A RAPID ENZYME IMMUNOASSAY FOR THE DETERMINATION OF ANTIBODIES TO RABIES VIRUS

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DECLARATION

This thesis is my original work and has not been presented for a degree in any other university.

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This thesis has been submitted for examination with our approval as university supervisors.

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SUMMARY

Rabies is world-wide in distribution. Despite significant scientific advances in its prevention and control, the disease has been spreading in most parts of the world where it continues to persist as a major public health problem. The disease is endemic in Kenya where the dog plays a major role in its transmission to man.

In view of the almost 100% mortality rate of human rabies, prevention becomes essential. Also in animals, rabies is a disease approaching a 100% case fatality rate, but it has been known since the days of Pasteur that some animals survive both natural and experimental infection, and that a healthy and infective carrier state may occur. Since efficient vaccines are now available, vaccination may be performed on a large scale in relevant animals and in high-risk human populations.

The emergence of highly efficient vaccines has to a large extent obviated the need to confirm adequate immune responses of vaccinated individuals by serum antibody determinations. Nevertheless, a rapid, specific and reproducible assay for the detection and accurate quantitation of antibodies to rabies virus would be highly desirable for the following purposes :

(i) evaluation of potency of vaccines,

(ii) evaluation of individual immune responses,

(iii) standardization of hyperimmune antiserum for therapeutic/prophylactic use,

(iv) determination of efficacy of large-scale vaccination programmes,

(v) aid in the diagnosis of rabies in suspected non-vaccinated cases,

(vi) comparative studies of rhabdoviruses.

The results obtained by the mouse neutralization test (MNT) and the rapid fluorescent focus inhibition test (RFFIT) have been recognized as true reflections of the protective potency of a serum, and can be expressed as international units per millilitre (I.U./ml) in comparison with an internationally recognized reference serum. Both tests suffer from poor reproducibility and demands for highly skilled man-power. The absolute requirements for live infective challenge virus, a large number of animals or tissue culture capability as well as special facilities and equipment render these two neutralization tests unsuitable for routine or large-scale use in most countries. Although promising, most of the replacement tests have been found to be inadequate largely because of poor correlation with the MNT and the RFFIT.

In this study, an inhibition enzyme immunoassay (INH-EIA) for the detection and quantitation of rabies antibodies was developed and evaluated. In this system, the interaction of specific enzyme-labelled antibodies with their antigen is inhibited by non-labelled antibodies of the same specificity. The antibody titre is expressed as that dilution of a serum sample which gives 50% inhibition. This can subsequently be converted to equivalents of I.U./ml calculated on the basis of an antirabies reference serum whose potency has been determined by the MNT or the RFFIT.

The INH-EIA was carried out by coating microtitre plates with a predetermined dilution of a rabies virus preparation. The IgG fraction of absorbed serum from a goat immunized with human diploid cell rabies vaccine from Institut Merieux, France, was conjugated with horseradish peroxidase and shown to be specific for rabies virus components. It was shown that the conjugate possessed antibody activities to both the glycoprotein and the ribonucleoprotein components of the virus when compared with known antiglycoprotein and antiribonucleoprotein sera obtained from other laboratories.

No inhibition was observed when dilutions of tissue culture homogenates and known negative sera from 7 different animal species were assayed.

A high ionic strength salt (1M KCl) buffer containing 2% polyethylene glycol 6000 was used as test serum diluent. The composition of the diluent would enhance antigen-antibody interactions and might consolidate these reactions, thus presumably allowing the estimation of even low affinity antibodies. The INH-EIA was performed sequentially allowing antibodies of the test serum to interact with antigen prior to the addition of conjugate. It is expected that such an assay would be capable of detecting both low and high affinity antibodies, and also low levels of antibody. The ability of the INH-EIA to detect low levels of antibody was evident when low but definite antibody activity (equivalent to 0.25 - 1.95 I.U./ml) was detected in 5 human sera obtained 7 days after primary intradermal vaccination with human diploid cell rabies vaccine. The assay gave a mean titre equivalent to 0.06 I.U./ml (range 0.04 - 0.08 I.U./ml) in 14 sera from non-vaccinated humans. The ability of the INH-EIA to detect low levels of antibodies should render the test suitable for the intra-vitam diagnosis of rabies. This became evident when a serum sample from a previously unvaccinated rabid goat, bled one week before death, gave an antibody titre corresponding to 2.0 I.U./ml, i.e. ten times the highest titre obtained in serum samples from non-vaccinated humans and animals.

The reproducibility of the INH-EIA was assessed by performing 15 complete titrations of each of two antirabies sera obtained from the World Health Organization (WHO) and the Institut Merieux. The WHO serum which had a potency of 10 I.U./ml as determined by the MNT, gave a mean titre correspondng to 8.7 I.U./ml (range 7.2 - 9.9, coefficient of variation $\pm$ 10%) calculated on the basis of the Institut Merieux serum. The Institut Merieux serum gave a mean titre corresponding to 174 I.U./ml (range 151.6 - 205.4 I.U./ml, coeff. var. $\pm$ 10%) when the WHO serum was used as a standard. The Institut Merieux serum had been titrated against an international standard by using the RFFIT. A mean titre equivalent to 194 I.U./ml (range 183 - 209, coeff. var. $\pm$ 6%) was found. Thus, excellent agreement was found between the INH-EIA and both the MNT and the RFFIT.

In the INH-EIA, duplicate single serum dilutions of 1:2 could be used to accurately quantitate rabies antibodies as long as the inhibition was within 20-75%. This procedure proved valuable in the screening of large numbers of serum samples from any animal species within a short time.

The highest antibody titre obtained in sera from non-vaccinated persons and animals was 0.2 I.U./ml. Consequently, this titre was chosen as a cut-off point for the differentiation of rabies antibody positive and negative

sera. Some of the sera from man and different animal species gave antibody titres above the equivalent of 0.2 I.U./ml. This was notable with sera obtained from goats, cattle, elephants and hyenas. Since no antirabies vaccination is carried out in these animals in Kenya, it is surmised that the animals may have been exposed to the rabies virus or to one of the rabies-related viruses.

It is concluded that INH-EIA is a highly reproducible, sensitive and specific method for the detection and quantitation of antibodies to rabies virus and correlates well with the internationally accepted MNT and RFFIT. The method allows the screening at a single serum dilution of large numbers of specimens from different animal species. It is therefore likely that the method will find considerable application in seroepidemiological studies of rabies.

The INH-EIA appears to be an attractive substitute for both the MNT and the RFFIT because of its high sensitivity and specificity, high reproducibility, ease of performance and interpretation, rapidity and low cost. Further investigations are needed to confirm whether the antibody titres obtained in the INH-EIA represent true reflections of various levels of protective immunity.

1. INTRODUCTION

Rabies is an acute central nervous system disease of domestic, wild animals and man that usually results in death. The disease follows infection with a virus in the family Rhabdoviridae.

Although rabies has been known since ancient times and significant scientific advances have been made towards its prevention and control, it still continues to persist as a major public health problem and remains one of the most dreaded of the human communicable diseases. The disease exacts a heavy toll in terms of human deaths and losses in cattle production especially in developing countries. Figures of 15000 human deaths per year have been reported worldwide (Warrel, 1977). In Latin America alone, an annual loss in cattle production of $250 million has been reported (Keymer, 1985).

Rabies is worldwide in distribution, with the exception of a few countries and areas that have historically been free of the disease. In the tropics, urban rabies, with the dog as the principal transmitter, is a major problem. This is in contrast to areas outside the tropics where wildlife rabies appears to be a more serious human and animal health problem (Acha and Arambulo, 1985). Nevertheless, even outside the tropics, outbreaks of urban rabies have occurred in recent

years following the incursion of infected, wild animals into urban settlements, such as raccoons in the northeastern United States and foxes in Germany (Acha and Arambulo, 1985).

In Kenya, rabies has been known to exist for a long time. The earliest confirmed rabies case occurred near Nairobi in 1912 in a dog which had been attacked and bitten by a jackal (Hudson, 1944). In man, the disease has been appearing in a sporadic form mostly in the Coast province, Eastern province and in Western Kenya (Siongok and Karama, 1985). Rabies is widespread all over Kenya in animals, but occurs in endemic forms in Kitui and Machakos districts of Eastern province (Kariuki and Ngulo, 1985).

Treatment of rabies has always been a major problem and a challenge to modern medicine. The treatment of rabid animals is not undertaken if clinical evidence of the disease is present. However, medical treatment of human rabies has been tried, but it has historically been symptomatic and futile (Beran, 1981). Due to failure in treatment, prevention remains the only alternative to the control of both human and animal rabies. Prevention of rabies can be achieved through mass immunization of animals, mainly dogs, and immunization of persons at a high risk of exposure.

Owing to the almost uniform fatality of clinical rabies, it is important to assess the immune response of both man and animals after antirabies vaccination. Many vaccine manufacturing companies recommend the testing for the

presence of virus neutralizing antibodies after antirabies vaccination. Determination of antibody titres after vaccination has several advantages. These includes the identification of poor responders and the subsequent administration of booster doses of vaccine where indicated. This is especially important for people who are regularly exposed to the rabies virus by the nature of their work. Moreover, the screening for rabies antibodies in dog populations would be of particular importance since the results would indicate the efficacy of rabies control programmes. In addition, a test for the detection of rabies antibodies in sera of both man and animals could be valuable in diagnosis. This is because intra-vitam results of immunofluorescence and virus isolation are often negative, especially late in the clinical course of rabies (Nicholson et al., 1985). In such cases, the presence of serum antibodies is the most confirmatory procedure, provided that no antirabies vaccination has been carried out.

The neutralizing antibodies are specific for the virus glycoprotein, which is considered the major protective antigen of the rabies virion (Cox et al., 1977). Although several methods have been developed for the detection of rabies neutralizing antibodies, the mouse neutralization test (MNT) remains the most widely used. The lack of sensitivity and poor reproducibility of results, the need for special equipment and specially trained personnel, as well as poor

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correlation with the MNT, have been some of the major problems with most of the newer techniques.

Although the mouse neutralization test has been in use for a long time, it still has some inherent difficulties. It is time-consuming, requires special facilities for keeping large numbers of animals and for the maintenance of a live challenge virus standard, involves the use of an unnatural challenge route, is difficult to standardize, and is relatively expensive (Van Der Marel and Van Wezel, 1981). In view of these inherent problems, the MNT is being supplanted by the rapid fluorescent focus inhibition test (RFFIT). The RFFIT is rapid, simple to perform, easy to interpret, and correlates well with the MNT (Smith _et al_., 1973; Louie _et al_., 1975). However, the need for a fluorescent microscope and special facilities for the maintenance of cell cultures and live infective virus renders RFFIT inapplicable in most developing countries. Moreover, the challenge virus standard (CVS) used in the technique is not readily available in most parts of the world. The need for an inexpensive, simple, rapid and reproducible test for the detection and quantitation of rabies antibodies still remains.

Enzyme immunoassays (EIA) developed by Engvall and Perlmann (1971) and Van Weemen and Schuurs (1971) are sensitive and simple techniques which have been shown to be useful for the detection of rabies virus antibodies (Atanasiu _et al_., 1980; Nicholson and Prestage, 1982). Although

preliminary studies have shown the test to correlate well with the MNT (Nicholson et al., 1985), it is still being evaluated. The expression of EIA titre in a comprehensible manner has remained elusive.

In the present study, attempts were made to establish a reproducible, sensitive, rapid, specific and inexpensive EIA technique for the detection and quantitation of rabies virus antibodies. The exercise consisted of titrating rabies virus antibodies in sera of vaccinated people and animals and included some rabies virus specific antiglycoprotein and antiribonucleoprotein sera. In addition, rabies antibody screening tests were performed at a single serum dilution in sera of both man and animals of unknown vaccination histories in an attempt to identify vaccinated and non-vaccinated persons or animals. In this test, the inhibition enzyme immunoassay (INH-EIA), antibody levels were expressed in terms of 50% inhibition titres, which were in turn converted to equivalents of I.U./ml based on a standard antirabies serum.

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2. REVIEW OF LITERATURE

2.1 AETIOLOGY OF RABIES

The disease rabies is caused by the rabies virus which is the type species of the genus *Lyssavirus* in the family Rhabdoviridae. More than sixty of these rhabdoviruses have been identified in plants, insects, fish and animals (Brown and Crick, 1977).

Until fairly recently, rabies virus was regarded as a serological entity bearing no relationship to other members of the rhabdovirus group. However, this concept of antigenic unity has been challenged by some workers who have shown that serological and morphological relatedness does exist between rabies virus and six other rhabdoviruses found on the African continent (Shope *et al*., 1970).

The first of these rabies-related viruses, the Lagos bat virus, was isolated from the brain of a frugivorous bat (*Eidolon helvum*) on Lagos island, Nigeria, in 1956 (Boulger and Potterfield, 1958). The second virus, the Nigerian horse virus, was isolated in 1958 in Ibadan, Nigeria, from the brain of a horse which had died from "staggers", a disease resembling rabies (Potterfield *et al*., 1958). The third and fourth members of the group, Obodhiang and Kotonkan viruses, are unusual in that they are insect-borne. Obodhiang virus was isolated in 1963 from pools of *Mansonia uniformis* mosquitoes in the Sudan (Schmidt *et al*., 1965). Kotonkan

virus was obtained in 1967 from Culicoides midges in Nigeria (Kemp et al., 1973). Three strains of the fifth virus, Mokola, were isolated in 1968 from viscera of shrews (Crocidura species) captured in Ibadan, Nigeria. In the same area, two isolates of Mokola virus were subsequently isolated from human beings (Kemp et al., 1972; Familusi et al., 1972). The sixth virus, Duvenhage, was found in South Africa in 1970. It was isolated from the brain of a man who had died, apparently from rabies, after being bitten by a bat (Meredith et al., 1971; Tignor et al., 1977).

The interrelatedness of these viruses has been shown by the following tests: complement fixation, neutralization, cross-protection, and fluorescent antibody tests (Tignor and Shope, 1972; Schneider et al., 1973; Tignor et al., 1973; Tignor et al., 1977). On the basis of these tests, Nigerian horse virus was shown to be more similar to rabies virus than were Lagos bat or Mokola viruses. Obodhiang and Kotonkan viruses were only distantly related to rabies virus, but more closely related to Mokola virus.

The serological tests showed that certain subdivisions within the rabies group could be made. Lagos bat, Nigerian horse, and Mokola viruses are regarded as new serotypes of rabies virus (Tignor and Shope, 1972). Tignor and Shope (1972) showed that mice vaccinated with a rabies virus of reduced virulence were only poorly protected against Mokola virus. From their experiment, it would appear that the

classical rabies vaccines may not be effective against these new serotypes of rabies virus. This is a disquieting observation since two strains of Mokola virus were isolated from children, one of whom died (Familusi _et al_., 1972).

To date, there is limited knowledge of the significance of these rabies-related viruses and whether or not they pose a threat to man or animals in certain parts of the world (Brown and Crick, 1977). Neither is it known whether these or other rabies-like viruses exist in the wildlife of other countries. Antibodies to Kotonkan virus have been found in man, cattle, rodent, and insectivore sera in northern Nigeria, and in cattle, sheep, and horse sera in the southern part of that country (Kemp _et al_., 1973).

Other reports from Zimbabwe indicate the occurrence of another rabies-like virus isolated from cats and a dog which had died from a disease with clinical signs suggestive of dumb rabies (Foggin, 1982). The isolates from the brains of the dead animals showed little or no neutralization by standard rabies antiserum (Foggin, 1982). The dog had been vaccinated six months previously with a potent inactivated rabies vaccine of tissue culture origin (Rabisin(R), Institut Merieux). It would therefore, appear that these isolates, while possessing some of the properties of rabies virus, were antigenically dissimilar to challenge virus standard (CVS) rabies virus. A disturbing fact arising from that discovery is that the rabies vaccines presently used in

Zimbabwe may not protect dogs from these rabies-like virus isolates. It is also likely that humans who may be exposed to the atypical rabies virus could develop disease in spite of the usual rabies vaccination. These Zimbabwe isolates were later identified as Mokola virus by the neutralization index tests in mice, by comparison with the other rabies serogroup viruses (Foggin, 1983).

From the Zimbabwe experience, it would be useful to use a polyvalent vaccine or an appropriately combined vaccine regimen in certain areas such as West Africa in which more than one virus of the rabies group has been isolated. The more recent isolation of Duvenhage in the Transvaal suggests that these considerations may apply equally to other parts of the African continent. To date, twenty-six isolations of the rabies-related viruses have been made from a variety of animals and from humans in two widely separated regions of Africa, and recently one isolation of Duvenhage virus was made in Europe (Foggin and Swanepoel, 1985).

2.1.1. The morphology of rabies virus

The virions of the rhabdoviruses are elongated or bacilliform with one end rounded and the other flattened. They are 130 to 300 nanometers (nm) in length by 70 nm in diameter with three-layered lipoprotein envelopes containing virus-specific peplomers and vesicular appendages at the flat ends of the particles (Brown and Crick, 1977). The virions

have been described as bullet-shaped. The envelope and membrane proteins enclose the long tubular nucleocapsids. The internal helices have 30 to 35 coils in the form of cylinders 50 nm in diameter by 150 nm long. The genomes are single molecules of single-stranded ribonucleic acid (RNA) with a molecular weight of 3.5 to 4.6 x 10^6 daltons (Sokol et al., 1969).

Purified rabies virions contain about 2 to 3% RNA and some carbohydrate, about 25% lipid, and more than 50% protein. Electrophoretic patterns show four major proteins: G, N, M_1, M_2 and one minor protein, L. The molecular weights of these structural proteins have been estimated, but vary depending on the virus strain examined. Protein L was estimated at 185 Kd, G at 67 Kd, N at 55.5 Kd, M_1 at 37.5 Kd, and M_2 at 25.8 Kd (Dietzschold et al., 1983). Lipid is found only in the outer virion membrane and is somewhat different in composition from the plasma membranes of the host cells. The virions have surface projections which consist of the G protein, a glycoprotein. The G protein is important in vaccine-induced immunization and in serological identification of rabies virus, as the specific epitopes against which neutralizing antibodies are formed are located on the glycoprotein (Crick and Brown, 1969; 1970; Wiktor et al., 1973a; Cox et al., 1977).

The rabies virus haemagglutinates goose erythrocytes, a factor which is important in some diagnostic procedures

(Halonen *et al.*, 1968; Gough and Dierks, 1971). The haemagglutinin is associated with the glycoprotein, but is inactive in glycoprotein which is dissociated from the viral envelope (Fenner, 1975-1976).

The N protein of the ribonucleoprotein appears to be shared by the *Lyssavirus* genus. It is believed to be responsible for the complement fixing and fluorescent antibody test reactions shared by these viruses (Wiktor *et al.*, 1973). The M_1 protein is considered to be adjacent to the ribonucleoprotein, and the smaller M_2 protein is believed to be associated with the viral lipids in the lipoprotein envelope. These latter two proteins are thought to be concerned with the organization of the ribonucleoprotein strand into its helical configuration and subsequently into the membrane of the bullet-shaped particles (Brown and Crick, 1977). The minor L protein is of uncertain location and function. However, Brown and Crick (1977) are of the opinion that if it is analogous to the L protein of vesicular stomatitis virus (VS), a rhabdovirus, then it may be associated with an enzyme which is involved in the replication of the ribonucleic acid. This has been demonstrated in the VS virus, but not in rabies virus.

2.2 OCCURRENCE AND DISTRIBUTION OF RABIES

2.2.1 The world picture

Rabies has been well documented through the period of recorded history. Since 500 B.C., several descriptions of

the disease could be found in the writings of Democritus, Aristotle, Hippocrates and others (Wiktor, 1985).

The disease is found all over the world and in all climates, in the tropics and in the arctic, though Hawaii, Britain, Panama and Australia appear at present to be free of rabies (Christie, 1981). Recent observations indicate that the disease is extending its range in its major reservoir hosts, moving eastward in Indonesia in dogs, northward and westward at a rate of about fifty miles annually in the United States in raccoons, westward in the United States in skunks, and southward in South America in Vampire bats (Beran, 1981). In central Europe, the disease has been extending through foxes and is moving across countries formerly free in western Europe (Acha and Arambulo, 1985). Recently, the disease has been threatening countries which are still free. These countries include the United Kingdom, Australia, New Zealand, Japan, Taiwan, and the islands of the Pacific ocean, where an outbreak in Guam in 1967 was controlled and the disease eradicated in 1968 (Beran, 1981).

Rabies is widespread over the continent of Africa and some authors are of the view that rabies virus evolved in the old world, Africa or Asia (Shope, 1982). They argue that the existence in Africa of at least five viruses serologically related to rabies virus supports the hypothesis of its African origin with the parallel evolution of several related viruses.

2.2.2. Rabies in Kenya

Rabies is widespread all over Kenya (Figure 1) and there is evidence that it existed even before the advent of the Europeans. The first laboratory confirmed case was in 1912 in a dog on the outskirts of Nairobi (Hudson, 1944).

Dogs, both domestic and wild, are the most important transmitters of rabies to man, constituting about 89% of all animal bites (Siongok and Karama, 1985). The cat is another significant animal contributing to about 10.3% of the bites, leaving only 0.7% for other animals. Some of the domestic animals reported to cause bites are cattle, horses, goats and sheep.

Wild animals are also reported to be involved in the spread of the disease. These animals include jackals, honey badgers, civet cats, hyenas, foxes, mongooses and rodents, the first two being the most important (Siongok and Karama, 1985).

The disease has in recent years spread to districts previously regarded rabies-free. In just one year, 1982, the disease was reported from 30 out of 41 districts in the country (Kariuki and Ngulo, 1985), with Kitui and Machakos districts reporting the highest number of cases.

There has been an increase in the number of rabies positive cases in animals presented for diagnosis at the Veterinary Research Laboratories (Kabete, Kenya). Over a period of six years (1976-1981), a total of 275 animal cases

Endemic

Sporadic case

Figure 1 : Rabies spread in Kenya by districts 1974-1983. Numbers refer to the year and month when cases were first reported. Unshaded areas with dates when first cases were reported represent those districts where more cases were reported after the first one (Kariuki and Ngulo, 1985).

were diagnosed (Siongok and Karama, 1985). There was a parallel increase in the number of human vaccinations and deaths from rabies in the same period (Table 1). It is possible that there might have been cases of admissions and deaths during 1977 and 1978, but records from all over the country were not readily available at the time of collecting the data (Siongok and Karama, 1985).

Table 1 : HUMAN VACCINATIONS, CASES AND DEATHS DUE TO RABIES IN KENYA (SIONGOK AND KARAMA, 1985)

Year	Vaccinated	Cases admitted	Died
1977	Not recorded	Not recorded	Not recorded
1978	" "	" "	" "
1979	" "	8	6
1980	1,736	6	69
1981	1,127	12	17
Total	2,863	26	92

2.3 RABIES VIRUS TRANSMISSION, MODE OF INFECTION AND PATHOGENESIS

Rabies virus is almost always transmitted via a bite of a rabid animal (Figure 2). Other routes of infection are possible but are of less significance. Aerosol transmission of rabies virus has been reported (Winkler et al., 1973; Kaplan, 1977). Occasional rabies infection follows a lick on an open wound or mucus membranes, but intact skin appears to be an effective barrier (Hattwick and Gregg, 1975; Warrel, 1976). Oral transmission by means of ingestion of rabies virus has been recorded, and this might explain natural infection in carnivores that frequently eat sick or dead rabid animals (Shah and Jaswal, 1976). The possibility of transplacental or milk-borne infection has been reported (Martell et al., 1973). Human-to-human transmission of rabies virus through a corneal transplant has also been recorded (Houff et al., 1979).

When the virus is injected into the peripheral tissues of a bite victim, it must reach the central nervous system (CNS) before it can cause symptoms of rabies. Baer, et al.,(1965)have shown convincingly that the virus does travel along peripheral nerves from the site of injection to the CNS and that passage along the nerves and the development of symptoms of rabies can be prevented by prior neurectomy.

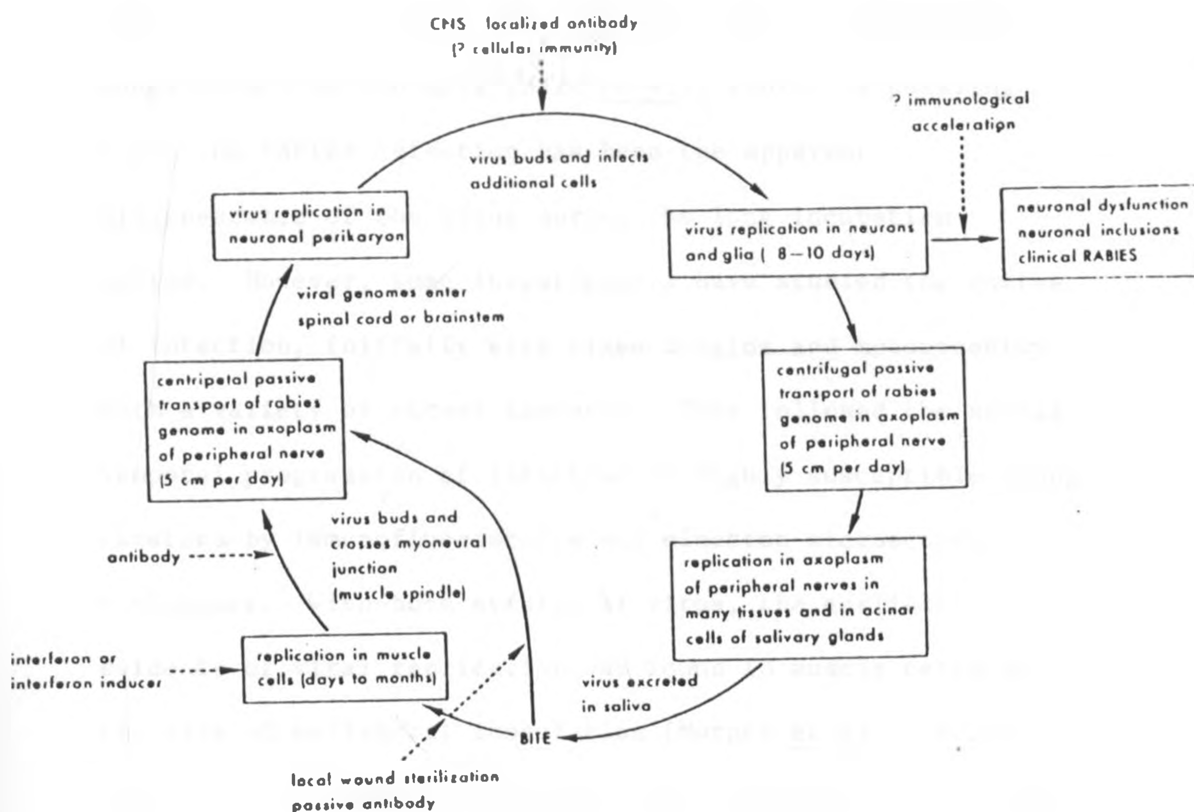

Figure 2 : Pathogenesis of rabies. Dotted arrows indicate points of potential modulation (Nathanson and Miller, 1982).

Following exposure, there is usually an incubation period of 30 to 60 days, but the symptom-free period may range from 6 to 270 days (Held et al., 1967). A puzzling aspect of rabies infection has been the apparent disappearance of the virus during the long incubation period. However, some investigators have studied the course of infection, initially with fixed strains and subsequently with a variety of street isolates. They followed the serial temporal progression of infection in highly susceptible young hamsters by immunofluorescence and electron microscopic techniques. With both strains of virus, the earliest evidence of viral replication was found in muscle cells at the site of peripheral inoculation (Murphy et al., 1973a). Electron micrographs demonstrated the budding of virus from myocyte plasma membranes into the extracellular space. This early replication in muscle may provide the requisite amplification for the virus to then successfully invade the peripheral nervous system (Murphy and Bauer 1974; Charlton and Casey 1979; Fekadu and Shaddock, 1984).

Sequential experiments indicate an orderly progression of infection from muscle to neuromuscular and neurotendonal spindles to peripheral nerves to CNS (Murphy et al. 1973a). Once the virus reaches the CNS, rapid spread occurs. It enters and replicates in nerve cells in all parts of the brain, including those areas which may be most concerned with behaviour or emotions. Infection of these areas of the brain

may explain the agitation of a rabid animal or man undergoing the furious phase of the disease (Murphy et al., 1973a).

Having replicated in the nerve cells, the virus next spreads outwards, again along peripheral nerves, and reaches many tissues of the body: kidneys, pancreas, adrenals, nasal mucosa, lachrymal glands and salivary glands among others (Murphy et al., 1973b). The arrival of the virus at so many sites can be demonstrated in diagnostic animal inoculation by immunofluorescence tests on saliva (Goldwasser et al., 1959; Vaughn et al., 1965; Fekadu and Shaddock, 1982), throat or tracheal secretions, tears, cerebrospinal fluid, corneal smears (Bhatt et al., 1974; Corey and Hattwick, 1975; Koch et al., 1975), and in skin biopsy specimens showing peripheral nerve fibrils (Bryceson et al., 1975). The arrival of the virus in the salivary glands allows its transmission to another victim via a bite, which is the main method of spread.

2.4 PREVENTION OF RABIES

Prevention of both human and animal rabies can be achieved through vaccinations with safe and potent rabies vaccines. Since the nervous tissue rabies vaccine of Pasteur, many other vaccine preparations have been used. These include the Fermi vaccine, the Semple vaccine, the duck embryo vaccine (Peck et al., 1956), and the suckling mouse brain vaccine (Fuenzalida, 1976).

Apart from the serious side effects produced by these early vaccines, the greatest concern has been their actual effectiveness in preventing rabies infection. None produces very high titres of serum neutralizing antibody, and most investigators agree that they are of highly questionable protective value after severe rabies exposure (Beran, 1981).

Probably the most important recent development in rabies prophylaxis since Pasteur's original vaccine has been the introduction of a concentrated vaccine produced from rabies virus grown in human diploid cells (Wiktor et al., 1969). The human diploid cell rabies vaccine (HDCV) has been thoroughly tested both in the laboratory and in the field. Numerous studies have demonstrated a dramatic increase in the levels of serum neutralizing antibody elicited by this vaccine in comparison with previously available vaccines (Wiktor et al., 1973b; Bahmanyar, 1974). However, the high cost of the HDCV and the inadequacy of current health delivery systems remain serious barriers to the control of human rabies in the developing countries. Another cell culture vaccine, the purified vero cell rabies vaccine (PVRV), is still in its trial stages. It is a more purified vaccine than the HDCV and when it finally becomes available in the market, may replace the more expensive HDCV (Roumiantzeff et al., 1985). Potent and safe tissue culture origin vaccines for animal immunization are available: Rabisin(R) (Institut Merieux, France) and Rabdomun(R) (Wellcome Ltd., Germany).

2.5 DETECTION OF ANTIBODIES TO RABIES VIRUS

Many techniques have been developed over the years for the assay of rabies antibodies. Their usefulness depends on their ability to detect virus neutralizing antibodies. The methods include : mouse neutralization test (Webster and Dawson, 1935), indirect fluorescent-antibody test (Thomas, 1975), passive haemagglutination test (Gough and Dierks, 1971), rapid fluorescent focus inhibition test (Smith et al., 1973), enzyme linked immunosorbent assay (Atanasiu et al., 1980), counterimmunoelectrophoresis (Diaz and Varela-Diaz, 1977), modified counterimmunoelectrophoresis (Diaz and Myers, 1980), radioimmunoassay (Wiktor et al., 1972) and complement fixation test (Kuwert et al., 1976). Although these techniques have been used, the mouse neutralization test (MNT) remains the most widely applied. The lack of sensitivity and poor reproducibility of results, the need for special equipment and specially trained personnel and poor correlation with the MNT, have been some of the major problems with the newer techniques.

To date, the mouse neutralization test (MNT) is the method specified by the WHO Expert Committee on Rabies (1984) as the official potency test for antirabies serum. Reproducibility of results with the MNT, however, is still questionable despite all efforts to standardize various parameters. This is because in vivo methods of antibody assay which utilize death of the host as an end point are inherently

imprecise because of the many uncontrollable factors, such as non-specific deaths, or susceptibility or resistance to lethal infections (Louie et al., 1975). Furthermore, the MNT involves the intracranial inoculation of mice with challenge virus which is an unnatural challenge route. The method also takes up to 14 days to complete, requires special facilities for keeping large numbers of animals, and is relatively expensive.

The need to replace the MNT is clear. The recently described rapid fluorescent focus inhibition test (RFFIT) of Smith et al.,(1973) appears promising because of its economical use of reagents and test sample, its completion in 24 hours, and its suitability for testing large numbers of samples. However, the need for a fluorescent microscope and special facilities for maintenance of cell cultures and live infective virus renders RFFIT inapplicable in most developing countries. In addition, the challenge virus standard used in the technique is not readily available in most parts of the world. The need for an inexpensive, rapid and reproducible test still remains. The recently described enzyme-linked immunosorbent assay (ELISA) has been shown to be a rapid, inexpensive and highly reproducible test for detection of rabies antibodies, and correlates well with both the MNT and RFFIT (Nicholson and Prestage, 1982; Nicholson et al., 1985). However, the accurate expression of ELISA titres in a comprehensible manner has remained elusive, and at the

present time, there is no consensus of opinion on the best way to express them (De Savigny and Voller, 1980; Malvano et al., 1982).

The ELISA test has become firmly established as an alternative to radioimmunoassay (RIA) for the detection and quantitation of antigens and antibodies both in research and diagnosis. The tests are similar to RIA, but instead of a radioisotope label an enzyme is used, usually alkaline phosphatase or horseradish peroxidase. Most commonly the enzyme-substrate reaction produces a colour change which can either be read visually or quantitated with a spectrophotometer. The antigen-antibody reaction takes place with one or the other firmly attached to a solid support such as a 96-well flat bottomed plate. This allows for the minimum of interference from non-immunological reactants which are mostly removed by washing procedures carried out at each stage of the test. The sensitivity of ELISA, which is comparable to that of RIA, is brought about through the amplifying activity of the enzyme conjugate which can process up to 100,000 molecules of substrate per minute (Yolken, 1982).

It has long been recognized that the protection afforded by post-exposure treatment is neither correlated with the time of appearance nor titre of actively induced rabies neutralizing antibody (Sikes et al., 1971). This may in part be because neutralization tests on "early" sera

measure mostly IgM which, unlike IgG, remain in the intact circulation and are non-protective (Turner, 1978). It is conceivable, therefore, that a test such as ELISA, which measures class-specific antibody, gives a better index of post-exposure protection than virus neutralization, especially if it measures other antibodies, for example lytic antibodies and those associated with antibody-dependent cell-mediated cytotoxicity which are of probable importance shortly after exposure (Nicholson et al., 1985).

The ELISA technique does have recognized disadvantages, for example the problem of non-specific binding. Care must be taken to ensure that test sera are not heat-inactivated as this significantly increases the amount of non-specific binding (Nicholson et al., 1985). Other drawbacks include the reduction in sensitivity for IgG caused by rheumatoid factor and high titred antigen-specific IgM which competes with IgG for antigenic binding sites. But by far, the greatest problem for developing countries is the supply of relatively pure antigen, which depends, for its availability, on technical expertise and well-equipped laboratory facilities not usually found in the third world.

3. MATERIALS AND METHODS

3.1 RABIES VIRUS ANTIGEN PREPARATIONS

Two rabies virus antigen preparations were used in this study. One was used for immunization of an experimental animal and the other for coating of microtitre plates. The rabies virus (Wistar Rabies PM W1381503-3M strain grown on human diploid cells) preparation used for immunizing an experimental animal was the Merieux Inactivated Rabies Vaccine. The lyophilized human diploid cell rabies vaccine (HDCV) batch No. W0578 was obtained through the courtesy of May & Baker, Nairobi, from the manufacturers, Institut Merieux in Lyon, France.

The other rabies virus antigen preparation used for coating of microtitre plates in inhibition enzyme immunoassay (INH-EIA), was an inactivated and concentrated rabies virus (Flury LEP strain grown on baby hamster kidney cells) liquid preparation with an antigenicity value of 25 I.U./ml. This preparation was obtained through the courtesy of Dr. C. Nderitu of Wellcome, Kenya Limited, from Wellcome laboratories in Germany.

3.2 PRODUCTION OF ANTISERUM TO RABIES VIRUS

A goat #380 was used for immunization with the human diploid cell rabies vaccine for a period of eighteen months.

The lyophilized vaccine was reconstituted in one millilitre of sterile distilled water and emulsified in Freund's complete adjuvant (DIFCO Laboratories, Detroit, USA) in a ratio of 1:3. The emulsified vaccine was then injected into lymph nodes according to the method described by Newbould (1965), and intramuscularly. Subsequent booster doses were emulsified in Freund's incomplete adjuvant and injected intramuscularly at two weeks intervals.

Test bleedings were done before each immunization until strong precipitin lines were observed when the antirabies serum was tested against the HDCV in immunodiffusion tests.

3.3 ABSORPTION AND SALT FRACTIONATION OF THE ANTIRABIES SERUM

The antirabies serum showed reactions with dilutions of human serum in Ouchterlony's double diffusion method, and was therefore absorbed in an attempt to remove that antibody activity. Initially, the antiserum was absorbed by the addition of normal human serum (NHS) insoluble immunosorbents to it several times. When this procedure could not remove the reaction, the absorption was continued by the addition of NHS to the antiserum. This procedure also failed to remove the reaction and as a result NHS was precipitated with 50% saturated ammonium sulphate according to the method described by Heide and Schwick (1978). The supernate obtained from the

precipitation showed reaction with the antirabies serum in Ouchterlony's double diffusion method. The supernate was therefore lyophilized and added in portions to the antirabies serum and the mixture left stirring overnight on a magnetic stirrer at $4^{o}C$. This procedure was repeated several times until the reaction disappeared.

Two hundred millilitres of the absorbed antirabies serum was diluted in saline to a final volume of 800 mls. The antiserum was then precipitated by the addition of 18% (w/v) anhydrous sodium sulphate. The mixture was centrifuged at 2000 xg for 15 minutes at room temperature and the resultant sediment (gammaglobulin fraction) suspended in a small volume of saline and dialysed against several changes of saline for 24 hours.

3.4 PREPARATION OF IgG FRACTION FROM IMMUNE SERUM

The IgG fraction was isolated from the antirabies serum according to the method described by Fey et al. (1976), using DEAE-cellulose chromatography (Cellex D, BIO-RAD Laboratories, Richmond, California, USA). Different molarities (0.01M, 0.02M and 0.04M) of phosphate buffer, pH 8.0, were used in a stepwise manner.

The rabies gammaglobulin fraction obtained as described in section 3.3 was dialysed against 0.01M phosphate buffer, pH 8.0, for 24 hours, and then concentrated to 66 mls using

"DIAFLO PM 30" ultrafilter (Amicon, Massachusetts). The fraction was then applied to the top of a DEAE-cellulose column and allowed to run through by gravity. Six millilitre fractions were collected with a fraction collector (LKB Bromma, Sweden). The 0.02M phosphate buffer followed by the 0.04M phosphate buffer were added to the column and fractions collected.

All the peak fractions were pooled separately and concentrated by ultrafiltration using "DIAFLO PM 30" ultrafilter with a cut-off point of 30,000 daltons. The protein content of each fraction was determined by reading the optical density (OD) at 280 nm in a Beckmann spectrophotometer model 25. The peak eluted by 0.01M phosphate buffer, pH 8.0, showed reaction with the human diploid cell rabies vaccine in Ouchterlony's double diffusion test and its IgG content was 44.75 mg/ml. The IgG content was calculated according to the formula $OD_{280}^{1\%} = 13.5$ for goat IgG given by Givol and Hurwitz (1969).

3.5 COUPLING HORSERADISH PEROXIDASE TO THE IgG FRACTION OF GOAT #380 ANTIRABIES SERUM

Coupling of horseradish peroxidase (HRPO) to the purified IgG fraction of goat #380 antirabies serum was done using the heterobifunctional reagent N-succinimidyl-3-(2-pyridyldithio) propionate (SPDP) (Pharmacia Fine Chemicals, Uppsala, Sweden) following the method described by Ishikawa *et al.* (1983).

3.6 SERUM SAMPLES USED IN INHIBITION ENZYME IMMUNOASSAY

3.6.1 Specific antiglycoprotein and antiribonucleoprotein sera

Two rabbit antisera against the rabies virus glycoprotein and ribonucleoprotein were obtained from the Wistar Institute, USA. A sheep antiglycoprotein serum was obtained from the National Institute for Biological Standards and Control, London. In addition to titrating the antisera in inhibition enzyme immunoassay, immunodiffusion tests were performed to check for the relationship between them, the goat #380 antirabies IgG fraction, and the concentrated rabies virus antigen preparation (Wellcome, Germany). In the immunodiffusion tests, the rabies virus antigen preparation was always placed in the central well with the antisera and the IgG fraction in the peripheral wells.

3.6.2. Standard antirabies sera

Human rabies immune globulin (Immogam rabies(R)) was obtained from the Institut Merieux in Lyon, France. It was a solution of human immune globulin obtained from donors immunized with the Merieux inactivated rabies vaccine (Wistar strain cultivated in human diploid cells). The antiserum had a potency of 150 I.U./ml.

An antirabies reference serum was obtained from Wellcome Laboratories in West Germany. It was a World Health Organization (WHO) international antirabies standard with a potency of 10 I.U./ml.

3.6.3. Human serum samples

Fourteen individuals were put on a course of pre-exposure immunization with the human diploid cell rabies vaccine (HDCV). They were injected with 0.1 ml HDCV intradermally on days 0, 7 and 28 and bled on days 0, 7, 28 and 64. Antibody titres at each bleeding were calculated based on the Institut Merieux antirabies serum.

One already immunized individual was given a booster injection with 0.1 ml HDCV intradermally and bled after every one or two days for a period of 25 days. Antibody titres were calculated at each bleeding on the basis of the Institut Merieux reference serum.

A total of 1,976 human sera from the Kenya Blood Bank and 418 from Norway were screened for rabies antibodies.

3.6.4. Dog sera

Dog serum samples used in screening for rabies antibodies were obtained from the Kenya Medical Research Institute (KEMRI) through the courtesy of Dr. Koimett. The 285 sera were from dogs bled in Machakos and Narok districts of Kenya. Eleven dog sera, also used in screening for rabies antibodies, were obtained from the University of Oslo, Faculty of Veterinary Medicine, Norway.

3.6.5. Wild animal sera

A total of 232 wild animal sera used in screening for rabies antibodies were obtained from the Wildlife Section of the Kenya Veterinary Research Laboratories, Kabete. The samples were from 14 different animal species and included 18 hartebeests, 25 Thomson gazelles, 20 wildebeests, 9 oryx, 16 buffaloes, 15 elands, 24 elephants, 18 waterbucks, 14 Grant gazelles, 29 zebras, 16 hyenas, 5 lions and 4 golden backed jackals. Nineteen camel sera were also included in this group.

3.6.6. Cattle and goat sera

A total of 192 cattle sera used in screening for rabies antibodies were obtained from the Kenya Meat Commission abbatoir at Athi River. Four hundred and twenty-nine goat sera used for rabies antibody screening were obtained from the Turkana district of Kenya.

All the sera screened for rabies antibodies had their antibody titres calculated if they gave percent inhibitions within the linear portion of the Institut Merieux reference serum curve.

3.7 IMMUNODIFFUSION TESTS

The microtechnique of Ouchterlony's (1948) double diffusion in agar described by Crowle (1973) was used with slight modifications. One percent (w/v) of purified Oxoid agar (Oxoid Ltd., England, Cat. No. 085-7297) in phosphate

buffered saline (PBS) with 0.1% sodium azide was used in the preparation of the gel. A dissociating agent, 0.1% Triton X-100, was incorporated into the gel in the tests involving the rabies virus specific antiglycoprotein and antiribonucleoprotein sera. A volume of 3.5 ml of molten agar was poured on a microscope slide (25 x 75 mm) to give a depth of approximately 2 mm. With a gel puncher (Gelman Instruments Co., Ann Arbor, Michigan, USA), wells of 4 mm diameter and 5 mm apart were cut in hexagonal patterns with central wells. The agar in the wells was removed by suction after punching. The central well was filled with 15 ul of antiserum or antigen while peripheral wells contained 15 ul of antigen or antiserum.

The slides were placed in a humid box and diffusion allowed to proceed for approximately 48 hours at room temperature. The precipitin bands were recorded and the slides washed with saline, rinsed with distilled water, then pressed as described by Weeke (1973). The pressed slides were washed overnight in 3% trisodium citrate solution, pH 8.5, to remove components not participating in the formation of specific precipitin reactions. The slides were rinsed with distilled water to remove salts, pressed, dried and stained with Coomassie Brilliant Blue (CBB) staining solution (Appendix 2) for 30 minutes, and destained using destaining solution for CBB stain (Appendix 3).

3.8 ENZYME IMMUNOASSAY

An inhibition enzyme immunoassay (INH-EIA) was used for the quantitation of rabies antibodies. This was carried out using polystyrene microtitre plates (Nunc. A/S, Denmark). The antigen preparation used for coating the plates was the inactivated and concentrated rabies virus described in section 3.1. Serum samples used in the assay are those described in section 3.6. The enzyme-immunoglobulin conjugate used in the assay is described in section 3.5.

The substrate for the enzyme consisted of 10 mg o-phenylenediamine (OPD) (Sigma Prod. No. P3888) per 11 ml of 0.05 M citrate/acetate buffer, pH 5.0, containing 0.1 ml of 1% hydrogen peroxide.

The optical density (OD) of each well was read with a "Minireader" MR 590 (Dynatech Microtitre Systems) equipped with a 410 nm interference filter, or after acidification, with a 490 nm interference filter.

3.8.1. The enzyme immunoassay procedures

Microtitre plates were coated with 1:200 dilution of the concentrated rabies virus preparation. The coating buffer was 1:100 PBS, containing 2% polyethylene glycol (PEG) (MW 6000) and 0.1% sodium azide. The test serum was added as the next layer whereby rabies antibodies, if present,

would combine with and block the antigenic sites on the virus. This interaction was then detected by the addition of the goat #380 antirabies virus antibody-enzyme conjugate. The titre was expressed as the reciprocal of that serum dilution which gave 50% inhibition units (INH 50% units).

The optimal conditions used for the assay were determined by repeated preliminary variations, such as changes in buffer and diluent compositions, time and incubation temperature. Chequer board titrations were carried out to determine the optimal dilutions of the immunoreactants used in the assay. The optimal dilutions of the antigen and conjugate were 1:200 and 1:2000 respectively.

Several controls were included in every plate. The Institut Merieux antirabies serum was always titrated in every plate and all serum antibody titres were calculated on its basis. Conjugate controls were included to check for the non-specific binding of the conjugate to the wells. Zero inhibition controls were included in every plate, and the OD's obtained from these controls were utilized in calculating percent inhibitions of serum dilutions.

The substrate control was not only used for monitoring spontaneous changes of the substrate but also for zeroing the "Minireader".

The INH-EIA was based on the principles described by Duermeyer (1980) with some modifications, and was carried out as follows :

1. The Nunc microtitre plate wells were coated with 100 ul of 1:200 dilution of the concentrated and inactivated rabies virus preparation. The plates were then incubated overnight at room temperature in a humid box. Plates not used immediately were stored frozen at $-20^{o}C$. When ready for use, they were thawn at $37^{o}C$.
2. The plates were washed three times at two minutes intervals by flooding them with EIA wash solution and emptied by tapping the plates several times onto layers of tissue paper.
3. One hundred ul of several dilutions of the Institut Merieux reference serum and test sera were run in duplicates. The serum samples were diluted in the serum diluent. For the screening tests, a single serum dilution of 1:2 was employed. The plates were shaken and incubated at room temperature overnight in a humid box.
4. The plates were washed as in step 2.
5. One hundred ul of goat #380 antirabies IgG-HRPO conjugate diluted 1:2000 in a conjugate diluent and containing 5% normal goat serum was added to all wells except for the substrate control wells. The plates were shaken and incubated for one hour at $37^{o}C$ in a humid box.
6. The plates were washed as in step 2 but this time 4 times instead of 3.

7. One hundred ul of OPD substrate were added to all wells and incubation done in the dark at room temperature. Colour development was monitored by reading the OD's at 10-20 minutes intervals with a 410 nm interference filter until the $OD_{410\ nm}$ of the zero inhibition was between 0.90 and 1.2. The enzyme activity was then stopped by adding 50 ul of 2N sulphuric acid to all wells and the plates shaken.
8. The absorbance at 490 nm was read.

3.8.2. Determination of 50% inhibition titres of serum samples

In the preliminary studies of the INH-EIA, some rabies antibody negative and positive serum samples were tested. The positive serum samples were from known vaccinated individuals, while the negative ones were from known non-immunized persons. These sera were titrated and their complete titration curves obtained. Their 50% inhibition titres were read from the curves as shown in Figure 3 (p. 41). In all the plates, the Institut Merieux antirabies serum was always titrated. All the serum samples from non-immunized individuals gave 50% inhibition titres of less than 10 which is the lowest titre that can be determined without extrapolation of the titration curves. All the serum samples used for screening were diluted in the serum diluent containing 4% PEG. The Institut Merieux antirabies reference serum and all positive sera were diluted in the serum diluent containing 2% PEG.

7. One hundred ul of OPD substrate were added to all wells and incubation done in the dark at room temperature. Colour development was monitored by reading the OD's at 10-20 minutes intervals with a 410 nm interference filter until the $OD_{410\ nm}$ of the zero inhibition was between 0.90 and 1.2. The enzyme activity was then stopped by adding 50 ul of 2N sulphuric acid to all wells and the plates shaken.
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A single serum dilution, 1:2, which discriminated well between sera from immunized and non-immunized individuals was chosen. These single 1:2 dilutions of the serum samples were run in duplicates and their 50% inhibition titres calculated on the basis of the Institut Merieux antirabies reference serum if they gave percent inhibitions within the linear portion of the reference serum curve.

The percent inhibition given by the 1:2 dilution of 28 known negative sera lay inside the linear portion of the reference curve, i.e. between 20 and 75%. Titres of those sera which gave percent inhibitions less than 20 could not be calculated since they lay outside the linear portion of the reference curve.

The percent inhibitions of most positive sera tested at 1:2 dilution lay well above the linear portion of the positive reference curve. The testing of the 49 known human positive sera at 1:2 dilution was done to check how they would compare with the known negative sera run at the same dilution.

The 50% inhibition titres of serum samples were calculated as follows :

a. Calculation of percent inhibition of serum dilution (average of duplicates) was done using the formula

$$\% \text{ inhibition} = 1.0 - \frac{OD_s}{OD_z} \times 100$$

where OD_s = absorbance (OD 490 nm) given by the serum dilution,

OD_z = absorbance (OD 490 nm) of zero inhibition.

b. Determination from the positive reference curve of that dilution of the positive reference serum which gave the same % inhibition as that of the serum sample dilution [Figure 4 (p. 42)]. If this dilution is Q and the titre of the positive reference serum is P (the dilution which gave 50% inhibition), the titre of the unknown serum sample was calculated as follows :

$$\text{Titre of serum} = \frac{P}{Q} \times \text{ dilution of the serum sample.}$$

Since the potency of the Institut Merieux antirabies serum was known, i.e. 150 I.U./ml, it was possible to calculate the number of 50% inhibition units equivalent to 1 I.U./ml. If the reference serum gave a 50% inhibition titre of 3,000, then 1 I.U./ml would be equivalent to 3000/150 = 20 50% inhibition units. This in turn made it possible to convert the 50% inhibition titres of the test sera to equivalents of I.U./ml. In the above example, I.U./ml of the test serum would be :

$$\text{I.U./ml} = \frac{\text{P/Q x dilution of serum sample}}{\text{50\% inhibition units corresponding to 1 I.U./ml of the reference serum}}.$$

Some sera were titrated and their 50% inhibition titres obtained from their complete titration curves. These titres were subsequently converted to equivalents of I.U./ml on the

basis of the Institut Merieux serum. If in the above example a serum sample gave a titre of 80 50% inhibition units, then its potency would be $\frac{80 \times 150}{3000} = 4$ I.U./ml.

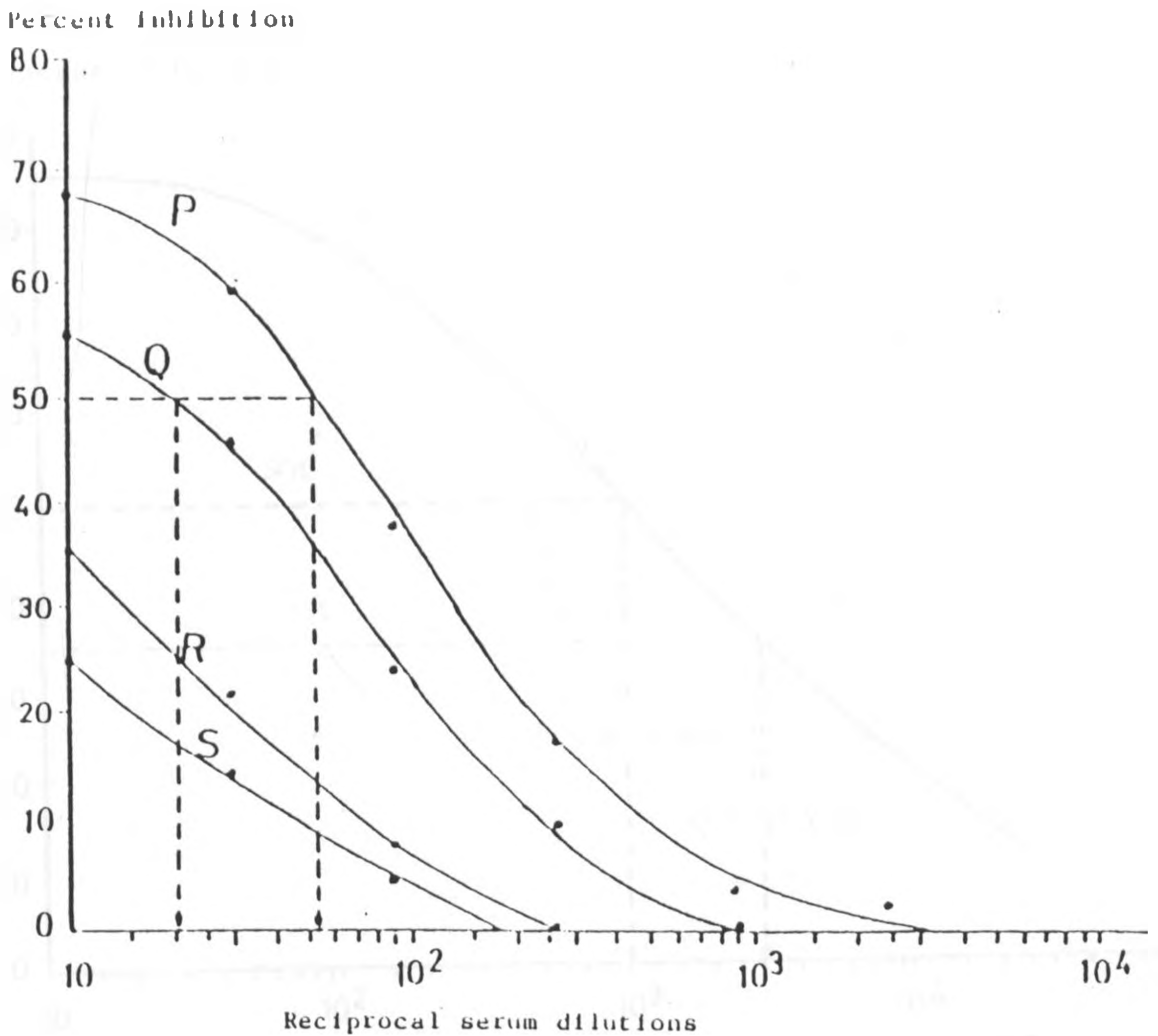

Figure 3 : The illustration shows how 50% inhibition titres were obtained from complete titration curves

Key : P, Q, R and S are serum samples

Their 50% inhibition titres are :

P = 54 R = < 10

Q = 22 S = < 10

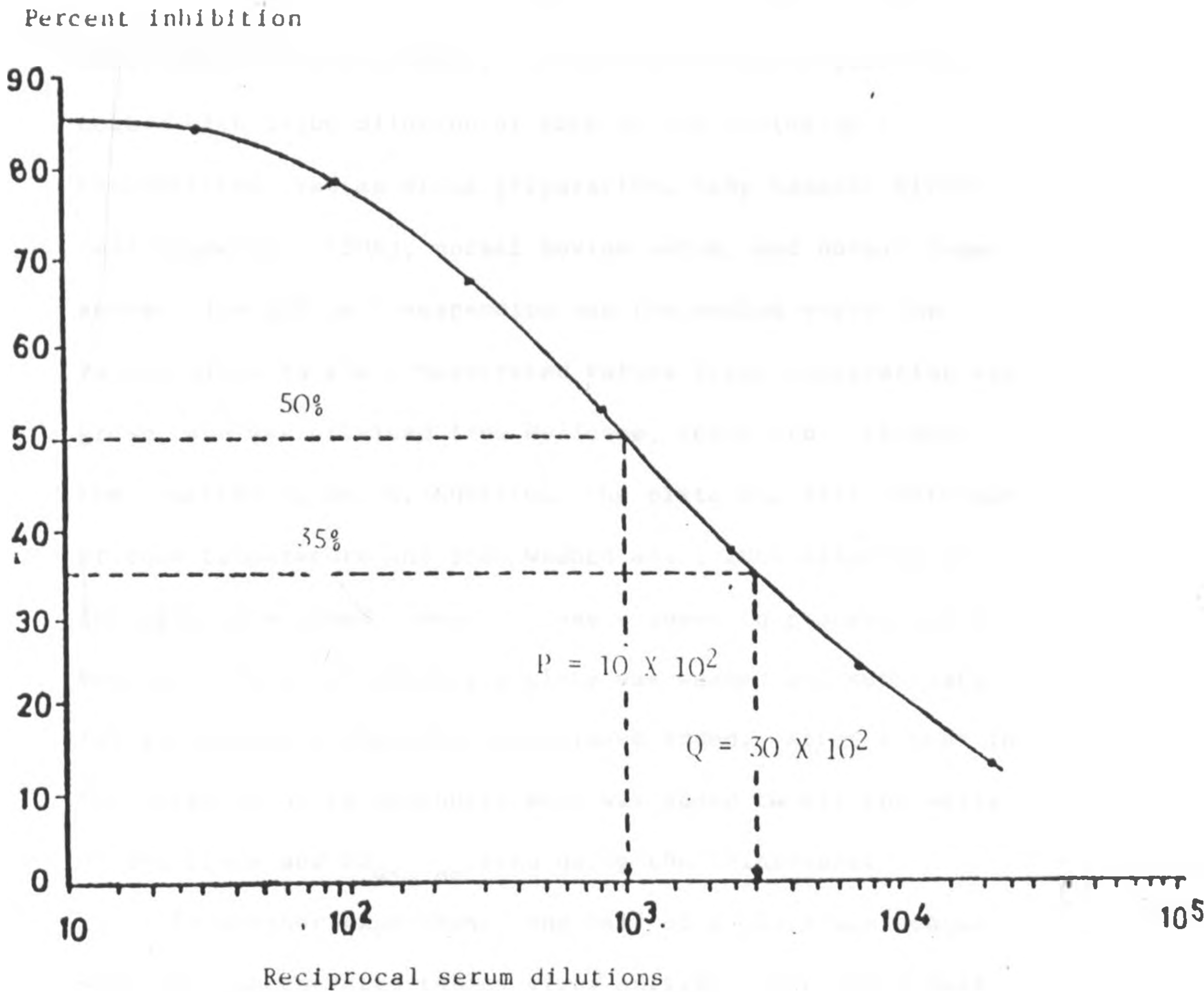

Figure 4 : The illustration shows an example of a positive reference curve used in calculating 50% inhibition titres on the basis of a single serum dilution (According to Duermeyer, 1980)

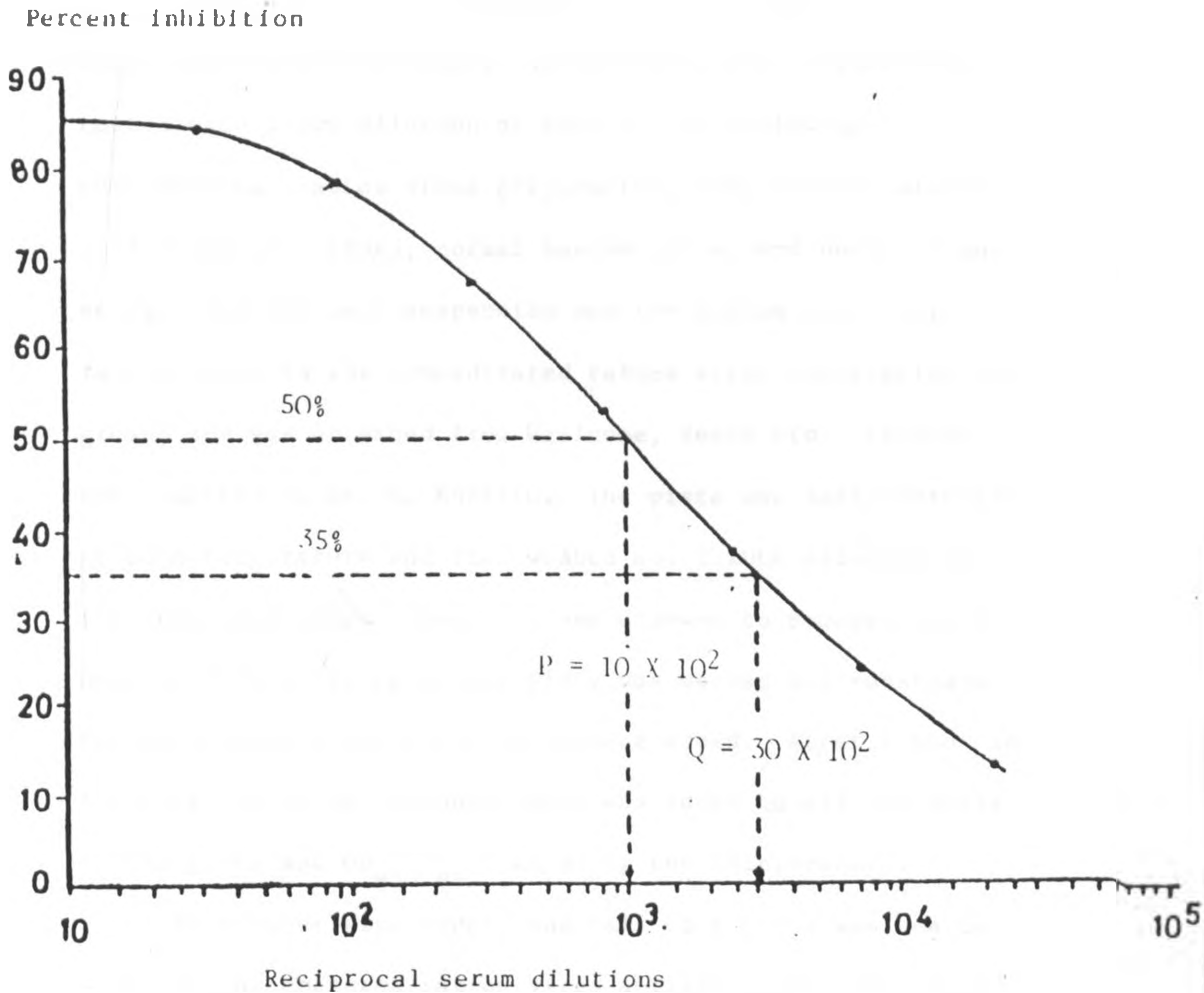

Figure 4 : The illustration shows an example of a positive reference curve used in calculating 50% inhibition titres on the basis of a single serum dilution (According to Duermeyer, 1980)

3.9 REACTIVITY OF GOAT #380 ANTIRABIES IgG - HRPO CONJUGATE IN ENZYME IMMUNOASSAYS

To test for the reactivity of the conjugate, some three experiments were performed. In the first one, a plate was coated with 1:200 dilution of each of the following : concentrated rabies virus preparation, baby hamster kidney cell suspension (BHK), normal bovine serum, and normal human serum. The BHK cell suspension was the medium where the rabies virus in the concentrated rabies virus preparation was grown, and was obtained from Wellcome, Kenya Ltd., through the courtesy of Dr. C. Nderitu. The plate was left overnight at room temperature and then washed and 1:2000 dilution of the conjugate added. Reaction was allowed to proceed for 1 hour at 37°C after which the plate was washed and substrate for the enzyme horseradish peroxidase added. After 1 hour in the dark, 50 ul 2N sulphuric acid was added to all the wells of the plate and $OD_{490\ nm}$ read using the "Minireader".

In another experiment, one half of a plate was coated with the concentrated rabies virus antigen. The other half was left uncoated. The plate was left at room temperature overnight, washed, and then rabies pre-immune and immune sera from a Sykes monkey #4 and a goat #864 were added in several dilutions in both the coated and non-coated halves of the plate. Both the Sykes monkey and the goat had been immunized with the concentrated rabies virus preparation. The plate was incubated at 37°C for 2 hours and then washed.

Conjugate and substrate were added as in the first experiment and $OD_{490\ nm}$ readings recorded for both the coated and non-coated halves of the plate.

In addition to the two experiments, sheep, goat, camel, dog, cattle, pig and chicken sera plus the BHK cell suspension were titrated in INH-EIA.

3.10 EVALUATION OF REPRODUCIBILITY OF THE INHIBITION ENZYME IMMUNOASSAY

The two antirabies sera, from Institut Merieux and WHO, were used for determining the reproducibility of the INH-EIA. The antirabies activity of the Institut Merieux serum was 150 I.U./ml while that of the WHO serum was 10 I.U./ml. The two antisera were titrated to obtain their complete titration curves and their 50% inhibition titres recorded. Fifteen titrations of the two were performed on five different occasions over a period of six months. The 50% inhibition titres of each serum were converted to equivalents of I.U./ml. The titres of one serum were calculated based on the other and vice-versa. If the WHO reference serum had a 50% inhibition titre of 120 units, then 1 I.U./ml of it would be equivalent to 120/10 = 12 50% inhibition units. If in the same experiment the Institut Merieux reference serum had a 50% inhibition titre of 3600 units, then this titre based on the WHO reference serum would be equivalent to 3600/12 = 300 I.U./ml.

The coefficient of variation of the 15 titres of each reference serum was calculated using the following formula :

$$\text{Coefficient of variation} = \frac{S}{\bar{X}} \times 100$$

where S = pooled standard deviation of the titres,

$\bar{X}$ = mean titre of the runs.

This formula is described by Thorner and Remein (1961).

4. RESULTS

4.1 SPECIFICITY OF GOAT #380 ANTIRABIES SERUM

Immunodiffusion analyses using this antiserum showed antibody activity against three components present in both the concentrated rabies virus antigen (Wellcome, Germany) and the human diploid cell rabies vaccine (Institut Merieux, Lyon, France). The antiserum originally showed antibody activity against one component present in normal human serum (NHS). The precipitin line formed between the antiserum and NHS showed a reaction of identity with one of the precipitin lines formed between the antiserum and the rabies virus preparations.

After extensive absorption of the antiserum, the single precipitin line against NHS disappeared and it showed only two precipitin lines against the two rabies virus preparations. The absorbed antiserum gave no reactions with sera from a large number of animal species which included sheep, goat, camel, dog, cattle, pig and chicken in immunodiffusion tests.

4.1.1. Reactivity of goat #380 antirabies IgG-HRPO conjugate in enzyme immunoassays

The optical densities ($OD_{490\ nm}$) obtained when a microtitre plate was coated with the same dilution (1:200) of BHK cell medium, normal bovine serum, normal human serum and a rabies virus preparation were 0.02 for the first three and 1.02 for the rabies antigen preparation.

The results obtained when one half of a microtitre plate was coated with 1:200 dilution of the concentrated rabies virus preparation and then rabies pre-immune and immune sera added in several dilutions in both halves, are shown in Table II. The results are in percent inhibitions of the conjugate by the various serum dilutions. In the non-coated half of the plate, $OD_{490\ nm}$ readings were zero. This was an indication that the conjugate could not react with both the pre-immune and immune sera in the noncoated half because of the absence of the rabies antigen, and that the conjugate did not bind non-specifically onto the plate. There was a clear difference in percent inhibitions between the rabies pre-immune and immune sera.

The sera from the 7 different animal species plus the BHK cell suspension gave virtually no inhibition when they were titrated in INH-EIA.

4.2 REPRODUCIBILITY OF THE INHIBITION ENZYME IMMUNOASSAY

In preliminary studies with the inhibition enzyme immunoassay, fifteen titrations of the two antirabies reference sera, obtained from the WHO and Institut Merieux, were performed on five different occasions over a period of six months. Both reference sera gave identical coefficient of variation of $\pm$ 10% over the period. The results obtained are summarised in Table III.

TABLE II : PERCENT INHIBITIONS OF GOAT #380 ANTIRABIES IgG-HRPO CONJUGATE BY RABIES PRE-IMMUNE AND IMMUNE SERA

Serum dilutions	Sykes monkey #4 rabies pre-immune serum	Sykes monkey #4 immune serum	Goat #864 rabies pre-immune serum	Goat #864 immune serum
1:10	8.4	94.4	22.9	96.6
1:50	10.6	87.7	3.9	93.3
1:250	5	53.3	0	82.1
1:1250	2.8	19.6	0	53.4

TABLE III : REPRODUCIBILITY OF THE INHIBITION ENZYME IMMUNOASSAY

Antibody titres (I.U./ml) of the two reference sera obtained on five different days and the titres of each serum calculated on the basis of the other

Day of assay	Institut Merieux reference serum	WHO reference serum
Day 1	193.5	7.7
	152.1	9.9
	168.4	8.9
Day 2	185.0	8.1
	202.1	7.5
	184.4	8.1
Day 3	205.4	7.2
	184.0	8.2
	166.7	9.0
Day 4	154.2	9.8
	151.6	9.9
	171.7	8.7
Day 5	163.4	9.2
	155.9	9.6
	171.7	8.8
	M.T. 174	8.7
	C.V. ± 10%	± 10%

M.T. = mean titre
C.V. = coefficient of variation

Four titrations of the Institut Merieux reference serum were performed at the Institut Merieux Laboratory in Lyon, France. The method employed for the titrations was the rapid fluorescent focus inhibition test (RFFIT), and the following titres, in I.U./ml, were obtained : 209, 192, 183, 193. The mean titre was 194 I.U./ml with a coefficient of variation of ± 6%.

4.3 RESULTS OF INHIBITION ENZYME IMMUNOASSAY OF SERUM SAMPLES DETERMINED ON THE BASIS OF COMPLETE TITRATION CURVES

4.3.1 Antibody titres (I.U./ml) of serum samples from individuals who underwent a pre-exposure immunization with the human diploid cell rabies vaccine and one serum sample from a rabid goat

Antibody titres of the 14 individuals who underwent a pre-exposure immunization with the human diploid cell rabies vaccine (HDCV) are given in Table IV. There was a definite increase in antibody titres by day 7 and a very good booster response by day 28 and 64.

The antibody titre of one serum sample from a goat bled one week before it died of rabies acquired from a dog bite, was 2.0 I.U./ml. This was a high antibody titre considering that the goat had not previously been vaccinated against rabies.

The antibody response of an individual who received a booster injection of 0.1 ml HDCV intradermally and bled after every one or two days for a period of 25 days is as depicted in Figure 5. An increase in antibody titre could be detected as early as 6 days after the booster injection.

TABLE IV : SERUM ANTIBODY TITRES (I.U./ML) OF INDIVIDUALS VACCINATED ON DAYS 0, 7 AND 28 WITH 0.1 ml HDCV INTRADERMALLY AND TITRES CALCULATED ON THE BASIS OF THE INSTITUT MERIEUX REFERENCE SERUM

Individual	Antibody titres (I.U./ml)			
	Day 0	Day 7	Day 28	Day 64
1	0.05	0.49	-	0.54
2	0.05	0.73	2.68	5.49
3	0.05	0.48	1.04	1.66
4	0.05	0.25	3.00	5.49
5	0.08	-	1.02	1.73
6	0.06	1.95	2.35	4.91
7	0.06	-	25.80	-
8	0.06	-	7.70	-
9	0.08	-	8.70	-
10	0.07	-	7.00	-
11	0.04	-	1.10	-
12	0.04	-	14.00	-
13	0.08	-	14.00	-
14	0.05	-	20.60	-

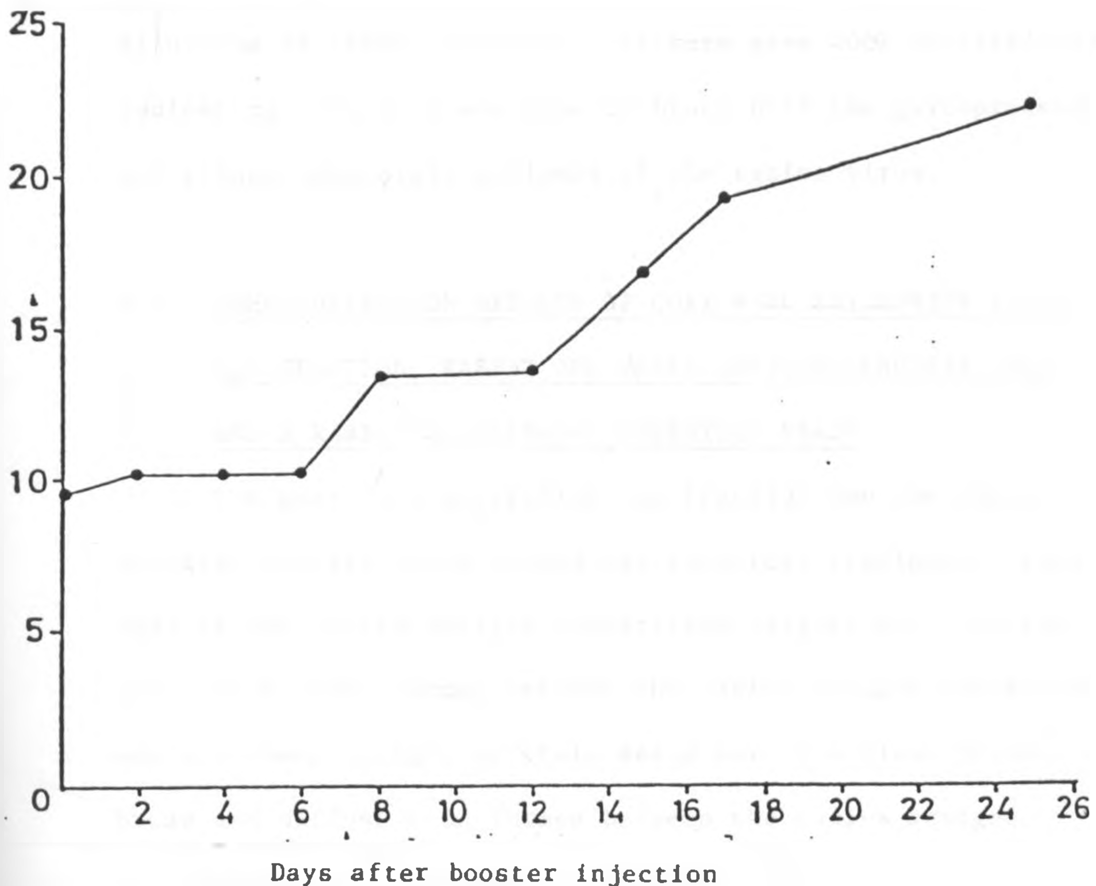

Figure 5 : Antibody response of an individual (K.L.) following a booster injection with 0.1 ml HDCV intradermally

4.3.2. Antibody titres obtained for the antiglycoprotein and antiribonucleoprotein serum samples

Antibody titres in I.U./ml obtained when the rabbit antiribonucleoprotein, rabbit antiglycoprotein and sheep antiglycoprotein sera were titrated in inhibition enzyme immunoassay were 2381, 21 and 38 respectively. The low dilutions of these "specific" antisera gave 100% inhibitions, indicating that each was able to block both the glycoprotein and ribonucleoprotein antigens of the rabies virus.

4.4 IMMUNODIFFUSION RESULTS OF GOAT #380 ANTIRABIES VIRUS IgG FRACTION, RABBIT AND SHEEP ANTIGLYCOPROTEIN SERA AND A RABBIT ANTIRIBONUCLEOPROTEIN SERUM

The goat #380 antirabies IgG fraction and the sheep antiglycoprotein serum formed two identical precipitin lines against the rabies antigen preparation (Figure 6). The two precipitin lines formed between the rabies antigen preparation and the sheep antiglycoprotein serum were identical to one broad and diffuse line formed between the rabies antigen preparation and the rabbit antiribonucleoprotein serum (Figure 6). The rabbit antiglycoprotein serum also formed two precipitin lines against the rabies antigen preparation which were identical to the two closely associated precipitin lines formed between the goat #380 antirabies IgG fraction

and the rabies antigen preparation (Figure 7). The rabbit antiribonucleoprotein serum formed one broad and diffuse precipitin line against the rabies antigen preparation. This line was identical to the two closely associated lines formed between the goat #380 antirabies IgG fraction and the rabies antigen preparation (Figure 9). One of the two precipitin lines formed between the rabies antigen preparation and the two antiglycoprotein sera disappeared on serial dilution of the antisera (Figures 7 and 8).

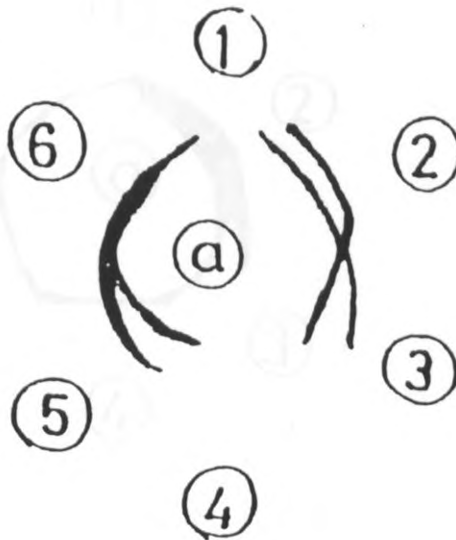

Figure 6 : Relationship between goat #380 antirabies virus IgG fraction, rabies virus, sheep antiglycoprotein serum, and rabbit antiribonucleoprotein serum in Ouchterlony's double diffusion test

Key :

a = concentrated rabies virus antigen preparation

2 = Goat #380 antirabies IgG fraction

3 = Sheep antiglycoprotein serum

5 = Sheep antiglycoprotein serum

6 = Rabbit antiribonucleoprotein serum

1 = Saline

4 = Saline

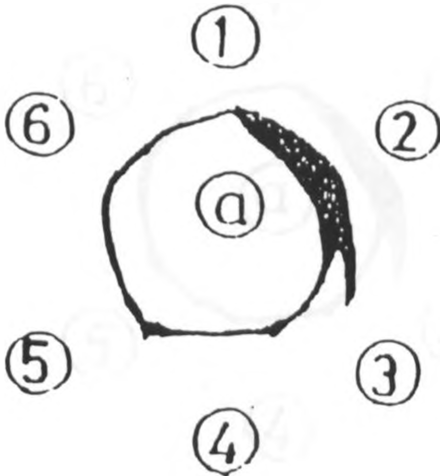

Figure 7 : Relationship between goat #380 antirabies virus IgG fraction, rabies virus and several dilutions of rabbit antiglycoprotein serum in Ouchterlony's double diffusion test

Key :

a = Concentrated rabies virus antigen preparation

2 = Goat #380 antirabies IgG fraction

3 = Rabbit antiglycoprotein serum (1:2)

4 = Rabbit antiglycoprotein serum (1:4)

5 = Rabbit antiglycoprotein serum (1:8)

6 = Rabbit antiglycoprotein serum (1:16)

1 = Rabbit antiglycoprotein serum (1:32)

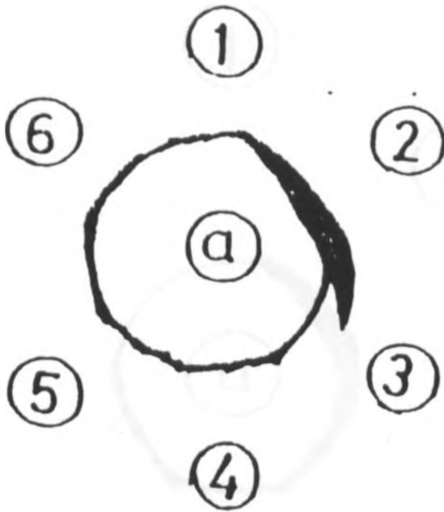

Figure 8 : Relationship between goat #380 antirabies virus IgG fraction, rabies virus and several dilutions of sheep antiglycoprotein serum in Ouchterlony's double diffusion test

Key :

a = Concentrated rabies virus antigen preparation

2 = Goat #380 antirabies IgG fraction

3 = Sheep antiglycoprotein serum (1:2)

4 = Sheep antiglycoprotein serum (1:4)

5 = Sheep antiglycoprotein serum (1:8)

6 = Sheep antiglycoprotein serum (1:16)

1 = Sheep antiglycoprotein serum (1:32)

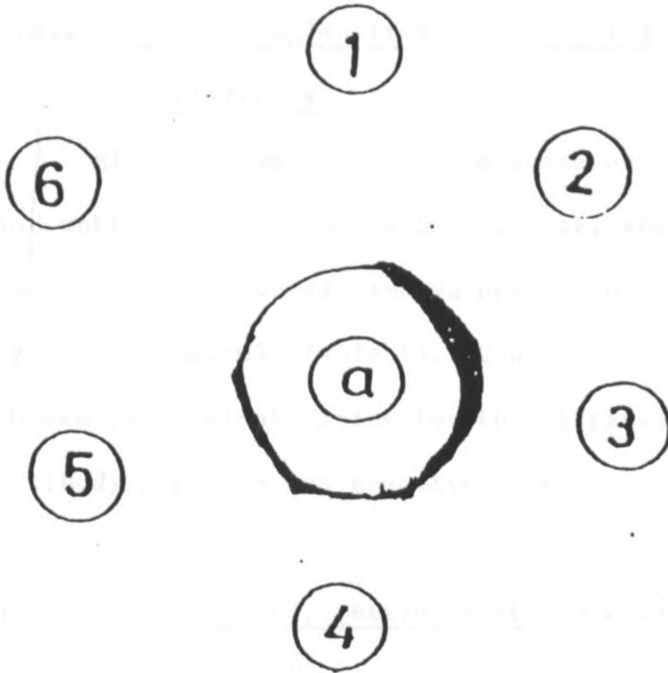

Figure 9 : Relationship between goat #380 antirabies virus IgG fraction, rabies virus and several dilutions of rabbit antiribonucleoprotein serum in Ouchterlony's double diffusion test

Key :

a = Concentrated rabies virus antigen preparation

2 = Goat #380 antirabies IgG fraction

3 = Rabbit antiribonucleoprotein serum (1:2)

4 = Rabbit antiribonucleoprotein serum (1:4)

5 = Rabbit antiribonucleoprotein serum (1:8)

6 = Rabbit antiribonucleoprotein serum (1:16)

1 = Rabbit antiribonucleoprotein serum (1:32)

4.5 ANTIBODY TITRES OBTAINED THROUGH TESTING OF SERA AT A SINGLE DILUTION

4.5.1 Serum samples from known vaccinated and unvaccinated individuals

All 28 serum samples from known unvaccinated individuals had antibody titres below 0.2 I.U./ml, whereas 89.8% of the 49 sera from known vaccinated persons had antibody titres above 0.2 I.U./ml (Table V). Consequently, 0.2 I.U./ml was chosen as a cut-off point for the differentiation of rabies antibody positive and negative sera.

4.5.2 Human, dog, cattle, goat and wild animal serum samples

Of the 418 human sera from Norway and 1,976 human sera from the Kenya Blood Bank, 98.3% and 97.5% respectively had antibody titres below 0.2 I.U./ml. None of the 11 dog sera from Norway had an antibody titre above 0.2 I.U./ml, whereas 37.2% of the 285 dog sera from Kenya had antibody titres above 0.2 I.U./ml. The percentages of the 192 cattle and 429 goat sera which had antibody titres above 0.2 I.U./ml were 19.3% and 9.6% respectively.

Some of the 232 serum samples from wild animals had high antibody titres. This was notable with the 24 elephant and 16 hyena sera where 75% of the former and 37.4% of the latter had antibody titres well above the cut-off point of 0.2 I.U./ml. Only 4.7% of the other wild animal sera had antibody titres above 0.2 I.U./ml.

These results, especially from the elephant and hyena sera, were of interest since no antirabies vaccination is carried out in these animal species in Kenya.

TABLE V : PERCENTAGE OF SERA FROM MAN AND DIFFERENT ANIMAL SPECIES GIVING A RANGE OF ANTIBODY TITRES (EQUIVALENT OF I.U./ML) DETERMINED AT SINGLE SERUM DILUTIONS AND CALCULATED ON THE BASIS OF THE INSTITUT MERIEUX ANTIRABIES SERUM

SERUM SAMPLES

Titre (Equivalents of I.U./ml)	HUMAN				ANIMAL						
	Kenya known pre-vaccination	Kenya blood bank	Norway	Kenya known post-vaccination	Kenya dog	Norway dog	Goat	Cattle	Elephant	Hyena	Other wild animals
	(28)*	(1,976)	(418)	(49)	(285)	(11)	(429)	(192)	(24)	(16)	(192)
0 - 0.1	92.7++	83.9	83.7	2.0	30.2	72.7	62.2	52.1	12.5	56.3	82.8
0.1 - 0.2	7.3	13.6	14.6	8.2	32.6	27.3	28.2	28.6	12.5	6.3	12.5
0.2 - 0.3		1.5	1.4	8.2	15.8		4.7	6.3	12.5	18.8	3.1
0.3 - 0.4		0.4		6.1	4.9		2.6	4.7	25.0	6.3	0.5
0.4 - 0.5		0.1		12.1	1.1		1.6	4.2	16.7	6.3	1.0
0.5 - 0.6		0.1	0.3	6.1	1.4		0.2	1.0			
0.6 - 0.7		0.1		8.2	2.5		0.2	1.6	12.5	6.0	
0.7 - 0.8		0.05		6.1	2.5		0.2	1.0	8.3		
0.8 - 0.9		0.05		14.3	3.2						
0.9 - 0.1		0.05		4.2	1.4						
> 1.0		0.15		24.5	4.4		0.1	0.5			0.1

* Indicates number of sera tested

++ Indicates percentage of sera with antibody titres within the given range

5. DISCUSSION AND CONCLUSIONS

The results of the mouse neutralization test (MNT) and the recently described rapid fluorescent focus inhibition test (RFFIT) (Smith et al., 1973) have been recognized as true reflections of the protective potency of a serum. The antibody titres obtained by these methods can be expressed as international units per millilitre (I.U./ml) in comparison with an internationally recognized reference serum. Both tests, however, suffer from poor reproducibility and demands for highly skilled man-power. The absolute requirements for live infective challenge virus, a large number of animals or tissue culture capability as well as special facilities and equipment, render these two neutralization tests unsuitable for routine or large-scale use in most countries. Although promising, most of the replacement tests have been found to be inadequate largely because of poor correlation with the MNT and the RFFIT. The MNT should a priori provide the most biologically relevant assessment of antibody activity, but it is more cumbersome, difficult and expensive than the other tests. The need for a rapid and inexpensive test which correlates well with the MNT is indicated.

The possibility of using enzyme immunoassays (EIA) as rapid, sensitive and inexpensive tools for detecting and quantitating rabies antibodies has been investigated (Atanasiu et al., 1980; Nicholson and Prestage, 1982). The

important advantages of EIA over other conventional tests have been their high sensitivities, safety, ability to measure IgG and IgM, low cost, avoidance of subjectivity, rapidity and reliability (Nicholson et al., 1985). In spite of these arguments in favour of the commonly used EIA, most investigators agree that it is very difficult to express the titre in a consistent way and at the present time, there is no consensus of opinion on the best way to express EIA results (De Savigny and Voller, 1980; Malvano et al., 1982).

In this study, an inhibition enzyme immunoassay (INH-EIA) for the detection and quantitation of rabies antibodies was developed and evaluated. The IgG fraction of absorbed serum from a goat immunized with human diploid cell rabies vaccine (HDCV) from Institut Merieux, France, was conjugated with horseradish peroxidase and shown to be specific for rabies virus components. It was shown that the conjugate possessed antibody activities to both the glycoprotein and the ribonucleoprotein components of the virus when compared with known antiglycoprotein and antiribonucleoprotein sera obtained from other laboratories. Further evidence of the specificity for rabies virus was obtained when no inhibition was observed when dilutions of tissue culture homogenates and known negative sera from 7 different animal species were assayed.

The high reproducibility of the INH-EIA was shown when two human antirabies sera, obtained from the WHO and Institut Merieux, were titrated fifteen times on five different

occasions. Both sera gave the same coefficient of variation of ± 10%. This is in sharp contrast to the large variations of ± 22%, ± 41% and ± 133% which have been reported for the MNT ((Chippaux et al., 1985a; Chippaux et al., 1985b; Van Der Marel and Van Wezel, 1981).

Several investigators have presented data suggesting that EIA, as a purely quantitative assay, might be influenced by the affinity of the primary antibodies (Engvall and Perlmann, 1972; Butler et al., 1978). Butler et al., (1978) hypothesized that the binding of the large secondary antibody-enzyme complex weakens the binding of low-affinity primary antibodies to the point of their displacement during the subsequent washing procedures. In the present INH-EIA, a high ionic strength salt (1M KCl) buffer containing 2% polyethylene glycol 6000, a nonionic polymer, was used as test serum diluent. The composition of the diluent enhances antigen-antibody interactions and might consolidate these interactions (Saunders, 1979; Hellsing, 1978), thus allowing the estimation of even low affinity antibodies. The procedure allows the antibodies in the test serum to interact with antigens prior to the addition of the conjugate. It is expected that this would reduce the dissociation of low affinity antibodies by the presumably high affinity antibodies of the conjugate.

Although the conjugate was prepared using presumably high affinity IgG isolated from the serum of a hyperimmunized

goat, it is possible that the actual affinity of the antibodies in the conjugate is low. Low affinity could have arisen by denaturation during the conjugation procedure and/or through the covalent binding of enzyme near the antigen binding sites. A conjugate of low affinity may possess considerable advantages in INH-ElA as it would not be able to compete as efficiently as high affinity antibodies for antigen. Accordingly, such an assay would be capable of detecting both low and high affinity antibodies, and also low levels of antibody. The only evidence available for the detection of low affinity antibodies and also of low levels of antibodies is the finding of definite antibody titres (equivalent to 0.25 - 1.95 I.U./ml) in the 5 human serum samples obtained 7 days after intradermal primary vaccination with 0.1 ml HDCV (Table IV). This deduction is drawn from the observations that the first antibodies to appear as early as seven days after immunization possess low affinity and high affinity antibodies are produced after continued immunization with antigen (Kabat and Mayer, 1971).

The 14 human pre-vaccination sera gave a mean antibody titre corresponding to 0.06 I.U./ml (range 0.04 - 0.08 I.U./ml) calculated on the basis of the Institut Merieux antirabies serum. It is surmised that these antibody titres may have been due to non-specific inhibitions or represented low levels of antibodies to rabies-related viruses. The same observation has been encountered in the MNT where in one study, a non-specific neutralizing antibody titre equivalent

to a mean of 0.1 I.U./ml was obtained in 16 human pre-vaccination sera (Aoki et al., 1975).

If one assumes that high affinity antibodies are more efficent than low affinity antibodies in neutralizing virus as is the case with antisera to tetanus and diphtheria toxins (Kabat and Mayer, 1971), it is evident that INH-EIA ought to favour the estimation of only high affinity antibodies in order to correlate well with the MNT and the RFFIT. The mean antibody titre corresponding to 8.7 I.U./ml obtained in the WHO serum by using INH-EIA compared closely with the mean antibody titre corresponding to 10.0 I.U./ml obtained in the same serum by using the MNT. The mean antibody titre corresponding to 174 I.U./ml obtained in the Institut Merieux serum by using INH-EIA, also compared closely with the mean antibody titre corresponding to 194 I.U./ml obtained in the same serum by using the RFFIT. The close correlation found between the results of the INH-EIA and the two neutralization tests was somewhat surprising if one assumes that both low and high affinity antibodies of all classes can be detected by the INH-EIA, while only high affinity antibodies might be expected to contribute to neutralization in the MNT and the RFFIT. However, the possibility exists that the antisera used in this comparative study contained antibodies of largely the same affinity, or had a similar ratio of high/low affinity antibodies. It is of interest, and rather unexpected, to note that neither the time of appearance nor titre of

actively induced rabies neutralizing antibody are significantly correlated with protection (Sikes *et al.*, 1971). In fact, it appears that antibodies may contribute in experimental situations to the exacerbation of the disease and give rise to more prominent pathological changes (Zlotnick and Grant, 1973).

While it is easy to envisage the role of antibody in the termination of infections which have a prominent viraemic phase, there is little evidence for its effect in the clearance of virus from a solid target organ (Ogra *et al.*, 1975; Bloom and Rager-Zisman, 1975). It is particularly difficult to envision an efficient elimination mechanism by antibody in the central nervous sytem (CNS) in spite of the real possibility of antibody production by plasma cells localized within the CNS. Nevertheless, antibody is likely to play an important role in the early stages of infection before localization of the virus in nerve tissue.

Little is known about the actual role of cellular immune responses in rabies. Doherty and Zinkernagel (1974) have shown that killer T cells are capable of lysing virally infected target cells *in vitro*. Wiktor *et al.* (1977) have described a similar T-cell killing of rabies infected cells. Antibodies may also participate in cell-mediated immune responses by providing the required specificity in antibody-dependent cell-mediated cytotoxicity (ADCC) in which K cells of the T series are the effector cells. These cells

have surface receptors for the Fc fragment of IgG molecules which have combined specifically with the virus or cells with expressed viral antigens (Heberman, 1983). Monocytes or macrophages and polymorphonuclear leucocytes also possess Fc receptors and may exercise cytotoxic reactivity against IgG-coated target cells.

It is possible that antibodies of the IgG class which are involved in cytotoxic reactions may possess low affinity for antigen. The actual concentration and specificities of these antibodies for viral antigens are not known. It is also uncertain whether antiribonucleoprotein or antiglycoprotein antibodies, or both, participate in cytotoxic reactions, and to what extent their quantitation would reflect the protective immunity of an individual. While the MNT and the RFFIT would be unable to detect these antibodies, the INH-EIA should enable their detection and quantitation. If they represent a significant proportion of the total amount of antibodies to rabies virus, their presence in a serum sample might result in poor correlation between INH-EIA and the neutralization tests, unless they appear in a constant ratio to neutralizing antibodies. However, if they also represent a significant contribution to the protection in a vaccinated individual, a test which detects all antibodies associated with protection would be desirable. It could therefore be hypothesized that the neutralization tests alone do not provide an accurate

parameter for the real immune status of an individual, and this might explain some of the discrepancies observed between neutralizing titre, time of appearance of neutralizing antibody and protection.

The considerations outlined above formed the basis for developing a test which should detect antibodies of various affinities and different specificities. It was therefore somewhat surprising to note the excellent correlation between two different human antisera to rabies virus, but this observation needs to be confirmed and expanded by comparative studies of sera obtained early and late during immunization using the MNT, the RFFIT and the INH-EIA. Unfortunately, such sera have been unavailable for this study. If the results should turn out in favour of the estimation of purely neutralizing antibody, assumed to be specific for the glycoprotein component (Cox et al., 1977), the INH-EIA can very easily be modified to serve this purpose. This can be done by either using pure viral glycoprotein antigen and a polyvalent conjugate, or a complex antigen using a pure monospecific conjugate.

The confirmation of the diagnosis of rabies during life would be useful for several reasons. The gravity of the patients prognosis would be more certain, and it would be easier to decide how to manage the patient, to institute proper precautions, and to immunize attendant medical staff quickly. The intra vitam diagnosis of rabies based on the

demonstration of rabies antigen in corneal impression smears and in cutaneous nerve fibres using immunofluorescence test have been reported (Schneider 1969; Smith et al., 1972; Bryceson et al., 1975). However, the techniques have been found to require experience and relatively sophisticated equipment, and not reliable (Bryceson et al., 1975). In addition, the immunofluorescence results and virus isolation have been found to be uncertain in later stages of the disease (Nicholson et al., 1985). In such cases, clinical history and signs, as well as the finding of very high serum neutralizing antibody titres is the most reliable confirmatory procedure providing that no antirabies vaccination has ever been carried out (Rubin et al., 1970; Hattwick et al., 1972; Porras et al., 1976). Therefore, the availability of a sensitive, rapid and specific method for the detection and quantitation of rabies antibodies is highly desirable in such cases. The present INH-EIA is capable of detecting antibodies of whatever specificity to viral proteins. This renders the method suitable for the detection of very low levels of antibodies to any of the major antigens and should therefore be applicable for the intra vitam diagnosis of rabies. Evidence for this assumption was obtained when an antibody titre, equivalent to 2.0 I.U./ml, ten times the highest titre detectable in any known pre-vaccination sera, was obtained in a serum sample from an unvaccinated rabid goat bled one week before death.

Another possible modification of the INH-EIA would be to use a conjugate specific for the ribonucleoprotein component, considered as the group-specific antigen for rabies and rabies-related viruses (Beran, 1981). Such modification would allow detection of antibodies to rabies-related viruses which would go undetected in a test designed to detect only neutralizing (antiglycoprotein) antibodies. This may be of considerable importance on the African continent where rabies-like viruses have been shown to cause a disease indistinguishable from rabies (Familusi *et al.*, 1972; Meredith *et al.*, 1971; Foggin, 1982).

In the present study, antibody titres above the highest detectable titre (equivalent to 0.2 I.U./ml) in any known pre-vaccination sera were obtained in sera from a variety of animal species which are not vaccinated against rabies in Kenya (Table V). This was notable in sera obtained from goats, cattle, hyenas, elephants and to a less extent in sera from other wild animal species. It was not possible to confirm what these reactors represented but it is conceivable that the animals may have been exposed to the rabies virus or to one of the rabies-related viruses. This possibility rests on the fact that the conjugate used possessed distinct antibody activity against the group-specific ribonucleoprotein and that a known antiribonucleoprotein serum showed strong inhibition in the INH-EIA.

Although presently the classical street virus is the only important cause of rabies, it seems a certainty that the number of infections by rabies-related viruses will increase with time. This will be more evident when classical rabies is controlled or reduced by vaccination which does not provide, or provides inefficient protection against the related viruses (Tignor and Shope, 1972; Foggin, 1982). Monoclonal antibodies directed against the strain-specific antigen(s) of the various strains of rhabdoviruses pathogenic for man and animals, are needed to define the epidemiological pattern of these infections, and to determine their distribution and spread in various populations.

All the 11 dog sera from Norway gave antibody titres below the highest detectable titre in any known pre-vaccination sera, ie. equivalent to 0.2 I.U./ml calculated on the basis of the Institut Merieux serum. This observation was to be expected because routine dog vaccination is not undertaken in rabies-free Norway (Beran, 1981). Of the 285 dog sera from Machakos and Narok districts where vaccination programmes have been carried out, 37.2% gave positive reactions for rabies antibodies with titres ranging from equivalents of 0.2 I.U./ml to and above 1.0 I.U./ml. Since antirabies vaccination is routinely carried out in dogs in Kenya, it is most likely that the reactors are a reflection of the efficiency of the programmes. Although rabies is considered a uniformly fatal disease, it has been

known since the days of Pasteur that some animals survive both natural and experimental infection, and that a healthy and infective carrier state may occur (Veeraraghavan et al., 1969; Fekadu, 1972; Fekadu, 1975; Afshar et al., 1972). On the basis of this observation, the possibility that one or more of the above reactors would be representative of chronic rabies virus carriers cannot be ruled out.

Only 2.5% of the 1,976 human sera from Kenya and 1.7% of the 418 human sera from Norway gave antibody titres above the equivalent of 0.2 I.U./ml. Although it has not been possible to obtain vaccination histories of these reactors, it is likely that they represent previous antirabies vaccinations.

The rationale for using a single serum dilution in the screening tests and the subsequent calculation of titres based on a positive serum reference curve has been investigated by a previous worker (Kyule, 1983). He showed that there is an excellent agreement (92.5%) between titres obtained from complete titration curves and those calculated on the basis of a positive serum reference curve if the serum dilutions give percent inhibitions within the linear portion of the positive serum reference curve.

It is concluded that INH-EIA is a highly reproducible, sensitive and specific method for the detection and quantitation of antibodies to rabies virus. The method is capable of detecting both neutralizing (antiglycoprotein) and antiribonucleoprotein antibodies. The antibodies against the

ribonucleoprotein are believed to be responsible for complement fixing reactions (Wiktor *et al.*, 1973a) and have also been shown to be associated with protection (Kuwert *et al.*, 1976). The use of a conjugate which also possesses antibody activity to ribonucleoprotein may therefore be justified in tests designed to estimate the level of protective immunity in an individual.

The INH-EIA permits rapid and reproducible determinations of antibody titres and allows the screening of large numbers of specimens from man and any animal species using single serum dilutions. It is therefore likely that the method will find considerable application in seroepidemiological studies of rabies and related infections. The method is rapid because of its completion in 12 to 18 hours and the availability of ready-for-use plates, it is easy to perform and interpret, and relatively inexpensive.

The INH-EIA appears to be an attractive substitute for both the MNT and the RFFIT because of its high sensitivity and specificity, high reproducibility, ease of performance and interpretation, rapidity and low cost. Further investigations are needed to confirm whether the antibody titres obtained in the INH-EIA represent true reflections of various levels of protective immunity.

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APPENDIX 1

DILUENTS AND THE SOLUTION USED IN ENZYME IMMUNOASSAY

1.1 Serum diluent

0.05 M Phosphate buffer pH 8.0	1000 ml
Potassium chloride	75.0 g
Benzoic acid	2.5 g
Di-sodium ethylenediaminetetraacetate	1.0 g
Tween 20	1.0 ml
Polyethylene glycol (PEG) (MW 6000)	20.0 g

pH adjusted to 7.5 with 4 M sodium hydroxide

1.2 Conjugate diluent

0.05 M Phosphate buffer pH 8.0	1000 ml
Potassium chloride	75.0 g
Benzoic acid	2.5 g
Di-sodium ethylenediaminetetraacetate	1.0 g
Tween 20	5.0 ml

pH adjusted to 7.5 with 4 M sodium hydroxide

1.3 Enzyme immunoassay wash solution

Phosphate buffered saline pH 7.4	10,000 ml
Tween 20	5.0 ml
Sodium azide	2.0 g

APPENDIX 2

COOMASSIE BRILLIANT BLUE 250R SOLUTION

Coomassie Brilliant Blue 250R	10 g
Ethanol	900 ml
Glacial acetic acid	200 ml
Distilled water	900 ml

APPENDIX 3

DESTAINING SOLUTION FOR COOMASSIE BRILLIANT BLUE STAINING SOLUTION

Ethanol	900 ml
Glacial acetic acid	200 ml
Distilled water	900 ml