DEVELOPMENT OF ALTERNATIVE APPROACHES FOR IN PROCESS QUALITY CONTROL OF RABIES VACCINE

Thesis

Submitted in partial fulfilment of the requirement for the degree of

MASTER OF VETERINARY SCIENCE

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VETERINARY VIROLOGY

By

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To

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Certified that the research work embodied in this thesis entitled **"Development of alternative approaches for in process quality control of rabies vaccine"** *submitted by* **Dr. Manoj Kumar, Roll No. 4738,** *for the award of* **Master of Veterinary Science** *degree in* **Veterinary Virology** *at Indian Veterinary Research Institute, Izatnagar, is the original work carried out by the candidate himself under my supervision and guidance.*

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(Manoj Kumar)

Abbreviations

ABLV	Australian Bat Lyssa Virus
BHK-21	Baby Hamster Kidney cells
BPL	Beta propiolactone
CADRAD	Centre for Animal Disease Research and Diagnosis
CDC	Centers for Disease Control and Prevention
CPE	Cytopathic effect
CVS	Challenge Virus Standard strain
cDNA	Complementry deoxy ribonucleic acid
EBLV	European Bat Lyssa Virus
ELISA	Enzyme linked immunosorbent assay
ERA	Evelyn Rokitniki Abelseth strain
FAT	Fluorescent Antibody Test
FAVN	Fluorescent Antibody Virus Neutralization Test
FBS	Fetal Bovine Serum
FITC	Fluorescein iso thiocyanate
G	Glycoprotein
GMEM	Glasgow minimum essential medium
Gs	Secretary Glycoprotein
HEP	High Egg Passage
I/P	Intra peritoneal route
IVRI	Indian Veterinary Research Institute
L	Polymerase
LEP	Low Egg Passage
M	Matrix protein
MNT	Mouse neutralization test
MOI	Multiplicity of infection
N	Nucleoprotein
mRNA	Messenger Ribonucleic acid
NAChR	Nicotinic Acetyl Choline Receptor
NCAM	Nerve cell Adhesion Molecule

NS	Non structural protein
OIE	Office International des Epizooties
P	Phosphoprotein
p75 NTR	p75 Neurotropin Receptor
PBS	Phosphate Buffer Saline
PM/K	Pittman Moore/Karamany strain
PV	Pasteur Virus strain
RFFIT	Rapid Fluorescence Focus Inhibition Test
RNA	Ribonucleic Acid
RNP	Ribonucleoprotein
RREID	Rapid rabies enzyme immuno diagnosis
SAD-B19	Street Alabama Dufferin B-19 strain
VNT	Virus Neutralization Test
WHO	World Health Organization

Units of Measurement/Notations

%	Percentage
ºC	Degree Celsius
Cat. #	Catalogue number
cm^2	Square centi metre
g	Gram
hr	Hour
LD_{50}	Lethal Dose 50
Log_{10}	Logarithmic scale 10
µl	Micro litre
M	Molar
min	Minutes
ml	Milli litre
M. Wt.	Molecular weight
nm	Nano metre
FFU	Fluorescent Focus Units
pH	Negative logarithm of Hydrogen ion concentration
rpm	Revolutions per minute
v/v	Volume/Volume

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Chapter I

Introduction

Rabies is one of the oldest, dreadful and highly contagious diseases known to mankind since early civilization dating back 5000 years. It is prevalent in all parts of world except Australia, New Zealand, Britain, Japan and Scandinavia and is transmitted through bite of rabid animals. Rabies is a severe and fatal viral disease affecting central nervous system of warm-blooded animals, including man (Van Regenmortel *et al.,* 2000). It is enzootic and sometimes epizootic in a variety of mammalian species, insectivorous and hematophagous bats (WHO, 1973). The virus is usually introduced by a bite wound. Although penetration can occur through intact mucous membranes and the digestive tract (Fischman, 1968), but not through intact skin. Airborne natural infection is also possible in exceptional circumstances, as in caves harboring large numbers of bats carrying the virus (Constantine, 1967).

In Asia, the main route of rabies virus transmission is through rabid dog bites which is responsible for 96-98% of death from rabies in human, other animal species like cat, cattle, monkey, mongoose etc. Serologic evidence of infection in bats has also been documented in Cambodia (Reynes *et al*., 2004). In Asia more than 2.5 billion people are potentially exposed to rabies infection, each year. An estimated 8 million people receive treatment after being exposed to animals that are suspected for rabies. The economic burden in Asia has been estimated to be US$ 563 million (96% of the total burden of rabies world wide) (WHO, 2004 and Knobel *et al.,* 2005). It has been estimated that every year 55,000 people die from rabies world wide out of this 31,000 (56%) of these deaths occurs in Asia, mainly (90%) in rural areas (WHO, 2004 and Knobel *et al.,* 2005).

It has remained an important cause of disease in animal reservoirs with significant human cases, especially in the developing countries (Haupt, 1999). In India, rabies is endemic

except the Lakshdweep, Andaman and Nicobar, Dadra and Nagar Haveli. States like Manipur, Meghalaya, Sikkim, Arunachal Pradesh, Mizoram and Nagaland have reported occasional cases of rabies while substantial deaths have been reported in all other states of India (Sharma, 1990). It has been estimated that approximately 20,000 persons die from rabies every year in India (Sudarshan *et al.,* 2007).

Rabies virus belongs to Genus: *Lyssavirus,* of the Family: *Rhabdoviridae* under the Order: *Mononegavirales* (Rupprecht *et al.,* 1995). The virus is bullet shaped, its genome is non-segmented, linear, minus-stranded RNA molecule of 11, 932 nucleotides long (Tordo *et al.,* 1986). Viral genome has five major genes, which code for five major viral proteins namely Nucleoprotein (N), Non-structural protein (NS or M1), Matrix protein (M2), Glycoprotein (G), and Polymerase (L) (Goto *et al.,* 1995). Among these proteins, the G protein is involved in cellular reception and is the only antigen that induces virus-neutralizing antibodies. Variability in the sequence of this protein appears to be responsible for the serotypic differences among Lyssaviruses (Rupprecht *et al.,* 1991). The virus replicates in cytoplasm of a variety of cells and released by budding. Specific cellular receptors for rabies virus are characterized only in neurons (Kawai and Morimoto, 1994). Although the virus replicates in a variety of cell lines like BHK-21, Vero, human diploid cells and other cell lines of non-neuronal origin, cytopathic changes are not observed in these cells (Bektemirova *et al.,* 1979).

The control of an infectious disease is made easier if there is an effective vaccine and efficient diagnosis. Works towards rabies diagnosis in the past concentrated on histopathological methods that exploited different staining processes for the detection of cytoplasmic virus inclusions, the Negri bodies (Tierkel and Atanasiu, 1996). Later, direct immunofluorescence (Goldwasser and Kissling, 1958) was used extensively and replaced staining techniques. In some cases, however virus isolation is necessary for which procedures used most frequently involve mice (Koprowski, 1966) or one of a number of different cell lines (King, 1996). Other virus detection methods include enzyme-linked immunosorbent assay (Perrin *et al.,* 1986), electron microscopy (Hummeler and Atanasiu, 1996) and Polymerase Chain Reaction (Tordo *et al.,* 1996). Flowcytometry has also been described for monitoring rabies infection in BHK-21 and C6 cell lines (Bordignon *et al.,* 2002 ; Anandan, 2006).

Mouse neutralization test (MNT) was considered as a standard test for assaying rabies antibody titers which consisted of neutralizing a constant dose of the previously titrated challenge virus with a series of different dilutions of antirabies serum, using mice as an indicator system (Koprowski, 1973). Following successful propagation of rabies virus in cell culture, these methods have been replaced by *in vitro* methods with their principle remaining the same as in mice. FAT (fluorescent antibody test) is considered to be a gold standard test for the detection and titration of rabies virus in culture. Rapid Fluorescent Focus Inhibition Test (RFFIT) has been of immense help in diagnosing rabies antibodies. In RFFIT, determination of antibodies is indicated by a reduction in number of fluorescent foci of virus infected cells. RFFIT has been shown to be more sensitive than the MNT in detecting virus neutralizing antibodies in post vaccinal sera (Smith *et al.,* 1973). RFFIT is more reliable and reproducible than a neutralization test in mice (Louie *et al.,* 1975, Fitzgerald, 1979). Therefore critical use of FAT and RFFIT for uniform quality control of rabies vaccine will help us in reducing the use of mice for potency assay.

The potency of rabies vaccine is generally determind *in vivo* by the NIH (National Institute of Health) test (Seligmann, 1973) recommended by WHO expert committee on rabies (WHO, 1984). This test is based on two vaccinations of mice followed by an intracerebral challenge with the CVS (Challenge Virus Standard) mouse brain strain of fixed rabies virus. The European pharmacopoeia test for animal vaccine, a simplified version of the NIH test, has also been used for rabies vaccine evaluation (Bijlenga, 1978). In this test mice are challenged by intracerebral injection two weeks after administration of single vaccine dose by intra peritoneal (I/P) route but this result in less sensitivity than NIH test (Barth et *al.*, 1988). For Veterinary rabies vaccine potency test, each dilution uses 10 mice with single immunization (OIE, 2004; Indian pharmacopoeia, 2007; Council of Europe, 2008) while vaccine for human use uses 16 mice (Wilbur and Aubert, 1996) and 18 mice (Farmacopeia Brasileira, 2004) in each dilution with two immunizations seven days apart.

Some times, this laborious *in vivo* vaccination-challenge procedure in mice shows poor reproducibility, mainly due to heterogenicity in mice and challenge procedures used (Barth *et al.,* 1988). For both practical and ethical reasons, replacement of *in vivo* potency test by more rapid and more reliable *in vitro* methods for potency control is highly desirable

(Rooijakkers E.J.M., 1996a). The NIH test is still the only potency test accepted by WHO for the batch release of human rabies vaccine (WHO, 2007).

Glycoprotein content is an indication of quantity of most important protective antigen of rabies virus. Quantitative PCR techniques will further help to quantify virus after inactivation, therefore will avoid handling of live virus as in mouse inoculation test. These *in vitro* assays will help to improve quality of vaccine and also in process monitoring of vaccine production. Further, majority of the laboratories in India and other developing countries are forced to handle rabies virus under inadequate biosafety and biosecurity conditions due to high demand of quality vaccine. Therefore production of vaccine with minimum efforts will reduce the exposure time of workers to such a fatal virus at the same time it will help in economic production of good vaccine.

Keeping all these points in mind the present study has been planed with following objective:

To evaluate a combination of *in vitro* tests for in process quality control and potency test of rabies cell culture vaccine.

Chapter II

Review of Literature

2.1 Rabies virus

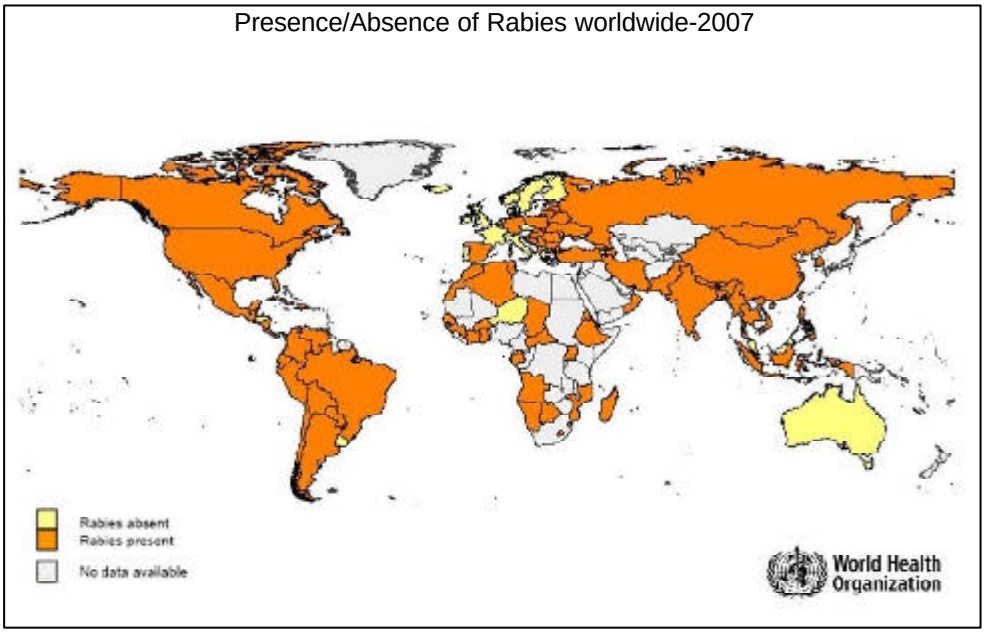
Rabies virus is a bullet shaped, enveloped, negative sense-RNA virus that belong to the genus *Lyssavirus* of the family *Rhabdoviridae* under the order *Mononegavirales* (Rupprecht *et al*., 1995; Pringle, 1997). It has an average dimension of 180x75 nm (WHO, 1973). The virus comprises of a helical nucleocapsid core with an outer lipoprotein envelope. The helically wound virus core consists of 30-35 coils, measuring 4.2-4.6 µm in un-wound form (Sokol *et al*., 1969). The outer lipoprotein envelope measures around 7.5-10 nm in thickness, and possesses glycoprotein (G protein) spikes of 10 nm length (Dietzschold *et al*., 1996) covering the entire surface, excluding the blunt end portion of the virion.

2.2 Virus classification

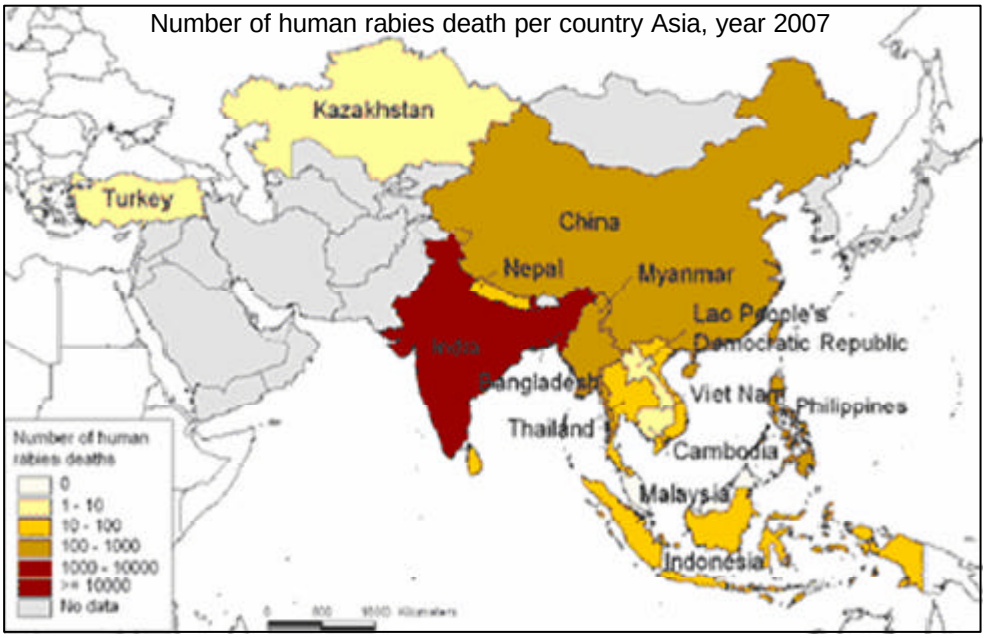
Based on cross protection tests and molecular biological analysis, seven distinct genetic lineages are distinguished within the genus *Lyssavirus*, namely the classical rabies virus (genotype-1/serotype-1), Lagos bat virus (genotype-2/ serotype-2), Mokola virus (genotype-3/serotype-3), and Duvenhage virus (genotype-4/serotype-4). The European bat lyssaviruses (EBLV1-genotype 5 and EBLV2-genotype 6) and Australian bat lyssavirus (ABLV-genotype 7) (Fekadu *et al*., 1988; Baer, 1991; Bourhy *et al*., 1993). This classification however may bound to evolve, particularly as surveillance for bat lyssaviruses is reinforced (Reynes *et al*., 2004). Already, four additional divergent lyssaviruses have been isolated in bats of Central Asia, East Siberia and the Black Sea region and proposed as new genotypes: Aravan virus, Khaujand virus, Irkut virus, and West-Caucasian bat virus (Botvinkin *et al*., 2003 and Kuzmin *et al*., 2003). The rabies related viruses (RRVs) have been found to cause illness indistinguishable

Global distribution of rabies

Presence/Absence of Rabies worldwide-2007



Number of human rabies death per country Asia, year 2007



from classical rabies (Smith, 1996). The laboratory strains of rabies virus (RV) includes Pasteur virus (PV), Pitman Moore (PM), Challenge virus standard virus (CVS), Flury low egg passage (LEP) and Flurry high egg passage (HEP) (Sacramento *et al*., 1992).The *Lyssavirus* genus also includes Obodhiang and Kontonkan viruses (Shope and Tesh, 1987).

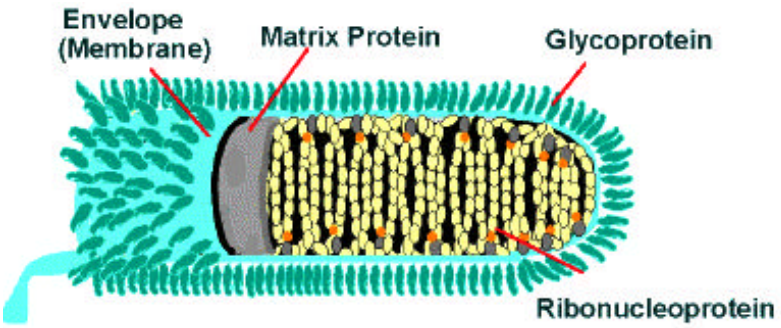
2.3 Viral genome

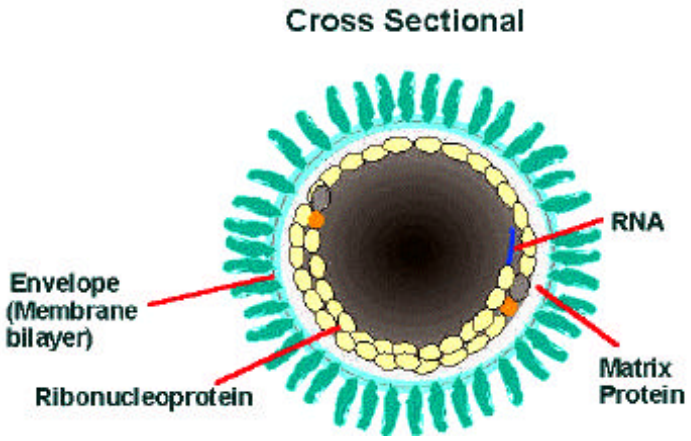
Rabies virus genome is a non-segmented, minus-stranded RNA molecule of 11, 932 nucleotides long (Tordo *et al*., 1986). Viral genome has five major genes, which code for five major viral proteins namely Nucleoprotein (N), Non-structural protein (P or NS or M1), Matrix protein (M2), Glycoprotein (G) which exists as a trimer, and Polymerase (L) (Goto *et al*., 1995). The order of the genes, namely, 3'-N-P-M-G-L-5', is highly conserved (**Fig.1**). The G protein is involved in cellular reception and is the only antigen that induces virus-neutralizing antibodies. Variability in the sequence of this protein appears to be responsible for the serotypic differences among lyssaviruses (Rupprecht *et al*., 1991). RNA viruses are characterized by a high mutation rate during replication due to the lack of proofreading and post replication error correction by the RNA polymerase (Domingo and Holland, 1994). It has emerged that from many studies that the nucleoprotein is relatively more conserved compared to the rest of the lyssavirus genome. Direct comparison demonstrates that the degree of conservation varies in the following order: nucleoprotein > matrix protein > glycoprotein > phosphoprotein (Bourhy *et al*., 1993).

2.4 Viral Proteins

2.4.1 Glycoprotein (G)

Rabies virus G protein is the only antigen capable of inducing and reacting with virus-neutralizing antibodies (Cox *et al*., 1977). The G protein, which forms spike projections of the virion are composed of homotrimers of single type which controls major aspects of host cell interaction, such as receptor recognition and receptor binding, host range, immunogenicity, neurovirulence (Dietzschold *et al.*, 2005), spread from the postsynaptic site to the presynaptic site (Etessami *et al*., 2000)and host adaptation (Badrane *et al*., 2001) as well as in the low pH-dependent membrane fusion that occurs in the endosomes for releasing the viral ribonucleoprotein into the cytoplasm (Thoulouze *et al*., 1998 and Tuffereau *et al*., 1998). It is





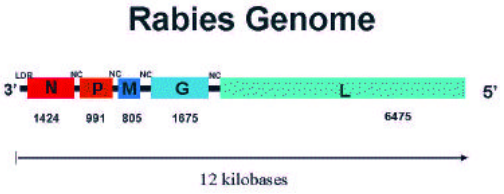


Fig.1: STRUCTURE OF RABIES VIRUS AND ITS GENOME

(http://www.rabies.net and CDC)

involved in cellular reception. Gene coding G protein has been cloned and sequenced (Anilionis *et al.*, 1981; Tordo *et al*., 1986; Conzelmann *et al*., 1990) and it contain a single open-reading frame encoding a 524 amino acid protein. The mature G Protein has 505 amino acids after the cleavage of the N-terminal 19- amino acid hydrophobic signal peptide. At the amino acid level there is approximately 90% homology among the rabies virus strains. Mutations at position 333, resulting in a substitution of glutamine or isoleucine for arginine, cause loss of virulence in adult immuno-competent mice regardless of the virus dose or route of inoculation (Seif *et al*., 1985; Tuffereau *et al*., 1998) and this arginine residue appears to be essential for G protein mediated fusion of the viral envelope with neurons (Morimoto *et al*., 1992). G protein has three structural domains: the C-terminal cytoplasmic domain, transmembrane domain and the external antigenic domain extending from the transmembrane to the N-terminus (Wunner, 1991). Although, all the strains of rabies virus are not antigenically similar owing to the difference in G protein ectodomain and, therefore, do not cross neutralize with antisera from other rabies viruses. Current vaccine strains are effective against phylogroup I viruses (Genotypes 1, 4, 5, 6 and 7) but not to phylogroup II (Genotypes 2 and 3), which have been in use since quite long (WHO, 2005). A soluble form of rabies virus G protein (Gs) is secreted from virus infected cells lacking 58 amino acids from the C-terminus (Dietzschold *et al*., 1983) and it is antigenically identical to the full length G protein but does not confer protection against lethal challenge with rabies virus (Dietzschold *et al*., 1983).

2.4.2 Nucleocapsid (N) Protein

The nucleoprotein (N) is highly conserved, abundant and a key structural component of the viral ribonucleoprotein core essential to viral propagation, constitutes the main target for rabies diagnosis and virus identification (Dean *et al*., 1996). Rabies virus Nucleocapsid protein consists of 450 amino acids. N protein is phosphorylated at the serine residue at position 389 (Dietzschold *et al*., 1987). The amino acid sequence of the N protein has been deduced from the primary nucleotide sequence of PV, CVS, ERA, and SAD-B19 strains (Tordo *et al*., 1986; Ertl *et al*., 1989; Conzelmann *et al*., 1990). It shows high degree (98% to 99.6%) of homology between the different N proteins of these fixed rabies strains that correlates with their antigenic similarity to each other and to several field rabies virus strains, as analyzed with MAbs (Dietzschold *et al*., 1988; Wunner *et al*., 1988). N protein has been shown to be a

major target antigen for T-helper cells that cross-react among lyssaviruses (Celis *et al*., 1988a; Celis *et al*., 1988b; Ertl *et al*., 1989).

2.4.3 Phosphoprotein (P)

Phosphoprotein (P) is also known as Non-structural Protein (NS) or M1 protein. It Contains 297 amino acids (Tordo *et al*., 1986; Conzelmann *et al*., 1990; Larson and Wunner, 1990) and it is phosphorylated (Sokol *et al*., 1969). The P protein is an essential co-factor of the L polymerase (Emerson and Yu, 1995) and is required for RNA encapsidation (Horikami *et al*., 1992; Curran *et al*., 1995). Both P and L are associated with the helical RNP (Harmon *et al*., 1985). P protein also incriminated for involving in the prevention of host cell type Interferon expression system (Brzozka *et al*., 2005).The P protein is important determinant in retrograde transport of the virus within axons after binding with dynein light chain LC8 (Jacob *et al*., 2000 and Raux *et al*., 2000)

2.4.4 Matrix (M) protein

M protein consists of 202 amino acids and it shares 91% to 94% sequence homology among rabies virus strains (Hiramatsu *et al*., 1993). Gaudin *et al*. (1991) reported the presence of a palmitic acid linkage in M protein. It has been proposed that the main function of M protein is to interact with the cytoplasmic domain of the G protein and the RNP during virus assembly and budding of virus by regulating the balance of virus transcription and replication and also interacts with the transmembrane spike of G protein (Knipe *et al*., 1977; Wagner *et al*., 1984; Wunner, 1991; Mebatsion *et al*., 1999; Finke *et al*., 2003).

2.4.5 RNA-dependent RNA Polymerase (L)

The L protein is the largest rhabdovirus protein and in rabies virus it contains 2,142 amino acids (Schubert *et al*., 1984; Tordo *et al*., 1988; Conzelmann *et al*., 1990). Its functions include RNA synthesis, capping, methylation, and polyadenylation of viral RNAs (Banerjee, 1987; Wagner, 1990; Wunner, 1991). In the L protein there are at least four stretches of amino acids that are conserved among rhabdoviruses and paramyxoviruses (Poch *et al*., 1990). These regions may be functional domains responsible for the multiple catalytic activities of the L protein.

2.5 Virus-cell interaction

Rabies virus replicates in a variety of cells. These cells can be neuronal and non-neuronal. Some of the neuronal cell receptors were known, namely p75 neurotropin receptor (p75 NTR), neuron adhesion molecule (NCAM) or nicotinic acetyl-choline receptor (NAChR) that may allow efficient entry of rabies virus into neurons (Lentz *et al*., 1982; Thoulouze *et al*., 1998; Tuffereau *et al*., 1998). Ubiquitous receptors on most cell types are more likely to exist, since a broad spectrum of non-neuronal cells from different species can be infected *in vitro* (Reagan and Wunner, 1985). After G protein mediated receptor binding, the rabies virus is probably internalized by receptor-mediated endocytosis. This forms a coated pit, which then fuses with a lysosome followed by release of the viral nucleocapsid into the cytosol (Gosztonyi, 1994). Virus replication predominantly occurs in areas of the cell rich in ribosomes.

The genome is transcribed into five viral mRNAs for the production of five major viral proteins viz. N, P, M, G and L and then the full-length plus-stranded RNA is produced as the template for the progeny viral genome (Dubois-Dalcq *et al.,* 1984). The viral envelope forms from host cisternal membranes, into which the G and M proteins are inserted (Murphy *et al*., 1973). The envelope also includes small amounts of some host proteins (Levy *et al*., 1994). In natural infections, the virus appears to accumulate in cytoplasmic cisterns, from where it is released by budding either by fusion of the cisternal membrane with the cell membrane or upon dissolution of the cell itself (Gosztonyi, 1994). Altered regulation of host genes may also be involved in the replication and spread of the virus (Prosniak *et al*., 2001). While many experiments indicate the major role of the G protein in RV spread from the postsynaptic site to the presynaptic site (Etessami *et al*., 2000), the RV P protein might be an important determinant of retrograde transport of the virus within axons (Jacob *et al*., 2000).

2.6 Virus replication

Rabies virus has an eclipse period of 6 to 12 hrs in BHK-21 cells and produces virus for several days without appreciable inhibition of cellular DNA, RNA or Protein synthesis (Tsiang and Atanasiu, 1971; Iwasaki *et al*., 1973; Wiktor, 1973; Matsumoto, 1974). A tissue culture test for the primary isolation of street rabies virus was evaluated using BHK-21 cells and also found that the addition of diethyl amino ethyl (DEAE) dextran promoted viral invasiveness of positive test samples (Rudd *et al*., 1980). Evidence of viral polypeptide synthesis

in rabies virus-infected cells has been limited to the detection of virus specific polypeptides in the cytoplasm of infected cells by immunofluorescent techniques (Tsiang and Atanasiu, 1971; Wiktor, 1973).

Kaplan *et al.* (1967) have studied the single-cycle growth curve of Pitman-Moore (PM) strain of fixed rabies virus in BHK-21 clone 13 cells. They reported that the presence of rabies antigen was first observed in about 50% of the cells in 8 to 9 hrs after infection, and there was a further lag period of 3 hrs before infectious intracellular virus was produced. Virus was first detected in the medium between 12 and 15 hrs after infection. From than on, the number of cells containing rabies antigen and those shedding infectious virus increased rapidly, reaching a plateau between 18 and 24 hrs after infection. Maximal yield of infectious virus was observed 48 hrs after exposure and at this time more viruses was found extracellularly than intracellularly. The growth pattern of the virus infected cell by FACS was showed a short eclipse phase at around 12 hrs followed by a steady increase in the proportion of infected cells up to 48 hrs and maximum at 48 hrs of infection and later the titer of virus declined sharply.(Anandan, 2006).

2.7 Diagnostic techniques for the detection of Rabies Virus

2.7.1 Mouse Inoculation Test (MIT) and Fluorescent Antibody Test (FAT)

Rabies diagnosis in the past were mainly focused on histopathological methods that exploited different staining processes for the detection of cytoplasmic virus inclusions, the Negri bodies (Tierkel and Atanasiu, 1996). Direct fluorescent antibody test (FAT) was discovered by Goldwasser and Kissling in 1958 and has proven to be a fast and one of the most reliable diagnostic tools for the routine diagnosis of rabies (Dean and Abelseth, 1973). It has been perfected to such an extent that many laboratories have opted to abandon the MIT (Rudd and Trimarchi, 1989). Chhabra *et al*. (2007) have found the sensitivity of FAT was 100% and it gave cent percent concordant results with MIT. False negative FAT results are not common but can occur due to inadequate sampling, faulty equipment, unsatisfactory conjugate, lack of control slide or lack of experience in reading the slide and hence it has been suggested that in a standard diagnostic centre for rabies, confirmation of FAT results may be

performed by MIT particularly in the samples which are negative by FAT. MIT has ability to detect small quantities of virus in the sample. Apart from that it has its applicability to partially decomposed specimens. It is practical, sensitive, reliable and technically non-demanding. Its main drawback, besides the inherent environmental and ethical issues with the use of live animals in the laboratory. MIT takes long time, typically 7 to 21 days before the result is obtained. MIT also requires a well-maintained animal house for continuous supply of mice and larger number of mice per sample and is labour intensive. The direct FAT is being employed as diagnostic technique because of its sensitivity, accuracy, and speed as recommended by WHO (Meslin *et al.*, 1996) and OIE (Shanker, 2009). Immunofluorescence was found in the form of characteristic apple green fluorescence. The correlation between FAT and mouse inoculation is 92-99%.

2.7.2 PCR and Real time PCR technique

The advantages of RNA detection methods are in the transferability of the technology to a wide variety of other sample types that may be unsuitable for the direct fluorescent antibody test, such as saliva and cerebrospinal fluid (Crepin *et al.*, 1998). TaqMan PCR is widely implemented as a detection and quantitation method for viruses. These tests have been developed for a number of negative strand RNA viruses, e.g., Hendra virus (Smith *et al.*, 2001), Rift velly fever virus (Garcia *et al.*, 2001), Respiratory syncytia virus (Hu *et al.*, 2003) and have been found to be sensitive and specific, rapid and require no post-PCR manipulation. PCR has been used for the confirmatory diagnosis of human rabies when other test could not be readily applied (Black *et al.*, 2002). A modified method of PCR, Reverse transcriptase PCR (RT-PCR) has been used to detect rabies in infected decomposed brain tissues (Nadin-Davis, 1998). Problems associated with sensitivity of the FAT and decomposed brain tissues have been overcome with the adaptation of RT-PCR. Sacramento *et al*. (1991) used PCR technique as an alternative method for the diagnosis and molecular epidemiology of rabies virus. Tordo *et al*. (1994) used PCR technology for Lyssavirus diagnosis. Heaton *et al*. (1997) studied hemi nested PCR assay for detection of six genotypes of rabies and rabies related viruses. Nadin-Davis (1998) worked on PCR protocols for rabies virus discrimination. Gupta *et al*. (2001) used single tube non-interrupted RT-PCR for the detection of RV in brain tissue. Heaton *et al*. (1999) and Picard *et al*. (2004) developed a hemi-nested RT-PCR method for

the specific determination of EBL-1 and compared with other diagnostic methods. Hughes et *al*., (2004) used TaqMan PCR based method for the detection of rabies virus in tissue sample. Recently, *in-situ* polymerase chain reaction has been used to detect the rabies virus RNA both in cell culture (neuroblastoma cells) as well as in brain tissues (Jayakumar *et al*., 2003 and Praveena *et al*., 2007).

2.8 Detection and quantification of rabies antibody

2.8.1 Rapid Fluorescent Focus Inhibition Test (RFFIT) and Mouse Neutralization Test (MNT)

Detection and quantification of rabies antibodies is intended in the first place for checking the immunity to rabies or effectiveness of rabies vaccines. Detection and quantification of virus neutralization rabies antibodies in the serum is based on inhibition of rabies infection in vivo in animals or in vitro in cell cultures (Atanasiu, 1973; Bourhy and Sureau, 1991). Several suitable procedures have been recommended for determination of titre of virus neutralization antibodies. Earlier Mouse virus neutralization test (MNT) was the only test available for the detection of rabies virus neutralization antibodies (Webster and Dawson., 1935). The methods most frequently used for quantification of immune response in vaccinated animals after rabies vaccination challenge are serum neutralization methods carried out on mice and in cell cultures (Smith *et al.*, 1996). WHO recommends in vivo virus neutralization test on mice (VNT) and in vitro rapid fluorescence focus inhibition test (RFFIT).

The VNT on mice is time consuming and too expensive for routine use in virological laboratories. Recently it has been replaced by sensitive, less expensive and more rapid in vitro tests. The RFFIT is highly sensitive and advantageous because of its low time demand. The application of the RFFIT for detection and quantification of rabies antibodies also requires an OIE standard (WHO, 1992). Later, Smith *et al.*, (1973) described RFFIT in which the determination of antibodies is indicated by a reduction in the number of fluorescent foci of virus infected cells. This method was adapted for use in micro plates in 1997 (Zalan *et al*., 1979). Further, fluorescent antibody virus neutralization test (FAVN test), an adaptation of RFFIT has also been developed and evaluated for the titration of neutralizing antibodies in the serum (Aubert *et al.*, 1996).

Ondrejkova et al. (2002) compared the standard diagnostic methods VNT on mice and RFFIT with the FAVN test and found that there are no significant differences with regard to their sensitivity, specificity and reproducibility. However, there were differences in rapidity of the tests, simplicity and easiness of preparation for them, costs of the reagents and equipment of laboratories. With regard to the preparation and performance of the test (RFFIT takes 24–48 hours) and the respective costs, RFFIT appeared better than FAVN. The RFFIT requires lower volumes of examined serum (0.1 ml) than the FAVN (0.2 ml), lower volume of virus, etc. A disadvantage of the RFFIT is the reading of results and their evaluation. The comparison of tests for detection and quantification of rabies antibodies showed no significant differences between RFFIT and the newer FAVN method, recommended by OIE for examination of animal sera. Both the methods allow identification of non-vaccinated animals with 100% accuracy. However, in certain group of immunosuppressive, poorly reacting vaccinated animals both the methods may provide "false positive and false negative" antibody response in those cases in which only one of them is used (*Briggs et al*., 1998). Because of this, the titer of rabies antibodies in such animals should be determined by two or three methods.

WHO recommends that the vaccinated animals are protected sufficiently when their level of rabies antibodies equal or exceed 0.5IU/ml (*Ondrejkova et al.,* 2002). Several serological tests have also been described including the test involving cell cultures (Smith *et al*., 1973; Zalan *et al*., 1979; Bussereau *et al.*, 1982; Smith, 1996). Perrin *et al*., (1986) developed a rapid rabies enzyme immunodiagnostic test (RREID) based on the use of anti nucleocapsid IgG in the enzyme linked immune assay. Esterhuysen *et al.,* (1995) developed liquid phase blocking ELISA. Tsun *et al*., (1977) measured the humoral antibody titer of rabies by Indirect Radioimmunoassay (RIA) and Indirect Fluorescent Antibody Test (IFAT) along with Rapid Fluorescence Focus Inhibition Test (RFFIT) and got that indirect radioimmunoassay (RIA) and indirect fluorescent antibody test (IFAT) is ten fold less sensitive than RFFIT. Katayama *et al.,* (1999) developed double antibody sandwich ELISA (DAS-ELISA) to detect the presence of rabies antigen in various tissue samples. ELISA indirectly evaluates the reaction of the viral glycoprotein with the rabies antibodies while MNT and RFFIT directly measure the reaction of whole viral antigens. MNT is tedious and complicated and hazardous during preparation of virus antigen while ELISA is simple and safe.

Inoue *et al*., (2003) prepared recombinant His tagged nucleoproteins (His-rNP) and expressed in E.coli as safe antigen for antibody detection by fluorescent ELISA (FELISA) and found good correlation with antibody titer detrmind by RFFIT. The sensitivity and specificity of FELISA was 91.7% and 100% respectively.

2.9 Rabies vaccine

Initially rabies vaccine was prepared from adult animal nerve tissue e.g. phenolised sheep brain vaccine by David Semple in 1911 (Meslin *et al.,* 1996; Bleck *et al*., 1997) and suckling mouse brain vaccine (Fuenzalida *et al.,* 1995). Rabies vaccines were also prepared in chicken embryos (Koprowski and Cox, 1948), duck embryos (Peck *et al.,* 1955) and human diploid cell lines (Hemachuda, 1999). The production of rabies vaccines for veterinary use now is based upon the use of continuous cell lines such as Baby Hamster Kidney (BHK-21) cells or a hamster embryo cell line (NIL2) (Pay *et al.,* 1985; Sharma, 1990; Sureau, 1992). B-propiolactone inactivated sheep brain vaccine, which was produced by Indian Veterinary Research Institute till recent past and the state veterinary biological units, however efficacious, possess problems due to animal ethics issues associated with use of sheep for its production (Anandan, 2006). Modern cell culture rabies vaccines are highly effective and cross-protective between strains of rabies virus but the high cost of rabies biologicals is still a problem, while new manufacturers and the use of intradermal vaccination schedules should bring down expenses. It might become more attractive if industry would manufacture multiple dose vials (5-10 ml when reconstituted) to be used by the public sector in mass campaigns. Therefore a potent cell culture based vaccine is the need of the hour. The quality of the vaccine need to be checked and verified as in majority of the cases it is used for curative purpose.

2.10 Quality control of vaccine

The potency of rabies vaccine is generally determind *in vivo* by the NIH test (Seligmann, 1973) recommended by WHO expert committee on rabies (WHO, 1992). Centers for Disease Control (CDC) has developed a modified method of NIH test (CDC/Rapid peripheral challenge (RPC) test) in which single dose of vaccination by intra muscular (I/M) route in place of intra peritoneal (I/P) route and challenged after four weeks using rabies CVS (Wunderli *et al.*, 2006). This test closely mimics the natural route of infection or vaccination. However, this in vivo vaccination-challenge procedure in mice is subject to poor reproducibility

i.e. laborious, expensive procedure and time consuming mainly due heterogenicity in mice and challenge procedures used (Barth *et al.*, 1988). For both practical and ethical reasons, replacement of in vivo potency test by more rapid and more reliable in vitro methods for potency assessment is highly desirable (Rooijakkers *et al.*,1996a).

Antibody assay in immunized mice used for the NIH test seems to be the best possible manner to determine the potency of inactivated rabies vaccines (Lazrowicz et *al.*, 1982). The potency may be determined serologically by measuring the neutralizing antibody titers induced after vaccination of mice by using a rapid fluorescent focus inhibition test (RFFIT) and correlation between the challenge test results and the mean titers can be determined by RFFIT. Although this method is faster and less painful for the animals, it is not widely used yet (Kramer et *al*., 2009).

The glycoprotein of rabies virus is the antigen on the surface of the virus which is responsible for inducing neutralizing antibody (Cox *et al*., 1977; Wiktor *et al.*, 1984). For this reason, it may be hypothesized that the glycoprotein content of different strain of rabies virus will correlate with their biological potency. Based on these concept, so far, the modified antibody binding test (ABT), (Barth *et al*., 1981; Barth *et al.*, 1990) and Single radial immunodiffusion test (SRID) (Ferguson and schild, 1982; Ferguson *et al.*, 1984; Ferguson and Health, 1992) were proposed as alternatives and glycoprotein content was estimated by multiple regression analysis in terms of equivalent unit per dose (EqU/dose) (Beranger *et al*., 1981). Roland *et al*., (1987) measured the glycoprotein content of PM (Pitman and Moore) strain of rabies virus by single radial immunodiffusion assay (SRID) and found good correlation. Their use is restricted to glycoprotein measurement by polyclonal antibody and it is not applicable to adjuvanted vaccine, however ELISA method is applicable.

The ELISA systems described so far for potency estimation were all based on polyclonal antibodies against glycoprotein(GP) or nucleoproteins(NP)(Atanasiu *et al*., 1980; Van der Marel and Van Wezel,1981; Thraenhart and Ramakrishnan,1989; Perrin *et al.*,1990) or MAb specific for glycoprotein (Lafon *et al*.,1985). Several ELISA methods have been developed (Lyng et *al.*, 1992) to measure only glycoprotein (Fournier *et al.*, 2003 and Gamoh *et al.*, 2003) or glycoprotein and nucleoprotein content (Rooijakkers, 1996b). Quantitative ELISA system for potency control were developed using monoclonal antibodies (MAbs) directed to

the glycoprotein or nucleoproteins of rabies virus (Rooijakkers *et al*., 1996b). Glycoprotein content was determined by a parallel line analysis (European pharmacopoeia) and was expressed in equivalent unit per dose. Specificity of the monoclonal antibody used in ELISA method, at least 2ng/ml of glycoprotein can be detected. It is likely that vaccines with a borderline titer or titer bellow the specification of 2.5 IU/dose should be detected (Fournier *et al*., 2003).

The protective effect of vaccination against the rabies virus is mediated by both humoral and cell mediated immunity (CMI) including virus neutralizing antibody (WHO, 1988) and CD8+ cytotoxic T lymphocytes (Wiktor, 1978; Morgeaux *et al*., 1989 and Kawano *et al.*, 1990). IL-2 seems to be very crucial in anti-rabies immunity as exogenous IL-2 acts as adjuvant for rabies vaccine (Perrin *et al.*, 1988; Nunberg *et al*., 1989) and IL-2 injected alone protect the animals infected with a lethal dose of wild rabies virus (Perrin *et al.*, 1988). Marie *et al.*, (1991), measured the potency of different rabies vaccines via CMI assessed by the production of IL-2 by $CD4^{+}CD8^{-}$ lymphocytes. IL-2 production by splenocytes from mice immunized with various vaccines was measured following *in vitro* stimulation with antigens from different rabies and rabies-related strains. IL-2 production was specific, reproducible and correlated with the vaccine protective activity as determined by the pre-exposure NIH test. It has been very difficult to identify a single method by which all necessary parameters of the different types of inactivated rabies vaccines can be satisfactorily measured (Barth *et al.*, 1988). Therefore in addition to the determination of glycoprotein content and virus neutralizing antibody level, the measurement of IL-2 could be favourably considered for the the evaluation of rabies vaccine potency, in place of the NIH test. However, antigenicity assay have also proven unreliable for estimating immunogenicity when comparing vaccines derived from different rabies strains or in assuring vaccine stability (Lyng *et al.*, 1992). Antigen concentrations with in a given preparation can not correlate with immunogenic potential, nor with the ability of a vaccine to stimulate a protective immune response (WHO, 1992). As a result of these observations, *in vivo* determination of vaccine potency is still recommended for release and stability of licensed vaccine for both human and veterinary use (Lyng *et al*., 1992 WHO, 1994).

NIH mouse protection test for potency determination of inactivated human rabies vaccine using 9 mice per dilution is in good agreement with the results obtained using 18 mice per dilution (Wlamir *et al*., 2009). This would result in 42-48% reduction in number of mice

used. It would be a reasonably fair approach to adapt *in vitro* approaches for in process quality control of rabies vaccine. After a minimal level of satisfaction based on *in vitro* test, NIH test can be performed for final potency testing of vaccine batches in order to reduce the use of mice. It is desired, if we can establish a correlation between numbers of fluorescent focus units (FFU) before inactivation of virus with antigenic value in terms of international units for vaccine as a rough estimate.

Chapter III

Materials and Methods

The present study was undertaken at Rabies laboratory, Division of Biological Products, IVRI, Izatnagar campus, with an aim to develop an alternative in vitro test for in process quality control and potency test of rabies vaccine. An overview of experimental design is given in flow chart on the next page. Facilities available at Division of Veterinary Biotechnology, Division of Veterinary Pathology and Center for Animal Disease Research and Diagnosis (CADRAD) were also utilized during the study. The details of the media, reagents, buffers and solutions used in the present study have been presented in Appendix or described at appropriate places.

3.1 Materials

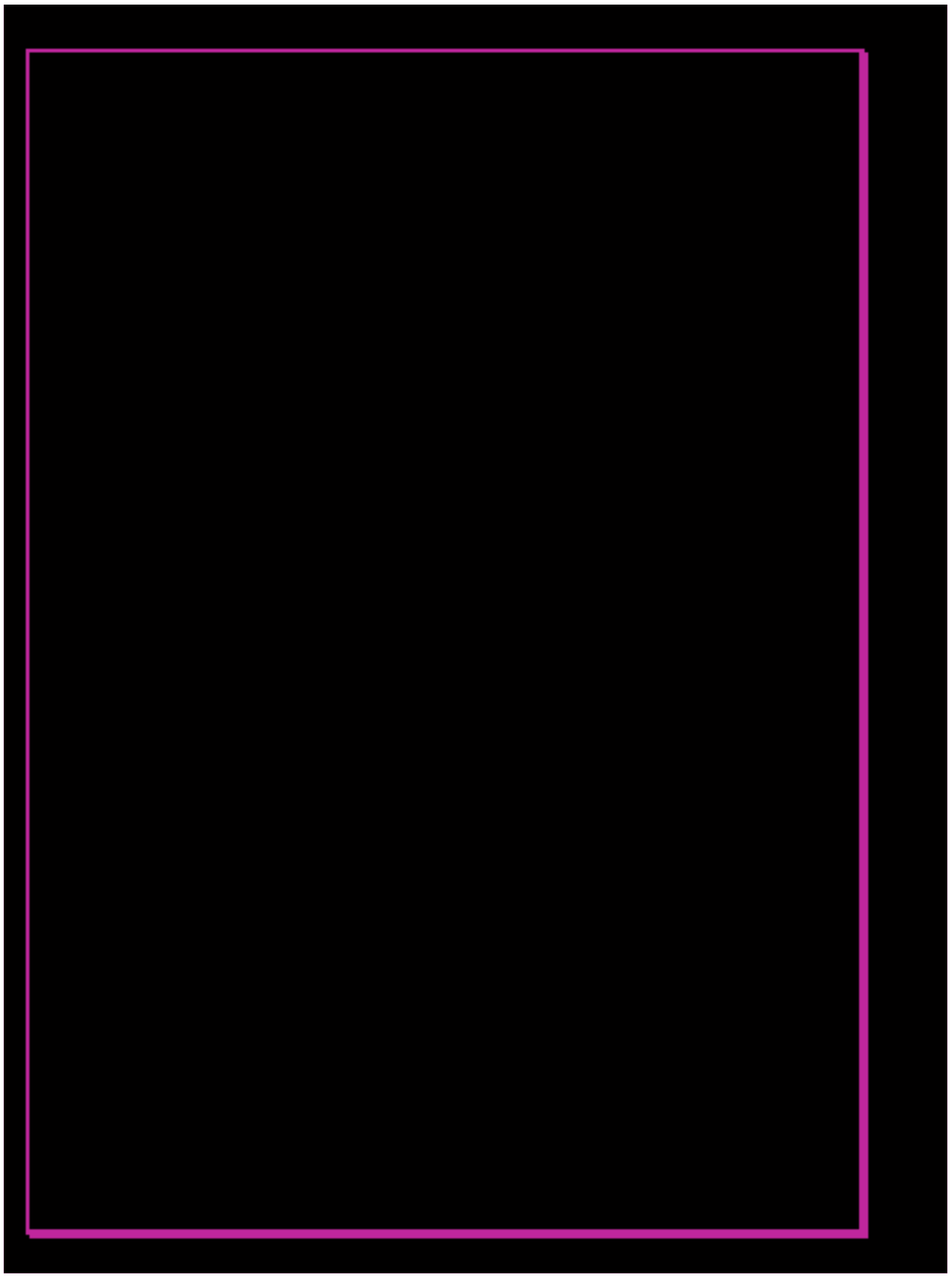
3.1.1 Cell line

BHK-21 clone 13 cells

The cell lines (BHK-21 clone 13 cells) used in the present study was received from the IVRI, Bangalore campus. BHK-21 clone 13 cells between passage No. 40 to 53 were used for the propagation of Pasteur virus (PV-11) strain of rabies virus. BHK-21 cells were propagated in Glasgow Minimum Essential Medium (G-6148, Sigma) containing 10% fetal bovine serum (41F668K, GIBCO) and subsequently maintained using 2% fetal bovine serum.

3.1.2 Vaccine virus

Pasteur virus (PV-11) strain of rabies virus is a fixed virus strain, which is one of the common vaccine strains adapted to grow in BHK-21 cell system. PV-11 strain of rabies virus having a titer of $3x10^6$ FFU/ml was maintained in Rabies laboratory, Division of Biological



Products, IVRI. This was further propagated in BHK-21 cells to produce working stock of virus. Rabies virus aliquots were stored at -70ºC till further use.

Challenge Virus Standard (CVS)

Challenge Virus Standard (CVS) rabies infected brain tissue (passage-2) maintained in Division of Biological Products, Indian Veterinary Research Institute, Izatnagar, was used in the present study for the potency assay.

3.1.3 Reference vaccine and Reference antibodies

Verorab (Sanofi Pasteur SA, France) vaccine having $\geq$2.5 IU/vial was used as reference vaccine and VINRIG (VINS, Bioproducts Ltd. India) anti rabies serum raised in equine having antibody titer $\geq$300 IU/ml was used as reference serum for antibody assessment in RFFIT.

3.1.4 Rabies anti-nucleocapsid FITC conjugate

Liquid Rabies anti-nucleocapsid FITC conjugate from VMRD (cat#210-28 RAB) and Bio-rad (cat#357-2114) was used for fluorescent antibody test (FAT) and Rapid fluorescent focus inhibition test (RFFIT) to detect and quantify virus and antibodies in BHK-21 cells.

3.1.5 Experimental animals

Mice

Swiss albino mice, 2-3 weeks old of either sex, weighing about 10-15 grams were procured from Laboratory Animal Research Section, IVRI. These were kept in polypropylene cages for 2 days prior to experiment for acclimatization. The animals were provided feed and water ad libitum.

Rabbit

Four female adults New Zealand white rabbits were procured from Laboratory Animal Research Section, IVRI. These were kept in rabies laboratory shed for 2 days prior to experiment for acclimatization. The animals were provided feed and water ad libitum.

3.1.6 Equipments and plastic wares

Microscopes

1. Inverted binocular microscope (Zeiss, West Germany)
2. Fluorescent microscope (Olympus)

Centrifuges

1. Bench top centrifuge- Hermle Z 360 K, Berthhold Hermle AG, Germany
2. Sorvall RC 5C plus high speed refrigerated centifuge (Depont, USA)

Glass and Plastic wares

1. Cell culture plates and flasks - Nunclon®, Denmark
2. Mini trays (6×12) 72 wells plate (Catalog no. 136528), Nunclon®, Denmark
3. Cell factory (5 stack cell chamber with 3180 cm^2 surface area)-Corning®
4. Disposable pipettes - Nunclon®, Denmark
5. Glassware- Borosil®

Other Equipments

1. Vortex mixer- Touch type mixer (Basco, India)
2. CO2 Incubators (Astec Co., Japan)
3. Micropipettes- single and multi channel
4. Thermal cycler (Mastercycler personal, Eppendrof, Germany)
5. Real Time PCR (MX 3000 P System, Stratagene, USA).
6. Microcentrifuge (Eppendorf, Germany)
7. Alpha imager gel documentation system (Alpha Innoecth Coproration, USA).

3.2. METHODS

3.2.1 Propagation of rabies virus

Preparation of working seed virus

Rabies virus (PV-11 strain) was propagated in BHK-21 clone 13 cells. Using 0.1 multiplicity of infection (MOI), rabies virus was co-cultivated with BHK-21clone 13 cells using 10% GMEM at 37ºC for 48 hrs in 25 and 75 cm^2 cell culture flasks. During the course of virus production, pH was adjusted to around 7.6 using 7.5% sodium bicarbonate solution and media was changed with GMEM containing 2% FBS. After 48 hrs of infection/co cultivation virus was harvested and frozen at -20ºC (Anandan, 2006). Virus harvest was thawed and aliquots were prepared and kept at -70^0C. Virus harvest was titrated and used for propagation and further up scaling of virus for production of experimental vaccine.

Propagation of virus for experimental vaccine

Rabies virus (PV-11 strain) was propagated in BHK-21 clone 13 cells. Using 0.1 multiplicity of infection (MOI), rabies virus was co-cultivated with BHK-21clone 13 cells in 10% GMEM at 37ºC for 48 hrs in 5 stacked cell factory (Fig.2a) and roller culture bottle. During the course of virus production, after 24 hrs media was changed with GMEM containing 2% FBS (pH-7.6). After 48 hrs of co cultivation, virus was harvested and frozen at -20ºC. Virus was thawed and centrifuged at 3000 rpm for 10 min. The cell free virus was aliquoted and stored at -70ºC for infectivity assay and quantification of rabies virus by different methods and preparation of experimental vaccine.

3.3 Quantification of rabies virus

3.3.1 Fluorescent Antibody Test (FAT)

Fluorescent antibody test (FAT) is the gold standard test for the detection of rabies virus in tissue smears and cell cultures. Characteristic apple green fluorescence is observed in positive samples. Rabies anti-nucleocapsid antibody conjugated with FITC was used as a tracing antibody in FAT. The protocol used for the titration of rabies virus (PV strain) in BHK-21 clone 13 cells using co-cultivation technique (Anandan, 2006) is given below in brief.

1. **Virus dilution:** virus dilutions was made using 8 eppendorf tubes in which 90µl of chilled 2% GMEM was taken. 10 µl of virus was added in the first tube and mixed thoroughly by vortexing (10 µl in 100 µl makes 1 $\log_{10}$ dilution). After mixing, 10 µl of diluted virus was transferred to the next tube; likewise serial $\log_{10}$ dilutions were made up to tube number-7. At each step use of a new sterile tip was ensured and the last tube was left undisturbed as 2% medium in it can be used as maintenance media for cell control wells.

2. BHK-21 cells were taken at a cell concentration of 4 x10^5 cells per ml in 10% GMEM in an eppendorf tube. Using a micro-channel pipette, 30 µl of cell suspension was added per eppendorf tube and mixed by gentle vertoxing.

3. From each eppendorf tube, 10 µl of sample were added to Terasaki plate (72 well mini trays cell culture plate) in three replicates in the same order from 10^{-1} to 10^{-7} using a micro-channel pipette.

4. Then Terasaki plate (Fig.2b) was covered with the lid, labeled and placed in a CO_2 incubator at 37ºC for 24 hrs.

5. After the incubation period of 21-24 hrs, the medium was aspirated completely and the cell monolayer was washed with phosphate buffer saline (pH-7.2).

6. Cells were fixed using 80% chilled acetone (80/20 in PBS) at -20ºC for 30 minutes.

7. Acetone was aspirated completely and the Terasaki plate was air dried at 37°C for 30 minutes.

8. After complete drying, Rabies anti-nucleocapsid antibody conjugated with FITC was added at the rate of 10 µl per well to cover the monolayer entirely.

9. Then Terasaki plate was incubated at 37ºC for 30 minutes in humid chamber.

10. Conjugate was aspirated and plates was washed with 1x FA rinsing buffer and then soak in 10 µl of 1x rinsing buffer for 10 minutes.

11. Rinsing buffer was aspirated and 10 µl of mounting fluid was added to all wells (50% Glycerine in 1x FA rinsing buffer, V/V).

12. Plate was observed under fluorescent microscope and fluorescent foci were counted for the determination of virus titre in terms of FFU/ml of virus sample.

13. Virus titre was calculated using Reed and Muench formula (Reed and Muench, 1938).

3.3.2. Mouse inoculation test (MIT)

Mouse inoculation test (MIT) is an *in vivo* test for rabies virus titration in mice. After a virus is propogated in either cell culture or in a suitable animal, we need to know the infectivity titre of the virus material obtained. This can be determined *in vivo* by inoculating increasing dilutions of the virus material to a susceptible host animal such as laboratory mice intra cerebrally. Based on mortality seen at different dilutions, the infectivity titre can be determined as the reciprocal of highest dilution showing 50% mortality in the inoculated mice. This is expressed as LD_{50} /ml (50% lethal dose) and can be calculated by using either Reed and Muench or Karber formula. The protocol used for the titration of rabies virus (PV-11 strain) in mice is given below in brief.

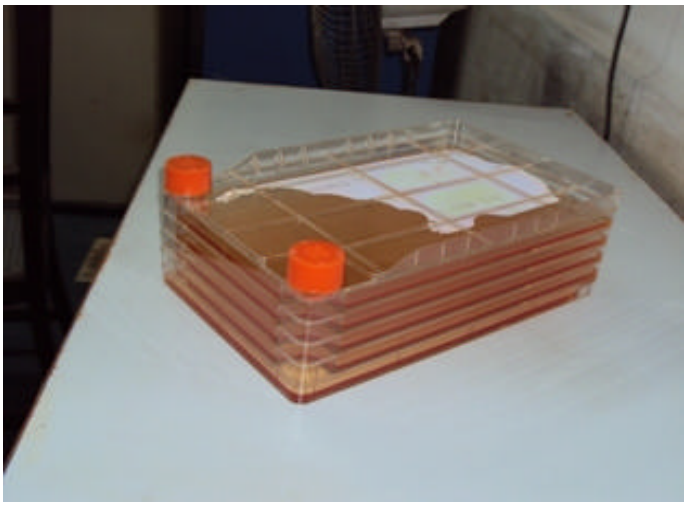


Fig.2a: Five stacked cell factory used for experimental rabies cell culture vaccine production.

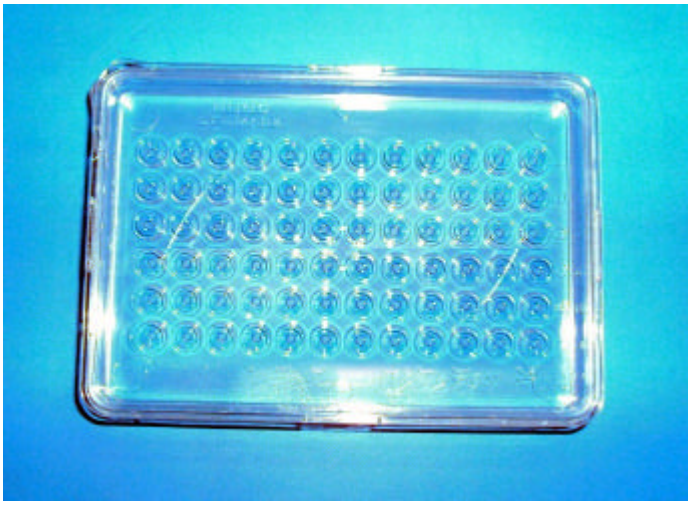


Fig.2b: Terasaki plate used for titration of rabies virus by fluorescent antibody technique.

1. **Virus dilution:** Virus dilutions was made using 9 eppendorf tubes in which 900µl of chilled sterile PBS was taken and 100 µl of virus was added in the first tube and mixed thoroughly (100 µl in 1000 µl makes $\log_{10}$ dilution). After mixing, 100 µl of diluted virus was transferred to the next tube; likewise serial $\log_{10}$ dilutions were made up to tube number-8. At each step a new sterile tip was taken and the last tube was left undisturbed as fresh PBS in it can be used for control group of mice.

2. Diluted virus was inoculated as 0.03 ml of each virus dilution intracerebrally into 2-3 wk old mice (3), starting from the highest dilution (10^{-8}) to lowest dilution (10^{-3}).

3. PBS only was inoculated in control group of mice (3).

4. All the mice were observed for clinical signs specific to rabies and death up to 14 days. Any death occurring with in 5 days of inoculation was considered as non-specific. Total rabies specific death in each dilution from 5th to 14th day was recorded.

5. Virus titer was calculated by Reed and Muench formula using a soft ware.

3.3.3. Polymerase Chain Reaction (PCR)

Preparation of virus dilution

900 µl of PBS was taken in 9 eppendorf tubes and 100 µl of virus was added in 1st eppendorf tube and vertex then 100 µl of virus was transferred to 2nd likewise serial $\log_{10}$ dilutions were made up to tube number-8. Suitable control was also kept.

Isolation of total RNA from rabies virus

Total RNA from rabies virus was isolated by Total RNA (tissue/cell culture) mini kit (Cat # IB47302, IBI Scientific, USA). The procedure as per manufactures instructions is described in brief.

1. 100 µl of virus from dilution 10^{-3} to10^{-8} and PBS only as control was taken in 1.5 ml eppendorf tube. Then 400 µl of RB buffer and 4 µl of B-propiolactone were added and shaked vigorously then incubated at room temperature for 5 minutes.

2. Added 500 µl of 70% ethanol and shaked vigorously and placed a RB column in 2 ml collection tube and transfered 500 µl of ethanol added mixture to RB column and centrifuged at 13000 rpm for 1 minute.

3. Discarded the flow through and again added 500 µl of remaining sample in RB column and centrifuged at 13000 rpm for 1 minute and again discarded the flow through.

4. Added 400 µl of W1 buffer in to RB column and centrifuged at 13000 rpm for 1 minute and discarded the flow through.

5. Added 600 µl of wash buffer in RB column and centrifuged at 13000 rpm for 1 minute and discard the flow through.

6. Centrifuged the RB column at 15000 rpm for 3 min to dry up the RB column. Placed the dried RB column in a clean 1.5 ml eppendorf tube.

7. Finally, 30 µl of nuclease free water was added to the center of RB column and kept for 3 minutes, then centrifuged at 15000rpm for 1 minute and RNA was eluated and collected in eppendorf tube and stored at -80^{0}C until further use.

Preparation of cDNA

cDNA synthesis was carried out using QuatiTect reverse Transcription Kit (Cat # 205311, QIAGEN) following the protocol recommended by the manufacturer. The procedure is described in brief:

1. 2 µl of total RNA, 2 µl of gDNA Wipeout buffer and NFW (variable) to make 14µl final volume were taken in 0.5 ml micro centrifuge tubes and incubated for 2 min at 42^{O}C. Then samples were quickly chilled on ice.

2. 20 µl reaction mixture was prepared by addition of following 6 µl of master mix reagents to all in the order given bellow.

	Reagents	**Amount**
	Quantiscript Reverse Transcriptase	1 µl
	Quantiscript RT 5X buffer	4 µl
	RT Primer Mix (10 mM)	1 µl

3. The reaction mixture was incubated at 42^{O}C for 30 min and then heated at 95^{O}C for 3 min to inactivate reverse transcriptase.

4. cDNA was then stored at -20^{O}C.

Amplification of N genes by PCR

Rabies viral genome quantification was carried out by PCR by using specific N gene primers was given in table.1a. The PCR was carried out by mixing the following contents to make final volume of 25.0 µl reaction mixture.

Table.1a: Primers used for amplification of N genes by PCR

Gene	Primer sequence	Product (bp)	Annealing Temp. (°C)	Reference
Rab 'N'	RAB/N/F410 5'ACTGATGTAGAAAGGGAATTG 3' RAB/N/R942 5'GAACGGAAGTGGATGAAATA 3'	533	50	Gupta *et al.*, 2001

Component	Volume
2.5X Master mix	10.0 µl
Primer Forward	1.0 µl
Primer reversed	1.0 µl
Template cDNA	2.0 µl
$MgCl_2$	1.0 µl
Nuclease free water	10.0 µl

The tube contents were mixed and centrifuged briefly to collect the contents. Then amplication was carried out using a thermocycler (Mastercycler personal, Eppendrof, Germany) by following the programme as given below.

Cycle	Time	Temperature
1X	2 min	94^0C
	30 s	94^0C
35X	30 s	50^oC
1X	1 min	72^0C
	10min	72^0C

Agarose Gel Electrophoresis

One percent agarose (Cat # RM273, Himedia, India) was prepared in 0.5x TBE buffer with ethidium bromide (0.5 µg/ml). The PCR product was run at 40V for 1 hr and then the gel was seen using Alpha imager gel documentation system and photograph was recorded.

3.3.4 Real Time PCR

Rabies viral genome detection and quantification by quantitative Real Time PCR (MX 3000 P System, Stratagene, USA) by using specific N gene primers is given in table.1b. Real Time PCR reaction was performed in 8 well PCR tubes using DyNAmo SYBR Green qPCR kit (Cat # F-400L, Finnzymes, Finland). The reaction mix was prepared by adding following reagents:

Table1b: Primers used for amplification of N genes by real time PCR

Gene	Primer sequence	Product (bp)	Annealing Temp. (°C)	Reference
RabN	F5'GGATTGAGCATCTATATTCAGC 3' R5'GAGGAACGGCGGTCTCCTG 3'	200	55	Reddy.V. Manjunatha, Ph.D Thesis, 2010, IVRI (Unpublished information)

- ☞ 10.0 µl of 2X cyber green master mix was taken in real time PCR tubes.
- ☞ 0.5 µl of forward primer (10 pM of final concentration).
- ☞ 0.5 µl of reverse primer (10 pM of final concentration).
- ☞ 2.0 µl of cDNA (~ 50 ng final concentration).

Nuclease free PCR grade water was added to adjust the final volume to 20 µl.

The reagents were mixed gently without creating bubbles. The tubes were placed in the plate of the instrument and run on PCR programme as given:

Cycle	Time	Temperature
1X	5 min	95ºC
	20 s	95ºC
40X	20 s	55ºC
	20 s	72ºC

Analysis of Real Time PCR results

The arithmatic mean of Ct values (m Ct) was calculated. Correlation between Ct values and different dilutions of rabies virus were estabilised by plotting standard curve.

3.4. Titration of challenge virus standard (CVS)

Rabies infected mice brain, maintained in rabies laboratory, Division of Biological Products, IVRI was used. 20% brain suspension was prepared in CVS diluent (pH-7.6) and stored at -80 °C. 10% brain suspension was prepared from 20% by using CVS diluent. The protocol for CVS titration in mice is given in brief:

1. **Virus dilution:** Virus dilution was made using 9 eppendorf tubes in which 900µl of chilled fresh PBS was taken and 100 µl of virus was added in the first tube and mixed thoroughly (10 µl in 100 µl makes 1 $\log_{10}$ dilution). After mixing, 100 µl of diluted virus was transferred to the next tube; likewise serial $\log_{10}$ dilutions were made up to tube number-8. At each step a new sterile tip was taken and the last tube was left undisturbed as fresh PBS in it can be used for control group of mice.
2. Diluted virus was inoculated at the dose rate of 0.03 ml per mice intra cerebrally to 2-3 week old mice (7 Nos) from dilutions 10^{-8} to 10^{-3}.
3. 0.03 ml of PBS was inoculated in control group of mice (7 Nos).
4. All the mice were observed for clinical signs specific to rabies and death up to14 days.
5. Virus titer was calculated by Reed and Muench (1938) formula using a soft ware.

3.5. Preparation of experimental rabies vaccine and quality control

3.5.1 Inactivation of virus harvest from cell culture

Thawed, clarified pre titrated virus harvest (2 liter) and stabilizer (200g sucrose and 0.375g glycine in distil water) was added at the ratio of 1:10. Then pH was adjusted to 8.5 with sterile 1N NaOH. Kept the virus harvest in water bath at 37°C and freshly prepared beta propiolactone (BPL) at the final dilution (1:4000) was added with intermittent shaking. The material was kept in water bath for 2 hrs under constant stirring for uniform mixing and then kept at 4°C for 24 hrs. Inactivation process was repeated once again in a separate vessel.This was subjected for test of residual infectivity.

3.5.2 Test for inactivation

The BPL inactivated material was tested for complete inactivation of virus using MIT and FAT.

Mouse inoculation test

The BPL inactivated vaccine was tested for complete inactivation in adults Swiss albino mice by intra cerebral route. The protocol used for MIT is given below in brief.

1. BPL Inactivated material and PV-11 virus is 10 fold diluted in sterile PBS (pH 7.2).
2. BPL inactivated material was inoculated intra cerebrally at the dose rate of 0.03 ml per mice in 2-3 weeks old mice (3).
3. PV-11 was inoculated intra cerebrally at the dose rate of 0.03 ml per mice in 2-3 weeks old mice (3).
4. PBS only is inoculated intra cerebrally at the dose rate of 0.03 ml per mice in 2-3 weeks old mice (4) in control group.
5. All mice were kept under observation up to 14 days for untoward reaction.

Fluorescent antibody test

1. 90 µl of 10% GMEM was taken in eppendorf tube and 10 µl of BPL inactivated material was added and mixed properly. Similarly PV-11 strain of rabies virus was 10 fold diluted in GMEM containing 10% FBS in eppendorf tube and 30 µl of BHK-21 cells was added to both tube and mixed properly.
2. Transfered 10 µl of sample in triplet in terasaki plate and plate was kept in CO_2 incubator for 24 hrs.
3. After the incubation period of 22-24 hrs, the spent medium was aspirated completely and the cell monolayer was washed with phosphate buffer saline (pH-7.2).
4. Cells were fixed using 80% chilled acetone (80/20 in PBS) at -20ºC for 30 minutes.
5. After fixing plate was stained according to protocol for FAT given in breif at 3.3.1

3.5.3 Freeze drying of inactivated virus

The 5ml material was filled in each vial and loaded in automated (Lyodry) freeze drier. The freeze drying was done as per the programme given in table.2.

3.5.4 Potency test of vaccine

NIH test is generally used for the *in vivo* potency test of rabies vaccine as recommended by WHO expert committee on rabies (WHO, 1984). This test is based on two vaccinations of mice one weeks apart followed by an intracerebral challenge with the CVS (Challenge Virus Standard) mouse brain strain of fixed rabies virus after 14 days of first vaccination. All mice are observed up to 28 days and potency calculated in terms of international units (IU) in comparison with reference vaccine. As per Office International des Epizooties (OIE), Indian pharmacopoeia (IP) and European pharmacopoeia (EP), single immunization with 10 mice per dilution is recommended. The protocol used for the potency test in mice was slightly modified due to none availability of sufficient mice of uniform quality.

1. Freeze dried test vaccine was reconstituted in 1ml of freshly prepared PBS and reference vaccine (Verorab) was diluted in 1.25 ml of distil water to make it $\geq$1 IU/ml.
2. Four, five fold dilutions of test vaccine were prepared as 1:10, 1:50, 1:250 and 1:1250.
3. Four, five fold dilutions of reference vaccine (Verorab) were also prepared like test vaccine.
4. 0.5 ml of test vaccine and reference vaccine (Verorab) was inoculated by I/P route to 12 mice per group and control group received only PBS.
5. On 14th day blood samples were collected from all immunized mice for antibody assessment, serum was separated aseptically and inactivated at 56^{o}C for 30 minutes.
6. After 14 days of immunization mice were challenged with challenge virus standard (CVS) using 0.03ml I/C. This virus was titrated subsequently and found to have about 300 LD_{50}/ mice.
7. All mice were observed till all the mice died in control group.
8. Potency of test vaccine was calculated in IU/ml by comparison with the reference vaccine.

Table.2

freeze drying data

Run started: Tue Mar 09 16:42:27 2010
COLUMN WIDTH = 6
NO SPACES AFTER COLUMNS

Product Name: Rabies Cell Culture 09-03-10
Product Number: Exp.1
Operator: Dr R. P. Sigh/Dr. Manoj Kumar
Recipe File Name: Rabies Cell culture 09-03-10.rcp

Variables to be retrieved from SuperTrend files: TSHELF_CAL, SHLF_SETPT, TCOND_CAL, VACUUM2, VAC_SETPT, TPROD_AVG, TP01_CAL, TP02_CAL, TP03_CAL, TP04_CALVariables to be retrieved from the Opto controller: CYCLE_TIME, CYCLE, PHASE, STEP

Date	Time	Run	Cycle	Phase	Step	Shelf	Setpt	CondVacuum	Setpt	TP Avg	TP01	TP02	TP03	TP04
03-09-2010	16:42:27	0	1.0	0	0	24.5	25.0	23.9738690	0	150.0	25.7-32768	25.3	25.8	
03-09-2010	17:12:28		1.0	1	1	13.5	12.8	-58.2488430	0	150.0	19.1-32768	20.6	22.7	
03-09-2010	17:42:28	59	1.0	1	1	4.9	5.3	-66.4463110	0	150.0	14.2-32768	16.0	18.6	
03-09-2010	18:12:28		1.0	1	1	-2.7	-2.3	-69.3438820	0	150.0	8.7-32768	11.2	14.6	
03-09-2010	18:42:28	119	1.0	1	1	-9.5	-9.8	-70.7438820	0	150.0	3.7-32768	6.3	10.3	
03-09-2010	19:12:28		1.0	1	2	-10.4	-10.0	-72.5438820	0	150.0	0.4-32768	3.3	6.9	
03-09-2010	19:42:28		1.0	1	2	-9.2	-10.0	-73.0412650	0	150.0	-0.9-32768	1.9	5.2	
03-09-2010	20:12:28	209	1.0	1	2	-9.4	-10.0	-73.1412650	0	150.0	-1.6-32768	1.2	4.1	
03-09-2010	20:42:28	239	1.0	1	2	-9.0	-10.0	-73.0412650	0	150.0	-1.7-32768	0.6	3.3	
03-09-2010	21:12:28	269	1.0	1	3	-11.8	-12.4	-72.8412650	0	150.0	-3.5-32768	-0.5	2.5	
03-09-2010	21:42:28	299	1.0	1	3	-15.1	-14.9	-72.7412650	0	150.0	-5.2-32768	-1.9	1.2	
03-09-2010	22:12:28		1.0	1	3	-17.3	-17.4	-72.5412650	0	150.0	-6.8-32768	-3.4	-0.3	
03-09-2010	22:42:28	359	1.0	1	3	-19.2	-19.9	-72.0412650	0	150.0	-8.5-32768	-5.1	-1.7	
03-09-2010	23:12:28		1.0	1	4	-19.2	-20.0	-72.4412650	0	150.0	-9.4-32768	-5.9	-2.8	
03-09-2010	23:42:28		1.0	1	4	-19.3	-20.0	-72.2412650	0	150.0	-9.8-32768	-6.2	-3.4	
03-10-2010	00:12:28	449	1.0	1	4	-19.1	-20.0	-72.3412650	0	150.0	-8.0-32768	-6.5	-3.8	
03-10-2010	00:42:28	479	1.0	1	4	-19.4	-20.0	-72.4412650	0	150.0	-10.2-32768	-6.8	-4.1	
03-10-2010	01:12:28	509	1.0	1	5	-22.1	-22.4	-72.1412650	0	150.0	-11.1-32768	-7.5	-4.6	
03-10-2010	01:42:28	539	1.0	1	5	-23.0	-24.9	-71.9412650	0	150.0	-12.9-32768	-8.8	-5.5	
03-10-2010	02:12:28		1.0	1	5	-27.2	-27.4	-71.9412650	0	150.0	-14.7-32768	-10.3	-6.9	
03-10-2010	02:42:28	599	1.0	1	5	-29.5	-29.9	-72.1412650	0	150.0	-16.6-32768	-11.7	-8.3	
03-10-2010	03:12:28	629	1.0	1	6	-30.1	-30.0	-72.6387950	0	150.0	-17.6-32768	-12.7	-9.3	
03-10-2010	03:42:28		1.0	1	6	-29.4	-30.0	-72.7387950	0	150.0	-18.0-32768	-13.1	-9.9	
03-10-2010	04:12:28	689	1.0	1	6	-30.2	-30.0	-72.4387950	0	150.0	-18.2-32768	-13.4	-10.3	
03-10-2010	04:42:28		1.0	1	6	-30.3	-30.0	-72.7387950	0	150.0	-18.3-32768	-13.6	-10.5	
03-10-2010	05:12:28	749	1.0	1	7	-31.9	-32.4	-72.6387950	0	150.0	-19.4-32768	-14.4	-9.0	
03-10-2010	05:42:28		1.0	1	7	-34.3	-34.9	-72.3387950	0	150.0	-21.0-32768	-15.6	-1.4	
03-10-2010	06:12:28		1.0	1	7	-37.0	-37.4	-72.4387950	0	150.0	-22.9-32768	-17.0	-4.5	
03-10-2010	06:42:28		1.0	1	7	-38.0	-39.9	-72.3387950	0	150.0	-24.7-32768	-18.6	-12.7	
03-10-2010	07:12:28		1.0	1	8	-40.2	-40.0	-73.2387950	0	150.0	-25.6-32768	-19.5	-15.5	
03-10-2010	07:42:28	899	1.0	1	8	-40.2	-40.0	-73.2387950	0	150.0	-25.9-32768	-18.0	-16.2	
03-10-2010	08:12:28		1.0	1	8	-40.1	-40.0	-73.2387950	0	150.0	-26.2-32768	-20.3	-16.6	

03-10-2010	08:42:28	959	1.0	1	8	-39.2	-40.0	-73.4387950	0	150.0	-26.4	-32768	-20.5	-16.8
03-10-2010	09:12:28	989	1.0	1	9	-40.2	-40.0	-73.7387950	0	150.0	-26.5	-32768	-20.8	-17.2
03-10-2010	09:42:28	1019	1.0	1	9	-39.2	-40.0	-73.9387950	0	150.0	-26.8	-32768	-21.0	-17.4
03-10-2010	10:12:28	1049	1.0	1	9	-39.6	-40.0	-73.9387950	0	150.0	-26.8	-32768	-21.2	-17.7
03-10-2010	10:42:28	1079	1.0	1	9	-40.1	-40.0	-74.1387950	0	150.0	-25.0	-32768	-21.3	-17.9
03-10-2010	11:12:28		1.0	1	10	-39.1	-40.0	-74.4387950	0	150.0	-27.1	-32768	-21.5	-16.0
03-10-2010	11:42:28	1139	1.0	1	10	-40.2	-40.0	-74.2387950	0	150.0	-27.1	-32768	-21.5	-18.1
03-10-2010	12:12:28		1.0	1	10	-40.2	-40.0	-74.2387950	0	150.0	-27.1	-32768	-21.6	-18.2
03-10-2010	12:42:28		1.0	1	10	-39.2	-40.0	-74.1387950	0	150.0	-27.1	-32768	-21.6	-18.2
03-10-2010	13:12:28	1226	1.0	3	0	-38.0	-40.0	-72.7387950	0	150.0	-27.0	-32768	-21.6	-18.2
03-10-2010	13:42:28	1256	1.0	6	1	-39.2	-40.0	-79.8	392	400	150.0	-26.6	-32768	-25.8	-22.8
03-10-2010	14:12:28	1283	1.0	6	1	-40.4	-40.0	-80.2	373	400	150.0	-26.1	-32768	-25.9	-21.1
03-10-2010	14:42:28	1313	1.0	6	1	-40.3	-40.0	-80.1	382	400	150.0	-25.8	-32768	-26.0	-20.5
03-10-2010	15:12:28	1343	1.0	6	1	-40.3	-40.0	-80.3	379	400	150.0	-25.5	-32768	-25.9	-20.4
03-10-2010	15:42:28	1373	1.0	6	1	-40.3	-40.0	-80.9	385	400	150.0	-25.4	-32768	-25.9	-22.7
03-10-2010	16:12:28	1403	1.0	6	1	-39.9	-40.0	-81.4	383	400	150.0	-25.4	-32768	-25.9	-21.2
03-10-2010	16:42:28	1433	1.0	6	1	-39.4	-40.0	-81.7	393	400	150.0	-25.3	-32768	-25.8	-21.7
03-10-2010	17:12:28	1463	1.0	6	1	-39.2	-40.0	-81.8	392	400	150.0	-25.2	-32768	-25.8	-20.7
03-10-2010	17:42:28	1490	1.0	6	2	-39.3	-39.8	-81.2	370	400	150.0	-25.0	-32768	-25.6	-20.2
03-10-2010	18:12:28	1520	1.0	6	2	-38.5	-39.1	-82.2	383	400	150.0	-24.6	-32768	-25.3	-18.0
03-10-2010	18:42:28	1550	1.0	6	2	-38.4	-38.5	-82.1	380	400	150.0	-24.2	-32768	-24.9	-19.6
03-10-2010	19:12:28	1580	1.0	6	2	-38.2	-37.9	-80.0	386	400	150.0	-23.7	-32768	-24.5	-19.2
03-10-2010	19:42:28	1610	1.0	6	2	-36.6	-37.3	-82.2	381	400	150.0	-23.1	-32768	-22.0	-20.8
03-10-2010	20:12:28	1640	1.0	6	2	-36.3	-36.6	-82.2	394	400	150.0	-22.6	-32768	-23.4	-18.9
03-10-2010	20:42:28	1670	1.0	6	2	-35.7	-36.0	-82.3	390	400	150.0	-20.0	-32768	-22.9	-19.8
03-10-2010	21:12:28	1700	1.0	6	2	-35.2	-35.4	-82.3	385	400	150.0	-21.5	-32768	-22.3	-17.7
03-10-2010	21:42:28	1730	1.0	6	3	-33.9	-34.8	-82.4	395	400	150.0	-20.9	-32768	-21.7	-18.5
03-10-2010	22:12:28	1760	1.0	6	3	-34.3	-34.1	-82.3	378	400	150.0	-20.4	-32768	-21.1	-19.4
03-10-2010	22:42:28	1790	1.0	6	3	-31.0	-33.5	-82.3	396	400	150.0	-19.8	-32768	-20.5	-17.9
03-10-2010	23:12:28	1820	1.0	6	3	-33.2	-32.9	-82.2	385	400	150.0	-19.2	-32768	-19.9	-17.8
03-10-2010	23:42:28	1850	1.0	6	3	-31.9	-32.3	-82.1	393	400	150.0	-18.6	-32768	-19.2	-15.3
03-11-2010	00:12:28	1880	1.0	6	3	-32.1	-31.6	-82.2	377	400	150.0	-16.0	-32768	-18.6	-14.7
03-11-2010	00:42:28	1910	1.0	6	3	-30.7	-31.0	-82.2	390	400	150.0	-17.4	-32768	-17.9	-24.2
03-11-2010	01:12:28	1940	1.0	6	3	-29.6	-30.4	-82.3	386	400	150.0	-16.7	-32768	-17.2	-24.7
03-11-2010	01:42:28	1970	1.0	6	4	-29.0	-29.8	-61.0	249	300	150.0	-16.1	-32768	-16.2	-25.5
03-11-2010	02:12:28	2000	1.0	6	4	-29.2	-29.1	-78.7	263	300	150.0	-15.5	-32768	-15.7	-25.4
03-11-2010	02:42:28	2030	1.0	6	4	-27.8	-28.5	-79.7	290	300	150.0	-15.2	-32768	-15.4	-24.7
03-11-2010	03:12:28	2060	1.0	6	4	-27.9	-27.9	-78.6	293	300	150.0	-14.7	-32768	-13.0	-22.6
03-11-2010	03:42:28	2090	1.0	6	4	-27.7	-27.3	-78.4	299	300	150.0	-14.3	-32768	-14.5	-21.5
03-11-2010	04:12:28	2120	1.0	6	4	-27.1	-26.6	-74.9	285	300	150.0	-13.9	-32768	-14.1	-20.4
03-11-2010	04:42:28	2150	1.0	6	4	-26.7	-26.0	-72.0	285	300	150.0	-13.5	-32768	-13.7	-19.6
03-11-2010	05:12:28	2180	1.0	6	4	-23.0	-25.4	-74.4	290	300	150.0	-13.1	-32768	-13.3	-19.1
03-11-2010	05:42:28	2210	1.0	6	5	-24.9	-24.8	-74.0	292	300	150.0	-12.8	-32768	-12.9	-18.5
03-11-2010	06:12:28	2240	1.0	6	5	-23.5	-24.1	-74.1	295	300	150.0	-12.4	-32768	-12.5	-18.0
03-11-2010	06:42:28	2270	1.0	6	5	-23.4	-23.5	-68.4	283	300	150.0	-10.0	-32768	-12.0	-17.6
03-11-2010	07:12:28	2300	1.0	6	5	-22.9	-22.9	-67.3	284	300	150.0	-11.6	-32768	-11.6	-17.1
03-11-2010	07:42:28	2330	1.0	6	5	-21.5	-22.3	-68.6	290	300	150.0	-11.2	-32768	-11.2	-16.6
03-11-2010	08:12:28	2360	1.0	6	5	-21.8	-21.6	-68.7	298	300	150.0	-10.8	-32768	-10.8	-14.0

freeze drying data

03-11-2010	08:42:28	2388	1.0	6	5	-21.6	-21.0	-63.3	286	300	150.0	-10.4	-32768	-10.3	-15.5
03-11-2010	09:12:28	2418	1.0	6	5	-21.0	-20.4	-63.0	296	300	150.0	-10.0	-32768	-8.0	-13.0
03-11-2010	09:42:28	2448	1.0	6	6	-19.6	-19.8	-62.0	287	300	150.0	-9.7	-32768	-9.5	-14.3
03-11-2010	10:12:28	2478	1.0	6	6	-18.6	-19.2	-64.2	298	300	150.0	-9.3	-32768	-9.1	-13.4
03-11-2010	10:42:28	2508	1.0	6	6	-19.1	-18.5	-61.1	290	300	150.0	-8.9	-32768	-8.8	-12.6
03-11-2010	11:12:28	2538	1.0	6	6	-17.4	-17.9	-58.0	284	300	150.0	-8.6	-32768	-8.3	-11.9
03-11-2010	11:42:28	2568	1.0	6	6	-16.6	-17.3	-61.1	292	300	150.0	-8.2	-32768	-7.9	-11.2
03-11-2010	12:12:28	2598	1.0	6	6	-14.0	-16.7	-59.3	287	300	150.0	-7.8	-32768	-7.5	-10.6
03-11-2010	12:42:28	2628	1.0	6	6	-15.4	-16.0	-57.9	287	300	150.0	-7.4	-32768	-7.1	-9.8
03-11-2010	13:12:28	2658	1.0	6	6	-15.8	-15.4	-57.2	287	300	150.0	-5.0	-32768	-6.7	-8.9
03-11-2010	13:42:28	2688	1.0	6	7	-14.9	-14.8	-54.0	166	200	150.0	-6.2	-32768	-5.8	-8.2
03-11-2010	14:12:28	2718	1.0	6	7	-13.5	-14.2	-59.9	190	200	150.0	-5.9	-32768	-5.5	-7.2
03-11-2010	14:42:28	2748	1.0	6	7	-13.8	-13.5	-58.1	190	200	150.0	-5.4	-32768	-4.9	-6.1
03-11-2010	15:12:28	2778	1.0	6	7	-13.4	-12.9	-58.2	189	200	150.0	-3.0	-32768	-4.5	-5.0
03-11-2010	15:42:28	2808	1.0	6	7	-12.1	-12.3	-58.1	186	200	150.0	-4.6	-32768	-2.0	-3.9
03-11-2010	16:12:28	2838	1.0	6	7	-11.9	-11.7	-60.1	197	200	150.0	-4.2	-32768	-3.6	-2.7
03-11-2010	16:42:28	2868	1.0	6	7	-11.2	-11.0	-58.0	192	200	150.0	-3.8	-32768	-3.2	-1.3
03-11-2010	17:12:28	2898	1.0	6	7	-9.9	-10.4	-59.3	186	200	150.0	-3.3	-32768	-2.7	-0.4
03-11-2010	17:42:28	2928	1.0	6	8	-10.1	-9.6	-61.0	197	200	150.0	-2.8	-32768	-2.2	1.1
03-11-2010	18:12:28	2958	1.0	6	8	-8.2	-8.3	-60.7	191	200	150.0	0.0	-32768	-1.4	1.8
03-11-2010	18:42:28	2988	1.0	6	8	-6.4	-7.1	-60.2	186	200	150.0	-1.2	-32768	-0.7	2.5
03-11-2010	19:12:28	3018	1.0	6	8	-6.1	-5.8	-61.3	194	200	150.0	-0.4	-32768	0.1	3.2
03-11-2010	19:42:28	3048	1.0	6	9	-4.8	-4.6	-59.8	184	200	150.0	0.5	-32768	1.0	3.9
03-11-2010	20:12:28	3078	1.0	6	9	-3.5	-3.3	-62.6	192	200	150.0	1.3	-32768	1.8	4.6
03-11-2010	20:42:28	3108	1.0	6	9	-1.5	-2.1	-61.7	186	200	150.0	2.2	-32768	2.7	5.4
03-11-2010	21:12:28	3137	1.0	6	9	-1.6	-0.8	-63.2	188	200	150.0	3.1	-32768	3.6	6.1
03-11-2010	21:42:28	3167	1.0	6	10	0.9	0.4	-63.6	97	100	150.0	4.4	-32768	4.9	7.0
03-11-2010	22:12:28	3197	1.0	6	10	0.9	1.7	-68.0	93	100	150.0	5.3	-32768	5.9	7.9
03-11-2010	22:42:28				10	2.3	2.9	-69.3	92	100	150.0	6.2	-32768	6.8	8.6
03-11-2010	23:12:28	3257	1.0	6	10	4.7	4.2	-69.4	92	100	150.0	7.1	-32768	7.7	9.4
03-11-2010	23:42:28	3287	1.0	6	11	5.1	5.4	-70.7	90	100	150.0	8.1	-32768	8.6	10.1
03-12-2010	00:12:28	3317	1.0	6	11	6.1	6.7	-72.4	91	100	150.0	9.0	-32768	9.6	10.9
03-12-2010	00:42:28	3347	1.0	6	11	8.5	7.9	-74.3	93	100	150.0	9.9	-32768	10.5	11.7
03-12-2010	01:12:28	3377	1.0	6	11	9.4	9.2	-76.3	96	100	150.0	10.9	-32768	11.4	12.5
03-12-2010	01:42:28	3407	1.0	6	12	10.1	10.4	-77.9	90	100	150.0	11.9	-32768	12.4	13.3
03-12-2010	02:12:28	3437	1.0	6	12	11.1	11.7	-79.7	98	100	150.0	12.8	-32768	13.3	14.1
03-12-2010	02:42:28	3467	1.0	6	12	12.1	12.9	-82.1	97	100	150.0	13.8	-32768	14.2	14.9
03-12-2010	03:12:28	3497	1.0	6	12	13.6	14.2	-84.3	96	100	150.0	14.8	-32768	15.1	15.6
03-12-2010	03:42:28	3527	1.0	6	13	15.1	15.4	-81.9	90	100	150.0	15.8	-32768	16.1	16.5
03-12-2010	04:12:28	3557	1.0	6	13	17.3	16.7	-83.4	94	100	150.0	16.7	-32768	17.0	17.2
03-12-2010	04:42:28	3587	1.0	6	13	18.4	17.9	-83.4	99	100	150.0	17.7	-32768	17.9	18.0
03-12-2010	05:12:28	3617	1.0	6	13	19.6	19.2	-83.5	97	100	150.0	18.7	-32768	18.8	18.8
03-12-2010	05:42:28	3647	1.0	6	14	20.9	20.4	-83.6	94	100	150.0	19.6	-32768	19.7	19.6
03-12-2010	06:12:28	3677	1.0	6	14	21.9	21.7	-84.6	95	100	150.0	20.6	-32768	20.7	20.4
03-12-2010	06:42:28	3707	1.0	6	14	22.7	22.9	-84.9	98	100	150.0	21.6	-32768	21.5	21.3
03-12-2010	07:12:28	3737	1.0	6	14	24.6	24.2	-85.3	92	100	150.0	22.5	-32768	22.4	22.0
03-12-2010	07:42:28	3767	1.0	6	15	26.7	26.7	-85.8	99	100	150.0	23.7	-32768	23.6	22.9
03-12-2010	08:12:28	3797	1.0	6	16	31.8	31.7	-87.3	97	100	150.0	26.5	-32768	26.1	24.9

freeze drying data

03-12-2010	08:42:28	3827	1.0	7	0	34.4	35.0	-86.5	90	100	150.0	29.3-32768	28.6	27.2
03-12-2010	09:12:28	3857	1.0	8	0	22.5	4.0	-80.4	67	0	150.0	27.5-32768	26.9	27.7
03-12-2010	09:42:28	3887	1.0	8	0	3.9	4.0	-81.9	51	0	150.0	15.9-32768	17.6	19.6
03-12-2010	10:12:28	3917	1.0	8	0	3.8	4.0	-83.7	48	0	150.0	14.1-32768	15.4	16.8
03-12-2010	10:42:28	3947	1.0	8	0	4.6	4.0	-83.6	44	0	150.0	12.9-32768	14.1	15.4

Run ended: Fri Mar 12 10:47:47 2010

3.5.5 Rapid fluorescent focus inhibition test (RFFIT) for antibody assessment

Rapid fluorescent focus inhibition test (RFFIT) is employed in diagnosis of rabies antibodies. The potency may be determined serologically by measuring the neutralizing antibody titer induced after immunization of mice and rabbit by using RFFIT. In RFFIT, determination of antibodies is indicated by a reduction in number of fluorescent foci of virus infected cells. RFFIT was performed following the protocol by Smith *et al*. (1996) with little modifications.The principle of RFFIT involved a neutralization step between a fixed quantity of virus and a serially 4-fold diluted homologous serum incubated at 37ºC for 1 hr or +4ºC overnight. The virus-serum mixture was then co-cultivated with BHK-21 cells and incubated for 24 hrs. Whatever rabies virus is left after neutralization will infect the cells and this can be detected in terms of FFD_{50}. The procedure followed for RFFIT is given below in brief.

Methods

Four New Zealand white breed of rabbits were obtained from Laboratory Animal Research Section, IVRI. After 2 days of acclimatization, immunization was done with two different doses of reference vaccine (Verorab) and test vaccine following a post bite schedule and bleeding of rabbits was done according to table given bellow.

Immunization and bleeding schedule of rabbit:

Rabbit immunization	Dose of vaccine	Day of immunization	Day of bleeding
Verorab	100 µl($\geq$0.5 IU)	0, 3, 7, 14, 28	0, 14, 21, 28, 35
	20 µl ($\geq$0.1IU)	-do-	- do-
Test vaccine	500µl($3x10^7$FFUeqvt.)	-do-	-do-
	100µl($6x10^6$FFUeqvt.)	-do-	-do-

Collection of blood:

Blood was collected as per above schedule from ear vein of rabbits. Serum was separated under aseptic condition in 500 µl of eppendorf tube and kept at -20ºC. Later serum was inactivated by keeping all serum samples in water bath at 56ºC for 30 minutes.

1. **Serum dilution:** 32 µl of GMEM containing 10% fetal bovine serum was taken in a row of 10 wells in a 384 well cell culture plate. 8 µl of serum sample was added in the first well, mixed thoroughly and 8 µl was transferred to the next well. Likewise, serial 4-fold dilutions were made up to the last well and finally 8 µl was discarded from the 9^{th} well and 10^{th} well have only media. Similarly 4 fold dilutions of reference serum were prepared.
2. **Virus dilution:** Rabies virus, PV strain having a titre of $1.22x10^7$ FFU/ml was diluted 1:100 in chilled 10% GMEM and mixed thoroughly.
3. 32 µl of diluted virus was added to all wells containing serially 4-fold diluted serum and virus control well. Then 384 well cell culture plate was incubated at 37ºC for 1 hr for virus neutralization in CO_2 incubator.
4. After the virus neutralization step, 32 µl of BHK-21 cells was added in all well and plate was incubated at 37ºC for 24 hrs in humidified chamber.
5. After the incubation period of 20-24 hrs, the spent medium was aspirated completely and cell monolayer was washed with phosphate buffer saline (pH-7.2).
6. Cells were fixed with 80% chilled acetone (80/20 in PBS) and plate was kept at -20ºC for 30 minutes.
7. Acetone was aspirated completely and the plate was air dried in room incubator at 37°C for 30 minutes.
8. After complete drying, 15 µl of rabies anti-nucleocapsid FITC conjugate (1/20 in PBS) was added to all wells.
9. Then plate was incubated at 37ºC for 30 minutes in humid chamber.
10. Aspirated the conjugate and plate was washed with PBS.
11. 30 µl of mounting fluid was added to all wells (50% Glycerine in PBS, V/V) and Plate was observed under fluorescent microscope.
12. Antibody titer in test serum was calculated by comparison with the reference serum in IU/ml.

3.6 Economics of Production of 5000 doses of Rabies cell culture vaccine based on certain assumption

Assumptions

- Depreciation on non recurring items is shown per annum.
- Uses cost is one month for similar three activities at a time.
- Electricity @ 5 Rs/unit

	Non recurring expenditure	**Total cost (Rs.)**	**Uses cost (Rs.)**
1.	Building depreciation cost@2% per annum	10,00000.00	900.00
2.	Vertical laminar flow @10%	1,00000.00	300.00
3.	CO_2 incubator@10%	2,50000.00	900.00
4.	Ultracentrifuge centrifuge@10%	2,50000.00	900.00
5.	Water bath@10%	25,000.00	800.00
6.	Freeze dryer @10%	25,00000.00	2,500.00
7.	Fluorescent microscope@10%	6,00000.00	1000.00
	Recurring expenditure		
1.	Cell culture medium (GMEM)		10000.00
2.	Fetal bovine serum		11000.00
3.	Reference standard		700.00
4.	Reference serum		70.00
5.	Anti nucleocapsid FITC conjugate		600.00
6.	Experimental animal		6000.00
7.	Micro tips and Pipette		50.00
8.	Micro titration plate (Terasaki plate)		200.00
9.	Electricity bill		3000.00
10.	Man power (Scientific, Technical, Supportive)		20000.00
11.	Roller bottle & Glass ware		4000.00
12.	Miscellaneous		1000.00
		Total cost =	61220.00

Production cost of vaccine = 61220/5000

= 12.24 Rs./dose

Chapter IV

Results

4.1 Propagation of Rabies virus (PV-11)

4.1.1 Preparation of working seed virus

Master seed of rabies virus (PV-11 strain) was co-cultivated in BHK-21 clone 13 cells using 0.1 multiplicity of infection (MOI) and harvested at 48 hrs. FAT was standardized in micro titration Terasaki plate and 10 fold dilution of virus sample harvest was titrated by Indirect FAT. Degree of fluorescence, in terms of fluorescence forming units (FFU) were observed under a fluorescent microscope. At $10^{-1}$and 10^{-2} virus dilutions almost all the cells were found to emit intense cytoplasmic fluorescence. On increasing virus dilutions, fluorescent foci were appreciated distinctly enabling FFU counts. At a virus dilution of 10^{-4} 5 infected cells could be detected showing rabies virus specific fluorescence. The intensity of fluorescence of infected cells between 10^{-1} dilution of virus to 10^{-4} dilution of virus is depicted in Fig.3. Titer of virus was found 4×10^{6} FFU/ml. This virus was used for further propagation and up scaling of virus for production of experimental vaccine.

4. 2 Propagation of virus for production and quality control of experimental vaccine

Rabies virus (PV-11 strain) was co cultivated in BHK-21 clone 13 cells with 0.1 MOI using cell factory and roller bottle. These were harvested at 48 hrs and pooled for the preparation of experimental vaccine. The cell free virus was subjected to quantification using FAT, MIT, Conventional reverse transcriptase PCR and real time PCR.

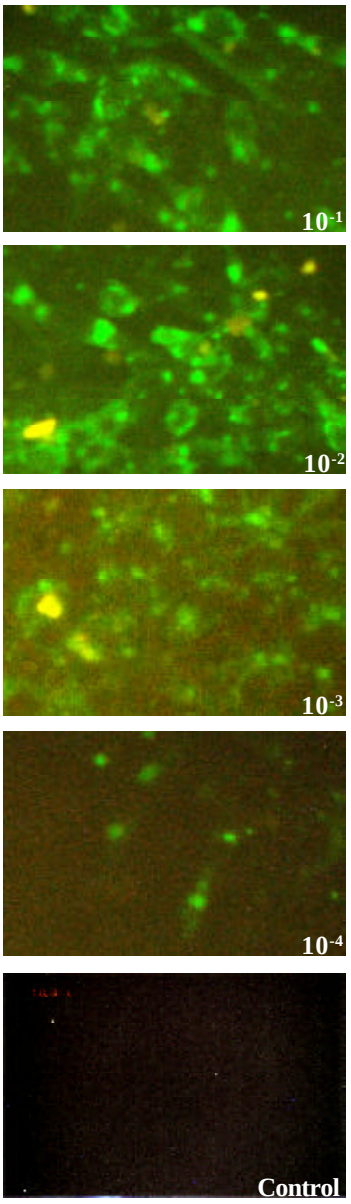


Fig.3: **FAT showing BHK-21 cells infected with 10 fold dilution of PV-11 strain of rabies virus.**

4.2.1 Fluorescent Antibody Test (FAT)

5 fold dilutions of pooled virus sample harvested at 48 hrs was titrated by FAT using Terasaki plate. Degree of fluorescence, in terms of fluorescence forming unit (FFU) was observed under a fluorescent microscope. At virus dilutions 1:5, 1:25, 1:125, 1: 625 almost all the cells were found to emit intense cytoplasmic fluorescence. On increasing virus dilution fluorescent foci were appreciated distinctly enabling FFU counts. At 1:15625 dilutions 7 infected cells could be detected showing rabies virus specific fluorescence. The intensity chart of fluorescence of infected cells between 1:5 dilution of virus to 1:15625 dilution of virus is depicted in above table. Same virus sample was titrated 3 times by indirect FAT and the average titer of virus was found to be 1.22×10^{7} FFU/ml.

Virus dilution	Pattern of Fluorescence
1:5	+ + + + +
1:25	+ + + + +
1:125	+ + + + +
1:625	+ + + +
1:3125	+ + +
1:15625	7 FFU

+ + + + + = Most cells showing fluorescence
+ + + + = Lot of cells showing fluorescence
+ + + = More than 50 cells showing fluorescence

4.2.2 Mouse Inoculation Test (MIT)

During the present investigation rabies virus was titrated in vivo in mice. For this 0.3 ml of 10 fold diluted virus (from10^{-8} to 10^{-1}) was inoculated intra cerebrally to each mice. The mice were observed for 14 days for rabies specific symptoms and death. Death after 5 day was only considered as positive. Virus titer was expressed in terms of LD_{50}/ml using Reed and Muench method. Observation charts is given in table 3.

Table.3: Death pattern of mice inoculated with PV-11 strain of rabies virus at different dilutions using 30 µl /mice by intracerebral route

Virus dilution	No. of mice per diution	Died	Survived	Accumulative value		% mortality (A/A+B)
				Death (A)	Survived (B)	
10^{-3}	3	3	0	9	0	100
10^{-4}	3	3	0	6	0	100
10^{-5}	3	3	0	3	0	100
10^{-6}	3	0	3	0	3	0
10^{-7}	3	0	3	0	6	0
10^{-8}	3	0	3	0	9	0
PBS	3	0	3	0	0	0

4.2.3 Polymerase Chain Reaction (PCR)

Rabies virus at different dilution (10^{-3} to 10^{-8}) was subjected to quantification by PCR with specific N gene primer and products was run in 1.0 % agarose showing specific products of 533 bp could be amplified and visualized up to virus dilution 10^{-6}(Fig.4) which subsequently become invisible at higher dilutions.

4.2.4 Real time PCR

During the present investigation titration of rabies virus was done using real time PCR by plotting standard curve between rabies virus dilution (10^{-3} to 10^{-8}) and Ct value. Ten fold dilution of pre titrated rabies virus was run in real time PCR and product specific amplification was seen up to 10^{-8} dilution which correspond to $5x10^{10}$ gene copies/ml and products Tm is 84^{o}C. Amplification plots and standard curve was obtained by using ct value and virus dilution (Fig. 5a, 5b).

4.2. 5 Comparative efficacy of FAT, MIT, PCR and real time PCR:

FAT was found to be equally specific and sensitive as mouse inoculation test (MIT). PCR was found to be about 50 times more sensitive then FAT and MIT. The real time PCR was found at least 1000 times more sensitive for detection of virus than FAT and MIT while 100 times more sensitive than PCR.The number of infectious virus particle (FAT and MIT) and gene copies/ml of sample are depicted bellow.

FAT	MIT	PCR	Real time PCR
1.22×10^{7}FFU/ml	$10^{7.02}LD_{50}$/ml	5×10^{8} gene copy/ml	5×10^{10} gene copy /ml

4.3. Inactivation of virus harvest

Virus was inactivated using B propiolactone (1:4000). Inactivation was checked by mice inoculation test (in vivo) and simultaneously by FAT (in vitro). All mice inoculated with inactivated virus and control survived while the mice inoculated with live virus died. This result was also supported by FAT in which control well and inactivated virus well did not show any specific fluorescence while live virus infected well showed specific fluorescence.

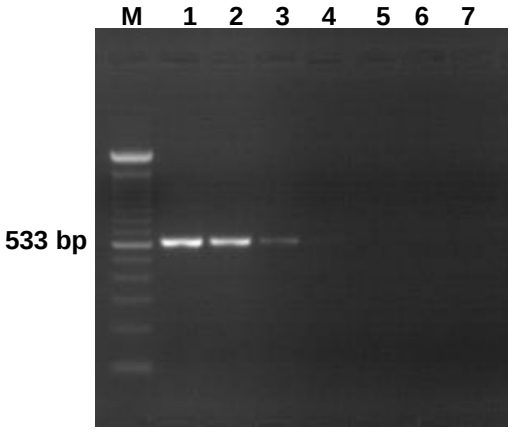


Fig.4: Photograph showing specific amplicon of N gene (533bp) of rabies virus from 10 fold diluted (10^{-3} to 10^{-8}) virus sample. Specific PCR products could be detected up to 10^{-6} dilution (lane-4) of virus which corresponds to $5x10^{8}$ genomic copies of virus/ml of sample.

Lane M : 100bp DNA ladder
Lane 1 : 10^{-3}
Lane 2 : 10^{-4}
Lane 3 : 10^{-5}
Lane 4 : 10^{-6}
Lane 5 : 10^{-7}
Lane 6 : 10^{-8}
Lane 7 : PBS

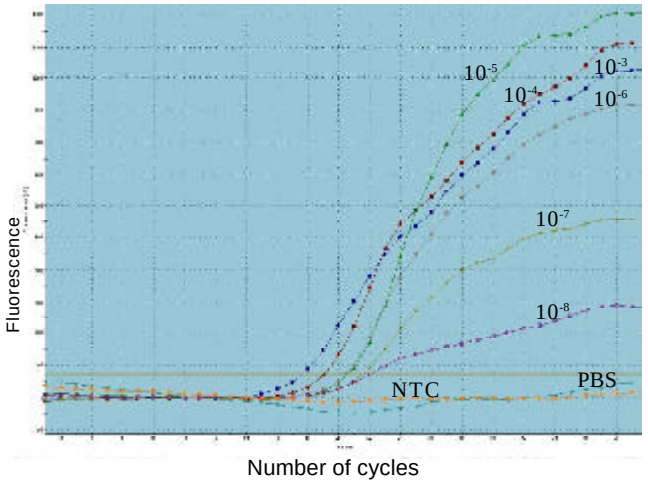


Fig5a: **Amplification plots of different dilutions (from 10^{-3} to 10^{-8}, PBS and NTC) of rabies virus genome using N gene (200bp) primer by Real time PCR. The Ct (threshold cycle) values on X axis indicated desired amplification of sample.**

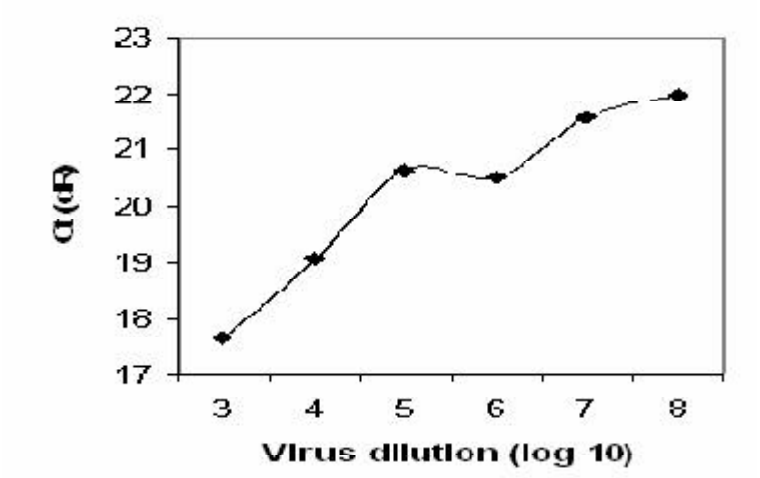


Fig5b: **Standard curve between Ct value and different virus dilutions (from 10^{-3} to10^{-8}) of rabies virus using real time PCR. The real time PCR could detect up to 10^{-8} dilution of virus which corresponds to 5×10^{10} gene copies/ml of virus sample.**

4.4. Potency assay of experimental vaccine:

During the present study, potency test of the experimental vaccine was done using mice and rabbit model. Subsequent upon immunization mice were subjected to RFFIT and challenge test while rabbit for RFFIT.

4.4.1 Titration of Challenge virus standard

Challenge virus standard (CVS) was titrated in mice and titer was found to be $10^{-5.4}$ LD_{50}/30µl of inoculum. Observation chart is given in table 4.

Table.4: Death pattern of mice inoculated with Challenge Virus Standard strain of virus at different dilutions using 30 µl /mice by intracerebral route.

Virus dilution	No. of mice per diution	Died	Survived	Accumulative value		% mortality (A/A+B)
				Death (A)	Survived (B)	
10^{-3}	7	7	0	18	0	100
10^{-4}	7	5	2	11	2	84.61
10^{-5}	7	4	3	6	5	54.54
10^{-6}	7	2	5	2	10	16.66
10^{-7}	7	0	7	0	17	0
10^{-8}	7	0	7	0	24	0
PBS	7	0	7	0	0	0

4.4.2 Potency test in mice

In the present study potency evaluation of test vaccine was done in mice and compared with reference vaccine (Verorab). The potency of test vaccine was adjusted to equal to reference vaccine (Verorab) ($\geq$2.5 IU/ml). In order to avoid non specific death data was taken as ratio of mice surviving on 5^{th} day of challenge and day at which all control mice died. Apart from this neutralizing antibody were assessed by RFFIT on the day of challenge (14^{th} days post immunization). Established correlation between mean antibody titer using RFFIT and survivability of mice after challenge. The present study indicated a direct correlation of survival rate with antibody titer in mice (Table. 5 and Fig.6a, 6b, 7).

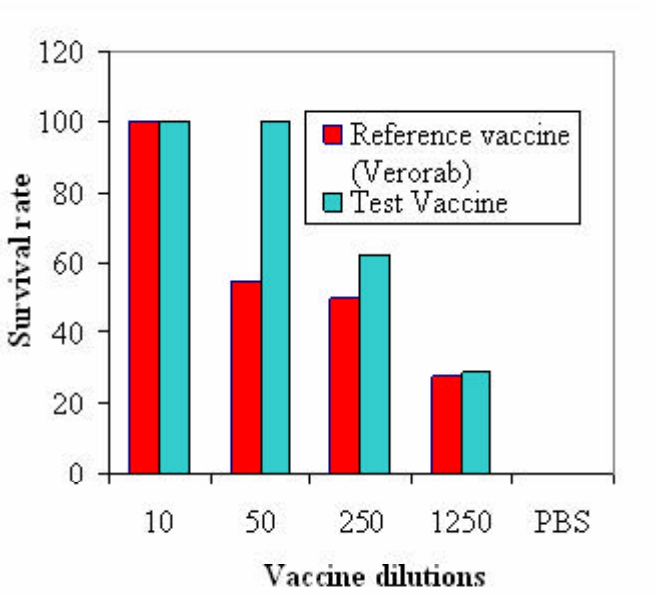


Fig.6a: Comparative efficacy of Test vaccine with Reference vaccine (Verorab) in mice at 4 different dilutions (10, 50, 250 and 1250). Survival rate in different groups was calculated on the day all the mice in control/PBS group died of specific symptoms of rabies.

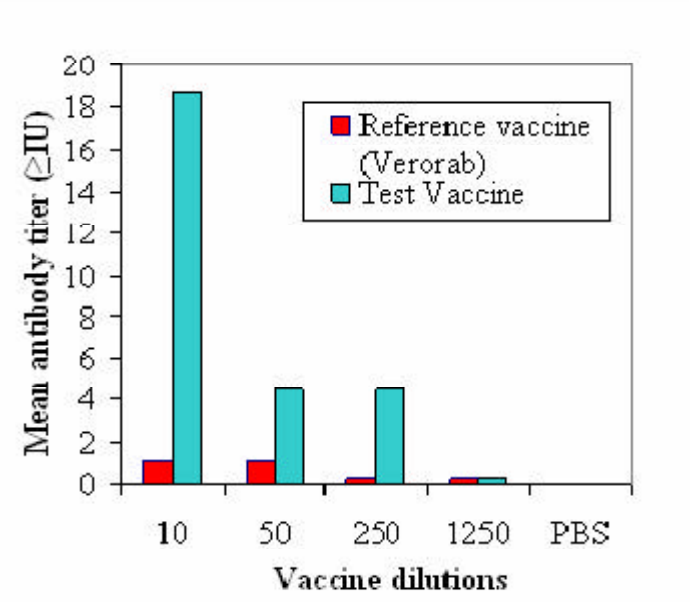


Fig.6b: Mean antibody titer of mice using RFFIT at 4 different dilutions (10, 50, 250 and 1250) of Reference vaccine (Verorab) and Test vaccine on 14th day.

Table.5: Comparative efficacy of test vaccine with reference vaccine (Verorab) in mice at 4 different dilutions (10, 50, 250 and 1250). Survival rate in different groups was calculated on the day all the mice in control/PBS group died of specific symptoms of rabies. Mean antibody titer of mice as measured by RFFIT was calculated from the sample collected on day 14.

Reference vaccine (Verorab)			Test Vaccine		
Dilutions (≥IU/mice)	**Survival rate**	**Antibody titer(IU/ml)**	**Dilutions (FFU eqvt.)**	**Survival rate**	**Antibody titer (IU/ml)**
1:10 (0.1IU)	100%	≥1.13	1:10 (3.0x 10^6)	100%	≥18.7
1:50 (0.02IU)	55%	≥1.13	1:50 (0.60x 10^6)	100%	≥4.54
1:250 (0.004IU)	50%	≤0.28	1:250 (0.12x 10^6)	62%	≥4.54
1:1250 (0.0008IU)	28%	≤0.28	1:1250 (0.024x 10^6)	28.6%	≥0.28
PBS (vaccine diluent)	0	0	0	0	0

4.4.3 Kinetics of antibody response in rabbit

Kinetics of antibody response was investigated at two different doses of reference (Verorab) and test vaccine following post exposure immunization schedule in rabbit using RFFIT. The pattern of antibody kinetics was more or less similar in ≥0.5 IU of reference vaccine and $3x10^7$ FFU of test vaccine with slight delayed immune response. At the same titer ≥0.1 IU of reference vaccine (Verorab) produced a similar antibody response as that of $6x10^6$ FFU of test vaccine. The antibody response became optimum in all the groups after 7 days of 5^{th} /final immunization (Table.6 and Fig. 8)

Table 6: Antibody kinetics of immunized rabbits using Test Vaccine and Verorab at two different doses following a post exposure schedule of immunization (days 0, 3, 7, 14 & 28).

Vaccine	Dose of vaccine	Antibody titer(=IU/ml) after immunization				
		0 day	14th day	21st day	28th day	35th day
Reference	100µl (≥0.5IU)	0	300	300	300	300
vaccine (Verorab)	20 µl (≥0.1IU)	0	75	75	75	300
Test vaccine	500µl (3×10^7FFU)	0	75	300	300	300
	100µl (6×10^6FFU)	0	75	75	75	300

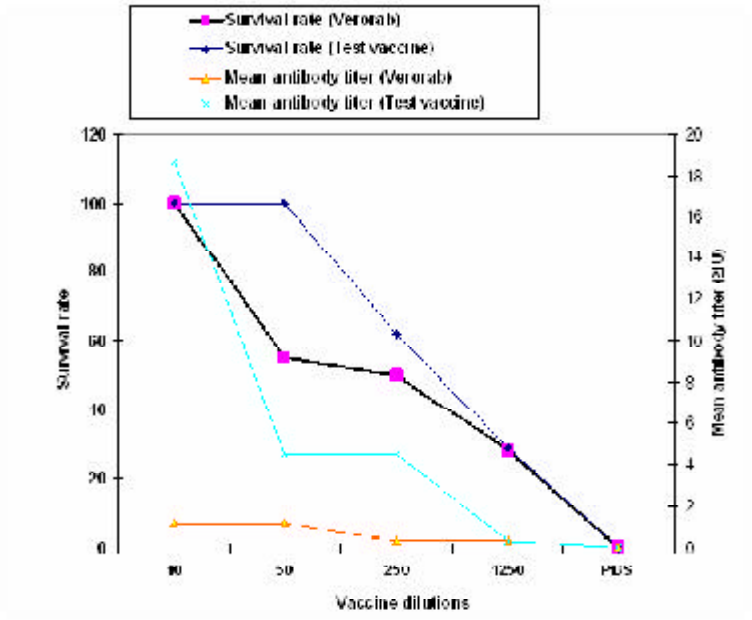


Fig.7: Comparative efficacy of Test vaccine with Reference vaccine (Verorab) at 4 different dilutions (10, 50, 250 and 1250) between survival rate and mean antibody titer on day 14 in different groups of mice.

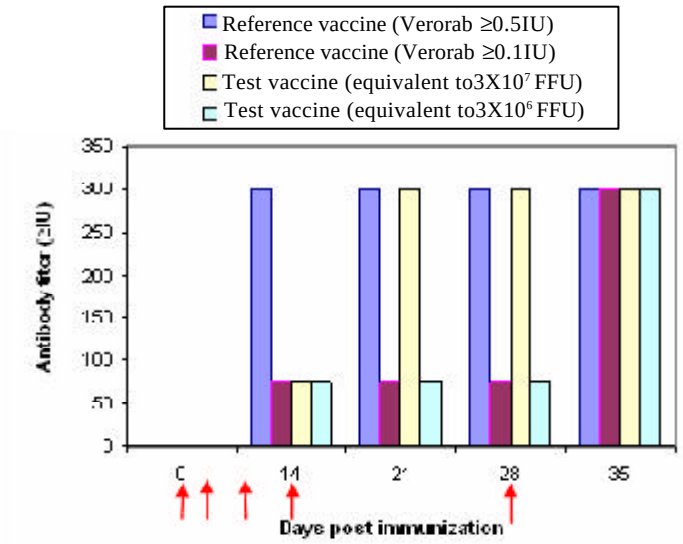


Fig.8: Antibody kinetics of immunized rabbits using Reference vaccine (Verorab) and Test Vaccine at two different doses following a post exposure schedule of immunization. Point of arrow (▲) indicates the days on which immunization (days 0, 3, 7, 14 & 28) took place.

Chapter V

Discussion

Rabies is a highly fatal viral zoonosis involving nervous system of warm-blooded animals including man. It is prevalent in all parts of the world except few island countries. The disease under Indian socio-economic and socio-cultural conditions is spread mainly by the bite of rabid dogs. Major geographical regions of our country are classified as having high endemic for rabies (WHO, 2001). Although the disease is known to mankind since several centuries, sincere efforts to protect the livestock population from this deadly disease have not been given due importance. Vaccination of the animals following a pre-exposure or post exposure vaccination schedule is the only way to prevent rabies and to protect the animals from this deadly disease.

The disease has been eradicated from many countries with large scale vaccination campaigns and controlled destruction of vectors of the disease (Pastoret *et al*., 1992). The reduced risk of exposure of rabies in canine population is automatically a solution to reduce rabies incidence in the human as well as domestic livestock population. The majority of the vaccines used in veterinary field at the moment are based upon the use of continuous cell lines such as BHK-21 cells or hamster embryo cell line (NIL2) (Pay *et al.*, 1985; Sureau, 1992). The inactivated cell culture vaccine is the only option for prophylactic and curative immunization against rabies. Due to fatal zoonotic disease and risk involved with the handling of virus, it is always desired to produce quality vaccine with a minimum handling/processing of live virus in laboratories with inadequate biosafety facilities. Furthermore due to inadequate infrastructure of cell culture work in the developing countries, it is sometimes difficult to get desired antigenic value in the vaccine preparations. In order to produce the vaccine with a desired antigenic value, cost of the per unit vaccine production increases resulting in a higher burden on people

with low paying capacity. The antirabies vaccination in large ruminants in India is carried out mainly as a therapeutic measure following a post bite vaccination schedule, subsequent upon dog bite.Under these circumstances the antigenic value of such a vaccine and quality assurance is of utmost importance. In field conditions, an efficacious vaccine with a proven antigenic value/antigenic mass is must for large ruminants considering its economic value and risks after exposure to infection. A critical observation from virus propagation to production of end product by reassuring certain in-process monitoring system is likely to reduce the production cost of the quality vaccine.

Keeping all these problems in mind, the present study ambises development of an alternative approach for in process quality control of rabies cell culture vaccine especially in terms of use of virus with known quality at predetermined MOI using FAT as basic technique. This involves propagation of PV-11 strain of rabies virus in BHK-21 cell line and preparation of working seed virus for the production of an experimental rabies vaccine. Further up scaling of rabies virus using 0.1 MOI of inoculum, an experimental vaccine was produced and evaluated for its efficacy in mice and rabbits using virulent challenge in mice and antibody response by RFFIT in both the species. The techniques adopted and evaluated in the present investigation are likely to reduce the use of mice in the routine rabies vaccine production process and also increase the efficacy of vaccine, especially in the small vaccine production units like state veterinary biological units. This will help the up scaling of rabies vaccine production in the country like India which has huge demand for rabies vaccine due to large number of post bite cases.

Quality of seed virus plays an important role in the vaccine production process. Master seed virus with a titer of 3×10^6 FFU/ml was taken and subsequently used for preparation of working seed virus with the titer 4×10^6 FFU/ml. This working seed virus was used for the production of an experimental batch of rabies vaccine with a known quantity of virus. For preparation of experimental vaccine, BHK-21 cells were infected using 0.1 MOI of virus and virus harvest was collected at 48 hrs of infection with an idea that this gives a maximum yield of virus (Anandan, 2006). The experimental batch of rabies vaccine was produced in cell factory and roller culture bottle. For comparison, the virus yield in cell factory was 3 times higher than the roller bottle (3×10^7 FFU/ml in cell factory against 10^7 FFU/ml in roller bottle). It is possible that the cell density and quality of cells is relatively higher in the cell factory as compared

to roller bottles. The virus harvest obtained at 48 hrs after inoculation was pooled and subject for virus quantification and inactivation.

In view of the development of alterative approach for quality control of rabies vaccine, the vaccine virus from experimental batch was quantified using various techniques Viz: Fluorescent antibody test (FAT), Mouse inoculation test (MIT), Polymerase chain reaction (PCR) and real time PCR. Comparative efficacy of indirect FAT with MIT indicated that both the test are equally sensitive. Therefore, these tests can replace each other based on the requirement and level of technical expertise available in the laboratory. The critical analysis of the virus inoculum having a virus titer of 1.22×10^7 FFU/ml by FAT was found to have $10^{7.02}$ LD_{50}/ml in MIT. This difference could be due to the fact that a part of virus inoculum oozes out during intra cerebral inoculation and also due to measurement errors using insulin syringe. These finding are in accordance with the available literature recommended by WHO and OIE (Chapman *et al*., 1973; Meslin *et al.,* 1996; Shanker, 2009). Some of these workers have found that there is a 90-99% correlation between FAT and MIT. Chabara *et al.* 2007 reported that sensitivity of FAT was 100% and it gives 100% concendance with MIT. False negative FAT results are not common but can occur due to inadequate sampling, faulty equipment, and unsatisfactory conjugate, lack of proper control and lack of experience. Application of FAT as a routine test will reduce use of live animals and time duration (2 days for FAT as against 14 days for MIT).

PCR technique is considered to be a sensitive tool for detection and quantification of virus. Therefore, we compared the reverse transcriptase PCR and real time PCR with the gold standard test like FAT and MIT. Rabies virus specific genome could be detected as 5×10^8 genomic copies/ml in PCR as against 5×10^{10} genomic copies/ml by real time PCR in a virus inoculum containing 1.22×10^7 FFU/ml. Findings indicated that conventional PCR techniques is 50 times more sensitive than FAT and MIT, while real time PCR is 1000 times more sensitive than FAT/MIT. The higher sensitivity of PCR technique could be because of the fact that FAT and MIT detect only infectious live virus particles where as PCR technique is directed towards viral RNA detection. Further freezing and thawing of a virus sample may reduce the live infectious virus titer to many folds. These in vitro assays will also help us to improve quality of vaccine and also in process monitoring of vaccine production. Both the PCR techniques

were directed towards the detection of N gene (Gupta *et al.,* 2006) of rabies virus which is considered to be most abundant and most reliable gene for virus detection and quantification (Dean *et al.,* 1996). It is also known established fact that PCR is more sensitive technique for detection of viral genome as compared to serological techniques using Peste des Petits Ruminants virus model (Saravanan *et al.,* 2004). We need to keep in mind that quantification of rabies antigen is not reliable for estimation of immunogenicity when vaccines derived from different rabies virus strains were compared (Lyng *et al.,* 1992). Antigen concentrations with in a given preparation could not correlate with immunogenic potential, nor with the ability of a vaccine to stimulate a protective immune response (WHO, 1992).

ß-propiolactone (BPL), an alkylating and virus inactivating agent is a most common inactivating agent used for inactivation of rabies virus in the vaccine production process. It has been indicated that in addition to inactivation of virus, BPL also reduces/eliminates the carcinogenic property of BHK-21 cells by strand break and nick of cellular DNA. The damage to the DNA structure by BPL modifies the biological properties of the purified cellular supernatant DNA appraised by its ability to serve as the template in vitro for different polymerases (Morgeaux *et al.,* 1993) and at the same time this does not interfere with the antigenic property of virus for induction of protective immune response. During the present investigation the experimental virus was inactivated using 1:4000 dilution of BPL. This inactivated virus was tested for any residual infectivity in BHK-21 cells using FAT to trace virus infectivity and by mouse inoculation test. Both the tests revealed that no residual live virus was present in vaccine sample.

Potency of experimental vaccine was tested using mouse model and the NIH test. For this assay, 4 dilutions of reference vaccine (Verorab) and test vaccine were inoculated by I/P route and mouse were challenged with challenge virus strain (CVS) of rabies virus. The potency of 1st dilution of reference vaccine was adjusted to $\geq$0.1 IU where as that of test vaccine was adjusted equivalent to 3.0 x 10^6 FFU. The findings indicated that the test vaccine is equally potent as the reference vaccine. It was also observed that 3 x 10^6 FFU equivalent of inactivated virus may be equivalent to $\geq$0.1 IU of reference vaccine. Therefore, in order to produce a veterinary vaccine of $\geq$1 IU, about 3 x 10^7 FFU infectious units of rabies virus particle may be required. This will fulfill the basic requirement in terms of antigenic value/antigenic mass. Antigenic

value would have been more precisely measured by us if, in case the International/National reference standards would have been used in place of reference vaccine as standard. This could not be possible due to non availability of these standards during the present study.

Antibody assay in immunized mice used for the NIH test seems to be the best possible means to determine the potency of inactivated rabies vaccines (Lazrowicz et *al.*, 1982). The potency may be determined serologically by measuring the neutralizing antibody titers induced after vaccination of mice using a rapid fluorescent focus inhibition test (RFFIT). Correlation between the challenge test results and the mean titers can be determined by RFFIT. Although this method is faster and less painful for the animals, it is not widely used yet (Kramer et *al.*, 2009). Based on this in present study the humoral immune response was assessed using RFFIT. The antibody response in different group of reference vaccine indicated that $\geq$0.2 IU induces the protective immunity of $\geq$1.13 IU/ml while that of about 1.2 x 10^5 FFU equivalent of test vaccine induces similar protective antibody response on 14^{th} day post immunization. In general the antibody response was higher in test vaccine as compared to respective dilution of reference vaccine. It is worth to mention here that reference vaccine was purified virus: wistar rabies PM/WI-138 1503-3M strain grown in Vero cells while test vaccine was a cell free whole culture of PV-11 strain of rabies virus grown on BHK21 cells. It is possible that in addition to virus particles, the non structural components of virus such as soluble glycoprotein (G_S) secreted by virus infected cells (Dietzschold *et al.*, 1983), Phosphoprotein (P) or Non structural protein (NS) (Sonoda *et al.*, 1993) and RNA dependant RNA polymerase (L) may also contribute the humoral immune response as well as the of the vaccine.

The major objective of present investigation was to develop an alternative approache for quality control of rabies vaccine which is intended to be used mainly in post bite cases. We adopted similar regime for immunization of rabbit and assessment of the humoral immune response using RFFIT. For this rabbits were immunized on 0, 3, 7, 14 and 28 days at two different doses of verorab ($\geq$0.5 IU and $\geq$0.1 IU/dose) and test vaccine (3 x 10^7 FFU equivalent and 6 x 10^6 FFU equivalents). The antibody kinetics of these animals on day 0, 14, 21, 28 and 35 indicated that all the animal show $\geq$75 IU/ml on 14^{th} days of immunization schedule. A higher early antibody response was observed in rabbit immunized with $\geq$0.5 IU on day 14^{th}, which subsequently became equal to test vaccine on day 21^{st} in higher dose of test vaccine.

The lower doses (≥0.1 IU/dose) reference vaccine and test vaccine (6 x 10^6 FFU eqvt.) had an identical antibody response showing protection on day 14 (≥75 IU/ml) which reached the peak (≥300 IU) on day 35 following 5^{th} and final dose of immunization. These finding suggest that although in majority of the cases, highest antibody titers are obtained after 4 initial immunizations (0, 3, 7, 14 days). However, the 5^{th} immunization may be beneficial in cases of vaccines with low antigenic value, as is possible in the absence of stringent quality control measure and also poor storage conditions in the countries where electric failures are common. This is more important in the rural areas of India which encounter frequent electric failures with inadequate transport conditions and poor awareness about the vaccine quality.

The present investigation revealed that FAT and RFFIT can replace MIT and Mouse virus neutralization test (MNT) respectively during in process quality control of rabies vaccine. Therefore critical use of FAT and RFFIT for uniform quality control of rabies vaccine will help us in reducing the use of mice for potency assay. Further in order to reduce the number of mice in NIH test/any similar test as recommended by Indian pharmacopoeia and European pharmacopoeia can be used only for the quality assurance of the finished products for release of vaccine batch. Looking at the incidence of vaccine failures in post bite cases of animals, we may focus more on potency of the vaccine with even a slight compromise on purity of antigen. The non purified vaccine which may have some non-structural proteins of virus origin may induce protective antibody response several fold higher than the affinity purified vaccine. The investigation would have been more interesting and precise with the availability of International reference standards (vaccine/serum).The availability of uniform quality of mice in adequate number may also have improved the authenticity of findings further. The production cost of vaccine was roughly estimated to be Rs. 13. Per dose which is one third price of exiting animal rabies vaccine. Use of different adjutants and their combinations may improve the potency of vaccine and may reduce the production cost of vaccine for use in post bite cases of large and small ruminants.

Chapter VI

Summary

Rabies is a highly fatal viral zoonotic disease of warm-blooded animals including man. The causative agent of rabies has been classified under the genus *Lyssavirus* of the family *Rhabdoviridae*. Now a day's rabies cell culture vaccines are being used extensively for treatment and prophylactic immunization against rabies in animals. The anti rabies vaccination in large ruminants in India is carried out mainly for treatment following a post bite vaccination schedule. Under these circumstances quality assurance of vaccine is of utmost significance. Keeping all above points in mind the present investigation was planned with an objective of development of an alternative approaches for in process quality control of rabies vaccine to minimize the use of mice and simplify vaccine production process.

In the present investigation, an experimental rabies vaccine was produced by growing and up scaling of working seed virus (PV-11 strain) with the titer $4x10^6$ FFU/ml at 0.1 MOI in BHK-21 cell using cell factory and roller bottle. The virus yield in cell factory was three times higher than roller bottle. Virus harvest collected at 48 hrs was pooled and quantified by Fluorescent antibody test (FAT) and Mouse inoculation test (MIT), PCR and real time PCR. The pooled virus inoculum having a virus titer of 1.22×10^7 FFU/ml by FAT was found to have $10^{7.02}$ LD_{50}/ml in MIT. The findings suggests that FAT was equally sensitive as MIT. Therefore, FAT can replace MIT based on the requirement and level of technical expertise available in the laboratory. We compared the sensitivity of PCR and real time PCR with the gold standard test like FAT and MIT for virus quantification. Findings indicated that real time PCR is at least 1000 times more sensitive than FAT and MIT. The higher sensitivity of PCR technique could be because of the fact that FAT and MIT detect only infectious live virus particles where as PCR technique is directed towards viral RNA detection irrespective of live virus particle.

Vaccine virus was inactivated using β–propiolactone (1:4000 dilution). This inactivated virus was tested for any residual infectivity in BHK-21 cells using FAT to trace virus infectivity and by mouse inoculation test. Both the test revealed that no residual live virus was present in vaccine sample after two cycle of inactivation.

Potency of test vaccine was evaluated using mice and rabbit model and compared with reference vaccine (Verorab). Potency of vaccine was evaluated using mouse model in NIH test. Four five fold dilutions of reference vaccine (Verorab) and test vaccine (1:10, 50, 250, 1250) were inoculated by I/P route and mouse were challenged with challenge virus strain (CVS) of rabies virus on day 14. It was observed that 3×10^6 FFU equivalent of inactivated virus may be equivalent to $\geq$0.1 IU of reference vaccine. Therefore, in order to produce a veterinary vaccine of $\geq$1 IU, about 3×10^7 FFU infectious units of rabies virus particle may be required. The findings also indicated that the test vaccine was equally potent as the reference vaccine. The potency of test vaccine was also determined serologically by measuring the neutralizing antibody titers induced after day 14 post immunization challenge of mice in NIH test using RFFIT. Findings Mean antibody titers in mice on day 14 post immunization was well correlated with survivability of mice following virus challenge. It seems, RFFIT can be used as back up test for potency estimation in case of failure of NIH test.

The major objective of present investigation was to develop an alternative approaches for quality control of rabies vaccine which is intended to be used mainly in post bite cases. We adopted similar regime for immunization of rabbit and assessment of the humoral immune response using RFFIT. For this rabbits were immunized on 0, 3, 7, 14 and 28 days at 2 different doses of reference vaccine ($\geq$0.5 IU and $\geq$0.1 IU/dose) and test vaccine (3×10^7 FFU equivalent and 6×10^6 FFU equivalents). The antibody kinetics of these animals on day 0, 14, 21, 28 and 35 indicated that all the animal show $\geq$75 IU/ml on 14^{th} days of immunization schedule. The finding also suggested that although in majority of the cases highest antibody titers are obtained after 4 initial immunizations (0, 3, 7, 14 days). However, the 5^{th} immunization may be beneficial in cases of vaccines with low antigenic value, as is possible in the absence of stringent quality control measures and also poor storage and transport conditions in the countries like India where electric failures are common.

The present investigation reveals that FAT and Rapid fluorescent focus inhibition test (RFFIT) can replace Mouse inoculation test (MIT) and Mouse virus neutralization test (MNT) respectively during in process quality control of rabies vaccine. Therefore critical use of FAT and RFFIT for uniform quality control of rabies vaccine will help us in reducing the use of mice for potency assay. However for final release of vaccine batches NIH test or similar test as recommended by Indian pharmacopoeia may be followed.

Chapter VII

Mini Abstract

Rabies is a highly fatal viral zoonotic disease of warm blooded animals including man. The present investigation aimed at critical investigations on development of alternative approaches for in process quality control of rabies vaccine.

During the present study, rabies virus (PV-11 strain) was propagated with known titer of working seed virus ($4x10^6$FFU/ml) at 0.1 MOI and harvested at 48 hrs. The pooled virus harvest was titrated by Fluorescent antibody test (FAT) and Mouse inoculation test (MIT). Findings suggested that both tests were equally sensitive for virus infectivity assay. PCR technique was also evaluated for quantification of virus. Quantitative real time PCR was found to be atleast 1000 times more sensitive than FAT and MIT. This may be due to the fact that MIT/ FAT detects only infectious live virus particles where as PCR detects copies of viral genome. Cell culture propagated virus was inactivated by β-propiolactone (BPL) and tested for any residual infectivity in BHK-21 cells using FAT and MIT. These test revealed that no residual live virus was present in vaccine sample.

Potency of test vaccine was evaluated using a test similar to National Institute of Health (NIH). Potency of test vaccine was adjusted equal to reference vaccine (Vero rab). Findings with NIH test and Rapid fluorescent focus inhibition test (RFFIT) indicated that mean antibody titers correlate well with survivability of mice following virulent challenge. Therefore, RFFIT can be used as back up test for potency estimation in case of failure of NIH test. Furthter, we adopted post bite immunization schedule for rabbits using 2 different doses of test and reference vaccines assessed for the humoral immune response using RFFIT. The antibody kinetics of these animals indicated that highest antibody titers ($\geq$300IU) were obtained after 4 initial immunizations (0, 3, 7, 14 days). However, the 5^{th} immunization was beneficial in cases of vaccines with low antigenic value. This is more important in developing countries like India due to poor storage condition on account of frequent electric failures and inadequate transportation facilities.

Chapter VIII

लघु सारांश

रेबीज एक बेहद घातक विषाणु जनित जूनोटिक बीमारी है, जो मुख्यतः गर्म खून वाले जानवरों में होता है। वर्तमान अध्ययन रेबीज रोग के टीके में गुणवत्ता नियंत्रण के लिए वैकल्पिक तरीकों के विकास पर गंभीर जांच करने के उद्देश्य से किया गया है। वर्तमान अध्ययन के दौरान रेबीज विषाणु (पी.वी.–11) का उत्पादन, ज्ञात टाइटर के बीज विषाणु (4×10^6 एफ.एफ.यू./एम.एल.) के 0.1 एम.ओ.आइ. संक्रमण से वी.एच.के.–21 कोशिका में किया गया और 48 घंटे पर एकत्रित किया गया। एकत्रित विषाणु का अनुमापन प्रतिदिप्त प्रतिपिण्ड परीक्षण तथा चूहे में संक्रमण परीक्षण द्वारा किया गया। वर्तमान अध्ययन से यह पता चलता है कि दोनो परीक्षण समान रूप से विषाणु संक्रमण परख के लिए संवेदनशील हैं। पी.सी.आर. तकनीक को भी विषाणु अनुमापन के लिए जांचा गया। रीयल टाइम पी.सी.आर., प्रतिदिप्त प्रतिपिण्ड परीक्षण तथा चूहें संक्रमण परीक्षण से एक हजार गुणा अधिक संवेदनशील पाया गया। इसका मुख्य कारण यह हो सकता है कि प्रतिदिप्त प्रतिपिण्ड परीक्षण तथा चूहे संक्रमण परीक्षण केवल संक्रमण विषाणु का पता लगाता है जबकि पी.सी.आर. विषाणु के जीनोम का पता लगाता है।

वी.एच.के. कोशिकाओं में उत्पादित रेबीज विषाणु वीटा प्रोपिओलेकटोन (वी.पी.एल.) के द्वारा निष्क्रिय किया गया और विषाणु निष्क्रियता की जांच वी.एच.के.–21 कोशिका में प्रतिदिप्त प्रतिपिण्ड परीक्षण तथा चूहे संक्रमण परीक्षण द्वारा किया गया। दोनो परीक्षणों से पता चला कि टीके के नमूने में कोई अवशिष्ट संक्रमण विषाणु नहीं थे। रेबीज टीके का शक्ति परीक्षण चूहों में एन. आई.एच. (नेशनल इंस्ट्टूट आफ हेल्थ) परीक्षण से किया गया और टीके की शक्ति को संदर्भ टीके के बराबर समायोजित किया गया। वर्तमान अध्ययन के अनुसार एन.आइ.एच. परीक्षण में चूहों की जीवितता और रेपीड फ्लूरिसेन्ट फोकस इन्हीबीसन टेस्ट (आर.एफ.एफ.आई.टी.) के द्वारा प्रतिपिण्ड टाइटर अच्छी तरह सहबंधित पाया गया। इसलिए एन.आइ.एच. परीक्षण के विफल हो जाने के समय शक्ति परीक्षण के आंकलन के लिए वैक्लिपिक तरीका आर.एफ.एफ. आई.टी. के रूप में इस्तेमाल किया जा सकता है। हमने खरगोश में रेबीज जानवरों के काटने के बाद टीकाकरण कार्यक्रम के आधार पर 2 अलग–अलग खुराकों में टीका लगाया और प्रतिरक्षा प्रतिक्रिया (एंटीबाडी टाइटर) का आकलन रेपीड फ्लूरिसेन्ट फोकस इन्हीबीसन टेस्ट द्वारा किया। इन जानवरों के प्रतिपिण्ड कैनेटीक्स के अनुसार उच्चतम प्रतिपिण्ड टाइटर ($\geq$300 IU) आरम्भिक चार टीकाकरण (0,3,7, 24 दिन) के बाद पाया गया। हांलाकि पांचवां टीकाकरण कम प्रतिजन के टीकों के मामलों में फायदेमंद होगा इसका महत्व सबसे अधिक भारत जैसे विकासशील देशों में है जहां अपर्याप्रप्त परिवाहन और लगातार बिजली की कमी के कारण टीके का सही भडारण एवं रख–रखाव नहीं हो पाता है।

Chapter IX

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Appendix

1) Glasgow Minimum Essential Medium (GMEM)

GMEM (G-6148, Sigma)	12.7 g
Tryptose phosphate broth (TPB)	3 g
Sodium bicarbonate	0.75 g
Gentamicin	50 mg
Triple distilled water (autoclaved)	900 ml

Medium is filter sterilized and 48 hrs (at 37ºC) tested media is used for making growth media (10% FBS) and maintenance media (2% FBS).

2) 7.5% $NaHCO_3$ solution

$NaHCO_3$	7.5 g
Distilled water (autoclaved)	100 ml

Filter sterilized using 0.22 µm syringe filters.

3) Phosphate buffer saline (PBS) (pH 7.2)

NaCl	8 g
KCl	0.2 g
KH_2PO_4	0.2 g
Na_2HPO_4	1.15 g
Distilled water	1000 ml

Mix and make the solution and sterilize it by autoclaving at 121°C/15 lbs pressure/15 min.

4) 50% Buffered glycerine (v/v)

PBS (pH 7.2)	100 ml
Glycerine	100 ml

Mix both the solutions thoroughly.

5) Acetone for fixation

Acetone	80 ml
PBS (pH 7.2)	20 ml

Stored at -20ºC, use chilled acetone for fixing the cell monolayer.

6) 4x F A rinse buffer (pH-9.0-9.5)

Na_2CO_3	11.4g
Na_2HCO_3	33.6 g
NaCl	8 g
Distilled water	1000 ml

Mix and make the solution and kept at room temperature in tightly stoppered container.