DEVELOPMENT OF DNA VACCINE ENCODING MODIFIED GLYCOPROTEIN GENE AGAINST RABIES

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ZARIA, NIGERIA

JUNE, 2008

DECLARATION

I declare that the work in this dissertation entitled DEVELOPMENT OF DNA VACCINE ENCODING MODIFIED GLYCOPROTEIN GENE AGAINST RABIES has been performed by me in the Department of Veterinary Surgery and Medicine under the supervision of Professors A.B. Ogunkoya, A. J. Nok and J.U. Umoh. The information derived from the literature has been duly acknowledged in the text and a list of references provided. No part of this dissertation has previously been presented for another degree or diploma at any university.

OSINUBI, M. O. V.	__________________	__________________
Name of Student	Signature	Date

CERTIFICATION

This dissertation titled "DEVELOPMENT DNA VACCINE ENCODING MODIFIED GLYCOPROTEIN GENE AGAINST RABIES" by OSINUBI, Modupe Olubusola Vivian meets the regulations governing the award of the degree of Doctor of Philosophy of Ahmadu Bello University, Zaria, and is approved for its contribution to scientific knowledge and literary presentation.

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DEDICATION

This work is dedicated to my husband Prof K. J. Osinubi, who initiated my postgraduate studies and stood by me throughout, giving all necessary support and encouragement needed, you are indeed a jewel of inestimable value, and also to our children Omokolade, Ayodeji and Oludamilola that God blessed us with.

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Now unto Him that is able to keep you from falling, and to present you faultless before the presence of His glory with exceeding joy, to the only wise God our Saviour, be glory and majesty, dominion and power, both now and ever. Amen. (Jude: 24 – 25)

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ABSTRACT

A study aimed at the identification and antigenic characterization of existing rabies virus strains in Nigeria with subsequent classification into their phylogenetic groups, and the development of an immunogenic DNA vaccine that will protect against such major circulating rabies variants in Nigeria was conducted. Plasmids encoding glycoprotein/modified glycoprotein genes of bat and dog rabies variants, respectively, were developed and tested for potency in mice using multiple administrations, single administration and various routes. From the source of sample collection, Seller's staining method was used to confirm rabies infection in the one hundred and sixty-five (165) samples collected. However, only fifty-one (51) samples, which accounted for 30.9% of the total samples, were found positive using direct fluorescent antibody (DFA) technique. Also, fourteen (14) of the positive samples which were prepared according to mouse inoculation test (MIT) technique and inoculated into pools of suckling mice did not kill them and no signs suggestive of rabies were observed. It was inferred that due to the deficiencies in preservation and transportation of the samples, the viability of the viruses could not be sustained. Ribonucleic acid (RNA) was extracted from 13 (25.5%) of the fifty-one (51) samples that were positive to DFA, using TRIZOL reagent. The integrity of the extracted RNA remained intact (quantitation range of 1.5 to 2.4 at A260/A280). Analysis of the phylogenetic relationships performed on the complete Nucleoprotein (N) coding gene established them to belong to the genotype 1, comprising of typical dog rabies virus originating from West Africa (Africa 2 group). Two samples appeared very closely situated to Africa 3 group, which are classified to be related to African mongoose and represents a wild life rabies variant. These two isolates could probably be suggested to be related to the wild life rabies variants. In the multiple intramuscular (i.m) vaccination schedules using the CDAG3, neutralizing antibody was detected in 60% of the vaccinated mice as early as day 21 which gradually increased after booster shots were administered. On challenge with street rabies variant belonging to the African 2, (West African dog rabies virus), all the mice in this group survived. In the subcutaneous (s.c) multiple administration group, neutralizing antibody was first noticed by day 62 after a booster had been given though only 20% of these had neutralizing antibody level greater than the

protective level of 0.5 IU/ml recommended by WHO. Out of the 60% that showed traces of antibody production, only 20% survived the challenge. While in the oral (p.o) route, neutralizing antibody was detected in just 20% and all the mice died after challenge. Multiple administration using s.c and p.o routes were found not protective in this research. CDAG3 single dose i.m route showed neutralizing antibody by Day 30 with 80% of the mice surviving the challenge. Similar observation was recorded for the single intra-dermal route (i.d) that received only one tenth of the DNA vaccine. Results obtained from WCBVG and controls using different administration regimens and routes did not show neutralizing antibodies and did not protect the mice from challenge. It was concluded that WCBVG does not protect against the street rabies strain. Statistical analysis using Fisher exact test – two tailed probability, showed that the i.m and the i.d routes were statistically significant compared with the control ($P < 0.01$). Also there was statistical significance when comparing i.m route to the s.c routes in multiple administration ($P < 0.05$). All mice that did not survive the challenge were tested for rabies antigen in their brains using the DFA and Direct Rapid Immunohistochemistry Technique (DRIT) and all were positive to the tests conducted.

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CHAPTER ONE

INTRODUCTION

1.1 The Rabies Virus

Rabies, which is an acute, progressive and fatal disease, is the 11th cause of human death due to infectious disease, having about 100% mortality rate (WHO, 2000). Human mortality due to rabies is estimated to be 60,000 deaths per year worldwide, mostly reported in Asia and Africa (WHO, 2005). It is a viral zoonosis that causes encephalomyelitis. The infection is caused by the viruses of the Rhabdoviridae family of the genus Lyssavirus, which has seven genotypes (Gt), and is maintained in reservoir mammals, mainly carnivores (dogs) and bats.

Genotype 1 comprises the classical rabies virus (RV) strains which are found in almost every country in the world. It is endemic in all continents but a few European countries, some islands, peninsulas and Antarctica are free of the fear of the disease, although imported infection is a universal risk.

Genotypes 2 to 7 include the rabies – related viruses (RRV), more specifically:

- Lagos bat virus (LBV), (Gt 2)
- Mokola (MOK) virus, (Gt 3)
- Duvenhage (Gt 4)
- European bat lyssavirus 1 (EBLV-1), (Gt 5)
- European bat lyssavirus 2 (EBLV-2), (Gt 6)
- Australian bat lyssavirus (ABLV), (Gt 7)

(Boulger and Porterfield, 1958; Bourhy *et al*., 1992; 1993; Amengual *et al*., 1997; Cliquet and Meyer, 2004).

Lyssaviruses are a group of negative – stranded non-segmented RNA viruses. The viral particle is bullet shaped and measures about 180µm in length and 75 µm in width. The viruses are made up of a helical ribonucleoprotein core (RNA) surrounded by an envelope of a helical structure which is extremely labile (Sokol *et al*., 1969).

Rabies virus also possesses a negative – stranded RNA genome of about 12Kb, and encodes five major proteins (Coslett *et. al*.., 1980; Holloway and Obijeski, 1980). The five major polypeptide components are of different molecular sizes forming the protein moiety of the virus: Nucleoprotein (N), phosphoprotein (P), matrix protein (M), glycoprotein (G) and polymerase (L) (Coslett *et al*., 1980; Nottidge, 2005). The surface shows a honeycomb arrangement with surface projections, having a knob-like structure at the distal end. Each projection is made up of a bi-layer membranous lipoprotein envelope penetrated by a single glycoprotein (G) protein (Dietzschold, 1987). The N, NS and L proteins together with the genomic ribonucleic acid (RNA) form a nucleocapsid which is enveloped by a membrane containing the transmembrane glycoprotein G, whose spikes are mainly responsible for the antigenicity of the virus. The M protein is located at the inner side of the membrane, providing a connection between the nucleocapsid and the cytoplasmic domain of the glycoprotein (Cox *et. al*., 1981). There is an existence of a pseudogene region (I between the G and L cistrons and heterogeneity of the intergenic regions (Tordo *et al*., 1986, 1988).

Many variants of the rabies virus have been identified (Smith, 1989; Nottidge, 2005). Previous antigenic studies performed on both the N and G have demonstrated the antigenic diversity of Lyssaviruses (Rupprecht *et al*., 1991; Smith, 1989). Kissi *et al*. (1995) in an attempt to compare the intrinsic and extrinsic genetic diversity of the

Lyssavirus genotypes, partially sequenced 69 rabies virus isolates from various parts of the world and compared them to 13 representative isolates of the Lyssavirus genotype. The analysis of their phylogenetic relationships, performed on complete nucleoprotein (N) coding gene (1350 bases), established that the rabies virus isolates belonged to genotype 1 and that at least 11 phylogenetic lineages in accordance with their geographical localization and species of origin. These lineages diverged mostly by the accumulation of synonymous mutations. Previous antigenic studies performed on both the nucleoprotein (N) and the glycoprotein (G) demonstrated the antigenic diversity of Lyssaviruses (Wiktor *et al*., 1980; Rupprecht *et al*., 1987; Smith, 1989).

The N protein of Lyssaviruses is a phosphorylated protein 450 or 451 amino acids long (Tordo *et al*., 1986a; Bourhy *et al*., 1993), which is synthesized in large amounts during cell infection. During morphogenesis, the N protein binds tightly to genomic RNA protecting it from ribonuclease action (Sokol *et al*., 1969). In the mature virion, the N protein constitutes the major component of the internal helical nucleocapsid. In rhabdoviruses, it is involved in the regulation of transcription and replication. It also plays an important role in the T helper response, in particular against challenge with lyssaviruses antigenically distant from the vaccinal strains (Dietzschold *et al*., 1987; Ertl *et al*., 1991).

1.2 Diagnosis of Rabies

Diagnosis of rabies based on clinical symptoms alone is difficult and unreliable, except when there are specific clinical signs of hydrophobia or aerophobia (WHO, 2005). The first clinical signs of rabies are non-specific, and the diagnosis is often confirmed later in the course of the disease or at postmortem (Ogunkoya, 1997).

Many techniques have been used in the past to make a diagnosis, but among recently used diagnostic techniques; molecular diagnosis has been adopted to detect viral nucleic acid in antemortem or postmortem specimens. Reverse – transcription polymerase chain reaction (RT-PCR) has been used successfully for detecting the viral nucleic acid of rabies virus in antemortem specimens. Molecular detection by RT-PCR has the highest sensitivity, but it requires standardization and very stringent quality control in order to avoid false results (Crepin *et al*., 1998). The RNA of rabies virus can be detected in several biological fluids and samples (e.g. saliva, cerebrospinal fluid, tears, urine, skin biopsy and brain samples). Saliva and neck-skin biopsy samples are the most widely – studied specimens for attempting antemortem diagnosis (Crepin *et al*., 1998, Smith *et al*., 2003). Nucleoprotein gene (N) is the most conserved rabies virus gene (Macedo *et al*, 2006), and analysis of this gene is the most suitable approach for the molecular diagnosis of rabies (Smith, 2002).

1.3 Epidemiology of Rabies

Though a number of countries throughout the world have been free from rabies for many years; some are reported to have eliminated the disease and in others it has reappeared after variable periods of time. The disease continues to pose both public health and economic problems of varying severity in all continents except Australia and Antarctica (Turner, 1976). The United States was recently reported to be free of canine rabies (Rupprecht and Tumphery, 2007).

Rabies is reported to be presented in most of the African continent (Abdussalam and Botton, 1974). Reliable information on its incidence is sparse but the available data suggests that the disease is particularly prevalent in North – Western and Central Africa where canine rabies is most commonly reported. In areas where the canine disease predominates the principal foci are in towns, infection being maintained in stray animals (Abdussalam and Botton, 1974). It is worth noting the vagaries of international reporting, though accurate in some cases and undoubtedly imprecise in others, demand a cautious interpretation of the available data and suggest that the world picture be viewed as an impression rather than a precise record (Turner, 1976).

In areas where canine rabies is reported to present a major problem, the wildlife disease is reported to be of much less concern, but is nevertheless irregularily reported in a wide variety of wild animals and their role cannot be completely ruled out (Turner, 1976). Some of the most extensive wildlife surveys in Africa have been concerned with bats but the African chiroptera are rarely implicated as major vectors as they are in many parts of the New World. Turner, (1976) assessed the world picture at that period and depicted man's domestic pet-dogs and cats as the major potential hazard for human infection. They reported dogs to be responsible for more than 80% of bite wounds in those seeking antirabies treatment in Africa, though there are however many gaps in the reporting which suggest that this is an underestimate. Domestic animals also provide the link with widelife rabies which remains an intractable problem, even in the more sophisticated countries, despite destructive assaults made so far on wildlife; this has not been outstandingly successful. However, with recent research on the ecology of the vectors, their immunization in the field and the use of antifertility agents in population reduction provides prospects for the future control of wildlife rabies.

In Nigeria, the rabies virus had been isolated from not only infected dogs, but also from clinically healthy and previously unvaccinated dogs (Aghomo *et al*., 1989) which is contrary to the belief that rabies is uniformly fatal in all species of animals (Rife, 1968). The isolation confirms the work of Fekadu and Baer (1980) as well as Fekadu *et al*. (1981) who reported recovery of animals from clinical rabies with intermittent shedding of the virus in their saliva over the course of 1 year (Aghomo *et al*., 1989). Equally, Fekadu *et al*. (1982) isolated rabies virus from a clinically healthy Ethiopian dog.

1.4 Statement of Problem

The occurrence of vaccine failure cases and cases of vaccine – induced rabies infection indicate that the Flury Low egg passage (LEP) vaccine may not be 100% effective. The occurrence of vaccine - induced rabies or vaccine failures could further complicate rabies problem in Nigeria, as not all vaccinated dogs may be protected. LEP vaccine appears safe for adult dogs but retains some pathogenicity for young puppies (Crick and Brown, 1976). It has been shown to compare favourably with the more recently developed tissue culture vaccines and with suckling mouse brain vaccines (Sikes, 1971), and a single dose of vaccine in dogs has been reported to provide a duration of immunity of more than three years (Tierkel *et al*., 1953). In 10 of the 14 reported cases of rabies associated with LEP vaccine, the dogs developed rabies between 5 months and 2 years post-inoculation (Okoh, 1982). An explanation for these failures among others include, manner of handling of the vaccines during production, the use of a non-viable vaccine due to prolonged exposure of the vaccine to high temperature and reconstitution of the vaccine for too long a period before inoculation. Other reasons are inadequacy of the Flury strain vaccine which is currently used in Nigeria for animal vaccination to provide protective immunity against same variants of local lyssaviruses as well as unusual pathogenicity and virulence of local variants. Also, it has been suggested that vaccinated dogs could respond poorly especially if they are immunocompomised at the time of vaccination (Osinubi *et al*, 1999, 2005).

Wiktor and Koprowski (1978) found that the use of rabies specific hybridoma monoclonal antibodies permits the detection of different specificities expressed by the viruses of the rabies group. This led to work done by Wiktor, et al., (1980) that showed evidence of antigenic differences within the lyssaviruses after the analysis of several strains of fixed and street rabies virus. These antigenic differences were also noticed by Sureau, et al., (1983), and have been suspected to be responsible for occasional vaccine failure even when the vaccine can be assumed to be potent and the treatment prompt (Hattwick, et al., 1972; CDC, 1976).

Wiktor, et al., (1980) found that homologous strains are closely related than heterologous strains. Equally Wiktor, et al., (1980) observed that rabies strains of viruses originating from the same geographical area showed identical or nearly identical patterns of reactivity. This was confirmed by Ogunkoya, et al., (2003) that showed a definite pattern of reactivity that was observed in the analysis of some Nigerian street rabies virus using monoclonal antibodies, which indicated that all the strains isolated were antigenically uniform. Failures of commonly used vaccines to protect Nigerian virus further strengthen Wiktor's suggestion, that vaccines made for use in different parts of the world should be prepared from prevailing viruses in such areas (Wiktor et al., 1984).

In line with these suggestions, a hunt for a circulating rabies virus variant suitable for vaccine production in Nigeria had been on (Ogunkoya et al., 2003). This study was planned to develop a vaccine using a Nigerian rabies strain.

1.5 Justification

Many vaccines currently used in developing countries fail to maintain detectable levels of neutralizing antibody. In Nigeria, it has equally been shown that Low egg passage (LEP) Flury strain vaccine used did not protect the dogs appreciably (Okoh, 1982; Nottidge, 1994), this was confirmed by Ogunkoya *et al*., (2003) which showed that only 54.3% of vaccinated dogs had neutralizing antibody. Various workers have reported rabies in vaccinated dogs in Nigeria (Okoh, 1982, 1983, 1984, Bobade *et al*., 1983; Okoh *et al*., 1985; Oboegbulum *et al*., 1987). And the isolates were found to be different antigenically from the field strains characterized by Okoh *et al*. (1988). This could suggest that there are many different local strains of rabies virus of unknown public healthy significance and probably explains the reason why the incidence has not declined appreciably despite efforts to control it, instead many cases of rabies with high exposure potential have been reported with rabies occurring in vaccinated dogs.

Despite efforts to control rabies, the disease continues to be a major scourge of dogs and cats in Nigeria (Umoh and Belino, 1979; Fagbami *et al*., 1981; Okoh, 1986). Thousands of dogs are vaccinated annually with the Flury low egg passage (LEP) vaccine strain, produced at VOM, Nigeria (Nawathe *et al*., 1981), but the prevalence of the disease in domestic animals has not declined (Durojaiye, 1984; Okoh, 1986: Nottidge, 1994: Ogunkoya *et al*., 2003). The use of inadequate vaccines suggests that an inexpensive, more immunogenic and protective vaccine that elicits long-term immunity after a single-dose vaccination could improve control of canine rabies in developing countries (Donald *et al*., 2003). A possible solution for the problems associated with controlling canine rabies, as stated above, could be a DNA vaccine.

Various groups have developed rabies DNA vaccine (Xiang *et al*., 1995; Ray *et al*., 1997; Bahloul *et al*., 1998; Lodmell *et al*., 1998; Biswas *et al*., 1999; Osorio *et al*., 1999; Perrin *et al*., 2000; Lodmell *et al*., 2001; Gupta *et al*., 2005; Bahloul *et al*., 2006; Gupta *et al*., 2006; Patial *et al*., 2007), and many have shown the effectiveness of pre-exposure DNA vaccination protection in mice and non-human primates against the rabies virus. (Xiang *et al*., 1994; Ertl *et al*., 1990; Bahloul *et al*., 1998;, Lodmell *et. al*., 1998; Ray *et al*., 1997; Lodmell and Ewalt, 2000; Lodmell *et al*., 2001) and post-exposure (Lodmell and Ewalt, 2001; Bahloul *et al*., 2006) mouse models and in non-human primates (Lodmell *et al*., 1998, Lodmell *et al*., 2001). Also previous studies have shown that DNA-based immunization with plasmids encoding the rabies virus glycoprotein (CVS, ERA and PV strains) protects mice against rabies (Bahloul et al., 1998; Jallet et al., 1999; Lodmell et al., 1998; Xiang et al., 1994).

The advantages of DNA vaccines as compared to conventional vaccines are manifold especially for developing countries as they are easy to manufacture and they provide long lasting immune responses (Patial *et al*., 2007). Other advantages that make them attractive include that they do not require a cold chain before delivery, since DNA plasmids are stable. Technology transfer of the vaccine production to developing countries, where rabies is endemic, could be easier and at cheaper cost for the vaccine than for the more stringent and cumbersome cell culture technologies. The more potent immune responses elicited by DNA vaccines may be beneficial in harsh conditions where dogs suffer from malnutrition and numerous diseases which depress their immune systems and unlike with conventional vaccines which suffer from the interference of maternally transferred antibodies, (Precausta *et al.*, 1982). DNA vaccines have been known to efficiently immunize younger puppies less than 3 months old (Wang *et al*., 1997)

If potency can be improved, then plasmid DNA vaccines may offer many advantages over classical technologies in terms of rapid development, stability, simplicity, economic value and lenght of immune responses. Based on these advantages of DNA immunization over the conventional method, the concept of this study was to develop an immunogenic DNA vaccine that will protect against the major circulating rabies variants in Nigeria.

1.6 Research Aim and Objectives

1.6.1 Aim

The aim of the research was to develop immunogenic deoxyribonucleic acid (DNA) rabies vaccines against circulating Nigerian rabies variants and evaluate the potencies of such vaccines.

1.6.2 Objectives

In order to achieve the above aim the following were proposed:

- Identification by antigenic characterization of existing rabies virus strains in Nigeria.

- Development of plasmid DNA vaccines for immunization against the circulating variants.
- Vaccination of mice with the plasmid DNA rabies vaccines using single and multiple administrations, and different routes.
- Evaluation of protectiveness of the plasmid DNA vaccines.

CHAPTER TWO

LITERATURE REVIEW

2.1 Historical Background

The word rabies originated about 3000 B.C. from the word "*rabha*" meaning "violence" (where the victim becomes violent). It has many synonyms world wide. Democrites in the 15th century BC called it "nerve fire" and Hippocrates later described it as hydrophobia (Wilkinson, 1988; Nottidge, 2005). The Greek named it *lyssa* meaning "violent". The Egyptian god sirus was depicted as a furious dog because dog rabies was very common at that time. In India during the 30th century B.C, the god of death had a dog as his companion and messenger.

In Nigeria, it was recognized by local people prior to 1912 in various dialectic names such as *digbolugi* (Yoruba), *ciwon kare* (Hausa), *ginnaji* (Fulani), e*bua idat* (Efik/Ibibio) and *ara nkita* (Igbo) (Nottidge, 2005). The first documented fatality in man bitten by a dog was in the 23rd century B.C. The first major outbreak of rabies in dogs in Africa was in Ethiopia in 1884 and in South Africa in 1893. In Nigeria, the first documented case in human was in 1912 and in dogs in 1925 (Boulger and Hardy, 1960).

2.2 The Rabies Virus

Rabies is a viral disease of the central nervous system leading to death of the affected animal in most cases (Haig, 1977). It is a zoonosis of certain mammal species, endemic in all continents. Only a few European countries, some islands and peninsulas and Antarctica, are free of the fear of rabies, although imported infection is a universal risk (Nottidge, 2005).

Rabies encephalomyelitis remains a serious public health concern (60,000 human deaths and 10 million post-exposure treatments reported per year) in both industrialised and developing countries (WHO, 1995). The etiologic agents belong to the *Lyssavirus* genus and *Rhabdoviridae* family (Tordo *et al*., 1998). Rabies is the first of seven genotypes, which represents the classical rabies viruses whereas the remaining groups (2 through 7) correspond to rabies – related viruses (Badrane *et. al*., 2001), five of which have caused fatal infection (Warrell *et al*., 1988; Hooper *et. al*., 1998). The Lagos bat virus is classified as Group 2. Two genotypes (3 and 4): Mokola virus isolates in shrews found in Ibadan, Nigeria (Shope *et al*., 1970) and *Duvenhage* virus in bats found in South Africa (Meredith *et. al*., 1971) are very occasionally found and only in Africa (Warrell *et al*., 1988). Two further genotypes (5 and 6) are European bat lyssaviruses (EBV 1 & 2) found in insectivorus bats across Europe. In addition, a lyssavirus recently isolated from a fruit bat known as flying foxes (*Pteropus alecto*) in 1996 (Hooper *et al*., 1998) and responsible for one human death in Australia was recently proposed to inaugurate a new lyssavirus Group 7.

Other rabies related viruses include Obodhiang isolated from mosquito in Sudan (Schmidt *et al*., 1965), Kotonkan from midges in Ibadan (Kemp *et al*., 1973) and Oulo-fato from dog and ground squirrels in West Africa (Bouffard, 1912; McMillan and Boulger, 1960).

The lyssavirus group is all morphologically, morphogenetically and pathogenetically similar, sharing cross-reacting antigens (Shope et al., 1970). The genus was divided into two phylogroups as a result of serological and genetic analyses (Badrane et al., 2001). Phylogroup I comprises all the viruses except Mokola virus, which is in phylogroup II with Lagos Bat virus. Based on this division, all phylogroup I genotypes have caused fatal rabies such as encephalitis in man, whereas Mokola virus has probably only caused three known human infections, one of which was fatal encephalitis, (Warrell et al., 1988). Experimentally, phylogroup II viruses are less pathogenic, and there is little if any cross-neutralisation with the phylogroup I lyssaviruses.

2.2.1 Description of the rabies virus

Lyssaviruses are a group of negative-strand non-segmented RNA viruses that are highly neurotropic and cause fatal encephalitis when the virus gains access to the central nervous system (CNS). Rabies virus is a prototype species of the genus (Coleman et al., 2004). The complete virus particle (Fig. 2.1) is bullet shaped and measures about 180 µm by 75 µm (Hummeler *et al*., 1967). In addition there are truncated particles which are significantly shorter than the standard virus length, but retain the bullet shaped morphology and pathogenicity. There are five (5) major polypeptide components of different molecular sizes forming the protein moiety of the virus; nucleoprotein (N), phosphoprotein (P), matrix protein (M), glycoprotein (G) and polymerase (L). The surface shows a honeycomb arrangement with surface projections (60µm – 70µm) having a knob-like structure at the distal end. Each projection is made up of a bi-layer membranous lipoprotein envelope penetrated by a single glycoprotein (G-protein) (Dietzschold, 1983; Nottidge, 2005).

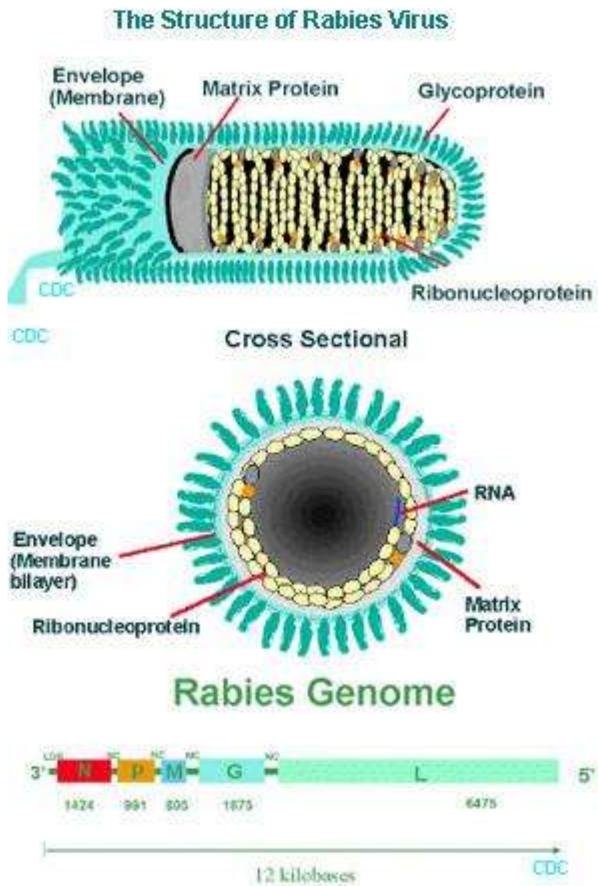


Fig. 2.1: Rabies Virus Structure.

The Rhadoviruses are made up of a helical ribonucleoprotein core (RNA) surrounded by an envelope of a helical structure which is extremely labile (Sokol *et. al.*, 1969). The phosphoprotein and polymerase are part of the RNA

The rabies glycoprotein and nucleoprotein are both key antigens in the induction of protection (Drings *et al.*, 1999). The glycoprotein (G) is a transmembrane protein that forms the spikes of the virus (Delagneau *et al.*, 1981). It forms trimers on the viral surface and determines the tropism of the virus by binding to receptors expressed on neurons. Glycoprotein G is the only viral protein that induces virus neutralizing antibodies (VNAb)

(Wiktor *et al*., 1973; Cox *et al*., 1977) and also protects against intracerebral (I.C) challenge (Perrin *et al*., 1985). Furthermore, the G protein stimulates T helper (Th or T_4) (Celis *et al*., 1988) as well as cytolytic T cells T(CTL) (MacFarlan *et al*., 1984; Wiktor *et al*., 1988; Ertl *et al*., 1990). The three-dimensional structure of G molecules is critical for both VNAb induction and protection, and G molecules must be anchored onto a viral or synthetic membrane, (Perrin *et al*., 1988). Animals immunized with subunit vaccines based on the rabies virus G protein are reported to be protected against challenge with rabies virus administered peripherally or directly into the central nervous system (Wiktor *et al*., 1984). Such neutralizing antibodies are known to prevent the spread of rabies virus into the central nervous system, (Shankar *et al*., 1991).

The Glycoprotein G is organized in peplomers on the membrane surface, and is encoded by an open reading frame (ORF) of 1575 nucleotides (524 amino acids, including the signal sequence), like all glycoproteins of rabies virus strains analyzed so far (Tordo *et al*., 1986; Anilionis *et al*., 1981; Yelverton *et al*., 1983; Prehaud *et al*., 1988), the overall amino acid homologies of these glycoproteins range from 88.2% (SAD B19/CVS) to 99.4% (SAD B19/ERA). The closely related ERA and SAD B19 proteins show 3 amino acids replacements (Bahloul *et al*., 1998).

The nucleoprotein (N) forms the major constituent of the internal ribonucleoprotein complex (RNP), by binding to the RNA and the L and NS proteins (Sokol *et al*., 1969). The N protein is more/highly conserved across genotypes than the G protein across lyssaviruses (Flemand *et al*., 1980). It stimulates Th cell production (Ertl *et al*., 1991) and induces cross-protection against intramuscular (i.m) challenge (Dietzschold *et al*., 1987). The role of rabies virus nucleoprotein (N) in protection against rabies was examined by

Sumner *et. al*. (1991) using recombinant vaccinia viruses expressing the N gene of the challenge virus standard strain (CVS). Two recombinants were isolated that expressed the rabies N gene in infected cells as determined by radioimmunoprecipitation and immunofluorescence microscopy with an anti-N monoclonal antibody. Mice inoculated intradermally and challenged showed high survival ratios and developed non-neutralizing antibodies against rabies nucleoprotein (N) gene.

The N protein is expressed in abundance in the host after infection by rabies virus and it primes and stimulates the VNAb production induces by classical vaccines (Dietzschold *et. al*., 1987). Monoclonal antibodies (MAbs) against the N protein (MAb-N) have been characterized extensively and shown to possess patterns of reaction that can distinguish isolates from different outbreak areas or from different species in the same area (Smith, 1989). However, this has to be interpreted with caution because while antigenic differences are useful markers of epidemiologically separate events, a finding of antigenic identity in isolates cannot be relied upon to indicate that the isolates are epidemiologically related.

Smith *et al*., (1992) showed the nucleotide sequence analysis of a 200 – bp (two hundred base pair) region of the N-gene of rabies virus differentiated unique genetic groups of rabies virus from samples collected in areas where dog rabies is enzootic in Asia, Africa, Europe, and the Americas. Patterns of nucleotide sequence identified for an outbreak area were conserved in samples collected over three decades. Samples taken from areas of Asia, Latin America, Europe and Africa enzootic for rabies in dogs were found to be antigenically similar to each other, but epidemiologic investigations of new outbreaks or unexpected cases of rabies arising from contact with dogs were hampered by inability to identify the source of the virus. Further complicating their investigation was the lengthy incubation period of rabies, which permitted the transport or movement of infected animals over long distances.

Molecular approach using alignment and pairwise analysis of limited regions of nucleotide sequence allowed the measurement of difference between virus isolates as well as established patterns of relatedness among isolates from geographically separate outbreak areas. It has been shown that taxonomic classification of the *Lyssavirus* genus of *Rhabdoviridae* depends on antigenic differences of the N protein (Arai *et al*., 1997).

2.2.2 Amino Acid Substitution

The exchange of a single amino acid in the antigenic site III (aa 330 to 357) of fixed strains of rabies virus glycoprotein may have significant effects on its biological behaviour. By the replacement of Arg (333) with Ile, Glu or Gln the interaction of the virus with specific neuronal cells is impaired and a significant loss of pathogenicity was observed (Dietzschold *et. al*., 1983; Seif *et. al*., 1985; Kucera *et. al*., 1985). The SAD B19 and the ERA strain have identical antigenic sites III.

As shown by revertants, single amino acid substitutions in regions other than known antigenic sites can influence the biological behavior of rabies virus e.g. an avirulent antigenic mutant, street Alabama gif (SAG-1) which has a mutant of 1-nucleotide substitution in amino-acid position 333 of the glycoprotein (Tuffereau *et. al*., 1989) when tested for its pathogenicity in adult mice was shown to be almost apathogenic for adult mice inoculated by intracerebral route (Seif *et. al*., 1985; Coulon *et. al*., 1983; Fiamand *et. al*., 1993).

2.2.3 Characteristics of the rabies virus

Rabies virus is very sensitive to environmental factors. It is rapidly destroyed by direct sunlight, ultraviolet irradiation, heat at $60^{o}C$ for five minutes, lipid solvents, (70% alcohol and ether), sodium deoxycholate, trypsin and common detergents. However, it is preserved at sub-zero temperature and in glycerin (Nottidge, 2005).

2.3 World Prevalence of Rabies

The Egyptian god, sirus was depicted as a furious dog because dog rabies was very common at that time (Nottidge, 2005). In India (30^{th} century B.C), the God of death had a dog as his companion and messenger. Rabies was also mentioned with respect and concern in the legal documents of Mesopotamia in the 23^{rd} century B.C., and ancient Chinese writings indicate that it was recognized in dogs centuries before the birth of Christ (Wilkinson, 1988). The disease is found in almost all parts of the world and the first documented fatality in man bitten by a dog was in the 23^{rd} century B.C. (Nottidge, 2005).

In Africa, the first major outbreak of rabies in dogs was in Ethiopia in 1884 and in South Africa in 1893. In Nigeria, the first documented case in human was in 1912 and in dogs in 1925 (Boulger and Hardy, 1960). Rabies still remains a fatal infection in man and animals. It is found all over the world except in some countries where there is strict quarantine system, rigorous eradication or natural barriers like mountains and rivers. Recently, the United States of America has been declared free of canine rabies (Rupprecht and Tumphery, 2007).

By 1995, the world estimate was about 70,000 deaths in humans per year, which are about 200 per day worldwide. However, there are only about 35,000 notifications per year (CDC, 2004). Animal rabies by 1995 was the 12th in rank in the WHO list of infectious and parasitic diseases which are major causes of death (WHO, 1994), which by 2000 it was rated the 11th cause of human death of infectious disease (WHO, 2000). It has been reported that 98% of human rabies cases occur in the developing countries of Asia, Africa and Latin America (Meslin *et al*., 1994 and Dressen, 1997).

Many developed countries of the world have accurate reports on the outbreaks of rabies. In Nigeria, the status of rabies is unknown as reported by WHO, (2006). Fagbami *et al*. (1981) found that rabies infections both in animals and humans are grossly under reported.

In Nigeria, rabies is a major health problem and is widespread (Nawathe, 1980; Ezebuiro *et al*., 1980). Official disease reporting is rudimentary. Hence information is lacking on the prevalence and distribution of rabies. No wildlife reservoir host has been clearly identified, although sporadic cases in wildlife have been reported (Okoh, 1976; Kasali, 1977). The domestic dog seems to be the major source of human exposure to rabies and perhaps also the reservoir host in Nigeria. Nawathe (1980) reported no wild animal (except one bat) submitted as a rabies suspect during a 3 year period of study. Reports from the National Veterinary Research Institute also show that, over a 10-year period, 95% of rabies cases submitted for diagnosis were of canine origin (Nawathe, 1980).

The relative roles of stray and owned dogs in the epidemiology of rabies in Nigeria follow the classical pattern in which the stray dog seems to be the disseminator among dogs, while the owned dog is the more important source of human exposure (Brobst *et al*., 1959). Owned dogs, regardless of rabies status, were involved in human bites 2.9 times more often than stray dogs. However, stray dogs involved in human bites or found acting strangely were 1.5 times more likely to be rabid than owned dogs. Thus, the stray dog seems to play a role in bringing the virus from one community to the other, while the owned dog is still the most likely source of human infection (Brobst *et al*., 1959). Most owned dogs in Nigeria are free-roaming, providing ample opportunity for contact with rabid strays.

Direct immunofluourescence staining, using both the regular conjugated anti-rabies globulin and a conjugated monoclonal antibody battery to lyssaviruses, was performed on brain samples of suspect animal, which generated data on the rabies situation in Kaduna State, in Northern Nigeria, (Ezeokoli and Umoh, 1989). From the data, it was discovered that there were more dogs, and consequently more rabies outbreaks in the Southern part of the state than in the North; though this did not appear to be associated with the religious beliefs of the local population. It was also observed that there seems to be a cyclic pattern of distribution of outbreaks, and 74.5% of dogs involved in bites had identifiable owners which confirmed the findings of Brobst et al., (1959). Seventy percent of dogs were older than one year and were not vaccinated (75.5%).

The seasonal and age-specific rates of infection of rabies follow classical trends. Cases seemed to cluster around April and September primarily, corresponding to breeding seasons for dogs in Zaria, with intense communal activities (including fights) conducive to the spread of infectious diseases, particularly bite-transmitted rabies. A similar September/October cluster has been observed in Accra, Ghana (Belcher *et al*., 1976).

Rabies has equally been isolated from healthy dogs (Aghomo and Rupprecht, 1990). The isolated viruses were passaged in mice and adapted to cell culture and they were also subjected to monoclonal antibody (MAb) characterization and anti-glycoprotein (G) MAbs. These confirmed them as true rabies virus isolates belonging to the same antigenic group. Though, antigenically distinct from the Flury Low egg passage (LEP) rabies vaccine virus (which is widely used throughout Nigeria for canine vaccination) previously characterized from Nigeria and also from the field strains characterized by Okoh *et al*., (1988).

Okoh (2000) reported rabies isolates from vaccinated dogs that died of rabies infection in the Plateau area of Nigeria which were characterized using MAbs showed that they were also rabies viruses and none of the isolates was identified as the LEP vaccine strain. The isolates had typical pattern of reactivity of isolates from unvaccinated dogs, indicating they were not derived from the vaccine viruses (i.e. they were not of vaccine strain). Equally preliminary studies conducted by Okoh *et al*., (1988) showed guinea pigs immunized with LEP Flury strain vaccine used in canine vaccination in Nigeria to be protected against five variant representatives in five of seven groups of 41 isolates of street rabies virus from Nigeria characterized by hybridoma monoclonal antibodies specific for the nucleocapsid and glycoprotein antigens of rabies viron. They were equally protected when challenged by challenge virus standard even when 1:125 dilution of the vaccine was used. All these strains of rabies viruses of unknown public health significance and control of all strains will require considerable research on the epidemiology and pathogenesis of lyssaviruses in Nigeria.

2.4 Transmission

Rabies is transmitted through the bite of a rabid animal. Transmission is usually from saliva of an infected animal, through bite from animal to animal or animal to man (Durojaiye, 1984). Dogs are the primary source of infection to humans and other domestic animals. Though the dog is moderately susceptible, it acts as a reservoir for urban rabies virus in developing countries.

Transmission can also occur through aerosol contamination of mucous membrane of the mouth or eye (corneal transplant) and bruised skin, or by aerosolized particles or a fresh wound licked by a rabid animal (Jawetz *et al*., 1980; Ojo, 1998; Rodostitis, 2000). Other possible routes of infection include through ingestion, placental (transplacental) and mother's milk (transmammary), (Constantine, 1966; Umoh and Belino, 1979).

2.5 Pathogenesis

Rabies virus is highly neurotropic and causes fatal encephalitis when the virus gains access to the central nervous system (CNS). Lyssavirus infections usually result from the bite of an infected animal. Subsequently, the virus infects local sensory and motor neurons (Tsiang *et al*., 1986; Shankar *et al*., 1991) and replicate locally in muscle cells or attach directly to nerve endings, in particular to nicotinic acetylcholine receptors at motor end plates. Attachment also occurs to several other neuronal receptors (Thoulouze *et al*., 1997). It rapidly ascends the nervous system to the brain; by entering the presynaptic nerve ending by endocytosis and may be associated with synaptic vesicles. Inside peripheral nerves, the virus is carried in a retrograde direction by fast axonal transport centripetally to the CNS. The virus remains intraneural throughout its passage and is experimentally inaccessible to extraneural antibodies. On reaching the CNS, there is massive viral replication on membranes within neurons and transsynaptic transmission of virus occurs from cell to cell. Glial cells are very rarely infected. Viral proteins accumulate in the cytoplasm appearing as inclusions, the classical Negri Bodies. Virions are now visible by electron microscopy in neurons (Ogunkoya, 1997)

Involvement of the limbic system and amygdaloid nuclei cause aggressive behaviour in animals. Hydrophobia in man is probably associated with brain stem infection (Warrell *et al*., 1976). Retrograde axonal transport from the brain, via somatic and autonomic efferent nerves, deposit virus in many tissues (Jackson *et al*., 2003) including skeletal and cardiac muscle, adrenal medulla where infection may be clinically significant, kidney, taste buds, respiratory tract, cornea, and nerve twiglets in the hair follicles. At this stage, productive viral replication occurs, with budding from outer cell membranes in the salivary, lacrimal and other glands, which permits the further transmission of rabies by bites to other mammals. Uncontrolled replication leads to disease and ultimately death (Jackson, 2003). There is no evidence of viraemia, but rabies virus is shed in human saliva, lacrimal and respiratory tract secretions, rarely in urine (Anderson *et al*., 1984) and in milk.

2.6 Clinical Signs of Rabies

2.6.1 Clinical rabies in animal and man

Warm – blooded animals are susceptible to rabies virus infection, and although clinical rabies usually ends in death, many infections are aborted and the hosts are asymptomatic (Beran 1984). The differences in rabies infection in reservoir host species, that is species involved in virus transmission in nature, foxes, skunks, dogs, cats and 'dead end' host species (cattle, horse, man) have more to do with behavioural patterns of each species when rabid than with the nature of the infection per se (Ogunkoya, 1997).

Precise and consistent topographic locations of rabies infection in the brain have been identified for the specific signs and symptoms at various stages of clinical illness in people and animals. Dogs and cats are major hosts of rabies worldwide, and are of epidemiological zoonotic importance while humans represent 'dead end' hosts. Clinical signs in these three species are as described.

2.6.2 Rabies in dogs

Dogs have remained the major hosts and are intermediate in susceptibility to the infection in developing countries (Tierkel 1959; Beran 1984). This means that dogs in comparison with many other animals are moderately susceptible to the disease. Dean *et al.* (1963) have estimated that one peripheral dog LD_{50} of street virus is approximately 86,000 intracerebral mouse LD_{50}.

Johnson (1971) once said that there has been no other virus so diabolically adapted to selective neuronal populations that can drive the host in fury to transmit the virus to another host animal or human than the rabies virus. In other words, biting desire reaches its peak at a time the titre of the virus becomes high in the saliva. Virus may appear in the saliva up to 7 days before symptoms appear.

2.6.2.1 Incubation period

According to Tierkel (1959), the incubation period is usually between 3 to 8 weeks, rarely exceeding 4 months but within a range of 10 days to 6 months. This was confirmed among 26 dogs that died of laboratory confirmed rabies in England during or after a 6-month quarantine period (Boulger, 1966).

The incubation period has been said to depend on the size of the viral inocula, the proximity of the wound to large nerves and the length of the neural path from the wound to the brain i.e. it may be shorter following bites on the face and head and longer when in the legs or the extremities. Also it may be shorter in small breed dogs as compared with large breeds (Beran, 1979).

Studies on the pathogenesis of rabies in the central nervous system (CNS) have shown that clinical signs were not observed until after several growth cycles of the virus had occurred and the virus had spread through the entire CNS with the involvement of numerous neurons (Johnson, 1965; Schneider, 1969).

The clinical signs may be variable in dogs at the onset but by the time the animal dies, there usually have been enough characteristic signs evident of the diseases. In dog rabies, there are classical/distinct or overlapping stages of the disease as follows.

1. Prodromal (change in mental attitude)
2. Furious
3. Dumb
4. Atypical

2.6.2.2 The prodromal stage

The prodromal or melancholy stage may or may not develop or it passes quickly enough that it may not even be noticed by the owners of the dogs and inexperienced veterinarians. Signs peculiar to this stage include change in affection; normally friendly dogs may become withdrawn, seeking one place of seclusion after another (usually dark places) and normally independent dogs may become highly affectionate and lick their owners' hands or faces. Dogs which are not confined may wonder long distances from their home premises. Other signs characteristic of this stage include:

(i) Dogs may disappear from home longer than usual.

(ii) They may have hallucinations (fly biting)

(iii) They may start to have changes in voice

(iv) They may spring at moving objects (broom attacking)

(v) They often develop claustrophobia

(vi) They may chew at the site of infection (hyperesthesia of the bite site).

(vii) Fever at this time may be moderate and usually disappears as symptoms progress. This stage last a few to 36 hours. The animals then go into either dumb or furious rabies stages or may just drop dead. (Beran, 1979; Ogunkoya, 1997).

2.6.2.3 Furious Rabies

This is the stage of rabies is described as aggressively mad dog. This is a serious community hazard. The clinical period may vary from so short as to be unnoticed, to a period of extreme excitation, exaggerated responses to stimulation and aggressive behaviours lasting 1-7 days.

The following symptoms are typical of this stage of the disease:

(i) Dogs are violent and irritable and attack anything or everything.

(ii) If let loose, they may wander, often going in a straight line as if being chased by something. They may stop only to attack a moving or non-moving inanimate object that gets within range of sight, sound or smell and then move on. During attacks, dogs apparently feel no pain, may lacerate their mouths or knock out their teeth in this frenzy. (Beran, 1984).

Frenzied dogs can inflict bites on many animals and humans before death. A furious dog in frenzy left 41 people and 9 other dogs bitten during a 4 hour period before it was killed, (Beran, 1979). The usual characteristic snarls and barks are absent during these attacks. They are usually silent when attacking.

(iii) During this furious period, dogs may show increased thirst and may drink, or try to drink before the muscles of deglutition are not yet paralyzed. There is usually no sign of hydrophobia. Some dogs, both male and female, show sexual arousal. In spite of these behavioural aberrations, most dogs continue to recognize their owners depending on the stage and respond in a quiet way to their master's voices and command, (Beran, 1984). This may be the reason why some owners unknowingly could handle their mad dogs and bring them to clinics without receiving bites.

(iv) Hallucinations may continue. Depraved appetite such as pica, self-mutilation from chewing inanimate objects may become evident.

(v) Extreme claustrophobia may be exhibited. Eyes of affected dogs when examined may show development of strabismus “fox-like squint” which may be unilateral or bilateral; convergent or divergent

(vi) Profuse salivation with the head characteristically held at a downward angle, causing drooling or frothing of tenacious saliva. Drooling in the mouth and the holding of the tail tightly between the rear legs have been described as characteristics of canine rabies through the ages.

(vii) As the symptoms progress, the change in voice also progresses due to paralysis of laryngeal muscles causing a “deep bay voice” Towards death, the rabies howls disappear.

(viii) There is progressive paralysis of muscles of coordination.

(ix) Though they may snap at those whom they knew if threatened, they do not commonly attack their masters. Rabid dogs generally recognize their homes and know the way back home. (Ogunkoya, 1997).

2.6.2.4 Dumb or paralytic rabies

Some authors have described the spinal cord and the lowest part of brain stem (medulla oblongata) as mainly affected in dumb rabies, as well as those parts of the cord transmitting motor impulses to the muscles and parts controlling sensory impulses of touch and temperature from the skin. Other authors have described dumb rabies as the last stage of furious. Transmission can result in paralytic or violent form (Baczunski, 1975).

Murphy (1985) associated it with the infection and dysfunction of neurons of the higher centres of the brain which were said to usually result in depression and coma. In dogs, the following symptoms are observed:

(i) Animals appear poisoned; this may follow melancholy or furious stage signs.

(ii) Inco-ordination resulting from ascending paralysis with paralysis of the jaw leading to the jaw being hung open as a result of paralysis of deglutition muscles. Thick mucoid and strongy saliva drooping from the animals' mouth is the result of the dog being unable to swallow.

(iii) The papillary changes seen in furious rabies may be observed.

(iv) The temperature may be normal or subnormal. It should be noted however that, in dumb rabies the paralysis is progressive. Paralysis usually begins with the lower jaw hanging, then progresses to the head and neck; then involves one or both hind legs causing the animal to sway or fall.

(v) Coma and death ensue from respiratory failure.

(vi) The clinical course is usually 1 to 3 days. (Ogunkoya, 1997).

2.6.2.5 Atypical or mongrel rabies

This is a mixture of furious and dumb rabies. The animal shows intermittent aggression which is usually followed by a temporary calmness. The degree of aggressiveness is lower than in the furious form. The animal lasts a little longer from onset to death than in furious rabies. This is the stage that most dogs are presented to the clinic (Ogunkoya, 1997).

2.6.3 Rabies in cats

Cats are highly susceptible to rabies (WHO, 1973) and about 75% of rabid cats show a pronounced furious phase before progressive paralysis sets in (Beran, 1984). They serve as secondary hosts and are seldom involved in intrapecific maintenance of transmission, (Kaplan, 1969; Beran *et al*. 1972 and Vaughn, 1975). Most cats in developing countries are not kept under confinement, so the status of rabies might be difficult to be established within the species.

In endemic areas where dogs are the maintaining reservoirs, the virus tends to spill over into cats. However, when the transmission cycle is broken in dogs by means of mass vaccination, the disease disappears in cats as well, (Beran *et al*., 1972). On the other hand, where both dog and wildlife transmissions are occurring, control of the transmission cycle in dogs may bring a previous unrecognized host.

For example, wildlife – cat transmission cycle came into focus in the U.S.A. and the continent of Europe (Vaughn, 1975). Cats, especially young males because of their nocturnal outdoor habit are frequently exposed to rabid animals. The incubation period is difficult to determine. It is however, considered that they are usually shorter than those in dogs, (Tierkel, 1959 and Vaughn, 1963).

2.6.3.1 Clinical signs in cats

The prodromal phase usually lasts less than 1 day and is characterized by low grade fever and pronounced change in behaviour. Affectionate cats may suddenly become apprehensive, withdrawn or temperamental. Independent active cats may become unusually solicitous. The furious or excitatory phase lasts 1 – 4 days but may persist up to 7 days.

Rabid cats may retreat into hiding places from which they spring out and attack when approached or when sought out by their owners. The attacks by rabid cats are particularly vicious, and because feline species seek out victims' jugular when attacking, they are particularly dangerous.

When not attacking they show muscular twitching or tremor pupillary dilatation and flashing eyes, mouths foaming with saliva, backs arched and claws protruded making a frightening sight. Unlike dogs, rabid cats appear not to recognize or respond to familiar persons, (Steel and Lester 1958; and Tierkel 1959).

The paralytic phase of the disease lasts 1 – 4 days and the cat shows incoordination most marked in the rear legs, and then developing into ascending paralysis. Paralysis of the muscles of the head and neck leads to hoarseness of the voice and drooling or foaming of saliva.

The paralytic or dumb form predorminates in few cases and the affected cats may purr, show unusual affection, or may hide until death ensues usually in a few hours to 1 – 3 days after clinical onset. In few cases, recoveries from the clinical disease have been reported, (Beran, 1984).

2.6.4 Rabies in man

Nearly all human rabies cases followed exposure to the infections through saliva of rabid animals. These were introduced either by direct bites or contact with freshly abraded skin or muous membranes (Leach and Johnson, 1940; Johnson, 1974; Hattwick and Gregg, 1975; Beran, 1984). Although five cases of human rabies are considered to have resulted from aerosol exposure, three in bat caves and two in laboratory animals accident (Kent and Finegold, 1960; Constantine 1962; Winkler *et. al*. 1973). In addition rabies has occurred in four persons, who received corneal transplant from persons who died from undiagnosed rabies encephalitis (Houff *et. al*. 1979, Anon 1981).

Clinical rabies in humans can be divided into five stages: incubation period, prodromal, acute neurological phase, coma and death, but few cases have been reported to recover (Bernard, 1984).

2.6.4.1 Incubation period

The incubation period has been reported to be as short as 7 days although could be as long as 19 years (Warrel, 1976). Less than 1% of the cases of well documented incubation periods have occurred between 1 – 5 years after exposure (Rubin *et al*., 1969; Hattwick *et al*., 1976; Held *et al*., 1976).

The incubation period is usually between 20 and 90 days and it is shorter if the site of bites is on the head (25 – 40 days) than when it is on an extremity (46 – 78 days) (Held *et al*., 1976). Incubation periods are usually shorter in children than in adults. Exposures on richly innervated areas as the face, neck, hand and especially finger-tips, lead more frequently to infection and shorter incubation period. Those on the trunk or proximal portions of the limbs may take longer time, unless such exposures are in direct proximity to major nerve trunks (Beran, 1984). In general, incubation period may also depend on the nature of the virus and the dose of inoculums

During the incubation period the patients are clinically well, except for symptoms limited to bite trauma. Aside, many patients experience varying degrees of anxiety associated with the prospect of impending fatal disease. However, during the few days, they may experience some pain, tingling sensation, and mild pruritis or paraesthesia of which usually begin at the site of bite. The sensations may remain fixed in location or progressively ascend towards the central nervous system. Invariably, trembling or weakness may appear in such affected limbs, (Beran 1984).

2.6.4.2 Prodromal period

In human, prodromal period is usually 24 to 48 hours but rarely this may extend to 1 week or longer. During this time, patients present vague symptoms of a state of being unwell, often with considerable apprehension.

Malaise, anorexia, low grade fever, fatigue, headache, restlessness and tension are all possible symptoms, (Bernard, 1984; Beran, 1984). In approximately 50% of patients, pain and paraesthsia occur at the site of exposure and if present, are usually the first rabies specific symptoms.

Other more severe but nonspecific and common symptoms occurring during the prodromal period include influenza like condition such as, sore throat in association with cough, gastritis, with nausea or vomiting or enteritis, (Dupont and Tarle, 1965). None of these manifestations are pathognomonic of rabies and in children or adults, in the absence of a history of exposure to the disease; they may not lead many attending physicians to suspect rabies. The presence of wounds and scars especially if there are symptoms referable to such lesions should always prompt physicians to inquire further about possible animal contacts, (Ogunkoya, 1997).

2.6.4.3 Acute neurologic phase

Onset of clinical rabies in most patients is marked by excitatory manifestations including increasing sensitivity to tactile, visual or auditory stimuli but obvious decrease reaction to local pain. There are hyperactivity, disorientation, hallucinations, seizures, bizarre behaviour and nuchal rigidity. Other symptoms are increased muscular tone, accelerated pulse, pupillary dilatation, increased lacrimation, perspiration and salivation.

In most cases, marked hyperactivity develops which lasts from hours to days, (Warrel, 1976). Hyperactivity is typically intermittent with 1 – 5 minutes agitation, thrashing, running, biting or other bizarre behaviour alternating with the period of calm (Babes, 1912; Hattwick, *et al*., 1972). Hyperactivity episodes may occur spontaneously or be precipitated by a variety of tactile, auditory, visual or other stimuli, (Beran, 1984).

The outstanding symptoms are dramatic or gradual onset of painful and spasmodic contractions of the muscles of deglutition, characteristically precipitated with contact of fluid which the patients face in an attempt to drink. This occurred in 30 – 50% of rabies cases, (Bernard, 1984). Ejection of fluid may be explosive with spluttering, coughing and entrance of fluid into the nasal passages or trachea. Such episodes are terrifying and building up a state of hydrophobia which could be stimulated by the taste, smell, sound or sight of liquids frequently including the patients own saliva, (Beran, 1981). The contractions may progress to involve jerky spasms of the diaphragm and chest muscles: the arms may be thrown upward, the head jerked backward, and the whole body arched in episthotonus. Choking resulting from the respiratory muscular spasms may become so severe that prolonged apnoea with cyanosis and gasping may ensue, (Beran, 1984). Some patients feel so much pain and constriction of the throat that they clutch at their necks, (Beran, 1984).

Stimuli in addition to liquids which may set off the spasms include foods of any type, drafts of air, especially across the face (aerophobia) or touching of the inside of mouth or throat, noises, and lights. Even though the patients may be able to continue to swallow solid boluses of food, liquid would induce painful contractions in the throat. Spasms of the throat increase in intensity and frequency until they may occur spontaneously or nearly continuously. At this time, patients may reach extremes of agitation and terror. They may struggle frantically and attack nearby persons, although they do not bite, generally, convulsion may ensue.

In a study of 38 human cases in the United States, 66% had difficulty in swallowing, 66% had excitement or agitation, 58% had paralysis or weakness, 34% had hypersalivation and 29% either hydrophobia or aerophobia during the clinical stages (Schneider, 1975; Bernard, 1984). In Nigeria, records are not enough for classification as above. But Warrel (1977) reported that all six clinical patients (100%) had hydrophobia. Generally, more than 90% of patients have some neurologic symptoms at the time of admission.

Between episodes of excitation, patients become calm and lucid, able to communicate and realize what is happening to them (Beran, 1984). The variable involvement of other nerves and portions of the brain lead to a wide variety of symptoms. Damage to the cranial neves may cause facial paralysis, squinting or drooping of eyelids, paralysis or swelling of vocal cords may lead to hoarseness, and cries from pain or fear may become more like barks. Salivation and lacrimation may be continuous and excessive, leading to a frothy flow from the open mouth and a tear-stained face. Urinary flow may fluctuate as posterior pituitary function becomes unbalanced (Ogunkoya, 1997).

Increased libido in male patient could occur: Temperature regulation may become erratic, leading to brain-damaging fevers of above 42^{o}C to hypothermia. Both respiration and heart beat may become irregular with cluster breathing alternating with period of apnea while fluctuating blood pressure has also beeen observed. Pneumonia, due either to the rabies virus or to infection secondary to inspired fluids or saliva may ensue in patients who survive several days (Ogunkoya, 1997).

Generally, unless the patient dies abruptly from respiratory or cardiac arrest, paralysis develops and becomes the most prominent neurologic problems. In 15 – 20% of cases the period of hyperactivity is either minimal or absent and paralysis dominates the entire clinical course (Pawan, 1939; Love, 1944; Chopra *et. al*., 1980). The paralysis may be diffused and symmetrical, maximal in the bitten extremity, or ascend as in Landry-Guillain Barre syndrome (Bernard, 1984).

The acute neurologic phase lasts 2 – 7 days with longer duration in severe paralytic forms. It ends either with sudden death or with the onset of coma. Throughout this period, the mental status continues to fluctuate, with periods of severe obtundation alternating with those of relative normality. Depressive or paralytic symptoms may become predominant at a point in the course of the clinical disease, even from onset in somewhat less than 20% of patient, (Knuti, 1929). In clinical rabies, characterized from onset by paralytic manifestations, principal involvement of the spinal cord occurs rather than the brain, (Beran, 1984). Onset of clinical disease is usually characterized by headache, high fever and sweating.

Constipation, anuria, drooling of saliva due to inability to swallow, and stiff neck, suggestive of meningitis may be indicative. Paralysis developed progressively involving legs, arms and trunks and may ascend as in Landry-Guillain Barre syndrome, (Hattwick *et al*., 1972). Finally, the muscles of swallowing and respiration become paralyzed. Patients usually remain lucid during the progression of paralytic systems. Hydrophobia is only rarely observed during this phase, and it is probably associated with terminal throat spasms, (Beran, 1984).

Delirium and coma precede death. Paralytic human rabies had been associated with vampire bat-transmitted rabies, (Pawan, 1939). But Warrel (1977) reported that it may be common in Nigeria.

2.6.4.4 Coma

Coma occurs 4 – 10 days after onset of symptoms and may last for hours or months (Bernard, 1984). In untreated patients, respiratory arrest usually occurs shortly after onset of coma and the patient dies. For those that received intensive support, the duration of illness average 25 days (Anderson *et. al*., 1984).

During the coma phase, a variety of fatal or potentially fatal complications occur, (Houff *et. al*., 1979).

2.6.4.5 Recovery

Rabies is nearly always fatal without proper postexposure prophylaxis (PEP) (CDC, 1999). So far, there are six known occurrence of human recovery after rabies infection, five of which were either previously vaccinated, (CDC, 1977) or received some form of PEP before the onset of illness (Porras *et. al*., 1976, Hattwick *et. al*., 1972, Alvarez *et. al*., 1994; Madhusudana *et. al*., 2002), only one of the five patients recovered without neurologic sequence, (Hattwick, 1972). The sixth case was unique because the patient received no rabies prophylaxis either before or after illness onset. Historically, the mortality rate among previously unvaccinated rabies patients has been 100% (Jackson, 2003). No specific course of treatment for humans has been demonstrated to be effective, but a combination of treatments, which might include rabies vaccine, rabies immune globulin, monoclonal antibodies, ribavirin, interferon – alpha, or examine, has been proposed, (Jackson, 2003). Given the lack of therapeutic utility observed to date, and because the patient (sixth case) had rabies-virus-neutralizing antibodies on diagnosis, the use of immune – modulators (e.g. rabies vaccine, rabies immune globulin, or interferon) were avoided. The patient was treated with therapeutic coma induction to produce a burst suppression combined with antiviral drugs, (Willoughby *et. al*, 2005). This is the first documented recovery from clinical rabies by a patient who had not received either pre-or postexposure prophylaxis for rabies, (CDC; MMWR, 2004). So far, this treatment has not been reproducible in other patients, (Hemachudha *et al*., 2006).

2.7 Diagnosis

2.7.1 Diagnosis of rabies in animals and man

2.7.1.1 Clinical diagnosis in animals.

A good history of any animal with mental or personality changes deserves caution, curiosity and proper investigation in order to rule out rabies. Questions that will shed more light into the case at hand should be asked, such as vaccination history of the animal, history of fight in the past 3 to 8 weeks and available information on the other animal(s) engaged in the fight. Also information on personality or appearance changes is considered necessary guidelines suggested assisting in examination of suspected animals, (Ogunkoya, 1997).

If nervous breakdown is present, the onset should be ascertained. When put in the run, animals may sway or fall. This shows that they are progressively experiencing nervous breakdown. (Paralysis in rabies commonly begins at the head and neck, then on one or both hind legs). If a nervous breakdown is established, symptoms which are typical of rabies will nearly always develop in the next 24 – 48 hours, (Ogunkoya, 1997).

2.7.1.2 Necropsy examination

In some cases of rabies, the stomach contents could be used as presumptive evidence for rabies. In rabies, unusual and unexpected objects such as sand, stones, iron materials, glasses etc. could be found in the stomach. They are evidences of depraved appetite which may be enough to confirm rabies in the dog when clinical signs were characteristic, (Ogunkoya, 1997).

2.7.1.2.1 Shipment of specimens to the Laboratory

Dogs or cats suspected of being rabid should be captured and confined for 10 days if possible, and the disease should be allowed to progress until fatal termination. The premature killing of such animals will reduce the accuracy of histologic laboratory diagnosis, since the development of Negri bodies in the brain is directly related to the length of clinical illness in rabies. This applies seriously to such countries e.g. Nigeria where examination of Negri bodies is the basis of diagnosis. In places where the immunofluorscence test facility is available, an animal does not have to be held for Negri bodies to develop. Immunofluorescing antigen will be detectable in the brain at any stage of the disease during which the virus can be transmitted. Therefore, a strongly suspected animal may be euthanized especially if it causes a severe bite or a bite on the head or face of the victim. The biting animal may then be decapitated for immediate shipment to the relevant laboratory (Ogunkoya, 1997).

A wild or stray animal that bites a human or another animal always be euthanized immediately and sent to the laboratory for examination. Whichever method of diagnosis a laboratory employs, it is reasonable to inoculate all specimens into suckling mice (Ogunkoya, 1997).

For effective shipment, the veterinarian must be familiar with the requirements of existing laboratories. For instance, some laboratories prefer to do a complete necropsy if possible. If so, the animal should be sent or taken to the laboratory intact. Most laboratories want the head of the animal refrigerated.

Specimens should be placed on wet ice and, if possible, delivered directly to the laboratory by a messenger. If the specimen is to be shipped, the head should be placed in a suitable watertight metal container, such as a clean paint can, and sealed tightly. The container should be placed in a larger water tight container, such as an ice chest and pack cracked ice around it. The shipment must be by the fastest means and the shipper is responsible for making sure that the package does not leak specimen or water from wet ice. Forms containing necessary information should accompany the specimen and the medical personnel are to be informed of the laboratory results, (Ogunkoya, 1997).

2.7.1.2.2 Glycerolated specimens

Parts of the brain tissue or salivary gland can also be sent to laboratories for examination in a 50% glycerin-saline, fresh or combination of both. In this case, the virus infectivity and antigen reactivity in the immunofluorescence test are preserved. Pieces of tissue approximately half inch square should be placed in the 50% glycerin-saline since smaller pieces may break up. Portions of brain should include parts of the hippocampus, cerebellum and cerebral cortex from each side. If possible, include a portion of the medulla or brain stem, (Ogunkoya, 1997).

In the 50% glycerin-saline method, refrigeration is not required. Glycerolated portions of brain do not usually produce satisfactory impression smears on a slide unless washed thoroughly in saline, as it is difficult to make the glycerolated brain adhere to glass. Therefore brain smears of the materials in question may first be made from unpreserved brain, and if desired, stained and sent along with the specimens in 50% glycerol-saline solution. Glycerolated specimens are best used in animal inoculation (Ogunkoya, 1997).

2.7.1.3 Laboratory diagnosis of rabies

The first attempts to diagnose rabies in suspected animals, especially dogs, were made by examining the stomach contents for ingested sticks and stones as stigmata of deranged behaviour (Beran, 1984). It was not until the late 1800's after Pasteur had shown that rabies could be transmitted by inoculation of central nervous system (CNS) tissue, that a search for brain lesions pathognomonic for rabies began (Fekadu and Smith, 1985). In 1892, Babes described perivascular accumulations of "embryonal" cells around blood vessels and neurons in brain tissue undergoing chromatolytic degeneration which he called "rabidic tubercules". These lesions, now known as Babe's nodules, are also found in other viral encephalitis and their presence was therefore not considered specific indication of rabies infection (Perl, 1975). The first diagnostic brain lesion was reported by Negri in 1903. He described an organism within neuronal cytoplasm, which he considered to be the specific "protozoa", etiologic agent of rabies. Although Negri "protozoa" were new and known to be accumulating rabies virus antigen, he was correct in his assertion that the Negri body antigen represents a specific and diagnostic lesion of the disease. Until 1958, when Goldwasser and Kissling introduced the detection of viral antigen by immunofluorescent antibody (IFA) method, Seller's stain for Negri body technique and biologic inoculation of viral isolates formed the basis for rabies diagnosis. After the introduction of IFA, different methods and techniques for rabies diagnosis were later developed.

In all, laboratory examinations used in diagnosis of rabies can be divided into two separate procedures: Antemortem and postmortem examinations.

2.7.1.4 Post-mortem diagnosis of rabies

2.7.1.4.1 Seller's staining method for the detection of Negri bodies:

This method has been found to be simple and economical. The procedure was described by Sellers, (1927). The Negri body development is related to the length of time an animal lives after it had developed the clinical signs of rabies. Although the observation of Negri bodies confirms the diagnosis of rabies, the inclusions are found in only 75 – 80% of specimens found positive by other more sensitive diagnostic methods. In some animal species, the sensitivity of this diagnostic method may even drop to 50%, (Baer *et. al*., 1968). The observation of non-specific inclusions in some animals, particularly cats and rodents, may also result in a number of false positive rabies diagnoses in these species, (Fekadu and Smith, 1985). Because of the low sensitivity and possibility of false-positive with Seller's test, its result cannot be relied upon singularly. It should be confirmed by the mouse inoculation test, (MIT) (Goldwasser, *et. al*., 1958; Fekadu and Smith, 1985) or any other more sensitive method.

2.7.1.4.2 Mouse inoculation tests for viral isolation:

This method was previously described by Koprowski (1973). Five weaning mice are usually inoculated intracerebrally and observed daily for 30 days for signs of rabies (trembling, humping, paralysis or prostration). A 7 to 20 day incubation period is expected in mice inoculated with street virus preparation, but incubation periods of 30 days or more are sometimes observed. The major problem with this technique is the length of incubation period which mostly is unsuitable, when an exposure has occurred that needs rapid diagnosis and confirmation. This test is expensive.

2.7.1.4.3 Direct immunofluorescence antibody (IFA) test for rabies virus:

The procedure for the IFA technique was first described by Goldwasser and Kissling (1958). When reacted with fluorescein conjugated rabies immune serum and illuminated with ultra-violet light, rabies antigen in infected tissues appear as brightly-coloured apple green or greenish yellow, round to oval intracellular accumulations. The antibody primarily responsible for staining in the IFA test is that directed against the nucleocapsid antigen of the virus. This antigen is believed to be the same as the classical Negri body, seen on histological examination of CNS tissue (Matsumoto, 1962). In addition to the larger stained bodies, infected tissues may contain smaller collections of antigen, which appear as granular or dust-like fluorescent particles or thread (Fekadu and Smith, 1985). Immunofluorescent rabies antigen may be detected in all parts of the CNS of infected animals, but because of the frequently uneven distribution of antigen, the most reliable diagnosis is made from tests which include examination of the medulla (brain stem), cerebellum and the hippocampus (Koprowski, 1973). Lately, antigen could be detected in the skin, salivary gland and other tissues of an infected animal (Beran, 1984).

Comparative studies of IFA and virus isolation techniques have shown a 97 – 98% correlation in diagnosis (Kissling, 1975). When a very small amount of antigen is present, however, the virus isolation technique is a more sensitive procedure. The major advantage of IFA is that the technique can detect rabies antigen in tissues, which may no longer contain infectious virus because of tissue decay or the presence of antibody (Fekadu and Smith, 1985). The equipments are rather costly and are not always available in laboratories of the developing countries (Sureau and Bourhy, 1988),

2.7.1.5 Ante-mortem diagnosis

Developed laboratory techniques are available that permit the rapid diagnosis of rabies in humans. Ante-mortem diagnosis is aimed at aiding the judgement of the physician in designing the best and most suitable clinical treatment for the patient, and protection of others who may have been exposed to the disease. The methods of antemortem diagnosis are based on previous studies of rabies pathogenesis (Perl, 1975). These studies of rabies virus showed it is centrifugally transmitted to the peripheral organs (peripheral nerves, cornea, salivary glands and other tissues near the CNS) at the early part of the disease. As a result, detection of viral antigen in IFA-stained impressions of corneal epithelium and frozen sections of skin biopsy (Schneider, 1969; Smith *et. al*., 1972) and isolation of virus from saliva and tracheal aspirates became possible and has been found reliable indicators of rabies infections of the CNS (Hattwick *et. al*., 1972).

The demonstration of a significant rise in titer of antibodies to rabies virus in serum, in the absence of passive or active immunization, and the appearance of antibody in cerebro-spinal fluid during the course of the infection are also satisfactory methods of diagnosis (Hattwick and Greg, 1975; Fekadu and Baer, 1980).

Laboratory techniques used in these procedures have all been described. The cornea impression technique was described by Schneider, (1969), nuchal biopsy (Smith and Blenden, 1972) and isolation of rabies virus from saliva by mouse inoculation, (Koprowski, 1973) or by inoculation of susceptible cell lines and detection of an intracellular viral antigen by IFA techniques, Goldwasser and Kissling (1958); Koprowski, (1973); Wiktor, (1973), had also been described. Cell systems were also successfully used to grow street rabies virus isolates in the marine neuroblastoma cell (NBC). Rapid fluorescent focus inhibition test (RFFIT) has been found to be sensitive, rapid, economical and reproduceable and has been used to measure neutralizing antibody in pre-and post-exposure anti-rabies prophylaxis, (Johnson, 1973).

Interpretation of results of antemortem diagnosis is difficult as well as delicate.Negative findings on all of the above-mentioned tests for example while certainly reducing the possibility that rabies virus is the cause of illness, cannot rule out rabies infection totally. Although a positive result on any of the currently used tests (detection of viral antigen by IFA in corneal impression, nuchal or CNS biopsy; virus isolation from saliva; antibody in serum or CSF of non-vaccinated patient) is an indication of rabies infection, care must be taken in making conclusions (Fekadu and Smith, 1985). The detection of virus antigen by IFA or virus isolation is predicated on peripheral spread of rabies virus, but death may occur before the spread of virus from CNS, (Baer, 1975). Also rabies prophylaxis and/or the immune response to infection may prevent virus spread or mask its presence in peripheral tissues, (Anon, 1983). In addition, the use of immunosuppressive drug or interferon treatment may susppress or delays the antibody presence in the CSF or a rise in serum titers despite obvious rabies infection. Other new methods are:

(i) The Rapid Rabies Enzyme Immuno-Diagnosis (RREID) test (Sureau and Bourthy, 1988). RREID was developed based on the enzyme linked immunosorbent assay (ELISA) technique, (Perrin *et al*., 1986)

(ii) The Dot Blot Hybridization techniques routinely used in molecular biology for the detection of specific nucleic acid sequences have been applied to rapid diagnosis of variety of infectious diseases (Lowe, 1986) including detection and identification of a panel of virus using specific DNA probes, (Berninger *et. al*. 1982; Dust *et. al*., 1983; Redfield *et al*., 1983; Hypia, 1985). This method is now being used in rabies diagnosis, (Ermine *et. al*., 1988).

(iii) The use of Monoclonal antibodies.Wiktor *et. al*., (1980) observed that the use of the rabies-specific hybridoma monoclonal antibodies permits the detection of different specificities expressed by viruses of the rabies group. It has also become possible to detect specific and cross-reacting antigens between and within the groups of rabies and rabies-related viruses. Also the differentiation between laboratory strains from street rabies is now possible.

2.8 Other Techniques Used in Diagnosis of Rabies

2.8.1 Reverse transcription polymerase chain reaction

Reverse transcription polymerase chain reaction (RT-PCR), which is a rapid and sensitive assay, has been used for detection of rabies virus and rabies – related viruses, (Heaton *et al*., 1997). Studies have shown that the nested and hemi-nested RT-PCR assays were more sensitive than the fluorescent antibody (FA) test. The rabies virus nucleoprotein (N) gene, which is highly conserved, has been used as the target for RT-PCR analysis in several molecular epidemiological studies, (Nadin-Davis *et al*., 1993, 1994; Smith *et al*., 1995; De Mattos *et al*., 1999, 1999; Bourhy *et al*., 1999; Ito *et al*., 1999, David *et al*., 2002).

RT-PCR (followed by nested PCR) was developed for diagnosis. Nested PCR is more sensitive than single – step PCR, but it increases the possibility of cross-contamination of samples and carry-over of amplified products. The identification of rabies virus variant isolates can be done by PCR analysis, (Arai *et. al.*, 1997).

2.8.2 Direct rapid immunochemistry test

Direct rapid immunohistochemistry test (DRIT) is used for the detection of rabies virus antigen. It is an unlicensed procedure designed for consideration as a potential confirmatory measure of the direct fluorescent antibody test according to the national standard operating procedure for the diagosis of rabies in animals (http://www.cdc.gov/ncidod/dvrid/rabies/professional/publications/DFA-diagnosis/DFA-protocol-b.htm). DRIT compliments the direct fluorescent antibody test.

2.9 Prevention and Treatment of Rabies

Rabies can be prevented by vaccination, both in humans and other animals. Virtually every infection with rabies was a death sentence, until Louis Pasteur and Emile Roux developed the first rabies vaccination in 1885. This vaccine was first used on a human on July 6, 1885 – nine-year old boy Joseph Meister (1876 – 1940) who had been mauled by a rabid dog, (Merck Manual, 1983).

Their vaccine consisted of a sample of the virus harvested from infected (and necessarily dead) rabbits, which was weakened by allowing it to dry for 5 to 10 days. Similar nerve tissue-derived vaccines are still used now in some countries, and while they are much cheaper than modern cell culture vaccines, they are not as effective and carry a certain risk of neurological complications, (Merck Manual, 1983).

The human diploid cell rabies vaccine (H.D.C.V.) was started in 1967. Human diploid cell rabies vaccines are made using the attenuated Pitman-Moore L503 strain of the virus. Human diploid cell rabies vaccines have been given to more than 1.5 million humans as of 2006. Newer and less expensive purified chicken embryo cell vaccine, and purified Vero cell rabies vaccine are now available. The purified Vero cell rabies vaccine uses the attenuated Wistar strain of the rabies virus, and uses the Vero cell line as its host, (Ogunkoya, 1997).

Some recent works have shown that during lethal rabies infection the blood-brain barrier (BBB) does not allow anti-viral immune cells to enter the brain, the primary site of rabies virus replication (Roy *et. al*., 2007). This aspect contributes to the pathogenicity of the virus and artificially increasing BBB permeability promotes viral clearance, (Roy and Hooper, 2007). Opening the BBB during rabies infection has been suggested as a possible novel approach to treat the disease.

2.9.1 Pre-exposure prophylaxis

Currently pre-exposure immunization has been used on domesticated and normal non-human populations. In many jurisdictions, domestic dogs, cats, and ferrets are required to be vaccinated. A pre-exposure vaccination is also available for humans, most commonly given to veterinarians and those traveling to regions where the disease is common, such as India. Most tourists do not need such a vaccination, just those doing substantial non-urban activities. However, should a vaccinated human be bitten by a carrier, failure to receive subsequent post-exposure treatment could be fatal, although post-exposure treatment for a vaccinated human is far less extensive than that which would normally be required by one with no pre-exposure vaccination, (Ogunkoya, 1997).

In 1984 researchers at the Wistar Institute developed a recombinant vaccine called V-RG by inserting the glycoprotein gene from rabies into a vaccinia virus, (Wiktor *et. al*., 1984). It is harmless to humans and has been shown to be safe for various species of animals that might accidentally encounter it in the wild, including birds (gulls, hawks, and owls) (Artois *et. al*., 1990).

V-RG has been successfully used in the field in Belgium, France, and the United States to prevent outbreaks of rabies in wildlife. The virus is stable under relatively high temperatures and can be delivered orally, making mass vaccination of wildlife possible by putting it in tasty baits. The plan for immunization of normal populations involves dropping bait containing food wrapped around a small dose of the live virus. The bait would be dropped by helicopter concentrating on areas that have not been infected yet. Just such a strategy of oral immunization of foxes in Europe has already achieved substantial reductions in the incidence of human rabies. A strategy of vaccinating "neighborhood dogs" in Jaipur, India, (combined with a sterilization program) has also resulted in a large reduction in the number of human cases, (Reece and Chawla, 2006).

2.9.2 Post-exposure prophylaxis

Treatment after exposure, known as post-exposure prophylaxis or "P.E.P.", is highly successful in preventing the disease if administered promptly, within fourteen days after infection. The first step is immediately washing the wound with soap and water, which is very effective at reducing the number of viral particles. In the United States, patients receive one dose of immunoglobulin and five doses of rabies vaccine over a twenty-eight day period. One-half the dose of immunoglobulin is injected in the region of the bite, if possible, with the remainder injected intramuscularly away from the bite. This is much less painful compared with administering immunoglobulin through the abdominal wall with a large needle, which is how it was done in the past. The first dose of rabies vaccine is given as soon as possible after exposure, with additional doses on days three, seven, fourteen, and twenty-eight after the first. Patients that have previously received pre-exposure vaccination do not receive the immunoglobulin, only the post-exposure vaccinations, though it is recommended for the antibody titer of such patients to be evaluated. The titer should measure up to the recommended value of 0.5IU/ml by WHO. Since the widespread vaccination of domestic dogs and cats and the development of effective human vaccines and immunoglobulin treatments, the number of recorded deaths in the U.S. from rabies has dropped from one hundred or more annually in the early twentieth century, to 1 – 2 per year, mostly caused by bat bites, which may go unnoticed by the victim and hence untreated, (Ogunkoya, 1997).

P.E.P. is effective in treating rabies because the virus must travel from the site of infection through the peripheral nervous system (nerves in the body) before infecting the central nervous system (brain and spinal cord) and glands to cause lethal damage. This travel along the nerves is usually slow enough that vaccine and immunoglobulin can be administered to protect the brain and glands from infection. The lenght of time this travel requires is dependent on how far the infected area is from the brain: if the victim is bitten in the face, for example, the time between initial infection and infection of the brain is very short and P.E.P. may not be successful, (Ogunkoya, 1997).

2.9.3 Induced coma treatment

In 2005, the case of Jeanna Giese, 15 year old girl who survived acute rabies, without pre or post exposure vaccination was reported, indicating the successful treatment of rabies through induction of a coma, (Willoughby *et al*., 2005). This treatment approach was based on the theory that rabies' detrimental effects were caused by temporary dysfunctions of the brain, and that the induction of a coma, by producing a temporary partial stop in brain function, would protect the brain from damage while the body builds up an immune response to the virus. After thirty-one days of isolation and seventy-six days of hospitalization, she was released from the hospital, having survived rabies. Later attempts to use the same treatment have failed.

The primary care physician of this case published in the April 2007 issue of Scientific American, noting that subsequent failures of what he calls the Milwaukee protocol did not use the cocktail of drugs used during the treatment. A point he makes for future research is the relationship of the virus to depletion of biopterin in the brain, (Rodney and Willoughby, 2007)

2.10 Strains Used for Vaccine Production

2.10.1 Evelyn-Rokitnicky-Abelseth (ERA) strain.

ERA strain is closely related to the SAD B19 strain which originated from a street virus (SAD, street Alabama Dufferin) isolated from a rabid dog and was attenuated by multiple passages in different cell types. They both originated from the same isolate but differ in cell culture history and ERA has retained a residual pathogenicity; it is nonpathogenic for a wide range of animal species when administered orally or intramuscularly, (Conzelmann *et al*., 1990).

Currently, the only complete nucleotide sequence of an attenuated rabies virus vaccine available in a gene bank is that of SAD B19. Because almost all commercially available attenuated rabies virus vaccines currently available on the market are SAD derivatives and cannot sufficiently be distinguished from each other on a genetic basis.

Since 1983, a successful field trial has been performed with the SAD B19 rabies virus vaccine strain, which immunizes foxes after oral application. No case of rabies has yet been reported which was caused by this vaccine strain. Safety and efficacy of the SAD B19 live vaccine have led to a drastic decrease of rabies cases and eradication of wildlife rabies, at least in parts of Europe, appeared realistic, (Schneider and Cox, 1983; Schneider *et al*., 1988).

The nucleoprotein gene (N gene) of SAD B19 encodes 450 amino acids. The deduced amino acid sequence differs in four residues from that of the PV strain (99.1% homology). Three of the substitutions are located in the amino terminal moiety of the protein and one in the carboxy terminal region. The corrected version of the ERA N protein (Ertl *et al*., 1989) contains two different amino acids compared to the SAD B19 sequence (99.6% homology).

ERA rabies virus and other attenuated rabies virus vaccines stimulate a strong immune response and are efficient live vaccines. Attenuated strain of ERA infects nonneuronal cells. It infects activated murine lymphocytes and the human lymphoblastoid Jurkat T-cell line in vitro. The lymphocytes are more permissive to the attenuated ERA rabies virus strain than the CVS strain. ERA viral replication induces apoptosis of infected

Jurkat T cells, and cell death is concomitant with viral glycoprotein expression, suggesting that this protein has a role in the induction of apoptosis, (Thoulouze *et. al*., 1997).

2.10.2 West Caucasian bat virus (WCBV)

During 2002, WCBV is one of the two new lyssaviruses (WCBV and Irkut) isolated from Eurasian bats. According to preliminary identification with anti-nucleocapsid monoclonal antibodies and comparison of limited N gene sequences, both may be considered as new genotypes of the lyssavirus gene, (Botvinkin *et al*., 2003). These viruses were named West Causasian bat virus (WCBV) and Irkut virus. The isolation of these viruses has been described earlier, (Botvinkin *et al*., 2003). WCBV was isolated from the brain of a common bent-winged bat (*Miniopterus schreibersi*) in Krasnodar region in July 2002 by intracerebral mouse inoculation technique (Koprowski, 1996). In mice, it caused fatal encephalitis, and typical cytoplasmic inclusions were detected in mouse brain impressions using either polyclonal or monoclonal anti-rabies virus antibodies. The coding region of the entire N gene consisted of 1350 nucleotides (nt) (450 deduced amino acids) (see Appendix XIX.)

WCBV demonstrated significant diversity compared to other lyssavirus representatives, (Ertl *et al*., 1991); Fu *et al*., 1994; Da Cruz *et al*., 2002), and remained distant from all other lyssaviruses however, most divergent representative of the genus according to N gene, was Mokola virus (MOKV) (Kuzmin *et al*., 2005).

The G gene of both Irkut and WCBV began with the signal peptide of 19 amino acids common to all lyssaviruses. The G of WCBV coded 506 amino acids. This is different from all other lyssavirus representatives, (Wunner, 2002). The ectodomain of both Irkut virus and WCBV consisted of 439 amino acids, and did not produce any gaps in an alignment with other lyssaviruses, as was also true for the cytoplasmic domain: all alignment gaps were produced along the endodomain only. R333, which was reported as an important factor of pathogenicity in a peripheral challenge, (Dietzschold *et al.*, 1983; Badrane *et al.*, 2001), was conserved in Irkut ectodomain, but not in WCBV, where it was substituted by E333. A potential glycosylation site was found in the WCBV ectodomain at position 334 as it had been published earlier for GT 2, (Badrane *et al.*, 2001). WCBV also showed to be the most diverse member at the nucleotide level and amino acid level, compare to other GTs.

It can be concluded that WCBV is the most divergent representative of the lyssavirus genus described to date, (Kuzmin *et al.*, 2005). The principal host of this virus is unknown. It was isolated from *Miniopterus schreibersi*, a broadly distributed colonial species. This bat species is known not to perform long-distance seasonal migrations, but it is quite mobile and, according to tracking data, the population occupying caves on both slopes of the Caucasian ridge is permanently intermixed, (Gazaryan, 1999). Thus, more distant movements appear probable. WCBV was apathogenic for 3-week-old mice by intramuscular and oral routes even when 106 mouse intracerebral lethal doses were given. Syrian hamsters challenged intramuscularly with the same virus dose developed typical rabies symptoms and all succumbed (100% mortality). It is worthy to note that WCBVC and phylogroup 2 lyssaviruses are not laboratory strains. They were isolated from a number of naturally infected mammalian species (MOKV was also isolated from humans), so there should be some adaptive mechanisms providing their natural circulation, (Kuzmin *et al.*, 2005).

Anti-rabies biologicals have been known to provide incomplete protection of experimental animals against European bat lyssaviruses (EBLVs). Limited protection against Lagos bat virus (LBV), and no protection against Mokola virus (MOKV) (Fekadu *et al*., 1988; Lafon *et al*., 1988; Mebatsion *et al*., 1992; Bahlou *et al*., 1998; Jallet *et al*., 1999; Nel *et al*., 2003). Thus the same lack of efficacy against WCBV is expected, (Kuzmin *et al*., 2005).

2.10.3 Low egg passage (LEP) Flury strain

The production and use of rabies vaccines in Nigeria have been described by Nawathe *et al*. (1981). Vaccination history in Nigeria is dated as far back as 1935 for the control of rabies by vaccination of dogs above 6 months old, the then Veterinary Department issued simple type phenolized sheep brain vaccine to be given in 2 doses a week apart. But the vaccine met with apparent neuroparalytic accidents and vaccine failures resulting in its withdrawal from the field within five years of its issue, (Thorne, 1954). No vaccine was available until 1956 when low egg passage (LEP) Flury vaccine grown in chicken embryos was developed and issued to the field. There has not been any major change except for the modification in the techniques to suit production in quantity. High egg passage (HEP) Flury vaccine was developed and issued to the field since 1971 for cats, cattle and horses.

The advent of the live rabies vaccines began with the adaptation of the Flury strain of rabies virus to day-old chickens and later to the chicken embryo. This strain was isolated by Johnson from a young girl who died of rabies in USA following an unusual exposure in which there was no evidence of bite, but a history of the licking of mucous membranes. The LEP was found to be avirulent for dogs when injected parenterally by Koprowski and Black, (1950). Further experiments of the rabies strains with dogs showed that such dogs developed good immunity of long duration.

Further studies with this live virus vaccine yielded positive results that gave the vaccine prominence and was licensed for U.S.A. and in many countries of the world. The vaccine was widely used in campaigns and routine veterinary practices. There were numerous demonstrations in the United States and other parts of the world that confirmed its safety and efficacy. But there were set backs as shown with puppies less than 11 weeks of age who do not respond well as older animals, (Kaebele, 1958) and that it may cause encephalitis in puppies under 3 weeks of age, (Steele, 1973). The biggest set back has been the short life, (Dean *et al*., 1964). In addition, numerous cases of vaccine-induced rabies in dogs, (Cabasso, 1962; Kappus, 1976; Constantine *et al*., 1978) and cats, (Bellinger *et al*., 1983) were reported for LEP strain vaccine.

2.11 Vaccine Failure

Despite all efforts to control rabies, the disease continues to be a major scourge of dogs and cats in Nigeria, (Umoh and Belino, 1979; Fagbami *et al*., 1981; Okoh, 1986; Nottidge, 1994; Osinubi *et al*., 1999; Ogunkoya *et al*., 2003). Thousands of dogs are vaccinated annually with the Flury Low Egg Passage (LEP) vaccine strain, produced at Vom, Nigeria (Nawathe *et al*., 1981), but the prevalence of the disease in domestic animals has not declined, (Durojaiye, 1984; Okoh, 1986).

Aghomo *et al*., (1990) determined the serological response of puppies from Nigeria to LEP rabies vaccine using two set of puppies from rabies-vaccinated and non-vaccinated bitches. Serum rabies virus neutralizing anitboides (VNA) measured using modified rapid fluorescent focus inhibition test RFFIT showed puppies from non-vaccinated bitches responded well to the vaccination with a progressive increase in VNA, whole in contrast, puppies from vaccinated bitches responded well to rabies vaccination late. Though, detectable maternal rabies VNA and rabies anti-ribonucleoprotein (RNP) antibodies decreased by 6 weeks postpartum. This indicated the poor response of puppies especially those from vaccinated bitches to LEP rabies vaccine. Other researchers have reported rabies in vaccinated dogs using LEP rabies vaccine in Nigeria, (Okoh, 1982; 1983; 1984; Nottidge, 1994, Ogunkoya *et al*., 2003).

Hattwick *et al*., (1972) suggested that the occurrence of street rabies virus in vaccinated dogs dying from the disease under one year of age could be due to failure of the LEP vaccine to protect against invading street variants. Though, it was not possible for them to determine the antibody titer in the dying vaccinated dogs, but occasional failures occur even when the vaccine can be assumed to be potent (CDC, 1976; Poterfield, 1981). The occurrence of apparent vaccine failure cases and apparent cases of vaccine-induced rabies infection indicate that the LEP Flury vaccine may not be 100% effective. This could further complicate rabies problem in Nigeria, as not all vaccinated dogs may be protected. LEP vaccine appears safer for adult dogs but retains some pathogenicity for young puppies, cats and cattle, (Crick and Brown, 1976). Though, LEP vaccine has been s hown to compare favourably with the more recently developed tissue culture vaccines and suckling mouse brain vaccines (Sikes *et al*.,, 1971), a single dose of the vaccine in dogs has been shown to provide a duration of immunity of more than three years, (Tierkel *et al*., 1953). Despite these, there are still reported cases of rabies in vaccinated dogs with LEP vaccine. A ten year survey/study (1970-1980) showed 584 cases of rabies confirmed by the NVRI (Okoh, 1980), out of which 14 were in vaccinated dogs. In 10 out of 14 reported cases associated with LEP vaccine, the dogs were observed to have developed rabies between 5 months and 2 years post-vaccination. Four cases occurred less than 30 days after first vaccination within a period when immune response is incomplete, so these may not be attributed to failure of vaccine to protect. But the previous 10 cases obviously were not protected by the LEP vaccine, (Okoh *et al.*, 1982).

Animals immunized before exposure are usually resistant to infection if neutralizing antibodies are present in their sera, (Crick and Brown, 1976). However, the presence of antibody in the serum of a person and presumably animal treated after exposure by vaccine with or without antiserum may not be a reliable indication of the protection effect of the therapy (WHO Expert Committee on Rabies, 1973), although it has been assumed that the rapid production of antibody is essential. This is because it is thought that once the virus has entered the central nervous system it is no longer accessible to circulating antibody. This could probably explain the remaining 10 cases.

Many reasons have been attributed to these failures of vaccinated dogs to be protected. Incorrect technique in performing the intramuscular injection, resulting in the inoculum being placed subcutaneously is probably one of the reasons for these failures, and the use of a non-viable vaccine; the causes of which include prolonged exposure of the vaccine to high temperature and reconstitution of the vaccine for too long a period before inoculation. It is recognized that no vaccine for this description will give 100% immunity, (Lawrence, 1956). Also vaccines administered under adverse conditions or improperly handled such as administering vaccine to immunologically depressed or incompetent animals.

Reasons why vaccination may fail to protect have been discussed by other researchers in Nigeria, (Nawathe, 1980; Anosa *et al*.,. 1980; Fagbami *et al*., 1981). They suggested that rabies in vaccinated dogs in the country maybe due to causes such as improper storage of vaccine, reversion to virulence of the vaccine virus and improper administration of vaccine. Cases have been reported in various parts of Nigeria, where they reported possibility of rabies infection to actually have been induced by the vaccine.

Antigenic differences in rabies viruses have been suspected to be also responsible for such failures, (Wiktor *et al*., 1980). Although the glycoprotein antigen of rabies virus is primarily responsible for induction of virus neutralizing antibodies and for protection against rabies virus infection, (Wiktor *et al*., 1973), differences in the nucleocapsid and glycoprotein antigens have been observed simultaneously in the natural variants of rabies virus(Wiktor *et al*., 1980; Wiktor and Koprowski, 1980; Schneider, 1982; Sureau *et al*., 1983; Sureau and Rollin, 1982) and such variants could be implicated in the failure of the vaccine treatment of exposed persons or of prophylactically immunized animals due to the lack of cross-reactivity between the strains used for vaccine production and the street virus prevalent in a given region (Wiktor *et al*., 1980; Schneider, 1982; Sureau *et al*., 1983). Antigenic differences among fixed and street rabies virus strains are detectable by the use of monoclonal antibodies (Wiktor and Koprowski, 1978; Schneider, 1982).

Analysis of several strains isolated from humans dying from rabies despite vaccine treatment indicates that some of the vaccine treatment failures could have resulted from antigenic differences between the vaccine strain and the infecting strain of virus, (Wiktor *et* *al*., 1980; Wiktor and Koprowski, 1980; Schneider, 1982; Sureau *et al*., 1983). Work done showing 41 street rabies viruses isolated including three from vaccinated dogs, (Okoh, 1983) and primarily from dogs involved in human exposure in Nigeria were characterized for their nucleocapsid glycoprotein antigenic properties using a panel of 80 monoclonal antibodies. Seven distinct groups of variants were identified, showing diversity in the Nigerian strain of rabies virus.

For rabies to be kept under control, it is recommended by World Health Organization (WHO) that 80-85% of the susceptible host be protected, but successes have not been achieved in this regard. Work done in Tunisia by Haddad (1987) showed very low serological response in dogs vaccinated with both inactivated experimental vaccine and commercial vaccines. Though, the work contradicted the successful immunization of dogs obtained by the National Program against Rabies launched in Tunisia 5 years prior to the research. Equally in Nigeria, the problem of achieving a rabies free environment does not only lie on rabies in vaccinated dogs but vaccinated dogs with LEP have been shown to poorly respond to the vaccine. Work done by Ogunkoya *et al*., (2003) showed only 54% serological response in dogs vaccinated with LEP Flurry strain vaccine. Though, doubt has sometimes been cast upon the protective effect of rabies antibodies in serum of animals and humans suffering from fatal rabies, who often produce high antibody titres. Nevertheless, a large number of laboratory experiments and field observations clearly demonstrate that cats and dogs which develop antibodies after vaccination and before challenge have a very high probability of surviving any challenge, no matter how strong the dose and which virus strain was used. It could therefore be concluded that rabies antibody titration can still be relied upon as a form of protection against the rabies disease, (Aubert, 1992).But the certainty of this needs to be proved.

Thus in attempt, to get 80-85% protected population as recommended by WHO, there is need to investigate into the development of an immunogenic and protective vaccine against the circulating rabies variants in Nigeria.

2.12 Rabies Vaccines

2.12.1 History of rabies vaccines

Over 100 years ago, in 1885, Louis Pasteur developed a crude nerve tissue vaccine for the postexposure treatment of rabies. This form of vaccination used dessicated infected tissue and was found to prevent rabies infection in a 9 year old boy named Joseph Meister. Following Pasteur's initial vaccine, inactivated vaccines were developed. This inactivated form of vaccine was produced by serial dilutions followed by sterilization with chemical agents that inactivated the virus. Further improvements to the inactivated virus vaccine developed through growing virus in various animal tissues and inactivation by UV light or phenol. Semple (derived from sheep or goat brain) vaccines examples of inactivated virus and still used throughout the world today. They are poorly efficacious and poorly tolerated. Side-effects include the demyelination of central or peripheral nervous system in 1 out of 3000, and are occasionally fatal. (http://www.rabies.com).

2.12.1.1 History of DNA vaccination

Many vaccines began as serendipitous discoveries. Louis Pasteur discovered attenuated vaccines when old cholera cultures lost their virulence when chickens were inoculated with aged cultures, they unexpectedly developed immunity to cholera.These were referred to as **First generation vaccines** which are whole – organism vaccines – either live and weakened, or killed forms (Alarcon *et. al*., 1999). Live, attenuated vaccines, such as smallpox and polio vaccines, are able to induce killer T-cell (Tc or CTL) responses, helper T-cell (T_H) responses and antibody immunity. However, there is a small risk that attenuated forms of a pathogen can revert to a dangerous form, and may still be able to cause disease in immunocompromised people. While killed vaccines do not have this risk, they cannot generate specific killer T-cell responses, and may not work at all for some disease, (Alarcon *et. al*. 1999). In order to minimize these risks, so-called **second generation vaccines** were developed. These are subunit vaccines, consisting of defined protein antigens or recombinant protein components. These, too, are able to generate T_H and antibody responses, but not killer T-cell responses.

DNA vaccines are **third generation vaccines**, and are made up of a small, circular piece of bacterial DNA (called a plasmid) that has been genetically engineered to produce one or two specific proteins (antigens) from a micro-organism. The vaccine DNA is injected into the cells of the body, where the "inner machinery" of the host cells "reads" the DNA and converts it into pathogenic proteins. Because these proteins are recognized as foreign, they are processed by the host cells and displayed on their surface, to alert the immune system, which then triggers a range of immune responses (Alarcon *et. al*., 1999;). This DNA vaccination was discovered by chance when a group of researchers in the United States observed that mouse skeletal muscle can take up naked DNA and express proteins encoded by the naked DNA was actually used as a control in their experiements whose objective was to identify lipids that enhance DNA delivery into skeletal muscle. When DNA encoding an influenza virus protein was injected into the skeletal muscles of mice, synthesis of the virus protein in the mouse muscle triggered an immune response resulting in protection of the mice from a subsequence influenza infection. These results, published in science in the year 1993, marked the beginning of **DNA vaccines** also known as **nucleic acid vaccines, polynucleotide vaccines** or **genetic vaccines,** (Rangarajan, 2002).

These DNA vaccines developed from "failed" gene therapy experiments. The first demonstration of a plasmid – induced immune response which was observed when mice inoculated with a plasmid expressing human growth hormone elicited antibodies instead of altering growth, (Tang *et. al*., 1992).

DNA vaccines which were first described in 1992 (Tang *et al*., 1992), have been shown to induce immune response to a variety of viral, bacterial and parasitic antigens. In addition, they have shown efficacy in treatment of allergic diseases, autoimmunity and tumor models, (Kowalczyk and Ertl, 1999).

DNA vaccines utilize naked DNA strands alone for immunization, without traditional proteins or carrier viruses. The genes encoding immugeneic proteins are inserted into a circle of bacterial DNA known as a plasmid. DNA vaccine uses just enough genes from the virus to activate the immune system. The plasmids are shot into muscle tissue using a gene gun, where they are taken up and expressed by cells. Surprisingly enough this method of immunization has been shown to work surprisingly well when it comes to eliciting an immune response against pathogens, yet it is still not clear exactly how the DNA vaccines stimulate an immune response. It must be also kept in mind that the response is till not as strong as that seen with traditional vaccines. Recently announced was the first DNA vaccine that was shown to prevent rabies in monkeys. All eight of the monkeys treated with the DNA vaccine before being given a lethal dose of the rabies virus survived. DNA vaccine is very inexpensive to make and it's very stable, so it does not require refrigeration. This makes it useful candidate in developing countries. But the new vaccine can only be used prior to exposure to rabies, and is not a replacement for post-exposure treatment with IgG and HDCV. Researchers said there are no immediate plans to conduct human clinical trials, due to questions concerning whether additional genes must be inserted to offer protection to humans, and whether it would be effective if it were administered after a person contracted rabies.

2.12.2 Method of Delivery of DNA vaccine.

DNA vaccines have been introduced into animal tissues by a number of different methods.

The two most popular approaches are injection of DNA in saline/buffer using a standard hypodermic needle, and gene gun delivery – injection in saline is normally conducted, intramuscularly (IM) (Xiang *et. al*. 1994; Osorio *et. al*., 1999, Perrin *et. al*., 2000) in skeletal muscle, or intradermally (ID) (Forg *et. al*., 1998; Lodmell *et. al*., 2006) with DNA being delivered to the extracellular spaces. Immune responses to this method of delivery can be affected by many factors, including needle type, (Sedegah *et. al*., 1994) needle alignment, speed of injection, volume of injection, muscle type, and age, sex and physiological condition of the animal being injected, (Alarcon *et. al*., 1999).

Gene gun delivery, the other commonly used method of delivery, ballistically accelerate plasmid DNA (pDNA) that has been adsorbed into gold or tungsten microparticles into the target cells, using compressed helium as an accelerant, (Alarcon *et. al*., 1999; Lewis and Babiuk, 1999). Alternative delivery methods have included aerosol instillation of naked DNA on mucosal surfaces such as the nasal (Cruz *et. al*., 2006) and lung mucosa (Lewis and Babiuk, 1999), and topical administration of pDNA to the eye (Daheshia, 1997) and vaginal mucosa (Lewis and Babiuk, 1999). Mucosal surface delivery has also been achieved using catronic lipsome – DNA preparations (Robinson and Pertmer, 2000) biodegradable microspheres (Alarcon *et. al*., 1999; Chen *et. al*., 1998) attenuated shigella or listeria vectors for oral (Fekadu *et. al*., 1996) administration to the intestinal mucosa (Sizemore *et. al*., 1995) and recombinant adenovirus vectors (Lewis and Babiuk, 1999).

The method of delivery determines the dose of DNA required to raise an effective immune response – Saline injections require variable amounts of DNA, from 100µg – 1mg, whereas gene gun deliveries require 100 to 1000 times less DNA than intramuscular saline injection to raise an effective immune response, (Fynan *et. al*., 1993). Generally, 0.2µg - 20µg are required, although quantities as low as 16ng have been reported, (Alarcon *et. al*., 1999). These quantities vary from species to species, with mice, for example, requiring approximately 10 times less DNA than primates, (Robinson and Pertmer, 2000). Saline injections require more DNA because the DNA is delivered to the extracellular spaces of the target tissue (normally muscle), where it has to overcome physical barriers (such as the basal lamina and large amounts of connective tissue, etc), before it is taken up by the cells, while gene gun deliveries bombard DNA directly into the cells, resulting in less "wastage", (Alarcon *et. al*., 1999; Robison and Pertmer, 2000)

2.12.3 Vectors used for DNA vaccine

DNA vaccines are bacterial vectors that carry a pathogen's gene under the control of a strong constitutively active promoter such as the one derived from cytomegalovirus (CMV), (Kowalczyk and Ertl, 1999). Inoculation of DNA vaccines into muscle or skin by a syringe or a propulsion device such as a gene gun results in uptake of the DNA into cells, followed by transcription and translation of the pathogen's gene and, consequently, an immune response composed of antibodies, T helper cells and cytolytic T lympohocytes (CTLs) (Tang *et al.*, 1992; Ulmer *et al.*, 1993; Xiang *et al.*, 1994).

Vectors employed for the construction of DNA vaccines are bacterial plasmids which are otherwise commonly used for a vitro expression of proteins in mammalian systems. Most of these vectors carry viral promoters that cause constitutive expression of antigen in a large variety of cell types. These include the human cytomegalovirus immediate/early promoter (CMV/IE), the Rous sarcoma virus (RSV) long terminal repeat (LTR) and the Simian virus (SV) 40 early promoter. The SV40 promoter is ~ 40 times weaker than the CMV promoter and only works in some systems, such as the one based on the rabies virus glycoprotein (Xiang et al., 1995), which causes cell death upon overexpression by a more potent promoter. It is also possible to induce a good immune response using the major histocompatibility (MHC) class I promoter (Xiang *et al.*, 1997) that drives expression in most cells.The MHC class II promoter, which causes antigen expression only in specialized cells, mainly of the immune system such as macrophages, dendritic cells and B cells, was also found to drive expression of vector-encoded antigens to levels sufficient for induction of an immune response. Nevertheless, vectors based on this promoter give significantly lower immune responses when compared with vectors carrying a viral or MHC class I promoter, (Xiang *et al*., 1997). Most DNA vaccine vectors contain an intron, which is an element that can increase expression of genes. This 'cassette' is followed by the gene encoding the antigen of interest flanked by the SV40 or bovine growth hormone 3'-untranslated region (BGH 3'-UTR) transcript termination/polyadenylation sequences. This part of the vector is often referred to as the transcriptional unit responsible for antigen synthesis. The other part is the plasmid backbone that contains an origin of replication (ori) enabling high-yield production in *Escherichia coli* along with an antibiotic resistance gene, such as ampicillin (not approved by federal agencies for use in humans) or kanamycin (a resistance market suitable for human vaccines), to confer antibiotic-selected growth in bacteria. This part of the plasmid backbone contains unmethylated CpG sequences that possess important immunomodulatory properties and provides an intrinsic adjuvant effect for DNA vaccines, (Krieg *et al*., 1995). It is thought that the magnitude of the immune response to DNA vaccines directly correlates with the level of antigen expression, measured in vitro upon transient transfection of cells.

2.12.4 Induction of immunity to DNA vaccines

The immune system requires three signals for activation of antigen-specific immune responses. Signal 1 is the antigen; in the case of DNA vaccines, this antigen is encoded by the transcriptional unit of the expression vector. Signal 2 is a co-stimulatory signal provided primarily by determinants of the B7 family, that is B7.1 or B7.2 molecules, which are expressed on mature, activated antigen-presenting cells (APCs) (Schwartz, 1992). Certain cytokines such as interleukin (IL)-2 can also provide signal 2 to B cells.

Signal 0, also called the danger signal (Matzinger, 1994) is a molecularly yet to be identified factor or a multitude of redundant factors that cause(s) activation of professional antigen-presenting cells, mainly dendritic cells, that in their immature (resting) stage are highly efficient at digesting and processing antigen but are fairly inefficient at stimulating T cells. The danger signal supposedly activates resting dendritic cells, which then upregulate express of MHC determinants and constimulatory molecules. Upon activation dendritic cells also start synthesis of cytokines and chemokines and gain the ability to migrate to lymphatic tissue, where stimulation of T and B cells take place.Antigen taken up by antigen-presenting cells without a signal 0, such as proteins released from apoptotic cells, is thought to remain immunologically silent or to induce tolerance.

In most DNA vaccinations, the plasmid is inoculated into either muscle or skin; immune responses have also been reported after intravenous or intranasal administration. Following i.m. injection, myocytes appear to be the predominant cell type transfected by the DNA, as was shown using vectors expressing reporter proteins, (Wolff *et al.*, 1990). Following skin inoculations, antigen expression occurs mostly in keratinocytes, (Raz *et al.*, 1994; Eisenbraun *et al.*, 1993). Although a potential role of keratinocytes in antigen presentation remains to be evaluated, all available evidence suggests that these nonmigratory cells, that is myocytes or keratinocytes, have little if any contribution to the stimulation of the naïve immune system by DNA vaccines.

2.12.4.1 Induction of humoral immunity by DNA vaccines

Inoculation of plasmid DNA has been reported to be an effective way of generating humoral immune responses for a variety of viral, bacterial and eukaryotic antigens. The first report of induction of an antibody response utilized particle bombardment of gold beads coated with DNA encoding the human growth hormone and the human α-1 antitrypsin, (Tang *et al*., 1992). This initial report using a so-called gene gun which propels DNA directly into cells was rapidly confirmed using simple intramuscular (i.m) or intradermal (i.d) injection for a diverse array of antigens.

In some cases, the DNA vaccine induced antibodies that neutralized the infectious agents and thus provided protection to challenge. Such antibodies have been detected in animals vaccinated with DNA encoding rabies virus glycoprotein and influenza hemagglutinin (HA) (Donnelly *et al*., 1995; Deck *et al*., 1997; Ulmer *et al*., 1994; Justewicz *et al*., 1995). In one of the studies the antibody response to the rabies virus nucleoprotein was tested. This antigen, if presented to the immune system as a recombinant protein or within the context of the rabies virus core, induces a potent B cell response that provides protection to peripheral viral challenge, (Condo *et al*., 1996; Fu *et al.*, 1997). Nevertheless, upon immunization of mice with a number of different vectors expressing the rabies virus nucleoprotein under the control of the CMV or SV40 promoter, only a marginal B cell response could be elicited after several booster immunizations, and no response was detected after a single immunization. B cells respond to extracellular antigen; they are oblivious to protein hidden behind cell membranes. DNA vaccines, as opposed to many viruses including rabies virus, are noncytopathic and thus fail to release antigen by causing cell death; they might therefore be unable to efficiently induce humoral responses to antigens that are maintained within the nucleus or the cytoplasma of transfected cells, this might be circumvented by reconstructing such antigens, for example adding a signal sequence that allows their secretion or display on cell membranes.

Neutralizing antibodies, the main correlate of protection for many infectious diseases, are generally directed against secreted or cell surface-expressed antigens. Nevertheless, such antigens often show substantial variability between different strains or even isolates of a pathogen due to selective pressure from the immune system. Nucleoproteins are more conserved and thus provide useful additions to broaden the spectrum of vaccine efficacy.

Although specific antibodies may be detected as early as 1 week after plamid injection (David *et al.*, 1993; David and Brazalot-Millan, 1997), generally genetic vaccines result in slowly rising antibody titers which reach peak levels 8-12 weeks after immunization. These titers then persist at high levels for several months. The unusual kinetics of the B cell response to DNA vaccination is presumably a reflection of the comparatively long-lasting persistence of vector DNA and of vector-encoded proteins in transfected cells. Once a B cell response has been initiated, the longevity of the vector-encoded antigen favors its deposition in the form of immunocomplexes on follicular dendritic cells. Such complexes are released when antibody titers decline and cause further activation of memory B cells.

The precocity and strength of the humoral response depends on the dose of vector DNA. Antibody levels may be further increased by a second DNA injection. Antibody titers can also be increased by using a traditional vaccine such as peptides or viral recombinants for booster immunization, (Okuda *et al.*, 1997; Schneider *et al.*, 1998; Sedegah *et al.*, 1998). Although DNA vaccine induce antibody titers that in many systems suffice to provide protection against challenge with a pathogen, as a rule, titers achieved by DNA vaccines are more persistent but otherwise well below those that can be elicited by a traditional subunit vaccine such as a recombinant virus. Notwithstanding, DNA vaccines are remarkably well suited to prime the antibody response, which upon subsequent booster immunization with a fairly low dose of a recombinant vaccine reaches titers exceeding those achieved by the vaccine given at optimal doses alone once or even repeatedly.

The isotypes of serum antibodies induced by DNA vaccination are generally IgG (predominantly IgG2a in mice), but serum IgM and IgA have also been detected, (Deck *et al*., 1997; Justewicz *et al*., 1995). Upon intramuscular (i.m) DNA vaccination, antibodies of the IgG2a isotype predominate, indicating stimulation of a Th 1 response

Antibody responses elicited by DNA vaccinations are influenced by a number of variables, including type of antigen encoded, location of expressed antigen (i.e. intracellular vs secreted); number, frequency and dose of immunizations; site and method of antigen delivery, etc. Recent kinetics of antibody response shows that humoral responses after a single DNA injection can be much longer-lived than after a single injection with a recombinant protein. DNA – raised antibody responses rise much more slowly than when natural infection or recombinant protein immunization occurs. It can take as long as 12 weeks to reach peak litres in mice, although boosting can increase the rate of antibody production. This slow response is probably due to the low levels of antigen expressed over several weeks, which supports both primary and secondary phases of antibody response, but DNA immunization is known to induce a qualitative superior response.

DNA vaccination generates an effective immune memory via the display of antigen-antibody complexes on follicular dendritic cells (FDC), which are potent B-cell stimulators. T-cells can be stimulated by similar, germinal centre dendritic cells. FDC are able to generate an immune memory because antibodies production “overlaps” long-term expression of antigen, allowing antigen-antibody immunocomplexes to form and be displayed by FDC, (Robinson and Pertmer, 2000).

2.12.4.2 T cell-mediated immune responses

2.12.4.2.1 Helper T-Cell Responses

DNA immunization is able to raise a range of TH responses, including lymphoproliferation and the generation of a variety of cytokine profiles. A major advantage of DNA vaccines is the ease with which they can be manipulated to bias the type of T-cell help towards a TH 1 or TH 2 response, (Feltquate, 1997). Each type of response has distinctive patterns of lymphokine and chemokine expression, specific types of immunoglobulins expressed, patterns of lymphocyte trafficking and types of innate immune responses generated.

2.12.4.2.2 Mechanistic basis for different types of T-cell Help

It is not understood how these different methods of DNA immunization, or the forms of antigen expressed, raise different profiles of T-cell help. It was thought that the relatively large amounts of DNA used in IM injection were responsible for the induction of TH1 responses. However, evidence has shown no differences in TH type due to dose, (Feltquate, 1997). It has been postulated that the type of T-cell help raised is determined by the differentiated state of antigen presenting cells. Dendritic cells can differentiate to secrete 1L – 12 (which supports TH1 cell development) or 1L – 4 (which supports TH2 responses) (Banchereau and Steinman, 1998). The type of T-cell help raised is influenced by the method of delivery and the type of immunogen expressed, as well as targeting of different lymphoid compartments, (Boyle *et. al*., 1996; Alarcon *et. al*., 1999). Generally, saline needle injections (IM or ID) tend to induce TH1 responses i.e DNA injected by needle is endocytosed into the dendritic cell, which is then stimulated to differentiate for TH1 cytokine production, (Jakob *et. al*., 1998) while the gene gun bombards the DNA directly into the cell, thus bypassing TH1 stimulation and raises TH 2 responses (Feltquate 1997; Boyle *et. al*., 1996). This is true for intracellular and plasma membrane – bound antigens, but not for secreted antigens, which seem to generate TH 2 responses, regardless of the method of delivery, (Sallberg *et. al*., 1997). Generally, the type of T-cell help raised is stable over time, and does not change when challenged or after subsequent immunizations, (Feltquat, 1997; Boyle *et. al*., 1996).

2.12.4.2.3 Cytotoxic T-cell Responses

One of the greatest advantages of DNA vaccines is that they are able to induce cytotoxic T lymphocytes (CTL) without the inherent risk associated with live vaccines – CTL responses can be raised against immunodominant and immunorecessive CTL epitopes, (Fu, *et.al*., 1997) as well as subdominant CTL epitopes (Chen *et. al*., 1998) in a manner which appears to mimic a natural infection. This may prove to be a useful tool in assessing CTL epitopes of an antigen, and their role in providing immunity.

Induction of cytolytic T lymphocytes (CTL) activity upon DNA vaccination was initially demonstrated after in vitro restimulation of lymph node lymphocytes or splenocytes isolated from vaccinated mice. This assay system primarily detects memory T cells. Activation of effector CTLs was subsequently confirmed by testing freshly isolated splenocytes for lysis of antigen-expressing target cells. CTLs were detectable upon DNA vaccination of mice and primates to a variety of antigens, including the rabies glycoprotein, (Xiang *et al*., 1994).

In some experimental systems, specific CTL activity could be detected as early as 3-6 days after DNA injection; their activity reached its maximal level by 12 days and was maintained for several months (David *et al*., 1995) or even for the lifetime of the animals. Booster immunization with a second dose of DNA or a recombinant vaccine expressing the same antigen enhanced the CTL response.

2.12.4.2.4 Interferon

Both helper and cytotoxic T-cells can control viral infections by secreting interferons. Cytotoxic T-cells usually kill virally infected cells. However, they can also be stimulated to secrete antiviral cytokines such as INF-y and TNF - <, which don't kill the cell but place severe limitations on viral infection by down-regulating the expression of viral components, (Franco, 1997). DNA vaccinations can thus be used to curb viral infections by non-destructive IFN – mediated control

2.12.5 DNA vaccines in comparison with traditional vaccines

As outlined above, DNA vaccines induce, upon de novo synthesis of antigen in transfected cells, a full spectrum of immune responses, including antibodies, T helper cells and cytolytic T cells. This is also achieved by live attenuated vaccines or viral recombinant vaccines. DNA vaccines lack the inherent risks of attenuated vaccines and, unlike recombinant vaccines, immune responses to the vaccine carrier are not an issue upon vaccination with plasmid DNA. DNA vaccines, unlike viral recombinants, can be used repeated for different immunogens. Inactivated vaccines and protein vaccines have minor side effects and induce antibodies and T helper cells, two immune mechanism that fully suffice for protection to many pathogens. Nevertheless, most inactivated vaccines or protein vaccines require addition of adjuvants which induce a local inflammatory reaction, which commonly correlates with the efficacy of the adjuvant, limits the use of many adjuvants in humans. DNA vaccines provide their own adjuvant through unmethylated CpG sequences present in the bacterial part of the vector. Inactivated vaccines and proteins commonly induce Th2 responses, which in the case of some infections such as those with respiratory syncitial virus might actually exacerbate disease upon infection. DNA vaccines, at least upon intramuscular immunization, induce Th1 responses. Peptide vaccines induce monospecific B or T cell responses.

Many of the traditional vaccines fail to induce immune responses in neonates, whereas DNA vaccines readily stimulate T and B cell responses in newborn animals, (Wang *et al.*, 1997; Bot *et al.*, 1996; 1997), While the immune response to most traditional vaccines is impaired in the presence of maternally transferred antibodies, (Xiang and Ertl, 1992; Harte *et al.*, 1982; Albrecht *et al.*, 1997). DNA vaccines given to neonates are not or only marginally affected by the dam's immune status, (Wang *et al.*, 1998; Manickan *et al.*, 1997; Hassett *et al.*, 1997).

For reasons not fully understood, DNA vaccines are exceedingly potent in priming the immune response. Experimental animals inoculated with a DNA vaccine develop very high immune responses upon booster immunization with a low dose of a traditional vaccine expressing the same antigen. This might be linked to the adjuvant effect of CpG sequences that by creating a unique cytokine milieu might favor activation of memory T helper cells.

2.12.6 Advantages and disadvantages of DNA vaccines

2.12.6.1 Advantages of DNA vaccines

DNA vaccines have a number of advantages over conventional vaccines including the ability to induce a wider range of immune response types, they stimulate a full spectrum, of immune responses as a natural infection including cytotoxic T lymphocytes (CTLs) generally not induced by protein vaccines and they generate exceptionally long-lasting immune responses, (David *et al*., 1993; Bahloul *et al.,* 2006).

DNA vaccines have the ability to induce both humoral and cell mediated immune response, they inherently favor the induction of cytotoxic cellular immunity since Ag can be presented through endogenous processing pathways in the context of class 1 MHC and have the ability to directly infect antigen presenting cells (APCs).

They have been used to generate monoclonal antibodies to help to identify the antigen of a pathogen that induces protective immunity, (Vanderzanden *et al*., 1998), the typical objective of genetic immunization is the isolation of polyclonal antibody containing sera or antibody – producing B cells which can be used to generate monoclonal antibody (MAb) – producing cell lines (either by immortalization or by fusion with myeloma cells to form hybridomas). The same vector can be used for both genetic immunization and antigen production.

One major advantage of DNA vaccines is the ease with which they can be generated, modified and purified. Generating a recombinant vaccine takes several months, whereas production of an attenuated pathogen can take years. Production of a recombinant protein, followed by purification, takes weeks. Generation of a DNA vaccine takes between 2-4 days; amplification and purification take less than 24 h, (Kowalczyk and Ertl, 1999).

DNA vaccines are inexpensive to mass-produce an important consideration for the production of vaccines for use in developing countries or in veterinary medicine. They are economical i.e. cost effectiveness, and this possibly minimizes the expense of vaccinating hundreds – of – thousands of dogs. They are equally easy to purify using simple and inexpensive procedures. All DNA vaccines can be produced using similar fermentation, purification, and validation techniques. This ability to use generic production and verification techniques simplifies vaccine development and production, (Weiner and Kennedy, 1999; Gurunathan and Seder, 2000).

They are extremely stable i.e thermostable; the DNA can be boiled, precipitated in ethanol and shipped across the globe at room temperature i.e they are stable at room/high temperature; they do not require cold chains, which are very difficult to maintain in less-developed countries. They can be stored dry or in an aqueous solution at room temperature. Thus, there is stability of vaccine for storage and shipping and can be used in the field without cold-chain protection as required for protein-based vaccines.

In addition, DNA vaccines are non-infectious. Thus far DNA vaccines have not shown any severe adverse reactions. There is little evidence for integration of DNA into the host cell genome, (Nichols *et al.*, 1995). Autoimmune reaction such as stimulation of persistent antibodies to DNA has not been observed. One study reported induction of antibodies to DNA upon DNA vaccination (Mor *et al.*, 1997); however, the titers were very low and transient. In our system even multiple immunizations with high does of DNA failed to elicit detectable antibodies to double or single-stranded DNA, (Xiang *et al*., 1995). In some systems, DNA vaccines seem to overcome genetic unresponsiveness, (Gerdts *et al*., 1997; Doolan *et al*., 1996). It is known that maternal neutralizing antibody interferes with active immunization with protein-based rabies vaccine (Xiang and Ertl, 1992), but it has been shown in neonatal mice (Wang *et. al*., 1998) that rabies DNA vaccine might also effectively immunize puppies possessing maternal anti-rabies neutralizing antibody.Thus, DNA vaccines were shown to induce immunity in neonatal animals, (Wang *et al*., 1997). Passive transfer of antibodies inhibits the immune response to DNA vaccines in adult mice, (Wang *et al*., 1998). But in neonatal mice, maternally transmitted antibodies or hyperimmune serum given iatrogenically failed to reduce the B cell response to DNA vaccines, (Wang *et al*., 1998). This suggests their potential usefulness for early childhood vaccination, which is currently unfeasible with traditional vaccines to many common infections due to the maternal antibody-mediated inhibition of the infant's own immune response to active immunization.

Since DNA vaccine plasmids can be constructed using simple recombinant DNA techniques and therefore is possible to co-inoculate multiple plasmids encoding different antigens of the same pathogen or different pathogens. They have the ability to maintain long-term persistence of immunogen, (Alarcon *et. al*. 1999). In vivo expression of DNA vaccines ensures protein more closely resembling normal eukaryotic structure, with accompanying post-translational modifications, (Alarcon *et. al*., 1999). The host cell provides the post-translational modifications to the antigen, allowing its production with the correct native conformation to live infection. Since pathogenic protein is synthesized in its native form inside the host cell, the chances of vaccine failure are minimal.

Clinical studies have shown DNA vaccines to be well tolerated; they provide their own adjuvant in the form of unmethylated bacterial CpG sequences (Klinman *et al*., 1996; Krieg *et al*., 1995; Yamamoto *et al*., 1992) that induce an innate immune response which in turn sponsors activation of an antigen-specific immune response. The main advantage of using DNA vaccines over traditional live ones is that there is no contamination with viruses that could be disseminated in the environemtn and reproduced in susceptible animals, (Cruz *et. al*., 2006)

2.12.6.2 Disadvantages of DNA vaccines

DNA vaccines have disadvantages. They result in rather slow rising antibody responses, which disallow their use for postexposure vaccination to certain infections such as with rabies virus. DNA vaccines persist for weeks to months at the site of inoculation; antigen is produced for several weeks. Although this is advantageous for enhancing the immune response in the case of an adverse reaction, the continued production of antigen that can only be terminated by surgical intervention will be problematic. Also since expression of the plasmid – encoded antigen appears to persist for long periods of time, there is a concern that unresponsiveness, rather than protective immunity, might result (Toda *et al*., 1997; Chen *et al*.)

They have the potential for atypical processing of bacterial and parasite proteins, and the development of secondary anti-DNA autoantibodies occurs, but not always and is usually transient. Antibodies to DNA can cause disease and are associated with systemic lupus erythematosus (Toda *et al*., 1997; Chen *et al*., 1998; Gregoriadis *et al*., 1997; Ishii *et al*., 1997; Sasaki *et al*., 1997).

The two most serious possible side effects of DNA vaccination are transformation due to integration of DNA into the host cell genome or the development of autoimmunity due to induction of T cells to cryptic epitopes. The major concern about DNA vaccines is whether the plasmid DNA integrates into the genome randomly, potentially leading to insertional mutagenesis. But neither of these has been observed. Nevertheless, as with every bioactive substance, these adverse events might be sufficiently rare to only become apparent after mass administration. One side effect that has been observed is the development of a mild inflammatory reaction at the site of an intramuscular (i.m) inoculation. This is common to all vaccines and thus poses only a minor concern for humans. For meat-producing livestock such as cattle, scarring muscle tissue is unwelcome, and an alternative route of DNA vaccination is required (Toda *et al*., 1997; Chen *et al*., 1998; Gregoriadis *et al*., 1997; Ishii *et al*., 1997; Sasaki *et al*., 1997).

One technical disadvantage of DNA vaccines is that their mode of administration currently involves an invasive procedure that is either an injection by syringe or the use of a gene gun, which is cumbersome for mass vaccination. Although some investigators reported induction of immunity upon intranasal application of DNA, the response was low. The development of optimized delivery vehicles such as microencapsulation or emulsion in cationic lipids will most likely eventually allow administration of DNA per os or by the intranasal route, (Toda *et al*., 1997; Chen *et al*., 1998; Gregoriadis *et al*., 1997; Ishii *et al*., 1997; Sasaki *et al*., 1997).

DNA vaccines are limited to protein antigens (hence excluding carbohydrate antigens: polyssacharide based subunit vaccine and adjuvants (unless added to DNA mixture), (Rangarajan, 2002).

Although induction of protective immune responses following DNA vaccination has been demonstrated for a variety of diseases, the mechanism of DNA vaccine action is still not completely understood, (Weiner and Kennedy, 1999; Gurunathan *et. al*., 2000). DNA vaccines deliver antigens to dendritic cells (DCs) and other professional antigen – presenting cells (APC) for T cell activation, (Akbari *et. al*., 1999; Condon *et. al*., 1996; Kirman and Seder, 2003; Porgador *et. al*., 1998). This mode of antigen delivery is not very efficient and induces only sub-optimal levels of immune response. This limitation appears primarily due to the low frequency of APC such as DCs that could be transfected by naked DNA and also their inability to engage innate immune effector mechanisms that are critical for the generation of adaptive immunity, (Gurunathan *et. al*., 2000; Kirman and Seder, 2003; Leitner *et. al*., 2005; Restifo *et. al*., 2000; Rodriguez and Whitton, 2000).

CHAPTER THREE

MATERIALS AND METHODS

3.1 Materials

3.1.1 Equipments

Specimen storage containers, (polypropylene jars/sample bottles), Microscope slides with cover slips (Teflon/pre-ringed well slides), Acetone fixation and post-stain rinse containers, Syringe filters (0.45µm), Incubator (37^{o}C), Humidified staining tray/chamber and Necropsy instruments.

Pipettes (200µl and 1000 µl,), Disposable pipette tips (Ranin pipette tips for pipetman 200µl and 1000µl), conical (15 and 50 ml), sterile polystyrene, nonpyrogenic, Sterile disposable surgical blades, Screw cap unbreakable test tubes (1.25mls), Microtest tubes (0.6 and 1.25ml), Sterile disposable cans, Biohazard discard and container, Bucket of ice, Closed refrigerated centrifuge.

Micropipette (pipetman 10µl, 20µl, 50µl and 100µl), Micropipette tips (10µl, 20µl, 50µl and 100µl), Pipette bulb, Micro tubes, sterile thin-wall thermocycler microfuge tubes (600µl), Centrifuge tubes (10ml, 15ml and 50ml), Dispo, 2ml Latex, Wash tube (2 ml), Sample collection tube (1.5 ml), Serum separator Tube Microtainer® brand, Natelson Blood collecting Tubes (Heparinized) Fisherbrand®, and Plastic container.

Thermalcycler (GeneAmp® PCR system 9700 and PTC – 100® Peltier), Centrifuge eppendorf® (centrifuge 5415c), PC system (computer) connected to sequencer, Sequencers (ABI PRISM 377 DNA sequencer and Applied Biosystems 3730/3730x/DNA Analyzer).

Microwave Oven, Conical beaker (1000 ml), Plastic beaker (500 ml), Glass cylinder (100ml), Mini Submarine Electrophoresis unit, Electrophoresis power supply controls (Hoefer EPS 301), Electronic measuring scale (Sartorius LP 6200s), Paraffin paper, Weighing balance, weighing paper. Fisher scientific Co. 09-898-12A 3x3”, Stainless steel spatula, Polaroid 667 black and white instant pack film, Fotodyne Polaroid camera, Face shield (uv Absorber) and Illuminator Fotodyne®,

CENTRI-SEP Columns containing: special fritted microfuge tube drygel, Microtube rack, Vortex Mixer, Vacuum Centrifuge, , Non-lint wipes, Sequencing plates, Combs, Clamps, Spacers, Serological pipets bulb, Stirrer/Hot plate steel plate, Rod – like magnets, Filtering jar, Sequence loading pipet and tips

Light Microscope with 20x and 40x objectives, Tissue-Tek slide staining kit; Fisher Cat.No.NC9479355 (T-Tek#25608902), Slide holder, 24-place; Fisher Cat. No. NC9418050 (T-Tek#2560886), Fisher Brand syringe 25mm 0.45mm filter, Fisher Scientific Cat.No.07-719D, Wheaton glass vials 8 ml, Fisher Scientific Cat.No.06-408C, BD 10 cc syringe 209604, Fisher Scientific Cat.No.14-823-2A, Pipeltor 200 µl Rainin:htt://www.rainin.com/p200, Corning cover glass 24 x 60, VWR Scientific Cat.No.48396-160, Microscope slides white frosted, Erie Scientific Co. Order No.10-226, polypropylene Falcon 352097 Fisher Scientific Cat.No.07-200-300, Tips – universal (1-200 µl vol.). Corning-Costar, Fisher Scientific Cat.No.07-200-300, VWR Pipet Bulb (0.05-100 ml), VWR Scientific Cat.No.53497-055, Serological pipette (1.0 ml), pyrex Corning 7078D-1, VWR Scientific Cat.No.53222-259, Serological pipette (5.0 ml), pyrex Corning 7078D-5, VWR Scientific Cat.No.53222-281, Serological pipette (10.0 ml), plastic, VWR Scientific Cat.No.20171-042, Serological pipette (25.0ml), Tissue culture plate lids (50/case). BD Falcon, Fisher Scientific, Cat.No.08-772-2B, Clock-Timer (Fisher Scientific; VWR Scientific), Timer (West Bend®), Drummond pipet-Aid and Serological pipette holder.

Scissors, forceps, Swabs sticks, Aluminium foil paper, Closed refrigerated centrifuge, Tuberculin syringe (0.5 cc) fitted with a 27- guage needle, ¼ - to 3/8 inch (1 – 1.5 cm) long, contained in a test tube with a gauze or cotton plug in the bottom, Insulin ½ cc syringes with 31G needles, Paper towels (polyethelene – backed), Mice cages with drinking water bottles, Sample boxes, Flask of ice, Flask/cooler, Surgical gloves (latex and nitrile), Laboratory coats/caps/shoe covers, disposable gowns, Labels, Test-tube rack, Discard pans, Animal forceps, Nose mask and Face shield/Safety goggles.

Biocontainment safety hood, C25 Incubator shake classic series, Q1A prep spin column, Q1AGEN – tip 500, Polyscience® water bath (37, 42 and 56^{o}C), Pressure vacuum, Centrifuge : Beckman Coulter, Avanti^{TM} Centrifuge J-301, sys. ID# 414546. Filter : Nalgene 90mm Filter top filter – 500, 1000ml; Nalgene 50mm Filter unit – 150 or 250 ml, UV Spectrophotometer, Autoclave and/or instrument sterilizer (Tuttnauer autoclave – steam sterilizer) tuttnauer – BRINKMANN 2540E.

Lab – Tek chamber slides with coverslip, sterile glass 8-well slide sterile plasticware or glassware, Cell culture flask (25, 75 and 150cm^2), Flasks T150 and T75, treated, non-pyrogenic polystyrene, sterile. Cryovials, 1 – 2 ml, Beaker – Tri-cornered polypropylene beakers, Coplin staining jar, Nalgene disposable filter, pore size 0.22 µm (500 and 125 ml), Hemacytometer (Neubauer).

Biological safety cabinet (BSC): NSF classification = class 11, Type A.

CO_2 water-jacketed incubator, Liquid nitrogen, Revco Refrigerated storage (4^oC refrigerator, - 20^oC and -80^oC freezers), Revco chest freezer (-80^oC), Ultima 11, Explosion proof, Polyscience, Inverted microscope (Zeiss, Axiovert 25 Nikon model TMS-F), Fluorescent microscope (Zeiss, Axioskop with 160x or 200x mag., :Zeiss, Axioskop 2 plus HBO 100, HAL 100, Centrifuge (Beckman Coulter, Allegra 6R bench-top centrifuge, 0 – 8000 rpm), Fisherbrand pipette/dispensers, rubber pipette fillers.

3.1.2 Supplies

Female mice (Hsd: 1CR (CD-1), Mice feed (LabDiet 500, Rodent diet).

Pools of suckling mice, (2- 4 days old). White Swiss mice. Brain samples (including the pons, medulla, midbrain, hippocampus and cerebellum).

3.1.3 Reagents

Acetone, FITC – conjugated anti-rabies antibodies (see Appendix II), Specificity controls (see Appendix III), Conjugate dilutant (see Appendix II), Counterstains (see Appendix II), Rinse/soak buffer (Sigma P3813) (see Appendix II), Mountant (0.05M Tris-buffered saline pH 9.0 with 20% glycerol, Trizma pre-set crystals, Sigma catalog #T6003; ACS grade glycerol, Sigma catalog # G7893).), MNA (mouse neuroblastoma cells) cells (Sigma P3813) and Immersion oil.

TRIZOL (U.S Patent No.5, 346,994; Cat No. 15596 – 026)

Reverse Transcription – Reaction mix (Appendix XV), 50x Tris-acetate – EDTA (TAE):

(10x: 0.4M Tris, 0.2M acetic acid, 10 mM EDTA, pH = 8.4, 1000 ml), Tris base (FW 121.1) 0.40M, Acetic acid (99.5%) 0.20M, EDTA solution (0.5M, pH 8.0, soln.3) 0.01M, Deionized H_20, 1 x Tris – acetate – EDTA (TAE) stock buffer (Dilution was made to 1 x before use to yield 40 mM Tris base, 20 mM Acetic acid, and 1 mM EDTA), Ethidium bromide 10 mg/ml (0.5µg/ml).

Loading buffer (5x, 25% Ficoll 400, 0.25% Bromophenol blue, 10 ml) containing: Deionized H_20, Ficoll 400/Glycerol, Bromphenol blue (FW 691.9). Agarose (certified TM molecular biology Agarose 500g), Molecular weight marker, XIV (1kb DNA Ladder 500µg/ml).(Solution in 10mM Tris – HCl, 1mM EDTA, pH 8.250µg/ml, and Decontaminants (Lysol IC., Quaternary disinfectant cleaner, concentrated (dilute 1 : 256) containing Didecyl dimethyl ammonium chloride 9.22%, n-Alkyl dimethyl benzyl ammonium chloride 6.14% and Inert ingredients 84.64%; total 100% (Fischer Scientific), 10% chlorox bleach).

Primers (00IF. 550F, 1066F, 550B, 1066B and 304), ExoSAP-IT® (containing Exonuclease 1 and shrimp alkaline phosphatase in a specially formulated buffer), Formamide Hi-Di™ (containing CAS 75-12-7, CAS 30-00-4), Sterile distilled deionized water., Big Dye® Terminator, Loading Dye, Formamide to Dextran Blue/EDTA (molecular Biology Grade, DNase RNase Protease free), Isopropanol, Hot water, ETOH and Polyacrylamide Gel Mix.

Luria Bertani (LB) medium, Ampicillin antibiotics (100 µg/ml), *E.coli* JM109 (2355751), S.O.C. medium, 70% ethanol, Buffers: PI (resuspension buffer) containing 50 mM Tris-cl, pH 8.0, 10 mM EDTA, 100 µg/ml RNase A; P2 (lysis buffer) containing 200 mM NaOH, 1% SDS (10/v); P3 (neutralization buffer) containing 3.0M potassium acetate pH 5.5,; FWB2 (QIA filter wash buffer) containing IM Potassium acetate pH 5.0; :QBT (equilibration buffer) containing 750mM NaCl, 50 mM MOPS, pH 7.0, 15% Isopropanol (v/v), 0.15% Triton® x-100 (v/v).; :QC (wash buffer) containing 1.0M NaCl, 50 mM MOPs. pH 7.0, 15% Isopropanol (v/v) ; QF (elution buffer) containing 1.25M NaCl, 50mM Tris-Cl, pH 8.5, 15% Isopropanol (v/v), QN (elution buffer) containing 1.6M NaCl, 50 mM MOPS, pH 7.0, 15% Isopropanol (v/v); TE containing 10 nM Tris-Cl, pH 8.0, 1 nM EDTA; EB containing 10 nM Tris-Cl, pH 8.5; N3 Containing guanidine hydrochloride, acetic acid; PB containing guanidine hydrochloride, Isopropanol; PE (Wash buffer) – add 220mls ethanol to 55ml Buffer PE to get 275ml, Plasmid DNAs = CDAG3 (10µg, 100 µg), WCBVG (10µg, 100 µg).

Isoflurane inhalation anaesthetic agent, Diagnostic conjugates (Fluorescein isothiocyanate; FITC), Conjugated anti-rabies virus antibodies, Centocor FITC-Anti-rabies Monoclonal Globulin; Fujirebio Diagnostics, Inc., Chemicon International, Acetone – Acetone AXO120-6 (500 ml), Gelatin – Bacto – Gelatin, Difco no. 0143 – 15-1 (100g).

Cell culture media: Distilled water, Fetal bovine serum 40nM filtered, Sodium bicarbonate, TN Minimum Essential Medium (MEM) (10x), 500 ml liquid –: containing Earle's salts (see Appendix V, for composition), MEM vitamin solution liquid (100x) 100 ml liquid prepared in 0.85% NaCl; pH 7.0 to 7.4, L-glutamine – 200nM (100x), 100 ml liquid = supplied at 29.2mg/ml in 0.85% NaCl, pH 4.7 to 5.6, Antibiotic – Antimycotic – (100x), 100 ml liquid (containg 10,000 units of penicillin (base), 10,000 µg of streptomycin (base), 25 µg of amphotericin B/ml utilizing penicillin G (sodium salt), Streptomycine sulfate, and amphotericin B as Fungizone®, Antimycotic in 0.85% saline.

Formation, 10% buffered; Fisher Brand, Order no.SF 100-4 (4L), Phosphate buffered saline (PBS); Fisher Scientific Cat.No.SH30256-02, Hydrogen peroxide 3%. Any commercially available 3% hydrogen peroxide, Primary antibody: CDC cocktail (50 ml) of mouse anti-rabies biotinylated monoclonal antibodies. Streptavidin-poeroxidase: 50 ml (71-00-38): Kirkgarrd & Perry Laboratories Inc, Amino-ethylcarbizole (AEC) substrate, Sigma no.A6926, (3-Amino 9-ethylcorbrazole tablets), N1 N1 Dimethyl formamide GR, EM Science, Cherry Hill, NJ, Thomas Scientific Order No.C279-A87, Acetate Buffer, 0.1M, pH 5.2, Polyscientific, Bay Shore, NY, (516) 586-0400. Cat. No.S140, Gills formulation #2, Fisher order No.CS401-45, ML, and Diluted 1:2 in distilled water. (Gill Hematoxylin stain), Gel/Mount, Biomeda Corp., Foster City CA, (aqueous/dry mounting) Fisher Scientific Cat.No.BM-M01, 20ml, TWEEN 80, polyethylene glycol, Sigma-Aldrich order no. P1754, Deionized water, Fisher Scientific, Cat.No.575232.

Sodium bicarbonate solution, 10ml liquid,7.5% (w/v). Trypsin-EDTA (Versense): 0.05% Trypsin, 0.53mM EDTA-4Na (10x), 100 ml liquid, Phosphate buffered saline (PBS): Formula number 4550: (ca $^{2+}$ and mg $^{2+}$ free) PBS, 0.0IM, pH 7.4 and Formula number 4588: PBS, 0.0IM, pH 7.4 – 7.6, (Sigma P3813) (See Appendix VI for composition), Dimethylsulfoxide (DMSO) – 5.0 ml sterile.

3.2 Methods

3.2.1 Collection of Samples and Transportation

3.2.1.1 Sample collection and handling

Fresh brain samples (including the pons, medulla, midbrain, hippocampus and cerebellum) were collected from suspected rabid dogs and cats from two states (Plateau and Kaduna) (though, samples were intended to be collected from different parts of Nigeria, they were unavailable). The samples were collected after the animals had died.

A total of 165 samples (163 dogs and 2 cats) were collected. The samples were stored in sample bottles and polypropylene jars depending on the sample size, and were then stored in the freezer at -20 ^{o}C before shipment. Each sample was stored in a separate bottle/jar and properly labelled with an identifying number on a tape.

3.2.1.2 Shipment of samples

The sample bottles containing the frozen brain specimens were packed in a foam padded box with frozen ice packs and sent to the Centers for Disease Control and Prevention, Edward Roybal Infectious Disease Laboratory – Rabies Section by DHL Courier Service. There was no dry ice available for the transportation, but instructions were given for the samples to be maintained under cold chain.

3.2.1.3 Laboratory handling of samples

The samples were received in the Rabies laboratory of the Edward Roybal Infectious Disease Laboratory, Centers for Disease Control and Prevention, Atlanta Georgia, and stored at -20^{o}C until use.

3.2.2 Techniques for Identification of Rabies Virus Antigen

3.2.2.1 Direct Fluorescent Antibody Testing

Direct fluorescent Antibody Testing commonly referred to as DFA or FAT was used for postmortem diagnosis of rabies in samples taken from brains of suspected dogs and cats. Fluorescence suggestive of rabies was examined at a 400x magnification. (See Appendices XVI and XVII for magnification and resolution). For each area of the brain examined, staining was graded by the amount of antigen present.

3.2.2.2 Conjugate titration

Conjugate (FITC conjugated anti-rabies conjugates) was reconstituted and titrated to determine a working dilution. A determination of a working dilution for a conjugate was made exactly as the diagnostic reagents used for test samples (same diluent, tubes, syringe filters, and counterstain concentration). Serial twofold dilutions (e.g. 1:10, 1:20 and 1:40) were prepared for all prospective conjugates. Two or more slides were stained for each dilution. The titration was read and recorded independently. The consensus of the last dilution providing crisp + 4 staining with minimal background fluorescence was used as the end-point dilution of the reagent (See Appendix III).

A more precise working dilution was obtained by preparing limited dilutions around the end-point dilution (for example, when the end-point of the initial titration was 1:80, the conjugate was retested at a dilution of 1:70, 1:80, 1:90, 1:100, 1:110, 1:120 and so on). The working stock of the conjugate was two steps more concentrated than the first dilution at which a fall-off in staining was observed (e.g. when the amount of antigen stained or the intensity of the stained antigent was diminished at 1:110 dilution, the working dilution of the conjugate was taken to be 1:90).

Because antigen presentation and antibody avidity and affinity vary with different rabies virus samples, and viral inclusions may appear quite different when stained with different reagents, titration results were confirmed by reaction with slides made from a second variant of rabies virus.

3.2.2.3 Specificity controls

The specificity control for an FITC labeled hyperimmune serum rabies reagent is an FITC labeled serum reagent produced in the same animal host as the rabies reagent (typically goat or horse). The control reagent was diluted to the same mg protein concentration as the rabies reagent.

3.2.2.4 Conjugate diluent

The conjugate diluent was a 0.0IM phosphate buffered isotonic saline solution at pH 7.4 to 7.6 (phosphate buffered saline (PBS) sigma P3813, a 0.0IM phosphate buffer, pH 7.4, with 0.138M NaCI and 0.0027M KCl). *No protein stabilizer (bovine serum albumin) was needed in the diluent.

3.2.2.5 Counterstains

Counterstains were added to the working dilution of the conjugate to provide contrast and lower background and to serve as a marker for accidental omission of the diagnostic reagent. Evans blue counterstain (0.5% in PBs, sigma, product # E-0133, for use in immunofluorescent assays) was aliquoted and stored at 4 ^{o}C. The amount of counterstain added to a conjugate was determined by titration when the working dilution of the conjugate was determined. The concentration was prepared by adding 2.5 microliters of 0.5% stock dye solution per ml of conjugate diluent.

3.2.2.6 Rinse/soak buffer

A phosphate buffer solution (PBS) formula of the same pH and molarity as the conjugate diluent was used as the rinse/soak buffer (sigma P3813 which is 0.01M phosphate buffer, pH 7.4, with 0.138M NaCl and 0.0027M KCl).

3.2.2.7 Mountant (0.05M Tris buffered pH 9.0 saline with 20% glycerol)

0.05M Tris/0.15M NaCl solution was prepared by dissolving 0.623 grams of Trizma pre-set crystals and 0.85 g NaCl in a total volume of 100 ml distilled water. It was filtered with 0.45µm filter and stored at room temperature. The mountant was added to the coverslip by dispensing with a syringe fitted with a 0.45 µm syringe filter.

3.2.2.8 Preparation of impression slides/smears

Samples were removed from the freezer (-20^{o}C) and allowed to thaw at room temperature in a safety hood. A smear or touch impression was made on slides which were prepared from the cut surface of the cross section that exposed multiple ascending and descending nerve tracts. A duplicate impression of the cross section was made for staining with the second reagent (because each brain areas is to be tested with FITC labeled anti-rabies reagents prepared from different sources i.e., two different monoclonal antibody reagents or a monoclonal antibody reagent and a hyperimmune serum reagent). Each sample brain area was of a size sufficient for examination of 40 separate views (microscopic fields) at a magnification of approximately 200x (i.e., making an impression in a 15mm diameter well or a smear of 10mm^2). Excess sample materials were blotted from the slides using paper towels.

3.2.2.9 Control slides

Control slides were prepared in the same manner as test/sample slides. Positive and negative control slides were prepared.

3.2.2.10 Drying and fixation

All the impressions/smears were air dried completely at room temperature in a safety biocontainment hood for about 15 – 30 minutes. When the tissue no longer appeared wet and glistening; slides from an individual test animal were combined in one container containing acetone for fixation. Positive and negative control slides were put in different separate containers of acetone and fixed at the same time as test slides to control the effect of acetone fixation on test performance. Slides were fixed for a minimum of 1hr at -20^{o}C explosion proof freezer.

3.2.2.11 Staining, rinsing and mounting

After the acetone fixed impression/smear control and test slides were air dried at room temperature, each anti-rabies conjugate was added by dispensing through a syringe fitted with a 0.45um low protein binding filter (the first 3 drops of the conjugate were discarded). The slides were arranged so that the positive control slide was the first to receive the conjugate (to control for any unexpected removal of antibody by filtration) and the negative control slide was the last to receive the conjugate (to control for adequate removal of excess fluorescein by the filter throughout conjugate application).

The slides were then incubated for 30 minutes at 37^{o}C in a high humidity chamber. After staining, excess conjugate was drained from the slides onto adsorbent paper and the slides were given a brief rinse under a stream of PBS, then immersed and soaked in PBS for 3 to 5 minutes (control slides and slides from each test animal were in a separate rinse container – coplin jars). The PBS was discarded and replaced and the slides were soaked for a second 3 to 5 minute interval.

Slides were carefully blotted to remove excess liquid, and then briefly air dried before mounting. Slides were mounted by dropping a small amount of 20% glycerol-tris buffered saline pH 9.0 onto coverslips arranged on absorbent paper. Stained slides were inverted over the coverslips. Excess mountant was wicked into the absorbent paper by applying light pressure to the back of the slides. A small volume syringe fitted with a 0.45 µm filter and small bore rubber tubing was used to dispense the mountant in small droplets onto the coverslips. Slides were read within 2 hours of mounting. Positive stained slides were then preserved for reference at refrigerator temperature. Positive samples were inoculated into pools of suckling mice to recover the virus and ribonucleic acid (RNA) was also extracted from the positive samples.

3.2.3 Ribonucleic Acid (RNA) Extraction

RNA extraction using TRIZOL was conducted to isolate total RNA from cells and tissues. RNAs were extracted from all the samples positive for direct fluorescent antibody (DFA) test i.e all the samples (ST) that showed +4 and +3 staining intensity/antigen distribution had their RNA extracted.

3.2.3.1 Preparation of RNase-free water

RNase-free water was commercially purchased and in its absence, it could be prepared by drawing water into RNase-free glass bottles. DEPC was added to 0.01% (v/v). It was left to stand overnight and autoclaved.

3.2.3.2 Homogenization of tissues

Screw cap unbreakable test tubes (1.25 ml) were labeled according to the sample numbers and 1 ml of TRIZOL reagent was dispensed into each tube. The brain samples were cut with sterile disposable surgical blades and placed in the corresponding labeled sterile disposable cans and weighed. Each sample was weighed between 50 – 100 mg; the samples were further cut into pieces in the cans using the disposable surgical blades. A different blade was used for each sample and disposed into the biohazard discard container after use. The tissues (brain samples) were then transferred into the corresponding labeled screw cap unbreakable tubes containing TRIZOL reagent and homogenized by pipetting up and down. The sample volume did not exceed 10% of the volume of TRIZOL reagent used for homogenization. The samples were kept on ice throughout the homogenization procedure.

3.2.3.3 Phase separation

The homogenized samples were incubated for 5 minutes at 15 to 30^{o}C to ensure the complete dissociation of nucleoprotein complexes; 0.2 ml of chloroform was added per 1 ml of TRIZOL reagent. The sample tubes were securely capped. The tubes were vigorously shaken by hand for 15 seconds and incubated at 15 to 30^{o}C for 2 to 3 minutes.

The samples were centrifuged at 10000 rpm (10,000xg) for 15 minutes at 4^{o}C. Following centrifugation, the mixture separated into a lower red, phenol – chloroform phase, an interphase, and a colorless upper aqueous phase. RNA remained exclusively in the aqueous phase (while DNA and protein remained in the organic phase) of which volume was about 60% of the volume of TRIZOL reagent used for homogenization.

3.2.3.4 RNA precipitation

New sets of screw cap unbreakable tubes were labeled and the aqueous phase was transferred into the fresh tube. RNA was precipitated from the aqueous phase by mixing with isopropyl alcohol. 0.5 ml of isopropyl alcohol was used per 1ml of TRIZOL used for the initial homogenization. The samples were incubated at 15 to 30^{o}C for 10 minutes and centrifuged at 12,000 rpm (12,000xg) for 10 minutes at 4^{o}C. The RNA precipitate was seen at this stage as a gel – like pellet on the side and bottom of the tube.

3.2.3.5 RNA wash

Supernatants were removed and RNA pellets were washed with 75% ethanol by the addition of 1 ml of 75% ethanol per 1 ml of TRIZOL reagent used for the initial homogenization. The samples were mixed by vortexing and centrifuged at 8000 rpm (8,000xg) for 5 minutes at 4^{o}C.

3.2.3.6 Redissolving, Quantitation and Expected Yields of RNA

RNA pellets were air-dried for 5 – 10 minutes, not allowing the pellets to completely dry (which greatly decreased its solubility). The RNA, were dissolved in

RNase-free water by passing the solution a few times through a pipette tip, and then incubated for 10 minutes at 56°C and stored at -70°C (Bracete et al, 1998). The RNA was quantified using spectrophotometric at A260/280 and on agarose gel electrophoresis.

3.2.4 Reverse Transcription Polymerase Chain Reaction (RT – PCR), Polymerase Chain Reaction (PCR) Amplification

RNA was first reverse transcribed into cDNA using a reverse transcriptase andthe resulting cDNA was used as templates for subsequent PCR amplification using primers specific for one or more genes. One-step RT – PCR where all reaction components were mixed in one tube prior to starting the reactions was used. This offered simplicity and convenience and minimized the possibility for contamination.

3.2.4.1 RT-PCR, PCR Reaction

Extracted RNA samples were removed from - 80°C and placed on ice to thaw, vortexed and pipetted up and down. Microtubes (600 µl) were labeled according to each sample. Appropriate cocktails/mixtures (RT and PCR Reactions) were made and mixed completely by tapping the tubes and centrifuged.

RT primer (forward only) 1 µl was added to each reaction tube, and 5 µl of RNA was added to the same set of tubes. The mixtures were vortexed well and centrifuged at 13,000 min-1 for 1 minute. They were heated at 94 °C for 2 minutes in a thermalcycler using the mastercycler protocol 94, and were then cooled on ice for 5 minutes. RT-Rxn mix 14 µl was added to each tube (volume 20 µl), incubated at 42°C for 90 minutes (mastercycler protocol 42 for 90min). They were vortexed and centrifuged and 80 µl of

PCR reaction mix was added to each RT tube (100µl total). The thermalcycler was preheated to 94^{o}C and samples loaded in it and ran on Mastercyder protocol (RabPCR A). The products were stored at -20^{o}C.

3.2.5 Agarose Gel Electrophoresis

Agarose gel electrophoresis was used to separate DNA fragments as small as 0.1 kb or less. The agarose concentration determines the position of the dye bands relative to a polynucleotide.

3.2.5.1 Preparation of running buffer

A 1 x Tris – acetate – EDTA (TAE) stock buffer was prepared by measuring 20mls of 50x Tris-acetate-EDTA (TAE) stock buffer into a 1000 ml beaker using serological pipet. The volume was made up to 1000 ml (1:50 dilution) with deionized water. The beaker was cap screw and solution mixed together.

3.2.5.2 Preparation of sample loading buffer

Deionized water (7.0 ml) was measured into a sterile 20 ml unbreakable beaker. Ficoll 400/Glycerol 2.5g and 25.0mg bromphenol blue (FW 691.9) were added and solution made up to 10.0 ml with deionized water.

3.2.5.3 Preparation of Agarose solution

Approximately 7 ml agarose solution per mm of gel thickness was prepared by measuring 50 ml of TAE 1x stock buffer into a glass conical beaker. Agarose (0.5 g) was measured with a clean spatula on a clean paraffin paper and weighed on an electronic scale. The weighed agarose was added to TAE water. The mixture was microwave to dissolve for about 60 – 90 seconds checking and mixing at intervals. (The mixture was not allowed to boil or foam). The solution was allowed to cool to about 50^oC and 2.5 µl (0.5 µg/ml of ethidium bromide was added and mixed (to observe separation during electrophoresis).

3.2.5.4 Casting of gel

The running tray was installed by firmly grasping the casting tray with one hand. With the other hand, one end of the running tray was placed against the foam pad at the bottom edge and the tray was pressed against the pad, and then lowered to rest on the bottom of the casting tray, seating the other end of the tray against the opposite foam pad.

The 12 well combs were prepared by fitting the two slots in the comb between the (loosened) thumb screw heads and the comb back; the screws were tightened until the comb was supported. The comb assemblying was seated on the run of the casting tray and the bottom was adjusted to about 1.0mm from the running tray. The screws were tightened to secure the comb. Two combs were prepared in a tray to run twice as many samples. The comb assembly was removed and casting assembly was placed on a leveling surface and leveled, using the spirit level on the running tray as a guide.

The agarose solution (cooled to 50^oC) was poured into the casting tray. The comb assembly was oriented so that the comb faced the nearest foam pad and seated on the tray rim. The comb was checked to be sure it was vertical so as to prevent well shape distortions. The second comb assembly was placed in the center of the tray. The gel was left for a minimum of 30 minutes to set (this can be hastened by putting in a refrigerator at 4^{o}C).

Once the gel was set, the comb was removed carefully by partially lifting and slightly tilting the comb at one end and then slowly withdrawn from the gel (care was taken not to pull the comb straight up which created a vacuum in the well, that may lift the gel out of the tray). The running tray and gel was removed by grasping the handles of the tray and pressing against one of the foam pads. Once the tray was cleared on the opposite pad, it was lifted out. The running tray and gel were transferred to the chilled base. (The base was chilled before use because of higher voltage settings used). Buffer chambers were filled with running buffer until the gel was submerged under approximately 1mm of solution. (This required about 220 ml).

3.2.5.5 Loading of samples

Small test tubes (0.6mls) were labeled according to sample numbers. In the test tubes, 5 µl of sample, 5µl of distilled deionized water and 2µl of loading buffer (1/6 of the final volume) were mixed together by pipetting up and down. Micro-pipette with tips were used to load each sample with care taken to avoid puncturing the well bottom or entrapping any bubbles, discarding each tip after use. Molecular weight marker 8 µl (which allowed accurate sizing of DNA fragments generated by PCR or restriction digest separated on agarose gel) was loaded into the first wells.

After loading all the samples and DNA ladder, the lid was placed so that the cathode (-vs black lead) was at the end nearest the sample well. (Nucleic acid samples migrate toward the anode, +ve, red lead). Color – coded leads were connected (red to red, and black to black to an approved power supply, such as the Hoefer EPS 2A200 Voltage was set at 150 V and timer for 15 minutes.

After the separation (electrophoresis run), the power supply was turned off, leads disconnected, lid of agarose unit removed. The gel was removed and transferred in a plastic container to the dark room where the gel was photographed by sliding the gel onto the transilluminator surface for maximum exposure, and sample viewed under short wave uv radiation 366-nm uv light can be used). The photographs were taken using Fotodyne ® Polaroid camera with Polaroid 667 black and white instant pack film. The films were developed for viewing (Ausubel et al., 1993; Sambrook et al., 1989).

3.2.6 Deoxyribonucleic Acid (DNA) Sequencing

DNA sequencing was used for the determination of the precise sequence of nucleotides in a sample of DNA (i.e., the biochemical method for determining the order of the nucleotide bases A, G, C and T in a DNA).

3.2.6.1 Purification and sequencing of PCR products

The Dye-terminator sequencing method was used. It was necessary to purify the PCR product from excess unreacted primers left over from the PCR reaction before sequencing. ExoSAP-IT® was used which treats PCR products ranging in size from less than 100 bp to over 20 kb with absolutely no sample loss by removing unused primers and nucleotides.

3.2.6.2 PCR Clean - up

Micro tubes (600 µl) were labeled according to sample numbers of the post – PCR reaction products. The samples were split according to the primers used. A sample was split into 2 (i.e., for both the forward and reverse primers). 5 µl of each sample was dispersed into the tubes according to the sample numbers and primers. EXOSAP – IT was removed from – 20^oC freezer and kept on ice throughout the procedure, 2 µl of the EXOSAP – IT was mixed with every 5 µl of post – PCR reaction (a higher quantity was used for samples of which 10 µl was used), product for a combined 7 µl reaction volume. The mixture was incubated at 37^oC for 15 minutes to degrade the remaining primers and nucleotides. The tubes were set into the thermalcycler at protocol exosap / exosap avv / exosap it dgs to incubate at 80^oC for 15 minutes to inactivate the EXOSAP-IT. Treated PCR products were stored at -20^oC for DNA sequencing.

3.2.6.3 Primer preparation

Primers (40 µm) were brought out of the freezer and placed on ice to thaw. To every 5µl of 40 µm primer intended to be used, 20 µl of RNase DNase free water was added (i.e., a 1:4 dilution).

3.2.6.4 Removal of dye terminators prior to sequencing (CENTRI–SEP PROTOCOL)

CENTRI-SEP columns were used for the fast and efficient purification of large molecules (proteins, nucleic acids, complex carbohydrates, etc) from small molecules (nucleotides, buffer salts, etc). The column design is based, on the description by Sambrook et al. (1989). The gel provided excellent recovery of DNA fragments >16 base pairs or 25-mer while removing >98% of salts, NTP's and other low-molecular – weight compounds.

CENTRI-SEP columns are recommended by Applied Biosystems Inc. for effective and reliable removal of excess Dye Deoxy^{TM} terminators from completed DNA sequencing reactions. The procedure below was used in conjunction with the Taq DyeDeoxy^{TM} and ABI Prism^{TM} terminator cycle sequencing kits, including those with Ampli Taq®, FS, used on ABI models 373A, 377A, 310, 3100, 3700 and 3730.

3.2.6.5 PCR sequencing reaction

Tubes containing the treated PCR products were removed from -20^{o}C and placed on ice to thaw. To every 5 µl sample tube, 1 µl of the corresponding primer (1:4) and 2 µl of Big Dye® were added, vortexed and centrifuged at 13,000 rpm for 1 minute. The tubes were placed into thermocycler under protocol mastercyder seqpcr rab/rabseq at 20 µl rxn for 3 hours. The samples were kept on ice for immediate processing.

3.2.6.6 Column hydration

The columns were labeled corresponding to the samples (treated PCR products). The columns were gently tapped to insure that the dry gel has settled in the bottom of the spin column. The top column caps were removed and the column reconstituted by adding 750 µl of distilled deionized water to each tube. The column end stopper was left in place for the column to stand up by itself. The column caps were replaced and gel hydrated completely by vortexing. The reconstituted columns were put in the refrigerator at 4^{o}C for at least 30 minutes.

3.2.6.7 Removal of interstitial fluid

Air bubbles present were removed from the column gel by inverting the column and sharply tapping the column, allowing the gel to slurry to the opposite end of the column. The columns were placed upright and the gel was allowed to settle, while in a microtube rack. After the gel had settled and free of bubbles, the top column cap was first removed and then the column end stopper was removed from the bottom. This was done for all the columns to be used. Excess fluid was allowed to drain by gravity into a wash tube (2 ml). For the fluid that did not flow immediately through the end of the column, a 2 ml latex pipet bulb was used to apply gentle air pressure to the top of the column to force the fluid to start through the column filter. The columns were allowed to stop draining on their own. The drained fluids were discarded.

The column and wash tubes were spinned in a variable speed in centrifuge at 5000 rpm for 3 minutes (can also speed at 7500 rpm for 2 minutes) to remove interstitial fluid. The position of the columns was tracked using the orientation mark molded into the column. Approximately 300 µl of fluid was collected from each sample. Any drop at the end of the column was blotted dry. Wash tubes and the interstitial fluids were discarded. Gel material was not allowed to dry excessively.

3.2.6.8 Sample processing

The columns were held to the light and the samples were carefully dispensed directly onto the center of the gel bed at the top of the column, without disturbing the gel surface. The sides of the column were not contacted with the reaction mixture or the sample pipet tip, as this can reduce the efficiency of purification and possibly run the analysis due to excess dyes. The columns were placed into the sample collection tube (1.5 ml) and both were placed into the rotor. Proper column orientation was maintained. The highest point of the gel media in the column was made to point toward the outside of the rotor. The columns and collection tubes were spinned at 5,000rpm for 3 minutes (could also spin at 7500 rpm for 2 minutes). The purified samples were collected in the bottom of the sample collection tube. The spin columns were discarded. The samples were dried in a vacuum centrifuge for 1 hour, and stored at -20^{o}C until ready to use.

3.2.6.9 Gels and loading

3.2.6.9.1 Preparation of loading dye

Five parts loading dye (250 ml) was mixed to one part (50mls) formamide to Dextran Blue.EDTA in a microtube (0.6ml), the mixture was homogenized by vortexing and then placed on ice.

3.2.6.9.2 Preparation of 10% ammonic buffer

0.1 g of ammonium persulfate was weighed into a 1.25 ml tube. 1 ml of deionized water was added to it and vortexed well. It was then placed in the refrigerator until when ready to use.

3.2.6.9.3 Making a sequencing gel

Into a clean glass conical beaker, water and urea were measured, added together and mixed according to the required quantity. Long Ranger gel solution and 10xTBE were added to the mixture and mixed. A rod-like magnet was placed in the mixture and placed on a hot plate stirrer (setting 7) and heated (at level $5^{1/2}$) for about 2 minutes in order to completely dissolve the mixture. The mixture was poured into a glass cylinder and made up to 100 ml with distilled deionized water. The mixture was filtered through a filtering jar connected to a pressure vacuum. Sequencing plates were washed and cleaned with hot water, deionized water and isopropanol. They were dried with non-lint wipes. Spacers were moistened and applied to the sides of the plate. The top plate was added and clamps applied one inch apart. 10% ammonic buffers, followed by Temed were added to the sequencing gel mixture. The mixture was immediately added in between the plates, banging on the plate to advance the front and prevent bubbles while adding the gel. The comb was inserted backward/spacer and clamped at top of plate. It was left for approximately 2 hours to allow for polymerization.

3.2.6.9.4 Sample preparation and gel loading (ready combs)

The sequencer was turned on ahead to warm up. To each of the sample, 3.5 µl of loading dye was added to the base of the tubes avoiding contact with the inner wall of the tubes with the pipette tips. The samples were vortexed for about 10 seconds and centrifuged for 30 seconds, and placed on ice. The samples were then denatured for 2 minutes at 94^{o}C, in a thermalcycler at mastercycler protocol seqheat. Samples were immediately placed on ice.

Loading tray (prepared sequenced gel) was cleaned and dried with ETOH and Type 11water. The comb (36 or 48 well) was inserted forward to create the wells; tray was fitted to the sequencer. Solution (TBE) was filled into the lower and upper containers of the sequencer, air bubbles were removed from the well and a pre-run test was carried out.

Samples (1.5 µl each) were loaded into each well loading the odd numbered wells first and allowing the sequence to run for about 5 minutes before loading the even numbered wells in order to track the samples. The sequencer was allowed to run for 8 hours.

3.2.6.10 Use of Applied Biosystems 3780/3730x/DNA Analyzer

The computer workstation was started before starting the Applied Biosystems analyzer. Monitor and computer were powered and logon dialog box opened and instrument turned on.

100µl of formamide was added to each samplex, vortexed and denatured in a thermalcycler at 94^{o}C for 2 minutes. 50 µl of each sample was loaded into the corresponding well. 50 µl of formamide was loaded into any empty well not containing sample. The plate was sealed with heat seal film; and loaded into the machine for sequencing which ran for about 3 hours.

3.2.6.11 Editing of sequences

The resulting sequencing was edited using Bioedit, pairwise alignment, end to side.

3.3 Technique for Virus Isolation

3.3.1 Mouse inoculation test

Mouse inoculation test was carried out to isolate rabies viruses.

All vessels and test tubes used for each specimen were labeled with the specimen number (brain sample numbers). Proper protective clothing (surgical gloves-doubled:

laboratory coats) were put on and all procedure for virus preparation was carried out under a negative – pressure hood (safety biocontainment hood).

3.3.2 Specimen preparation

400 µl of MEM was dispensed into each labeled unbreakable screw cap tube (1.25 ml), using scissors and forceps.Pieces of brain tissues were removed from each sample and weighed. Different/separate scissors and forceps were used for all the samples.

The brain tissues were added to the medium in the tube, minced and ground to a paste with sterile wooden swabs end, by twisting and rubbing the two sticks together until the brain samples has completely dissolved. Another 400 µl of the medium is added to the paste and mixed together further. The MEM was cooled to $4^{o}C$ before use. A 20% homogenate was made for each sample. The unbreakable screw cap tubes containing the suspensions were centrifuged at 150 – 200xg for 5 minutes at $4^{o}C$.

3.3.3 Dilution

After centrifuge, the samples were carefully removed from the centrifuge, and supernantants were pipetted out and filtered into new labeled sterile unbreakable screw cap tubes. Two sets of labeled sterile unbreakable screw cap tubes were labeled for each sample, 10^{-1} for the first set and 10^{-2} for the second set. 900 µl of MEM was dispensed into each tube. To the first set of tubes for 10^{-1} dilution, 100 µl of each suspension was added to the corresponding tube, after vortexing the suspension, and again the tubes were capped and vortexed well to ensure proper mixture, then 100 µl was transferred from each tube into their corresponding labeled. Second set of tubes for 10^{-2} dilution, vortexed well and kept on ice. 0.5 cc tuberculin syringes with 27-guage needle were labeled according to the samples number and vortexing the second sets of tubes (10^{-2} dilution) again, 20 µl of each sample was aspirated into the syringes equaling the number of suckling mice for each virus sample (e.g. if sample. A is to be inoculated into a pool of 16 suckling mice, then 16 different tuberculin syringes containing 20 µl of the virus suspension is prepared. A syringe per mouse was used. The syringes were placed in a test tube with gauze in the botton and were kept in a ziplock plastic bag which was kept in a flask/cooler containing ice and were then transferred to the animal facility where the mice were housed. The remaining unused supernantant at different dilutions and the neat were frozen at -80^{o}C for future reference.

3.3.4 Inoculation of mice

At the animal facility, where the mice were housed, the work surface was covered with paper towels to avoid contamination, and proper protective clothing (laboratory coat, double gloves, caps, shoe cover) were worn with additional nose mask and face shield. The cages were labeled with specimen/sample numbers, experiment and protocol numbers, type/strain of tissue inoculated, volume of inoculum, route of inoculation, age of mice, and the date of inoculation.

The syringe was held perpendicularly, with needle up and the tube and gauze still in place, and syringe was tapped to remove any air bubbles by rising to the needle which is then expelled from the syringe into the gauze in the tube. Care was taken not to expel the air bubbles into the air to avoid any aerosol accidental infection. Using animal forceps, each suckling mouse of about 3-4 day old was removed from the cage and held stomach down, and inoculated intracerebrally by insertion of the needle through the cranium at a point about halfway between the ear and eye just off the midline of the skull, 20 µl of the specimen was injected into each mouse. This was repeated for all the mice for all the samples prepared (an average of 15 – 16 suckling mice per mother was used for each sample). The mice were checked to ensure that none died of trauma. The mice were well supplied with food and water and observed daily for 30 days for specific signs of rabies in mice including ruffling of the fur, humping, trembling lack of coordination of the bind legs, ascending paralysis, prostration, slow movements and lack of responsiveness to stimuli and lack of milk in the stomach.

3.4 Plasmid DNA Vaccine Preparation and Inoculation

3.4.1 Modification of Virus

A reverse genetics system was developed for the rabies virus strain ERA (Evelyn – Rokitnicki – Abelseth) using a T7 RNA polymerase, containing an eight amino acid nuclear location signal at the N terminal derived from the SV40 large autogene plasmid which facilitated virus recovery . The glycoproyein gene was switched with the matrix protein gene, creating a reshuffled virus ERAgm (gene order N-P-G-M-L). The rearranged ERAgm rabies virus and the parental ERA (gene order N-P-M-G-L) were replicated in infected BSR cells (Wu and Rupprecht, 2008). A derivative virus was recovered ERAg3 (with a mutation at the amino acid 333 in glycoprotein by substituting the amino acid Arginine (AGA) with Glutamine (GAG) (WU *et al*, 2005). The attenuated virus was inoculated into laboratory animals using mice (according to MIT as previously described) to determine its pathogenicity. It was then amplified (RT-PCR), sequenced and ran on gel electrophoresis as previously described.

3.4.2 Purification of Plasmid DNA

Preparation of LB (Luria – Bertani) medium.

10 g tryptone, 5 g yeast extract, and 10 g NaCl were dissolved in 800 ml distilled water, pH was adjusted to 7.0 with 1N NaOH and volume adjusted to 1 liter with distilled water. The prepared medium was sterilized by autoclaving, ampicillin antibiotics 1ml/1000mls medium was added.

3.4.3 Preparation of Buffers

- Buffer PI: RNase A solution was added to Buffer P1, and mixed. LyseBlue reagent was added and mixed by using the one vial LyseBlue (which was spinned down briefly before use) per bottle of Buffer P1 to achieve a 1:1000 dilution. This was stored at 2 – 8^{o}C.
- Buffer PE: ethanol (96-100%) was added to Buffer PE and mixed.
- Buffers P2 and N3: were checked for salt precipitation. Any precipitation was redissolved by warming to 37^{o}C, and mixed without shaking Buffer P2 vigorously.

3.4.4 Isolation of Insert

3.4.4.1 Wizard PCR Preps DNA Purification System

Purification of DNA from Low –Melting –Temperature Agarose method was used to separate the PCR products by electrophoresis in a TAE agarose gel containing ethidium bromide using standard protocol.

Following electrophoresis, the DNA band was quickly excised from the gel using a clean, sterile scapel (i.e the band was visualized with a medium/long wavelength ≥ 300nm, UV light and was excised quickly to minimize exposure to UV light). The band was isolated in approximately 300µl = 300mg or less of agarose.

The 300µl (300mg) of agarose slice was transferred to a 1.5ml microcentrifuge tube and incubated at 70^0C until the agarose was completely melted. The PCR Preps resin was thorouhly mixed before removing an aliquot. Any crystals or aggregates present were dissolved by warming the resin to 25-37^0C for 10 minutes, and cooled to 30^0C before use. One (1ml) of resin was added to the melted agarose slice; and thoroughly mixed for 20 seconds.

3.4.4.2 Purification Using a Vacuum Manifold

For each gel sample, one wizard^R Minicolumn was prepared. The provided syringe barrel was attached to the Luer-Lok^R extension of each Minicolumn. The tip of the Minicolumn/syringe barrel assembly was inserted into the vacuum manifold. The resin/DNA mix from above was pipetted into the syringe barrel and a vacuum was applied to draw the resin/DNA mix into the Minicolumn. The vacuum was broken to the Minicolumn, 2ml of 80% isopropanol was added to the syringe barrel to wash the column, and a vacuum was reapplied to draw the solution through the Minicolumn. The resin was dried by continous drawing of a vacuum for 30seconds after the solution has been pulled through the column. The syringe barrel was removed and the Minicolumn transferred to a 1.5ml microcentrifuge tube. The Minicolumn was centrifuged at 10,000xg in a microcentrifuge for 2 minutes to remove any residual isopropanol. The Minicolumn was transferred to a new microcentrifuge tube, 50µl of TE buffer was applied to the Minicolumn and left for 1 minute (the DNA remained intact on the Minicolumn for up to 30 minutes). The Minicolumn was centrifuged for 20 seconds at 10,000xg to elute the DNA fragment. The Minicolumn was removed and discarded. The purified DNA was stored in the microcentrifuge tube at 4^0C or -20^0C (Sambrook *et al.*, 1989; York *et al.*, 1993).

3.4.5 Cloning of Products with pcDNA3.1 (-) Vectors

3.4.5.1 Ligation

The vector was briefly centrifuged and ligation reaction was set up using restriction enzymes (Pst 1 and Kpn 1). The reaction was 5µl plasmid, 2µl buffer, 0.5 kpn1, 0.5 pst 1 and 12µl deionized water giving a total volume 20µl. The ligation buffer was vigorously vortexed before addition. The reaction was mixed by pipetting gently and incubated at 16^0C overnight.

3.4.5.2 Transformation: Transformation of JM109 High Efficiency Competent Cells

The ligated product was transformed into bacterium (*E.coli* JM109) for propagation. The LB/ampicillin plates were prepared. Ligation reaction was briefly centrifuged, and 2µl of the reaction was added to a sterile 1.5ml tube on ice. JM109 high efficiency competent cells was removed from -80^{0}C freezer and placed on an ice bath for 6 minutes to thaw. The cells were mixed by gently flicking the tube; 50µl of cells was carefully transferred to the ligation reaction tubes and incubated on ice for 20 minutes. The cells were then heat-shocked for 45-50 seconds in a water bath at exactly 42^{0}C and immediately returned to ice for 2 minutes, 950µl room temperature SOC medium was then added to the ligation reaction transformation and incubated for 1.5 hours at 37^{0}C with shaking (-150rpm) in an incubator shaker. 100µl of each transformation culture was plated onto selective agar (LB plus ampicillin) to select for bacteria that has the plasmid of interest. The plates were incubated overnight at 37^{0}C (Mezei and Storts, 1994; Zhou *et al*., 1995; Online edition of Promega Corporation, 2005).

3.4.5.3 Selection of bacteria with plasmid

Individual colonies was picked and inoculated into about 2-3 mls of medium (LB plus ampicillin), and incubated overnight in an incubator shaker at 37^{0}C, 260nm.

The plasmid was harvested using Maxiprep to obtain large quantities of plasmid containing the glycoprotein G. Cloning was confirmed by PCR and DNA sequencing.

The same procedure was followed for the cloning and purification of glycoprotein G of the parental virus of West Caucasian Bat Virus (WCBV) rabies virus.

3.4.6 Growth of bacterial cultures in tubes or flasks

Into a 10 ml centrifuge tube, 3 ml of LB medium containting antibioties was dispensed under a biocontainment safety hood. A single colony was picked from a freshly streaked selective plate and inculated into the medium. It was then incubated for 12 – 16 hours at 37^{o}C with vigorous shaking at 260 nm in an incubator shaker. Bacterial cells were harvested by centrifugation at >8000 rpm (6800xg) in a conventional, table – top microcentrifuge for 2 – 3 minutes at room temperation (15 – 25^{o}C). Pelleted bacterial cells were kept on ice.

3.4.7 Plasmid DNA purification using Q1A prep spin miniprep kit and a microcentrifuge

The protocol was designed for purification of up to 20 µl of high-copy plasmid DNA from 1 – 5 ml overnight cultures of *E.coli* in LB (Luria-Bertani) medium. Buffer P1 was removed from the refrigerator and vigorously shaken to ensure Lyse Blue particles have completely dissolved and then kept on ice. The pelleted bacterial cells were resuspended in 250 µl Buffer P1 by pipeting up and down and vortexing until no clumps remained, and transferred to a microcentrifuge tube (Buffer P1 was kept on ice throughout its use). 250 µl of Buffer P2 was added and thoroughly but gently mixed by inverting the tube 4 – 6 times until the solution became viscous and slightly clear. The lysis reaction was not allowed to proceed for more than 5 minutes. The solution turned blue as a result of the LyseBlue added to Buffer P1 thereby giving the mixture a homogeneous colored suspension. 350 µl of N3 was added and mixed immediately and thoroughly by inverting the tube 4 – 6 times or more as required till the solution became cloudy and traces of blue disappeared leaving homogenous colorless suspension which indicated that the SDS has been effectively precipitated. It was then centrifuged for 10 minutes at 13,000 rpm (≈17,900xg) in a table-top microcentrifuge. A compact white pellet was formed. Supernatants from the previous step was applied to the Q1A prep spin column by pipetting and centrifuged for 30 – 60 seconds. The flow through was discarded. The Q1A prep spin column was washed by adding 500µl Buffer PB and centrifuged for 30 – 60 seconds. The flow – through was discarded (this step was carried out to remove trace nuclease activity when using end A+ strains such as the JM series, HB 101 and its derivatives, or any wild-type strain, which have high levels of nuclease activity or high carbohydrate content.

The Q1A prep spin column was washed by adding 750 µl Buffer PE and centrifuged for 30 – 60 seconds. The flow-through was discarded and centrifuged for an additional 1 minute to remove residual wash buffer. (Residual ethanol from Buffer PE may inhibit subsequent enzymatic reactions).

The Q1A prep column was placed in a clean 1.5ml microcentrifuge tube. DNA was eluted by the addition of 50 µl Buffer EB (10mM Tris-Cl, pH 8.5) to the center of each Q1A prep spin column, allowed to stand for 1 minute and centrifuged for 1 minute. The Q1A prep was discarded, and DNA collected in the lower microcentrifuge tube. The DNA was quantified by running on agarose gell electrophoresis (as previously described) and stored at -20^{o}C.

3.4.8 Amplification of Plasmid DNA

Purified plasmid DNA was thawed at room temperature, centrifuged for 60 seconds at 13,200rpm and placed on ice immediately. *E.coli* JM109 bacterium (2355751) was removed from -80^{o}C and placed on ice for about 6 minutes to thaw. 50µl of E.coli was added gently to 5µl of purified plasmid DNA sample in a microcentrifuge tube (1.25 ml) and left on ice to incubate for 20 – 30 minutes. This was followed immediately by putting into hot water bath at 42^{o}C for 45 seconds and placed back on ice for 2 minutes. 25µl of s.o.c medium (an enriched LB medium) was added and put into the incubator shaker for 1 hour at 225 nm at 37^{o}C.

After 1 hour, 150 ml of LB medium (plus ampicillin) was dispensed into 2 different flasks and 150 µl each of the bacteria plus plasmid was added to each flask and both flasks were put into the incubator shaker overnight at 260 nm at 37^{o}C.

3.4.9 Plasmid or Cosmid DNA Purification Using Q1AGEN Plasmid Midi and Maxi Kit

This protocol was designed for preparation of up to 100 µg of high – or low – copy plasmid or cosmid DNA using the Q1AGEN – plasmid midi kit, or up 500 µg using the Q1AGEN plasmid maxi kit. For the Q1AGEN – tip 100, the expected yields are 75 – 100 µg for high – copy plasmids and 20 – 100 µg for low – copy plasmids. For the Q1AGEN – tip 500, the expected yields are 300 – 500 µg for high – copy plasmids and 100 – 500 µg for low – copy plasmids.

Bacterial cells were harvested by centrifugation at 6000xg for 15 minutes at 4^{o}C Buffer P1 was removed from the refrigeration and vigorously shaken to ensure complete resuspension of LyseBlue particles, and kept on ice. The bacterial pellet was resuspended in 10 ml Buffer P1 by pipetting up and down and vortexing until no cell clamps remained.

Buffer P2 was put into water bath at 37^{o}C to dissolve any formed precipitate, 10 ml of Buffer P2 was added to the suspended bacterial pellet and mixed thoroughly by vigorously inverting the sealed tube 4 – 6 times until the cell suspension turned from blue to a homogenous colored suspension and incubated at room temperature (15 – 25^{o}C) for 5 minutes. The lysis reaction was not allowed to proceed more than 5 minutes. 10ml of chilled buffer P3 was added and mixed immediately and thoroughly by vigorously inverting 4 – 6 times and incubated on ice for 20 minutes (which enhanced precipitation). This gave a fluffy white material like appearance and the lysate became less viscous. The precipitated material contained genomic DNA proteins, cell debris, and KDS. The lysate was mixed thoroughly to ensure even potassium dodecyl sulfate precipitation. The mixture was filtered using pressure vacuum.

Q1AGEN-tip 500 was equilibrated by applying 10mls Buffer QBT, and the column was allowed to empty by gravity flow. This was allowed to drain completely. The supernantant from the filter was applied to the Q1AGEN-tip and allowed to enter the resin by gravity flow. The Q1AGEN-tip was washed with 30mls Buffer QC twice by allowing the buffer to move through by gravity flow. DNA was eluted with 15mls Buffer QF and the eluate was collected in 50ml tube and was precipitated by the addition of 10.5mls (0.7 volumes) room temperature isopropanol, mixed and centrifuged immediately at $\geq$ 15,000xg for 30 minutes at 4^{o}C. The supernatant was carefully decanted and DNA pellet was washed with 5ml of room – temperature 70% ethanol, and centrifuged at $\geq$ 15,000xg for 10 minutes. The supernatant was carefully decanted by pipetting out without disturbing the pellet. The pellet was air-dried for 5 – 10 minutes and DNA redissolved in a suitable volume of buffer (TE Buffer, pH 8.0 or 10 mM Tris-Cl, pH 8.5).

3.4.10 Determination of yield

DNA concentration was determined by both uv spectrophotometry at 260nm and quantitative analysis on an agarose gel as previously described. The Plasmid DNA was stored at -20^{o}C until when used.

3.5 Vaccination of Mice with Plasmid DNA

3.5.1 Preparation of Mice

The different plasmids DNA (CDAG3, WCBVG) were handled separately but with the same approach. Seventy-five, 3 week old Hsd: 1CR (CD-1) female mice were purchased and supplied by a Charles River Laboratories.

The mice were housed in cage boxes in group of five mice per cage and kept in the animal facility of the Centers for Disease Control and Prevention (CDC), Atlanta, Georgia, United States of America. A one week acclimatization period was observed during which the mice were observed for any sign of distress or ill-health. They were kept on water and LabDiet 500 Rodent diet on a daily basis. During the acclimatization period, baseline serum samples were collected from all the mice.

3.5.2 Pre-vaccination Blood (serum) collection

The mice were anaesthesized using isoflurane inhalation anaesthetics, and approximately 2ml of blood was collected at each bleeding from a mouse through retroorbital bleeding route using heparinized blood collecting tubes. The blood was immediately transferred into serum separator tube. The blood was centrifuged at 11,000 rpm for 15 – 30 minutes to separate the serum from the blood. The clear serum was collected into clean microcentrifuge tubes by pippeting and stored at -20^{o}C.

3.5.3 Vaccination of mice

For each vaccine, the mice were divided into different groups of five mice per group. For the multiple shots, the following routes – intramuscular, subcutaneous and oral were used (Fig. 3.1). Each route constituted a group of five mice and the control group also had five mice per group for the different routes.

For the single dose inoculation, the intramuscular and intradermal routes were used with five mice for each vaccine group and five mice for control group for each route.

Three doses of 100 µg each of the plasmid DNA at 3 weekly interval was given to each mouse in the multiple shots dose regimen, while only a single once and for all shot of 100µg of the plasmid was given to the single shot intramuscular group and 10µg given to the single intradermal group, (Figs 3.2 and 3.3).

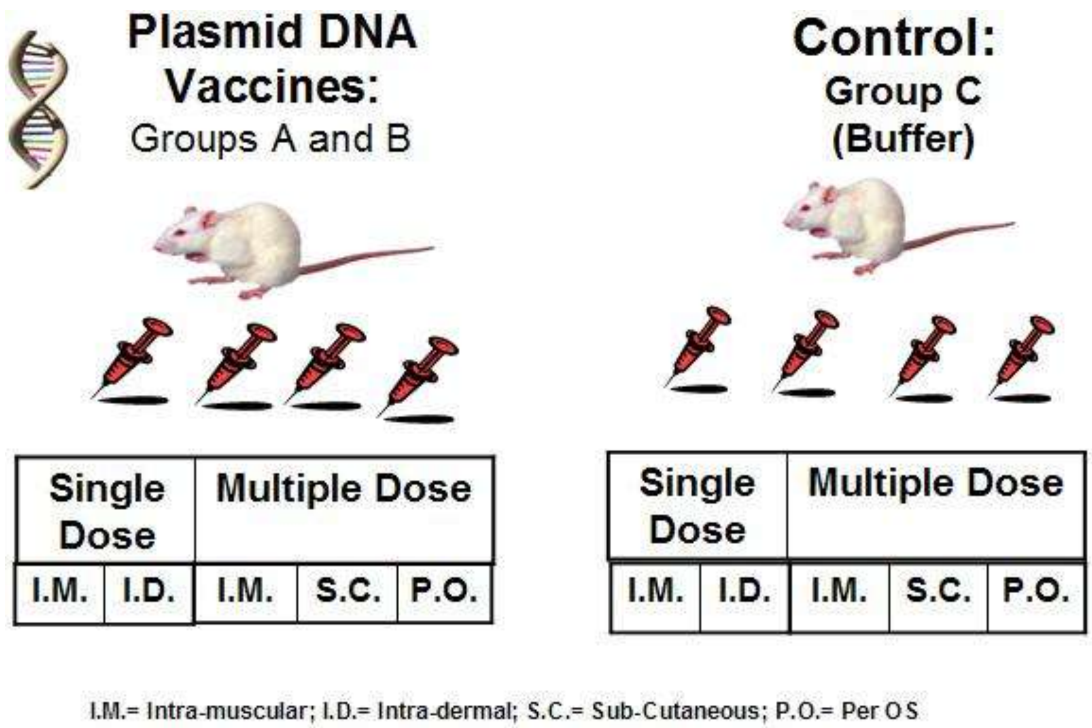


Fig 3.1: Experimental Design I: Vaccination of mice using different routes, single and multiple administrations

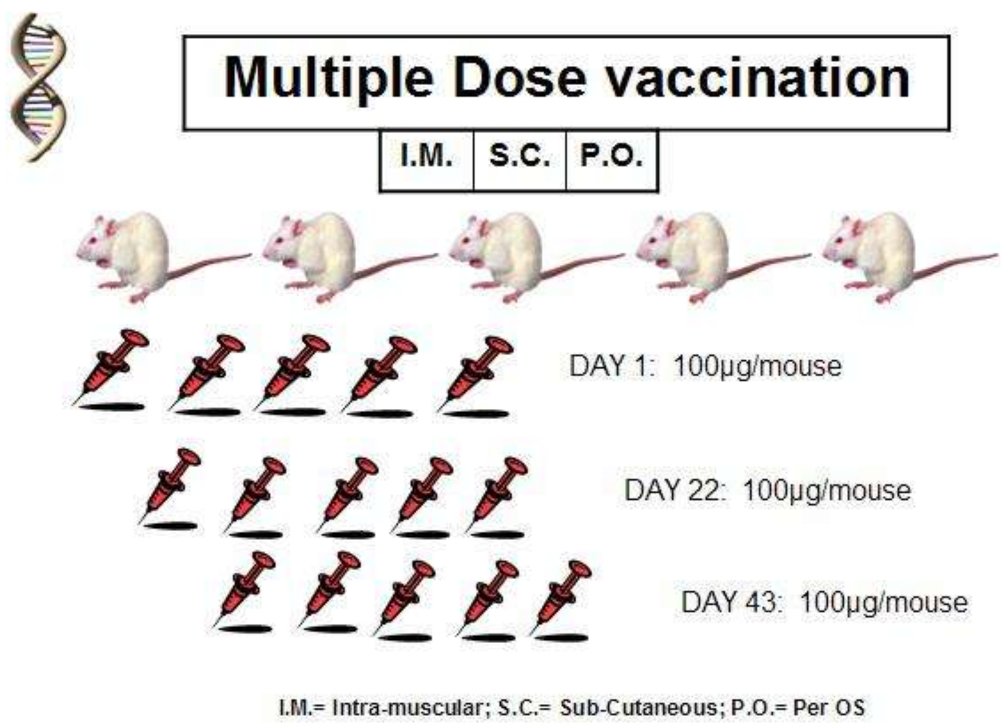


Fig 3.2: Experimental Design II: Vaccination of mice using multiple administrations and different routes

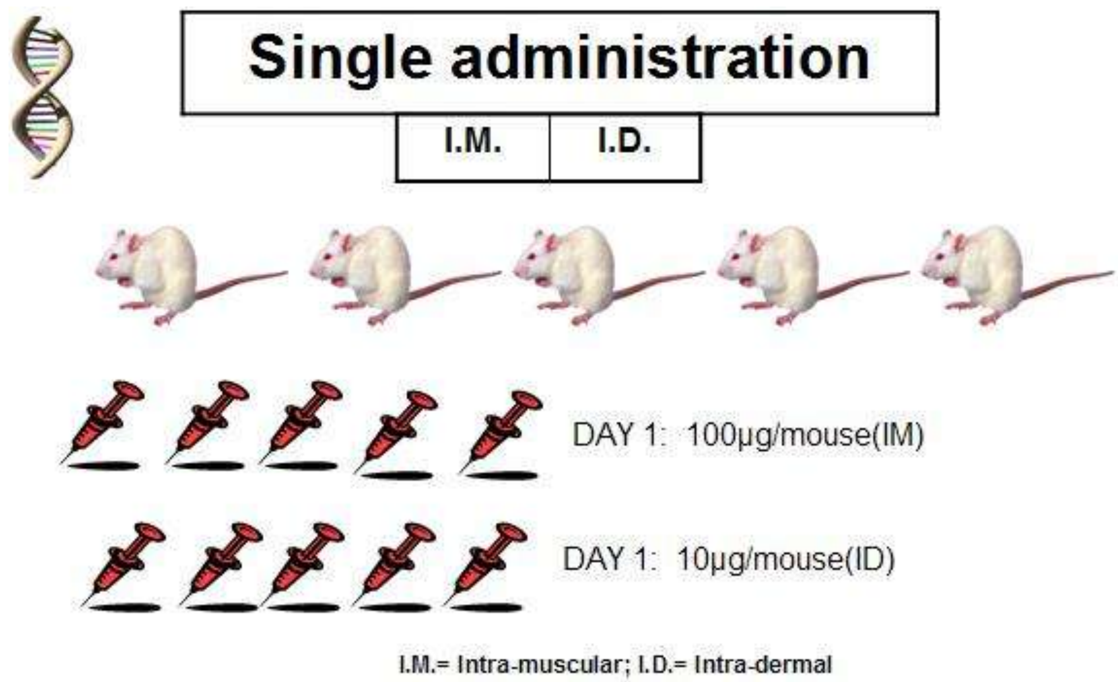


Fig 3.3: Experimental Design III: Vaccination of mice using single administration, intramuscular and intradermal routes

3.5.4 Site of inoculation

The thigh muscle was used for the intramuscular route, neck skin fold for the subcutaneous, the ear pinnae for the intradermal and oral route was given through the mouth.

3.5.5 Blood Collection

Blood sample was collected on day 0 from all the mice and on days 7 and 30 after inoculation for the single shot while blood samples were taken on a three weekly interval in the multiple dosing schedules. The serum was separated and stored at -20^{o}C as previously described.

3.6 Determnation of Rabies Neutralizing Antibody

3.6.1 Rapid Fluorescent Focus Inhibition Test (RFFIT)

RFFIT is a neutralization test that measures antibody induced by exposure to rabies virus antigens. It was used for determining rabies virus neutralizing antibody.

3.6.1.1 Mouse neuroblastoma cells

The cell culture line of mouse neuroblastoma (MNA) cells used at the CDC, was originally obtained from the Wistar Institute, Rabies Section, Philadelphia PA, USA.

3.6.1.2 Rabies challenge virus

Rabies challenge virus standard (CVS-11) strain used at the CDC may be obtained from the laboratory of standards and testing, Division of Manufacturing and Product

Quality, Office of compliance and Biological Quality, Center for Biologics Evaluation and Research, Food and Drug Administration, Bethesda, U.S.A.

3.6.1.3 Reference serum standard

Standard Rabies Immune Globulin may be obtained from the Laboratory of standards and Testing, Division of Manufacturing and Product Quality, Office of Compliance and Biological Quality, Center for Biologics Evaluation and Research, Food and Drug Administration, Bethesda. U. S. A. The reference serum for national laboratories is the National Institute for Biological standards and control, Herts. The serum will have a predetermined unit value that should be used to obtain the dilution necessary for a laboratory stock containing 2 IU/ml.

3.6.1.4 Preparation of the challenge virus and back titration

The amount of virus to be used in the test should be 50 FFD_{50}/0.1 ml. Using MEM-10 as diluent for CVS-11, a 50ml centrifuge tube containing 50FFD_{50}/0.1 ml was prepared. 1.0 ml of the 50FFD_{50}/0.1 ml was allowed for each slide in the test (for example, if running 10-test serum, 11 ml of CVS-11 was prepared in MEM-10 i.e., 10-test serum plus 1 control slide). 2-serial 10-fold dilutions (1:10 and 1:100) of CVS-11 from the 50FFD_{50}/0.1 tube were made to give 5.0 FFD_{50} and 0.5 FFD_{50}/0.1ml, respectively. 2 vials -1(1:10) and -2(1:100) dilutions were labeled and 0.9ml of MEM-10 was added to each. A pipetman was used to transfer 0.1 ml of 50FFD_{50} to the 1:10 vial, mixed several times and tip discarded. 0.1ml was transferred from the -1 vial to the -2 vial and mixed several times.

Preparation of the mouse neuroblastoma (MNA) cells was done just prior to use, 20 to 30 minutes before the end of the 90-minute incubation period.

3.6.1.5 Preparation of Rabies conjugate – FITC

Dilution factor predetermined and printed on the package insert was confirmed prior to use. The conjugate was rehydrated and diluted according to the package directions. Stock solutions of conjugate were stored as frozen aliquots in -20^{o}C freezer, while conjugate diluted to the working dilution was stored at +4^{o}C. The working dilution of the conjugate was filtered with a $\leq$ 0.45µm filter. Conjugate diluent used is 0.01M phosphate buffered saline solution at pH 7.4 to 7.6 with 0.138M NaCl and 0.0027M KCl.

Evans blue counterstain 0.5% was aliquoted and stored at +4^{o}C, amount added to the conjugate was determined by titration when the working dilution of the conjugate was determined. Cells appeared noticeably red due to counterstain which was not so strong as to diminish the specific green fluorescence. Evans blue concentration of 0.00125% was prepared by adding 2.5 microliters of 0.5% stock dye solution per ml of conjugate diluent.

3.6.1.6 Preparation of growth media for mouse neuroblastoma cells

Mouse neuroblastoma cells prefer an acidic medium with increased vitamins. Eagles minimum essential medium (EMEM) was supplemented with 2x vitamins, 2x glutamine and 10% fetal bovine serum (FBS). Penicillin (100 units/ml), streptomycin (100µg/ml, and amphotericin B (0.25µg/ml) were also added. The final concentration of sodium bicarbonate was 0.75mg/ml. The media was optimized for cell growth in closed culture. (The lids of the flasks were completely tightened). Open flask cultures were incubated in an atmosphere of 0.5% $C0_2$ at 37°C and 90% humidity. Higher $C0_2$ levels required a higher concentration of sodium bicarbonate in the medium.

3.6.1.7 Preparation of standards: seed virus suspension

One 3-day-old 150 ml flask (T150) culture of mouse neuroblastoma (MNA) cells was trypsinized. 30 x 10^6 cells were resuspended in a 50-ml conical centrifuge tube in 2.7 ml of Eagle's minimum essential medium supplemented with 10% fetal bovine serum (MEN-10). 10 x 10^6 infectious units of CVS-11 rabies virus was added and vortex – mixed once. The cells and virus were incubated for 15 minutes at 37°C, the cells were vortex-mixed once. Ten ml of MEM-10 was added, vortex – mixed, and cells centrifuged at 500 g for 10 minutes. Supernatant was discarded. The cells were resuspended in 30ml of growth medium (MEM-10) and transferred to a 150-ml flask the flask was gently rocked to mix the cell suspension, and then three 8-well tissue culture chamber slides were prepared by pipetting 0.2 ml of the cell suspension into one well of each slide. The flask and slides were incubated at 37°C in a humidified incubator with 0.5% carbon dioxide ($C0_2$). Flask was incubated as a closed culture (cap tightened).

At 20, 40 and 64 hours after incubation, one slide was acetone – fixed and stained using an immunofluorescence technique to determine the virus infectivity. The supernatant was harvested 24 hours after the cells reached 100% infectivity (typically 40 hours after infection). Supernatant was transferred to a 50-ml centrifuge tube, and centrifuged at 4000 rpm for 10 minutes. The suspernatant was distributed into 0.5 ml aliquots and stored at -80°C.

3.6.1.8 Titration of seed virus suspension

One aliquot of the seed virus was thawed and an 8-serial 10-fold dilution (10^{-1} to 10^{-8}) in MEM-10 was prepared. 0.1 of each virus dilution was distributed into one well of an 8-well tissue culture chamber slide. 0.2 ml of MNA cells suspended in MEM-10 (concentration 5 x 10^4 cells per 0.2 ml) was added to each well. The cells and virus were mixed by gently rocking the slide, then incubated at 37°C in a humidified incubator with 0.5% CO_2 for 40 hours. The slide was acetone-fixed and stained using an immunofluorescence technique. Evidence of virus infection was observed at the 10^{-6} dilution of virus, indicating a virus stock suspension containing at least 1 x 10^6 infectious units per 0.1 ml.

3.6.1.8 Preparation of stock virus suspension

A 30 x 10^6 MNA cells was infected with 10 x 10^6 infectious units of the seed virus preparation. Supernatant was harvested 24 hours after the cells reached 100% infectivity (typically 40 hours after infection). The supernatant was distributed into 0.5ml aliquots and stored at -80°C.

3.6.1.9 Titration of stock suspension

One aliquot of the stock virus was thawed and a 6 – serial 10-fold dilutions (10^{-1} to $10^{6)}$ in MEM-10 was prepared. 0.1 ml of each virus dilution was distributed into one well of an 8-well tissue culture chamber slide. 0.2 ml of MNA cell suspended in MEM-10 (concentration 1 x 10^5 cells per 0.2 ml) was added to each well. Then incubated at 37°C in a humidified incubator with 0.5% CO_2 for 90 minutes. The cells and virus suspension was mixed by gently rocking the slide, and incubated at 37°C in a humidified incubator for 20 hours. The slide was acetone fixed and stained using an immunofluorescence technique.

It is worth noting that each well of an 8-well tissue culture chamber slide contained 25 to 50 distinct microscopic fields when observed at 160 – 200 times magnification. One unit of virus for the RFFIT was determined as the dilution at which 50% of the observed microscopic fields contained one or more foci of infected cells (the focus-forming dose, FFD_{50}). The stock virus suspension contained at least 1 x 10^4 FFD_{50} per 0.1ml (i.e. the well with cells infected with the 10^{-4} dilution of the virus contained at least one focus of infected cells in 50% of the observed microscopic fields). A stock virus suspension of this titre was then diluted to $10^{-2.3}$ to obtain a challenge virus.

3.6.1.10 Reconstitution of the standard rabies immune globulin (SRIG)

Each vial of the U.S.Standard Rabies Immune Globulin contains a pre-determined number of international units (IU) of rabies antibody. For use in a rabies serum neutralization test, this serum should be diluted to 2 IU per ml. The current lot of SRIG used at the centers for Disease Control and Prevention Rabies Laboratory is Lot R-3 containing 59 IU's per vial and was used as an example for reconstitution below.

Added to a separate sterile polypropylene tube, 2.0 ml of sterile distilled water was added using a volumetric pipette (or equivalent). 1.0 ml of this water was transferred and lypholized product rehydrated, mixing several times to bring into solution. The 1.0 ml water/immune globulin was transferred to a second sterile 50ml tube. The remaining 1.0 ml of sterile water was used to rinse the sides of the vial 2 times with 0.5 ml, transferring the 0.5 ml to the rehydrated tube after rinse.

The 2.0 ml water/immune globulin was left at room temperature for 4 hours to assure complete rehydration. This was diluted to 2 IU per ml in PBS (Ca^{2+} and Mg^{2+} free) with 0.2% Difco gelation (by adding 27.5 ml of PBS/Gelatin diluent to the 2.0 ml of undiluted standard serum).

The immune globulin was placed in the refrigerator at 4°C overnight to ensure a thorough mixing of the product. (It is optional to stir the immune globulin overnight). 0.2ml per 1.0 cryovial was aliquoted (sarstedt, corning and Nunc) and frozen at -80°C.

3.6.1.11 Titration of reference serum

A replicate titration by RFFIT was performed, and the range and standard deviation were calculated. The acceptable titer for reference serum is an end-point titer of 1:200.

3.6.1.12 Propagation and maintenance of MNA monolayer cell culture

The subpassage (splitting) of stock mouse neuroblastoma cell (MNA) culture flasks was performed when the cells were at or near confluency, normally 3 to 4 days. Flasks (T75) with confluent or nearly confluent monolayer for subpassage was selected and examined prior to use. The media was carefully removed from the flask using 25 ml pipette taking care not to disturb the cell monolayer. The cell monolayer was rinsed with 5ml of PBS 4550 (Ca^{2+} and Mg^{2+} free). The flask was gently rocked back and forth to wash the residual medium from the cells. The wash step was repeated 2 ml of Trypsin – EDTA was added using 5ml pipette, and flask incubated for 5 minutes at room temperature or 2 to 3 minutes at 37°C. The cells were observed during the incubation. When the cells began to detach from the surface of the flask, the flask was held with one hand and the side and bottom of the flask was gently tapped with the palm of the hand to completely dislodge the cells.

8ml of fresh MEM-10 using a 10 ml pipette was added to the cell suspension as soon as possible to inhibit the enzymatic activity of the trypsin. The cells were dispersed by gently pipetting them up and down enough times to break apart clumps and produce an even suspension.

The resuspended cells were seeded by transferring 2ml into new culture flasks containing 23 ml of MEM-10 (transferring 2.0 ml of the cell suspension to a flask is a 1.5 split ratio; adding 1.0ml of cells is a 1.10 split). The cells were dispersed by gently swirling the flask, the flask was incubated at 37^{o}C with or without CO_2 (the flask lid was completely tightened).

Cell culture flasks of MNA cells for RFFIT were used within 4 days. Each subcultured flask was labeled with cell type (MNA), date of subcultured, new passage number and the initials of the person that splitted the flask.

3.6.1.13 Procedure for RFFIT

The mice sera were heat inactivated at 56^{o}C for 30 minutes. The test sera were diluted in MEM-10, serum end – point titrations were tested at 8-serial 5-fold dilutions (1:5 to 1:390625) and some at 4-serial 5 fold dilutions (1:5 to 1:625). Using an 8-well Tissue – Tek slide, one slide was used per test sample, and in some other instances, 2 test samples were used per slide.

The slides were labeled with corresponding test sera number. One slide per test serum was used when determining the end-point titer. The dilution factor was 8-serial 5-

fold, while in other instances, one slide was shared per 2 test sera with the dilution factor being 4-serial 5-fold. 0.075ml of MEM-10 was added to the first well using a microtiter pipette. This was the 1:5 dilution of the test serum, 0.1ml of MEM-10 was added to the seven other wells of the slide in the case of 8-serial 5-fold dilution and to the three other wells of the slide for 4-serial 5-fold dilution. 0.05 ml of test serum was added to the first well (1:5 dilution). The serum and MEM-10 was mixed several times avoiding creating bubbles. 0.025ml of the 1:5 dilution well (serum – MEM-10) was transferred to the second well (1:25 dilution) and the transfer of 0.025 ml to each consecutive well up to the final dilution (1:390625 for 8-serial 5-fold dilution and 1:625 for 4-serial 5-fold dilution) continued, discarding 0.025ml at the end. All the serum samples were prepared as described above.

3.6.1.14 Preparation of the Control Slide

A control slide with a reference serum control, a virus back titration and a cell control were prepared. 0.075 ml of MEM-10 was added to the first well of the reference serum dilution on the left of the slide. This was the 1:5 dilution of reference serum 0-1ml of MEM-10 was added to the remaining wells of the reference serum dilution wells (1:25 – 1:625) and to the 3 wells of the virus back titration. The cell control well received 0.2 ml of MEM-10. 0.05ml of reference serum containing 2 IU/ml was added to the 1:5 dilution well on the bottom left of the slide (well 1). This was mixed several times and 0.025 ml of the 1:5 dilution of the reference serum (well 1) was transferred to the 1:25 dilution well (well 2) and this was continued through to the 1:625 dilution well (well 4), and 0.025ml was discarded at the end.

The challenge virus was prepared and back titrated, (as previously described). 0-1ml of the $0.5FFD_{50}$, $5.0FFD_{50}$, and $50FFD_{50}$ of virus were added to sequential chambers (i.e. wells 2, 3, and 4 on the right of control slide respectively). 0.1ml of the virus preparation containing $50FFD_{50}$/0.1ml was added to all chambers of the test sera and reference serum dilutions. Virus was not added to the cell control well on the bottom right of the control slide.

All the slides were incubated for 90 minutes at 37^oC in a CO_2 incubator with 0.5% CO_2. Mouse neuroblastoma (MNA) cells were prepared prior to use, 20 to 30 minutes before the end of the 90-minute incubation, as previously described. After producing an even suspension of MNA cells in 10 ml of MEM-10, the 10ml cell suspension was transferred to a 25 ml conical centrifuge tube (this contains an approximate yield of 5.0×10^5 cells per ml when suspended in 32 ml of MEM-10). 0.2 ml of the 5.0×10^5 cells/ml was added to each chamber of the slides, starting with the cell control well on the bottom right corner of the control slide. The slides were incubated for 20 hours at 37^oC in a 0.5% CO_2 incubator.

3.6.1.15 Fixation of slides

After 20 hours, the supernatant was decanted and the chamber sides were removed from the slides, they were then dip rinsed in phosphate buffered saline (PBS), formula number 4588 and followed by dip rinsing in cold acetone (-20^oC). The were fixed for a longer 30-minute incubation in fresh -20^oC acetone in a freezer. Following 30-minute fixation, the slides were removed from acetone and allowed to air dry at room temperature for 10 minutes.

3.6.1.16 Staining of slides

Rabies conjugate (with working dilution previously determined) was added to each chamber monolayer sufficient to cover the entire monolayer (approximately 100 µl per well). The slides were incubated in a humidity chamber (slides were placed on a tray with a moistened paper towel and covered with the lid of a 96 well microtiter plate) at 37^{o}C for 30 minutes.

Following 30 – minute incubation, the conjugate was decanted from the slides and rinsed twice in PBS (4588) for 3 to 5 minutes each rinse. They were then dip rinsed in distilled water and placed in a slide holder on cold packs for reading using a fluorescent microscope.

3.6.1.17 Reading of slides

Each of the 8-well Tissue – Tek slide chambers contains 25 to 50 distinct microscopic fields when observed at 160 to 200 times magnification. Twenty (20) low-power (160 – 200x) microscope fields were observed in each chamber, and the number of fields that contained fluorescing cells were counted starting in one of the corners of the well. The control slide was read first, and the number of positive fields (fields with fluorescing foci) was recorded on the RFFIT data sheet. Also the test serum slides were observed and the numbers of positive fields were recorded on the RFFIT data result sheet. The 50% end-point titres for control slide and each serum was calculated and determined.

3.7 Development and preparation of Challenge Virus

A challenge virus was developed and titrated for use as chanllenge of the inoculated mice. A sample of central nervous system (CNS) from Chad, which is a representative of African 2 dog virus, was used for the development of a challenge virus. The dog brain samples from Chad (numbers: 2006 – 70 and 2006 – 45) that had previously been sequenced were homogenized. Supernatant was added to MNA cell suspension and percentage (%) infection was monitored.

Slides were made and stained to check for infectivity. The percentage infectivity (% infection of mouse neuroblastoma cells MNA cells) was monitored for one week. When virus number (% infection MNA cells) reached 90%, 30 million of virus infected MNA cells was added to 2 T150 flasks (virus 70 and 45) plus 25 ml of MEM-10 each (5^{th} passage P5).

Supernatant was collected after 48 hours for virus titration and 25 ml of fresh MEM-10 added to each flask. Supernatant was titrated, 7 serial 10 fold, starting with undiluted supernatant. The T150 flasks were passage four days, later, 1.0 ml of infected MNA cells were each (virus 70 and 45) added to 2-0 ml of fresh MNA cells in T75 flasks and monitored for percentage infection (P6). Supernatant was collected for titration after 72 hours and each flask passage the 7^{th} time (P7). 2ml of infected MNA cells was added to 2ml fresh cells to F75 flasks and monitored for percentage infection. Supernatant was collected for titration 48 to 72 hours (see Appendix XIV for titration). Supernatant was collected for titration, flasks were refed with 20ml fresh MEM-10. Twenty-four hours later, the supernatant was titrated. Each flask was passaged (P8) and 2ml of infected MNA cells was added to 2ml fresh cells to T75 flasks, monitored for percentage infection and supernatant collected for titration 48 to 72 hours. 2 ml infected plus 2 ml fresh MNA cells plus 16ml MEM-10 was passaged, and supernatant collected, flasks refed with 20 ml MEM-10.

Finally, supernatant was collected 48 hours later and flasks were discarded. A virus titer of 10 to the 7/ml was obtained (highest titer virus) and stored (see Appendix XIV). The virus supernatant was stored at -80 in aliquots of 0.5 ml/tube.

3.7.1 Inoculation of mice (virus titration challenge in mice)

The neat virus was removed from -80^{o}C and thaw on bucket of ice. Dilutions of 10^{-1}, 10^{-2} and 10^{-3} were made in MEM-10 by adding 100 µl of neat virus into 900µl of MEM-10, vortexed (to get 10^{-1}) and 100µl was pipetted from the 10^{-1} dilution and added to 900 µl of fresh MEM-10 and vortexed well to get the 10^{-2} dilution. Similarly, 100 µl was pipetted from 10^{-2} into fresh 900 µl of MEM-10 and vortexed to get 10^{-3} dilution.

Three week old mice were housed in batches of 5 mice per cage. Each mouse in cages A, B, C and D was inoculated with 50 µl of neat, 10^{-1} , 10^{-2} and 10^{-3} virus dilution respectively. The mice were observed for signs of rabies for 21 days.

3.7.2 Inoculation virus challenge of vaccinated mice

Mice inoculated with single dose of the prepared plasmid DNA vaccines were challenged on day 30 following vaccination with 50 µl of 10^7 TCID 50/ml titre at 10^{-1} virus dilution per mouse while mice that received multiple doses were similarly challenged, 3 weeks post second booster dose with each mouse receiving 50 µl of 10^{-1}

dilution of virus. The mice were observed for signs of rabies on mice for a period of 21 days and beyond.

3.8 Direct Rapid Immunohistochemistry Test

Direct Rapid Immunohistochemistry Test (DRIT) is used for the detection of rabies virus antigen. It is an unlicensed procedure designed for consideration as a potential confirmatory measure of the direct fluorescent antibody test according to the national standard operating procedure for the diagosis of rabies in animals (http://www.cdc.gov/ncidod/dvrid/rabies/professional/publications/DFA-diagnosis/DFA-protocol-b.htm). All the mice that died after the challenge using rabies virus were subjected to DRIT and DFA as previously described.

3.8.1 Preparation of reagents

Phosphate buffered saline with 1% TWEEN-80 (TPBS). TPBS is made with a combination of PBS, with 1% tween-80 added to 1 litre of PBS. The mixture was shaken until RWEEN-80 was completely dissolved into a solution (i.e., 1:100 ratio). Alternatively the mixture can be put on a stirrer if available.

3.8.2 Hematoxylin

Gills formulation #2 was diluted 1:2 in distilled water. For a 250 ml dish, 125 ml hematoxylin was added to 125 ml deionized water, and gently mixed.

3.8.3 Peroxidase substrate: amino-ethylcarbizole (AEC)

Stock solution: One 20 mg table of 3-amino 9-ethyl carbazole (AEC) was dissolved in 5ml of N1 N1 dimethylformamide in a glass wheaton jar, labeled ‘AEC stock’, dated and stored in the refrigerator (4^oC) for 1 to 2 months. This was cove red with foil before putting into the refrigherator because it is sensitive to light.

3.8.4 Working dilution: This was prepared with each test p;rior to stanining.

7 ml of acetate buffer was added to 15 ml centrifuge tube using a 10 ml plastic pipette. Then 0.5 ml of AEC stock soluition was added using a 1 ml pyrex (glass) pipet. Also 0.075 ml (75µ) of 3% hydrogen peroxide was added.

The mixture was filtered using a 10 ml syringe with syringe filter (0.45 µm) into a separate 15ml centrifuge tube. This mixture once made is only stable for 2-3 hours.

3.8.5 Procedure for DRIT

Routine touch impressions of the suspected CNS tissues from mice that died after challenge were made on labeled glass microscope slides. Also touch impressions of standard positive and negative controls were made. The slides were air-dried for 5 minutes at room temperature. The slides were immersed in 10% buffered formalin at room temperature for 10 minutes then removed and dip-rinsed several times to wash off any excess fixative in wash buffer TPBS (PBS with 1% tween 80). The slides were immersed in 3% hydrogen peroxide for 10 minutes. Excess hydrogen peroxide was removed by dip-rinsisng slides in TPBS. The slides were transferred for another rinse (after dipping, excess buffer was shaken off, and blotted from slide edges surrounding the impression).

One slide was done at a time leaving the remaining humidity chamber (or using the plastic top to a 96-well plate or another simple cover over slides, on a moistened paper towel, on lab bench top) at room temperature with p;rimary antibody-biotinylated anti-rabies MAb for 10 minutes. Enough of this primary antibody was added by drop to cover the impression.

After incubation, excess conjugate was shekn off. Slides were dip-rinsed in TPBS, excess buffer is shaken off and blotted from slide edges surringing the impression Slides were incub ated with strep;tavidin-peroxidase comples, adding enough by drop to cover the impression, in numidity chamber at room temperature for 10 minutes. After incubation, excess is shaken off. Then they were dip-rinsed with TPBS. Excess buffer was shaken off and blotted from slide edges surrounding the impression. The slides were incublated with peroxidase substrate, amino-ethylcarbizone (AEC). Enough of the AEC was added to the slide by drop to cover the impression in a humidity chamber at room temperature for 10 minutes. After incubation,. Excess substrate was shaken off, and slides were dip-rinsed in deionized/distilled water to ensure removal of excess stain. The slides were then transferred to fresh distilled water to rinse.lides were mounted with ater-solution mounting medium and covered with cove r-slip. When multiple slides were stained, they were left in the deionized/distilled water rinse before cover-slipping.

The slides were viewed by light microscope, using a 20x objective to scan the field, and a 40x objective for higher power inspection and results were recorded.

3.9 Statistical Analysis

Results obtained from all different times of administration and routes were subjected to statistical analysis using Fisher exact test – two tailed probability test.

CHAPTER FOUR

RESULTS

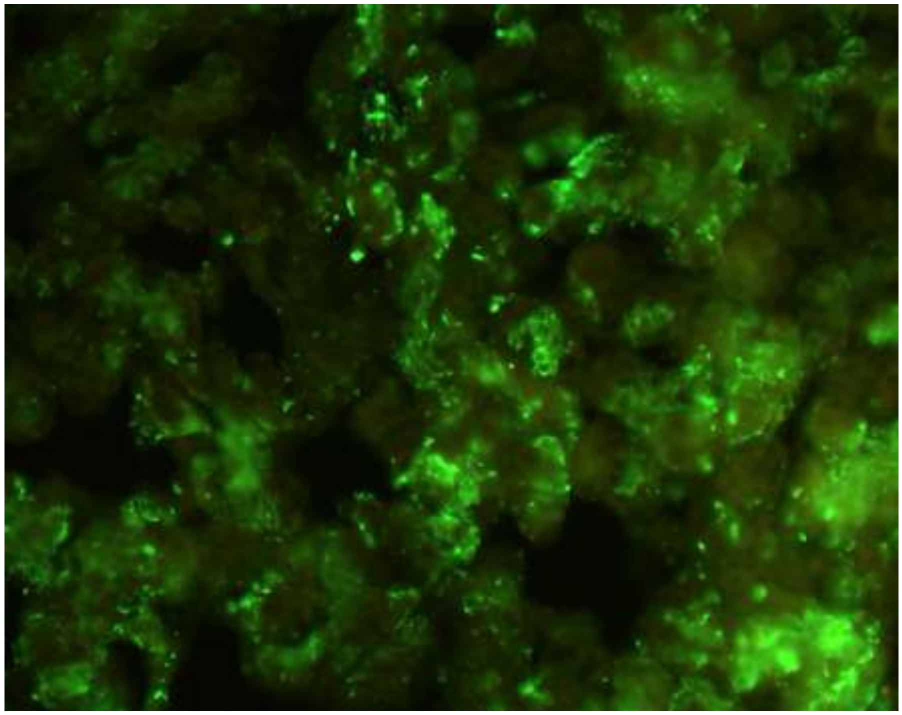
4.1 Postmortem Diagnosis of Rabies in Animals by Direct Fluorescent Antibody Testing

4.1.1 Staining intensity

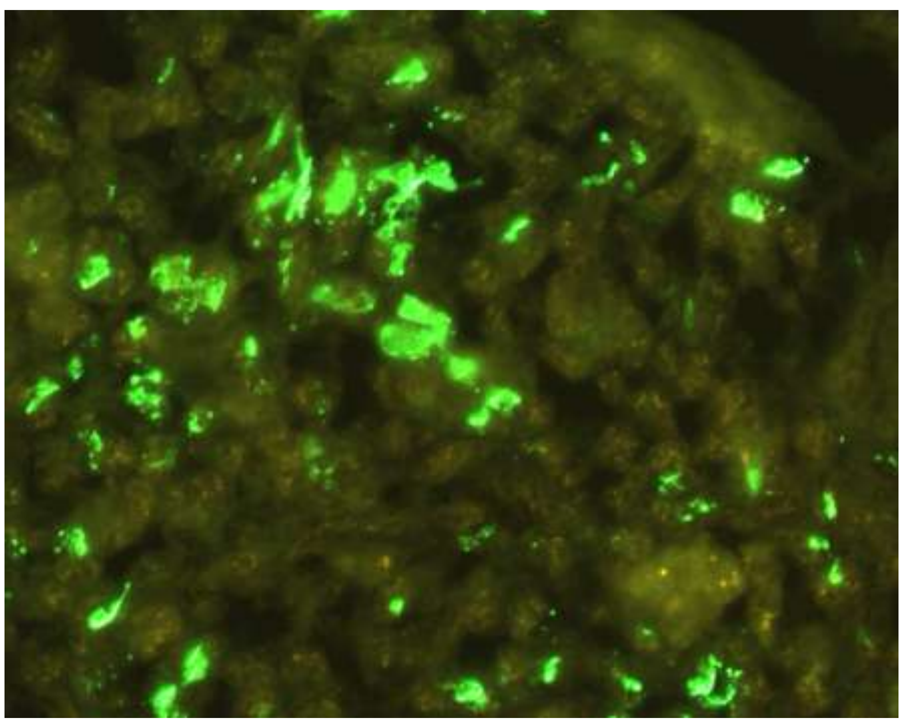
A minimum of 80 total fields were examined: 40 fields stained with reagent 1 (CENTOCOR FITC Anti-Rabies Monoclonal Globulin) and 40 fields stained with reagent 2 (Monoclonal antibody FITC conjugate). Observations made for each test slide were recorded as staining intensity/antigen distribution (e.g. +4/ + 2). Staining intensity was graded from +4 to +1. Of the one hundred and sixty-five samples tested, fifty one were found to have +4 to +3 staining intensity/antigen distribution and considered positive which accounted for just 30.9% of the total samples. Whereas from the source of sample collection, all the samples were considered positive to rabies infection using Seller's staining method, even though Seller's staining has been reported to be only 70 – 75% accurate (Beran, 1984). Positive control slides in all the tests contained staining of +4 intensity (i.e., a glaring, apple green brilliance) (Plate 4.1); this intensity was observed in 29 of the samples (Table 4.1), slightly diminished staining intensity (i.e., a slight loss of glare) was graded as +3 intensity (Plates 4.2) and this was observed in 22 samples, while noticeably dull stain was graded +2 to +1 which was observed in 53 samples (Plates 4.3). No staining was observed in 61 samples (Plates 4.4) and the negative control slides (Plate 4.4).

Table 4.1: Direct Fluorescent Antibody Testing (Staining Intensity) of Brain Samples from Dogs suspected to have died from Rabies.

Staining Intensity	Number of Samples	Percentage (%)
+4	29	17.6
+3	22	13.3
+2	53	32.1
+1	61	37.0
TOTAL	**165**	**100**

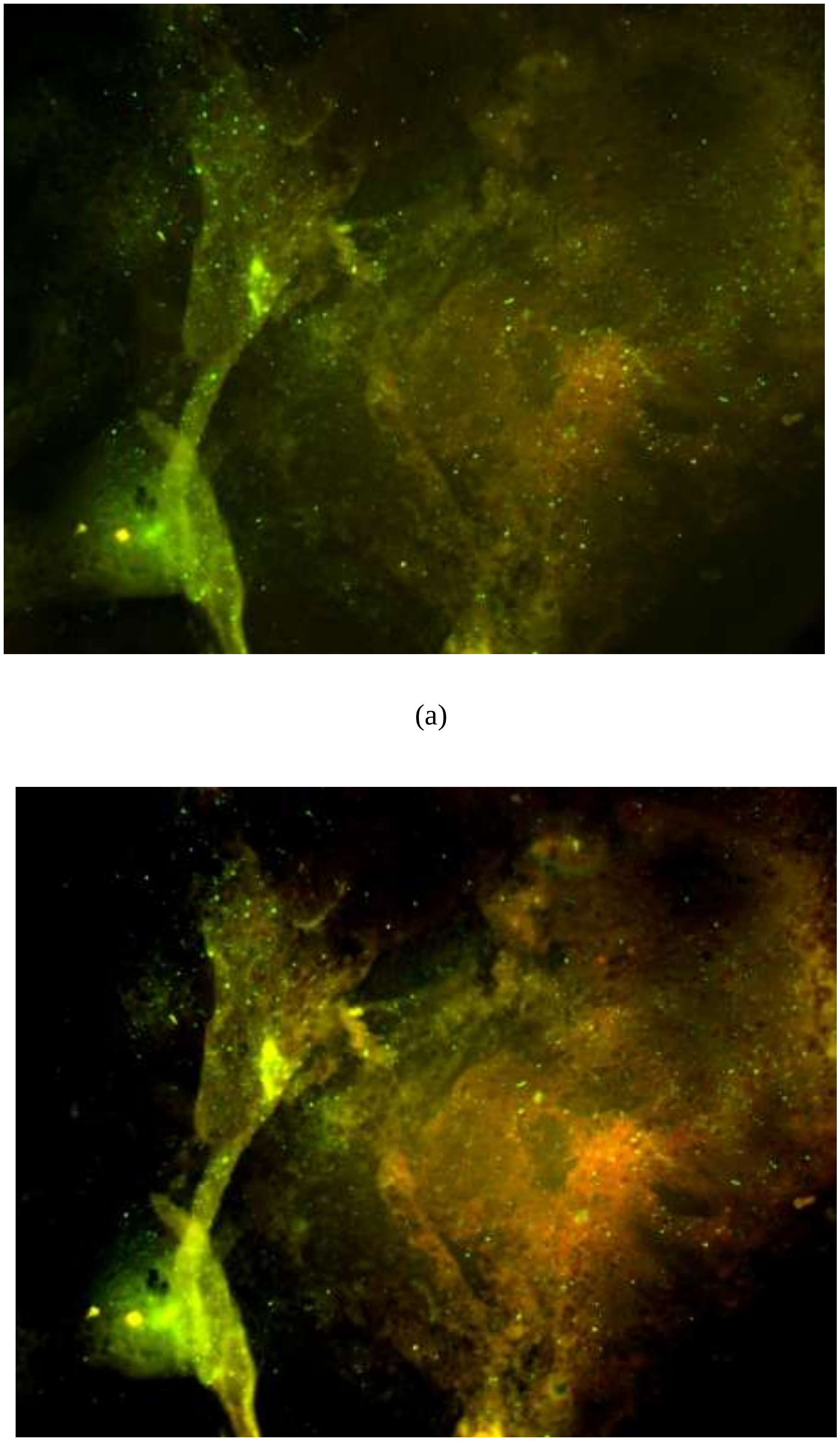


(a)



(b)

Plate 4.1 (a-b): DFA positive test results showing +4 staining intensity



(a)

(b)

Plate 4.2 (a-b): DFA positive test results showing +3 staining intensity

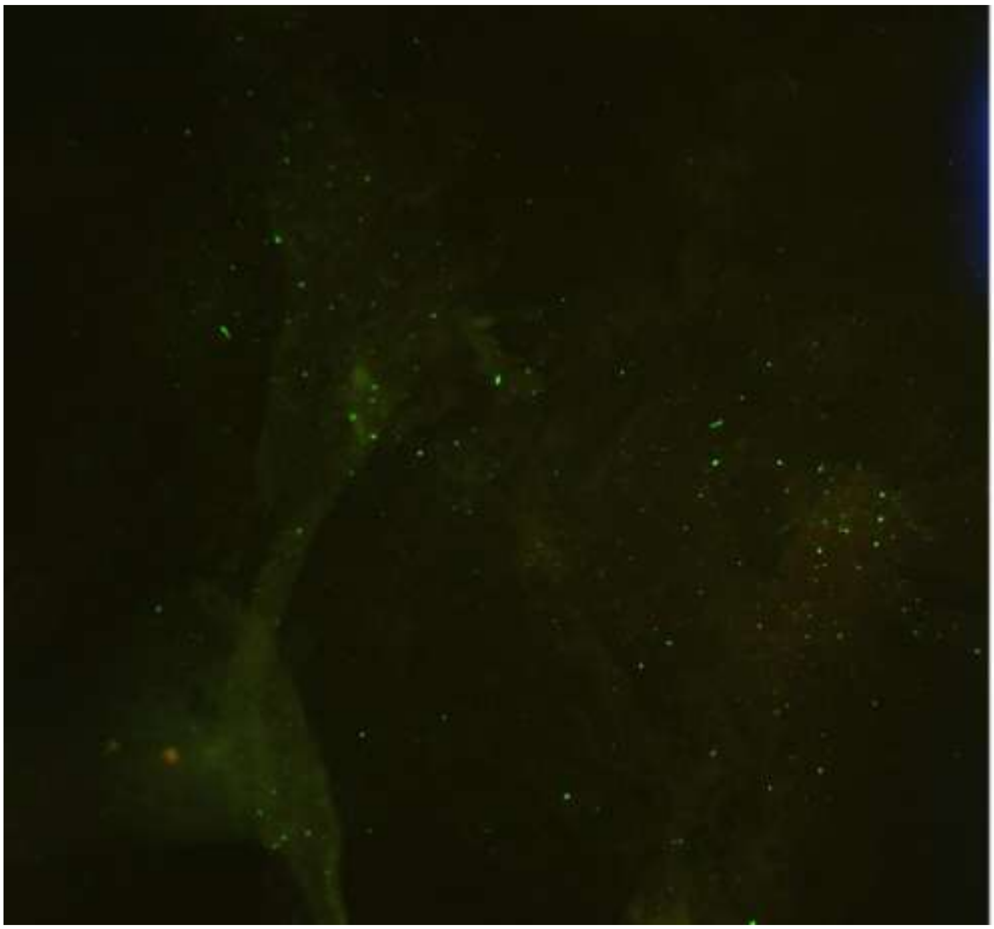
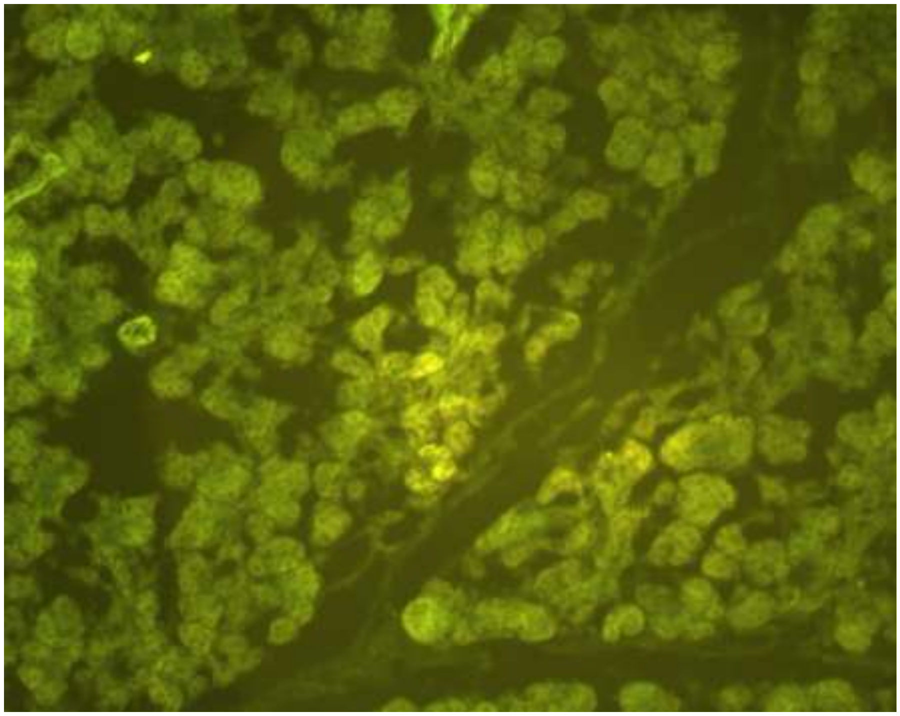
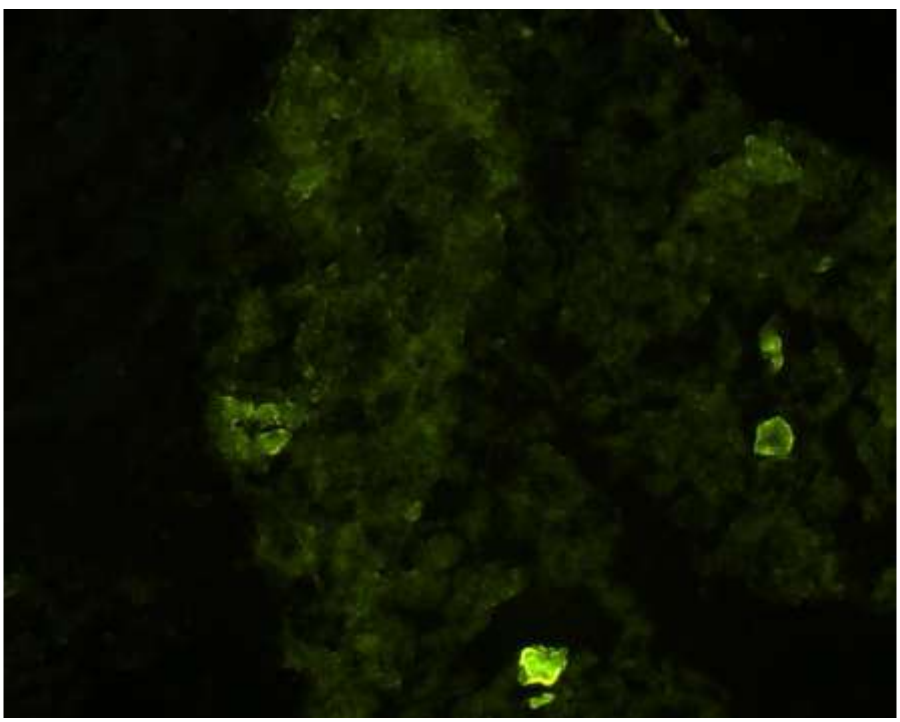


Plate 4.3: DFA test result showing +2/+1 staining intensity



(a)



(b)

Plate 4.4 (a-b): DFA test results showing no staining

4.1.2 Antigen distribution

A massive infiltration of large and small inclusions of varying shape in almost every area of the impressed was graded as +4 (Plate 4.5); this was observed in positive control and in 40 of the samples (Table 4.2), while +3 denoted for inclusions of varying size and shape found in almost every microscopic field, with vary number of inclusions per field but inclusions were numerous in most fields was observed in 11 of the samples (Plates 4.6), +2 was used for samples (50) with inclusions of varying size and shape present in 10% to 50% of the microscopic fields which most fields contained only a few inclusions (Plates 4.7) . And +1 was used for inclusions of varying size and shape present in < 10% of the microscopic fields with only a few inclusions found per field (usually one or two inclusions per field) (Plate 4.8), this was observed in just three (3) of the samples. No staining was observed in 61 of the samples (Plates 4.4).

Table 4.2: Direct Fluorescent Antibody Testing (Antigen Distribution) of Brain Samples from Dogs Suspected to have died from Rabies

Antigen (Ag) Distribution	Number of Samples	Percentage (%)
+4	40	24.2
+3	11	6.7
+2	50	30.3
+1	3	1.8
No Ag observed	61	37.0
TOTAL	**165**	**100**

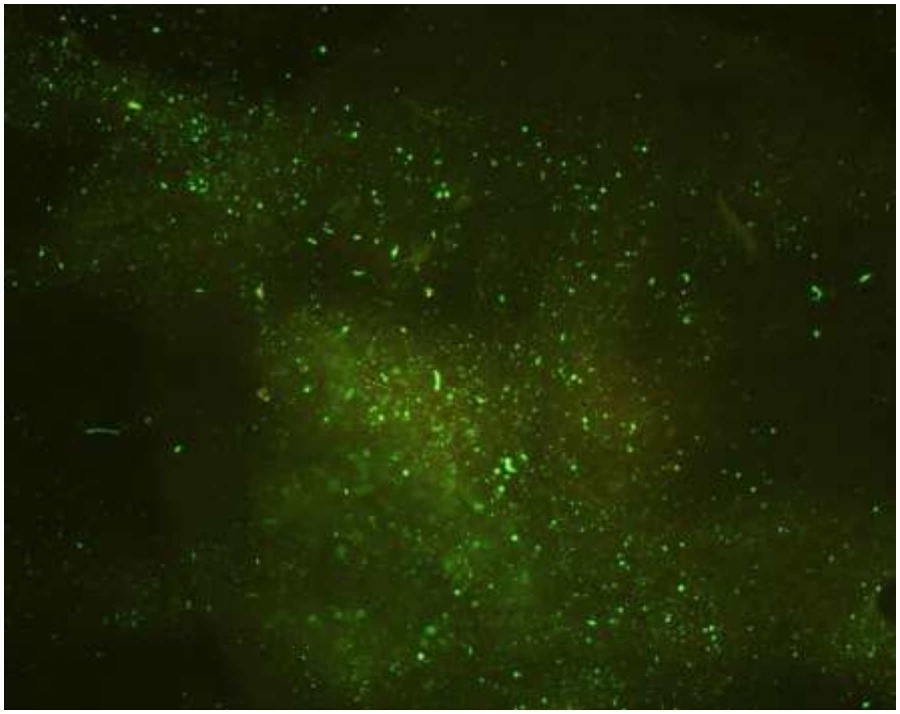


Plate 4.5: DFA test result showing +4 antigen distribution

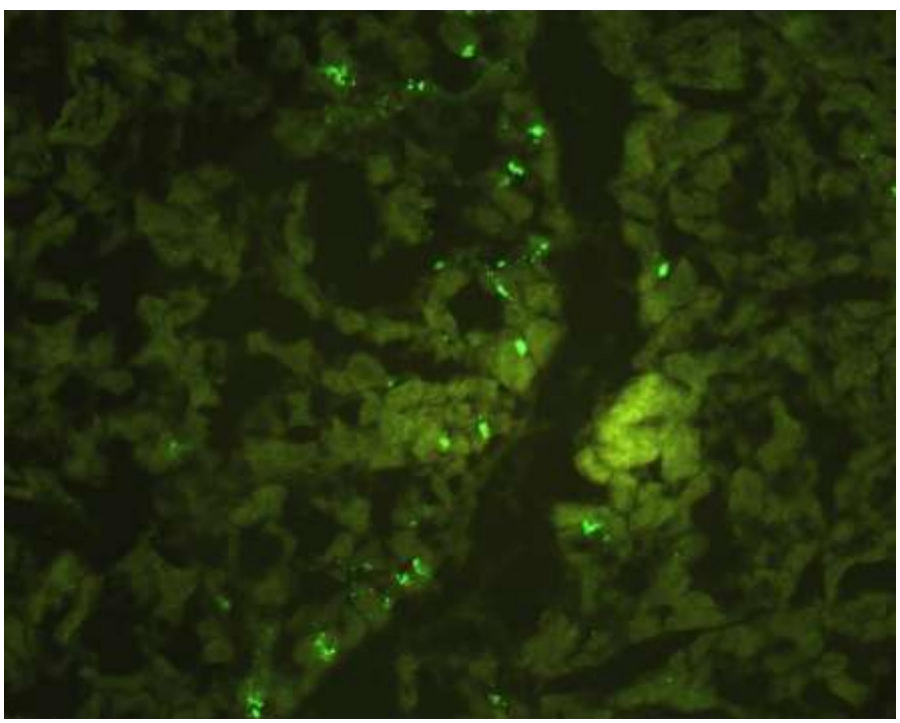


Plate 4.6: DFA test result showing +3 antigen distribution

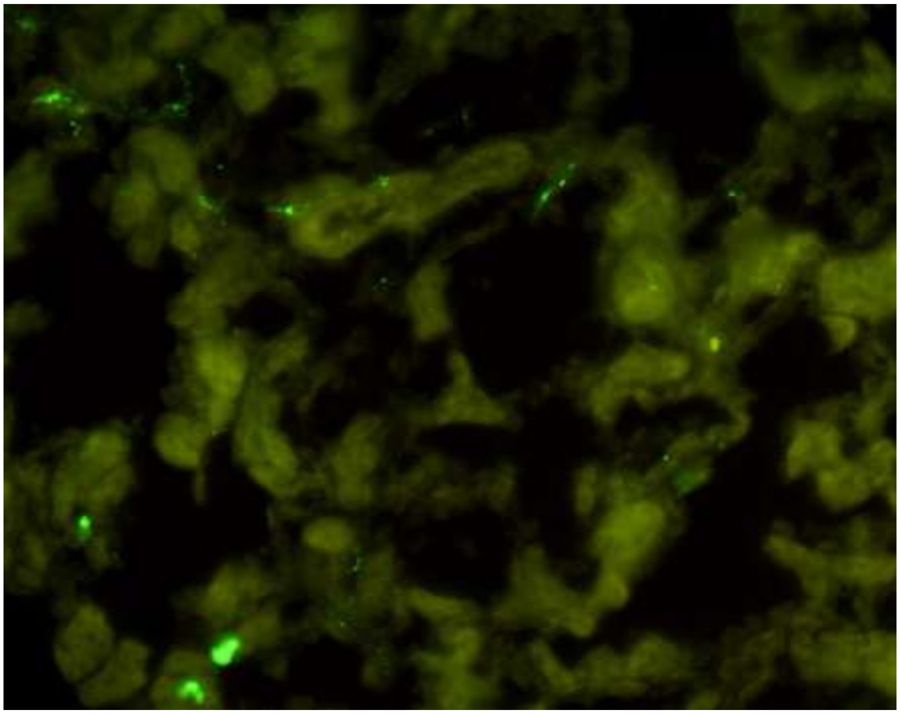


Plate 4.7: DFA test result showing +2 antigen distribution

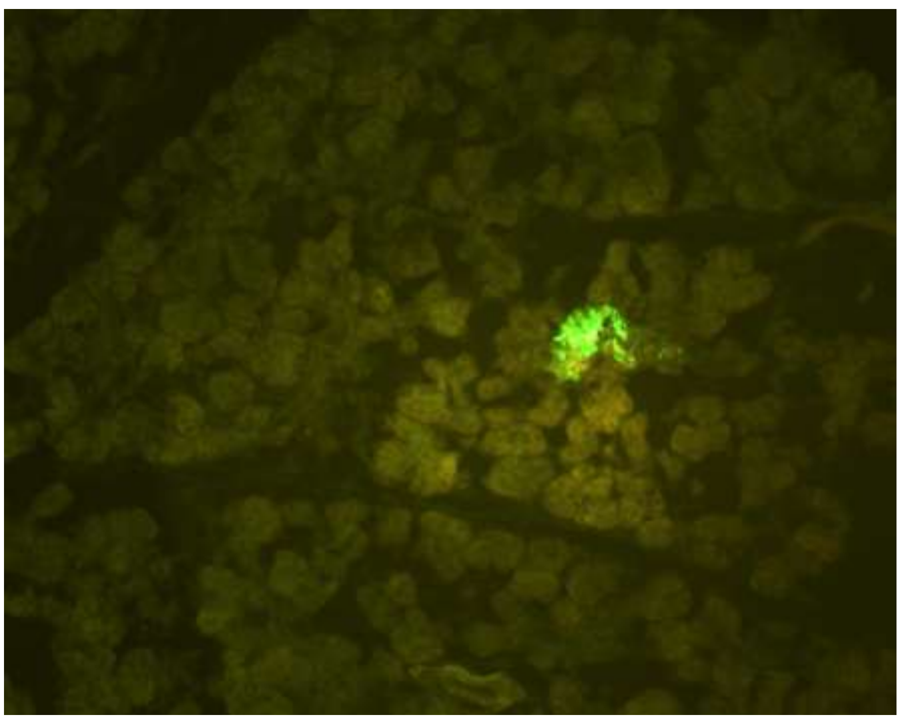


Plate 4.8: DFA test result showing +1 antigen distribution

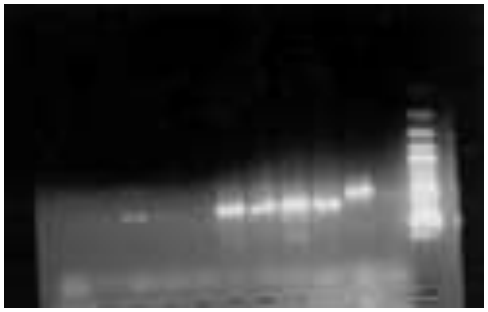
4.2 Mouse Inoculation Test

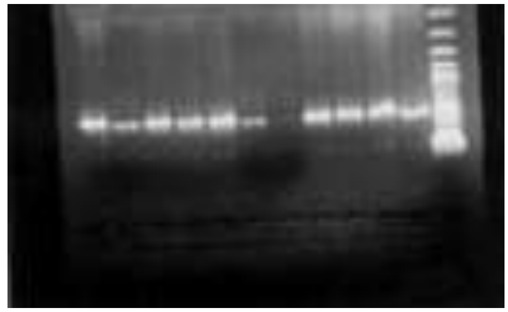
A total of 14 samples were prepared according to mouse inoculation test (MIT) techniques and inoculated into pools of suckling mice as described in the technique and observed for signs of rabies for at least 21 days. From sample RD17, 1 mouse was found dead within 48 hours of inoculation. During a period of about 5 – 6 days 1 mouse each was lost from the group of mice inoculated with samples ZAD2, NLLB, RD39, RD44 and RD181. No signs suggestive of rabies or any further death were observed in the rest of the groups (Raw data in Appendix XXVII).

4.3 Ribonucleic Acid Extraction

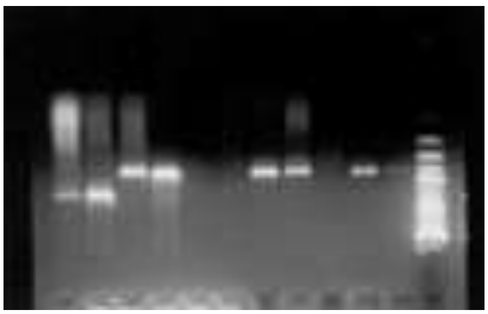
Out of the fifty-one (51) samples, total RNA was successfully isolated from thirteen (13) of the samples.

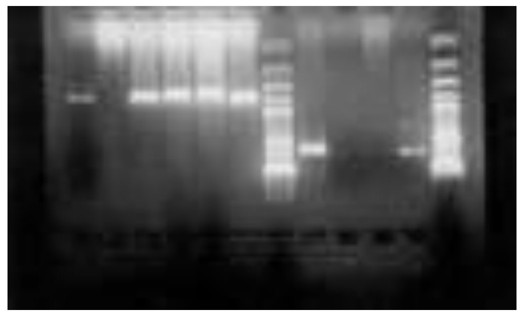
Quantitation of the extracted RNAs had a range of A260/ A280 > 1.5 to 2.4 (Table 4.3) when diluted into TE buffer solutions. Sample NLLB had A260/A280 < 1.6 (i.e., 1.5. The result of reverse transcription – polymerase chain reaction (RT-PCR), polymerase chain reaction (PCR) that was carried out on the extracted RNA to identify the rabies genes (Plates 4.9), through DNA sequencing was used to classify the isolates into their phylogenetic group. Analysis of the polygenetic relationships, performed on the complete nucleoprotein (N) coding gene, established them to belong to the genotype 1(Fig 4.1) comprising of typical dog rabies virus with their geographical location pointing to West Africa and the animal species as dog (Fig. 4.2). The nucleotide (ntd) sequences generated in this study had 450 amino acids (1350 ntds) (Appendix XXV). Further classification showed that the strains originate from the West African dog rabies (Figs 4.2). Samples RD44 and RD42 are very closely situated to Africa 3 group (African mongoose) based on their phylogenetic classification on the phylogenetic tree (Fig 4.2).





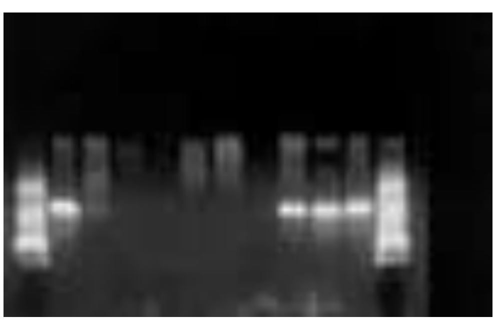
(a) **(b)**

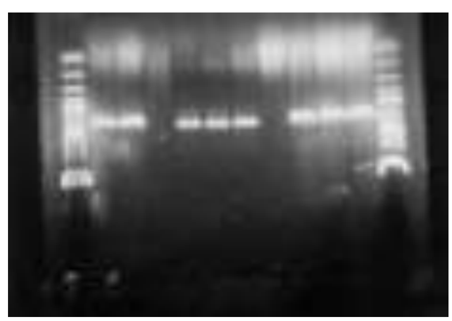




(c) **(d)**

(e) **(f)**





(g) **(h)**

Plate 4.9(a-h): Gel Electrophoresis of Dog Brain Specimens from Nigeria.

The key to the plates (a-h) are given below.

<u>Key to Plate 4.9</u>

The discription of the plates are from right to left starting with the marker and followed by the samples (wells 2 to 12).

Plate	1	2	3	4	5	6	7	8	9	10	11	12
a	Marker	RD173	ZAD4	ZAD1	ZAD2	ZAD8	ZAD10	RD39	RD44	RD67	RD163	RD132
b	Marker	ZAD1	ZAD2	ZAD8	ZAD10	RD39	RD44	RD67	RD132	RD163	RD194	NL16
c	Marker	RD42	RD44	RD67	RD132	RD137	RD163	RD193	RD194	NL16	NL16	ZAD1
d	Marker	RD194	NL16	RD173	ZAD4	Marker	ZAD1	ZAD2	ZAD8	ZAD10	RD7	RD39
e	Marker	RD7	RD17	NL		RC1	SRC		RD113	RD132	RD181	
f	Marker	RD7	RD17	NL		RC1	SRC		RD113	RD132	RD181	
g	Marker	RD7	RD17	NL		RC1	SRC		RD113	RD132	RD181	
h	Marker	ZAD2	ZAD8	ZAD10	RD7	RD39	RD42	RD44	RD67	RD132	RD137	Marker

Primers used

Plate a (wells 2 and 3): 550F-304B, (wells 4 to 12): 001F-1066B

Plate b (wells 2 to 12): 550F-304B

Plate c (wells 2 to 10): 1066F-304, wells (11 and 12): 001F-550B

Plate d (wells 2 to 5): 001F-1066B, wells (7 to 12): 1066F-304

Plate e (wells 2 to 4, 6, 7, 9 to 11): 1066F-304B

Plate f (wells 2 to 4, 6, 7, 9 to 11): 001F-550B

Plate g (wells 2 to 4, 6, 7, 9 to 11): 550F-304

Plate h (wells 2 to 11): 001F-550B

Table 4.3: Quantitation of Extracted RNAs

Sample Number	A260/A280
ZAD1	1.8
ZAD2	2.2
ZAD4	1.8
ZAD8	2.0
ZAD10	1.9
RD42	1.8
RD44	2.4
RD132	2.4
RD181	2.2
RD194	1.9
NLLB	1.5
NL16	2.1
RD137	1.8

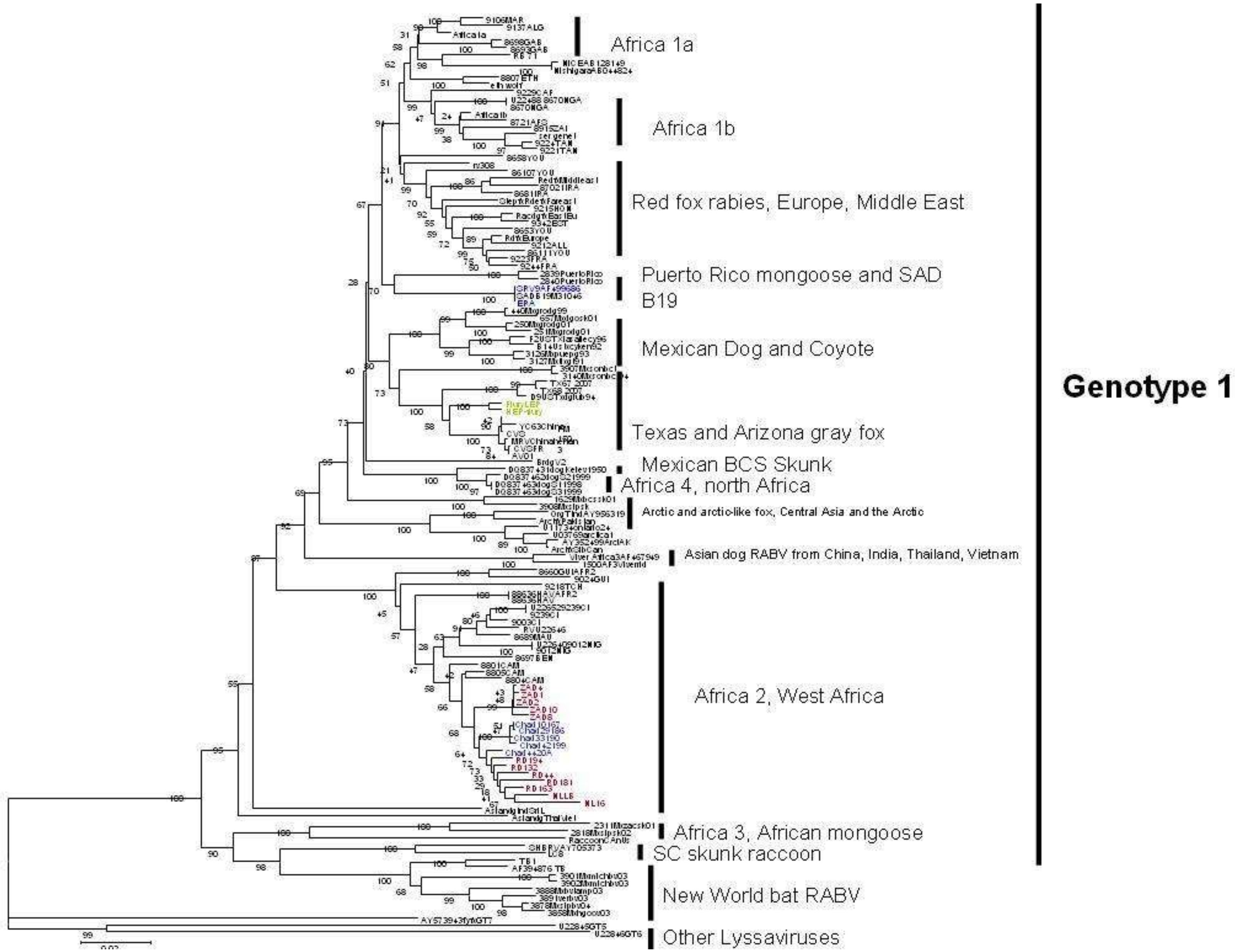


FIG 4.1: Phylogenetic Tree Classification of Rabies Virus into Phylogroup Using Nucleoprotein Gene.

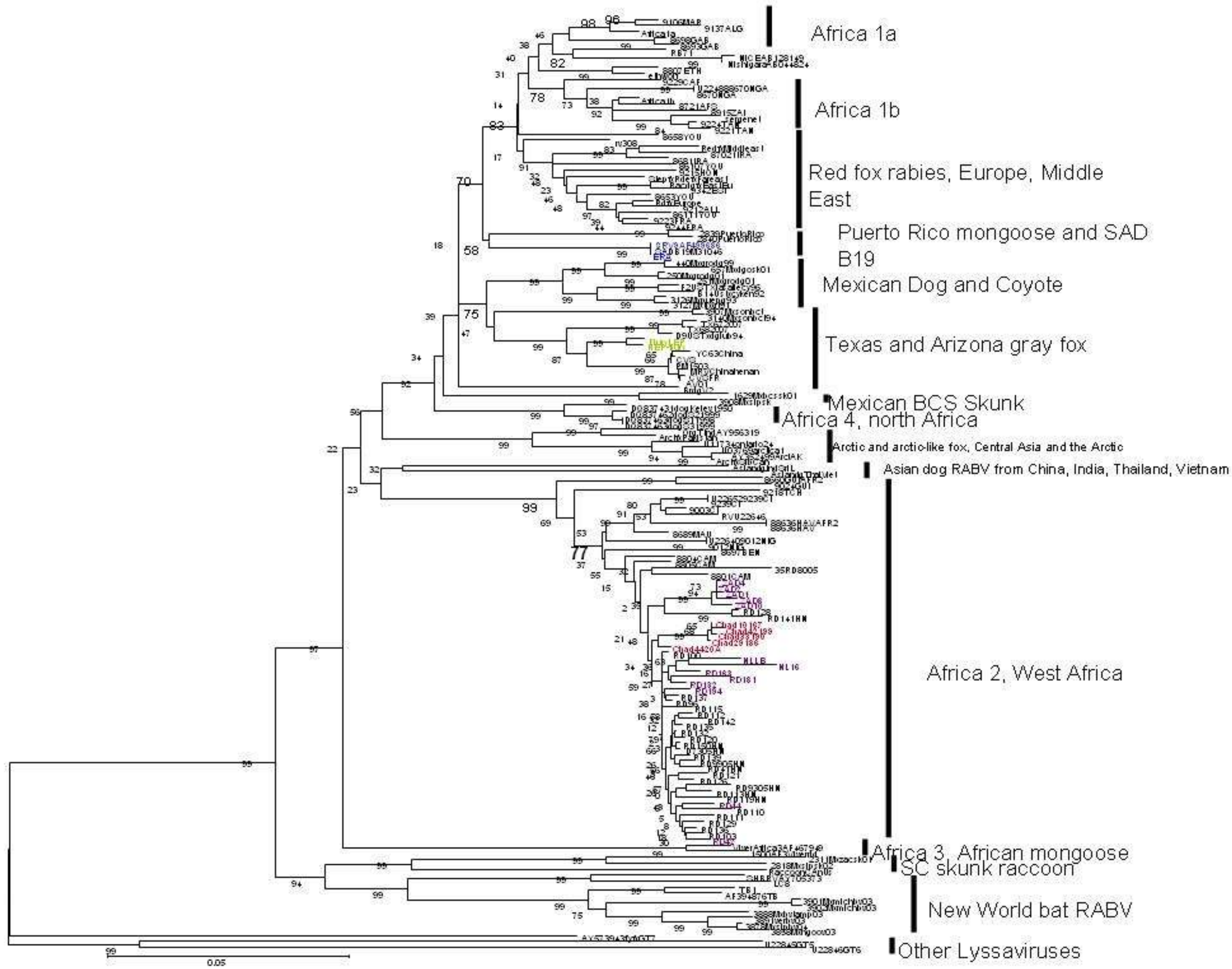


FIG 4.2: Phylogenetic Classification of Rabies Virus Showing the Origin of the Different Variants.

4.4 Vaccine, Vaccination and Challenge

4.4.1 Vaccine

For the purpose of these studies, the Plasmid DNA vaccine made from modified Glycoprotein gene of the Evelyn – Rokitnicki – Abelseth ($ERAg^3$) was referred to as $CDAG_3$ (Plates 4.10a, 4.10b and 4.12) and those from the Glycoprotein gene of the West Caucasian Bat Virus (WCBV) was referred to as WCBVG (Plates 4.11a and 4.11b).

Serum from blood collected from all the mice prior to inoculation of either vaccine or from control using buffer, that is, on day 0 (DO) had titers ≤ 5 or 11 with a geometric mean titer (GMT) of O which served as the baseline data.

Plate 4.10a: Gel Electrophoresis of CDA-G3 Vaccine

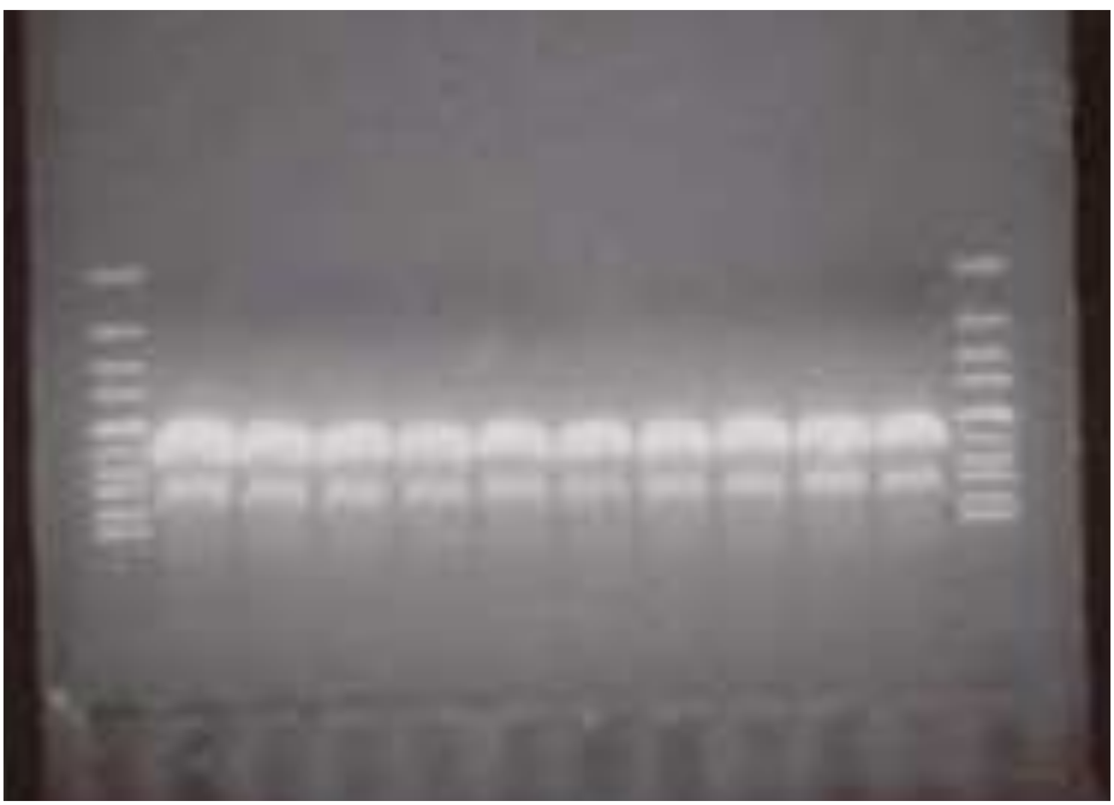


Plate 4.10b: Gel Electrophoresis of CDA-G3 Vaccine

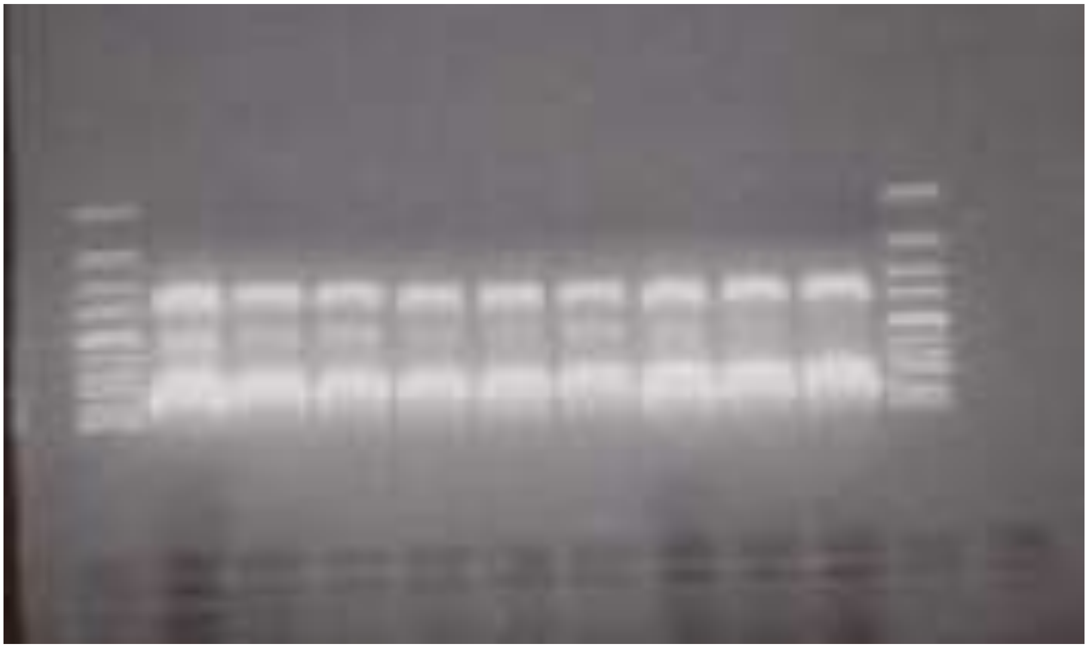


Plate 4.11a: Gel Electrophoresis of WCVBG Vaccine

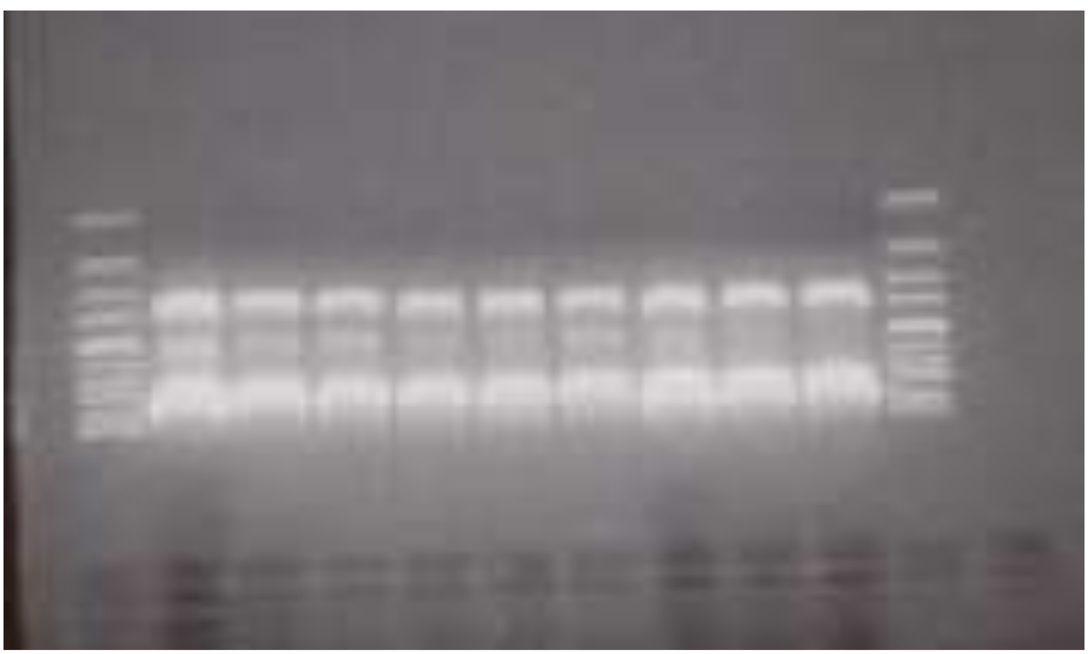


Plate 4.11b: Gel Electrophoresis of WCVBG Vaccine

Plate 4.12: Gel Electrophoresis after Plasmid Purification of CDAG3

4.4.2 Vaccination and challenge

4.4.2.1 The multiple administration regimens

In the multiple administration intramuscular vaccination schedule using the CDAG3 where 3 shots of 100 µg of the plasmid encoding the modified glycoprotein G of the ERA strain was administered at 3 weekly interval, neutralizing antibody was detected in sixty percent (60%) of the vaccinated mice as early as day 21 without receiving a booster shot and the antibody titer gradually continued to increase until after the second booster (Fig. 4.3).

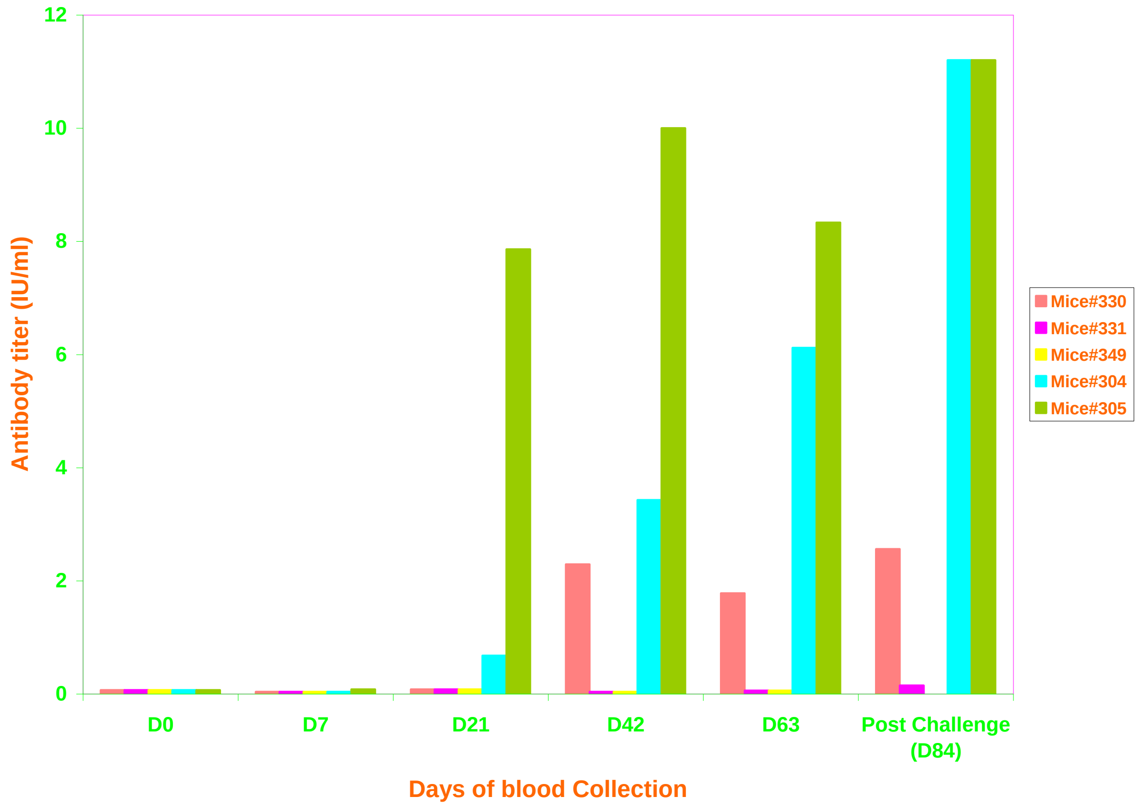


Fig: 4.3a: Effect of Multiple Administration of CDA-G3 Vaccine using Intra-Muscular (IM) route: Animal to Animal variation.

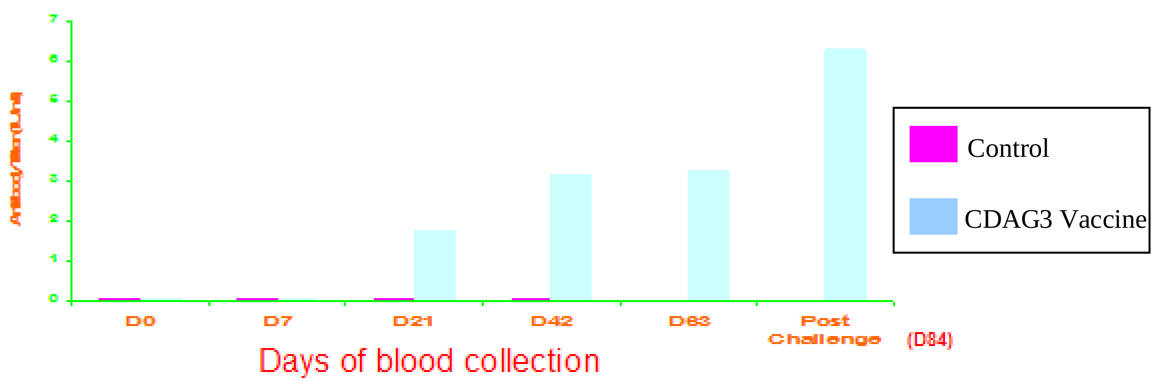


Fig: 4.3b: Effect of Multiple Administration of CDA-G3 Vaccine using Intra-Muscular (IM) Route.

Evidence of neutralizing antibodies was detected using the Rapid Focus Fluorescent Inhibition Test (RFFIT). In the course of the experiment, forty percent (40%) of the experimental subjects did not show any traceable neutralizing antibody using RFFIT. The detectable antibody in the serum of the vaccinated subjects shown as early as day 21 (D21) post inoculation had a GMT of 232.6 (Table 4.4), the GMT increased to 602.56 by day 42 receiving the first boost of the vaccine on day 22 and this increased consistently by day 63 with a GMT of 812.83 after receiving the second boost on day 43. The experimental animals were challenged on day 63 and observed. Having challenged the mice with 50 µl of 10^7 $TCID_{50}$/ml titre street rabies virus strain from Chad, a closely related variant to the Nigerian rabies street virus belonging to the African 2, West African dog rabies virus(Fig 4.2) . All the mice in this group survived the challenge (Fig. 4.4) even including the forty percent (40%) that did not show detectable neutralizing antibody prior to the challenge. One mouse was lost in this group during the D63 bleeding.

Table 4.4: Antibody titre (IU/ml) of mice vaccinated with CDAG3 Plasmid DNA rabies vaccine (encoding modified glycoprotein gene) using multiple administrations and different routes.

	Days of Blood Collection.																
	D0		First Inoc.	D 7		D 21		1st Boost	D 42		2nd Boost	D 63		Challenge	Death post Challenge	Post Challenge	
	Titre	IU/ml		Titre	IU/ml	Titre	IU/ml		Titre	IU/ml		Titre	IU/ml		No of days	Titre	IU/ml
Sample Number																	
CDAG3 i/m 330	≤ 11	≤ 0.07		≤ 5	≤ 0.04	≤ 11	≤ 0.08		320	2.29		320	1.78		Survived	320	2.56
CDAG3 i/m 331	≤ 11	≤ 0.07		≤ 5	≤ 0.04	≤ 11	≤ 0.08		≤ 5	≤0.04		≤ 11	≤ 0.06		Survived	19	0.15
CDAG3 i/m 349	≤ 11	≤ 0.07		≤ 5	≤ 0.04	≤ 11	≤ 0.08		≤ 5	≤ 0.04		≤ 11	≤ 0.06		Survived *		
CDAG3 i/m 304	≤ 11	≤ 0.07		≤ 5	≤ 0.04	95	0.68		480	3.43		1100	6.12		Survived	1400	11.2
CDAG3 i/m 305	≤ 11	≤ 0.07		≤ 11	≤ 0.08	1100	7.86		1400	10		1500	8.33		Survived	1400	11.2
Geometric mean titre	0			0		323.6			602.6			812.8				333	
CDAG3 s/c 350	≤ 11	≤ 0.07		≤ 5	≤ 0.04	≤ 11	≤ 0.08		8	0.06		≤ 11	≤ 0.06		12		
CDAG3 s/c 351	≤ 11	≤ 0.07		≤ 5	≤ 0.04	≤ 11	≤ 0.08		≤ 5	≤0.04		≤ 11	≤ 0.06		11		
CDAG3 s/c 346	≤ 11	≤ 0.07		≤ 5	≤ 0.04	≤ 11	≤ 0.08		320	2.29		125	1.25		Survived	290	2.32
CDAG3 s/c 352	≤ 11	≤ 0.07		≤ 11	≤ 0.08	≤ 11	≤ 0.08		13	0.09		19	0.11		11		
CDAG3 s/c 353	≤ 11	≤ 0.07		≤ 11	≤ 0.08	≤ 11	≤ 0.08		≤ 5	≤ 0.04		≤ 5	≤ 0.03		14		
Geometric mean titre	0			0		0			32.11			48.98					
CDAG3 pos 371	≤ 11	≤ 0.06		≤ 5	≤ 0.04	≤ 11	≤ 0.08		320	2.29		≤ 5	≤ 0.03		16		
CDAG3 pos 372	≤ 11	≤ 0.06		≤ 11	≤ 0.08	≤ 11	≤ 0.08		≤ 5	≤ 0.04		≤ 5	≤ 0.03		15		
CDAG3 pos 373	≤ 11	≤ 0.06		≤ 11	≤ 0.08	≤ 11	≤ 0.08		≤ 5	≤ 0.04		≤ 5	≤ 0.03		11		
CDAG3 pos 374	≤ 5	≤ 0.03		≤ 11	≤ 0.08	≤ 11	≤ 0.08		≤ 11	≤ 0.08		≤ 11	≤ 0.06		12		
CDAG3 pos 375	≤ 11	≤ 0.06		≤ 11	≤ 0.08	≤ 11	≤ 0.08		≤ 5	≤ 0.04		≤ 5	≤ 0.03		12		
Geometric mean titre	0			0		0			320.0			0					

* i.e died during last bleeding.

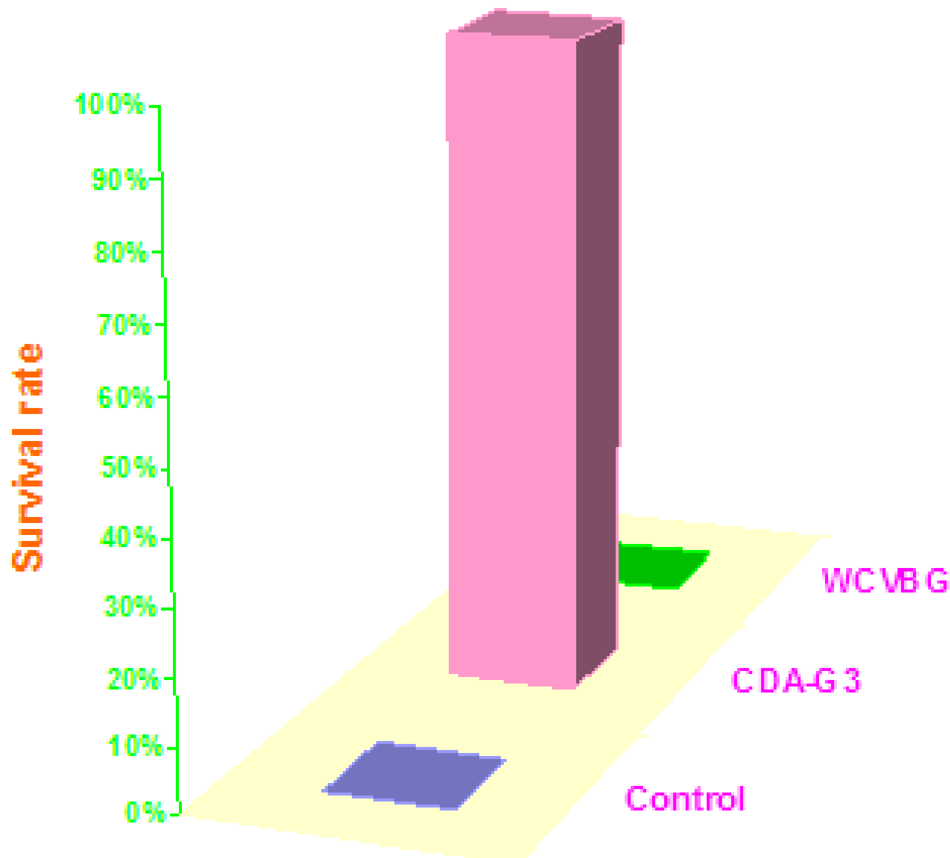


Fig: 4.4: Survival Rate Post Challenge after Multiple Administrations of Vaccines using Intra Muscular (IM) Route.

The antibody titer post challenge in the mice remained the same or increased in the individual mouse and even the mouse that did not show any detectable antibody titer prior to challenge had some detectable level (0.15 iu/ml) though not up to the recommended level by World Health Organization (WHO) (0.5 iu/ml). The GMT was 333. (Table 4.4)

In the subcutaneous multiple administration group where mice were inoculated with 3 shots of 100 µg of plasmid encoding the modified glycoprotein G of ERA strain (CDAG3) at 3 weekly intervals using the dorsal neck skin fold. Detectable neutralizing antibody was first noticed after a booster was given in 60% of the experimental animals (Fig. 4.5), though only 20% of these had level (2.29 IU/ml) greater than the recommended protective level of 0.5 IU/ml by WHO. This level decreased after the second booster (1.25 IU/ml) before increasing again after the challenge (2.32 IU/ml). The detectable antibody titer noticed after the first boost on day 22 (D22), had a GMT of 32.11 (Table 4.4) which increased to 48.98 on D63 after the second boost. Following challenge after D63, only 20% survived the challenge while the remaining 80% died between eleven (11) and fourteen (14) days post challenge. For the survival, the antibody titer 45days post challenge showed 2.32 IU/ml which is over fourfold the expected recommended WHO level, the titer increased and almost doubled the level recorded on D63 (1.25 iu/ml). Forty percent in this subcutaneous group that showed some traceable antibody level did not survive the challenge.

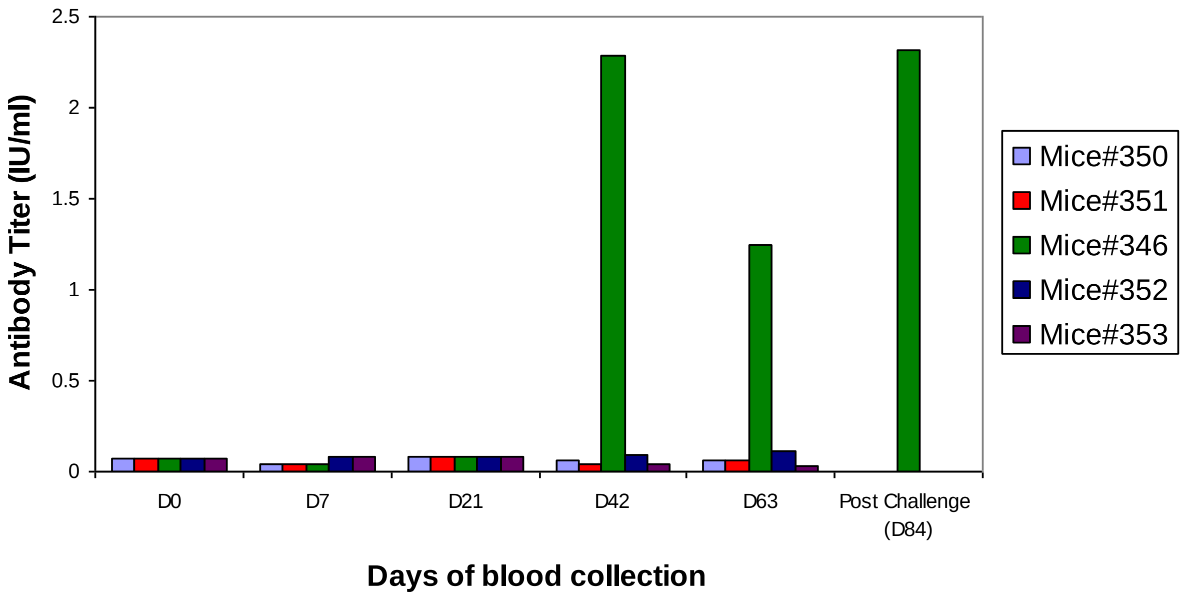


Fig. 4.5a: Effect of Multiple Administration Sub-cutaneous (sc) Vaccination using CDA-G3 Vaccine: Animal to Animal Variation

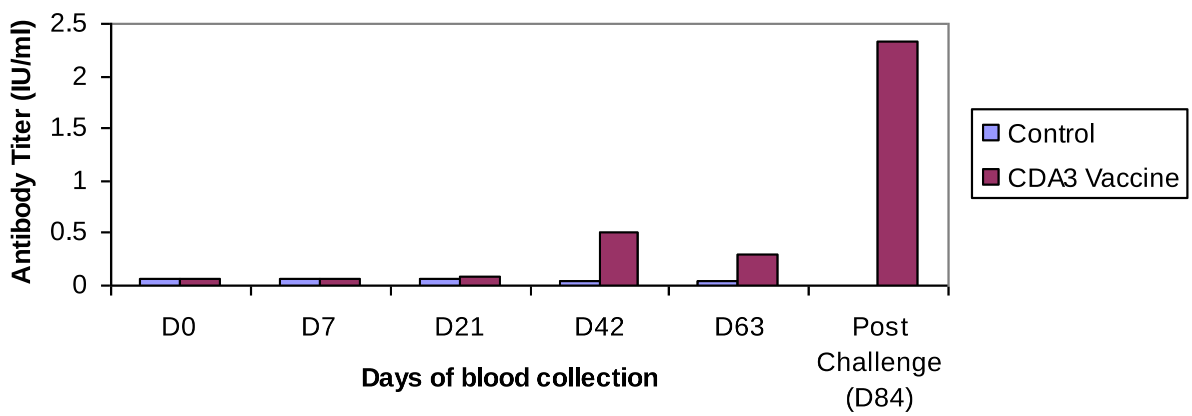


Fig. 4.5b: Effect of Multiple Administration Sub-cutaneous (sc) Vaccination using CDA3 Vaccine

Three shots of 100 µg of CDAG3 was administered at 3 weekly interval for the oral route group, the only neutralizing antibody detected was in 20% of the experimental subject after receiving primary and first booster doses, the antibody recorded was not confirmed following the second booster (Fig. 4.6), and no significant neutralizing antibody was seen on D63. The traceable antibody level that was detected in 20% of the experimental subjects on D42 had a titer of 320 (2.29 iu/ml), but by D63 even after receiving a second boost earlier, no traceable antibody was detected (Table 4.4). On challenge, all the mice in this group did not survive they died between 11 and 16 days following the challenge.

Multiple administration of WCBVG, for the intramuscular, subcutaneous and oral routes, had antibody titers that remained $\leq$ 5 or 11 with GMT of zero (0) (Table 4.5). All the mice died between days 11 and 18 following challenge. Results obtained from vaccination of mice with plasmid encoding the glycoprotein of WCBV (WCBVG) using different routes and shots did not produce neutralizing antibodies and did not protect the mice from challenge.

Controls for multiple administrations with buffer solution using intramuscular, subcutaneous and oral routes did not show neutralizing antibody and all GMT constantly remained zero (0) throughout the study period (Table 4.6). Post challenge, all the mice in all the control groups died between days 9 and 12 (Fig 4.7)

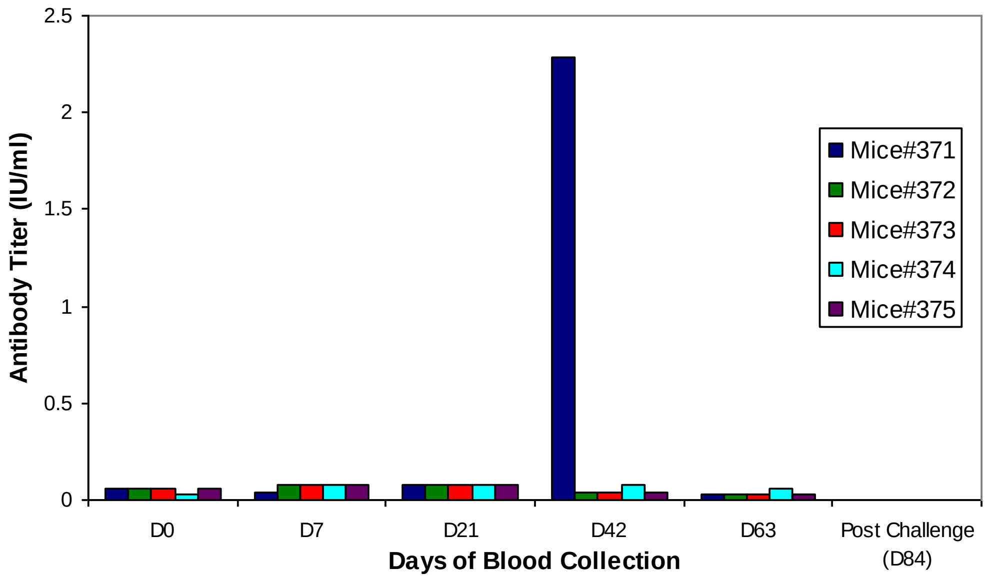


Fig. 4.6a: Effect of Multiple Administration Oral Vaccination using CDA-G3 Vaccine: Animal to Animal Variation

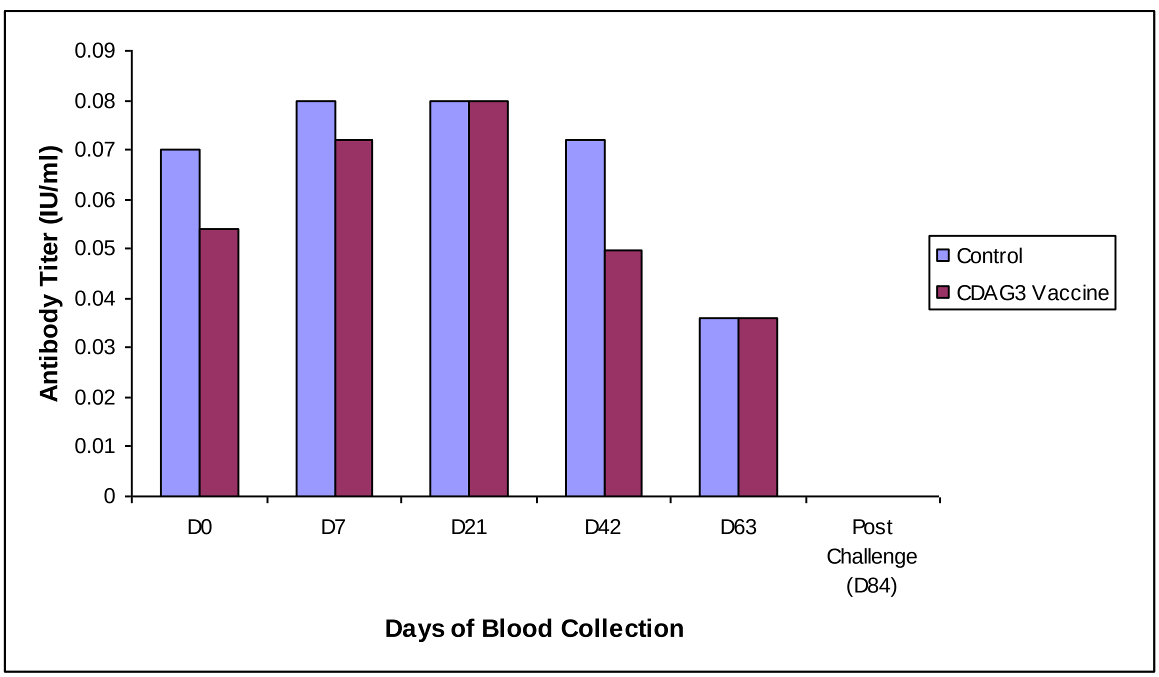


Fig. 4.6b: Effect of Multi-dose Oral route (PO) Vaccination using CDA-G3 Vaccine

Table 4.5: Antibody titre if mice inoculated with WCBVG Plasmid DNA (encoding glycoprotein glycoprotein gene) using multiple dose regimen and different routes

Days of Blood Collection.

Sample Number	Baseline D0 Titre	Baseline D0 IU/ml	First Inoc	D 7 Titre	D 7 IU/ml	D 21 Titre	D 21 IU/ml	1st Boost	D 42 Titre	D 42 IU/ml	2nd Boost	D 63 Titre	D 63 IU/ml	Challenge	Death post Challenge No of days	Post Challenge Titre	Post Challenge IU/ml
WCBVG i/m 306	≤ 11	≤ 0.07		≤ 5	≤ 0.04	≤ 5	≤ 0.04		≤ 5	≤ 0.04		≤ 5	≤ 0.03		11	None. All died	
WCBVG i/m 367	≤ 11	≤ 0.07		≤ 5	≤ 0.04	≤ 11	≤ 0.08		≤ 5	≤ 0.04		≤ 11	≤ 0.06		11		
WCBVG i/m 308	≤ 11	≤ 0.07		≤ 5	≤ 0.04	≤ 11	≤ 0.08		≤ 5	≤ 0.04		≤ 5	≤ 0.03		12		
WCBVG i/m 356	≤ 11	≤ 0.07		≤ 11	≤ 0.08	≤ 11	≤ 0.08		≤ 5	≤ 0.04		≤ 11	≤ 0.06		18		
WCBVG i/m 310	≤ 11	≤ 0.07		≤ 5	≤ 0.04	≤ 11	≤ 0.08		≤ 5	≤ 0.04		≤ 11	≤ 0.06		11		
Geometric Mean Titre	0			0		0			0			0					
WCBVG s/c 338	≤ 5	≤ 0.03		≤ 5	≤ 0.04	≤ 11	≤ 0.08		≤ 5	≤ 0.04		≤ 11	≤ 0.06		18		
WCBVG s/c 381	≤ 11	≤ 0.07		≤ 5	≤ 0.04	≤ 11	≤ 0.08		≤ 5	≤ 0.04		≤ 5	≤ 0.03		12		
WCBVG s/c 357	≤ 11	≤ 0.07		≤ 11	≤ 0.08	≤ 11	≤ 0.08		≤ 5	≤ 0.04		≤ 5	≤ 0.03		11		
WCBVG s/c 397	≤ 5	≤ 0.03		≤ 5	≤ 0.04	≤ 11	≤ 0.08		≤ 5	≤ 0.04		≤ 11	≤ 0.06		12		
WCBVG s/c 396	≤ 11	≤ 0.07		≤ 5	≤ 0.04	≤ 11	≤ 0.08		≤ 5	≤ 0.04		≤ 5	≤ 0.03		12		
Geometric Mean Titre	0			0		0			0			0					
WCBVG po 376	≤ 5	≤ 0.03		≤ 11	≤ 0.08	≤ 11	≤ 0.08		≤ 5	≤ 0.04		≤ 5	≤ 0.03		11		
WCBVG po 377	≤ 11	≤ 0.06		≤ 11	≤ 0.08	≤ 11	≤ 0.08		≤ 5	≤ 0.04		≤ 5	≤ 0.03		11		
WCBVG po 378	≤ 5	≤ 0.03		≤ 11	≤ 0.08	≤ 11	≤ 0.08		≤ 5	≤ 0.04		≤ 5	≤ 0.03		14		
WCBVG po 379	≤ 11	≤ 0.06		≤ 11	≤ 0.08	≤ 11	≤ 0.08		≤ 5	≤ 0.04		≤ 5	≤ 0.03		11		
WCBVG po 380	≤ 11	≤ 0.06		≤ 11	≤ 0.08	≤ 11	≤ 0.08		≤ 5	≤ 0.04		≤ 5	≤ 0.03		12		
Geometric Mean Titre		0		0		0			0			0					

Table 4.6: Antibody titre of mice inoculated with buffer using multiple administration and different routes

	Days of Blood Collection.		First Inoc.					1st Boost			2nd Boost			Challenge			
	Baseline D0			D 7		D 21			D 42			D63			Death post challenge	Post Challenge	
Sample Number	Titre	IU/ml		Titre	IU/ml	Titre	IU/ml		Titre	IU/ml		Titre	IU/ml		No of days	Titre	IU/ml
Control i/m 317	≤ 11	≤ 0.07		≤ 11	≤ 0.08	≤ 11	≤ 0.08		≤ 5	≤ 0.04		≤ 5	≤ 0.03		12	None. All died	
Control i/m 318	≤ 11	≤ 0.07		≤ 5	≤ 0.04	≤ 5	≤ 0.04		≤ 5	≤ 0.04		≤ 5	≤ 0.03		11		
Control i/m 319	≤ 11	≤ 0.07		≤ 11	≤ 0.08	≤ 5	≤ 0.04		≤ 5	≤ 0.04		≤ 5	≤ 0.03		11		
Control i/m 320	≤ 11	≤ 0.07		≤ 5	≤ 0.04	≤ 11	≤ 0.08		≤ 5	≤ 0.04		≤ 5	≤ 0.03		10		
***	≤ 11	≤ 0.07															
Geometric Mean Titre	0			0		0			0			0					
Control s/c 326	≤ 11	≤ 0.07		≤ 5	≤ 0.04	≤ 11	≤ 0.08		≤ 5	≤ 0.04		≤ 11	≤ 0.06		10		
Control s/c 358	≤ 11	≤ 0.07		≤ 11	≤ 0.08	≤ 11	≤ 0.08		≤ 11	≤ 0.08		≤ 11	≤ 0.06		10		
Control s/c 359	≤ 11	≤ 0.07		≤ 11	≤ 0.08	≤ 11	≤ 0.08		≤ 5	≤ 0.04		≤ 5	≤ 0.03		9		
Control s/c 360	≤ 11	≤ 0.07		≤ 5	≤ 0.04	≤ 5	≤ 0.04		≤ 5	≤ 0.04		≤ 5	≤ 0.03		11		
Control s/c 383	≤ 11	≤ 0.07		≤ 5	≤ 0.04	≤ 5	≤ 0.04		≤ 5	≤ 0.04		≤ 5	≤ 0.03		10		
Geometric Mean Titre	0			0		0			0			0					
Control po 361	≤ 11	≤ 0.07		≤ 11	≤ 0.08	≤ 11	≤ 0.08		≤ 11	≤ 0.08		≤ 5	≤ 0.03		11		
Control po 362	≤ 11	≤ 0.07		≤ 11	≤ 0.08	≤ 11	≤ 0.08		≤ 11	≤ 0.08		≤ 5	≤ 0.03		9		
Control po 363	≤ 11	≤ 0.07		≤ 11	≤ 0.08	≤ 11	≤ 0.08		≤ 5	≤ 0.04		≤ 5	≤ 0.03		9		
Control po 364	≤ 11	≤ 0.07		≤ 11	≤ 0.08	≤ 11	≤ 0.08		≤ 11	≤ 0.08		≤ 5	≤ 0.03		11		
Control po 365	≤ 11	≤ 0.07		≤ 11	≤ 0.08	≤ 11	≤ 0.08		≤ 11	≤ 0.08		≤ 11	≤ 0.06		12		
Geometric Mean Titre	0			0		0			0			0					

Table 4.7: Antibody titre of mice vaccinated with CDAG3 and WCBVG Plasmid DNA Rabies vaccine (encoding modified glycoprotein gene and glycoprotein gene respectively) using single intra-muscular inoculation.

	Days of Blood Collection. Baseline D0		Inoculation	D 30		Challenge	Death post Challenge	Post Challenge	
	Titre	IU/ml		Titre	IU/ml		No of days	Titre	IU/ml
Sample Number									
CDAG3 i/m A	≤ 5	≤ 0.05		≤ 5	≤ 0.05		14		
CDAG3 i/m B	≤ 5	≤ 0.05		320	3.2		Survived	125	1
CDAG3 i/m C	≤ 5	≤ 0.05		320	3.2		Survived	125	1
CDAG3 i/m D	≤ 5	≤ 0.05		340	3.4		Survived	45	0.36
CDAG3 i/m E	≤ 5	≤ 0.05		60	0.6		Survived	16	0.13
Geometric Mean Titre	0			215.03				57.54	
WCBVG i/m A	≤ 5	≤ 0.05		≤ 5	≤ 0.05		14		
WCBVG i/m B	≤ 5	≤ 0.05		≤ 5	≤ 0.05		12		
WCBVG i/m C	≤ 5	≤ 0.05		≤ 5	≤ 0.05		14		
WCBVG i/m D	≤ 5	≤ 0.05		≤ 5	≤ 0.05		Survived	Later euthanized.	
WCBVG i/m E	≤ 5	≤ 0.05		≤ 5	≤ 0.05		14		
Geometric Mean Titre	0			0					
Control i/m A	≤ 5	≤ 0.05		≤ 5	≤ 0.05		10		
Control i/m B	≤ 5	≤ 0.05		≤ 5	≤ 0.05		10		
Control i/m C	≤ 5	≤ 0.05		≤ 5	≤ 0.05		11		
Control i/m D	≤ 5	≤ 0.05		≤ 5	≤ 0.05		12		
Control i/m E	≤ 5	≤ 0.05		≤ 5	≤ 0.05		11		
Geometric Mean Titre	0			0					

Table 4.8: Antibody titre (IU/ml) of mice vaccinated with CDAG3 and WCBVG Plasmid DNA Rabies vaccines (encoding modified glycoprotein gene and glycoprotein gene respectively) using single intradermal vaccination.

	Days of Blood Collection										
	Baseline D 0		Inoculation	D 7		D 30		Challenge	Post Challenge		
	Titre	IU/ml		Titre	IU/ml	Titre	IU/ml		No of days post..	Titre	IU/ml
Sample Number											
CDAG3 i/d 394	≤ 5	≤ 0.03		≤ 5	≤ 0.04	≤ 5	≤ 0.04		15		
CDAG3 i/d 395	≤ 5	≤ 0.03		≤ 5	≤ 0.04	320	1.78		Survived	170	1.36
CDAG3 i/d 399	≤ 5	≤ 0.03		≤ 5	≤ 0.04	25	0.14		Survived	60	0.48
CDAG3 i/d 398	≤ 5	≤ 0.03		≤ 5	≤ 0.04	85	0.47		Survived	230	1.84
Geometric Mean Titre	0			0		88.44				132.84	
WCBVG i/d 386	≤ 5	≤ 0.03		≤ 5	≤ 0.04	≤ 11	≤ 0.06		Survived	Later euthanized	
WCBVG i/d 387	≤ 5	≤ 0.03		≤ 5	≤ 0.04	≤ 11	≤ 0.06		14		
WCBVG i/d 388	≤ 5	≤ 0.03		13	0.09	≤ 11	≤ 0.06		14		
Geometric Mean Titre	0			12.88		0					
Control i/d 323	≤ 5	≤ 0.03		≤ 5	≤ 0.04	≤ 5	≤ 0.04		10		
Control i/d 325	≤ 5	≤ 0.03		≤ 5	≤ 0.04	≤ 5	≤ 0.04		10		
Control i/d 329	≤ 5	≤ 0.03		≤ 5	≤ 0.04	≤ 5	≤ 0.04		9		
Geometric Mean Titre	0			0		0					

4.4.2.2 The single administration regimen

In the CDAG3 intramuscular route, antibody levels were detected on day 30 (D30) with 80% of the experimental animals showing antibody titers of 0.6, 3.2, 3.2 and 3.4 IU/ml with a GMT of 215.03 (Table 4.7). The 80% of the experimental animals (Fig 4.8) that had measureable antibody survived the intramuscular challenge (Fig. 4.9). In this study, single administration intramuscular route was shown to induce protective antibody which was still maintained after challenge. All the 80% that survived the challenge had titers of 0.13, 1.0, 1.0 and 0.36 respectively 30 days after challenge and a GMT of 57.54.

Similar observation was recorded for the single dose intradermal route that received just 10 μg of the DNA vaccine into the inner ear pinnae using injection needle, having 75% survival post challenge (fig 4.10). The 75% of the experimental subjects had neutralizing antibody titer of 0.14, 0.47 and 1.78 with a GMT of 88.44 while the titers 30 days (D30) post challenge were 0.48, 1.84 and 1.36, respectively (Table 4.8) with an increase of GMT to 132.84. The antibody titre measured 21 days post challenge increased when compared to the titre measured before the challenge. The 25% that did not survive the challenge died 15 days post challenge.

WCBVG single administration intramuscular and intradermal routes did not show detectable neutralizing antibody titer and had a geometric mean titer (GMT) of zero (0). However, following challenge, 20% of the mice from the intramuscular route and 33% from intradermal route survived despite no traceable detected antibody level (Table 4.7). The twenty percent (20%) from the single intramuscular administration group (fig 4.11) and thirty-three percent (33%) (Fig 4.12) from the single administration intradermal group that survived the challenge were eventually euthanized because of the persistent showing of clinical signs suggestive of rabies (circling and restlessness), even after 45 days post challenge.

Control Group for the single dose using intramuscular and intradermal routes did not show any antibody titer, all neutralizing antibody titer levels were ≤ 5 or 11 with GMT of zero (0), and all the mice died between 9 and 12 days (Fig 4.7) following challenge for both groups (Tables 4.7 and 4.8).

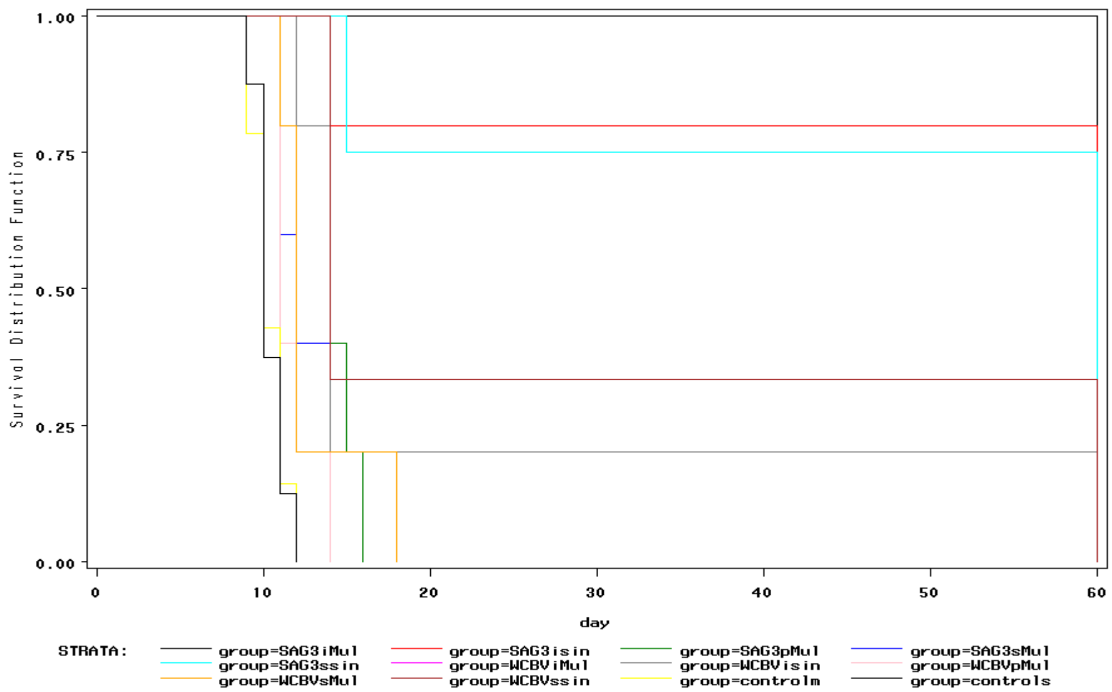


Fig 4.7: Survival Distribution Function of DNA Vaccines and Control

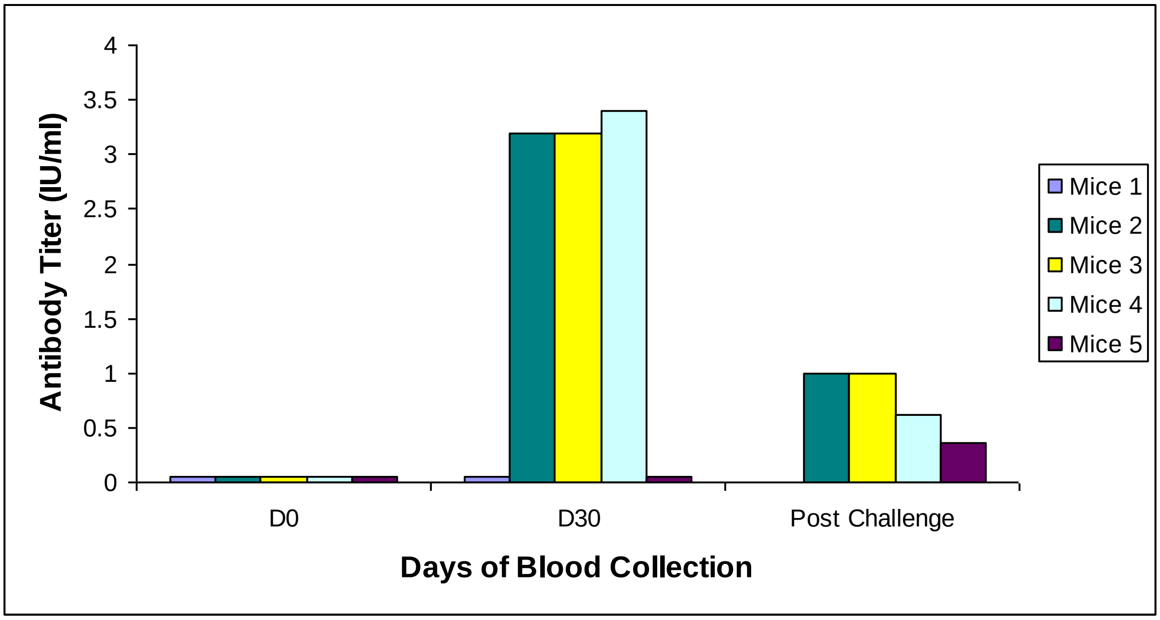


Fig 4.8a: Effect of Single Dose Intra muscular (IM) Vaccination using CDA-G3 Vaccine: Animal to Animal Variation

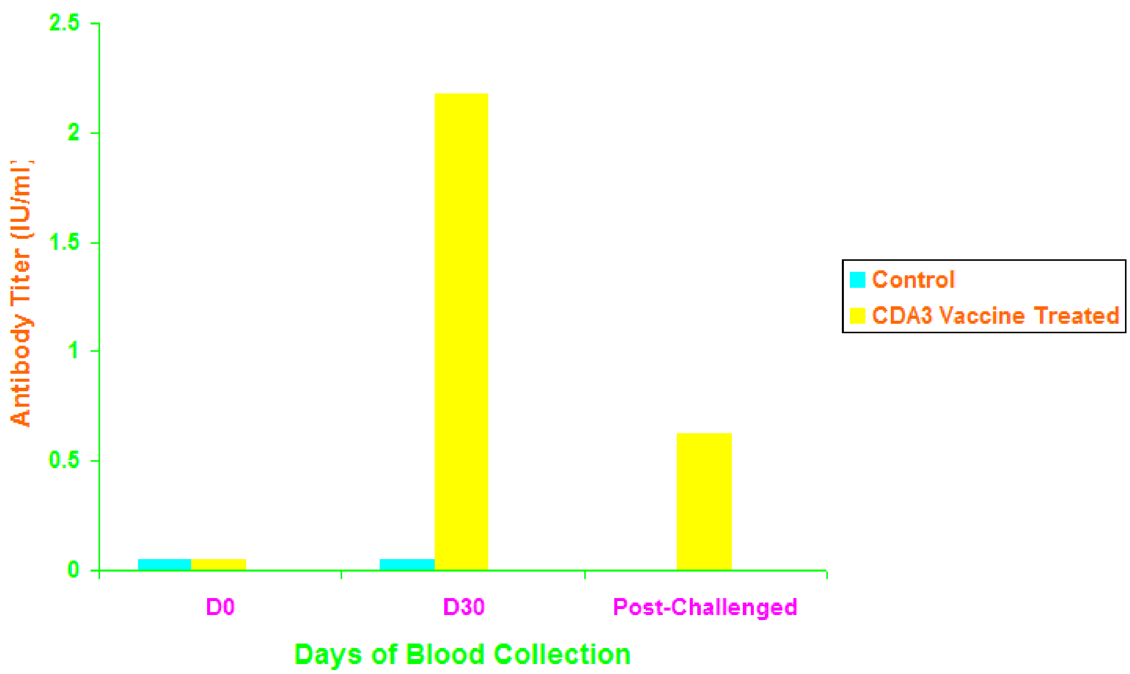


Fig 4.8b: Effect of Single Dose Intra muscular (IM) Vaccination using CDA-G3 Vaccine

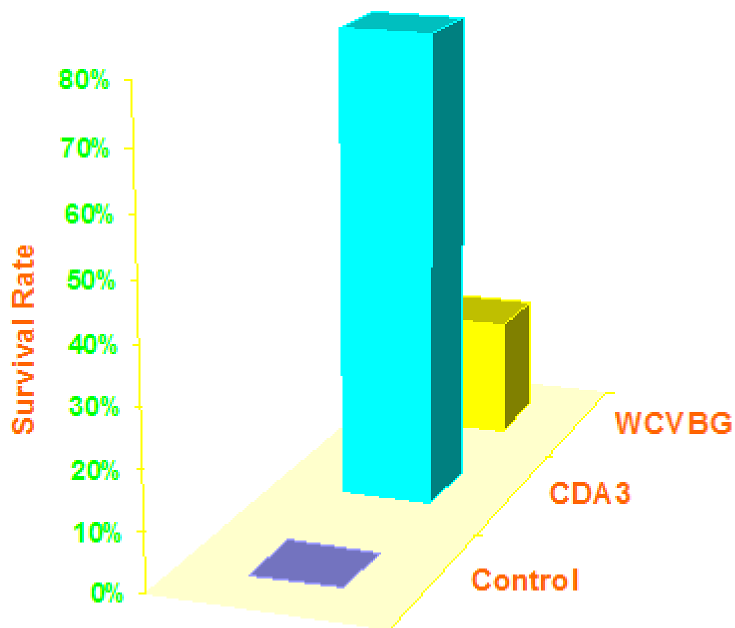


Fig 4.9: Survival Rate Post challenge after Administration of Single Dose Intra muscular (IM) Vaccines

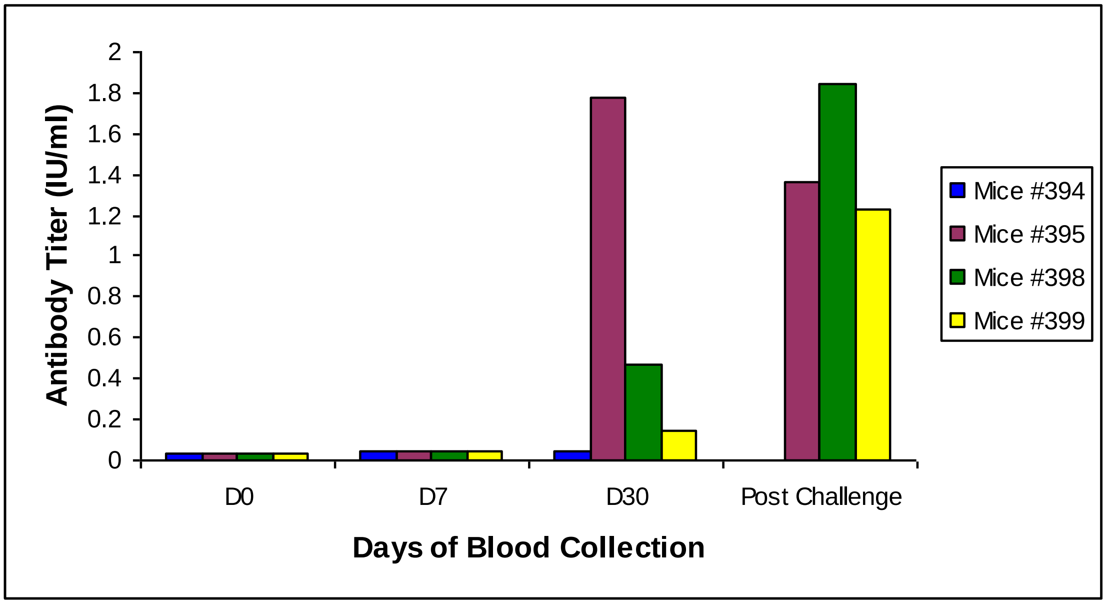


Fig 4.10a: Effect of Single Dose Intra dermal (ID) Vaccination using CDA-G3 Vaccine: Animal to Animal Variation

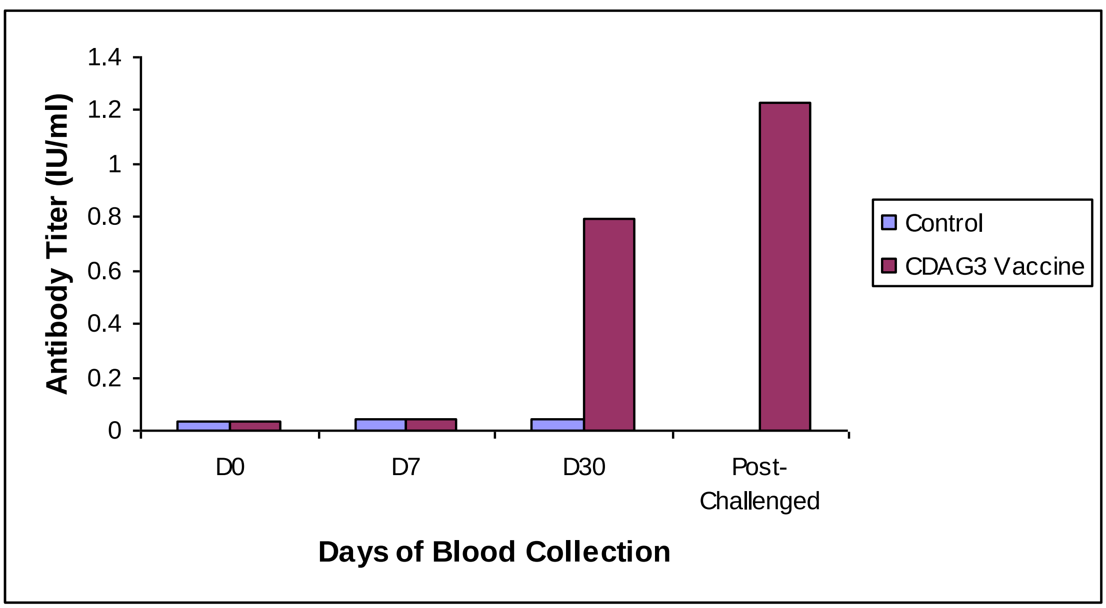


Fig 4.10b: Effect of Single Dose Intra dermal (ID) Vaccination using CDA-G3 Vaccine

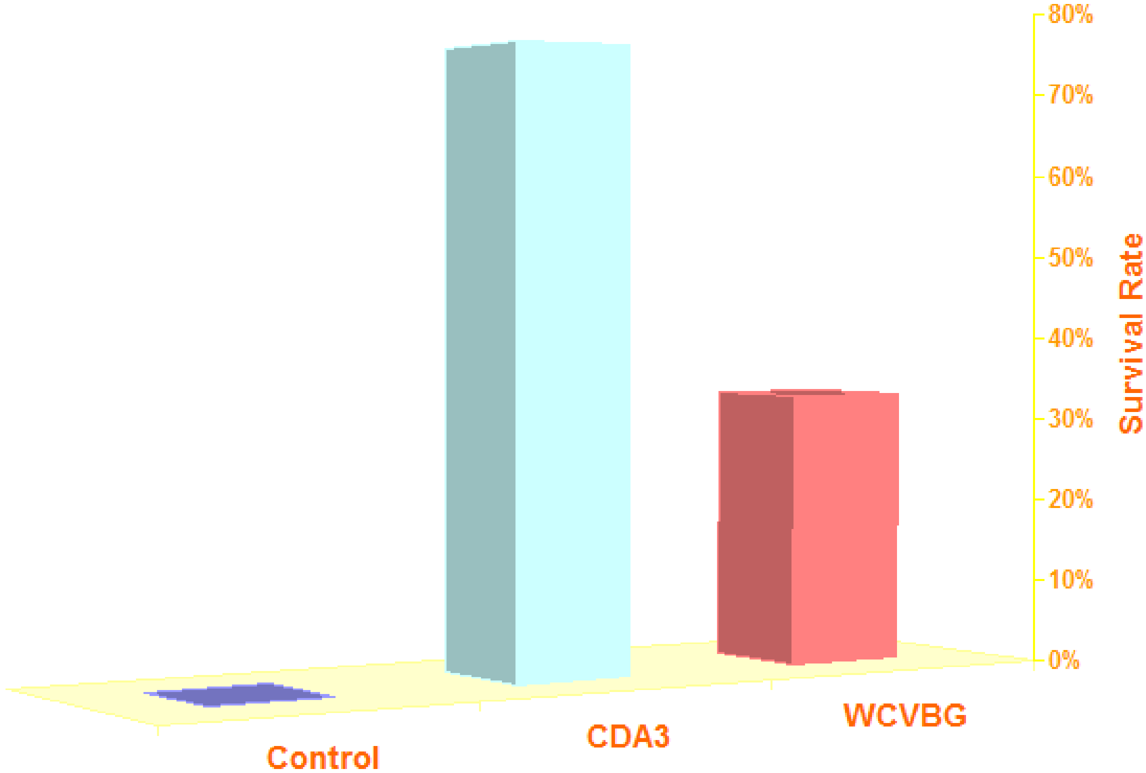


Fig 4.11: Survival Rate Post challenge after Single Dose Intra dermal (ID) Administration of Vaccines

4.5 Direct Rapid Immunohistochemistry Test and Direct Fluorescent Antibody Technique

All mice that did not survive the challenge regardless of the number of shots given or the route of administration which had their brains removed were tested (using DRIT and DFA) for detection of rabies antigen. The impression slides made from these brain samples were positive to both of the tests conducted (Plates 4.13 – 4.14).

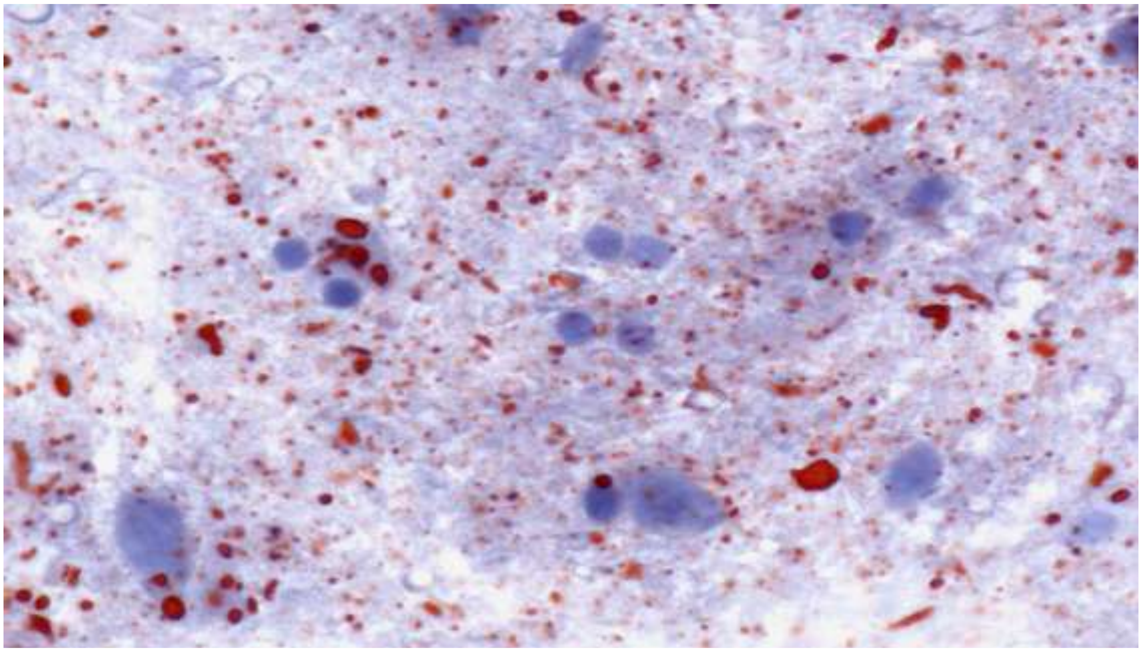


Plate 4.13a: Direct Rapid Immunohistochemistry technique (DRIT) Positive

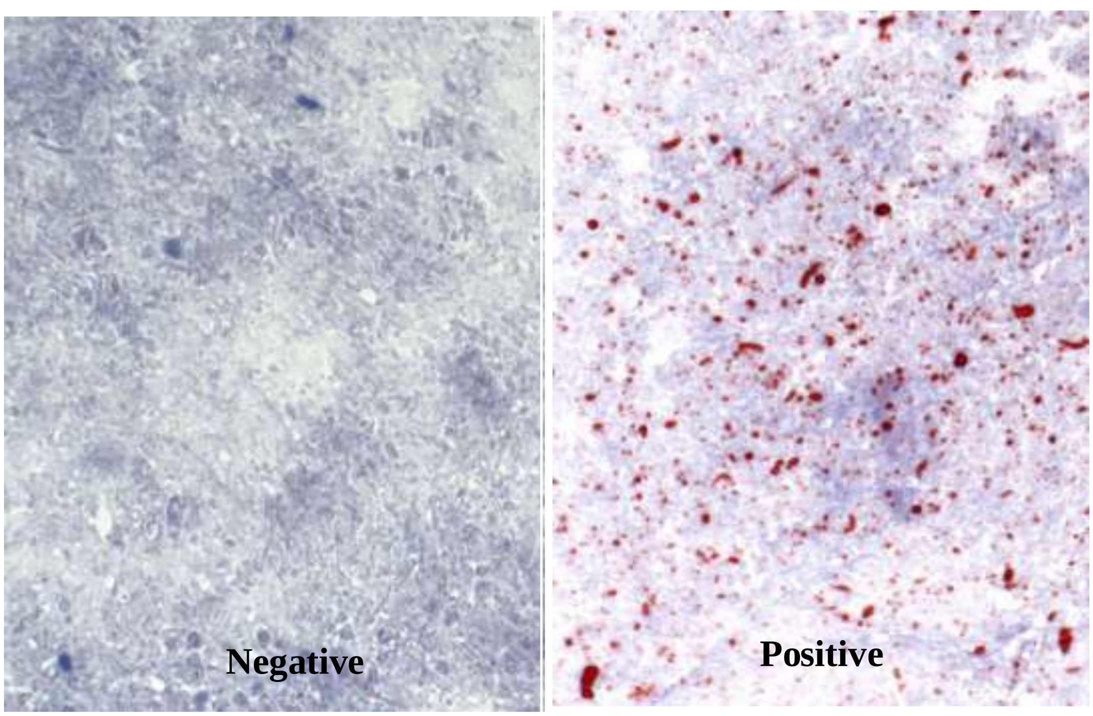


Plate 4.13b: Direct Rapid Immunohistochemistry Test (DRIT)

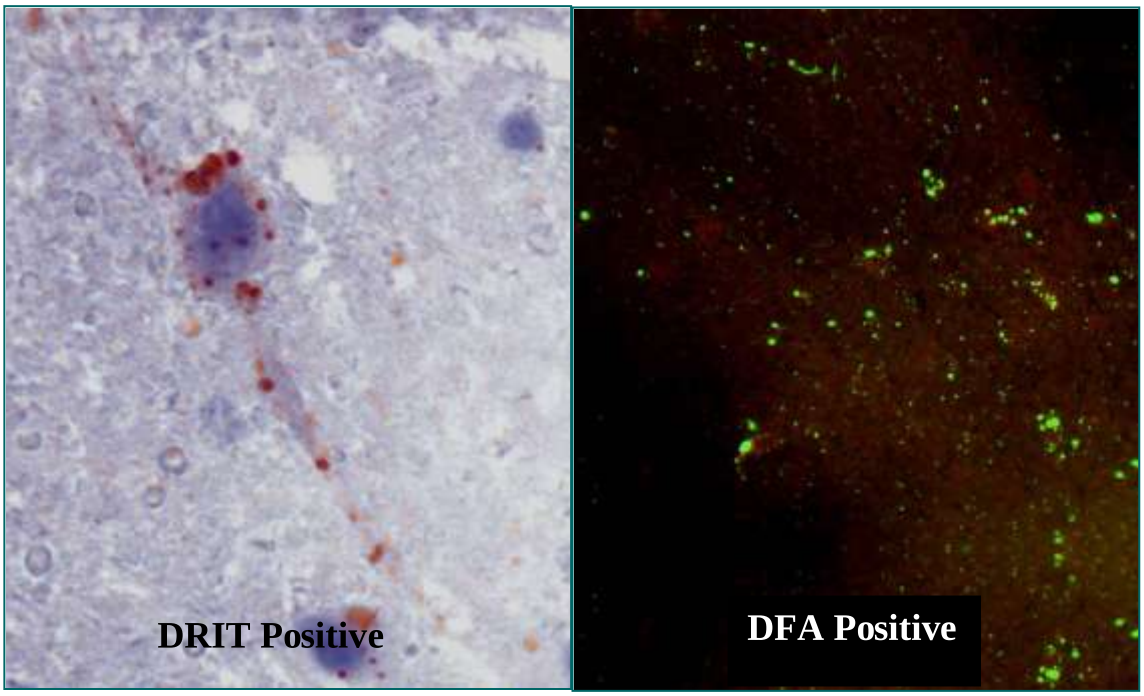


Plate 4.14: DRIT and DFA Positive Results (+4 Staining Intensity)

4.6 Statistical Analysis

Statistical analysis using Fisher exact test – two tailed probability, showed that the intramuscular (IM) and the intradermal (ID) routes were statistically significant compared to the control ($P<0.01$). This shows that both routes are efficient routes of immunization using the DNA vaccine. Also there was statistical significance when comparing intramuscular route (IM) to the subcutaneous (SC) routes in multiple dose regimens ($P< 0.05$). This goes to show the superiority of the IM route over the SC route.

CHAPTER FIVE
DISCUSSION

5.1 Discussion

Rabies virus in the brains of infected animals produces intracytoplasmic inclusions of various shapes. A single microscopic field may contain dust – like particles of <1µm in diameter and large, round to oval masses and strings 2 - 10µm in diameter (CDC protocol, 2007). When specifically stained with FITC labeled antibody, these inclusions appeared smooth, with very bright margins, and a somewhat less intensely stained central area. In this study, these inclusion types were also observed in the positive samples. FITC labelled antibody was used, where +4 to +3 staining intensity/antigen distribution was considered diagnostic for rabies infection, while weakly dull stain and weakly staining inclusions were not considered diagnostic for a rabies infection, even though it has been reported that diminished staining intensity may be as a result of denaturation of rabies virus antigen, non-specific binding of antibody to components of inflamed tissue or artifacts of tissue decomposition (CDC Protocol, 2007).

Direct fluorescent antibody (DFA) technique has more than ninety percent accuracy and is the first line of approach in the diagnosis of rabies in the laboratory where the study was carried out, so DFA became unavoidably the first choice used in diagnosis. Although, Seller's staining was the method of diagnosis used from the source of sample collection, this method has earlier been shown to be only 50% - 75% accurate, (Seller, 1927; Baer et al., 1968; Fekadu and Smith, 1985). While in this study, only 30.9% of the total samples positive to Seller's staining technique were found positive to DFA. The discrepancy of about 70% between results in this study using DFA with the

Seller's staining from source could be attributed to one or more of the following reasons: the first being the manner of storage; the samples were stored at source at -20^{o}C for 8 – 12 months before collection.

It is recommended that samples should be tested as soon as possible when samples are still fresh (Ogunkoya, 1997) because smears made from frozen and thawed tissue are not as satisfactory as those made from tissue that has not been frozen. This was confirmed in this study as observed in the results, where samples were stored for a long period and as such the discrepancy in results between the Seller staining method and the DFA used for confirmation.

Also the condition of storage and transportation could have had an effect on the stored samples. Ideally, samples should be constantly stored at -70^{o}C and transported on dry ice, but the samples used in this study were not only stored at -20^{o}C but also had the problem of inconsistent power (electricity) supply. Availability of dry ice is difficult in Nigeria, which left us with no option than to send the samples on ice packs with instructions that the cold chain should be maintained on transit. This work has shown that method of preservation and system of transportation can reduce potency by 70%. For samples to be preserved, especially if it is impossible to work on them immediately or as soon as possible, it is therefore strongly suggested to store them at -70^{o}C with a constant power supply or in liquid nitrogen or transport on dry ice. Currently, this looks impossible in Nigeria, so it calls for research to look into alternate means of preservation and transportation of samples for diagnosis and isolation of rabies viral antigen.

Equally, each of the 14 samples that were prepared and inoculated into mice did not kill the mice and no signs suggestive of rabies were observed. This does agree with previous findings of Goldwasser, et al., (1958), Koprowski, (1973) and Fekadu and Smith, (1985), that described the Mice Inoculation Test (MIT) as a sensitive test and suggested its use as a confirmatory test for rabies diagnosis. The non consistency of this result with earlier authors maybe attributed to condition of the samples before inoculation as earlier stated. This hampered the original design of this study, which was to isolate Nigerian strains of rabies, classify them and use as model for DNA vaccine development. Thus, Evelyn – Rokitnicki – Abelseth (ERA) rabies virus was used. It is suggested that the possibility of using the Nigerian variants in a future study should be looked into.

Ribonucleic acid (RNA) was extracted from all the samples that showed +4 and +3 staining intensity/antigen distribution, using TRIZOL. Out of the fifty-one (51) samples, RNA was successfully extracted from only 13 samples. Reasons for failure to successfully extract RNA could be due to RNase contamination (Chomczynski, 1993), but during the studies, as practised in the laboratory where the studies were carried out, all adequate precautions were taken in preventing RNase contamination. Other contributing factors of extraction failure could be the tissue degradation. About 92% tissue depreciation was recorded probably due to poor storage and adverse conditions and inefficient means of transportation. Tissues are recommended to be immediately processed or frozen at -60 to -70^{o}C after removal from the animal according to Ogunkoya, 1997, but the samples used in the study were stored at -20^{o}C for 20 – 28 months before transportation on ice pack for use. The results obtained amounted to a great economic loss and wasted effort, though similar finding was recorded by Ogunkoya (1997) in which all the viruses were lost on transit from Nigeria to India.

The integrity of the extracted RNA from the thirteen samples remained intact. Their quantitation had a range at A260/A280 of 1.6 to 2.4 when diluted in TE buffer solution, except for sample NLLB which had a value of 1.5 i.e. <1.6. This could be as a result of the extracted RNA of this one sample being partially dissolved or to incomplete dissolution of the final RNA pellet prior to spectrophotometry analysis (Wilfinger *et al.*, 1997; Fox, 1998), or probably as a result of the aqueous phase being contaminated with the phenol phase. Reverse-Transcription Polymerase Chain Reaction (RT-PCR) was used to detect the viral nucleic acid in the extracted samples. This agrees with work done by previous authors (Heaton *et al.*, 1997, Crepin *et al.*, 1998, Smith *et al.*, 2003) that showed the detection of RNA of rabies virus in several biological fluids and samples using RT-PCR.

Analysis of the phylogenetic relationships performed on the complete nucleoprotein (N) coding gene, established them to belong to the genotype 1 comprising of typical dog rabies virus (fig 4.1) with their geographical location pointing to West Africa and the animal species as dog (Fig. 4.2). Work done by Kissi *et al.* (1995), in an attempt to compare intrinsic and extrinsic genetic diversity of the lyssavirus genotypes using analysis of phylogenetic relationships, performed on the complete nucleoprotein coding gene (1350 bases), established that the rabies virus isolates all belonged to genotype 1(fig 4.1) and this agrees with findings in this work. The N protein of lyssaviruses has phosphorylated protein of 450 or 451 amino acids (aa) long (Tordo *et. al*., 1986a; Bourhy *et. al*., 1993) which is synthesized in large amounts during cell infection. The nucleotide (ntd) sequences generated in this study also had 450 amino acids (1350 ntds) (Appendice XXV). The viruses were classified into African Gp 2, (Tordo *et al.*, 1988 and Bourhy *et al.*, 1993). Further classification showed the strains to originate from the West African dog rabies. Samples RD44 and RD42 (fig.4.2) are very closely situated to Africa 3 group based on their phylogenetic classification on the phylogenetic tree. Africa 3 viruses are further classified to be related to African mongoose which represents a wild life rabies variant. These two isolates could probably be suggested to have a relationship with the wild life rabies variants or probably originated from the wild

Findings in this work show that rabies is present in Nigeria and this agrees with previous authors who confirmed the endemicity of rabies in Nigeria, (Turner, 1976, 1976; Okoh, 1982; Okoh *et. al*., 1988; Aghomo, 1990; Umoh *et al.*, 1990; Ogunkoya, 1997; Okoh, 2000; Ogunkoya *et. al.*, 2003; Nottidge, 2005) but, also playing down on the role of wild life rabies which up till now is not well understood in Nigeria. It is becoming clearer that wild life rabies is coming into the cycle of urban rabies. Therefore planning for rabies control in the urban areas without due consideration to its control in the wild may be counter productive.

Rabies zoonosis can be prevented by vaccination of humans before or after exposure. However, a more radical approach is possible, involving the elimination of the principal vector/reservoir by vaccinating dogs. And the vaccine must be effective, safe and inexpensive. The goal of finding an effective vaccine has been the focus of recent researchers, (Bahloul *et al*., 1998; Fodor *et al*., 2000; Bahloul *et al.*, 2006; Cliquet *et al*., 2007). This work was embarked upon based on the same focus using modern technology.

In line with this goal, a hunt for a circulating rabies virus variant suitable for vaccine production in Nigeria had been on, (Ogunkoya *et al*., 2003). This study was planned to develop a vaccine using a Nigerian rabies strain. But failure to isolate a virus from samples collected from Nigeria led to a modification in the study, where a vaccine virus strain Evelyn-Rokitnicki-Abelseth (ERA) that had stood a test of time in protecting animals against the rabies virus infection was used. It is non-pathogenic for a wide range of animal species when administered orally or intramuscularly, (Conzelmann *et al*., 1990). ERA rabies virus has been found to stimulate a strong immune response and is an efficient live vaccine. All these qualities aided our choice of using the virus.

With the suspicion of the role of wild life rabies involvement in urban rabies cycle in Nigeria, It was felt that a vaccine made for use in Nigeria should be made out of a wild rabies variant and challenged with a street rabies virus to check for cross reactivity or protection. The choice of using the West Caucasian Bat Virus (WCBV) was based on the reasons that WCBV is one of the two new lyssaviruses recently isolated from Eurasian bats (Botvinkin *et al*., 2003). It was isolated from the brain of a common bent-winged bat (*Miniopterus schreibersi*) in Krasnodar region in July 2002 by intracerebral mouse inoculation technique. This virus has demonstrated significant diversity compared to other lyssavirus representatives, (Da Cruz *et al*., 2002), and though it remained distant from all other lyssaviruses however, the most divergent representative of the genus according to N gene, was Mokola virus (MOKV) (Kuzmin *et al*., 2005). The glycoprotein gene was used without modification to develop a vaccine since this is just a pilot study using this new virus.

Multiple administration intramuscular vaccination using the CDAG3 showed that the vaccine did not only induce humoral but probably also cellular immune responses which resulted in protection against intramuscular challenge with the rabies virus. This agrees with findings of Xiang *et al*. (1994) that showed the role of cellular immune response in protection against challenge with rabies virus. Their work showed that plasmid vector expressing the rabies virus glycoprotein injected intramuscularly in mice for induction of rabies virus – specific immune responses developed rabies virus glycoprotein – specific cytolytic T cells, lymphokine – secreting T helper cells of the TH1 subset, and rabies virus-neutralizing antibodies and such mice were fully protected against a subsequent challenge with rabies virus (Xiang *et al*., 1994). The protection of these mice is likely to be due to cellular immune response.

The antibody titer measured following challenge was still maintained 21 days after, and half of the 40% that did not show detectable neutralizing antibody prior to challenge, had measurable neutralizing antibody up to 0.15IU/ml after the challenge. However the level was lower when compared to the standard value of 0.5 IU/ml recommended by World Health Organization. However, it was still protective. This humoral response post challenge is due to anamnestic response by the mice to the challenge by the street rabies variant because they had previously been primed by the vaccinations. Meanwhile the geometric mean titer (GMT) for this group dropped from 812.83 on D63 prior to challenge to 333 twenty-one days post challenge. Not because of reduction in antibody production, but due to the response of the 20% which had previously not shown any humoral response prior to challenge but had detectable neutralizing antibody response post challenge.

The 20% that died in this group did not die due to the challenge but due to technical errors during bleeding.

Intramuscular injection route was favourably considered for the following reasons; (i): ease of using needle injection seems more appropriate than gene gun delivery for mass vaccination campaigns for dogs, in developing countries, (ii) intramuscular injection of DNA vaccines has been demonstrated to be efficient in various animal species (Perrin *et al.*, 1999; Cruz *et al*., 2006; Bahloul *et al*., 2006).

Osorio *et al*. (1999) determined that the intramuscular route of rabies DNA vaccination is an effective route to vaccinate using multiple shots. They found that after the dogs received primary and booster intramuscular immunization with a rabies DNA vaccine, they were protected against challenge with a wild-type dog rabies virus. In this study, where mice vaccinated with multiple shots of plasmids encoding modified glycoprotein gene of the rabies virus intramuscularly were found to be 100% protected against challenge with a street rabies virus.

Also considering the number of injections, various protocols using two – four intramuscular injections have been experimented and found that at least two intramuscular injections, each of 100 µg of plasmid encoding the rabies virus glycoprotein were sufficient to induce high VNAb (virus neutralizing antibody) titers against the Pasteur virus (PV) strain, the challenge virus standard (CVS strain) and a wild – type rabies virus isolated from rabid dogs (Xiang *et al*., 1997; Bahloul *et al*., 1998; Bahloul *et al*., 2006). Three shots of 100 µg each of the plasmid encoding the modified rabies glycoprotein gene given to the mice at 3 weekly interval in this study fully protected against challenge.

In the subcutaneous multiple administration group, the reason for the inconsistency cannot be immediately explained, there might be need to further investigate into what could have caused the inconsistent trend. Though, reason probably could be due to slow absorption in subcutaneous route.

Out of the 60% that showed some traces of antibody production, only 20% survived the challenge. The level of antibody produced was not high enough to protect the mice against challenge. The level of antibody shown by the 20% that survived the challenged increased after the challenge, nearly doubling the level recorded on D63 prior to challenge. The challenge by the street rabies virus contributed to this increased based on a boosted anamnestic reaction. The subcutaneous route has been shown to be a poor route for administration of DNA vaccine due to the poor low response recorded among the vaccinated experimental animals. It has previously been observed by researchers (Ulmer *et al*., 1994) that regardless of the route of administration, the plasmid has to reach the nuclear compartment of the transfected host cell in order to be transcribed into messenger RNA (mRNA). It is then translated in the cytosol into a viral antigen, which will be processed and presented to the immune system. The muscle cells is thought to produce the viral antigen, which will be taken up by the more specialized antigen presenting cells

(APC_5) especially bystander dendritic cells, to trigger the immune response, (Ulmer *et al*., 1994). These cells are minimal at the subcutaneous site, and when such route is used, a lot of barrier such as connective tissues and subcutaneous fat are encountered before the plasmid reaches the host cells. This could have contributed to the low response observed in this route.

Another hypothesis considers that the antigen is primarily produced by resident dendritic cells directly transfected after intramuscular injection, which are absent in subcutaneous injection. Results of studies of Osorio *et al*. (1999) and Perrin *et al*. (1999) clearly showed that methods of delivery and sites of vaccination influence neutralizing antibody responses of dogs immunized with different DNA vaccines. This has equally been observed in this study. The response through the intramuscular route was found to be superior to the subcutaneous route. Subcutaneous route was considered as an alternative route of injection because there is reduction of pain at the injection site and due to the large space; large amounts of antigen can be deposited without compromising the surrounding tissues.

In the oral multiple administration, following challenge, all the mice died indicating absence of protection. The antibody titer that was detected in 20% of the mouse after the first booster could have been an error during laboratory techniques of detecting antibodies, and due to the size of the experimental subject, there was not enough serum to repeat the particular test. There is need for further investigation into the possibility of using the oral route as an alternative route of administration especially in this part of the world because of the advantage it holds in the possibility of immunizing stray dogs which are predominant in our environment.

Observations were made in respect to difficulties encountered during the experiment in administration of the cloned genes to the experimental subjects using the oral route. Further work need to be done on the use of bait to administer or improve the technique in administration in order to validate this route of administration. Though mice in this group had the longest range of days (11-16) it took them to die after the challenge, they still were not protected.

In the past 40 years, great progress has been made in the development of oral vaccines against rabies, (Wandeler, 1991). The primary focus of these efforts has been towards the application in control against wildlife rabies in Europe and North America, by the strategic distribution of vaccine laden baits, (Stohr and Meslin, 1996; Macinnes *et al.,* 2001). Adaptation of this to our environment in controlling stray dogs will go a long way in reducing the incidence of rabies in Nigeria. Though for these activities, self-replicating virus vaccines are needed to contact the oral mucosa of a diversity of mammalian carnivores because large amounts of inactivated antigens are required for minimal protection, (Rupprecht *et al*., 1992; 2005). Current vaccines production methods are cost-prohibitive to produce these products, which may require milligram concentrations of purified antigens, such as the rabies virus glycoprotein (G) at both high density and in a similar orientation as intact viral particles (Dietzschold and Schnell, 2002). However, there is need to look further into this route of administration, as it will play a very important role in the control of rabies in stray dogs that maintain and spread the disease in Nigeria.

$CDAG_3$ single dose intramuscular route showed neutralizing antibody by day 30 (D30) with 80% of the experimental animals surviving the intramuscular challenge. In this study, single administration intramuscular route was shown to induce protective antibody which was still maintained after challenge. One application of DNA vaccines encoding rabies virus glycoprotein has been established to provide protection against rabies in several experimental animals using the intradermal route (Xiang *et al*, 1995; Ray *et al*., 1997; Bahloul *et al*., 1998; Lodmell *et al*., 1998; Osorio *et al*., 1999; Perrin *et al*., 1999 and Lodmell *et al*., 2001). Ray *et al*. (1997) evaluated two plasmid DNA vaccines expressing the glycoprotein (G) gene of the challenge virus standard (CVS) rabies virus for their ability to elicit neutralizing antibody and protect mice against lethal rabies virus challenge found that a single inoculation protected 100% of the vaccinated mice which had neutralizing antibody titer $\geq$ 1.40 prior to virus challenge. Similar results were also recorded using the single intramuscular route in this study, but this is the first time such results will be recorded using plasmid encoding modified glycoprotein G of the rearranged proteins.

Similar observation was recorded for the single dose intradermal route that received just 10 μg of the DNA vaccine into the inner ear pinnae using injection needle, having 75% survival post challenge. The antibody titre measured 21 days post challenge increased when compared to the titre measured before the challenge. This has established prove that the intradermal route elicits a sustained antibody production. Mechanism(s) accounting for the superiority of the ear pinna as a vaccination site are ascribed to its unique immunological features which focus the concentration of processed antigen in a restricted area that is connected with a major draining lymph node. It is thought that the concentration of processed antigen results in an enhanced stimulation of T-lymphocytes by antigen – loaded dendritic cells (langerhans cells in ear pinnae) (Forg *et al*., 1998). Previous reports have already stressed the superiority of the ear pinnae over muscle tissues as a site for DNA vaccination, since it is very rich in dendritic cells (Lodmell *et al*., 2003; Forg *et al*., 1998)

It can be concluded that vaccines made from the glycoprotein G of the bat lyssavirus strain (WCBV) does not protect against the street rabies strain. Previous work done by Perrin *et al*., (1999) also showed that beagles immunized intramuscularly with plasmid encoding the rabies virus (PV strain) glycoprotein, neutralizing antibodies against both wild-type rabies virus and European Bat lyssaviruses (EBLV1 and EBLV2) were low. Equally, Kuzmin`*et al.*, (2005) concluded in their work that public health awareness needed to be raised, and attention to be paid to the protective efficacy of commercially available rabies vaccine, as well as immune globulin reactivity, with non-rabies lyssaviruses. They reported that anti-rabies biologicals provide incomplete protection of experimental animals against European bat lyssavirus (EBLVs) of genotypes 5 and 6, limited protection against Lagos bat virus (LBV) belonging to lyssavirus genotype 2, and no protection against Mokola bat virus (MOKV) belonging to the lyssavirus genotype 3. WCBV is reported to be limitedly related to LBV and MOKV.

Many studies have equally shown the difficulty in cross protection between lyssaviruses (Nel *et al*., 2003). In this study, the plasmid DNA vaccine made from Glycoprotein G of West Caucasian Bat lyssavirus could not protect against a street dog rabies strain from Chad, an Africa virus of the lyssavirus genotype 1.

In general, from the results obtained from this study, all the routes have shown protection except the oral route.In the multiple administrations, the intramuscular route had the highest protection (100%) of mice vaccinated using CDAG3 vaccine. This route showed superiority over the subcutaneous route and oral route. Further, using only one shot of the vaccine both the intramuscular and intra-dermal routes showed appreciably protection, even though only a tenth (10 µg) of the intramuscular dose (100 µg) was given to the ID route. Economically, the single shot regimen has shown to be cost effective by using only one shot compared to the three shots used in the multiple shots regimen, which is just approximately 33% of the multiple total dose used in the multiple dose regimen. Single dose intra-dermal route appears to be the most cost effective where only about 3% of the vaccine dose was utilised and yet it still protected efficiently.

In comparison, using WCBVG both single and multiple shots did not protect mice against challenge using the street dog rabies virus. There was no cross protection within these genotypes. With the suspected role of wild life in urban rabies cycle world wide, for example, a human death was recently reported in Kenya who was bitten by a bat. So, there is the need to investigate into cross protectively within all the genotypes of the lyssaviruses.

Results of all mice that did not survive the challenge regardless of the number of shots given or the route of administration, including all the controls indicate that the vaccinated mice and control injected with buffer solution were not protected against the challenge by the street rabies virus and they all died of the rabies infection.

Although the DFA test is the one most frequently used to diagnose rabies, DRIT which is an unlicensed procedure was equally used as a measure of the DFA test, according to the national standard operating procedure for the diagnosis of rabies in animals (http://www.cdc.gov/ncidod/dvrd/rabies/professional/publications/DFA_diagnosis/DFA-protocol-b.htm). DRIT was tried to determine its sensitivity and specificity because apart from being a cheaper test to run, it requires the use of less expensive and sophisticated equipment and takes shorter time to complete a run (one hour). This technique will be of a great advantage to our environment.

Positive results in both tests confirm the presence of rabies antigen and suggest that the mice were actually not protected by the vaccine and could not survive the challenge with the street rabies variant

CHAPTER SIX

CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusion

Analysis of the phylogenetic relationships performed on the complete nucleoprotein (N) coding gene of the existing rabies virus strains identified in Nigeria established them to belong to the genotype 1, comprising typical dog rabies virus with their geographical location pointing to West Africa and the animal species as dog

Plasmid encoding modified glycoprotein gene of the Evelyn – Rokitnicki – Abelseth (ERA) rabies virus has been shown in this study to be safe and efficient when challenged with the the street rabies virus belonging to the West African dog rabies strain. These results indicate that DNA vaccines could be a solution for providing developing countries with an inexpensive vaccine that is simple to prepare and is highly efficacious with excellent stability.

With the suspected role of wild life in urban rabies cycle world wide, there is the need to join with other researchers to investigate into research that will bring about cross protection within the genotypes of all the lyssaviruses, especially genotype 3.

Currently in Nigeria, it appears impossible to store and maintain samples at -70^{o} C with constant power supply. This calls for research to look into alternate means of preservation and transportation of samples for diagnosis and isolation of rabies viral antigen.

6.2 Recommendations

Based on the advantages DNA vaccine has over the conventional vaccine and on the practical results obtained from this study, it is recommended that:

- ✓ The vaccine be tried in target animals such as the dog, fox, cat etc.
- ✓ The intramuscular route which showed good results over other routes despite the number of shots (i.e., single and multiple shots) should be adopted and tried in the target animals (dogs, foxes e.t.c.).
- ✓ Due to the efficacy of the DNA vaccine tried in this study, it is suggested that it may effectively replace LEP Flury strain rabies vaccine currently used in Nigeria.
- ✓ Further research is suggested to be conducted into the dose-effect using single intramuscular dose regimen because of the ease of administering intramuscular injection than intra-dermal especially during mass vaccination.
- ✓ More work be carried out using the Direct Rapid Immunohistochemistry Technique (DRIT), especially in the field to ascertain its sensitivity and specificity, and hence be recommended for use for the diagnosis of rabies in Nigeria.

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APPENDICES

Appendix I

Test Results of Direct Fluorescent Antibody Test

Staining intensity/antigen distribution. Rabies virus in the brains of infected animals produces intracytoplasmic inclusions of various shapes. A single microscopic field may contain dust-like particles of <1 usm in diameter and large, round to oval masses and strings 2 to 10 um in diameter. When specifically stained with an FITC labeled antibody, these inclusions appear smooth, with very bright margins, and a somewhat less intensely stained central area. Observations made for each test slide will recorded as staining intensity/antigen distribution (e.g. +4/+2).

Staining intensity is graded from +4 to +1. Positive control slides in all tests should always contain staining of +4 intensity (a slight loss of glare) is graded as +3 intensity and may occur in test samples positive for rabies when sample handling has not been optimal. Noticeably dull stain is graded +2 to +1 and cannot be considered as diagnostic for a rabies infection without confirmation of specificity. Even though diminished staining intensity may be the result of denaturation of rabies virus antigen, diminished staining may also result from non-specific binding of antibody to components of inflamed tissue or artifacts of tissue decomposition.

Antigen distribution. For each area of the brain examined, staining is graded by the amount of antigen present as follows:-

+4, a massive infiltration of large and small inclusions of varying shape in almost every area of the impression.

+3, inclusions of varying size and shape are found in almost every microscopic field, the number of inclusions per field varies, but inclusions are numerous in most fields.

+2, inclusions of varying size and shape are present in 10% to 50% of the microscopic fields and most fields contain only a few inclusions.

+1, inclusions of varying size and shape are present in <10% of the microscope fields and only a few inclusions are found per field (usually only one or two inclusions per field).

Appendix II

List of Commercially Available Reagents

A. FITC Labeled Anti-Rabies and Control Reagents

Fujirubio Diagnostics, Inc. 1 0 800 – 342 – 9225.

CENTOCOR FITC Anti-Rabies Monoclonal Globulin

Catalog #800-090. A mixture of two IgG2a monoclonal antibodies.

Chemicon International, Inc. 1 – 800 – 437 – 7500

Light Diagnostics Rabies DFA Reagent (Monoclonal antibody FITC conjugate). Catalog #5100. A micture of two 1gG1 monoclonal antibodies and one IgG2 monoclonal antibody.

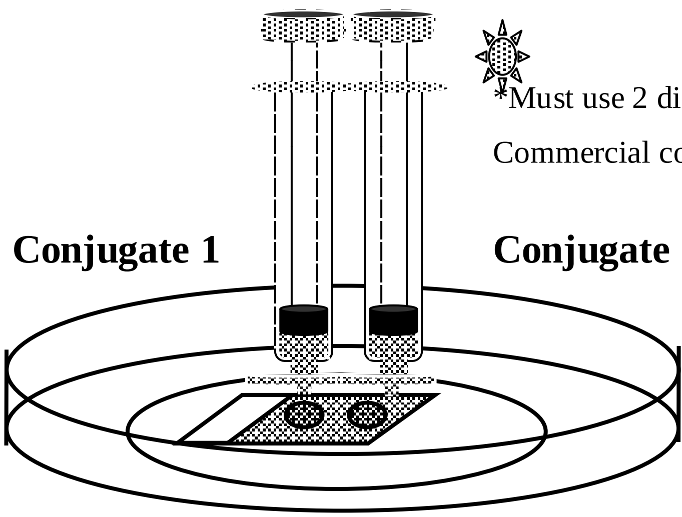
Light Diagnostics Rabies Negative Control (Monoclonal antibody FITC conjugate). Catalog #5102. A micture of IgG1 and IgG2 monoclonal antibodies.

Light Diagnostics Rabies DFA Reagent II (Monoclonal antibody FITC conjugate). Catalog #5500. Contains the same two IgG2a monoclonal antibodies as Centocor #800-090.

Light Diagnostics Rabies Polyclonal DFA Reagent (Goat IgG FITC conjugate). Catalog #5199. Goat hyperimmune serum (IgG fraction).

Light Diagnostics Rabies Negative Control

Catalog #5202. FITC labeled normal goat serum (IgG fraction).



*Must use 2 different conjugate preparations.

Commercial conjugates are listed above.

B. Immerson Oil

Fisher Scientific 1 -800 -766 -7000

Cargille Laboratories Immersion Oil for Fluorescence Microscopy; Type DF.

C. Conjugate Diluent

0.01M phosphate buffered isotoric saline solution at pH 7.4 to 7.6

:phosphate buffered saline (PBS) sigma P3813 (is a 0.0IM phosphate buffer solution with pH 7.4, with 0.138MNcl and 0.027M KCL).

No protein stabilizer

:Bovine serum albumin.

D. Counterstain

-Evans blue counterstan (0.5% on PBS, sigma, product #E-0133(.

0.00125%.

E. Rinse/soak buffer (APBS formula of same PH and molarity ass the conjugate diluent)

:Sigma P3813 (0.0IM phosphate buffer, pH 7.4, with 0.138 MNacl and 0.0027 MKCL).

Appendix III

Interpretation of Results

Appendix IIIa: Complete Tests

If the positive and negative control give the appropriate results, tissue condition and adequate sample tissue was tested. The test can be considered complete or incomplete based on observed patterns of staining.

Negative – No specific staining in the test slides with 2 different anti-rabies conjugates.

Positive – clearly positive with both anti-rabies conjugates (+3-4 staining and +2-+4 antigen distribution).

Appendix IIIb: Incomplete Tests

{<10% of examined fields.

Typical Inclusions {>10% with sparse
{ Distribution 1 – 2 per field.

{weak intensity < +3

{Inclusion morphology but with +4 intensity.
{regularly sized uniformed texture.
{
Atypical {Fluorescing bacteria; might mask small amounts
{of rabies specific staining.
{
{Particulate or free fluorescein; might mask
{small amounts of rabies specific staining

Discordant results with 2 reagents or 2 readers.

Appendix IV

Composition of Earle's Salts

Components	Molecular Weight	Concentration (mg/L	Molarity (mM)
INORGANIC SALTS			
Calcium chloride (caC12) (Anhyd.1)	111	2000.00	18.0
Potassium Chloride (KC1)	75	4000.00	53.0
Magnesium Sulfate (MgSo4)	120	976.70	8.13
Sodium Chloride (NaC1)	58	68000.00	1172.4
Sodium Phosphate-H20 (NaH2PO4-H20)	338	1400.00	10.1
OTHER COMPONENTS			
D-glocuse	180	10000.00	55.5
Phenol Red	398	100.00	0.251
AMINO ACID			
L-Arginine-HC1	211	1260.00	6.00
L-Cystine-2Na	286	286.00	1.00
L-Histidine-HC1-H20	210	420.00	2.00
L-Isoleucine	131	520.00	4.00
L-Leucine	131	520.00	4.00
L-Lysine-HC1	183	720.00	3.97
L-Mwthionine	149	150.00	1.01
L-Phenylalanine	165	320.00	1.94
L-Threonine	119	480.00	4.03
L-Tyrosine-2Na-2H20	263	520.00	1.99
L-Valine	117	460.00	3.93
VITAMINS			
D-Ca Pantothenate	477	10.00	0.02
Choline Chloride	140	10.00	0.071
Folic Acid	441	10.00	0.022
i-Inositol	180	20.00	0.111
Niacinamide	122	10.00	0.081
Pyridoxal HC1	204	10.00	0.49
Riboflavin	376	1.00	0.002
Thiamine HC1	337	10.00	0.029

Appendix V

Composition of PBS 4550/4588

Phosphate buffered saline (PBS): Two PBS formulas are used for REFIT:

a) Formula number 4550: (Ca^{2+} and Mg^{2+} free) PBS, 0.01M, pH = 7.40 +/- 0.05; osmolality = 285 +/1.15, sterile, clear, coloriess, 1000 m1/each.

FORMULATION

Sodium choloride ……………………………………	8.00 gm
Potassium chloride …………………………………..	0.2 gm
Sodium Phosphate Dibasic Anhydrous ……………..	1.15 gm
Potassium phosphate Monobasic Abnhydrous ……..	0.21 gm
Water Reagent-Grade Type I, QS ………………….	1000.0 ml

Adjust to pH 7.4 with Hydrochloric Acid or Sodium Hydroxide, (for rinsing cell monolayer, aliquot into 10ml volumes and store at 4C).

b) Formula number 4588: PBS, 0.01M, pH 7.4 – 7.6, pH = 7.50 +/-0.01; osmolality – 290 +/-20, sterile, clear, coloriess 1000 ml/each.

Formulation:

Sodium Choloride ……………………………………..	8.50 gm
Potassium phosphate Monobasic ……………………..	0.23 gm
Potassium Phosphate Dibasic ……………………….	1.46 gm
Water. Reagent-Grade Type I, QS ………………….	1000.0ml

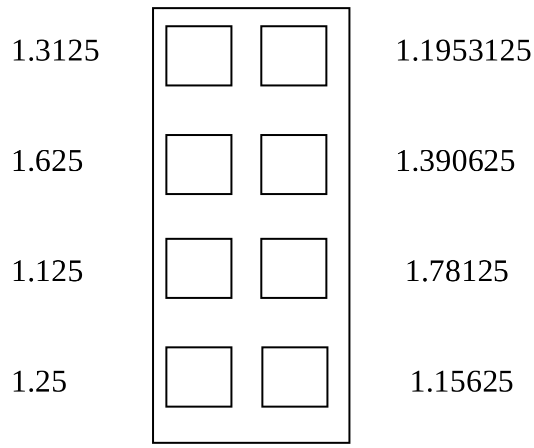
Adjust to pH 7.50 with Hydrochloric Acid or sodium Hydroxide.

Appendix VI

Flow Chart - The basic steps in RFFIT procedure

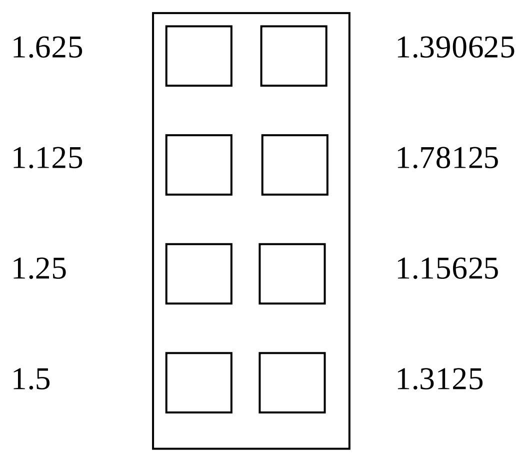
1. Heat inactivation of serum at $56^{o}C$
2. Serum dilutions in MEM-10

Dilutions of test serum in MEM-10 (0.lml) using an 8-well tissue culture chamber slide



3. Addition of rabies challenge virus standard (CVS-11)

Add 0.1 ml virus/well



4. Incubation of virus-serum mixture for 90 minutes

Incubate $37^{o}C$ 0.5% CO_2

5.	Addition of cells Mouse neuroblastoma cells (MNA)	Add 0.2ml MNA cells at 0.5×10^6 cells per ml
6.	Incubation of virus-serum-cell mixture	Incubate37^{o}C, 0.5% CO_2 for 20 hour ↓
7.	Fixation of slides	Dip-rinse slide in phosphate buffered seline (PBS) Fix in cold Acetone at -20oC for 30 minutes. ↓
8.	Direct Fluorescent Antibody (DFA) staining	Air dry slides and add DFA stain to each well
9.	Incubation of DFA stained slides	Incubate 30 mintes at 37oC in a humidity chamber
10.	Observation of slides under Fluorescence microscope	Observe for Fluorescing Cels
11.	Record results.	
12.	Determination of titer or IU	

Calculations

Using the control slide and test serum values from the previous example, the test serum end-point titre and international units can be calculated.

Determination of 50% end-point titres of serum:

The serum neutralization end-point titer is defined as the dilution factor of the highest serum dilution in which there is a 50% reduction in the number of fluorescing foci.

The 50% end-point titre of serum can be made by determining the number of fluorescing foci at each dilution, and then use the cumulative totals in the Reed-Meunch formula:

Appendix VII

Calculation of Fields Containing Infected Cells

(a) Calculate the percentage of fields containing infected cells.

Serum Dilution	No. of fields containing Infected cells	Fields containing infected cells	Fields containing no infected cells	Percentage of fields containing infected cells
1:5	0/20	0	88	0/88=0
1:25	0/20	0	68	0/68=0
1:125	0/20	0	48	0/48=0
1:625	0/20	0	28	0/28=0
1:3125	12/20	12	8	12/20=60
1:15625	20/20	12	0	32/32=100
1:79125	20/20	52	0	52/52=100
1:390625	20/20	72	0	52/52=100

(b) Using the method of Reed and Muench, calculate the difference between the logarithm of the starting dilution and the logarithm of the 50% end-point dilution (difference of logarithms) from the formula:

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

In this example the starting point dilution (the dilution showing an infectivity next below 50%) is 625, the dilution factor is 5.

Hence, the difference in logarithms is:

$$\frac{50-0}{60-0}\, x\ 0.69897 = 0.582475$$

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

Log (reciprocal of 50% end-point dilution)

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

= 2.79588 + 0.582475 = 3.38 (approx.)

Hence, log (50% end-point dilution) = -3.38 and the 50% end-point dilution = $10^{-3.38}$ = (1:2399).

Appendix VIII

Determination of the Potency of Test Serum in International Units (IU) per ml

The results of the RFFIT can be expressed as a serum titer or in International Units (IU) of antibody. In either case, a reference serum of known titer is required. When the results are to be reported as a titer, the reference serum is used as a control to insure the sensitivity of the test. It should demonstrate approximately the same titer determined in previous testw.

When the test serum results are expressed in IU, the calculation is dependent on the number of IU in the reference serum. The reference serum is diluted to contain 2 IU/ml and tittered along with the test serum. The ED_{50} titers of the reference serum and the test serum are then related in the following formula for calculation of IU/ml in the test serum:

$$\begin{matrix}\text{Number of} \\ \text{IU/ml}\end{matrix} = \frac{\text{End - point tite r of the test serum}}{\text{End - point tite r of the reference}} \, x \begin{matrix}\text{2 IU/ml in the} \\ \text{referenceserum}\end{matrix}$$

Example from above:

Test serum titer = 2399

Reference serum titer = 200

$$\begin{matrix}\text{Number of IU/ml} \\ \text{in the test serum}\end{matrix} = \frac{2399}{200} \, x \; 2 \; IU/ml$$

$$= 12 \text{ x } 2 \text{ IU/ml}$$

$$= 24 \text{ IU/ml}$$

Appendix IX

Determination of FFD_{50} of Challenge Virus

1. Calculate the percentage of fields containing infected cells

Virus Dilution	**No. of fields containing infected cells**	**Fields containing infected cells**	**Field containin no infected cells**	**Percentate of fields containing infected cells**
10^{-1}	20/20	70	0	70/70=100
10^{-2}	20/20	50	0	50/50=100
10^{-3}	20/20	30	0	30/30=100
10^{-4}	8/20	10	12	20/22=45
10^{-5}	2/20	2	30	2/32=6
10^{-6}	0/20	0	50	0/50=0

2. Using the method of Reed and Muench. Calculate the difference between the logarithm of the starting dilution and the logarithm of the 50% end-point dilution (difference of logarithms) from the formula:

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

In this example the starting point dilution (the dilution showing an infectivity next below 50%) is 10-4, and the dilution factor is 10.

Hence, the difference in logarithms is:

$$\frac{50 - 45}{100 - 45} \, x\, 1 = 0.091$$

3. Since the infectivity decreases with increasing dilution, the 50% end-point dilution is lower than the starting point dilution and is calculated by substracting the difference of logarithms as follows:

Log (reciprocal of 50% end-point dilution)

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

$= \text{Log } 10^{-4} – 0.091 = 3.91$ (approx.)

Hence, long (50% end-point dilution) – 3. 91

and the 50% end-point dilution = $10^{-3.91}$.

<u>Appendix X</u>

Titers of challenge virus used in the RFFIT (Reed-Muench)

Number of fields with fluorescing foci/Total number of fields

<table>
<tr><td>Challenge dose (50 FFD$_{50}$/0.1ml)</td><td colspan="25">20/20 →</td></tr>
<tr><td>1:10 (-1)</td><td>10/20</td><td>11/20</td><td>12/20</td><td>13/20</td><td>14/20</td><td>15/20</td><td>16/20</td><td>17/20</td><td>18/20</td><td>19/20</td><td colspan="15">20/20 →</td></tr>
<tr><td>1:100 (-2)</td><td colspan="10">0/20 →</td><td>0/20</td><td>1/20</td><td>2/20</td><td>3/20</td><td>4/20</td><td>5/20</td><td>6/20</td><td>7/20</td><td>8/20</td><td>9/20</td><td>10/20</td><td>11/20</td><td>12/20</td><td>13/20</td><td>14/20</td></tr>
<tr><td>FFD$_{50}$</td><td>10</td><td>15</td><td>15</td><td>17</td><td>19</td><td>25</td><td>26</td><td>26</td><td>27</td><td>30</td><td>32</td><td>32</td><td>35</td><td>40</td><td>43</td><td>47</td><td>51</td><td>59</td><td>68</td><td>80</td><td>100</td><td>150</td><td>150</td><td>170</td><td>190</td></tr>
</table>

Acceptable range

Appendix Xb

Acceptable range of fields with fluorescing foci for the control reference serum.

Standard rabies immune globulin; Lot R-3 (2 IU/ml):

<u>Dilution</u>

1:5	0 of 20 infected fields
1:25	0 of 20 infected fields
1:125	4 to 8 infected fields of 20
1:625	20 of 20 infected fields

Appendix XI

50% serum End-point Titres Corresponding to the Numbers of Fluorescing Foci (calculated by the Reed-Muench method)

Numbers of Fields with Fluorescing Foci/Total fields

Serum dilution

1:5 1:25	0/20 20/20	1/20 20/20	2/20 20/20	3/20 20/20	4/20 20/20	5/20 20/20	6/20 20/20	7/20 20/20	8/20 20/20	9/20 20/20	10/20 20/20	11/2020/20	12/20 20/20	13/20 20/20	14/20 20/20	15/20 20/20	16/20 20/20	17/20 20/20	18/20 20/20	19/20 20/20	20/20 20/20
	11	11	10	9	9	8	7	7	6	5	<5	<5	<5	<5	<5	<5	<5	<5	<5	<5	<5

1:25 1:125	0/20 20/20	1/20 20/20	2/20 20/20	3/20 20/20	4/20 20/20	5/20 20/20	6/20 20/20	7/20 20/20	8/20 20/20	9/20 20/20	10/20 20/20	11/20 20/20	12/20 20/20	13/20 20/20	14/20 20/20	15/20 20/20	16/20 20/20	17/20 20/20	18/20 20/20	19/20 20/20	20/20 20/20
	56	54	50	50	45	42	40	36	33	29	25	21	19	17	16	14	13	13	13	12	...

1:125 1:625	0/20 20/20	1/20 20/20	2/20 20/20	3/20 20/20	4/20 20/20	5/20 20/20	6/20 20/20	7/20 20/20	8/20 20/20	9/20 20/20	10/20 20/20	11/20 20/20	12/20 20/20	13/20 20/20	14/20 20/20	15/20 20/20	16/20 20/20	17/20 20/20	18/20 20/20	19/20 20/20	20/20 20/20
	280	270	250	250	230	210	200	180	170	145	125	110	95	85	75	70	70	65	60	60	…

1:625 1:3125	0/20 20/20	1/20 20/20	2/20 20/20	3/20 20/20	4/20 20/20	5/20 20/20	6/20 20/20	7/20 20/20	8/20 20/20	9/20 20/20	10/20 20/20	11/20 20/20	12/20 20/20	13/20 20/20	14/20 20/20	15/20 20/20	16/20 20/20	17/20 20/20	18/20 20/20	19/20 20/20	20/20 20/20
	1400	1300	1300	1200	1100	1100	1000	900	800	700	625	540	480	440	390	360	340	320	320	290	…

1:3125 1:15625	0/20 20/20	1/20 20/20	2/20 20/20	3/20 20/20	4/20 20/20	5/20 20/20	6/20 20/20	7/20 20/20	8/20 20/20	9/20 20/20	10/20 20/20	11/20 20/20	12/20 20/20	13/20 20/20	14/20 20/20	15/20 20/20	16/20 20/20	17/20 20/20	18/20 20/20	19/20 20/20	20/20 20/20
	7000	6800	6300	6000	5700	5400	5100	4600	4200	3600	3125	2700	2400	2200	1900	1800	1600	1600	1500	1500	…

1:15625 1:78125	0/20 20/20	1/20 20/20	2/20 20/20	3/20 20/20	4/20 20/20	5/20 20/20	6/20 20/20	7/20 20/20	8/20 20/20	9/20 20/20	10/20 20/20	11/20 20/20	12/20 20/20	13/20 20/20	14/20 20/20	15/20 20/20	16/20 20/20	17/20 20/20	18/20 20/20	19/20 20/20	20/20 20/20
	34800	33489	31950	30313	28571	26718	24747	22652	20432	18086	15625	13500	12000	10000	10000	9500	8500	8000	7500	7500	…

1:78125 1:390625	0/20 20/20	1/20 20/20	2/20 20/20	3/20 20/20	4/20 20/20	5/20 20/20	6/20 20/20	7/20 20/20	8/20 20/20	9/20 20/20	10/20 20/20	11/20 20/20	12/20 20/20	13/20 20/20	14/20 20/20	15/20 20/20	16/20 20/20	17/20 20/20	18/20 20/20	19/20 20/20	20/20 20/20
	174692	167448	159750	151565	142857	133591	123735	113263	102160	90434	78124	67491	59744	53887	49326	45687	42724	40269	38206	36450	…

Appendix XII

Titration of Stock Virus (CVS-11 for RFFIT)

Titration volume (0.1 ml)	**Virus dilution**	**Number of fields with fluorescing foci total # of fields**	**Percent of infected cells per single field**
	-5 (1:100,000)	0/20	0 infected cells
Dilution with 10 of 20 infected fields	-4(1:10,000)	10/20	0 to infected cells/field
	-3(1:1000)	20/20	1 to 5 infected cells/field
	-2(1:100)	20/20	10 to 50 infected cells/field
	-1(1:10)	20/20	

The -4 dilution of virus contains at least one focus of infected cells in 50% of the observed microscope field (160 – 200x) = 1 FFD_{50} /0.1ml

The stock virus suspension should be diluted 10^{-23} to obtain a challenge dose of 50 FFD_{50} in 0.1ml.

4.0 log of dilution with 0 to 1 infected cells per field

$$-\frac{1.7}{2.3} \text{log of 50 dilution of virus required to achieve 50 } FFD_{50} \text{ in 0.1ml}$$

$10^{2.3}$ = 1:200

To calculate the amount of challenge virus suspension needed for each text:

Each slide will receive 0.8ml virus (100ul per well, 8 wells per slide).

Calculate using 1.0ml virus per slide. If the test run is 16 slides, need 16ml of virus suspension.

Add 80 µl of stock virus to 16 ml MEM-10 (1:200 dilution)

Each well receives 100µl virus suspension.

Appendix XIII

Tissue-Tek staining tray set-up and reagent exchange for Direct Rapid Immunohistochemistry Technique (DRIT).

Staining dish reagent	Number of change required
- Formalin ---------------------------	Change out after 2 runs or once a week.
- TPBS -------------------------------	Change out with each test.
- 3% Hydrogen peroxide ----------	Change out with each test.
- Deionized/distrilled water (dH2O)--	Change out with each test.

Appendix XIV

Composition of RT-PCR, PCR Reaction Mix

Composition of RT – Reaction Mix

Reagent	Amount per Rxn, µl		# Rxn's	Total Volume, µl
DEPC H_20 ET Buffer (5x) dNTPs (10mM)	7.5 4.5 2.2	x x x	20 20 20	146 90 44
Total	14	X	20	280

Aliquots were made ahead of time (i.e., 10 reactions per tube) and were stored in -20^oC .14.8 µl total volume per reaction.

Transcriptase and RNase component of RT-Reaction Mix

Reagent	Amount per Rxn, µl		# Rxn's/tube		Total Volume, µl
Reverse Transcriptase RNase Inhibitor	0.4 0.4	x x	Depends on # of rxn required		Added to each tube

Composition of PCR Rxn mix

Reagent	Amount per Rxn, µl		# Rxn's	Total Volume, µl
DNase – Rnase free H_20 RT Buffer Tris 8.3 (10x) Forward Primer Reverse Primer Ampli Tag	74.5 8.64 1.1 1.5 0.54	X s x x x µl	# of rxn	Added to each tube
Total				

Appendix XV

Primers used for RT-PCR and PCR Amplification Reaction.

Primers used (10µm for RT-PCR, 40µm for PCR Amplification Rxn)

001F: ACGCTTAACGAMAAA

550F: ATGTGYGCTAAYTGGAGYAC

1066F: GARAGAAGATTCTTCAGRGA

550B: GTRCTCCARTTAGCRCACAT

1066B: TCYCTGAAGAATCTTCTYTC

304: TTGACGAAGATCTTGCTCAT

Appendix XVI

Composition of Polyacrylamide Gel Mix

Composition of Polyacrylamide Gel Mix

Reagent	**Amt for 50ml**	**Amt for 100ml**
Urea (electrophoresis grade) BP 169 – 212	18g	36g
Long Ranger gel solution (containing acrylamide)	5.4ml	10.8ml
Water (Deionized)	25ml	50ml
10x TBE	5.0ml	10ml
Temed	35µl	70µl
100% APS (ammonium persulfate buffer)	250µl	500µl

Appendix XVII

Preparation of growth media

Preparation of growth media

Reagents	Volume (ml)	
	1L (1000ml)	**500ml**
Distilled H_20	740	370
MEM vitamin solution (100x)	20	10
Eagle MEM (10x)	100	50
Fetal bovine serum	100	50
Glutamine (100x)	20	10
Antibiotic – Antimycotic (100x)	10	5
Sodium bicarbonate (7.5%)	10	5

The medium was filtered using 0.22µm filter and stored at 4^oC.

Appendix XVIII

Results of Titration of Virus Used for Challenge

Titration of various supernatant (% infected MNA cells per well/dilution)

From neat to 7 serial 10 fold dilutions (100ul volume).

Chad dog virus isolates 2006 – 45 supernatant titrations on MNA cells:

-7	0	0	0	0	0	0	
-6	0	0	0	0	0	0	
-5	0	0	0	5%	5%	0	
-4	0	0	0	5%	5%	5%	
-3	<5%	<5%	5%	5%	10%	10%	
-2	10%	10%	10%	25%	25%	20%	
-1	20%	25%	25%	60%	60%	50%	
Neat	40%	50%	40%	90%	60%	80%	
	2006-45(A)	2006-45(B)	2006-45	2006-45	2006-45	2006-45	2006-45
Date	18th Nov	18th Nov	19th Nov	21st Nov	23rd Nov	25th Nov	27th Nov

Chad dog virus isolates 2006 – 70 supernatant titrations on MNA cells:

-7	0	0	0	0	0	0	
-6	0	0	0	0	0	0	
-5	0	0	0	5%	5%	0	
-4	0	5	5	10%	5%	5%	
-3	5%	10%	10%	50%	10%	10%	
-2	20%	25%	25%	25%	25%	20%	
-1	40%	50%	70%	90%	70%	60%	
Neat	70%	80%	90%	100%	100%	90%	
	2006-70(A)	2006-70(B)	2006-70	2006-70	2006-70	2006-70	2006-70
Date	18th Nov	18th Nov	19th Nov	21st Nov	23rd Nov	25th Nov	27th Nov

Virus suspernatant stored -80 Forma Freezer (FEO 02463) Rack 35B box 14

Samples labeled 2006-70 Chad Dog 21-Nov-07

42 aliquots at 0.5ml each.

Tiration of various supernatant (% infected MNA cells per well/dilution)

From neat to 7 serial 10 fold dilutions (100ul volume).

Chad virus isolate 2006-45

-7	0	0	0	0	0
-6	0	0	0	0	0
-5	0	0	0	5%	5%
-4	0	0	0	5%	5%
-3	<5%	<5%	5%	5%	10%
-2	10%	10%	10%	25%	25%
-1	20%	25%	25%	60%	60%
Neat	40%	50%	40%	90%	90%
	2006-45(A)	2006-45(B)	2006-45(19th)	2006-45	2006-45
Date	18th Nov	18th Nov	19th Nov	21st Nov	23rd Nov

Chad virus isolates 2006-70

-7	0	0	0	0	0
-6	0	0	0	0	0
-5	0	0	0	5%	5%
-4	0	5	5	10%	5%
-3	5%	10%	10%	25%	10%
-2	20%	25%	25%	50%	25%
-1	40%	50%	70%	90%	70%
Neat	70%	80%	90%	100%	100%
	2006-70(A)	2006-70(B)	2006-70	2006-70	2006-70
Date	18th Nov	18th Nov	19th Nov	21st Nov	23rd Nov

		Virus No. (% infection MNA cells)	
Passage No.	Date	2006-70	2006-45
1.	31-Oct-07	10%	5%
2.	01-Nov-07	25%	10%
3.	03-Nov-07	70%	50%
4.	05-Nov-07	90%	90%
07-Nov-07: Added 30 million virus infected MNA cells to 2 T150 flasks (70 and 45) + 25 ml of MEM-10 each. P5			
09-Nov-07: Collected supernatant for virus titration; added 25 ml fresh MEM-10 each flask.			
09-Nov-07: Titration of supernatant, 7 serial 10 fold, starting with undiluted supernatant.			
11-Nov-07:FSR virus titration slides, Infectivity low, <5% infected cells in undiluted well. Both ended at 1:10.			
13-Nov-07: Passage of T150 flasks. Added 1.0ml of infected MNA cells each (virus 70 and 45) to 2.0ml of fresh MNA cells in T75 flasks. Monitored % infection (P6).			
Passage 7 (from 12-Nov)	16-Nov-07	100%	100%
16-Nov-07: Collect supernatant for titration. Passage each flask (P7). Add 2ml of infected MNA cells to 2ml flesh cells to T75 flasks, monitor % infection, collect supernatant for titration 48 to 72 hours.			
		Virus No. (% infection MNA cells)	
Passage No.	Date	2006-70	2006-45
1.	31-Oct-07	10%	5%
2.	01-Nov-07	25%	10%
3.	03-Nov-07	70%	50%

Step wise Preparation of Challenge Virus

		Virus No. (% infection MNA cells)	
Passage No.	Date	2006-70	2006-45
1.	31-Oct-07	10%	5%
2.	01-Nov-07	25%	10%
3.	03-Nov-07	70%	50%
4.	05-Nov-07	90%	90%
07-Nov-07: Added 30 million virus infected MNA cells to 2 T150 flasks (70 and 45) + 25 ml of MEM-10 each. P5			
09-Nov-07: Collected supernatant for virus titration; added 25 ml fresh MEM-10 each flask.			
09-Nov-07: Titration of supernatant, 7 serial 10 fold, starting with undiluted supernatant.			
11-Nov-07:FSR virus titration slides, Infectivity low, <5% infected cells in undiluted well, both ended at 1:10.			
13-Nov-07: Passage of T150 flasks. Added 1.0ml of infected MNA cells each (virus			

70 and 45) to 2.0ml of fresh MNA cells in T75 flasks. Monitored % infection (P6).			
Passage 7 (from 12-Nov)	16-Nov-07	100%	100%
16-Nov-07: Collect supernatant for titration. Passage each flask (P7). Add 2ml of infected MNA cells to 2ml flesh cells to T75 flasks, monitor % infection, collect supernatant for titration 48 to 72 hours.			
Titration of supernatant from 16-Nov (% infection) 48 hours			
		2006-07	2006-45
	Neat	50%	40%
	-1	25%	20%
	-2	10%	10%
	-3	5%	5%
	-4	0	0
18-Nov-07: Ccollected supernatant for titration; refed flasks with 20 ml fresh MEM-10			
19-Nov-07: Titration of supernatant from 18-Nov; and sup. Collected 19-Nov, Neat, 7 serial 10 fold dilutions in MEM-10.			
20-Nov-07: Passage each flask (P8). Add 2 ml of infected MNA cells to 2 ml flesh cells to T75 flasks, monitor % infection, collect supernatant for titration 48 to 72 hours.			
23-Nov-07: Collect supernatant for virus titration (2 flasks); passage 2 ml infected cells plus 2ml fresh MNA cells + 16ml MEM-10.			
25-Nov-07: Collected supernatant and refed flasks (2); 20ml MEM-10.			
FINAL: 27-Nov-07 collected supernatant and discarded flasks (2)			

Titration of various supernatant (% infected MNA cells per well/dilution)

From neat to 7 serial 10 fold dilutions (100ul volume).

-7	0	0	0
-6	0	0	0
-5	0	0	0
-4	0	0	0
-3	5%	<5%	<5%
-2	10%	10%	10%
-1	25%	20%	25%
Neat	40%	40%	50%
	2006-45(19th)	2006-45 (A)	2006-45 (B)
-7	0	0	0
-6	0	0	0
-5	0	0	0
-4	5%	0	5
-3	10%	5%	10%
-2	25%	20%	25%
-1	70%	40%	50%
Neat	90%	70%	80%
	2006-70(19th)	2006-70 (A)	2006-70 (B)

Appendix XIX

Fluorescence Microscope

The Manufacturers offer many equipment options. Appendix contains a discussion of oculars, objective lens, filter sets, and light sources and their effect on instrument performance. At a minimum, all rabies diagnostic laboratories should have a reflected light (incident light) fluorescence microscope with high quality objective lenses. Both magnification and numeric aperture (NA) must be considered in lens selection. Although image size increases with magnification, both resolution and image brightness are related to the NA of the objective lens, and brightness decreases with magnification. For example, a high quality 20 X dry objective with an NA of 0.75 provides a brighter image over a larger field of view wth no loss of resolution as compared to a dry 40 X objective with an NA of 0.75. The use of immersion oil increases image brightness by preventing the loss of emitted light in the airspace between coverslip and dry objective. Although not every slide must be observed with a 40 X oil objective of high NA, resolution of very fine dust like inclusions and recognition of some types of non-specific staining is aided by examination with this type of lens. An oil immersion lens requires high quality immersion oil. The oil should be chosen that produces the least autofluorescence and thus the best contrast between FITC and tissue (e.g., Cargille type DF). The oil should have the same refractive index as glass (1.515).

Appendix XX

Glycoprotein gene sequence from the ERA rabies virus

1 atggttcctc aggctctcct gtttgtaccc cttctggttt ttccattgtg ttttgggaaa
61 ttccctattt acacgatacc agacaagctt ggtccctgga gcccgattga catacatcac
121 ctcagctgcc caaacaattt ggtagtggag gacgaaggat gcaccaacct gtcagggttc
181 tcctacatgg aacttaaagt tggatacatc ttagccataa aaatgaacgg gttcacttgc
241 acaggcgttg tgacggaggc tgaaacctac actaacttcg ttggttatgt cacaaccacg
301 ttcaaaagaa agcatttccg cccaacacca gatgcatgta gagccgcgta caactggaag
361 atggccggtg accccagata tgaagagtct ctacacaatc cgtaccctga ctaccactgg
421 cttcgaactg taaaaaccac caaggagtct ctcgttatca tatctccaag tgtggcagat
481 ttggacccat atgacagatc ccttcactcg agggtcttcc ctagcgggaa gtgctcagga
541 ttggacccat atgacagatc ccttcactcg agggtrcttcc ctagcgggaa gatgcccgag
601 aatccgagac tagggatgtc ttgtgacatt tttaccaata gtagagggaa gagagcattc
661 aaagggagtg agacttgcgg ctttgtagat gaaagaggcc tatataagtc tttaaaagga
721 gcatgcaaac tcaagttatg tggagttcta ggacttagac ttatggatgg aacatgggtc
781 gcgatgcaaa catcaaatga aaccaaatgg tgccctcccg atcagttggt gaacctgcac
841 gactttcgct cagacgaaat tgagcacctt gttgtagagg agttggtcag gaagagagag
901 gagtgtctgg atgcactaga gtccatcatg acaaccaagt cagtgagttt cagacgtctc
961 agtcatttaa gaaaacttgt ccctgggttt ggaaaagcat ataccatatt caacaagacc
1021 ttgatggaag ccgatgctca ctacaagtca gtcagaactt ggaatgagat cctcccttca
1081 aaagggtgtt taagagttgg ggggaggtgt catcctcatg tgaacggggt gtttttcaat
1141 ggtataatat taggacctga cggcaatgtc ttaatcccag agatgcaatc atccctcctc
1201 cagcaacata tggagttgtt ggaatcctcg gttatccccc ttgtgcaccc cctggcagac
1261 ccgtctaccg ttttcaagga cggtgacgag gctgaggatt ttgttgaagt tcaccttccc
1321 gatgtgcaca atcaggtctc aggagttgac ttgggtctcc cgaactgggg gaagtatgta
1381 ttactgagtg caggggccct gactgccttg atgttgataa ttttcctgat gacatgttgt
1441 agaagagtca atcgatcaga acctacgcaa cacaatctca gagggacagg gagggaggta
1501 tcagtcactc cccaaagcgg gaagatcata tcttcatggg aatcacacaa gagtgggggt
1561 gagaccagac tgtgaggact ggccgtcctt tcaacgatcc aagtcctgaa gatcacctcc
1621 ccttgggggg ttctttttga aaaaaaacct gggttca

Appendix XXI

GLYCOPROTEIN GENE NUCLEOTIDE SEQUENCE OF THE WEST CAUCASIAN BAT VIRUS

1 atggcttcct actttgcgtt ggtcttgaac gggatctcta tggttttcag tcaaggtctt
61 ttcccccttt acactatccc tgaccatctg ggaccatgga cccccataga tctaagtcac
121 cttcactgcc cgaacaatct ttatactgat gcctcttatt gtacaactga acaaagcata
181 acctacacag agttgaaggt cggatcatct gtgtcacaaa aaatccccgg atttacatgt
241 acgggggtaa gaactgaatc tgtaacatat accaactttg ttggctatgt gactaccacg
301 ttcaagaaaa aacactttcc tcctaaatcc agggactgta gagaggcgta tgagaggaag
361 aaagcaggag atcctagata tgaagagtct ttagcccacc catatcctga caacagttgg
421 ctgagaacag tgactacaac aaaggattcc tgggtgatca tcgagcccag tgtagtggag
481 ttagatatat acacaagtgc cttgtattca cctcttttca aggatggaac atgttcaaaa
541 tctagaacat attcccccta ctgtccaacc aatcatgact tcaccatttg gatgccagag
601 agtgaaaaca taagatctgc ctgtaatctg ttttccacaa gtagagggaa actagtcagg
661 aaccgcacat ccacctgcgg gattatcgat gagagagggc tgttcagatc agttaaagga
721 gcatgcaaaa tatcaatatg cggtaggcag ggaatccgtt tagtggatgg aacttggatg
781 tcttttagat actcagagta cttacctgtg tgttctccat cacagctgat caacacgcac
841 gacatcaagg tcgatgagct ggagaatgct atagttttag acttgattag gaggagagaa
901 gaatgtcttg acaccctaga aacaattttg atgtcaggat ctgtgagtca caggaggctg
961 agtcatttca gaaagctggt tccaggatct gggaaggctt actcttatat aaacggcacc
1021 ttaatggaat cagatgctca ctacatcaag gtagagaatt ggtcagaggt catcccacac
1081 aaaggatgtc tcatggtcgg gggcaaatgc tatgagccag tcaatgatgt gtatttcaac
1141 gggatcattc gggattcaaa taatcagatc ttgatacctg agatgcagtc cagtcttctc
1201 agagaacatg ttgacctgtt gaaggctaat atagttccgt tcaggcatcc aatgttactt
1261 aggtccttca catctgacac tgaagaagat atcgtcgagt ttgtcaaccc tcatctccaa
1321 gatacccaga agttggtgtc agatatggat ctcgggttat cagactggaa gagatatcta
1381 ctaattggat ctttggccgt aggaggagtg gtagcaatct tattcatcgg aacatgttgt
1441 ctgagatgta gagcagggag aaacagaaga acaatccgat ccaatcatag gtcattgtcc
1501 catgacgtgg tgttccataa agataaggat aaagtgatta cttcttggga atcttacaag
1561 ggacaaactg cccaataa

Appendix XXII

ENTIRE NUCLEOTIDE SEQUENCE OF THE WEST CAUCASIAN BAT VIRUS

1 atggattctg aacacattgt gtttagggtc agaaatgaaa tagtgactct caaacccgaa
61 gtgatatccg accagtatga atataaatat cctgccatta cagataagaa aaaaccctcc
121 ataacactcg ggagggctcc cgatctgagc attgcctaca ggtcgattct gtcagggttc
181 aatgctgcta agttggatcc agatgatgtg tgctcctatc tagcagctgc tatgccattg
241 ttcgaagggg tttgcccaga ggattggatc agttacggga tcattatagc aaggaaggga
301 gacaagatta accctagtca cctggtagat ataatgagga cagaagtgga aggtaactgg
361 tctcaatctg gaggcgctga cgtgactaga aatcccaccg ttgctgagca tgcctccctg
421 gtaggtctcc tcctctgcct gtatagattg agcaaaatag tgggccaaaa tacagccaac
481 tacaagacaa atgttgcaga caggatggaa caaatattcg aaacagcccc ttttgtcaaa
541 atcatagagc accacacact gatgacaact cacaagatgt gtgccaattg gagtaccatt
601 ccaaacttta gatttcttgt tggaacctat gacatgtttt tctcaagaat tgaccattta
661 tattctgctt tgagggtcgg cacagttgtc actgcatatg aagactgtac cgggcttgta
721 tcgttcactg cttttctcaa acagataaac ttgtcagcaa gagatgcaat cctatacttt
781 ttccataaga attttgaaga agaaatcagg agaatgttcc gtcccaatca ggagactgct
841 gttccccact cttatttcat ccactttaga tctttgggtc ttagcgggaa gtctccatac
901 tcctccaatg cagtgggtca tgtgttcaac ttgattcact ttgtgggatg ctacatgggg
961 caagtaaggt ctctaaatgc gacagtcatt cagacatgtg ccccccacga aatgtctgtt
1021 ttgggaggtt atttggggga ggaattcttt ggaaaaggta cgtttgagag aagattcttt
1081 agagatgaaa gagagttgca ggatcatctt gaggcagaag aggccaagat agacattgct
1141 ctggcagatg acgccacagt agactccgga gatgaggatt tctacggcgg agagtcgagg
1201 agcccagagg cagtctataa tagaatcatc atgaacaagg ggagactcaa gaagttacac
1261 ataaagaggt atagatctgt gagctctaat catcaagccc gacccaatac ttttgcagag
1321 tttctaaata aggtttattc ggatgacaat tgagttaaac tgcaagtatt gaaatgaaaa
1381 aaacatcaac acccctcttc catcttctgt ctcgaagatg agcaagagtc ttattcaccc
1441 cagtgatctc cgggcaggct tagctgacat agaaatggca gatgagactg tcgatctggt
1501 ttacaagaat ctgtcagaag gacaagctca cttgcaaggg gaacccttttg atatcaaaga
1561 ccttccagag ggagtctcca aattacaaat ctcagataat gtgagaagtg acacctcacc
1621 caatgaatac tcagatgaag acgatgagga aggagaagat gagtacgaag aagtctatga
1681 ccctgtgagt gctttccaag attttctaga cgagacgggt tcgtacctca tctctaagct
1741 aaagaaggga gaaaagatca agaagacttg gtctgaggtc tctagagtga tctatagtta
1801 cgtcatgagt aactttcctc ctagaccgcc taaaccaaca acaaaagata tcgctgttca
1861 ggctgatctc aagaaaccaa atgagattca gaagatctcc gaacataaat ctaagtcaga
1921 accttcaccc agagaacctg tagtagagat gcataagcat gccactcttg aaaatcctga

1981 ggatgatgaa ggagctctcg aaagtgaaat agctcaccaa gtcgcagaga gttactccaa
2041 aaagtataag ttcccatcta agtcctcagg tatttttctc tggaattttg aacaactgaa
2101 gatgaacttg gatgatattg tccaggttgc aagaggtgtt ccggggataa gtcaaattgt
2161 tgagaggggt ggtaagctcc ctttgagatg tatgcttggt tatgttggat tagaaacttc
2221 taagagattc aggtctctgg tcaaccaaga caagttgtgt aaactgatgc aagaggatct
2281 taatgcctac tcggtctcat ccaacaatta gaggaataga aaggattaat ttgtaacatt
2341 gggagatccg cttggtttca atggaagcaa gcaaattcaa gaatttcaca actcacaaga
2401 ttggagaagt cttcccataa tgaaaaaaac taacacccct catcatgaat ttcctaagga
2461 aaatgatgaa aacatgccgt gatgacgagt cttccaagcc tttggatcct tcagcccctc
2521 ctgatgacga tgacttgtgg cttcctcctc cagaatatgt tcctcttcat gaaatctcct
2581 ctaaagggaa cacacgcaat ttctgcatca gcggagaagt taagatatgt agtcccaatg
2641 gatactcctt caagatcatc cgtcacatcc tccgatcttt tgagagcgtt tactctggga
2701 atcgaaggat gataggtttg gtcaaggtgg tcatcgggct aaccctgtct gggagtccag
2761 tccccgaggg catgaactgg gtttataaac ttagaaagac tcttgtgttc cagtggtcta
2821 attccagcgg gcccttggaa ggggaagagt tagagtactc tcaagaaatc acatgggatg
2881 atgactctga gtatgtaggg ctgcagatca gagtcaatgc aaaacaatgc cacattgcag
2941 ggagatcctg gtgtgtgaac atgaactccc gtgcctgcca attgtggtct gacatgacct
3001 taaaaactca acaatctgaa gaagatgagc acacctcagt tctcatagaa tgagaattta
3061 tcctcacaag cagattatca catcctgcaa catcttgagc aatagaacaa tttagtaacg
3121 gtgccctatc atagctgcag atataggagc ctacgatcaa cttttttttct tgtgacgatg
3181 aactcaactg tgaaaaaaac tgactctcag aagctccagc atgaaggact taagagacaa
3241 catccctcat ctcagagaaa tggcttccta ctttgcgttg gtcttgaacg ggatctctat
3301 ggttttcagt caaggtcttt tccccccttta cactatccct gaccatctgg gaccatggac
3361 ccccatagat ctaagtcacc ttcactgccc gaacaatctt tatactgatg cctcttattg
3421 tacaactgaa caaagcataa cctacacaga gttgaaggtc ggatcatctg tgtcacaaaa
3481 aatccccgga tttacatgta cgggggtaag aactgaatct gtaacatata ccaactttgt
3541 tggctatgtg actaccacgt tcaagaaaaa acactttcct cctaaatcca gggactgtag
3601 agaggcgtat gagaggaaga aagcaggaga tcctagatat gaagagtctt tagcccaccc
3661 atatcctgac aacagttggc tgagaacagt gactacaaca aaggattcct gggtgatcat
3721 cgagcccagt gtagtggagt tagatatata cacaagtgcc ttgtattcac ctcttttcaa
3781 ggatggaaca tgttcaaaat ctagaacata ttccccctac tgtccaacca atcatgactt
3841 caccatttgg atgccagaga gtgaaaacat aagatctgcc tgtaatctgt tttccacaag
3901 tagagggaaa ctagtcagga accgcacatc cacctgcggg attatcgatg agagagggct
3961 gttcagatca gttaaaggag catgcaaaat atcaatatgc ggtaggcagg gaatccgttt
4021 agtggatgga acttggatgt cttttagata ctcagagtac ttacctgtgt gttctccatc
4081 acagctgatc aacacgcacg acatcaaggt cgatgagctg gagaatgcta tagttttaga
4141 cttgattagg aggagagaag aatgtcttga caccctagaa acaattttga tgtcaggatc
4201 tgtgagtcac aggaggctga gtcatttcag aaagctggtt ccaggatctg ggaaggctta

4261 ctcttatata aacggcacct taatggaatc agatgctcac tacatcaagg tagagaattg
4321 gtcagaggtc atcccacaca aaggatgtct catggtcggg ggcaaatgct atgagccagt
4381 caatgatgtg tatttcaacg ggatcattcg ggattcaaat aatcagatct tgatacctga
4441 gatgcagtcc agtcttctca gagaacatgt tgacctgttg aaggctaata tagttccgtt
4501 caggcatcca atgttactta ggtccttcac atctgacact gaagaagata tcgtcgagtt
4561 tgtcaaccct catctccaag atacccagaa gttggtgtca gatatggatc tcgggttatc
4621 agactggaag agatatctac taattggatc tttggccgta ggaggagtgg tagcaatctt
4681 attcatcgga acatgttgtc tgagatgtag agcagggaga aacagaagaa caatccgatc
4741 caatcatagg tcattgtccc atgacgtggt gttccataaa gataaggata aagtgattac
4801 ttcttgggaa tcttacaagg gacaaactgc ccaataacaa ggtattgaat tcttcaaggg
4861 agaatcaagc acctagaaga gttataagct ctcaagagac atgcgttctt ttgagttgta
4921 tatcttcttg caatacaaaa tatggaatgc agggtactca atcagataaa ttcagaatgg
4981 tgctttgaga ggaaacaatt atggtagcat tacactggac actggagaat atctccaaaa
5041 gcgattgctc tctttgataa acatatccat ctaggaggaa ttggtgtttt ctccgctagt
5101 ctatttctag ctggttcatg gtacttttga tgctataacc tcgtattata ggacctcaca
5161 gttcggttta ctgggactta cagataatca tctcatctcc ttcttaagca tctgctggga
5221 ttttaatgca tgagggcagt gacagtgtgc aataatgcgg atacaatact aactaactgg
5281 tttgatagga cttgtcttgt gtgtttcatt taccctagat gccccatcag tagaagcata
5341 tatctttgtt tagtatccca tgctgttaaa tgttggcgcc aaagatatca aggagagaga
5401 aaagctaact aggcactaca atcgaagtcg acaacccctt tgaacagggg acatgaaagc
5461 agcatggttg cccctcgagg gaactctcct ccaatataca atctgcagtc tatactgtat
5521 ctaacaggtt aatgaaaaaa acattaatca gaaactgaag cacccgccag atacaggacc
5581 aaatcacagt tgtcacatca ggaaattcac catacacaca gacgtgccta tccaaggaga
5641 taacacccct actttcagat tattaattac caagttcttc tcgttcttgc ttataaacca
5701 tgtgga

Appendix XXIII

Nucleotide sequence of Nucleoprotein gene of Nigerian dog rabies variants

		* 20 * 40 * 60
NL 16	:	ATGGATGCCGACAAGATTGTATTCAAAGTCAATAATCAGGTGGTTTCCTTGAAGCCAGAGAT
NLLB	:	ATGGATGCCGACAAGATTGTATTCAAAGTCAATAATCAGGTGGTTTCCTTGAAGCCAGAGAT
RD44	:	ATGGATGCCGACAAGATTGTATTCAAAGTCAATAATCAGGTGGTTTCCTTGAAGCCAGAGAT
RD132	:	ATGGATGCCGACAAGATTGTATTCAAAGTCAATAATCAGGTGGTTTCCTTGAAGCCAGAGAT
RD163	:	ATGGATGCCGACAAGATTGTATTCAAAGTCAATAATCAGGTGGTTTCCTTGAAGCCAGAGAT
RD194	:	ATGGATGCCGACAAGATTGTATTCAAAGTCAATAATCAGGTGGTTTCCTTGAAGCCAGAGAT
RD	:	ATGGATGCCGACAAGATTGTATTCAAAGTCAATAATCAGGTGGTTTCCTTGAAGCCAGAGAT
ZAD1	:	ATGGATGCCGACAAGATTGTATTCAAGGTCAATAATCAGGTGGTTTCCTTGAAGCCAGAGAT
ZAD2	:	ATGGATGCCGACAAGATTGTATTCAAGGTCAATAATCAGGTGGTTTCCTTGAAGCCAGAGAT
ZAD4	:	ATGGATGCCGACAAGATTGTATTCAAGGTCAATAATCAGGTGGTTTCCTTGAAGCCAGAGAT
ZAD8	:	ATGGATGCCGACAAGATTGTATTCAAGGTCAATAATCAGGTGGTTTCCTTGAAGCCAGAGAT
ZAD10	;	ATGGATGCCGACAAGATTGTATTCAAGGTCAATAATCAGGTGGTTTCCTTGAAGCCAGAGAT
SADB1871	:	ATGGATGCCGACAAGATTGTATTCAAAGTCAATAATCAGGTGGTCTCTTTGAAGCCTGAGAT

		80 * 100 * 120 *
NL 16	:	ATCAATATGAGTACAAATACCCTGCTATCAAAGACTTGAAGAAGCCCAGTATAACCTTAGGG
NLLB	:	ATCAATATGAGTACAAATACCCTGCTATCAAAGACTTGAAGAAGCCCAGTATAACCTTAGGG
RD44	:	ATCAATATGAGTACAAATACCCTGCTATCAAAGACTTGAAGAAGCCCAGTATAACCTTAGGG
RD132	:	ATCAATATGAGTACAAATACCCTGCTATCAAAGACTTGAAAAAGCCCAGTATAACCTTAGGG
RD163	:	ATCAATATGAGTACAAATACCCTGCTATCAAAGACTTGAAAAAGCCCAGTATAACCTTAGGG
RD194	:	ATCAATATGAGTACAAATACCCTGCTATCAAAGACTTGAAGAAGCCCAGTATAACCTTAGGG
RD	:	ATCAATATGAGTACAAATACCCTGCTATCAAAGACTTGAAGAAGCCCAGTATAACCTTAGGG
ZAD1	:	ATCAATATGAGTACAAATACCCTGCTATCAAAGACTTGAAGAAGCCCAGTATAACCTTAGGG
ZAD2	:	ATCAATATGAGTACAAATACCCTGCTATCAAAGACTTGAAGAAGCCCAGTATAACCTTAGGG
ZAD4	:	ATCAATATGAGTACAAATACCCTGCTATCAAAGACTTGAAGAAGCCCAGTATAACCTTAGGG
ZAD8	:	ATCAATATGAGTACAAATACCCTGCTATCAAAGACTTGAAGAAGCCCAGTATAACCTTAGGG
ZAD10	:	ATCAATATGAGTACAAATACCCTGCTATCAAAGACTTGAAGAAGCCCAGTATAACCTTAGGG
SADB1971	:	ATCAATATGAGTACAAGTACCCTGCCATCAAAGATTTGAAAAAGCCCTGTATAACCCTAGGA

		* 160 * 180 * 200
NL 16	:	CGATTTAAACAAAGCATACAAGTCAGTTTTATCAGGCATGAATGCTGCCAAGCTTGATCCTG
NLLB	:	CGATTTAAACAAAGCATACAAGTCAGTTTTATCAGGCATGAATGCTGCCAAGCTTGATCCTG
RD44	:	CGATTTAAACAAAGCATACAAGTCAGTTTTATCAGGCATGAATGCTGCCAAGCTTGATCCTG
RD132	:	CGATTTAAACAAAGCATACAAGTCAGTTTTATCAGGCATGAATGCTGCCAAGCTTGATCCTG
RD163	:	CGATTTAAACAAAGCATACAAGTCAGTTTTATCAGGCATGAATGCTGCCAAGCTTGATCCTG
RD194	:	CGATTTAAACAAAGCATACAAGTCAGTTTTATCAGGCATGAATGCTGCCAAGCTTGATCCTG
RD	:	CGATTTAAACAAAGCATACAAGTCAGTTTTATCAGGCATGAATGCTGCCAAGCTTGATCCTG
ZAD1	:	CGATTTAAACAAAGCATACAAGTCAGTTTTATCAGGTATGAATGCTGCCAAGCTTGATCCTG
ZAD2	:	CGATTTAAACAAAGCATACAAGTCAGTTTTATCAGGTATGAATGCTGCCAAGCTTGATCCTG
ZAD4	:	CGATTTAAACAAAGCATACAAGTCAGTTTTATCAGGTATGAATGCTGCCAAGCTTGATCCTG
ZAD8	:	CGATTTAAACAAAGCATACAAGTCAGTTTTATCAGGTATGAATGCTGCCAAGCTTGATCCTG
ZAD10	:	CGATTTAAACAAAGCATACAAGTCAGTTTTATCAGGTATGAATGCTGCCAAGCTTGATCCTG
SAD1971	:	CGATTTAAATAAAGCATACAAGTCAGTTTTGTCAGGCATGAGCGCCGCCAAACTTAATCCTG

220 * 240 * 260 *

NL 16	:	TGTTCCTATTTGGCAGCTGCGATGCAGCTCTTTGAAGGGACATGTCCTGAAGACTGGACCAG
NLLB	:	TGTTCCTATTTGGCAGCTGCAATGCAGCTCTTTGAAGGGACATGTCCTGAAGACTGGACCAG
RD44	:	TGTTCCTATTTGGCAGCTGCGATGCAGCTCTTTGAAGGGACATGTCCTGAAGACTGGACCAG
RD132	:	TGTTCCTATTTGGCAGCTGCGATGCAGCTCTTTGAAGGGACATGTCCTGAAGACTGGACCAG
RD163	:	TGTTCCTATTTGGCAGCTGCGATGCAGCTCTTTGAAGGGACATGTCCTGAAGACTGGACCAG
RD194	:	TGTTCCTATTTGGCAGCTGCAATGCAGCTCTTTGAAGGGACATGTCCTGAAGACTGGACCAG
RD	:	TGTTCCTATTTGGCAGCTGCGATGCAGCTCTTTGAAGGGACATGTCCTGAAGACTGGACCAG
ZAD1	:	TGTTCCTATCTGGCAGCTGCGATGCAACTCTTTGAAGGGACATGTCCTGAAGACTGGACCAG
ZAD2	:	TGTTCCTATCTGGCAGCTGCGATGCAACTCTTTGAAGGGACATGTCCTGAAGACTGGACCAG
ZAD4	:	TGTTCCTATCTGGCAGCTGCGATGCAACTCTTTGAAGGGACATGTCCTGAAGACTGGACCAG
ZAD8	:	TGTTCCTATCTGGCAGCTGCGATGCAACTCTTTGAAGGGACATGTCCTGAAGACTGGACCAG
ZAD10	:	TGTTCCTATCTGGCAGCTGCGATGCAACTCTTTGAAGGGACATGTCCTGAAGACTGGACCAG
SADB1971	:	TGTTCCTATTTGGCAGCGGCAATGCAGTTTTTTGAGGGGACATGTCCGGAAGACTGGACCAG

* 300 * 320 * 340

NL 16	:	TCTTGATTGCAAGAAAGGGAGACAAGATCACCCCAGATTCTCTTGTGGAGATCAAGCGTACA
NLLB	:	TCTTGATTGCAAGAAAGGGAGACAAGATCACCCCAGATTCTCTTGTGGAGATCAAGCGTACA
RD44	:	TCTTGATTGCAAGAAAGGGAGACAAGATCACCCCAGATTCTCTTGTGGAGATCAAGCGTACA
RD132	:	TCTTGATTGCAAGAAAGGGAGACAAGATCACCCCAGATTCTCTTGTGGAGATCAAGCGTACA
RD163	:	TCTTGATTGCAAGAAAGGGAGACAAGATCACCCCAGATTCTCTTGTGGAGATCAAGCGTACA
RD194	:	TCTTGATTGCAAGAAAGGGAGACAAGATCACCCCAGATTCTCTTGTGGAGATCAAGCGTACA
RD	:	TCTTGATTGCAAGAAAGGGAGACAAGATCACCCCAGATTCTCTTGTGGAGATCAAGCGTACA
ZAD1	:	TCTTGATTGCAAGAAAGGGAGACAAGATCACCCCAGATTCTCTTGTGGAGATCAAGCGTACG
ZAD2	:	TCTTGATTGCAAGAAAGGGAGACAAGATCACCCCAGATTCTCTTGTGGAGATCAAGCGTACG
ZAD4	:	TCTTGATTGCAAGAAAGGGAGACAAGATCACCCCAGATTCTCTTGTGGAGATCAAGCGTACG
ZAD	:	TCTTGATTGCAAGAAAGGGAGACAAGATCACCCCAGATTCTCTTGTGGAGATCAAGCGTACG
ZAD1	:	TCTTGATTGCAAGAAAGGGAGACAAGATCACCCCAGATTCTCTTGTGGAGATCAAGCGTACG
SADB1971	:	TTGTGATTGCACGAAAAGGAGATAAGATCACCCCAGGTTCTCTGGTGGAGATAAAACGTACT

360 * 380 * 400 *

NL 16	:	AGGAAACTGGGCTCTAACAGGAGGTATGGAACTGACGAGAGACCCCACGGTTTCTGAACATG
NLLB	:	AGGAAACTGGGCTCTAACAGGAGGTATGGAACTGACGAGAGACCCCACGGTTTCTGAACATG
RD44	:	AGGAAACTGGGCTCTAACAGGAGGTATGGAACTGACGAGAGACCCCACGGTTTCTGAACATG
RD132	:	AGGAAACTGGGCTCTAACAGGAGGTATGGAACTGACGAGAGACCCCACGGTTTCTGAACATG
RD163	:	AGGAAACTGGGCTCTAACAGGAGGTATGGAACTGACGAGAGACCCCACGGTTTCTGAACATG
RD194	:	AGGAAACTGGGCTCTAACAGGAGGTATGGAACTGACGAGAGACCCCACGGTTTCTGAACATG
RD	:	AGGAAACTGGGCTCTAACAGGAGGTATGGAACTGACGAGAGACCCCACGGTTTCTGAACATG
ZAD1	:	AGGAAACTGGGCTCTGACAGGAGGTATGGAACTGACGAGAGACCCCACGGTTTCTGAACATG
ZAD2	:	AGGAAACTGGGCTCTGACAGGAGGTATGGAACTGACGAGAGACCCCACGGTTTCTGAACATG
ZAD4	:	AGGAAACTGGGCTCTGACAGGAGGTATGGAACTGACGAGAGACCCCACGGTTTCTGAACATG
ZAD8	:	AGGAAACTGGGCTCTGACAGGAGGTATGGAACTGACGAGAGACCCCACGGTTTCTGAACATG
ZAD10	:	AGGAAACTGGGCTCTGACAGGAGGTATGGAACTGACGAGAGACCCCCCGGTTTCTGAACATG
SADB1971	:	AGGGAATTGGGCTCTGACAGGAGGCATGGAACTGACAAGAGACCCCACTGTCCCTGAGCATG

* 440 * 460 * 480

NL 16	:	GTCGGTCTGCTCTTGAGTCTGTATAGGTTGAGCAAAATATCAGGACAAAACACAGGCAACTA
NLLB	:	GTCGGTCTGCTCTTGAGTCTGTATAGGTTGAGCAAAATATCAGGACAAAACACAGGCAACTT
RD44	:	GTCGGTCTGCTCTTGAGTCTGTATAGGTTGAGCAAAATATCAGGACAAAACACAGGCAACTA
RD132	:	GTCGGTCTGCTCTTGAGTCTGTATAGGTTGAGCAAAATATCAGGACAAAACACAGGCAACTA
RD163	:	GTCGGTCTGCTCTTGAGTCTGTATAGGTTGAGCAAAATATCAGGACAAAACACAGGCAACTA
RD194	:	GTCGGTCTGCTCTTGAGTCTGTATAGGTTGAGCAAAATATCAGGACAAAACACAGGCAACTA
RD	:	GTCGGTCTGCTCTTGAGTCTGTATAGGTTGAGCAAAATATCAGGACAAAACACAGGCAACTA
ZAD1	:	GTCGGTCTACTCTTGAGTCTGTATAGGTTGAGCAAAATATCAGGACAAAACACAGGCAACTA
ZAD2	:	GTCGGTCTGCTCTTGAGTCTGTATAGGTTGAGCAAAATATCAGGACAAAACACAGGCAACTA
ZAD4	:	GTCGGTCTGCTCTTGAGTCTGTATAGGTTGAGCAAAATATCAGGACAAAACACAGGCAACTA
ZAD8	:	GTCGGTCTGCTCTTGAGTCTGTATAGGTTGAGCAAAATATCAGGACAAAACACAGGCAACTA
ZAD10	:	GTCGGTCTGCTCTTGAGTCTGTATAGGTTGAGCAAAATATCAGGACAAAACACAGGCAACTA
SADB1971	:	GTCGGTCTTCTCTTGAGTCTGTATAGGTTGAGCAAAATATCCGGGACAAAACACTGGTAACTA

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                     500         *          520     *        540          *

NL 16      :  ACATTGCAGATCGGATAGAGCAGATTTTTGAGACAGCCCCTTTCGTTAAAATTGTGGAACAT
NLLB       :  ACATTGCAGATCGGATAGAGCAGATTTTTGAGACAGCCCCTTTCGTTAAAATTGTGGAACAT
RD44       :  ACATTGCAGATCGGATAGAGCAGATTTTTGAGACAGCCCCTTTCGTTAAAATTGTGGAACAT
RD132      :  ACATTGCAGATCGGATAGAGCAGATTTTTGAGACAGCCCCTTTCGTTAAAATTGTGGAACAT
RD163      :  ACATTGCAGATCGGATAGAGCAGATTTTTGAGACAGCCCCTTTCGTTAAAATTGTGGAACAT
RD194      :  ACATTGCAGATCGGATAGAGCAGATTTTTGAGACAGCCCCTTTCGTTAAAATTGTGGAACAT
RD         :  ACATTGCAGATCGGATAGAGCAGATTTTTGAGACAGCCCCTTTCGTTAAAATTGTGGAACAT
ZAD1       :  ACATTGCAGATCGGATAGAGCAGATTTTTGAGACAGCCCCTTTCGTTAAAATTGTGGAACAT
ZAD2       :  ACATTGCAGATCGGATAGAGCAGATTTTTGAGACAGCCCCTTTCGTTAAAATTGTGGAACAT
ZAD4       :  ACATTGCAGATCGGATAGAGCAGATTTTTGAGACAGCCCCTTTCGTTAAAATTGTGGAACAT
ZAD8       :  ACATTGCAGATCGGATAGAGCAGATTTTTGAGACAGCCCCTTTCGTTAAAATTGTGGAACAT
ZAD10      :  ACATTGCAGATCGGATAGAGCAGATTTTTGAGACAGCCCCTTTCGTTAAAATTGTGGAACAT
SADB1971   :  ACATTGCAGACAGGATAGAGCAGATTTTTGAGACAGCCCCTTTTGTTAAAATCGTGGAACAC
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                      *         580          *      600        *          620

NL 16      :  GATGACAACTCATAAAATGTGCGCTAACTGGGAGACAATGGGGACCTTCCGGGTCTTGGCCG
NLLB       :  GATGACAACTCATAAAATGTGCGCTAACTGGGAGACAAGACCGAGCTTCAGCTTCTTGAGAG
RD44       :  GATGACAACTCATAAAATGTGCGCTAACTGGGAGAAGATACGTTACGTCAAATTTGGGGCCG
RD132      :  GATGACAACTCATAAAATGTGCGCTACTGGGAGTACTATACCGAACTTCCGATTCTTGGCCG
RD163      :  GATGACAACTCATAAAATGTGCGCTAACTGGGAGACCATACCGAACTTCCGATTCTTGGCCG
RD194      :  GATGACAACTCATAAAATGTGCGCCAATTGGAGTACCATACCCAACTTCCGATTCTTGGCCG
RD         :  GATGACAACTCATAAAATGTGCGCTACTGGGAGCACAAAACCGAGCTTCCGATTCTTGAGCG
ZAD1       :  GATGACCACTCATAAAATGTGCGCTAATTGGAGTACCATACCGAACTTCCGATTCTTGGCCG
ZAD2       :  GATGACAACTCATAAAATGTGCGCTAATTGGAGTACCATACCGAACTTCCGATTCTTGGCCG
ZAD4       :  GATGACAACTCATAAAATGTGCGCTAATTGGAGTACCATACCGAACTTCCGATTCTTGGCCG
ZAD8       :  GATGACAACTCATAAAAATGGTGCTAATTGGAGTACCATACCGAACTTCCGATTCTTGGCCG
ZAD10      :  GATGACAACTCATAAAATGTGCGCTAACTGGGAGACAATACCGAACTTCCGATTCTTGGCCG
SADB1971   :  AATGACAACTCACAAAATGTGTGCTAATTGGAGTACTATACCAAACTTCAGATTTTTGGCCG
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                     640         *          660           *    680            *

NL 16      :  CCCATGTTTATCAACGGGATTCCGAAAATATCTTCAGCCACCTGCGTGGGCACAGTAGTCAC
NLLB       :  AACTTGTATTTCTCCCGGATTGAGCATCTATATTCAGCTATCAGAGTGGGCACAGTAGTCAC
RD44       :  GACATGTATTTCTCCCGGATTGAGCATCTATATTCACCTATCAGAGTGGGCACAGTAGTCAC
RD132      :  GACATGTATTTCTCCCGGATTGAGCATCTATATTCAGCTATCAGAGTGGGCACAGTAGTCAC
RD163      :  GACATGTATTTCTCCCGGATTGAGCATCTATATTCAGCTATCAGAGTGGGCACAGTAGTCAC
RD194      :  CACATGTTTTTCTCCCGGATTGAGCATCTATATTCAGCTATCAGAGTGGGCACAGTAGTCAC
RD         :  GACATGTGTTTCTCCCGGATTGAGCATCTATATTCAGCTATCAGAGTGGGCACAGTAGTCAC
ZAD1       :  CACATGTATTTCTCCCGGATTGAGCATCTATATTCAGCTATCVAGCTGGGCACAGTGGTCAC
ZAD2       :  GACATGTATTTCTCCCGGATTGAGCATCTATATTCAGCTATCAGAGTGGGCACAGTGGTCAC
ZAD4       :  GACATGTATTTCTCCCGGATTGAGCATCTATATTCAGCTATCAGAGTGGGCACAGTGGTCAC
ZAD8       :  GACATGTATTTCTCCCGGATTGGGCATCTTTATTCAGCTATCAGAGTGGGCACAGTGGTCAC
ZAD10      :  GACATGTATTTCTCCCGGATTGAGCATCTATATTCAGCTATCAGAGTGGGCACAGTGGTCAC
SAD1971    :  GACATGTTTTTCTCCCGGATTGAGCATCTATATTCAGCAATCAGAGTGGGCACAGTTGTCAC
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                      *         720          *     740         *        760

NL 16      :  AAGATTGCTCTGGGCTGGTGTCGTTCACAGGGTTCATAAAACAGATAAATCTCACTGCAAGA
NLLB       :  AAGATTGCTCTGGGCTGGTGTCGTTCACAGGGTTCATAAAACAGATAAATCTCACTGCAAGA
RD44       :  AAGATTGCTCTGGGCTGGTGTCGTTCACAGGGTTCATAAAACAGATAAATCTCACTGCAAGA
RD132      :  AAGATTGCTCTGGGCTGGTGTCGTTCACAGGGTTCATAAAACAGATAAATCTCACTGCAAGA
RD163      :  AAGATTGCTCTGGGCTGGTGTCGTTCACAGGGTTCATAAAACAGATAAATCTCACTGCAAGA
RD         :  AAGATTGCTCTGGGCTGGTGTCGTTCACAGGGTTCATAAAACAGATAAATCTCACTGCAAGA
ZAD1       :  AAGATTGCTCTGGACTGGTGTCCGTTACAGGGTTCATAAAACAGATAAACCCCACTGCAAGA
ZAD2       :  AAGATTGCTCTGGGCTGGTGTCATTCACAGGGTTCATAAAACAGATAAATCTCACTGCAAGA
ZAD4       :  AAGATTGCTCTGGGCTGGTGTCATTCACAGGGTTCATAAAACAGATAAATCTCACTGCAAGA
ZAD8       :  AAGATTGCTCTGGGCTGGTGTCATTCACAGGGTTCATAAAACAGATAAATCTCACTGCAAGA
ZAD10      :  AAGATTGCTCTGGGCTGGTGTCATTCACAGGGTTCATAAAACAGATAAATCTCACTGCAAGA
SADB1971   :  AAGACTGTTCAGGACTGGTATCATTTACTGGGTTCATAAAACAAATCAATCTCACCGCTAGA
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                780        *         800        *         820         *

NL 16     :     ACTATATTTCTTCCACAAGAACTTTGAAGAAGAGATACGAAGAATGTTCGAGCCAGGGCAAG
NLLB      :     ACTATATTTCTTCCACAAGAACTTTGAAGAAGAGATACGAAGAATGTTCGAGCCAGGGCAAG
RD44      :     ACTATATTTCTTCCACAAGAACTTTGAAGAAGAGATACGAAGAATGTTCGAGCCAGGGCAAG
RD132     :     ACTATATTTCTTCCACAAGAACTTTGAAGAAGAGATACGAAGAATGTTCGAGCCAGGGCAAG
RD163     :     ACTATATTTCTTCCACAAGAACTTTGAAGAAGAGATACGAAGAATGTTCGAGCCAGGGCAAG
RD        :     ACTATATTTCTTCCACAAGAACTTTGAAGAAGAGATACGAAGAATGTTCGAGCCAGGGCAAG
ZAD1      :     ACTATATTTCTTCCACAAGAACTTTGAAGAAGAGATACGAAGAATGTTCGAGCCAGGGCAAG
ZAD2      :     ACTATATTTCTTCCACAAGAACTTTGAAGAAGAGATACGAAGAATGTTCGAGCCAGGGCAAG
ZAD4      :     ACTATATTTCTTCCACAAGAACTTTGAAGAAGAGATACGAAGAATGTTCGAGCCAGGGCAAG
ZAD8      :     ACTATATTTCTTCCACAAGAACTTTGAAGAAGAGATACGAAGAATGTTCGAGCCAGGGCAAG
ZAD10     :     ACTATATTTCTTCCACAAGAACTTTGAAGAAGAGATACGAAGAATGTTCGAGCCAGGGCAAG
SADB1971  :     ACTATATTTCTTCCACAAGAACTTTGAGGAAGAGATACGAAGAATGTTTGAGCCAGGGCAAG
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                      *         860        *     880          *    900

NL 16     :     GTTCCTCACTCCTATTTCATTCACTTCCGCTCGTTGGGTCTGAGCGGGAAGTCCCCGTATTC
NLLB      :     GTTCCTCACTCCTATTTCATTCACTTCCGCTCGTTGGGTCTGAGCGGGAAGTCCCCGTATTC
RD44      :     GTTCCTCACTCCTATTTCATTCACTTCCGCTCGTTGGGTCTGAGCGGGAAGTCCCCGTATTC
RD132     :     GTTCCTCACTCCTATTTCATTCACTTCCGCTCGTTGGGTCTGAGCGGGAAGTCCCCGTATTC
RD163     :     GTTCCTCACTCCTATTTCATTCACTTCCGCTCGTTGGGTCTGAGCGGGAAGTCCCCGTATTC
RD194     :     GTTCCTCACTCCTATTTCATTCACTTCCGCTCGTTGGGTCTGAGCGGGAAGTCCCCGTATTC
RD        :     GTTCCTCACTCCTATTTCATTCACTTCCGCTCGTTGGGTCTGAGCGGGAAGTCCCCGTATTC
ZAD1      :     GTCCCTCACTCCTATTTCATTCACTTCCGCTCGTTGGGTCTGAGCGGGAAGTCCCCGTATTC
ZAD2      :     GTCCCTCACTCCTATTTCATTCACTTCCGCTCGTTGGGTCTGAGCGGGAAGTCCCCGTATTC
ZAD4      :     GTCCCTCACTCCTATTTCATTCACTTCCGCTCGTTGGGTCTGAGCGGGAAGTCCCCGTATTC
ZAD8      :     GTCCCTCACTCCTATTTCATTCACTTCCGCTCGTTGGGTCTGAGCGGGAAGTCCCCGTATTC
ZAD10     :     GTCCCTCACTCCTATTTCATTCACTTCCGCTCGTTGGGTCTGAGCGGGAAGTCCCCGTATTC
SADB1971  :     GTTCCTCACTCTTATTTCATCCACTTCCGTTCACTAGGCTTGAGTGGGAAATCTCCTTATTC
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```
                      920        *         940   *      960        *

NL 16     :     CAGTAGGTCATGTATTCAATCTCATTCACTTTGTTGGATGTTATATGGGTCAGGTCAGGTCC
NLLB      :     CAGTAGGTCATGTATTCAATCTCATTCACTTTGTTGGATGTTATATGGGTCAGGTCAGGTCC
RD44      :     CAGTAGGTCATGTATTCAATCTCATTCACTTTGTTGGATGTTATATGGGTCAGGTCAGGTCC
RD132     :     CAGTAGGTCATGTATTCAATCTCATTCACTTTGTTGGATGTTATATGGGTCAGGTCAGGTCC
RD163     :     CAGTAGGTCATGTATTCAATCTCATTCACTTTGTTGGATGTTATATGGGTCAGGTCAGGTCC
RD194     :     CAGTAGGTCATGTATTCAATCTCATTCACTTTGTTGGATGTTATATGGGTCAGGTCAGGTCC
RD        :     CAGTAGGTCATGTATTCAATCTCATTCACTTTGTTGGATGTTATATGGGTCAGGTCAGGTCC
ZAD1      :     CAGTAGGTCATGTATTCAATCTCATTCACTTTGTTGGATGTTATATGGGTCAAGTCAGGTCC
ZAD2      :     CAGTAGGTCATGTATTCAATCTCATTCACTTTGTTGGATGTTATATGGGTCAAGTCAGGTCC
ZAD 4     :     CAGTAGGTCATGTATTCAATCTCATTCACTTTGTTGGATGTTATATGGGTCAAGTCAGGTCC
ZAD8      :     CAGTAGGTCATGTATTCAATCTCATTCACTTTGTTGGATGTTATATGGGTCAAGTCAGGTCC
ZAD10     :     CAGTAGGTCATGTATTCAATCTCATTCACTTTGTTGGATGTTATATGGGTCAAGTCAGGTCC
SADB1971  :     CTGTTGGTCACGTGTTCAATCTCATTCACTTTGTAGGATGCTATATGGGTCAAGTCAGATCC
```

```
                      *         1000       *     1020      *          1040

NLA 16    :     GACGGTCATTGCTGCATGTGCTCCTCATGAGATGTCTGTCCTAGGGGGTTATCTAGGGGAAG
NLLB      :     GACGGTCATTGCTGCATGTGCTCCTCATGAGATGTCTGTCCTAGGGGGTTATCTAGGGGAAG
RD44      :     GACGGTCATTGCTGCATGTGCTCCTCATGAGATGTCTGTCCTAGGGGGTTATCTAGGGGAAG
RD132     :     GACGGTCATTGCTGCATGTGCTCCTCATGAGATGTCTGTCCTAGGGGGTTATCTAGGGGAAG
RD163     :     GACGGTCATTGCTGCATGTGCTCCTCATGAGATGTCTGTCCTAGGGGGTTATCTAGGGGAAG
RD        :     GACGGTCATTGCTGCATGTGCTCCTCATGAGATGTCTGTCCTAGGGGGTTATCTAGGGGAAG
ZAD1      :     AACGGTCATTGCTGCATGTGCTCCTCATGAGATGTCTGTCCTAGGGGGTTATCTAGGGGAAG
ZAD2      :     AACGGTCATTGCTGCATGTGCTCCTCATGAGATGTCTGTCCTAGGGGGTTATCTAGGGGAAG
ZAD4      :     AACGGTCATTGCTGCATGTGCTCCTCATGAGATGTCTGTCCTAGGGGGTTATCTAGGGGAAG
ZAD8      :     AACGGTCATTGCTGCATGTGCTCCTCATGAGATGTCTGTCCTAGGGGGTTATCTAGGGGAAG
ZAD10     :     AACGGTCATTGCTGCATGTGCTCCTCATGAGATGTCTGTCCTAGGGGGTTATCTAGGGGAAG
SADB1971  :     AACGGTTATTGCTGCATGTGCTCCTCATGAAATGTCTGTTCTAGGGGGCTATCTGGGAGAGG
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                 1060        *         1080        *        1100        *
NL 16     :  GGAAAGGGCACATTT-GAAAGAAGATTCTTTAGAGACGAAAAAGAGCTCCAAGAATATGAGG
NLLB      :  GGAAAGGGCACATTT-GAAAGAAGATTCTTTAGAGACGAAAAAGAACTCCAAGAATATGAAG
RD44      :  GGAAAGGGCACATTT-GAAAGAAGATTCTTTAGAGACGAAAAAGAACTCCAAGAATATGAGG
RD132     :  GGAAAGGGCACATTT-GAAAGAAGATTCTTTAGAGACGAAAAAGAACTCCAAGAATATGAGG
RD163     :  GGAAAGGGCACATTT-GAAAGAAGATTCTTTAGAGACGAAAAAGAACTCCAAGAATATGAGG
RD194     :  GGAAAGGGCACATTT-GAAAGAAGATTCTTTAGAGACGAAAAAGAGCTCCAAGAATATGAGG
RD        :  GGAAAGGGCACATTT-GAAAGAAGATTCTTTAGAGACGAAAAAGAACTCCAAGAATATGAGG
ZAD1      :  GGAAAGGGCACATTC-GAGAGAAGATTCTTTAGAGACGAAAAAGAACTCCAAGAGTATGAGG
ZAD2      :  GGAAAGGGCACATTC-GAGAGAAGATTCTTTAGAGACGAAAAAGAACTCCAAGAGTATGAGG
ZAD4      :  GGAAAGGGCACATTC-GAGAGAAGATTCTTTAGAGACGAAAAAGAACTCCAAGAGTATGAGG
ZAD8      :  GGAAAGGGCACATTC-GAGAGAAGATTCTTTAGAGACGAAAAAGAACTCCAAGAGTATGAGG
ZAD10     :  GGAAAGGGCACATTC-GAGAGAAGATTCTTTAGAGACGAAAAAGAACTCCAAGAGTATGAGG
SADB1971  :  GGGAAAGGGACATTT-GAAAGAAGATTCTTCAGAGATGAGAAAGAACTTCAAGAATACGAGG
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                   *        1140        *        1160        *        1180
NL 16     :  TTGACGAAGACTGATGTGGCACTGGCAGATGACGGAACCGTCAACTCTGATGATGAGGATTA
NLLB      :  TTGACGAAGACTGATGTGGCACTGGCAGATGACGGAACCGTCAACTCTGATGATGAGGATTA
RD44      :  TTGACGAAGACTGATGTGGCACTGGCAGATGACGGAACCGTCAACTCTGATGATGAGGATTA
RD132     :  TTGACGAAGACTGATGTGGCACTGGCAGATGACGGAACCGTCAACTCTGATGATGAGGATTA
RD163     :  TTGACGAAGACTGATGTGGCACTGGCAGATGACGGAACCGTCAACTCTGATGATGAGGATTA
RD194     :  TTGACGAAGACTGATGTGGCACTGGCAGATGACGGAACCGTCAACTCTGATGATGAGGATTA
RD        :  TTGACGAAGACTGATGTGGCACTGGCAGATGACGGAACCGTCAACTCTGATGATGAGGATTA
ZAD1      :  TTGACAAAGACTGATGTGGCACTGGCAGATGACGGAACCGTCAACTCTGATGATGAGGATTA
ZAD2      :  TTGACAAAGACTGATGTGGCACTGGCAGATGACGGAACCGTCAACTCTGATGATGAGGATTA
ZAD4      :  TTGACAAAGACTGATGTGGCACTGGCAGATGACGGAACCGTCAACTCTGATGATGAGGATTA
ZAD8      :  TTGACAAAGACTGATGTGGCACTGGCAGATGACGGAACCGTCAACTCTGATGATGAGGATTA
ZAD10     :  TTGACAAAGACTGATGTGGCACTGGCAGATGACGGAACCGTCAACTCTGATGATGAGGATTA
SADB1971  :  CTGACAAAGACTGACGTAGCACTGGCAGATGATGGAACTGTCAACTCTGACGACGAGGACTA
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                 1200        *          1220        *       1240        *
NL 16     :  GTGAGACCAGGAGTCCTGAAGCCGTTTATACTCGGATCATGATGAACGGAGGGCGACTAAAG
NLB       :  GTGAGACCAGGAGTCCTGAACCCGTTTATACTCGGATCATGATGAACGGAGGGCGACTAAAG
RD44      :  GTGAGACCAGGAGTCCTGAAGCCGTTTATACTCGGATCATGATGAACGGAGGGCGACTAAAG
RD132     :  GTGAGACCAGGAGTCCTGAACCTGTTTATACTCGGATCATGATGAACGGAGGGCGACTAAAG
RD163     :  GGGAGACCAGGAGTCCTGAACCTGTTTATACTCGGATCATGATGAACGGAGGGCGACTAAAG
RD194     :  GTGAGACCAGGAGTCCTGAAGCCGTTTATACTCGGATCATGATGAACGGAGGGCGACTAAAG
RD        :  GTGAGACCAGGAGTCCTGAAGCCGTTTATACTCGGATCATGATGAACGGAGGGCGACTAAAG
ZAD1      :  GTGAGACCAGGAGTCCTGAAGCCGTTTATACTCGGATCATGATGAACGGAGGGCGACTAAAG
ZAD2      :  GTGAGACCAGGAGTCCTGAAGCCGTTTATACTCGGATCATGATGAACGGAGGGCGACTAAAG
ZAD4      :  GTGAGACCAGGAGTCCTGAAGCCGTTTATACTCGGATCATGATGAACGGAGGGCGACTAAAG
ZAD8      :  GTGAGACCAGGAGTCCTGAACCCGTTTATACTCGGATCATGATGAACGGAGGGCGACTAAAG
ZAD10     :  GTGAGACCAGGAGTCCTGAAGCCGTTTATACTCGGATCATGATGAACGGAGGGCGACTAAAG
SADB1971  :  GTGAAACCAGAAGTCCGGAGGCTGTTTATACTCGAATCATGATGAATGGAGGTCGACTAAAG
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                   *     1280        *         1300        *        1320
NL 16     :  CATAAGGAGATATGTTTCAGTCAGTTCCAATCATCAAGCTCGCCC-AAACTCATTCGCTGGG
NLLB      :  CATAAGGAGATATGTTTCAGTCAGTTCCAATCATCAAGCTCGCCC-AAACTCATTTGCTGGG
RD44      :  CATAAGGAGATATGTTTCAGTCAGTTCCA-TCATCAAGCTCGCCCCAAACTCATTTGCTGAG
RD132     :  CATAAGGAGATATGTTTCAGTCAGTTCCAATCATCAAGCTCGCCC-AAACTCATTTGCTGAG
RD163     :  CATAAGGAGATATGTTTCAGTCAGTTCCAATCATCAAGCTCGCCC-AAACTCATTTGCTGAG
RD194     :  CATAAGGAGATATGTTTCAGTCAGTTCCAATCATCAAGCTCGCCC-AAACTCATTCGCTGAG
RD        :  CATAAGGAGATATGTTTCAGTCAGTTCCAATCATCAAGCTCGCCC-AAACTCATTTGCTGAG
ZAD1      :  CATAAGGAGATATGTTTCAGTCAGTTCCAATCATCAAGCTCGCCC-AAACTCATTTGCCGAG
ZAD2      :  CATAAGGAGATATGTTTCAGTCAGTTCCAATCATCAAGCTCGCCC-AAACTCATTTGCCGAG
ZAD4      :  CATAAGGAGATATGTTTCAGTCAGTTCCAATCATCAAGCTCGCCC-AAACTCATTTGCCGAG
ZAD8      :  CATAAGGAGATATGTTTCAGTCAGTTCCAATCATCAAGCTCGCCC-AAACTCATTTGCCGAG
ZAD10     :  CATAAGGAGATATGTTTCAGTCAGTTCCAATCATCAAGCTCGCCC-AAACTCATTTGCCGAG
SADB1971  :  CATACGGAGATATGTCTCAGTCAGTTCCAATCATCAAGCCCGTCC-AAACTCATTCGCCGAG
```

1340

NL 16	:	CAAAACATATTCTAGTGACTCA	: 1350
NLLB	:	CAAAACATATTCTAGTGACTCA	: 1350
RD44	:	CAAAACATATTCTAGTGACTCA	: 1350
RD132	:	CAAAACATATTCTAGTGACTCA	: 1350
RD163	:	CAAAACATATTCTAGTGACTCA	: 1350
RD194	:	CAAAACATATTCTAGTGACTCA	: 1350
RD	:	CAAAACATATTCTAGTGACTCA	: 1350
ZAD1	:	CAAAACATATTCTAGTGACTCA	: 1350
ZAD2	:	CAAAACATATTCTAGTGACTCA	: 1350
ZAD4	:	CAAAACATATTCTAGTGACTCA	: 1350
ZAD8	:	CAAAACATATTCTAGTGACTCA	: 1350
ZAD10	:	CAAAACATATTCTAGTGACTCA	: 1350
SADB1971	:	CAAGACATATTCGAGTGACTCA	: 1350

Appendix XXIV

Sample of Record sheet for RFFIT

Appendix XXV: Record sheet for inoculation of mice for virus isolation 1/3

1244 suckling mice

Experiment No:	**1244**	Date:	23-Aug-07	Virus:	Strain & Passage:	Nigerian isolates	Final LD50
Preparation:	20% homogenate	diluted 1:100	Diluent: MEM medium				
Volume:	20 ul	Route:	IC	Age: 2-3 days	Protocol:	1421 Mouse	

Inoc. Date:		**Aug**									**Sep**																														**Oct**
23 Aug 2007		23	24	25	26	27	28	29	30	31	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	
		0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	
ZAD1	n	14	14	14	14	14	14	14	14	14	14	14	14	14	14	14	14	14	14	14	14	14	14																		
	s																																								
n=14	d																																								
	e																																								
comments																																									
ZAD2	n	15	15	15	15	14	14	14	14	14	14	14	14	14	14	14	14	14	14	14	14	14	14																		
	s																																								
n=15	d																																								
	e																																								
comments																																									
ZAD4	n	14	14	14	14	14	14	14	14	14	14	14	14	14	14	14	14	14	14	14	14	14	14																		
	s																																								
n=14	d																																								
	e																																								
comments																																									
ZAD8	n	12	12	12	12	12	12	12	12	12	12	12	12	12	12	12	12	12	12	12	12	12	12																		
	s																																								
n=12	d																																								
	e																																								
comments																																									
NLLB	n	15	15	15	15	14	14	14	14	14	14	14	14	14	14	14	14	14	14	14	14	14	14																		
	s																																								
n=15	d																																								
	e																																								
comments																																									

n number of normal animals in cage
s number of animals with mild signs (i.e. paresis, ataxia) to be observed for progression of illness
d number of animals found dead
e number of animals with signs that were euthanized
PEP 24 hours post infection

Appendix XXV: Record sheet for inoculation of mice for virus isolation 2/3

1244 suckling mice

Experiment No:	**1244**	Date:	23-Aug-07	Virus:		Strain & Passage:	Nigerian isolates	Final LD50
Preparation:	20% homogenate	diluted 1:100	Diluent: MEM medium					
Volume:	20 ul	Route:	IC	Age: 2-3 days		Protocol:	1421 Mouse	

Inoc. Date: **23 Aug 2007**		**Aug** 23	24	25	26	27	28	29	30	31	**Sep** 1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
		0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38
	n	16	16	16	16	16	16	16	16	16	16	16	16	16	16	16	16	16	16	16	16	16	16																	
RD7	s																																							
n=16	d																																							
	e																																							
comments																																								
	n	13	12	12	12	12	12	12	12	12	12	12	12	12	12	12	12	12	12	12	12	12	12																	
RD17	s																																							
n=13	d																																							
	e																																							
comments																																								
	n	13	13	13	13	13	13	13	13	13	13	13	13	13	13	13	13	13	13	13	13	13	13																	
RD39	s																																							
n=13	d																																							
	e																																							
comments																																								
	n	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8																	
RD42	s																																							
n=8	d																																							
	e																																							
comments																																								
	n	14	14	14	14	14	13	13	13	13	13	13	13	13	13	13	13	13	13	13	13	13	13																	
RD44	s																																							
n=14	d																																							
	e																																							
comments																																								

n number of normal animals in cage
s number of animals with mild signs (i.e. paresis, ataxia) to be observed for progression of illness
d number of animals found dead
e number of animals with signs that were euthanized
PEP 24 hours post infection

Appendix XXV: Record sheet for inoculation of mice for virus isolation 3/3

1244 suckling mice

Experiment No:	**1244**	Date:	23-Aug-07	Virus:	Strain & Passage:	Nigerian isolates	Final LD50
Preparation:	20% homogenate	diluted 1:100	Diluent: MEM medium				
Volume:	20 ul	Route:	IC	Age: 2-3 days	Protocol:	1421 Mouse	

Inoc. Date:		**Aug**									**Sep**																							
23 Aug 2007		23	24	25	26	27	28	29	30	31	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
		0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32
RD132	n	11	11	11	11	11	11	11	11	11	11	11	11	11	11	11	11	11	11	11	11	11	11											
	s																																	
n=11	d																																	
	e																																	
comments																																		
RD137	n	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5											
	s																																	
n=5	d																																	
	e																																	
comments																																		
RD181	n	11	11	11	11	11	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10											
	s																																	
n=11	d																																	
	e																																	
comments																																		
RD194	n	14	14	14	14	14	14	14	14	14	14	14	14	14	14	14	14	14	14	14	14	14	14											
	s																																	
n=14	d																																	
	e																																	
comments																																		
comments																																		

n number of normal animals in cage
s number of animals with mild signs (i.e. paresis, ataxia) to be observed for progression of illness
d number of animals found dead
e number of animals with signs that were euthanized
PEP 24 hours post infection

Appendix XXVI: Record sheet for vaccination of mice with DNA vaccine 1/2

1247 DNA in mice

Experiment No: **1247** Date: 11-Sep-07 Virus: Strain & Passage: Nigerian isolates Final LD50

Preparation: Diluent: MEM medium

Volume: 50-100 ul Route: im and sc Age: 5 weeks Protocol: 1421 Mouse

Inoc. Date:		**Sep**			**F**			**M**		**W**		**F**			**M**		**W**		**F**			**Oct**		**W**		**F**			**M**		**W**		**F**			**M**		**W**		**F**		
11 Sep 2007		11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
		0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40
CDAG3	n																																									
i.m.	s																																									
100ul	d																																									
n=5	e																																									
comments																																										
CDAG3	n																																									
s.c.	s																																									
100ul	d																																									
n=5	e																																									
comments																																										
CDAG3	n																																									
p.o.	s																																									
100ul	d																																									
n=5	e																																									
comments																																										
CDAG3	n																																									
i.d.	s																																									
10ul	d																																									
n=5	e																																									
comments																																										
CDAG3																																										
i.m.																																										
100ul																																										
n=5																																										
comments																																										

n number of normal animals in cage
s number of animals with mild signs (i.e. paresis, ataxia) to be observed for progression of illness
d number of animals found dead
e number of animals with signs that were euthanized
PEP 24 hours post infection

Appendix XXVI: Record sheet for vaccination of mice with DNA vaccine 2/2

1247 DNA in mice

Experiment No: **1247** Date: 11-Sep-07 Virus: Strain & Passage: Nigerian isolates Final LD50

Preparation: Diluent: MEM medium

Volume: 50-100 ul Route: im and sc Age: 5 weeks Protocol: 1421 Mouse

Inoc. Date:		**Sep**			**F**			**M**		**W**		**F**			**M**		**W**		**F**			**Oct**		**W**		**F**			**M**		**W**		**F**			**M**	**W**		**F**			
11 Sep 2007		11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
		0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40
WCBVG	n																																									
i.m.	s																																									
100ul	d																																									
n=5	e																																									
comments																																										
WCBVG	n																																									
s.c.	s																																									
100ul	d																																									
n=5	e																																									
comments																																										
WCBVG	n																																									
p.o.	s																																									
100ul	d																																									
n=5	e																																									
comments																																										
WCBVG	n																																									
i.d.	s																																									
10ul	d																																									
n=5	e																																									
comments																																										
WCBVG																																										
i.m.																																										
100ul																																										
n=5																																										
comments																																										

n number of normal animals in cage
s number of animals with mild signs (i.e. paresis, ataxia) to be observed for progression of illness
d number of animals found dead
e number of animals with signs that were euthanized
PEP 24 hours post infection