COMPARISON OF ANTIBODY RESPONSE TO SINGLE SITE AND MULTIPLE SITES INJECTION USING ANTIRABIES VACCINE IN DOGS

BY
OSINUBI, MODUPE OLUBUSOLA VIVIAN
DVM (1990) A.B.U.. ZARIA

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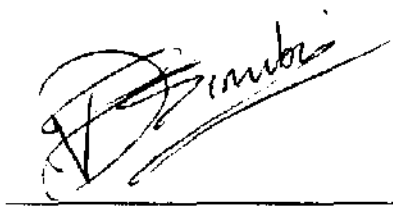
DECLARATION

The work presented in this Thesis is original and carried out under the supervision of DR. A.B. OGUNKOYA, Department of Veterinary Surgery and Medicine and PROF. J.U. UMOH of Department of Veterinary Public and Preventive Medicine, Ahmadu Bello University, Zaria.

The work of other investigators was referred to and acknowledged. No part of this Thesis has previously been submitted for a degree or diploma.



OSINUBI, MODUPE OLUBUSOLA VIVIAN
Ahmadu Bello University, Zaria.
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CERTIFICATION

The thesis entitled "Comparison of Single Site with Multiple Sites Injection using Antirabies Vaccine in Dogs" by OSINUBI, M. O. V. meets the regulations governing the award of Master of Science of Ahmadu Bello University and is approved for its contribution to knowledge and literary presentation.

Dr. A. B. Ogunkoya
Department of Veterinary
Surgery and Medicine.

Date 20/11/98

Prof. J. U. Umoh
Department of Veterinary
Public Health and Preventive
Medicine.

Date Nov 20, 1998.

Prof. D. Ogwu
Head of Department,
Veterinary Surgery and Medicine

Date 20/11/98

Prof. J. Olayemi
Dean, Postgraduate School

Date 11/08/99

DEDICATION

This work is dedicated to my husband, Dr. K. J. Osinubi of the Department of Civil Engineering, Ahmadu Bello University, Zaria, who has been a very great source of encouragement and also to our children Omokolade Oluwafemi Osinubi and Ayodeji Oluwaseun Osinubi for their patience, understanding and support.

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ABSTRACT

A total of eighteen (18) dogs were immunized with the live attenuated low egg passage Flury strain rabies vaccine and killed Flury strain rabies vaccine using one, two and three inoculation sites for the recommended dose of each vaccine (in divided doses). Pre-immunization sera were collected from all the dogs while post-immunization sera were collected on days 3, 7, 10, 14 and 28 from all the dogs and analysed for viral neutralization antibody using mouse neutralization test (MNT).

The two-site dose regimen of the killed vaccine induced measurable antibodies in all the subjects from day 3 post vaccination. This regimen proved to be superior to the single site standard dose regimen, which did not induce measurable antibodies until 7 days post vaccination. The antibody titres for single regimen were less than those of the two site regimen throughout the period of observation. The same condition was also observed when the live attenuated vaccine was used.

The levels of neutralizing antibody production achieved with triple-site regimen were four times higher than those of the two-site regimen, irrespective of the type of vaccine used. All the dogs given the triple site regimen had measurable amount of antibody by day 28 post vaccination. A higher production of antibody was observed when live attenuated antirabies vaccine was used as indicated by an earlier protection than when the killed antirabies vaccine was used.

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CHAPTER ONE

INTRODUCTION

Rabies, an acute viral encephalities, is one of the oldest communicable diseases in recorded history. It was known in the ancient civilization of the Nile, Euphrates and Hindus River Valleys. Available evidences show that the code of Hammurabi of the 23rd century B.C. laid down rules, regulations and punishment on dog owners whose dogs were associated with bite wounds and consequently the spread of rabies to human beings (Tierkel, 1958).

This singular act established the understanding of the old civilization on the epidemiology and zoonotic nature of rabies. Tierkel (1958) and James (1973) further expatiated on the historical background of rabies in relation to old superstitious beliefs and the reality of the disease. Presently in the world, rabies is well documented and it is still one of the unconquered important zoonotic diseases, especially in the underdeveloped countries (Acha and Arambulo III, 1985).

Rabies trend in view of human fatality established canine rabies. Between 1989 and 1992 dogs accounted for 97.8% of fatal human rabies reported worldwide (World Health Organization, 1992). In the industrialized nations, risk to human beings has been minimized. Mills (1997) reported that 20,000 - 100,000 people die of rabies yearly, out of these , 91.3% fatal cases occurred in developing countries. In Nigeria, rabies is endemic and dog has been implicated as the animal that maintains and spreads the disease (Umoh and Belino, 1979; Fagbami *et al.*, 1981; Ogunkoya, 1997).

Previous efforts to treat rabid dogs and human beings proved

unsuccessful despite all types of approaches employed until Pasteur *et al.*, (1881) successfully introduced the use of immunization through vaccination. Consequently, the focus on rabies pre and post exposure treatment , control and its possible eradication has been directed at building a herd immunity in dogs through the use of immunization. This has been carried out, especially in developed countries, with some level of success. However, in Nigeria, rabies control and eradication programme has not been successful despite the introduction of the low egg passage (LEP) Flury strain vaccine produced by the Nigerian Veterinary Research Institute (NVRI) over thirty-seven years ago. Oboegbulem *et al.* (1987) as well as Okoh (1981) reported cases of rabies occurring in dogs vaccinated with LEP. This led to various studies meant to discover why Nigeria is not achieving a herd immunity in urban dogs. Inspite of the studies conducted, Ogunkoya *et al.* (1992) reported that there is substantial evidence to show that the disease is on the increase.

Serological efforts aimed at finding out the causes of rabies in vaccinated animals revealed that 12.5% of 42 dogs vaccinated with LEP using the conventional one site injection failed to show a measurable antibody response (Harry and Adeiga, 1981). Also a field serological survey of 140 dogs vaccinated with the LEP Flury strain vaccine reported by Ogunkoya (1989) revealed a 45.7% immuno response of the dogs and this was found to be less in younger animals than older age groups. This low sero-conversion and consequential vaccine failure might be due to factors associated with the vaccination procedure, the age of animal, the incompatibility of infecting viruses etc. as reported by Schneider *et al.* (1972), Wiktor (1978) as well as Bunn *et al.* (1984) and confirmed by Thraenhart (1988) and Umoh *et al.* (1990).

The search for higher sero-conversion in patients led to the report of Turner *et al.* (1976) that a significant improvement in the dynamics of antibody response was recorded when Human Diploid Cell Rabies Vaccine (HDCV) doses were split and given on several sites of the body in humans as compared to the conventional one site. It was explained that the influenced positive dynamics of immuno-response was as a result of a greater number of lymph nodes stimulated at the same time through the various sites. This has been confirmed by Warrell *et al.* (1983, 1984, 1985), Bakliac *et al.* (1985) as well as Vodopija *et al.* (1988) who reported that multiple site inoculation is superior to the conventional single site injection. Consequently, the World Health Organization (WHO) in 1984 approved the multiple site immunization system in humans.

Presently in Nigeria, the mode of immunization in dogs is through the conventional single site injection. However, as a result of the success of multiple site inoculation in humans the idea was conceived to carry out a similar test on dogs in order to observe their response antibody formation.

The aim of the study was to investigate the efficacy of multiple site inoculation of antirabies vaccine in dogs. In order to achieve this aim the following objectives were considered :

- vaccination of 3 groups of dogs using single site, double site and triple site regimens, for the same dose of anti-rabies vaccine;

- determination of the viral neutralization antibody in serum samples from each group of dogs.

CHAPTER TWO

REVIEW OF LITERATURE

2.1 The Rabies Virus.

Rabies virus is a member of the genus Lyssavirus within the family Rhabdoviridae, a group of more than 80-bullet-shaped viruses, infecting many vertebrate, invertebrate and plant hosts (Crick and Brown, 1976). This group has a single strand of non-segmented genomic RNA of negative polarity with a nucleoprotein core surrounded by a lipid bilayer (Madore and England, 1977).

The ribonucleic acid (RNA) carries the coding capacity for the five viral proteins (Lecocq *et al.*, 1985) and it is linked with about 1,800 molecules of a nucleoprotein N to form a helical ribonucleoprotein which is lightly bound within the nucleocapsid. This is surrounded by a lipoprotein envelope (lipid + M protein) through which the surface spikes (glycoprotein , G) protrude. Two additional proteins, L and NS, are associated with the nucleocapsid (Madore and England, 1977).

The glycoprotein is the virion component responsible for induction of virus - neutralizing antibody and protection of animals against challenge. It is also responsible for the stimulation of specific T cells which express helper, suppressor or cytotoxic activities, and determination of virulence. Because of its multiple immunogenic functions, the rabies virus glycoprotein is potentially suitable for vaccination purposes (Dietzschold et *al.*, 1983).

Although rabies virus is essentially neurotropic, it will replicate in a wide variety of cells both in the intact animal and in tissue culture (Clark, 1980). However, it replicates poorly in the striated muscle near the

site of inoculation and unless it is inactivated by natural or active immune response the virus enters the nervous system, after which it is considered to be invulnerable to the immune response induced by vaccination because the blood-brain barrier restricts the passage of neutralizing (NA) antibodies and of immune cells (Murphy and Bauer, 1974).

2.2 Transmission

Dogs are the major source of infection to man and animals. Throughout the middle ages there were many references to rabid wild animals which destroyed farm animals and invaded cities, but the dog continues to be the source of the disease insofar as one can gather from historical writings (James, 1973). In Nigeria, 99% of rabies victims contracted the disease from dogs (Fagbami *et al.*, 1981). Earlier, Boulger and Hardy (1960) reported that dogs accounted for over 80% of bite wounds in humans while Umoh and Belino (1979) reported about 70% of cases of animal rabies in Nigeria are due to dog bites. Cats come next to dogs in the frequency of rabies cases but they are rarely incriminated independently of dogs (James, 1973). Nawathe (1980) reported that rabies in cows, horses, pigs, cats, goats, etc. is a spill-over from dogs rabies. The spread of the disease among species apart from bite wounds could also be attributed to intraspecific transmission that appears to include possible inhalation, ingestion and perhaps vertical transmission of rabies virus (Centre for Disease Control, 1969; 1974)

2.3 Vaccine and Vaccination

Vaccination using vaccines seem to be the most successful control programme of rabies. Due to the fact that rabies epidemics have occurred on every continent except Australia and Antarctica, the invention of a vaccine

was a landmark event. In the late 1800s the French physician, Louis Pasteur made suspensions from brain tissue infected with rabies and injected those suspensions into the brains of uninfected animals, each time inducing the disease. Pasteur discovered he could protect dogs against rabies by injecting them with suspensions of dried, minced spinal cords from rabies-infected rabbits (Mills, 1997).

Whatever, domestic animals, a major or occasional vector of rabies still constitute the principal contamination agent for man, whose protection is based on their vaccination (Mackowiak, 1974), this led to the production of commercial vaccines which were widely used during the late 1920s. Many communities undertook mass immunization programs, some of which were successful while others failed (James, 1973).

Vaccination of dogs against rabies was re-introduced in 1935, using the sample - typed phenolized sheep-brain vaccine and, up to 1940, dogs over six months of age were vaccinated with two doses of vaccine given a week apart, (Thorne, 1954). Vaccination using the LEP Flury strain vaccine was introduced in 1956. This led to the advent of the live rabies vaccine with the adaptation of the Flury strain of rabies virus to day-old chickens and later to the chicken embryo. Johnson (1965) isolated the Flury strain from a young girl who died of rabies following an unusual exposure in which there was evidence of no bite but a history of the licking of mucous membranes. The virus was passed intracerebrally through 136 transfers of day-old chicks. Later Koprowski and Cox (1948) propagated the Flury strain virus in chicken embryos. Virus from the 40th - 50th chicken embryo passage was designated Low egg passage (LEP) and found to be avirulent for dogs when injected parenterally (Koprowski and Black, 1950). Further experiments with dogs showed they developed good immunity of long duration. The successful

3-year study of immunity proved that the live vaccine protected longer than the inactivated vaccines (Tierkel *et al.*, 1953; Brown *et al.*, 1973). Rabies vaccines for dogs have cycled from inactivated nervous tissue origin vaccines to modified live vaccines and now back to inactivated vaccines, but primarily of tissue culture origin. This evolution been a concern for safety rather than potency. Immunogenicity tests have indicated that with some vaccines, the route of administration is critical. Likewise, the age at which to vaccinate is equally important, and data indicated that the poor response seen in young dogs is due to some factors other than interference from maternal antibody (Bunn, 1985).

Many types of rabies vaccines have been produced since the original vaccine was developed by Pasteur in 1881 and several reviews have dealt with the history of the vaccine. The vaccines that are available today for use in dogs can be divided into 3 groups: the modified live virus (MLV) vaccines, the inactivated nervous tissue origin (NTO) vaccines and the inactivated cell culture origin (CCO) vaccines which are summarized in Table 2.1.

While it is generally believed that inactivated vaccines are stable than MLV vaccines, MLV vaccines usually contain considerably more virus that the minimum immunizing dose giving them adequate stability when properly stored. Inactivated vaccines, on the hand, may contain little antigen in excess of the minimum amount (Wiktor and Koprowski, 1978).

TABLE 2.1 STRAINS OF VIRUS USED IN VACCINE PRODUCTION

MODIFIED LIVE VIRUS	NERVOUS TISSUE ORIGIN	CELL CULTURE ORIGIN
Flury (LEP, HEP)	CVS	CVS
SAD	Pasteur	SAD
Kelev	51	Pasteur
Vnukovo	91	Flury

Source: Kaeberle, 1958; Sikes, 1969 and Crick, 1973.

Where:

CVS - Challenge virus strain, HEP - High egg passage

LEP - Low egg passage, SAD - Street Alabama Dufferin and includes ERA

2.4 Age of Vaccination

Kaeberle (1958) reported that dogs 11 - 16 weeks of age responded better to Flury LEP, or HEP vaccine, than dogs from 5 - 10 weeks of age. (81% protection from challenge versus 38% protection from challenge, respectively). He confirmed that puppies less than 11 weeks of age did not respond and that it may cause encephalitis in puppies under 3 weeks of age. Today, LEP is not recommended for animals under 3 months of age. An age relationship was confirmed by a study conducted at the National Veterinary Services Laboratories, U. S. A., where three groups of 20 dogs from sero-negative dams were vaccinated with Flury LEP vaccine with ages ranging from 3 - 3½ months, 4½ - 5 months and 6 - 6½ months, and a result showing geometric mean titre (GMT) of 1:15, 1:28 and 1:91 was achieved respectively for the different groups (Bunn, 1984). But it is not feasible to vaccinate dogs only after they are 6 months old since this would leave a large part of the canine population susceptible to rabies (National Association of State Public Health Veterinarians, Inc., U.S.A., 1983).

2.5 Route of Vaccination

The immunogenicity of MLV rabies vaccine is enhanced if the vaccines are given intramuscularly (i/m) instead of subcutaneous (s/c) (Johnston *et al.*, 1957; Brown *et al.*, 1973). The superiority of the I/m route was evident in a three year duration of immunity test conducted with Flury - HEP vaccine in which there was a 100% challenge survival in dogs given a full dose of vaccine while only 59% of the dogs vaccinated through s/c survived (Brown *et al.*, 1973). Though the i/m route has been shown to be better than s/c for stimulating an immune response for most types of vaccines, but for safety reasons, the site should be confined to the muscles of the thigh. I/m inoculation into the muscles close to the spine has been suggested as an influencing factor in cases of vaccine induced rabies (Cabasso, 1962).

2.6 Duration of Immunity

The duration of immunity confirmed by the vaccines have shown that dogs can be protected for one, two or three years, while it can be assumed that a dog of more than 6 months of age will be immunized for the length of time claimed by the manufacturer. The same assumption cannot be made for a dog that is vaccinated when less than 6 months of age. In the United States of America, the National Association of State Public Health Veterinarians recommends that all vaccines should be administered at 3 months of age and again one year later (National Association of State Public Health Veterinarians, Inc., 1983).

2.7 Detection of Antibodies against Rabies Virus

Various methods, which are discussed below, have been used to detect antibodies against rabies virus. Currently, virus-neutralizing antibodies (VNA) is being used as the method of choice for checking the immunity response after vaccination (Thraenhart and Kuwert, 1977; Sureau, 1988). In

1891, Babes, and in 1902, Kraus and Kreissl (Webster, 1944), demonstrated that persons taking vaccine treatment developed neutralizing properties in their blood. This effect was shown by combining blood serum from the patient with brain-virus emulsions and injecting the mixture intracerebrally into rabbits. If the animals so injected died of rabies, the blood was said to contain no rabicidal or protective properties whereas if they remained alive, the blood was said to contain such properties. Antibody to rabies virus is not found in vaccinated individuals only but also in the naturally infected individuals. It may be found in the serum on the first day of onset of illness (Bell, 1966; Lodmell *et al.*, 1969), but its presence prior to onset is more difficult to substantiate, especially in naturally infected animals. The antibody is mainly of IgM and IgG classes of immunoglobulins (Fujisaki *et al.*, 1968; Cho *et al.*, 1972; Gough and Dierks, 1971) with the IgM (19S neutralizing antibody) occurring earlier than the IgG (Fujisaki *et al.*, 1968). The production of neutralizing antibody is a property of the envelope glycoprotein of rabies virus (Wiktor *et al.*, 1973; Schneider *et al.*, 1973).

The various tests used in measuring antibody against rabies virus include:

(a) Virus Protection Test in Mice.

This is one of the two tests recommended by the World Health Organisation (Thraenhart *et al.*, 1977; Atanasiu, 1973). It was developed by Webster and Dawson (1935) and was used for measuring antibodies against rabies virus (Webster, 1936). The present serum neutralization test in mice using a variable serum dilution and a constant virus dose arose from the above. In this test, dilutions of the serum are mixed with a constant virus. After 90 minutes incubation at 37°C, the mixture is chilled, and 0.03 ml is injected

intracerebrally into 3-week old mice (Atanasiu, 1973). Though, this is the most commonly used test for measuring antibody it has the disadvantage of requiring a two-week period for its completion (Thomas *et al.*, 1963).

(b) Complement Fixation Test (CFT) (Sever, 1962).

This test requires pure concentrated antigen for detection of the CF antibody. This is not used routinely.

(c) Direct Fluorescent Antibody Test (FAT) in Cell Culture.

King *et al.* (1965) used fluorescent antibody (FA) staining in chick embryo fibroblasts infected with fixed virus to determine rabies antibody. This test has the advantage of being completed in 72 hours. Debbie *et al.* (1972) used a tissue culture (TC) FA technique for the measurement of rabies-neutralizing antibody. They performed the tests with BHK-21 cells infected with ERA vaccine virus strain or into a Flurry HEP rabies virus strain on Lab Tek TC chamber slides. The vero cell line infected with a Flurry HEP was also used. They obtained a more consistent result with ERA virus and BHK-21 cells than with any other combination. The results were found to be reliable and comparable to the standard mouse serum neutralisation test.

(d) Indirect Fluorescent Rabies Antibody Test (IFAT).

Goldwasser and Kissling (1958) reported on the specificity of this technique for detection of antibodies against rabies virus. This test has an advantage in that it can be completed in one day and has been used for screening for antibodies vaccinated individuals. (Thomas et al., 1963; Grandien and Espmark, 1974).

(e) Gel Diffusion Method.

This method as reported by Kubes (1965) can be used to identify unknown antibodies. This also, offers a rapid method for identification of immune serum. The main limitation is that a relatively high concentration of rabies virus is needed to prepare the precipitating antigen. Details of this test have been described by Lepine (1973).

(f) Serum Neutralization by the Plaque-Reduction Technique.

It has been described by Sedwick and Wiktor (1967). Five to six days are required for completion of this test.

(g) Hemagglutination-Inhibition (Halonen *et al.*, 1968) and Passive hemagglutination Tests (Gough and Dierks, 1971).

These have been used to measure antibody. Passive hemagglutination, apart from requiring purified and concentrated virus, might not work for all animals, e.g. the test is not good for rabbit serum. It can, however, be completed in one day.

(h) Fluorescent Focus Inhibition Test (FFIT).

Lennette and Emmons (1971) reported the use of FFIT by the California State Department of Health. In this test serial dilutions of the test serum are mixed with a constant amount of LEP Flury strain rabies virus. The mixture is inoculated on a BHK-21 confluent monolayer and incubated for 4 days before the cells were harvested and stained by FA. The presence or absence of virus in the BHK-21 cells was noted. Inhibition indicates positive serum.

(i) Rapid fluorescent Focus Inhibition Test (RFFIT) (Smith, 1976). This

is the second of the antibody induction tests recommended by WHO (Thraenhart, 1988). This test is similar to and was designed based on the FFIT of Lennette and Emmons (1971). In this test BHK-21/135 cells sensitized with DEAE-Dextran (to a final concentration of 10 µg/ml) are added to already incubated serum-virus mixture in Lab Tek TC chamber slides. After a 24-hour incubation period, the resulting monolayer is stained by FA. This test measures the ability of the serum antibody to block the infection of the BHK-21 clone 13S with a tissue culture BHK-21 adapted CVS-11 rabies virus. This test needs only 3 days for completion but the main drawback is the necessity of a specialized laboratory with facilities for tissue culture, infectious rabies virus and fluorescence microscopy, thus can only be carried out in a few centres in some countries (Thraenhart, 1988).

j) Enzyme - Linked Immunoadsorbent Assays (ELISA).

ElISA application for measuring specific antibodies is common place in human and veterinary medicine. However, the technique has not yet gained widespread acceptance in rabies serology inspite of a variety of assay systems described in the literature (Atanasiu *et al.*, 1977; 1978; Atanasiu and Perrin, 1979; Nicholson and Prestage, 1982; Sureau *et al.*, 1982; Thraenhart and Kuwert, 1977; WHO expert committee on rabies, 1984).

The Essen-Ig-Elisa was proposed as a fast and simple antibody induction test, which can be used after post-exposure treatment with inactivated tissue-culture vaccines at vaccination centres in tropical areas (Thraenhart, 1990).

Conventional ELISA tests can be carried out with a minimum of

equipment. However, only serum samples can be investigated in all currently used tests. The use of blood without centrifugation would be advantageous.

(k) Rabies Blood-Drop ELISA (RBDE).

This is the test for the determination of rabies virus antibodies by use of one drop of blood without centrifugation. It is a very simple inexpensive test which needs no equipment (Thraenhart, 1990).

(l) Rabies Agglutination Test (RAT).

It is a simple rapid test based on the capability of specific antibodies to agglutinate sensitized latex beads. The RAT proposed is based on the capability of specific rabies neutralizing antibodies to agglutinate latex beads previously coated with purified rabies virus (Sureau *et al.*, 1988).

Another way of assessing immune status of vaccinated animals is by challenging the animals with virulent virus and observing the signs of or mortality due to rabies. This had been recognized quite early by Galtier (1881), Pasteur *et al.* (1882), Roux and Nocard (1888), Moncet (1898) and Krasmitski (1902) in their experiments with animals have been used ever since to assess the potency of vaccines. This is the basis for pre exposure immunization against rabies. There have been several reports of reduction in incidence or control of rabies by pre exposure immunization of animals (Johnson, 1945; Koprowski *et al.*, 1955; Carneiro *et al.*, 1955). Resistance to infection correlates well with the presence of neutralizing antibody (Koprowski and Black, 1952; Fenje, 1960; Otto and Heyke, 1962).

2.8 Control

Immunization of domestic animals represents the single most effective means in the control of rabies in man. Whatever the origin of the disease, wildlife or street rabies, domestic animals such as dogs, cats, cattle and horses are the unfailing primary vector for human exposure (Winkler, 1975). Vaccination is effective in preventing rabies in animals and subsequent human exposure. The importance of vaccination procedures for domestic animals is substantiated by recent upsurge in the number of documented cases of animal rabies (Winkler, 1975).

Investigation as to the possibility of vaccinating dogs against rabies began about 100 years ago at the Lyon Veterinary College under the guidance of Balard. Pasteur studied with him for a short time before going to Paris. Pasteur was the first to demonstrate that dogs could be successfully immunized against rabies (Pasteur, 1886) and since his days, it has been known that vaccination against rabies is the efficient strategy for the control of this deadly disease (Thraenhart and Marcus, 1990). Despite all these, this disease of antiquity continues to persist as a major public health problem in this age of high technology. Even with significant scientific break throughs and advances in its prevention and control, rabies remains one of the most dreadful and gruesome of the human communicable infectious diseases. In fact, in the last decade, the gap has widened between available technology and knowledge to prevent and control the disease and the application for the benefit of the general population, particularly in the poor developing countries where rabies persists to cause numerous human deaths and unmeasurable human anguish and suffering (Mills, 1997).

2.9 Rabies despite Vaccination

Although unvaccinated animals are more likely to transmit rabies, vaccinated animals can also do so, if the vaccination was ineffective for any reason. Despite the control measure taken in Nigeria, rabies has been reported in vaccinated dogs (Ikede and Adeyefa, 1982; Bobade *et al.*, 1981) which constitutes a very great danger to the public and veterinarians in particular, who are at a very high risk of contacting the disease(Oduye and Aghomo, 1985). In Nigeria, there has been several reports of rabies occurring in dogs vaccinated with LEP as a result of failure of vaccination protection. Adeiga and Harry (1981) found that 12.5% of the 42 dogs vaccinated with LEP failed to show measurable antibody response. Oboegbulem *et al.* (1987) also estimated a vaccination failure rate of 2.5 cases per 10,000 doses of LEP.

Thraenhart and Marcus (1990) attributed vaccine failures to be due to low content of rabies virus in relation to the total protein content and that factors that influence the safety and potency of rabies vaccines include:

i. Selection of a virus strain with a high degree of cross-reactive-antigenicity and immunogenicity.

ii. Virus multiplication in a substrate without substances disturbing the health of the vaccine.

iii. Purification and concentration of the immunizing antigens.

iv. Vaccine production of uniform quality. They then concluded that every batch of the rabies vaccine has to be controlled before release. This control comprises identification, safety, potency and stability of the vaccine (Thraenhart, 1988).

Adeiga and Harry (1981) attributed poor vaccine transportation and

storage as a probable cause for the vaccination failure. The route of injection is also important, the subcutaneous route has been widely used but it is apparent that the intramuscular route results in better protection of longer duration (Tierkel *et al.*,1953).

Koprowski (1980) summarised in his work that there is marked variation among field strains of rabies virus. The reactivity of the viruses with a large panel of monoclonal antibodies (MAbs) enables the grouping of the variants in antigenically similar classes. Although he observed that there are marked antigenic differences among several of the field isolates and viruses used for vaccine production, it is uncertain whether vaccination failures observed in humans and animals can be ascribed to these differences.

Webster (1936) earlier reported that the usage of commercial vaccines which were in production and have been widely used since late 1920s but many communities who undertook mass immunization programs, found that some were successful while others failed, so it soon became apparent that results were inconsistent, which left much to be desired. As early as 1930, it was suspected that the vaccines were lacking in antigenicity but it was not proven until Webster demonstrated in laboratory mice that most of the commercial rabies vaccines used for dogs, as well as those prepared for human use, lacked antigenicity. Habel (1940) confirmed Webster's findings and developed an assessment test to determine the potency of the vaccine. This is the well-known Habel test. It was apparent from Habel's studies that many of the strains used for vaccine production were impotent. Many of these strains were traced to the Old Pasteur fixed virus. Further, it was apparent that the strains had changed in the course of propagation, using different animals and methods, and they were no longer useful in

vaccine production. Today, government control agencies require that all manufacturers use only potent virus strains in the production of vaccine and that each lot of vaccines be tested for antigenicity.

Work done in Tunisia showed that following a single injection for primovaccination, it is possible that long before 2 year-period for booster dose is reached, the dog population immunity has become too low due to a low antibody response. Haddad *et al.* (1988) showed that inspite of good antigenic titres, neither of the two vaccines used in National Program for Rabies Control in March 1982 with collaboration of WHO, could induce an antibody titre comparable with international norms. Furthermore, a booster injection a year after primovaccination did not induce an anamnestic response (Haddad *et al.*, 1988). In line with this, Blancou *et al.* (1983) cited an example of a 6 year old female alsatian adequately vaccinated at 3 months old (with a tissue-culture vaccine inactivated with ß-propiolactone without adjuvant) with booster given annually for 4 years (antigenic value > 1 µ/dose according to the NIH test) but 5 months after the 6th injection, the dog showed first signs of rabies after having been bitten by a fox and later died with confirmed rabies diagnosis (FAT, MNT). Serum from blood sample taken on day of death contained no significant traces of rabies antibodies (titre < 0.05 iµ/ml) and globulin rate was 7% (as opposed to an average 12 or 13% in normal dogs). This confirmed that the dog had not adequately responded to the rabies antigen).

There is also the problem of inapparent rabies, for instance, in 1932, an apparently healthy dog was reported to have bitten other dogs; the biting dog remained healthy for 6 months whereas the bitten dog came down with rabies (Anon, 1932). Another apparently healthy dog in Kaduna bit two other dogs that later became rabid while the biting dog lived for over a year

(Anon, 1935). This has brought about a strong feeling that latent rabies exists probably as a result of poor antibody response to vaccination.

2.10 Multiple Sites Vaccination Schedules

A significant breakthrough towards a more efficient use of rabies vaccine was the discovery that the dynamics of antibody response could be influenced by splitting the vaccine dose and giving it at several sites in the body, rather than just at one site. Thus, a greater number of groups of lymph nodes are included in the immune response, resulting in a more reliable, rapid and high-titred antibody response. Multi-site intradermal application of Human Diploid Cell Rabies Vaccine (HDCV) was first attempted by Turner *et al.* (1976), with the majority of the clinical work subsequently done by Warrell *et al.* (1985) in Thailand, where two doses of the vaccine were split into four injections of 0.5 ml and applied to four different sites on day 0 and by day 14, a GMT of 32-0 iμ/ml using RFFIT method (RFFIT, n=15) was obtained. Vodopija *et al.* (1990) also used a three-dose regimen of Purified Chick-Embryo Cell (PCEC) culture rabies vaccine, in which the first dose was split into two 0.5 ml injections and given bilaterally into the deltoids of both arms. Sero-conversion was induced in all tested subjects (n = 30) by day 14. In a controlled comparative trial of 4 different regimens of Foetal Bovine-Kidney Cell (FBKC) vaccines plus the standard WHO regimen as control the 4-site application of two doses of vaccine induced a sero-conversion rate of 80% by day 10 and 100% by days 14, 30 and 45 compared to the standard schedule which had a sero-conversion rate of 84% on day 14 and 100% on days 30 and 45 (Baklaic *et al.*, 1985). Other works done on multiple priming include the 8-site intradermal method ("8-0-4-0-1-1") using HDCV and PCECV where 0.1 ml of reconstituted vaccine was given on day 0 at each of 8 sites intradermally over deltoid, lateral thigh, suprascapular region and lower quadrant of the abdomen, while on day 7,

0.1 ml of vaccine is given at each of 4 sites over deltoid and thighs and on days 28 and 90, 0.1 ml of the vaccine is given at one site, over deltoid. By day 7, antibody was detected in 88% of the patients and up to 1 year there was a 100% sero conversion in all patients tested (Warrell *et al.*, 1983; 1984; 1985; Suntharasamai et *al.*, 1987).

Similarly, a 2-site intradermal method ("2-2-2-0-1-1") was employed using purified vero cell vaccine (PVRV), purified primary chick embryo cell vaccine (PCECV) and Purified Duck Embryo Vaccine (PDEV). Rabies neutralizing antibodies in all tested patients sera were > 0.5 iμ/ml after one year. (Phanuphak *et al.*, 1987; WHO, 1992; Charanasri *et al.*, 1992; Suntharasamai *et al.*, 1994; Khawplod *et al.*, 1995).

2.11 Interferon

Interferon is a class of small soluble proteins produced and released by cells invaded by virus, which induce in non-infected cells the formation of an antiviral protein that inhibits viral multiplication (Dorland's pocket medical dictionary).

Since 1972, improvements and modifications have been introduced in the development of the original inactivated rabies vaccine for human use was produced (Atanasiu *et al.*, 1974) thus numerous tests have been performed to demonstrate its potency in raising high levels of antibody, T cell mediated response, interferon and protection in laboratory animals (Atanasiu and Tsiang, 1980). The ability for rabies vaccines to induce interferon is one major factor which might play a decisive role in post exposure treatment (Baer *et al.*, 1982). Results obtained by Atanasiu and Tsiang (1980) showed that levels of interferon titres are correlated with protection tests in mice. These results were in accordance with those obtained by Baer *et al.*

(1977) which also found that those vaccines which did not protect were unable to induce interferon in the mouse and concluded that rabies vaccine potency tests should include evaluation of interferon production as well as resistance to street rabies challenge in mouse.

Furthermore, it was stated that in both mice and sub-human primates (rhesus monkeys), post exposure vaccination with HDCV was ineffective in significantly reducing mortality but the addition of either locally-applied interferon inducer, or exogenous interferon, resulted in a reduction of rabies mortality to zero or nearly zero (Baer *et al.*, 1977).

Recombinant (alpha) interferon became available many years ago and was recently licensed for human use. The local application of either recombinant murine (alpha) interferon in mice or recombinant human (alpha) interferon in rhesus monkeys, along with human diploid vaccine, resulted in a significantly reduction in mortality (Baer *et al.*, 1977).

Kumato and Akira (1980) reported the production of interferon as early as 1 hr after vaccination and level reached a peak between 3 and 6 hours and then decline rapidly after 12 hours, while Nicholson *et al.* (1980) observed peak interferon titres to be 17 - 25 hours after vaccination. However, Kuwert *et al.* (1980) showed that high titres of interferon activity as measured in the serum of vaccines they used developed 6 - 7 hours after primary immunization with a 100% response and concluded that interferon production thus seems to depend firstly upon the product used, and secondly upon the vaccine dose and eventually antigen mass applied in primary immunization. Higher dose proved to yield higher interferon concentration in the sera than those of lower dose thus it is dose dependent.

CHAPTER THREE

MATERIALS AND METHODS

3.1 Pre-Immunization Preparation

Eighteen dogs of 7 to 8 weeks of age were bought and housed for an eight week acclimatization period. The dogs were fed on a diet of ground maize and ground soya beans cooked with palm oil and table salt. During this time, detailed clinical and laboratory evaluations including blood screening for parasites and faecal examination for helminths were carried out on the dogs which were identified with collars and tags.

The dogs were subjected to endo and ectoparasitic control using Pyrantel Pamoate (Pfizer product) and Praziquantel (Bayer product) at the dose of 5 mg/kg per dog orally. They were also subjected to ectoparasitic control using Spot On[2] (deltametrin) at the dose rate of 1 ml/10 kg applied topically. These were repeated every 6 - 8 weeks.

Furthermore, all the dogs were vaccinated using a polyvalent vaccine (Canine distemper, Hepatitis, Leptospirosis, Parvovirus enteritis and Para-influenza - (DHLP & P))(Fort Dodge[3]) at 8 weeks, 12 weeks and 16 weeks of age.

3.2 Pre-Immunization Screening

3.2.1 Serum Collection and Screening

About 10 ml of blood was collected from each dog prior to immunization and each sample was kept in a separate sterile clean test tube. The test tubes containing the blood samples were centrifuged at 150 g for 20 minutes after which serum from each sample was pipetted into a separate clean bijou bottle and stored at - 4° C. These were analysed

for neutralization antibodies against rabies using mice. Only dogs whose serum samples did not show any measurable antibody as shown by the mouse neutralization test results were used.

3.3 Virus Preparation

Brain tissue from a dog positive for rabies antigen in accordance with the Fluorescent Antibody Technique (FAT) was used to make a suspension of the virus. A 10% suspension was made by weighing 1.0 gm of the brain tissue and placing it in 9.0 ml of 1% minimum essential medium (1% MEM). The suspension was centrifuged at 500 g for 20 minutes. The supernatants were used for titration in mice.

3.4 Virus Titration in Mice

In order to adapt the virus, it was passed twice in mice. Tenfold dilutions of the supernatant from the virus suspension were made in 1% MEM up to 10^{-9} dilution. Each of 6 weanling mice was inoculated intracerebrally with 0.03 ml of each dilution and observed for a period of 28 days. Mortality were recorded while the 50% endpoint dilution was calculated using the Reed and Muench (1938) method as shown in Appendix A. The lethal dose (LD_{50}) was found to be $10^{-4.88}$

3.5 Vaccines

Two types of vaccine were used. Fort Dodge(R) antirabies vaccine with an official name 'Killed Flury strain rabies virus' with serial number 19936A was procured from Bluebatcoy Ltd., Kaduna. The second type of vaccine is the Antirabies Vaccine for dogs with official name "Live attenuated Flury strain rabies virus: chick embryo origin, low egg passage" with bach number 05 was procured from the National Veterinary Research Institute (NVRI), Vom. The vaccines were stored at 4°C until

used.

3.6 Grouping of Dogs

The dogs were grouped into 3 (A, B, C) of 6 dogs each as shown in Fig. 3.1. Group A was subdivided into 3 groups (AF,AV and AC) of 2 dogs each, while groups B and C were also divided into 3 groups (BF, BV and BC) and (CF, CV and CC), respectively, with 2 dogs in each group.

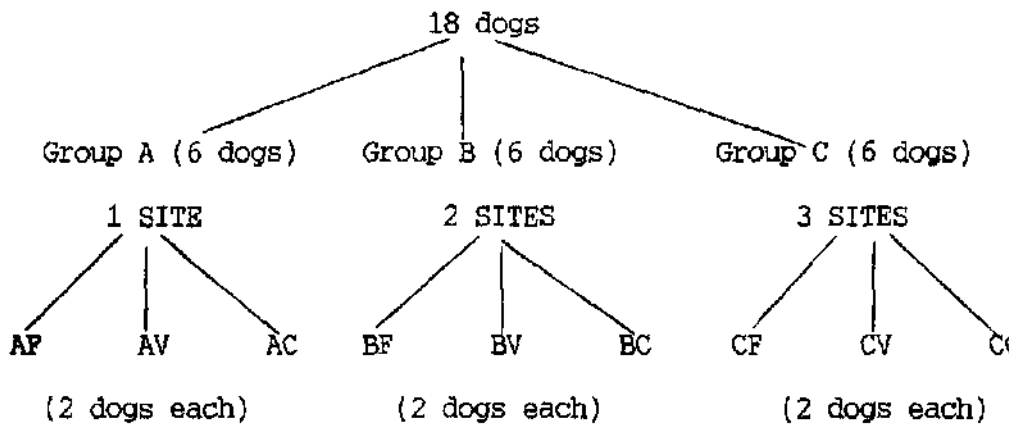

Fig. 3.1 Grouping of dogs for experiment.

3.7 Immunization

Group A dogs were vaccinated using only one site of F-vaccine (i.e. Killed Flury strain antirabies virus) for AF, V-vaccine (i.e. Live attenuated Flury strain antirabies virus) for AV and distilled water which served as control for AC. Groups B and C dogs were injected using two sites and three sites, respectively, for the same dose of each vaccine as in group A (i.e. 1 ml of F – vaccine and 2.5 ml of V – vaccine which were split accordingly with respect to the number of sites used). The V-vaccine was reconstituted according to the manufacturer's instruction before use.

One vastus lateralis muscle was used in each dog for single site

injection using 1 ml for AF dogs and 2.5 ml for AV dogs; two (one on each limb) were used for two sites injection by splitting the doses into 0.5 ml and 1.25 ml at each site for BF and BV dogs, respectively, while for the three sites injections both vastus lateralis muscles and one bicep muscle were used by injecting approximately 0.3 ml and 0.8 ml at each site for CF and CV dogs, respectively. The intramuscular route was used for injections.

3.8 Serum Collection

Blood samples were drawn via the jugular vein of all the dogs 3, 7, 10, 14 and 28 days post vaccination. The samples were each centrifuged at 150 g for 20 minutes after which the serum was pipetted into separate clean bijou bottles and stored at - 20^0 C before use in neutralization test.

3.9 Serum Neutralization Test

This was carried out at the Virology Department of the University College Hospital (U.C.H.), Ibadan. Six mice were used for each sample of the serum. A constant-virus, varying -serum method was used to measure serum-neutralization of the rabies virus. That is, a standardized amount of virus ($64LD_{50}$) was combined with various dilutions of each serum sample. The samples were analysed for serum antibody titre and protection using mouse neutralization test (MNT). Results (see Appendices B and C) were expressed in terms of a serum titre, defined as the dilution factor of the highest dilution of serum which neutralizes a standard amount of virus (see Appendix D for reagents and equipment used).

All the serum samples were inactivated at 56^0 C for 30 minutes. Serum dilution was carried out by making a 1:5 dilution of each serum by adding 1 ml of each of the sample to 4 ml of 1% MEM. Serial twofold dilutions of the 1:5 dilution of the sera were prepared through 1:80 (by serially

transferring 0.5 ml of each dilution of serum into 0.5 ml MEM). Pretitred passaged virus was diluted in MEM to contain $64LD_{50}$, which was found to be 1.8 (i.e. log of 64) order of the magnitude back of the end point titre of $10^{-4.88}$.

A two tenth (0.2 ml) of each serum dilution was combined with 0.2 ml of $64LD_{50}$ of the passaged virus in prelabelled test tubes. The test tubes were incubated at 37^0 C for 90 minutes after which they were kept at 4^0 C (Atanasiu, 1973).

From each serum virus mixture, 0.03 ml was inoculated intracerebrally into each of 6 three week old mice. Each batch of 6 inoculated mice was placed in a separate cage. The mice were observed daily for 28 days for any signs of rabies infection (cycling, convulsion, ataxia and paralysis) and death. The 50% mortality end point dilution for each serum sample was calculated using the Reed and Muench (1938) method (see Appendix E).

3.10 Calculation of Geometric Mean Titre (GMT)

The GMT values were calculated by computing the mean of the common logarithms to the base 10 (log_{10}) of the titres (see Appendix F) and then reading the antilogarithms of the mean (see Appendices G and H).

CHAPTER FOUR

RESULTS

4.1 Killed Flury Strain Antirabies Vaccine

A normal standard dose regimen with killed Flury strain rabies virus using the conventional method did not show an appreciable measurable antibodies by day 28 in all the subjects as shown in Table 4.1. A geometric mean titre (GMT) of 13.7 was recorded on day 28.

TABLE 4.1 NEUTRALIZATION OF RABIES VIRUS BY SERUM SAMPLES OF DOGS VACCINATED WITH KILLED FLURY STRAIN ANTIRABIES VACCINE ACCORDING TO THE NUMBER OF INJECTION SITES USING MOUSE NEUTRALIZATION TEST (MNT).

GROUPS	GEOMETRIC MEAN TITRE (GMT)				
	DAY 3	DAY 7	DAY 10	DAY 14	DAY 28
A	0	8.1	8.7	10.6	13.7
B	8.1	10.8	12.2	14.1	29.2
C	24.8	51.9	61.7	> 80	100

A, B and C - 1, 2 and 3 sites, respectively.

DAY - Day of post immunization serum collection from dogs

The antibody titres varied depending on the serum collection days post vaccination as shown in Table 4.1. There was no measurable antibody till day 3 post vaccination, while GMT value of 8.1, 8.7 and 10.6 were recorded on days 7, 10 and 14, respectively.

Results obtained when the two site regimen of the same vaccine was

used showed antibody titres in all the subjects from day 3 with a GMT value of 8.1. The serum samples collected for the same regimen on days 7, 10 and 14 post vaccination showed increases in the GMT values of 10.8, 12.2 and 14.1, respectively. On day 28 post vaccination, antibody titre with GMT value of 29.2, which is double the value for the single site regimen on the corresponding day, was recorded as shown in Table 4.1.

In the triple site regimen, reasonable levels of antibody titres were recorded from day 3 with a GMT value of 24.8, which is close to the value obtained on day 28 for the two site regimen. The GMT values increased from day 7 with a value of 51.9 to values greater than 80 and 100 on days 14 and 28, respectively.

4.2 Live Attenuated Flury Strain Antirabies Vaccine

Results obtained when the live attenuated Flury strain antirabies vaccine was used are summarised in Table 4.2. For the conventional method, antibody titres with GMT value of 7.9 was recorded on day 3 post vaccination and the value increased to 8.4, 9.0, 11.2 and 12.9 on days 7, 10, 14 and 28, respectively.

Results recorded for the two site regimen showed the same pattern of increasing antibody titre with increase in the number of days post vaccination. GMT values of 8.4, 11.2 and 12.9 were recorded on days 3, 7 and 10 post vaccination, which equalled the values recorded on days 7, 14 and 28, respectively, for the conventional single site dose regimen as shown in Table 4.2. An antibody titre with GMT value of 15.1 was recorded on day 14 post vaccination and this value increased to 25.4, which is double the one site regimen value of 12.9, on day 28 post vaccination.

The triple site dose regimen showed a reasonable level of antibody titre with GMT value of 50.1 on day 3 post vaccination. The GMT values increased with increase in days post vaccination up to a maximum value of 100 on day 28 as compared with values of 12.9 and 25.4 recorded for the single and double site regimens, respectively.

All the controls for the different sites regimen injected with distilled water did not show any trace of antibody. Consequently, the GMT values equalled zero.

TABLE 4.2 NEUTRALIZATION OF RABIES VIRUS BY SERUM SAMPLES OF DOGS VACCINATED WITH LIVE ATTENUATED FLURY STRAIN ANTIRABIES VACCINE ACCORDING TO THE NUMBER OF INJECTION SITES USING MOUSE NEUTRALIZATION TEST (MNT).

GROUPS	GEOMETRIC MEAN TITRE (GMT)				
	DAY 3	DAY 7	DAY 10	DAY 14	DAY 28
A	7.9	8.4	9.0	11.2	12.9
B	8.4	11.2	12.9	15.1	25.4
C	50.1	65.3	70.8	> 80	100

A,B and C - 1, 2 and 3 sites, respectively.

DAY - Day of post immunization serum collection from dogs.

CHAPTER FIVE

DISCUSSION

Results of studies conducted by Clark *et al.* (1975) showed that the phase of immune response critical to the development of protection against fatal rabies virus infection is unknown while Sikes et *al.* (1971) reported that high levels of serum neutralizing antibodies have been used as a measure of protection in rabies. This remains the most valid procedure for evaluating the protective potential of human and animal rabies vaccines and treatment regimens (i.e. estimation of serum antibody levels in immunized unexposed persons and animals).

Immune response is directly related to the protection of human or animal victims. Thus, high levels of rabies neutralizing antibody in the serum have on different occasions been regarded as protections (Sikes *et al.*, 1971).

Potency tests for rabies vaccines have been classified into direct or vaccination-challenge tests; semi-direct tests that evaluate the immunoglobulin response following vaccination; and indirect tests such as the antibody-binding tests which do not involve introduction of the vaccine into animals or man (Soulebot *et al.*, 1974).

Direct tests are impractical for the large variety of domestic animals which receive rabies vaccinations, mainly because of the difficulty and cost of that would be incurred . Consequently, laboratory animal models are used in potency tests for rabies vaccination and the results are applied to human and animal vaccination.

Mouse neutralization test (MNT), one of the tests used for measurement of rabies antibody is designed to measure antibodies which prevent infection by rabies virus. The result of MNT for rabies antibody reflect the degree of protection against infection with the virus and it has been observed to give a good level of protection (Wallis and Francis, 1981).

The methodology adopted in this study is in accordance with the work of authors mentioned above. Mouse neutralization assay was developed based on the principle that the serum is mixed with the virus and incubated under appropriate conditions (see paragraph 3.9) before introduction into a susceptible host system. The presence of unneutralized virus can be detected by lack of viral neutralization as evidenced by death or illness. Thus death of the mouse is the end point, which indicates a negative result (Lennette and Emmons, 1971).

In this study, negative results were recorded in all the serum - viral mixtures of all the controls as well as in serum collected 3 days post vaccination of one site dose regimen using the killed Flury strain antirabies vaccine. This indicated no presence of antibodies in the sera collected from the controls and thus no neutralization took place. Furthermore, the fact that quite a number of mice inoculated with the mixture of dog sera and rabies virus did not develop the disease while all the controls came down with the disease indicated that some sera contained sufficient antibodies that neutralized the virus. The mice dying after being inoculated with serum collected from dogs 3 days post vaccination using killed Flury strain antirabies vaccine most probably indicated that the serum did not or incompletely neutralised the virus in the serum - virus mixture. This is in agreement with the observations of Lennette and Emmons (1971).

The varying death times for the dead mice can be attributed to the serum titres used which could not neutralize all the virus particles while the quantity neutralized varied with different titres. This is in agreement with the procedure of Reed and Muench (1938) in the calculation of the 50% end point mortality of serum. The serum samples that did not neutralize the virus sufficiently could not protect the mice and hence death of the mice occurred at varied times after inoculation. For serum sample mixtures with very low viral particles as observed earlier using live attenuated Flury strain antirabies vaccine the mice probably survived at the early stage as a result of other protective body mechanisms like interferon which was able to limit the spread of the virus into adjacent cells until sufficient immune response was mounted to get rid of the organisms. These forms of other mechanisms were not in focus in this study.

But it has been observed by Baer and Yager (1977) that protection following rabies vaccination was more closely correlated with the level of interferon than the antibody levels at the initial stage. However, with serum viral mixtures containing large quantities of unneutralized virus particles there was no protection. It is believed that the mice that survived were inoculated with viral/serum mixtures in which the antibody titres were high enough to completely neutralise the virus. Detection of antibody in the serum samples of vaccinated dogs showed that all the vaccines were immunogenic (Figs. 5.1 and 5.2).

Roitt (1975) reported that killed organisms (dead viruses) that have been inactivated provide a safe antigen for immunization. However, the immunity conferred is often inferior to that resulting from live organisms due to the fact that replication of living microbes confronts the host with a larger and more sustained dose of antigen. Ideally, immunity would be

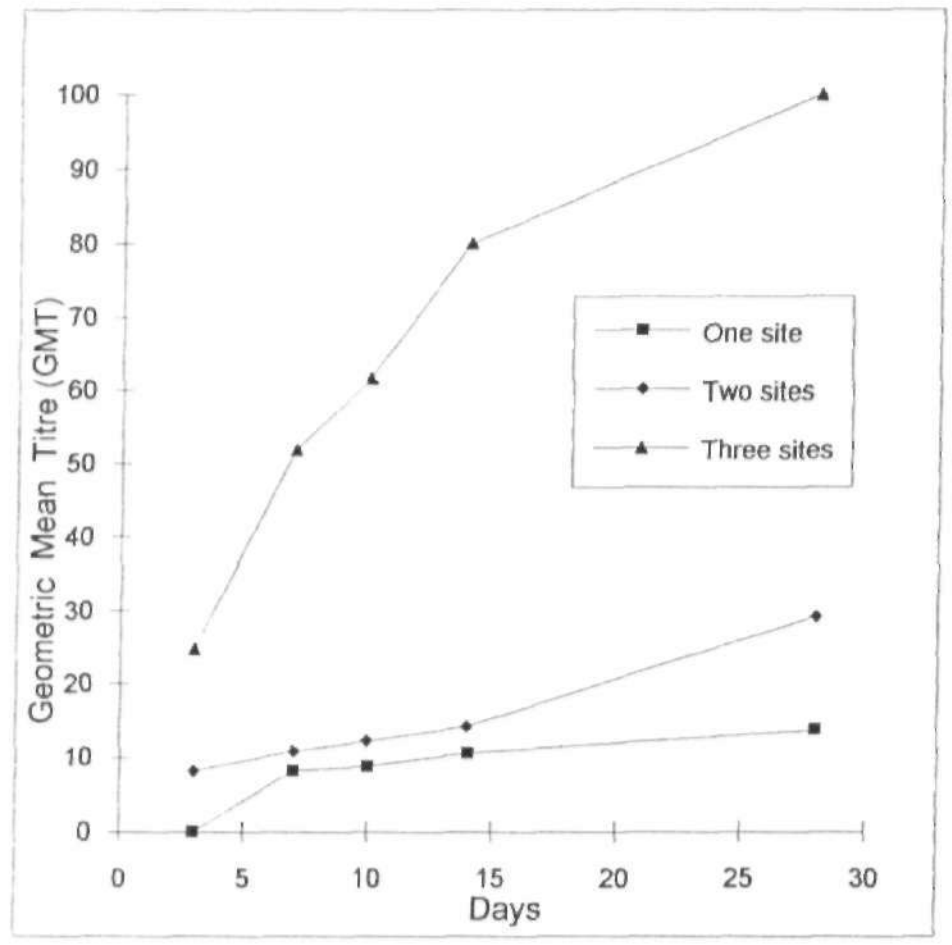

Fig. 5.1 Comparison of the neutralization of rabies virus by serum samples obtained from dogs at various days post vaccination using killed Flury strain antirabies vaccine

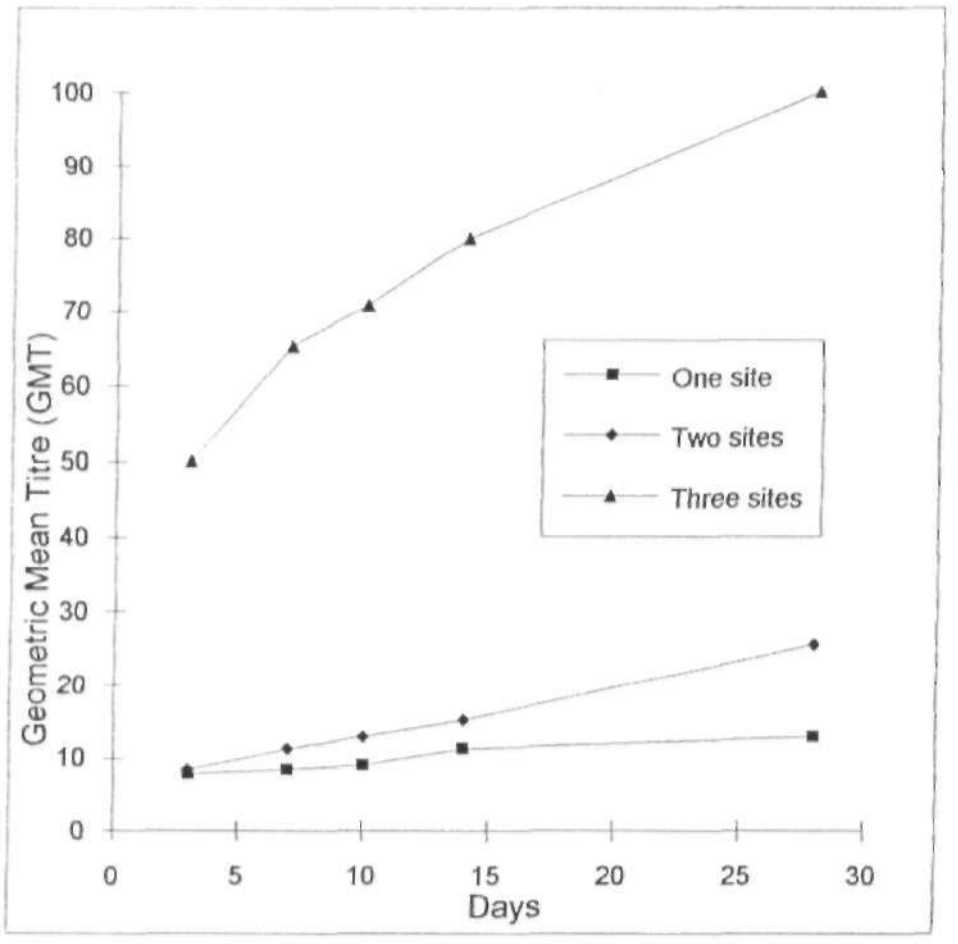

Fig. 5.2 Comparison of rabies virus by serum samples obtained from dogs at various days post vaccination using live attenuated Flur.y strain antirabies vaccine.

best established by infection with a modified but live (attenuated) form of the vaccine which would multiply at the site of the natural infection without producing disease. This pattern was observed in this study where results obtained when live attenuated Flury strain antirabies vaccine was used showed higher antibody titres up till about 18 - 19 days post vaccination using one and two injection sites (Figs. 5.3 and 5.4) and 14 days post vaccination using three injection sites (Fig. 5.5). Also, live attenuated Flury strain antirabies vaccine appeared to stimulate earlier and more protective serum than the killed Flury strain antirabies vaccine (Figs. 5.3 - 5.5).

It was previously observed by Roitt (1975) that live viruses served as more persistent antigens than the killed virus and so stimulated more antibody producing cells. However with both vaccines as the days increased, protection was observed in this work to have increased also. The explanation of this could not be unconnected with the clonal expansion of the neutralising antibody producing cells was proportional to the number of days post vaccination as reported by Roitt (1975).

Protection level can be seen to be related to the number of site regimen as shown in Figs. 5.1 and 5.2. For the killed and live attenuated Flury strain antirabies vaccines, the serum samples from single site regimen had less antibody titre than the two sites regimen, which in turn had less antibody titre than the three site regimen. The reason for these occurrences might be due to suggestion made by Black (1993), that the more the sites that were used for inoculation, the more the number of B-lymphocytes that were stimulated to form neutralising antibodies. This is in agreement with earlier reports made by Turner *et al.* (1976), Warrell

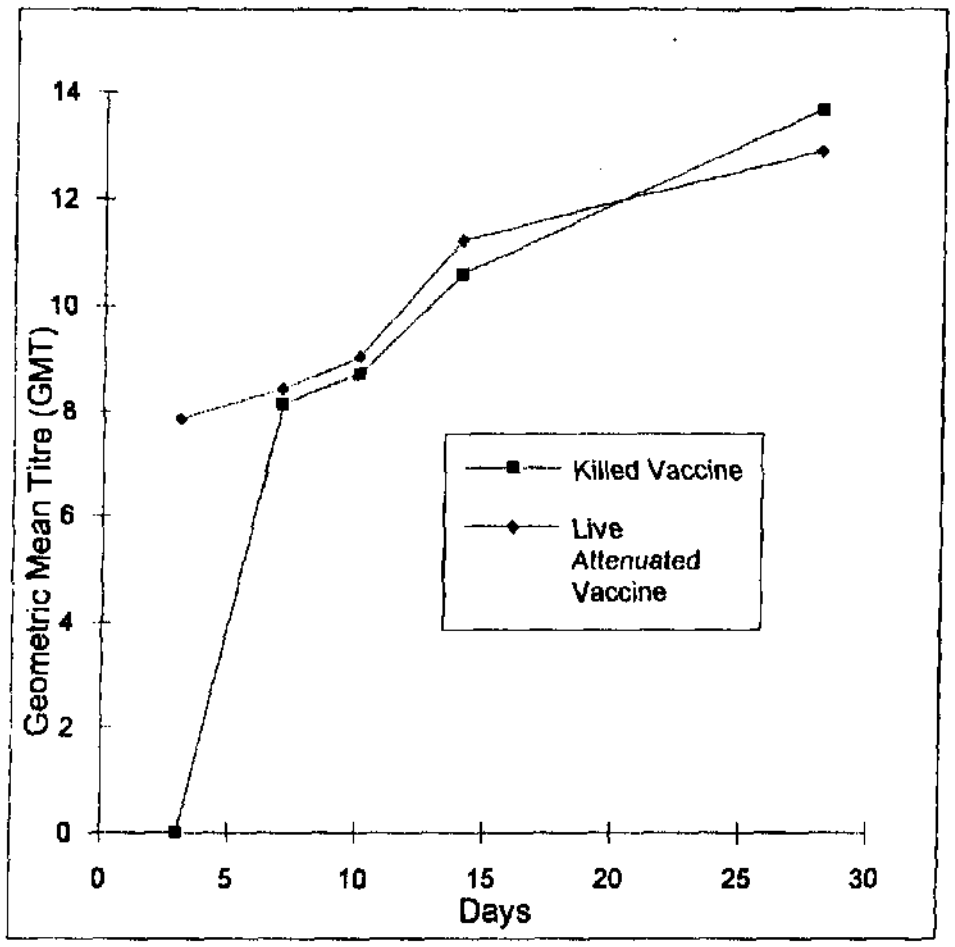

Fig. 5.3 Comparison of the neutralization of rabies virus by serum samples obtained from dogs at various days post vaccination using one injection site.

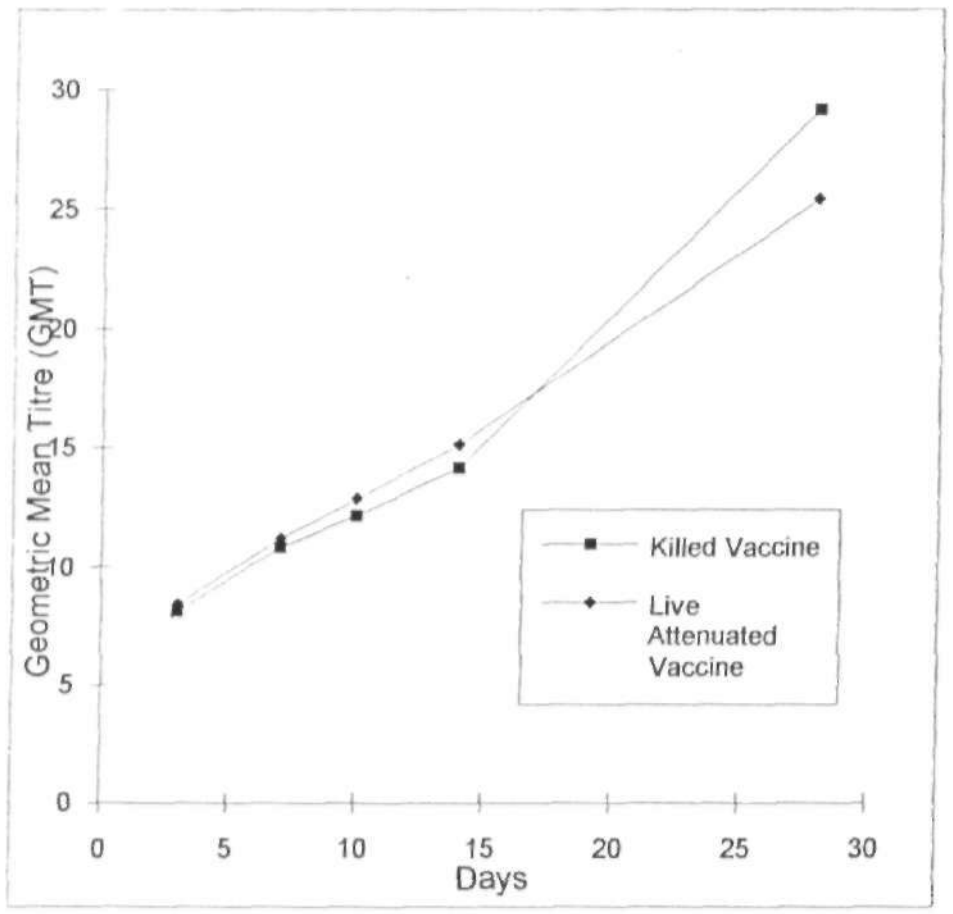

Fig. 5.4 Comparison of the neutralizationof rabies virus by serum samples obtained from dogs at various days post vaccination using two injection sites.

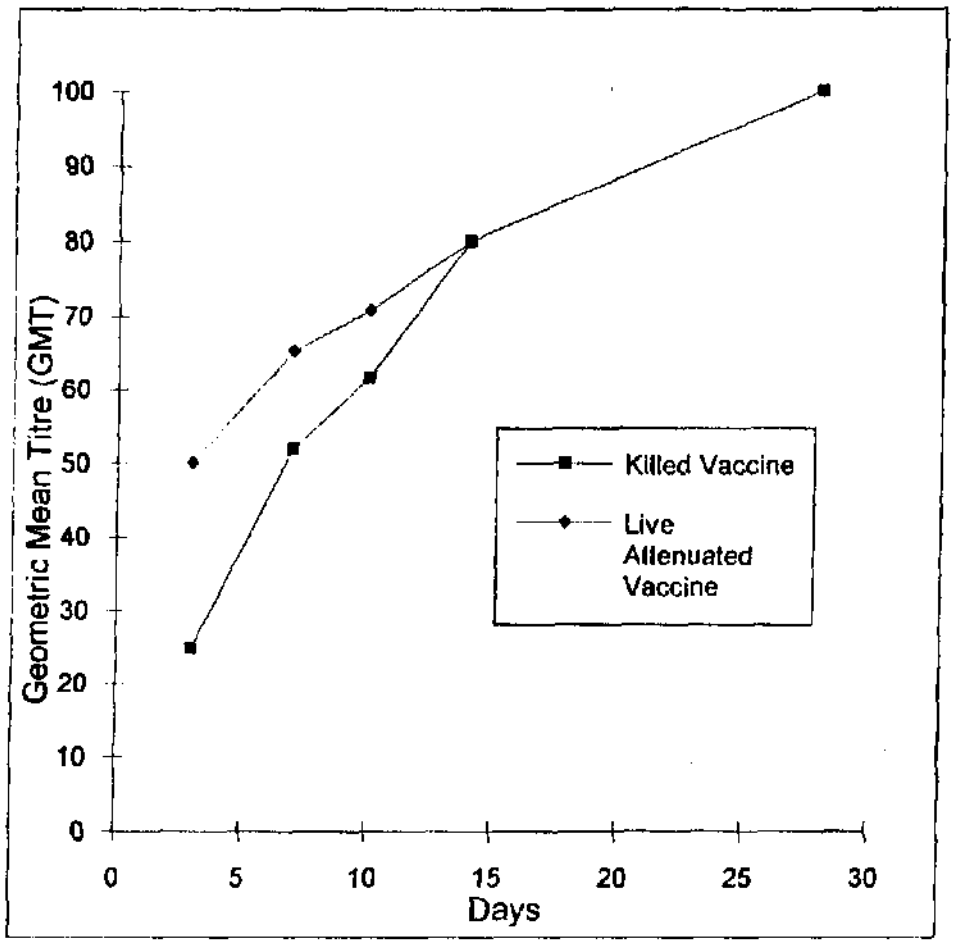

Fig. 5.5 Comparison of the neutralization of rabies virus by serum samples obtained from dogs at various days post vaccination using three injection sites.

et al. (1984, 1985), Bakliac *et al.* (1985) and Vodopija *et al.* (1988).

A clone of cells producing antibodies were initiated at each inoculation site. This can be supported by the fact that when the killed Flury strain antirabies vaccine was used there was no virus neutralization by antibodies in mice given dog serum samples obtained up to day 3 post vaccination in the single site vaccination regimen. Protection was observed as from day 3 in the two site regimen where more cells were involved in producing antibodies. This equalled that of day 7 in the single site regimen (Fig. 5.1). This antibody production doubled the one provided by sera from single site inoculation regimen by day 28. Also, serum samples collected 7 days post inoculation from dogs with triple site regimen protected over 50% of the mice and by day 28 all the inoculated mice survived (Fig. 5.1).

Similar patterns were observed for the live attenuated Flury strain antirabies vaccine as shown in Fig. 5.2. This form of immuno-response has been reported (Turner *et al.*, 1976; Warrell *et al.*, 1983 and Baklaic, *et al.*, 1985) in human beings. These findings were in agreement with those of Roitt (1975) who reported that injection of small amounts of antigen leads to the production of high - affinity antibodies whereas larger amounts of antigen give more antibody of lower affinity. He further explained that when an appropriate number of antigen molecules are bound to the antibody receptors on the cell surface, the lymphocyte will be stimulated to develop into an antibody - producing clone.

However, when small amounts of antigen are present, only the lymphocytes with high - affinity antibody receptors are able to bind sufficient antigen for stimulation to occur which will lead to daughter cells producing high-affinity antibody. With increasing concentration of

antigen, even antibodies with relatively low affinity will bind more antigen i.e. at high doses of antigen, lymphocytes with lower affinity anitbody receptors will also be stimulated. These are more abundant than those with receptors of high affinity, therefore with respect to this study carried out, the concept of splitting a dose into divided doses and inoculating at different sites lead to the reduced concentration of antigen at each site which in turn resulted to stimulation of lymphocytes with high affinity antibody receptors.

Recorded results show that neutralising antibodies produced post vaccination increased to appreciable levels in the serum and this progressed steadily irrespective of the number of sites used for administering the vaccine. A 100% protection offered to the mice by serum samples obtained 28 days post vaccination from triple site vaccination regimen, is an evidence that multiple site antirabies vaccination provide high level of protection for dogs as shown in Figs. 5.1 and 5.2.

Crick and Brown (1974) reported that the mode of immunization based on the route and number of inoculation sites could influence the results of potency tests, such that in tests using multiple inoculations a relatively poor vaccine could appear to be quite potent. On the basis of this enhanced protection through multiple site inoculation and taking into consideration the multiple problems that lead to vaccine and vaccination failure, the multiple site mode of inoculation may reduce the rate of vaccine failure.

CHAPTER SIX

CONCLUSION AND RECOMMENDATION

6.1 Conclusion

The two-site dose regimen of the killed vaccine induced sero-conversion in all the subjects from day 3 post vaccination. This regimen proved to be superior to the single site standard dose regimen, which did not show measurable antibody until 7 days post vaccination. The antibody titres for single regimen were less than those of the two site regimen throughout the period of observation. The same condition was also observed when the live attenuated vaccine was used. The levels of neutralizing antibody production achieved with triple-site regimen showed a four-fold increase in serum titre over the two-site regimen, irrespective of the type of vaccine used. A 100% sero-conversion was achieved by day 28 post vaccination for the three-site regimen. An enhanced production of antibody was observed when live attenuated antirabies vaccine was used as indicated by an earlier production than when the killed Flury strain antirabies vaccine was used. The more the site of vaccination, the more the antibodies produced.

6.2 Recommendation

This study has shown that the use of the multiple site mode of inoculation leads to increased antibody production. Therefore, it is recommended for use in the administration of antirabies vaccines in dogs.

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APPENDICES

APPENDIX A: CALCULATION OF TITRES

Calculation of the 50% Mortality Endpoint of the Virus Assuming the following results were obtained

Virus Dilution	Deaths Per No. Inoc.	Cumulative Deaths	Cumulative Survivors	Mortality Ratio	% Mortality
$10^{-5}(1:10^5)$	6/6	13	0	13/13	100
$10^{-6}(1:10^6)$	5/6	7	1	7/8	88
$10^{-7}(1:10^7)$	2/6	2	5	2/7	29
$10^{-8}(1:10^8)$	0/6	0	11	0/11	0

Then the titre can be calculated using Reed and Muench (1938) Method.

Interpolation = $\frac{\text{\% Mortality Greater than 50\% - 50\%}}{\text{\% Mortality Greater than 50\% - \% Mortality Less than 50\%}}$

Substituting $\frac{88 - 50}{88 - 29} = \frac{38}{59} = 0.6$

Interpolative value is then multiplied with the negative $\log_{10}$ of the dilution ratio.

Negative $\log_{10}$ of the dilution ratio of 10 = -1

Interpolative value = x 0.6

Corrected Interpolative value = - 0.6

(2) Then find the end-point dilution associated with 50% mortality: which is located between 10^{-5} and 10^{-7} dilution.

(3) The $\log_{10}$ of the 50% end-point dilution is then estimated by adding the corrected interpolative value to the $\log_{10}$ of the dilution above 50%.

$$-6 + (-0.6) = -66.$$

(4) The 50% end-point dilution is estimated at $10^{-5.6}$.

(5) The 50% titre is estimated as $10^{5.6}$

(6) The lethal dose (LD_{50}) used in the study was found to be $10^{-4.88}$

APPENDIX B: EXPERIMENTAL RESULTS OF MOUSE NEUTRALIZATION TESTS (STUDY 1)

	Undiluted	1/10	1/20	1/40	1/80
F1-3	6/6	6/6	6/6	6/6	6/6
F1-7	5/6	5/6	6/6	6/6	6/6
F1-10	5/6	5/6	6/6	6/6	6/6
F1-14	5/6	5/6	5/6	5/6	6/6
F1-28	5/6	4/6	5/6	5/6	5/6
V1-3	5/6	5/6	6/6	6/6	6/6
V1-7	5/6	5/6	5/6	6/6	6/6
V1-10	5/6	5/6	5/6	5/6	6/6
V1-14	5/6	4/6	5/6	5/6	5/6
V1-28	4/6	4/6	4/6	5/6	5/6
F2-3	5/6	5/6	6/6	6/6	6/6
F2-7	5/6	5/6	5/6	6/6	6/6
F2-10	5/6	4/6	5/6	6/6	6/6
F2-14	3/6	3/6	5/6	6/6	6/6
F2-28	0/6	0/6	1/6	5/6	6/6
V2-3	5/6	5/6	5/6	6/6	6/6
V2-7	=> Lost in	Transit			
V2-10	4/6	4/6	4/6	5/6	6/6
V2-14	3/6	3//6	4/6	5/6	6/6
V2-28	2/6	2/6	3/6	4/6	5/6
F3-3	1/6	2/6	3/6	4/6	5/6
F3-7	0/6	1/6	1/6	1/6	5/6
F3-10	0/6	1/6	1/6	1/6	4/6
F3-14	0/6	0/6	0/6	1/6	2/6
F3-28	0/6	2/6	0/6	0/6	0/6
V3-3	1/6	1/6	2/6	2/6	3/6
V3-7	0/6	0/6	0/6	2/6	3/6
V3-10	*0/6	*1/6	*4/6	*6/6	6/6
V3-14	0/6	0/6	1/6	2/6	0/6
V3-28	0/6	0/6	0/6	0/6	0/6
All Controls	6/6	6/6	6/6	6/6	6/6

APPENDIX C: EXPERIMENTAL RESULTS OF MOUSE NEUTRALIZATION TESTS (STUDY 2)

	Undiluted	1/10	1/20	1/40	1/80
2F1-3	6/6	6/6	6/6	6/6	6/6
2F1-7	5/6	5/6	5/6	6/6	6/6
2F1-10	5/6	5/6	5/6	5/6	6/6
2F1-14	5/6	4/6	4/6	5/6	6/6
2F1-28	2/6	2/6	4/6	5/6	5/6
2V1-3	5/6	5/6	6/6	6/6	6/6
2V1-7	5/6	5/6	5/6	6/6	6/6
2V1-10	5/6	5/6	5/6	5/6	6/6
2V1-14	4/6	4/6	5/6	5/6	5/6
2V1-28	3/6	4/6	5/6	5/6	5/6
2F2-3	5/6	5/6	5/6	5/6	6/6
2F2-7	4/6	4/6	4/6	4/6	5/6
2F2-10	3/6	3/6	4/6	4/6	5/6
2F2-14	3/6	3/6	4/6	4/6	4/6
2F2-28	1/6	0/6	1/6	5/6	5/6
2V2-3	5/6	5/6	5/6	5/6	6/6
2V2-7	4/6	4/6	5/6	5/6	5/6
2V2-10	4/6	4/6	4/6	4/6	5/6
2V2-14	4/6	3/6	4/6	4/6	5/6
2V2-28	2/6	2/6	3/6	2/6	5/6
2F3-3	1/6	2/6	3/6	4/6	5/6
2F3-7	2/6	2/6	1/6	4/6	5/6
2F3-10	1/6	1/6	1/6	1/6	2/6
2F3-14	0/6	0/6	0/6	1/6	1/6
2F3-28	0/6	0/6	0/6	0/6	0/6
2V3-3	0/6	0/6	2/6	2/6	3/6
2V3-7	0/6	0/6	2/6	0/6	3/6
2V3-10	0/6	0/6	0/6	1/6	3/6
2V3-14	0/6	0/6	1/6	2/6	0/6
2V3-28	0/6	0/6	0/6	0/6	0/6
All Controls	0/6	0/6	0/6	0/6	0/6

APPENDIX D

A. **Reagents**
- 1% minimum essential medium (1% MEM).
- Titrated street rabies virus with a two passage in mice.
- Sera of unknown antibody status.
- Mice (six/cage).

B. **Equipment**
- Syringes (1 ml) and needles.
- Mice cages with beddings, mice cubes and water.
- Animal forceps.
- 37^0 C water bath.
- 56^0 C water bath.

APPENDIX E: CALCULATION OF A 50% MORTALITY END-POINT DILUTION FOR A SERUM ASSUMING THE FOLLOWING RESULTS WERE OBTAINED

Serum Dilution	Constant Virus Dose	Deaths per No. Inoc.	Cumulative Deaths	Cumulative Survivors	Mortality Ratio	% Mortality
1:10	+ Virus	4/6	4	5	4/9	44.4
1:20	+ Virus	5/6	9	3	5/12	75.0
1:40	+ Virus	5/6	14	2	14/16	87.5
1:80	+ Virus	5/6	19	1	19/20	95.0

Then the titre can be calculated using Reed and Muench Method (1938).

$$\text{Interpolation Formula} = \frac{\% \text{ Mortality Greater than 50\%} - 50\%}{\% \text{ Mortality Greater than 50\%} - \% \text{ Mortality less than 50\%}}$$

$$\text{Substituting} = \frac{75.0 - 50}{75.0 - 44.4} = \frac{25}{300.6} = 0.82$$

(1) Interpolative value is then multiplied with the negative $\log_{10}$ of the dilution ratio.

Negative $\log_{10}$ of the dilution ratio of 2 = -0.3

Interpolative value = 0.82

Corrected Interpolative value -0.25

(2) The end-point dilution associated with 50% mortality is between

1:10 = 10^{-1} and 1:20 = $10^{-1.30}$

(3) The $\log_{10}$ of the 50% end-point dilution is then estimated by subtracting the corrected interpolative value from the $\log_{10}$ of the dilution greater than the 50% end-point.

- 1.30 - (- 0.25)

- 1.30 + 0.25

- 1.05

(4) The 50% end-point dilution is estimated to be $10^{-1.05}$

OR $\frac{1}{10^{1.05}} = \frac{1}{11.22}$

(5) The 50% serum titer is estimated to be $10^{1.05}$ OR 11.22.

APPENDIX F: CALCULATION OF GEOMETRIC MEAN TITRES

Geometric mean titres (GMT) were calculated by computing the mean of the common logarithms to base 10 ($\log_{10}$) of the titres and reading the reading the antilogarithms of the mean.

Example using hypothetical results obtained from a series of sera

Serum Titres	log
7.59	0.88
8.71	0.94
	1.82 / 2 = 0.91

Geometric mean titre = Antilog of 0.91 = 8.13

APPENDIX G: SERUM TITRES FROM MOUSE NEUTRALIZATION TEST (MNT) USING KILLED FLURY STRAIN ANTIRABIES VACCINE ACCORDING TO THE NUMBER OF INJECTION SITES

NO.OF SITE(S) USED	POST IMMUN. SERUM COLLECTION DAYS FROM DOGS	SERUM TITRE FOR DOG 1	SERUM TITRE FOR DOG 2	GEOMETRIC MEAN TITRE
AF1	3	0	0	0
AF1	7	7.59	8.71	8.12
AF1	10	8.13	9.33	8.71
AF1	14	8.71	12.88	10.59
AF1	28	11.22	16.60	13.65
BF2	3	7.59	8.71	8.12
BF2	7	8.13	14.45	10.84
BF2	10	9.12	16.60	12.16
BF2	14	10.96	18.22	14.13
BF2	28	28.18	30.20	29.17
CF3	3	21.88	28.18	24.83
CF3	7	48.98	54.95	51.88
CF3	10	53.70	70.79	61.66
CF3	14	>80	>80	>80
CF3	28	100	100	100

ALL CONTROL
AC, BC AND CC : GMT = 0

APPENDIX H: SERUM TITRES FROM MNT USING LIVE ATTENUATED FLURY STRAIN ANTIRABIES VACCINE ACCORDING TO THE NUMBER OF INJECTION SITES

NO.OF SITE(S) USED	POST IMMUN. SERUM COLLECTION DAYS FROM DOGS	SERUM TITRE FOR DOG 1	SERUM TITRE FOR DOG 2	GEOMETRIC MEAN TITRE
AV1	3	7.59	8.13	7.85
AV1	7	8.13	8.71	8.41
AV1	10	8.71	9.33	9.02
AV1	14	11.22	11.22	11.22
AV1	28	12.88	12.88	12.88
BV2	3	8.13	8.71	8.41
BV2	7	LOST	11.22	11.22
BV2	10	11.48	14.45	12.88
BV2	14	13.80	16.60	15.14
BV2	28	21.88	29.51	25.41
CV3	3	47.86	52.48	50.12
CV3	7	64.57	66.07	65.31
CV3	10	70.79	70.79	70.79
CV3	14	>80	>80	>80
CV3	28	>80	100	> 80 - 100

ALL CONTROL
AC, BC AND CC : GMT = 0

LOST - Lost in transit