EVALUATION OF THREE DIAGNOSTIC METHODS FOR RABIES VIRUS DETECTION IN FRESH, FROZEN ARCHIVED AND CITRATE BUFFER TREATED FORMALIN-FIXED DOG BRAIN TISSUES

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AUGUST, 2018

DECLARATION

I declare that the work in this Dissertation entitled "**Evaluation of Three Diagnostic Methods for Rabies Virus Detection in Fresh, Frozen Archived and Citrate Buffer Treated Formalin-Fixed Dog Brain Tissues"**has been carried out by me in the Department of Veterinary Microbiology. The information derived from the literature has been duly acknowledged in the text and a list of references provided. No part of this dissertation was previously presented for another degree or diploma at this or any other institution.

God'spower Richard OKOH ____________________ ____________________

Signature Date

CERTIFICATION

This dissertation entitled "**EVALUATION OF THREE DIAGNOSTIC METHODS FOR RABIES VIRUS DETECTION IN FRESH, FROZEN ARCHIVED AND CITRATE BUFFER TREATED FORMALIN-FIXED DOG BRAIN TISSUES"** by God'spower Richard OKOH meets the regulations governing the award of the degree of Masters of Science of the Ahmadu Bello University, and is approved for its contribution to knowledge and literary presentation.

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DEDICATION

This dissertation is dedicated to God Almighty for making this work a reality.I am also pleased to dedicate this thesis to my Wife, Barr. MrsEsther AyeniOkohand my children, Ruby ObehiOkoh and Michael OsemegbeOkoh, may God continue to bless you all.

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ABSTRACT

Rabies is recognized as a global zoonosis yet it is remarkably neglected, despite thehigh fatality of the disease following onset of clinical signs. It remains a threat under-appreciated by healthcare practitioners in many endemic areas, often owing to lack of post-mortem evaluation and public health reporting. This study aimsto evaluate rabies virus detection by Direct Fluorescent Antibody Test (DFAT), Enzyme Linked Immuno-Sorbent Assay (ELISA) and Rapid Immuno-Diagnostic Test (RIDT) in fresh, frozen archived and citrate buffer treated formalin-fixed dog brain tissues.A total of fifty confirmed DFAT positive and negative dog brain tissues preserved at -20°C were collected from the archives of the Central Diagnostic Laboratory, National Veterinary Research Institute, Vom, Nigeria for analysis using DFAT, ELISA and RIDT. This study also evaluated the rabies status of fresh brain specimens from 40 rabies-suspected cases. A comparable brain specimen from each case was fixed in neutral buffered formalin and examined by the DFAT following treatment with sodium citrate buffer. We found 96% agreement of ELISA and DFAT for rabies antigen detection [Cohen's kappa coefficient (κ) = 0.834; 95% C.I.,Concordance coefficient =78%: 95% C.I.] and 54% agreement of RIDT and DFAT (κ = 0.170; 95% C.I., concordance coefficient= 17%: 95% C.I.). The sensitivities of ELISA and RIDT were 95.5% and 47.6% compared to DFAT respectively, while the specificities of ELISA and RIDT were 100% and 87.5% respectively. ELISA performed better than RIDT and recorded equivalent result with DFAT. Following treatment with sodium citrate buffer, DFAT on formalin-fixed brain tissues exhibited a sensitivity of 100% in comparison to DFAT on fresh-tissues. No false positive resultwas obtained in formalin-fixed DFAT procedure, demonstrating 100% specificity. There was no apparent difference

in the intensity of fluorescence between DFAT on fresh samples and formalin-fixed DFAT (κ = 1.000; 95% C.I.,concordance = 98%; 95% C.I.). The ELISA is as reliable a diagnostic method as the DFAT which is the gold standard for rabies diagnosis. It has an advantage of being able to analyze large number of samples at the same time, making it more suitable for epidemiological studies and for laboratories that cannot perform DFAT. The unsatisfactory result of RIDT in this study reiterates the need to perform an adequate test validation before it can be used in the laboratory for rabies diagnosis.Rabies antigen retrieval from formalin-fixed brain tissues with heated sodium buffer and its subsequent detection by DFAT is as valid and reliable a procedure for rabies diagnosis as the DFAT on fresh samples.Formalin could therefore, be used henceforth to fix tissues of rabies suspected cases for routine diagnosis, transportationor archival purposes.

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LIST OF ABBREVIATIONS

AR: Antigen Retrieval

CDC: Centres for Disease Control and Prevention

CI: Confidence Interval

CNS: Central Nervous System

DFAT: Direct Fluorescent Antibody Test

ELISA: Enzyme Linked Immuno-Sorbent Assay

ICTV: International Committee on Taxonomy of Viruses

OIE: Office International des Epizooties

RABV: Rabies Virus

PBS: Phosphate Buffered Saline

RIDT: Rapid Immuno-Diagnostic Test

RNA: Ribonucleic Acid

RNP: Ribonucleoprotein

WHO: World Health Organisation

CHAPTER ONE

INTRODUCTION

1.1 Background of the Study

Rabies is a central nervous system (CNS) disease that is almost invariably fatal following the onset of clinical signs. The causative agent is rabies virus (RABV), a negative-sense, single stranded ribonucleic acid (RNA) virus of approximately 12 kilo base pairs (kbp) (Zhou *et al*., 2013) with cylindrical morphology (bullet-shaped) and is the type species of the *Lyssavirus* genus of the Rhabdovirus family, which has a relatively simple, modular genome organization and encodes five structural proteins: a RNA-dependent RNA polymerase (L), a nucleoprotein (N), a phosphorylated protein (P), a matrix protein (M) and an external surface glycoprotein (G) (Finke and Conzelmann, 2005). Neuro-invasiveness and neurotropism are the main features that define the pathogenesis of rabies (Dietzschold *et al*., 2008).

Globally, estimates show that human death due to endemic dog-mediated rabies is most prevalent in Asia, with the highest occurrence and mortalities recorded in India. Next to Asia is Africa; however, absence of dependable data has led to uncertainty in the estimation of the disease burden (Mshelbwala *etal*., 2013). India has the most prevalent rate of human rabies in the world, basically due to large number of stray dogs (Menezes, 2008). Rabies which is known to be endemic in Nigeria has the domestic dog as the primary host of the causative virus (Barecha *etal*., 2017). It first occurred in the country in humans in 1912 and was first diagnosed in the laboratory in a dog in 1925 (Boulger and

Porterfield, 1958), since then, human and animal rabies cases have been reported in all the regions and ecological zones of Nigeria annually (Atuman *etal*., 2014; Tekki *etal*., 2016).

Rabies which constitutes substantial public health problem is transmitted following a deep bite or scratch by an infected animal(Kularatne*et al*., 2016). Dogs are the main host and transmitter of rabies to both animals and humans. They are the cause of human rabies deaths in Asia and Africa in more than 95% of all cases (WHO, 2017). Rabies virus is one of the most significant zoonotic pathogen of bat origin (Kia*et al*., 2013). Bats are the source of most human rabies deaths in the Americas (Escobar*et al*., 2010). Bat rabies has also recently emerged as a public health threat in Australia and Western Europe. Human deaths following exposure to foxes, raccoons, skunks, jackals, mongooses and other wild carnivore host species are very rare. Transmission can also occur when infectious material (usually saliva) comes into direct contact with human mucosa or fresh skin wounds (Carter and Saunders, 2007). Human-to-human transmission by bite is theoretically possible but has never been confirmed. Rarely, rabies may be contracted by inhalation of virus-containing aerosol or via transplantation of an infected organ (Srinivasan, *et al*., 2005). Ingestion of raw meat or other tissues from animals infected with rabies is not a confirmed source of human infection (World Health Organization, 2016).

Because rabies affects the central nervous system, it is nearly always associated with behavioral changes that may manifest in many different ways. Classically, rabies has been described as having a prodromal phase followed by either an excitive furious form (hyperactivity), or a paralytic dumb form with profuse salivation in dogs (Fekadu, 1991). In the early symptom (prodomal) stage of rabies infection, the dog will show only mild signs of CNS abnormalities. This stage will last from one to three days. Most dogs will

then progress to the furious stage, the paralytic stage, or a combination of the two, while others succumb to the infection without displaying any major symptoms. Furious rabies is characterized by extreme behavioral changes, including overt aggression and attack behavior. Paralytic rabies, also referred to as dumb rabies, is characterized by weakness and loss of coordination, followed by paralysis. The following are some of the symptoms in dogs: pica, fever, seizures, paralysis, hydrophobia, dropped jaw, inability to swallow, change in tone of bark, muscular lack of coordination, unusual shyness or aggression, excessive excitability, constant irritability, changes in attitude and behavior, paralysis in the mandible and larynx, excessive salivation (hyper-salivation), or frothy saliva (William *et al*., 2011). Initial symptoms of rabies in humans are often nonspecific such as fever and headache (Giesen *et al*., 2015). As rabies progresses and causes inflammation of the brain and/or meninges, symptoms can include slight or partial paralysis, anxiety, insomnia, confusion, agitation, abnormal behavior, paranoia, terror, and hallucinations, progressing to delirium and coma (Cotran *et al*., 2005; Giesen *et al*., 2015). The person may also have hydrophobia (World Health Organization, 2016). Death usually occurs within days of the onset of these symptoms.

Rabies can be difficult to diagnose, because, in the early stages, it is easily confused with other diseases or with aggressiveness (Dacheux *et al*., 2010). History and clinical signs gives only indication of disease, however, definite diagnosis of rabies relies on the laboratory demonstration and identification of virus or its specific components(Mani and Madhusudana, 2013).Tests that can be applied to formalin-fixed tissues benefit from freedom from sample preservation concerns and risk associated with transporting and processing samples containing infectious virus(Wunnerand Jackson, 2010). A potential

limitation of the procedure to work with formalin-fixed preparation is the inability to cultivate and amplify the virus from an inactivated sample (Shankar, 2009). Microscopic examination of specimens is one of the laboratory routines that allows for the rapid identification of rabies virus-specific antigen, irrespective of geographical location and condition of the host. Direct fluorescent antibody test (DFAT) is the 'gold standard' method for diagnosing rabies and its use has been recommended by the World Health Organization (WHO) (Tekki*et al*., 2016). However, Fooks *et al*. (2009), noted that decomposed samples can affect the sensitivity and specificity of DFAT and this may be due to thedifficulty to differentiate specific fluorescence due to N antigen from nonspecific fluorescence which may result from bacterial contamination (Mani and Madhusudana, 2013).Reliable results are obtained only when fresh brain tissue is used; however, DFAT can also be applied to specimens preserved in 50% glycerol saline after rigorous washing of the specimens with normal saline (Durr *et al*., 2008).Immunofluorescence method applied to formalin fixed tissues was significantly less sensitive than the DFAT method on fresh brain tissues. With recent modifications on the method to achieve better immunofluorescence (Warner *et al*., 1997, 1999), the procedure is approaching comparable sensitivity with DFATon fresh tissues (Whitfield*et al*., 2001). Arecently described method for the detection of RABV antigen from postmortem samples is the rapid immunodiagnostic test (RIDT), a useful method for rabies diagnosis without the need for laboratory equipment(Eggerbauer*et al*., 2016). This immune-chromatographic lateral flow strip test is a one-step test that facilitates low-cost, rapid identification of viral antigen. Though tests based on immune-chromatographic techniques can be used as a rapid screening test in animals, they need to undergo considerable improvement and evaluation

on human clinical samples, before they can be recommended for use in the diagnosis of human rabies(Mani and Madhusudana, 2013).Apart from the recently developed RIDT(Mani and Madhusudana, 2013) for rabies antigens detection, a validated Enzyme Linked Immuno-Sorbent Assay (ELISA) also known as Rapid Rabies Enzyme Immuno-Diagnosis (RREID) for the rapid post-mortem detection of antigen in brain samples is also available. It has been used to evaluate large number of specimens under field conditions in different countries (Bourhy *et al*., 1989; Morvan *et al*., 1990) and its use is recommended by the World Health Organization (World Health Oraganization, 2005). An added advantage is that partial decomposition of the brain will not affect the test result (Duong *et al*., 2016).

1.2 Statement of the Research Problem

Rabies is recognized as global zoonosis yet remains remarkably neglected, despite unmatched lethality. In 2010, approximately, 26,000 people died from rabies, down from 54,000 in 1990. Most of these deaths occurred in Asia and Africa (Lozano *et al*., 2012). An estimated 20,000 people die every year from rabies in India (more than a third of the global toll) (Harris, 2012). As of 2015, China had the second-highest number of cases (approximately 6,000), followed by the Democratic Republic of the Congo (5,600) (Hampson *et al*., 2015). In Asia and in parts of the Americas and Africa, dogs remain the principal host(WHO, 2017). Mandatory vaccination of animals is less effective in rural areas, especially in developing countries where pets may not be privatelykept and their destruction may be unacceptable(Ogun*et al*., 2010). More than 10,000 Nigerians are exposed to rabies annually, and about 1,000 annual cattle mortality are reported (Tekki*et*

al., 2016). In Nigeria, human and animal rabies cases are on the increase annually despite the availability of vaccines for its control (Ogunkoya, 2008; Hambolu*et al*., 2014).

Rabies remains a threat underappreciated by healthcare practitioners in many endemic areas, often owing to lack of rapid diagnostic tools, post-mortem evaluation, and public health reporting(Lembo*et al*., 2010). Although most veterinary laboratories in Africa have sufficient personnel capacity to diagnose rabies in animals, routine diagnosis is often limited by lack of laboratory equipment and reagents (Mallewa *et al*., 2007; Nel, 2013).

In much of the developing world, rabies surveillance and diagnosis in domestic and wild animals are severely constrained (Lembo *et al*., 2006). The mouse inoculation test (MIT) is a virus isolation method usually for confirmation with sensitivity of almost 100% (Ehizibolo *et al*., 2009). However, Velleca and Forrester (1981) reported that in rare cases, false positives may occur, and it takes a period of 21 days before the result may be available which may be too late to be valuable in treatment decisions for humans (Ehizibolo *et al*., 2009). The direct microscopic examination by the Seller's staining test (SST), which due to its low sensitivity and reliability (Tierkel and Atanasiu, 1996) had long been substituted with the more sensitive, specific and reliable DFAT in most rabies laboratories globally. Decomposed samples can, however, reduce the sensitivity and specificity of the DFAT (Fooks*et al*., 2009) and the need for an ultraviolet (UV) microscope makes the test very expensive to perform in developing countries (Dürr *et al*., 2008; Ehizibolo *et al*., 2009). Difficulties in obtaining diagnostic results from field material due to inadequate sample preservation and lack of rapid tests have led to widespread under-reporting of the disease (Lembo *et al*., 2006). Consequently, the true public health impact of rabies has been greatly under-estimated and political commitment

for its control has been lacking (Coleman *et al*., 2004). There is a need for more economical and user friendly method of preservation and diagnosis, particularly for use in developing countries. Therefore, this study sought to analyze rabies virus detection by DFAT, ELISA and RIDT in fresh, frozen archived and citrate buffer treated formalin-fixed dog brain tissues.

1.3 Justification for the Study

There are 60,000 deaths from rabies annually. There is a higher prevalence in rural areas and it disproportionately affects children. Rabies is usually fatal after symptoms develop (World Health Organisation, 2016).

Rabies urgently requires strengthening of new and existing diagnostic methodology in order to overcome the threat it poses (Singathia *et al*., 2012). It is pertinent to note that accurate laboratory diagnosis of rabies in an animal has a direct effect on human treatment (Messenger *etal*., 2003). Therefore, rapid and accurate diagnosis of rabies is vital to human post-exposure prophylaxis, steer epidemiologic surveillance and provide adequate information for the design of rabies control programs (Ehizibolo *et al*., 2009).

The DFAT has been regarded as the 'gold standard' method for rabies diagnosis for many years despite the numerous limitations associated with this technique (Dürr *et al*., 2008; Fooks*et al*., 2009). Enzyme Linked Immunosrbent Assay (ELISA) is suitable for analysing samples not preserved in good conditions (Morvan *et al*., 1990). It is rapid, easy to use, and relatively safe because they do not require the use of infectious virus, making them suitable for use in developing countries (Dacheux *et al*., 2010).The RIDT is rapid and simple, and does not require any special equipment or technical expertise(Mani and Madhusudana,

2013).The RIDT can be implemented in frontline laboratories to improve the surveillance and control of rabies in remote places from which the transport of samples to a central laboratory would be difficult or even impossible or where classical rabies laboratory diagnosis using recommended techniques cannot be established for financial or logistical reasons(Duong*et al*., 2016).

To ensure reliable results, the brain tissues to be tested must be preserved by chilling or freezing. The transportation of the tissues to the rabies laboratory often presents difficulties, since facilities for refrigeration are usually limited in tropical countries(Umoh and Blenden, 1981; Aguiar*et al*., 2013; Duong *et al*., 2016). Even when ice or dry ice and insulated packages are available, the delays involved in transportation often result in deterioration of the tissues en route, which increases the likelihood of false negative results.Because of these difficulties, many specimens are not submitted for laboratory examination, even though it is important that decisions on post-exposure prophylaxis should, whenever possible, be based upon the results of laboratory tests(Umoh and Blenden, 1981; Duong *et al*., 2016). Also, as specimens are submitted only from areas thought to be accessible to laboratory services, any surveillance data derived arehighly biased.

There is a great need for a chemical method of tissue preservation that would allow storage for extended periods without freezing and would still be compatible with conventional tests and the recently developed rapid tests such as ELISA and RIDT. If this were available, a higher proportion of decisions regarding post-exposure immunization could be based upon laboratory data; cumulative surveillance data would be morerepresentative and meaningful; and the infectionhazard to laboratory workers would be reduced(Umoh and

Blenden, 1981). It wasgenerally felt that chemical preservation with formalinmakes DFAT examination virtually impossible. However, with the recent modification to achieve better immunofluorescence (Warner *et al.*, 1997), this procedure may now be approaching comparable sensitivity to DFAT on fresh tissue.

1.4 Aim of the Study:

To evaluate rabies virus detection by DFAT, ELISA and RIDT in fresh, frozen archived and citrate buffer treated formalin-fixed dog brain tissues.

1.5 Objectives of the Study

- To detect rabies virus antigen in frozen archiveddog brain tissues using DFAT, ELISA and RIDT.
- To retrieve rabies virus antigen from formalin-fixeddog brain tissues usingsodium citrate buffer.
- To detect rabiesantigen in fresh and citrate buffer treated formalin-fixed dog brain tissues using DFAT.
- To detect rabies antigen in formalin-fixeddog brain tissues using ELISA and RIDT.
- To evaluate and compare the results obtained in fresh andcitrate buffer treated formalin-fixed brain tissues.

1.6 Research Questions

- What is the detection rate of DFAT, ELISA and RIDT in frozen archiveddog brain tissues?

- Can sodium citrate buffer retrieve rabies virus antigen from formalin-fixeddog brain tissues?
- What is the detection rate of DFAT in fresh and citrate buffer treated formalin-fixed brain tissues of dog?
- What is the detection rate of ELISA and RIDT in formalin-fixedbrain tissues of dog?
- What are the sensitivity and specificity of DFAT on citrate buffer treated formalin-fixed brain tissues compared to DFAT on fresh brain tissues?

CHAPTER TWO

LITERATURE REVIEW

2.1. The Rabies Virus

Rabies virus (RABV) is a neurotropic virus that causes rabies in humans and animals(Dietzschold *et al.*, 2008). Rabies transmission can occur through the saliva of infected animals and less commonly through contact with human saliva (WHO, 2013). Rabies virus, like many rhabdoviruses, has an extremely wide host range. In the wild it has been found infecting many mammalian species, while in the laboratory it has been found that birds can be infected (Carter and Saunders, 2007; Baby *et al.*, 2015).

Rabies viruses may be categorized as either fixed (adapted by passage in animals or cell culture) or street (wild type). The use of monoclonal antibodies and genetic sequencing to differentiate street RABVs has been helpful in identifying viral variants originating in major host reservoirs throughout the world and suggesting the likely sources of human exposure when a history of definitive animal bite was otherwise missing from a patient's case history(Rupprecht, 1996).

The RABV has a cylindrical morphology and is the type species of the *Lyssavirus* genus of the *Rhabdoviridae* family. Rabies virus belongs to the order *Mononegavirales*, viruses with non-segmented, negative-stranded RNA genomes(Healy *et al.*, 2013). Within this group, viruses with a distinct "bullet" shape are classified in the *Rhabdoviridae* family, which

includes at least three genera of animal viruses, *Lyssavirus*, *Ephemerovirus*, and *Vesiculovirus* (CDC, 2011). Based on recent phylogenetic evidence, lyssaviruses are categorized into seven major species, viz:*rabies virus* (species 1), *Lagos bat virus* (species 2), *Mokola virus* (species 3), *Duvenhage virus* (species 4), *European Bat lyssaviruses* type 1 and 2 (species 5 and 6), and *Australian batlyssavirus* (species 7) (Badrane*et al.*, 2001).Other species in the genus include *Aravan lyssavirus, Bokeloh lyssavirus, Ikoma lyssavirus, Irkut lyssavirus, Khujand lyssavirus, Shimoni bat lyssavirus, and West Caucasian bat lyssavirus* (Kuzmin *et al.*, 2005;Rupprecht*et al.*, 2017).Two other putative lyssaviruses do not yet have taxonomic status. One is Lleida bat lyssavirus (LLEBV), identified previously by only a partial genome sequence, while isolation attempts continue (Aréchiga*et al.*, 2013). The other, soon to be submitted to the ICTV for review, is the most recently described Gannoruwa bat lyssavirus (Gunawardena*et al.*, 2016).

All transcription and replication events take place in the cytoplasm inside a specialized “virus factory”, the Negri body. These are 2–10 μm in diameter and are typical for a rabies infection and thus have been used as definite histological proof of such infection(Albertini*et al.*, 2008).

2.1.1 Structure of rabies virus

The RABV is a negative-sense, non-segmented, single-stranded RNA virus measuring approximately 60 nm wideand 180 nm long. It is composed of an internal protein core or nucleocapsid, containing the nucleic acid, and an outer envelope, a lipid-containing bilayer covered with trans-membrane glycoprotein spikes(Drew, 2004; Figure 2.1).

The virus genome encodes five proteins associated with either the helical ribonucleoprotein (RNP) complex or the viral envelope. The L (polymerase), N (nucleoprotein), and P (phospoprotein) proteins comprise the RNP complex, together with the viral RNA(Rupprecht, 1996; CDC, 2011; Figure 2.2). In the RNP, genomic RNA is

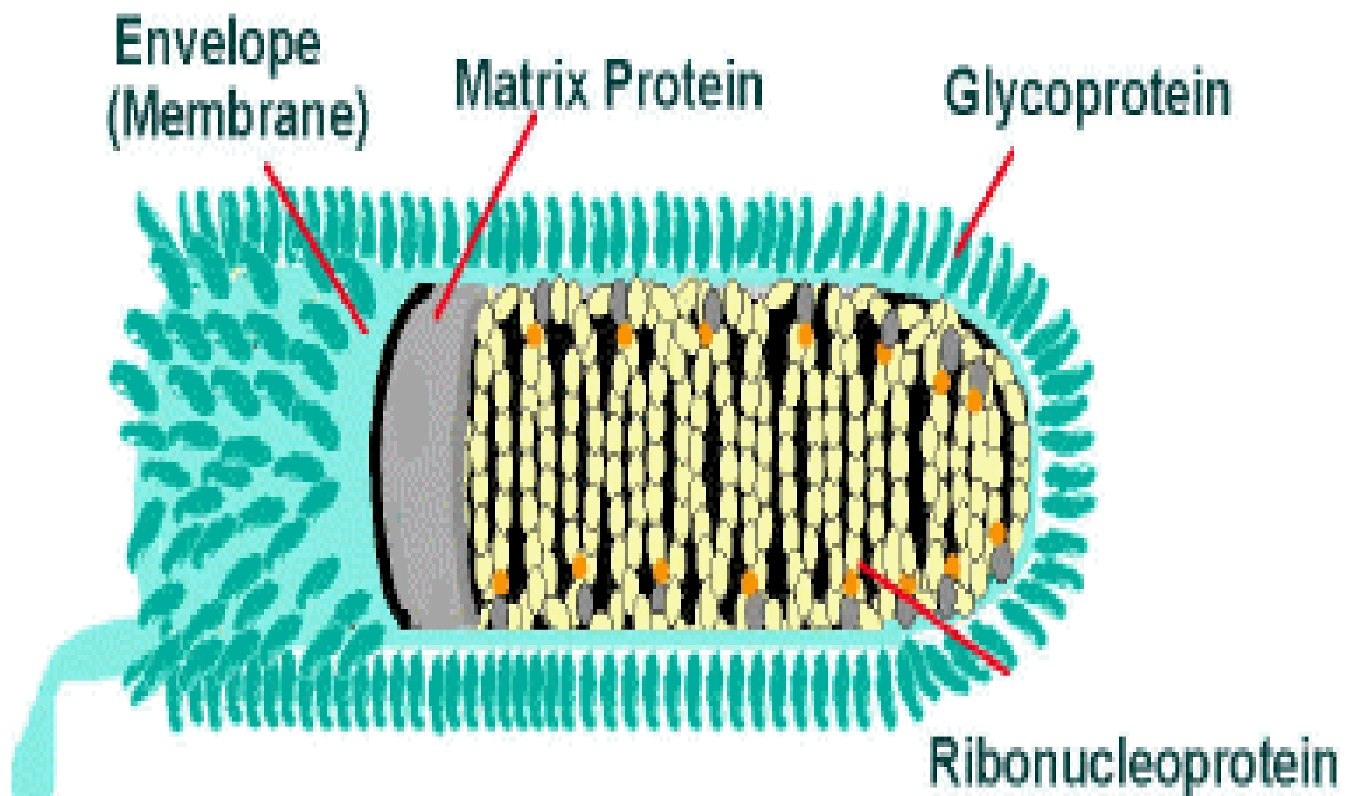


Figure 2.1:Cross-Sectional Diagram of the Rabies Virion (CDC, 2011).

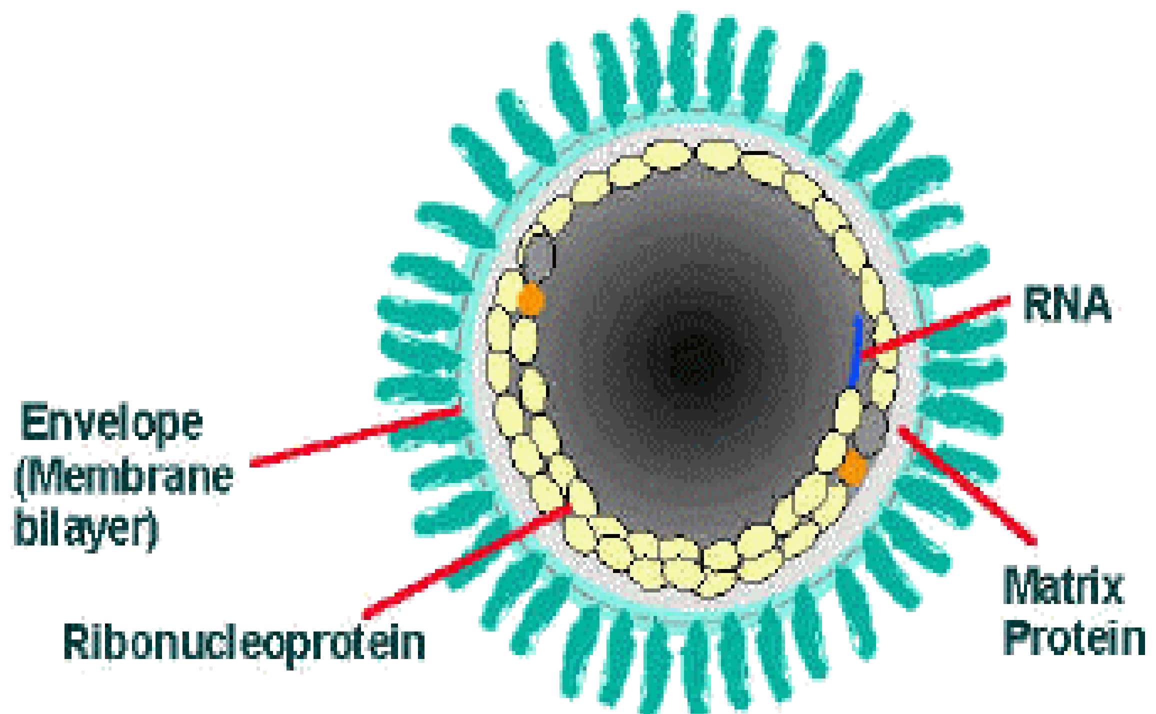


Figure 2.2: The Cross-Sectional Diagram Demonstrates the Concentric Layers: Envelope Membrane Bilayer, M Protein, and Tightly Coiled Encased Genomic RNA (CDC, 2011).

tightly encased by the nucleoprotein. These aggregate in the cytoplasm of virus-infected neurons and compose Negri bodies, the characteristic histopathologic finding of rabies virus infection(Albertini *et al*., 2008). The M (matrix) and G (glycoprotein) proteins are associated with the lipid envelope.

The G protein forms approximately 400 trimeric spikes that cover the outer surface of the virion envelope and is the only RABV protein known to induce virus-neutralizing antibody(Orciari*et al*., 2015). The M protein is associated both with the envelope and the RNP and may be the central protein of rhabdovirus assembly(Rupprecht, 1996; Orciari *et al*., 2015).

2.2 Transmission

The commonest way of rabies transmission is by the bite of an infected mammal(Consales and Bolzan, 2007). Bites by rabid animals generally inoculate virus-laden saliva through the skin into muscle and subcutaneous tissues (Warrell and Warrell, 2004). Other inoculation routes are rare. Rabies virus entry occurs through wounds or direct contact with mucosal surfaces. The virus cannot cross intact skin(Consales and Bolzan, 2007). The risk of rabies infection by a bite (5%-80%) is at least 50 times greater than that by a scratch (0.1%-1%) (Hemachudha*et al*., 2002). Mortality after untreated bites by rabid dogs ranges from 38% to 57% and depends on the severity and location of the wound as well as on the

presumed virus concentration in the saliva (Hemachudha *et al*., 2002; Warrell and Warrell 2004).

Bat virus might be more infectious when inoculated superficially into the epidermis since it replicates more rapidly in non-neuronal cells and at lower temperatures than do dog rabies viruses(Messenger*et al*., 2002). Percutaneous infection probably occurs during unnoticed skin contact, which may result in a minute bite. The route of viral entry into epithelial nerves and eventually into the central nervous system (CNS) is unknown (Consales and Bolzan, 2007).

Inhalation of aerosolized RABV occurred accidentally in laboratories of vaccine production (Consales and Bolzan, 2007)or in caves inhabited by numerous infected bats (Gibbons, 2002). Infection through the digestive tract has also been reported (Centers for Disease Control, 1999).

Contact with animal vaccines may be significant when attenuated vaccine is used. In these situations, rabies prophylaxis is necessary. The handling and skinning of infected carcasses can be of risk for workers in refrigeration plants and butchers' shops, and veterinarians(Consales and Bolzan, 2007).The contact with infected people could be a potential risk for their relatives and health workers when unprotected direct contact with secretions from a patient containing viable virus occurs (Fekadu*et al*., 1996;Consales and Bolzan, 2007).

There are many reports of organ transplantation involved in the transmission of rabies. The most frequent cases have been observed in corneal transplantation (Jadavi*et al*., 1996). The most recent case reported was of a German patient and occurred in 2005 (Hellenbrand*et*

al., 2005).In 2004, the Centers for Disease Control (CDC) in the United States confirmed the first case of rabies transmission through solid organ transplantation by testing autopsy samples after the death of four patients who received organ transplants (two kidney receptors, one liver receptor, and one receptor of an arterial segment) from the same donor. Subsequently, it was learned that a bat had bitten the donor (Srinivasan*et al*., 2005). Three other cases of rabies-related organ transplantation were reported in Germany in 2005. These three patients received lung, kidney and kidney/pancreas transplants following the donor's death (Hellenbrand *et al*., 2005).Before transplantation, the donor (if possible) and his relatives and friends should be questioned as to any history of physical contact with bats or bites by them or other mammals anywhere in the world. Patients with such a history should not be accepted as donors, even if post-exposure prophylaxis was carried out. There are no suitable screening tests to distinguish whether potential donors are infected(Consales and Bolzan, 2007). It has been recommended that donors, particularly those with neurological symptoms, should be screened for rabies (Dietzschold and Koprowski, 2005).

So far, cases of rabies infection via blood transfusion have not been reported and rabies viraemia has not been demonstrated in animals or men (Consales and Bolzan, 2007). There are no evidences that apparently healthy blood donors can transmit rabies, even if they incubate the infection(Consales and Bolzan, 2007). The one-year deferral of donation following post-exposure rabies prophylaxis remains a reasonable precaution (JPAC, 2005). In Brazil, the Sanitary Surveillance Center (ANVISA) has decided that the interval between rabies post-exposure treatment and blood donation should be one year and that for rabies pre-exposure vaccination, 4 weeks (Consales and Bolzan, 2007).

The RABV migrates along peripheral nerves (via the fast axonal transport system) towards the CNS at about 50-100 mm per day. This movement is strictly retrograde, which indicates infection is via sensory and motor nerves. Invasion of the CNS by the RABV glycoprotein may not occur via the sensory nerve pathway (Mazarakis*et al*., 2001).

The incubation period or eclipse phase varies from two weeks to six years (average: 2 to 3 months) according to the amount of viral inoculum and the inoculation site. Bites on the head, face, neck and hands, particularly together with bleeding, offer the highest risk and are generally associated with a shorter incubation period(Consales and Bolzan, 2007). The RABV can stay in the muscle tissue for long periods and in certain circumstances, its long persistence may provide an opportunity for host immune clearance and post-exposure treatment (Hemachudha *et al*., 2002; Warrell and Warrell 2004).

2.3 Pathogenesis

Scratches or bites by infected animals generally inoculate virus-laden saliva through the skin into muscle and subcutaneous tissues (Consales and Bolzan, 2007). Other routes of infection (such as airborne transmission, contamination of mucous membrane or broken skin, transplantation of tissues or organ) are rare (Warrell, 2003). During the incubation period the virus can replicate locally in muscle cells or attach directly to nerve endings (Warell and Warell, 2004; Figure 2.3). Having gained access to peripheral nerves, it travels in a retrograde direction within the axoplasm. When the virus reaches the central nervous system, there is massive replication on membranes within neurons. Direct transmission of virus occurs from cell to cell across synaptic junctions (Warell and Warell, 2004). At the onset of illness when evidence of neuronal dysfunction appears, there is little or no

apparent histopathological change (Warell and Warell, 2004). Centrifugal spread of virus from the central nervous system in somatic and autonomic nerves deposits virus in many tissues, including skeletal and cardiac muscle, adrenal glands, kidney, retina, cornea, pancreas, and nerves around hair follicles (Jackson *et al.*, 1999). Productive viral replication with budding from plasma membranes takes place predominantly in the

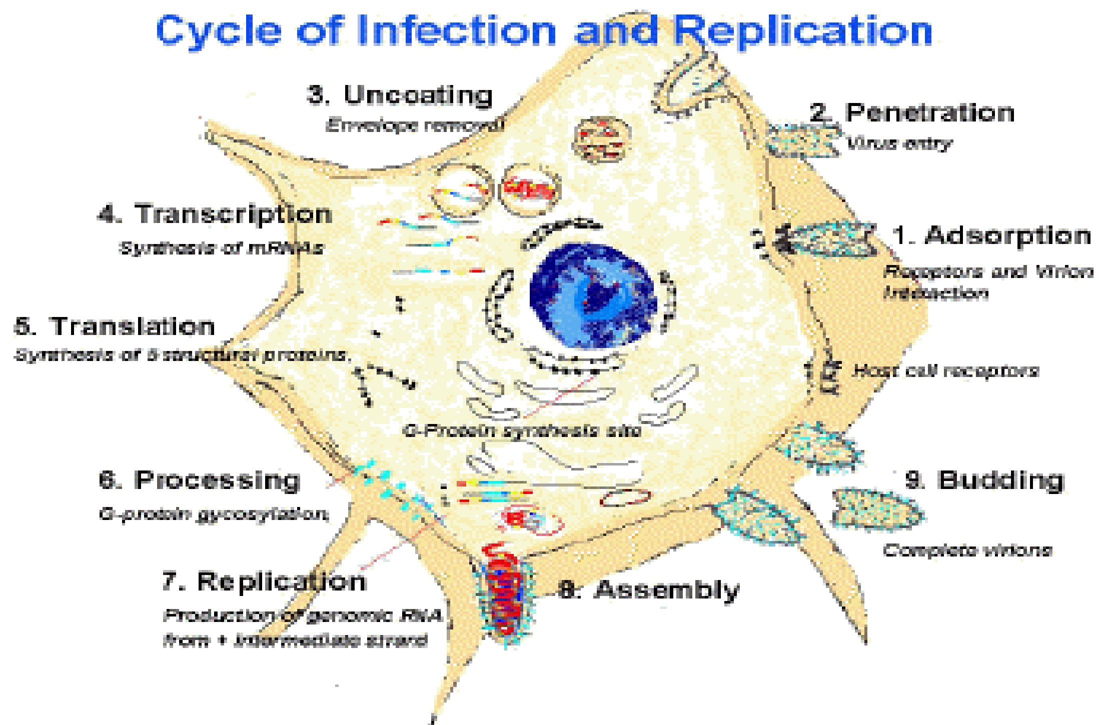


Figure 2.3: The Cycle of Infection and Replication for the Rabies Virus (CDC, 2011).

salivary glands, excreting virus that is transmissible to other mammals (Warell and Warell, 2004).

2.3.1 Viral invasion of cells

Local replication of virus in striated muscle at the scratch/bite site, before any contact with nervous tissue, could account for long incubation periods. In skunks, antigen was detectable in muscle for 2 months after inoculation (Charlton *et al.*, 1997). Virus is soon detectable experimentally at local motor or sensory nerve endings and, after superficial inoculation, in epithelial layers (Murphy, 1977).

Rabies virus can infect a great variety of neuronal and non-neuronal cells in vitro. Non-specific viral attachment to several types of cell-surface receptors including carbohydrates, phospholipids, and sialylated gangliosides have been demonstrated. Specific binding occurs at neuromuscular junctions, where virus co-localises with the nicotinic acetylcholine receptor (Lewis*et al.*, 2000). Binding at this postsynaptic site is competitive with cholinergic ligands, including the snake venom neurotoxin-bungarotoxin, which shows sequence homology with the envelope glycoprotein of rabies virus. Concentration of virus at this site increases its chances of entering the axon terminal across the synaptic cleft. Rabies virus attaches specifically to two other receptors on neuronal cell membranes:

the neural cell adhesion molecule (Thoulouze *et al*., 1998) and the p75 neurotropin receptor (p75NTR) (Langevin *et al*., 2002). Two neurotransmitter receptors in the central nervous system, for N-methyl-D-aspartate subtype R1 and gamma aminobutyric acid (GABA) have been suggested as possible receptors for rabies virus (Gosztonyi and Ludwig, 2001). Rabies genotype 1 and EBLV type 2 (genotype 6) bind avidly to p75NTR, but EBLV type 1 (genotype 5) and the other lyssaviruses do not (Tuffereau *et al*., 2001). Rabies virus binds to mammalian but not to avian p75NTR, which is consistent with the lack of rabies pathogenicity in birds. It has been suggested that binding to this receptor might not only enable entry into a cell but also facilitate fast axonal transport (Mazarakis *et al*., 2001; Wong *et al*., 2004).

Attachment of the viral glycoproteins (G) to host receptors mediates Clathrin-mediated endocytosis of the virus into the host cell. Rabies virus enters cells by adsorptive endocytosis into endosomes (Xiangjie *et al*., 2010). Soon after infection, virus may be associated with synaptic vesicles, since it co-localises with synapsin I, or with early acidic endosomes, in which case viral glycoprotein could fuse with the endosomal membrane releasing the core ribonucleoprotein complex into the cytosol (Lewis*et al*., 2000). The Lyssavirus glycoprotein has a unique reversible fusion inactive state at low pH, so it can resist fusion and could remain intact in vesicles (Gaudin, 2000).

2.3.2 Rabies virus replication

The fusion of the RABV envelope to the host cell membrane (adsorption) initiates the infection process. The interaction of the G protein and specific cell surface receptors such

as the nicotinicacetylcholine, neural cell adhesion molecule and p75 neurotropin receptormay be involved (Finke and Conzelmann, 2005).

The RABV genome is single-stranded, antisense, nonsegmented, RNA of approximately 12 kb. There is a leader-sequence (LDR) of approximately 50 nucleotides, followed by N, P, M, G, and L genes (Albertini*et al*., 2011).

After adsorption, the virus penetrates the host cell and enters the cytoplasm by pinocytosis (via clathrin-coated pits). The virions aggregate in the large endosomes (cytoplasmic vesicles). The viral membranes fuse to the endosomal membranes, causing the release of viral RNP into the cytoplasm (uncoating) (Lewis *et al*., 2000; CDC, 2011). Because lyssaviruses have a linear single-negative-stranded ribonucleic acid (RNA) genome, messenger RNAs (mRNAs) must be transcribed to permit virus replication(Albertini *et al*., 2011).

A viral-encoded polymerase (L gene) transcribes the genomic strand of rabies RNA into leader RNA and five capped and polyadenylated mRNAs, which are translated into proteins (Finke*etal*., 2000). Translation which involves the synthesis of the N, P, M, G and L proteins, occurs on free ribosomes in the cytoplasm. Although G protein synthesis is initiated on free ribosomes, completion of synthesis and glycosylation (processing of the glycoprotein), occurs in the endoplamsic reticulum (ER) and Golgi apparatus(CDC, 2011). The intracellular ratio of leader RNA to N protein regulates the switch from transcription to replication. When this switch is activated, replication of the viral genome begins. The first step in viral replication is synthesis of full-length copies (positive strands) of the viral genome. When the switch to replication occurs, RNA transcription becomes “non-stop”

and stop codons are ignored (CDC, 2011). The viral polymerase enters a single site on the 3′ end of the genome, and proceeds to synthesize full-length copies of the genome. These positive strands of rabies RNA serve as templates for synthesis of full-length negative strands of the viral genome (Palusa*et al*., 2012).

During the assembly process, the N-P-L complex encapsulates negative-stranded genomic RNA to form the RNP core, and the M protein forms a capsule, or matrix, around the RNP. The RNP-M complex migrates to an area of the plasma membrane containing glycoprotein inserts, and the M-protein initiates coiling. The M-RNP complex binds with the glycoprotein, and the completed virus buds from the plasma membrane(Finke and Conzelmann, 2003; Okumura and Harty, 2011). Within the central nervous system (CNS), there is preferential viral budding from plasma membranes. Conversely, virus in the salivary glands buds primarily from the cell membrane into the acinar lumen. Viral budding into the salivary gland and virus-induced aggressive biting-behavior in the host animal maximize chances of viral infection of a new host (CDC, 2011).

2.3.3 Transport of rabies virus to the brain during infections

Rabies virus migrates along peripheral nerves towards the central nervous system (CNS) at about 50–100 mm per day via the fast axonal transport system (Tsiang, 1993). Because this movement is strictly retrograde, it is used experimentally to track neural pathways (Graf *et al*., 2002). Infection is thought to be via sensory as well as motor nerves, because antigen was detected in sensory nerve endings and dorsal root ganglia soon after peripheral inoculation in several studies (Murphy, 1977; Tsiang, 1993).

However, Mazarakis and colleagues (2001) have shown that rabies glycoprotein invasion of the central nervous system apparently does not occur via this sensory nerve pathway. Using a rabies-glycoprotein-pseudotyped lentivirus vector, which is effectively transported in a single neuron but cannot cross synapses, they showed that injection of muscle with the lentivirus resulted in transgene expression in the ventral horn of the spinal cord. Injection of skin resulted in transgene expression in dorsal root ganglia but not further into the dorsal horn of the spinal cord. Injection into the dorsal horn of the spinal cord, however, produced transgene expression in the relevant dorsal root ganglia but not in the skin (Mazarakis *et al.*, 2001). This finding shows that rabies-glycoprotein-pseudotyped lentivirus is retrogradely transported in neurons, and thus it can pass to the spinal cord through the motor nerves, but not via this pathway in peripheral sensory nerves. If any lentivirus entered sympathetic motor nerves supplying the skin, it might reach the sympathetic ganglia, but not the spinal cord in this experiment.

Further studies showed the abolition of this retrograde transport by substitution of the arginine-333 residue on the glycoprotein (Wong *et al.*, 2004)). Two groups of researchers found independently that, like some other virus proteins, rabies phosphoprotein can interact with the light-chain 8 (LC8) protein, a highly conserved cytoplasmic component of dynein cargo-binding complex and myosin V, and an inhibitor of neuronal nitric oxide synthase. LC8 also co-localises with viral ribonucleoprotein in vitro. The myosin V actin-based motor complex drives cytoplasmic vesicular transport in the endoplasmic reticulum, so LC8 binding to rabies proteins indicates its possible involvement in viral pathogenesis early in the cycle of neuronal infection (Jacob *et al.*, 2000; Raux *et al.*, 2000). The association of rabies phosphoprotein with the LC8 component of dynein would enable

axonal retrograde transport of the viral ribonucleoprotein complex, as predicted by Murphy (Murphy, 1977). However, retrograde transport of virus can still occur if the phosphoprotein binding site is deleted, hence, binding of viral proteins to the LC8 molecule is not essential for pathogenesis (Desmaris *et al*., 2001; Mebatsion, 2001; Lalli and Schiavo, 2002).

In theory, if on entry to a neuron the virus in an acidic endosome fused with the vesicle membrane, the liberated naked nucleocapsid (including the phosphoprotein) could be attached to dynein via LC8; if the virus remained intact, the envelope glycoprotein could be the ligand for vesicular transport through the p75NTR (Mazarakis *et al*., 2001). Free nucleocapsids and whole virions in vesicles within axons have been shown in electron micrographs (Gosztonyi, 1979). This process is found with herpes simplex virus, the components of which are transported separately along microtubules, as either nucleocapsids or glycoprotein within vesicles (Miranda-Saksena *et al*., 2000). Whether either mechanism is used by rabies virus remains to be proven.

2.3.4 Spread of rabies virus within the CNS

Viral replication is intraneuronal, but the mechanism of interneuronal spread is unknown. Sequential transcription, viral messenger ribonucleic acids (mRNAs) are capped and polyadenylated by polymerase stuttering in the cytoplasm (John *et al*., 2002). Replication presumably starts when enough nucleoprotein is present to encapsidate neo-synthetized antigenomes and genomes. The ribonucleocapsid binds to the matrix protein and buds via the host endosomal sorting complexes required for transport (ESCRT) which occurs at the plasma membrane, releasing new virions (Sonja *et al*., 2007). The fact that budding of

virus is very rarely seen at synapses by electron microscopy suggests that infectious naked nucleocapsids are transferred across synapses (Gosztonyi*et al.*, 1993). However, interneuronal infection is dependent on the presence of viral glycoprotein, which suggests that intact virus can cross the synapse (Etessami *et al.*, 2000). The mechanism of uptake is unknown, but one possibility is through the synaptic vesicle recycling system or tubule-guided viral movement (Lewis*et al.*, 2000). Among street viruses, the dynamics and effects of infection can be influenced by the particular strain, since dog rabies viruses are specific for neurons, whereas bat strains can also infect astrocytes (Warell and Warell, 2004).

2.3.5 Effect of rabies virus on neuronal function

In human beings, the symptoms of encephalitis and even death can occur with only minor histopathological changes. Rabies virus must have some profound effects on the functions of infected and some uninfected neurons (Warell and Warell, 2004). Few abnormalities of organelle structure are seen on electron microscopy in neurons infected with street virus (Murphy, 1977). Minor electroencephalographic changes during animal infection indicate neuronal dysfunction (Tsiang, 1993). Although magnetic resonance imaging (MRI) shows a range of abnormalities as the human encephalitis progresses, no consistent pattern has yet emerged. Abnormalities of neurotransmitter functions affecting serotonin, opioid, GABA, and muscarinic acetylcholine transmission have been found experimentally, in some cases in specific brain areas, and not always associated with the presence of virus (Jackson, 2002). Results show no clear explanation of the limbic-system dysfunction suggested by the classic clinical features.

Changes in neurotransmitter functions could lead to failure of brain networking and regulation of responses. The involvement of excitatory amino acids in neuronal toxicity is a possibility. Many non-competitive antagonists of N-methyl-D-aspartate have an antiviral effect on viral replication (Tsiang, 1993). Surprisingly, one of these, ketamine, specifically inhibits transcription of the rabies virus genome (Lockhart*et al*, 1992). The effect of infection on the function of neuronal membrane ion channels could be reduction of normal inhibitory events (Iwata *et al.*, 2000). Apoptosis could contribute to pathogenesis since fatal infection of mice is associated with apoptosis of T cells invading the brain and neuronal preservation. By contrast, neuronal apoptosis occurs in non-lethal infection, with development of an immune response (Jackson, 2002; Baloul and Lafon, 2003). The role of nitric oxide toxicity in neuronal dysfunction in rabies is not clear, but it could be related to the LC8 inhibition of neuronal nitric oxide synthase, through interaction with the viral phosphoprotein (Jacob *et al.*, 2000; Jackson, 2002). Although rabies infection progressively decreases host gene expression overall, a few genes are upregulated, some associated with the interferon response, host-cell protein synthesis, synaptic vesicle function, and neuron growth and spread, even in some uninfected or non-neuronal cells (Prosniak *et al.*, 2003). One hypothesis on the cause of death is therefore that short-circuiting of normal neural pathways results from the formation of new interneuronal connections (Prosniak *etal.*, 2001). Another hypothesis is that disruption of neuronal metabolism ends in the exhaustion of metabolic pools (Dietzschold *et al.*, 2001).

2.3.6 Neuro-inflammation and the blood brain barrier

The nervous system (NS) intrinsically limits the inflammatory response following injury; however, inflammation is still triggered in the NS by most infections. In contrast to most

encephalitic viruses, RABV triggers a more limited inflammatory response. In fact, the more pathogenic the strain is, the smaller the inflammatory response (Baloul and Lafon, 2003). It is unclear how exactly RABV is able to limit the inflammatory response, but it has been suggested that the ability correlates to the differences between classical and non-classical strains. By comparing the amount of viral RNA and 18 cytokine mRNAs in twelve different brain regions of dogs infected with classical and non-classical rabies, Laothamatas *et al*.(2008) was able to determine key differences in the inflammatory response triggered. The differences were found early on in infection, with non-classical RABV infected dogs having higher levels of both interleukin-1beta and interferon-gamma (INF-γ) and lower levels of viral RNA. Dogs infected with classical RABV had much higher levels of viral mRNA in brain tissue. Later in infection, there wasn't much difference between the viral RNA and cytokine mRNA levels in dogs infected with the different strains (Laothamatas *et al*., 2008). The increased neuro-invasiveness of the classical strain probably correlates to the decreased immune response in comparison with the non-classical strain. Strength of the neuro-inflammitory response might also correlate to the permeability of the blood brain barrier (BBB).

The permeability of the BBB in rabies infection is important as permeability relates to host cell survival. A more permeable membrane leads to an increased chance of host survival as it allows for passage of more immune cells into the NS. It has been shown that BBB permeability is increased in laboratory attenuated strains of RABV but until experiments carried out by Chai *et al*.(2014), the underlying mechanisms were a mystery. Tight junction proteins (TJPs) are critical to maintaining the BBB, so Chai *et al*.(2014) examined the expression of three TJPs in mice infected with a wild type RABV strain, a lab

attenuated strain, or a sham infection. Immunohistochemistry and subsequent Western Blots of mouse brain tissue showed that the TJP expression in the wild type strain infected mice closely resembled that of the sham injected mice, while mice infected with the lab attenuated strain showed significantly lower expression levels. This suggested a correlation between the loss of TJPs in brains of mice infected with the attenuated RABV and the increased permeability of the BBB in these mice. However, this loss of TJ expression is not due directly to attenuated RABV infection as neither the attenuated nor the wild type strains are able to infect brain micro-vascular endothelial cells (BMECs) in vitro (Chai *et al*., 2014). Chai *et al*. (2014) were only able to change the expression pattern of TJPs after exposing cultured BMECs to extracts taken from brains of mice infected with the attenuated RABV strain. This suggested that it was a response triggered by the virus rather than the virus itself that caused the down regulation of TJPs. This led to the use of a mouse cytokine/chemokine magnetic bead panel to determine if there were differences in the cytokine/chemokine levels in brains of mice infected with either the wild type or attenuated strain.

The brains of mice infected with the attenuated strain had higher levels of cytokines/chemokines and a genetic network map was created for each strain (Chai *et al*., 2014). The network induced by attenuated strain contains 26 focus molecules while the wild type strain network only has 12. IFN-γ is at the center of the network, which corresponds to the findings of Laothamatas *et al*. and their non-classical strain induction of higher levels of INF-γ (Laothamatas *et al*., 2008; Chai *et al*., 2014). Upon further investigation of INF-gamma's role in BBB permeability, Chai *et al*. (2014) found that silencing INF-γ causes a decrease in TJ and an increased permeability of the BBB

suggesting that the attenuated strain interferes with normal INF-γ signaling. This is another example of the balance RABV has to strike. The higher levels of INF-γ in attenuated strains could come from changes in protein binding like Prehaud *et al*. (2010) found in the PDZBS of the G protein. It could be something completely different. Further investigation of this issue is important for understanding RABV and how to stop RABV infection.

RABV has a two pronged strategy to evading the immune system. First, RABV uses the host's natural defense of the NS from excessive immune response to its advantage by making it extremely difficult for lymphocytes to do their job. RABV is able to create a neuroevasive environment in the peripheral nervous system and the spinal cord by upregulating the expression of surface proteins that inhibit T cell activity such as B7-H1 (Lafon *et al*., 2005; Megret *et al*., 2007; Lafon *et al*., 2008). Once it reaches the brain RABV is able to avoid triggering an inflammatory response therefore keeping the lymphocytes out of the brain (Chai *et al*., 2014). Second, RABV is able to promote neurite growth and prevent cell mediated apoptosis (Prehaud *et al*., 2010). This allows virions to utilize the host's nervous system as a transportation system to the brain.

2.4Immune Response to Rabies Virus- Vaccination and Infection

2.4.1 The immune response to rabies vaccination

The most important role of rabies vaccine is in the induction of sustained antibody response with help of CD4+ T lymphocyte activation. Rabies is an exception because it is generally thought that cytotoxic T cells are more important to clear viral infections from tissues than antibodies. Moreover, activation of CD8+ T cells induces a pathological reaction which is clinically associated with paralysis. This should probably discourage the

use of live vaccines such as DNA vaccine or recombinant virus as post exposure vaccines (Lodmell and Ewalt, 2001), because of the strong risk in mounting a strong deleterious CD8 response in nervous system (NS). Nevertheless, these new generation vaccines are suitable for pre exposure vaccination regimens because of the robustness of live vaccination. The inactivated post exposure vaccines that induce mainly B cells activation with the help of CD4+ T cells are the most appropriate choice to preserve the integrity of NS. Post exposure vaccines probably confer protection because they prime an immune response in the periphery in secondary organ. Activated lymphocytes, CD4, antibody secreting plasmocytes and possibly antibodies can migrate into the NS parenchyma.

Although there have been a small number of cases of survivors following infection with rabies (Warell and Warell, 2004; Willoughby *et al*., 2005), the vast majority of humans who develop rabies, die as a consequence of infection. Despite this, vaccination is highly effective at preventing disease when administered before or shortly after exposure to virus. Current vaccines consist of inactivated virus grown in continuous cell-lines. Pre-exposure vaccination, such as that given to health-care and laboratory workers, and travelers to rabies-endemic areas, is administered in three doses intramuscularly at 0, 7 and 28 days. Immunoglobulin M (IgM) is detectable within 4 days post-inoculation with IgG appearing at day 7 (Johnson*et al*., 2010). Follow-up studies have shown that responses persist for up to 2 years after vaccination and passive transfer studies suggest that it is IgG that provides the most effective protection against disease probably because of the inability of IgM to penetrate tissue (Turner, 1978; Johnson *et al*., 2010). Prompt post-exposure treatment or prophylaxis (PEP) is the only effective treatment for the prevention of rabies. Unlike pre-exposure vaccination, the regimens recommended by the World Health Organisation

(WHO) for PEP consist of intensive re-exposure to vaccine over a short period of time. A standard course would be repeated intramuscular inoculation at 0, 2, 7, 14 and 28 days (Warell and Warell, 2004). Vaccine potency is a key factor in judging the effectiveness of RABV vaccines and usually reflects both the antigen content of a particular batch and the titre of antibody induced following inoculation. The key parameter measured in humans or animals, is the titre of neutralizing antibody induced following vaccination. It has also been shown that Rabies human diploid cell vaccine elicits cross-neutralising and cross-protecting immune responses against European and Australian bat lyssaviruses (Brookes *et al.*, 2005). Two neutralization assays are used to measure this, the rapid fluorescent focus inhibition test (RFFIT) and the fluorescent antibody neutralization assay (FAVN) (Cliquet *et al.*, 1998; Johnson*et al.*, 2010).

2.4.2 The immune response to rabies infection

The primary correlate of protection, as demonstrated by the effectiveness of post-vaccination to virus challenge and numerous experimental studies, is the presence of neutralizing antibody (Hooper *et al.*, 1998). In experimental models of infection using gene knock out (k.o) mice, those strains that were susceptible to attenuated strains of rabies virus were those that lacked all T and B cells or only B cells, whilst mice only lacking CD8+ T cells were not susceptible (Hooper *et al.*, 1998). Surprisingly, infection can produce adaptive immune response, often late in the clinical course, that fails to control the disease. The majority of human rabies cases do not have detectable antibody responses until some days after the development of acute disease. In a study of rabies admissions in Thailand only 3 of 11 cases had detectable neutralizing antibody to the viral glycoprotein (Kasempimolporn *et al.*, 1991). Even in these cases the titres were particularly low,

varying from 0.26 to 3.42 international units per ml serum (IU/ml). In six of these patients, where cerebrospinal fluid (CSF) was investigated, no anti-rabies antibody was detected. A similar review of human cases in the USA showed that none of the patients had detectable neutralizing antibody against rabies on presentation to hospital, although over half seroconverted within 10 days (Noah *et al*., 1998). Of 14 patients who were investigated for antibody in the CSF, only 2 had measurable levels. In the first of these cases anti-rabies antibody titres only appeared at day 16 after hospital admission (Solomon *et al*., 2005). In the second case, the appearance of antibodies within both serum and CSF occurred over a week after admission to hospital. A previous case of human rabies in the UK, infected with a related virus, European bat lyssavirus type 2, had no detectable anti-viral response at any time during hospitalization (Nathwani *et al*., 2003).

The vast majority of cases end in the death of the patient and the absence of protective antibody at the site of virus replication is likely to contribute to these poor rates of survival. A more rapid response to infection, particularly before the virus has entered the brain and the ability of that antibody to enter the CNS would be expected to enhance survival or limit spread of virus. Passive transfer of monoclonal antibodies that can inhibit cell-to-cell spread of virus has been observed and suggests that antibodies can contribute to viral clearance from the CNS although the development of such strategies in man have not been successful (Dietzschold *et al*., 1992). During infection, it is unclear from where the antigen that drives the antibody response is derived, either from virus in the periphery or from virus in the CNS. Again rodent experimental models of infection have severe limitations in understanding natural infection. In a Syrian hamster model of infection with virulent virus, IgG2 antibody was detected as early as 5 days post-challenge (Coe and Bell, 1977).

However, the virus was injected by the intra-peritoneal route, and efficiently induced rabies within 8 days. We have observed the rapid induction of neutralizing antibodies (within 5 days) to footpad inoculation (Johnson *et al*., 2007). There have been no studies to define the isotype of these antibodies and in the serum the earliest antibody is likely to be IgM. A caveat to these studies is that the level of virus used is likely to be considerably higher than that transmitted in saliva. However, such titres of virus are needed to ensure consistent induction of disease in a majority of the animals inoculated.

In addition to the adaptive immune response, host defense against the early stages of infection is provided by the innate immune response (Haller *et al*., 2006). To investigate the innate immune response within the brain to lyssavirus infection, key transcripts indicative of innate defences were measured in a mouse model system (Marquette *et al*., 1996; Camelo *et al*., 2000; Baloul and Lafon, 2003; Wang *et al*., 2005; Johnson *et al*., 2006). Following infection with Rabies virus, transcript levels for type 1 interferons (IFN-a and -b), the inflammatory mediator (cytokines) interleukin 6 (IL-6), IL- 1, and TNF-α as well as the antiviral protein Mx1 and chemokines (CCL-5, CXCL-10) increased in the brains of mice. Intracranial inoculation resulted in the early detection of virus replication and rapid expression within the brain of the innate immune response genes. Transcripts for type 1 IFNs declined as the disease progressed. Peripheral, extraneural inoculation delayed the host response until virus entered the brain, but then resulted in a large increase in the level of IFN-b, IL-6 and Mx1 transcripts. Induction of this response was also observed following infection with the related European bat lyssaviruses, a group of zoonotic viruses capable of causing fatal, rabies-like disease in mammalian species (Johnson *et al*., 2006).

2.4.3 Rabies virus immunosuppression on the host

The reasons for the hosts' limited responsiveness to infection could reflect a number of factors. The neurotropism of the virus results in the vast majority of replication occurring firstly in the dorsal root ganglion and then progressively through the CNS. Traditionally this has been considered an immune-privileged location and thus not under the same level of immune-surveillance as other organs.

Furthermore, the ability of the immune system to induce inflammation (encephalitis) is tightly regulated to prevent bystander damage that could leave the host with neuronal deficit. This dogma is changing with a better understanding of the lymphatic drainage of the brain and spinal cord (Cserr and Knopf, 1992), and the identification of cell trafficking within the CNS (Goldman *et al.*, 2006). However, this alone cannot explain the failure to deal with rabies virus as a number of other viral infections including Herpes viruses (Mettenleiter, 2003) and Borna disease virus (BDV) (Morimoto *et al.*, 1998) can infect the brain and in the case of BDV persistently, yet are effectively controlled by a functional immune system.

Another explanation could be that the infectious dose administered during a bite is too small to trigger immune responses enabling the virus to infect local sensory nerves. This could well happen in many circumstances. Bats appear to be efficient transmitters of rabies but the level of virus in saliva is barely measurable using RT-PCR methods. It has been suggested that a bat variant of rabies found only in North America could be adapted to transmission at low doses and favour replication at temperatures below 37°C (Morimoto *et al.*, 1996). This may better reflect surface skin temperature and there is increasing evidence that the sub-dermal inoculation route is a more effective means of infecting experimental

animals with European bat lyssaviruses when directly compared to intramuscular inoculation.

A final explanation for the lack of antibody could result from immune-suppression induced by the virus. Mechanisms of both systemic immune-suppression and immune inhibition at the cellular level have been proposed. Observations of lymphoid depletion as a result of rabies virus infection were made in the late 1980s (Perry *et al*., 1990). This lymphopenia appeared to affect all lymphoid tissue including the thymus, spleen and lymph nodes and all cell types (Cardenas *et al*., 1995). The cause proposed for this effect was adrenal hormone toxicity of lymphoid tissues, as mice adrenalectomized prior to infection did not suffer similar depletion.

Further evidence for immunosuppression was manifest as reduced cell mediated responses to both the mitogen concanavalin A (Hirai *et al*., 1992) and the rabies virus specific antigen (Perrin *et al*., 1996). Recent research has suggested that rather than a hormonal imbalance causing systemic immunosuppression, cytokines may be responsible, and specifically, production of tumour necrosis factor alpha (TNF-α) in the CNS during infection (Marquette *et al*., 1996). This is supported by the improved survival of TNF-α receptor knockout mice in response to infection with neurovirulent rabies virus (Camelo *et al*., 2000). However, TNF-α transcription increases in the brain of mice infected with avirulent strains (Wang *et al*., 2005) and we have not observed increases in serum TNF-α in mice infected with both RABV and EBLV-2 (VLA, unpublished data). Also, many of these immunological perturbations are only observed in mice once disease develops.

In the absence of large-scale virus replication and damage to neurons, it is unclear how the immune response could be suppressed, especially in draining lymph nodes that might encounter antigen shortly after a biting event. Some have suggested that both lymphocytes (Thoulouze *et al*., 1997) and macrophages (Nakamichi *et al*., 2004) are susceptible to infection, and infection of these cells has a profound effect on the cytokine expression profiles seen.

A further possible immunosuppressive mechanism within virally infected cells has been identified. Recent studies have now demonstrated that in vitro, RABV is able to suppress the action of interferons (Vidy *et al*., 2005; Brzozka *et al*., 2006). This is mediated by the phosphoprotein that binds STAT isoforms, prevents their accumulation in the cytoplasm and disrupts interferon signaling. It has also been shown to inhibit translocation of STAT dimers into the nucleus and initiating transcription of interferon and interferon-inducible genes (Vidy *et al*., 2007). However, numerous studies have shown that inflammatory cytokines are produced within the CNS during infection suggesting there may be other pathways active in vivo that can compensate for this suppression (Saha and Rangarajan, 2003; Wang *et al*., 2005). It is unclear from these in vivo studies whether the source of CNS cytokines is directly due to RABV infection (i.e. neurons) or is generated by bystander cells or populations entering the CNS.

In conclusion, the immune privileged status of the CNS and the blood–brain barrier (BBB) could explain some of the delays in the development of a protective response. Virus replication only increases once RABV has entered the CNS so the level of antigen in the periphery is likely to be limited. Also, although there is now clear evidence for antigen presentation in the CNS and rapid drainage from the spine and CNS to local lymphoid

tissue (Knopf *et al.*, 1998), this could produce a delay in presentation of antigen to appropriate B cells and it seems unlikely that spleen is a relevant site for immune responses to rabies virus. Once B cells are stimulated and the process of maturation begins there is then the challenge to deliver antibodies or plasmacytoid cells back into the CNS. Again, experimental models have shown infiltration of both T and B cells into the dorsal root ganglia, spinal cord and brain (Johnson *et al.*, 2007). However, the majority of these cells are T cells, and most appear to be destined to apoptose shortly after entering the CNS (Baloul and Lafon, 2003). Furthermore, there are intrinsic complexities in enacting immune responses in the CNS. These include tight regulation of major histocompatibility expression (Irwin *et al.*, 1999), high levels of Fas-mediated apoptosis in lymphocytes and the expression by neuronal cells of immunosuppressive factors. The BBB remains intact during rabies virus infection (Roy *et al.*, 2007) and would likely exclude antibodies, although this does not appear to be complete as one report has suggested that a neutralizing monoclonal antibody can clear virus from the CNS when given intravenously (Dietzschold, 1993). Variation between virus strains may play a role in the rate of spread through the CNS, BBB permeability and the speed of development of the immune response. A number of studies have suggested that the virus suppresses the adaptive immune response. There is certainly compelling evidence that the viral phosphoprotein inhibits interferon responses although infection is associated with the increase in gene transcripts for interferon-inducible genes. It is likely that inhibition is transitory and provides a short delay in the host response that gives the virus a “headstart”.

However, there is a paradox in that the later stages of infection where viral burden is at its highest and encephalitis is at its most severe appears to be the one point where the host

immune system is at its most active. The suppression of peripheral immune responses is more controversial as it is not clear how the virus can achieve this in organs such as the spleen where no virus is present and there are no known soluble factors generated by the virus that could mediate this. A single article has investigated that presentation of RABV antigen in association with immune-stimulating complexes (Charlton *et al.*, 1997). This study showed the uptake of antigen by marginal zone macrophages in the spleen following intravenous injection and the subsequent development of antibody responses in the presence or after depletion of macrophages by clodronate liposome treatment. There have been no studies looking at the antigen presentation of rabies virus or B cell maturation in response to anti-rabies vaccination. This suggests that rabies virus infection may prove a useful paradigm in the investigation of all aspects of antibody development and could identify the source of infiltrating lymphocytes present during the later stages of infection. It will also have practical benefits in understanding the lack of immune development during infection and identification of strategies that might improve the induction of immunity by post-exposure prophylaxis (Charlton *et al.*, 1997).

2.5 Laboratory Examination of Rabies Virus

2.5.1 Detecting rabies virus: mouse inoculation tests

Historically and in the research setting, RABV infection is identified by infecting cells and detecting virus. This can be done either through the mouse inoculation test (MIT) or by inoculation of samples onto cultures of murine neuroblastoma or other cells (rapid tissue culture infection test, RTCT) (Maniand Madhusudana, 2013; Duong *et al.*, 2016).Following intracerebral inoculation of mice aged 3–4 weeks; MIT test results are

available after an incubation period of up to 28 days. Some strains are associated with a longer incubation period. In laboratories with cell culture facilities and an appropriate level of bio-containment, the RTCT provides results within 24–48 h, which is far quicker than intracerebral inoculation(Dacheux and Bourhy, 2015). Although it is more sensitive to toxic or bacterial contaminants, its sensitivity is comparable to that ofthe MIT (Duong *et al.*,2016). As RABV does not cause any cytopathic effect, the detection of the virus must therefore be evidenced by DFAT. In addition, MIT requires animal facilities to produce mice (or a supplier that can quickly provide animals of a suitable age in sufficient numbers), as well as animal facilities with a high level of bio-containment (ASL3) to maintain the inoculated animals. Animal ethics regulations also recommend avoiding the use of animals when an efficient cell culture system exists. As results are usually required urgently and due to animal protection issues, the World Health Organization (WHO, 2013), as well as the World Organization for Animal Health (OIE, 2012), now recommend replacing the MIT with the isolation of RABV in cell culture whenever possible.

2.5.2 Detecting Rabies viral RNA in samples

The international health organizations do not currently consider molecular assays to be reference techniques for the post-mortem diagnosis of RABV in humans and animals. However, they are recommended for intra-vitam diagnosis in humans. Although they require laboratory technicians to be trained and validated in molecular techniques, a PCR assay has been shown to be more sensitive than DFAT for the detection of lyssaviruses(Picard-Meyer*et al.*, 2004).

I. Reverse-transcriptase PCR (RT-PCR)

RT-PCR is the method most often used to detect RABV RNA for intra vitam diagnosis of rabies in humans. Saliva samples or skin biopsies taken at the nape of the neck (being careful to include hairfollicles)(Dacheux*et al.*, 2008) can be tested using this technique. The test reaches100% sensitivity when at least three successive saliva samples arecollected at 3- to 6-h intervals (due to irregular viral shedding) and tested (Dacheux *et al.*, 2008). Positive results can be obtained as soon as the patient isadmitted. Human and animal brain samples collected by variousmethods can also be tested with this technique, even when theyhave been kept at relatively high ambient temperatures or when they have become degraded(David*et al.*, 2002; Rojas *et al.*, 2006; Duong *et al.*, 2016). Several sets of more or lessconsensual primers for RABV have been developed. In some cases,primers specific for variant types of RABV have been developed forprecise and geographically limited purposes, especially research.Although time- and resource-intensive, the sequencing of PCRamplicons can improve the specificity of the technique. Specificityis very high when the technique is implemented rigorously, butfalse-negatives and false-positives may occur. RT-PCR is highlysusceptible to cross-contamination in the operational setting,unless standardization and procedures are stringent for the PCRitself and for the sample extraction and reverse transcription of the RNA(Hanlon and Nadin-Davis, 2013).Other promising virus detection techniques have been developed, such as loop-mediated isothermal amplification (LAMP)(Muleya*et al.*,2012; Duong *et al.*, 2016) and nucleic acid sequence-based amplification (NASBA) (Wacharapluesadee *et al.,* 2011). These are suited to developing settings as they require less sophisticated equipment and are less costly (Duong *et al.*, 2016).

II. Real-time reverse transcriptase PCR (RT-qPCR)

These assays are based on the transcription of viral RNA to cDNA before amplification (RT). This phase is followed by PCR, which uses specific primers and probes or a dye to provide real-time quantification of DNA. These assays reduce the risk of cross contamination thanks to closed tubes and show an improved sensitivity compared to conventional RT-PCR protocols (Hayman*et al.*, 2011; Duong *et al.*, 2016). However, as the probes used are highly specific for known sequences, sequence mismatch between the primer/probe sequences and the target viral sequence may adversely affect the sensitivity of the test, leading to false-negative results. Because the PCR fragments generated are usually very short, thesequencing of the amplicons may provide diagnostic confirmation but cannot be used for in-depth molecular phylogenetic analyses(Mey*et al.*, 2016).

2.5.3 Detecting rabies viral antigens

a) Direct fluorescent antibody test (DFAT)

Direct fluorescent antibody test is the main assay used worldwide; it is the WHO and OIE recommended gold standard for the diagnosis of rabies in fresh or frozen brain samples. The latter are important in tropical countries, as preserving fresh samples at 4-8°C is often a challenge(Centers for Disease Control and Prevention, 2011; Duong *et al.*, 2016). This assay is based on attaching fluorescein isothiocyanate (FITC) to polyclonal antibodies targeting the RABV ribonucleocapsid, or monoclonal antibodies targeting the RABV nucleoprotein (N). If the targeted RABV antigen is present in the sample fixed on a slide,antibodies attach to it, remain attached despite washing, and canbe observed using a fluorescence microscope(Mani and Madhusudana, 2013). Results are available within 1–2 h and are expressed as positive or negative. The sensitivity and specificity of DFAT nears 99% in an experienced laboratory, but is extremely observer-dependent(Robardet *et al.*,

2013; Duong *et al*., 2016). At least two observers must spend enough time on each slide once the quality of the sample has been ensured. This test is best performed on fresh brain samples; the reliability of this assay to diagnose rabies in degraded animal brain samples or corneal smears is low(Mani and Madhusudana, 2013; Duong *et al*., 2016).

b) Enzyme linked immuno-sorbent assay (ELISA)

Enzyme Linked Immuno-Sorbent Assay (ELISA) techniques have been adapted to detect RABV antigens in samples using monoclonal or polyclonal antibodies. These use microplates coated with purified polyclonal or monoclonal anti- RABV IgG targeting the nucleocapsid. Several versions of these assays have been developed (RREID, WELYSSA, etc.) and some have been commercially available for some time (Perrin *et al*., 1992; Xu*et al*., 2007; Duong *et al*., 2016)They have been shown to be sensitive and specific and can be applied even to partially degraded brain samples(Mani and Madhusudana, 2013). Test results can be evaluated qualitatively with the naked eye. However, these techniques are less sensitive than DFAT (96% agreement between DFAT and RREID test results)(Meslin*et al*., 1996). For this reason, they should not replace DFAT in laboratories where DFAT is already performed. These are now implemented in a very limited number of countries, using homemade reagents (Duong *et al*., 2016).

c) Rapid immuno-diagnostic test (RIDT)

The Rapid Rabies Ag Test Kit is a lateral flow device based on a qualitative chromatographic immunoassay developed for the detection of RABV antigen in fresh animal brain tissue (e.g., canine, bovine, raccoon dog). This type of new RIDT appears

suited to the field and frontline laboratories. It is sensitive andspecific for brain samples from any animal and it's highly dependent on theantibodies (polyclonal or monoclonal) used. This test has a sensitivity ranging from 91.7% to 96.9% and a specificity ranging from 98.9% to 100% when compared to a fluorescent antibody test (Kang *et al*., 2007; Yang*et al*., 2012). Furthermore, a result can be obtained within 15– 20 min, including the preparation time. This test appears to be very accurate in detecting RABV antigen, but other tests are required if questionable results are obtained Duong *et al*., 2016). However, the RIDT kit requires further validation before it can be recommended for use by either the OIE or WHO. At this stage, the RIDT should be implemented (1) for research purposes only, or (2) in frontline laboratories to improve the surveillance and control of rabies in remote places from which the shipment of samples to a central laboratory would be difficult or even impossible (or where classical rabies laboratory diagnosis using recommended techniques cannot be established for financial or logistical reasons) (Duong *et al*., 2016). Many different products using the same methodology are commercially available and these exhibit highly variable intrinsic properties (Duong *et al*., 2016; Eggabauer *et al*., 2016). Laboratories aiming to use the RIDT should therefore evaluate the kit carefully and assess the specificity and sensitivity or should rely on available evaluations performed by international reference laboratories before routine use (Duong *et al*., 2016).

d) Direct rapid immunohistochemistry test (DRIT)

This promising method was developed recently by the US Centers for Disease Control and Prevention (CDC). It is based on the detection of rabies N protein in brain smears fixed in formalin, using highly concentrated monoclonal antibodies in the presence of streptavidin

peroxidase and a substrate coloring agent. Test results are available within 1 h. This test can be implemented in the field and no fluorescence microscope is required (Dürr*et al*., 2008;Fooks*et al*., 2009; Madhusudana*et al*., 2012). The estimated sensitivity and specificity approach 100% when compared to a fluorescent antibody test (Lembo *et al*., 2006; Madhusudana *et al*., 2012). This method can also beused for samples that have been frozen or preserved in glycerol (Duong *et al*., 2016).Cost-effective indirect immunochemistry assays have been developedthat can also provide indications on the RABV variant(Dyer *et al*., 2013). A major concern, however, is access to uninterrupted supplies of controlled batches of monoclonal antibodies, which are only available through a few laboratories specialized in rabies diagnosis (Duong *et al*., 2016).

2.5.4 Detecting rabies antibodies

The detection of antibody in serum in the absence of a history of rabies vaccination, or in cerebrospinal fluid (CSF), provides indirect evidence of rabies infection. However, the interpretation of test results may be difficult, since the host immune response may vary among individuals: the sensitivity and negative predictive value of antibody detection methods in rabies patients is very poor,(Mani and Madhusudana, 2013; Wasniewski*et al*., 2014) as patients with suspected rabies overwhelmingly die before they can mount an antibody response(Duong *et al*., 2016). The antibody response is only detectable in the blood (or CSF) after 8–10 days, (Johnson *et al*., 2010) while the majority of human rabies deaths occur around six days after the onset of clinical signs(World Health Organization, 1999; Duong *et al*., 2016). The poor yield of this technique has been shown in a series of human rabies cases(Noah *et al*., 1998). These tests are thus better suited to assess the protection of laboratory or veterinary workers or of pets before trans boundary travel, or to

check for the appropriate immune response in patients receiving post exposure prophylaxis (PEP) as part of research (vaccine evaluation, seroprevalence studies) (Duong *et al.*, 2016).

2.6 Preservation of Tissues for Rabies Diagnosis

During the transportation or storage of suspect material for diagnosis (animal heads, brain or other tissue samples), no risk of human contaminationshould arise: brains must be placed in a leak-proof rigid container (animal heads will be wrapped in absorbent material) (Shankar, 2009). An explosion proof -20°C freezer is required for storage; long term sample storage requires a freezer at -70°C. Frost-free freezers should not be used. Heat cycles in frost-free freezers will denature proteins in reagents and specimens and may compromise test results. If laboratory is within close distance, ice packs containing dry ice can be used to preserve or transport fresh sample to the laboratory (Shankar, 2009).

When it is not possible to send refrigerated samples, other preservation techniques may be used. The choice of the preservative is closely linked to the tests to be used for diagnosis(Shankar, 2009): If the specimen has been preserved in a formalin solution, DFAT may be used only after the specimen has been treated with a proteolytic enzyme such as proteinase K prior to staining to expose antigenic sites that formerly were masked by bonds resulting from fixation (Warner *et al.*, 1997). Selection and increasing the concentration of well suited anti-rabies monoclonal antibodies in DFAT has also been used to improve rabies antigen detection in formalin fixed tissues (Warner *et al.*, 1999; Wurner and Jackson, 2010). Confirmatory and further characterization tests such as virus isolation

cannot be performed on formalin-fixed material due to inactivation of the virus and conventional reverse transcription polymerase chain reaction (RT-PCR) is problematic becauseviralRNAisknownto degrade during formalin fixation(Bustin,2002). However, Coertse*et al*. (2011) applied a real-timeRT-PCR method on formalin fixed brain tissue and it was possible to confirmrabies and obtain phylogenetic information thatindicated a close relationship between this virus and the canid rabies virus variants from another province (KwaZulu-Natal) in South Africa.

Infectivity at room temperature may be extended for several days if brain material is kept in a mixture of 50% glycerol in phosphate buffered saline (PBS)(Dürr*et al*., 2008). It does not protect against titre decline due to thermal conditions and therefore, because rabies is thermolabile, the virus titer will decline during glycerol/PBS storage(Shankar, 2009). Under normal transport conditions in the tropics, this protection may only be effective for a matter of several days. Therefore, whenever possible samples in glycerol/saline should be kept refrigerated. As the virus is not inactivated by glycerol/PBS, all laboratory tests can be used on these samples(Dürr *et al*., 2008; Shankar, 2009).

2.7 Rabies Antigen Retrieval from Formalin-Fixed Tissues

Formaldehyde, originally discovered to be a tissue fixative by Ferdinand Blum in 1893, has been used widely to the present day (Gatta*et al*., 2012; Howatand Wilson, 2014). As a fixative, formalin is made up of commercial concentrated formalin (37–40% solution of formaldehyde) diluted to a 10% solution (3.7– 4% formaldehyde). Formalin has several advantages over alcohol, particularly the superior preservation of morphological detail(Shi *et al*., 1997). Because of the long history of the use of formalin as the standard fixative for

tissue processing in histopathology, most of the criteria for pathological diagnosis have been established by the observation of formalin-fixed, paraffin-embedded tissue sections stained with hematoxylin and eosin. For this reason, although many other fixatives exist and some may be superior for immunohistochemistry (IHC),none has supplanted formalin in general use(Shi*et al.*, 1997).

With the recognition that formalin fixation causes major chemical changes in antigens by unknown mechanisms, several attempts were made to modify the methods of fixation and a variety of modified fixatives have been proposed for preservation of certain antigens (Taylor and Cote 1994; Howat and Wilson, 2014). However, no ideal fixative has been found that can be used universally in immunohistochemistry. In addition, the modified fixatives do not closely reproducethe preservation of morphology achieved by formalin fixation. A change of fixative, therefore, may also mean changing the histopathological criteria employed for diagnosis. A direct consequence would be loss of the benefit of the rich collection of archival paraffin tissues the world over(Shi *et al.*, 1997). These were the primary thoughts behind the development of novel antigen retrieval (AR) technique, to maintain formalin as the standard fixative for both morphology and immunohistochemistry (Shi *et al.*, 1997;Srinivasan*et al.*, 2002).

Biochemical studies of the chemical reaction between protein and formalin by Fraenkel–Conrat and co-workers in the 1940s indicated that hydrolysis of cross-linkages between formalin and protein is limited by certain amino acid side chains, such as imidazol and indol,but that these crosslinkages can be reversed by high-temperature heating (120°C) or strong alkaline treatment(Fraenkel-Conrat and Olcott, 1948; Thavarajah*et al.*, 2012). This observation formed the basis for the development of AR techniques in 1991 (Shi *et al.*,

1991; 1992). A simple method of either heating the routinely fixed paraffin-embedded tissue sections in water or immersing the routinely formalin-fixed, acid decalcified, celloidin-embedded tissue sections in a NaOH–methanol solution yielded dramatic retrieval results (Shi *et al*., 1991,1992, 1993a). The AR technique has been used increasingly and has demonstrated its value in many studies (Shi *et al*., 1993b; 1997; De Matos*et al*., 2010;Okoye and Nnatuanya, 2015).

2.7.1 Factors that can influence rabies antigen retrieval

I. The effect of heating

The fact that high-temperature heating is the most important factor for retrieval of antigens masked by formalin fixation was demonstrated by Shi *et al*. (1991) and in many subsequentapplications of AR–IHC in pathology (Taylor *et al*.1996;Alelú-Paz *et al*., 2008;Otali*et al*., 2009). Higher temperature in general yields better results of AR–IHC. For example, Kawai *et al*. (1994) found that heating at 90°C for 10 minutes was more effective than heating at 60°C for 120 minutes. Different heating methods have been used for AR–IHC, such as autoclaving (Bankfalvi *et al*., 1994; Pons *et al*., 1995), pressure cooking (Miller and Estran 1995;Pileri *et al*., 1997), water bath (Vinod *et al*., 2016), microwaving (MW) (Erber *et al*., 1996; Hussaini *et al*., 2013), and steam heating (Pasha *et al*. 1995; Ramos-Vara and Beissenherz, 2000). Again, the temperature achieved by these methods appears to be the critical variable.

II. The effect of pH.

Another important factor is the pH of the AR solution (Shi *et al*., 1995; 1997). Although some antigens yield satisfactory results by AR treatment with the use of distilled water,

other antigens require heating in buffers of specific pH to obtain the strongest intensity of staining(Shi*et al*., 2001). A few antigens yielded satisfactory results only when buffers in a limited pH range were used. For example, an extracellular immunostaining pattern when the MAb to thrombospondin was used on archival paraffin sections could be achieved only by AR–IHC with the use of low-pH (pH 1–2) AR solution(Grossfeld *et al*., 1996; Shi *et al*., 1997).

III. The effect of molarity

Suurmeijer and Boon (1993b) tested the effect of molarity by using aluminum chloride solution with concentrations ranging from 0.5% to 4% for AR–IHC staining of vimentin on archivalparaffin sections. They indicated that the best stainingfor vimentin was achieved with the use of 4% aluminumchloride. For citrate and other buffers in widespread use for AR, molarity is not a major issue (Shi *et al*., 1997).

IV. The antigen retrieval solution.

Although the chemical componentof AR solution may play a role as a possible co-factorin the heating procedure, thus far no single chemical has been identified that is both essential and best for AR. To determine the ideal AR solution, Shi *et al*. (1997) recommended using a “test battery,” because different antigens may require different conditions for retrieval. It may be possible to obtain an equivalent intensity of staining with several different AR solutions if the pH value of the AR solution and the heating conditions are optimized, using the “test battery” method. For example, Katoh and Breier (1994) found that equally good results of p53 immunostaining by the AR–IHC method can be obtained with either normal saline, citrate buffer, or distilled water. Hazelbag *et al*. (1995) tested a broad panel

of MAbs against a variety of keratins on formalin-fixed, paraffin-embedded tissues with different AR solutions. They concluded that microwave heating in either “TUF” or a simple detergent, DET, in a 0.05% solution containing anionogenic (15–30%) and non-ionogenic (5–15%) surfactants (Dish Clean; Bosman Chemie, Heijningen, The Netherlands) before immunostaining could yield similar results. On the basis of the study of AR–IHC and pH, Shi *et al*. (1995) demonstrated three types of antigens based on pH values of the AR solution, which was supported by recent studies concerning protein structure, indicating that the complexity of protein structure is not as complex as the function of protein (Alberts *et al*., 2002).

2.7.2 Mechanism of rabies virusantigen retrieval

Antigen Retrieval (AR) has shed some light on our understanding of the mechanism(s) of protein-formalin interactions. First of all, it showed that the modification of protein structure by formalin is reversible under certain conditions, such as high-temperature heating or strong alkaline treatment (Shi *et al*., 2011). Shi *et al*. (2001) suggested a possible mechanism of the AR technique, i.e., loosening or breaking of the cross linkages caused by formalin fixation. Mason and O’Leary (1991) demonstrated that the process of crosslinkingdoes not result in discernible alteration of protein secondary structure. Therefore, the AR technique may take advantage of the fact that the crosslinking of protein produced by formalin fixation may “protect” formalin-modified epitopes from denaturation during the heating procedure. Suurmeijer and Boon (1993a) summarized the possible mechanism of AR as follows: (a) breaking of the formalin-induced cross linkage between epitopes and unrelated proteins; (b) extraction of diffusible blocking proteins; (c) precipitation of protein; and (d) rehydration of the tissue sections, allowing better

penetration of antibody and increasing accessibility of epitopes. In contrast to the "breaking crosslinking" theory, Cattoretti *et al*. (1993) proposed a "protein denaturation" theory on the basis of their observation that some antigens or endogenous enzymatic activities might be lost after heating AR treatment.To understand the mechanism(s) of AR, the first step may be to study the exact alterations in the three dimensional structure of proteins caused by formalin fixation. Because antigen–antibody recognition is often dependent on the three-dimensional structure of protein, it is likely that the mechanism of AR may be based on a re-modification of the formalin modified protein structure (Shi *et al*., 1997).

Crosslinking is a special form of chemical modification of protein (Spicer and Davis, 2014), which can be defined as a process involving the joining of two molecular components by covalent bonds achieved by the use of crosslinking reagents, such as tissue fixatives (Shi *et al*., 2001). Traditionally, the process of tissue fixation has been divided into two stages: the primary reaction is an addition reaction between amine and an aldehyde (e.g., formalin) or ketone (e.g., acetone), $R\text{-}NH_2 + HCHO \rightarrow R\text{-}NH\text{-}CH_2OH$,followed by a secondary condensation reaction: $R\text{-}NH\text{-}CH_2OH + H_2N\text{-}CO\text{-}R' \rightarrow R\text{-}NH\text{-}CH_2\text{-}NHCO\text{-}R' + H2O$ (Shi *et al*., 1997). Both aldehyde and ketone are carbonyl compounds that can react with amino groups of proteins to form Schiff bases (Spicer and Davis, 2014). This addition reaction of acetone may explain some of the enhancement of AR–IHC for tissues fixed in fixatives other than formalin (Howat and Wilson, 2014).

The secondary, tertiary, and quaternary structures of proteins are dependent on the primary structure or the amino acid sequence. In general, if the primary structure of protein is still intact it may be possible torecover its original three-dimensional conformation under an

optimal retrieval system (Shi *et al.*, 2011). Because cross-linkages are complicated processes that depend on a variety of conditions such as pH, temperature, conditions of tissue and fixation, they may lead to a variety of protein alterations (Alberts *et al.*, 2002).

Antibodies recognize specific epitopes localized in a particular spatial configuration within the protein molecule. This is particularly true for discontinuous antigenic determinants, which are composed of residues from different parts of the amino acid sequence (Shi *et al.*, 1997; Arrueboet *al.*, 2009). A change in protein conformation caused by formalin fixation may mask the tissue antigenicity. The AR method may lead to a re-modification of the protein structure (induced by high-temperature heating or by non-heating procedures, such as strong alkaline treatment) and re-establish the three-dimensional structure of protein in its native condition, or very close to that state(Shi *et al.*, 1997).This "modification–re-modification" hypothesis has been supported by previous studies. Using microwave heating followed by trypsin digestion, Szekeres *et al*. (1994; 1995) found a decrease in the nuclear staining of ER and MIB1, which may correspond to a sensitivity of the nuclear Ki-67 to enzyme digestion, as the antigenic structure was modified by microwave AR heating in such a way that the epitope was exposed for the subsequentalteration by enzyme treatment. Some studies have demonstrated that if a fixed tissue section shows satisfactory immuno-staining without AR, it may become worse after AR treatment; the converse may also be true (Shi *et al*., 2011; Katoh, 2016). This might mean that if an antigenic structure is formalinresistant, i.e., without modification after fixation, it may be modified by heating. Therefore, the immuno-reactivity may be worse after AR heating unless the antigenic structure is also heat-resistant.Morgan *et al*. (1994) suggested a hypothesisconcerning the possible mechanism of AR, focusingon tissue-bound calcium ions. These authors

suggestedthat the calcium complex formation with protein in their formalin-fixed state may mask the antigen, which may need a considerable amount of energy, i.e., high-temperature heating and calcium chelation (citrate), to release the calcium from this cage-like calcium complex. To test this hypothesis, they added excess calcium ions in a mixed solution of 50 mM $CaCl_2$ with either Ethylenediaminetetraacetic acid (EDTA), Ethylene glycol tetraacetic acid (EGTA), or citrate buffer (calcium chelators) as the AR solution for autoclave heatingtreatment, and obtained a negative staining for MIB1. The same negative result could also be obtained by a sequential use of AR solution and 50 mM $CaCl_2$ solution in heating the tissue sections. They suggest that there is inhibition of AR by either competitive (mixed $CaCl_2$ with AR solution) or non-competitive (sequential use of AR solution and $CaCl_2$ solution) inhibition. They also tried to repeat the AR procedure after inhibition of $CaCl_2$ and obtained partially recovered immuno-staining intensity. When the concentration of $CaCl_2$ was reduced to 10 mM for inhibition, the inhibition of MIB1 staining was completely reversed.

Shi *et al*. (1997) conducted a test of AR–IHC in the presence of $CaCl_2$. They found that (a) The same negative staining of MIB1 could be obtained by using $CaCl_2$ alone as the AR solution; (b) not all antibodies showed a negative result, e.g., the intensity of AR–IHC for keratin remained the same after use of $CaCl_2$; and (c) re-heating the slides in Tris-HCl buffer, pH 1.5, restored a stronger positive stainingfor both MIB1 and p53. Although the fact thatcalcium interferes with AR (at least for some antigens) is consistent with the hypothesis that calcium-induced modification of protein may play a role in the retrieval process, it does not prove that binding of endogenous calcium is responsible for widespread loss of antigenicity in formalin fixation. Another issue that arouses suspicion

of the calcium-binding hypothesis is the fact that the same intensity of AR with MIB1 can be obtained by a variety of AR solutions other than citrate buffer or with any calcium chelating agents. In particular, a stronger intensity can be produced by using a low pH or a higher pH of Tris-HCl buffer for AR of MIB1 (Shi *et al*. 1995). It is necessary to study the superstructure of protein, particularly the antigenic structure, under various conditions, such as normal (frozen), after formalin fixation, and after AR treatment, to understand the exact mechanism of AR.On the basis of this study, more effective AR techniques may be developed (Shi *et al*., 1997).

CHAPTER THREE

MATERIALS AND METHODS

3.1Study Area

The experiment was conducted at the National Veterinary Research Institute, Vom. Vom is one of the towns in Plateau state, central Nigeria, situated on the Jos Plateau near the source of the Kaduna River, 18 miles (29 km) southwest of Jos town. Vom is located at latitude 9° 44' 00" N and longitude 8° 47' 00" E. (National Geospatial-Intelligence Agency, Bethesda, MD, USA, 1995-2012).

3.2 Specimen Collection

Dog brain tissues (DFAT positive and negative) preserved at -20°C were collected from the archives of the Central Diagnostic Laboratory, National Veterinary Research Institute, Vom, for this study. The tissues were thawed at room temperature. Approximately 1g of brain tissue samples were cut and homogenized in 10ml PBS (pH 8.5). Supernatants were carefully collected after centrifuging at 1000 rpm for 20 minutes. All homogenates and supernatants were stored at -20°C until used.

Fresh dog brain specimens were also collected as paired samples from rabies suspected cases that were received for postmortem detection of rabies in the Central Diagnostic Laboratory, National Veterinary Research Institute, Vomover a period of three months. Samples were collected from different anatomic locations of the brain, viz: hippocampus,

cerebellum, and brain stem respectively. One portion of each paired sample was prepared for fresh fluorescent antibody testing and the other portion was fixed in 10% neutral buffered formalin (NBF) at room temperature for a minimum period of three days. The tests were performed in blinded schedules. The DFAT evaluations of the rabies status of fresh tissue samples and those of formalin- fixed tissue samples were maintained independently until all tests were completed.

3.3 Sample Size

A total of 90brain samples (50 archived,40fresh/formalin-fixed)werecollected for this study basedon convenience.

3.4 Detection of Rabies Virus Antigen in Frozen Archival Dog Brain Tissues Using Direct Fluorescent Antibody Test, Enzyme Linked Immuno-Sorbent Assay and Rapid Immuno-Diagnostic Test

3.4.1 Direct fluorescent antibody test.

Direct fluorescent antibody test (DFAT) was done according to Dean*etal*. (1996) and Office International des Épizooties (OIE) (2013). Impression smear was made on appropriately labeled pre-cleaned slides by turning the slides over the assembled portion of the brain tissues. Positive and negative controls were set up separately. The slides were air dried at room temperature and fixed by placing in a coplin jar containing acetone at -20°C for 30 minutes. The slides were removed from the acetone and then air-dried at room temperature. The fixed slides were transferred to a humidified chamber and a drop of 150µl of fluorescein-labeled monoclonal anti-rabies immunoglobulin (Fujirebio Diagnostics, Inc., U.S) was used for staining. These were then incubated at 37ºC for 30 minutes. After incubation, the slides were washed in Phosphate Buffered Saline (PBS) (pH

8.5), two changes for 5 minutes. The slides were then air-dried at room temperature and arranged in a slide carrier. A drop of 50% mounting buffered glycerol and a cover slip were applied on each smear. The slides were visualized under a fluorescent microscope (Zeiss International, Germany). Presence of Bright/dull/dim apple green oval or ellipsoid fluorescing intracellular accumulations was considered positive. Using the positive and negative control slides to compare, fluorescence was scored by two separate individuals using a three-plus scoring system (scores were as follows: 3 +++ bright apple green fluorescence; 2 ++ dull apple green fluorescence; 1 + dim but detectable apple green fluorescence). Positivity of impression smears of the various parts of the brain was compared to determine the distribution of rabies virus antigen across the fresh brain.

3.4.2 Enzyme linked immuno-sorbent assay (Sandwich ELISA)

Approximately 1g of brain tissue sample was cut and homogenized in 10ml PBS (pH 8.5). Supernatants were carefully collected after centrifuging at 1000 rpm for 20 minutes. The test was carried out according to the manufacturer's instruction (MyBioSource, U.S, MBS9391984). Briefly, in the micro-ELISA strip plate, two wells were left as negative control and another two wells as positive control. Negative and positive controls in a volume of 50µl were added to the negative and positive control wells respectively. In sample wells, 10µl sample and 40µl Sample dilution buffer were added. 100µl of HRP-conjugate reagent was added to the positive control, negative control and sample wells and then mixed very well by gentle shaking. The plate was incubated for 60 minutes at 37°C after covering with an adhesive strip. Following incubation, the test wells were washed manually with 1:20 pre diluted washing buffer solution (400µl) by carefully peeling off the adhesive strip and washing 5 times. At each washing step, the wash solution was decanted

after resting for one minute. After the last wash and decanting, any remaining wash solution was removed by aspirating. The plate was inverted and blotted against clean paper towels. Fifty microliter of Chromogen Solution A and 50µl Chromogen Solution B were added to each well (shielded from light) and mixed by gently shaking. The plate was then incubated at 37℃ for 15 minutes. Fifty microliter of the stop solution (Appendix I) was added to each well to terminate the reaction and the wells observed for colour change.

The optical density (OD) of the test wells were then read at 450nm using a microtitre plate reader (Thermo ScientificMultiskan FC, Finland) ensuring that the bottom of the wells was clean prior to reading. Assay was carried out within 15 minutes after adding stop solution. The critical value (cut off) was calculated as the average OD value of negative control + 0.15. The sample is canine rabies virus positive if the OD value ≥ cut off. Scoring was done based on the values of the sample OD and the cut off.

3.4.3Rapid immuno-diagnostic test

The test was done according to the manufacturer's instruction (Quickings, China, SHQB17-G135). Briefly, swab stick was inserted into 10% brain tissue fluid homogenates (prepared as described earlier) until saturated and then placed into the assay buffer tube where it was thoroughly agitated to ensure good sample extraction. The cassette was taken out from the foil pouch and placed horizontally. Gradually, 3 drops of sample extraction were dripped into the sample hole using disposable dropper. The result was interpreted in 5-10 minutes.

The presence of both control band and test band on the strip (whether test band is clear or vague) was considered positive. The test and control lines on the strips were separately

classified by two individuals using a three-plus scoring system representing the intensity of the reaction in the test line area.

3.5 Rabies AntigenRetrieval Using Sodium Citrate Buffer and the Detection of Rabies Virus Antigen in Citrate Buffer Treated Formalin-Fixed and Fresh Tissues by Direct Fluorescent Antibody Test.

3.5.1 Preparation of sodium citrate buffer (0.01M; pH 6.0) for rabies antigen retrieval

Sodium citrate (2.35g)and citric acid (0.42g) were dissolvedin 200ml of distilled water.Then, the total volume of the mixture was made up to one litre with distilled water in a volumetric flask. The sodium citrate buffer was heated to 92°C in a water bath.

3.5.2 Direct fluorescent antibody test on citrate buffer treated formalin-fixed and fresh brain tissues

Formalin fixed brain tissues were removed and washed three times with PBS (pH 8.5). To prepare smears, the brain stem, hippocampus and cerebellum were crushed together in a mortar after which impression smears were made on clean slides. The slides were air dried at room temperature and then fixed by placing in a coplin jar containing acetone at -20°C for 30 minutes. The slides were removed and air dried at room temperature. The slides were then immersed in the staining dish containing the pre-heated sodium citrate buffer (0.01 M; pH 6.0) and incubated at 92°C for 30 minutes in a water bath. The staining dish was then removed and slides were allowed to cool at room temperature for 20 minutes. Thereafter, slides were rinsed once in PBS (pH 8.5) and air dried at room temperature. The slides were transferred to a humidified chamber and a drop of 150µl of fluorescein-labeled monoclonal anti-rabies immunoglobulin (Fujirebio Diagnostics, Inc., U.S) was used for staining. These were then incubated at 37ºC for 30 minutes. After incubation, the slides were washed three times with Phosphate Buffered Saline (PBS) (pH 8.5). The slides

were then air-dried at room temperature and arranged in a slide carrier. A drop of 50% mounting buffered glycerol and a cover slip were applied on each smear. The slides were visualized under a fluorescent microscope (Zeiss International, Germany). Presence of Bright/dull/dim apple green oval or ellipsoid fluorescing intracellular accumulations was considered positive. Fluorescence was scored by two separate experienced individuals using a three-plus scoring system (scores were as follows: 3 +++ bright apple green fluorescence; 2 ++ dull apple green fluorescence; 1 + dim but detectable apple green fluorescence). Direct fluorescent antibody test was also carried out on formalinized brain tissues without treatment with sodium citrate buffer as a control.

Direct Fluorescent Antibody Test on fresh brain tissues was done as earlier described for DFAT on frozen archived brain specimens.

3.6Enzyme Linked Immuno-Sorbent Assay (Sandwich ELISA) And Rapid Immuno Diagnostic Test on Formalin-Fixed Dog Brain Tissues

The ELISA and RIDT were conducted as described earlier after thoroughwashing of the formalinized brain tissues (3X) for 2 minuteswith PBS (pH 7.2).

3.7 Data Analysis

The intensity of the fluorescence was counted and given one point per cross (+: 1 point; ++: 2 points; +++: 3 points). The concordance coefficient and simple Cohen's kappa coefficient value were used for statistical comparison of the diagnostic tests. The concordance coefficient values were expressed as percentage. The kappa value of agreement levels was interpreted as follows: Poor agreement ≤0.20, fair agreement =0.20-0.40, moderate agreement =0.40-0.60, good agreement =0.60-0.80, and very good agreement ≥0.80. The confidence interval was calculated by assuming a binomial

distribution. All statistical procedures were done using the MedCalc Software (MedCalc Software bvba, Version 17.8).

CHAPTER FOUR

RESULTS

4.1 Detection of Rabies Virus Antigen in Frozen Archived Dog Brain Tissues Using Direct Fluorescent Antibody Test, Enzyme Linked Immuno-Sorbent Assayand Rapid Immuno-Diagnostic Test.

A total of 50 DFAT confirmed cases (44 positive and 6 negative) offrozen archived dog brain tissues were tested with DFAT, ELISA and RIDT. Out of the 44 DFAT positive frozen archivedbrain samples tested, 44 (100%) tested positive by DFAT (Appendix II),42 (95.5%) tested positive by ELISA (Appendix III) and 21(47.7%) tested positive by RIDT (appendix IV) (Table 4.1).Table 4.2 shows the sensitivity and specificity of ELISA and RIDT relative to DFAT for rabies antigen detection in frozen archived brain samples. Two (4%) samples that were negative for rabies antigen by DFAT were positive by ELISA. Twenty-two (44%) samples that were positive by DFAT were negative by RIDT while 1 (2%) sample that was negative by DFAT was positive by RIDT. However, we found 96% agreement (42 positives and 6 negatives) of ELISA and DFAT and 54% agreement of RIDT and DFAT (20 positives and 7 negatives). Compared to DFAT, the Sensitivities of ELISA and RIDT were 95.5% and 47.6% respectively while the specificities of ELISA and RIDT were 100% and 87.5% respectively. The simple Cohen's kappa coefficient for ELISA relative to the DFAT was found to be 0.834 (95% C.I. 0.613-1.0). For RIDT, the Kappa value was 0.170 (95% C.I. 0.003- 0.337). The concordant scores of the various techniques was shown in figure 4.1.

Table 4.1: Rabies Antigen Detection in Frozen Archived Dog Brain Tissues by Direct Fluorescent Antibody Test, Enzyme Linked Immuno-Sorbent Assayand RapidImmuno-Diagnostic Test

	$DFAT_a$	$DFAT_b$	%	ELISA	%	RIDT	%
Positive	44	44	100	42	95.5	21	47.7
Negative	6	6	100	6	100	6	100

Key: $DFAT_a$: Direct Immuno-Fluorescent Test before testing; $DFAT_b$: Direct Immuno-Fluorescent Test after testing;ELISA: Enzyme Linked Immuno-Sorbent Assay; RIDT: Rapid Immuno-Diagnostic Test; %: Percentage.

Table 4.2: Sensitivity and Specificity of Enzyme Linked Immuno-Sorbent Assayand Rapid Immuno-Diagnostic Test for Rabies Antigen Detection in Frozen Archived Brain Samples Against The 'Gold Standard' Direct Fluorescent Antibody Test

		ELISA			RIDT		
		P	N	TOTAL	P	N	TOTAL
DFAT	P	42	2	44	20	22	42
	N	0	6	6	1	7	8
Total		42	8	50	21	29	50
Sensitivity				95.5%			47.6%
Specificity				100%			87.5%

Key: P: positive; N: negative; DFAT: Direct Immuno-Fluorescent Test; ELISA: Enzyme Linked Immuno-Sorbent Assay; RIDT: Rapid Immuno-Diagnostic Test.

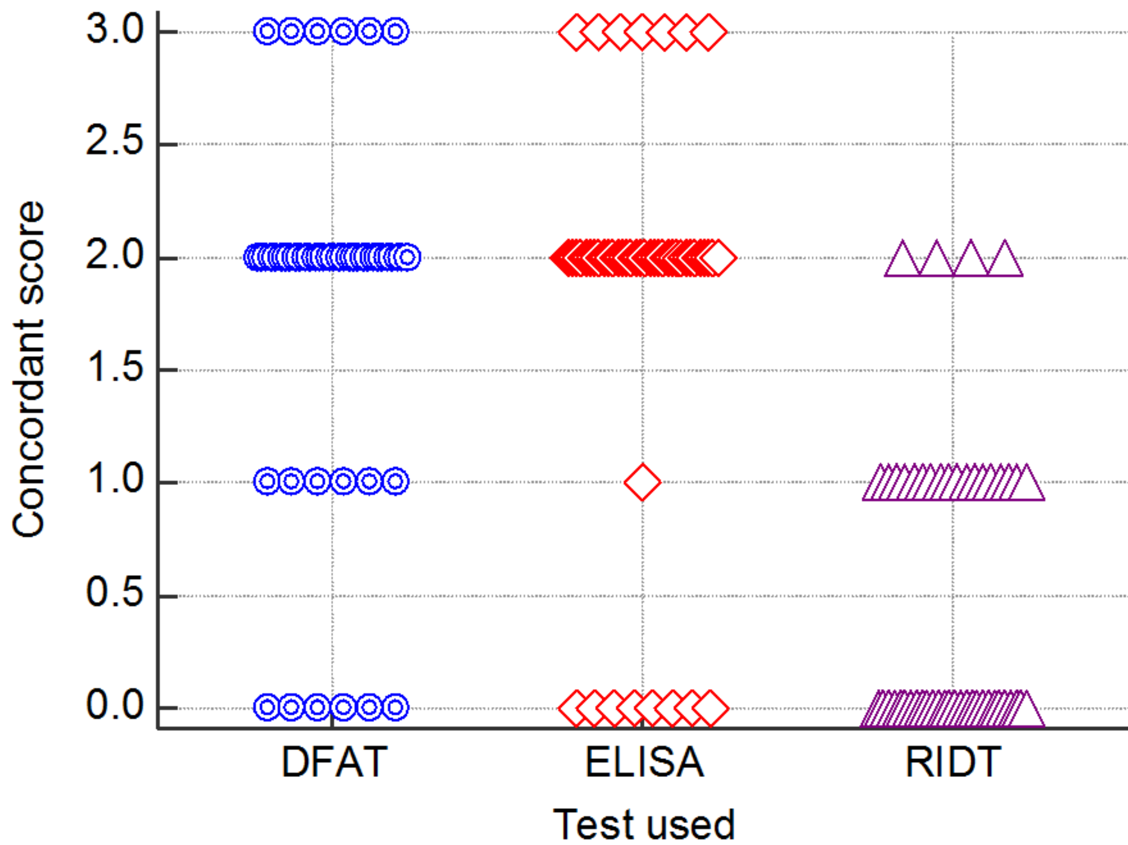


Figure 4.1: Concordant Results for Detection of Rabies Antigen Using Direct Fluorescent Antibody Test, Enzyme Linked Immuno-Sorbent Assayand Rapid Immuno-Diagnostic Test in Frozen Archived Dog Brain Tissues.

Key: DFAT: Direct Immuno-Fluorescent Test; ELISA: Enzyme Linked Immuno-Sorbent Assay; RIDT: Rapid Immuno-Diagnostic Test.

The concordance coefficients for ELISA and RIDT relative to DFAT were 78% (95% C.I. 0.6366 to 0.8654) and 17% (95% C.I. 0.05138- 0.2752) respectively.

4.2 Rabies Antigen Retrieval Using Sodium Citrate Buffer and The Detection of Rabies Virus Antigen in Citrate Buffer Treated Formalin-Fixed and Fresh Tissues byDirect Fluorescent Antibody Test

The combined results of the preliminary experiment on citrate buffer treated formalin-fixed tissues were presented in Table 4.3. Out of the 40 samples tested, 32 (80%) brain samples were positive by DFAT after treatment with sodium citrate buffer and none tested positive without treatment with sodium citrate buffer. The results were not affected by the length of time the tissues were left in formalin. Similarly, fresh brain tissues (Hippocampus, brain stem and cerebellum) were positive in 32 (80%) cases out of the 40 cases tested using DFAT for the presence of rabies virus antigen (Tables 4.3 and 4.4).

4.3 Detection of Rabies Virus Antigen in Formalin-Fixed Brain Tissues byEnzyme Linked Immuno-Sorbent Assayand Rapid Immuno-Diagnostic Test

Results of ELISA and RIDT on formalin-fixed tissues showed that six (43%) brain tissues tested positive by ELISA and no positive result was recorded for RIDT(Table 4.5).

4.4 Comparison of Direct Fluorescent Antibody Teston Fresh and Citrate Buffer Treated Formalin-Fixed Dog BrainTissues

As shown in Table 4.6, 32 brain tissues were positive for rabies antigen by both the DFAT on fresh tissue and the DFAT on formalin-fixed tissue. Thus, DFAT on formalin-fixed tissue exhibited a sensitivity of 100% in comparison to DFAT on fresh-tissue.

Table 4.3: Rabies Virus Antigen Detection Using Direct Fluorescent Antibody Test in Formalin-Fixed and Fresh Dog Brain Tissues

	$DFAT_a$	$DFAT_b$	$DFAT_c$
No. tested	40	40	40
Positive	0	32	32
Percentage (%)	0	80	80

Key: $DFAT_a$:DFAT on formalin-fixed dog brain tissues without treatment withheated sodium citrate buffer; $DFAT_b$: DFAT on formalin-fixed dog brain tissues after treatment withheated sodium citrate buffer;$DFAT_c$:DFATon fresh dog brain tissues

Table 4.4: Rabies Virus Distribution in Fresh Brain Tissues

Part of brain examined	No. Examined	No. Positive	Percentage positive (%)
Hippocampus	40	32	80
Brain stem	40	32	80
Cerebellum	40	32	80

No.: Number

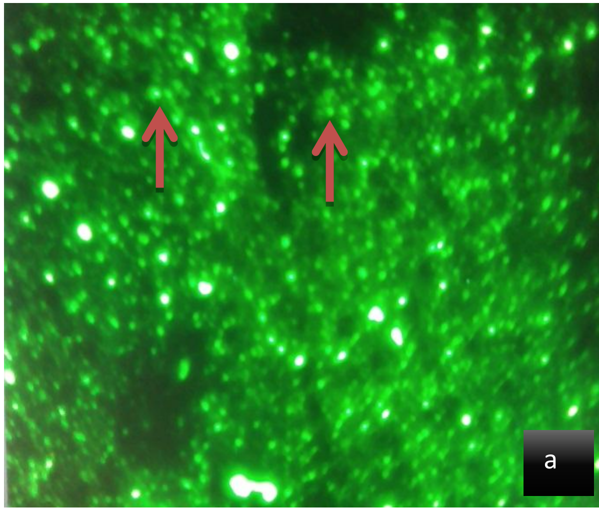
Table 4.5: Rabies Antigen Detection by Enzyme Linked Immuno-Sorbent Assay and Rapid-Immuno-Diagnostic Test

	ELISA	RIDT
No. tested	14	14
No. Positive	6	0
Percentage (%)	43	0

Table 4.6: Sensitivity and Specificity Comparison of Direct Fluorescent Antibody Teston Fresh and Formalin-Fixed Tissues

	DFAT on formalin-fixed sodium citrate treated tissues		
	Positive	Negative	TOTAL
DFAT on Fresh samples (positive)	32	0	32
DFAT on Fresh samples (negative)	0	8	8
TOTAL	32	8	40
Sensitivity	100%		
Specificity	100%		

No false positive results were obtained in formalin-fixed DFAT procedure, demonstrating 100% specificity. We found 100% agreement (32 positives and 8 negatives) between the two techniques. Although the size of fluorescing particles in fresh tissue DFAT was larger than those of formalin-fixed DFAT,there was no apparent difference in the intensity of fluorescence in DFAT on fresh sample and formalin-fixed DFAT (concordance = 98%; 95% C.I. 0.9660 to 0.9903) (Plate I). The concordant score of the various techniques was shown in figure 4.2. The strength of agreement between DFAT on formalin-fixed sodium citrate treated tissues and DFAT on fresh tissues was very good (Cohen's kappa coefficient value= 1.000; 95% C.I. 1.000-1.000).



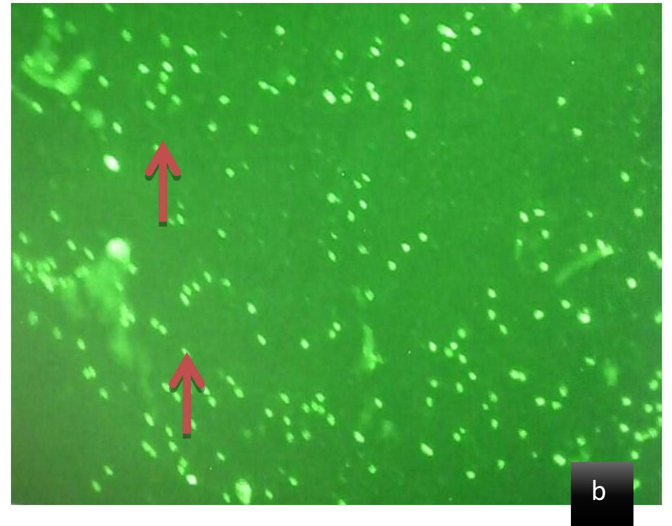


Plate I:a. Apple green fluorescing intracellular accumulations (DFAT Positive) (arrows) of fresh brain smear (Hippocampus); b. Apple green fluorescing intracellular accumulation (Positive) (arrows) of formalin-fixed brain smear after treatment with heated sodium citrate buffer (0.01M, pH 6.0-6.2) and stained with fluorescein-labeled monoclonal anti-rabies immunoglobulin ×400

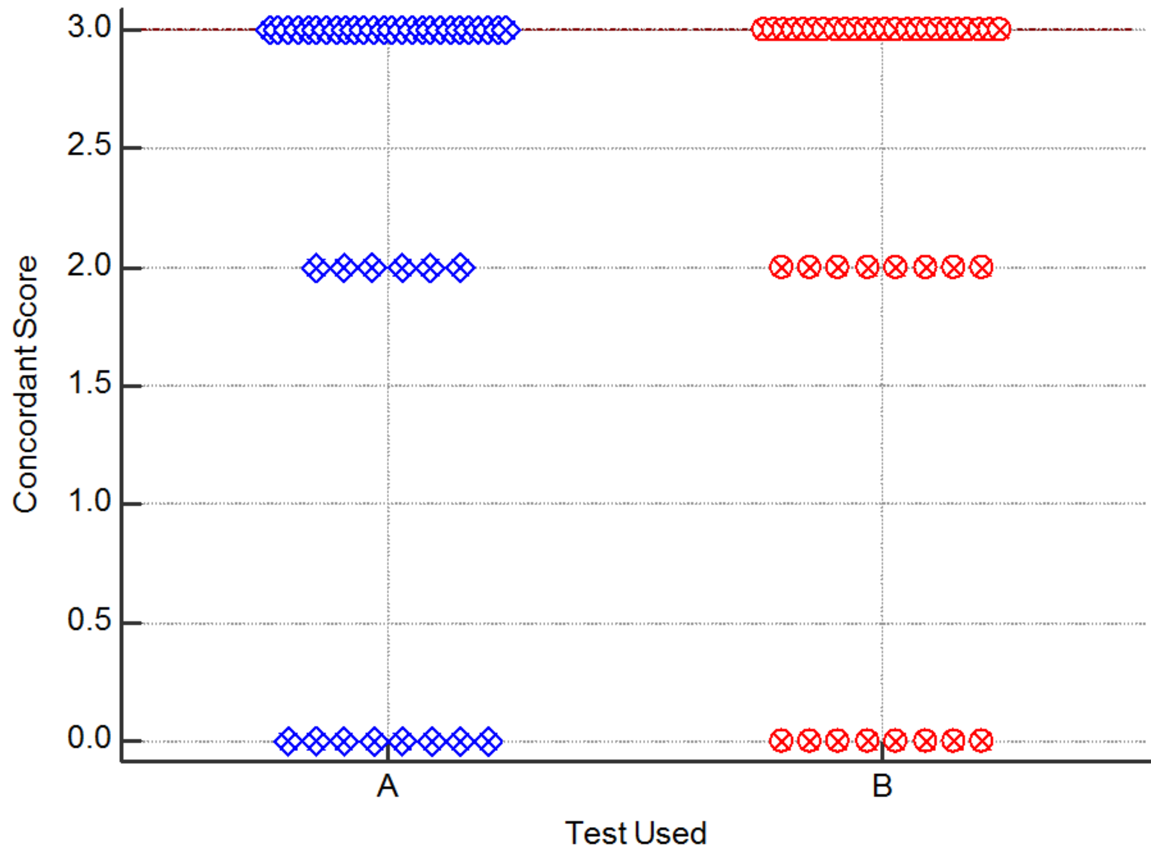


Figure 4.2: Rabies Virus Detection in Fresh and Formalin-Fixed Sodium Citrate Buffer Treated Dog Brain Tissues

Key: A= DFAT on fresh tissues; B= DFAT on formalin-fixed treated tissues

CHAPTER FIVE

DISCUSSION

The 'gold standard' method for diagnosing rabies worldwide is the direct fluorescent antibody test (DFAT), which is recommended by the World Health Organization (WHO) and OIE (WHO, 1992; OIE, 2008; Tekki, 2016). The main advantages of DFAT are its high sensitivity and specificity, even on fixed specimen (Whitfield *et al*., 2001) and that results can be obtained within 3~4 hours (Duong *et al*., 2016). Despite the detectable advantages of the DFAT in diagnosing rabies, complementary diagnostic methods that can be as reliable as the DFAT are needed. Any false negative results may lead to death and widespread under-reporting of the disease while false positive results can lead to unnecessary post exposure prophylaxis (Lembo *etal*., 2006; Yang *et al*., 2012). Consequently, the true public health impact of rabies will be greatly under-estimated and political commitment for its control would be lacking (Coleman *et al*., 2004).

In this study, 100% of the frozen archived brain specimens tested positive for rabies virus antigen by DFAT, 95.5% tested positive by ELISA and 47.7% tested positive by RIDT. Similar findings have been recorded by other researchers; Whitfield *et al*. (2001) recorded 66.9% positive result by DFAT in brain specimens. Of the 1253 specimens analyzed in a trial by Perrin and Sureau (1987), 651 were positive in both the DFAT and the ELISA. Three different studies conducted by Yang *et al*. (2012) Sharma *et al*. (2015) and Audu *et al*. (2017)gave 17%, 64.7% and 16% positive results respectively by RIDT on brain samples. Generally, the accuracy of rabies diagnosis is dependent on the quality of the sample (Fooks *et al*., 2009; Cliquet *et al*., 2010), the type of anti-rabies conjugate used

(Robardet *et al.*, 2013), virus antigen distribution in the brain and areas of the brain tested (Bingham and van der Merwe, 2002).

The present study evaluated the efficacy of RIDT to be used under laboratory and field condition for rabies diagnosis and obtained sensitivity and specificity of 47.6% and 87.5% respectively. This however, contradicts the findings of Nishizono *et al.* (2008) who reported a sensitivity of 95.25% and a specificity of 88.9% using a type I RIDT kit which recognizes epitope II and III of the nucleoprotein of rabies virus. Similarly, Kang *et al* (2007) recorded a high sensitivity and specificity of 91.7% and 100% respectively. This variation in the sensitivity and specificity of RIDT was observed by Eggerbauer *et al.* (2016) who compared six commercially available RIDTs for diagnostic and analytical sensitivity, as well as their specificity and concluded that the sensitivity and specificity considerably varied with different test kits. Also, none of the test kits investigated proved to be satisfactory, although the results somewhat contradict previous studies, indicating batch to batch variation. Therefore, the low sensitivity and specificity of RIDT recorded in the present study could be attributed to poor quality control and relatively low detection limit of the test kit used.

The ELISA is usable even on autolysed or partially degraded brain samples for rabies diagnosis. Results can be observedorinferred qualitatively with the naked eyes and a large number of rabies suspected samples can be tested at the same time using ELISA (Mani and Madhusudana, 2013; Duong *et al.*, 2016). However, false positive result due to cross reactivity with other antigens with very similar epitopes had been recorded (Perrin and Sureau, 1987). In this study, the sensitivity and specificity of ELISA were shown to be 95.5% and 100% respectively. This is in complete agreement with earlier studies (Saxena

et al., 1989; Miranda and Robles 1991; Perrin *et al*., 1992; Oelofsen and Smith, 1993). More recently, Xu *etal*. (2007) used a modified ELISA technique known as WELYSSA and reported sensitivity and specificity of 97% and 99.9% respectively.In the present study, 96% agreement was observed between DFAT and ELISA. The very good strength of agreement between the ELISA and DFAT (Concordance coefficient= 78%; Kappa= 0.834) implies that ELISA is as reliable as the DFAT and can be used in laboratories that cannot perform DFAT.

In this study, fresh brain tissues were positive in 80% of cases tested for the presence of rabies virus antigen by DFAT. Although, the intensity of fluorescence showed no apparent difference in the different parts of the brain tested, the size of fluorescence varied and ranged from large oval to small dust like particles. This finding was also observed by Raju *et al*. (2008) who also suggested that thorough examination of impression smears of various anatomic location of the brain is needed for a reliable diagnosis by DFAT.

Several researchers have also made attempts to detect rabies virus antigen in formalin-fixed tissues using DFAT staining procedures (Bourhy and Sureau, 1990; Warner *et al*., 1997; Whitfield *et al*., 2001). All of these studies reported challenges in the tests without the use of antigen retrieving agents. In this study, 80% of formalin-fixed tissues tested positive for rabies virus antigen by DFAT after treatment with heated sodium citrate buffer (pH 6.0). All the formalin-fixed tissues tested negative to rabies virus antigen by DFAT without sodium citrate buffer treatment. This shows that sodium citrate buffer can be routinely used to retrieve rabies virus antigen for DFAT in cases where only formalin-fixed tissues are available or when preservation by freezing is difficult and/or impossible.

Formalin fixation forms protein cross-links that mask the antigenic sites in tissues, thereby giving weak or false negative result for DFAT rabies detection (Warner *etal*., 1997). Antigen retrieval with heated sodium citrate buffer breaks these protein cross-links and exposes antigenic sites, allowing antibody to bind. The results presented in this study demonstrated 100% sensitivity and specificity of the formalin-fixed DFAT procedure.This agrees with the finding of Whitfield *etal*. (2001) who used proteinase K as the antigen retrieving agent andrecorded 99.8% sensitivity and 100% specificity forformalin-fixed DFAT procedure.However, a lower sensitivity of formalin-fixed DFAT following enzymatic digestion with trypsinwas reportedin previous study(Umoh and Blenden, 1981). This suggests that sodium citrate buffer (pH 6.0) could be a better alternative to enzyme digestion for the retrieval of rabies virus antigen inDFAT procedure on formalin-fixed brain tissues.

Formalin-fixed DFAT would be useful in instances where rabies is not considered in the diagnosis of an encephalitic condition until after tissue samples have been placed in buffered formalin (Umoh and Blenden, 1981; Mani and Madhusudana, 2013). The technique should also be especially useful in developing countries where poor refrigeration facilities and tropical heat combine to cause rapid deterioration of tissue samples. It is also important to note that the shipping and handling of formalin-preserved tissues is safer for all persons who come into direct or indirect contact with them(Umoh and Blenden, 1981).

Due to shortage of the test kits, 14 brain samples were randomly selected from the 40 formalin-fixed brain specimens and tested using ELISA and RIDT without pre-treatment with sodium citrate buffer. Out of the 14 formalinized brain specimens, 6 (43%) tested positive for rabies virus by ELISA and none tested positive by RIDT. The poor

performance of both techniques on formalin-fixed brain tissues could be attributed to the blockage of antigenic sites by formalin thereby making antigen-antibody binding difficult. A careful perusal of literature reveals that no attempt had been made to retrieve and detect rabies antigen in formalinized tissues using ELISA and RIDT.

A concordance coefficient of 98% (95% C.I. 0.9660 to 0.9903) was recorded for DFAT on fresh and formalin-fixed brain tissues which indicate no apparent difference in the intensity of fluorescence of DFAT on fresh sample and formalin-fixed DFAT.The Cohen's kappa coefficient value of DFAT on formalin-fixed tissue relative to DFAT on fresh tissue was 1.000 (95% C.I. 1.000-1.000) which shows a very good strength of agreement between the two techniques. These further attest to the accuracy and reliability of formalin-fixed DFAT.

CHAPTER SIX

CONCLUSION AND RECOMMENDATIONS

6.1 Conclusion

- In this study, 100% of the frozen archival brain specimens tested positive for rabies virus antigenby DFAT, 95.5% tested positive by ELISA and42% tested positive by RIDT.
- The sensitivity and specificity of ELISA for rabies antigen detection in frozen archival dog brain tissues were shown to be 95.5% and 100% respectively whereasthe sensitivity and specificity of RIDTwere 47.6% and 87.5% respectively.
- There was 96% agreement between DFAT and ELISA and 54% agreement between DFAT and RIDT for rabies virus detection in archived dog brain tissues.
- The simple Cohen's kappa value of agreement for ELISA relative to the DFAT was found to be very good 0.834; 95% C.I. 0.613-1.0) whereas the Kappa value of agreementbetween RIDT and DFATwas very poor (0.170; 95% C.I. 0.003- 0.337).
- The concordance coefficient for ELISA relative to DFAT was high (78%; 95% C.I. 0.6366 to 0.8654) whereas that of RIDT relative to DFAT was low(17%; 95% C.I. 0.05138- 0.2752).
- The low sensitivity and specificity of RIDT recorded in the present study could be attributed to relatively low detection limit of the test kit used.
- The ELISA is as reliable a diagnostic method as the DFAT which is the gold standard for rabies diagnosis and can be used in laboratories that cannot perform

DFAT.Also,ELISA has an advantage of being able to analyze large number of samples at the same time, making it more suitable for epidemiological studies.

- Fresh brain tissues were positive in 32 (80%) cases out of the 40 cases tested for the presence of rabies virus antigen.
- Although sodium citrate buffer has been used for antigen retrieval from formalin-fixed tissues in Immunohistochemistry, this study provides new information on the retrieval of rabies virus antigen with sodium citrate buffer for DFAT on formalinized tissues.
- In this study, 80% of formalin-fixed tissues tested positive for rabies virus antigen by DFAT after treatment with heated citrate buffer (pH 6.0) whereas all the fixed tissues tested negativeby DFAT without citrate buffer treatment.
- This study also demonstrated 100% sensitivity and specificity of the formalin-fixed DFAT procedure for rabies virus detection.
- 43% of formalinized brain tissues tested positive for rabies virus by ELISA and none tested positive by RIDT.
- The performance of ELISA and RIDT for rabies virus detection on formalin-fixed tissues was very poor.
- Although the size of fluorescing particles in fresh tissue DFAT was larger than those of formalin-fixed DFAT, there was no apparent difference in the intensity of fluorescence in DFAT on fresh sample and formalin-fixed DFAT (concordance = 98%; 95% C.I. 0.9660 to 0.9903).

- The strength of agreement between DFAT on formalin-fixed and DFAT on fresh tissue was very good (Cohen's kappa coefficient value= 1.000; 95% C.I. 1.000-1.000).
- This study shows that formalin-fixed DFAT is as valid and reliable procedure for rabies diagnosis as the DFAT on fresh samples and would be useful in cases where storage facilities are not available.

6.2 Recommendations

- Enzyme Linked Immuno-Sorbent Assay could be used as a complementary test to DFAT to prevent false negative results and also in laboratories that cannot perform DFAT.The unsatisfactory result of RIDT in this study reiterates the need to perform an adequate test validation before it can be used in the laboratory for rabies diagnosis.
- Sodium citrate buffer could be routinely used to retrieve rabies virus antigen for DFAT in cases where only formalin-fixed tissues are available or when preservation by freezing is difficult and/or impossible.
- Formalin could therefore, be used henceforth to fix tissues of rabies suspected cases for routine diagnosis,transportationor archival purposes.
- There is need to develop more rapid techniques that are compatible with chemical method of tissue preservation especially in developing countries where facilities for refrigeration are usually limited.

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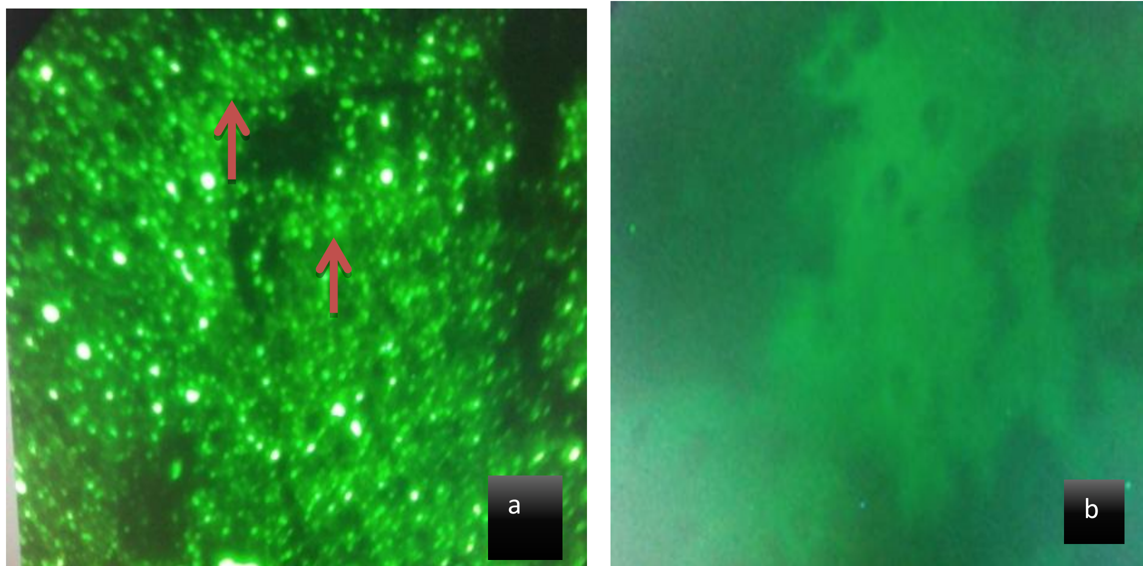
APPENDICES

APPENDIX I: Qualitative Canine Rabies Virus (RV) ELISA Kit (Reagent Provided)

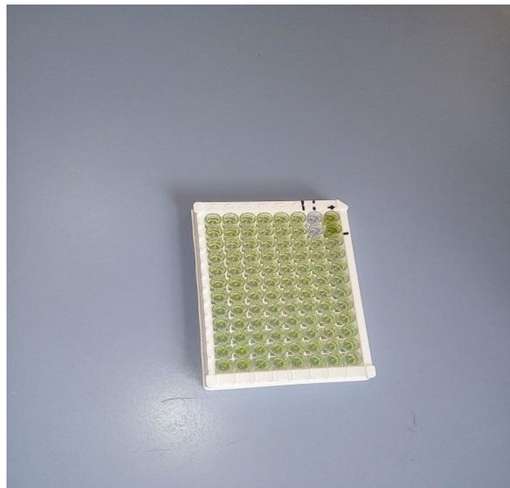
Items	Materials	96 Tests
1	Microelisa Striplate	12*8 strips
2	Positive control	0.5 ml/vial
3	Negative control	0.5 ml/vial
4	Sample diluent	6.0 ml
5	HRP-Conjugate reagent	10ml
6	20X Wash solution	25 ml
7	Chromogen Solution A	6.0 ml
8	Chromogen Solution B	6.0 ml
9	Stop Solution	6.0 ml
10	Closure plate membrane	2
11	User manual	1
12	Sealed bag	1

Appendix II:Direct Fluorescent Antibody Test Results on Frozen Archived Dog Brain Tissues

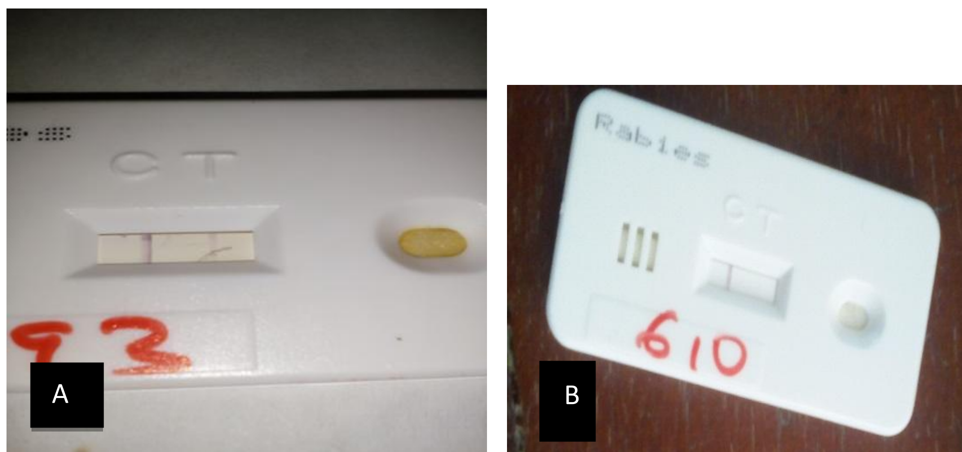


a. Apple green fluorescing intracellular accumulation (DFAT positive) (arrows) of frozen archiveddog brain smear and stained with fluorescein-labeled monoclonal anti-rabies immunoglobulin ×400; b. Frozen archived dog brain smear showing no fluorescence (DFAT negative).

Appendix III:ELISA plate showing yellow coloration after addition of stop solution to each well to terminate the reaction



Appendix IV:Rapid Immuno-Diagnostic Test result on Frozen Archival Dog Brain Tissues



Results of RIDT after applying the brain sample extraction into the sample hole of the test cassette. (A) Positive result showing both control and test bands on the test strip; (B) Negative result shows only the control band on the test strip.