OIE Virtual Training Series on Rabies Serology for SAARC Region

11-13 October 2021

FINAL TRAINING REPORT

Veterinary College, Bengaluru, India

Organized by

World Organisation for Animal Health (OIE) Regional Representation for Asia and the Pacific, Tokyo Japan

and

KVAFSU-CVA Rabies Diagnostic Laboratory
OIE Reference Laboratory for Rabies
Department of Veterinary Microbiology
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Introduction

Rabies is a viral zoonotic disease that affects the central nervous system invariably leading to death. Although fatal, it is 100% vaccine-preventable disease. Approximately 99% of rabies infections are acquired by bite of an infected dog. Africa and Asia contribute to over 99% of human rabies deaths that occur in the world and the vast majority (about 60%) of these are in Asia. Due to under-reporting and uncertainty, this number is likely to be a gross under-estimate. In the SAARC region, dogs are the main reservoirs of rabies. The burden of rabies is primarily on human health but disease control must be focused on the animal source. Dog-mediated human rabies can be eliminated by tackling the disease at its source: "the infected dogs".

Considering the seriousness of the disease and its endemicity, especially in African and Asian continents, the Tripartite Alliance which includes the Food and Agriculture Organization of the United Nations (FAO), the World Organisation for Animal Health (OIE), and the World Health Organization (WHO) along with the Global Alliance for Rabies Control (GARC) have launched "Zero by 30: The Global strategic plan to end human deaths from dog mediated rabies by 2030". This goal can be achieved through focusing on the areas that include human pre- and post-exposure prophylaxis, mass dog vaccination, surveillance and monitoring, laboratory diagnostic capability, public awareness risk communication and legislations.

The OIE Regional Representation for Asia and the Pacific (RRAP) in collaboration with the Karnataka Veterinary, Animal and Fisheries Sciences University (KVAFSU) - Commonwealth Veterinary Association (CVA) - Rabies Diagnostic Laboratory (KVAFSU-CVA-RDL) organized the Virtual Training Series on Rabies Diagnosis for SAARC Region on 5-6 November, 2020 to enhance animal rabies diagnostic surveillance in the region. In all, 24 laboratory diagnosticians/veterinarians working on animal rabies surveillance/control from Bangladesh, Bhutan, India, Maldives, Nepal, Pakistan, and Sri Lanka attended the virtual training. The training focused on the occipital foramen approach for brain sampling, its processing, transportation and preservation, and basic diagnostic techniques such as lateral flow assays (LFA) and direct fluorescent antibody techniques (DFA). One of the recommendations from the training was to continue providing virtual trainings on other topics such as serological diagnosis / surveillance that could be used for post-vaccination monitoring and certification of pet travels in South Asia. As countries are upscaling mass dog vaccination, especially in the free roaming dogs, it has become imperative to assess the effectiveness of such campaigns periodically using serological monitoring tools. Therefore, as a follow up, the OIE RRAP in collaboration with the KVAFSU-CVA RDL organized this virtual training on rabies titled "**OIE Virtual Training Series on Rabies Serology for SAARC Region"** during 11-13 October 2021 on *Zoom*®. The focus of this training was on serological diagnosis, specifically on Rapid Fluorescent Focus Inhibition Test (RFFIT) as a diagnostic tool for monitoring post-vaccination immune status in vaccinated population of dogs and for certification of movement of pet animals, particularly dogs and cats. In addition to skill development, this also aimed to bring harmonization in the SAARC region with respect to diagnosis and surveillance of rabies. This virtual training series on serology aimed at supporting SAARC MSs with knowledge and skills for monitoring post-vaccination immune status in vaccinated population and for certification of movement of pet animals, particularly dogs as we continue our actions to eliminate dog-mediated human rabies cases by 2030.

Training Objectives

The virtual training was organized with the following objectives:

- Enhance understanding of the principles and techniques of cell culture, virus maintenance and the Rapid Fluorescent Focus Inhibition Test (RFFIT) for rabies serology.
- Enhance laboratory capacity of SAARC MSs for rabies serology in general and RFFIT in particular.
- Enhance rabies prevention and control activities in the SAARC MSs through use of better serological techniques.
- Develop networking and coordination between rabies diagnostic/serology laboratories in SAARC MSs and the KVAFSU-CVA RDL, OIE Reference Laboratory for Rabies.
- Identify key knowledge gaps and issues related to rabies sero-surveillance and control in SAARC MSs.

Topics and methodology

The participants were trained on the OIE recommended Rapid Fluorescent Focus Inhibition Test (RFFIT) – a serological test, over three days period of 3 hours session each (Annexure 1).

As countries are upscaling mass dog vaccination, especially in the free roaming dogs, it has become imperative to assess the effectiveness of such campaigns periodically using serological monitoring tools. The WHO recommends that a minimum of 0.5 IU per ml of rabies antibody titre in people vaccinated against rabies as representation of sufficient immunity to protect against rabies infection. The same measure is used in dogs and cats to confirm a satisfactory response to vaccination. Since neutralizing antibodies are the main components of immune response to rabies virus, the gold standard tests are virus neutralization tests (VNT) i.e., Fluorescence Antibody Virus Neutralization (FAVN) test and Rapid Fluorescent Focus Inhibition Test (RFFIT). Of late, enzyme-linked immunosorbent assays (ELISAs) are also acceptable tests to detect binding antibodies but these do not detect the neutralizing antibodies. In view of this, the training program was focused on providing knowledge and skill to perform RFFIT and in turn a uniform antibody monitoring system in the SAARC region.

The training was undertaken using power point presentations, pre-recorded videos, and live interactive sessions with the experts from the OIE reference laboratory. The participants actively participated in the Q & A session that made the training more interactive and interesting.

Summary outputs

The training was officially opened after a series of opening keynote addresses made by Dr Hirofumi Kugita, Regional Representative; OIE RRAP; Dr H.D. Narayanaswamy (Vice Chancellor) KVAFSU; Dr S. Abdul Rahman from CVA; Dr Praveen Malik, Animal Husbandry Commissioner from the Department of Animal Husbandry and Dairying, Government of India (GOI); and Dr Girish Kumar, In-charge Dean of Veterinary college, Bengaluru. All the speakers appreciated the joint initiative by OIE and KVAFSU in undertaking this training and pledged their support to enhance rabies control activities in the SAARC region for achievement of the global goal of 2030. A total of 20 laboratory diagnosticians / veterinarians working on animal rabies surveillance/control from six Member States of South Asian Association for Regional Cooperation (Bangladesh, Bhutan, India, Nepal, Pakistan, and Sri Lanka)

attended the virtual training along with observers and RRAP / KVAFSU staff (Annexure 2). The participants were trained on the OIE recommended Rapid Fluorescent Focus Inhibition Test (RFFIT) as a serological tool for monitoring post-vaccination immune status in vaccinated population and for certification of movement of pet animals, particularly dogs. Dr Isloor, supported by his staff, took lead in imparting the training using theory and practical sessions including videos to demonstrate the RFFIT diagnostic techniques. The participants were quizzed with questions (Annexure 3) taken out from the training contents in the form of *mentimeter* quiz sessions to test their knowledge and how much they learnt at the training. Also, at the end of training sessions on each day, participants actively participated in the Q&A session (Annexure 4) that made the training more interactive and interesting.

The training was well received with 50% of the trainees assessing the training webinar as highly satisfactory, respectively (Annexure 5). Likewise, 44% of the trainees reported high impact on their technical knowledge on rabies. Some specific feedback that the trainees provided in the training evaluation included requests to consider providing physical laboratory training on rabies diagnosis as soon as travel restrictions are lifted so that the theory knowledge could be put into practice and request for training on ELISA and molecular diagnostic techniques for rabies diagnosis.

Copies of the presentations, videos and other materials can be accessed at https://rr-asia.oie.int/en/events/oie-virtual-training-series-on-rabies-serology-for-saarc-region/

Introduction of the KVAFSU-CVA Rabies Diagnostic Laboratory

Shrikrishna Isloor, Sharada, R. and D. Rathnamma

The Veterinary College, Bengaluru was established on 25th July 1958 and became a constituent college of the University of Agricultural Sciences, Bengaluru in 1965. It became part of the newly established Karnataka Veterinary, Animal and Fisheries Sciences University (KVAFSU), Bidar from April 2005. The college is located on the Bengaluru to Hyderabad highway at a distance of about 28 KM from the Kempegowda International Airport. The main focus of the Veterinary College is teaching, research and extension and is collaborating with various overseas universities and institutes including the Centre for Disease Control and Prevention (CDC), Atlanta, US, Animal and Plant Health Agency (APHA), UK, Virginia Tech, Blacksburg, University of Minnesota, Kansas State University, Ohio State University, US, University of Purdue, Curtin University of Technology, Perth, Western Australia and INSERM, France. In addition, the Veterinary College has been involved in animal rabies related research activities for the last 40 years. With the establishment of the dedicated KVAFSU-CVA Rabies Diagnostic Laboratory in 2013 (as a part of collaborative project involving KVAFSU, CVA and Crucell Holland B.V., Netherlands), a BSL-II Laboratory, the support for animal rabies research activities, especially diagnosis of animal rabies was strengthened. Furthermore, the laboratory was twinned with the Animal and Plant Health Agency (APHA), Weybridge, UK and CDC, Atlanta, GA, US under the OIE Twinning programme with the mandate of 'Strengthening the diagnosis of Rabies in India'. The laboratory has also developed a network of rabies diagnostic facilities as per the standard detection methods in other parts of the country. In addition to these, molecular studies such as PCR / LN 34 real-time PCR assay for the pan Lyssa viruses are being carried out for understanding the molecular epidemiology of rabies virus lurking in different parts of India. The phylogenetic analyses based on complete N and G gene sequences has revealed that, some sequences from southern parts of India cluster closely with sequences of rabies virus isolates from Sri Lanka. The rabies viral N and G protein amino acid sequences showed that the viral isolates recovered belong to genotype 1 and are similar.

In addition to the diagnosis and research activities, capacity building of veterinarians from different parts of the country, involved in rabies diagnosis is being undertaken by holding regular workshops and training programmes on rabies diagnosis. Since the establishment of the Laboratory, about 450 veterinarians from different regionally representative states of India have been trained. This has been done in collaboration with the support from CVA, CDC, Atlanta and APHA, UK.

The laboratory was designated as the 'OIE Reference Laboratory for rabies' on 16th June 2020 and it can serve the needs of not only India but other South Asian and South East Asian countries with respect to animal rabies research related activities and can serve as a centre of excellence in animal rabies, with main areas of focus being diagnosis of animal rabies and surveillance. It can undertake statutory diagnostic services and provide support to research projects for rabies and rabies-related viruses in the region. The expertise available at the laboratory could provide international test and disease consultancy services in the region.

Additionally, the laboratory will be able to provide consultancy in disease surveillance on rabies and non-rabies lyssaviruses, control strategies and diagnostics.

The laboratory will act as a national hub for animal rabies in India. Additionally, the laboratory will work in close collaboration with the WHO Collaborating Centre for Reference and Research in Rabies at the National Institute of Mental Health and Neurosciences (NIMHANS), Bengaluru as part of a One Health rabies strategy for the elimination of dog-mediated human rabies in India. Furthermore, the laboratory will continue to collaborate with other OIE Reference Laboratories for rabies, and in the future will also work more closely as part of a community of practice with other OIE Reference Laboratories for rabies.

The laboratory is also committed to offer excellent in-house or out-reach training in diagnostics, epidemiology as a tool to design and improve control measures. As an OIE reference centre, the activities of the OIE with reference to diagnosis and control of rabies in animals will be supported in addition to offering consultancy and training, quality assurance testing, inter-laboratory comparisons and provide assistance in developing quality standards in the region. The training can be provided on site at Veterinary College, KVAFSU, Hebbal, Bengaluru or can also be organized in the other regional laboratories and veterinary universities. In general, training can cover various diagnostic approaches such as immunological, molecular and cell culture methods, epidemiological and strategies for active surveillance followed by evaluation of strategies to control rabies in an area. The laboratory also collaborates with the public and private sectors in the field of development and evaluation of diagnostic assays and applied research.

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Introduction to Rabies Serology and RFFIT

Shrikrishna Isloor, Sharada, R. and D. Rathnamma

The main application of serology for rabies is to determine responses to vaccination in domestic animals, particularly in connection with international travel, or for monitoring mass vaccination campaigns in dogs and other wildlife reservoir species. The measurement of rabies antibodies has typically involved virus neutralization (VN) tests to detect rabies virus neutralizing antibodies. Enzyme linked Immunosorbent Assays (ELISAs) are now also recognized as acceptable tests to detect binding antibodies. A strong but not strict correlation in levels is observed between these two different antibody detection methods. Depending on the nature of ELISA tests, there can be variable sensitivity and specificity. In contrast to ELISAs, poor quality sera can cause cytotoxicity in VN tests, which could lead to false positive results. Depending on the intended purpose, both tests are useful for detecting responses to vaccination if appropriate cut-offs are used. However, ELISAs are currently not fit-for-purpose tests for certifying international movement of animals or trade. Serological surveys have also been used to provide information on infection dynamics of lyssaviruses in bats although standardization of serological tests for bats is still needed. In accordance to Chapter 3.1.17 of the OIE Terrestrial Manual, the VN and ELISAs are the recommended tests for monitoring immune status in individual animals or populations post-vaccination in the framework of rabies control. For the purposes of measuring antibody responses to vaccination prior to international animal movement or trade, only VN methods (FAVN test and RFFIT) are acceptable.

A. Virus Neutralization Tests in Cell culture

1. Fluorescent Antibody Virus Neutralization test (FAVN)

The principle of the FAVN test is the in-vitro neutralization of a constant amount of rabies virus ('challenge virus standard' [CVS-11] strain adapted to cell culture) before inoculating BHK 21 cells susceptible to rabies virus.

The serum titre is the dilution at which 100% of the virus is neutralized in 50% of the wells. This titre is expressed in IU/ml by comparing it with the neutralizing dilution of the OIE reference serum of dog origin under the same experimental conditions. The WHO standard 2 for rabies immunoglobulin [human] No. 2, or an internal control calibrated against the international control may also be used to calculate the IU/ml titre of test sera.

Generally, the minimum measurable neutralizing VN antibody titre considered to represent a reasonable level of seroconversion is 0.5 IU per ml. The same measure is used in dogs and cats to confirm an adequate response to vaccination prior to international travel. However, within the framework of monitoring mass vaccination campaigns, a single cut-off level of seropositivity may not be universally applicable among different species. This microplate method uses 96-well plates, and is an adaptation of the technique of Smith *et al.* (1973). The FAVN test and the rapid fluorescent focus inhibition test (RFFIT) give equivalent results.

2. Rapid Fluorescent Focus Inhibition Test (RFFIT)

Residual virus is detected using a standard fluorescence microscope. The serum neutralization end-point titre is defined as the dilution factor of the highest serum dilution at which 50% of the observed microscopic fields contain one or more infected cells (i.e. a 97% reduction in the virus inoculum). This value may be obtained by mathematical interpolation. Alternatively, a 100% neutralization titre may be determined by recording the highest serum dilution at which 100% of the challenge inoculum is neutralized and there are no infected cells in any of the observed fields. For both titration methods, the titre of antibody in the test serum (in IU/ml) can be obtained by comparison with the titre of the recognized reference serum standard included in each test.

B. Enzyme Linked Immuno-Sorbent Assay (ELISA)

Enzyme Linked Immuno-Sorbent Assays provide a rapid serological test that avoids the requirement to handle live rabies virus. These tests detect antibodies that can specifically bind to rabies virus antigens, primarily the rabies virus glycoprotein and nucleoprotein. None of the available direct, indirect or competitive ELISAs is validated for international animal movement or trade. However, ELISAs are a useful tool for monitoring rabies vaccination campaigns in wildlife species provided they are properly validated for this purpose. A commercial ELISA kit has been recommended for monitoring rabies vaccination campaigns in foxes and raccoon dogs.

Cell Culture Media Preparation

Rathnamma, D., Kavitha, G., Hridya, S.V. and Vinay, C.P.

Cell culture is one of the major techniques in the life sciences. It is the general term used for the removal of cells, tissues or organs from an animal or plant and their subsequent placement into an artificial environment conducive to their survival and/or proliferation. Basic environmental requirements for cells to grow optimally are: controlled temperature, a substrate for cell attachment, and appropriate growth medium and incubator that maintains correct pH and osmolality. The most important and crucial step in cell culture is selecting appropriate growth medium for the *in vitro* cultivation. A growth medium or culture medium is designed to support the growth of microorganisms and cells. Cell culture media generally comprise an appropriate source of energy and compounds which regulate the cell cycle. A typical culture medium is composed of a complement of amino acids, vitamins, inorganic salts, glucose, and serum as a source of growth factors, hormones, and attachment factors. In addition to nutrients, the medium also helps maintain pH and osmolality.

	Media Type	Examples	Uses
Natural media	Biological Fluids	plasma, serum, lymph, human placental cord serum, amniotic fluid	
	Tissue Extracts	Extract of liver, spleen, tumors, leucocytes and bone marrow, extract of the bovine embryo and chick embryo	
	Clots	coagulants or plasma clots	
Artificial media	Balanced salt solutions	PBS, DPBS, HBSS, EBSS	Form the basis of complex media
	Basal media	MEM DMEM	Primary and diploid culture
	Complex media	RPMI-1640, IMDM	Supports a wide range of mammalian cells

Artificial media

Artificial or synthetic media are prepared by adding nutrients (both organic and inorganic), vitamins, salts, O_2 and CO_2 gas phases, serum proteins, carbohydrates, cofactors. Different artificial media have been devised to serve one or more of the following purposes:

1) immediate survival (a balanced salt solution, with specific pH and osmotic pressure)
2) prolonged survival (a balanced salt solution supplemented with various formulation of organic compounds and/or serum)
3) indefinite growth
4) specialized functions

Artificial media are grouped into four categories:

i. Serum containing media

Foetal bovine serum is the most common supplement in animal cell culture media. It is used as a low-cost supplement to provide an optimal culture medium. Serum provides carriers

or chelators for labile or water-insoluble nutrients, hormones and growth factors, protease inhibitors, and binds and neutralizes toxic moieties.

ii. Serum-free media

Presence of serum in the media has many drawbacks and can lead to serious misinterpretations in immunological studies. A number of serum-free media have been developed. These media are generally specifically formulated to support the culture of a single cell type and incorporate defined quantities of purified growth factors, lipoproteins, and other proteins, which are otherwise usually provided by the serum. These media are also referred to as 'defined culture media' since the components in these media are known.

iii. Chemically defined media

These media contain contamination-free ultra-pure inorganic and organic ingredients, and may also contain pure protein additives, like growth factors. Their constituents are produced in bacteria or yeast by genetic engineering with the addition of vitamins, cholesterol, specific amino acids, and fatty acids.

iv. Protein-free media

Protein-free media do not contain any protein and only contain non-protein constituents. Compared to serum-supplemented media, use of protein-free media promotes superior cell growth and protein expression and facilitates downstream purification of any expressed product. Formulations like MEM, RPMI-1640 are protein-free and protein supplement is provided when required.

Basic Components of Culture Media

Culture media contain a mixture of amino acids, glucose, salts, vitamins, and other nutrients, and available either as a powder or as a liquid form from commercial suppliers. The requirements for these components vary among cell lines, and these differences are partly responsible for the extensive number of medium formulations. Each component performs a specific function, as described below:

a. Buffering systems

Regulating pH is critical for optimum culture conditions and is generally achieved by one of the two buffering systems:

Natural buffering system

In a natural buffering system, gaseous CO_2 balances with the CO_3/HCO_3 content of the culture medium. Cultures with a natural buffering system need to be maintained in an air atmosphere with 5-10% CO_2, usually maintained by a CO_2 incubator. A natural buffering system is low-cost and non-toxic.

HEPES: Chemical buffering using a zwitterion, HEPES, has a superior buffering

capacity in the pH range 7.2-7.4 and does not require a controlled gaseous atmosphere. HEPES is relatively expensive and toxic at a higher concentration for some cell types. HEPES has also been shown to greatly increase the sensitivity of media to phototoxic effects induced by exposure to fluorescent light.

b. Phenol red

Most of the commercially available culture media include phenol red as a pH indicator, which allows constant monitoring of pH. During the cell growth, the medium changes color as pH is changed due to the metabolites released by the cells. At low pH levels, phenol red turns the medium yellow, while at higher pH levels it turns the medium purple. Medium is bright red for pH 7.4, the optimum pH value for cell culture.

c. Inorganic salts

Inorganic salts in the media helps to retain the osmotic balance and help in regulating membrane potential by providing sodium, potassium, and calcium ions.

d. Amino acids

Amino acids are the building blocks of proteins, and thus are obligatory ingredients of all known cell culture media. Essential amino acids must be included in the culture media as cells cannot synthesize these by themselves. They are required for the proliferation of cells and their concentration determines the maximum achievable cell density. L-glutamine, an essential amino acid, is particularly important. L-glutamine provides nitrogen for NAD, NADPH and nucleotides and serves as a secondary energy source for metabolism. L-glutamine is an unstable amino acid that, with time, converts to a form that cannot be used by cells, and should thus be added to media just before use. Caution should be used when adding more L-glutamine than is called for in the original medium formulation since its degradation results in the build-up of ammonia, and ammonia can have a deleterious effect on some cell lines. L-glutamine concentrations for mammalian cell culture media can vary from 0.68 mM in Medium 199 to 4mM in Dulbecco's Modified Eagles's Medium. Invertebrate cell culture media can contain as much as 12.3 mM L-glutamine.

e. Carbohydrates

Carbohydrates in the form of sugars are the major source of energy. Most of the media contain glucose and galactose, however, some contain maltose and fructose.

f. Proteins and peptides

The most commonly used proteins and peptides are albumin, transferrin, and fibronectin. They are particularly important in serum-free media. Serum is a rich source of proteins and includes albumin, transferrin, aprotinin, fetuin and fibronectin. Albumin is the main protein in blood acting to bind water, salts, free fatty acids, hormones, and vitamins, and transport them between tissues and cells. The binding capacity of albumin makes it a suitable remover of toxic substances from the cell culture media.

g. Fatty acids and lipids

They are particularly important in serum-free media as they are generally present in serum.

h. Vitamins

Many vitamins are essential for growth and proliferation of cells. Vitamins cannot be synthesized in sufficient quantities by cells and are therefore important supplements required in tissue culture. Again serum is the major source of vitamins in cell culture, however, media are also enriched with different vitamins making them suitable for a particular cell line. The B group vitamins are most commonly added for growth stimulation.

i. Trace elements

Trace elements are often supplemented to serum-free media to replace those normally found in serum. Trace elements like copper, zinc, selenium and tricarboxylic acid intermediates are chemical elements that are needed in minute amounts for proper cell growth. These micronutrients are essential for many biological processes, *e.g.*, the maintenance of the functionality of enzymes.

j. Antibiotics and anti-fungals

Although not required for cell growth, antibiotics are often used to control the growth of bacterial and fungal contaminants. Routine use of antibiotics for cell culture is not recommended since antibiotics can mask contamination by mycoplasma and resistant bacteria. Moreover, antibiotics can also interfere with the metabolism of sensitive cells. Penicillin-streptomycin-amphotericin B preparations are the most commonly used combinations in cell culture media preparation. Plasmocin can eliminate mycoplasma contamination.

k. Serum in media

Serum is a complex mix of albumins, growth factors and growth inhibitors. Serum is one of the most important components of cell culture media and serves as a source for amino acids, proteins, vitamins (particularly fat-soluble vitamins such as A, D, E, and K), carbohydrates, lipids, hormones, growth factors, minerals, and trace elements. Serum from fetal and calf bovine sources are commonly used to support the growth of cells in culture. Fetal serum is a rich source of growth factors and is appropriate for cell cloning and for the growth of fastidious cells. Calf serum is used in contact-inhibition studies because of its lower growth-promoting properties. Normal growth media often contain 2-10% of serum. Supplementation of media with serum serves the following functions:

- Serum provides the basic nutrients (both in the solution as well as bound to the proteins) for cells.
- Serum provides several growth factors and hormones involved in growth promotion and specialized cell function.
- It provides several binding proteins like albumin, transferrin, which can carry other molecules into the cell. For example: albumin carries lipids, vitamins, hormones, etc. into cells.

- It also supplies proteins, like fibronectin, which promote the attachment of cells to the substrate. It also provides spreading factors that help the cells to spread out before they begin to divide.
- It provides protease inhibitors which protect cells from proteolysis.
- It also provides minerals, like Na+, K+, Zn2+, Fe2+, etc.
- It increases the viscosity of the medium and thus, protects cells from mechanical damages during agitation of suspension cultures.
- It also acts a buffer.

Preparation of Cell Culture Media

General requirements

The Baby Hamster Kidney 21 (BHK-21) cell line was maintained using following media and reagents.

- Dulbecco's Modified Eagle's Medium
- Sodium bicarbonate
- Antibiotic solution- Penicillin (10,000 units), Streptomycin (10mg) and Amphotericin B (25µg/ ml)
- Foetal bovine serum (FBS)
- Tryptose Phosphate Broth (TPB) (Autoclaved)
- Phenol Red

Ingredients required for cell culture media preparation (DMEM)

- 0.1 N HCl
- Double distilled water
- Filtration assembly
- Membrane filter
- Vacuum pump
- Syringes
- pH paper
- Micropipettes and tips
- 15 mL Falcon tubes

Procedure

A. Preparation of Dulbecco's Modified Eagle's Medium (DMEM)

1. The DMEM is prepared by adding 15.6 g powdered DMEM to 970 ml of double distilled water and supplemented with 2.2 g/L of sodium bicarbonate.
2. One per cent antibiotic solution {penicillin (10,000 units), streptomycin (10mg) and Amphotericin B (25µg/ ml)}, sterile 10 per cent tryptose phosphate broth (TPB) (autoclaved) and 11 mg/L phenol red is added and volume is made upto 1000 mL.
3. The medium is then mixed, filtered through positive pressure with membrane filter of size 0.22 µm.

Filtration of DMEM through membrane filter assembly

4. The pH of the filtered media is checked using pH paper and the pH is adjusted to ± 7.2 with 0.1 N HCl.

Adjustment of pH

5. The filtered media is kept for sterility check.
6. Once the media pass the sterility check, it is stored at 4°C.

B. Preparation of Maintenance Medium (MM)

1. The DMEM prepared is supplemented with 2 per cent of foetal bovine serum (FBS) for the preparation of MM.
2. The prepared media is labeled and stored at 4°C.

Maintenance medium (MM)

C. Preparation of Growth Medium (GM)

1. The DMEM prepared is supplemented with 10 per cent of FBS for the preparation of GM.
2. The prepared media is labeled and stored at 4°C.

Preparation of cell lines for Rapid Fluorescent Focus Inhibition Test (RFFIT)

Sharada, R., Dilip, L. and Tilak Chandan, S.

Cell lines used for RFFIT

Baby Hamster Kidney 21 (BHK 21) cell line maintained in KVAFSU-CVA Rabies Diagnostic Laboratory, OIE Reference Laboratory for Rabies, Dept. of Veterinary Microbiology, Veterinary College, Bengaluru is used and an aliquot from lowest passage is revived and sub-cultured.

Revival of BHK 21 cells

General requirements

- Growth medium (GM)
- An aliquot of cryopreserved BHK 21 cells
- Centrifuge
- CO_2 Incubator
- Micropipette and tips
- T-25 flask
- Marker pens
- Water bath
- Centrifuge tubes

Procedure

1. An aliquot of cryopreserved BHK 21 cells from lowest passage stored at -80 °C is revived by thawing the cryovial containing frozen cells by gentle agitation in a water bath at 37±1 °C for 2-3 mins.

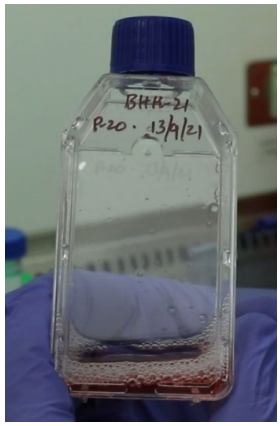
Cryovial containing frozen cells at -80 °C

2. The cryovial is removed from water bath and decontaminated by spraying with 70% Iso-propyl alcohol.
3. The contents (1 mL of cryopreserved cells) are transferred to sterile 15 ml centrifuge tube containing 2 mL of GM for centrifugation at 1000 RPM for 2-5 minutes at room temperature.

Centrifugation at 1000 RPM

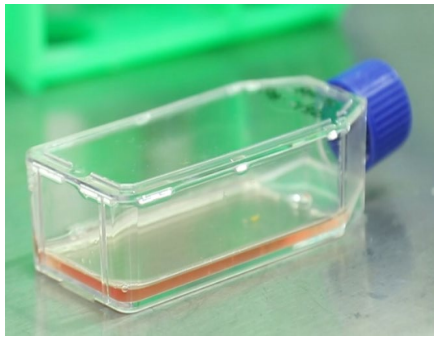
4. The supernatant is discarded and the cell pellet is suspended in 2 ml of GM.
5. The cell suspension is centrifuged again to remove the residual dimethyl sulfoxide (DMSO).
6. The step no. 4 is repeated.
7. The cell suspension is transferred into T-25 containing 4 ml of GM and is incubated at 37±1 °C at 4-5 per cent CO_2 concentration.

T-25 flask containing suspended cells in GM



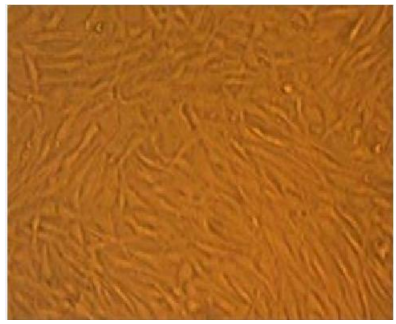
8. The cell monolayer is examined after 24-25 hours for checking cell growth.

Cell monolayer in T-25 flask after incubation



Microscopic observation of Cell monolayer with 30 % confluency after 24 hrs (200X)

Microscopic observation of Cell monolayer with 80-90 % confluency after 48 hrs (200X)



Subculture of BHK 21 cells

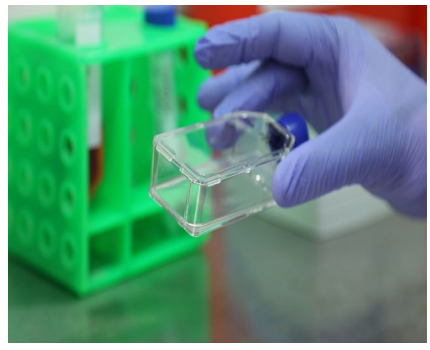
General requirements

- Trypsin EDTA
- GM
- T 25 flask
- T25 flask containing 80 % BHK 21 cells monolayer
- Micropipettes and tips

Procedure

1. Once the confluent monolayer is formed, the cells are sub-cultured.
2. The spent medium is removed and the cells monolayer is washed with GM to remove the dead cells, serum components and accumulated metabolic wastes.
3. Trypsinization of the monolayer is done to get the associated cells in dissociated and discrete form.
4. About 0.5-1 ml of trypsin EDTA is added to T-25 and kept at room temperature for 1-2 minutes.

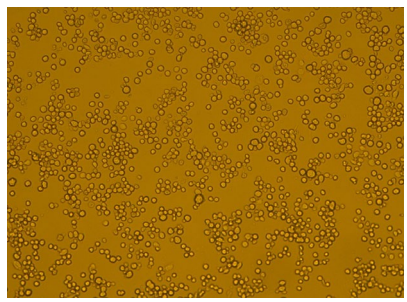
Trypsinization of the monolayer



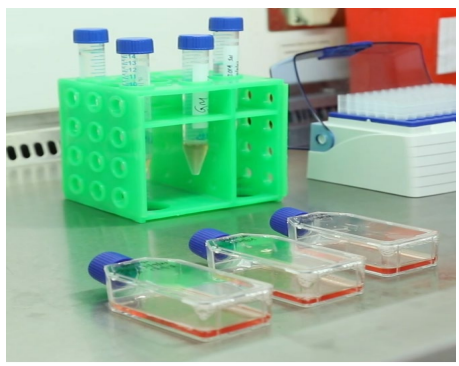
5. The trypsin-EDTA is discarded and again the step 4 is repeated.
6. The trypsin-EDTA is discarded and 3 mL of GM is added to the flask.
7. The trypsinized cells are detached from T-25 flask surface. The cells are suspended in GM and mixed by pipetting.

Detachment of BHK-21 cells from T-25 flask

Microscopic observation of detached BHK 21 cells (200X)



8. One mL of the suspended cells is distributed into each of the three T-25 flasks (split ratio 1:3).



9. The volume in each of the flask is made up to 6 mL with GM.
10. The flasks are incubated at 37±1 °C at 4-5 per cent CO_2 concentration.
11. The cell monolayer is examined after 24-25 hours for checking cell growth.

Preservation of BHK 21 cells

General requirements

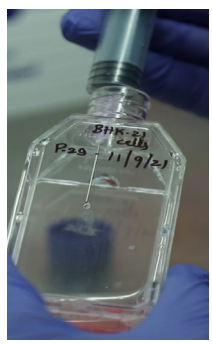
- Trypsin EDTA
- GM with 30 % FBS
- T25 flask containing 80 % BHK 21 cells monolayer
- Micropipettes and tips
- Cryovials

- Cryobabies
- Syringe
- DMSO
- Cryocooler
- - 80 °C freezer

Procedure

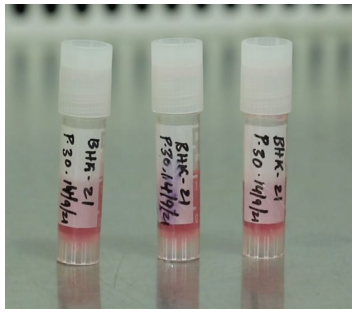
1. Once the confluent monolayer is formed, the cells are cryopreserved.
2. The spent medium is removed and the cell monolayer is washed with GM to remove the dead cells, serum components and accumulated metabolic wastes.
3. Trypsinization of the monolayer is done to get the associated cells in dissociated and discrete form.
4. About 0.5-1 ml of trypsin EDTA is added to T-25 and kept at room temperature for 1-2 minutes.
5. The trypsin-EDTA is discarded and again the step 4 is repeated.
6. The trypsin-EDTA is discarded and 3 mL of GM containing 30% FBS is added to the flask.
7. The trypsinized cells are detached from the T-25 flask surface and cells are suspended by pipetting.
8. The cell suspension is added with 10% DMSO (0.3 mL of DMSO added to T-25 flask containing 3 ml of cell suspension) drop by drop using sterile syringe.

Drop by drop addition of DMSO



9. The cell suspension containing 10% DMSO is aliquoted into cryovials (1 mL each). These aliquots are labeled, arranged in cryocooler and kept at -80 ± 2 ºC.

Aliquots of BHK 21 cells to be cryopreserved



Strains of virus, infection of cell culture, harvesting and titration of virus used for RFFIT

Shrikrishna Isloor, Kavitha, G. and Dilip, L.

Strains of rabies virus used for RFFIT

Use of live rabies virus (RABV) in the RFFIT method necessitates performance in a Biosafety Level (BSL) 2 or 3 laboratory. The BHK-21 cells adapted PV 3462 (Dr. Larghi's) strain of rabies virus obtained from Quality Control Rabies diagnostic laboratory of Pasteur Institute of India, Coonoor, Tamil Nadu or CVS-11 strain of rabies virus obtained from National Institute of Mental Health and Neurosciences (NIMHANS, Bengaluru) are used for the RFFIT. The virus seed lots are stored at -80±-2 °C and an aliquot from seed lot is grown, harvested and titrated. For the test 100 $TCID_{50}$ of virus per 100 µl shall be used.

Infection of cell culture

The BHK 21 cell monolayer with 80 per cent confluence is used for seed virus inoculation. The monolayer is seeded with Rabies virus and incubated at 37°C for viral adsorption for 90 minutes in CO_2 incubator at 37°C under 5 percent CO_2 tension. The viral inoculum is removed and the growth medium is added and incubated at 37°C in CO_2 incubator under 5 per cent CO_2 tension for two days.

For the calculation of virus inoculum to be seeded, use the formula:

$$\text{Volume of virus stock to be added} = \frac{\text{multiplicity of infection (MOI) x number of cells}}{\text{Virus titre}}$$

Generally, the BHK 21cells are infected with a lyssavirus at a multiplicity of infection (MOI) of 0.1.

Harvesting of virus

After two days of incubation, the virus propagated in the cell culture is harvested in a cryovial, aliquoted and stored in – 80°C freezer until further use. To get a second and subsequent virus harvest, 6 mL of maintenance medium is added to the infected monolayer and the flask is incubated again for 2 days in CO_2 incubator. With the same monolayer, maximum 4-5 harvests can be done in an interval of 2 days in between 2 harvests. All the harvests are titrated.

Titration of the harvested virus

Titration of the harvested virus is carried out to estimate the infectious unit of virus and stored at -80°C.

Materials and Equipments

Harvested Rabies virus lots
Micropipettes and sterile tips
Inverted microscope with Fluorescence attachment
96 well microtitre plate
PBS pH 7.2 ±0.2

CO_2 incubator
Anti-rabies N-protein monoclonal antibody based-FITC conjugate
Bacteriological incubator
Tissue paper

Procedure:

- The harvested virus is serially diluted using the cell culture growth medium as diluent and log dilutions from 10^{-1} to 10^{-6} are prepared.
- Five sterile centrifuge tubes are labeled starting from 10^{-1} to 10^{-6}.
- 900 µL of GM is taken into all tubes and 100 µL of harvested virus is added to first test tube (Table 1).
- After mixing, 100 µL from tube no.1 is transferred to tube no.2 and similar serial dilutions are carried out till tube no.6 and finally 100 µL is discarded from the last tube.

Table 1: Dilution of virus

Tube no.	**1**	**2**	**3**	**4**	**5**	**6**
Virus	100µl	**10x serial dilution**	→			
Diluent	900µl	900µl	900µl	900µl	900µl	900µl
Dilution	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}

- Five wells are maintained for each dilution (5 replicates).
- All these diluted viruses are added into 6 different wells in a microtitre plate in 5 replicates.

Table 2: Layout of microtitre plate for virus titration

	1 10^{-1}	**2** 10^{-2}	**3** 10^{-3}	**4** 10^{-4}	**5** 10^{-5}	**6** 10^{-6}						
A							**CC**	**V C**				
B							**CC**	**V C**				
C							**CC**	**V C**				
D							**CC**	**V C**				
E							**CC**	**V C**				

- The cells in tissue culture flask which had formed a monolayer of BHK- 21 cells are trypsinized and a homogenized cell suspension is made using 5 mL of growth medium. 50 µL of cell suspension is added to each well containing diluted virus suspension.

- The cell control (CC) and virus control (VC) are maintained by adding 50 µL of cell suspension with 100 µL of GM and with 100 µL of neat virus with GM respectively.
- The plate is incubated in CO_2 incubator at 37 ºC ± 1 ºC and 4-5 per cent CO_2 for 48 hrs.
- After 48 hours, the cell culture fluid (supernatant) from all the wells is removed and 100 µL of 70 per cent chilled acetone in double distilled water is added to all the wells.
- The plate is kept in -20ºC freezer for 60 minutes.
- After 60 minutes, acetone is removed using multi-channel pipette and the plate is air dried for 2-5 minutes.
- 50 µL of working dilution (1:15) of anti-rabies N-protein monoclonal antibody based-FITC conjugate is added.
- The plate is incubated at 37ºC for 60 ± 5 minutes.
- At the end of incubation, the mercury lamp of the fluorescent microscope is put on.
- After incubation, the plate is removed, the contents in all the wells are discarded and washed gently with 1x PBS with pH 7.2 ± 0.2 for 1-2 minutes (200 µL/well).
- The wash step is repeated twice to remove the residues of unbound conjugate.
- The plate is air dried for 2-3 minutes at room temperature and is observed under 10X and 20X objective magnification of fluorescent microscope.

Reading and recording of results

- All the fields in each of the wells are examined using Fluorescent microscope and all or none principle is adapted to evaluate the presence or absence of viral inclusions as apple green colored fluorescent particles in the cytoplasm of cells.
- The observations at various dilutions of virus is recorded (Table 2).

Interpretation of results:

- The virus titre is estimated by using Reed-Muench method (1938) in terms of $TCID_{50}$.

Table 3: Titration of virus using Reed-Muench method

Virus dilution	Infection ratio	Infected	Uninfected	Accumulated values		Infection ratio (I/I + U)	Percent (I/I+ U)*100
				Infected (I)	Uninfected (U)		
10^{-1}	/5						
10^{-2}	/5						
10^{-3}	/5						
10^{-4}	/5						
10^{-5}	/5						
10^{-6}	/5						

The 50% end point is considered for infection. The Proportionate Distance (PD) is calculated as below:

$$\text{Proportionate Distance (PD)} = \frac{\text{Infectivity above 50 \% - 50}}{\text{Infectivity above 50\% - Infectivity below 50 \%}}$$

- The log of the dilution above 50 per cent is considered. Hence, the 50 per cent end point is calculated in the following way:

(log ID50) = (log dilution above 50 per cent) + (Proportionate Distance × log dilution factor)

- Therefore, **$TCID_{50}$ is calculated as 10^{-x}/ 0.1 ml**
- The end point dilution is the dilution that will infect 50 per cent of the test units inoculated referred as one $TCID_{50}$.
- So, **100 $TCID_{50}$ = $10^{-(x-2)}$/ 0.1 ml**
- Antilog (x-2) is the dilution factor.
- So, the original virus stock is diluted by (antilog (x-2)) times to get 100 $TCID_{50}$ virus. This is used in RFFIT procedure.

Rapid Fluorescent Focus Inhibition Test (RFFIT)

Shrikrishna Isloor, Kavitha, G. and Dilip, L.

The Classical RFFIT was developed at the Communicable Disease Center {now the United States Centers for Disease Control and Prevention (CDC)} in 1973 by J. Smith, P. Yager and G. Baer to provide an alternative to the *in vivo* mouse neutralization test (MNT) for demonstrating the presence or absence of rabies virus neutralizing antibodies (RVNA) in serum. Several steps of the MNT procedure were preserved or used as the basis of the RFFIT. In essence, the *in vitro* (RFFIT) method was developed to mimic the *in vivo* (MNT) method. Use of live rabies virus (RABV) in the method necessitates performance in a Biosafety Level (BSL) 2 or 3 laboratory. The RFFIT results are primarily used:

- To determine the immunization status of individuals who have undergone pre- or post-exposure prophylaxis against RABV.
- For the evaluation of vaccines new schedules of rabies pre- or post-exposure prophylaxis.
- As demonstration of response to rabies vaccination for animals scheduled for importation to so-called rabies-free countries.
- For the management of plasma donors used in the production of rabies immunoglobulin (RIG).
- For the evaluation and calibration of newly developed serological tests.

The RFFIT is rarely used for the diagnosis of rabies in humans, as neutralizing antibodies appear in the cerebrospinal fluid (CSF) and in the serum of infected patients very late in the course of the disease. Modifications of the classical RFFIT led to the development of the FAVN test.

Materials and equipments

1. Test serum samples
2. BHK-21 cell lines
3. Titrated virus lots of PV 3462 (Dr. Larghi's) strain of rabies virus. For the test, 100 $TCID_{50}$ of virus per 100 µl is used.

4. 96-well tissue culture plate
5. Growth medium
6. PBS pH 7.2±0.2

7. Variable channel Micro-pipettes (1-10 µl, 20-100 µl and 20-1000 µl)
8. Sterile disposable microtips (1-10µl, 20-200µl and 1000µl)
9. Sterile Petri plates
10. Biosafety cabinet
11. Reference anti-rabies serum: Commercially available ERIG preparation calibrated against second international reference serum having a potency of 2 IU/mL (obtained from National Institute of Biological Standards, UK).
12. CO_2 incubator
13. Inverted Microscope with fluorescence attachment
14. Anti-rabies nucleoprotein IgG FITC conjugate
15. 70 % Chilled acetone with double distilled water

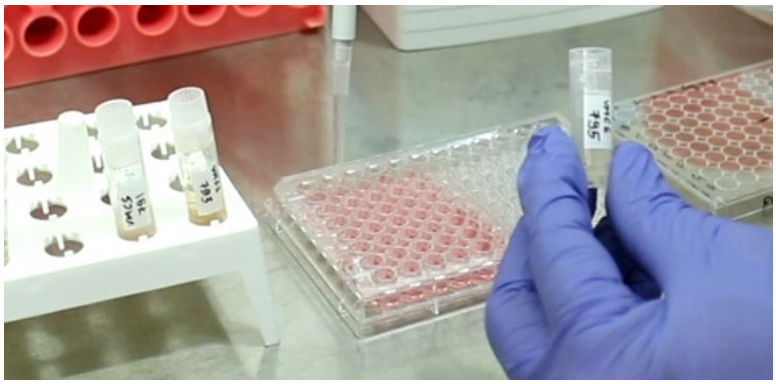
Procedure

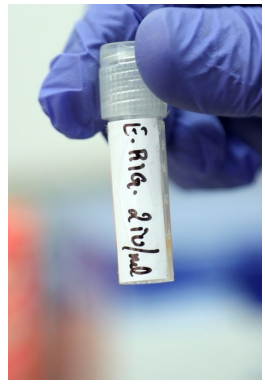
- The biosafety cabinet is kept ready for carrying out the procedure of RFFIT.
- All serum samples received are transferred to cryovials, labeled properly and subjected to heat inactivation of complements at 56±2 °C for 30-32 mins in the water bath.

Heat inactivation of serum samples at 56±2 °C

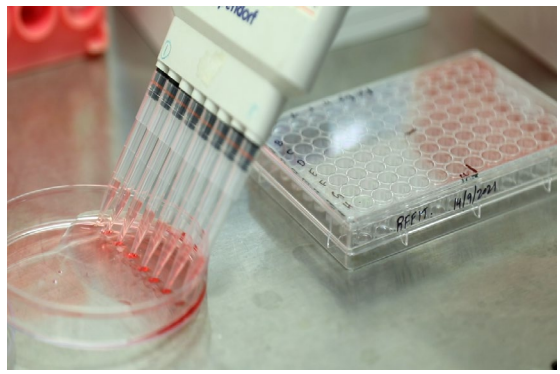
- A template of 96-well microtiter plate depicting all the test samples, cell and virus controls as shown in the **Table** at the end of this chapter.
- 100 µL of GM is added to each sample well and virus control wells (quadruplets); 200 µL of GM is added to cell control wells (quadruplets).

- 100 µL of serum under test is added to the first well (1:2) and mixed well by pipetting.
- After mixing, 100 µL from first well is transferred to the second well (1:4) and similar serial dilutions are carried out till eighth well (1:256) and finally 100 µL is discarded from the eighth well.
- Serial dilutions of reference serum (ERIG) are made similar to that of serum under test.

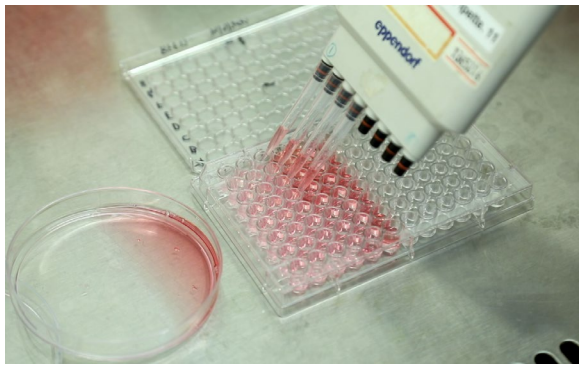




- The stock virus diluted in GM to contain 100 $TCID_{50}$ of virus per 100µL is prepared in sterile disposable Petri plates.
- 100µL of 100 $TCID_{50}$ of virus is added to each of the serum dilutions, including the reference serum and also to virus control (VC).

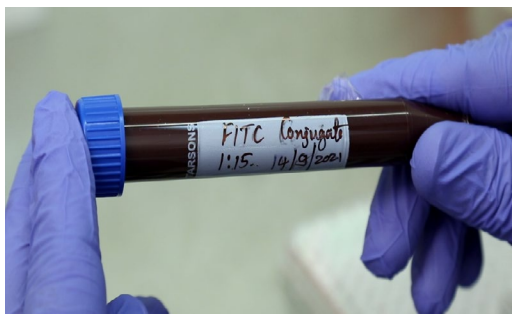


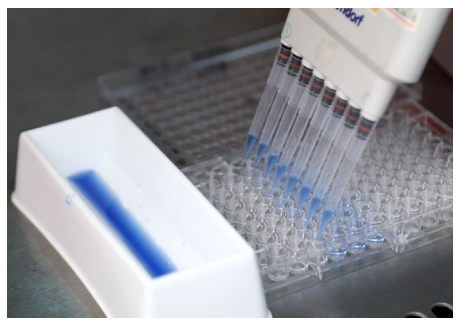
- The plate is incubated in CO_2 incubator for 60-65 min at 37±1 ºC and then brought back to the biosafety cabinet.
- The BHK-21 cell monolayer in a T-25 flask is trypsinized by using trypsin-EDTA and these cells are re-suspended in 5 mL of GM.
- 50 µL of cell suspension is added to each well of the plate containing serum and virus mixture; also to cell control and virus control wells.



- The plate is incubated in a CO_2 incubator at 37±1 ºC for 48-50 hours.
- At the end of 48-50 hours, the cell culture fluid from all the wells is removed, the cells are fixed in the wells of the plate by adding 100 µL of 70 per cent chilled acetone per well and kept at -20 ± 2˚C for 30-35 minutes.

- After 30-35 minutes, acetone is removed using multi-channel pipette and the plate is air dried.
- The cells are stained by adding 50 µL of pre titrated 1:15 diluted anti-rabies nucleoprotein IgG FITC conjugate and incubated in CO_2 incubator at 37±1 ºC for 60 ±5 mins.



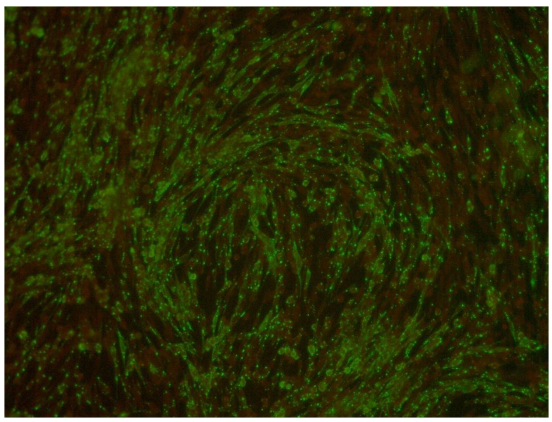


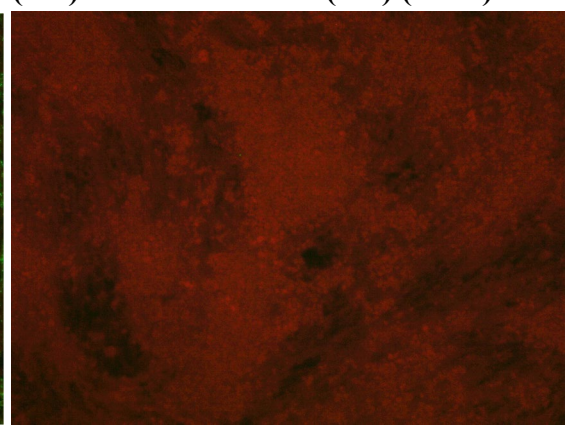
- At the end of the incubation, the mercury lamp of the inverted microscope is put on.
- After incubation, the plate is removed from the incubator, the contents of the plate are discarded and the wells are washed gently with 1x PBS with pH 7.2 ± 0.2 for 1-2 minutes (200 µL/ well).
- The wash step is repeated twice to remove the residues of unbound conjugate.
- The plate is air dried for 2-3 minutes at room temperature.

Reading and recording of results:

- The observations are made under Fluorescent microscope with 100X and 200X magnifications.
- Before taking the readings of individual samples, the readings of cell control and virus control wells are taken.

Microscopic observation of Virus Control (VC) and Cell Control (CC) (200X)





Microtiter plate layout for RFFIT

Sample	1	2	3	4	5	6	7	8	9	10	Ref.S	Controls
A	1:2	1:2	1:2	1:2	1:2	1:2	1:2	1:2	1:2	1:2	1:2 Ref.S	VC
B	1:4	1:4	1:4	1:4	1:4	1:4	1:4	1:4	1:4	1:4	1:4 Ref.S	VC
C	1:8	1:8	1:8	1:8	1:8	1:8	1:8	1:8	1:8	1:8	1:8 Ref.S	VC
D	1:16	1:16	1:16	1:16	1:16	1:16	1:16	1:16	1:16	1:16	1:16 Ref.S	VC
E	1:32	1:32	1:32	1:32	1:32	1:32	1:32	1:32	1:32	1:32	1:32 Ref.S	CC
F	1:64	1:64	1:64	1:64	1:64	1:64	1:64	1:64	1:64	1:64	1:64 Ref.S	CC
G	1:128	1:128	1:128	1:128	1:128	1:128	1:128	1:128	1:128	1:128	1:128 Ref.S	CC
H	1:256	1:256	1:256	1:256	1:256	1:256	1:256	1:256	1:256	1:256	1:256 Ref.S	CC

Interpretation of RFFIT

Dilip, L. and Hridya, S.V.

Interpretation of RFFIT results

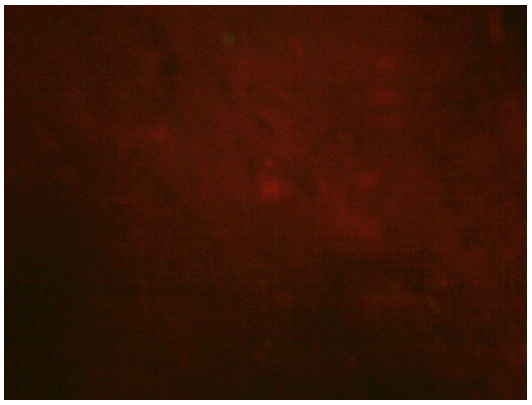
- **Validity of the test:** Fluorescent foci should be observed in all the virus control wells but not in all the cell control wells.
- All the wells containing serum dilutions are observed and looked for the presence or absence of fluorescence. The highest dilution of serum at which complete neutralization of virus *i.e.* absence of fluorescent foci of the monolayer is taken as end point dilution.
- Similarly, the end point for the reference serum is recorded.
- The neutralizing antibody titer of the serum under test is calculated using the formula:

$$\text{Titre (IU/ml)} = \frac{\text{Highest dilution of test serum showing complete neutralization X Unitage of reference serum}}{\text{Highest dilution of reference serum showing complete neutralization}}$$

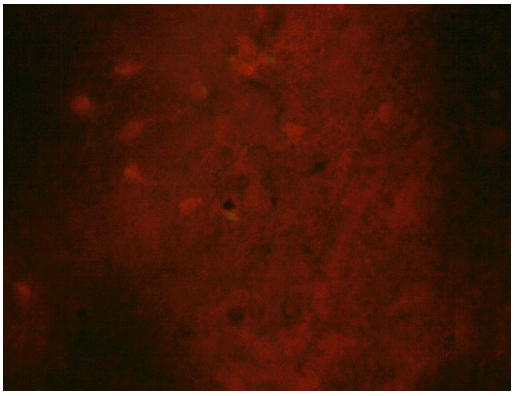
- A titre of equal to or greater than 0.5 IU/mL is considered as an indicative of adequate protection against rabies.
- Limit of Detection: The test can detect a minimum 0.5 IU/ml of Rabies Virus Neutralizing Antibody titer.
- RFFIT reports are prepared and then issued.

Microscopic observation of complete neutralization of virus at various dilution of serum (200X)

1:2

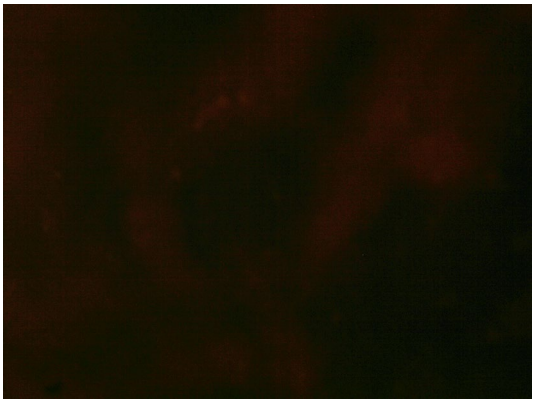


1:4



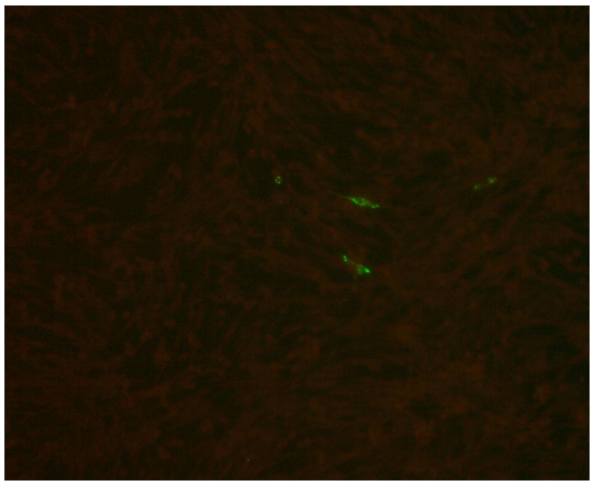
1:8

1:16



Microscopic observation of apple green fluorescing foci due to incomplete neutralization of virus at various dilutions of serum (200X)

1:32



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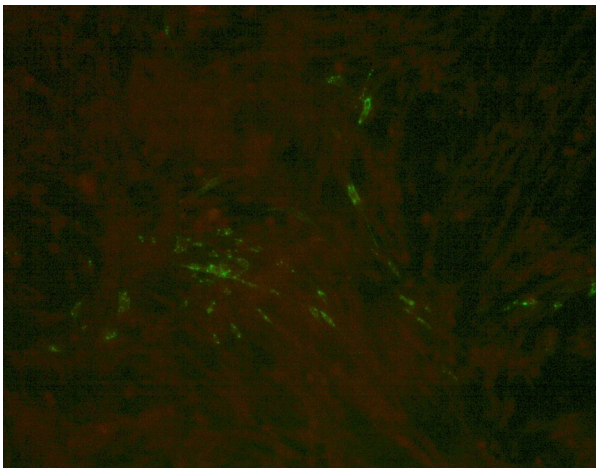

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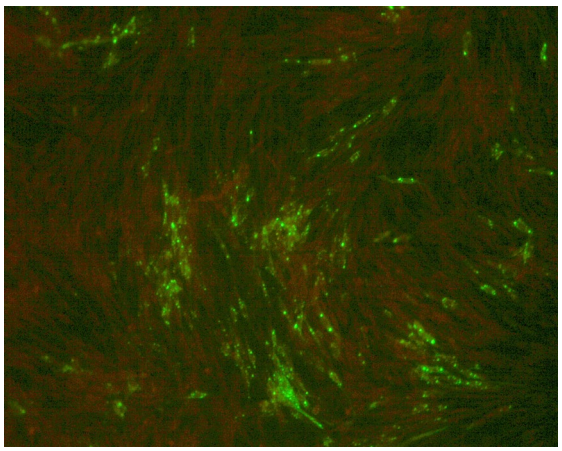

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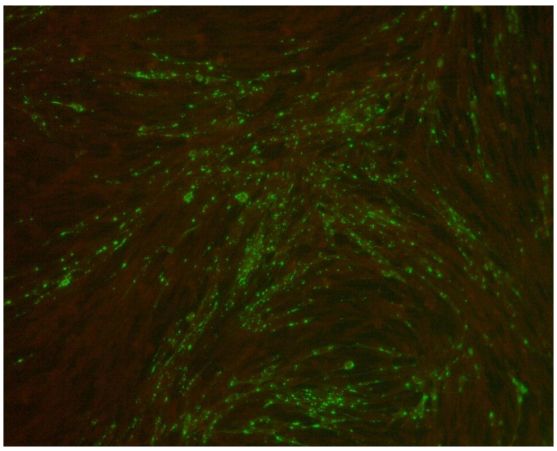


Format for submission of serum samples for RFFIT

KARNATAKA VETERINARY, ANIMAL AND FISHERIES SCIENCES UNIVERSITY, BIDAR
KVAFSU-CVA RABIES DIAGNOSTIC LABORATORY
OIE REFERENCE LABORATORY FOR RABIES
DEPARTMENT OF VETERINARY MICROBIOLOGY
VETERINARY COLLEGE, HEBBAL, BENGALURU – 560 024

FORMAT FOR SUBMISSION OF SERUM SAMPLES FOR RFFIT

1	Owner's Name, Address & Contact Details (E-mail, Phone No.)	
2	Name of the Animal	
3	Species, Gender	Male / Female / Intact / Neutered
4	Breed	
5	Age	
6	Date of Birth	
7	Colour	
8	Microchip No.	
9	Date of Last Vaccination	
10	Date of Serum sample collected	
11	Date of Serum sample submitted	
12	Vaccination Details (Scanned copy to be enclosed)	
13	Name of the Clinician with KVC No., address and contact details	

Note: Serum samples to be collected under aseptic conditions and to be sent under cold conditions.

An amount of Rs. 3000/- per serum sample be paid either in cash or Demand Draft (DD). The DD shall be drawn in favour of 'Assistant Comptroller, Veterinary College, Hebbal, Bengaluru

Signature of the clinician

Format for reporting of RFFIT result

KARNATAKA VETERINARY, ANIMAL AND FISHERIES SCIENCES UNIVERSITY, BIDAR
KVAFSU-CVA RABIES DIAGNOSTIC LABORATORY
OIE REFERENCE LABORATORY FOR RABIES
DEPARTMENT OF VETERINARY MICROBIOLOGY
VETERINARY COLLEGE, HEBBAL, BENGALURU – 560 024

TEST REPORT OF RABIES ANTIBODIES IN ANIMAL SERUM SAMPLE

01	Owner's Name & Address	
02	Clinician's Name & Address	
03	Animal's Name	
04	Species and Gender	
05	Breed and Colour	
06	Date of Birth and Age	
07	Microchip No. / Details	
08	Date of Last Vaccination	
09	Date of Serum Collection	
10	Date of Receipt of Serum	
11	Condition of Sample	
12	Test Performed	
13	Date of starting the test	
14	Date of completion of the test	

TEST RESULT:

SL. NO.	SAMPLE ID	LAB ID	TEST PROTOCOL	RESULTS
1	-	VMCS No.	Laboratory Techniques in Rabies, 4th Edition, 1996 (Published by WHO) SOP No. RDL 022, DOI 02.09.2019	± 0.27 IU/ml

Note:
- A minimum titre of 0.5 International Unit / ml is considered as sufficient by WHO / OIE
- The Result applies exclusively to the given material analyzed

Reviewed by:

1. (Shrikrishna Isloor)
Laboratory Director

2. (Sharada, R.)
Quality Manager

Authorized by:

(D. Rathnamma)
Biosafety Officer

Annexure 1. Training agenda

Day 1: Monday, 11 October, 2 PM

Topic	Speaker	Japan time	Duration
Session 1: Opening Session			
Introduction and welcome	Dr. Kinzang Dukpa, OIE RRAP	2:00 PM	10 min
Keynote remarks	Dr. Hirofumi Kugita, OIE Regional Representative for Asia and the Pacific		
	Dr. H.D. Narayanaswamy, Hon'ble Vice Chancellor, KVAFSU, Karnataka	2:10 PM	5 min
	Dr S. Abdul Rahman, Executive Director, CVA	2:15 PM	5 min
	Dr. Praveen Malik, Animal Husbandry Commissioner, Government of India	2:20 PM	5 min
Training objectives and outline of the training	Dr. Shrikrishna Isloor, Lab. Director, KVAFSU-CVA RDL	2:25 PM	5 min
Presiding official's remarks	Dean, Veterinary college, KVAFSU, Hebbal, Bengaluru	2:30 PM	5 min

Session 2: Introduction and follow up of previous training			
Participant introduction via *Mentimeter*®	OIE	2:35 PM	10 min
Follow-up actions taken following the virtual training on rabies diagnosis of November 2020	OIE/countries	2:45 PM	10 min
An introduction to the KVAFSU-CVA RDL's capacity for rabies diagnosis	KVAFSU	2:55 PM	10 min
Q & A		3:05 PM	10 min
Comfort break		3:15 PM	10 min
Session 3: Introduction to RFFIT and cell culture media			
Introduction to rabies serology and RFFIT	Dr. Shrikrishna Isloor Dr. D. Rathnamma Dr. Sharada, R. KVAFSU-CVA RDL	3:25 PM	15 min
Theory session on cell culture media	Dr. D. Rathnamma KVAFSU-CVA RDL	3:40 PM	15 min
Practical demonstration / video on cell culture media	Dr. Kavitha, G., Dr. Hridya, S.V., Dr. Vinay, C.P., KVAFSU-CVA RDL	3:55 PM	45 min
Q & A		4:40 PM	15 min
Training wrap-up for Day 1	OIE RRAP	4:55 PM	05 min

Day 2: Tuesday, 12 October, 2 PM

Topic	Speaker (s)	Japan time	Duration
Session 4: Preparation of cell lines for Rapid Fluorescent Focus Inhibition test (RFFIT)			
Introduction and welcome	OIE RRAP	2:00 PM	5 min
Theory session on cell lines used for RFFIT, their revival, subculture, maintenance and preservation	Dr. Sharada, R. KVAFSU-CVA RDL	2:05 PM	15 min
Mentimeter® session	OIE RRAP	2:20 PM	10 min
Practical demonstration / video on Cell lines used for RFFIT, their revival, subculture, maintenance, and preservation	Mr. Dilip, L., Dr. Tilak Chandan, S. KVAFSU-CVA RDL	2:30 PM	45 min
Q & A		3:15 PM	15 min
Comfort break		**3:30 PM**	**10 min**
Session 5: RFFIT – Methodology			
Theory session on strains of virus used for RFFIT, infection of cell culture, harvesting and titration of virus	Dr. Shrikrishna Isloor KVAFSU-CVA RDL	3:40 PM	20 min
Practical demonstration / video on strains of virus used for RFFIT, infection of cell culture, harvesting and titration of virus	Mr. Dilip, L., Dr. G. Kavitha KVAFSU-CVA RDL	4:00 PM	45 min
Q & A		4:45 PM	15 min
Training wrap-up for Day 2	OIE RRAP	5:00 PM	05 min

Day 3: Wednesday, 13 October, 2 PM

Topic	Speaker/s	Japan time	Duration
Session 6: RFFIT- Interpretation			
Introduction and welcome	OIE RRAP	2:00 PM	5 min
Theory session on Interpretation of RFFIT	Mr. Dilip, L. KVAFSU-CVA RDL	2:05 PM	15 min
Mentimeter® session	OIE RRAP	2:20 PM	10 min
Practical demonstration / video on Interpretation of RFFIT	Mr. Dilip, L., Dr. Hridya, S.V. KVAFSU-CVA RDL	2:30 PM	45 min
Q & A		3:15 PM	15 min
Comfort break		**3:30 PM**	**10 min**
Session 7: Way forward			
What are the minimum requirements to set up RFFIT in a laboratory?	Dr. Sharada, R. KVAFSU-CVA RDL	3:40 PM	15 min
Networking of national rabies diagnostic laboratories in the SAARC – a concept	Dr. Shrikrishna Isloor KVAFSU-CVA RDL	3:55 PM	10 min

Discussion		4:05 PM	15 min
Closing remarks : KVAFSU - Dr M. Narayanaswamy, Associate Director of Extension. OIE RRAP	All	4:20 PM	15 min

Note: **OIE RRAP** – OIE Regional Representation for Asia and the Pacific (Tokyo); **KVAFSU** – Karnataka Veterinary, Animal and Fisheries Sciences University, Bengaluru; **CVA** - Commonwealth Veterinary Association; **RDL** - Rabies Diagnostic Laboratory

Annexure 2. List of participants

	Country	**Name**	**Designation**	**Remarks**
1	Bangladesh	Dr Provat Chandra Saha	Senior Scientific Officer, Rabies Vaccine Section, LRI, DHAKA)	Country participant
2	Bangladesh	Dr Mohammad Mufazzal Hossain	Senior Scientific Office, Veterinary Public Health, Livestock Research Institute, Mohakhali, Dhaka	Country participant
3	Bangladesh	Dr Shukes Chandra Badhy	Upazila Livestock Officer (L/R Post), Central Disease Investigation Laboratory, Dhaka	Country participant
4	Bangladesh	Dr Md. Shohidul Islam Khokon	Upazila Livestock Officer (L/R Post) DLS, Attached- Preventing Anthrax and Rabies in Bangladesh through Surveilance and Response (PARB) Project, Dhaka	Country participant
5	Bhutan	Dr. Sonam Jamtsho	Deputy Chief Veterinary Officer, TVH & SVL Phuntsholing	Country participant
6	Bhutan	Dr. Tshewang Gembo	Deputy Chief Veterinary Officer, TVH & SVL Gelephu	Country participant
7	Bhutan	Dr. Lungten Dorji	Sr. Laboratory Technician, TVH & SVL Nganglam	Country participant
8	Bhutan	Ms. Kelzang Lhamo	Laboratory Technician, NCAH Serbithang	Country participant
9	India	Dr. Harsh Kumar Sharma	Assistant Professor, Faculty of Veterinary Sciences and Animal Husbandry, UT of J& K	Country participant
10	India	Dr. Debalina Mitra	LO, DAHD	Country participant
11	Nepal	Dr Krishna Raj Pandey	Senior Veterinary Officer Veterinary Laboratory, Surkhet	Country participant
12	Nepal	Dr Manish Man Shrestha	Veterinary Officer National Vaccine Production Laboratory, Kathmandu	Country participant
13	Nepal	Dr Manju Maharjan	Veterinary Officer Central Veterinary Laboratory, Kathmandu	Country participant
14	Nepal	Dr Suraj Subedi	Veterinary Officer Central Veterinary Laboratory, Kathmandu	Country participant

15	Pakistan	Dr Muhammad Numan	Senior Veterinary Officer (Research), Veterinary Research Institute, Lahore	Country participant
16	Pakistan	Dr Mobeen Sarwar	Assistant Disease Investigation Officer, Provincial Disease Diagnostic Laboratory, Punjab	Country participant
17	Sri Lanka	Dr. (Mrs.) S. Puvanendiran	Veterinary Research Officer	Country participant
18	Sri Lanka	Dr. G.D.N. Kumarasinghe	Veterinary Investigation Officer	Country participant
19	Sri Lanka	Dr. (Mrs.) Amutha Prabhaharan	Veterinary Investigation Officer	Country participant
20	Sri Lanka	Dr. (Ms.) K.V.I.N. Kumaravithana	Veterinary Investigation Officer	Country participant
21	India	Dr Abdul Rahman	Commonwealth Veterinary Association	Invited Speaker
22	India	Dr H.D. Narayanaswamy	Hon'ble Vice Chancellor, KVFSU, Karnataka	Invited Speaker
23	India	Dr Praveen Malik	Animal Husbandry Commissioner, Government of India	Invited Speaker
24	India	Dr Girish Kumar	In-charge Dean, Veterinary college, KVAFSU, Hebbal Bangalore	Invited Speaker
25	India	Dr M Narayanaswamy	Veterinary Colloege, Bangalore Associate Director of Extension	Invited Speaker
26	India	Prof. Shrikrishna Isloor	Veterinary College, KVAFSU, Hebbal, Bengaluru. Professor	Trainer
27	India	Dr Rathnamma Doddamane	KARNATAKA VETERINARY AND ANIMAL SCIENCES UNIVERSITY, BIDAR, INDIA	Trainer
28	India	Dr G. Kavitha	KVAFSU-CVA RDL	Trainer
29	India	Dr Hridya Susan Varughese	Veterinary College Bangalore PhD student	Trainer
30	India	Dr Vinay C.P.	Veterinary College, Bangalore	Trainer
31	India	Dr Sharada Ramakrishnaiah	Veterinary College, KVAFSU, Hebbal, Bengaluru	Trainer
32	India	Dr Tilak Chandan s Vastrad	KVAFSU	Trainer
33	India	Mr Dilip L	KVAFSU CVA Rabies Diagnostic laboratory Technical manager	Trainer
34	India	Harshitha N	Data entry operator Veterinary College, KVAFSU, Bengaluru	Trainer
35	India	Dr Yathiraj Sreenivasa	Lakeside Veterinary Hospital and Research Centre	Observer
36	India	Dr Krithiga, N.	KVAFSU	Observer
37	India	Dr Shyama Sundar, K.A.	KVAFSU	Observer

38	India	Dr Sangeeta Jadhav	KVAFSU	Observer
39	Japan	Nijiho Kawaguchi	Miyazaki University	Observer
40	Japan	Tiangang Xu	Regional Project Officer, OIE RRAP	Observer
41	Japan	Kaho Shinozaki	Obihiro University of Agriculture and Veterinary Medicine	Observer
42	Japan	Hirofumi Kugita	Regional Representative, OIE RRAP	Organiser
43	Japan	Kinzang Dukpa	Regional Project Coordinator, OIE RRAP	Organiser
44	Japan	Lesa Thompson	Regional Project Officer, OIE RRAP	Organiser
45	Japan	Izumi Goto	Administrative Officer, OIE RRAP	Organiser
46	Bhutan	Pasang Tshering	OIE Consultant, OIE RRAP	Organiser

Annexure 3. Questions for the *mentimeter* quiz (answers in bold)

Day 2: 12th October 2021

1. Neutralizing anti rabies vaccinal antibody titre equal to or greater than the following international unit (I.U.) Is considered adequate for protection against rabies in dogs.
   Answer: **0.5** / 1 / 5 / 10
2. The following test used for assessing anti rabies vaccinal antibody titre
   Answer: ELISA / FAVN / RFFIT / **All three**
3. The percentage of Foetal bovine serum (FBS) used in preparation of Growth medium for cell culture system in RFFIT is
   Answer: 1 / 2.5 / 5 / **10 %**
4. BHK 21 cell lines used in virus neutralization test for rabies are derived from
   Answer: **Hamster** / Rabbit / Guinea pig / Dog
5. Di Methyl Sulfoxide (DMSO) is used for the following purpose in cell culture
   Answer: Revival of cells / **Preservation of cells** / Subculturing of cells / All three
6. Rabies Diagnostic Laboratory located in which of the following countries is the latest OIE Reference Laboratory for Rabies ?
   Answer: China / Israel / **India** / South Korea
7. The following protein of the rabies virus is invariably targeted for immune - diagnostic purposes
   Answer: **Nucleoprotein** / Phosphoprotein / Matrix protein / Glycoprotein
8. Which part of the brain samples is recommended for post mortem immune diagnosis of rabies
   Answer: Brain stem / Cerebellum / **Both brain stem and cerebellum** / Cerebrum

Day 3: 13th October 2021

1. Which of the following enzymes is used for subculturing of cell culture monolayer.
   Answer: Rennin / Pepsin / **Trypsin** / None

2. The cell line used in the RFFIT is derived from
   Answer: Pancreas / Brain / **Kidney** / Liver

3. What method of infection of cell culture with the rabies virus is employed in RFFIT learnt during this training programme ?
   Answer: Adsorptive / **Co-cultivation** / Both adsorptive & Co-cultivation / None

4. Titration of the rabies virus is done by employing following rate of dilution of virus
   Answer: 2 fold / 5 fold / **10 fold** / 100 fold

5. Which is the counterstain used in making the conjugate for RFFIT ?
   Answer: Methylene blue / **Evan's blue** / Gill's hematoxylin / Crystal violet

6. After addition of cell suspension to diluted serum – Virus mixture, the microtiter plate should be incubated at 37 ºC in 5 % Co_2 tension for how long ?
   Answer: 18-20 hrs / 28-30 hrs / 38-40 hrs/ **48-50 hrs**

7. Limit of detection of RFFIT (interms of IU) you learnt is
   Answer: 0.25 / **0.5** / 0.75 / 1.0

8. The titre of of virus used in RFFIT is
   Answer: 1 $TCID_{50}$ / 10 $TCID_{50}$ / **100 $TCID_{50}$** / 1000 $TCID_{50}$

Annexure 4. Summary of questions and answers

Note: Answers as provided by Dr Shrikrishna Isloor and Dr Sharada of KVAFSU. For further queries, please directly contact Dr Isloor at kisloor@gmail.com or rdlkvafsucva@gmail.com

Day 1 (2021/10/11)

1. For large animals, the whole head is being submitted to my laboratory. Is there any SOP to collect head of large animals in field?
- Even for large animals, the occipital foramen approach can be employed whenever the carcasses are presented. As far as the collection of head from the large animals is concerned, a horizontal deep incision be made at the site anterior to the first cervical vertebrae but posterior to the occipital protuberance. The head may be completely severed / decapitated by extending this deep incision. The decapitated head need to be put in a suitable ziplock cover (preferably 2 such covers to be used) and transported in cold chain to the nearest diagnostic facility
- Details for occipital foramen method of brain sampling can be accessed here.

2. Do you suggest brain sample collection at the field level outside a biosafety cabinet?
   - Yes, it can be done by the occipital foramen approach which is very simple and user friendly. Please refer the presentation and videos from the virtual training organized in November 2020 for more details.

3. What do we do if we find negative result for rabies though the lateral flow assays (LFA)? Do we need to conduct further tests to reconfirm before issuing the result to the client?
- LFA though not a referred test for diagnosis, it is the only field based user friendly test for

diagnosis of Rabies. Hence, results by LFA need to be confirmed by direct fluorescent antibody (DFA) and/or PCR. Completely or partially putrified brain samples are not fit for testing by DFA but partially putrified brain samples can be tested by LFA. Even if negative on LFA, consider testing further by DFA and/or PCR.

4. How long can we keep prepared growth and maintenance media in stock?
- In our laboratory, normally the growth medium is prepared just before use by adding FBS / serum to the maintenance medium. Whereas, we preserve maintenance medium at 4 °C for up to two weeks.

5. Can we use Eagle's MEM instead of DMEM?
- Eagles MEM can be used but ensure that first the cells get adapted to this medium before bulk usage.

6. In our laboratory, we use the following protocol for medium (for MSR and BHK cells): autoclaving Nissui's Eagles MEM along with TPB in DD water, and then after sterilization of this basic medium, we make growth or maintenance media as required by adding the remaining sterile ingredients to it. Can we prepare the media here like this too?
- Generally, we do not autoclave ingredients of cell culture media, since heating may inactivate the biological activity. Distilled water and TPB are sterilized by autoclaving.
- However as for the preparation of Nissui's Eagles MEM for cell culture is concerned, follow the manufacturer's instructions as to autoclave / filter sterilize the same as they have both types of media.

Day 2 (2021/10/12)

7. What is the concentration of trypsin EDTA used?
- It is 0.25 % Trypsin EDTA

8. What should the cell density be for revival and sub-culture?
- 1 million cells per ml for revival and 0.5 million cells per ml for subculture

9. Can Maintenance medium be used in the washing step after thawing?
- Growth medium is preferred for washing since the serum concentration is higher in this which will maintain and support the survival of cells better

10. What is the maximum passage level for cells to be used for tests? What is the limit for subculturing a cell line?
- Avoid sub-culturing beyond 60-65 times. With subculturing, cells age and with ageing cells may lose some of the original properties and develop genetic aberrations. Usually, in our laboratory, we will go up to 65 subculturing.

11. Do we do manual jerk shaking after addition of trypsin to the flask?
- We may do gentle manual jerking for detachment of the cell monolayer.

12. What % of EDTA is used (with 0.25% trypsin)?
- It is 0.02 % EDTA.

13. Can we use dry bath incubator instead of a water bath?
- Avoid using a dry bath, as heat dissipation / conduction is efficient and faster in a water bath. Efficient and faster heat dissipation is important during thawing of frozen cells.

14. Can we incubate without CO_2 using some other reagents in the media?
- If you use non-porous capped Tissue culture flasks, then CO_2 supplementation is not mandatory. 5% CO_2 supplementation supports cells to undergo faster proliferation and is supportive to cells – this mimics *in-vivo* cell proliferation.

15. Can we discard spent media in sodium hypochlorite solution?
- Yes, you can. We use NaOH solution.

16. We keep the white lights of the room switched on while the UV lighting on inside the biosafety cabinet. Do you recommend keeping the whole room dark during/shortly after using the UV light, to avoid photo reactivity?
- There is no need to turn off the lights in the room but within the BSC ensure that white light is turned off and that shutters are closed.

17. Can we use larger size flasks (larger than T25) for growing cell lines, to have a greater stock of working cell lines?
- Yes, you can choose the flask size based on your needs.

18. Please clarify the storing temperatures for conjugates and other reagents used for the rabies test.
- It is important to aliquot conjugates and other reagents on delivery and refreeze. Repeated thawing and refreezing will affect the reagents. It is also important to make aliquots of serum. As serum samples for international movements of pet animals have to be tested (for anti-rabies vaccinal antibodies) in a batch, such test serum samples be frozen on arrival at the laboratory.

19. How long can rabies virus strains be stored at -80 °C without change in the aliquot strength?
- There will be a gradual reduction in the titre over a period though temperature is maintained at -80 °C throughout. We have been preserving the positive brain tissues for the past 8 years without losing its infectivity. Whereas we have preserved BHK21 cell culture harvest for a period of 5 years at -80 °C but encountered significant reduction in the titre although it was infective.

Day 3 (2021/10/13)

20. Can we use 1 IU as unit of reference serum? Does 2 IU reference serum should always give inhibition up to 1:8 dilution?
- There is no hard and fast rule that it has to be 2 IU, but we need to ensure that after comparing its performance with WHO reference serum. You may also start with 1 IU. Yes, if 2 IU reference serum used in the first well of a column in the microtitre plate with 1:2 dilution of serum, then, the next well (second well) in the same column corresponds to 1:4 dilution of serum and titre of serum will be 1 IU. Accordingly, the third well in that column corresponds to 1:8 dilution of serum and titre of the serum will be 0.5 IU. Universally, 0.5 IU of anti rabies vaccinal anti body titre is considered sufficient to confer protection against rabies. Therefore, 2 IU reference serum at 1:8 dilution corresponds to 0.5 IU, the cut off.

21. How do we calculate the minimal variation?
- Determine the standard deviation of IUs of the reference serum samples after 20 repetitions. The lower the deviation, the higher the precision of test.

22. What is the maximum IU detectable by RFFIT?
- IU refers to International units. The neutralizing antibody titre is expressed in IU/mL. You may go as high as the dilution can be made, but in our lab, we go up to 1:256 (corresponds to 64 IU) dilution (H row, the last row in the microtitre plate).

23. Regarding microchips, details may not be present in rural dogs/pets in parts of India.
- Microchip numbers are required for international movement of pets, but they are not required for routine sero-monitoring of anti-rabies antibodies to monitor mass dog vaccination campaigns.

24. Should I cover the plate with film during microscopy?
- Do not cover the plate while reading the results as there is no biosafety concern, since the cells are fixed and virus inactivated after fixing with 70 % chilled acetone.

25. Can we use FITC green light during examination, or is it mandatory to use a blue filter?
- Contact your microscope supplier to provide the filter suitable for FITC conjugate.

26. Is there any non-specific fluorescence during RFFIT?
- Beginners may get confused between specific and non-specific fluorescence. If washing is not done properly, one may have non-specific fluorescence. Non-specific binding of conjugates is very low with most of the conjugates as they are monoclonal antibody based.

27. If the titer is below than 0.5 IU i.e. below 1:2 dilution (1st well), then what can we say about the exact figure (e.g. may be 0.27 IU or something else)?
- At this rate of dilution, you can't specify anything below 0.5 IU. If you design the layout and dilutions differently, can answer this. At the most we can say that titre of antibodies is less than 0.5 IU

28. The diagnostic antibody is N protein based while the protective antibody is G protein based. Is there a correlation between these?
- There is paucity of information correlating N and G protein based diagnostics. However, both N and G proteins can be assessed for diagnosis, but now it is invariably N protein which is assessed in laboratories. For immunodiagnostic purposes N protein is targeted as it is immunodominant and usually found in abundance.

29. What do we do if different observers read the fluorescence different?
- You may have to repeat the test. Evaluators' competency needs to be proven. It is easy to say a sample is positive if you see even one viral inclusion; it is more difficult to say it is negative as you need to assess the complete well.

30. Is there a chance that RFFIT values less than 0.5 are still protective for rabies?
- RFFIT does not detect the CMI response. So RFFIT could be <0.5 IU with some immunity present. KVAFSU is now researching this topic.

31. Do we need to decontaminate the exhaust air coming from the biosafety cabinet to keep the external-to-the-lab-environment safe from viral aerosols?
- Some biosafety cabinets have a burn-off capacity, so even if any zoonotic pathogens pass through, they will be inactivated. This is not necessary for rabies virus. HEPA filters will effectively filter the air. It is recommended to have biosafety cabinets tested every year – maintenance is very important.

32. Can we use sodium hypochlorite instead of sodium hydroxide (NaOH)? Does sodium hydroxide have some advantages over hypochlorite?
- NaOH has been shown to be the best to inactivate the pathogens especially in presence of organic materials

33. How do you manage biological waste and its safe disposal after lab activity is finished?
- It is important to decontaminate biological materials (e.g tissue papers used for cleaning), e.g. with 2% NaOH. You can also secondarily decontaminate by autoclaving before discard. The discard is sent to an outsourced agency which manages for its final disposal.

34. What type of commercially available ELISA kit is used for rabies identification?
- Indirect quantitative ELISA kits targeting N protein are available commercially.

35. Can we send the serum samples from lab personnel to KVAFSU to test their immune status?
- KVAFSU-CVA laboratory personnel get their serum samples tested at the WHO Collaborating Centre for human rabies in Bengaluru, India (NIMHANS). Since the KVAFSU became an OIE RL last year, the government in India also asked them to test human samples (brain/serum). Dr Isloor will get back to participants on this point.

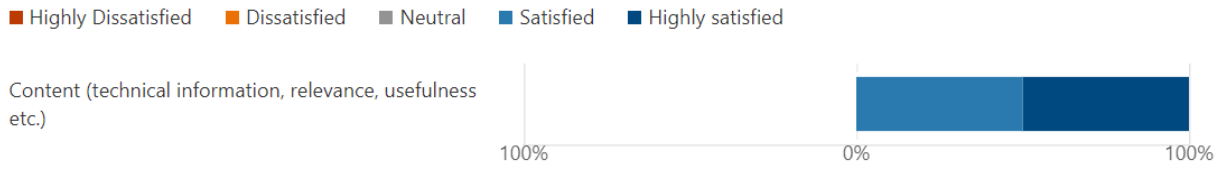
36. Is it required to have an MTA (material transfer agreement) to send samples to a reference laboratory?
- Yes, it is required. KVAFSU-CVA RDL can share the MTA format. Note that the MTA is required even within India (even if just in Bengaluru!)

Annexure 5. Training evaluation

Total responses = 16

1. Overall assessment of the webinar

Highly Satisfied: 8 Satisfied: 8 Neural: 0 Dissatisfied: 0 Highly Dissatisfied: 0



2. How would you rate the impact this webinar will have on:

… your technical knowledge on the subject

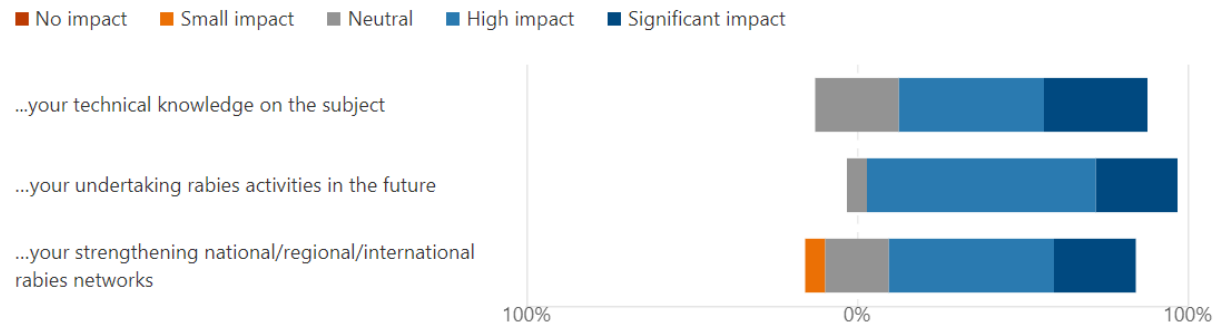
Significant impact: 5 High impact: 7 Neutral: 4 Small impact: 0 No impact: 0

… your understanding rabies activities in the future

Significant impact: 4 High impact: 11 Neutral: 1 Small impact: 0 No impact: 0

… your strengthening national/regional/international rabies networks

Significant impact: 4 High impact: 8 Neutral: 3 Small impact: 1 No impact: 0



3. Please rate how useful you found for each of the technical sessions

Introduction to rabies serology and RFFIT

Very Useful: 12 Useful: 4 Neutral: 0 Not very useful: 0 Not Useful: 0

Theory session on cell culture media

Very Useful: 12 Useful: 3 Neutral: 1 Not very useful: 0 Not Useful: 0

Practical demonstration/video on cell culture media

Very Useful: 10 Useful: 5 Neutral: 1 Not very useful: 0 Not Useful:0

Theory session on cell lines used for RFFIT, their revival, subculture, maintenance, and preservation

Very Useful: 13 Useful: 2 Neutral: 1 Not very useful: 0 Not Useful: 0

Practical demonstration/video on Cell lines used for RFFIT, their revival, subculture, maintenance, and preservation

Very Useful: 9 Useful: 4 Neutral: 2 Not very useful: 1 Not Useful: 0

Theory session on strains of virus used for RFFIT, infection of cell culture, harvesting and titration of virus

Very Useful: 8 Useful: 7 Neutral: 1 Not very useful: 0 Not Useful: 0

Practical demonstration/video on strains of virus used for RFFIT, infection of cell culture, harvesting and titration

Very Useful: 8 Useful: 6 Neutral: 1 Not very useful:1 Not Useful: 0

Theory session on Interpretation of RFFIT

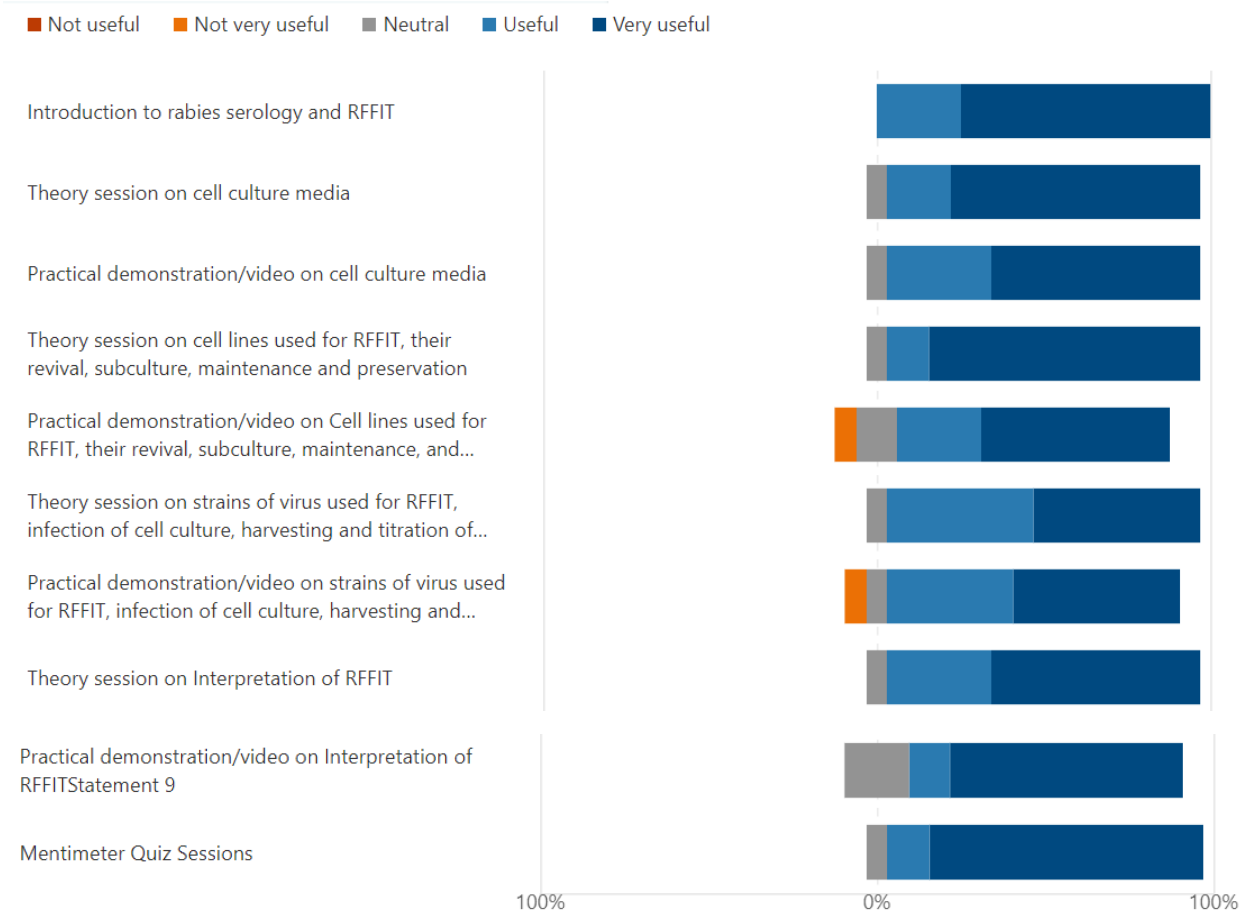
Very Useful: 10 Useful: 5 Neutral: 1 Not very useful: 0 Not Useful: 0

Practical demonstration/video on Interpretation of RFFIT

Very Useful: 11 Useful: 2 Neutral: 3 Not very useful: 0 Not Useful: 0

Mentimeter Quiz Sessions

Very Useful: 13 Useful: 2 Neutral: 1 Not very useful: 0 Not Useful: 0



4. Webinar logistics

Invitations received in due time

Highly Satisfied: 13 Satisfied: 3 Neutral: 0 Dissatisfied: 0 Highly Dissatisfied: 0

Registration procedures

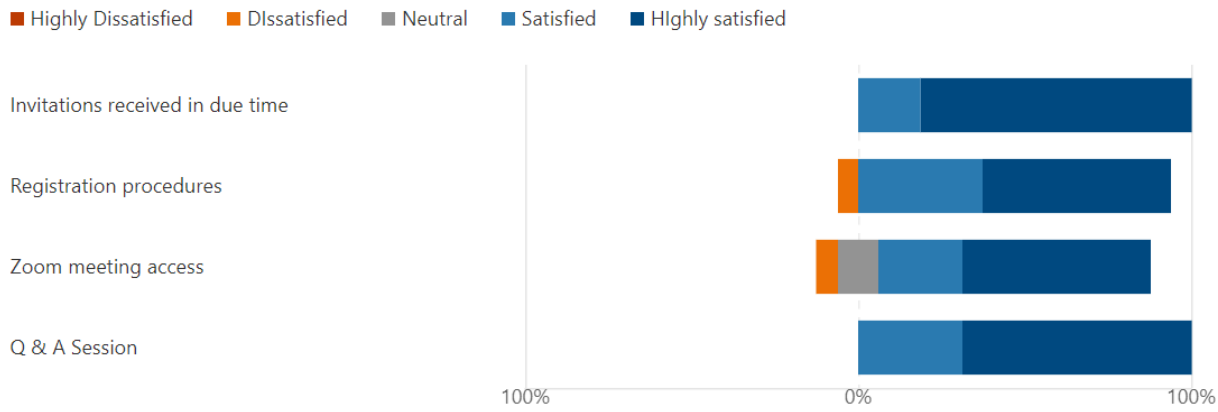
Highly Satisfied: 9 Satisfied: 6 Neutral: 0 Dissatisfied: 1 Highly Dissatisfied: 0

Zoom meeting access

Highly Satisfied: 9 Satisfied: 4 Neutral: 2 Dissatisfied: 1 Highly Dissatisfied: 0

Q & A Session

Highly Satisfied: 11 Satisfied: 5 Neutral: 0 Dissatisfied: 0 Highly Dissatisfied: 0



5. What did you like about this training?

- Very descriptive presentations, informative and clear and loud voice quality
- More exposure regarding rabies diagnosis and networking which very useful but requires hands on training by physical training program.
- Detailed presentation on technique
- Although it was virtual, all the lectures and videos were very good and interesting.
- RFFIT is a relatively new technique in Bhutanese context. It was useful to learn this technique first hand from OIE reference laboratory experts
- Almost the entire techniques were new to me which enabled me to appreciate every part of it in awe.
- Theoretical knowledge meeting the practical needs. Practical sessions including the video demonstration was very informative. Q & A sessions was also very good with clear responses from mentors.
- Highly technical and well elaborative topics/presentations by Dr Isloor and Dr Sharada
- Theoretical knowledge was informative
- Laboratory training like Rabies Serology in virtual media is like something better than nothing so let's go on for physical training for best output of training.
- All the speakers are well aware on their subject matter and did their very best to give their knowledge to us.
- This training is very useful. The contents are very good and way of presentation is very good. Time management was perfect.
- Clear presentations, Practical videos, Excellent interpretations, Clear concept delivery, Wonderful Q&A sessions, Excellent time mgmt.
- Despite the limitations of online training, the trainers tried to explain the theory behind the serological procedures in detail. The live video demonstrations are quite helpful to understand the actual procedure.
- It was very important for us to strengthening our rabies diagnosis activities in future.

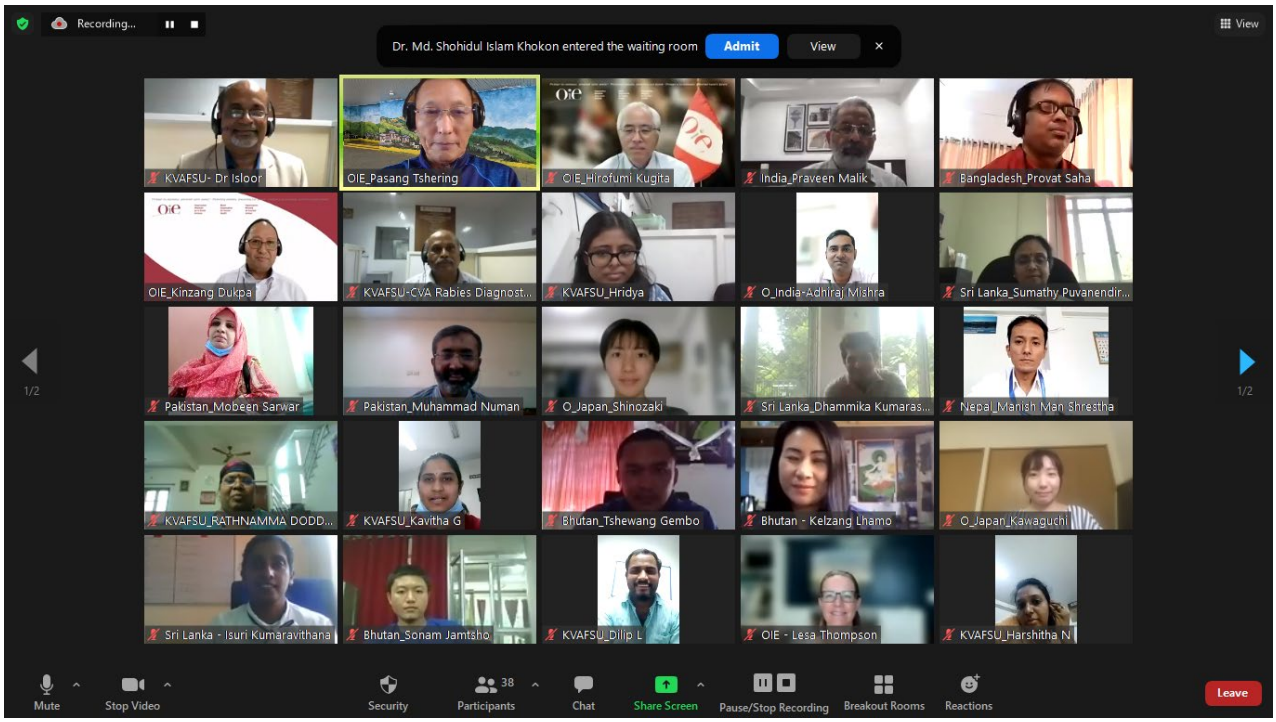
6. What other topics on rabies diagnosis would you like to see covered in the future trainings?
   - Physical training program as early as possible
   - Physical training needed.
   - If the meeting starts little bit early, it would be better. Each day ends around 2pm and little bit tired. If arrangements are made to end around 1pm it would be better
   - Sessions are hurried. More days could be included in coming trainings so that each sessions receives more time.
   - Taking in stock of the availability of the lab equipment that would be required while employing the techniques viz RFFIT
   - Practical hands on will be very helpful.
   - More time will be highly beneficial.
   - To learn practical techniques physical training is more important than virtual training
   - Physical training will be better than virtual one for laboratory training.
   - It would be better if can repeat the difficult parts, like RFFIT theory part to get clear idea for the persons who are new to this.
   - Need to attach physical training about this training which is very useful to us. Also upload the presentations and video clips to our mail that is useful to us to share the knowledge to other related veterinarians in our region
   - Please deliver slowly on the part of core calculation part. (But we may get it once we have the videos)
   - It would have been better if more time were given explaining the calculations with multiple examples. (Not done on day 2 but well described on day 3 with interpretation part)
   - To complete the training, one hands on training session in the laboratory itself is essential. Otherwise, it is not possible for any trainee to build a laboratory and start doing serological training.
   - The quiz or *mentimeter* session was quite interactive and help to lessen the boredom of online training. Instead of one quiz per day, one quiz session after completion of each topic may be organized to recap the key learning points.
   - Everything in this training was perfect.

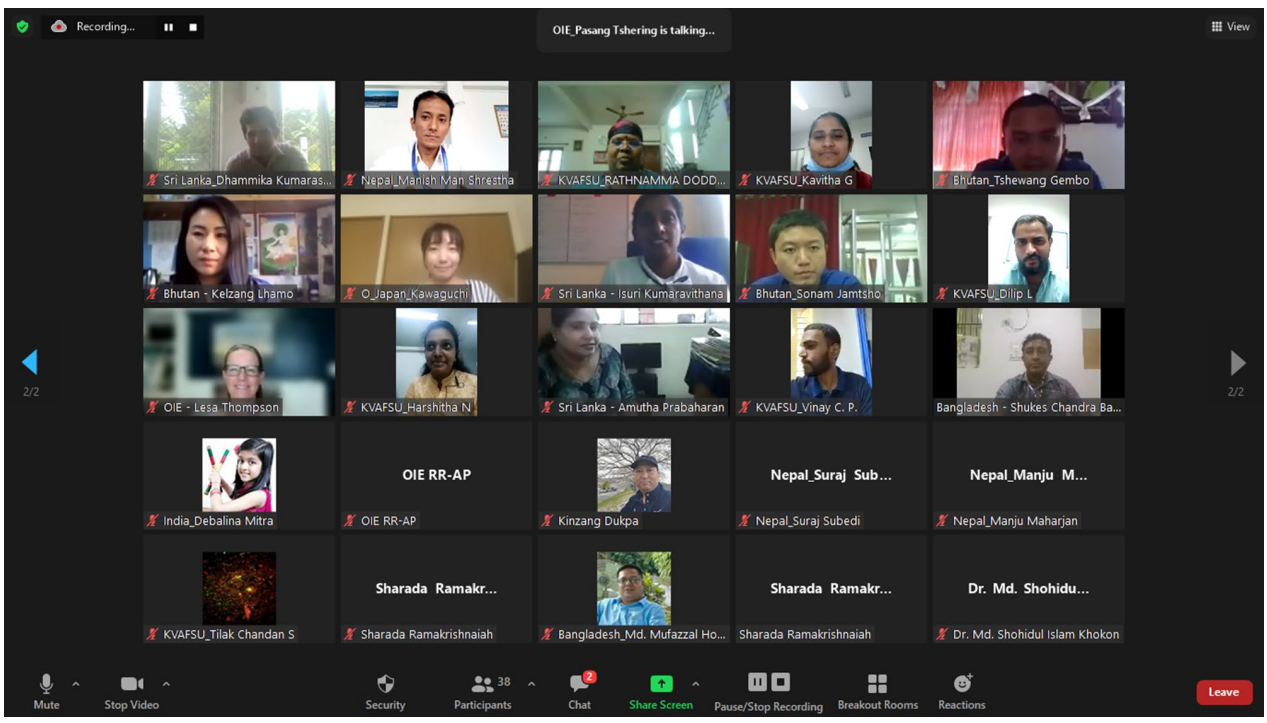
7. What other topics on rabies would you like to see covered in the future trainings?
   - PCR, Rabies virus titration, Rabies related viruses in south east Asia , ELISA
   - Vaccination strategy to have maximum coverage adopting latest software and gadgets.
   - More detail information on troubleshooting and molecular biology of Rabies.
   - Rabies vaccine production
   - Also cover please Real time PCR and conventional PCR in next trainings
   - Molecular Diagnosis of Rabies
   - Rabies diagnosis in live animals. Tests related to rabies diagnosis such as ELISA, Real time PCR and Conventional PCR need to be explored.
   - FAVN remained untouched. May be to avoid confusion with RFFIT. If FAVN is important (cost effective, easier than RFFIT or has some other significant advantages over RFFIT) then it could be a possible topic for future training. But ELISA for rabies sero-monitoring & sero-surveillance would be my topic of interest for near future training.
   - 'Dog number enumeration pre MDV', 'Dog Population Management' and ' Use of Technology/Web based APPs in managing MDV' may be covered in future trainings.

8. List TWO MOST important task you will do following this training to support post-vaccination monitoring of dog rabies vaccination in your country.

- Start cell culture in lab and start RFFIT
- Initiate cell culture facility
- Extension activities for awareness and try to establish basics in rabies laboratory in my region.
- I cannot start RFFIT now but planning to have ELISA to contribute in post-vaccination monitoring of dog rabies vaccination.
- Convince policy makers to establish a laboratory in our department. Share the information with other labs.
- 1. Push to institute RFFIT test in laboratory. 2. Collection of serum samples following mass vaccination.
- Promote brain sampling adopting occipital foramen technique.
- DFA tests regularly in Rabies diagnosis and maintain a Rabies bank. Planning and Implement RFFIT in my laboratory.
- Will try level best to pace towards strengthening of rabies diagnostic facilities.
- I want to start FAVN and REFIT test in my lab. But after physical training it will be more Clearfield When we perform test with our hands.
- I will talk with higher Authority for initiation of all this facility in our lab and initiate these facilities
- At present I am not involved in rabies control activities in my lab. Therefore, if there's any program in post vaccination monitoring I will voluntarily give my support. Will share my knowledge with my colleagues.
- Sero surveillance and vaccine efficacy
- Since I don't have the RFFIT technology at place in Nepal, I would just recap the videos and ppts you will share and get myself ready for physical training. I wonder with the cost of FITC conjugate. I wish KVAFSU team in this Dasahara Festival for successful production of the conjugate at low cost in short time so that all of us could be benefited. Recently, we have conducted an ELISA test for monitoring the antibody status of vaccinated dogs and will keep it up.
- Formulation and revision of National and State level Rabies Control Action Plan. Monitoring of National and State level Rabies Control Action Plan.

Annexure 6. Training in pictures





Videography by: Srushti Media, Bengaluru, India

Visual editing by: Vaibhav Isloor

Team "KVAFSU-CVA Rabies Diagnostic Laboratory" thanks to you all !!!

From Left to Right: **Arya, R. S., Hridya, S. V., Sheela, Sharada, R., Nagaraja, C. S., Isloor, S., Kavitha, G., Harshitha, N., Dilip, L., Vinay, C. P., Tilak Chandan, S. and Mahadevappa.**