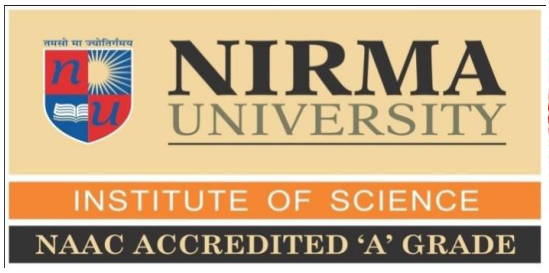
Determination of effect of pre-exposure immunization against Rabies virus using *in vitro* assay

A dissertation thesis submitted to Nirma University in partial fulfillment for the Degree of

MASTER OF SCIENCE

IN

MICROBIOLOGY



Submitted by

Pankti Jayant Dave (15MMB008)

Charmi Ketan Mehta (15MMB010)

Under the guidance of

Mr. Pankaj Kalita

Dr. Heena Dave

Dr. Vijay Kothari

INSTITUTE OF SCIENCE

NIRMA UNIVERSITY

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Thank you,

Date :

Place : Ahmedabad

Pankti Dave

Charmi Mehta

CONTENTS

ABBREVIATIONS

- WHO: World Health Organization
- CDC: Centre for Disease Control
- ATCC: American type culture collection
- NIBSC: The National Institute for Biological Standards and Control
- i.m.: Intra muscular
- MEM: Minimum Essential Medium
- FITC: fluorescein isothiocyanate
- CVS-11: Challenge Virus Strain-11
- BHK-21: Baby Hamster Kidney-21
- FFD_{50}: Median fluorescent focal dose
- $TCID_{50}$: Tissue culture infectivity dose
- PBS: phosphate buffered saline
- µL : microliter
- mg : milligram
- mL : milliliter
- FBS: Fetal Bovine Serum
- EDTA: Ethylenediaminetetraacetic Acid
- BSA: Bovine serum albumin
- IPA: Isopropyl alcohol
- DMSO: Dimethyl sulfoxide
- PEP: Post-exposure porphylaxis
- rpm: Rotation per minute
- DFA: Direct fluorescent antibody
- NA: Not Applicable

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ABSTRACT

Rabies is a fatal infectious viral disease following the onset of clinical symptoms. Rabies virus is a strictly neurotropic virus that propagates in the nervous system of the infected host from the site of entry (usually due to a bite) up to the site of exit (salivary glands). In recent years a considerable progress has been made in understanding the molecular biology, structure and function of rabies virus. A quick and easy procedure for the diagnosis of rabies is the use of suitable dye for the detection of Negri bodies which has been replaced in most laboratories by the Fluorescent Antibody Test. It is most widely used and most accurate method for diagnosing rabies infection in animals and humans. One of the techniques is a Virus neutralization test which uses the principle of neutralization of a constant dose of a previously titrated challenge virus infecting the cells by the antibody in a dose dependent manner. Hence the correct application of inactivated tissue culture-derived vaccines (Rabipur®) is highly effective at preventing the development of rabies. In this study, pre-exposure immunization was performed in 2 subjects. The result depicted high antibody generation which was expressed in IU/mL. It was observed that pre-exposure immunization of subject resulted in a steep increase in the antibody titre against rabies.

1.0 INTRODUCTION

1.1 Rabies

Rabies is a viral infectious disease transmitted to humans by the animals already suffering from it. The animals mainly responsible for causing rabies are racoons, bats, dogs, skunks and foxes. In 99% of cases, domestic dogs are responsible for virus transmission to humans. Generally, rabies is caused when the human comes in contact with the saliva of rabied dogs. This may also occur by other means such as bites, scratches, non-bites exposure and human to human transmission (CDC, 2016). Other two forms of the disease:

- People with furious rabies exhibit signs of hyperactivity, excitable behavior, hydrophobia (fear of water) and sometimes aerophobia (fear of drafts or of fresh air). Death occurs after a few days due to cardio-respiratory arrest.
- Paralytic rabies accounts for about 30% of the total number of human cases. This form of rabies runs a less dramatic and usually longer course than the furious form. Muscles gradually become paralyzed, starting at the site of the bite or scratch. A coma slowly develops, and eventually death occurs. The paralytic form of rabies is often misdiagnosed, contributing to the under-reporting of the disease (WHO, 2017).

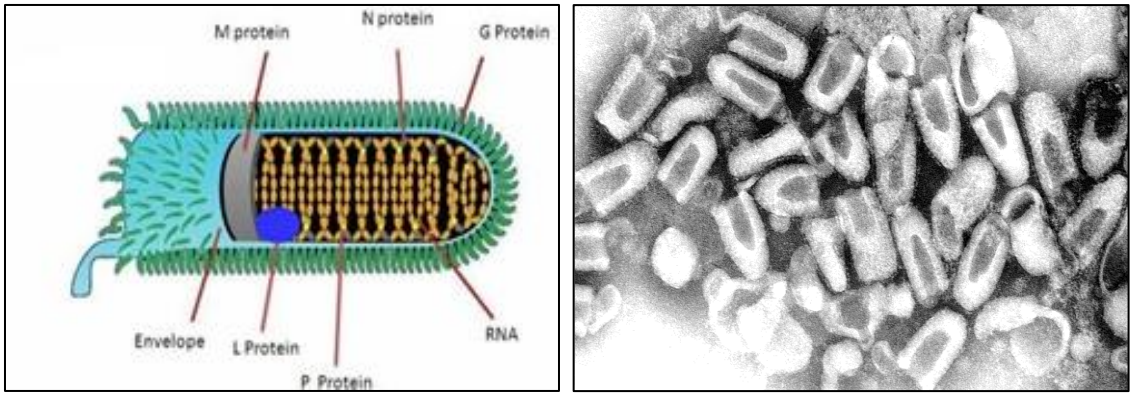
It has been reported that 95% of human deaths in Asian and African countries occurred because of rabies (Table 1). However not much information is available till date and seemingly it is the most neglected disease so far. The victims include rural and vulnerable populations, poor people and children belonging to age group of 5-14 years. Though effective human vaccines and immunoglobulins exist for rabies, they are not easily available or accessible to those in need.

Table 1: Annual Mortality Rate for Rabies (Dreesen, D.W., 1997**)**

Sr. No.	Country	Deaths/million population
1.	USA	0.023
2.	S Africa	0.1
3.	Thailand	4.5
4.	Vietnam	4.7
5.	Indonesia	11.0
6	Bangladesh	18.0
7.	India	35.5

1.2 Rabies virus

Rabies virus is a type species of the *Lyssavirus* genus of *Rhabdoviridae* family. It is an enveloped virus with cylindrical morphology (Yusuf, Erlangga., 2015) and possesses ssRNA which encodes 5 genes whose order is highly conserved: Nucleoprotein (N), Phosphoprotein (P), Matrix protein (M), Glycoprotein (G) & Viral RNA Polymerase (L).



a. **b.**

Figure 1: a. Structure of rabies virus and b. Electron micrograph of rabies virus

All *Rhabdoviruses* have two major structural components; helical ribonucleoprotein core (RNP) and surrounding envelops. The proteins associated with RNP are P and L. The glycoprotein has around 400 trimeric spikes, which are tightly arranged on the surface of the virus. The replication and transcription are mediated by virus nucleoprotein. If the nucleoprotein is not phosphorylated, both

viral transcription and replication are reduced (CDC, 2015; Hicks, D. J., *et. al.*, 2012).

After binding to the receptor, rabies virus enters its host cells by the endosomal transport pathway. Inside the endosome, the pH is low which induces the membrane fusion process that enables the viral genome to reach the cytosol. The receptor binding and membrane fusion are catalyzed by the glycoprotein G which plays a critical role in pathogenesis which makes new viral protein. The viral polymerase can only recognize ribonucleoprotein and cannot use free RNA as template. The cis-acting sequences regulate the transcription on the virus genome by protein M. This protein is essential for virus budding and regulates the fraction of mRNA production to replication (Wu, X. *et al*., 2002). After the occurance of infection, the activity of the polymerase switches to replication to produce full-length positive-strand RNA copies. These complementary RNAs are used as templates to make new negative-strand RNA genomes. They are packaged together with protein N to form ribonucleoprotein which then can form new viruses (CDC, 2015).

Dependent upon factors such as the location of virus entry and viral load, the incubation period for rabies is typically 1–3 months but may vary from 1 week to 1 year. As the virus spreads to the central nervous system, progressive and fatal inflammation of the brain and spinal cord develops (Meslin, Kaplan, Koprowski 1996).

1.3 Immunization against rabies

Immunization is the process by which an individual is made immune or resistant to an infectious disease by the administration of a vaccine. In order to stimulate the body's own immune system and to protect the person against subsequent infection or disease vaccines play an important role (Meslin, Kaplan, Koprowski 1996)

Immunization is a specific tool for controlling and eliminating life-threatening infectious diseases and is estimated to avert between 2-3 million deaths each year. It is the most cost-effective health investments that make it accessible to even the most hard-to-reach and vulnerable populations (WHO, 2015). It has clearly

defined target groups which can be delivered effectively through outreach activities; and vaccination does not require any major lifestyle change. Immunization is the process whereby a person is made immune or resistant to an infectious disease, typically by the administration of a vaccine. Vaccines stimulate the body's own immune system to protect the person against subsequent infection or disease.

Hence immunization is must against rabies which can be achieved by below mentioned two ways:

1) Pre-exposure immunization

People at increased risk of exposure to rabies, such as veterinarians, animal handlers, rabies laboratory workers, spelunkers, and rabies biologics production workers should be offered rabies vaccine.

The vaccine should be considered for:

- People whose activities bring them into frequent contact with rabies virus or with possibly rabid animals.
- International travelers who may come in contact with animals in parts of the world where rabies is most common.

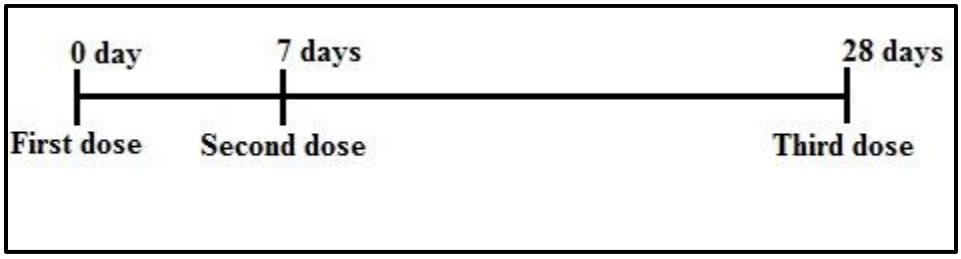


Figure 2: Pre-exposure Schedule for Rabies Vaccination

For laboratory workers and others who may be repeatedly exposed to rabies virus, periodic testing for immunity is recommended, and booster doses should be given as needed (CDC, 2009).

2) Post-exposure prophylaxis (PEP)

It is the immediate treatment of a bite victim after rabies exposure which prevents virus entry into the central nervous system that results in imminent death. PEP is achieved by extensive washing and local treatment of the wound as soon as

possible after exposure; a course of potent and effective rabies vaccine that meets WHO standards; and the administration of rabies immunoglobulin (RIG), if indicated. Effective treatment soon after exposure to rabies can prevent the onset of symptoms and death (CDC, 2009).

RABIPUR®

- Composition: One vial of powder and solvent for solution for injection for one immunization dose (1 ml) contains inactivated rabies virus (strain flury LEP), potency > 2.5 IU.
- Host system: primary chicken fibroblast cell cultures
- Other ingredients: TRIS-(hydroxymethyl-)aminomethane, sodium chloride, EDTA (Titriplex III), potassium-L-glutamate, polygeline, saccharose, water for injections
- Storage and shelf life

Rabipur should be stored at +2 to +8 °C. It should not be used after the expiry date printed on the pack and container. The vaccine should be used immediately after reconstitution. This vaccine conforms to the World Health Organization (WHO) requirements and contains no preservative. The antibody concentration achieved by the immunization falls gradually; booster doses are therefore required to maintain immunity (WHO, 2006)

Figure 3 : Rabipur® Vaccin

Table 2: Appropriate Rabies Treatment Based on Different Categories of Exposure

Exposure category	Type of exposure		Treatment schedule
	Contact with a rabid or suspected rabid* wild or domestic animal	**Contact with an inoculated animal carcass**	
I	Touching/feeding animals, but clearly no contact with their saliva; patient's skin undamaged prior to and during contact	Touching inoculated carcass; skin intact	No treatment necessary. In cases of uncertainty, immunization to be administered as per schedule B (Table 3).
II	Animal has nibbled or licked exposed skin of the patient – Contact with saliva –Superficial, nonbleeding, Scratches made by the animal, with the exception of scratches on the head, neck, shoulder region, arms and hands (see exposure grade III)	Touching inoculated carcass; skin damaged	Immediate treatment as specified in schedule B. In cases of uncertainty, simultaneous administration of vaccine and immunoglobulin (active and passive immunization) should be administered as specified in schedule C (Table 3). If the animal proves to be healthy after examination, it is advisable to continue treatment as in schedule A. Check patient's immunity against tetanus.

Exposure category	Type of exposure		Treatment schedule
	Contact with a rabid or suspected rabid* wild or domestic animal	**Contact with an inoculated animal carcass**	
III	All bites – Bleeding scratches – All scratches on the head, neck, shoulder region, arms, and hands – Contact of patient's mucous membrane with animal saliva (e. g. licking, spray)	Contact of inoculated carcass with mucous membrane or fresh skin wound	Initiate immediate simultaneous administration of vaccine and immunoglobulin (active and passive immunization) as specified in schedule C (Table 3). If the animal proves to be healthy after examination, it is advisable to continue treatment as in schedule A. Check patient's immunity against tetanus.

Table 3: Pre-exposure and Post-exposure Immunization Treatment of Individuals with no or Inadequate Immunity

Schedule A Immunization prior to exposure	Schedule B Immunization after exposure	Schedule C Simultaneous prophylaxis after exposure
One injection of Rabipur i.m. on days: 0, 7, and 21 or 28	One injection of Rabipur i.m. on days: 0, 3, 7, 14, 28 OR 1 dose of Rabipur to be given into the right deltoid muscle, 1 dose into the left deltoid muscle on day 0; 1 dose to be applied on days 7 and 21. In small children the vaccine is to be given into thighs.	Give Rabipur as in schedule B If no rabies immunoglobulin is available at the time of the first vaccination, it must be administered no later than 7 days after the first vaccination.

The antibody concentration achieved by the immunization falls gradually; booster doses are therefore required to maintain immunity.

Booster doses

The International recommendations are as follows: For persons at continuous risk, evaluation of rabies virus neutralizing antibody titres by RFFIT is performed every 6 months. For persons at frequent risk, the WHO recommends antibody titre estimations every year, whereas the ACIP advocates testing every 2 years. If titres are below 0.5 IU/ml at any time, one booster dose should be administered. Considering the long term satisfactory antibody titres observed with Rabipur, if serological tests cannot be conducted due to cost considerations or inaccessible medical facilities, a booster dose one year after primary immunization followed by one dose every 5 years would be advisable.

1.4 BHK-21 CELL LINE

Table 4: General Characteristics of BHK-21 cell line

Organism	*Mesocricetus auratus*, hamster, Syrian golden
Tissue	kidney
Cell Type	fibroblast
Morphology	fibroblast
Culture Properties	adherent
Derivation	Derived from baby hamster kidneys of five unsexed, 1-day-old hamsters in March, 1961, by I.A. Macpherson and M.G.P. Stoker.
Applications	The World Organization for Animal Health (OIE) uses these cells for routine diagnosis of rabies.

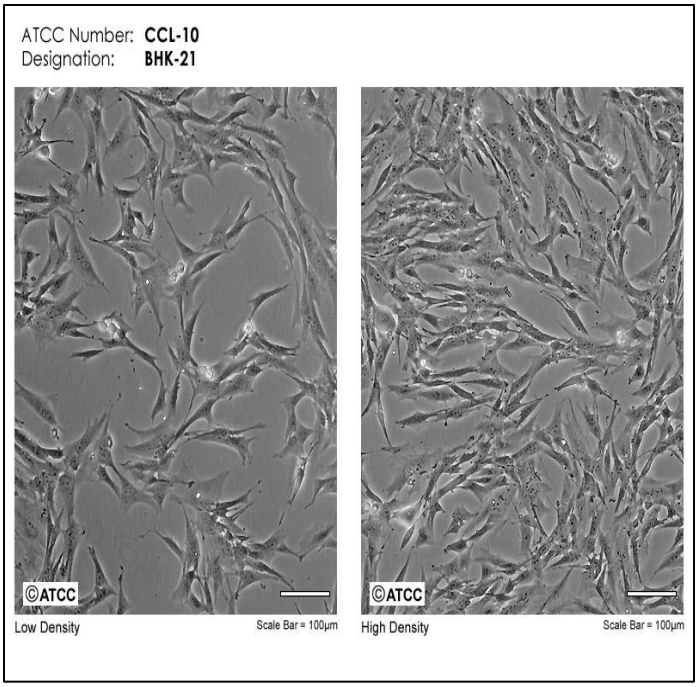


Figure 4: Morphology of BHK-21 cell lin

2.0 OBJECTIVES

- To learn a technique which specifically identifies antibodies generated against the vaccine over a period of time.
- Demonstration of the specificity of the antibody response.

3.0 MATERIALS

Table 5: List of Reagents and Consumables

Sr. No.	Reagents and Consumables	Make
1.	MEM culture media	Sigma
2.	Trypsin	Sigma
3.	Phenol Red	Sigma
4.	EDTA	Sigma
5.	Glucose	Sigma
6.	Sodium Pyruvate	Sigma
7.	Sodium Bicarbonate	Sigma
8.	Fetal Bovine Serum	Hyclone
9.	Penicillin-Streptomycin solution	Hi-media
10.	Trypan Blue	Sigma
11.	Hydrochloric Acid	SDFCL
12.	Sodium Hydroxide	Merck
13.	Sodium Chloride	Sigma
14.	Potassium Chloride	Sigma
15.	Sodium di-hydrogen Phosphate	Sigma
16.	di-Sodium Hydrogen Phosphate	Sigma
17.	Bovine serum albumin	Merck
18.	Rabies Polyclonal DFA Reagent (Goat IgG FITC Conjugate)	Millipore
19.	Acetone Assay: 99.8%	Merck
20.	2nd WHO International Standard for Antirabies immunoglobulin, Human	NIBSC, UK
21.	Purified water	In-house
22.	Autoclaved purified water	In-house
23.	Sterile serological pipettes (5, 10, 25 mL)	Eppendorf
24.	Micropipettes (2-20, 5-50, 10-100, 20-200, 30-300, 100-1000 µL)	Thermo

Sr. No.	Reagents and Consumables	Make
25.	Tissue culture flask (T_{75} cm^2, $T_{175}cm^2$)	Nunc
26.	Centrifuge tubes (15, 50 mL)	Tarson
27.	Sterile petriplates	Hi-media
28.	Neubaurer's chamber	NA

Table 6: List of Instruments

Sr. No.	Instruments	Make
1.	Inverted microscope	CDIC
2.	Compound microscope	MEJI
3.	Fluorescence microscope	Zeiss
4.	CO_2 incubator	Hera cell
5.	Biosafety cabinet	ESCO
6.	Cooling centrifuge	Thermo
7.	Water bath	Grant
8.	Refrigerators (2-8˚C)	Godrej
9.	-20 ˚C deep freezer	Thermo
10.	-70 ˚C deep freezer	Thermo
11.	1˚C cooler	Nalgene

4.0 PROCEDURE

4.1 Vial revival of BHK-21 cell line:

- 4 mL of sterile media was taken out in a 15 mL centrifuge tube to dilute the concentration of DMSO.
- Cryovial was withdrawn from the liquid nitrogen can and was immediately placed in a 1˚C cooler.
- 1˚C cooler was the transferred to the working lab and the thawing of vial was done by slight agitation in a water bath at 37˚C.
- Vial was thawed till the small amount of ice crystal was observed. Note: only the cryovial cap has to be kept outside the water and only lower half of the cryovial was submerged.
- Content of the vial was taken out and added to the 15 mL centrifuge tube containing 4mL of sterile media.
- The tube was then centrifuged at 1500 rpm for 5 minutes at 25˚C.
- Meanwhile $T_{75}cm^2$ tissue culture flask was labeled with the following details: Cell line name, Passage No. and Date.
- 13mL of sterile media was added to the tissue culture flask.
- After centrifugation the supernatant was discarded and the pellet was resuspended in 2 mL fresh sterile media with proper mixing.
- 2 mL cell suspension was then transferred to the tissue culture flask and was incubated in a humidified CO_2 incubator under the following conditions: Temperature: 37˚C; CO_2: 5%.
- The flask was observed under an inverted microscope for active growth condition, cell confluency, absence of bacterial and fungal contamination (European Pharmacopeia 5.0).

4.2 Subculturing of BHK-21 cell line:

- The media bottle was wiped out with, Trypsin-EDTA solution tube with 70% IPA under sterile conditions before using in Biosafety Cabinet.

- Tissue culture flask of cells was taken from CO_2 incubator and the flask was observed under an inverted microscope for active growth condition, cell confluency, absence of bacterial and fungal contamination.
- The cells were detached from the flask by using 0.25% Trypsin-EDTA solution.
- 5 mL of Trypsin-EDTA solution was added gently at the side walls of the flasks to remove the serum. The flask was shaken slowly 1-2 times and immediately Trypsin-EDTA solution from the flask was removed.
- 5 mL of Trypsin-EDTA solution was again added gently to the side walls of the flask. The Trypsin-EDTA solution from the flask was removed when the cells started detaching from the flask.
- The cells were resuspended in 5 mL fresh media in the flask and mixed properly.
- 200 µL of cell suspension was removed for cell counting and cell count was performed as mentioned below:
    - 180 µL of Trypan blue added in 2 mL centrifuge tube.
    - Add 20 µL of cell suspension was added and mixed gently.
    - The Neubauer chamber was loaded with 10 µL of the above suspension and the cell counting was performed under the compound microscope.
    - Calculation: 1) % Viability = Avg. of live cells/Avg. of total cells * 100
      2) Cell count = Average cells x Dilution Factor x 104/mL
      3) Total cells = Re-suspended volume * cells/mL
- A new flask was labeled with the following details: Cell line name, Passage No. and Date. Note: Passage No.was changed in the increasing numerical order.
- Based on the cell count the cells were resuspended in the new tissue culture flask as per requirement.
- The flask was observed for active growth condition, cell confluency, absence of bacterial and fungal contamination in the flask by an inverted microscope (European Pharmacopeia 5.0).

4.3 VIRUS PROPAGATION

- A $T_{75}cm^2$ flask was placed with appropriate amount of cells in a CO_2 incubator. Observe the flask was observed regularly till it attained confluency.
- The flask was trypsinized using 0.5% trypsin and the cells were resuspended in complete media and 200 µL was removed for cell counting and cell counting was performed.
- Cells were seeded as per requirement in a new flask to be used for virus infection.
- Next day flask was observed for confluency and used media was discarded from the flask.
- 5 mL of virus suspension was added in the flask and flask was incubated in CO_2 incubator for 90 minutes with occasional shaking.
- After incubation, virus suspension was removed from the flask and 15 mL fresh media was added.
- The flask was incubated in CO_2 incubator for 72 hours.
- After completion of incubation, the infected flask was kept in -70°C deep freezer for overnight freezing. Next day, flask was freeze thawed and suspension was centrifuged at 4000 rpm for 20 mins at 4°C.
- Virus was harvested when all cells are infected.
- After centrifugation, suspension was collected and stored at -70°C for further use (Wiktor, T. J., 1964).

4.4 IMMUNIZATION OF SUBJECTS

- After completing routine health checkup, two healthy subjects were screened for antibody titre in blood against rabies virus.
- The subject with antibody titre below 0.15 IU/mL was selected for immunization study and Rabipur® vaccine was given and blood samples were collected to check antibody titre as per schedule mentioned in Table 7.

Table 7: Immunization and Sample Collection Schedule

Date	Vaccine dose	Sample collection
16.02.17	Day 0	Pre dose
23.02.17	Day 7	Day 7
02.03.17	-	Day 14
09.03.17	-	Day 21
16.03.17	Day 28	Day 28
31.03.17	-	Day 42

4.5 SERUM SEPARATION

- Collected blood was kept at room temperature for 30 minutes to form complete clot and centrifuged at 3000 RPM for 15 minutes at 15°C temperature.
- After centrifugation, the serum (supernatant) was collected carefully with micropipette in 50 mL centrifuge tube (European Pharmacopeia 5.0).

4.6 RAPID FLUORESCENT FOCI INHIBITION TEST ASSAY (RFFIT)

Principle:

Virus neutralization tests are the most widely used assays for rabies antibody. The assay uses the principle of neutralization of a constant dose of the previously titrated challenge virus infecting BHK-21 cell line, by the antibody in a dose dependent manner. The neutralization is checked by using a secondary FITC conjugated antibody against the rabies virus nucleoprotein. The comparison of the 50% reduction of fluorescent fields with respect to positive control indicates the 50% end point dilution. Potency of the test sample/test material is determined by taking the ratio of 50% end point dilution of test to that of standard multiplied by the initial potency of reference standard.

- To propagate BHK-21 cells, 2.0 - 4.0 × 106 BHK-21 cells were seeded in a T_{75} cm^2 tissue culture flask containing 15 mL complete MEM (having 10% FBS) culture media. The flask was incubated for 1 - 3 days in humidified CO_2 incubator under following condition. Temperature: 37 ± 1ºC, CO_2: 5% ± 0.5%
- A single cell suspension was prepared, for which the supernatant was discarded from $T_{75}cm^2$ tissue culture flask containing confluent culture of BHK-21 cells. The confluent layer was washed with 5 mL of 0.25% Trypsin-EDTA solution to

remove dead cells and cell debris, the Trypsin-EDTA solution was discarded from it and again 5 mL of 0.25% Trypsin-EDTA solution was added to detach the cells. When the cells started to detach from the flask, 0.25% Trypsin-EDTA solution was discarded from it.

- The cells were resuspended in 5 mL of complete MEM (having 10% FBS) media, and almost 200 µL of cell suspension was taken for counting. The cell suspension was diluted in such a way to obtain final cell concentration of 1 $x10^5$ cells/mL with complete MEM (having 10% FBS) media. 200 µL of cell suspension was pipetted out of 96 wells flat bottom tissue culture plate. The plate was incubated until monolayer was observed in each well of the plate (24 hours) in humidified CO_2 incubator at 37 ± 1ºC and CO_2: 5% ± 0.5%
- The NIBSC Standard was diluted (30 IU/mL) with serum free MEM such that to bring it at 2 IU/mL.
- The Standard further was further diluted 10 times taking 100 µL in 900 µL serum free MEM. Further the standard was diluted by 1:2, 1:4, and 1:8 and so on, such that 50% end point virus neutralization can be observed. The dilutions were performed in round bottom plate.
- The test serum was inactivated by incubating at 56°C in serological water bath for 30 minutes. The test serum was diluted in eppendorf tube in the range of 10-100 times before serial dilution in plate if required. Further the test serum sample was diluted to 1:2, 1:4, 1:8 and so on, such that 50% end point virus neutralization could be observed. The dilutions were performed in round bottom plate.
- 220 µL of standard and 110 µL of samples were added in the first wells of the plate as shown in the plate layout.
- 110 µL of plain serum free MEM was added in respective wells. 110 µL from the first well was taken out using multichannel pipette and was added to the second well to dilute it two fold. After gentle mixing 110 µL from that well was taken and added to the next successive well till required.
- Three wells were marked for each virus control, cell control, 10 times diluted virus and 100 times diluted virus control having only 110 µL serum free MEM (no antibodies).

- After completion of the serial dilution of test serum sample a previously titrated CVS-11 stock virus vial was taken and diluted in serum free MEM such that the titre was between 30 FFD_{50} to 300 FFD_{50}. 110 µL of this diluted virus was added to each well having test serum. Also 110 µL of it was added to the virus control wells. 110 µL of serum free MEM was added to the cell control (no virus).
- Further this virus was diluted 10 and 100 times and 110 µL of it was added in wells which were designated as 10 times and 100 times diluted virus in the below layout.

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
A	Std 1:2	Std 1:2	Std 1:2	S1 1:2	S1 1:2	S1 1:2						
B	Std 1:4	Std 1:4	Std 1:4	S1 1:4	S1 1:4	S1 1:4						
C	Std 1:8	Std 1:8	Std 1:8	S1 1:8	S1 1:8	S1 1:8						
D	Std 1:16	Std 1:16	Std 1:16	S1 1:16	S1 1:16	S1 1:16						
E	Std 1:32	Std 1:32	Std 1:32	S1 1:32	S1 1:32	S1 1:32						
F	CC	VC	VC I	VC II								
G	CC	VC	VC I	VC II								
H	CC	VC	VC I	VC II								

Note: NIBSC standard: Std, Serum sample: S1, Cell control: CC, Virus control: VC, Virus control (1:10): VC I, Virus control (1:100): VC II

Figure 5: Representative Scheme of RFFIT assay plate

- The plate was incubated for 90 minutes in humidified CO_2 incubator at 37 ± 1ºC and CO_2: 5% ± 0.5%.
- The supernatant was discarded from the 24 hrs. incubated BHK-21 seeded 96 wells flat bottom tissue culture plate.200 µL of suspension was transferred from the round bottom plate into the respective wells of the BHK-21 seeded plate.
- The plate was incubated for 22 hrs. in humidified CO_2 incubator at 37 ± 1ºC and CO_2: 5% ± 0.5%.
- The supernatant was removed and the wells were washed with 200 µL of 80% Acetone solution twice. 200 µL of 80% acetone was added to each well and the plate was kept at -20 ± 5°C for 30-45 minutes for fixation.

- The supernatant was removed and the plate was kept at room temperature for 1 hour for air drying.
- 1:30 times dilution of FITC conjugated antirabies antibody (DFA Reagent) was prepared and 65 µL of it was added to each well. It was made sure that the solution covered the monolayer uniformly without any dead space. The plate was incubated for 45 minutes in humidified CO_2 incubator at 37 ± 1ºC and CO_2: 5% ± 0.5%.
- Note: This step was performed in dark as DFA reagent is light sensitive.
- After incubation, FITC conjugated antirabies antibody (DFA Reagent) was discarded and the wells were washed thrice with 200 µL of 1XPBS. It was made sure to remove the PBS wash content after tapping the plate inverted on tissue towel pad.
- The plate was observed under Fluorescence microscope under blue filter, which showed infected stained cells fluorescing bright green. The control wells were observed first. Then the standard dilutions were observed (Meslin, Kaplan, Koprowski, 1996; Smith *et.* al., 1973).
- During observation, the wells were scored for infectivity by dividing the well shown in Figure 6.

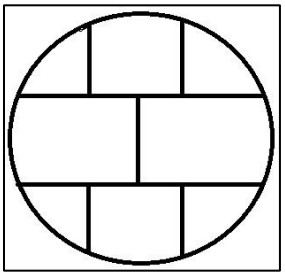


Figure 6: Eight field scoring of well

- Determine the dilution of the reference preparation and the dilution of the test preparation that reduce the number of fluorescent fields by 50%. Calculate end point titre using Reed and Muench formula. Calculate the potency as below:
  - **Use the below formula to obtain the difference of logarithm**
    - Difference of log: $\frac{\text{50\% - infectivity below 50\%}}{\text{Infectivity above 50\% - infectivity below 50\%}}$ X log of dilution factor

- Log (reciprocal of 50% end-point dilution) = Log (reciprocal of starting point dilution) + difference of Log
- End point titre = Antilog (reciprocal of 50% end-point dilution) * dilution factor

➢ **Calculate the potency as below for samples with unknown potency:**

- Potency(IU/mL): $\frac{\text{Endpoint titre of sample}}{\text{Endpoint titre of Ref. Std}} \times 2$ IU/mL

(Reed LJ, Muench H., 1938)

5.0 RESULTS AND DISCUSSION

After performing staining, plates were observed under the fluorescence microscope (**10X**). The cells in cell control wells did not show any fluorescence (Figure 7a). The cells infected with CVS-11 virus showed green fluorescence and decrease in fluorescence was observed in virus control (1:10) and virus control (1:100) as shown in figure 7b, 7c and 7d respectively.

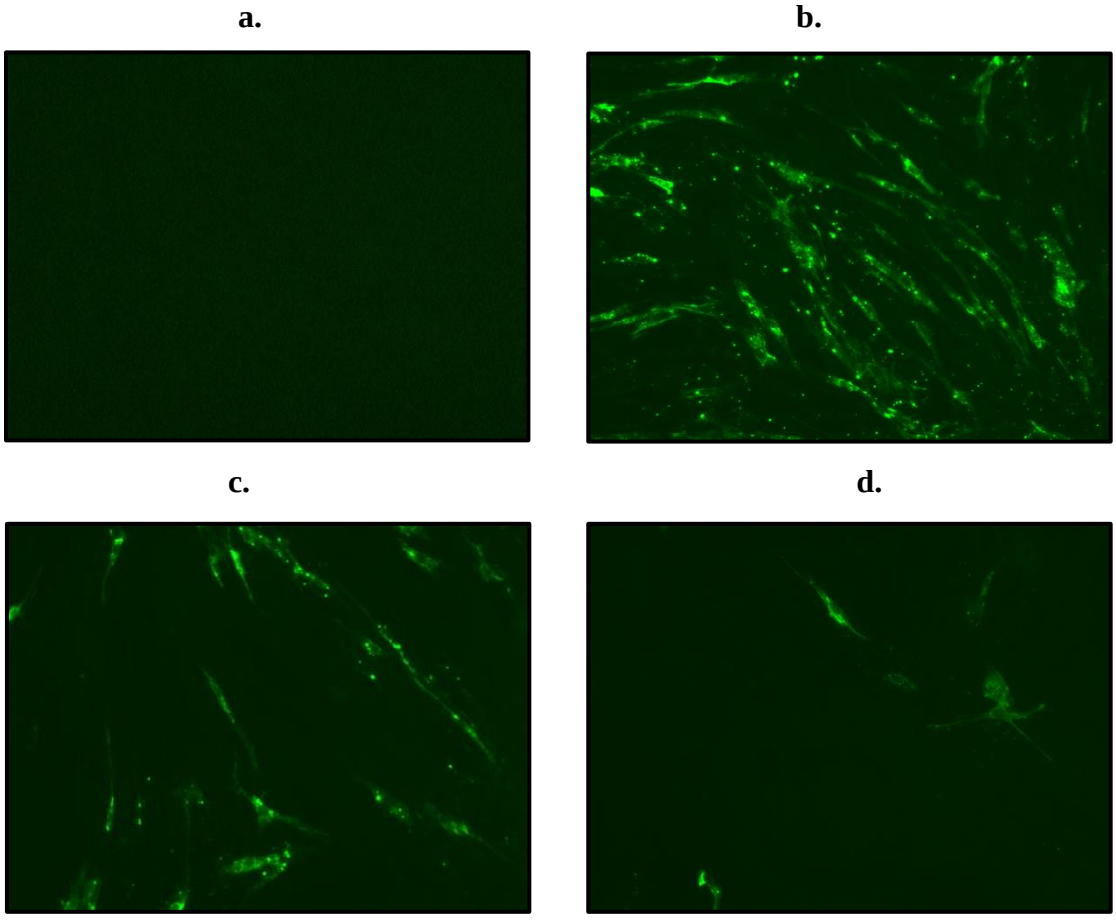


Figure 7: Images of Control well under fluorescent microscope; a. cell control, b. virus control, c. virus control (1:10), d. virus control (1:100)

5.1 Screening and selection of subjects

Two subjects (Subject 1 and Subject 2) were screened for the antibodies against rabies virus in blood. Among two subjects, antibody titre of subject 1 was found to be 6.78 IU/mL (Table 8) which was higher than the selection criteria of 0.15 IU/mL and thus subject 1 was rejected for immunization study. After investigation

of subject 1, we came to know that the subject was already immunized with vaccination in childhood and this could be the reason for the high antibody titre.

Table 8: Screening Results of Subject 1

NIBSC (Subject 1)				Serum (Subject 1)			
Dilution	**Infected**	**Non-infected**	**%**	**Dilution**	**Infected**	**Non-infected**	**%**
1:2	0	24	0.00	1:2	0	24	0.00
1:4	5	19	20.83	1:4	0	24	0.00
1:8	14	10	58.33	1:8	5	19	20.83
1:16	21	3	87.50	1:16	16	8	66.66
1:32	24	0	100.00	1:32	24	0	100.00
Titre in serum sample: 6.78 IU/mL							

Due to higher antibody titre, only booster dose was preferred to gain a specific antibody titre which can protect the subject while handling with the rabies virus. After booster dose, antibody tire in serum sample was increased to 12.31 IU/mL (Table 9) on day 7 which will be sufficient titre for protection of subject against rabies virus.

Table 9: Antibody Titre on Day 7 in Subject 1 after Booster Dose

NIBSC (Subject 1)				Serum (Subject 1)			
Dilution	**Infected**	**Non-infected**	**%**	**Dilution**	**Infected**	**Non-infected**	**%**
1:2	00	24	0.00	1:2	04	20	16.66
1:4	01	23	4.16	1:4	10	14	41.66
1:8	11	13	45.83	1:8	15	09	62.50
1:16	21	03	87.50	1:16	20	04	83.33
1:32	24	00	100.00	1:32	24	00	100.00
Titre in serum sample: 12.31 IU/mL							

The antibody titre in the serum of Subject 2 was found to be 0.11 IU/ ml (Table 10) which was lower than the selection criteria of 0.15 IU/mL. So, subject 2 was selected to study the effect of pre-exposure immunization as per schedule mentioned in Table 7. In past, the subject did not get any exposure against rabies virus (live or dead) which is confirmed by the obtained result.

Table 10: Screening Results of Subject 2

NIBSC (Subject 2)				Serum (Subject 2)			
Dilution	**Infected**	**Non-infected**	**%**	**Dilution**	**Infected**	**Non-infected**	**%**
1:2	0	24	0.00	1:2	5	19	20.83
1:4	5	19	20.83	1:4	13	11	54.16
1:8	14	10	58.33	1:8	19	5	79.16
1:16	21	3	87.50	1:16	21	3	87.50
1:32	24	0	100.00	1:32	24	0	100.00
Titre in serum sample: 0.11 IU/mL							

5.2 Immunization results of selected subject

According to the immunization schedule discussed above (Table 7) the antibody titre of the serum samples collected on pre dose and days 7, 14, 21, 28 and 42 were obtained as 0.20 IU/ml, 1.98 IU/ml, 23.66 IU/ml, 25.36 IU/ml, 29.39 IU/ml and 29.45 IU/ml respectively (Table 11-16). The results obtained (Table 17) showed the significant increase in the antibody titre. This significant increase is the result of pre-exposure vaccination. Initially after the first dose of vaccination the antibody titre increased in comparison to the predose sample antibody titre. A subsequent increase was found when the booster doses were administered to the subject 2 on days 14 and 28 respectively (Figure 8). Hence from these results one can observe that pre-exposure vaccination resulted into rise in immunity of the individual which leads to overall protection to the veterinarians, animal handlers, rabies laboratory workers, spelunkers, and rabies biologics production workers.

Table 11: Antibody Titre in Pre dose Serum Sample

NIBSC (Pre dose)				Serum (Pre dose)			
Dilution	**Infected**	**Non-infected**	**%**	**Dilution**	**Infected**	**Non-infected**	**%**
1:2	00	24	0.00	1:2	00	24	0.00
1:4	01	23	4.16	1:4	01	23	4.16
1:8	11	13	45.83	1:8	10	14	41.66
1:16	21	03	87.50	1:16	22	02	91.66
1:32	24	00	100.00	1:32	24	00	100.00
Titre in serum sample: 0.20 IU/mL							

Table 12: Antibody Titre in Serum Sample after Day 7

NIBSC (7 day)				Serum (7 day)			
Dilution	**Infected**	**Non-infected**	**%**	**Dilution**	**Infected**	**Non-infected**	**%**
1:2	00	24	0.00	1:2	00	24	0.00
1:4	07	17	29.16	1:4	05	19	20.83
1:8	14	10	58.33	1:8	15	09	62.50
1:16	21	03	87.50	1:16	22	02	91.66
1:32	24	00	100.00	1:32	24	00	100.00
Titre in serum sample: 1.98 IU/mL							

Table 13: Antibody Titre in Serum Sample after Day 14

NIBSC (14 day)				Serum (14 day)			
Dilution	**Infected**	**Non-infected**	**%**	**Dilution**	**Infected**	**Non-infected**	**%**
1:2	00	24	0.00	1:2	00	24	0.00
1:4	06	18	25.00	1:4	00	24	0.00
1:8	13	11	54.16	1:8	06	18	25.00
1:16	21	03	87.50	1:16	11	13	45.83
1:32	24	00	100.00	1:32	21	03	87.50
Titre in serum sample: 23.66 IU/mL							

Table 14: Antibody Titre in Serum Sample after Day 21

NIBSC (21 day)				Serum (21 day)			
Dilution	**Infected**	**Non-infected**	**%**	**Dilution**	**Infected**	**Non-infected**	**%**
1:2	00	24	0.00	1:2	00	24	0.00
1:4	06	18	25.00	1:4	00	24	0.00
1:8	13	11	54.16	1:8	02	22	8.33
1:16	21	03	87.50	1:16	10	14	41.66
1:32	24	00	100.00	1:32	20	04	83.33
Titre in serum sample: 25.36 IU/mL							

Table 15: Antibody Titre in Serum Sample after Day 28

NIBSC (28 day)				Serum (28 day)			
Dilution	**Infected**	**Non-infected**	**%**	**Dilution**	**Infected**	**Non-infected**	**%**
1:2	00	24	0.00	1:2	00	24	0.00
1:4	05	19	20.83	1:4	00	24	0.00
1:8	14	10	58.33	1:8	04	20	16.67
1:16	21	03	87.50	1:16	09	15	37.50
1:32	24	00	100.00	1:32	18	16	75.00
Titre in serum sample: 29.39 IU/mL							

Table 16: Antibody Titre in Serum Sample after Day 42

NIBSC (42 day)				Serum (42 day)			
Dilution	**Infected**	**Non-infected**	**%**	**Dilution**	**Infected**	**Non-infected**	**%**
1:2	00	24	0.00	1:2	00	24	0.00
1:4	07	17	29.16	1:4	00	24	0.00
1:8	14	10	58.33	1:8	04	20	16.66
1:16	21	03	87.50	1:16	09	15	37.50
1:32	24	00	100.00	1:32	20	04	83.33
Titre in serum sample: 29.45 IU/mL							

Table 17: Summary of Antibody Titre in Serum Sample

Sr. No.	Sample ID (serum)	Titre in serum sample for IU/mL
1.	Pre dose	0.20
2.	Day 7	1.98
3.	Day 14	23.66
4.	Day 21	25.36
5.	Day 28	29.39
6.	Day 42	29.45

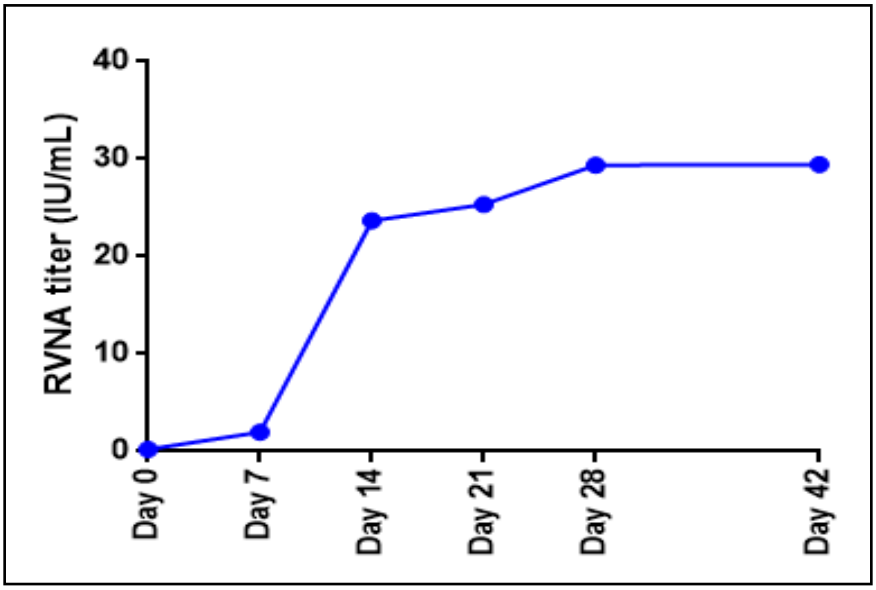


Figure 8 : Antibody Generation Profile after Immunization

5.3 Specific antibody response

The antibody response of man and animals to many bacteria and virus antigens are associated with the major immunoglobulin classes, IgM and IgG. In primary immunization, neutralizing activity in serum is usually associated first with transient IgM synthesis and later with a more durable and quantitatively greater IgG response by contrast response to secondary immunization is predominantly by IgG (Figure 9)

As stated rabies virus IgM were detected already three days after the first vaccination. The IgM-antibody concentration increased to a maximum at the 22nd day. Rabies virus antibodies of the IgG-class were found in the serum at the 7th day. A steep increase of the rabies virus IgG-antibodies was observed from day 10 post vaccination to a maximum between the 30th and 40th day (Thraenhart O., 1979).

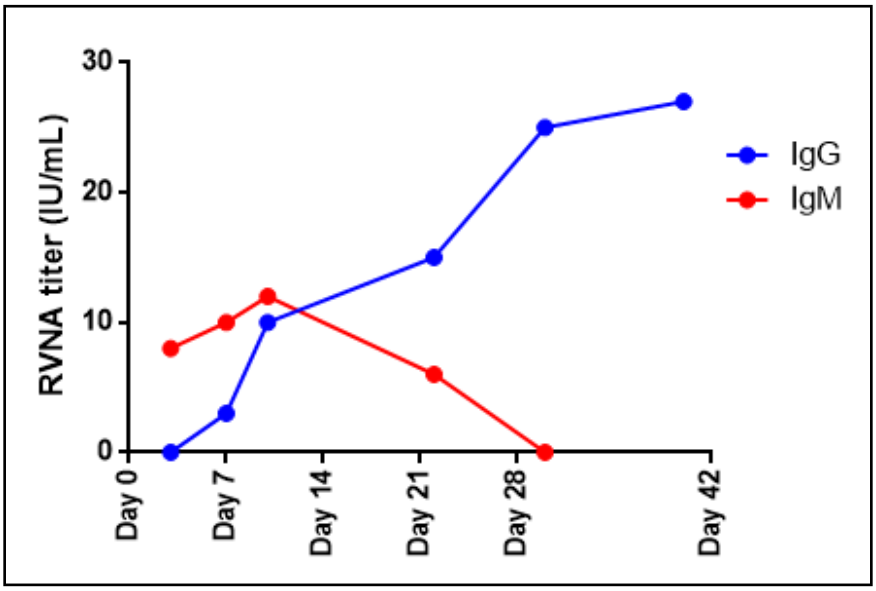


Figure 9: IgG and IgM Response after Immunization

5.4 Future developments and challenges

- An improved understanding of rabies pathogenesis would be useful in the development of novel therapies for human rabies.
- The increased use of experimental studies performed in natural animal models would be informative.
- There needs to be a more concerted effort to control dog rabies using mass vaccination of dogs.
- Commitment of governments with the allocation of the necessary resources.
- The high cost of rabies biologicals, including rabies vaccine and rabies immune globulin, is a barrier for effective human rabies prevention and remains an important future challenge.

6.0 CONCLUSION

In the current study we have reached to a conclusion that pre-exposure immunization plays an important role in enhancing the immune response against the specific antigen which provides protection to the individual by generating antigen specific antibody response. This response generated boosts the immune system by generation of memory cells. The successful adaptation of the virus to the mammalian nervous system explains why rabies is fatal in almost all cases. Thus it has been depicted through our studies that pre-exposure immunization is a necessity to individuals prone to rabies virus.

APPENDICES

- **10 X PBS (Phosphate Buffered Saline)**

CHEMICAL	For 100 mL
Sodium dihydrogen phosphate	0.23 ± 0.02 g
di-sodium hydrogen phosphate	1.15 ± 0.1 g
Sodium chloride	8 ± 0.02 g
Potassium chloride	0.2 ± 0.02 g

- 0.23 ± 0.02 g NaH_2PO_4 was weighed and dissolved in 70 mL of purified water in 250 mL glass bottle.
- 1.15 ± 0.1 g Na2HPO4 was added into it and dissolved to mix.
- 8 ± 0.02 g of NaCl was added into it and dissolved to mix.
- 0.2 ± 0.02 g of KCl was added into it.
- All the materials were dissolved properly.
- The volume was made up to 100 mL with purified water.
- It was autoclave and stored at room temperature and used within 1 month.

- **1 X PBS (Phosphate Buffered Saline)**

- 30 mL of 10X PBS was taken in 500 mL glass bottle.
- 270 mL of purified water was added to the bottle to make 300 mL of 1X PBS solution.
- It was stored at 2-8 ºC and used within 1 week.

- **0.25% Trypsin-EDTA solution**

CHEMICAL	For 200 mL
Glucose	200 ± 0.2 mg
EDTA	39.45 ± 0.2 mg
Trypsin	500 ± 0.2 mg
1 X PBS	Upto 200 mL

- 50 mL 1X PBS was taken in 250 mL glass bottle.
- 500 ± 0.2 mg of Trypsin was weighed and added into it.
- 200 ± 0.2 mg of glucose was weighed and added in to it.
- 39.45 ± 0.2 mg of EDTA was weighed and added in to it.
- The materials were dissolved properly.
- A pinch of Phenol red was aaded as pH indicator.
- The volume was made up to 200 mL by adding 1X PBS.
- It was filtered through 0.22 µm syringe filter.
- It was aliquoted in 50 mL sterile tarson tube.
- It was stored at -20 ± 5 ºC and used within 1 month.

➢ **Incomplete Minimum Essential Medium Eagle (MEM) 1000 mL**

CHEMICAL	For 1000 mL
MEM	Dried powder for 1 L
Sodium bicarbonate	2.2 ± 0.2 g
Sodium pyruvate	110 ± 0.2 mg
Penicillin- Streptomycin	10 mL
Purified water	Upto 990 mL

- 2.2 ± 0.2 g of sodium bicarbonate and 110 ± 0.2 mg of sodium pyruvate was weighed and added in the 1.0 L sterile glass bottle.
- 400 mL of autoclaved purified water was added into it.
- It was mixed completely.
- The readymade mixture of MEM powder from the pack was added (carefully without spilling) and the ingredients were mixed till they dissolved completely.
- The volume of the media was made up to 990 mL by adding autoclaved purified water.
- The bottle was checked to ensure whether all the contents have dissolved or not.
- The pH was checked and adjusted to 7.2 ± 0.2 using 1N NaOH /HCl, if required.
- The bottle was taken under the laminar airflow hood and the content was filtered through a disposable 0.22 µm filtration assembly.

- 10 mL of 100X Penicillin - Streptomycin solution was added to obtain final concentration of 1X Penicillin- Streptomycin.
- The bottle was stored at 2°C to 8°C, used within 1 month.

➢ **NIBSC Standard (30 IU):**

- (2nd WHO International Standard for Antirabies immunoglobulin, Human)
- Lyophilized ampoule of NIBSC standard stored at -70 ± 5°C was taken and reconstituted in 1 mL autoclaved purified water. It was mixed gently to ensure minimal foaming.
- The aliquots of the solution were prepared in PCR tubes and stored at -70 ± 5°C after labeling.

➢ **10% BSA:**

- 1 ± 0.1 gm of cell culture grade BSA was weighed in a 50mL centrifuge tube and was dissolved in 10mL of 1X PBS.
- The solution was filtered using 0.22 µm syringe filter.
- It was stored at 2°C to 8°C and used within 1 month.

➢ **0.2% Serum-free MEM:**

- 49 mL of MEM incomplete media was taken in a 50 mL centrifuge tube.
- 1 mL of 10% BSA solution was added to the centrifuge tube. It was mixed and used freshly prepared.

➢ **Complete MEM (10% FBS):**

- 90 mL of MEM incomplete media was taken and 10 mL of FBS was added to it.
- It was mixed and used fresh.

➢ **80% Acetone:**

- 80 mL of acetone was taken in 250 mL glass bottle.
- 20 mL of purified water was added to the above and mix properly.

- It was stored at -20 ± 5 ºC and used within 1 week.

➢ **FITC conjugated antirabies antibody (DFA Reagent)**

- 7.25 mL of 1X PBS was taken in 15mL centrifuge tube.
- 250 µL of FITC conjugated antirabies antibody (ready to use DFA Reagent) was added to the above. It was mixed properly.

➢ **1N NaOH:**

- 0.4 g of NaOH was weighed and added to 7mL of purified water in 15 mL centrifuge tube.
- It was dissolved properly and the volume was made upto 10 mL.
- The same was used for adjusting pH of media if required.

➢ **1N HCl:**

- 1 mL of concentrated HCl was taken in a 15 mL tarson tube.
- 11 mL of purified water was added to it and mix properly.
- The same was used for adjusting pH of media if required.

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