addition of 14 µl RTRX cocktail to each reaction tube (RTRX cocktail contains RTRX mix, plus reverse transcriptase and RNase inhibitor) briefly vortexed and centrifuged.

5. The thermocycler block was preheated to 42°C before placing the tubes into the thermocycler. The tubes were incubated at 42°C for 90 min with 4°C hold. At the end of the 90 minutes incubation, complimentary DNA (cDNA) was synthesized (if the sample was positive for rabies RNA) which further all was used for the PCR reaction.

Polymerase Chain Reaction (PCR): In a separate clean area where the RNA was added a PCR Pre-mix/cocktail Buffer was prepared (as previous described for every 5.4 reactions) by adding 372.6 µl molecular grade water, 43.2 µl of Tris buffer pH 8.3, 2.7 µl (5 U/ µl) Taq polymerase (Amplitaq™ PE Biosystems# N880153), 5.4 µl of 20uM no degenerated or 40uM degenerated forward Primer 1 (in this particular case 001degFw or 921degFw) and 6.75 µl of 20uM no degenerated or 40uM degenerated Primer 2 (in this particular case 921degB, 550degB and 304B) were used, and labelled appropriately. Notice that at the end we had three separated PCR cocktails 1) 001-921degB; 2) 001-550degB and 3) 1066degFw-304B.

1. The RT reaction tubes were removed from the thermocycler, and immediately placed them on ice.

2. 80 µl PCR cocktail was added to each of sample reaction tubes accordingly and centrifuged briefly.

3. The tubes were placed into the thermocycler preheated at 94°C and ran 40 cycles of PCR (1 minute at 94°C followed by 40 cycles of 94 °C for 30 seconds, 37°C for 30 seconds, and 72°C for 90 seconds; with hold at 4°C followed by 7 minute extension at 72°C).

4. At the end three RT-PCR products/amplicons (using the above mentioned PCR cocktail/primer sets) for each sample were synthesized.

Hemi-nested PCR: (Reamplification of the primary PCR reactions with primers, using only one internal primer and one of primers used for the primary RT-PCR).

1. Initially the hemi-nested PCR buffer premix was prepared in advance by adding (for every 5.4 reactions tubes) 372.6 µl molecular grade water, 43.2 µl 1 M Tris buffer 8.3, 2.7 µl Taq polymerase (Amplitaq), 71.6 µl of RTRX buffer (without enzymes and protector RNase inhibitor), 6.75 µl of forward hemi-nested primer and 6.75 µl of primer reverse hemi-nested primer in the same concentrations as for the primary RT-PCR, e.g. 20uM for no degenerated and 40uM for degenerated primers.

2. 10 µl of the primary RT-PCR product was removed from each reaction tube in a designated area where cDNA was handled, and added it to a tube containing 90 µl of hemi-nested PCR cocktail Pre-mix.

3. Hemi-nested reaction was ran with the same cycle parameters that were used for the primary RT-PCR.

Gel electrophoresis and detection of amplicons

Gel Electrophoresis for detection of amplicons was carried out in a separate laboratory for handling cDNA samples. PCR products were never opened in an area where RNA extraction or RT is performed.

1. NuSieve 3:1 (4% agarose) which is an excellent medium for high resolution separation and visualization of short amplicons of 400 bp or less required for diagnostic RT-PCR was used. To prepare the NuSieve 3:1 agarose; 2 grams of agarose was added to a polypropylene flask containing 110 ml of de-ionized water, and mix by swirling. Using a microwave, the agarose was melted completely. Cool to approximately 45 °C by running tap water along the outside wall of the flask until just warm to touch.
2. About 30 ml of the agarose gel solution was added to a mold. Two gel combs were placed into the agarose gel and was allowed to solidify approximately 30 minutes at room temperature before the combs were removed.
3. 1x TBE (Tris, boric acid, EDTA pH 8.4 at 25°C) buffer was added to the surface of the solidified gel and the gel was placed into an electrophoresis chamber
4. 10 µl of gel red (DNA dye) was added to 1ml of loading dye (blue, purple and yellow)
5. Then 7 µl of (6x) loading dye (blue, purple and yellow) was mixed with 20 µl of DNA 100 bp ladder (molecular weight markers) and added 10 ul of the molecular weight marker mixture to the first and last wells of the agarose gel after removing the combs.

6. A 7 µl of (6x) loading dye was also mixed with 20 µl of each RT-PCR/ hemi- nested PCR products and the rabies positive control sample. The mixture for each sample was added to the remaining wells of the gel, each position of the wells for each sample was recorded on a record book.
7. After all samples have been loaded; the gel was subjected to electrophoresis at 150 V for 15-20 minutes in which the dyes moves towards the negative pole
8. Specific amplicons were checked by exposing the gel to a high wave UV light source and the results were documented by photographing.
9. Samples were considered positive (“rabies specific nucleoprotein amplicons detected”) when all control measures (molecular weight maker and the controls) were met and a band of the expected size is present in the lane ran for the sample. Nucleic acid sequencing is required to confirm that the band represents cDNA from rabies virus.
10. A sample was considered negative (“Nucleic Acid Not Detected”) if all control measures were in place/met and yet there is no band present in the lane ran for the sample.- All positive results were confirmed by nucleotide sequencing, and the sequences obtained were identified via comparison with sequences available in the CDC rabies laboratory and public domain (GenBank).

Purification and Sequencing PCR

As noted earlier, each sample has three RT-PCR products. Each product was made with two primer sets as indicated (001degFw-921degB; 001degFw-550degB and 1066degFw-304B). For each of the products we set up two sequencing reactions.

1. Initially; 10 μ l each of the three RT-PCR products (amplicons) for every sample was taken into two separate tubes (labelled for forward and backward/reverse primers).
2. then to each tube 4 μ L of EXOSAP-IT single stranded DNase enzyme (which destroy all double stranded DNA) was added into the tubes.
3. Then the tubes were then click spin for 30 seconds to mix the content (enzyme + cDNA/amplicon).
4. Followed by placing the tubes into thermocycler PCR machine and set at two steps. Step 1 was set at 37°C for 15 minutes (this was to allowed the EXOSP-IT enzyme destroy all double stranded DNA from the unused primers and proteins in the initial RNA to cDNA process). The second step was set at 80°C for 15 minutes (this was to destroy the EXOSP-IT enzyme itself after finishing its work at step 1) so that only pure products were left.
5. At the end of the 30 minutes in the above steps, the tubes were removed and 2 μ l of the corresponding primers were added. For example for amplicon number 001-921 we set up 2 tubes (forward and backward containing 10 μ l of the amplicon) then 2 μ l of primer 001degFw diluted at 3.2 mM was added to 1st tube while 2 μ l of primer 921degB diluted at 3.2 mM added to the 2nd tube).
6. Then 4 μ l of the Big dye (containing regular and defective nucleotides labelled with 4 different fluorochromes + Taq Polymerase + salts) were also added to each reaction tubes.
7. The tubes containing the mixture (amplicons + primers +Big dye) were returned into the thermocycler PCR machine and ran 25 cycles in the following setting: the denaturalization temperature at 96°C for 10 seconds, the annealing temperature at 50°C

for 5 seconds and the polymerization temperature at 60°C for 4 minutes all in 25 cycles. At the end of this sequencing PCR cycles, millions of short and long chain DNA strands are formed. These products were used for purification and DNA sequencing.

Purification of sequencing PCR products: The ‘Sephacrose’ which is pore microfilters that allowed the movement and retention of small molecules into the pores and allowed the passage of big molecules between the sephacrose molecules was used for the purification process. The small molecules were the unused nucleotides, small fragments of amplicons etc not used during the sequencing PCR. The big molecules were the formed long chain single stranded DNA of PCR product that will be passed and seen in the capillary sequencer.

1. Initially 730 µL of sterile cell culture Grade A water was added to the sephacrose powder and centrifuged at 4000 rpm for 3 minutes in order to remove water from the sephacrose.
2. the tube containing the sephacrose were removed from the centrifuge and 20 µl of each sequencing PCR products (which looks pinkish due to small, big molecules and the big dye) were added to the corresponding tube/sephacrose, then centrifuged at 4000 rpm for 4 minutes.
3. The tubes were then removed from the centrifuge and 50 µl of formamide (which separates the strands of the DNA to be visible in the capillary sequencer) were added to each tube and mixed thoroughly.
4. The mixture were then removed and transferred into a 96 well plate and placed into the PCR machine and set at denaturalization temperature of 94°C for 2 minutes to allow the single strand DNAs to be linear.

5. The plate was then transferred to sequencing machine after putting fresh buffers required for sequencing analysis in the machine. An automatic sequencer 3700 DNA analyzer (Applied Biosystems) connected to a computer program was used according to manufacturer's recommendations

APPENDIX VIII: COMPLETE PROTOCOL FOR MODIFIED RFFIT TEST ON BATS SERA

Heat inactivation

Heat inactivation (HI) was deemed necessary to destroy heat labile components such as complements. In addition to complement inactivation, heat inactivation was believed to inactivate adventitious microbial contaminants such as mycoplasmas. The HI was carried out by initial click spinning of each bat serum followed by heat inactivation in water bath (at 56⁰C) for 30 minutes. Then the sera were removed and vortexed for 1 minute then clicked spin again and proceed to the micro titre RFFIT procedure or kept until used.

Modified RFFIT procedure

1. Four well glass slides were labeled (1 slide each per test serum, and 1 slide each per serum positive S, and back titration C, controls) and arranged on wetted towel paper placed on a flat metal tray (figure 3.4).

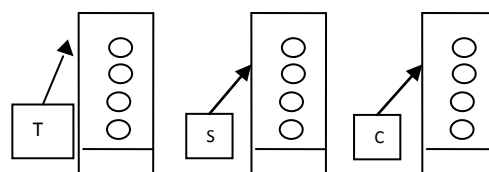


Figure 1a: 4 well slides set up for test serum T, positive serum S, and back titration C, controls

2. Using micropipette and 20 μ l pipette tips, 12 μ l of Eagle's minimum essential media (MEM) was dispensed on each well 1st – 4th on the test slides and positive serum control slide. On the cell plus virus control (back titration) slide (C) only on the 1st – 3rd

wells was 12 μ l each of the MEM being placed, but the 4th well (being sera negative control well) had 24 μ l MEM being placed.

3. Then a 5-fold serial dilution was done by taking 3 μ l of each corresponding test serum using micropipette and 10 μ l pipette tips and placed on the 1st well of the corresponding slide, pipette-mixed with the MEM (totaling 15 μ l) which became 1:5 dilution, 3 μ l was then removed from the 1st well to the 2nd, pipette-mixed to become 1:25 dilution, then 3 μ l was removed and transferred to the 3rd well (1:125) pipette-mixed and 3 μ l transferred to the 4th well pipette-mixed. Then 3 μ l was removed and discarded with the tips into waste container containing disinfectant (Isopropanol). Same was done for all the test samples.

4. The same pattern was also done in the positive serum control slide (S) using standard rabies immune globulin (SRIG). Note for the S slide, a known serum containing Challenge Virus Standard CVS-11, antibody was used which was diluted at 1:286 i.e 3.5 μ l CVS/ml of MEM).

5. This step was followed with the addition of a known virus (lyssavirus) to be tested for, and Duvenhage virus was tested for, and was added, following appropriate dilution as follows:

6. To prepare the working dilution of the virus, 10mls of MEM was aspirated using pipetting device and placed in 15ml conical tube, we then took 7 μ l of the Duvenhage virus and added to the 10ml MEM in the tube, this represent the working dilution of the virus (50FFD = 50 fluorescent focal dose). 12 μ l each of the working virus (Duvenhage virus) dilution was added to each well of all the test slides and the positive serum control slide (S) making a final volume of 24 μ l in each well.

7. For the cell plus virus (C+V) control slide, we added 12µl of the working dilution virus into the first well while 1:10 (5FFD) and 1:100 (0.5FFD) viral dilutions were added to the 2nd and 3rd well respectively. Nothing was added to the 4th well of the C+V slide which already had 24µl MEM. To prepare the 1:10 and 1:100 dilution used in the 2nd and 3rd wells respectively of the C+V slide, 900µl each were placed in two separate tubes. Then 100µl of the working virus dilution was added to the first tube to make 1:10 virus dilution, then using a new tip, 100µl of the 1:10 dilution was removed and added to the second tube to form the 1:100 virus dilutions. Then 12µl each of the working virus dilution, then 1:10 and 1:100 dilutions were added accordingly (into 1st, 2nd and 3rd wells) in the C+V control slide as mentioned above.

8. All the slides were then incubated at 37⁰C in humid chamber for 90 minutes followed by addition of cell line (to facilitate growth of un-neutralized virus after initial serum + virus incubation)

9. Mouse neuroblastoma (MNA) cell line was used. But the working dilution of the MNA was prepared at least 10 minutes before the end of the 90 minutes incubation period of the serum plus virus.

10. To prepare the cell working dilution, we took out a 1:5 MNA cell line from the incubator, examined the viability of the monolayer of the cell line and pipette out all the MEM using pipetting device. Then we used 7 ml of PBS pH 7.4 to wash the surface of the cell from the top gently to the bottom and aspirate out immediately. We changed another pipette and PBS to wash again the second time. Then we took 3 ml of trypsin and spread over the surface of the cell and placed the conical flask flat for 3 minutes to allow time for the monolayer of the MNA to peel off. At the end of the 3 minutes the flask was rock gently by holding with the right hand and hitting the left palm gently.

Then 7 ml of MEM was added to wash the bottom of the flask towards down making 10ml total volume (3ml trypsin + 7 ml MEM + Cell) that forms the working dilution of the cell, which were aspirated and used immediately at the end of the 90 minutes incubation period of the serum + virus.

11. By the end of the 90 minutes incubation of the serum + virus, the slides were taken out of the incubator and placed back in the hood and 24µl of the working MNA cell dilution were added to all the wells undergoing the test including the test slides, positive control slide (S) and the C+V control slides. After addition of the MNA cell line, the slides were returned back to the incubator under humid chamber at 37⁰C for 44 hours.

12. At the end of the 44hour incubation period, the slides were removed and discarded all the media/liquid then slides were dip rinsed in PBS pH7.4 then acetone and arranged in slides holder (taking one slide at a time).

13. Then all the slides in slides holder were placed in coplin jar containing cold acetone and fixed in acetone for 30 minutes at -20⁰ C freezer.

14. At the end of the 30 minutes fixation the slides were arranged on a wetted paper towel placed on flat metal tray and stained with Rabies anti – rabies monoclonal FITC conjugate (Fujirebio) at 1:40 dilution using 2 – 3 drops of the stain.

15. After staining the slides were incubated for 30 minutes with the stained under CO₂ (humid chamber) condition at 37⁰C.

16. At the end of the incubation the slides were removed and arranged in slide holders after blotting the excess stain then immersed in coplin jar containing PBS pH 7.5 for 5 minutes twice.

17. At the end of the second washing in PBS slides were then placed on dry paper towels on the trays, allowed to air dried for 10 minutes and then examined under fluorescent microscope at x20 magnification (Zeiss, Axioskop with up to x160 was used).

18. Ten examination fields were viewed for every well and presence of fluorescing apple green particles suggest the sample is negative. But absence of fluorescing apple green particles particularly in less than 5 of the 10 examination fields indicate presence of neutralizing antibodies and the sample was considered positive for Duvenhage virus. Results were recorded.

APPENDIX IX: COMPLETE PROTOCOL FOR STANDARD RFFIT TEST ON HUMAN SERA

RFFIT Test Procedure: The test involves the following steps

Heat Inactivation: Heat inactivate (HI) human serum at 56⁰C for 45 minutes This was carried out by initial click spinning of each serum followed by heat inactivation in water bath (at 56⁰C) for 45 minutes. Then the sera were removed and vortexed for 1 minute then clicked spin again and proceed to the standard RFFIT procedure or kept refrigerated at -4⁰C until ready for the procedure.

Dilution of test serum: An eight chamber tissue-tek slide was gotten and 4 chambers each were reserved for a test serum for a four serial 5-fold dilutions (1:5 to 1:625) as shown in the chart below (figure 3.5a). Using pipette and 100 μ l pipette tip, a 75 μ l of Eagle's Minimum Essential Media (MEM-10) was placed into the 1st chamber and 100 μ l each of the MEM into the 2nd, 3rd and 4th chambers for each labeled test serum. A 50 μ l of the test serum was then added to the 1st chamber pipette-mixed about three times then 25 μ l of the mixture was removed and added to the 2nd chamber pipette-mixed and same volume 25 μ l was removed from the 2nd to the 3rd then the 4th chamber in the same way. After mixing the 4th chamber 25 μ l, was removed and discarded with the tips into the liquid waste container leaving 100 μ l volume of the mixture in each chamber diluted at 1:5 to 1:625 (1st to 4th chamber for each test serum). New pipette tip was used for each test serum. Virus (CVS-11) working dilution was later added into the mixture.

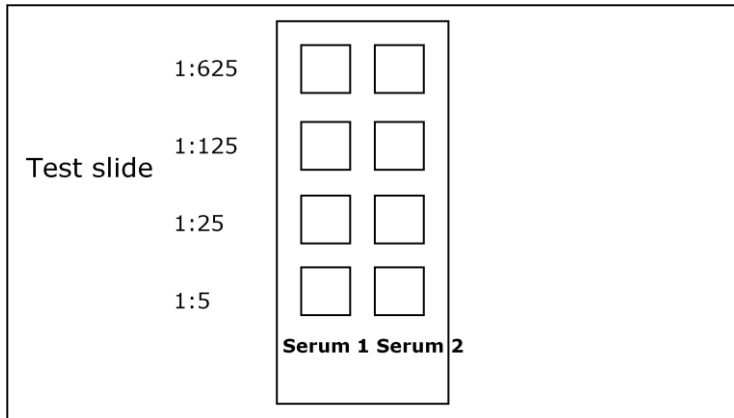


Figure 1b: Two test serum 8 chamber titration slide

Preparation of the control slide: As in the test serum, an eight chamber tissue-tek slide was gotten and 4 chambers (1st column) were reserved for reference (positive) serum (S) while the 2nd column chambers for virus back titration / Cell + Virus (C+V) see chart below (figure 3.5b) On the 1st (S) column addition of MEM-10 and positive serum 4 serial 5-fold dilution was made as in test serum dilution above. For the C+V column, 200 μ l of the MEM-10 was placed on the 1st (cell control chamber while 100 μ l each of the MEM-10 into the 2rd, 3rd and 4th chambers (Chart 3.1b) . A 100 μ l of the virus working dilution (50 Fluorescent Foci Dose FFD) was later added into S chambers, 2nd chamber of C+V column while 1:10 and 1:100 dilution of the virus into 3rd and 4th chambers.

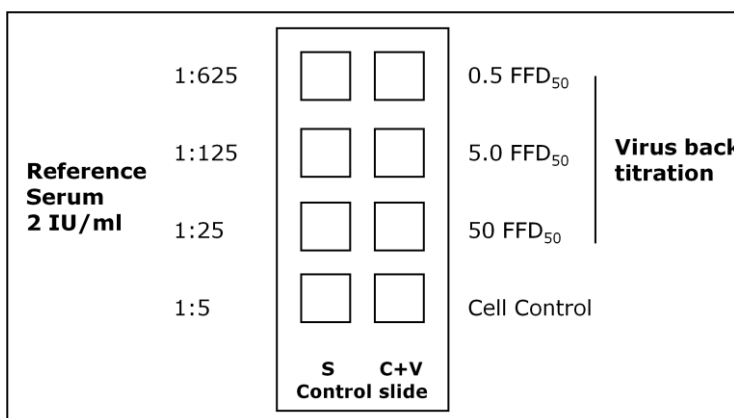


Figure 2b: Positive serum (S) and virus back titration (C+V) control slide

Addition of the rabies Challenge Virus Standard (CVS-11): As recommended in the CDC RFFIT protocol (CDC, 2006), we used MEM-10 as diluent for CVS-11 virus, A 1:200 dilution of CVS-11 in MEM-10 placed in a 50 ml centrifuge tube will yield working virus suspension containing 50 FFD₅₀ / 0.1 ml. Sometimes we made addition of 80µl of the stock CVS-11 into 16ml of the MEM-10 to a 50FFD suspension/working dilution sufficient for 16 slides.

Usually, 100 µl of the working dilution was added to each well (chamber) of the test slides, the positive serum wells and the 2nd well of the C+V column. But only 100µl of the 1:10 and 1:100 dilution of the working virus dilution were added to the 3rd and 4th wells of the C+V column respectively. No virus was added into the 1st (cell control) well. The 1:10 and 1:100 were prepared by placing 900 µl each into 2 separate screw cap tubes then 100 µl of the working virus dilution was added into the 1st tube, this forms the 1:10 dilution. Then 100µl of the 1:10 dilution was removed and added to the 2nd tube, this forms the 1:100 dilutions which were used.

Following addition of the CVS-11 virus the mixture (serum + virus) were incubated at 37⁰C in humid chamber for 90 minutes before addition of the cell line (Mouse Neuroblastoma, MNA cell line).

Preparation and Addition of Mouse Neuroblastoma, MNA Cells:

To prepare the cell working dilution, we took out a 1:5 (2-day old) monolayer MNA cell line or 1:10 (3-day old) monolayer MNA cell line from the incubator, examined the viability of the monolayer of the cell line and pipette out all the MEM using pipetting device. Then we used 7 ml of PBS pH 7.4 to wash the surface of the cell from the top gently to the bottom and aspirate out immediately.

We changed another pipette and PBS to wash again the second time. Then we took 3 ml of trypsin and spread over the surface of the cell and placed the conical T75 flask flat for 3 minutes to allow time for the monolayer of the MNA to peel off. At the end of the 3 minutes the flask was rock gently by holding with the right hand and hitting the left palm gently. Then 7 ml of MEM was added to wash the bottom of the flask towards down making 10ml total volume (3 ml trypsin + 7 ml MEM). A healthy 3-day-old monolayer of MNA cells in a T75 flask contains approximately 15×10^6 cells. Further dilution was necessary to give 5.0×10^5 cells/ml.

To achieve this, the 10ml total volume (3 ml trypsin + 7 ml MEM) was further dissolved in 20 ml MEM in a 50ml centrifuge tube. This final volume (30 ml) forms the MNA cell working dilution allowing approximately 2.0 ml of 5.0×10^5 cells/ml for each 8-chamber slide in the test. A 0.2 ml (200 μ l) of the 5×10^5 cells / ml was added to each chamber of the slides at the end of the 90 minutes (serum + virus) incubation, starting with the cell control on the bottom right corner of the control slide giving approximately 100,000 MNA cells per well. Following addition of the MNA cells, the mixture (serum + virus + cell) were returned and incubated at 37°C under humid chamber for 20hours. At the end of the 20hours incubation, staining was conducted.

Note: 1:5 (2-day old) or 1:10 (3-day old) MNA cell line are prepared by the following steps. A 23 mls and 24mls of MEM-10 were added each into two separate new empty T75 flasks. Then 2ml and 1 ml of the total 10ml (3 ml trypsin + 7ml MEM-10) of the previous existing MNA cell line were added into 23ml and 24ml MEM in the two separate T75 flasks to forms a final volume of 25mls each. The 23ml flask was the 1:5 dilutions while 24ml flask was the 1:10 dilution. These flasks were incubated at 37°C

for 48 hours (for 1:5) and 72 hours (for 1:10) to yield a 2-day and 3-day old MNA monolayer cells respectively for use or for another run.

Testing the viability of the MNA cell line was done using inverted microscope to check for death, floating or interrupted/immature monolayer before used. Absence of any of the above defects allows the MNA monolayer cell good candidate for use in the subsequent procedure.

Fixation and staining of the slides: By the end of the 20 hours incubation, the samples were taken to the hood and the mixture were discarded and the chambers were detached and discarded into waste container. The slides (one at a time) were dip rinsed 3 times in PBS pH 7.5 (no. 4886) then in cold acetone, each in small plastic beakers and finally arranged on a slide holder. The slide holder with the slides were then placed into coplin jar containing cold acetone and taken into freezer and left for a minimum period of 30 minutes to fix the cells. At the end of the 30 minutes fixation the slides were removed and arranged moistened paper towel on a metal tray. The slides were allowed to air-dried for 10 minutes then each well was stained with 1:40 dilution of anti-nucleocapsid monoclonal FITC rabies antibody, Fujirebio Inc. USA. The stained slides were incubated at 37 °C for 30 minutes in humid chamber. At the end of the 30 minutes incubation, the slides were removed excess stain discarded then arranged on slides holders and dropped in coplin jar containing PBS pH 7.5 (no.4886) to wash for 5 minutes twice. After the washing steps, the slides were arranged on a dried paper towel on a metal tray, air-dried for 10 minutes and examined under fluorescent microscope for the presence or absence of fluorescing virus particles.

Examination of slides and Interpretation of results: Each of the 8-well Tissue-Tek slide chambers contains 25 to 50 distinct microscopic fields when observed at 160 to

200 times magnification. Twenty examination fields were viewed for each well under a fluorescent microscope (Zeiss, Axioskop with up to 200X was used). The numbers of fields which contain fluorescing cells (even if it was one particle/cell) were counted starting in one of the corners of the well as depicted in figure 3.6. The number of positive fields out of 20 fields/ chamber examined was recorded on a RFFIT results data sheet as exemplified for the control slide in figure 3.7. Presence of less than 10 infected fields/chamber per test serum in 1:5 dilution (suggesting presence of neutralizing antibodies), was considered positive for rabies antibody.

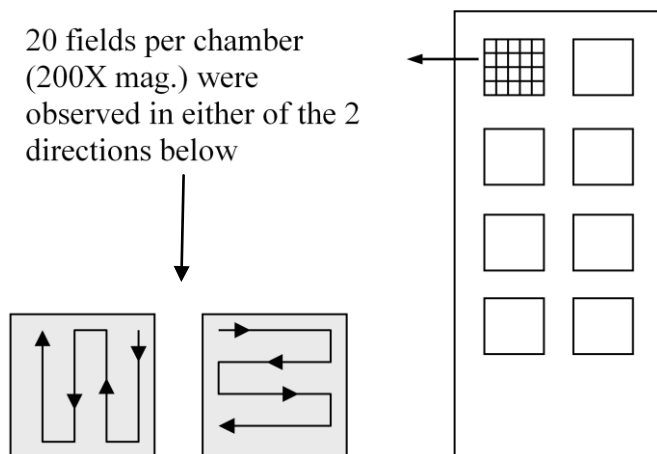


Figure 3b: Mode of reading stained slides in RFFIT procedure

RFFIT data result sheet

S			C + V
1:625	20	5	0.5 FFD ₅₀
1:125	6	20	5.0 FFD ₅₀
1:25	0	20	50 FFD ₅₀
1:5	0	0	Cell Control


Control slide

Figure 4b: RFFIT data result sheet for the control slide

Determination of the potency of test serum in international units (IU) per ml: The potency (titre) of the test serum antibody was calculated based on the Reed-Muench Method (Reed and Muench, 1938) as described in the antibody titre table (Appendix C) contained in the CDC Standard Operating Procedure for RFFIT for determining rabies virus neutralizing antibody (CDC, 2006). Using the expression below:

$$\text{Number of IU/ml} = \frac{\text{End-point titre of the test serum}}{\text{End-point titre of the reference}} \times 2 \text{ IU / ml in the reference serum}$$

APPENDIX X: Ahmadu Bello University Teaching Hospital Ethical committee's approval



Ahmadu Bello University Teaching Hospital
P.M.B. 06, Shika - Zaria, Kaduna State, Nigeria. 069-876305
website: www.abuth.org abuthshika@yahoo.com Abuthshika@gmail.com

Chairman of Board:
Chief Medical Director: DR. LAWAL KHALID, MBBS, FMCS, FWACS, FRCS(ED) mni
Chairman, Medical Advisory Committee: DR. ABDULLAHI MOHAMMED, MBBS, FWACP FICS
Director of Administration: BARR. ISHAK BELLO, LL.B, BL, LL.M, PGDM, AHAN, FCAI

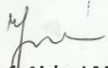
Our Ref:
ABUTH/HREC/TRG/36 13th August, 2012


Dr Garba Ahmed
Dept of Vet. Public Health & Preventive Medicine,
Faculty of Veterinary Medicine,
Ahmadu Bello University,
Zaria.

ETHICAL CLEARANCE

Your application for ethical clearance on the research proposal titled. "EPIDEMIOLOGY, G.I.S. MAPPING AND MOLECULAR CHARACTERIZATION OF RABIES VIRUS IN DOGS AND BATS IN NIGER STATE, NIGERIA." refers.

This is to convey ethical approval for you to commence the study. The ABUTH Scientific and Health Research Ethics Committee requires an annual update from the principal investigator.


Prof. Aisha I Mamman
Asst. Dean Postgraduate Studies
For: Chairman, ABUTH HREC



APPENDIX XI: Niger State Ministry of Health Ethical Committee's approval



NIGER STATE HOSPITALS MANAGEMENT BOARD
GENERAL HOSPITAL, MINNA

ADDRESS:
No. 1 Hospital Road,
P.M.B. 2, Minna
Niger State, Nigeria.
Tel: 0816999223
E-mail: genhosp.minna@yahoo.com

25th January, 2012

The Head of Department,
Veterinary Public Health & Preventive Medicine,
Ahmadu Bello University Zaria, Nigeria.

Through: **Dr. Garba Ahmed,**
PhD Student, A.B.U, Zaria.

Sir,

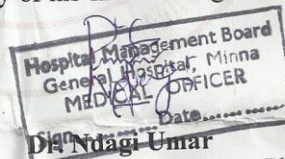
RE: DR. GARBA AHMED (PhD/VET-MED/04908/2009-2010)
RESEARCH, ETHICS AND PUBLICATION COMMITTEE'S APPROVAL
TO CONDUCT RESEARCH

The bearer, a PhD student from your department sought for permission to conduct research on Rabies relating to dogs, bats and human subjects in Niger State, Nigeria. Of particular interests are questionnaire surveys, hospital records of human rabies and dog bite injuries, as well as collecting blood sera from occupational group that slaughter dogs (butchers) / dog handlers in the state.

The committee, after going through his proposal 'Epidemiology, G.I.S Mapping and Molecular Characterization of Rabies Virus in Dogs and Bats in Niger State, Nigeria' has given him the approval to conduct his research.

The hospital management will like a copy of his final findings to be submitted to the committee.

Thank you for your cooperation.



Dr. Ndagi Umar
(B. Pharm, M.Pharm, Pharm. D)
Secretary REPC
For: Head Hospitals Services

Cc - Centers for Disease Control and Prevention, Atlanta, USA
- National Veterinary Research Institute, Vom- Nigeria

APPENDIX XII:50% Serum End-point Titers Corresponding to the Numbers of Fluorescing Foci (calculated by the Reed-Muench Method)

XII 50% Serum End-point Titers Corresponding to the Numbers of Fluorescing Foci (calculated by the Reed-Muench Method)																					
Serum dilution		Number of Fields with Fluorescing Foci/Total fields																			
1:5 1:25	0/20	1/20	2/20	3/20	4/20	5/20	6/20	7/20	8/20	9/20	10/20	11/20	12/20	13/20	14/20	15/20	16/20	17/20	18/20	19/20	20/20
	20/20	20/20	20/20	20/20	20/20	20/20	20/20	20/20	20/20	20/20	20/20	20/20	20/20	20/20	20/20	20/20	20/20	20/20	20/20	20/20	20/20
	11	11	10	9	9	8	7	7	6	5	<5	<5	<5	<5	<5	<5	<5	<5	<5	<5	<5
1:25 1:125	0/20	1/20	2/20	3/20	4/20	5/20	6/20	7/20	8/20	9/20	10/20	11/20	12/20	13/20	14/20	15/20	16/20	17/20	18/20	19/20	20/20
	20/20	20/20	20/20	20/20	20/20	20/20	20/20	20/20	20/20	20/20	20/20	20/20	20/20	20/20	20/20	20/20	20/20	20/20	20/20	20/20	20/20
	56	54	50	50	45	42	40	36	33	29	25	21	19	17	16	14	13	13	13	12	--
1:125 1:625	0/20	1/20	2/20	3/20	4/20	5/20	6/20	7/20	8/20	9/20	10/20	11/20	12/20	13/20	14/20	15/20	16/20	17/20	18/20	19/20	20/20
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	280	270	250	250	230	210	200	180	170	145	125	110	95	85	75	70	70	65	60	60	--
1:625 1:3125	0/20	1/20	2/20	3/20	4/20	5/20	6/20	7/20	8/20	9/20	10/20	11/20	12/20	13/20	14/20	15/20	16/20	17/20	18/20	19/20	20/20
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1:78125 1:390625	0/20	1/20	2/20	3/20	4/20	5/20	6/20	7/20	8/20	9/20	10/20	11/20	12/20	13/20	14/20	15/20	16/20	17/20	18/20	19/20	20/20
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	174602	167448	159750	151585	142857	133991	123738	113283	102160	90434	78124	67491	59744	53887	49326	45687	42724	40269	38206	36450	--

APPENDIX XIII: Amino acids sequences of the 3 positive dog brain samples from Niger State, Nigeria

>252_Minna_2012

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>276_Bida_2012

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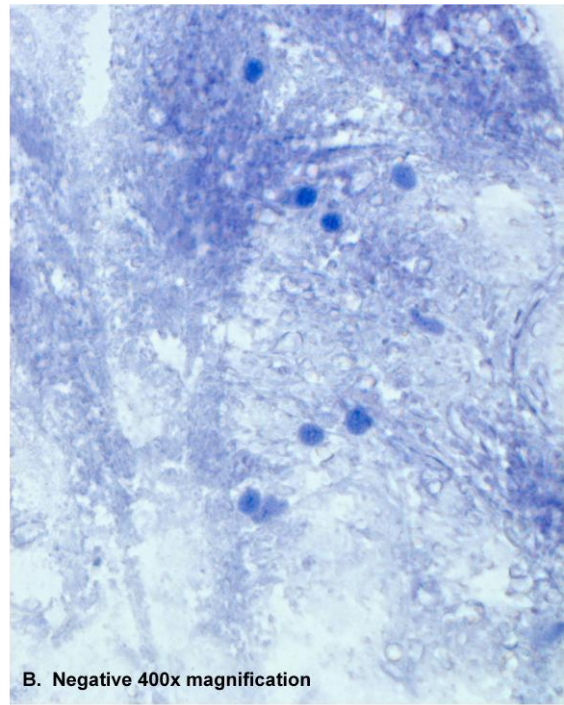
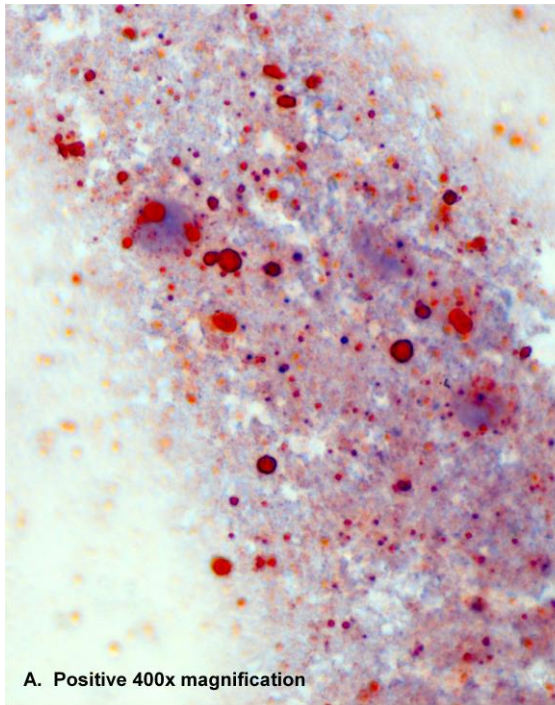
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GAAGCCGTTTATACTCGGATCATGATGAACGGAGGGCGACTAAAGAGA
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CAAACCTCATTTGCCGAGTTCCTAAACAAAACATACTCTAGTGA CTCA

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ATGGATGCCGACAAGATTGTATTCAAAGTCAATAATCAGGTGGTTTCCT
TGAAGCCAGAGATTATTGTGGATCAATATGAGTACAAATACCCTGCTAT
CAAAGACTTGAAGAAGCCCAGTATAACCTTAGGGAAAGCCCCCGATTT
AAACAAAGCATAACAAGTCAGTTTTATCAGGCATGAATGCTGCCAAGCT
TGATCCTGACGATGTATGTTCCCTATTTGGCAGCTGCGATGCAGCTCTTT
GAAGGGACATGTCCTGAAGACTGGACCAGCTATGGAATCTTGATTGCA
AGAAAGGGAGACAAGATCACCCCAGATTCTCTTGTAGAGATCAAGCGT
ACAGATATAGAAGGGA ACTGGGCTCTAACAGGAGGTATGGA ACTGACG
AGAGACCCACGGTTTCCGAACATGCATCCTTAGTCGGTCTGCTCTTG
AGTCTGTATAGGTTGAGCAAAATATCAGGACAAAACACAGGCAACTAC
AAGACAAACATTGCAGATCGGATAGAGCAGATTTTTGAGACAGCCCCT

TTCGTTAAAATTGTGGAACATCATACCCTGATGACAACTCATAAAATGT
GCGCTAATTGGAGTACCATAACCGAACTTCCGATTCTTGGCCGGAACAT
ACGACATGTATTTCTCCCGAATTGAGCATCTATATTCAGCTATCAGAGT
GGGCACAGTAGTCACTGCTTATGAAGATTGCTCAGGGCTGGTGTTCGTT
CACAGGGTTCATAAAACAGATAAATCTCACTGCGAGAGAAGCGATACT
ATATTTCTTCCACAAGAAGCTTTGAAGAAGAGATACGAAGAATGTTCGA
GCCAGGGCAGGAGACTGCGGTTCCCTCACTCCTATTTCAATCACTTCCG
CTCGTTGGGTCTGAGCGGGAAGTCCCCGTATTCGTCAAATGCAGTAGG
TCATGTATTCAATCTCATTCACTTTGTTGGATGTTATATGGGTCAGGTC
AGGTCCTTAAATGCGACGGTCATTGCTGCATGTGCTCCTCATGAGATG
TCTGTCCTAGGGGGTTATCTAGGTGAAGAGTTTTTTGGAAAGGGCACA
TTTGAAGAAGATTCTTTAGAGACGAAAAGAAGCTCCAAGAATATGAG
GCAGCTGAATTGACGAAGACTGATGTGGCACTGGCAGATGACGGAACC
GTCAACTCTGATGATGAGGATTACTTTTCCGGTGAGACCAGGAGTCCT
GAAGCCGTTTATACTCGGATCATGATGAACGGAGGGCGACTAAAGAGA
TCTCACATAAGGAGATATGTTTCAGTTAGTTCCAATCATCAAGCTCGCC
CAAACCTCATTTGCTGAGTTTCTAAACAAAACATATTCTAGTGACTCA

APPENDIX XIV: The direct rapid immunohistochemistry test (DRIT) slide on brain sample touch impressions A= Positive. B= Negative



The direct rapid immunohistochemistry test (DRIT) on fresh brain touch impressions
A. Positive red specific staining identification of rabies virus nucleocapsid
B. Negative

APPENDIX XIV: The direct rapid immunohistochemistry test (DRIT) slide on brain sample touch impressions A= Positive. B= Negative

APPENDIX XV: Rabies virus virion, is a bullet shaped with five genomic components
(Source: Warrell and Warrell, 2004).

