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Comparative Evaluation of Direct Rapid Immuno-Histochemical Test (DRIT) with Direct Fluorescent-Antibody Test (DFAT) for laboratory diagnosis of animal Rabies in Ethiopia

By:

Sintayehu Abedla

Addis Ababa University College of veterinary Medicine and Agriculture
Department of veterinary microbiology, immunology and public health

Jun, 2021

Bishoftu, Ethiopia

Comparative Evaluation of Direct Rapid Immuno-Histochemical Test (DRIT) with Direct Fluorescent-Antibody Test (DFAT) for laboratory diagnosis of animal Rabies in Ethiopia

A Thesis Submitted to the College of Veterinary Medicine and Agriculture of Addis Ababa University in partial fulfillment of the requirements for the degree of Master of in Veterinary Microbiology

By:

Sintayehu Abdela

Jun, 2021

Bishoftu, Ethiopia

Addis Ababa University College of Veterinary Medicine and Agriculture Department of Microbiology, Immunology and Veterinary Public Health

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As members of the Examining Board of the final MSc open defense, we certify that we have read and evaluated the Thesis prepared by Sintayehu Abdela, titled "Comparative Evaluation of Direct Rapid Immuno-Histochemical Test (DRIT) with Direct Fluorescent-Antibody Test (DFAT) for laboratory diagnosis of animal Rabies in Ethiopia" and recommend that it be accepted as fulfilling the thesis requirement for the degree of Masters of: Masters of Science in veterinary microbiology

Dr.Kebede Amenu (DVM, MSc, PhD Associate Professor)
Chairman _____ _____
Signature Date

Dr.
External Examiner _____ _____
Signature Date

Dr. Debebe Ashenafi (DVM, MVSc)
Internal Examiner _____ _____
Signature Date

Hika Waktole (MSc, MBA; Assistant Professor)
Major Advisor _____ _____
Signature Date

Dr. Gezahegne Mamo (DVM, MSc, PhD Associate Professor)
Co- Advisor _____ _____
Signature Date

Dr. Abraham Ali (DVM, MSc, PhD candidate)
Co- Advisor _____ _____
Signature Date

Professor, Yakob Hailu (DVM, MVSc, PhD)
Department chairman

_____ _____
Signature Date

SIGNED STATEMENT OF DECLARATION

I declare that this thesis is my original work and that all sources of material used for this thesis have been duly acknowledged. This thesis has been submitted in partial fulfillment of the requirements for an advanced MSc degree at Addis Ababa University, College of Veterinary Medicine and Agriculture and is submitted at the University/College library to be made available to borrowers under rules of the Library. Once again I declare that this thesis is not submitted to any other institution anywhere for the award of any academic degree, diploma, or certificate.

Name: Sintayehu Abdela Signature: _____

College of Veterinary Medicine, Bishoftu

Date of Submission

AKENOLOGEMENT

Above all, I am grateful to the Almighty Lord of the Universe. I'd like to express my sincere gratitude to my principal advisor, Hika Waktole, for his overall activities in providing necessary intellectual guidance and devoting time to correcting this proposal paper; I'd also like to thank my co-advisor, Dr. Gezahegne Mamo, who guided me throughout this thesis, and also Dr. Abraham Ali, who also serves as my co-adviser. For his professional assistance, I am grateful to my colleague Dr. Desalegn Getahun. I'd like to express my gratitude to my family as well. Also Cristina Hile and Dr. Nato Hundessa, two of my closest friends, were instrumental in providing support and thought-provoking suggestions during the project. I'd like to express my gratitude from the bottom of my heart to. I'd like to express my heartfelt gratitude to my dearest friend Solomom Teferi for his unwavering support in making this project a success. I'd also like to express my gratitude to the Ethiopian Public Health Institute, which covered all of my tuition during my studies. I'm grateful for the following once more. My whole team, Dr. Baye Ashenef, Enedalekachew Gerema, Mesefene A, Amberber Adamu, Dr. BayeAshenef, Garuma Getahune, Henok Freed, Yirgalem Tadese and employees working in the zoonotic case team and Vaccine production section, laboratory animal section and rabies laboratory diagnosis team. I want to thank the Ethiopian public health institute, especially the zoonotic disease research case team, for supplying the reagents and permitting the necessary facilities needed to perform the laboratory tests and for all the technical and financial support, too.

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LIST OF ABBREVIATION

CDC	Centers for Disease Control and Prevention
CNS	Central nervous system
DFAT	Direct fluorescent antibody testing
FAT	Fluorescent antibody test
FITC	Fluorescein Isothiocyanate
ICTV	International Committee on Taxonomy of Viruses
MIT	Mice inoculation technique
PEP	Post-exposure prophylaxis
RABV	Rabies Virus
RNA	Ribonucleic acid
RT-PCR	Reverse Transcriptase -Polymerase chain reaction
RVG	Rabies virus glycoprotein
DRIT	Direct rapid Immunohistochemical Test
ELISA	Enzyme-Linked Immunosorbent Assay
PNS	peripheral nervous system
IQC	Internal quality control
%	present
WHO	World Health Organization

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ABSTRACT

Direct fluorescent antibody test (DFAT) is used as a gold standard method for rabies virus detection. The present study aimed was to compare and evaluate DRIT with Direct Fluorescent Antibody Test (DFAT) to use equivalently as one of rabies virus diagnosing methods in areas where DFAT is not accessible. The method is based on the capture of rabies nucleoprotein (N) antigen in brain smears using a cocktail of biotinylated monoclonal antibodies specific for the N protein and color development by streptavidin peroxidase-amino ethyl carbazole and counterstaining with hematoxylin. The test was performed in parallel with the standard DFAT and mice inoculation test (MIT) using 100 brain specimens from various species of animals. The majority of them were dogs (n=88), followed by cats (n =8), cattle (n =3), and donkey (n =1), and also from those samples that were tested by DRIT and DFAT, we randomly selected and tested 12 brain samples by MIT. The results indicated that 63% of the tests were positive by DFAT and 64% were positive by DRIT. A slight difference was observed in such a way that one sample was negative by DFAT but positive by DRIT and MIT. The DRIT provides powerful, economical tool for rabies diagnosis to improve existing rabies surveillance, prevention and control programs in Ethiopia. Although further laboratory and field examinations are essential, our findings were providing and remark the potential value of the DRIT for countries with limited diagnostic resources.

Keywords: *DRIT, Rabies, MIT, DFAT, Surveillance*

1. INTRODUCTION

Rabies is a vaccine-preventable, zoonotic, viral disease. Once clinical symptoms appear, rabies is almost 100% fatal. In up to 99% of cases, domestic dogs are responsible for rabies virus transmission to humans. However, rabies can affect both domestic and wild animals. It is spread to people and animals through bites or scratches, usually via saliva("The cell biology of rabies virus: using stealth to reach the brain," 2010).It is presented to cause a large number of deaths in animals and humans each year. Human rabies is present in 150 countries and territories and on all continents (Barecha *et al.*, 2017). Of these countries, Ethiopia is one of the worst affected country (Hampson *et al*., 2015).

According to Tariku Jibat et al, (2018) research suggestions in most rabies endemic countries, reliable documents of incidence data on rabies and rabies exposure are missing (T. J. Beyene et al., 2018). However, estimates of burden have always been uncertain due to the absence of reliable data. The official record usually underestimates the actual number of human rabies cases and hence the actual burden. For example, in sub-Sahara African countries such as Tanzania, the occurrence of human rabies predicted based on active surveillance information on bite occurrences was up to 100 times larger than the officially reported number of deaths (Taylor and Nel, 2015).

In Ethiopia, the national annual assessments from official records present 12 exposure cases per 100,000 population and 1.6 rabies deaths per 100,000 populations. However, the true numbers are expected to be higher as many cases are not reported (T. J. Beyene et al., 2018). Recently, rabies is a considerable disease that has been detected for many years in Ethiopia. The first rabies epidemic in Ethiopia reported in Addis Ababa in August 1903. It is believed that communication between the public and animal health sector was not enough to inexistent considering reporting and control of rabies cases(Pieracci *et al.*, 2016).

At present time, the Direct Fluorescent Antibody Test (DFAT) is mentioned as the gold-standard test by the World Organization for Animal Health (OIE) and World Health Organization (WHO) (Tekki *et al.*, 2016)for rabies detection; however, the limitation of use of DRIT in developing countries is that the method is technically demanding and needs the use of a fluorescence microscope, which is expensive and difficult to maintain, and it also needs a specific rabies conjugate as well as technical expertise. Thus, there is a necessity for a rapid diagnostic test that has comparable sensitivity and specificity as fluorescent antibody test (FAT), which is economical and can be adapted to field as well as laboratory conditions in resource-constraint countries (G. Singh *et al.*, 2017). The DRIT is an experimental assay, which is used in limited field trials in Africa, such as Nigeria and South Africa. It has been demonstrated to be as specific and sensitive as the gold standard direct fluorescent antibody test (DFAT) (Coetzer *et al.*, 2014).

In Ethiopia, the rabies surveillance system is very week due to the lack of rabies laboratory, and also the burden of rabies is under expected or under-reported. DFAT is currently available only in three regions of Ethiopia because establishing DFAT in all regions requires high-level expertise, expensive laboratory equipment, reagents, and sophisticated laboratory setups. As in all other endemic countries, and establishment of a diagnostic laboratory set-up as the priority for the improvement of national rabies control program is needed. Any technical advances that make a diagnosis of rabies more rapid, accurate, and cost-effective, will consequently facilitate recruiting such programs in resource-limited countries.

According to Middel*et al*. (2017), DRIT was presented to possess a sensitivity and specificity of 100% and 98.2%, respectively. Moreover, positive and negative test agreement was reported to be 98.3% and 99.1%, respectively, with an overall test agreement of 98.8%. The typical cost to check a sample was $3.13 CAD for materials, and hands-on technical time to finish the test was estimated at 0.55 h. Also, the DRIT procedure was proposed to be accurate, inexpensive, fast, and simple to perform a

great tool for monitoring the progression of rabies incursion. DRIT not only features a diagnostic efficacy adequate to that of the gold standard DFAT, but is additionally faster, cheaper, and easier to elucidate by an inexperienced reader (Middel *et al*., 2017).

These findings highlight the versatility of the DRIT as a potential WHO and OIE accredited rabies diagnostic assay (DRIT SOP).The authors attempted to demonstrate which of the existing laboratory techniques, being applied for rabies diagnosis worldwide, has unique advantages over the other by comparing the gold standard laboratory technique DFAT and DRIT so that the result of the study would serve to trigger government officials both in the ministry of health and agriculture to take further action in scaling up the suitable laboratory diagnostic techniques, provided the limited laboratory set up. For internal quality purpose, the researchers agreed to perform mice inoculation test (MIT) by selecting 12 randomly selected brain samples and inoculated into a mice brain tissue. The rationale behind performing MIT was that there are indeterminate conditions, usually 1% while performing both DRIT and DFAT.

In situations where skills and facilities for other tests [e.g., cell culture, polymerase chain reaction, (PCR)] are not available, MIT could be served as an optional confirmatory technique. In a country like Ethiopia, where rabies is highly widespread, besides with very limited access for laboratory diagnosis of rabies, the result of this research would have several implications like enhancing the rabies laboratory based surveillance in Ethiopia so its suitability regarding the time taken, technical ease, user acceptability, and the stability of the DRIT to perform the test makes the present study crucial.

Objective:

To compare and evaluate the Direct Rapid Immunohistochemistry test (DRIT), with Direct Fluorescent Antibody Test, (DFAT) for detection of rabies virus

2. LITERATURE REVIEW

2.1. Disease Definition

Rabies is a viral infection that mainly spreads through a bite from an infected animal. It is an RNA virus of the rhabdovirus family. Without early treatment, it is usually fatal. The virus can affect the body in one of two ways: It enters the peripheral nervous system (PNS) directly and migrates to the brain. It replicates within muscle tissue, where it is safe from the host's immune system. From here, it enters the nervous system through the neuromuscular junctions. Once inside the nervous system, the virus produces acute inflammation of the brain, followed by Coma and death. There are two types of rabies. Furious or encephalitic rabies: This occurs in 80 percent of human cases. The person is more likely to experience hyperactivity and hydrophobia. Paralytic or "dumb" rabies: Paralysis is a dominant symptom(Hankins and Rosekrans, 2004).

2.2. The Etiology

Rabies is caused by negative-strand RNA infections having a place in the class Lyssavirus, family Rhabdoviridae of the arrange Mononegavirales. Infections are separated on the premise of objective characteristics, such as the sort of nucleic corrosive, replication procedure, genomic organization, relative size, and morphology (Rupprecht *et al*., 2002). (Dietzgen et al., 2011)In addition to the classical rabies infection, Mekola and Lagos bat was confined from the bat in Ethiopia (Mebatsion *et al.*, 1992) (Deressa *et al*., 2010).The virus is sensitive to lipid solvents (cleanser solution, ether, chloroform, and acetone), 70% ethanol, iodine arrangements, and quaternary ammonium compounds. Other significant properties are resistance to drying, rehashed solidifying and defrosting, relative steadiness at pH 5-10, and sensitivity to pasteurization temperatures and ultraviolet light the infection is promptly inactivated by p-propiolactone but is safer to 0.25- 0.594 phenol used in Sempie-type vaccines, where a few days are required to get total inactivation(Wu *et al*., 2017).

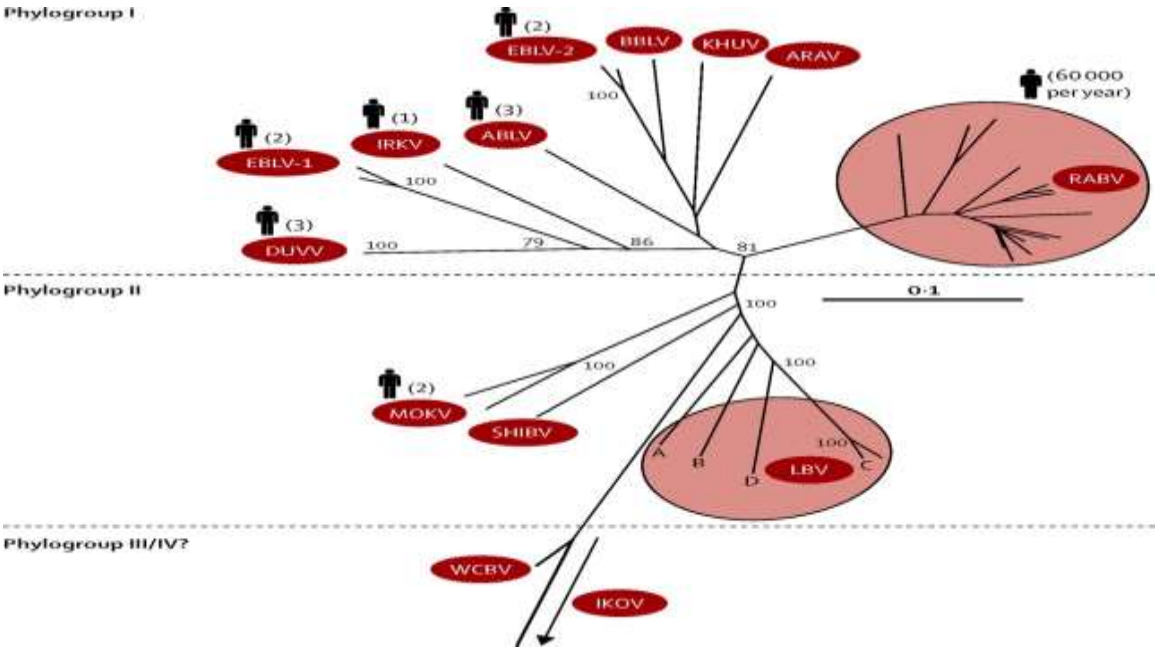


Figure 1: Phylogenetic tree of the Lyssavirusphylogroups and their respective species

Sources: (Ceballos *et al*., 2013)

Nucleoprotein sequences (405 nucleotides) were aligned with Cluster and the phylogenetic tree was visualized using Tree View version 3.2. Bootstrap values at relevant nodes are shown. According to the proposed antigenicity of each group of isolates, the viruses are divided into different phylogroups. Where available, accession numbers for sequences are rabies virus (RABV AY102999, AY062068, AY103008, AY062069, AY102993, AY352514, AY330735, AY062090, AY062070, AY062047), Lagos bat virus (LBV EF547459, EF547449, EF547447, GU170202), West Caucasian bat virus (WCBV EF614258), Shimoni bat virus (SHIBV GU170201), Mokola virus (MOKV AY062074, AY062077), Duvenhage virus (DUVV AY062079), European bat lyssavirus type 1 (EBLV-1 AY062088, EF157976), Irkut virus (IRKV EF614260), Australian bat lyssavirus (ABLV AF418014), European bat lyssavirus type 2 (EBLV-2AY062091, AY062089), Bokeloh bat lyssavirus (BBLV JF311903), Khujand virus (KHUV EF614261), Aravan virus (ARAV EF614259), and Ikomalyssavirus (IKOV JX193798). Several sequences within the phylogeny are unpublished and as such do not have accession numbers(Meslin *et al*., 1996).

2.3. Morphological characteristics of the viral agent

Rabies virus is bullet-shaped, 180 x 75 nm in size, with one end rounded or conical and the other plane or concave. The genome that occurs in the central core is un-segmented, linear, negative-sense, 10-12 kilobase, single-stranded RNA enclosed within a helically wound nucleocapsid. RNA- dependent RNA polymerase enzyme, which is essential for the initiation of replication of the virus, is enclosed within the virus in association with the rib nucleoprotein core. The latter is surrounded by a viral membrane (matrix protein), which may beinvaginatedat the plane end. The viral membrane in turn is surrounded by a lipoprotein envelop that carries knoblike spikes made up of glycoprotein G. The spikes do not cover the plane end of the virus and may be released from the envelope by treatment with lipid solvents or detergents. Rabies virus is sensitive to alcohol and lipid solvents (e.g. ether, chloroform, and acetone), iodine preparations, quaternary ammonium compounds, soap, and detergents. It can be inactivated by phenol, formalin, beta-propiolactone, sunlight, ultraviolet irradiation, and heat at 50°C for one hour or 60°C for 5 minutes. The virus dies at room temperature but can survive for weeks when stabilized by 50% glycerol. It can remain stable for several days at 0-4°C and can be preserved at -70°C or by lyophilization (freeze-drying)(Wunner, 2003).

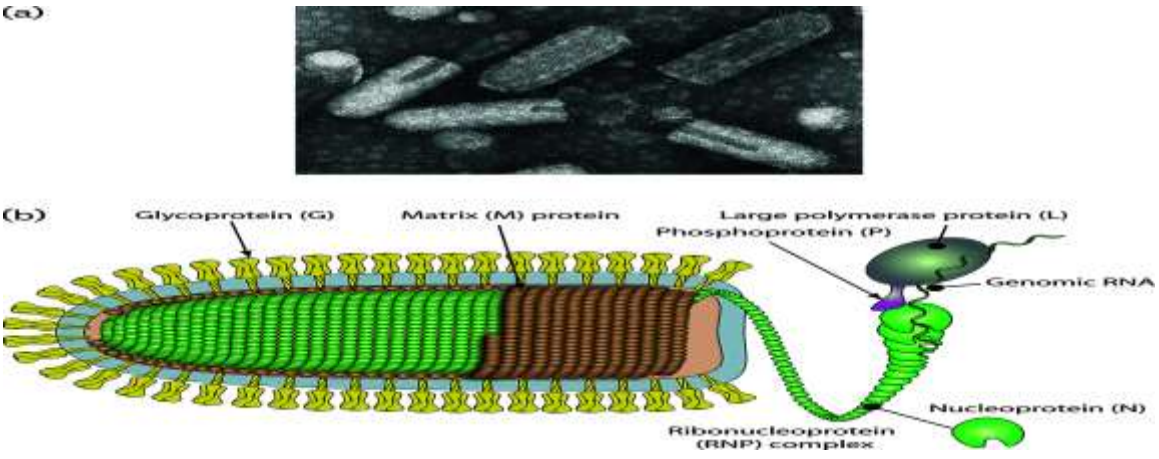


Figure 2: Morphological characteristics of the viral agent.

(a) Negative-contrast electron micrograph of vesicular stomatitis Indiana virus particles. The bar represents 100 nm (courtesy of P. Perrin).

(b) Schematic illustration of a rhabdovirusvirion and ribonucleocapsid structure. Unravelingof the RNP is

Source:(Walker *et al*., 2018)

2.4. Rabies Virus Genome Organization and Transcription

Rhabdovirus negative sense (–) single-stranded RNA genomes range from 10.8 to 16.1 kb. Almost all Rhabdovirus genomes are un-segmented but Rhabdoviruses with bi-segmented genomes also are familiar. Terminal non-coding regions are partially complementary. Genomes usually encode five major structural proteins but may alsoencode additional (accessory) proteins either in additional genes or as alternative ORFs within the structural protein genes(Walker *et al*., 2018)**].**

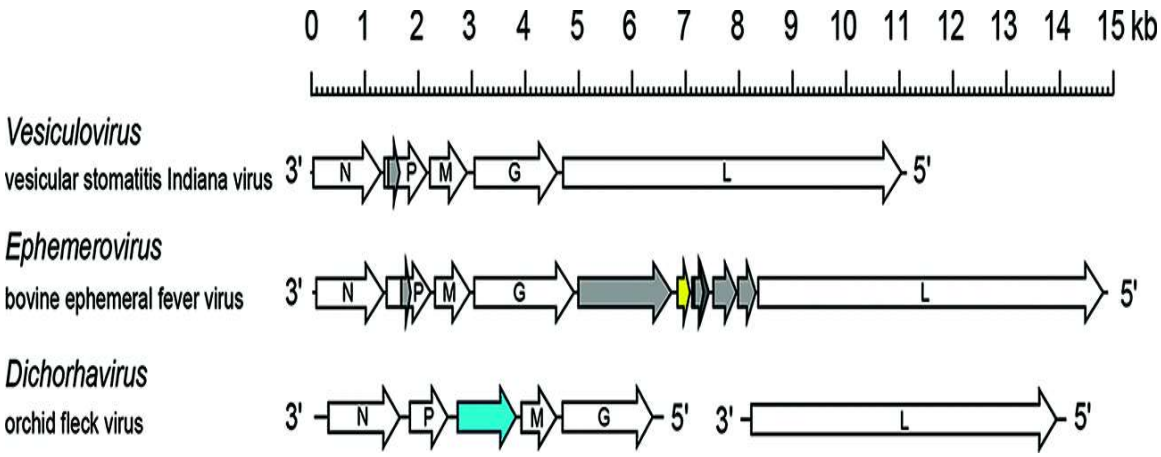


Figure 3: Schematic representation of rhabdovirus genome organization.

Exemplifying variations in architecture and the number and location of accessory genes' Arrows indicate the position of long ORFs. Alternative ORFs occur within some genes; only ORFs (≥180 nt) that appear likely to be expressed are shown. ORFs encoding viroporin (yellow) and movement proteins (blue) are shown

Source: (Walker *et al*., 2018)

2.5. Epidemiology and Geographic distribution

Rabies is a deadly zoonotic disease that spreads worldwide and is transmitted mostly by carnivores to humans and livestock. It is known to cause a large number of deaths in humans and animals each year. Human rabies is prevalent in 150 countries and territories and on all continents (Barecha *et al.*, 2017). Tens of thousands of people die every year from rabies. Globally, it is estimated that the death rate in humans (due to endemic dog rabies) is the highest in Asia, with the highest death rate recorded in India. This is closely followed by Africa. However, burden estimates were always anecdotal due to a lack of reliable data (Taylor and Nel, 2015).

The epidemiology of rabies varies in numerous parts of the globe, with two patterns being recognized. In sylvatic rabies, infection is maintained in wildlife reservoirs in countries like North America and Europe. This pattern contrasts thereupon in much of Asia, Africa, and Latin America, where urban rabies is maintained as an epizootic infection in the domestic dog, and human disease is much more common. Viral strains differ among various animal hosts. Human-to-human transmission may also occur (Pastoret and Brochier, 1998).

In Africa, evidence suggests that the primary cycle of rabies virus maintenance is among domestic dogs, although other carnivores may be involved as groups that need no maintenance. This result indicates that mass vaccination targeting domestic dogs will have the greatest effect in reducing the risk of infection in all other species including humans, livestock, and wildlife. The role of bats and other carnivores in the transmission of rabies in humans in Africa appears to be minimal (Blitzer *et al*., 2012).

In Ethiopia, thousands of people become infected with rabies every year and an estimated 2,700 people die; it is one of the highest rates of rabies death in the world, but the true number of deaths from rabies is unknown because the disease is underreported and laboratories for diagnosing rabies have not been established. Moreover, few places in Ethiopia offer life-saving preventative treatment of human rabies (PEP) and most people

do not have the means to reach a large hospital for treatment. Additionally, people's awareness of what to do if a dog bites them is low, and people often do not seek medical help (Ramos *et al.*, 2015).

The domestic dog plays a pivotal role in rabies transmission, with 85-95% of rabies cases in humans being described as being bitten by dogs due to their close association with humans. According to a WHO report, ten million people are bitten by animals and are considered for the prevention and treatment of rabies worldwide annually. Of this number, approximately 55,000 people die from this disease annually (Hambolu *et al.*, 2014).

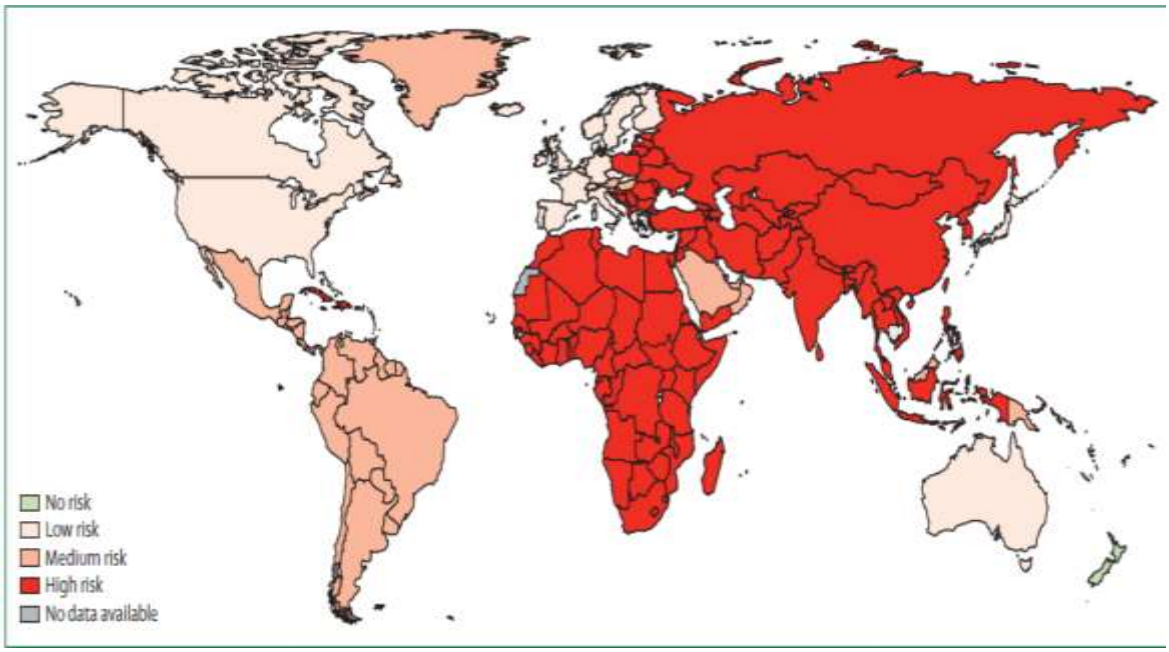


Figure 4:WHO rabies risks map

WHO Data show from most of the cases of human rabies occurs in Africa and Asia. Attempts to accurately map the distribution of rabies risk or incidence show the absence of quantitative data and the irrelevance of political boundaries in the control of disease with animal reservoirs. In low-risk areas, pre-exposure immunization is recommended for individuals who will come into contact with bats. In medium-riskareas, pre-exposure

immunization is recommended for individuals who will come into contact with bats and other wildlife. In high-risk areas, pre-exposure immunization is recommended for individuals who will come into contact with domestic animals such as dogs, and other rabies vectors (Fooks *et al.*, 2014).

Sources:(Fooks *et al*., 2014)

2.6. Routes of Transmission

Rabies is most typically transmitted to humans and other animals via the bite of a rabies-infected animal or contamination of scratch wounds by virus-infected saliva. The number of viruses reaching the lesion could be considering transmission; as an example, within the case of humans when a bite needs to penetrate clothing, the saliva is also retained within the fabric and is prevented from entering the wound. Potential non-bite modes of transmission include contamination of a pre-existing wound by saliva, cerebral cerebrospinal fluid, tears, or nerve tissue from a suspect or known rabid animal or person, contact of mucosa with the saliva of an infected animal, or via organ transplantation from an infected donor. On rare occasions, human rabies has been acquired by inhalation of airborne viruses in laboratories working with the live rabies virus and in caves with innumerable bats (Niezgoda *et al*., 2003).

2.6.1. Direct contact/Bite transmission of rabies

Rabies is mainly transmitted by the bite of a rabid animal that contains the rabies virus within the saliva. The virus may also be transmitted via direct contact of fresh wound or intact mucosal surface (eyes, nose, mouth, and vaginal canal) with infectious saliva or neural tissue, by licks of infected animals, and transdermal scratches contaminated with infectious material. The virus cannot penetrate the intact skin. The disease agent is maintained and transmitted by a spread of various host species and widespread among doggies. Besides the very fact that dog bites are a significant unhealthiness that will cause physical trauma to victims and considerable cost to communities, they'll function as a

route for the transmission of rabies to the victim especially if bitten by a rabid dog (Niezgoda*et al.*, 2003).

2.6.2. Aerosol transmission

Transmission of rabies by inhalation of virus-containing aerosol is rare but has been reported and might be a possible hazard for laboratory workers. Inhalation of an aerosolized rabies virus during homogenization of fixed virus within the laboratory had resulted in two human rabies cases. This means the likelihood of aerosol transmission of the rabies virus. Aerosols generated within the laboratory or caves containing numerous Brazilian free-tail bats have rarely caused human rabies. Rarely, rabies may be contracted by inhalation of virus-containing aerosols in laboratories during which materials that contain highly concentrated live RABV are handled or in caves with a high density of rabies-infected bats. Wild carnivore species and bats present the next risk for rabies transmission than other wildlife, as they're the reservoirs of RABV (Johnson *et al*., 2006)

2.6.3. Human to human transmission

Srinivasan et al., 2005 study shows that it is also possible, but quite rare, that individuals may get rabies if infectious material from a rabid animal, like saliva, gets directly into their eyes, nose, mouth, or a wound. Casual contact, like touching an individual with rabies or contact with non-infectious fluid or tissue (urine, blood, and feces) doesn't constitute an exposure and doesn't require post-exposure prophylaxis. Additionally, contact with someone who is receiving rabies vaccination doesn't constitute rabies exposure and doesn't require post-exposure prophylaxis. the sole well-documented cases of rabies caused by human-to-human transmission occurred among eight recipients of transplanted corneas, and recently among three recipients of solid organs from undiagnosed organ donors (patient died of neurological symptoms, but later was confirmed to own died of rabies) to the recipients has been documented within u. s.. Human-to-human transmission of rabies through other solid organ tissue transplants (e.g. liver, kidney, lung, pancreas, iliac artery) has also been reported and documented within the USA, Germany, and other countries. Although rabies virus has been isolated from a range of tissues and body fluids, including bodily fluid, saliva, tears, and urine sediment,

there's some anecdotal reports of suspected transmission of rabies from human-to-human are inadequately documented in rabies endemic areas (Srinivasan *et al.*, 2005).

2.7. Pathogenesis of animal

Once the infection is established within neuronal cells the virus usurps host cell machinery to reach cell bodies in the spinal cord or brainstem or in sensory ganglia (eg, dorsal root ganglia) where replication occurs. The motility of RABV along neuronal processes has been exploited to map neuronal circuitry(Ginger *et al*., 2013). The virus is transported by fast axonal transport through the spinal cord to the brain. Until this stage, the patient shows no clinical signs. Host detection of virus intracellularly occurs through the retinoic acid-inducible gene 1 pathway (Hornung *et al.*, 2006) through the detection of cap structures on the virus mRNAs and through Toll-like receptors.

This detection stimulates an early interferon response that is antagonized by the virus phosphoprotein.The mechanism of interferon antagonism seems to be conserved between many of the lyssaviruses. Once in the CNS, the virus replicates extensively and clinical disease develops. Notably, fatal encephalitic rabies might not necessarily be accompanied by substantial inflammation and this feature could, in part, be due to the genetics of the infecting virus. This variability in the inflammatory response is also seen in cases of dog rabies. Furthermore, neuronophagia can vary substantially and neuronal apoptosis does not seem to have an important role in rabies encephalitis in human beings(Jackson *et al.*, 2008). Nonetheless, extensive infection of the brain leads to centrifugal dissemination of the virus through neurons to distant sites throughout the body. Of particular importance in reservoir, species is the spread of the virus to peripheral sites that release the virus into the oral cavity via the salivary glands. Rabies virus antigen staining of the taste buds has also been observed (Freuling *et al*., 2009).

The salivary glands are innervated from the parasympathetic nervous system via the submandibular ganglion and glossopharyngeal nerves, by sympathetic innervation via the superior cervical ganglion, and by afferent innervation. Ultrastructural studies suggested

that the virus is able to travel from the brain to peripheral sites by budding on synaptic or adjacent plasma membranes of dendrites with budding also occurring, albeit less often, from the plasma membrane of the perikaryon. Detection of virus particles engulfed by an invaginated membrane of an adjacent axonal terminus suggested transneuronaldendroaxonal movement. However, alongside this finding, the virus has also been reported to bud directly into the intercellular space, which along with hyper-salivation and the aggressive behavior often associated with infection, promotes onward transmission of the virus to new hosts(Charlton and Casey, 1979).

The cause of death as a result of infection with rabies virus has not been irrefutably established. Overwhelming virus replication in the nervous system leads to many systemic complications, including multi-organ failure. Experimental studies show strong evidence for upregulation of interferon's, cytokines, and chemokine's in the CNS in response to infection with rabies virus. Transcriptomic studies have shown the up-regulation of many interferon-inducible genes and this up-regulation has been supported by immunohistological demonstration of chemokine production in neurons. Such production drives an influx of immune cells into the CNS, particularly T cells. However, in the case of rabies virus infection, this influx does not control infection and the host invariably dies. In the absence of therapeutic options no mechanisms exist to ameliorate rabies virus replication once the virus reaches the brain. Evidence for an immune response is not detected until the virus has entered the CNS. How the virus avoids immune surveillance in the periphery is unknown, but might be attributable to the low level of replication early in the infection or activation of immune suppression in the periphery(Lafon, 2011).

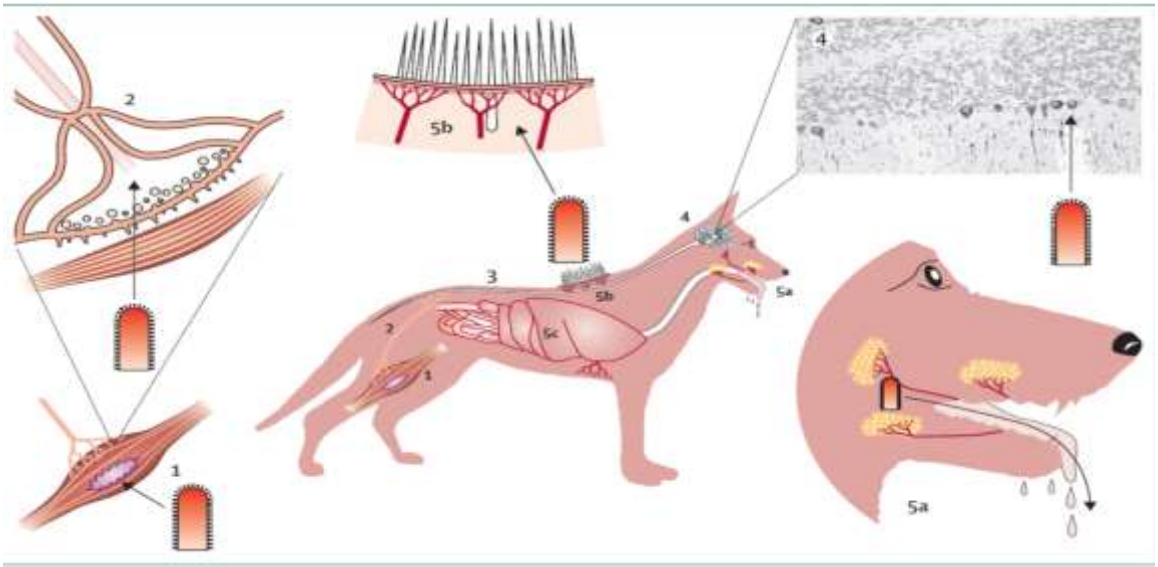


Figure 5: Pathogenesis of rabies virus.

1) Virus enters muscle tissue of host through a bite wound, then 2) enters the peripheral nervous system (PNS) via neuromuscular junction, and then 3) travels from PNS to spinal cord and brain. 4) Virus enters the brain and undergoes extensive replication leading to neuronal dysfunction (slide shows virus in Purkinje cells of cerebellum 40 x magnifications). 5a) the virus replicates in salivary glands and is excreted in saliva, 5b) enters peripheral nerves of the skin and Purkinje cells, and 5c) spreads from the brain to infect many tissues and organs in the host(Jackson, 1994)

Sources: (Fooks *et al.*, 2014).

2.8. Incubation Period

The incubation period of rabies in dogs varies from a week to many months. The incubation period apparently depends upon several factors which include the site of exposure (inoculation), the dose, and the virus strain. In a study of incubation periods, dogs experimentally infected with various doses of street-rabies viruses to simulate natural infection were observed for at least -two years. In the dogs which died of rabies, the incubation periods were 7-125 days, depending on the dose and strain used; the incubation periods were inversely proportional to the virus dose. In another experiment,

the longest incubation period in an experimentally infected dog was 8, 5 months(Fekadu, 1993).

2.9. Clinical features in animals

Clinical signs of rabies in animals will vary depending on the effect of the virus on the brain. Typical signs include sudden behavioral changes and progressive paralysis leading to death. In some cases, however, an animal may die rapidly without demonstrating significant clinical signs. Rabies infection in animals is classically divided into three major stages: prodromal, furious, and paralytic. Classical descriptions may be misleading, however, as rabies can be quite variable in its presentation. Not all animals pass through all of the clinical stages and the animals may vacillate between stages (Salomao *et al*., 2017).

Prodromal phase During the prodromal period, which lasts ~1–3 days, animals show only indistinguishable nonspecific signs, which intensify rapidly. Usually, they stop eating and drinking, withdraw, maybe lethargic, or showing fever, vomiting, and anorexia. Furious Phase the second phase of infection usually lasts 2 to 4 days and not all rabid animals experience it. This is the classic "mad-dog syndrome," although it may be seen in all species. There is rarely evidence of paralysis during this stage. The animal becomes irritable and with the slightest provocation, may viciously and aggressively use its teeth, claws, horns, or hooves. The posture and expression are one of alertness and anxiety, with pupils dilated. Noise may invite an attack. Such animals lose caution and fear of people and other animals, animals with this form of rabies may demonstrate sudden behavior changes and attack without provocation. As the disease progresses, muscular incoordination and seizures are common. Death results from progressive paralysis(Hemachudha et al., 2003)**.**

Paralytic phase (dumb rabies) the phase of infection usually lasts for 2 to 4 days. Animals with this form of rabies may be depressed or unusually docile. The animal will often have paralysis, generally of the face, throat and neck, causing abnormal facial expressions,

drooling and inability to swallow Dropping of the lower jaw is common in dogs. Owners frequently examine the mouth of dogs and livestock searching for a foreign body or administer medication with their bare hands, thereby exposing themselves to rabies. These animals may not be vicious and rarely attempt to bite. Paralysis may affect the body, first affecting the hind legs. The paralysis progresses rapidly to all parts of the body, and coma and death follow in a few hours (Wunner, 2003).

2.10. Species Affected and Reservoirs

All mammals are susceptible to rabies and can transmit the RABV, but there is great interspecies variability that exists among mammals in the capability of acting as reservoirs. The primary reservoir for rabies is carnivorous mammals throughout the world. Below 10% of the documented rabies cases occur in domesticated animals including cats, cattle, and dogs predominantly Jindal and. Raccoons, skunks, bats, and foxes are the wild animals from which a huge proportion of rabies cases are reported every year. Wildlife is the main reservoir for the disease. A spatial model to predict the emergence of rabies in raccoon has been developed(R. Singh *et al*., 2017).

The RABV circulates with two epidemiological cycles, which are interrelated i.e. urban and sylvatic cycle, having basically pet mutts, cats and wild well evolved creatures like fox, raccoon, jackal, wolf, pester, mongoose and bats, etc., as vectors/reservoirs, separately Blackwood. Be that as it may, both cycles may cover in a few topographical circumstances. Primarily community and stray puppy populace keeps up the urban cycle and spill-over to pet mutts makes extra burden to human with a chance of rabies. One or at the foremost two species act as a vector for RABV in a specific geological zone. In India, pooches and jackals were the major vector or supply. In urban regions of our country, pooches are the most stores and transmit the infection through chomp to people as well as creatures. More imperative components for the food of the stray puppy populace in India are likely the terrible waste arrangement and open butcher offices. Once more urbanization and the development of ghetto zones advance make favorable conditions (R. Singh *et al.*, 2017).

2.11. Rabies Virus Diagnostic Techniques

Ordinarily, the brain is collected taking after the opening of the skull in a necropsy room, and the fitting tests are collected. This step may be perilous on the off chance that research facility specialists are not completely prepared, or beneath field conditions. In such cases, there are two conceivable strategies of collecting a few brain tests without opening the cranium(Shankar, 2009).Occipital foramen course and Retro-orbital course are the major procedures for a brain Test collection from the rachidian bulb, the base of the cerebellum, hippocampus, cortex, and medulla oblongata (Shankar, 2009).

2.11.1. Clinical examination of rabies in animal

It is critical to examine all known exposures of domestic animals to a possibly raging creature. Household creatures other than mutts that chomp other household creatures are not ordinarily considered as possibly raging unless they are showing signs consistent with the malady. Distinguishing and appropriately dealing with these exposures incredibly decreases the hazard of consequent creature presentation to other out-of-control residential and wild creatures. A wild creature that has possibly uncovered a household creature to rabies ought to be tried for rabies at whatever point conceivable (Tepsumethanon *et al*., n.d.).

2.11.2. Sample collection and shipment guidelines

The specimen useful for correct diagnosis of rabies in animals is especially the brain and salivary glands. Though it's risky to gather the ante mortem specimens, if required, saliva and corneal smears is also collected as already described. The better course, however, is to allow the animal to die natural death unless otherwise required (see microscopic examination). Facilities for removal of the animal brain and salivary glands don't seem to be available within the laboratory and hence the entire brain or salivary glands should be sent to the laboratory after post-mortem (Mbilo *et al*., 2020).

2.11.3. The FAT florescent antibody test

The sample for rabies diagnosis is the head of the suspected animal. The brain is extracted for sampling. Animals submitted for rabies testing should be euthanized while maintaining the integrity of the brain for recognition of the anatomical parts by lab 12 personnel. For prompt laboratory testing, submitted specimens should be stored and transported under refrigeration(World, Health, Organization, 2013). The gold standard confirmatory test is the Fluorescent Antibody Test (FAT). Biosafety level 2 facilities are adequate for routine diagnosis. Personnel working with the virus should receive pre-exposure prophylaxis. FAT is the gold standard test and the standard procedure in public health labs around the world(World , Health, 2018). It provides a reliable diagnosis in 98–100% of cases for all rabies virus strains if a potent conjugate is used. FAT is normally positive after inoculation of brain tissue, saliva or CSF in cell culture, or after intra-cerebral inoculation in mice or in suckling mice (Veera *et al.*, 2005).It uses a fluorescent dye conjugated to rabies antiserum and a fluorescent microscope and is the quickest (2 hours), most reliable method available, both for diagnostic and research purposes. Results normally display oval apple-green negri bodies diagnostic of rabies). Results of FAT can be available within two hours of submission of the specimen while results of mice inoculation can take up to 28 (Mucheru, 2015).

2.11.4. Direct rapid immunohistochemical test DRIT

To avoid the need for expensive fluorescently labeled antibodies and fluorescent microscopes, a histochemical test (direct rapid immunohistochemical test; DRIT) that allows the use of low-cost light microscopy has been developed. DRIT can detect rabies antigen in fresh brain impressions within 1 h. Development of DRIT will enable transfer of this diagnostic capability to endemic regions where cost precludes laboratory confirmation. Alongside FAT, the OIE recommends the use of a virus isolation test, particularly when FAT results are equivocal and for cases of human exposure. In-vitro isolation tests can be used to grow the virus from suspect samples with the added value of virus isolation for downstream characterization and research activities(World Health Organization, 2018).

2.11.5. Histological tests identification

Histological examination of brain sections or impression smears by light microscopy, in detection of characteristic Negribodies, has been considered standard method of rabies diagnosis for many years. Under light microscopy these Negri bodies are round or oval, acidophilic and strongly refringent, staining pink with haematoxylin-eosin and red with Mann's stain. Staining of an unfixed tissue smear by Seller's method allows diagnosis in less than one hour; whereas Mann's test generally involves fixing the tissue and embedding it in paraffin, with test results within three days. These techniques have the advantage that the laboratory equipment needed to perform them is inexpensive and any need to keep specimens cold after fixation is avoided. Whichever staining method is used, the evidence of infection is provided by intra cytoplasmic acidophilic bodies.These histological methods, especially the Seller's method, can no longer be recommended because they have very low sensitivity and should be abandoned. Additionally, false negatives results may occur because not all individuals develop Negri bodies and false positive results may occur if nonspecific inclusion bodies are present. Presence of Negri bodies in positive cases varies from 10 to 65% .This is why this technique has gradually been replaced by use of the fluorescent antibody test. The OIE no longer recommends histopathology for diagnosis of rabies(Mani and Madhusudana, 2013).

2.11.6. Detection of the replication of rabies virus after inoculation

Virus replication tests detect the infectivity of a tissue suspension in cell cultures or in laboratory animals. They should be used if the FAT gives an uncertain result. Two techniques can be employed for this purpose: the mice inoculation technique (MIT) and cell culture inoculation test(Mani and Madhusudana, 2013).

2.11.7. Mouse Inoculation Test (MIT)

All samples are subjected to the Fluorescent Antibody Test (FAT) as a first procedure and subsequently, all cases which are suspicious and all cases with a history of human contact are inoculated into mice (Bishop *et al.*, 2003). This test only uses fresh samples including brain, salivary glands, liver, spleen pancreas and nuchal skin (Yousaf et al., 2012). It is used in cases of inconclusive FAT tests and Involves intra-cerebral

inoculation of brain tissue from rabies-suspect animals into infant mice. When the test is positive, a large amount of virus can be isolated from a single mouse brain for strain identification purposes. It takes long (28 days). To reduce this time, the World Health Organization Expert Committee on Rabies recommends that sufficient animals should be inoculated to allow for the sequential daily euthanasia of one or two of these mice(WHO, 2004).

2.11.8. Tissue Culture Inoculation

Cell culture is as sensitive as the mouse inoculation test. Once a cell culture unit exists in the laboratory, this test should replace the mouse inoculation test as it avoids the use of live animals, is less expensive and gives more rapid results. It is often advisable to carry out more than one type of test on each sample, particularly when there has been human exposure. Neuroblastoma cells e.g. N2a, CCL-131 in the American Type Culture Collection (ATCC) 1 are highly susceptible to infection with lyssaviruses. The cells are grown in Dulbecco's modified Eagle's medium (DMEM) with 5% fetal calf serum (FCS), incubated at 36°C with 5% CO2. Cell culture tests may be undertaken in multi-well plastic plates, multi-chambered glass slides or on glass cover-slips. Presence of rabies virus in the cells is revealed by the FAT. The result of the test is obtained after at least 18 hours (one replication cycle of virus in the cells); generally incubation continues for 48 hours (10) or in some laboratories up to 4 days(Webster, 1987).

2.11.9. Enzyme-Linked Immunosorbent Assay (ELISA)

ELISA that detects rabies antigen is useful for large surveys and is a variable of the immunochemical test(Xu *et al.*, 2007). Principles of the Rapid Rabies Enzyme-Immunodiagnostic (RREID) test are similar to FAT. Rabies antiserum is conjugated to an enzyme which gives a color reaction when reacted with its substrate. Specificity and sensitivity should be investigated before use and should be combined with other tests such as FAT for confirmation(Nicholson and Prestage, 1982).

2.11.10. Detection of rabies virus RNA

For a large number of samples, as in an epidemiological survey, the polymerase chain reaction (PCR) can provide rapid results in specially equipped laboratories. Samples used include saliva, urine or cerebrospinal fluid. The test is suitable for the typing of rabies virus isolates and for describing their variability within and among regions. Although it is expensive and requires experienced technicians, it utilizes tissue samples in any condition (Yousaf *et al*., 2012).

2.11.11. Virus isolation in Rapid Tissue Culture Infection Test (RTCIT*)*

Virus isolation by *in-vitro* technique (RTCIT) is done by using highly susceptible cell line murine neuroblastoma (NA-C 1300), neuroblastoma cell line *e.g.*CCL-131(Singathia et al., 2012). It can be used for testing saliva and cerebrospinal fluid samples from living individuals as well as for testing brain and salivary gland tissues from *post mortem*. Virus isolation in cell culture can be used in cases where immunofluorescence is inconclusive. After overnight incubation (18hr.) at37^{o}C in 5%CO2, the cells are fixed, stained with an anti-nucleocapsid antibody conjugated to FITC and observed under fluorescent microscope.RTCIT has progressively replaced the MIT because it is less expensive as it avoids the use of live animals, relatively sensitive, easy to undertake and can substantially reduce the timerequired to obtain results from 30 days in MIT to 4 days. Several comparative studies between the RTCIT and both MIT and FAT have shown that the RTCIT is at least as sensitive as the MIT in demonstrating rabies virus in human and animal tissues, but others have found it to be less sensitive than MIT. Several laboratories have now replaced the MIT with RTCIT as the routine test for rabies diagnosis. But problem with this test is that a negative result in samples from a live animal is not conclusive, many animals have been found to be rabies virus positive in the brainbut negative in saliva samples; even clinical patients with rabies may be negative for virus in biopsy, saliva or CSF. It is often advisable to carry out more than one type of test on each sample, particularly when there has been human exposure(Singathia *et al*., 2012).

2.12. Rabies exposure management in animals

It is important to investigate all known exposures of domestic animals to a potentially rabid animal. Domestic animals other than dogs that bite other domestic animals are not usually considered as potentially rabid unless they are exhibiting signs compatible with the disease. Identifying and properly handling these exposures greatly reduces the risk of subsequent animal exposure to other rabid domestic and wild animals. A wild animal that has potentially exposed a domestic animal to rabies should be tested for rabies whenever possible.

2.12.1. Wound Management

2.12.1.1. Wound Cleaning

Since the rabies virus enters the other exposed animal body through a bite or scratch, it is unavoidable 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 exposed animal presents late to veterinary clinic. This can be done by prompt and gentle thorough washing with soap or detergent and flushing the wound with running water for 10 minutes. If soap and detergent are not immediately available wash with running water for at least 10 minutes. Avoid direct touching of wounds with bare hands. Considering the importance of this step the veterinary clinics should have wound washing facilities.It should be noted that the immediate washing of the wound is a priority. However, the bite victim animals should not be deprived of the benefit of wound toilet as long as there is an unhealed wound, which can be washed even if the owner of animal reports late. The maximum benefit of the wound washing is obtained when fresh wound is cleaned immediately. Suturing of wound should be avoided as far as possible. If unavoidable, minimum loose sutures should be applied after adequate local treatment the wound(World Health Organization, 2013).

2.12.2. Antibiotic Prophylaxis

Common pathogens associated with animal bites include Staphylococcus, Streptococcus, Pasteurella and anaerobic bacteria which are common in the mouth of animals. Wounds that become infected within 24 hours of the bite are often caused by Pasteurella. This fastidious gram-negative bacterium can cause septicemia, meningitis, and endocarditis, thus Antibiotic should be considered for all bites and Pen strep is a choice of treatment against those pathogens(World Health Organization, 2013).

2.13. Prevention and control of rabies

2.13.1. Dog management

Past rabies elimination strategies included the reduction of population density through culling, based on the rationale that rabies transmission is density-dependent with disease density increasing proportional to host density. However studies have shown that culling is an ineffective means of elimination and mass vaccination is most efficacious to reduce disease incidence in all species. The Bangladesh canine rabies elimination program focuses primarily on dog bite management and mass dog vaccination to reduce the incidence of human deaths. Since 2011, dog vaccines have been administered in 58 of the 64 districts, combined with local capacity building and knowledge transfer, resulting in a 50% decrease in human rabies deaths, demonstrating the effectiveness of mass vaccination(world health organization, 2005)(Briggs *et al*., 2005).Coordinated actions for eliminating human rabies in the Americas began in 1983, with technical cooperation from PAHO on regional programmes and the operation of epidemiologic surveillance system (*Siepi*). Achievements have been due largely to strong cooperation between the health and the agricultural sectors, as well as with regional and international organizations, public and private agencies and nongovernmental organizations. Since the introduction of the programme, the number of human rabies cases has dropped 95% and declined in dogs by 98%. Elimination of dog-mediated rabies is feasible given political will, adequate resources and diligent programme management. The progress of elimination strategies must be continuously controlled and evaluated at regional and national levels, in order to monitor subsequent effectiveness and ensure optimization of the use of financial and

human resources. Successful follow-up and evaluation requires data, rendering the need for rabies to be a disease of obligatory notification(world health organization, 2005)(Briggs *et al*., 2005).

2.13.2. Rabies surveillance

Although rabies is notifiable in many countries, surveillance is often weak and official reporting of disease incidence in humans and animals remains inadequate and incomplete. It is increasingly accepted that the available data underestimate the true incidence. And that in many cases the true quantitative burden of rabies is best displayed using estimates. As control programs and systems improve, effective surveillance results in more robust data and the incidence of dog-transmitted human rabies has been shown to decline(Blanton *et al.*, 2012). This is the situation in many countries in the Region of the Americas where disease incidence has decreased by 98%. Rabies virus in dogs is endemic, or local to, Ethiopia where approximately 1.7 human deaths per 100,000 people are reported every year (highest reported in Africa). Human rabies is a reportable disease in Ethiopia.

The Public Health Emergency Management (PHEM) Center of the Ethiopian Public Health Institute (EPHI), is responsible for collecting, aggregating, and analyzing data on 24 nationally notifiable diseases and conditions which includes human rabies. However, animal rabies surveillance and diagnostic testing capacity is limited and often neglected compared to the more economically important livestock diseases. Limited animalrabies surveillance and laboratory diagnostic testing occurs in Addis Ababa but is largely representative of its ten sub cities and its surroundings. Surveillance of both human and animal rabies is essential to detect high-risk areas and outbreaks quickly and to monitor the use of vaccine. The main purpose of public health surveillance may be to demonstrate the presence and distribution of the disease in humans and animals as part of control improve awareness of the situation or, ultimately, document the absence of disease (Deressa *et al*., 2010).

2.13.3. Rabies Vaccination

Rabies has a vaccine but not treatment, so prevention by pre exposure vaccine is best for source animal and high risk population. mass vaccination of dog population have greater role so, should be carried out as much as possible in the world mostly in developed country. However, rabies remains endemic to many parts of the developing world where the resources of appropriate PEP are limited, the infrastructure and facility are inadequate, and, most importantly, awareness about rabies is lacking. In these cases, inexpensive, safe, and effective vaccines are urgently needed. This situation is even more pronounced given the fact that the most important and probably the only practical way to control rabies globally is the mass vaccination of dogs as well as wildlife reservoirs. The vaccine is given as pre exposure and post exposure with or without rabies immunoglobulin and it have no contraindication for pregnant but for immune suppressed. Generally, mass vaccination of dogs, proper post exposure management, appropriate surveillance system, and increasing the awareness of the community about the disease needs special attention for prevention and control of the disease, vaccination as prevention is better than curing especially for rabies due to its fatality(K. Beyene *et al.*, 2018).

Post Exposure Prophylaxis (PEP): In Ethiopia nerve tissue vaccine and cell culture vaccines are in use as PEP for rabies exposure. ***PEP using Nerve Tissue Vaccine (NTV)*** WHO advises against the production and use of nerve tissue vaccines due to the likelihood of severe adverse reactions and lower immunogenicity compared to cell culture-derived vaccines? While there has been progress with countries phasing out the use and production of nerve-tissue vaccines – most recently in Pakistan and Mongolia – their use and production in some countries continues (Ethiopia, Bolivia, Honduras and El Salvador). In Ethiopia NTV is produced locally in Ethiopian Public Health Institute (EPHI). ***Nervous Tissue /Fermi-Type/ Anti-Rabies Vaccine for Human use*** The vaccine consists of 5% aqueous suspension of brain tissue from sheep inoculated with fixed rabies virus, inactivated with Phenol. The vial contains 100ml.The vaccine works by causing the body to protect itself against rabies. The body makes antibodies which fight the rabies virus and ensure the production and maintenance of high levels of virus

neutralizing antibodies. The vaccine is given to people after they have been exposed to rabies virus. This vaccine must not be used for pre exposure vaccination purpose. This indication must be based upon a number of factors assessed by an experienced physician. If the bites are severe, treatment should be started as soon as possible, but may be stopped if the animal is seen to be healthy ten days after the bite. If for any reason the rabies suspected animal is disappeared during the observation period before the 10th day, treatment must be given in full course. If the rabies suspected animal is killed, treatment should be started as soon as possible and maybe interrupted only after rabid suspected animal examination of brain has eliminate all suspicion of rabies.

2.14. Status in Ethiopia

Rabies is one of the most severe infectious diseases in Ethiopia; many cases diagnosed in various parts of the country. The dog is the species most responsible for human exposure; over 98% of human cases and vaccinations are due to the bite of rabid or suspected rabid dogs. Most of the treatments are due to stray dogs that bite and escape and are not available for observation. Most of the people who die of rabies are under 40 years of age. Among adults, the majority are males (Fekadu, 1982). Persistence, and to some extent expansion of the overall rabies situation in the regions of the country indicate inadequacy of control activities. The various constraints that are responsible for the situation include: Not a priority disease, insufficient surveillance systems, inadequate resources due to lack of political support, lack of a national policy and a comprehensively coordinated national rabies control program, weak inter-sectarian coordination, and dog population management programs, non-implementation of technically sound strategies, inadequate research and development and absence of health education as well as lack of public awareness and cooperation (Alie, 2015).

3. MATERIALS AND METHODS

3.1. Study Area

The study was conducted in Addis Ababa city administration, Addis Ababa, the capital city of Ethiopia, covers an area of 530 km2 and is divided into 10 sub-cities. Ethiopian public health institute is located at Gulele sub-city. Currently, the institute is focusingon priority disease research and strengthening the national public health laboratory services in the country. It is also the technical hand of the Federal Ministry of Health. So, all dogs inflicting bite including laboratory diagnosis of rabies in and around Addis Ababa is managed through the national diagnostic center; which is Ethiopia Public Health Institute (EPHI) (Figure).

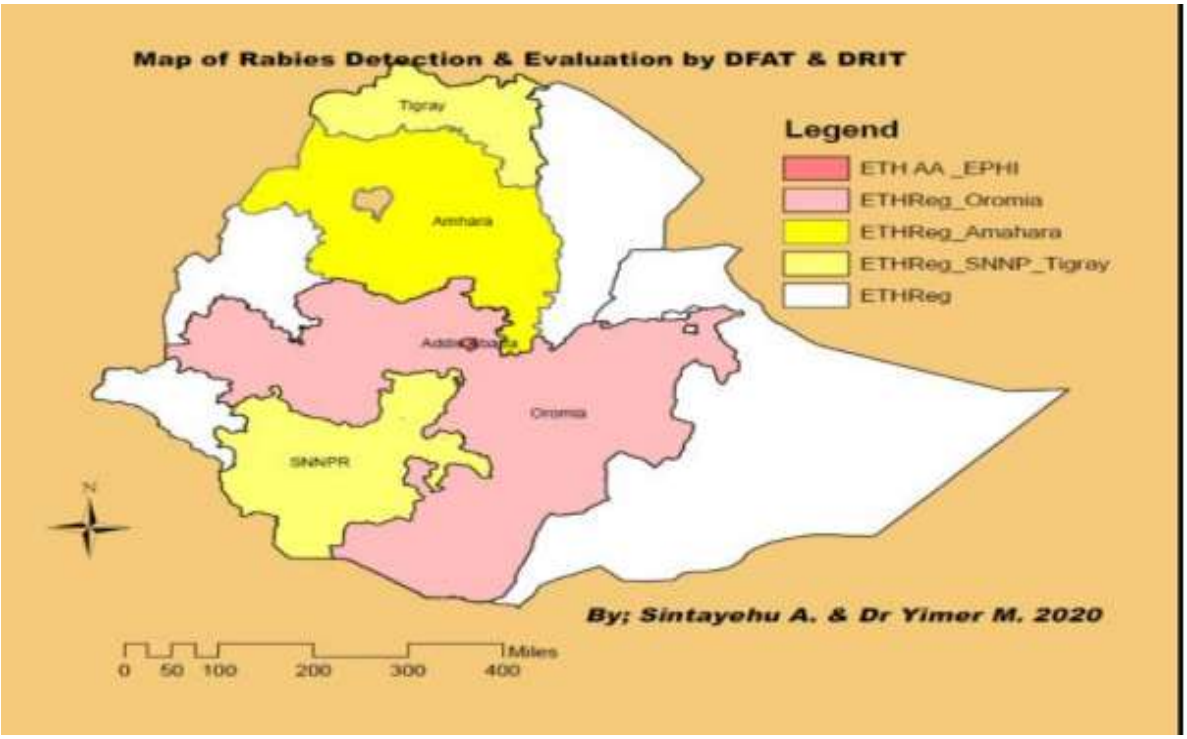


Figure 6: Map of study areaof rabies detection and evaluation by DFAT and DRIT.

3.2. Study Design and Sample size

An experimental study was conducted, a complete of 100 brain specimens were collected during the study period (December 2019 – June 2020) from various regions and different species of animals and brought to the Ethiopian public health institute rabies diagnostic laboratory.

3.3. Sampling Strategy

Ethiopian public health institute is that the only institute responsible for rabies national diagnostic center. Rabies suspected Brain samples were brought from different animal species and different states of the country. The sample submitted to the EPHI laboratory then the post mortem examination was carefully collected and kept frozen at -20oC until tested then brain smears were prepared additionally Positive control smear also prepared next the smeared slid was subjected to Fluorescent Antibody Test (FAT) and Direct Rapid Immunohistochemical Test (DRIT). The mouse inoculation test was also used in cases of in-vivo trial and further laboratory confirmation. DFA is a gold standard for rabies diagnosis.

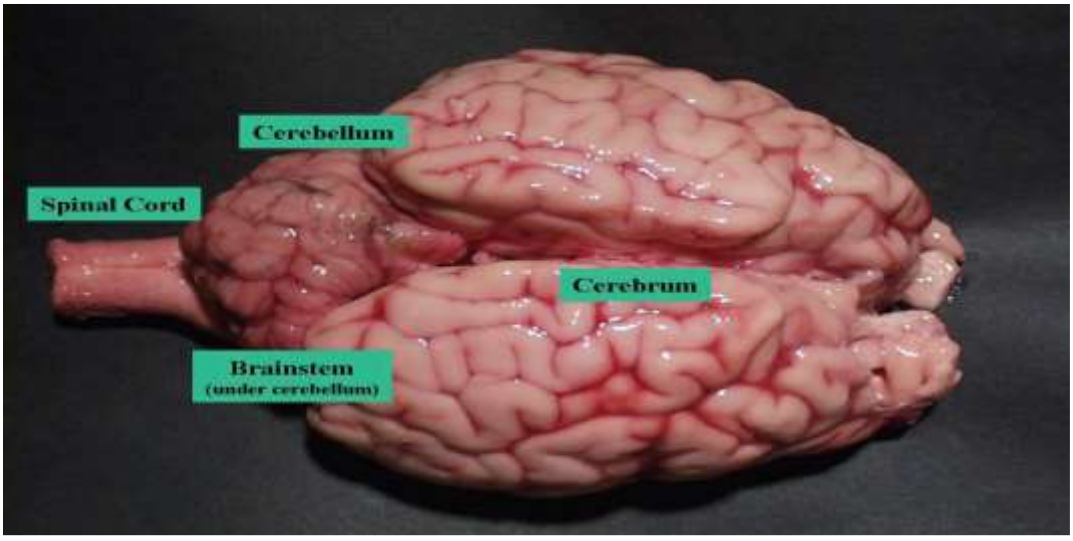
3.4. Laboratory Analysis

3.4.1. Sample Collection, preservation, and Storage

An animal was euthanized for rabies testing by any humane method that does not damage the head, including barbiturate and non-barbiturate injectable or gases. The carcass was refrigerated immediately following death to retard decomposition and autolysis of the brain. Because the animal species, site of exposure, variant of rabies virus and time and cause of death can all affect the terminal distribution of rabies virus in the brain of an infected animal, multiple areas of two to three regions of the brain must be examined to achieve reliable results. Consequently, the intact head of the animal constitutes the ideal specimen for most species. The entire body of a bat was submitted to avoid risk of loss of brain during decapitation of this very small animal and to facilitate identification of the

bat species for important epizootiologic considerations. For large livestock, such as cattle and horses, shipping of the entire head to a diagnostic laboratory/center poses special problems. For these animals, portions of the brainstem and cerebellum was removed by the veterinary (Trimarchi and Nadin-Davis, 2007).

Preservation technique was used in the event dispatch of the material under cold chain is not feasible. Isolation of virus is not possible from formalin preserved samples or those transported at room temperature. Vitality of virus was extended for several days by storing the materials in a mixture of 50% glycerol in phosphate buffered saline (PBS) however it may not protect the virus/fragment against declining titer, due to thermal fluctuations. Therefore, as far as possible low temperature should be maintained while transporting glycerol/saline preserved samples. As the virus is not inactivated by glycerol/PBS, all laboratory tests can be used on these samples. Samples are also collected, stored and frozen in deep freeze until application of test to demonstrate anti rabies antibodies (Singathia *et al.*, 2012). An explosion proof 20c° freezer is required for fixation of impression / smear slides and storage of acetone and other reagents; long term sample storage requires a freezer at -70°C. Frost-free freezers should not be used. Heat cycles in frost-free freezers will denature proteins in reagents and specimens and may compromise test results(Meslin *et al*., 1996).



3.4.2. Direct fluorescent antibody test (DFAT)

DFA is a gold standard test was performed according to CDC guidelines. Briefly, the impression smears was made on labeled glass slides from hippocampus and brain steam and brain cerebrum. The smears was dried in the air and fixed with chilled acetone for 4 hours. For internal quality control (IQC), positive and negative impression smears was made from rabid and normal mouse brains respectively. The fixed slides was stained with rabies conjugated anti-nucleocapsid (Bio-Rad, France) and incubated for half an hour at 37°C in a humid chamber. Finally, the slides was examined under UV microscope (Zeiss) using a 40X objective. Brain smears showing green fluorescent dotted particles as punctuate foci of varying sizes within or outside neurons were considered as positive (Prabhu *et al.*, 2018)

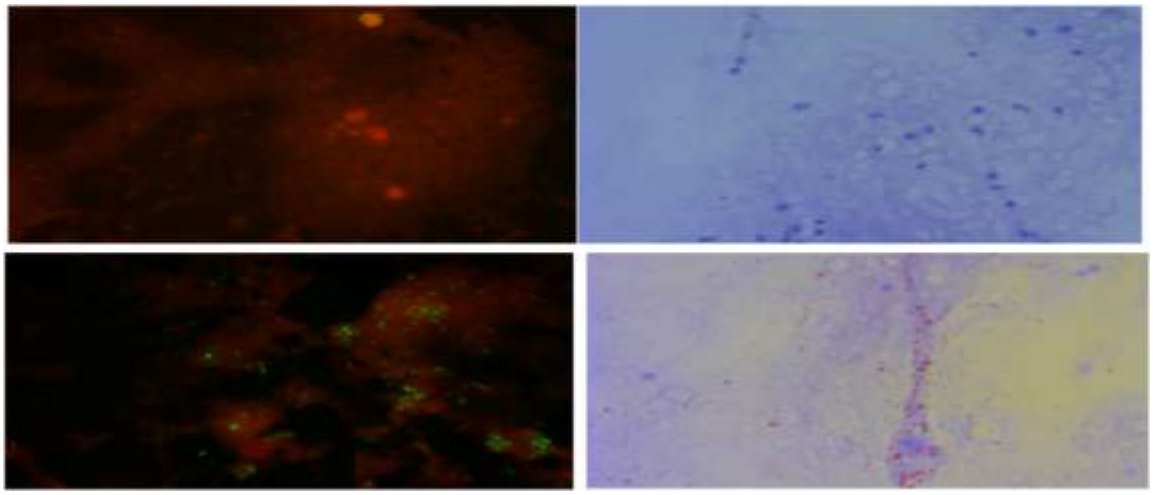


Figure 7: Brain impression from a non-rabid dog (top two panels) or a dog suspected of rabies (bottom two panels).

Subjected to direct fluorescent antibody (DFA) test using anti-rabies virus nucleocapsid protein IgG-FITC conjugate (left two panels) or to direct rapid immunochemistry test with (DRIT) usingbiotinylated mouse anti-rabies monoclonal antibodies and streptavidin-peroxidase, with hematoxylincounterstain (right two panels) Scale: 200×.

Sources:(Prabhu *et al*., 2018)

3.4.3. Direct Rapid Immunohistochemical Test(DRIT)

A center for Disease Control, USA was used as per the instructions given in the manual for the same samples as used in the DFAT. Briefly, the touch impression smears was air-dried at room temperature (RT) and then fixed in 10% buffered formalin for 10 minutes. Fixed slides was washed thoroughly with tween phosphate buffer saline (TPBS; PBS with 1% tween 80) then immersed in 3% hydrogen peroxide (H2O2) for 10 minutes. Excess H2O2 was removed by dip-rinsing in fresh TPBS The slides was incubated with monoclonal antibody cocktail in humid chamber for 10 minutes at room temperature (RT), washed by dip-rinse in TPBS and then incubated with streptavidin-peroxidase complex for 10 minutes in humid chamber at RT. This was followed by washing with TPBS. Slides then incubated with fresh peroxidase substrate, aminoethylcarbizole (AEC) with the working dilution in a humidity chamber at RT for 10 minutes. After washing with distilled water, the slide was counterstained with hematoxylin for 2 minutes. Finally, the slides was examined under light microscope (Olympus) using a 20X objective to scan the field and a 40X objective for higher power inspection of red inclusion body against a light blue background (Patrick *et al*., 2019)

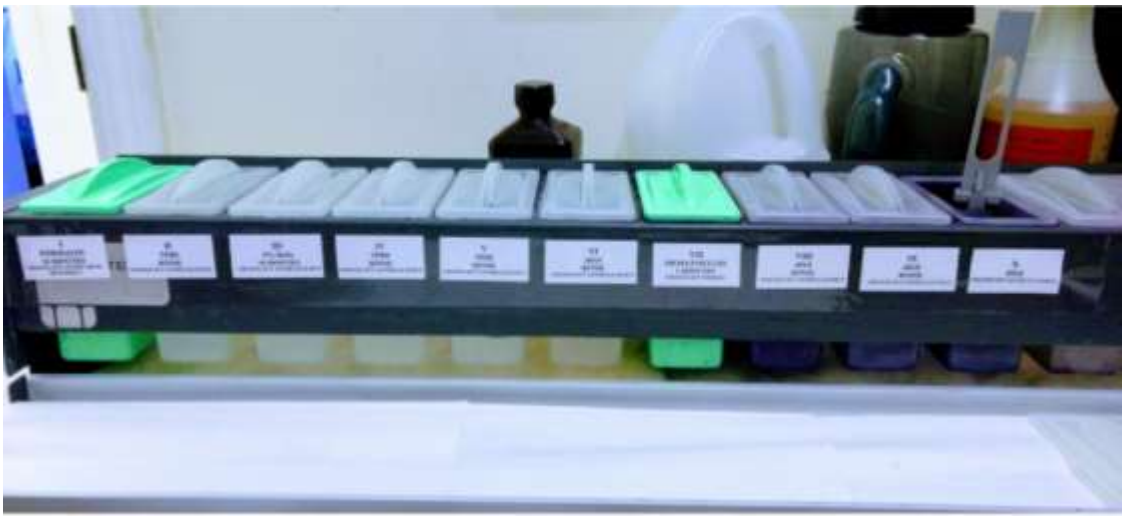


Figure 8: Setup of 10 slide staining dishes with reagents for testing.

The dishes are labeled with reagent name in the order needed to follow the protocol

Sources:(Patrick *et al*., 2019)

3.4.4. Mice Inoculation Test (MIT)

Five to ten mice, 3-4 weeks old (12-14 g), or a litter of 2-day-old newborn mice, were inoculated intracerebrally. It is recommended, though not strictly essential, to use specific pathogen-free (SPF) mice. The inoculum was clarified supernatant of a 20% (w/v) homogenate of brain material (cortex, Ammon's horn, cerebellum, medulla oblongata) in an isotonic buffered solution containing antibiotics. To reduce animal pain, mice were anesthetized when inoculated. The young adult mice were observed daily for 28 days, and every dead mouse is examined for rabies using the FAT. For street fox rabies strains, deaths due to rabies generally begin 9 days post-inoculation. For faster results in newborn mice, it is possible to check one baby mouse by FAT on days 5, 7, 9, and 11 post-inoculation. This in-vivo test is quite expensive, particularly if SPF mice are used, and should be avoided where possible. It does not give rapid results (compared with in-vitro inoculation tests), but when the test is positive, a large amount of virus can be isolated from a single mouse brain for strain identification purposes. Another advantage of this low-tech test is that it can be easily and practicably be applied in situations where skills and facilities for other tests (e.g. cell culture) are not available (Trimarchi and Nadin-Davis, 2007)

3.5. Data Analysis

The results obtained from DFAT, DRIT and MIT were entered in a Microsoft Excel 2010 spreadsheet. Sensitivity was calculated using the formula [True Positive (TP)/(True Positive (TP)+False Negative (FN))]×100, where TP was the number of specimens with true-positive results as based on the reference test and FN was the number of specimen with false-negative results. Specificity was defined as [True Negative (TN)/ (True Negative (TN) +False Positive (FP))] ×100, where TN was the number of specimen with true-negative results and FP was the number of specimen with false positive results. Confidence intervals for sensitivity and specificity were computed with the online MEDCALC® easy-to-use statistical software

3.6. Ethical Clearance

The study was approved by the zoonosis research case team of the institute. All clinical and laboratory investigations were conducted according to the principles expressed in the declaration of Helsinki 1975, as revised in 1983.

4. RESULTS

A total of 100 samples were collected and tested by the two techniques from different species of animal and from different regions of the country. The majority of the samples were from dogs (n = 88), followed by cats (n = 8), cattle (n = 3) and donkey (n = 1). Additionally, we randomly selected and tested 12 brain samples by MIT (Table 2) technique from those samples that were tested by DRIT and DFAT for internal quality control of the tests. Among the total samples tested, we found similar results, i.e., 63% were positive by DFAT and 64% were positive by DRIT. A slight difference was observed in a way that one sample was negative by DFAT but positive by DRIT and MIT (Table 2, Figure 11).

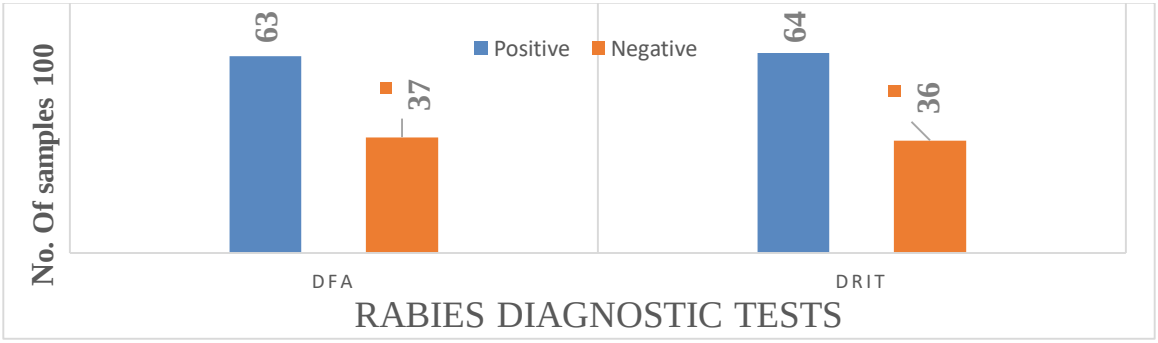
Table 1: Results of rabies virus diagnosis by DFAT and DRIT

Sample scores(n)	DFAT test result		DRIT test results		Correlation
	Positive	Negative	Positive	Negative	
Dog (88)	**56**	**32**	**57**	**31**	**99%**
Cat (8)	**5**	**3**	**5**	**3**	**100%**
Cattle (3)	**1**	**2**	**1**	**2**	**100%**
Donkey (1)	**1**	**0**	**1**	**0**	**100%**
Total(100)	63	37	64	36	100%

Table 2: Diagnosis result of MIT for rabies virus detection

Sample scores and	number of MIT test		Total
	Positive	Negative	
Dog (9)	**4**	**5**	**9**
Cat (2)	**1**	**1**	**2**
Cattle (1)	**1**	**0**	**1**
Total (12)	6	6	12

Figure 9: Comparison results of rabies diagnostic tests DFAT and DRIT



5. DISCUSSION

The rabies virus can be diagnosed by using laboratory techniques such as DFAT, DRIT, PCR, MIT, and others. From those tests, DFAT is used as a gold standard method; however, DFAT is currently available only in three regions of Ethiopia, because establishing DFAT in all regions requires high-level expertise, expensive laboratory equipment, reagents, and sophisticated laboratory setups. So that, to strengthen the surveillance system in our country; we believed that it is important to assess, evaluate, and apply other less costly, accessible, and easier laboratory tests that can equivalently be sensitive and specific with DFAT. Thus, this study aimed to compare and evaluate DRIT with DFAT to be equivalently used as one of the rabies diagnosing methods in areas where DFAT is not accessible. Since, DFAT is a very expensive type of method that requires highly trained expertise, sophisticated laboratory setup, and costly reagents it becomes challenging to be accessible to all regions of Ethiopia.

As WHO emphasizes that rabies prevention and control requires an integrated and functional surveillance system that is supported by easily applicable and extensively accessible laboratory tests. Thus, our study aimed to compare and evaluate a simple and easy alternative laboratory test other than DFAT; such test can be used for the diagnosis of rabies and will help to strengthen the rabies surveillance system in animals. Laboratory testing is desirable; however, it is extremely limited in are source-poor country and rural areas of endemic. Animal surveillance requires in the disease endemic setting with high caseloads may therefore be satisfied by resorting to syndrome case definitions and history of a dog bite. The findings of our study strongly prove that DRIT should potentially be used as an alternative diagnostic test for rabies in areas where DFAT laboratory setups are not available.

The study results were compared and evaluated with the gold standard test and out of the total 100 animal samples tested using DRIT and DFAT, 64% and 63% tested positive, respectively. In the current study, we found that DFAT had 100% sensitivity and

specificity as compared to DRIT. This could be due to the variation in prevalence of rabies in study areas. In this study, high Positive predictive value was seen, indicating high prevalence of rabies in the study area. Herein, 88% of animals tested represented dogs. This is similar to previous studies done in Ethiopia that reported higher proportion of dog specimen test (Deressa et al., 2010)) which stress the point that domestic dog remains responsible for human exposure and thus circulation and maintenance of rabies in the study area. Higher rabies prevalence among dogs was also documented elsewhere the positivity among submitted samples tested using DFA stood at 63%. In comparison, a slight variation in positivity (64%) was seen in samples tested using DRIT, showing a significant agreement between DFA and DRIT results. This is in line with previous reports elsewhere Nevertheless, there was one (1.56%) false negative by DFA as compared to DRIT, with some variation in specificity. found DRIT to be reliable in capturing false negative results of DFA

Our finding was comparable and in line with the study conducted by G. Singh et al. (2017) who have demonstrated that the sensitivity and specificity of DRIT were 100%. The positivity among submitted samples tested using DFAT stood at 63%. In comparison, a slight variation in positivity (64%) was seen in samples tested using DRIT, showing a significant agreement between DFA and DRIT results. This is in line with previous reports elsewhere (Dürr*et al.*, 2008). There were 1.56% false-negative results showed by DFAT as compared to DRIT, with some variation in specificity; however, this is happened due to sample decomposition.

Thus, DFAT is best done on fresh brain specimens than DRIT; the reliability of this method to diagnose rabies in decomposed animal brain samples is low (Mani and Madhusudana, 2013). In such cases, the DRIT would be a superior test that is less sensitive to microscope issues and provides accurate results. DRIT is simple to perform, though the numbers of steps are more than DFAT. DFAT requires air-dried smear at least two hours for fixation in cold acetone (Dürr*et al*., 2008), whereas DRIT requires only 10 minutes for fixation by using formalin. This also has another advantage as formalin inactivates the virus without impacting the antigenicity, whereas DFAT use acetone as a

fixative, which does not completely inactivate the virus as presented by the infectivity of acetone fixed tissue for neuroblastoma cells; if available, would be a potential biohazard to laboratory personnel (Prabhu *et al*., 2018). DRIT requires only light microscope, which isabout10 times cheaper than the fluorescent microscope that is used in DFAT. Also, DFAT requires a refrigerator, due to storage condition of the kit, various chemical reagents, as well as for the acetone fixation, which is a critical point for the field use. Another concern about DFAT is the need for incubator (Madhusudana *et al*., 2012).

6. CONCLUSION

The DRIT showed sensitivity and specificity equivalent to those of the DFAT.DIRT is simple to perform and does not require expensive equipment and sophisticated laboratory setup or infrastructure. The earlier reports and the present study proved that the time is taken to perform the test; technical ease, user acceptability, and the stability of the DRIT laboratory condition make DRIT a very suitable laboratory test to strengthen the rabies surveillance in Ethiopia. To conclude, DRIT should be an alternative approach for rabies diagnostic testing, which can be done to decentralize rabies laboratories in the field or in those areas that have no access to DFAT. Taking the findings in this study, similar recommendation is made here to facilitate PEP decision making and thus reduce unnecessary deaths and/ or associated financial and psychological costs in Ethiopia context. Therefore, efforts should be made to expand laboratory tests in the country. Despite with DRIT being less expensive than DFA ref, further considerations should be taken to enhance laboratory access in resource constrained settings like Ethiopia. However, any DRIT scale up effort should take into account its feasibility.

7. RECOMMENDATION

- To use this valuable new diagnostic test proper training and technology transfer is a priority
- The use of DFA in combination with DRIT has been recommended.
- Furth studies and evaluation will be require around the technique

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8. ANNEXES

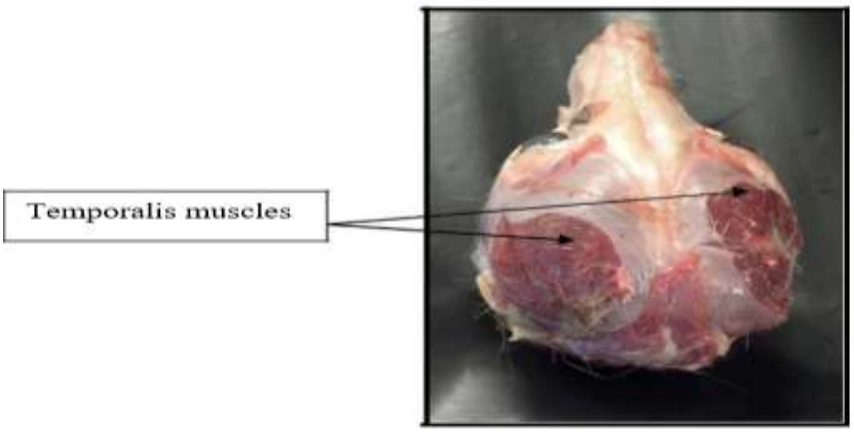
Annex 1: pictures captured during work three part of the brain part for detection of rabies virus

Annex 2: Direct Fluorescent Antibody Test (DFAT) post mortem procedure

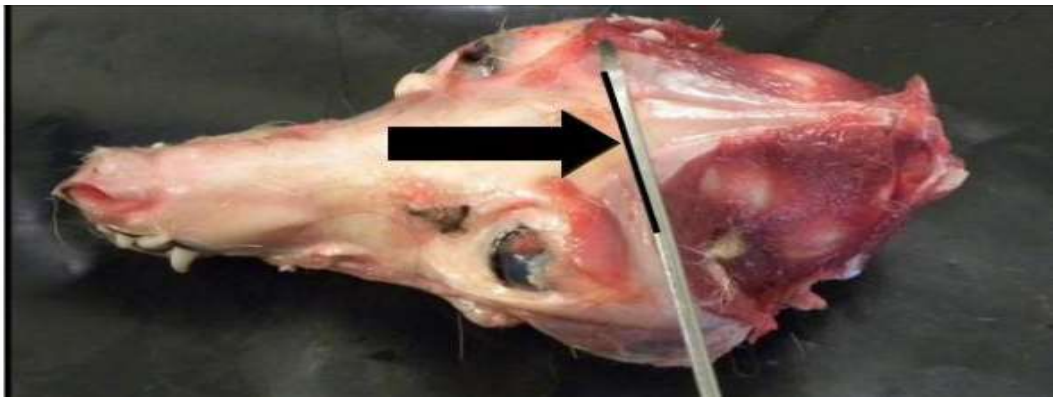
2.1: Removal of the brain and sampling of Ammon's horn, brainstem and cerebellum**.**

Before handling, it is essential to make sure that all the members of the staff in contact with a suspected sample are vaccinated against the rabies virus. In addition, each operator must use the following PPE:

1. Tyvek overall
2. Disposable protective face filter mask (FFP2D or equivalent) that fits the face correctly
3. Protective goggles or a visor against splashes from contaminated material
4. Disposable shoe covers
5. Disposable nitrile or latex gloves; in this case, it is advisable to wear more than one pair of gloves (one on top of the other)
6. Disinfect the autopsy table with 3% solution of sodium hypochlorite (concentration that ensures a proper disinfection).
7. Secure the animal firmly to the autopsy table or, better, decapitate the animal and hold the head tightly with forceps.
8. Remove the skin of the skull to expose the temporalis muscles

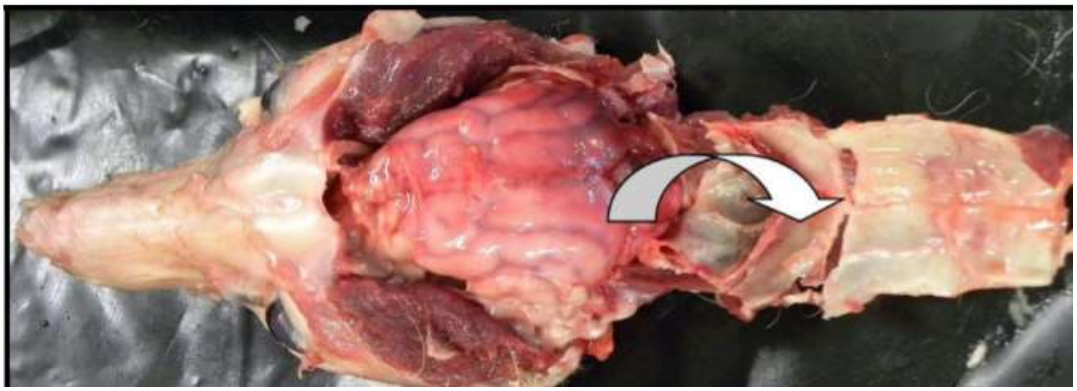


Open the skull at the eye level and cut along the temporal bone (picture 1, 2 and 3



Picture 1-First incision at the eye level

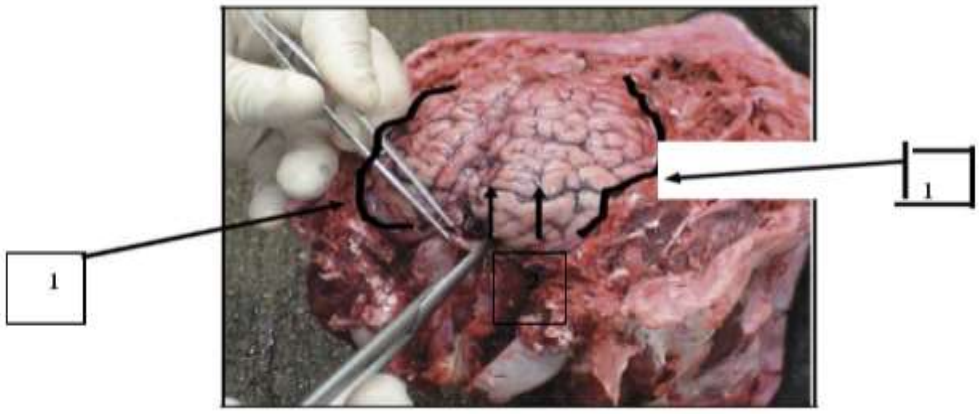
Picture 2- Incision at the temporal bone



Picture 3- Exposure of the brain

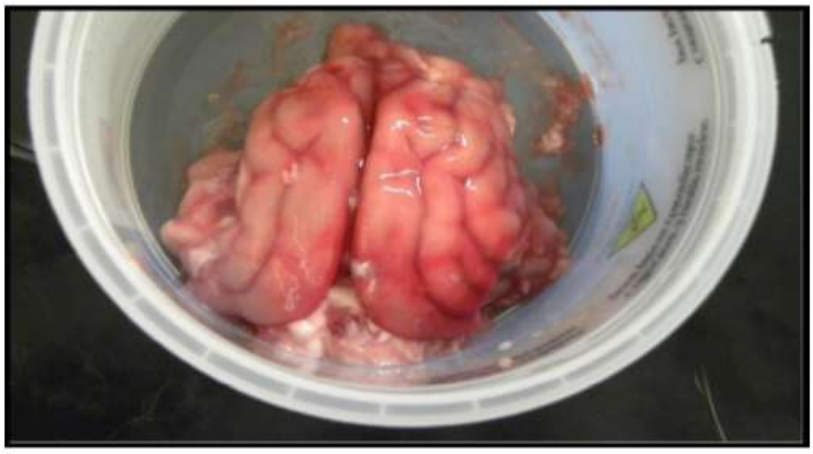
Prepared by IZSVe (FAO Rabies reference centre) within the framework of the RESOLAB Rabies Subnetwork – March 2013

Once the skull has been removed, use fresh instruments (sterile) to remove the meninges, Make an incision in the meninges, starting from the median region and then on each side of the longitudinal sinus



Make a second incision perpendicular to the first one and push the meningeal flaps upward and backwards (step 1 on the picture below). Using sterile instruments cut through the medulla with a scalpel as low as possible and lift up the brain, proceeding from back to front and successively severing the cranial nerves (step 2 on the picture above).

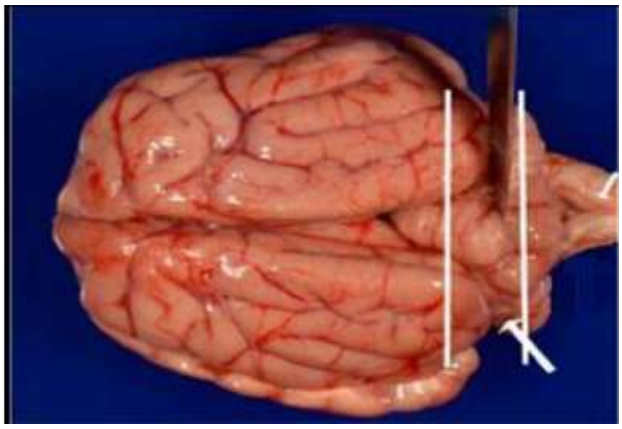
At the end of the operation, roll the brain gently forward into a sterile plastic container so that it rests on its upper surface.



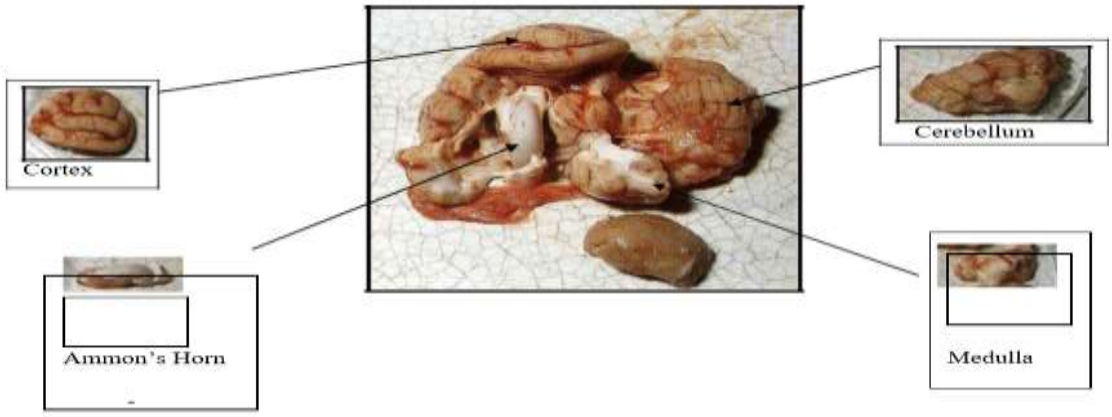
Prepared by IZSVe (FAO Rabies reference centre) within the framework of the RESOLAB Rabies Subnetwork – March 2013

Cut across the brain transversely, starting from the base behind the optic chiasma and proceed towards the lower third of the cerebral hemisphere. The third ventricle appears on the cut surface;

Ammon's horn is seen as a whitish fold resembling a large bean cut transversely, and can then be removed.



A small section of Ammon's Horn, medulla and cerebellum are cut and placed in a container marked with the number of the sample and the name of the species.



N.B: the sample is not suitable if it is mouldy. However, it is advisable to analyze and take into account the condition of the sample during the interpretation of the results. In the case of a negative sample, it could be a false negative (deterioration of antigen). It is then advisable toperform a second sampling (whenever possible), or to specify the uncertainty of the result in the analysis results.

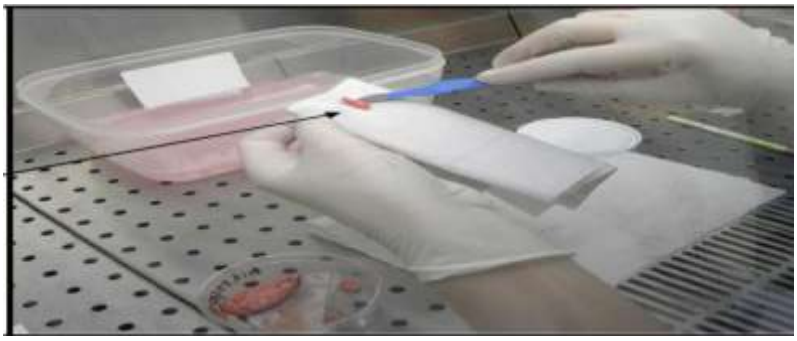
Prepared by IZSVe (FAO Rabies reference centre) within the framework of the RESOLAB Rabies Subnetwork – March 2013

2.2 Preparation of test slides:

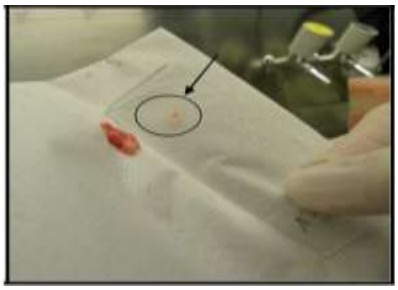
At least one slide is prepared for all terrestrial mammals and at least 2 slides are prepared for each domestic sample. Positive controls consist in one slide impressed with a brain of a mouse infected by a CVS-11 challenge. The negative control consists in one slide impressed with a brain of a healthy mouse. Each step of the manipulation must be carried out under Biosafety cabinet class II.

2.2.1 Impression method: The brain must be frozen.

- Cut a small section of brain tissue and place it on one clean paper towel.



- A clean microscope slide is then place on the cut surface of the section and pressed gently downwards with just enough pressure to create a slight spread of the exposed surface of the tissue against the Slide.
- According to the size of the section, 3-4 impressions can be made on the slide.

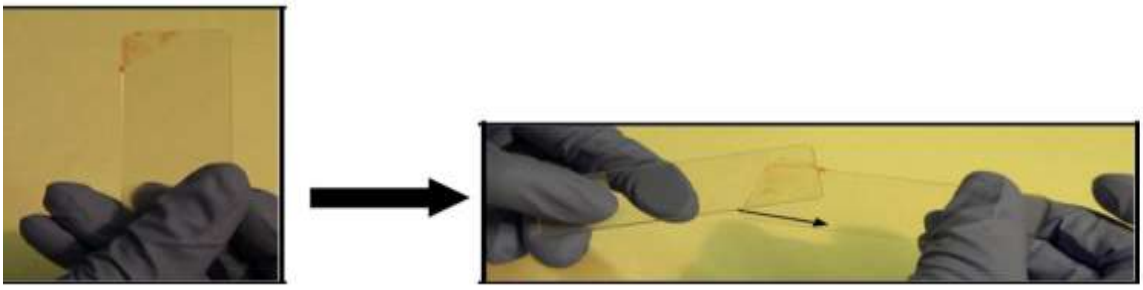


- Remove any excess brain tissue from the slides by blotting on a clean paper towel.

- Allow the slides to air dry for 10 minutes.

2.2.2 Smear method

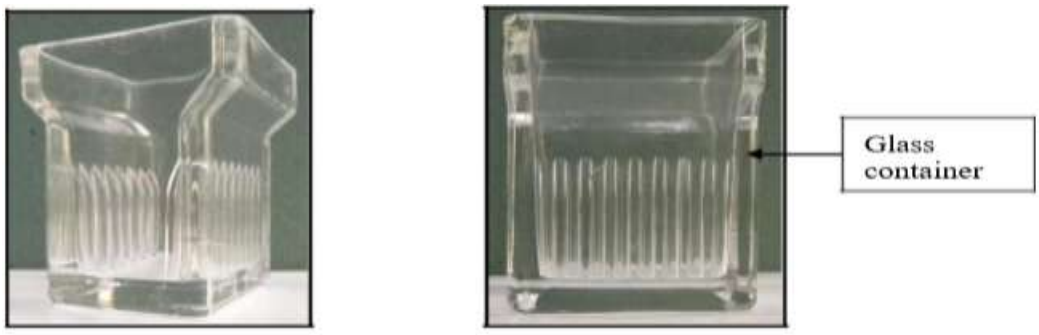
- Place a small section of brain tissue on one end of the slide.



- Another slide is used to crush the section of tissue against the first slide.
- Spread the sample homogeneously over a small portion of the slide.
- Care should be taken not to use too large a tissue-section, as these results in an excessively thick film, making proper staining and microscopic examination impossible.

2.3 Fixation

- Once dried, both test slides and positive/negative control slides are immerse in slide racks (glass container) filled with cold pure acetone and fixed at room temperature for 1 hour (under chemical cabinet).
- Use a Hellendhal staining jar (previously stored in a freezer to prevent it from breaking during the addition of acetone) for fixing.
- Hellendhal staining jar



Prepared by IZSVe (FAO Rabies reference centre) within the framework of the RESOLAB Rabies Subnetwork – March 2013

N.B: The positive control should be fixed in separate containers to avoid transfer of tissues from sample slides to the positive control during fixation.

- Remove the slides from acetone and air-dry the slides at room temperature for 30 minutes (under chemical cabinet).

2.4 Immuno-fluorescent staining

- Calculate the total volume of the antibody you need multiplying the number of slides that will be analyzed by the amount that will be distributed on each slide (20 µl). (Number of slides x 20= total volume (µl) of antibody needs for the work session).
- At this volume, add the required amount of Evan's blue in order to obtain 1:20 dilution. (For example, for 100 µl total volume of antibody, 5 µl of Evan's blue at 1% will be added). 6. Add 20 µl of conjugate and Evan's blue on each slide. Incubated slides 30 minutes for at 37°C in a humidified chamber to avoid drying of the conjugate.

2.5 Washing

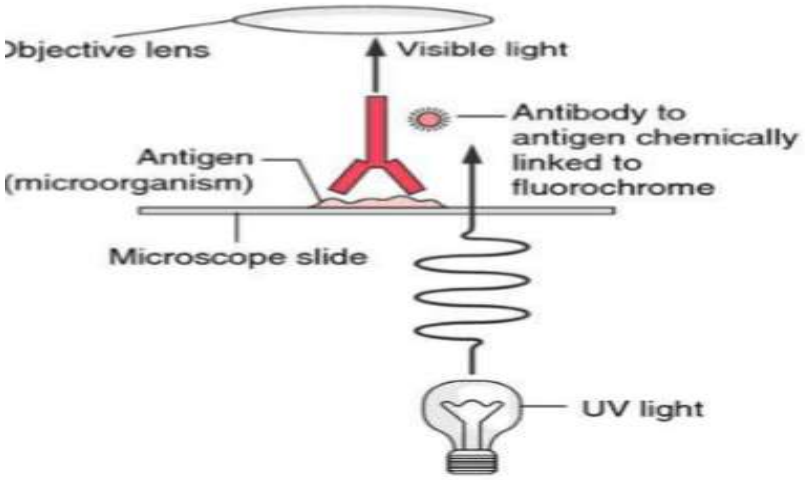
- After incubation, slides are washed twice with PBS (5 min for each wash) and once with distilled water for a few seconds. Drain the slides on filter paper. 9. When dry, one drop of glycerol (at 30% in PBS) is added on each slide 10. Carefully place the cover slip avoiding the formation of bubbles.

2.6 Microscope reading

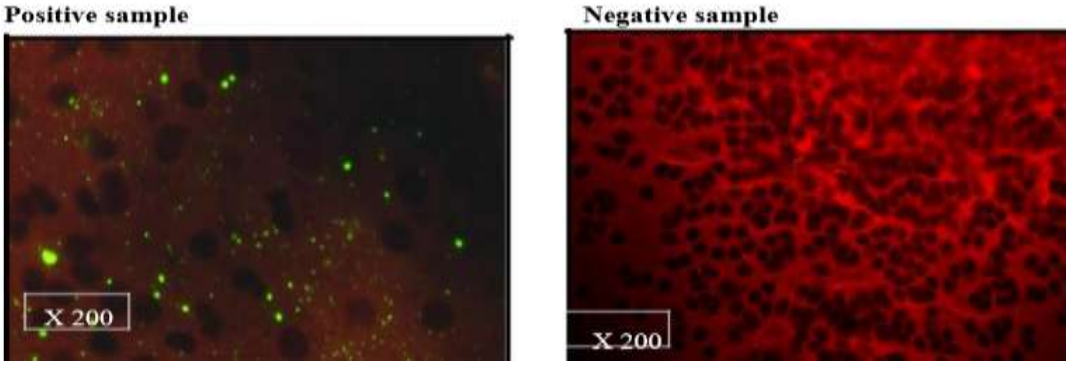
- Fresh, frozen or glycerolated brain material may be examined. The procedure consists in allowing a labeled antibody (fluorescein isothiocyanante) to react with a specific antigen (if present) and in observing the reaction under a fluorescence microscope.

Principle of Immunofluorenscence:

- This diagnostic test is highly accurate and guarantees high sensitivity and specificity. The results are available after 1 and ½ - 2 hours from reception of the carcass. There are a large number of polyclonal and monoclonal commercial antibodies.
- The laboratory is free to choose the one that works best, paying attention to the dilution to apply before use.
- The use of the anti-rabies nucleocapsid conjugate (polyclonal) corresponds to the protocol in use at the IZSVe laboratory (FAO Reference Centre for Rabies).
- These polyclonal antibodies are targeted against the ribo-nucleoprotein complex of the Rabies virus.



- Control slides are examined before the test slides to ensure the equipment is operating satisfactorily and slides are properly stained.
- Positive control and test slides containing rabies antigen will contain brilliantly fluorescing green or greenish-yellow structures.
- A diagnosis session is considered valid provided that: - Fluorescence is detected on the positive control slide - No fluorescence is detected on the negative control slide



Materials, equipment and reagents

Materials:

Samples of brain to be tested for rabies diagnosis Brain of mice infected with CVS -11 (positive control for terrestrial mammals diagnosis) Brain of uninfected mice (negative control for terrestrial mammals and bats diagnosis)

Equipment:

- ✓ Slides and cover slips
- ✓ Micropipettes with sterile tips
- ✓ Box slide folder
- ✓ Optical microscope equipped for fluorescence reading
- ✓ Thermostat at 37°C (± 2°C)
- ✓ Lab coat, face mask, goggles and gloves
- ✓ Class II Safety cabinet Laminar Flow

Chemicals

- ✓ Sterile PBS (pH= 7.2-7.4); (Keep refrigerated at 4°C)
- ✓ NaCl g 8.0 KH2PO4 g 0.2 KH2PO4 ● 12 H2O g 2.9 KCl g 0.2
- ✓ Sterile distilled water
- ✓ Undiluted acetone
- ✓ Fluorescent conjugate (Lyophilised, absorbed anti-rabies nucleocapsid conjugate)

  N.B: after re-suspension, the solution can be stored for 15 days at 4°C in the dark glycerol in PBS (30%)

  N.B: The solution is discarded if not clear and colourless or if contaminated by molds.
- ✓ Evan's blue solution

Annex 3: Direct Rapid Immunohistochemistry Test (DRIT) procedure

Safety

All persons involved in rabies testing should receive pre-exposure immunization with regular serologic tests and booster immunizations as necessary unimmunized individuals should not enter laboratories or areas where rabies work is conducted. All tissues processed must be disposed of as medical waste and all activities related to the handling of animals and samples for rabies diagnosis should be performed using appropriate biosafety practices to avoid direct contact with potentially infected tissues or fluids (CDC and National Institutes of Health, Biosafety in Microbiological and Biomedical Laboratories, 4th edition, U.S. Government Printing Office, 1999). Personnel working with rabid animals and tissues from rabid animals e.g. brain, are at risk of rabies infection through accidental injection or contamination of mucous membranes with rabies virus contaminated material and by exposure to aerosols of rabies infected material. All manipulations of tissues and slides should be conducted in a manner that does not aerosolize liquids or produce airborne particles. Barrier protection is required for safe removal of brain tissue from animals submitted for rabies testing. At a minimum, barrier protection during field necropsy should include eye protection e.g. safety glasses, face shield; and surgical gloves. When processing multiple animals in a central location Personal Protective Equipment (PPE) should include: heavy rubber gloves, laboratory gown and waterproof apron, boots, surgical masks, protective sleeves, and a face shield. Fume hoods or biosafety hoods are not required, but they provide additional protection from odor, ectoparasites, and bone fragments.

Brainstem/cranial cord collection for rabies testing

Make a ventral midline incision from the symphysis of the mandible to several centimeters caudally beyond the larynx. Sever the musculature attachments of the tongue rostrally and on both sides, proceeding caudally to free the larynx, trachea, and esophagus (as if preparing to remove the “pluck“ or tongue, esophagus, trachea, lungs, and heart in

one piece) and retract to expose the ventral surface of the spinal column and associated musculature. Palpate to identify the atlanto-occipital joint and dissect to expose the tough connective tissue located on the ventral surface of the joint. Although tough, the connective tissue is thin and directly overlays cerebrospinal and the spinal cord. With the tip of the scalpel blade, carefully cut through the connective tissue (but not the spinal cord) and work the tip of the scalpel down both sides of the joint, while flexing the joint to gain better access. The exposed brainstem/spinal cord tissue may then be severed as far caudally and rostrally as possible to yield central nervous system tissue suitable for rabies testing. Samples may be placed in screw cap vials, preferably unbreakable (i.e. not glass), or other suitable containers, such as ointment tins. Consideration should be given to adequate sample information (such as species, a unique identifying number, date, animal location, etc.). Samples may be refrigerated until testing if they will be tested within several days. Otherwise, the samples should be frozen, and kept frozen during storage and shipment, until they are tested. To avoid cross contamination of samples; each specimen should be handled on a clean work surface with new disposable gloves. All instruments used during necropsy, dissection, and slide preparation must be properly disposed. Instruments not in use should be kept in closed storage. Only those instruments in use for processing a single sample should be exposed. Maintain test samples for 3 months. Representative positives can be subsampled and used as controls. All positive brain samples should be sent to the CDC for epidemiologic typing, and for other purposes, along with 10% of negatives for confirmation. Unacceptable deterioration or decomposition of a sample is a qualitative assessment of the condition of each sample upon arrival in the laboratory or at the time of testing.

Observation: results should be recorded on the RIT Result Sheet (page 14).

Good: Optimum brainstem/cranial cord, fresh, no tissue decomposition.

Fair: Slight tissue decomposition but identifiable as brainstem/cranial cord; may be some discoloration around the periphery but stable tissue at the core of the sample.

Poor: Substantial green color, discoloration, liquefaction, desiccation, or an unrecognizable gross anatomy. Substantial green color, liquefaction, desiccation, or an

unrecognizable gross anatomy can indicate an unsatisfactory sample. A substantial loss of tissue during staining and washing or the presence of bacteria on the stained slide may also indicate sample deterioration.

If negative results are obtained on deteriorated tissue, the test report should state only that the condition of the sample is such that tests cannot rule out the presence of rabies virus in the specimen. The negative findings should not be mentioned, since this is often misinterpreted as a negative diagnosis. Positive test results are reported as such. Samples with indeterminate results and all positive samples should be sending to the CDC in Atlanta for confirmation and typing.

Equipment and Reagents (The use of trade names is for laboratory comparison only and does not imply endorsement by PHS)

Equipment/Supplies:

- ✓ Light Microscope: with 20x and 40x objectives
- ✓ Tissue- Tek slide staining kit; Fisher Cat. No. NC9479355 (T-Tek #25608902)
- ✓ Slide holder, 24-place; Fisher Cat. No. NC9418050 (T-Tek #25608868)
- ✓ Fisher Brand syringe: 25mm 0.45 Fm filter, Fisher Scientific Cat. No. 09-719D.
- ✓ Wheaton glass vials 8ml, Fisher Scientific Cat. No. 06-408BC
- ✓ BD 10cc syringe: 209604, Fisher Scientific Cat. No. 14-823-2A
- ✓ Pipettor 200Fl Rainin: http://www.rainin.com/ P200 Pipetman pipet 50-200Fl.
- ✓ Corning cover glass 24x60, VWR Scientific Cat No. 48396-160
- ✓ Microscope slides white frosted, Erie Scientific Co. Order No. 10-226.
- ✓ Centrifuge tube (15 ml), polypropylene Falcon 352097 Fisher Scientific Cat. No. 14-959-70C
- ✓ Tips-universal (1 – 200 Fl vol.) Corning-Costar: Fisher Scientific Cat No. 07 200-300VWR Pipet Bulb (0.05 – 100 ml), VWR Scientific Cat. No. 53497-055
- ✓ Serological pipet (1.0 ml), Pyrex Corning 7078D-1, VWR Scientific Cat. 53222-259

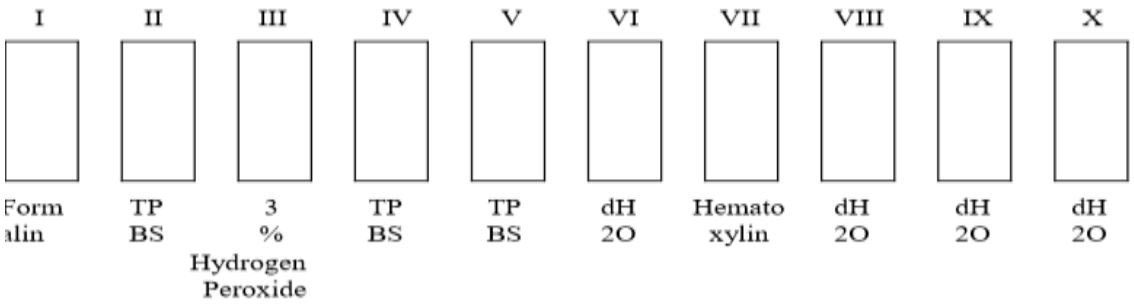
- ✓ Serological pipet (5.0 ml), Pyrex Corning 7078D-5, VWR Scientific Cat. No. 53222-281
- ✓ Serological pipet (10.0 ml), Plastic, VWR Scientific Cat. No. 20171-042
- ✓ Tissue culture plate lids (50/case), BD Falcon, Fisher Scientific, Cat. No. 08-772-2B
- ✓

Reagents

***Note**: All reagents can be stored at room temperature unless noted otherwise.

- ✓ Formalin: 10% buffered; Fisher Brand, Order no. SF 100-4 (4L)
- ✓ Phosphate buffered saline (PBS); Fisher Scientific Cat. No. SH30256.02
- ✓ Hydrogen peroxide 3%: Use any commercially available 3% hydrogen peroxide e.g. Walgreen Drug Store; CVS Pharmacy, Grocery Store brand, etc.
- ✓ Primary antibody: CDC cocktail (50 ml) of mouse anti-rabies biotinylated monoclonal antibodies, store at 4oC. Supplied by CDC; ready-to-use.
- ✓ Streptavidin-Peroxidase: 50mL (71-00-38): Kirkegaard& Perry Laboratories Inc; ready-to-use, store at 4oC.
- ✓ Amino-ethylcarbizole(AEC) substrate: SIGMA no. A6926.
- ✓ N,N, Dimethyl formamide GR, EM Science: Cherry Hill, NJ. Thomas scientific Order No. C279-A87
- ✓ Acetate Buffer:0.1M, pH 5.2, Poly scientific, Bay Shore, NY, (516) 586-0400. Catalog No. S140.
- ✓ Gills formulation #2: Fisher order no. CS401-4D, 4L, Diluted 1:2 in distilled water
- ✓ Gel/Mount:BioMeda Corp., Foster City CA, (aqueous/dry mounting) Fisher Scientific Cat. No. BM-MO1, 20ml
- ✓ TWEEN 80: Polyethylene glycol, Sigma-Aldrich order no. P1754.
- ✓ Deionized water**:** Fisher Scientific, Cat. No. S75232

Tissue-Tek staining tray set-up and reagent exchange.



Staining dish number:

i. Formalin – change out after 2 runs or once a week.
ii. TPBS – change out with each test.
iii. 3% Hydrogen peroxide – change out with each test.
iv. TPBS – change out with each test.
v. TPBS – change out with each test.
vi. Deionized/distilled water (dH2O) – change out with each test.
vii. VII. Hematoxylin– change out once a week.
viii. dH2O – change out with each test.
ix. dH2O – change out with each test.
x. dH2O – change out with each test.

Preparation of reagents for staining dish

i. Formalin, 10% buffered; ready-to-use
ii. TPBS:
Phosphate buffered saline with 1% tween-80 (TPBS) TPBS (PBS with 1% tween-80) = to 990 ml of PBS add 10 ml Tween-80. Shake until tween-80 is completely into solution.
iii. 3% hydrogen peroxide; ready-to-use
iv. TPBS:

v. TPBS:

vi. Deionized/distilled water (dH2O); ready-to-use,

vii. Hematoxylin

Gills formulation #2 diluted 1:2 in distilled water.

The staining dish will hold 250 ml of solution (125 ml hematoxylin + 125 ml deionized water).

viii. Deionized/distilled water (dH2O)

ix. Deionized/distilled water (dH2O)

x. Deionized/distilled water (dH2O)

Protocol for the Direct Rapid Immunohistochemical Test(DRIT) Streptavidin-biotin peroxidase staining technique for diagnosis of rabies virus

1. Make routine touch impressions of suspect CNS tissues on labeled glass microscope slides (include standard positive and negative controls).
2. Air-dry slides for 5 minutes at room temperature.
3. Immerse slides in 10% buffered formalin at room temperature for 10 minutes. Dish I.
4. Remove and dip-rinse slides several times to wash off any excess fixative in wash buffer TPBS (PBS with 1% tween 80). Dish II.
5. Immerse slides in 3% hydrogen peroxide for 10 minutes. Dish III.
6. Remove excess hydrogen peroxide by dip-rinsing slides in TPBS, Dish IV.Transfer slides to the next rinse Dish V (after dipping, shake off excess buffer, and blot excess buffer from slide edges surrounding the impression). Work with one slide at a time leaving the remaining slides immersed within TPBS rinse.
7. Incubate slides in a humidity chamber (e.g. may use the plastic top to a 96-well plate or another simple cover over slides, on a moistened paper towel, on lab bench top) at room temperature with primary antibody - biotinylated anti-rabies mAb for 10 minutes (add enough of this primary antibody by drop to cover the impression).

8. After incubation shake off excess conjugate. Dip-rinse slides with TPBS, Dish V (shake off excess TPBS and blot buffer from slide edges surrounding the impression).Can use this same wash buffer through step 10
9. Incubate slides with streptavidin-peroxidase complex (add enough of this complex to the slide by drop to cover the impression) in a humidity chamber at room temperature for 10 minutes. After incubation shake off excess.Dip-rinse slides with TPBS, Dish V (shake off excess buffer and blot excess buffer from slide edges surrounding the impression).
10. Incubate slides with peroxidase substrate, amino-ethylcarbizole (AEC) – prepare the working dilution just prior to use.Add enough of this substrate to the slide by drop to cover the impression in a humidity chamber at room temperature for 10 minutes. After incubation, shake off excess substrate.
11. Dip-rinse slides in deionized/distilled water, **Dish VI.**
12. Counterstain with Gills Hematoxylin (diluted 1:2 with deionized/distilled water) for 2 minutes, **Dish VII**.
13. Immediately dip-rinse this stain from slides with deionized/distilled water Dish **VIII**. Make a second dip-rinse of slides with fresh deionized/distilled water (**Dish IX**) to ensure removal of excess stain.
14. Transfer slides to fresh distilled water **Dish X**.
15. Mount slides with water-soluble mounting medium and cover-slip. (Work with one slide at a time, shake off excess deionized/distilled water and blot excess from slide edges surrounding the impression).
16. Do not allow slides to air-dry prior to cover-slipping. If multiple slides are stained, they may sit in the deionized/distilled water rinse before cover slipping.
17. View slides by light microscopy, using a 20x objective to scan the field, and a 40x objective for higher power inspection (rabies virus antigen appears as red inclusions against the blue neuronal background).
18. Record results.