ANNA UNIVERSITY : CHENNAI 600 025

BONAFIDE CERTIFICATE P-1833

Certified that this project report "**EVALUATION OF TISSUE CULTURE ANTI-RABIES VACCINE (TCARV) IMMUNIZATION RESPONSE ON HUMAN SERA SAMPLES BY *IN VIVO* AND *IN VITRO* METHODS**" is the bonafide work of **C.VIGNESH** who carried out the project work under my supervision.

SIGNATURE 30/4/07

(Dr.P.Rajasekaran)

HEAD OF THE DEPARTMENT

Department of Biotechnology
Kumaraguru College of Technology
Coimbatore.

SIGNATURE 30/4/07

(Dr.N.Saraswathy)

SUPERVISOR

Department of Biotechnology
Kumaraguru College of Technology
Coimbatore.

P 1833

ANNA UNIVERSITY : CHENNAI 600 025

BONAFIDE CERTIFICATE

Certified that this project report "**EVALUATION OF TISSUE CULTURE ANTI-RABIES VACCINE (TCARV) IMMUNIZATION RESPONSE ON HUMAN SERA SAMPLES BY *IN VIVO* AND *IN VITRO* METHODS**" is the bonafide work of **K.KANNAN** who carried out the project work under my supervision.

SIGNATURE 30/6/7

(Dr.P.Rajasekaran)

HEAD OF THE DEPARTMENT

Department of Biotechnology
Kumaraguru College of Technology
Coimbatore.

SIGNATURE

(Mr.M.Shanmuga Prakash))

SUPERVISOR

Department of Biotechnology
Kumaraguru College of Technology
Coimbatore.

CERTIFICATE OF EVALUATION

College: KUMARAGURU COLLEGE OF TECHNOLOGY

Branch: INDUSTRIAL BIOTECHNOLOGY

Semester:Eighth Semester

S.NO	Name of the students	Title of the Project	Name of the Supervisor with Designation
01	**C.Vignesh**	**EVALUATION OF TISSUE CULTURE ANTI-RABIES VACCINE (TCARV) IMMUNIZATION**	**Dr.N.Saraswathy Senior Lecturer.**
02	**K.Kannan**	**RESPONSE ON HUMAN SERA SAMPLES BY *IN VIVO* AND *IN VITRO* METHODS.**	**Mr.M.ShanmugaPrakash, Lecturer.**

The report of the project work was submitted by the above students in partial fulfillment for the award of Bachelor of Technology in Industrial Biotechnology of Anna University were evaluated and confirmed to be report of the work done by the above students and evaluated.

(INTERNAL EXAMINER)

(EXTERNAL EXAMINER)

PASTEUR INSTITUTE OF INDIA

COONOOR – 643 103. THE NILGIRS

PROJECT CERTIFICATE

This is to certify that the project entitled **"EVALUATION OF TISSUE CULTURE ANTIRABIES VACCINE IMMUNIZATION RESPONSE ON HUMAN SERA SAMPLES BY IN-VIVO (MOUSE NEUTRALIZATION) AND IN-VITRO (REFFIT AND MIXED HEMADSORPTION) TEST"** has been carried out by **Mr. C. VIGNESH** final year student of B.Tech. Industrial Biotechnology of Kumaraguru College of Technology, Coimbatore . The work has been carried out at Pasteur Institute of India, Coonoor under the guidance of Dr. A. Premkumar Research Officer which has been approved by the undersigned.

Dr.A. PREM KUMAR
Research Officer
Pasteur Institute of India,
Coonoor - 643 103.

Dr. N. ELANGESWARAN
DIRECTOR,
PASTEUR INSTITUTE OF INDIA,
COONOOR – 643 103.

PASTEUR INSTITUTE OF INDIA
COONOOR – 643 103. THE NILGIRS

PROJECT CERTIFICATE

This is to certify that the project entitled **"EVALUATION OF TISSUE CULTURE ANTIRABIES VACCINE IMMUNIZATION RESPONSE ON HUMAN SERA SAMPLES BY IN-VIVO (MOUSE NEUTRALIZATION) AND IN-VITRO (REFFIT AND MIXED HEMADSORPTION) TEST"** has been carried out by **Mr. KANNAN KALIAPPAN** final year student of B.Tech. Industrial Biotechnology of Kumaraguru College of Technology, Coimbatore . The work has been carried out at Pasteur Institute of India, Coonoor under the guidance of Dr. A. Premkumar Research Officer which has been approved by the undersigned.

Dr.A. PREM KUMAR
Research Officer
Pasteur Institute of India.
Coonoor – 643 103.

Dr. N. ELANGESWARAN
DIRECTOR,
PASTEUR INSTITUTE OF INDIA,
COONOOR – 643 103.

ACKNOWLEDGEMENT

We express our deep sense of gratitude to **Dr.P.Rajasekaran**, Head of the Department, Department of Industrial Biotechnology, Kumaraguru College of Technology, Coimbatore for his guidance and help in all ways throughout the project work.

We would like to express our sincere thanks to our guide, **Dr.Premkumar**, Faculty, Pasteur Institute of India, Coonoor for his unfailing guidance and support throughout the work and thereafter. Also the institute for lending us their time, space and resources.

Our heartfelt gratitude to our internal guides **Dr.N.Saraswathy**, Senior Lecturer, **Mr.M.Shanmuga prakash**, Lecturer, Kumaraguru College of Technology, Coimbatore for their continuous assistance. We also thank the Faculties, Kumaraguru College of Technology, Coimbatore for their valuable suggestions and critisms.

We record our thanks to our parents, friends and classmates for their timely help and moral support.

Finally, We remember the kind help from all the non-teaching members of the Department of Industrial Biotechnology.

(KANNAN KALIAPPAN)

(VIGNESH. C.)

ABSTRACT

The antibody levels of the sera samples of rabid dog bitten and immunized patients, were determined by *in vivo* (MNT), and *in vitro* (RFFIT,MH test) methods. In the MNT, 60 mice were used at different dilutions in which 21 were dead , 29 were showing severe rabies symptoms and 10 were alive. In RFFIT, complete neutralization was observed in sample II in all dilutions where as in sample III no neutralization was seen. In samples I and IV, the amount of fluorescence increases as the dilutions increase due to the decline in the antibody level. In the Rapid Fluorescent Focus Inhibition Test (RFFIT) and Mouse Neutralization Test (MNT) the amount of antibodies combining with the glycoprotein of the virus surface are measured. In Mixed Hemadsorption test the antibodies combine with the virus – coded glycoprotein antigen of the surface of rabies – infected cells. In MH test, the amount of antibodies could be determined from the diameter of the zone. The zone with 5 mm diameter is considered to be the most efficient. Out of the these three experiments it is found that RFFIT is more sensitive than MNT and MH test, since the antibody titre could be measured accurately.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ELIZA -Enzyme Linked Immunosorbent Assay

CVS-Challenge virus standard

M-Matrix protein

G-Glycoprotein

N-Nucleoprotein

RNA-Ribo Nucleic acid

nm- nanometer

L-Transcriptase protein

NS-Transcriptase associated protein

INTRODUCTION

1. INTRODUCTION

Rabies is a viral Zoonotic disease of mammals, including humans, which causes encephalomyelitis. Rabies is induced by neurotropic viruses of the *Lyssavirus* genus, *Rhabdoviridae* family. So far it is known that the genus *Lyssavirus* includes 7 genotypes (*Bourhy et al., 1993*). The number of genotypes is likely to increase (*Kuzmin et al., 2003*). The genotype 1 comprises classical rabies virus responsible for most human rabies cases and it is distributed in almost every country throughout the world. The virus is maintained in mammalian reservoirs, mainly domestic and wild carnivores as well as bats. Dogs represent the major rabies reservoir in developing countries. The onset occurs after infection of the rabies virus nearly always through exposure to a rabid animal. In humans, the onset symptoms are headache, fever, malaise, apprehension, diarrhea, and sometimes tingling at the bite site. As the disease progresses, the person may develop furious rabies which consists of periods of aggressive behavior (biting, thrashing) followed by calm. The individual experiences muscle spasms, convulsions, hydrophobia (the fear of water), delirium accompanied by hallucinations. Paralysis develops later in the disease. Twenty percent of rabid persons develop paralytic rabies in which paralysis dominates the clinical picture. In these persons, the aggressive behavior characteristic of furious rabies is not evident. Both forms of rabies culminate in coma then death, which is often due to respiratory paralysis or cardiac arrest.

Rabies occurs worldwide with an estimated 40,000 human deaths annually. About 54 percent of the hydrophobia cases were admitted in India. Overall hospitalization rate was 0.81 per 100,000 population. It was significantly higher in 5-14 year old than in other age groups and in males

than in females (p <0.0009). Cases occurred round the year. Almost 96 percent cases (206/215) gave history of animal exposure, 13 days to 10 years (median 60 days) before hospitalization. Animals involved were stray dog (193/206 = 90 percent), pet dog, cat, jackal, mongoose, monkey and fox. Most of cases were never vaccinated (78 percent) or inadequately vaccinated (44 percent); only 4 percent each received appropriate wound treatment, or rabies immunoglobulin (Singh et al., 1998).

1.1. TRADITIONAL VACCINES FOR RABIES

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

1.1.1. HUMAN DIPLOID CELL VACCINE

According to Dreesen and David (1980) today human diploid cell vaccines (HDCV), also an inactivated virus vaccine, are used

almost exclusively by the developed world for pre and post vaccination of Rabies. Virus can be harvested from infected human diploid cells, concentrated by ultra filtration and is inactivated by chemicals such as beta propiolactone. The vaccine product consists of a sterile, stable, freeze-dried suspension of rabies virus. Human diploid cell vaccine gives high neutralizing antibody after 10 days, and carries a lower burden of side-effects as those derived from neuronal tissue. Because the product contains trace amounts of animal by-products, antibiotics, and human serum albumin, systemic allergic reactions are possible such as local reactions, swelling, and reddening and have been reported. Antibodies are given to reduce the viral burden and enhance the effectiveness of treatment since it has been documented that vaccination alone may not be enough to prevent rabies.

1.1.2. ANIMAL VACCINES

In 1983, a vaccinia-rabies glycoprotein (V-RG) recombinant virus vaccine was developed that has proven to be an effective oral immunogenic in raccoons and various other important reservoir species. Vaccine advantages include improved thermo stability and an inability to cause rabies.

1.1.3.DNA VACCINES

DNA vaccines utilize naked DNA strands alone for immunization, without traditional proteins or carrier viruses. The genes encoding immunogenic proteins are inserted into a circle of bacterial DNA known as a plasmid. DNA vaccine uses just enough genes from the virus to activate the

immune system. The plasmids are shot into muscle tissue using a gene gun, where they are taken up and expressed by cells. Surprisingly enough this method of immunization (Fu and Wasi, 1986) has been shown to work surprisingly well when it comes to eliciting an immune response against pathogens, yet it is still not clear exactly how the DNA vaccines stimulate an immune response. the new vaccine can only be used prior to exposure to rabies, and is not a replacement for post-exposure treatment with IgG Researchers said there are no immediate plans to conduct human clinical trials, due to questions concerning whether additional genes must be inserted to offer protection to humans, and whether it would be effective if it were administered after a person contracted rabies.

1.1.4. TISSUE CULTURE BASED ANTI- RABIES VACCINE

Currently, Vero cell lines of the African monkey have been used in the production of Anti Rabies vaccine. The inactivated virus is grown in vero cell lines, which are then extracted and purified by various down stream processing methods in order to commercialize the vaccine for human use.(Trabelsi, et al., 2006).

Pasteur Institute of India, Coonoor is one of the leading Institutes in the production of Anti-rabies Vaccine for the Expanded Programme of Immunization of Govt. of India. This Institute started functioning as Pasteur Institute of Southern India, on April 6, 1907 and the Institute took a new birth as the Pasteur Institute of India (registered as a society under the Societies Registration Act 1860) and started functioning as an autonomous body under the Ministry of Health and Family Welfare, Government of India, New Delhi from the February 10, 1977. The affairs of the institute are managed by a governing body.

1.2 Treatment for rabies

The best post-exposure treatment after attacks by a rabid animal or one suspected of being rabid consists of cleansing the bite wound by flushing with soap and water, and starting an immunization protocol consisting of 5 doses of vaccine administered on days, 0,3,7,14,30 as soon as possible. The wounded areas should not be closed by suturing.

In 1881 Louis Pasteur published the first report on the successful treatment of a nine year old boy (who was severely bitten by a rabid dog) by vaccination with the rabbit spinal cord vaccine. Vaccination remained the sole treatment of exposed individuals until 1954.

In 1983, significant progress was made in the preparation and delivery of Rabies vaccine by adapting cell line as source of rabies antigen instead of animal source. Vaccines prepared in cell cultures are now widely available, and have been shown to combine safety with high immunogenicity. These vaccines prevent the development of rabies encephalitis mainly by inducing formation of neutralizing antibodies (Baer,1984). Successful immunization must be demonstrated by detecting adequate levels of neutralizing antibody in the serum of a vaccinated person.

Many laboratory tests have been evaluated for measuring antibody against rabies. Experience has shown that different tests may measure different types of antibody in the serum of rabies- immunized or naturally infected animals or humans. The serum may contain mixtures of immunoglobulins induced by either the rabies virus ribonucleoprotein internal antigens or the glycoprotein surface antigens.

Tests like the neutralization, such as Rapid Fluorescent Focus Inhibition Test (RFFIT), Mouse Neutralization Test (MNT) measure antibodies combining with the glycoprotein of the virus surface. Tests such as Mixed Hemadsorption and the Cytotoxicity test, employing the virus–coded glycoprotein antigen of the surface of rabies – infected cells also measure protecting antibodies.

The Mouse Neutralization Test (MNT) was developed by Webster and Dawson in 1935. It is the accepted reference method for all other antibody tests. The MNT is an in-vivo procedure, used mainly for the assay and potency testing of therapeutic anti–rabies serum and immunoglobulin, but it is also applicable to any serum containing rabies antibody. Thus, it can be used to determine the antibody titres of human sera collected during the therapeutic trials of different vaccines. In this test, after immunization with serum dilutions in mice, the death of the mouse is taken as the end-point. In-vivo results are difficult to reproduce because of uncontrollable factors, such as susceptibility or resistance to lethal infections and non-specific deaths. The MNT is time- consuming, expensive, needs large number of animals, and employs live rabies virus, which is at time dangerous to handle.

The Rapid Fluorescent Focus Inhibition Test (RFFIT) was developed by the California State Department Of Health, USA (Smith., 1973). The RFFIT is an *in vitro* test for antibody assay, which employs fluorescent- antibody stained foci of rabies virus growth in cell culture as an end-point. The RFFIT is performed in cell culture [Smith et al., 1973] and are depicted as International Units per ml (IU/ml)

RFFIT, as in-vitro test, has been standardized and is routinely performed at Pasteur Institute of India, Coonoor since 1995. Hundreds of sera samples have been screened. It is highly reproducible, Sensitive, specific and easy to perform.

The Mixed Hemadsorption Test (MH) originally described for rabies by Espmark, et al., 1978. It measures antibodies combining with antigens on rabies–infected cells equivalent to the glycoprotein antigen at the exterior of the budding virus. It is a sensitive, reliable, and technically convenient method as it has a capacity for testing many sera in the same run.

The objective is

(i) To evaluate tissue culture anti-rabies vaccine (TCARV) immunization response on human sera samples by Mouse Neutralization Test (MNT), Rapid Fluorescent Focus Inhibition Test (RFFIT) and Mixed Hemadsorption Test.

(ii) To compare, analyze and evaluate the merits and demerits of *in vivo* and *in vitro* tests.

LITERATURE REVIEW

2. LITERATURE REVIEW

2.1. Structure of Rabies Virus

The rabies virus is a negative-sense, non-segmented, single-stranded RNA virus measuring approximately 60 nm × 180 nm. It is composed of an internal protein core or nucleocapsid, containing the nucleic acid, and an outer envelope, a lipid-containing bilayer covered with Tran membrane glycoprotein spikes. The RNA is non- infectious, having a molecular weight of 4.6 x 10^6 Dalton. The entire genome of the PV strain of rabies virus has now been sequenced and shown to contain 11, 932 nucleotides (Tordo and Pouch, 1988).

The virus genome encodes five proteins associated with either the ribonucleoprotein (RNP) complex or the viral envelope (figure.1). The L (transcriptase), N (nucleoprotein), and NS (transcriptase-associated) proteins comprise the RNP complex, together with the viral RNA. These aggregate in the cytoplasm of virus-infected neurons and compose Negri bodies, the characteristic histopathologic finding of rabies virus infection. The M (matrix) and G (glycoprotein) proteins are associated with the lipid envelope. The G protein forms the protrusions that cover the outer surface of the virion envelope and is the only rabies virus protein known to induce virus-neutralizing antibody.

2.2. Transmission and Reservoir

The rabies virus is transmitted to other animals through close contact with saliva from infected animals. Bite is the main mode of virus transmission; scratching and licking on broken skin and mucous membrane.

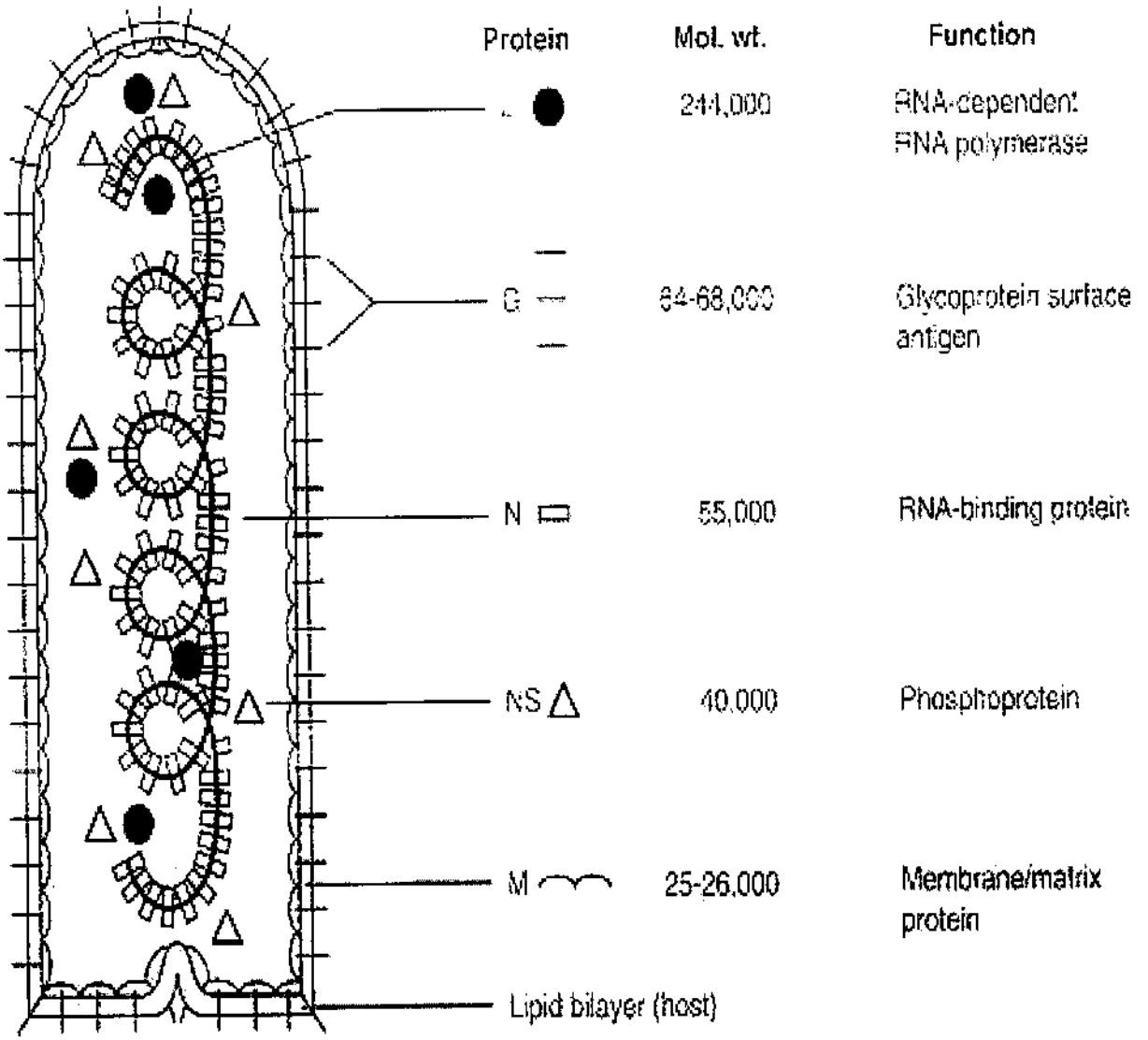

Figure 2.1.1. Virion structure of rabies virus.

can also transmit the disease. The transport of the virus from the site of virus entry occurs through the neuronal pathway into the brain which is the preferential site of virus replication. Once the virus reaches the central nervous system, it replicates massively.

The virus is then transported to many tissues, such as skeletal and cardiac muscles, adrenal glands, kidneys, retina, cornea, etc, through the nerves. Productive viral replication takes place predominantly in salivary glands, excreting virus transmissible to other mammals. The rabies virus is circulating in different susceptible populations of mammal species, which constitute the virus reservoir, allowing the maintenance of on epidemiological cycle of rabies in a certain geographical area. In most industrialized areas (USA, Canada, Europe), the main reservoirs of rabies are wild mammals (mainly raccoon, skunk, fox and bat in the USA and Canada and the red fox and raccoon dogs in Europe). In developing countries in Africa, Asia and South America dogs represent the major rabies reservoir, transmitting rabies to other animals and to humans as well. Different independent epidemiologic cycles exist in bats, involving different genotypes of *Lyssavirus* (in particular genotype 1 in the Americans, genotypes 5 (EBL1) and 6 (EBL2) in Europe and genotype 7 in Australia).

2.3. Pathogenesis

After virus inoculation through animal bite, local replication of virus at the site of entry may occur for a long time (Fekadu, 1988). The virus enters into peripheral nerves at the infection site and replicates in non nervous tissues (muscle cells). After uptake into peripheral nerves, the virus then moves along the nerve axons to the central nervous system (CNS) (Jackson, 2002). After infection develops within the CNS, the rabies virus

disseminates rapidly by centrifugal spread to peripheral nerves, which may lead to invasion of highly innervated sites of various tissues, including the salivary glands. The immune response to naturally acquired virus is slow and generally, specific rabies neutralizing antibodies are not detected before the onset of illness, leading to a fatal outcome in most cases (Fekadu, 1988).

2.4 Incubation Period

The incubation period (the time from initial viral exposure at first demonstration of clinical signs) is dependent upon viral dose, route and strain as well as the site of inoculation. Concentrated inoculum of virus produces a short period of incubation and a rapid course of the disease, before spread of rabies virus throughout the brain. Limited data are available on incubation periods after natural infection. Data available for rabid cats indicate an incubation period ranging from 9 days to 6 months, but generally being between 4 and 6 weeks. For dogs, this period ranges from a week to several months (Fekadu, 1988). Long incubation periods observed in naturally infected animals may be due to infection with very small amounts of virus (Fekadu, 1988).

For transmission of rabies, the key factor is the presence of the virus in saliva and salivary glands in particular, before the period where detectable clinical signs are recorded. It has been documented that dogs can excrete rabies virus in the saliva up to 14 days before clinical signs appear (Fekadu, 1988). Those data are crucial since transmission of rabies may unknowingly occur and therefore no preventive measures are taken because the animal appears healthy.

Serologic tests provide clinicians, epidemiologists, and research virologists with powerful tools for studying rabies virus. Detecting antibody may be useful in diagnosing rabies early in the clinical course of the disease, in providing an estimate of vaccine efficacy, and in assessing disease prevalence in areas of enzootic rabies.

The immune response to the varying doses, routes, and types of human vaccines (HEP Flurry, Harris or Sample) has been determined by the Neutralization Test (Grandien .,1971). This test was used by Rubin et al.,1971, to compare the human antibody response to vaccination with Duck embryo or HEP Flurry vaccines. Other studies have been conducted to determine the presence of neutralizing antibodies in individuals administered anti-sera soon after their exposure to rabies (Bahmanyar et al., 1976).

The neutralization test has also been used to determine the antibody response in animal vaccine studies. In one study, dogs were vaccinated with various rabies vaccines and the neutralization test was used to determine antibody in the pre-vaccination sera and sera taken 43 and 60 days after vaccination and one day before challenge (Khawplod et al., 2007).

Koprowski and Black (1952) reported the decline in antibody titer and resistance to challenge 2 years after the administration of Chick- embryo adapted rabies virus to dogs. In 1954, Koprowski and Black reported on the antibody response of guinea pigs vaccinated with Flurry Strain LEP.

Coyne et al., in 2001 reported a correlation between antibody response, vaccine dosage, and resistance to challenge in dogs, mice, guinea pigs, and hamsters. In this study of 162 dogs, 39.5% had antibody and

resisted challenge, 1.9% had antibody but did not resist challenge, and 64.3% without antibody died when challenged. This test has also been used to determine neutralizing antibody in foxes, skunks, raccoons, bats, and opossums (Sikes et al., 1971).

Introduction of Immunofluorescence into the diagnostic virology laboratory in the 1950s permitted the development of a number of in-vitro tests for measuring rabies antibodies. The development of a stable fluorochrome by Riggs et al., permitted the widespread application of the fluorescent antibody method introduced by Coons and Kaplan (1996).

The Standard procedure recommended at the Seventh meeting of the WHO Expert Committee on rabies was the Mouse Neutralization Test (MNT) and the Plaque Reduction assay. Since then, Plaque Reduction methods have been super-seeded by Rapid Fluorescent Focus Inhibition test (RFFIT), which are more convenient. Although, the MNT is still widely used as a reference test, RFFIT has been shown to be at least as sensitive as the MNT in measuring virus – neutralizing antibodies, and results have also been shown to correlate well with other tests such as the soluble antigen fluorescent antibody test, passive haemagglutination and radio-immuno assays. Reported differences in the potencies of rabies antibody preparations, as measured by the MNT and RFFIT, were not confirmed in a collaborative study carried out under the aegis of WHO.

RFFIT, as a method, where in Smith et al., 1973, described anti-rabies virus neutralization antibody in sera transudates can be detected.

At the workshop held at the Bureau of Biological, in 1975,

Bethesta, Mary Land (USA) discussed the concept of using the amount of antibody produced in immunized animals as an index of the potency of the immunizing agent; this can be estimated by RFFIT.

Human Cerebrospinal fluid and animal sera have been tested by the RFFIT and Mouse Neutralization Test [Smith et al., 1973] and there seems to be no significant difference between the antibody titers by either method.

Guillemin et al., (1981) compared Mouse Neutralization Test (MNT) and RFFIT using sera of different animal species including man after vaccination. They used a reference serum raised in a horse after two injections of rabies vaccine. A correlation of 0.96 between MNT and RFFIT was found.

Haase et al., (1985) assayed several lots of RIG (Rabies Immunoglobulin) and RIS (Rabies Immuno horse Serum) from different procedures for rabies antibodies by the standard Mouse Neutralization Test (MNT) and the RFFIT. Only for some of the tested lots were the results found to be comparable. For others, especially RIG lots, significant differences were found.

Fitzgerald et al., (1979) made a comparative study, which was undertaken by seven laboratories in six countries, on antibody determination by different methods such as Mouse Neutralization Test (MNT), RFFIT, Plaque Reduction Test (PRT), and Complement Fixation Test (CFT) in sera of persons vaccinated with HDCS rabies vaccine (Gerstl, et al.,1988). They reported that MNT, RFFIT, PRT, and CFT were unable to detect antibodies

in HDCS vaccines until 7 days after the first vaccination. After 7 days, 5 to 7 out of the 8 patients gave positive results with the MNT, RFFIT, and PRT. With CFT's, only one serum was found positive on day 7.

Monica Grandien and Ake Espmark in 1978 described the advantage and adequacy of the Mixed Hemadsorption (MH) test for rabies antibody determination. They compared the results obtained by MH test with neutralization values obtained from different types of rabies vaccines. There was a good correlation of results for both methods.

MATERIALS AND METHODS

3.MATERIALS AND METHODS

3.1 Mouse Neutralization Test

The basic method for assaying the virus neutralization activity of a serum has a series of steps which includes mixing the serum with virus, Incubating the virus-serum mixture under appropriate conditions and introducing the mixture into a susceptible host system in which the presence of un-neutralized virus can be detected. With the Mouse Neutralization (MN) test, the lack of viral neutralization is evidenced by death or illness. In this type of MN test, a standardized amount of virus is combined with various dilutions of serum.

3.1.1 Materials Required

Preparation of Serum

Different Patient's blood were allowed to clot and the serum was tested soon after collection, prolonged storage at 4^0 C may result in degradation of the antibody and multiplication of contaminating organisms, therefore if storage required, the sample should be kept frozen until tested. Repeated freezing and thawing may also cause a decrease in the amount of detectable antibody.

Diluents

2% Serum Saline – (1ml fetal bovine serum in 49 ml phosphate buffered saline.

Challenge Virus Strain

For the rabies neutralization test, a standard rabies challenge virus (CVS – Challenge Virus Standard) was used. This strain was collected from PII, Coonoor. The freeze dried stock was reconstituted with suitable diluents (Fetal Bovine Serum)

Laboratory Mice

The laboratory mice were used for a variety of viral and serological tests because of its susceptibility and availability and it is recommended as the test animal for determining rabies neutralizing antibody. For the MN test, Swiss – Albino mice of 11 – 14 g were used.

Co_2 incubator

The serum-virus mixture were incubated at 37°c in a co_2 incubator which containing 4% co_2.

3.1.2 Test Procedure

Inactivation of Sera

Sera samples (I, II, III, and IV) collected from different individuals were used in this test. 2% serum saline was taken as diluents. 0.6 ml of 2% serum saline was added to small glass tubes. 0.2 ml of the serum sample I was added to the tube to give 1:4 dilutions. Sample II and Sample IV were diluted to 1:16 by mixing 0.1ml of the sample with 1.5ml of 2% serum saline in the tubes. 0.3ml of the sample III (Negative Serum) was taken. It should not be diluted with 2% serum saline. The diluted serum

samples were kept in a rack and then placed in a water bath at 56^0C for 30 minutes for inactivation to remove the viral inhibitory agents.

Virus Dilutions

Virus dilutions were carried out similar to that of the RFFIT test, which can be described later.

Reference serum

Reference serum (a serum of known titer that has previously been calibrated against the International Standard for rabies immunoglobulin and then stored in 1 – 2 ml aliquots at -20^0 C or below) was also tested in a similar way.

Serum – virus Neutralization

0.5ml of virus was added to 0.5ml of each of the desired serum dilutions. For the negative sample, 0.2ml of virus was added to 1:2 and 1:4 dilutions and 0.1ml was added to the neat sample.The serum – virus dilutions were then agitated and incubated at 37^0C for 90 minutes in $C0_2$ incubator. After this incubation, the dilutions were placed in an ice-bath for ten minutes.

Dilution of Sera samples

The inactivated sera samples were diluted as per the tables shown below.

dilutions / sample (ml)	1:4	1:8	1:16	1:32
Sample I	0.5	0.5	0.5	0.5
Diluents	0.5	0.5	0.5	0.5

Table 3.1.2.1.1.SERIAL DILUTION FOR INACTIVATED SAMPLE I

dilutions / sample (ml)	1:32	1:64	1:128	1:256	1:512	1:1024
Sample I	0.5	0.5	0.5	0.5	0.5	0.5
Diluents	0.5	0.5	0.5	0.5	0.5	0.5

Table 3.1.2.1.2. SERIAL DILUTION FOR INACTIVATED SAMPLE II

dilutions → / sample (ml) ↓	1:2	1:4
Sample III	0.1	0.05
Diluents	0.1	0.15

Table 3.1.2.1.3. SERIAL DILUTION FOR INACTIVATED SAMPLE III

dilutions → / sample (ml) ↓	1:32	1:64	1:128
Sample IV	0.5	0.5	0.5
Diluents	0.5	0.5	0.5

Table 3.1.2.1.4. SERIAL DILUTION FOR INACTIVATED SAMPLE IV

Inoculating the mixtures into mice

0.03ml of each virus – serum dilutions were inoculated intracerebrally into 3 – 4 week old mice.

3.1.3.Observation

The mice were observed daily for 14 days for signs of rabies. 50% mortality end-point of the serum–virus series was calculated.

3.2 RAPID FLUORESCENT FOCUS INHIBITION TEST(RFFIT)

3.2.1 Requirements

CO_2 incubator or suitable air proof container for CO_2 atmosphere and ordinary incubator for 37^0 C. Inverted microscope with large stage. U-V inverted microscope (NIKON).Microtitre plates with lid containing 96 flat- bottom wells suitable for tissue culture use. Water bath which can be maintained at 56^0 C and 37^0 C ± 0.5^0 C. Eppendorf adjustable volume pipettes for 100- 1000 µ and 10- 100 µl with respective tips. Glassware – bottles, test tubes, graduated pipettes of 1 ml, 5ml and 10 ml capacity. Tissue culture medium: AT047–Minimum Essential Medium (MEM) with Earle's salts, L-Glutamine, Non-Essential Amino Acid (NEAA) supplemented with 10% Tryptose Phosphide Broth (TPB), 10% Fetal bovine serum, neomycin and fungi zone. Fetal bovine serum, heat inactivated and screened for Mycoplasmas and viruses. Screw – Cap Vials (polycarbonate or polystyrene) suitable for storing the cells in liquid nitrogen. Laminar – flow cabinet with vertical flow.

3.2.2. Test Procedure

Inactivation of Sera

Serum samples collected from different patients were used in this test to estimate the neutralizing antibody titer against rabies virus. Growth medium was taken as diluents. 300 µl were added to small glass tubes or plastic eppendorf tubes, which were numbered serially. 100 µl of the serum samples were added to the tube to give 1:4 dilutions of the test serum samples.

The last two tubes contained the WHO Reference Serum and Negative Serum control respectively. The WHO Reference Serum ampoule was taken out from -70^0 C freezer and 100 µl was added to 1.9 ml of diluents to give 1:20 dilution. The reference serum contained 10 IU/ml. The Negative serum control was diluted to 1:4. The diluted serum samples were kept in a rack and then placed in a water bath at 56^0 C for 30 minutes for inactivation to remove the inhibitory agents that interfere the antigen - antibody neutralization reaction.

Dilution of serum samples

Flat–bottom microtitre plate having 96 wells was sterilized by exposure to U-V rays for 60 minutes. 100 µl of Growth medium was added to rows B to H. No medium was added to wells in row A. One row of wells from A to H was used for each serum sample. 100 µl of 1:4 dilution of serum sample was added to row A. The same 1: 4 dilution of serum sample was added to row B, which already contained 100 µl growth medium, to obtain 1: 8 dilutions. Using the same tip, the contents in well B was mixed

and 100 µl transferred to well C to make up to 1:16 dilution. After mixing, 100 µl from well C was transferred to next well. This was continued till well H and there after mixing 100 µl was discarded. In this way the serum sample was diluted in two-fold steps from 1:4 in well A to 1:512 in well H. The WHO Reference Serum was diluted in two-fold steps from 1:20 in well A to 1:2560 in well H. The Negative Serum control was diluted from 1:4 to 1:8 kept in two wells.

Virus Control

Three rows of 5 wells each were used for virus controls. 100 µl of medium was added to each well.

Virus Dilutions

PV 3462 strain of rabies virus grown in Neuroblastoma-2A cell was used as challenge virus. It was distributed in 200 µl aliquots in Eppendorf tubes and stored at -70^0 C.

The challenge dilution 10^{-3} and next two dilutions 10^{-4} and 10^{-5} are kept in an ice bath for 10 minutes.

The challenge dilution of virus was added in 100 µl amounts to all the serum wells. The microtitre plate was covered with the lid and incubated at 37°c for 90 minutes.

The virus titration was performed in each test to confirm the $CCID_{50}$ (cell culture infective dose) of virus used in each test using the dilution of virus 10^{-3} to 10^{-5}.

Incubation of Serum- Virus Mixtures

The plates were incubated in CO_2 incubator at 37^0 C for 90 minutes in an atmosphere of 4% CO_2.

Propagation and Addition of Neuro-2A cells

An MD bottle of Neuro-2A cells was used when the cell monolayer was 3 to 4 days old. The surface of the monolayer cells were aspirated with 5ml growth medium and suspended in it. (Eagle Minimum Essential Medium [MEM], see appendix I).The cells were diluted to contain 25,000/ 0.05ml (one drop). This quantity of cells was added to all the wells with dropper.

Virus Titration

Before addition of Neuro-2A cells, the 100 µl of virus dilutions 10^{-3}, 10^{-4}, 10^{-5} was added into the wells.

Cell Control

One drop of cell suspension and 200 µl of medium were added to each of these wells in row of two wells. The plates were incubated for 48 hours in CO_2 incubator at 37^0C in an atmosphere containing 4% CO_2.

Dilutions	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}
SampleVirus (ml)	0.1	0.5	4.0	0.1	0.1
+	+	+	+	+	+
Growth Medium (ml)	0.9	4.5	36.0	0.9	0.9

Challenge Dilution(10^{-3})

Table3.2.2.1. Serial dilutions of Rabies Virus for Challenging Mice and RFFIT

Fixation of cells

At the end of the incubation, the contents of each well Tissue Culture Fluid (TCF) in the microtitre plates were removed using eppendorf pipette and 100 µl of 70% acetone in PBS was added to every well. The plates were chilled at -20^{0}C deep freeze for half-an-hour.

Staining with Rabies Conjugate

The fixative was removed with a pipette and one drop of rabies conjugate (Fluorescein Iso-ThioCyanate) diluted at 1:25 dilution (working dilution) was added to each well and the plate was further incubated at 37^{0}C for 2 hours. The conjugate was removed and the wells were washed gently with distilled water once. Two drops of 10% mounting glycerol buffer, pH 9.0 was added to all the wells.

3.2.3 Observation

The plates were observed in an inverted U-V microscope (NIKON) using transmitted U-V light and B-2 condenser filter. Neutralization was indicating the absence of virus specific fluorescence in the cells. Absence of neutralization was indicating the presence of numerous foci of virus-specific fluorescence.

Fig 3.2.3.1. RAPID FLUORESCENT FOCUS INHIBITION TEST

1: 4 Serum dilutions in a small tube

⇩

Inactivate at 56^0 C water bath

⇩

Dilute the Serum samples in a microtitre plate

⇩

Add 0.1 ml of rabies virus (challenge dilution 10^{-3}) to all the wells.

⇩

Incubate in CO_2 incubator for 90 minutes for Serum – virus Neutralization

⇩

Add Neuro – 2A cells to all the wells

⇩

Incubate the plate in Co_2 incubator for 48 hrs. Remove TCF and add 50% acetone buffer for fixation

⇩

Remove Fixation & add working dilution of Rabies Conjugate for Staining

⇩

Wash with distilled water & mount with glycerol buffer

⇩

Observe under Microscope

3.3 Mixed Hemadsorption Test

3.3.1 Requirements

Monolayer of Vero cells grown in $75cm^3$ bottles, Rabies Virus Strain – PV 3462, 2% Fetal Calf Serum growth medium, 0.75% agar, Test Serum, Round Whatman Filter Paper Disc (5mm).

3.3.2 Test Procedure

Two bottles ($75cm^3$) with monolayer of Vero cells were used in this test. One bottle was used for the test and another for the control. The growth medium in two bottles was decanted. The monolayer of Vero cells were infected with rabies virus strain. Infection can be done by adding 2ml of virus and 2ml of growth medium to each bottle. These bottles were kept for adsorption in an incubator for 90 min.

After incubation, 20ml of Eagle's MEM supplemented with 2% fetal calf serum was added to each bottle, and further incubated for 3 days at 37^0C.Round Whatman filter paper discs (5mm) were soaked with 20µl test serum. For control, Phosphate Buffered Saline (PBS) was used. The infected tissue culture was washed once in PBS and overlaid with 20ml of 0.75% agar (at a temperature of 40 - 42^0C) containing 5% calf serum. The test serum paper discs were applied on the surface of the agar and the bottles were kept for 2 days at room temperature to allow antibodies to diffuse through the agar and attach to the surface of the infected cells. During this time, the bottles were left upside down to prevent any fluid in the bottle from reaching the test system. The agar sheet was then removed by the rapid inserting of 10ml of PBS at the edge of the agar.

RESULTS

4. RESULTS

4.1 INTRODUCTION

Blood sera collected from different individuals were used to determine antibody against rabies virus. Detection was carried out using Mouse Neutralization Test (MNT) and Rapid Fluorescent Focus Inhibition Test (RFFIT) and Mixed Hemadsorption (MH) Test. Whenever an antibody titre of test serum is determined by any method, a reference serum of known titre calibrated against the International Standard is also run in parallel along with test serum. The values obtained were expressed in International Units/ml, in comparison with international reference serum.

The main objective of this study was to evaluate immunization response on human sera samples by *in vivo* (MNT) and *in vitro* (RFFIT and Mixed Hemadsorption) test. This study was aimed at standardization and validation of RFFIT method on Neuro-2A cell system for neutralizing antibodies detection and to find the more sensitive method and consequently use it as routine test in the laboratory. The test serum I, II, III, IV and reference serum were diluted into two- fold dilutions, to achieve 50% end-point within the range of these dilutions.

4.2.DETECTION OF ANTIBODY BY MOUSE NEUTRALIZATION TEST (MNT)

For MNT test, the dilutions (1:4, 1:8, 1:16, 1:32, 1:64, 1:128, 1:256, 1:512) were inoculated intracerebrally (fig.4.2.1) into healthy Swiss albino mice(fig.4.2.1.2). (5 mice per dilution). The mice were kept at standard environmental condition and fed as per the laid down criteria and

observed each day and seen for any symptoms *viz.* paralysis of limbs, hunched back, tail rising, shivering, etc (Fig.4.2.1.3,4.2.1.4). Those mice that die during the first 4 days were recorded as non- specific deaths, whereas mice that die during the next 10 days were recorded as dying of rabies. Number of mice survived/dead was recorded each day. The results obtained by MNT test are given in the Table 4.2.2.

4.3.DETERMINATION OF ANTIBODY LEVEL BY RAPID FLUORESCENCE FOCUS INHIBITION TEST

The Rapid Fluorescent Focus Inhibition Test (RFFIT) is rabies – virus neutralization test run in cell culture to determine the rabies antibody level in human sera. The principle is that rabies- specific antibodies present in the test serum samples will neutralize the challenge virus, thereby preventing infection of the mouse neuroblastoma cell monolayer.

The Rapid Fluorescent Focus Inhibition Test (RFFIT) is probably the most widely accepted alternative to the MNT. Immuno-fluorescence staining rather than cytopathic effect is used as an indicator of viral growth. (Fig.4.3.1.1 & 4.3.1.2). In the absence of replicating viral antigen, specific fluorescence will not be observed. Conversely, the presence of specific fluorescence indicates viral replication and therefore absence of neutralizing antibody. The RFFIT takes only 24 hr and is both sensitive and accurate. The results obtained in RFFIT method performed are given in the Table.4.3.2.

4.4.DETECTION OF ANTIBODY BY MIXED HEMADSORPTION TEST

The Mixed Hemadsorption Test is shown to be an adequate test to evaluate the immunization response. The MH test measures antibody directed against virus – surface antigens in an infected cell monolayer. Vero cells infected with the rabies virus strain adapted to tissue culture produced a Hemadsorption phenomenon. Under appropriate conditions, rabies – specific antibodies were allowed to diffuse from soaked filter paper discs placed on the agar. After 48 hr reaction, the agar layer was removed. The round zones of Hemadsorption were observed for the presence of specific antibodies. Table.4.4.1 summarizes the results of MNT, RFFIT, and Mixed Hemadsorption methods.

4.5. COMPARISON OF MNT, RFFIT, MH TEST

Four different sera samples were collected from four different rabid dog bitten patients. Depending upon the concentration of antibodies, the sera samples were diluted with suitable diluents (2% serum saline). As the dilution increases, the concentration of anti body decreases and so the resistance to the antigen virus level drops gradually. At higher dilutions, the challenge virus is partially neutralized and hence it leads to infection, when inoculated in mice, in Mouse Neutralization Test. At higher dilutions, the mortality rate of the mice increases. MNT could be compared with the RFFIT where the presence of antibody level could be determined from the fluorescence level. Therefore the amount of anti bodies could be determined by using the reference sample. The N- neutralized samples indicate the presence of antibodies whereas the +-(non-neutralized samples) indicate the

absence of antibodies. In the Mixed hemadsorption test, the Z- zone indicates the presence of antibodies while the NZ- no zone indicates the absence of antibodies. Table 4.5.1. Shows the comparison of MNT, RFFIT and MH Test.

Fig.4.2.1.Intracerebral Inoculation in Swiss – Albino Mouse

Fig.4.2.1.1. Comparison of heathy mouse and mouse showing rabies symptoms

Fig.4.2.1.2. Normal Mouse (Swiss - Albino)

Fig.4.2.1.3.Paralysed mouse

Fig.4.2.1.4. Hunched back with Tail raising

Sample Identity	Dilution	Mice No.	Days Of Observation													
			1	2	3	4	5	6	7	8	9	10	11	12	13	14
I	8	1	/	/	/	/	/	/	/	/	/	/	/	/	/	/
		2	/	/	/	/	/	/	/	/	/	/	/	/	/	/
		3	/	/	/	/	/	/	/	/	/	/	/	/	/	/
		4	/	/	/	/	/	/	/	/	/	/	/	/	/	/
		5	/	/	/	/	/	/	/	/	/	/	/	/	/	/
	16	1	/	/	/	/	*/	*/	S	S	D					
		2	/	/	/	/	/	/	/	/	/	/	/	/	/	/
		3	/	/	/	/	/	/	/	/	/	/	/	/	/	/
		4	/	/	/	/	/	/	/	/	/	/	/	/	/	/
		5	/	/	/	/	/	/	/	/	/	/	/	/	/	/
	32	1	/	/	/	/	*/	*/	*/	S	S	D				
		2	/	/	/	/	/	*/	*/	S	S	D				
		3	/	/	/	/	/	/	/	/	/	/	/	/	/	/
		4	/	/	/	/	/	/	/	/	/	/	/	/	/	/
		5	/	/	/	/	/	/	/	/	/	/	/	/	/	/
II	256	1	/	/	/	/	/	/	/	/	/	/	/	/	/	/
		2	/	/	/	/	/	/	/	/	/	/	/	/	/	/
		3	/	/	/	/	/	/	/	/	/	/	/	/	/	/
		4	/	/	/	/	/	/	/	/	/	/	/	/	/	/
		5	/	/	/	/	/	/	/	/	/	/	/	/	/	/
	512	1	/	/	/	/	/	/	/	/	/	/	/	/	/	/
		2	/	/	/	/	/	/	/	/	/	/	/	/	/	/
		3	/	/	/	/	/	/	/	/	/	/	/	/	/	/
		4	/	/	/	/	/	/	/	/	/	/	/	/	/	/
		5	/	/	/	/	/	/	/	/	/	/	/	/	/	/
	1024	1	/	/	/	/	/	*/	*/	*/	S	S	D			
		2	/	/	/	/	/	/	/	/	/	/	/	/	/	/
		3	/	/	/	/	/	/	/	/	/	/	/	/	/	/
		4	/	/	/	/	/	/	/	/	/	/	/	/	/	/
		5	/	/	/	/	/	/	/	/	/	/	/	/	/	/
III	Neat	1	/	/	/	*/	*/	S	S	D						
		2	/	/	/	*/	*/	*/	S	S	D					
		3	/	/	/	*/	*/	*/	S	S	D					
		4	/	/	/	*/	*/	*/	S	S	D					
		5	/	/	/	*/	*/	*/	S	S	D					
	2	1	/	/	/	*/	S	S	D							

		2	/	/	/	'/	S	S	D							
		3	/	/	/	'/	'/	S	D							
		4	/	/	/	'/	'/	'/	S	D						
		5	/	/	/	'/	'/	'/	S	D						
	4	1	/	/	/	'/	S	S	D							
		2	/	/	/	'/	S	S	D							
		3	/	/	/	'/	S	S	D							
		4	/	/	/	'/	'/	S	S	D						
		5	/	/	/	'/	'/	S	S	D						
IV	32	1	/	/	/	/	/	/	/	/	/	/	/	/	/	/
		2	/	/	/	/	/	/	/	/	/	/	/	/	/	/
		3	/	/	/	/	/	/	/	/	/	/	/	/	/	/
		4	/	/	/	/	/	/	/	/	/	/	/	/	/	/
		5	/	/	/	/	/	/	/	/	/	/	/	/	/	/
	64	1	/	/	/	/	/	/	/	/	/	/	/	/	/	/
		2	/	/	/	/	/	/	/	/	/	/	/	/	/	/
		3	/	/	/	/	/	/	/	/	/	/	/	/	/	/
		4	/	/	/	/	/	/	/	/	/	/	/	/	/	/
		5	/	/	/	/	/	/	/	/	/	/	/	/	/	/
	128	1	/	/	/	/	'/	'/	'/	S	S	D				
		2	/	/	/	/	'/	'/	'/	S	S	D	/	/	/	/
		3	/	/	/	/	/	/	/	/	/	/	/	/	/	/
		4	/	/	/	/	/	/	/	/	/	/	/	/	/	/
		5	/	/	/	/	/	/	/	/	/	/	/	/	/	/

Table.4.2.2. Daily Observation Chart for Mouse Neutralization Test (MNT)

Mice used for Inoculation: Swiss Albino Mice of 11- 14 gm weight

/ -- Remain Healthy

'/ -- Doubtful one

S – Showing Rabies Symptoms

D – Died of Rabies Symptom

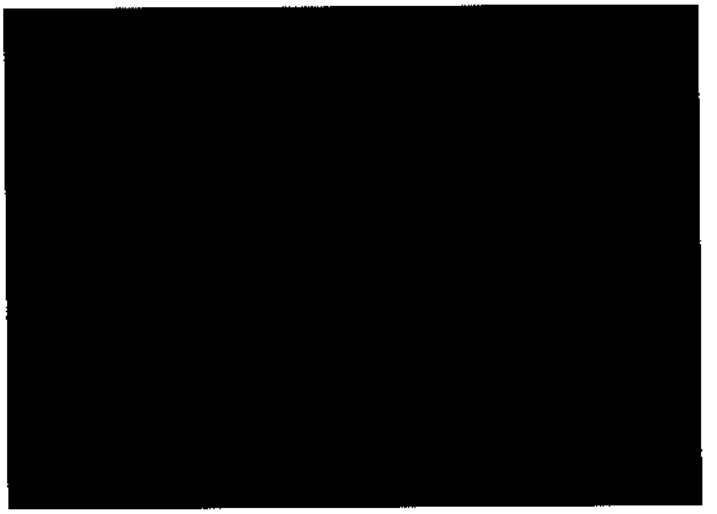

Fig.4.3.1.1. Absence of Fluorescence indicates presence of rabies specific antibodies (Virus neutralized)

Fig.4.3.1.2. Presence of fluorescence indicates absence of rabies specific antibodies (Virus Unneutralized)

dilution → / serum no ↓	512	256	128	64	32	16	8	4
WHO Reference	2560 +	1280 +	640 +	320 +	160 +	80 N	40 N	20 N
SampleI	+	+	+	+	+	N	N	N
Sample II	N	N	N	N	N	N	N	N
Sample III	+	+	+	+	+	+	+	+
Sample IV	+	+	+	N	N	N	N	N
Control	VC 10^{-5} +	VC 10^{-5} +	VC 10^{-5} +	VC 10^{-5} +	VC 10^{-4} +	VC 10^{-4} +	VC 10^{-4} +	VC 10^{-4} +

Table 4.3.2. Observation Chart for RFFIT.

N - Absence of Fluorescence i.e., Cent Percent Neutralization (Presence of Rabies Virus Neutralizing Antibody)

+ - Presence of Fluorescence i.e., Absence of Rabies Virus Neutralizing Antibody

VC - Virus Control of Dilutions 10^{-3} (Challenge Dilution), 10^{-4}, and 10^{-5}

Serum No.	*Antibody Titre (IU/ml)*
Sample I	2
Sample II	64
Sample III	<0.5
Sample IV	8

Table 4.3.3. Determination of amount of antibody using RFFIT method

Calculation

The highest dilution of the serum under test that completely neutralizes the challenge virus, as evidenced by the absence of infected cells in the RFFIT test, is the Antibody titre. This is expressed in International Units/ml and is determined by comparing the neutralization end- point of the serum under test with that of the standard reference serum.

$$\text{Antibody titre of Serum (IU/ML)} = \frac{\text{Antibody concentration of reference Serum}}{\text{Endpoint Dilution of Reference Serum}} \times \text{end point dilution of test serum}$$

The WHO Reference Serum (10 IU/ml) was neutralized in 1: 80 dilutions. From this, the titre of the test serum was calculated in IU/ml.

Calculation

Sample I: (16/80) x 10 = 2 (IU/ML)

Sample II: (512/80) x10 = 64 (IU/ML)

Sample III: (32/80) x10 = 0.4 (IU/ML)

Sample IV: (64/80) x10 = 8 (IU/ML)

Serum Sample	Dilution	OBSERVATION
Sample I	8	Z
	16	Z
	32	NZ
Sample II	256	Z
	512	Z
	1024	Z
Sample III	NEAT	NZ
	2	NZ
	4	NZ
Sample IV	32	Z
	64	Z
	128	NZ

TABLE 4.4.1. OBSERVATION CHART FOR MH TEST

Z -- Round Zones of Hemadsorption indicates presence of rabies specific antibodies

NZ -- No Zone indicates absence of rabies specific antibodies.

Serum Sample	Dilution	MH Test	In-Vitro RFFIT	Rabies Antibodies (RFFIT) (IU/ml)	In- Vivo MNT
Sample I	8	Z	N	2	5/5 alive
	16	Z	N		4/5 alive
	32	NZ	+		3/5 alive
Sample II	256	Z	N	64	5/5 alive
	512	Z	N		5/5 alive
	1024	Z	Not Known		4/5 alive
Sample III	Neat	NZ	Not Known	<0.5	5/5 dead
	2	NZ	Not Known		5/5 dead
	4	NZ	+		5/5 dead
Sample IV	32	Z	N	8	5/5 alive
	64	Z	N		5/5 alive
	128	NZ	+		3/5 alive

Table 4.5.1. **COMPARISION OF THE RESULTS OF MH TEST , RFFIT AND MNT**

RFFIT -- Rapid Fluorescent Focus Inhibition Test

MNT -- Mouse Neutralization Test

MH -- Mixed Hemadsorption

Z -- Round Zones of Hemadsorption indicates presence of rabies specific antibodies

NZ -- No Zone indicates absence of rabies specific antibodies

DISCUSSION

5. DISCUSSION

The Mouse Neutralization Test (MNT) has been considered the standard method for measuring the rabies neutralizing antibody level recommended by the World Health Organization but the disadvantage of this method is the use of animals for experiments and time taken. In recent years many reference laboratories such as Pasteur Institute of India, Coonoor, has been used Rapid Fluorescent Focus Inhibition Test (RFFIT) for detecting or measuring rabies antibody.

The MNT has been accepted as a standard method for determination of rabies antibody titer by WHO. A constant amount of rabies challenge virus standard strain was mixed with serial dilution of the test serum. Following an incubation period to permit virus neutralization (if antibodies present) aliquots of each dilution was inoculated intracerebrally into mice. 5 mice per dilution were used in this test. In MNT, the mortality rate of the mice increases as the dilutions of the sample sera increases. In this test 60 mice were used at different dilutions in which 21 were dead, 29 were showing severe rabies symptoms and 10 were alive.

In the present work, RFFIT for rabies antibody estimation has been standardized in which mouse neuroblastoma cell line, Neuro-2A and tissue culture and microtitre plates were used .The RFFIT has been internationally recognized as a standard in- vitro test for measuring the virus neutralizing antibodies. CVS (Challenge Virus Standard) strain of rabies virus was used as challenge virus and the infected cells were indirectly detected by staining with Fluorescent Iso-ThioCyanate (FITC) – conjugated rabies antibody in

microtitre plate. The plates were observed under UV microscope. The presence of fluorescence indicates the absence of viral specific antibodies whereas the absence of fluorescence indicates the presence of viral specific antibodies. The amount of antibodies could be measured by using the reference serum sample.

In Mixed Hemadsorption test, the zone formation indicates the presence of antibodies. The absence of zone indicates the absence of anti bodies. In this test, the amount of antibodies could be determined from the diameter of the zone. The zone with 5 mm diameter is considered to be the most efficient. Hence the RFFIT was found to be more sensitive than the MNT and Mixed Hemadsorption Test, as the amount of antibody level could be determined exactly.

The Mixed Hemadsorption Test was performed in $75cm^3$ bottles with agar – covered monolayer of rabies – infected cells. Rabies – specific antibodies were allowed to diffuse from soaked filter paper discs on the agar. After a 48 hr reaction, the agar layer was removed. Round zones were obtained for the presence of specific antibodies.

Rabies antibody data obtained by the MNT, RFFIT, and Mixed Hemadsorption Test were compared. There was a good correlation between the results obtained by the three methods. The experiments presented here speculated the applicability of Neuro-2A on RFFIT, avoiding the use of different cell lines for the same purpose. The standardization of RFFIT using this cell line demonstrated a strength correlation between the two techniques

(MNT & RFFIT). RFFIT method standardized on Mouse Neuroblastoma cell line for neutralizing antibodies detection turns the diagnosis easier and less expensive, when it was compared with those obtained by Chick Embryo Related (CER) cell line performed by Cardoso, Queiroz da Silva et al in 2006.

Compared with the MNT test, Mixed Hemadsorption test offers the advantage of being technically more convenient because of its capacity for testing numerous sera in a single run.

Since the time required to perform RFFIT was shorter than MNT, it was suggested that the RFFIT could be substituted for the standard MNT. Added to this, RFFIT can adequately measure antibody levels of patients being vaccinated.

CONCLUSION

6. CONCLUSION

The aim of our study was to detect the level of rabies antibody in the human sera and to compare three laboratory tests for that purpose: MNT (Mouse Neutralization Test), RFFIT, and Mixed Hemadsorption Test.

The present study as shown in the results demonstrated that the antibody determination by RFFIT method performed on Neuro-2A cell system showed a good correlation with that of conventional Mouse Neutralization test (MNT) and Mixed Hemadsorption Test.

The MH test for rabies antibodies was especially suited for laboratories in which this type of test is routinely used for other purposes, e.g. rubella, respiratory syncytial virus, herpes simplex, varicella and certain auto-antibodies.

As RFFIT is an *in vitro* test where it does not need to be observed for a longer time and also does not need the use of animals, RFFIT can be performed in any standard virology laboratory in microtitre plates and tests can be completed within 30 hours.

Finally, the great advantage of RFFIT method performed on Neuro-2A described here indicated that RFFIT was more sensitive than the other two methods and it was suitable for measuring the rabies neutralizing antibody in routine work.

APPENDICES

APPENDICES

APPENDIX I

EAGLE'S MINIMUM ESSENTIAL MEDIUM:

A tissue culture medium developed by H. Eagle containing vitamins , amino acids, inorganic salts and serous enrichments and dextrose.

10% tryptose phosphate broth, 10% fetal bovine serum with antibiotics fungi zone – 0.5% and neomycin 1% , pH adjusted to 7.2 with $NaHCO_3$.

APPENDIX II

FETAL CALF SERUM:

Human cells and tissues are grown in a culture form and in order for the cells or tissue to grow and proliferate , a source of nutrients , hormones and growth factors must be added. The useful supplement is fetal calf serum- also known as fetal bovine serum- a product that is cruelly derived from the fetuses of cows found pregnant at slaughter. Serum is the blood without any clotting factors and fetal calf serum especially, is considered to be rich source of nutrients.

It has been estimated that around half a million liters of raw FCS is produced each year world wide which equates to the harvesting of more than one million bovine fetuses annually, some sources have suggested that the actual figure may be closer to two million fetuses per

year.

METHOD OF COLLECTION:

After slaughter and bleeding of the cow at an abattoir, the mother's uterus containing the calf fetus is removed during the evisceration process(removal of mother's internal organs) and transferred to the blood collection room. A needle is then inserted between the fetus's ribs directly into its heart and the blood is vacuumed into a sterile collection bag. This process is aimed at minimizing the risk of contamination of the serum with micro organisms from the fetus and its environment. Only fetus over the age of three months are used otherwise the heart is considered too small to puncture. Once collected, the blood is allowed to clot at room temperature and the serum separated through a process known as refrigerated centrifugation.

REFERENCES

REFERENCES

1. Bahmanyar, M., Fayaz , A. and Nour-Salehi , SH. (1976) 'Successful protection of humans exposed to rabies infection'- JAMA 236,pp. 2751-2754.
2. Bourhy, H., Kissi, B., Tor, N., (1993) 'Molecular diversity of the Lyssavirus genus'- Virology, Vol.194,No.1,pp. 70-81
3. Briggs, DJ., Smith, JS., Mueller, LF., Schwenke, J., Davis, R.D. and Gordon, RC. (1998) 'A comparison of two serological methods for detection the immune response after rabies vaccination in dogs and cats being exported to rabies free areas'- Biologicals, Vol.26,pp. 347-355.
4. Byron, S. and Berlin, MD. (1990) 'Rabies vaccine adsorbed neutralizing antibody titres after 3 – dose- pre exposure vaccination'- American J., Vol. 80,pp. 476- 477.
5. Cardoso, TC., Queiroz da Silva, LH., S E L da Silva., Albas, A., Pardo, PE., Tanaka, AH., Cossy, LB. and Perri, SHV.(2006) 'Chicken embryo related (CER) cell line for quantification of rabies neutralizing antibody by fluorescent focus inhibition test'- Biologicals, Vol.34, pp.29- 32.
6. Cox, JH., Dietzschold, B. and Schneider, LG. (1977) 'Rabies virus glycoprotein. II. Biological and serological characterization'- Infect. Immun.Vol.16, pp. 754-759.
7. Coyne, MJ., Burr, JH., Yule, TD., Harding, MJ., Tresnan DB. And McGavin. (2001) 'Duration of immunity in dogs after vaccination or naturally acquired infection'-Vet Rec.,Vol.149,No.17,pp.509-15.
8. Dreesen, G and David, H .(1980) 'A Global Review of Rabies Vaccines for Human Use',- Vaccine Vol.15. pp.52-56.
9. Dietzschold, B., Gore M., Marchadier, D., Niu, HS., Bunschoten , HM., Otvos, L Jr., Wunner, WH., Ertl, HC., Osterhaus, AD. and Koprowski, H. (1990) ' Structural and immunological characterization of a linear virus-neutralizing epitope of the rabies

virus glycoprotein and its possible use in a synthetic vaccine.',-Virol. Vol.64,No.8,pp. 3804-3809.
10. Fekadu, M. (1988) 'Pathogenesis of Rabies Virus Infection in Dogs',- Reviews of Infectious Diseases Vol. 10, No.44,pp. 678-683.
11. Fitzgerald, EA. (1978) 'Laboratory evaluation of the immune response to rabies vaccine',- J., Biological Standardization Vol. 6,pp. 101- 109.
12. Fitzgerald, EA . (1979) 'A collaborative study on the testing of rabies immunoglobulin (human) by the mouse neutralization test (MNT) and the Rapid Fluorescent Focus Inhibition Test (RFFIT)',- J., Biological Standardization Vol. 7,pp. 67- 72.
13. Fu, ZF. and Wasi, C. (1986) 'Rabipur Proceedings', -Vaccine Vol.15,pp.51
14. Gerstl, F., Kurhz, I., Kot Zina, M and Dostal V. (1988) 'The difference in potency of anti-rabies immunoglobulin evaluated by the mouse neutralization test (MNT) and rapid fluorescent focus inhibition test (RFFIT)',-Report of the WHO consultation on rabies, pp.1160.
15. Grandien, M., (1971) 'Evaluation of tests for rabies antibody and analysis of serum responses after administration of three different types of rabies vaccines',- J., clinical microbiology Vol. 5,pp. 263-267.
16. Grandien, M. and Espmark, JA. (1974) 'Follow – up of antibody responses after post – exposure rabies immunization',- Symposia series in immunobiological standardization Vol. 21,pp. 243- 252.
17. Guillemin, F. (1981) 'Comparison of two methods for the determination of rabies neutralizing antibodies'. –J., Biological Standardization Vol 9,pp. 147- 156.
18. Haase, M., Seinsche, D. and Schneider, W. (1985) 'The mouse neutralization test in comparison with the RFFIT: differences in the results in rabies antibody determinations',- J., Biological Standardization Vol.13,pp. 123- 128.
19. Kaprowski, H. and Black, J. (1952) 'Studies on chick embryo adapted rabies virus;Duration of immunity in vaccinated dogs,- Exp Biol MedJul Vol.80,No.3,pp.410-5.
20. Khawplod, P., Wilde, H., Benjavongkulchai, M., Sriaroon, C. and Chomchey, P. (2007) 'Immunogenicity study of abbreviated rabies preexposure vaccination schedules',- J.TravelMed.Vol.14,No.3,pp.173-6.
21. Kuzmin, IV., Orciari, LA., Arai, YT., Smith, JS., Hanlon, CA.,

Kameoka, Y. and Rupprecht , CE. (2003) 'Bat lyssaviruses (Aravan and Khujand) from Central Asia: phylogenetic relationship according to N, P and G gene sequences'.-Virus Res Vol. 2, pp. 65-79.
22. Louie, RE., Dobkin, Mb., Meyer, P., Chin, B., Roby, RE., Hamonar, AH. and Cabasso, VF. (1975) 'Measurement of rabies antibody: comparison of the mouse neutralization test (MNT) with the rapid fluorescent focus inhibition test (RFFIT)',- J.,of Biological Standardization Vol. 3,pp. 365- 373.
23. Coons, FX., Kaplan, MM. and Koprowski, H. (1996) 'A rapid fluorescent focus inhibition test (RFFIT) for determining rabies virus-neutralizing antibody',- In: laboratory techniques in Rabies Vol.4,pp. 181- 192.
24. Monica Grandien, W. and Ake Espmark. (1978) 'The advantage and adequacy of the Mixed Hemadsorption test for rabies antibody determination',- Development of Biological Standardization Vol. 40,pp. 237– 242.
25. Riggs, JL., Seiwald, RJ., Burckhalter, JH., Downs, CM. and Metcalf, TG. (1958) 'Isothiocyanate compounds as fluorescent labeling agents for immune serum',-American J., Vol.34,No.6,pp.1081-97.
26. Rubin, RH., Dierks, RE., Gough, P., Gregg, MB., Gerlach, EH and Sikes, RK. (1971) 'Immunoglobulin response to rabies vaccine',-Lancet.Vol.2,pp.626-628.
27. Singh, J., Jain, DC., Bhatia, R. and Ichhpujani, RL. (1998) 'Epidemiological characteristics of rabies in India and surrounding areas',-IndianPediatr,Vol.38,No.12,pp.1354-60.
28. Smith, JS., Yager, PA. and Baer, GM. (1973) 'A rapid tissue culture test for determining rabies neutralizing antibody',- In: Laboratory Techniques in Rabies Vol. 23, pp.253- 357.
29. Trabelsi, K., Rourou, S., Loukil, H., Majoul, S. and Kallel, H.(2006) 'Optimization of virus yield as a strategy to improve rabies vaccine production by Vero cells in a bioreactor',-J Biotechnol. Vol.121,No.2,pp.261-71.
30. Tordo, N., Poch, O., Ermine, A. and Keith, G.(1986) 'Primary structure of leader RNA and nucleoprotein genes of the rabies genome: segmented homology with VSV',-Nucleic AcidsRes.Vol.14,No.6,pp.2671-2683.
31. Webster, WA., Charlton, KM. and Casey, GA. (1935) 'Persistent infections of a field strain of rabies virus in murine neuroblastoma (NA-C1300) cell cultures',-Can J Vet. Res.Vol.53,No.4,pp.445-448.