स्वच्छ भारत
एक कदम स्वच्छता की ओर

सत्यमेव जयते

Azadi Ka Amrit Mahotsav

Ministry of Health and Family Welfare
Government of India

Rabies
General Aspects & Laboratory Diagnostic Techniques
2022

National Centre for Disease Control
Directorate General of Health Services

World Health Organization Collaborating
Centre for Rabies Epidemiology

Ministry of Health and Family Welfare
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MESSAGE

Rabies is a viral zoonotic disease with neurological manifestations. Although once clinical signs appear disease is almost 100% fatal. Rabies is entirely preventable if timely and appropriately managed.

As India is working towards elimination of Rabies by 2030, laboratory confirmation of clinically suspected cases of Rabies is essential to estimate the true burden of the disease. Efforts to enhance human and animal rabies surveillance, guidance for clinical management and post exposure prophylaxis are important public health measures for prevention and control of Rabies. In this direction National Rabies Control Program has already formulated policy documents viz National Guidelines on Rabies Prophylaxis (2019) and National Action Plan for Rabies Elimination by 2030 (2021) with contributions from various stakeholders in human, veterinary, wildlife sector etc.

Diagnosis of human and animal Rabies is a challenge due to the lethality of the Rabies virus and difficulties encountered to obtain the samples for undertaking laboratory diagnosis. However, strengthening Rabies diagnostics at various levels is essential for the country while envisaging a plan to eliminate Rabies. A definitive, reliable diagnosis of Rabies in humans and animals requires appropriate laboratory structure with adequate biosafety measures. National Centre for Disease Control having apex national referral rabies laboratory (WHO Collaborating Centre for Rabies Epidemiology) also helps in capacity building and hand holding for various rabies laboratories in medical and veterinary sector strengthened under National Rabies Control Program.

Confirmation of diagnosis-especially in paralytic/atypical cases, characterization of causative agent/molecular epidemiology will improve the capacity of professionals in diagnosis and management of Rabies which will eventually help in Elimination of Rabies from the country. I acknowledge the efforts of experts for revising the first edition and bringing out the second edition of "Rabies: General Aspects and Laboratory Diagnosis" with the objective to provide detailed technical guidance especially with regard to laboratory diagnosis of Rabies country-vide.

(Dr. Sujeet Kumar Singh)

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Message

I am immensely pleased to know that NCDC is publishing a manual on "Rabies: General Aspects and Laboratory Diagnostic Techniques (Second Edition)". We all know that rabies is endemic in both humans and animals in most countries in Asia with dog mediated rabies being the predominant source of the infection. However, it is often under-reported and is therefore, considered to be a neglected disease. Although rabies poses significant public health concerns, there is a general lack of ground level surveillance and insufficient rabies diagnostic capacity across the regions.

Globally, the most cost-effective strategy for preventing rabies in people is by eliminating rabies in dogs and wildlife animals through animal vaccinations. Continuous advocacy is being done under National Rabies Control Program by the National Centre for Disease Control for prevention and control of rabies in animals, humans and wildlife. Detection of the virus or some of its specific components using recommended standard laboratory tests is therefore, the only way to undertake a reliable diagnosis of rabies for its control.

"Rabies: General Aspects and Laboratory Diagnostic Techniques (Second Edition)" is a document providing epidemiological aspects and laboratory techniques for Rabies in detail to help medical and veterinary laboratories to strengthen diagnostic capabilities in their respective States. This will contribute in timely diagnosis, management and control of Rabies.

I hope this updated manual will help in rabies capacity-building initiatives by NCDC and will be equally valuable for professionals working in Rabies Laboratories in the country.

I congratulate NCDC team for this endeavor.

Btripathi
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EXECUTIVE SUMMARY

Manual on Rabies: General Aspects and Laboratory Diagnostic Techniques (Second Edition) is an updated version of the previous edition published in 2007 to incorporate recent advances in the field of Rabies Diagnostics. This manual describes Laboratory techniques with step wise procedure in detail to enable medical and veterinary professionals to set up the diagnostic facilities for strengthening National Rabies Control Program (NRCP) and a step forward in direction of operationalizing "National Action Plan for dog- Mediated Rabies Elimination from India" by 2030. Due emphasis has been given to biosafety and quality assurance aspects which are integral parts of the laboratory system.

The second edition of "Rabies General Aspects and Laboratory Diagnostic Techniques" has been updated to include the latest information in the field of rabies. In the first chapter on the "Introduction," the phylogenetic classification of Lyssaviruses has been updated to include all known lyssavirus species to date. The epidemiological aspects have also been updated to reflect the latest information on animals transmitting rabies in India, modes of transmission with special reference to organ transplantation and data from various agencies on animal bites and rabies. The section on "Prevention of Rabies in Humans" has been updated with regards to National Guidelines for Rabies Prophylaxis-2019. A one page detailed protocol for rabies postexposure prophylaxis after an animal bite has been provided.

The section on "Laboratory Diagnosis of Rabies" has been updated to include the latest globally accepted techniques with an emphasis on reducing animal models in rabies diagnosis. The newer staining methods with higher sensitivity such as Direct Fluorescent Antibody Test (DFAT) and Direct Rapid Immunohistochemistry Test (DRIT) have been emphasized in detail in this manual. Details about collecting antemortem and postmortem specimens have been updated. A single-page workflow has been included with details for each test as per the experience of referral laboratories on Rabies in the country. Nucleic acid amplification tests have been updated to include non-rabies lyssavirus diagnosis also. Algorithms for antemortem and postmortem laboratory diagnosis of rabies have been included which are adopted from WHO Technical Review Series 2018 on Rabies.

The chapter on "Biosafety and Biosecurity" has been revised to include risk assessment-based precautions for different rabies laboratory techniques. The chapter on External Quality Assurance Scheme (EQAS) has an updated section on quality control aspects of various laboratory tests. The annexures now include details of human and animal rabies laboratories, examples of sample requisition proformas, and the laboratory reporting format of NRCP.

We would like to acknowledge all the contributors of first edition (2007)

- Dr. Shiv Lal, Additional Director General & Director NCDC,
- Dr. R.L Ichhpujani, Additional Director & NPO IDSP,
- Dr Veena Mittal, Joint Director & Head, Zoonosis Division,
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The contributions of Mr R.K. Pandey and Ms Babita Singhal, NCDC Delhi in designing the Annexures and artwork in the second edition is also acknowledged.

For further improvement, comments/suggestions on the manual are invited by the Centre for Arboviral and Zoonotic Diseases (CAZD), National Centre for Disease Control, Delhi (email id – nicdzoonosis@yahoo.com).

Table of Contents

RABIES: GENERAL ASPECTS

1.1 Introduction

Rabies is a zoonotic viral disease which causes encephalomyelitis in virtually all the warm-blooded animals including man. The causative agent is found in domestic and wild animals and is transmitted to other animals and to humans through close contacts with their saliva (i.e., bites, scratches, licks on broken skin and mucous membranes). Around 96% of all rabies transmission to humans is due to exposure to infected rabid dogs leading to human death. Man is dead end of the infection and hence does not play any role in transmission to new hosts. In most of the developing countries, dogs are the principal reservoirs of rabies (canine rabies) whereas, sylvatic rabies involving animals such as foxes, racoons and coyotes are principal reservoirs of this disease in developed countries.

Rabies has terrified man since antiquity. The fear is by no means unfounded since the disease is invariably fatal and perhaps the most painful and horrible of all communicable diseases in which the sick person is tormented at the same time with thirst and fear of water (hydrophobia). Till date no treatment has succeeded in curing hydrophobia. Despite great strides in the prevention of rabies, with few exceptions, the disease is no less a global problem. The number of human deaths globally due to dog-mediated Rabies is estimated to be 59000 annually, with an associated loss of 3.7 million Disability Adjusted Life Years (DALYs) [1]. The overall economic cost of dog-mediated rabies was estimated to be USD 8.6 billion[1]. Most of the deaths are estimated to have occurred in Asia (59.6%) and Africa (36.4%) [1]

1.2 Causative agent

Rabies virus belongs to the family *Rhabdoviridae* and genus *Lyssavirus* (Lyssa: Greek: rabies). Lyssaviruses are single-stranded, negative-sense RNA viruses with a genome size of approximately 12 kb that encodes five proteins: the nucleoprotein (N), the phosphoprotein (P), the matrix protein (M), the glycoprotein (G) and the large protein or polymerase (L).

Previ u ously the genus was divided into four serotypes (1– 4) by antigenic cross-reactivity with sera and monoclonal antibodies, which correspond to the following species (serotypes):

1. *Rabies virus* (RABV)
2. *Lagos bat virus* (LBV)
3. *Mokola virus* (MOKV)
4. *Duvenhage virus* (DUVV)

The demarcation criteria for lyssavirus species include:

Genetic distance, with a threshold of 80–82% nucleotide identity for the complete N gene, which provides better quantitative resolution than other genes, or 80–81% nucleotide identity for concatenated coding regions of the N+P+M+G+L genes. In general, all isolates belonging to the same species have higher identity values than the threshold, except the viruses currently included in the Lagos bat lyssavirus species. Some authors have therefore suggested that this species be subdivided into several genotypes. In the absence of other sufficient demarcation characteristics, however, Lagos bat lyssavirus has not been separated into several species, as the representatives segregate into a monophyletic cluster in most phylogenetic reconstructions.

- Topology and consistency of phylogenetic trees obtained with various evolutionary models;
- Antigenic patterns in reactions with nucleocapsid mAbs (preceded by serological cross-reactivity and definition of lyssavirus serotypes with polyclonal antisera); and,
- When available, additional characteristics, such as ecological properties, host, geographical range and pathological features.

To accommodate the growing variety of "rabies-related" viruses, the genus Lyssavirus was established under the auspices of the International Committee on the Taxonomy of Viruses. The "genotypes" served as a basis for the taxonomy of lyssavirus but were refined to satisfy the official rules of the International Committee, which apply to more complex entities, such as viral species. The genus has been subdivided into three phylogroups on the basis of genetic distances and serological cross reactivity. There is also one related, unclassified virus species,

Kotalahti bat lyssavirus which is most closely related to European Bat Lyssavirus-2. Currently, the International Committee on the Taxonomy of Viruses recognizes 17 Lyssavirus species (Table 1). The lyssavirus particle has a bullet-shaped form, 100–300 nm in length and 75 nm in diameter. The rabies virion is composed of five structural and functional units (Figure 1).

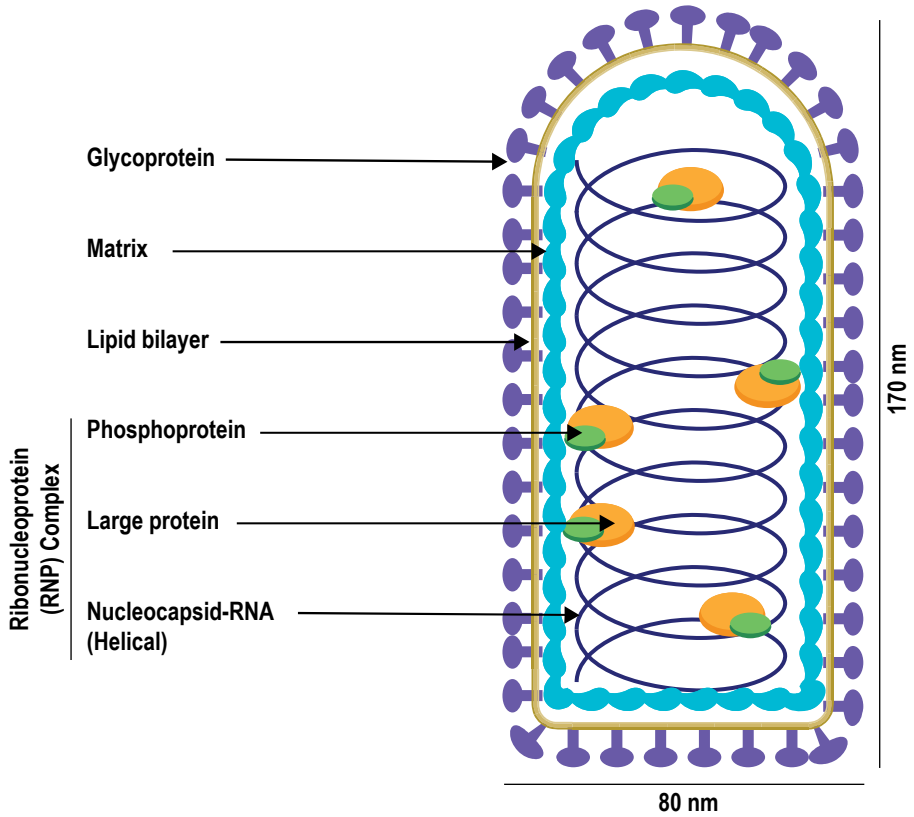


Figure 1. Structural and functional units of rabies virus

i. The outer envelope covered with spike-like projections (10 nm in length) corresponding to G-protein trimers which recognise specific viral receptors on susceptible cell membranes; Hence pathogenicity of Lyssavirus is attributed to protein G.

ii. The internal helically packaged ribonucleocapsid, which is composed of the genomic RNA intimately associated with protein N, polymerase L and its cofactor protein P (formerly named M1). The ribonucleocapsid complex ensures genome transcription and replication in the cytoplasm.

Finally, protein M (formerly named M2) occupies an intermediate position between the ribonucleocapsid and the envelope and is responsible for virus budding and the bullet-shaped morphology.

Table 1. Viruses currently included in the genus Lyssavirus[2]

Phylogroup	Lyssavirus species	Mammalian species most frequently infected	Vaccine protection?	Human fatalities reported (*[c])	Continent
I	Rabies lyssavirus	All mammals, predominantly dogs	Yes	Yes, (59000 human deaths/ year)	All
	Duvenhage lyssavirus	Egyptian slit-faced bat /undefined	Yes	Yes (3)	Africa
	European bat 1 lyssavirus	Serotine bat	Yes	Yes (2)	Europe
	European bat 2 lyssavirus	Daubenton's bat	Yes	Yes (2)	Europe
	Bokeloh bat lyssavirus	Natterer's bat	Yes	No	Europe
	Kotalahti bat lyssavirus [a]	Brandt's bat	-	No	Europe
	Aravan lyssavirus [b]	Lesser mouse-eared bat	Yes	No	Eurasia
	Irkut lyssavirus	Greater tube-nosed bat	Yes	Yes (1)	Eurasia
	Khujand lyssavirus [b]	Whiskered bat	Yes	No	Eurasia
	Australian bat lyssavirus	Black-flying fox and related spp.	Yes	Yes (3)	Australasia
	Australian bat lyssavirus	Yellow-bellied sheath-tailed bat			
	Gannoruwa bat lyssavirus	Indian flying fox	Yes	No	Asia
	Taiwan bat lyssavirus [a]	Japanese house bat	Yes	No	Asia
	Lagos bat lyssavirus	Numerous frugivorous bat species and occasional spillover to domestic dogs and cats	No	No	Africa

Phylogroup	Lyssavirus species	Mammalian species most frequently infected	Vaccine protection?	Human fatalities reported (*[c])	Continent
II	Mokola lyssavirus	Shrews *(Crocidura spp.)*, domestic cats and rodents	No	Yes (2)	Africa
	Shimoni bat lyssavirus[b]	Commerson's leaf-nosed bat	No	No	Africa
	Ikoma lyssavirus[b]	African civet	No	No	Africa
III	Lleida bat lyssavirus [b]	Common bent-winged bat	No	No	Europe
	West Caucasian bat lyssavirus [b]	Comman bent-winged bat	No	No	Europe
	West Caucasian bat lyssavirus [b]	Comman bent-winged bat	No	No	Europe

1.3 Susceptibility of rabies virus to physical and chemical agents

The rabies virus is highly resistant against cold, dryness and decay. However, it is susceptible to the action of various physical and chemical agents. This knowledge is most useful in management of animal bite wounds for inactivating the virus. Table 2 describes the susceptibility patterns of rabies virus.

Table 2. Susceptibility of rabies virus

Inactivation of rabies virus

- At 60ºC within 35 seconds (sensitive to pasteurization and boiling)
- At pH < 4 and > 10
- By action of oxidizing agents, most organic solvents, surface acting agents, quaternary ammonium compounds, proteolytic enzymes, ultraviolet rays and X-rays
- Soaps and detergents
- Alcohol

Preservation of rabies virus

- By freeze drying
- At ultra-low temperatures (≤ - 20 °C)
- Glycerine

1.4 Epidemiology

1.4.1 Global status

Globally rabies has been reported from all continents except for Australia and Antarctica. Several (>50) countries are currently free of rabies [3]. However, in the endemic countries the disease is not uniformly distributed. Areas free of disease, of low and high endemicity and areas with epizootic outbreaks can be found in many countries. In Africa and Asia (with few important exceptions such as Japan and Singapore), rabies is prevalent in almost whole of the territory with a stable pattern. Most of the countries of Americas and Europe report occurrence of disease in limited or border areas [4].

Dog mediated rabies has been eliminated from Western Europe, Canada, United States of America (USA), Japan and some Latin American countries[5]. Australia and many Pacific Island nations have always been free from dog-mediated rabies [6].

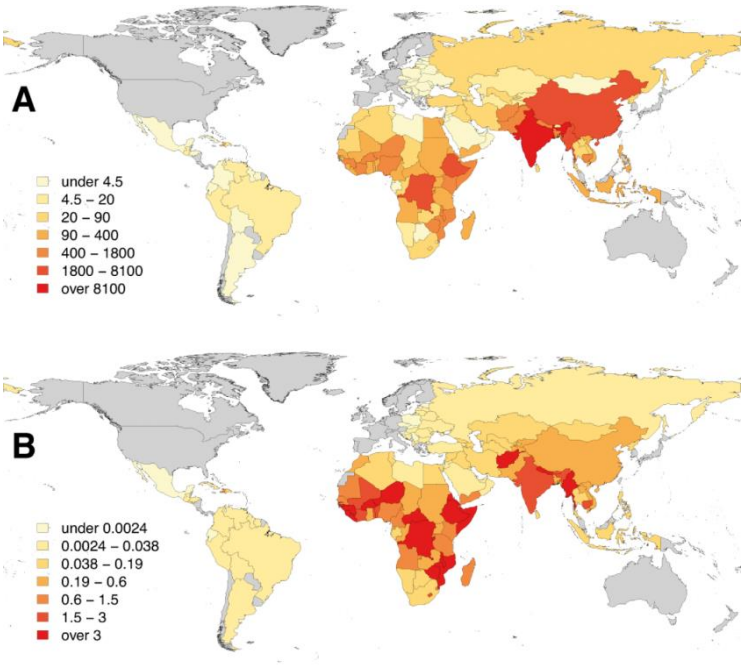


Figure 2. Global status of rabies. A: Human deaths from rabies; B: Death rates per capita (per 100 000 population); countries shaded in grey are free from canine rabies. [2]

Asia. An estimated 35172 human deaths (59.6% of global deaths) and loss of approximately 2.2 million DALYs occur per year in Asia due to dog-mediated rabies. India accounts for the most deaths in Asia (65% of human rabies deaths) and globally (36% of human rabies deaths) [1].The cost of PEP is highest in Asia, with estimates up to US$ 1.5 billion per year [2].

Africa. In Africa, an estimated 21476 human deaths occur each year due to dog-mediated rabies (36 % of global human deaths), with a loss of 1.34 million DALYs [1, 2].

Central Asia and the Middle East. The disease burden due to dog-mediated rabies is estimated to be 1875 human deaths and 14310 DALYs in Central Asia and 229 human deaths and 1875 DALYs per year in the Middle East [1, 2].

1.4.2 Rabies in India

Rabies is responsible for significant morbidity and mortality in India. The disease is endemic in the country, human and animal cases of rabies are reported from all over the country throughout the year except for Andaman & Nicobar and Lakshadweep Islands [7, 8]. In India, about 96% of the morbidity and mortality due to Rabies is associated with dog bites. Although Rabies affects people of all age groups, children are the most vulnerable which constitutes 40% of the people exposed to dog bites in Rabies-endemic areas. As per WHO estimates, India accounts for 36% of the global and 65% of the human Rabies deaths in the Southeast Asia region. “National Action Plan for Dog Mediated Rabies Elimination from India by 2030” is a multisectoral collaborative plan formulated by NRCP in consultation with various stakeholders [9]. In addition, few states in India are working towards the rabies elimination goals [10].

As per WHO-APCRI study it was estimated that 20,562 human deaths are due to Rabies occur annually in India. [7] The annual incidence of animal bites was estimated to be 1.7% (17.5 million per year). [11]

As per the Million Deaths Study 2012, India has an estimated 12700 deaths due to furious Rabies [12]. As per the Central Bureau of Health Intelligence, the trend of the number of

human Rabies cases reported a declining trend in deaths due to rabies through the last decade. Based on vaccine utilization it is estimated that almost 2.3 million people annually receive post exposure prophylaxis against rabies following bite or exposure to rabid or suspected rabid animal. Due to widespread underreporting and uncertain estimates, it is likely that this number is a gross underestimate of the true burden of disease. Consistent efforts are being undertaken to strengthen surveillance and improve reporting of Rabies Deaths in India under National Rabies Control Programs.

The cases occur throughout the year. Majority of the rabies cases are associated with dog bites. Cats, wolf, jackal, mongoose and monkeys are other important animals transmitting rabies in India (Table 3). Bat rabies has not been conclusively reported from India. However, serological evidences of exposure of bats to rabies or related lyssavirus are available [13].

Table 3. Animals transmitting rabies in India

Frequent	Occasionally		Not reported
Dogs	Cats Monkeys Cattle & buffaloes Mongoose Foxes, wolves & jackals Sheep & goats	Bears Pigs Donkeys Horses Camels Spotted Deer [14] Squirrel** [15]	Bats * Rodents * Birds

Note: All exposures in wild are considered as Category III exposures.
* Bite by bats or rodents do not ordinarily necessitate rabies vaccination in India. However, bites by bats or rodents in unusual circumstances may be considered for vaccination in consultation with an expert in the field of rabies.
**One case reported from India.

1.5 Mode of transmission

Rabies virus is predominantly neurotropic and kills the host in short period after it has entered the neurons. Before death, virus reaches salivary glands and is excreted in saliva. Rabies virus gains entry by contaminated saliva to another host when licking is adequate on pre-existing breach or bite of the rabid animal creates a mechanical breach of the skin .(Table 4). Organ/ tissue (cornea) for transplantation should not be collected from suspected/ confirmed rabies or Rabies-like encephalitis cases.

Table 4. Modes of transmission

Common	Rare
● Bites from infected animals ● Licks on broken skin and mucous membrane ● Scratches	● Inhalation ● Organ transplantation (Cornea) [16] ● Ingestion

1.6 Pathogenesis

On entering into human body through wounds or direct contact with mucosal surfaces, the rabies virus either multiplies at local site of inoculation in non-nervous tissues or directly enters peripheral nerves and travels by retrograde axoplasmic flow to the central nervous system prior to its spread towards brain via the nerves (Figure 3). Within the brain, virus spreads from infected to contiguous cells. There may be regional differences in the intensity with which areas of brain become infected. The main areas affected are usually the cerebellum, hypothalamus, hippocampus and scattered neurons in the reticular formation. It may be that aggression in rabies is related to the presence of virus in mid brain raphe nuclei and medial hypothalamus, since these are the two inhibitory centres of aggressive behaviour. It may also be that the distribution of virus in the brain has a bearing as whether the disease becomes the manifest in dumb or classical furious rabies. It does not follow the haematogenous spread. The movement of the virus is extremely slow (15–100 mm per day) which results into a long incubation period. The virus then moves from CNS via anterograde axoplasmic flow within peripheral nerves and reaches salivary glands and other organs. The virus is widely disseminated throughout the body at the time of clinical onset. This has practical implications as organ transplantation has resulted in transmission of the disease to the recipient.

1.7 Incubation period

The average incubation period for rabies in humans is estimated between 30-90 days. It ranges between 2 weeks to 6 years. Because of the wide range of incubation period, post- exposure prophylaxis should be given as soon as possible, however, it should not be denied to persons reporting late. Factors which may influence the length of the incubation period include the

site of bite, the amount of virus in saliva of the biting animal, strain of virus, age and immune status of the victim. It is shorter in case the bite is closer to brain and massive dose of virus has been inoculated.

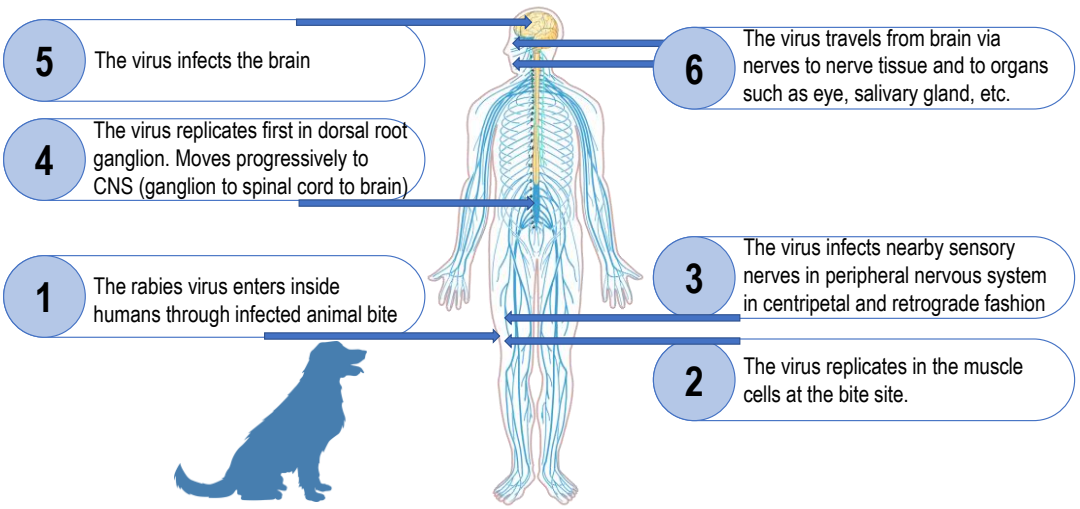


Figure 3. Pathogenesis of rabies

1.8 Immune response in rabies infection and vaccination

1.8.1 Immunity in rabies infection

Since rabies virus is strictly neuroinvasive, little amount of antigen is present in the early stages of the disease hence the humoral response to viral antigens in the infected host is negligible until late in the course of infection and remains low until the terminal phase of the disease. High levels of antibody appear in serum and in cerebrospinal fluid (CSF) in advanced stage of death and only at death and in cases where illness is naturally or artificially prolonged. Virus neutralizing antibodies are produced in response to the massive amount of viral antigen that is generated through widespread infection of the CNS and made accessible to the host's reticuloendothelial system.

The critically important cell-mediated immune response to rabies virus infection is, perhaps the most puzzling of the host's total immune response to rabies virus infection. Helper T Lymphocytes which play an essential role in supporting B cell production of antibodies and Cytotoxic T-Lymphocytes (CTLs) which function independently in cell mediated viral clearance are both key responses in the cell mediated immunity derived from viral antigens. The function of CTLs is to destroy target cells that display virus induced changes on their surfaces. However, in spite of their importance in viral clearance, T lymphocytes appear to be suppressed in animals infected by pathogenic street viruses. As a result of virus induced immunosuppression the disease increases in severity and mortality rises.

It is clear from various studies that both B and T cells play an important role in virus clearance.

1.8.2 Immunity following vaccination

Immunisation with attenuated live and inactivated rabies virus vaccines induces humoral and cell mediated immune responses that develop to functional levels in 7–10 days. Vaccine–induced antibody in animals and humans is regarded as a key factor in the prophylactic protection of animals and humans. Hence RIG, which provides immediate passive immunity with proven effectiveness over the initial few weeks after exposure in humans, is recommended in post–exposure treatment of humans to cover up the initial period before development of antibodies due to active immunisation. The antibodies help in controlling the spread of rabies virus infection as they are capable of effectively neutralizing virus that is present in intercellular spaces or in body fluids, and it may bind to virus expressed on the cell surface, allowing complement or antibody dependent cellular cytotoxicity to mediate killing of infected cells.

The efficacy of post exposure immunisation and long-term effects of vaccine induced prophylaxis against rabies seems also to be linked to the stimulation of a strong CTL response. Induction of CTLs and other effector T cells during infection with live attenuated rabies virus vaccine strains and in response to immunisation with inactivated virus is consistent with the observation that T lymphocytes, and CTLs in particular are essential for protection against a lethal dose of rabies virus.

1.9 Clinical features and diagnosis in humans

The clinical features can be described under three broad groups (Table 5)

- Prodromal phase
- Excitation phase
- Paralytic phase

Table 5. Clinical features of rabies in humans

Phase	Clinical Features
Prodromal Phase	• Discomfort or pain at the site of bite • Numbness and tingling in limb • Elevated Temperature • Malaise • Headache • Sore Throat • Priapism
Excitation Phase	• Restlessness, Tremors • Pharyngeal and laryngeal spasms • Fear of Water (hydrophobia) • Terror and excitement • Respiratory arrhythmias • Cardiac arrhythmias • Hypertension • Fear/ convulsions on exposure to light, air & sound • Intense thirst and dehydration • Change in tone of voice • Death
Paralytic Phase	• Restlessness, convulsions • Flaccid and Limp muscles • Unconsciousness • Death

The first symptom to appear may be pain and tingling in the affected limb, especially around the site of bite. This is seen in 35-65% cases. Hydrophobia (fear of water) is the best-known symptom of this disease. Hydrophobia is pathognomonic feature of rabies which is erroneously considered synonymous with rabies. It is usually the only neurologic abnormality

found in patients presented with furious rabies. This is due to a violent jerky contraction of the diaphragm and accessory muscles of inspiration that is triggered by the patient's attempts to swallow liquid and by a variety of other stimuli such as strong current of air, loud noise and bright light. Hydrophobia is usually not associated with pain in neck or throat. It is also not a conditioned reflex caused by aspiration of liquid into trachea. About 20% of the patients who present with paralytic form of rabies do not have hydrophobia. In these patients, diagnosis can only be established by laboratory confirmation. This is important as rabies has been documented from USA in cluster of human rabies cases associated with transplantation of solid organs from a misdiagnosed rabies patient. Rabies cases have also been documented following corneal transplantation.

Clinical Case. A suspected clinical case of rabies in humans is defined as: an acute neurological syndrome (i.e. encephalitis) dominated by forms of hyperactivity (furious rabies) or paralytic syndromes (paralytic rabies) progressing towards coma and death, usually by cardiac or respiratory failure, typically within 7–10 days of the first signs if no intensive care is instituted. These may include any of the following: aerophobia, hydrophobia, paresthesia or localised pain, dysphagia, localised weakness, nausea or vomiting.

A proper epidemiological correlation and laboratory confirmation is essential for diagnosis of rabies as failure to do so may result in misdiagnosis of rabies death as viral encephalitis caused by more common viruses. For surveillance purposes, rabies cases can be classified as follows:

Suspected Case. Death of a human with a history of dog bite a few weeks/months preceding death. Wherever available, the details of such cases should be shared in a line list– Name, Age, Gender, Address.

Probable Case. A suspected human case plus history of exposure[1] to a (suspect[2] / probable[3]) rabid animal.

[1] **Exposure** is usually defined as a bite or scratch from a Rabies-susceptible animal (usually dogs). It could also be lick exposure to open wounds, abrasion, mucous membranes of the patient.

[2] A **suspect rabid animal** is a Rabies-susceptible animal (usually dogs) which presents with any of the following signs at the time of exposure or within 10 days following exposure: unprovoked aggression (biting people or animals or inanimate objects), hypersalivation, paralysis, lethargy, abnormal vocalization, or diurnal activity of nocturnal species. Whenever the history of mentioned signs cannot be elicited, the history of exposure to a Rabies-susceptible animal would be considered adequate.

[3] A **probable rabid animal** is a suspect rabid animal (as defined above) with additional history of a bite by another suspect / probable rabid animal and/or is a suspect rabid animal that is killed, died or disappeared within 4-5 days of observing illness signs.

Laboratory-confirmed case. A suspect or a probable human case that is laboratory-confirmed by one or more of the following tests:

1. Detection of Rabies viral antigens by direct fluorescent antibody test (FAT) OR by ELISA in clinical specimens, preferably brain tissue (collected postmortem).
2. Detection by FAT on skin biopsy (antemortem) OR FAT positive after inoculation of brain tissue, saliva or CSF in cell culture, or after intracerebral inoculation in mice or in suckling mice
3. The detectable Rabies-neutralizing antibody titer in the CSF or serum of an unvaccinated person
4. Detection of viral nucleic acids by PCR on tissue collected postmortem or intra vitam in a clinical specimen (brain tissue or skin, cornea, urine or saliva).

Radiology also plays an important role in diagnosing the rabies especially magnetic resonance imaging regardless of clinical type, performed with adequate precautions for potentially infectious patients, can be useful. Abnormal, ill-defined, mildly hypersignal T2 images involving the brainstem, hippocampus, hypothalamus, deep and subcortical white matter and deep and cortical grey matter are evident. Gadolinium enhancement may appear clearly only in later stages, when patients lapse into a coma. Such patterns can help differentiate rabies

from other viral encephalitis, not in terms of location, but in the appearance of the T2 image and in the pattern of contrast enhancement, when compared with consciousness status. Computerized tomography of the brain is of little diagnostic value.

1.9.1 Differential diagnosis

Before the appearance of hydrophobia and in those cases where it does not manifest, rabies needs to be differentiated from other clinical conditions (Table 6).

1.10 Rabies in animals

All warm-blooded animals are vulnerable to rabies infection, and the species susceptibility is affected by factors such as viral variant, quantity of virus inoculated, and the site of bite. Younger animals like puppies are usually more vulnerable to rabies infection than older ones and can transmit disease. [17]

1.10.1 Clinical features in dogs

After an incubation period of around three months (range 10 days to 6 months), dog may manifest one or more of the following clinical features. There may be change in behavior of dog, change in bark tone, change in feeding habits, the animals may go off feed and eat abnormal objects. They may develop fever, vomiting, excessive salivation, paralysis of lower jaw, anxiety, restlessness, convulsions, paralysis leading to death within 5-7 days on onset of disease. There is however no hydrophobia in animals.

Rabies in dogs is also classified as dumb (predominantly paralytic manifestation with docile behavior of animal) or furious (mainly convulsions and aggressive behavior with greatly exaggerated biting tendencies).

Clinical rabies in dogs has to be differentiated from other diseases which manifest with similar features. These include distemper, hepatitis, epilepsy, poisoning, brain tumours and head injury.

Table 6. Differential diagnosis of rabies in humans

Phase	Clinical Manifestations
Initial Phase	● Lockjaw ● Encephalitis ● Hysteria
Paralytic phase	● Acute polyneuritis ● Poliomyelitis ● Belladona poisoning ● Delerium tremens
Post vaccination	● Rabies post vaccinal encephalomyelitis ● Gullian Barre Syndrome

1.10.2 Clinical features in cats

Rabid cats show extreme aggressiveness, great sensitivity to touch/noise, profuse salivation and may attempt to attack dog or even man.

1.10.3 Clinical features in cattle

In cattle, rabies is manifested as abnormal movements of posterior extremity, foamy yellow froth and decrease in yield of milk. Also, there will be other changes such as aggression, pruritis, ataxia, tenesmus, hepersalivation, tendency to attack and head butting in bulls. Consumption of milk and meat from rabid cattle is not advised. However, no human rabies cases resulting from consumption of milk and meat of rabid animal have been documented. Pasteurisation, boiling and cooking kills the virus.

1.11 Prevention of rabies in humans

Because of the long incubation period, which is typical in most cases of human rabies, it is possible to institute prophylactic post-exposure treatment. This must be started at the earliest to ensure that the individual will be immunized before the rabies virus reaches the CNS.

1.11.1 Decision to treat

In a rabies endemic country like India, where there is sustained dog-to-dog transmission every animal bite is potentially suspected as a rabid animal bite, the treatment should be started immediately after exposure. To bring out uniformity globally, the National guidelines for Rabies prophylaxis include classification of animal bite exposure broadly based on WHO recommendations should be followed (Table 7). [18]

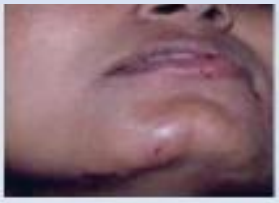
Although unvaccinated animals are more likely to transmit rabies, vaccinated animals can also do so if the vaccination of the biting animal was ineffective for any reason. The risk of dog being infected with rabies is greatly reduced when it appears healthy and there is confirmed history of vaccination with minimum of two immunisations with potent rabies vaccine in last two years. The treatment should be started immediately after the bite. The treatment may be modified if the suspected dog or cat involved in the incident is healthy after a 10-day observation and PEP can be converted to pre-exposure prophylaxis (PrEP) by skipping the vaccine dose on day 14 and administering it on day 28 while using IM regimen (ESSEN Schedule).

While using ID route of administration complete course of vaccination should be given irrespective of the status of the animal. The observation period is valid for dogs and cats only. Bite by all wild animals should be treated as category III exposure. It should be noted that bites by rats, mice, squirrel, hare and rabbits seldom require treatment. Bat rabies has not been conclusively proved in India and hence exposure does not warrant treatment. But there is a serological evidence of lyssavirus infection among bats in Nagaland, India and also, Gannoruwa bat lyssavirus has been isolated from the brains of Indian flying foxes in Sri Lanka.

The animal bite victims are generally managed at primary health centres with limited resources for primary wound management and rabies prophylaxis. As there is no remedy available for the rabies after appearance of clinical signs, rabies may result in death of almost all patients unless there is timely and appropriate case management. Palliative care of rabies patients is an integral part of rabies control and management guidelines. Palliative care by

trained healthcare workers should be accessible to all patients with confirmed rabies. World Health Organization's guide for palliative care can be referred for further reading. In exceptional cases, aggressive management may be considered. The aggressive management of rabies cases should only be done at reference centres with well trained teams of experts who have experience in managing rabies patients using ethically pre-accepted protocols.

Table 7. National Guidelines for recommended post-exposure treatment against rabies based on categories of exposure

Category	Type of Exposure	Recommended Post-Exposure Prophylaxis
I	• Touching or feeding of animals • Licks on intact skin • Contact of intact skin with secretions/excretions of rabid animal/human case	• None, if reliable case history is available • Wash Exposed area with Water & Soap and apply Antiseptic
II	• Nibbling of uncovered skin • Minor scratches or abrasions without bleeding 	• Proper wound management • Rabies vaccine
III	• Single or multiple transdermal bites or scratches,  • Licks on broken skin • Contamination of mucous membrane with saliva (i.e., licks) 	• Wound Management • Rabies Immunoglobulin • Rabies Vaccine

It is re-emphasised that the treatment should be started immediately after the exposure, but it should not be denied to person reporting late for treatment. There are three components of prevention of rabies in man. All three components carry equal importance, and one should not be given undue importance, or utter neglect, at the cost of other two components. Physician must attempt to provide the animal bite victim the benefit of all three of these as per category of bite. The three components are

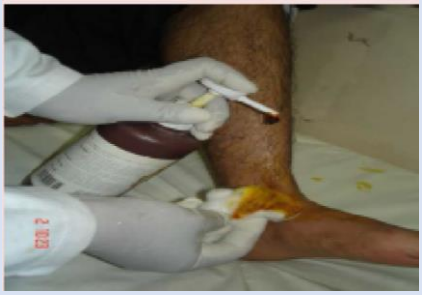
- Management of animal bite wound(s)
- Passive immunization with Rabies Immunoglobulin (RIG)
- Active immunization with Anti-rabies Vaccine (RABIES VACCINE)

1.11.2 Management of animal bite wound (s)

Wound toilet: Since the rabies virus enters the human body through a bite or scratch, it is imperative to remove as much saliva, and thereby the virus, from the wound as is possible by an efficient wound toilet that should not involve additional trauma. Since the rabies virus can persist and even multiply at the site of bite for a long time, wound toilet must be performed even if the patient reports late (Table 8).

The recommended first-aid procedures include immediate, thorough flushing and washing of all wounds with soap and water and application of Povidone Iodine or Antiseptic having virucidal activity. Tetanus prophylaxis should be given as per national guidelines. To prevent sepsis in the wound, a suitable course of an antibiotic may be prescribed. **Suturing** of wound should be avoided as far as possible. If unavoidable, minimum loose sutures should be applied after adequate local treatment along with proper instillation of ERIG or HRIG in the wound.

Table 8. Wound management in case of animal bite

Do's	Act	Effect
Physical	Wash **all wounds** with running water 	Mechanical removal of virus from the wound
Chemical	Wash **all wounds** with soap and water, apply antiseptic 	Inactivation of Virus
Biological	Infiltrate **immunoglobulin** into the **depth and around the wound(s)** in **Category III** exposures 	Neutralization of the virus
Don'ts		
● Do not touch the wound(s) with the bare hands ● Do not apply irritants like soil, chilies, oil, lime, herbs, chalk, betel leaves, etc.		

1.11.3 Passive Immunisation

Equine Rabies Immunoglobulin (ERIG): The anti-rabies serum provides passive immunity in the form of ready-made anti rabies antibody to tide over the initial phase of the infection. Anti-rabies serum or RIG has the property of binding with the rabies virus, thereby resulting in the loss of infectivity of the virus.

Human Rabies Immunoglobulins (HRIG): HRIG are free from the side effects encountered in a serum of heterologous origin, and because of their longer half-life, are given in half the dose of equine anti-rabies serum. The anti-rabies sera should always be brought to room temperature (20–25°C) before use.

Dose of Rabies Immunoglobulins (**RIG)**: The dose of equine anti-rabies serum is 40 IU per kg body weight of patient and is given after testing of sensitivity, upto a maximum of 3000 IU. The ARS produced in India contains 300 IU per ml. The dose of the human rabies immunoglobulins (HRIG) is 20 IU per kg body weight (maximum 1500 IU). HRIG does not require any prior sensitivity testing. HRIG preparation is available at a concentration of 150 IU per ml.

1.11.4 Active Immunisation

Active immunisation is achieved by administration of safe and potent Cell Culture Vaccines (CCVs). The vaccination schedule as per the National Guidelines for Rabies Prophylaxis, 2019 (by National Rabies Control Program, National Centre for Disease Control, Delhi) is provided in (Table 9). The protocol for deciding the course of rabies post-exposure prophylaxis in case of an animal bite victim is given in Figure 4.

Table 9. Vaccination routes and their respective schedules for post exposure prophylaxis (PEP) and pre-exposure prophylaxis (PrEP) for rabies virus (Adapted from NRCP)

Type of Prophylaxis	Route of Administration*	Dose of Vaccine	Day of Dose	Injections Per Visit	No. of Visits
Post Exposure Prophylaxis	Intradermal	0.1 ml per dose	Day 0, 3, 7 and 28	2	4
	Intramuscular	1 entire vaccine vial	Day 0, 3, 7, 14 and 28	1	5
Pre-exposure Prophylaxis	Intradermal	0.1 ml per dose	Day 0, 7, and 21 or 28	1	3
	Intramuscular	1 entire vaccine vial	Day 0, 7, and 21 or 28	1	3
Re-exposure	Intradermal	0.1 ml per dose	Day 0 & 3	1	2
	Intramuscular	1 entire vaccine vial	Day 0 & 3	1	2

***Site of Injection.**

- **Adults:** Deltoid Muscle
- **Infants and Small Children:** Anterolateral Thigh

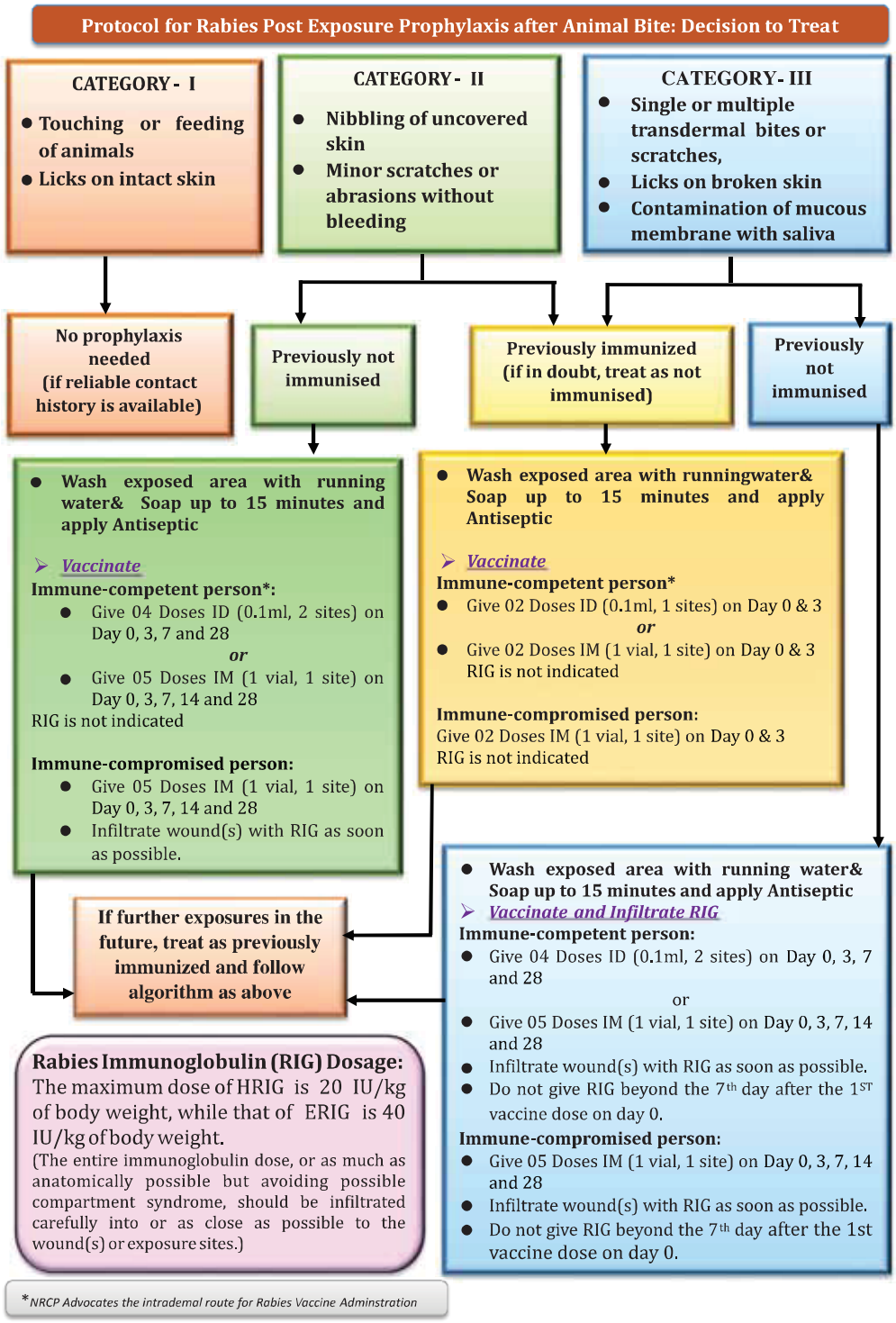


Figure 4. Protocol for rabies postexposure prophylaxis after animal bite

1.11.5 Management of re-exposure in previously vaccinated individuals

If re-exposed, persons who have previously received full pre-exposure or post-exposure treatment (either by IM or ID route) vaccine should be given for only two booster doses, intramuscularly/intradermally (0.1 ml at two sites) on days 0 and 3, but no rabies immunoglobulin. Only adequate wound washing would be required in case of re-exposure where animal bite victim has documented proof of complete PEP or PrEP within last three months. People who have previously received full post- exposure treatment with neural tissue vaccine (NTV) or vaccine of unproven potency or cannot document complete previous PEP or PrEP treatment should be treated as fresh case or given full PEP.

1.11.6 Pre-exposure prophylaxis

Pre-exposure prophylaxis may be offered to high-risk group like laboratory staff handling the virus and infected material, clinicians and para-medicals attending to hydrophobia cases, veterinarians, animal handlers and catchers, wildlife wardens, quarantine officers and travellers from rabies free areas to rabies endemic areas. Total three doses are recommended for pre-exposure prophylaxis.

- In case of IM route 1 full vial to be given on days 0, 7, and day 21 or 28.
- In case of ID route, 0.1 ml on one site to be given on days 0, 7, and on either day 21 or 28.

Note: High-risk groups should have their neutralizing antibody titres checked every 6 months during the initial two years period after the primary vaccination. If it is less than 0.5 IU/ml, a booster dose of vaccine should be given. Subsequently, sero-monitoring is recommended every two years. Vaccine-induced immunological memory persists in most cases for years. A booster would be recommended only if rabies virus neutralizing antibody titres have dropped to less than 0.5 IU/ml. Vaccinated Individuals on being exposed to RABV after successful pre-exposure immunization would require only two booster injections of vaccine given on day 0 and day 3. There is no need for RIG.

Laboratory Techniques for Diagnosis of Rabies

Laboratories are essential for disease surveillance, and most epidemiological surveillance systems require a laboratory component for the confirmation. These serve both for the routine confirmation of clinical syndromes and rapid confirmation of the causative agent in outbreaks. The diagnosis of Rabies is essentially clinical however role of rabies laboratory is undisputed and warranted in many situations.

Importance of Laboratory Confirmation of Human Rabies

- Confirmation of clinical diagnosis-especially in paralytic/atypical cases
- Patient Management/Barrier Nursing/Disinfection of ICU facilities
- Prophylactic vaccination to relatives, clinical & nursing staff
- Surveillance and estimation of disease burden
- Confirmation/Monitoring of disease-free status
- Characterization of causative agent/molecular epidemiology with regards to future scope for inclusion of surveillance of non-rabies lyssavirus in National Rabies Control Programme/National Action Plan for Rabies Elimination

2.1 Consultation, collection, packing, and transportation of specimens

2.1.1 Consultation

Because of the hazards associated with handling specimens and complex laboratory diagnostic modalities for Rabies, testing must be done with prior consultation.

First, contact your state health department regarding any sample submissions with details of suspected human rabies cases. See the list of referral laboratories with contact details for the nearest laboratory in your area.

If, after the consultation, it is deemed necessary to send human samples for testing to the Rabies Laboratory at the referral laboratory, the Rabies Duty Officer can answer to the

questions regarding the likelihood of a case, optimum samples and timing, sampling techniques, serial specimens required and shipping, etc

2.1.2 Collection of specimen

All samples from suspected Rabies cases should be considered potentially infectious. All specimens should be collected in a primary leak-proof container and must be securely sealed before transport to the designated laboratory.

2.1.2.1 Antemortem specimens

The person collecting the samples like serum, saliva, CSF, skin biopsy etc. from suspected Rabies cases should handle them carefully with all aseptic universal and standard precautions and appropriate PPE. The animals must be restrained before the collection of antemortem specimens to avoid any chances of a bite. No diagnostic tests are available to detect the rabies virus before the onset of clinical disease. Several tests and multiple samples are necessary to diagnose rabies antemortem (before death) in humans, as no single test is sufficient. From a suspected clinical case, the antemortem specimens that may be collected include saliva, corneal impression, skin biopsy, hair follicles, blood (unvaccinated), and cerebrospinal fluid. The specimens are collected as follows:

Saliva

1. Saliva (around 500 µl) should be collected using a sterile eyedropper pipette and transferred to a small sterile container that can be sealed securely. No preservatives or additional material should be added into it. Laboratory tests to be performed include detection of rabies RNA (by reverse transcription and polymerase chain reaction, RT-PCR, of extracted nucleic acids). If possible, collect three specimens in total: one specimen daily on three consecutive days (not three specimens on the same day).
2. Wet a sterile swab with tissue culture medium or physiological saline and remove excess medium by squeezing on the sides of the vial. Swab under the tongue, rinse in the tissue culture medium or physiological saline containing 2% normal horse serum (NHS) in a small sterile container. Rabies Tissue Culture Infection Test (RTCIT) is based on the

ability of the rabies virus to infect some cell lines in vitro, and saliva can be used as a sample for the same.

3. Tracheal aspirates and sputum are not suitable for rabies tests.

IMPORTANT - Due to dehydration/hydrophobia, collection of the saliva sample is sometimes difficult

Corneal Smear

1. Retract the eyelids with the thumb and one finger and press a clean marked slide against the cornea.
2. Prepare two impression on each slide, applying sufficient pressure to get the smear carefully.
3. Avoid exerting too much pressure as it may damage the eye.
4. Air-dry the impression for 10-15 mins at room temperature.
5. Treat with chilled acetone and process further for Direct Fluorescent Antibody Test (DFAT).

IMPORTANT - The DFAT of corneal impressions is rarely reliable in most clinical settings, and it is not recommended as a routine test because of the risk of corneal scarification, particularly in patients with causes of encephalitis other than rabies.

Skin Biopsy/ Nuchal Skin Biopsy

Section of skin, 5-6 mm in diameter and ≈5-7 mm in depth, must be taken from the nape of the neck (Figure 5). It is vital that the specimen should contain hair follicles and should be of sufficient depth to include the cutaneous nerves at the base of hair follicles. An excision or punch biopsy may be collected. Place the skin biopsy onto, and cover with, a piece of sterile saline-moistened gauze. This keeps the specimen from drying out. Place the gauze with the biopsy into a screw-top container. No fixative is required. The sample can be used for DFAT/ DRIT for detecting viral antigens (by using a cryostat to cut thin frozen sections of biopsy specimens for making impression smears), RT-PCR for detecting viral nucleic acid, and RTCIT for virus isolation.

IMPORTANT: Peripheral nerves in hair follicles within the dermis are one potential target that may be used for antemortem diagnosis.

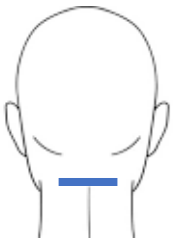


Figure 5. Nape of the neck from where nuchal skin biopsy should be taken for the rabies diagnosis

Serum and Cerebrospinal Fluid (CSF)

2-3ml (at least 0.5 ml) each of serum and CSF should be collected in a sterile vial with all aseptic precautions. If no vaccine or rabies immune serum has been given, the presence of antibody to rabies virus in the serum can be diagnosed. Antibody to rabies virus in the CSF suggests a rabies virus infection regardless of the immunization history. Laboratory tests for antibodies includes ELISA, indirect immunofluorescence, and virus neutralization. CSF samples can also be processed for RTCIT and molecular technique(NAAT-PCR).

2.1.2.2 Post-mortem specimens

These include the brain, spinal cord, salivary glands, etc., all of which should be handled carefully and treated as highly infected material. Protective clothes like laboratory coats, rubber aprons, thick rubber gloves, masks, and plain goggles should be used at the time of autopsy as well as during the handling of infectious material/ collection of samples.

Brain and Salivary Glands

Facilities for removing the animal brain and salivary glands are not available in the laboratory; hence, the whole brain or salivary glands should be sent to the laboratory after autopsy. The most convenient method of brain sample collection in animals without the need to break open

the skull is the Occipital foramen route of brain sampling, and this is one of the WOAH recommended sampling techniques for rabies diagnosis. The brain stem can be sampled easily with this method if it is impossible to send the entire brain, pieces from Ammons horn of hippocampus, cerebrum, cerebellum, pons, and medulla may be included. Many times, the caretakers do not agree to a full post-mortem. In such cases, the Vim-Silverman needle may be used to collect a small piece of brain sample. Small sections of both the cerebellum and the cerebrum should be submitted. Place the specimen in a wide mouth screw-top container (NOT GLASS) and submerge half the specimen in 50% glycerol saline and half in sterile PBS). If glycerol saline is not available: freeze and send at the earliest. A brain specimen is a preferred specimen for post-mortem diagnosis. However, if unavailable, clinicians may obtain a post-mortem nuchal skin biopsy for rabies diagnosis as described before. The sample can be used for DFAT for viral antigens by using a cryostat to cut thin frozen sections of biopsy specimens, RT-PCR and RTCIT.

IMPORTANT: Formalin fixed samples are not suitable for DFAT , RT-PCR, and RTCIT.

2.1.3 Labelling

All the specimens, e.g., slides and vials, must be labelled with the number of specimens, name of the patient, animal species, type of preservative used, etc., and should be collected only after consultation with the laboratory. Permanent markers should be used. The parcels should also be appropriately labelled, and relevant information to be enclosed for the following (Consult Annexure 3: Sample Requisition Proforma)

1. Human (Hydrophobia/ Paralytic): Name, Age, Gender, Treatment taken, Exposure to animal etc. may be enclosed.
2. Animal: The species and breed of animal, contact with other animals, symptoms, mode and date of death, vaccination status etc.

2.1.4 Packing and transportation

While packaging and transporting the suspected rabies sample, the following should be kept in mind

1. All samples should be considered potentially infectious.
    a. Nuchal skin samples are best shipped frozen (at -20 °C or below) but can be shipped simply with ice packs (2-8 °C) as well.
    b. Saliva samples should be shipped frozen (at -20 °C or below)
    c. Brain samples should be frozen, or half preserved in 50% glycerol–saline solution (if freezing is not readily available) and half in sterile PBS. Brain samples should never be preserved in formalin.
2. Please ensure the maintenance of cold chain 2-8 °C or -20 °C, whichever is applicable. If immediate transport is not possible, samples should be stored frozen at -20 °C or below.
3. All specimens should be collected in a primary container that is watertight and leak-proof and must be securely sealed (tape around the cap will ensure that the containers do not open during transit).
4. The primary container should be put in a secondary container, such as a zip-lock plastic bag with insulating material between the primary and secondary containers. The secondary container should be put into a rigid outer packaging box during transport.
5. The reference laboratory must be contacted before the shipment of samples with suspected rabies virus infection.
6. The sample should be sent in a double-enclosed waterproof container with cooling and absorbent materials. All of these should be placed in a leak-proof outer container in observance of the national guidelines
7. Utmost urgency should be exhibited in the transportation of these specimens because any undue delay, especially in tropical climates, will wither away the cooling effect of ice and result in the putrefaction of the sample, making it unsuitable for testing.
8. Sample to be transported by priority mail/courier to minimize transportation time.

2.2 Laboratory Tests

Many tests have been reported from time to time for detecting rabies antigen/ virus, assessing rabies antibodies, and studying the morphology and growth characteristics of the rabies virus. Using these tests in a diagnostic laboratory depends on the availability of appropriate facilities. The tests may be for detecting rabies antigen in ante-mortem specimens like corneal

impression, CSF or saliva, etc., or post-mortem specimens, including brain and salivary glands.

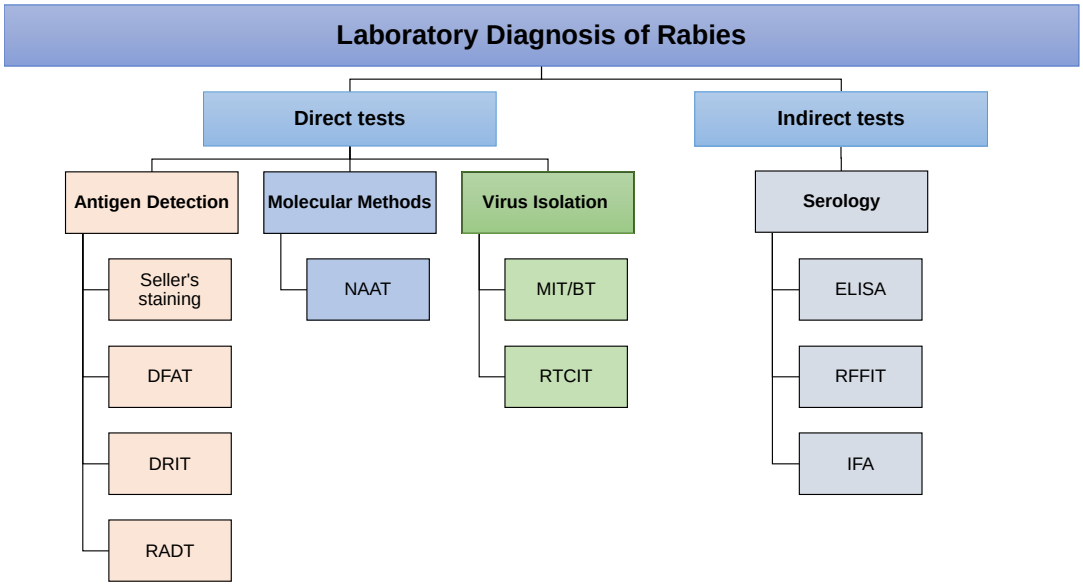


Figure 6. Types of laboratory diagnostic tests for rabies.

DFAT – Direct Fluorescence Antibody Test; DRIT – Direct Rapid Immuno-histochemistry Test; RADT – Rapid Antigen Detection Test; NAAT – Nucleic Acid Amplification Test; MIT – Mouse Inoculation Test; BT – Biological Test; RTCIT – Rabies Tissue Culture Inoculation Test; ELISA – Enzyme-Linked Immuno-Sorbent Assay; RFFIT – Rapid Fluorescent Focus Inhibition Test; IFA – Indirect Fluorescence Antibody Test.

2.2.1 Seller's staining for detection of Negri Bodies

Principle. Several viral infections are associated with the presence of inclusion bodies in the infected cells. These inclusion bodies may be intranuclear or intracytoplasmic – acidophilic or basophilic in nature depending on their reaction with the stains used. The presence of rabies infection can be demonstrated by the presence of intracytoplasmic inclusion bodies commonly known as Negri bodies. The intracellular nature of Negri bodies may be altered in impression smears due to the rupture of cells when these may be found both intracellular and

extracellular. The Negri bodies are acidophilic in nature and seen as pink to purplish-pink in colour in differential stains that use basic fuchsin or eosin with methylene blue as their base.

Note: Since the sensitivity of Seller's staining technique is low and as this test is usually done for academic/teaching purposes, it is recommended that test with better sensitivity such as DFAT (gold standard) or DRIT may be employed for laboratory confirmation of rabies wherever possible.

Reagents and Equipment

1. Seller's Stain
2. Light microscope
3. Glass slide (5 x 7.5 cm and 2.5 x 7.5 cm)
4. Scissors and forceps
5. Filter paper

Preparation and Staining of Slides.

The brain sample received in the laboratory may be unpreserved or preserved in 50% glycerol saline or 10% formalin saline. For demonstration of Negri bodies the unpreserved specimen can be processed as such. The formalin preserved specimen can be used for histopathology. The glycerinated specimen can be used for making impression smears and biological test like unpreserved specimen, but the presence of glycerine interferes with the adherence of tissue to the glass slide. Therefore, the glycerine must be washed off by immersing the tissue pieces in physiological saline for 30 minutes or more during which at least three changes of physiological saline are required to wash off the glycerine.

The procedure for preparation of impression and its staining is as follows:

1. With a pair of scissors make a longitudinal incision about 3-5 cm from the occipital pole into the dorsal surface of cerebral hemisphere approximately 2 cm lateral to midline of the brain.

2. Cut through to lateral ventricle.
3. Widen the opening to expose the hippocampus on ventricle floor.
4. The hippocampus can be seen as white, glistening, semi cylindrical and curved body.
5. Cut out a small piece of hippocampus (0.5-1 cm) and place it on a spatula/ filter paper with cut surface facing upwards.
6. Place the filter paper on glass slide (5 x 7.5 cm)
7. Lightly sponge the cut surface with the edge of a filter paper to remove blood.
8. Press a clean microscope slide on the tissue piece on spatula/filter paper to get an impression.
9. Make at least 3 impressions on each slide.
10. While the impressions are wet, flood the impression with working stain.
11. Stain for 2-3 seconds.
12. Quickly wash with tap water by gently flushing the slide.
13. Air dry the impressions.
14. Examine under oil immersion.

Observations. Following may be observed (Figure 7)

- Nerve cells – Blue cytoplasm and dark blue nucleus
- Stroma – Pink
- Erythrocytes – Copper coloured
- Negri bodies – Magenta to dark red with dark blue or black inner granules.

Similarly, impressions can be prepared from cortex and cerebellum and examined for Negri bodies.

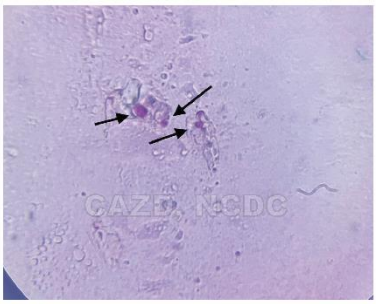


Figure 7. Seller's stain: Negri Bodies observed under 1000x magnification.

2.2.2 Direct Fluorescent Antibody Test (DFAT)

Principle. When mixed and kept under optimum conditions, Rabies-specific antibodies and antigens combine to form an antigen-antibody complex. This complex is not visible to the naked eye. Fluorescent dyes, like fluorescein isothiocyanate (FITC), can be used to visualize this antigen-antibody complex under a fluorescence microscope. To achieve this, the FITC is tagged with an anti-rabies antibody to form the conjugate. As the conjugate directly binds to the antigen, the process is called the direct fluorescent antibody test (DFAT).

Reagents and Equipment.

1. Fluorescent Microscope
2. BOD Incubator (37 °C)
3. Anti-Rabies Nucleocapsid mAb conjugated with Fluoroisothiocynate (FITC).
4. Phosphate-buffered saline (PBS) pH 7.5
5. Acetone (kept at -20 °C overnight or -80 °C for one hour)
6. Mounting medium. Prepare 50% glycerol PBS (50% glycerol & 50% PBS) pH adjusted to 8.5-9.0 as coverslip mounting medium.

   NOTE. The concentration of glycerol should be standardized by each laboratory by adjusting the percentage of glycerol and PBS.

Preparation of Impressions . Impression from the rabies suspected brain sample is prepared as follows

1. Prepare two impressions of approximately 1 cm diameter, about 1.5 cm from each end of the labelled slides.
2. Prepare touch impressions by gently pressing clean glass slides against tissue obtained after necropsy on a blotting paper.
3. Prepare at least four slides each from the cerebrum, hippocampus, midbrain, cerebellum, and medulla of the brain.
4. Remove excess tissue by blotting several times. Allow impressions to air dry for approximately 45-60 min.

   IMPORTANT - Immediately use the impressions for DFAT. If storage is required, store the impressions slides at -20 ℃ in a slide box, ensuring that slides are not touching each other.

Preparation of control slides. Control slides are prepared as follows

1. **Positive control slides.** Prepare two impressions of approximately 1 cm diameter about 1.5 cm from each end of the labelled slides using rabid mouse brain (RMB) or a brain sample of a laboratory-confirmed rabies positive case.
2. **Negative control slides.** Prepare two impressions of approximately 1 cm diameter about 1.5 cm from each end of the labelled slides using a normal mouse brain (NMB) or a brain sample of a laboratory-confirmed rabies negative case.

Protocol. The workflow for assay procedure is given in Figure 8. Brief protocol for DFAT is as follows:

1. Immerse the slides in Coplin jars containing chilled acetone in a deep freeze at –20 °C to –25 °C or -80 °C for one hour. Keep for 4 hrs to overnight. Drain off the acetone and store slides at –20 °C till stained.
2. Take out the impression slides from deep freezer and dry at room temperature for 20 minutes. Include known positive and negative impressions also.
3. Mark the outline of the impression area with grease marking pencil or wax pen. Flood the impression area with freshly prepared conjugate (as per the kit literature) with the help of a Pasteur pipette.

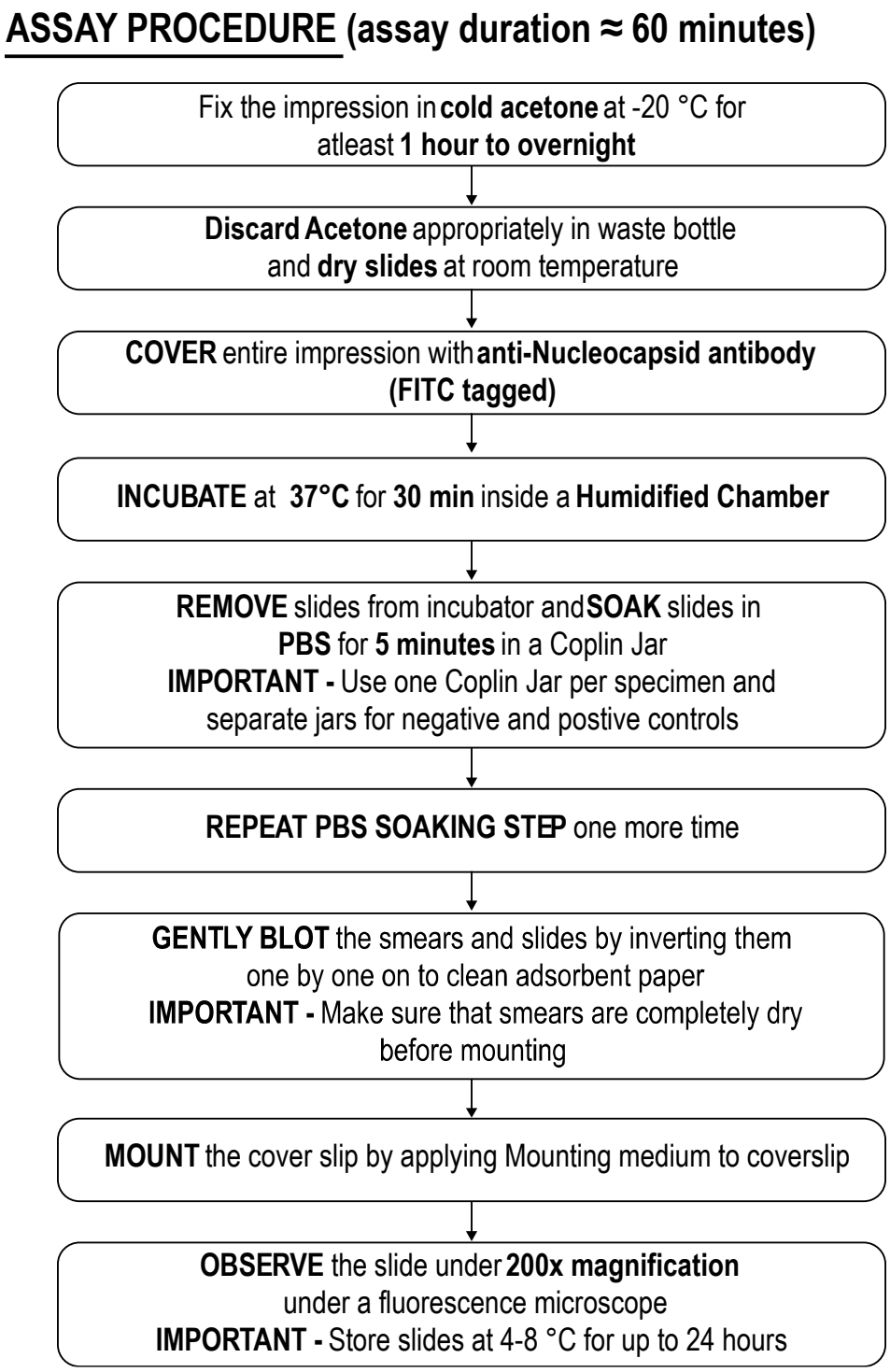


Figure 8. Workflow for performing DFAT assay on brain sample of suspected rabies case.

4. Place the slides in a chamber with moist filter paper at the bottom (Humidified Chamber). Cover the chamber and keep at 37°C for 30 minutes.
5. Wash the slides in 0.01 M phosphate buffered saline (pH 7.5) for 5 minutes.
6. Repeat washing.
7. Remove from buffer and dip in a jar of distilled water for 5 minutes with gentle shaking (Washing can also be performed by gentle agitation using a magnetic stirrer).
8. Remove from distilled water and dry the slides at room temperature. Mount in 50% buffered glycerol. Examine under a microscope with ultraviolet source of light (fluorescent microscope), the known positive, known negative and test slides.

Observations. Rabies antigen is seen as fine dusty particles emitting bright to dull apple green fluorescence (Figure 9).

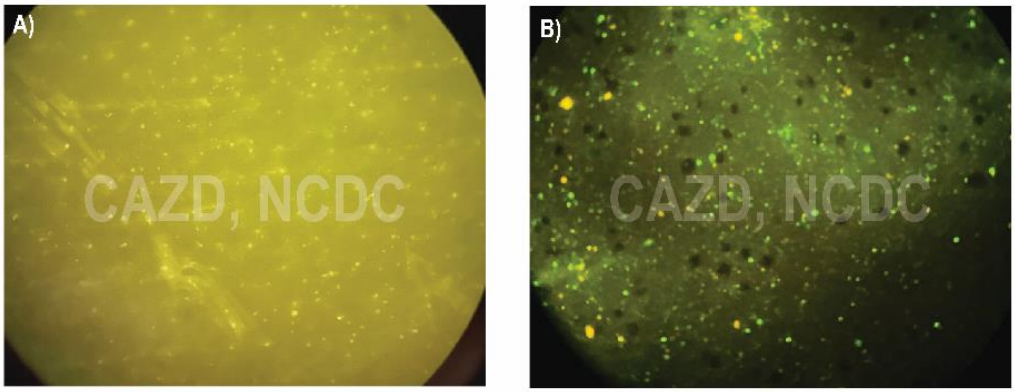


Figure 9. Fluorescence micrograph (400x magnification) of DFAT assay slides of A) normal brain impression, and B) rabid brain impression.

2.2.3 Direct Rapid Immunohistochemistry Test (DRIT)

Principle. DRIT detects rabies antigen in the sample based on specific antigen-antibody reaction followed by detection using a compound microscope. The formaldehyde fixed smear

is incubated with polyclonal or monoclonal anti-rabies antibodies which are labelled with biotin moiety. The unbound antibodies are washed away. The biotin moiety has a significant affinity towards streptavidin. The slide is then incubated with streptavidin conjugated with horseradish peroxidase (HRP). After removing unbound reagent, the slide is incubated with chromogen substrate (amino-ethyl carbazole, AEC) in presence of Hydrogen Peroxide (H_2O_2). The substrate is converted into an insoluble red precipitate which is visible under compound microscope.

Reagents and Equipment.

1. Kit - Direct Rapid Immuno-histochemistry test biotin streptavidin HRP system
2. Phosphate-buffered Saline (PBS)
3. 10% Buffered Formalin (BF)
4. 3% Hydrogen Peroxide (H_2O_2)
5. Wash Buffer – Tween PBS (TPBS)
6. AEC substrate working solution
7. Compound microscope

Impression Preparation. Prepare impressions as explained in section 2.2.2.

Preparation of Control Slides. Prepare control slides as explained in section 2.2.2.

Protocol. The workflow for the assay procedure is given in Figure 10. Brief protocol for DRIT is as follows:

1. Fix the impression by immersing slides in 10% Neutral Buffered Formalin (NBF) at room temperature for 10 minutes in a Coplin jar.
2. Dip-rinse the slides in fresh TPBS at least two times in a Coplin jar. Change the TPBS in between the rinsing.
3. Transfer slides to fresh TPBS and proceed for next step one slide at a time leaving the other slides in TPBS.
4. Immerse the slides in 3% H_2O_2 for 10 minutes in a Coplin jar at room temperature.

Note: This step is important to neutralize any endogenous peroxidases in the sample thus reducing artifacts.

ASSAY PROCEDURE (assay duration ≈ 60 minutes)

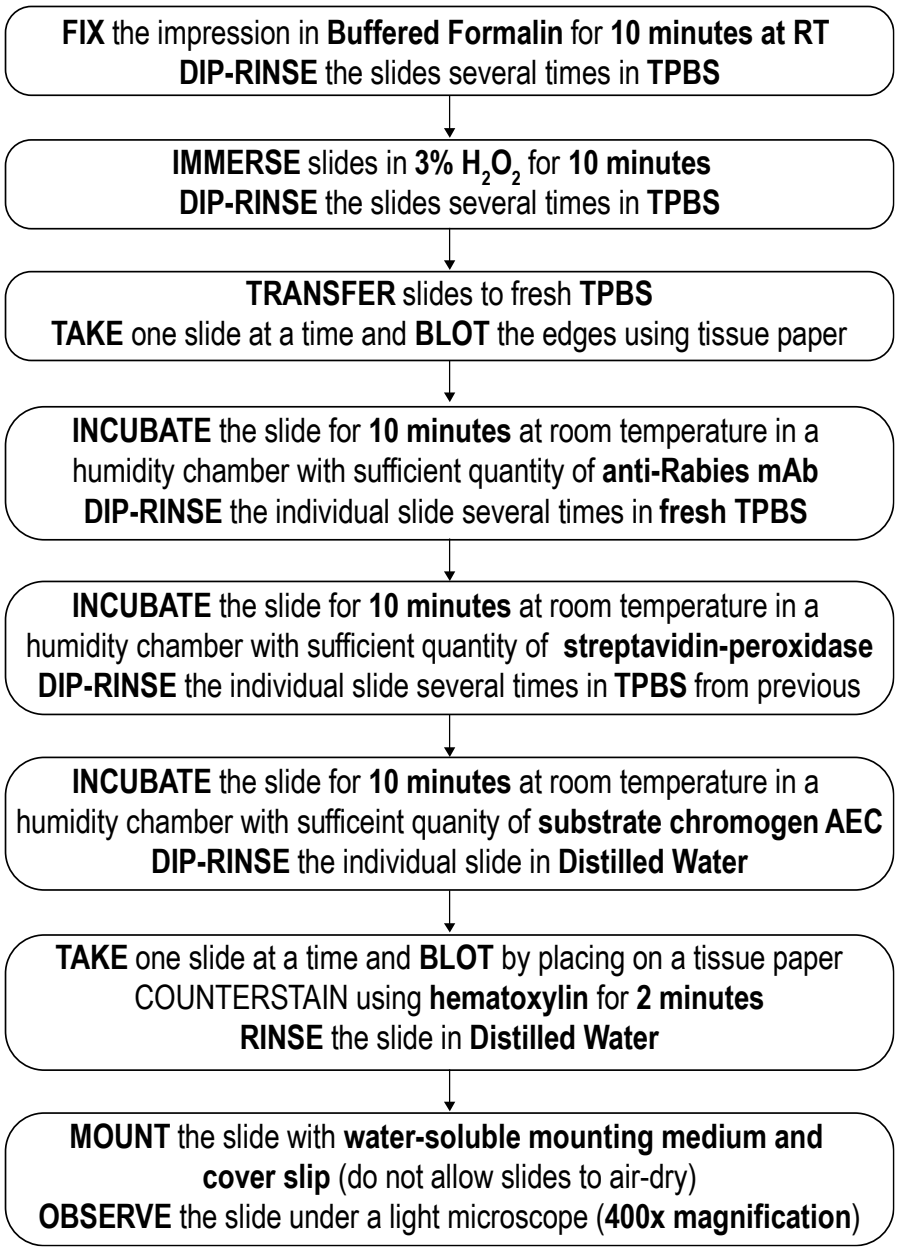


Figure 10. Workflow for performing DRIT assay on brain sample of suspected rabies case.

5. Dip-rinse the slides in fresh TPBS at least two times in a Coplin jar. Change the TPBS in between the rinsing.
6. Transfer slides to fresh TPBS and proceed for next step one slide at a time leaving the other slides in TPBS.
7. Blot the edges of the impression area using a tissue paper and place the slides inside a humidified chamber.
8. Incubate slides for 10 minutes at room temperature with sufficient quantity of biotinylated anti-rabies antibodies to cover the entire impression area.
9. Dip-rinse the slides in fresh TPBS at least two times in a Coplin jar. Change the TPBS in between the rinsing.
10. Incubate slides for 10 minutes at room temperature with sufficient quantity of streptavidin conjugated HRP to cover the entire impression area.
11. Dip-rinse the slides in fresh TPBS at least two times in a Coplin jar. Change the TPBS in between the rinsing.
12. Incubate slides for 10 minutes at room temperature with sufficient quantity of AEC chromogen substrate to cover the entire impression area.
13. Dip-rinse the slides in fresh TPBS at least two times in a Coplin jar. Change the TPBS in between the rinsing.
14. Counterstain with sufficient quantity of Gills Hematoxylin for 2 minutes at room temperature.
15. Dip-rinse the slides in distilled water at least two times in a Coplin jar.
16. Mount the slides in water-soluble mounting medium and observe under a compound microscope.

Observation. Rose-red inclusions visible on a blue neuronal background are an indicative of presence of rabies antigen in the impression of suspected sample (Figure 11).

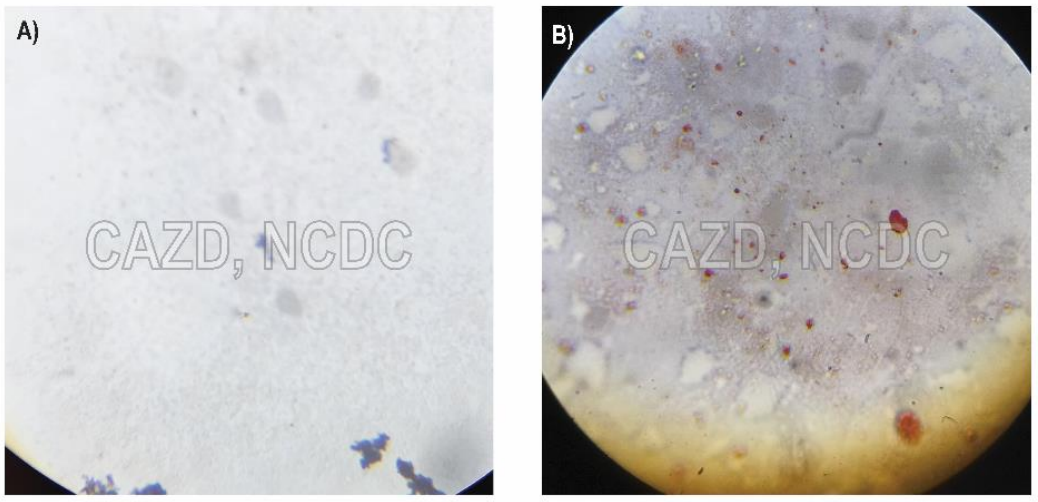


Figure 11. Brightfield Microscopic Image (400x magnification) of the DRIT staining of A) Normal brain impression, and B) Rabid brain impression.

2.2.4 Rapid Antigen Detection Test (RADT)

Principle. The rapid antigen detection test (RADT) is based on immunochromatographic principle in lateral flow format. The assay employs a lateral flow device which has an anti-rabies antibody immobilized on a nitrocellulose membrane [19]. The reagent has a secondary anti-rabies antibody which forms a complex with the virus present in the sample. The secondary antibody is usually tagged with a reporter dye or colloidal gold. The co mplex from the prepared sample moves chromatographically in the lateral flow device till it is captured by the immobilized anti-rabies antibody. This results in formation of a coloured band at the site of immobilized anti-rabies antibody in lateral flow device. The band can be observed visibly. A second immobilized antibody captures a control protein from the sample. This control should be clearly visible to interpret the assay results.

Reagents and Equipment.

1. Kit – Rabies Rapid Antigen Test Kit (The kit is recommended for canine, bovine, and racoon).
2. Phosphate-buffered Saline (PBS)
3. Pestle and Mortar or a tissue homogenizer

Sample preparation. A 10% brain homogenate is needed for the RADT and the collected brain sample should be processed as follows:

1. Collect 1 gram of brain tissue preferably a pooled brain sample which included parts of brain stem and cerebellum.
2. Add the sample to the grinding tube of tissue homogenizer or a pestle.
3. Prepare a fine homogenate of the brain tissue ensuring that overheating is avoided during use of tissue homogenizer.
4. Add 9 mL of PBS to the homogenate prepared above to obtain 10% brain homogenate.

Protocol. The workflow of the assay procedure is provided in the Figure 12. Brief protocol of RADT is as follows:

1. Ensure that all the samples and reagents have been equilibrated to room temperature before starting the procedure.
2. Use the provided disposable swab to take a small quantity of sample from 10% brain homogenate.
3. Insert the swab into the Assay Diluent tubes provided with the kit.
4. Mix the swab sample with Assay Diluent gently ensuring that the sample is mixed thoroughly with the assay diluent. Keep the mixture unperturbed for 10 seconds.
5. Ensure that the test device is kept on a flat and dry surface. A blotting paper/filter paper can be used to ensure this.
6. Using the disposable dropper provided in the kit, add 4 drops of the sample into the sample hole of the test device.

7. Observe the device carefully. A lateral movement of the sample should be visible in the result window.

   NOTE – in case the lateral flow is not visible, add one more drop of the sample.

8. Interpret the results within 5-10 minutes and not later than 20 minutes.

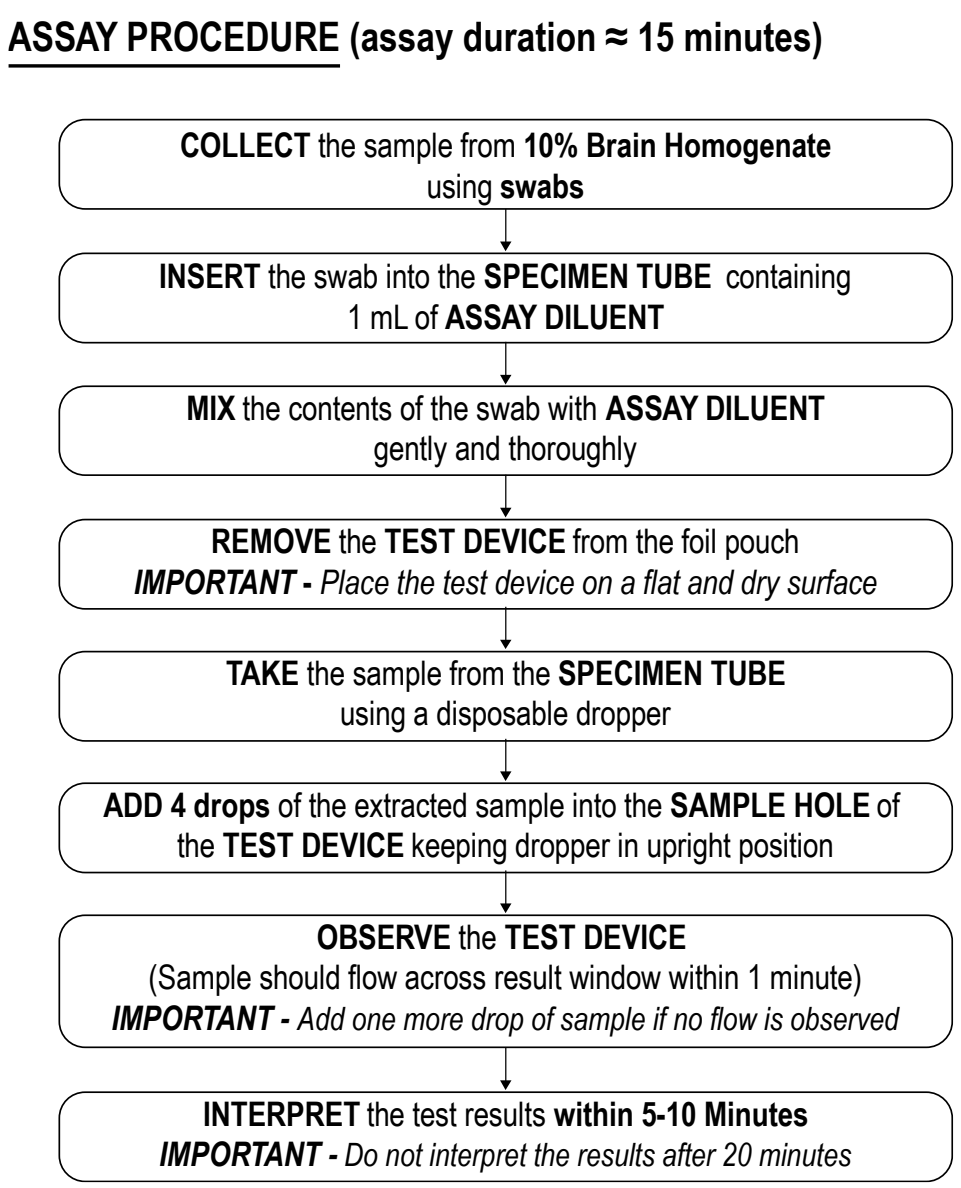


Figure 12. Workflow for performing RADT assay on brain sample of suspected rabies case.

Observation. Various outcomes of RADT and their interpretation is given in Figure 13. The presence of both positive and control lines within 5-10 minutes is considered as a positive result. Presence of only control line is considered as a negative result. No line visible in both test and control and line only visible in test but not in control is considered as an invalid result.

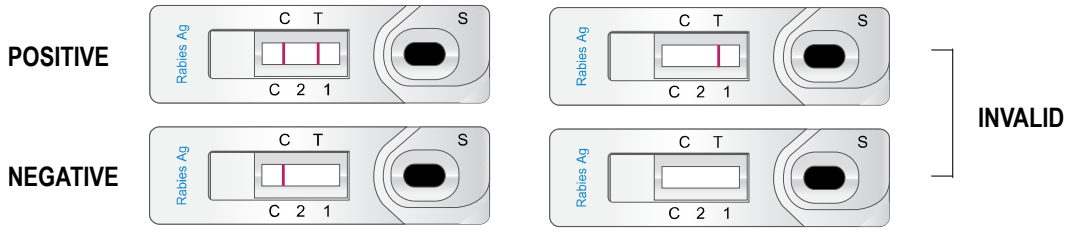


Figure 13. Interpretation of results for RADT on suspected rabies brain sample.

2.2.5 Nucleic Acid Amplification Tests (NAAT)

Principle. Nucleic Acid Amplification Tests (NAAT) are based on the detection of genomic material of the target organism in the sample. Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) can be used for Rabies RNA detection in suspected samples after proper standardization and optimization using appropriate controls. Rabies suspected brain sample is the preferred specimen. The sample is first homogenized and processed for extracting RNA. The extracted sample is then tested by a pan-lyssavirus nested PCR protocol in which the first reaction uses degenerate primers to amplify RNA of all lyssaviruses followed by a second reaction using both pan-lyssavirus degenerate primers and rabies-specific primers (Figure 14). The protocol confirms the presence of rabies and non-rabies lyssavirus RNA in the suspected brain sample. However, saliva/ nuchal skin biopsy may also be received in antemortem diagnosis, which needs to be processed for RNA extraction as per standard protocol.

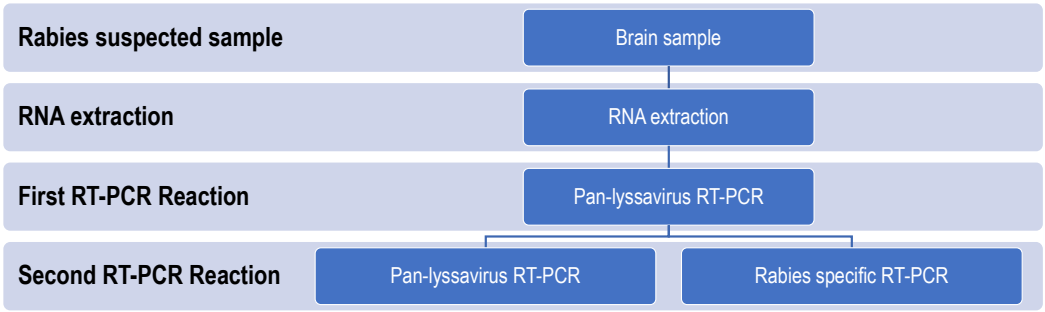


Figure 14. Nucleic Acid Amplification Test (NAAT) protocol for rabies suspected sample based on nested RT-PCR

2.2.5.1 Extraction of RNA from the rabies suspected brain sample

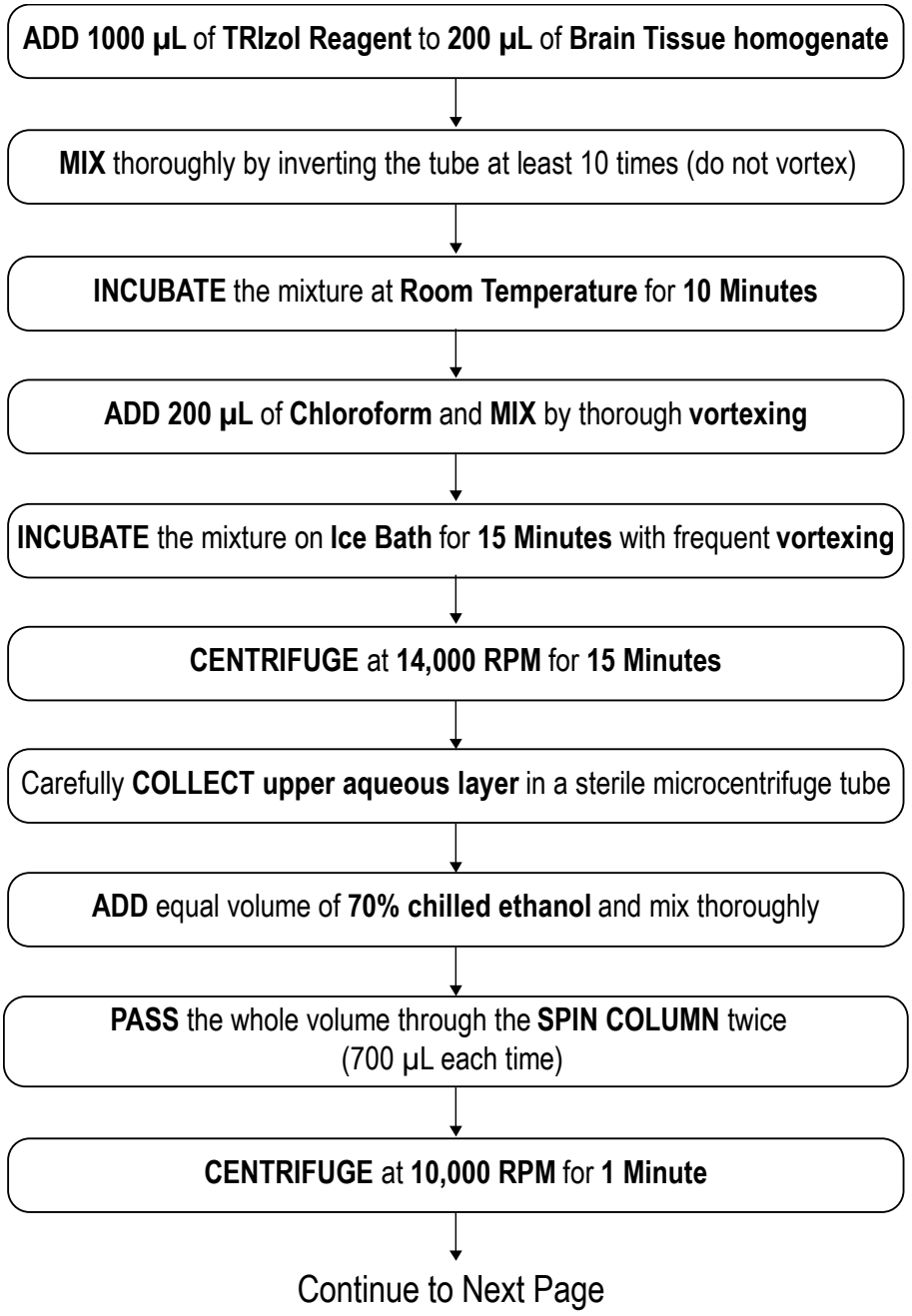
Reagents and Equipment.

1. RNA extraction kit
2. TRIzol™ reagent
3. Cold 70% ethanol (molecular grade) kept at -20 °C for overnight
4. Chloroform (molecular grade)
5. Pestle and Mortar or a tissue homogenizer

Sample preparation. A 10% brain homogenate is needed for the NAAT and the collected brain sample should be processed as follows:

1. Collect 1 gram of brain tissue preferably a pooled brain sample which included parts of brain stem and cerebellum.
2. Add the sample to the grinding tube of tissue homogenizer or a pestle.
3. Prepare a fine homogenate of the brain tissue ensuring that overheating is avoided during use of tissue homogenizer.
4. Add 9 ml of PBS to the homogenate prepared above to obtain 10% brain homogenate.

Workflow. The workflow of extracting RNA from suspected rabies brain sample is provided in Figure 15.



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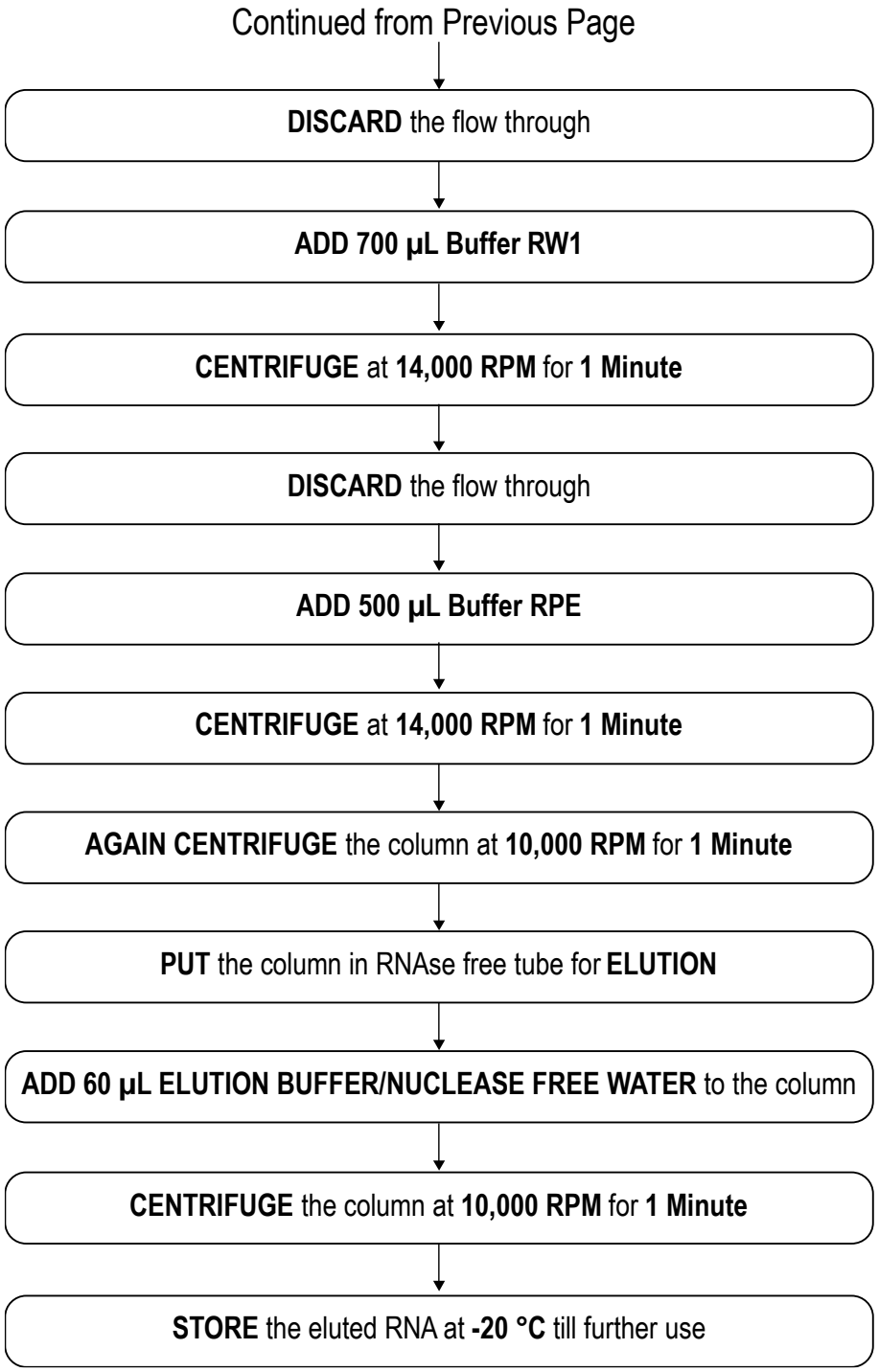

Figure 15. Workflow for extracting RNA from suspected rabies brain sample using in-house standardized protocol at CAZD, NCDC.

2.2.5.2 Hemi-nested conventional RT-PCR

The hemi-nested conventional RT-PCR protocol is based on pan-lyssavirus RT-PCR protocol from WHO Rabies Laboratory Manual. This assay is based on using degenerative primers to cover most of the genotypes of viruses belonging to lyssavirus species.

Reagents and Equipment.

1. Details of primers required for the protocol are provided in Table 10 [20, 21].

Table 10. Primers for hemi-nested pan-lyssavirus RT-PCR (Target– N gene)

Primer	Direction	Sequence	Nucleotide position#	Conc. (pmol/µL)
JW12UNI	F	ATGTAACACC**Y**CTACAATG	55-73	3.75
JW6UNI*	R	A**R**TT**V**GC**R**CACAT**Y**TT**R**TG	660-641	3.75
JW10UNI*	R	GTCAT**Y**A**RW**GT**R**TG**R**TG**Y**TC	636-617	3.75
JW10(P)	R	GTCATTAGAGTATGGTGTTC	636-617	3.75

Position according to the reference Pasteur virus genome (M13125)
*Degenerate primers [R – Purine (A/G), V – Not T (A/C/G), W – Weak (A/T), Y – Pyrimidine (C/T)]

2. OneStep RT-PCR kit (Qiagen)

   Note: National Reference Rabies Laboratory (Centre for Arboviral and Zoonotic Diseases and WHO Collaborating Centre for Rabies Epidemiology, NCDC Delhi) has extensive experience with the kit mentioned above. Any other equivalent kit can be used only after standardization.
3. Positive control (RNA extracted from a tissue-culture based rabies vaccine)
4. PCR Thermocycler
5. Molecular lab with clear demarcation between clean area, extraction area, template addition area, and control addition area
6. Gel casting system, gel electrophoresis system, gel documentation system

Protocol.

1. **Master mix preparation.** The master mix is prepared as per the kit literature with optimized concentration of primers. The reaction mixture setup for each reaction is provided in Table 11 for both first and second reaction of pan-lyssavirus hemi-nested RT-PCR and rabies specific RT-PCR to be done in parallel. Master mix should be made in a clean room/area.

2. **Template addition.** The template is added in template addition room/area. The template is added as per the information in Table 11. Each assay must include a non template control (NTC) with template replaced with nuclease free water.

3. **Positive control.** Each assay must include a positive control and the positive control should be handled in a separate control addition room/area.

Table 11. Reaction mixture setup for pan lyssavirus hemi-nested RT-PCR based on OneStep RT-PCR kit*

FIRST REACTION (pan-lyssa) volume per reaction (µL)		SECOND REACTION (pan-lyssa and rabies) volume per reaction (µL)	
Reagent	**Vol.**	**Reagent**	**Vol.**
5x Qiagen OneStep RT-PCR buffer	5.0	5x Qiagen OneStep RT-PCR buffer	5.0
dNTP mix (10 mM of each dNTP)	1.0	dNTP mix (10 mM of each dNTP)	1.0
Qiagen OneStep RT-PCR Enzyme mix	1.0	Qiagen OneStep RT-PCR Enzyme mix	1.0
Forward Primer JW12UNI (3.75 pmol/µL)	3.0	Forward Primer JW12UNI (3.75 pmol/µL)	0.5
Reverse Primer JW6UNI (3.75 pmol/µL)	3.0	Reverse Primer JW10UNI/ J10(P) (3.75 pmol/µL)	0.5
Nuclease free water	11.0	Nuclease free water	16.0
Master mix volume per reaction	**24.0**	**Master mix volume per reaction**	**24.0**
Template (Extracted RNA/Controls)	1.0	Template (1st rxn PCR product/ Controls)	1.0
Total volume per reaction	**25.0**	**Total volume per reaction**	**25.0**

*Sample format for reaction setup. Volumes may be optimized as per the enzyme used.

4. **RT-PCR reaction.** RT-PCR reaction is a single-step reaction based on kit literature from OneStep RT-PCR kit (Qiagen)**.** The optimized reaction conditions for both first and second reaction of pan-lyssavirus nested RT-PCR and rabies specific RT-PCR are provided in Table 12.

   Note: These conditions must be optimized in each lab using suitable controls.

Table 12. Cycling conditions for pan lyssavirus hemi-nested RT-PCR based on OneStep RT-PCR kit*

STEP	FIRST REACTION			SECOND REACTION		
	Temp	*Time*	*Cycles*	Temp	*Time*	*Cycles*
Reverse Transcription	50 °C	30 min	1	-	-	-
Initial Denaturation	95 °C	15 min	1	95 °C	15 min	1
Denaturation	94 °C	30 s	45	94 °C	30 s	35
Annealing	45 °C	45 s		45 °C	10 s	
	50 °C	15 s		50 °C	15 s	
Extension	72 °C	1 min		72 °C	30 s	
Final extension	72 °C	10 min	1	72 °C	10 min	1
Hold	4 °C	∞	n/a	4 °C	∞	n/a

*Sample format for reaction conditions. Reaction conditions may be optimized as per the enzyme used.

Analysis. A positive PCR result is observed in the form of a bright band of the expected size of 606 bp (first reaction) and 582 bp (second reaction) if the negative and positive controls work as expected. The interpretation of the results is as shown in Table 13. Figure 16 shows results of NAAT for suspected rabies brain sample received at CAZD, NCDC.

Table 13. Interpretation of Nucleic Acid Amplification Test (NAAT) results for rabies suspected sample tested by nested RT-PCR protocol

Pan-lyssavirus RT-PCR (Reaction 1)	Pan-lyssavirus RT- PCR (Reaction 2)	Rabies specific RT-PCR (Reaction 2)	Inference
+	+	+	Rabies RNA detected
+	+	-	Non-rabies lyssavirus RNA detected*
-	-	-	Sample is negative for lyssavirus RNA

*Sample along with extracted RNA may be shared with CAZD, NCDC for further characterization

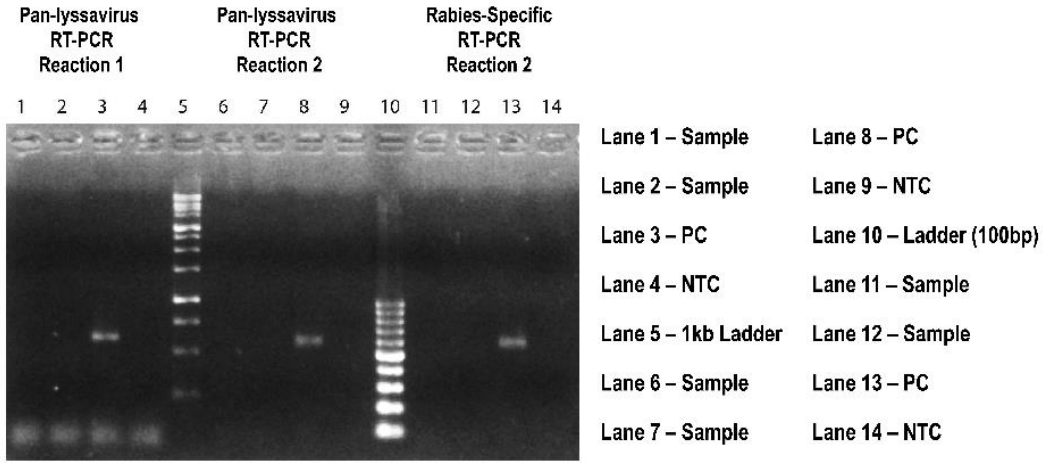

Figure 16. Documented gel of NAAT assay for suspected rabies sample.

2.2.5.3 Realtime RT-PCR assay

The realtime RT-PCR assay for detection of lyssavirus specific RNA is based on LN34 RT-PCR assay protocol from WHO Rabies Laboratory Manual [21]. This assay is based on using combination of degenerative primers and probes to detect RNA of most of the species belonging to lyssavirus species.

Reagents and Equipment.

1. Details of primers required for the protocol are provided in Table 14. The working solutions of primers and probes are prepared as provided in Table 15.

Table 14. Oligonucleotide sequences for primers and probes of LN34 realtime RT-PCR assay for detection of lyssavirus RNA

Primer/Probe*	Sequence	Nucleotide Position**
Probe LN34	FAM-AA+C+ACCY+C+T+ACA+A+TGGA-BHQ1	59 - 75
Probe LN34lago	FAM-AA +C +ACTA +C +T+ACA+A +TGGA-BHQ1	59 - 75
LN34Fwd1	ACGCTTAACAACCAGATCAAAGAA	1 - 24
LN34Fwd2	ACGCTTAACAACAAAATCADAGAAG	1 - 25
LN34Rev	CMGGGTAYTTRTAYTCATAYTGRTC	140 - 164

*Details of modifications

- LNA-modified bases are indicated by a plus preceding the base in the sequence (e.g. +A, +G, +C, +T)
- Degenerate primers [R – Purine (A/G), V – Not T (A/C/G), W – Weak (A/T), Y – Pyrimidine (C/T), M – Amino (A/C)]

**Position relative to the Lyssavirus full genome

Table 15. Working mixture for primers and probes of LN34 realtime RT-PCR assay for detection of lyssavirus RNA

Primer/Probe	Working solution
Probe	LN34 and LN34lago in 2:1 ratio
Forward Primer	LN34Fwd1 and LN34Fwd2 in 1:1 ratio
Reverse Primer	LN34Rev as it is

2. An internal control (β-actin) may be used to ascertain the quality of the sample. The details of primers and probes for the β-actin assay are provided in Table 16.

3. OneStep realtime RT-PCR kit – Ag-Path ID One-Step RT-PCR Kit [Life Technologies, Catalogue no. 4387391]

   Note: Any other equivalent kit can be used only after standardization.

Table 16. Primers and probes for β-actin realtime RT-PCR assay

Primer/Probe	Working mixture
β-actin Probe	HEX-TCCACCTTCCAGCAGATGTGGATCA-BHQ1
β-actin Forward Primer	CGATGAAGATCAAGATCATTGC
β-actin Reverse Primer	AAGCATTTGCGGTGGAC

Protocol.

1. **Master mix preparation.** The master mix is prepared as per the kit literature with optimized concentration of primers. Master mix should be made in a clean room/area.
2. **Template addition.** The template is added in template addition room/area. Each assay must include a non template control (NTC) with template replaced with nuclease free water. **2-5 µl templae can be used based on standardization.**
3. **Positive control.** Each assay must include a positive control and the positive control should be handled in a separate control addition room/area.
4. **RT-PCR reaction.** RT-PCR reaction is a single-step reaction based on kit literature. The optimized reaction conditions for both first and second reaction of pan-lyssavirus nested RT-PCR and rabies specific RT-PCR are provided in Table 17.

   Note: These conditions must be optimized in each lab using suitable controls.

Table 17. Reaction conditions for LN34 realtime RT-PCR assay for detection of lyssavirus RNA*

STEP	FIRST REACTION		
	Temp	*Time*	*Cycles*
Reverse Transcription	50 °C	30 min	1
Initial Denaturation	95 °C	10 min	1
Denaturation	94 °C	15 s	45
Annealing/Extension*	56 °C	30 s	

**Sample format for reaction conditions. Reaction conditions may be optimized as per the enzyme used.

**Capture fluorescence signal at FAM channel for lyssavirus-specific RNA and HEX channel for host-specific β-actin RNA (internal control)

Analysis. Analyse the results as per the laboratory-specified protocol for interpretation of realtime PCR results. Any manual threshold and baseline adjustments need to be recorded and explained. As a reference, the Ct values provided in the Table 18 can be used for interpreting the results of the assay. In case any inhibition is observe for internal control, a diluted sample may be used for troubleshooting the assay.

Table 18. Interpretation of LN34 assay results based on the Ct values

Assay	Ct value	Inference	Action
B-actin	<33	Positive	No action required
	33-45	Inconclusive	Possible inhibition. Repeat the assay.
	Not detected	Fail	Poor sample/extraction. Repeat sample processing and assay.
LN34	<35	Positive	No action required.
	35-45	Inconclusive	Additional tests required.
	Not detected	Negative/Inconclusive	Sample is negative if internal conteol passes the test.

2.2.6 Enzyme Linked Immunosorbent Assay (ELISA)

Principle. Enzyme Linked Immunosorbent Assay (ELISA) can be used to demonstrate rabies antibodies in human and animal (dogs, cats, fox) serum. The assay is a quantitative indirect ELISA which uses WHO's (human samples) and WOAH's (animal samples) traceable standards for quantification of antibodies. The microwells are coated with rabies glycoprotein extracted from inactivated and purified rabies membrane. Therefore, the antibodies detected are specific to rabies glycoprotein.

Reagents and Equipment.

1. Kits – following kits are used for detecting anti-rabies antibodies using ELISA
    a. Human samples – PLATELIA™ RABIES II ELISA Kit (BioRad, Catalogue number 355-1180).

b. Animal samples – PLATELIA™ RABIES II ELISA Kit ad usum veterinarium (BioRad, Catalogue number 355-0180).

Note: National Reference Rabies Laboratory (Centre for Arboviral and Zoonotic Diseases and WHO Collaborating Centre for Rabies Epidemiology, NCDC Delhi) has extensive experience with the kits mentioned above which are also specified in WHO TRS no 1012 (2018). Any other equivalent kit can be used only after standardization.

2. The reagents preparation as per the kit literature are provided in Table 19.

Table 19. Preparation of reagents before performing anti-rabies IgG ELISA for human and animal samples

Reagent	Preparation
Wash Solution (WS)[1]	Dilute **REAGENT R2** 1/10 in distilled water (100 mL R2 in 900 mL DW)
Conjugate[1]	Dilute **REAGENT R7** 1/10 in freshly prepared WS (0.4 mL R7 in 3.6 mL of WS)
Substrate[1]	Dilute **REAGENT R9** 1/11 in **REAGENT R8** (0.36 mL R9 in 3.64 mL R8)
Negative Control[2]	Dilute **REAGENT R3** 1/100 in **REAGENT R6** (3 µL R3 in 297 µL R6)
Positive Control [2,3]	Dilute **REAGENT R4a** 1/100 in **REAGENT R6** (3 µL R4a in 297 µL R6)
S6 control[2]	Dilute **REAGENT R4b** 1/100 in **REAGENT R6** (3 µL R4b in 297 µL R6)
S5 control[2]	Dilute **S6 CONTROL** 1/100 in **REAGENT R6** (150 µL S6 control in 150 µL R6)
S4 control[2]	Dilute **S5 CONTROL** 1/100 in **REAGENT R6** (150 µL S5 control in 150 µL R6)
S3 control[2]	Dilute **S4 CONTROL** 1/100 in **REAGENT R6** (150 µL S4 control in 150 µL R6)
S2 control[2]	Dilute **S3 CONTROL** 1/100 in **REAGENT R6** (150 µL S3 control in 150 µL R6)
S1 control[2]	Dilute **S2 CONTROL** 1/100 in **REAGENT R6** (150 µL S2 control in 150 µL R6)

[1] Prepare dilutions in appropriate volume containers
[2] Prepare dilutions in a dilution plate
[3] The concentration of PC is 0.5 EU/mL

3. ELISA microwell plate reader with 450 nm and 620 nm filters.
4. ELISA microwell plate washer.
5. Incubator (37 °C) humidified and without CO_2.
6. Variable volume multichannel pipette with sterile disposable tips (20-200 µL).
7. Variable volume single channel pipette (2-20 µL, 20-200 µL, and 100-1000 µL) with sterile disposable tips.

8. Graduated cylinder
9. Distilled water
10. Appropriate PPE (latex gloves, lab coat)
11. Vortex tube mixer
12. Discarding jar with 1% sodium hypochlorite solution.
13. Timer

Sample preparation. Sample preparation is same for both human and animal sera.

1. Use only clear, non-hemolyzed samples. Allow blood samples to clot and separate the serum. Store serum at 2-8°C.
   Note. If testing is to be delayed for more than 5 days, store samples at -20°C or colder temperature.
2. Prepare 1:100 dilution of serum samples (3 µL Sample in 297 µL Reagent R6 in a dilution plate)

Protocol. The test is performed as per the protocol from the kit literature. All controls should be put up in duplicate for the quantitative anti-rabies IgG ELISA. The workflow for performing anti-rabies IgG ELISA on human and animal serum samples is given in Figure 17.

Validation Criteria. The validation criteria for anti-rabies IgG ELISA are given as follows:

1. OD NC(i) < 0.05- The OD of each individual negative control must be lower than 0.05.
2. 0.300 < OD PC(i) < 1.200- The OD of each individual positive control must be between 0.300 and 1.200.
3. OD of Standards- OD values of the standards must increase in the following way: S1<S2<S3<S4<S5<S6.
4. 0.7≤OD S3/ODR4a≤1.3 – the ratio between S3 standard mean OD and R4a mean OD must be in between 0.7-1.3

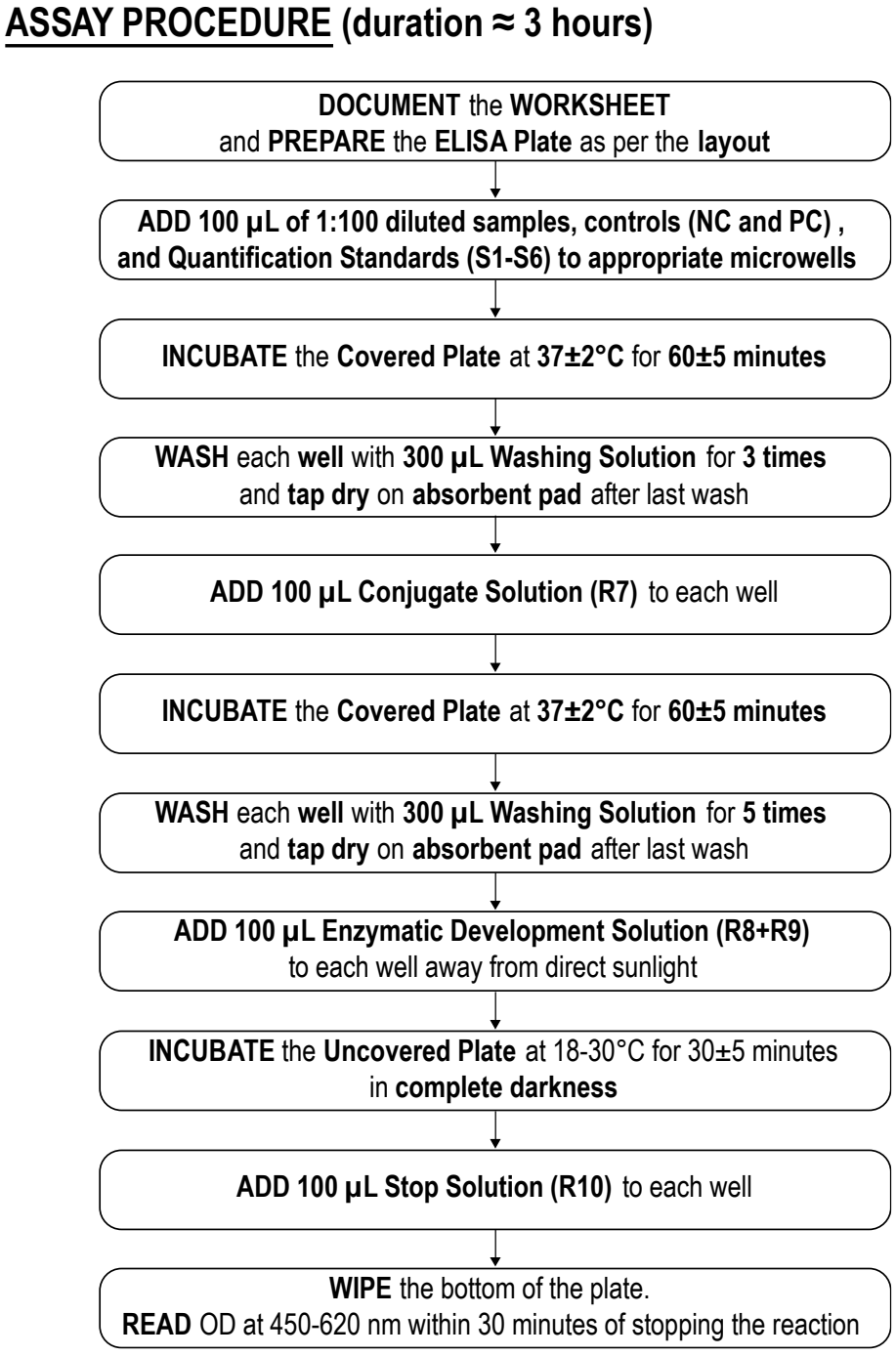

Figure 17. Workflow for performing anti-rabies IgG ELISA on human and animal serum samples.

Calculation and Result Interpretation. The quantification of anti-rabies IgG antibodies in Serum uses linear regression method based on OD values of the samples to obtain the titer. An excel-based tool is available with CAZD, NCDC to do this calculation which can be obtained from official sources. The results of the test are interpreted from either OD values or titers as shown in Table 20.

Table 20. Interpretation of anti-rabies IgG ELISA results based on OD values of samples or titer of samples

OD of sample	Titer of sample	Interpretation of the result
OD sample > S6	X > 4 EU/ml	**High Seroconversion**
S3 ≤ OD sample ≤ S6	0.5 EU/mL – X – 4 EU/mL	**Sufficient Seroconversion**
S1≤ OD sample ≤ S3	0.125 EU/mL– X – 0.5 EU/ml	**Insufficient Seroconversion**
OD sample < S1	X <0.125 EU/mL	**No Detectable Seroconversion**

2.2.7 Rapid Fluorescent Focus Inhibition Test (RFFIT)

Principle. The Rapid Fluorescent Focus Inhibition Test (RFFIT) is a rabies virus neutralisation test carried out in cell culture to determine the rabies virus antibody level in human or animal sera. Immunofluorescence staining is used as an indicator of viral growth. The RFFIT takes 20 hours and is sensitive and accurate in the hands of well-trained laboratory personnel. The protocol for animal samples is slightly different and is available in Manual of Diagnostic Tests and Vaccines for Terrestrial Animals 2022. [22]

The RFFIT is performed by mixing dilutions of test sera with a constant amount of rabies virus (50-50% Fluorescing Foci Doses (50 FFD_{50}/100 µl) in a multi chambered slide. After allowing the mixture to react in a CO_2 incubator at 37°C for 90 minutes, Baby Hamster Kidney (BHK)/ mouse neuroblastoma (MNA) cells in Eagle's minimum essential medium with 10% fetal bovine serum (MEM-10) are added to each serum-virus mixture.

The serum-virus-cell cultures are incubated for 20 hours in a CO2 incubator at 37°C. The cultures are removed from the incubator, washed, fixed and then stained with an anti-rabies conjugate and observed under a fluorescence microscope for the presence of fluorescing cells; 20 microscopic fields (160X – 200X) are read for each serum dilution and compared against the virus control (50 FFD_{50}/ 100 µl) which should contain 18 to 20 field with fluorescing cells. A reduction of 50% or more in the number of fields containing fluorescing cells indicates that there was enough antibody present to neutralise the virus.

Reagent and Equipment

A. Reagents

1. Conjugate – Anti rabies nucleocapsid conjugate (FITC)
2. Acetone
3. Gelatin
4. Cell Culture Media: Minimum Essential Medium containing Earle's salts but no L. Glutamine or sodium bicarbonate
5. Fetal Bovine Serum (FBS)/ Fetal Calf Serum (FCS)
6. Distilled water
7. Fungizone
8. HEPES Buffer
9. L-Glutamine
10. Antibiotic Antimycotic solution
11. Sodium bicarbonate
12. Trypsin-EDTA solution
13. Phosphate Buffered Saline (PBS), 0.01M, pH 7.4 + 0.5
14. Dimethyl Sulfoxide (DMSO)

B. Glassware and plastic ware

1. Cell culture flask
2. Lab-Tek Chamber slides with cover slip

3. Disposable sterile plastic pipettes
4. Centrifuge tube
5. Cryovials
6. Millipore disposable filter (pore size 0.22µ) 250 ml; 500 ml
7. Hemocytometer

C. Equipment

1. Biological safety cabinet
2. CO_2 incubator (Required setting: 37 °C with 5% CO_2)
3. Freezer
4. Inverted microscope
5. Fluorescent Microscope
6. Refrigerated centrifuge (0 – 8000 rpm)
7. Water bath

D. Standards and references

1. Baby Hamster Kidney 21/ Mouse Neuroblastoma 2A Cells (BHK/MNA)
2. Rabies virus-challenge Virus Standard (CVS Strain)
3. Reference serum standards diluted 2 I.U./ml.

Procedure

Preparation of seed virus

1. Trypsinise a three-day old 25 ml culture flask of BHK/MNA cells.
2. Resuspend cells in 3 ml of MEM-10 (10% MEM with FCS) at a final concentration of 30 x 10^6 cells per 3 ml.
3. Add 1 ml of virus (CVS) 10 x 10^6 Infectious Units per ml.
4. Incubate for 15 minutes at 37 °C.
5. Add 10 ml of MEM with 10% FCS.

6. Centrifuge at 1000 rpm for 10 minutes, discard the supernatant.
7. Add 30 ml of MEM-10 medium.
8. Transfer to 150 ml flask.
9. Gently rock this flask.
10. Take 6 ml of suspension from the flask.
11. Put 200 µl suspension in each well of 3 Lab-Tek chamber slides and incubate for 20 hours, 40 hours, 64 hours.
12. Incubate the flask and slides at appropriate temperature and CO_2 level in a humidified incubator.
13. Stain the first slide after 24 hours and observe.
14. Stain the second slide after 40 hours and observe.
15. Stain the third slide after 64 hours and observe.
16. Harvest the supernatant after reacing approximately 70% infectivity.
17. Centrifuge in 50 ml tube at 4000 g or 8000 rpm for 10 minutes.
18. Aliquot supernatant in 0.5 ml volume.
19. Store at – 80 °C.

Titration of seed virus suspension

1. Thaw one aliquot of seed virus (0.5 ml volume).
2. Prepare 8 serial tenfold dilutions (10^{-1} to 10^{-8}) in MEM-10.
3. Distribute 100 µl of each virus dilution into one well of an 8 well tissue culture chamber slide.
4. Add 200 µl of BHK/MNA cells suspended in MEM-10 to each well.
5. Mix the cells and virus by gentle rocking the slide.
6. Incubate at appropriate temperature and CO_2 level in a humidified incubator for 40 hours.
7. Acetone fix and stain the slide using an immunofluorescence technique
8. Evidence of virus infection should be observed at 10^{-6} dilution of virus (so that virus stock suspension contains 1 x 10^{-6} infectious units per 100 µl)

Preparation of stock virus suspension

1. Infect 30 x 10^6 BHK/MNA cells with 10 x 10^6 infectious units of seed virus
2. Harvest the supernatant (after 24 hours) after the cells reach 100% infectivity
3. Distribute the supernatant into 0.5 ml aliquots
4. Store at – 80°C

Titration of stock suspension

1. Thaw one aliquot of the seed virus
2. Prepare 6 serial tenfold dilutions (10^{-1} to 10^{-6}) in MEM-10
3. Distribute 100 µl ml of each virus dilution into one well of an 8 well tissue culture chamber slide
4. Add 200 µl ml of BHK/MNA cells suspended in MEM-10 (concentration 1 x 10^5 cells per 100 µl) to each well
5. Mix the cells and virus suspension by gently rocking the slide
6. Incubate at appropriate temperature and CO_2 level in a humidified incubator for 20 hours
7. Acetone fix and stain the slide using immunofluorescence technique

Procedure for performing RFFIT

1. Heat inactivate (HI) human serum at 56°C for 45 minutes. Animal serum is HI at 56°C for 30 minutes.
2. Dilute the test sera in MEM-10. Serum end-point titration are routinely tested at 8 serial folds dilutions (1:5 to 1:390625).
3. Using an 8-well Tissue-Tek slide, use one slide per test sample. Alternately, serum can be screened per slide

End-point titration

1. Label slides with corresponding test sera number. Use 1 slide per test serum when determining end-point titer. The dilution factor is 8-serial five-fold.
2. Add 100 µl of MEM-10 to the first well using a microtiter pipette. This will be the 1:5 dilution of test serum.
3. Add 100 µl of MEM–10 to the seven other wells of the slide.
4. Add 25 µl of test serum to the first well (1:5 dilution). Mix the serum and MEM–10 thoroughly avoiding bubbles.
5. Transfer 25 µl of the 1:5 dilution well (serum MEM–10) to the second well (1:25 dilution) and continue to transfer 25 µl to each consecutive well up to the final dilution (1:390625), discarding 25 µl at the end.
6. Prepare all serum samples as described above.

Preparation of the control slide

1. Prepare a control slide with a reference serum control, a virus back titration and a cell control.
2. Add 100 µl of MEM–10 to the first well of the reference serum dilution on the left of the slide. This will be the 1:5 dilution of reference serum.
3. Add 100 µl of MEM–10 to the remaining wells of the reference serum dilution wells (1:25 – 1:625) and to the 3 wells of the virus back titration. The cell control well receives 200 µl of MEM – 10.
4. Add 25 µl of reference serum containing 2IU/ml to the 1:5 dilution well on the bottom left of the slide (well 1).
5. Mix thoroughly and transfer 25 µl of the 1:5 dilution of reference serum (well 1) to the 25 µl dilution well (well 2) and continue through to the 1:625 dilution well (well 4) discarding 25 µl at the end.

Preparation of the challenge virus and back titration

1. The amount of virus to be used in the test should be 50 FFD_{50}/100 µl.

2. Using MEM–10 as diluent for CVS–11, prepare a 50 ml centrifuge tube containing 50 FFD_{50}/100 µl. Allow 1.0 ml of the FFD_{50}/100 µl for each slide in the test. For example, if running 10-test serum, prepare 11 ml of CVS–11 in MEM–10 (10-test serum plus 1 control slide).
3. Make 2-serial 10-fold dilutions (1:10 and 1:100) of CVS-11 from the FFD_{50}/ 100 µl tube to give 5.0 FFD_{50}/100 µl and 0.5 FFD_{50}/100 µl, respectively. Label as vial–1 (1:10) and vial–2 (1:100) dilutions, add 900 µl of MEM – 10 to each vial. Transfer 100 µl of 50 FFD_{50}/100 µl to the 1:10 vial, mix thoroughly and discard the tip. Transfer 100 µl of solution from the vial– 1 to the vial– 2 and mix thoroughly.
4. Add 100 µl of the 0.5 FFD_{50}, 5.0 FFD_{50}, and 50 FFD_{50} of virus dilutions to sequential chambers
5. Add 100 µl of the virus preparation containing 50 FFD_{50}/100 µl to all chambers of the test sera and reference serum dilutions.
6. Incubate all slides for 90 minutes at 37°C in a humidified incubator with 5% CO_2.

Preparation of the BHK/MNA Cells

1. Prepare the cells just prior to use, 20 to 30 minutes before the end of the 90 minutes, even suspension of MNA/BHK cells in 10 ml of MEM 10%.
2. Adjust the cell count to give 5.0 x 10^5 cell/ml.
3. Add 0.2 ml of the 5.0 x 10^5 cell/ml to each chamber of the slides, starting with the cell control well on the bottom right corner of the control slide.
4. Incubate the slides for 20 hours at appropriate temperature and CO_2 level in a humidified incubator.

Fixation of slides

1. Decant the supernatant and remove the chamber slides from the slides.
2. Dip rinse in phosphate buffered saline (PBS).
3. Dip rinse in cold acetone (-20°C).
4. Fix for a longer 30-minute incubation in fresh –20°C acetone in a freezer.

5. Following 30-minute fixation. Remove slides from acetone and allow them to air dry at room temperature for 10 minutes.

Staining of slides

1. Add rabies conjugate (working dilution previously determined) to each chamber monolayer sufficient to cover the entire monolayer (approximately 100µl per well).
2. Incubate the slide in a humidity chamber (e.g., place slides on a tray with a moistened paper towel and covered with the lid of a 96 well microtiter plate) at 37°C for 30 minutes.
3. Following 30-minute incubation, decant conjugate from slide and rinse in 2X -PBS for 10 minutes each rinse.
4. Dip rinse in distilled water and place slides in a slide holder on cold packs for reading using a fluorescent microscope.

Interpretation

1. Each of the 8-well Tissue Tek slide chambers contains 25 to 50 distinct microscopic fields when observed at 160 to 200 magnification.
2. Observe 20 low-power (160-200X) microscope fields in each chamber, and count the number of fields which contain fluorescing cells. Begin to count fields starting in one of the corners of the well.
3. Read the control slide first. The cell control should have no fields with fluorescing cells, i.e. no virus infected cells. Of the 20 fields inspected in each well of the virus back titration, the 50 FFD_{50}/100 µl should have at least 18 to 20 positive fields, the 5 FFD_{50}/100 µl should have 10 to 20 positive fields, and the 0.5 FFD_{50}/100 µl should have less than 10 positive fields.

An example of the number of positive fields observed on a reference and test serum slide are given below:

- **Control slide:** 20 fields are observed per well and the number of positive fields (fields with fluorescing foci) are recorded on the RFFIT data sheet.
- **Test serum:** The test serum slides are observed, and the number of positive fields are recorded on the RFFIT data result sheet.
- **Calculations:** Using the control slide and test serum values from the previous example, the test serum end-point titre and international units can be calculated.

Determination of 50% end point titres of serum

The serum neutralisation end-point titer is defined as the dilution factor of the highest serum dilution in which there is a 50% reduction in the number of fluorescing foci. The 50% end point titre of serum can be made by determining the number of fluorescing foci at each dilution, and then use the cumulative totals in the Reed-Muench formula:

1. Calculate the percentage of fields containing infected cells as per Table 21.

2. Using the method of Reed & Muench, calculate the difference between the logarithm of the starting dilution and the logarithm of the 50% end-point dilution (difference of logarithms) from the formula:

$$\left(\frac{50\% - Infectivity\ next\ below\ 50\%}{Infectivity\ next\ above\ 50\% - Infectivity\ next\ below\ 50\%}\right) \times logarithm\ of\ dilution\ factor$$

   In this example the starting point dilution (the dilution showing an infectivity next below 50%) is 625, the dilution factor is 5. Hence, the difference in logarithm is:

$$\frac{50 - 0}{60 - 0} \times 0.69897 = 0.582475$$

3. Since the infectivity is increasing as the dilution increases, the 50% end point dilution is higher than the starting point dilution and is calculated by adding the difference of logarithms as follows:

$$Log\ (reciprocal\ of\ 50\%\ end\ point\ dilution) + difference\ of\ logarithms$$

   In this example,

$$2.79588 + 0.582475 = 3.38\ (approx)$$

4. Hence, log (50% end point dilution) = - 3.38 and the 50% end – point dilution = $10^{-3.38}$ = (1:2399)

Table 21. Calculation of percentage of fields containing infected cells.

Serum dilution	No.of fields containing infected cells	Fields containing infected cells	Field containing no infected cells	% of fields containing infected cells
1:5	0/20	0	88	0/88 = 0
1:25	0/20	0	68	0/68 = 0
1:125	0/20	0	48	0/48 = 0
1:625	0/20	0	28	0/28 = 0
1:3125	12/20	12	8	12/20 = 60
1:15625	20/20	32	0	32/32 = 100
1:79125	20/20	52	0	52/52 = 100
1:390625	20/20	72	0	72/72 = 100

Determination of the potency of test serum in international units (IU) per ml

The results of the RFFIT can be expressed as a serum titer or in International Units (IU) of antibody. In either case, a reference serum of known titer is required. When the results are to be reported as a titer, the reference serum is used as a control to insure the sensitivity of the test. It should demonstrate approximately the same titer determined in previous tests.

When the test serum results are expressed in IU, the calculation is dependent on the number of IU in the reference serum. The reference serum is diluted to contain 2 IU/ml and tittered along with the test serum. The ED_{50} titers of the reference serum and the test serum are then related in the following formula for calculation of IU/ml in the test serum:

$$Test\ serum\ (IU/mL) = \frac{End - point\ titer\ of\ the\ test\ serum}{End -\ \ point\ titer\ of\ the\ reference} \times 2$$

2.2.8 Rabies Tissue Culture Infection Test (RTCIT)

Principle. Rabies Tissue Culture Infection Test (RTCIT) is based on the ability of rabies virus to infect some cell lines in vitro. Murine neuroblastoma 2A (MNA) has been shown to be the most sensitive cell line for this method. However, Baby Hamester Kidney (BHK) cell line is easy to maintain and commonly employed for RTCIT. Various rabies suspected samples (brain tissue, saliva, or CSF) can be tested using RTCIT. The samples are processed and added to the cell culture. The growth of the virus in the cell culture can be ascertained by a sensitive technique such as DFAT.

Reagents and Equipment

1. BHK/MNA cell line
2. Sterile tissue culture tubes
3. MEM Medium + 10% FCS
4. TPVG solution
5. Humidified CO_2 incubator
6. Inverted fluorescence microscope
7. Biosafety cabinet (Class 2)

Brain tissue preparation

1. Take approximately 0.5 gm of tissue from the hippocampus of the brain.
2. To this add 5 ml of PBS containing antibiotic to make a 10% w/v suspension.
3. Vortex mix vigorously and allow to settle for at least 1 hour at 4°C.
4. Withdraw the upper clear layer and dilute 10-fold with MEM medium supplemented with 10% fetal calf serum.
5. Add 100 units/ml penicillin and 50µg/ml of streptomycin. Shaken or vortex mix vigorously and allow to settle for at least 1 hour at 4°C.

CSF. Can be inoculated directly after treatment with antibiotics.

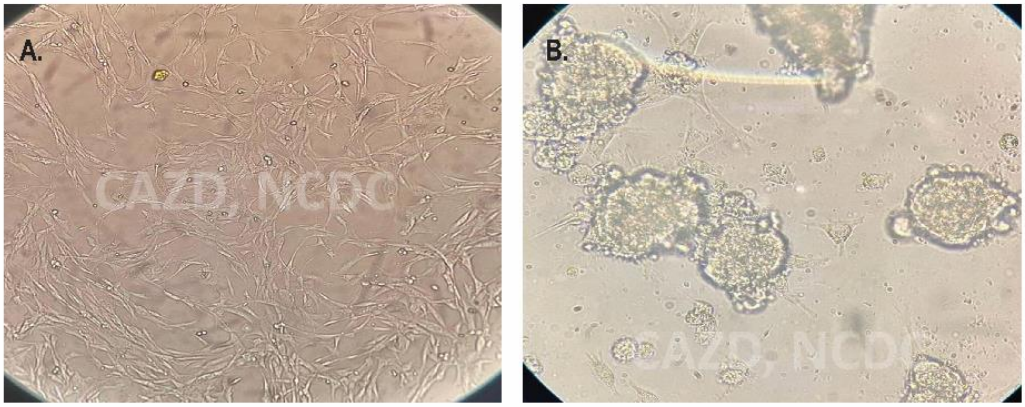

Figure 18. Baby Hamster Kidney 21 cell lines, A) no cytopathic effect; B) cytopathic effect seen

Cell suspension. BHK/MNA cells are grown for maintenance at 35 – 37°C in 25 cm^2 plastic culture bottles/tubes containing 5 ml of MEM-10 media. For inoculation of samples, the subculture is done in 25 cm^2 culture tube with 5 ml MEM supplemented with 10% FCS media.

Procedure

1. For each specimen take 2 ml of cell suspension in a culture bottle.
2. Add 4 ml of the 1% suspension (in MEM + 10% FCS) to each of the tubes containing cells.
3. With each batch use a known positive control and negative control.
4. Incubate the tubes at 37°C for four days.
5. The tubes should be checked daily for contamination.
6. After four days of growth, take Teflon slides and keep in the acetone for 2–5 minutes at room temperature.
7. Air dry the Teflon slides in laminar flow.
8. The fluid is separated in sterile tubes from infected cell culture tubes and stored at –70°C or liquid Nitrogen. Approximately 0.5 ml fluid is left in the cell culture tube.
9. Gently tap the tubes and by repeated forceful pipetting attempt to detach the cells.
10. Inoculate the resuspended cells with the help of the Pasteur pipettes in the wells of Teflon coated slides including the test samples cells, positive control and negative control.
11. Air dry the drops for 2–3 hours in the laminar flow.

12. Immerse the slides in Coplin jar containing chilled acetone in a deep freeze at –20°C to –25°C for 4 hours to overnight.
13. Take out the slides from chilled acetone and dry at room temperature for 20 minutes.
14. Pour 1 – 2 drops of rabies anti-nucleocapsid FITC conjugate with the help of Pasteur pipette.
15. Place the slides in moist chamber at 37°C for 30 minutes.
16. Wash the slide in 0.01 M phosphate buffer saline (pH 7.5) twice for 5 minutes each.
17. After this give two washings with distilled water for 5 minutes each.
18. Take out the slides from distilled water and dry at room temperature.
19. Mount in 50% buffered glycerol.
20. Examine the slides under a fluorescent microscope.

2.2.9 Mouse Inoculation Test (MIT)/Biological Test (BT)

Principle. The growth of rabies virus, just like any other virus takes place only in living tissues. These may be animals, developing chick embryo or tissue culture. Rabies virus has been found to be pathogenic to all mammals when given by intracerebral route. However, the animals commonly used for experimental studies of rabies are the mouse, rat, guinea pigs, hamster, rabbit, and dog. The white laboratory mice are susceptible to infection and are easy to handle in routine laboratory examination of specimens. Suckling mice are more susceptible than the adult but a regular supply of these may not be possible in all the laboratories. The presence of rabies virus in any specimen can be detected by intracerebral inoculation of specimen into mice and observing the animals for sufficient period for the development of sickness and death due to rabies.

Reagents and Equipment

1. 2% Normal horse serum (NHS) in distilled water.
2. 3-4 weeks old mice and mice cages.
3. Centrifuge.
4. Centrifuge tubes.
5. Pestle mortar, tissue grinder or omni mixer.

6. Scissors and forceps.

Procedure

Brain.

1. Aseptically collect 3-4 gms or pieces of areas of the brain, i.e. hippocampus, cerebrum, cerebellum, medulla, pons etc.
2. By weighing calculate the exact weight of the tissue pieces.
3. Homogenise these pieces to make a fine paste with the help of a pestle mortar/tissue grinder/ electric grinder/omni mixer.
4. Add enough chilled distilled water containing 2% inactivated normal horse serum (serum should be collected from horses not vaccinated against rabies) to make a 10% suspension. Mix thoroughly while adding the diluent.
5. Transfer to a sterile 15 ml centrifuge tube.
6. Centrifuge at 1000-1500 rpm for 5 minutes.
7. Collect the supernatant with the help of pipette in a Bijou bottle/half tube kept in ice bath.
8. Add 100 units/ml penicillin and 50 µg/ml of streptomycin and keep for 30 minutes.
9. Inoculate into 3-4 weeks old white mice by intracerebral route with ¼" long 26/27 gauze needle in 0.03 ml dose/mice. Use at least eight mice for each inoculum.
10. Observe for 21 days for signs like roughening and loss of lustre of the fur, tremor, hyper-excitability, arching of the back, convulsions, paralysis of the hind legs and death.
11. Check any mouse dying after 72 hours by rabies antigen.

Salivary Gland

Grind the salivary glands with the help of sterile sand or sterile coarse glass powder in a pestle and mortar and repeat the processes 5 to 11 as described above.

2.3 Algorithms for laboratory diagnosis of rabies

Algorithm for laboratory diagnosis of rabies in antemortem and postmortem samples from humans is given in Figure 19 and Figure 20, respectively.

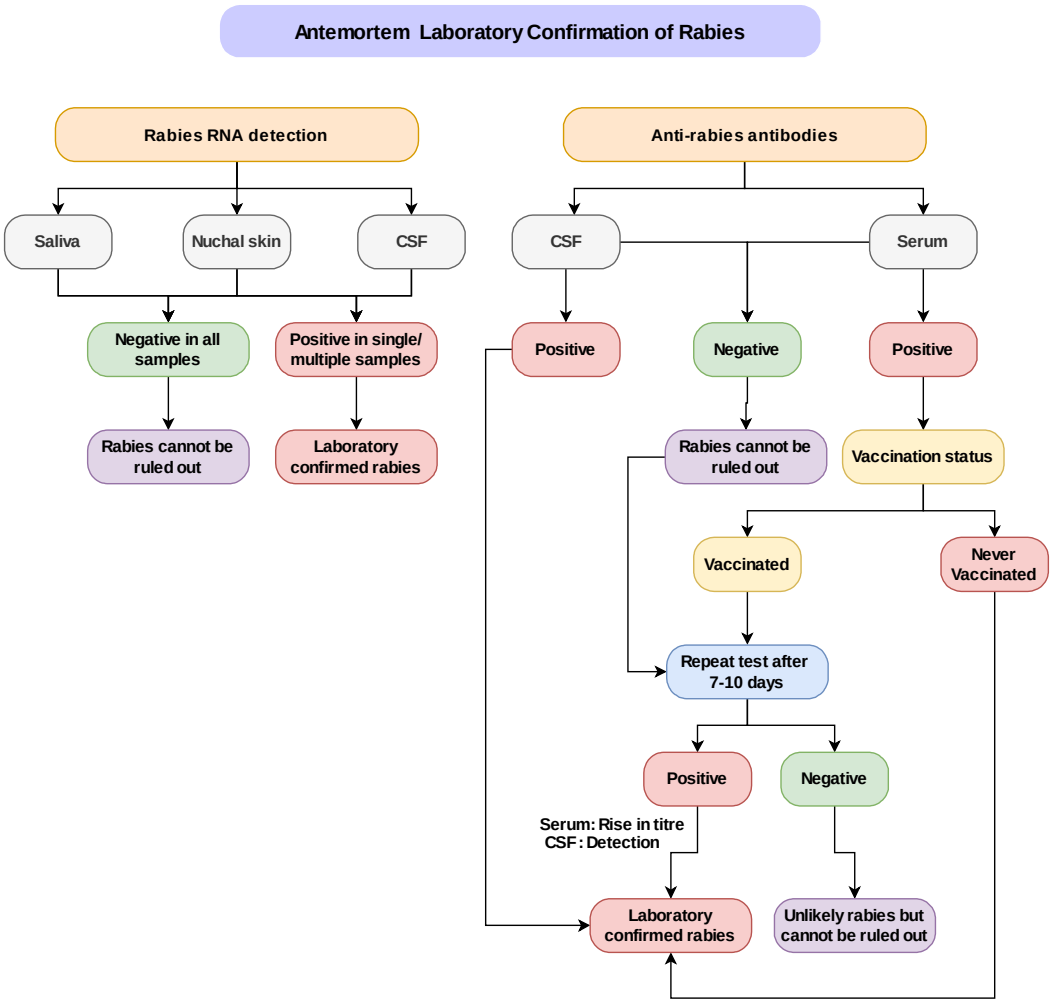

Figure 19. Algorithm for antemortem laboratory diagnosis of rabies in humans [23]

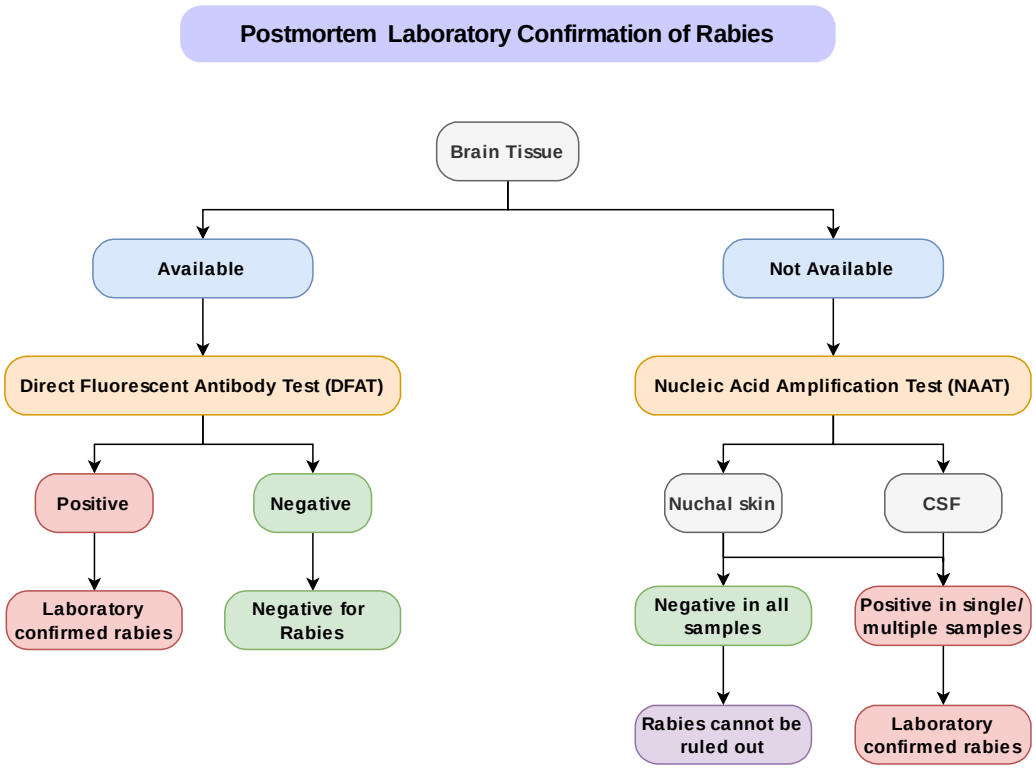

Figure 20. Algorithm for postmortem laboratory diagnosis of rabies in humans [23]

BIOSAFETY IN RABIES LABORATORY

Safety in rabies laboratory demands utmost importance because of the lethality of the disease and the property of the virus to gain entry into the body through a discontinuity in the skin howsoever minute it may be or through the mucous membrane of the worker. Extreme caution has to be exercised in the laboratory to prevent contracting infection with rabies virus which is present in high titres in various organs/tissues e.g. brain, salivary glands, spinal cord, as well as in almost all body fluid e.g. saliva, urine, tears etc. all of which can come in close contact with the laboratory worker. Though transmission through aerosols is possible it is not a usual mode of transmission of infection, yet its probability should always be kept in mind in the laboratory where high concentration of virus can result due to handling and processing of large number of specimens.

Laboratory workers should be aware of the dangers of working with virus and should also know how to tackle the emergencies originating from accidents in laboratories. This occupational risk can be negated through safe laboratory design, safe equipment, protective clothing, thorough and repeated training, appropriate pre and post exposure immunization and above all good laboratory practices which remains the fundamental of safety and even now, irreplaceable. Attention to and proficient use of work practices, safety equipment and engineering controls are also essential. Various components of laboratory safety are discussed here under in brief.

3.1 Laboratory design and risk assessment

Laboratory exclusively utilized for rabies diagnosis and entry to it should be restricted to only those persons who are vaccinated and have seroconverted. Within the laboratory, areas should be clearly demarcated for different procedures. Risk assessment is an important responsibility for laboratory in-charges of healthcare settings. The information identified by risk assessment will provide a guide for the selection of appropriate biosafety levels for various class of activities/tests done (Table 22).

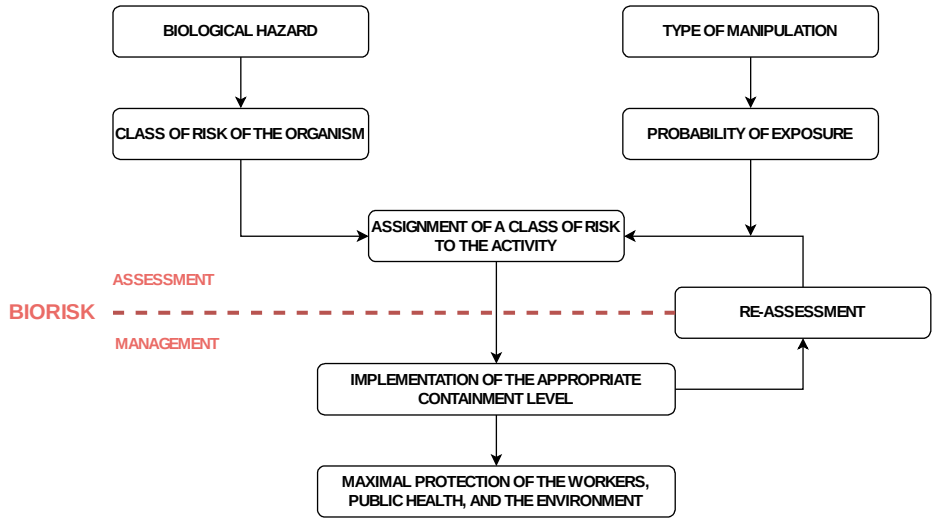

Figure 21. Bio-risk assessment and Management in Rabies laboratory

Table 22. Biocontainment level and recommended precautions for various rabies laboratory techniques

Laboratory Technique	Risk assessment*	Minimum Bio-containment level**	Type of PPE
ELISA for Anti-Rabies Antibody titre estimation	Low	BSL -2 lab with BSL-2 practices (no biosafety cabinet required)	Laboratory Coat/Gown, Gloves
FAT	Low to Moderate	BSL- 2 lab with BSL 2 practices	Laboratory Coat/ Gown, Gloves
RADT	Low to Moderate	BSL -2 lab with BSL-2 practices	Laboratory Coat/Gown, Gloves, eye protection
DRIT	Low to Moderate	BSL-2 Lab with BSL-2 practices	Lab Coat/Gown, Gloves
NAAT	Low to Moderate	BSL-2 Lab with BSL-2 practices	Lab Coat/Gown, Gloves, eye protection glasses, aerosol containment carriers required for centrifugation of infectious materials.
RTCIT/ RFFIT/ Necropsy	Moderate to High	BSL-2 lab for virus propagation with BSL-3 practices	Safety glasses, face shield, N95 mask closed – front gown, dedicated footwear (boots) and heavy rubber gloves

*Risk assessment based on activity exposure risk, technical proficiency, and PrEP vaccination status
**Additional precautions (such as BSL-2 with BSL-3 practices) should be considered when working with lyssaviruses other than rabies virus and while handling large quantities

3.2 Safety equipment

Air borne rabies infection has been demonstrated hence all procedures while handling potentially infectious material (live virus) which generate aerosols (high speed mixing, centrifugation, pipetting etc.) should be carried out in biosafety hoods with laminar flow of air under negative pressure. Centrifugation and mixing should be carried out in tightly closed containers. The handling/manipulations of known or potentially infectious materials (live virus) should be done within the biosafety cabinets instead of open benches. Certifications of operational integrity of containment equipment (biosafety hoods) by service provider should be done at time of installation and annually thereafter. Re-certification after repairs or filter changes, whenever moved

3.3 Training of manpower and authorised personnel

The technical persons working in the laboratories should be sufficiently trained in the handling of pathogens. These persons should receive training either in an already established rabies laboratory or under direct supervision of the trainer. The supporting staff should also be made well conversant with the handling and proper disposal of infected material. There are various instances where the laboratory worker has to be extra cautious and have to select appropriate PPE and safety equipment while dealing with any material in rabies laboratory especially where specimens may be potentially having rabies virus unless proved otherwise. “RESTRICTED AREA” and “AUTHORISED PERSONNEL Entry (Vaccinated Persons only)” signboards should be prominently displayed at the entry of the laboratory.

3.4 Good Laboratory Practices

Human error, poor laboratory practices and misuse of equipment cause the majority of laboratory accidents and related infections. It is emphasized that universal precautions and good laboratory practice (GLP) is fundamental to laboratory safety and can only be supplemented by safety equipment. Hazardous chemicals like Beta-propiolactone used for inactivation of rabies virus and production of vaccines are highly carcinogenic and should always be handled with care under hood. Transfer of infectious material/ hazardous chemical should be carried out by use of propipettes.

3.5 Collection and transport of specimens

All samples from suspected Rabies cases should be considered potentially infectious. All specimens should be collected in a primary leak proof container and must be securely sealed before transport in designated laboratory.

3.5.1 Antemortem specimens

a. **Human.** The person collecting the samples like serum, saliva, CSF, skin biopsy etc from suspected Rabies case should handle them carefully with all aseptic and standard precautions and appropriate PPE.
b. **Animals.** The animals must be restrained before collection of antemortem specimens to avoid any chances of a bite. All samples should be collected carefully with all aseptic and standard precautions and appropriate PPE.

3.5.2 Postmortem specimens

These include brain, spinal cord, salivary glands etc. all of which should be handled carefully and treated as highly infected material.

Protective clothes like laboratory coats, rubber aprons, thick rubber gloves, mask and plain goggles should be used at the time of autopsy as well as during handling of infectious material/ collection of samples.

3.6 Dissecting equipment and glassware

Instruments like scissors, forceps etc. used for collection and processing of postmortem specimens should be sharp to avoid undue pressure on gloves. No niched glassware is permitted.

3.7 Disinfection and disposal

After completion of the work, the working area should be cleaned with suitable disinfectant and waste should be segregated and treated to make them non-infectious (autoclaving/chemical disinfection/incineration) as per Central Pollution Control Board

"Guidelines for Management of Healthcare Waste as per the Biomedical Waste Management Rules, 2016"[24]. Dissecting Equipment and Glassware should be properly sterilized after the work is done. The carcass of the small animals should be placed in plastic leakproof bags, sealed and incinerated. The bedding of infected animals may also be incinerated.

3.8 Pre-exposure prophylaxis

All the persons working in rabies laboratory and handling infectious material from suspected rabies case, rabies virus or rabid animals should be administered pre-exposure prophylaxis to protect them against contracting the disease.

Total three doses are required for Pre-exposure prophylaxis. In case of IM route one full vial to be given on days 0, 7 and either day 21 or 28. In case of ID route 0.1 ml to be given on days 0, 7 and either day 21 or 28.

High risk groups should have their neutralizing antibody titres checked every 6 months during the initial two years period after the primary vaccination. If it is less than 0.5 IU/ml a booster dose of vaccine should be given. Subsequently, sero-monitoring is recommended every two years. Vaccine-induced immunological memory persists in most cases for years, a booster would be recommended only if rabies virus neutralizing antibody titres have dropped to less than 0.5 IU/ml.

3.9 Post-exposure management

Every effort should be made to avoid occurring of mishaps in the laboratory due to negligence by strictly following the safety precautions. Spill management kit should be readily available in the laboratory However, in case of accident where the spillage of virus/ infected material from suspected rabies cases occurs following action may be initiated:

a. Working area should be immediately and meticulously cleaned by using proper disinfectants.
b. The broken glass pieces should be picked up with the help of forceps and discarded in appropriate containers for disinfection. One must be cautious about picking of fine

pieces of glass which can sometimes penetrate even thick rubber gloves and spill must be managed as per spill management protocol of the laboratory.

c. Any accidental inoculation or even suspicion of the same onto a person must be immediately reported to the supervisor/ health care authorities.

d. The soiled area on the body must be immediately washed with plenty of soap and water to wash off as much of the virus as is possible. Care must be taken not to increase the existing trauma. However, if the spillage is only in the eyes, wash the eyes thoroughly and repeatedly with plenty of water.

e. On exposed sites other than eyes, apply an antiseptic which is readily available in the laboratory. These include any quaternary ammonium compound, spirit, ethanol, tincture, iodine, savlon, dettol etc.

f. As far as possible suturing of the wound be avoided. If it is inevitable, minimum number of stitches should be applied to bring the skin in apposition. Local and parenteral administration of antibiotics shall depend upon the condition of the wound.

g. The post exposure immunization shall depend upon the immunization status of the person. Combined serum and vaccine therapy shall be indicated in persons depending upon type of exposure who are not immunized or who have not responded satisfactorily to previous immunization against rabies. Two booster doses depending upon type of exposure of anti-rabies vaccine shall be needed by those who have been previously immunized satisfactorily.

QUALITY ASSURANCE IN RABIES LABORATORY

Quality assurance is the sum of all those activities that ensure reliability and accuracy of the emergent results or data. This automatically ensures a good credibility of the testing laboratory and enhances user's confidence in the laboratory results.

4.1 Components of Quality Assurance

Quality assurance has two components: Internal Quality Control (IQC) and External Quality Assessment (EQAS). IQC monitors the daily precision and accuracy of methodologies, personnel, and instruments. EQAS maintains long term accuracy (Table 23).

4.2 Factors influencing quality

Many factors can influence the quality of the laboratory results. These can be grouped into pre-analytical, analytical and post-analytical.

i) Pre-analytical factors. Of the various pre-analytical factors that influence the quality of the laboratory results, the knowledge of right investigation to perform, right selection of sample, right time of collection and requirement of sequential samples is absolutely must. Subsequent to this is collection of the right clinical specimen in adequate quantities using right technique. If the sample cannot be immediately processed it should be stored and transported using recommended methods. Needless to mention that right labelling of the specimen and sending it to the right laboratory with prior intimation and duly filled clinical proforma which performs that particular investigation is very essential for good quality reporting.

Table 23. Quality controls required for various rabies laboratory tests for each batch

Test	Positive control	Negative control
Seller's stain	Impression of mouse brain infected with CVS/ brain infected with rabies virus (laboratory confirmed)	Normal mouse brain impression
DFAT	Impression of mouse brain infected with CVS/ brain infected with rabies virus (laboratory confirmed)	Normal mouse brain impression
DRIT	Impression of mouse brain infected with CVS/ brain infected with rabies virus (laboratory confirmed)	Normal mouse brain impression
ELISA	Positive controls provided in the kit One sample with known titre (inhouse)	Negative control provided in the kit
RFFIT	Control slide Titered reference serum control Virus reference serum control Virus back titration Cell control	-
NAAT	Positive Control (inactivated rabies virus/ vaccine strain)	-

ii) Analytical factors. The staff in the rabies laboratory should be trained & committed. Good quality reagents and reliable equipment should be used including positive and negative controls with each test run Standard operating procedures (SOPs) should be followed for all the tests. Details on collection, storage and transport of clinical samples, selection and timing of appropriate clinical specimen, inoculation/test procedures, risk assessment and biosafety levels for various tests, precautions, validation, reporting and interpretation of results used in Rabies laboratory should be available in SOPs for various laboratory techniques. Top management should authorize SOPs and revised as and when required/ every two years. It should not only be available to all on the laboratory work benches, but instructions given there in should also be strictly adhered to, to assure uniformity. Record keeping/ Documentation of sample submitted/ working sheets/ analysis that is done in the laboratory should be enforced.

iii) Post-analytical factors. Of the post-analytical factors that can influence quality, right recording of the results is most important. Right interpretation of the results should be written on the report. Reporting of the laboratory results is the most crucial aspect in quality. Test reports should be given on a pre-designed, pre-tested format, written legibly or typed, highlighting the abnormal findings and interpreted in a simple language. The report given to the patient about a laboratory test should be short, clear, unambiguous and should not leave any room for making errors in its interpretation.

4.3 External Quality Assessment Scheme (EQAS)

An EQAS organizing laboratory can run the programme successfully by gaining the confidence of participating laboratories, by maintaining confidentiality of the individual reports, avoiding provocative statements and identifying unsatisfactory performers in groups. In addition, it should provide educational opportunities/ refresher trainings to participating laboratories. It should act as adviser and guide and not a fault-finding agency.

The purposes of a quality assessment programme are:

- To provide assurance to both physicians and the general public that laboratory diagnosis is of good quality
- To assess and compare the reliability of laboratory performance on a national scale
- To identify common errors
- To encourage the use of uniform procedures
- To encourage the use of standard reagents
- To stimulate the implementation of internal quality control programme
- To network the laboratories performing or intending to perform rabies diagnosis

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ANNEXURES

Annexure 1. Buffers, Reagents, and Stains

1. Sellers Stain

Composition.

Solution A

Methylene blue* CI - 52015	1.0 gm
Methanol (acetone free)	100 ml

Mix by using a pestle and mortar and store in a tightly stoppered bottle at 4°C
*Any make of dyes can be used if the dye content is not less than 85%

Solution B

Basic fuchsin* CI - 42510	1.0 gm
Methanol (acetone free)	100 ml

Mix by using a pestle and mortar and store in a tightly stoppered bottle at 4°C
*Any make of dyes can be used if the dye content is not less than 92%

Working stain.

1. Mix 2 volumes of Solution A and 1 volume of Solution B in properly stoppered container.
2. Keep the stain for 24 hours before use.
3. Stain can be stored for a longer period if evaporation of methanol is prevented.

Adjustment of Stains

With properly prepared stain usually no adjustment is required. However, sometimes the smear may stain too red or blue. Adjustment of the stain may be made by adding methylene blud or basic fuchsin solution. It may be standardised by staining slides for known positive brain.

2. 10% formal saline

Composition.

Formalin	10 ml
Physiological saline	90 ml

Prepare freshly just before the use.

3. Zenker's fluid

Composition.

Potassium dichromate	2.5 gms
Mercuric chloride	5.0 gms
Distilled water	100 ml

4. Bouin's Fixative

Composition.

Saturated solution of picric acid (1.2%)	70 ml
Formalin (40%)	25 ml
Glacial acetic acid	5 ml

5. Phosphate Buffered Saline (pH 7.5)

Composition.

NaH_2PO_4	0.4 gms
Na_2HPO_4	2.5 gms
NaCl	8.5 gms

Dissolve in 1000 mL of distilled water

6. 50% Buffered Glycerol

Composition.

0.2M PBS (pH 8.5)	50 mL
Glycerol (neutral)	50 mL

7. 2% Normal Horse Serum Distilled Water

Composition.

Normal horse serum (sterile)	10 mL
Distilled water (sterile)	490 mL
Mix the contents and add the following	
Penicillin G sodium	1,25,000 units
Streptomycin sulphate	50 mg

Distribute in 50 mL quantity in sterile screw-capped vials, store at 4°C

Annexure 2. National Referral/Regional Laboratories for Rabies Diagnosis in India (Human and Animal)

*List of existing Laboratories for diagnosis of Human Rabies**

S.N.	Name	Contact Details	Address
1	Centre for Arboviral and Zoonotic Diseases, National Centre for Disease Control, Delhi	+91-11-20832481 nicdzoonosis@yahoo.com	22, Sham Nath Marg, Civil Lines, New Delhi, Delhi 110054
2	National Institute of Mental Health and Neuro Science, Bangaluru S , Karnataka	+ 91-080-26995201, 26995202	Hosur Rd, near Bangalore Milk Dairy, Lakkasandra, Laljinagar, Wilson Garden, Bengaluru, Karnataka 560029
3	AIIMS, jodhpur, Rajasthan	+91-87645 05002	Basni Industrial Area, MIA 2nd Phase, Basni, Jodhpur, Rajasthan 342005
4	Infectious Disease and Beliaghata General Hospital, Kolkata, Govt. of WB	+91- 033 2353 6071 /2371/0033	57, Beleghata Main Rd, Subhas Sarobar Park, Phool Bagan, Beleghata, Kolkata, West Bengal 700010
5	Viral Research and Diagnostic Laboratory, Govt medical College, Amritsar, Govt. of Punjab	+91-183 242 6918	Medical Enclave, Amritsar, Punjab 143001
6	Regional Institute of Medical Science, Imphal, Manipur	+91-385 241 4629	Lamphel Rd, Lamphelpat, Imphal, Manipur 795004
7	State Public Health and Clinical Laboratory, Trivandrum, Govt. of Kerala	+91-11-23978046	Red Cross Road, Near General Hospital Road, Rishimangalam, Pattoor, Thiruvananthapuram, Kerala 695024
8	Rangaraya Medical College, Kakinada, Govt of Andhra Pradesh	+91-884 236 3401	Pithampuram, Road, Kakinada, Andhra Pradesh 533001
9	Haffkine Institute for Training, Research & Testing, Mumbai, Maharashtra	+9122-24160947 ,24160961 ,24160962	40/W, Acharya Dhonde Marg, Parel Village, Parel, Mumbai, Maharashtra 400012

*Network of laboratories is being expanded under NRCP Program

*List of existing Laboratories for diagnosis of Animal Rabies**

S.N.	Name of institute	Contact Details	Address
1	Centre for Arboviral and Zoonotic Diseases, National Centre for Disease Control, Delhi	+91-11-20832481 nicdzoonosis@yahoo.com	22, Sham Nath Marg, Civil Lines, New Delhi, Delhi 110054
2	KVAFSU-CVA Rabies Diagnostic Laboratory, WOAH (formerly OIE) Reference Laboratory for Rabies Department of Veterinary Microbiology, Veterinary College, Bengaluru Veterinary and Animal Sciences University.	09449992287/ 080-29532287 kisloor@gmail.com edlkrafsuera@gmail.com	Hebbal, Bengaluru-560024, Karnataka

S.N.	Name of institute	Contact Details	Address
3	Institute of Animal Health and Veterinary Biologicals	080 2341 1502, diriahvb@gmail.com	Bellary Rd, Bengaluru, Karnataka 560024
4	ICAR - National Institute of Veterinary Epidemiology and Disease Informatics	080 2309 3110, director.nivedi@icar.gov.in	Ramagondanahalli, Post Box No. 6450, Yelahanka, Bengaluru, Karnataka 560064
5	Tamil Nadu Veterinary and Animal Sciences University	9940534047, tirumurugaan.k.g@tanuvas.ac.in	Madras Veterinary College Campus Chennai- 600 007, Tamil Nadu
6	State Institute for Animal Diseases (Department of Animal Husbandry, Kerala)	9446557186, swapnasusan2003@yahoo.co.in	Pacha P.O., Palode, Thiruvananthapuram-695 562, Kerala
7	College of Veterinary and Animal Sciences, Kerala Veterinary and Animal Sciences University, Mannuthy	9447668796, vinodkumar@kvasu.ac.in	Mannuthy, Thrissur, 680651 Kerala
8	College of Veterinary and Animal Sciences, Kerala Veterinary and animal Sciences University, Pookode	09495014780, prasanna@kvasu.ac.in	Pookode, Wayanad Kerala- 673576
9	Scientific and Technical Manager Mission Rabies Office	9972449007, gowri@missionRabies.com	Flat# B-C2, Veterinary Hospital Complex,Tonca, Miramar, Panaji, Goa 403002
10	Bombay Veterinary College, Maharashtra Veterinary and Animal Sciences University.	9167493932, rpharande@gmail.com	Parel, Mumbai-400012 Maharashtra
11	College of Veterinary Science & A. H. Anand Agricultural University	9228309371, bbbhanderi@aau.in	Anand -388 110, Gujarat
12	Guru Anga Dev Veterinary and Animal Sciences University	9888466676, cksingh@usa.net	Ludhiana, Punjab – 141002
13	ICAR- Indian Veterinary Research Institute (IVRI)	9897806926, karam.singh@rediffmail.com	Izzatnagar – 243122, Bareilly, UP
14	College of Veterinary Science Assam Agricultural University	94355-58695, duttajyotib@gmail.com	Khanapara campus, Guwahati - 781 022, ASSAM
15	Disease Diagnostic Unit, Dept. of Animal Husbandry, State Govt. of Goa	+91-832 243 7245	Pashusamvardhan Bhavan, MG Rd, patto, Panaji, Goa 403001

*Network of laboratories is being expanded under NRCP Program

Annexure 3. Sample Requisition Proformas

Government of India
NATIONAL CENTRE FOR DISEASE CONTROL
22-SHAMNATH MARG, DELHI-110054
Centre for Arboviral and Zoonotic Diseases
WHO COLLABORATING CENTRE FOR RABIES EPIDEMIOLOGY

Proforma for Anti Rabies antibody estimation (Human)
(Kindly send 2ml of serum/5ml of clotted blood in a plain vial)
All tests done free of cost

Hospital Name
Name
Sex
Residential address

CR/Reg.No
Age

Address/Area where person is bitten:

Date of admission
Fate of binning animal

Biting animal : Pet/Stray dog/Any Other
Alive/Dead/Unknown/Clinically rabid/ Laboratory confirmed rabid

Number of persons bitten by animal
Date of bite No. of bites
Type of bites: Superficial / Deep
Management of Wound
Date

Site of bite
Category of Bite: I II III
Washed with soap/water Antiseptic / Cauterization/
Antirabies serum around wound/ Stitches/Nil

Who advised you to come to anti-rabies clinic
Self/Relative/Health worker/ Neighbour/ Friend/Previously treated patient

Treatment given at the anti-rabies clinic	Details	Date
Wound washed in the clinic	Yes/No	
Local instillation of ARS	Yes/No	
Wound suturing	Yes/No	
Antiseptic/Cauterization	Yes/No	
Route of vaccine	ID/IM	
Any deviation from Schedule		
Vaccine (Brand name and schedule details		
Vaccination Document Available	Yes/No	

Clinical Symptoms	Details	Date of onset
Fever		
Local paraesthesia		
Breathlessness		
Paralysis		
Convulsions		
Hydrophobia		
Any other relevant details		
Treatment given		
Any other Close Contacts of hydrophobia case		
Date of Death		

Previous History :-

P/H/O IMMUNIZATION

(a) **Immunized**

(b) **Incomplete Immunized / No documents Available**

(c) **Unimmunized**

Specimens of suspected case given to laboratory	Collection Date	Transportation Date
Blood		
CSF		
Skin biopsy		
Saliva		
Corneal smear		
Brain		
Any other		

Radiological investigations (CT/MRI)

Remarks

Doctor's Name & Signature with seal

NATIONAL CENTRE FOR DISEASE CONTROL

(DIRECTORATE GENERAL OF HEALTH SERVICES)

22, SHAM NATH MARG, NEW DELHI – 110054

CENTRE FOR ARBOVIRAL AND ZOONOTIC DISEASES

Proforma For Anti Rabies Antibody Titer Estimation(Dogs & Cats)

Name of the Hospital/Clinic sending the sample:

Name of the Veterinarian: Contact Details:

Details of Animal Owner

Name: Contact Details:

Adress

Details of Animal Microchip No.

Species of the animal: Place of Birth:

Type: Pet/Stray Age/DOB: Weight:

Sex: Breed:

Colour: Hospital ID:

History of Rabies in mother or siblings Yes/No/Unknown

If yes, Specify?

Purpose of visit to the Hospital/Clinic

Whether the animal is registered under Municipal Corporation : Yes / No

Deworming Done : Yes / No. If Yes, When was the last given

Clinical Symptoms in Dogs	Details	Date of Onset
Change in Behavior		
Off feed:		
Fever:		
Weight loss:		
Paralysis:		
Anorexia:		
Diarrhoea/Vomitting:		
Date & Time of death:		

Details and address of person/animal bitten by suspected rabid dog:	Date of Bite	Whether anti-rabies treatment started or not in them:

Vaccination Status : Vaccinated/Not Vaccinated/Irregularly vaccinated/unknown

Name of vaccine (ARV of any other vaccination)	Date	Route of Administration	If ARV, Vaccination whether pre/post-exposure prophylaxies.	Any other details

Date of collection of serum/blood :

Any bite history or being bitten by other animals (if Yes mention)

Date: Species : Site of Bite:

Any symptoms:

Signature of the Veterinarian

Date

NATIONAL CENTRE FOR DISEASE CONTROL
(DIRECTORATE GENERAL OF HEALTH SERVICES)
22, SHAM NATH MARG, NEW DELHI – 110054

CENTRE FOR ARBOVIRAL AND ZOONOTIC DISEASES

WHO COLLABORATING CENTRE FOR RABIES EPIDEMIOLOGY

Proforma for a case of Hydrophobia (Human)

Name of Hospital:

Sr. No.: Name:

Age: Sex:

Complete Address

Date of admission: **Date of animal bite:**

Animal species **Dog/Cat/Monkey/Mongoose/Others specify**

Type: Pet/Stray **Fate of animal: Alive/Killed/Unknown**

Bite : Provoked/unprovoked/ not known **Outcome in other persons bitten :Alive/Dead/Not known**

No. of other persons bitten by same animal within one week of the bite :

Type of Exposure

1. **Licks on intact skin**
2. **Single or multiple bites with bleeding**
3. **Licks on broken skin**
4. **Minor scratches or abrasions without bleeding**
5. **Nibbling of uncovered skin**
6. **Contamination of mucus membrane with saliva**

Duration between bite & local treatment of wound 0/1/2/3/4/5/6/7/More days

Local treatment taken before coming to anti-rabies clinic Yes/No/Not known

If Yes, tick whichever is applicable Washed with water/Washed with soap and water /Any other specify

Who advised you to come to anti-rabies clinic Self/Relative/Health worker/ Neighbour/ Friend/Previously treated patient

Previous History :-

P/H/O IMMUNIZATION

(a) **Immunized**

(b) Incomplete Immunized / No documents Available

(c) Unimmunized

Treatment given at the anti-rabies clinic	Details	Date
Wound washed in the clinic	Yes/No	
Local instillation of ARS	Yes/No	
Wound suturing	Yes/No	
Antiseptic/Cauterization	Yes/No	
Route of vaccine	ID/IM	
Any deviation from Schedule		
Vaccine (Brand name and schedule details		
Vaccination Document Available	Yes/No	

Clinical Symptoms	Details	Date of onset
Fever		
Local paraesthesia		
Breathlessness		
Paralysis		
Convulsions		
Hydrophobia		
Any other relevant details		
Treatment given		
Any other Close Contacts of hydrophobia case		

Specimens of suspected case given to laboratory	Collection Date	Transportation Date
Blood		
CSF		
Skin biopsy		
Saliva		
Corneal smear		
Brain		
Any other		

Radiological investigations (CT/MRI)

Death Cause of Death :
Date of Death :

Signature of Doctor

Annexure 4. Laboratory Reporting Format (NRCP)

National Rabies Control Program
National Centre for Disease Control
Ministry Of Health and Family Welfare
Government of India

Monthly report on Laboratory Diagnosis of Rabies (Human/Animal)

Name & Address of the Laboratory:

Name of the in charge:

Contact Number: Email ID:

Period of Reporting: Date of Reporting:

A. Summary of Report on Diagnostic Services

Tests Available: Sellers/FAT/dRIT/PCR/ Viruse Isolation/ Rapid test

	Specimen	Number Tested	Number Positive	Remarks
Human	CSF			
	Brain Tissue			
	Saliva			
	Any Other			
Dog	Brain			
Cat	Brain			
Monkey	Brain			
Other Animals (Please Specify)				

B. Summary of Anti Rabies antibody Titres

Tests Available: ELISA (Name of the Kit: _____) /RFFIT/ any other (Specify)

	Specimen		Number Tested	Titre		Remarks
				>0.5 IU/ml	<0.5 IU/ml	
Humans	CSF					
	Blood	After Complete Vaccination				
		Partial Vaccination				
		No Vaccination				
Animals	Blood (post Vaccination)					

Reporting Officer

Line List of Positive Patients (Only Human)

S.No.	Name	Age	Sex	Contact Number	Village	Sub District/ Taluk/Blck / mandal	District	State	Biting Animal	Name of Test	Specimen Type	Date Of	
												Sample Receipt	Result Declaration

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ABBREVIATIONS

°C	degree centigrade
µg	Microgram
µl	Microlitre
AEC	Amino-ethyl Carbazole
BF	Buffered Formalin
BHK	Baby Hamster Kidney Cells
bp	Base pairs
CCV	Cell Culture Vaccine
cDNA	Complementary Deoxyribo Nucleic Acid
CNS	Central Nervous System
CSF	Cerebrospinal Fluid
CTLs	Cytotoxic T-lymphocytes
CVS	Challenged Virus Strain
DFA	Direct Fluorescent Antibody
DFAT	Direct Fluorescent Antibody Test
DNA	Deoxyribo Nucleic Acid
dNTP	Deoxynucleoside Triphosphate
DRIT	Direct Rapid Immunohistochemistry Test
ELISA	Enzyme linked Immunsorbent Assay
EQAS	External Quality Assessment Scheme
ERIG	Equine Rabies Immunoglobulins
FBS	Fetal Bovine Serum
FCS	Foetal Calf Serum
FITC	Fluorescein Isothiocynate
gm	Gram
H_2O_2	Hydrogen Peroxide
HRIG	Human Rabies Immunoglobulins
HRP	Horseradish Peroxidase
ID	Intradermal
IFAT	Indirect Fluorescence Antibody Test
IM	Intramuscular
IQC	Internal Quality Control
IU	International Units
KD	kilodaltons
l	litre
M	Molar
MEM	Minimum Essential Medium
min	minute
MIT	Mouse Inoculation Test
ml	millilitre
mM	millimolar
MNA	Mouse Neuroblastoma
NAAT	Rapid Antigen Amplification Test
NBF	Netrual Buffered Formalin
ng	nanogram
NHS	Normal Horse Serum
nSm	nanometre
NMB	Normal Mouse Brain
NRCP	National Rabies Control Programme
NTC	No Template Control
NTV	Neutral Tissue Culture Vaccine
OIE	Office International des Epizooties
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
pH	Hydrogen Ion Concentration
PPE	Personal Protective Equipment
PPE	Post Exposure Prophylaxis
PrPE	Pre Exposure Prophylaxis
RADT	Rapid Antigen detection test
RABV	Rabies Virus
RFFIT	Rapid Fluorescent Focus Inhibition Test
RIG	Rabies Immunoglobulins
RMB	Rabid Mice Brain
RNA	Ribose Nucleic Acid
RNase	Ribonulease
rpm	Revolutions per Minute
RTCIT	Rabies Tissue Culture Infection Test
RT-PCR	Reverse Transcriptase Polymerase
sec	second
SOP	Standard Operating Procedure
ssRNA	Single Stranded Ribonucleic Acid
TPBS	Tween Phosphate Buffered Saline
UV	Ultraviolet Light
WHO	World Health Organization
WOAH	World Organization for Animal Heath

Rabies is one of the oldest recognized zoonotic diseases, with an almost 100% case fatality rate after the onset of the clinical symptoms. India is endemic to dog-transmitted rabies. National Centre for Disease Control, Delhi launched "National Action Plan for Dog Mediated Rabies Elimination from India by 2030" in 2021. NAPRE is a multi-sectoral collaborative initiative requiring extensive laboratory support to confirm most clinical rabies cases.

This manual is part of the national capacity building and strengthening of the human and animal rabies laboratory network by NCDC, Delhi. This publication highlights the contemporary understanding of rabies, its aetiology, epidemiology, wound management, prophylaxis, clinical diagnosis, laboratory diagnosis, biosafety, and quality assurance aspects. This manual aims to aid laboratory professionals from both human and veterinary fields in establishing rabies diagnostic facilities and help eliminate dog-mediated rabies from India by 2030.

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