

**Evaluation of Fluorescent Antibody Test for Detection of Rabies Virus
Antigen in Fresh, Frozen and Formalin-Fixed Brain Tissue Specimens in
Suspected Rabid Animals in Ethiopia**

BY

TIHITINA TESFAYE, B.Sc

Department of Microbiology, Immunology and Parasitology, Faculty of Medicine,
Addis Ababa University



December, 2017

**Evaluation of Fluorescent Antibody Test for Rabies Virus Antigen Detection
in Fresh, Frozen and Formalin-Fixed Brain Tissue Specimens in Suspected
Rabid Animals in Ethiopia**

SUPERVISORS

1. Dr. Yimtubezenash Woldeamanuel, MD, M. Sc, PhD

(DMIP, School of Medicine, AAU),

2. Dr. Daniel Asrat, MD, M. Sc, PhD

(DMIP, School of Medicine, AAU)

CO-SUPERVISOR

**1. Dr. Nishanwork Wondafrash (B.Sc,M.Sc) Directorate of Parasitology,
Bacteriology and Zoonosis, EPHI**

**A thesis submitted to the school of graduate studies, Addis Ababa University
in partial fulfillment of the requirements for the Master degree in Medical
Microbiology.**

December, 2017

ACKNOWLEDGEMENTS

My heartfelt gratitude and respect goes to Dr. Yimtubezinash Woldeamanuel, my main advisor, for her excellent scientific guidance, supervision, critical evaluation of the thesis draft and immediate feedback with valuable comments and suggestions throughout the course of my work. My sincere appreciation also goes to Dr. Daniel Asrat my co-advisor, for his scientific input and encouragement.

I would like to acknowledge Addis Ababa University, School of Medicine, College of Health Science, Department Of Microbiology, Immunology and Parasitology for giving me a chance to conduct such type of study.

My special thanks also go to Ethiopian Public Health Institute. I would like to thank all the staff members of Parasitological, Bacteriology and Zoonosis Disease Directorate, for their assistance during the work; and for giving permission to use different reagents and materials. Vaccine and diagnostics production directorate for their supports and encouragement.

I would like also to address my thanks to all who helped me during specimen collection, laboratory investigation, statisticians who advised me for data analysis and management.

Table of contents

List of contents	Page No.
ACKNOWLEDGEMENTS.....	I
Table of contents	II
Abbreviations	IV
List of Figures	V
List of Tables	V
Abstract.....	VI
CHAPTER I:	1
INTRODUCTION.....	1
1.1 Background	1
1.2. Statement of the problem	2
1.3. Literature review.....	4
1.3.1. Historical Perspective	4
1.3.2. Virological characteristics of rabies virus.....	5
1.3.3 Pathogenesis of Rabies	6
1.3.4. Epidemiology and Risk Factors	7
1.3.5 Clinical presentation	9
a. Prodromal stage.....	9
b.Classical (furious) phase	9
c.Paralytic (dumb) phase.....	9
1.3.6. Laboratory Methods for Isolation and Identification of rabies virus	11
1.4. Significance of the study	14
1.5. Objectives of the study	15
CHAPTER II:	16
MATERIALS AND METHODS.....	16
2.1. Study design, Period and Area	16
2.1.1. Study design.....	16
2.1.2. Study area and period.....	16
2.2. Sample size and Sampling Technique	16

2.3. Sample Collection, Handling and Transport	16
2.3.1 Tissue preparation	17
2.3.2 Fluorescent antibody test (FAT)	17
2.3.3 Cell culture.....	17
2.4. Data processing and analysis	18
2.5. Quality Control.....	18
2.6. Study Variables	19
2.6.1. Dependent variable	19
2.6.2. Independent variables	19
2.7. Ethical Consideration	19
CHAPTER III:	20
RESULTS	20
3.1. Demographic characteristics of suspected rabid animals	20
3.2. Clinical characteristics of suspected rabid animals.....	22
3.3. FAT result	23
3.4. Comparison of FAT: Frozen and formal fixed specimen vs. Fresh specimens examined	24
3.5 Duration of trypsin treatment	27
3.6 FAT for Quality control specimens.....	28
3.7 Cell Culture.....	28
CHAPTER IV:	29
DISCUSSION.....	29
Conclusion	33
Recommendations.....	34
References	35
ANNEXS	43

Abbreviations

AAU:	Addis Ababa University
CNS:	Central Nervous System
FAT:	Fluorescent antibody test
EPHI:	Ethiopian public health institute
FITC:	Fluorescein isothiocyanate
GBS:	Guillain-Barre syndrome
IF:	Immunofluorescence
OIE:	Office International Des Epizootics
RABV:	Rabies Virus
RNA:	Ribonucleic acid
SPSS:	Statistical package for social sciences
WHO:	World Health Organization

List of Figures

Page No.

Figure 1.1 Structure of Rabies virus (Adopted from Charles <i>et al</i> , 2002)	6
Figure 1.2 H&E stain of Negri bodies in the cytoplasm of nerve cell (Beigh, 2016)	12
Figure 1.3 Impression smear obtained from animals suspected of having rabies showing apple green fluorescence in neurons. Direct FAT (Dong <i>et al.</i> , 2012).....	13

List of Tables

Table 3.1a. Demographic characteristics of suspected rabid animals during March 2017 to July 2017	20
Table 3.2. Clinical signs in positive cases of rabid animals (n=32), EPHI, March 2017 to July 2017.....	23
Table 3.3: Fresh, Formalin fixed and Frozen brain tissue specimen grading result using Fluorescent antibody technique	24
Table 3.4. Percentage sensitivity and specificity of FAT in fixed brain specimen	25
Table 3.5. Percentage sensitivity and specificity of FAT in frozen brain specimen	26
Table 3.6. Comparison of FAT between Frozen and Fresh brain tissue specimens obtained from different animals	27
Table 3.7. Effect of duration of trypsin treatment on the results of IF staining for rabies antigen in formalin-fixed brain tissue samples after incubated at 37 °C.	27

Abstract

Background: Rabies is an infectious and preventable viral disease of mammals. It is caused by several *Lyssavirus* species, a group of negative sense RNA viruses, and most often transmitted through the bite of a rabid animal, commonly dogs. Many diagnostic methods have been used to detect rabies virus antigen. The preferred method for routine diagnosis of rabies in fresh or frozen brain tissues is the fluorescent antibody test.

Objective: The aim of this study was to evaluate fluorescent antibody test for rabies detection in fresh, frozen and formalin-fixed brain tissue specimens in Ethiopia

Methods: The study was carried out on rabies-suspected brains of animals which were collected from March 2017 to July 2017 at Ethiopian Public Health Institute. The detection of rabies antigen was done in brain impressions from three areas of the brain: hippocampus, cerebellum, and brain stem. One portion of each paired specimen was prepared for fresh, frozen and formalin fixed FAT. It was treated with globulin labeled with fluorescein isothiocyanate. Specific aggregates of rabies virus antigen was detected by their fluorescence using a reflected light (incident light) fluorescence microscope.

Results: The sensitivity of fixed brain tissue compared with fresh FAT was 96.9% and specificity was 100%. Frozen specimen gave similar sensitivity and specificity with fresh FAT which was 100%. The association between Formalin fixed tissue sample & Fresh tissue sample when it is done using FAT had p -value 0.257, frozen tissue sample & Fresh tissue sample had p value 1.00 and Formalin fixed tissue sample & frozen tissue sample had p value 0.257 with no significant difference.

Conclusion: Rabies detection in animals can be accomplished from diagnosis of rabies from fixed brain tissues which offers similar sensitivity as detection of rabies in impression smears.

Key words: Rabies, fluorescent antibody test, Ethiopia

CHAPTER I:

INTRODUCTION

1.1 Background

Rabies is an acute, virulent, progressive, and an ancient disease that affects all warm-blooded animals. It is caused by viruses in the family *Rhabdoviridae*, genus *Lyssavirus* (Kuzmin *et al.*, 2005). Rabies is a viral zoonosis, causing fatal encephalitis and is widely prevalent in developed as well as developing countries. Several specific Rabies virus (RABV) variants have been characterized from different mammalian hosts, such as dogs, foxes, mongooses, and other carnivores, and bats (Sudarshan *et al.*, 2007). In the developing world, RABV infections contribute most significantly to the public health burden of rabies (Nel *et al.*, 2007).

The transmission requires introduction of rabies virus from saliva or infected neural tissue into bite wounds or open cuts in skin or mucous membranes. The animal usually contracts rabies from the bite of an infected animal. Humans are recognized as dead end host for rabies virus. Under some circumstances transmission could be possible through inhalation of large amounts of aerosolized rabies virus or through organ transplantation from rabies infected patients, but transmission between humans is extremely rare (Hellenbrand *et al.*, 2005; Srinivasan *et al.*, 2005; Wyatt, 2007).

More than 95% of human rabies cases are due to dog bites and the rest associated with cat, fox and other carnivores. Rabies is endemic in developing countries of Asia and Africa, and human mortality from these endemic canine rabies exceeds 30,000 and 23,000, respectively (Knobel *et al.*, 2005).

History of bite, scratch and/or contact with a rabid animal and furious and/or paralytic form of the disease leads to suspicion of Rabies in humans. However history and clinical signs gives only indication of disease and definite diagnosis of rabies relies on the laboratory demonstration and identification of virus or its specific component (Madhusudana *et al.*, 2004).

In many parts of Ethiopia at different period of time, outbreaks of rabies in dogs were reported including those reported in 1984 in -Tigray, Begemder, Gojjam, and Wollo (Admasu *et al.*, 2014).

In Ethiopia domestic dogs are the principal reservoir of rabies (Yimer *et al.*, 2002). During 1999-2002 an increase in the incidence of canine rabies was observed in Addis Ababa city (Newayeselassie *et al.*, 2004; Abebe *et al.*, 2003). There is lack of accurate data regarding rabies prevalence and fatality rate because individuals who are exposed to rabies virus often see traditional healers for the diagnosis and treatment of the disease (Deressa *et al.*, 2010).

The situation in Ethiopia showed that the fatal human cases during the period between 2001-2009 were 386 humans with annual range of 35 to 58. The overall post exposure treatment for humans was 17,204 within and around Addis Ababa. During the same period, 20,414 suspected rabid animals were clinically examined (Deressa *et al.*, 2010).

There are different diagnostic methods available for diagnosis of rabies. Histopathological techniques, such as the Sellers Stain technique (Tierkel *et al.*, 1996), were used to determine the presence of Negri bodies as rabies virus-specific antigen. However, in a positive rabies cases the presence of Negri bodies varies from 10 to 65% (Fekadu, 1988; Leighton *et al.*, 1983; Sellers, 1927). Additionally, false positive results may occur if non-specific inclusion bodies are present. Because of false negative and positive results, this technique has gradually been replaced by use of the fluorescent antibody test which is highly specific and sensitive method. (Dean *et al.*, 1996) Therefore, the present study was undertaken to evaluate the fluorescent antibody test for rabies antigen detection in fresh, frozen and formalin-fixed brain tissue specimens from clinically rabies-suspected cases of dogs, feline, bovine and donkey.

1.2. Statement of the problem

Although human rabies has been eliminated or controlled to a great extent in some countries, Rabies remains a major public-health problem in most regions of the world, especially in developing countries where dogs play a principal role as a reservoir and transmitter of the disease to humans (Dacheux, 2010; WHO, 1992). Rabies affects all mammals and is always fatal after clinical disease develops (WHO, 2005). Among all infectious diseases prevalent in the world Rabies ranks seventh in which human life is lost. The disease accounts for up to 75000 human deaths each year in canine rabies-endemic regions of the world (Wyatt, 2007). It kills large number of animals and humans in many countries every year. The highest numbers of cases are reported in Africa and Asia, particularly the Indian sub-continent. In Ethiopia, it has been

shown that there is a high incidence of Rabies in humans and domestic animals (Jemberu *et al.*, 2013). Historically, histopathological techniques such as the Sellers Stain technique (Tierkel *et al.*, 1996) were used to determine the presence of Negri bodies as rabies virus-specific antigen. However, 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. In a positive rabies cases, the presence of Negri bodies varies from 10 to 65% (Leighton *et al.*, 1983; Fekadu, 1988; Sellers, 1927). This is why this technique has gradually been replaced by use of the fluorescent antibody test. The Office International Des Epizootics (OIE) no longer recommends histopathology for diagnosis of rabies (OIE, 2000). The routine diagnostic method used is fluorescent antibody test on brain impression smears in fresh or formalin fixed nervous tissue samples. The FAT is the sensitive and preferred method for the detection of the rabies antigen in fresh samples (Meslin *et al.*, 1996). However, in many situations, only formalin-fixed tissue is available for post-mortem diagnosis due to lack of laboratory facilities or submission of fixed rather than fresh tissues to the laboratory (Abreu *et al.*, 2012; Warner *et al.*, 1997).

Currently, there exists only one referral laboratory (EPHI) for rabies diagnosis service, which is based on animal clinical observation under quarantine period and laboratory confirmation. The laboratory confirmation is done using Fluorescent Antibody test (FAT) commonly through diagnosis of fresh brain tissue samples from areas of brain stem, hippocampus and cerebellum, and Mouse Inoculation Test (MIT) as a confirmatory test. There is no established diagnosis on human rabies at the EPHI except clinical observation and Pre Exposure Prophylaxis (PEP). The production of the Fermi type vaccine started in Ethiopia since 1944 E.C. and even though it is not recommended by WHO the vaccine is still in use due to the lack of modern vaccine production (Hurisa *et al.*, 2013). Annual consumption of the vaccine is more than 36,000 doses for human and 12,000 doses for animals. Currently, EPHI is working on tissue culture vaccine. Each year, thousands of people are infected with rabies in Ethiopia and an estimated 2,700 people die, one of the highest rabies death rates in the world— but the true number of deaths caused by rabies is unknown because the disease is underreported and rabies diagnostic laboratories are not established.

1.3. Literature review

1.3.1. Historical Perspective

Historically, rabies has been known to mankind since more than 4 millenniums (Théodoridès, 1986). Rabies is one of the oldest and most feared zoonotic diseases. Rabies is mentioned in several ancient literatures, such as the paper by Aristotle (300BC) that notes rabies as one of the diseases that affects dogs and any animal that the dog bites.

Nearly all the available historical texts on the origins of disease associated human rabies with canine rabies, and dogs have been regarded as the main vector of this zoonosis. Nevertheless, rabies has long been reported for many centuries in wild animals, and the latest advances in molecular epidemiology have proved that rabies virus has indeed existed in various species of animals for millennium (Bourhy *et al.*, 1999).

The Indian *Susrutasamhita* of the 1st Century AD states that in dogs, jackals, hyenas and tigers the tail, jaw and shoulders droop, and the animals drink a lot (Théodoridès, 1986). Arab and Persian authors of the 11th and 15th Centuries add the wolf, fox and beech marten to the list of rabid wild mammals (Théodoridès, 1986). This information indicates clearly that, since ancient times, rabies has affected dogs as well as wild carnivores, but nothing is known of the original vector of the disease. Early authors never cite bats as animals affected, and it was the conquistadors of South America who first reported bats as possible vectors of the virus (Baer, 1991).

Laboratory techniques in rabies were started as early as 1800 BC, for the first time Zinke demonstrated that the infection could be transmitted to a normal animal after inoculating with saliva from a rabid animal (Mani *et al.*, 2013). In 1903, Adolchi Negri was the first person who provided description of rabies virus infection using light microscope. Histological examination of brain sections or impression smears by light microscopy, in detection of characteristic Negri bodies, has been considered standard method of rabies diagnosis & provided a basis for diagnosing rabies for about 100 years (Trimarchi *et al.*, 2007).

In Ethiopia rabies is an important disease that has been recognized for many centuries. According to Richard Pankhurst, the first and only recorded of rabies case in Addis Ababa occurred in August 1903 (Pankhurst, 1990).

1.3.2. Virological characteristics of rabies virus

Rabies is a central nervous system (CNS) disease that is almost invariably fatal. The causative agent is rabies virus (RV), a negative-stranded RNA virus.

Taxonomically, rabies viruses belong to the *Rhabdoviridae* family, a constituent of the order *Mononegavirales* which is highly stable helical nucleocapsid and morphologically, a bullet shaped (typical rod) neurotropic RNA lyssa virus (Cox *et al.*, 1980; Kawai, 1977).

In the genus lyssa virus of which rabies virus (RABV) is the prototype, there are several species of the virus including Rabies virus, Lagos bat virus, Mokola virus, Duvenhage virus, European bat lyssa virus, and Australian bat lyssa virus, all capable of causing rabies (Kuzmin *et al.*, 2010).

The genome of the Rabies virus is a single molecule of linear, 11 to 15 Kb in size, negative sense, single stranded RNA genome that is encased in an envelope along the surface of the virus; there is a tight arrangement of trimeric spikes constructed of glycoproteins. The 12-kb negative-strand RNA comprises five genes encoding 1) the nucleoprotein (N), 2) the phosphoprotein (P), 3) the matrix protein (M), 4) the transmembrane spike glycoprotein (G) and 5) the “large” protein (L) (Figure 1.1). All rhabdoviruses have two major structural components; helical ribonucleoprotein core (RNP) and surrounding envelope. The two proteins, P and L are associated with RNP. The glycoprotein forms approximately 400 trimeric spikes, which are tightly arranged on the surface of the virus. The virus nucleoprotein (N) plays critical role in replication and transcription. Both viral transcription and replication are reduced, if the nucleoprotein is not phosphorylated (Wu *et al.*, 2002).

Rhabdoviruses cell surface receptors are not identified but some researches indicate the phospholipids, especially phosphatidyl serine as the cell surface receptor molecule. After endocytosis, pH-dependent fusion with the membrane of the endocytic vesicle occurs (Yousaf *et al.*, 2012). The polymerase which is carried by the virus makes five individual mRNA for each protein. These mRNAs are capped, methylated and polyadenylated. The polymerase then transcribes the negative-sense genomic RNA into positive sense strand. The switch between transcription and replication of genomic RNAs are controlled by the level of N protein. (Wu *et al.*, 2002).

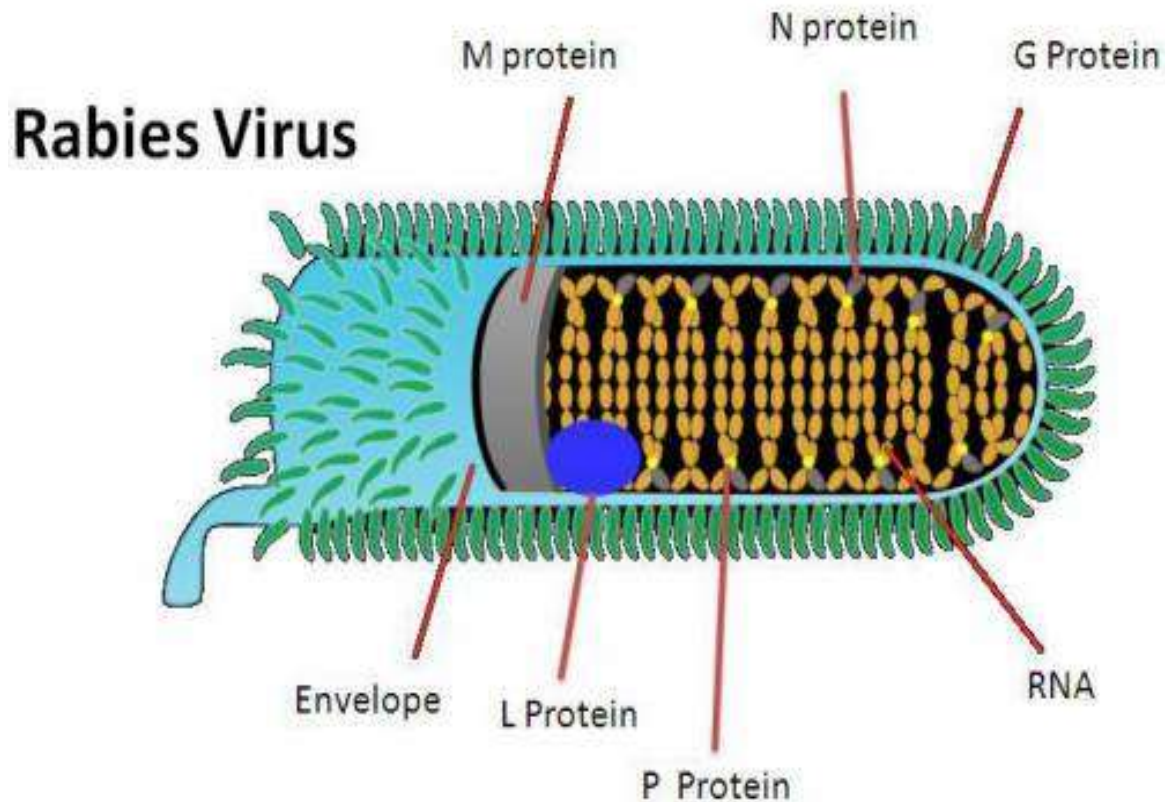


Figure 1.1 Structure of Rabies virus (Adopted from Charles *et al*, 2002)

1.3.3 Pathogenesis of Rabies

Rabies is an infectious disease caused by a virus that is transmitted from infected animals to humans. Several specific Rabies virus (RABV) variants have been characterized from different mammalian hosts, such as dogs, foxes, mongooses, other carnivores, and bats (Sudarshan *et al.*, 2007). The vast majority of rabies cases are transmitted through a dog bite (Knobel *et al.*, 2005). In addition to this, rabies virus could be transmitted from saliva or infected neural tissue into bite wounds or open cuts in skin or mucous membranes, inhalation of large amounts of aerosolized rabies virus and through organ transplantation from rabies infected patients (Wyatt, 2007). Transmission between humans is extremely rare and though very small amounts of virus could enter the blood stream when the bite occurs, it would be unable to replicate there, so the blood from a rabid animal is not considered infectious (Hellenbrand *et al.*, 2005; Srinivasan *et al.*, 2005; Wyatt, 2007).

RABV entry into cells involves receptor-mediated endocytosis, membrane fusion at acidic pH, and release of the virus RNP into the cytoplasm, involving its transition from a supercoiled state to a relaxed form which can serve as a template for the associated polymerase. The viral RNA appears to be completely shielded by the N protein and not accessible to small cellular compounds, including RNA-binding proteins like RNases or small (interfering) RNAs (Albertini *et al.*, 2008).

The ability to reach the CNS from a peripheral infection site, referred to as “neuroinvasiveness,” largely determines the virulence of the virus. A key strategy of the virus therefore is to avoid direct cytotoxicity, innate immunity, and inflammation to conserve the integrity of the neuronal network and to gain time to reach the CNS (Dietzschold *et al.*, 2008; Finke *et al.*, 2005; Schnell *et al.*, 2010).

The highest estimates of direct mortality due to rabies are transmission most commonly through the bite of a rabid animal. After a bite by a rabid animal, the virus enters the peripheral nervous system of human and it begins to multiply in the area near the entry site. It then travels along the afferent nerves toward the central nervous system where brain is the center of multiplication (Li *et al.*, 2005). When the virus reaches the brain, it rapidly causes encephalitis, the prodromal phase, and is the beginning of the symptoms and it is irreversible for medical treatment after the on-set of clinical manifestation of the disease. The incubation period lasts a varying amount of time; it can range from days to years, but the average length is 3-8 weeks (Deressa *et al.*, 2010). Compared to other viral encephalitides, little inflammation is observed in rabies and symptoms appear to arise from neuronal dysfunction rather than damage (Li *et al.*, 2005).

1.3.4. Epidemiology and Risk Factors

Rabies virus is found worldwide and has been described since ancient times. Rabies is generally considered to be a fast-moving trans boundary disease that does not respect borders and is the most important human zoonosis causing tens of thousands of deaths per year, mostly in children (Rupprecht *et al.*, 2008; Steele *et al.*, 2001; WHO, 2005).

It kills large number of animals and humans in many countries every year. Among all infectious diseases prevalent in the world, rabies ranks seventh in which human life is lost (Wyatt, 2007). The highest numbers of cases are reported in Africa and Asia, particularly the Indian-sub

continent (Burki, 2008). This situation reflects the relative lack of systematic control and prevention initiatives, including surveillance and response systems. In India, because vaccine-preventable diseases are the first public health priority especially in children, rabies and other zoonoses tend to be neglected, as they are not seen as the responsibility of either human or veterinary health care providers (John *et al.*, 2011).

Rabies causes at least 24,000 deaths per year in Africa (Cleaveland *et al.*, 2002). In Africa, human rabies has decreased over the past 10 years (WHO, 2010). Studies predicting the true number of human cases using indirect measures demonstrate that in Africa rabies is also under-reported because of poor surveillance and reporting structures and is diagnosed only clinically in most countries, as few have facilities for laboratory confirmation (Dodet, 2008;Knobel *et al.*, 2005).

There is a high Incidence of Rabies in humans and domestic animals in Ethiopia, as shown in the reports of high death rates in poor rural communities and children (Jemberu *et al.*, 2013). The presence of many stray dogs and cats in urban areas of Ethiopia can be sufficient to create significant risks for the spread of rabies in major cities and towns. The wide spread and abundant wild life populations have also a role in sylvatic (wildlife) rabies (Fekadu *et al.*, 1982). Infants and children are more vulnerable to rabies infection than other age groups due to the fact that they mostly play with dogs and pets (Deressa *et al.*, 2010). Overall poor knowledge of this disease in the country could also be a risk factor for rabies transmission and death (Tschopp *et al.*, 2016).

Rabies is still present in Europe, but the human rabies has disappeared from many European countries. Canine rabies has been eliminated from many regions through veterinary service initiatives, including the mandatory registration and vaccination of dogs and requirements for responsible dog ownership (Blanton *et al.*, 2012; CDC, 2007).

1.3.5 Clinical presentation

I. Animals

The incubation period of canine rabies varies from days to many months. The length of incubation period apparently depends on several factors, including the site of exposure (inoculation), the infecting dose and the virus strain (Fekadu *et al.*, 1982). There are three distinct clinical stages of rabies in animals.

a. Prodromal stage: The initial clinical signs are often nonspecific and which usually lasts for about 1-3 days. During this phase, the dog's behavior may change which includes fearfulness, restlessness, anorexia or an increased appetite, vomiting, diarrhea, a slight fever, dilation of the pupils, hyper reactivity to stimuli and excessive salivation. Animals often have behavioral and temperament changes, and may become either unusually aggressive or uncharacteristically affectionate.

b. Classical (furious) phase: Eventually, the prodromal stage is followed by a period of severe agitation, aggressiveness, confusion, or hallucinations. The dog is most dangerous at this stage because of its urge to bite anything it encounters. In most cases, an altered phonation (a characteristic high pitched bark) develops. The dog has difficulty swallowing because of spasms and paralysis of the pharyngeal muscle, causing the animal to drool. Death may follow convulsions even without the paralytic stage of the disease.

c. Paralytic (dumb) phase: Dumb rabies occurs when the classical phase is extremely short or absent. The most characteristic sign is the "dropped jaw" caused by paralysis of the masseter muscles. This stage is characterized by the inability to swallow, leading to a typical sign of foaming saliva around the mouth. The animal often makes choking sounds as if a bone were stuck in its throat. Some animals may develop paralysis beginning at the hind extremities. Eventually, complete paralysis is followed by death. These cases are often clinically indistinguishable from Guillain-Barre syndrome (GBS) (Gadre *et al.*, 2010; Hemachudha *et al.*, 2005; Udawat *et al.*, 2001)

II. Humans

1. Prodromal period

After a bite by a rabid animal, the virus enters the peripheral nervous system of human and it begins to multiply in the area near the entry site. It then travels along the afferent nerves toward the central nervous system. The duration of this period is 2-10 days. Nonspecific symptoms and signs develop. The common clinical sign in this stage include;pain,nausea, vomiting, fever, myalgia, insomnia and headach.

2. Acute neurologic period

The acute neurological signs include patients showing intermittent agitation and confusion. Signs of autonomic dysfunction including hydrophobia,hypersalivation , aerophobia, dyspnea , photophobia and piloerection . Other signs include fever, muscle fasciculation, hydrophobia,photophobia and convulsions. Cranial nerve involvement and ophthalmoplegia, facial weakness and dysphagia are also reported in patients in this stage. The duration of this period is 2-7 days Patients may die immediately or may progress to paralysis.

3. Paralytic rabies

Another form of rabies, paralytic rabies, is also known as dumb rabies or apathetic rabies, because the patient is relatively quiet compared with a person with the furious form. In the paralytic rabies cases, patients show signs of flaccid paralysis, urinary incontinence, and abdominal discomfort. The acute signs ended with abrupt death or progression into coma before death. Twenty percent of patients do not develop the furious form.

4. Coma

This begins within 10 days of onset with various duration. Without intensive supportive care, respiratory depression, arrest, and death occur shortly after coma. (CDC, 2004; Jackson, 1999; Senthilkumaran *et al.*, 2015, Susilawathi *et al.*, 2012; Wilson *et al.*, 1975)

1.3.6. Laboratory Methods for Isolation and Identification of rabies virus

There are different laboratory techniques available for diagnosis of rabies each having their own limitations and failures. Adolchi Negri was the first person who provided description of rabies virus infection using light microscope (Markson, 1969). Histological examination of brain sections or impression smears by light microscopy, in detection of characteristic Negri bodies, has been considered standard method of rabies diagnosis and provided a basis for diagnosing rabies for about 100 years (Trimarchi *et al.*, 2007).

Negri bodies are aggregates of viral proteins, intra-cytoplasmic, eosinophilic inclusions in rabies-infected tissue which are specific for rabies virus infection (Negri *et al.*, 1903). The staining techniques are not specific since they merely detect affinity for acidophilic stains. 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 (Figure 1.2).

However in this technique false negatives results may occur because not all individuals develop Negri bodies and false positive results may occur if nonspecific inclusion bodies are present (Leighton *et al.*, 1983; Fekadu *et al.*, 1988). In a positive rabies cases, the presence of Negri bodies varies from 10 to 65% (OIE,2000). Due to these limitations, histopathological technique has gradually been replaced by use of the fluorescent antibody test. The OIE no longer recommends histopathology for diagnosis of rabies.

The fluorescent antibody test (FAT) is the most widely used method for diagnosing rabies infection in animals and humans which is recommended by both WHO and OIE. Because the FAT is specific, and compared to the histological demonstration of Negri bodies has a high degree of sensitivity, it became a standard diagnostic procedure and is considered the gold standard for rabies diagnosis (Dean *et al.*, 1996) (Figure 1.3).

In 1958, Goldwasser and Kissling developed the first direct immunofluorescence for diagnosis of rabies and it became a routine diagnostic method in the 1970's. The test is particularly sensitive with fresh specimens, but it can also be used in fixed tissues after treatment with trypsin or other enzymes (Dean *et al.*, 1996; Whitfield, 2001). Brain tissue samples, smears or cells are treated with anti-rabies serum or globulin labelled with fluorescein isothiocyanate. The main advantage of this test is that results can be obtained within 2 hours, but the requirement of specialized

laboratories, trained personnel, fluorescence microscope and rabies conjugate makes this technique relatively expensive. However, The FAT provides a reliable diagnosis in 98-100% cases (Dean *et al.*, 2009). Immunofluorescent staining of rabies antigen in Formalin-fixed brain tissue was first reported by Johnson *et al.*, 1980.

In Ethiopia, EPHI is the only laboratory for diagnosis of rabies infection in which the laboratory has been using the FAT as the sole diagnostic method.

Other techniques for rabies diagnosis include:-direct visualization by electron microscopy, direct or indirect FAT, virus cultivation in cell lines, mouse inoculation test (MIT), immunohistochemistry, enzyme immunoassay, molecular hybridization, reverse transcription polymerase chain reaction (RT-PCR); demonstration of specific antibodies in cerebral fluid and dRIT(direct rapid immunohistochemical test) (Dean *et al.*, 1996; Koprowski, 1996; Tierkel *et al.*, 1996; Meslin *et al.*, 1996)

The confirmatory test among these several diagnostic methods is virus isolation (VI) by inoculating cell cultures with brain homogenates suspected to harbor RABV

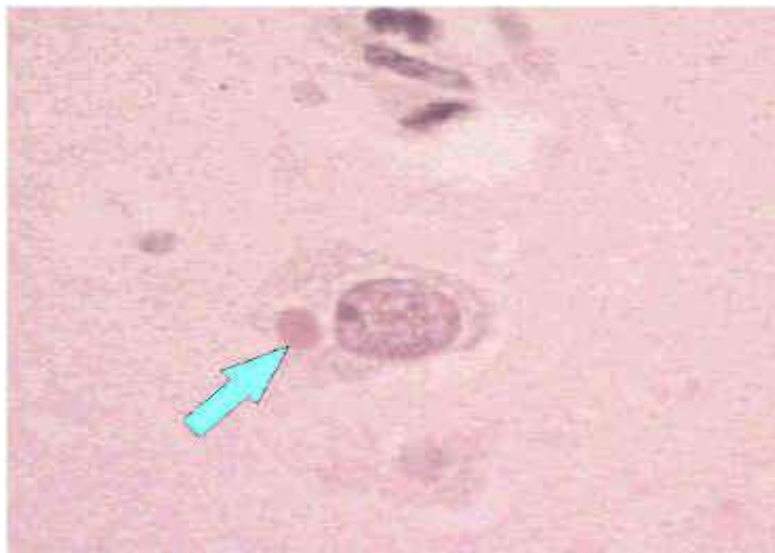


Figure 1.2 H&E stain of Negri bodies in the cytoplasm of nerve cell (Beigh, 2016)

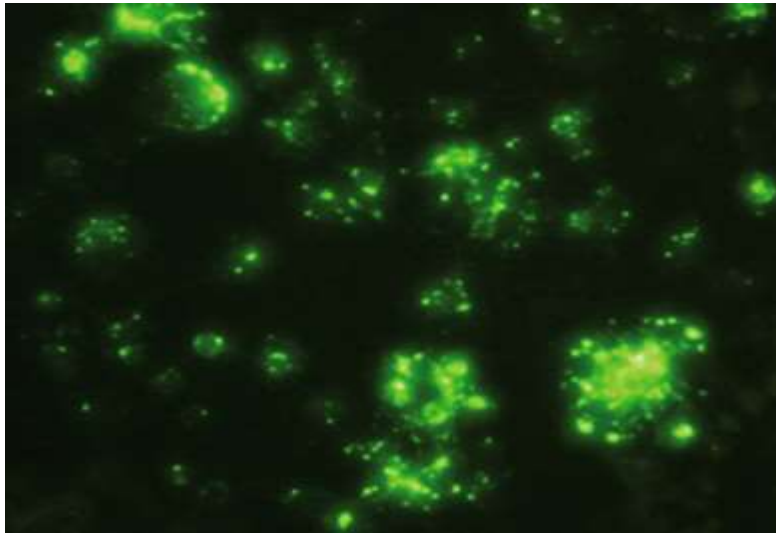


Figure 1.3 Impression smear obtained from animals suspected of having rabies showing apple green fluorescence in neurons. Direct FAT (Dong *et al.*, 2012)

1.3.7. Prevention of Rabies virus

Canine rabies has been eliminated from many regions through veterinary service initiatives, including the mandatory registration and vaccination of dogs and requirements for responsible dog ownership (Blanton *et al.*, 2012; CDC, 2007). Oral vaccination campaigns for wildlife have also removed the threat of sylvatic rabies from carnivores in some areas (Muller *et al.*, 2012).

Controlling and preventing rabies in dogs is crucial to preventing the disease in humans (Coleman *et al.*, 1996). Showcase initiatives have demonstrated that the elimination of canine rabies from Africa and Asia is epidemiologically and practically feasible, through mass vaccination and enforcement of responsible dog ownership (Kaare *et al.*, 2009; Zinsstag *et al.*, 2009).

Rabies can be prevented before the latent symptoms can develop, which consists of giving a person an injection of rabies immune globulin and injection of rabies vaccine as soon as possible after the bite or exposure to saliva from an infected animal. Human rabies immune globulin is used or injected at the bite area immediately because it attacks the virus and slows down or stops viral progression through the nerves (Fogelman *et al.*, 1993).

The people who are considered as high risk group need pre-exposure prophylaxis. These groups includes; people whose activities bring them in contact with rabies virus or rabid animals such as veterinarians, animal handlers and laboratory workers; international travelers likely to come in

contact with animals in the rabies prevalent areas. All these groups should be treated with rabies vaccines to avoid the chances of sudden exposure leading to infection.

Effective rabies control and prevention programmes require reliable information on disease occurrence, therefore they should be guided by modern epidemiological insights and driven by laboratory-based surveillance (Rupprecht *et al.*, 2006). Access to modern rabies vaccines and immunoglobulins, and creating awareness among the people in high-risk countries could also be important factors in the prevention of Rabies.

1.4. Significance of the study

The laboratory diagnosis of rabies has fundamental importance so that control measures can be taken, and human treatment decisions may be recommended. Some of the laboratory methods for detecting rabies virus antigen in a brain sample taken at necropsy include: - microscopic examination for Negri bodies (MEN), mouse inoculations test (MIT), and fluorescent antibody tests (FAT) (Dean *et al.*, 1996; Koprowski, 1996; Tierkel *et al.*, 1996). The virus might also be found in other tissues such as the salivary gland, skin (tactile facial hair follicles) and corneal impression smears, even though detection is less efficient (Barecha *et al.*, 2017). Laboratory confirmation of the clinical suspicion of rabies is vital for initiating prompt and appropriate infection control, public health measures and in prescribing anti-rabies treatment after exposure. On the other hand in cases lacking clinical suspicion or laboratory confirmation, such as samples from apparently healthy rabies carrier dogs; the prescribed treatment is generally delayed or interrupted. Therefore, this laboratory diagnosis must be trustworthy in terms of sensitivity and specificity, and also be quickly obtainable.

Diagnosis of clinical rabies is difficult and is often not made until after death of the animal, so early diagnosis of rabies in animals is necessary for timely administration of post-exposure prophylaxis to human beings.

FAT is the most widely used method for diagnosing rabies infection in animals and humans and recommended by both WHO and OIE. Because FAT is sensitive, specific, and easy to perform, it became a standard diagnostic procedure and is the preferred test for rabies diagnosis (Dean *et al.*, 1996; Smith, 1999). The test is particularly sensitive with fresh specimens but occasionally brain tissues from suspected rabid animals are still submitted in formalin, although this has been

discouraged for a number of years. However, the test can also be modified for testing in formalin-fixed material (Reid *et al.*, 1983; Warner *et al.*, 1999).

In cases where there is difficulty in sample transportation, or refrigeration facilities are inadequate or not continuously functioning, the technique of IF staining of smears prepared from formalized brain tissue after treatment with trypsin can be a useful adjunct to other diagnostic methods. It also makes working safer where special facilities are absent.

Therefore, the present study was aimed at comparing FAT for the diagnosis of rabies on fresh, frozen and formalin fixed brain tissues specimens and to evaluate the performance of FAT on these three clinical samples.

1.5. Objectives of the study

1.5.1. General objective

- To evaluate fluorescent antibody test for rabies detection in fresh, frozen and formalin-fixed brain tissue specimens in Ethiopia

1.5.2. Specific objective

- To evaluate the sensitivity and specificity of FAT on frozen brain samples and formalin-fixed brain samples compared with fresh brain samples obtained from different animals
- To determine optimal duration of trypsin treatment for maximizing result of rabies antigen detection by IF staining in formalin-fixed brain tissue samples

CHAPTER II:

MATERIALS AND METHODS

2.1. Study design, Period and Area

2.1.1. Study design

A cross sectional study was conducted.

2.1.2. Study area and period

The study was carried out at Parasitology, Bacteriology and Zoonosis Directorate of Ethiopian Public Health Institute (EPHI), and Vaccine and Diagnostics Production Directorate of EPHI from March 2017-July 2017. EPHI is the only referral laboratory for rabies diagnosis service, which is based on animal clinical observation under quarantine period & laboratory confirmation. The laboratory confirmation is done using Fluorescent Antibody test (FAT).

2.2. Sample size and Sampling Technique

FAT was done on 41 animal brain tissues which were collected from March 2017-July 2017. All fresh animal brain tissue that has been submitted to the Ethiopian public Health Institute during the study period was included in the study. Convenient sampling technique was employed.

2.3. Sample Collection, Handling and Transport

Whole brains were collected after natural death or from animals sacrificed during any stage of the clinical syndrome. Only brain tissues were examined in this study. The majority were from dogs, few others were from cats, ox, and donkey. Standard diagnosis of rabies virus infection is ideally based on examination of at least three areas of the brain: hippocampus, cerebellum, and brain stem (Fekadu *et al.*, 1986; Smith, 1999). Therefore, in the present study diagnosis of brain tissue samples from areas of brain stem, hippocampus and cerebellum were included. Separate test from the same brain tissue specimen was done. Fresh brain tissue specimens were processed as soon as the brain is dissected while frozen specimens were kept at -20°C and the third specimen were fixed in formalin for FAT analysis.

2.3.1 Tissue preparation

The brain tissues examined in this study was collected as paired specimens. One portion of each paired specimen was prepared for fresh, frozen fluorescent antibody testing and the other portion prepared for formalin-fixed by PI and trained laboratory personnel. The fresh/frozen specimens from brain impressions were made at necropsy or cryostat-sectioned specimens that were fixed in acetone as previously described (Goldwasser *et al.*, 1958). The tissues were held in buffered formalin (10% formalin) for a period of 96 hours. The formalin fixed tissue was digested in 0.25% trypsin for 90 minutes.

2.3.2 Fluorescent antibody test (FAT)

The FAT was performed according to the procedure described by the OIE and WHO (OIE, 2000; WHO, 1992). In brief, from fresh, frozen and fixed specimens thin sections of brain tissue on slides was fixed in cold acetone (-20°C) for 1 hour to overnight. After three successive wash with phosphate buffer saline (PBS), the slides were incubated with sufficient quantity (0.45 µl) of fluorescein isothiocyanate (FITC) conjugate (see Annex III).

After rinsing with PBS, the slides were air-dried and mounting buffered glycerin was applied. The slides were examined under cover slips at magnification of 400x using a fluorescent microscope. Positive and negative controls were run together with the test samples. The slide showing specific fluorescence confirmed a positive result.

2.3.3 Cell culture

As a single negative test on fresh material does not rule out the possibility of infection, cell culture was carried out simultaneously. BHK-21 cell lines (CDC, Atlanta) were used for cell culture titration (in vitro) of harvest virus suspension. A monolayer culture of susceptible cells is inoculated with a pool of several CNS tissues, including the brain stem. FAT carried out after appropriate incubation demonstrated the presence or absence of viral antigen as described by Webster (1987).

2.3.4. Comparison of FAT results

The sensitivity and specificity of FAT results of frozen and formalin preserved trypsin-digested specimens vs. fresh specimens were calculated with the following formula:

FAT Result Frozen/Fixed specimen	FAT result Fresh Specimen		Total Number
	Positive (True Positive-TP)	Negative (False positive-FP)	
Positive (number)	Negative (False Negative-FN)	Negative(True negative-TN)	Total Test positive
Negative (number)	Total	Total	Total Test Negative
			Total

$$\text{Sensitivity} = \frac{TP}{TP+FN} \times 100$$

$$\text{Specificity} = \frac{TN}{TN+FP} \times 100$$

All the specimens were maintained independently for fresh, frozen and formalin fixed FAT until all test results were completed. After the independent evaluation was completed, the results were compared.

2.4. Data processing and analysis

All data were entered and analyzed with SPSS 20 version of statistical software. For statistical analysis, a sample was defined as positive when at least brilliant apple green shining inclusions/dots/particles of varying sizes and shapes were present in 10% to 50% of the microscopic field. To compare the result of the percentage of sensitivity and the agreement between methods (Fresh/frozen & fixed FAT); two by two table for each test against the standard was employed as described by Noordhui Zen *et al.*, (1997).

2.5. Quality Control

Along with the test specimen, known specimen positive for rabies virus (challenge virus) and a known negative specimen was performed in parallel. In addition, all specimens negative for FAT were processed for cell culture using BHK-21 cell lines (Webster, 1987).

2.6. Study Variables

2.6.1. Dependent variable

- Correct diagnosis of rabies

2.6.2. Independent variables

- Specimen types (fresh, frozen & fixed tissues)
- Brain areas from which specimens were taken

2.7. Ethical Consideration

This research was performed after ethical approval from the DREC of DMIP and approval from the Department of Microbiology, Immunology and Parasitology (DMIP), and ethical clearance obtained from EPHI, Scientific and Ethical Review Committee. Permission to use the samples was obtained from Parasitology, Bacteriology and Zoonosis Directorate of EPHI.

CHAPTER III:

RESULTS

3.1. Demographic characteristics of suspected rabid animals

A total of 41 brain samples from suspected cases of rabies from different species of animals including dog (n=35) (85.4%), feline (cat) (4.9%) (n=2), bovine (ox) (n=3)(7.3%) and donkey (n=1)(2.4%) were collected during the study period from different geographical locations of the country. Among the animals included in this study, majority were males (Table 3.1a). And the Age distribution of the animals 36(87.8%) were >1 years of age (Table 3.1b).

Among the 41 animal species examined, 33(80.5%) were owned while 8(19.5%) were street animals. 38 out of 41 (7.3%) of the animals were not vaccinated. Among the suspected rabies cases included in this study, the majority of the animals were dead at presentation (Table 3.1b).Based on the regional distribution; 61% animals were from Addis Ababa, 31.7% from Oromia, 4.9% from Amhara region and 2.4% from SNNP regions (Figure.3.1)

Table 3.1a. Demographic characteristics of suspected rabid animals during March 2017 to July 2017

Animal species	Sex of the animal		Total	Percent (%)
	Male	Female		
Dogs	31	4	35	85.4
Feline	1	1	2	4.9
Bovine	2	1	3	7.3
Donkey	1	0	1	2.4
Total	35	6	41	100.0

Table 3.1b. Demographic Characteristics of suspected rabid animals during March 2017 to July 2017

	Categories	Frequency	Percent (%)
Ownership status	Owned	33	80.5
	Street	8	19.5
Vaccination status	Yes	3	7.3
	No	38	92.7
Animal presentation	Dead	32	78.0
	Alive	9	22.0
Age of animals	<1 year	5	12.2
	>1 year	36	87.8

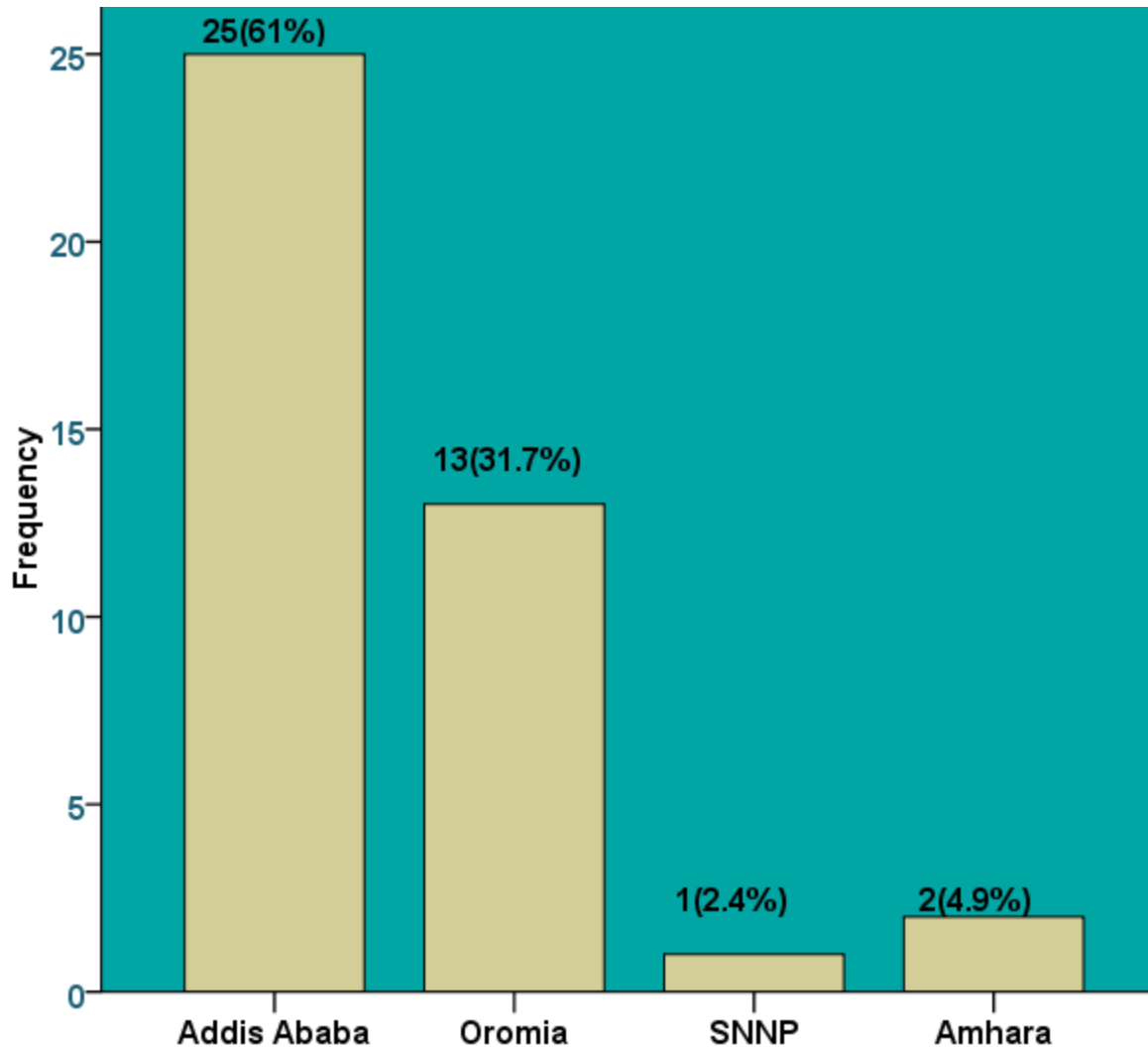


Figure 3.1. Distribution of suspected rabid animals based on Geographical location

3.2. Clinical characteristics of suspected rabid animals

Among 41 suspected rabid animal brains examined by FAT 32 (78%) were positive for rabies. Based on the history obtained from owner, the clinical characteristics of rabid animals are presented in Table 3.2. The most common clinical symptom observed was anorexia (87.5%), followed by behavioral change (56.3%) and hypersalivation (46.8%) (Table 3.2).

Table 3.2. Clinical signs in positive cases of rabid animals (n=32), EPHI, March 2017 to July 2017

Symptom	No. of animals	Percent (%)
Anorexia	28	87.5
Behavioral change	18	56.25
Hyper salivation	15	46.88
Paralysis	9	28.13
Frequent micturition	7	21.88
Nervous sign	16	50
No visible symptoms	4	12.5

3.3. FAT result

a. Fresh brain samples

Among 41 fresh brain specimens examined by FAT, 32/41(78%) were positive for rabies while the rest 9/41(22%) were negative (Table 3.3). Among the positive fresh brain tissue specimen of dogs (n=28/35), 25 showed grade +3 and 3 showed grade +2 results. All the positive fresh brain tissue specimens of feline, bovine and donkey showed grade +3 results (Table 3.3).

b.Frozen brain samples

Among the 41 frozen brain specimens examined by FAT 32/41(78%) were positive for rabies while the rest 9/41(22%) were negative (Table 3.3).Among the positive frozen brain tissue specimen of dogs (n=28/35), 24 showed grade +3 and 4 showed grade +2 results. All the positive frozen brain tissue specimens of feline, bovine and donkey showed grade +3 results (Table 3.3).

c. Formalin fixed brain samples

Among 41 formalin fixed brain specimens examined by FAT, 31/41(75.65%) were positive for rabies while the rest 10/41(24.4%) were negative (Table 3.3). Among the positive formalin fixed brain tissue specimen of dogs (n=27/35), 24 showed grade +3 and 3 showed grade +2 results. All the positive frozen brain tissue specimens of feline and donkey showed grade +2 and +3 results, respectively, while bovine specimens showed grade +3 and +2 results (Table 3.3).

Table 3.3: Fresh, Formalin fixed and Frozen brain tissue specimen grading result using Fluorescent antibody technique

Species	Fresh tissue		Frozen tissue		Formalin fixed tissue	
	Positive	Negati.	Positive	Negative	Pos	Neg
Dog (n=35)	(n=25)=+3 3 (n=3)=+2	7	(n=24)=+3 (n=4)=+2	7	(n=24)=+3 (n=3)=+2	8
Feline (n=2)	(n=1)=+3	1	(n=1)=+3	1	(n=1)=+2	1
Bovine (n=3)	(n=2)=+3	1	(n=2)=+3	1	(n=1)=+3 (n=1)=+2	1
Donkey (n=1)	(n=1)=+3	0	(n=1)=+3	0	(n=1)=+3	0
41	32	9	32	9	31	10

*Grade +3 (see Annex IV)

Grade+2 (see Annex IV)

Grade+1: (see Annex IV)

3.4. Comparison of FAT: Frozen and formal fixed specimen vs. Fresh specimens examined

The sensitivity and specificity FAT were calculated according to the method described by Mausner & Bahn (Mausner, 1974) using the following formula:

Percentage sensitivity= $\frac{\text{no. of true positives}}{\text{Total no. of true positives + false negatives}} \times 100$

Percentage specificity = $\frac{\text{no.of true negatives}}{\text{Total no. of true negatives + false positives}} \times 100$

True positive: A specimen that was positive for rabies antigen by direct IF examination of fresh specimen, and remained positive after frozen/formalin fixed.

True negative: A specimen that was negative for rabies antigen by direct IF examination of fresh specimen, and remained negative after frozen/ formalin fixed.

False positive: A specimen that was negative for rabies antigen by direct IF examination of fresh specimen, but gave a positive result after frozen/formalin fixed and treated with trypsin.

False negative: A specimen that was positive for rabies antigen by direct IF examination of fresh specimen, but gave a negative result after frozen/formalin fixed and treated with trypsin.

I. Sensitivity and specificity of FAT: fresh specimen vs. formalin fixed specimen (Table 3.4)

Percentage sensitivity= $\frac{\text{no. of true positives}}{\text{Total no. of true positives + false negatives}} \times 100$

$31/31+1 \times 100 = 96.9\%$

Percentage specificity = $\frac{\text{no.of true negatives}}{\text{Total no. of true negatives + false positives}} \times 100$

$9/9+0 \times 100 = 100\%$

Table 3.4. Percentage sensitivity and specificity of FAT in fixed brain specimen

FAT result	Fresh specimen		Total
	Positive	Negative	
Fixed specimen			
Positive	31 (TP)	0 (FP)	31
Negative	1 (FN)	9 (TN)	10
Total	32	9	41

II. Sensitivity and specificity of FAT: fresh specimen vs. frozen specimen (Table 3.5)

Percentage sensitivity = $\frac{\text{no. of true positives}}{\text{Total no. of true positives} + \text{false negatives}} \times 100$

Total no. of true positives + false negatives

$$32/32+0 \times 100 = 100\%$$

Percentage specificity = $\frac{\text{no. of true negatives}}{\text{Total no. of true negatives} + \text{false positives}} \times 100$

Total no. of true negatives + false positives

$$9/9+0 \times 100 = 100\%$$

Table 3.5. Percentage sensitivity and specificity of FAT in frozen brain specimen

FAT result	Fresh specimen		Total
	Positive	Negative	
Frozen specimen			
Positive	32 (TP)	0 (FP)	32
Negative	0 (FN)	9 (TN)	9
Total	32	9	41

In frozen specimen both the sensitivity and specificity of FAT is similar with FAT of fresh specimen which is 100 % (Table 3.6).

Out of the 32/41 specimens found to be positive with conventional test 31/41 yielded a positive result after trypsin digestion of the formalin-preserved part of the brain. 9/41 specimens which were negative with the conventional FAT also gave negative results with digested, formalin-preserved brain. And in a frozen specimens, 32/41 specimens were found to be positive like that of FAT of fresh specimen and 9/41 specimens were negative similar to the fresh specimen (Table 3.6).

Table 3.6. Comparison of FAT between Frozen and Fresh brain tissue specimens obtained from different animals

Species	Frozen tissue		Fresh tissue		Correlation (%)
	Positive	Negative	Positive	Negative	
Dog (n=35)	28	7	28	7	100
Feline (n=2)	1	1	1	1	100
Bovine (n=3)	2	1	2	1	100
Donkey (n=1)	1	0	1	0	100
	32	9	32	9	

3.5 Duration of trypsin treatment

In formalin-fixed tissue, the minimum duration of trypsin treatment that resulted in optimal fluorescence was 90 min at 37 °C (Table 3.8).

Table 3.7. Effect of duration of trypsin treatment on the results of IF staining for rabies antigen in formalin-fixed brain tissue samples after incubated at 37 °C.

Animal	Fresh tissue	Formalin-fixed tissue: duration of trypsin treatment (min)					
		0	30	60	90	120	150
Dog	-	-	-	-	-	-	-
Dog	3+	-	2+	2+	2+	2+	2+
Bovine	3+	-	2+	2+	3+	3+	3+
Donkey	3+	-	2+	2+	3+	3+	3+
Bovine	3+	-	2+	2+	2+	2+	2+
Dog	2+	-	-	-	-	-	-
Feline	3+	-	1+	2+	2+	2+	2+
Dog	3+	-	2+	3+	3+	3+	3+
Dog	3+	-	3+	3+	3+	3+	3+
Dog	3+	-	2+	3+	3+	3+	3+
Dog	3+	-	2+	2+	3+	3+	3+
Dog	3+	-	3+	3+	3+	3+	3+
Dog	2+	-	2+	2+	2+	2+	2+
Dog	3+	-	3+	3+	3+	3+	3+
Dog	3+	-	2+	2+	3+	3+	3+
Dog	3+	-	3+	3+	3+	3+	3+
Dog	3+	-	3+	3+	3+	3+	3+
Dog	3+	-	2+	3+	3+	3+	3+
Dog	2+	-	1+	2+	2+	2+	2+

*Grade +3 (see Annex IV)

Grade+2 (see Annex IV)

Grade - (see Annex IV)

3.6 FAT for Quality control specimens

Along all tissue specimens done, 30 known positive samples and 30 known negative samples that has been done using conventional method were processed with the test specimens and all the 30 (100%) known positive samples gave positive results when it is done using FAT. And all the 30 known negative samples gave negative results using FAT. The suspected rabies cases (local strains) were checked for sensitivity on BHK-21 cell line on both positive and negative controls.

3.7 Cell Culture

From a total of 41 fresh specimens examined using FAT, 9/41(22%) were negative. All the nine negative samples were cultured for rabies virus using BHK-21 cell lines and the result showed all were negative for rabies virus antigen.

CHAPTER IV:

DISCUSSION

Rabies is a major public-health problem in most regions of the world, especially in developing countries (WHO, 1992; Dacheux, 2010). The disease accounted for up to 75000 human deaths each year in canine rabies-endemic regions of the world (Wyatt, 2007). In Ethiopia rabies is one of the major public health burdens in which human lives are lost (Deressa *et al.*, 2010).

In the present study, the majority of animal species suspected for rabies infection were dogs (85.4%) in agreement with a study done in which more than 95% of human rabies cases were due to dog bites (Knobel *et al.*, 2005). Another study also showed that 91.8 % of the reported cases were in dogs (Ramos *et al.*, 2015). Among the 41 animal species examined in the current study, 80.5% were owned while 19.5% were street animals similar to another study conducted in which 78.4% were owned and 21.6% were street animals (Reta *et al.*, 2014). According to a retrospective study done in and around Addis Ababa from the rabies suspected cases, only 3.6% of animals had vaccination while the rest 96.4% were unvaccinated (Reta *et al.*, 2014). Similar finding was observed in the present study in which the majority of the dogs (94.7%) were unvaccinated and few were vaccinated (7.3%).

In regards to the age of the animals, 12.2% of the study animals were less than 1 year old, while 87.8% were older than 1 year which is similar to the previous study done in Metahara, Oromia and Afar regions where the majority of the study animal's age was greater than one year old (73.7%) (Tschopp *et al.*, 2016).

In the present study, the most reported symptom in suspected rabid animal was anorexia (87.5%), followed by behavioral changes (50%) and hyper salivation (46.8%). Similar findings have been reported in which anorexia and behavioral changes were found to be in 94.4% and 55.5% of rabid cases, respectively (Beigh *et al.*, 2016; Soria *et al.*, 1988). In this study other clinical characteristics such as paralysis, frequent micturation, nervousness were observed in 28.13%, 27.7% and 50% rabid cases, respectively. Similar findings have been reported in studies conducted elsewhere (Soria *et al.*, 1988; Srinunthapanth *et al.*, 1985; Beigh *et al.*, 2016).

Many diagnostic methods are being used to detect rabies virus antigen. FAT is the standard test for the detection of rabies virus antigen in fresh tissue and it can also be used in Formalin fixed brain tissues (Goldwasser *et al.*, 1958).

A study conducted by Abraham *et al.*,(2010) using animals' brain tissue samples of 2517 from dogs, cats, cattle, horses, donkeys, Shoats(young pig), hyenas and monkeys and examined for rabies using FAT, 76.9% were positive for rabies antigen. Similar finding was observed in the present study which included dogs, cats, bovine and donkey, where 78% were positive for rabies antigen using FAT technique.

In the present study, out of the 32 specimens which were found to be positive with fresh FAT, 31 yielded a positive result after trypsin digestion of the formalin-preserved brain tissue. This is in agreement with a study conducted by Barnard *et al.*,(1982) which compared the immunofluorescence obtained with formalin preserved, trypsin-digested specimens with the fluorescence obtained with undigested and glycerine-preserved specimens tissues (Barnard *et al.*, 1982). The result showed out of the 41 specimens which was found to be positive with conventional tests, 40 yielded a positive result after trypsin digestion of the formalin-preserved brain tissue and thirteen specimens negative with the conventional FAT also gave negative results with digested, formalin-preserved brain tissues (Barnard *et al.*, 1982). In another study where direct comparison of immunofluorescent staining of fresh tissue from 23 known rabies positive animals was done out of 21 smears of formalin fixed trypsin digested specimens, 2 failed to give a positive result (Umoh *et al.*, 1985).

According to Peggy (1979), the intensity and specificity of FA staining of measles when the trypsin digestion method was used to detect viral antigens present in hamster brain following experimental infection with measles virus, the results were comparable to those obtained with acetone-fixed, freshly frozen tissues that had been sectioned with a cryostat. Trypsin digestion enhanced the intensity of specific immunofluorescence in all virus-containing specimens examined. Use of pepsin and trypsin treatment of Formalin-fixed brain tissue enhance in unmasking rabies antigenic sites. According to study done by Frances *et al.*, (1983) specimens who were negative by mouse inoculation were negative after trypsin or pepsin-trypsin digestion.

The present study revealed that detection of rabies virus in fresh and formalin-fixed tissues was in agreement in 96.9% of the cases using FAT, similar to a previous study done where agreement revealed more than 99.8% of the cases using the same technique (Whitfield *et al.*, 2001). Also a study which suggested FAT on formalin fixed tissue as complementary to standard diagnostic techniques (Johnson *et al.*, 1980).

In the present study, FAT of formalin fixed tissue had a Sensitivity of 96.9% and specificity of 100%. In a similar study, FAT of formalin-fixed specimens exhibited a sensitivity of 99.8% in comparison to FAT of fresh tissue specimens. No false positive results were obtained in formalin-fixed FAT procedures (Whitfield *et al.*, 2001). In another study conducted by Umoh *et al.*, (1981) FAT of formalin fixed tissue had Sensitivity of 92% after treatment with trypsin. The sensitivity is little lower than the present study which has 96.9 this could be due to difference in quality of the sample, virus antigen distribution, and areas of the brain tested. Rabies virus antigen is not uniformly distributed in the brains of infected animals. In both studies the specificity was 100%.

In the present study the false negative results observed in the smears in the blind-coded reading were seen only on specimens that were known to contain low levels of rabies antigen (2+), which is similar to a previous study and this could be due to the reason that tissues that tended to wash away could infer some degree of autolysis (Umoh *et al.*, 1981).

In the current study from a total of 30 known positive and 30 known negative controls which run together with test specimen, all (100%) positive controls gave positive results and all (100%) negative controls gave negative results with FAT similar in findings performed by Rahman *et al.*, where no antigen expression was detected in the negative control of the buffalo's brains (Rahman *et al.*, 2015).

In the present study out of 41 tissue specimens diagnosed 9 gave negative results by FAT of fresh tissue. All the 9 negative FAT tissues were cultivated in BHK-21 cell line and FAT was done. All 9 (100%) of tissues cultivated in BHK-21 cell line gave negative result after FAT was done. BHK- 21 cells do present an adequate sensitivity and provide an adequate alternative, yielding reliable and faster results in a similar previous study (Fabio *et al.*, 2001).

Finding of the present study revealed the minimum duration of trypsin treatment that resulted in optimal fluorescence was 90 min incubated at 37°C which is in contrast to another study which

had optimal duration of 60 min (Umoh *et al.*,1981) .This could be due to difference in the concentration of trypsin.

FAT of Fresh tissue is the standard test for rabies diagnosis but this could not always be true especially in developing countries where rabies diagnostic facility is limited or refrigeration facilities are inadequate and also in areas where there is difficulty in sample transportation, fresh tissue for rabies diagnosis may not be available. Therefore, this study was conducted to assess the detection rate of IF staining of smears prepared from formalized brain tissue after treatment with trypsin.

Conclusion

Rabies is a fatal zoonotic disease and its clinical manifestations are unpredictable. Therefore a reliable rabies diagnostic test must detect viral antigen. In areas where adequate preservation of fresh material is not possible, the examination of formalin preserved, trypsin-digested brain seems to be more specific and more rapid examination.

The results of deep freezed FAT were similar in sensitivity and specificity with that of fresh specimen. The results of IF staining of rabies antigen in formalin-fixed CNS tissues treated with trypsin are comparable in sensitivity and specificity with results obtained on fresh or frozen specimens of the same tissues. Thus, fixation and preservation of CNS tissues in 10% formalin should be considered in situations where preservation by freezing is difficult. FAT on formalin fixed tissue can be used as an alternative to FAT on fresh tissue with the similar sensitivity, when only formalin-fixed tissue is available for post-mortem diagnosis. The technique should not replace standard methods, but is an acceptable procedure when no other possibilities exist.

Recommendations

Based on the findings of the present study, the following recommendations are made:

I. Policy makers (Ministry of Health & Ministry of Livestock and fishery)

- Demonstration on how to preserve brain tissues using 10% formalin in different regions and administrative cities of the country as an alternative sample where fresh sample is not available
- In areas where there is refrigerator, deep freeze sample is recommended as a means of brain tissue sample preservative
- Use of cell cultures may be used as a confirmatory test for FAT in the diagnosis of rabies

II. For future research

- Detection of the sensitivity & specificity of FAT in stored and preserved samples using large sample size
- Research using other diagnostic techniques for Rabies
- Further study to characterize the genotype of the collected samples of rabies virus by molecular techniques, so that to identify the dominant type in Ethiopia.

Limitation of the study

- Small number of rabies suspected cases (brain tissue specimens) were included in the study

References

- Abebe P, Yimer E, Newayeselassie B, Bekele A, Zewde B, Beyene M. (2003) A study on the prevalence of Animals Rabies in Addis Ababa during 1999-2002. *Ethiop. Vet. J*; 7:69-77.
- Abreu CC, Nakayama PA, Nogueira CI, Mesquita LP, Lopes PF, Wouters F, Varaschin MS, Bezerra PS. (2012) Histopathology and immunohistochemistry of tissues outside central nervous system in bovine rabies. *J Neurovirol.*; 20(4):388-397.
- Admasu P, Mekonnen Y, Gebrewold G. (2014) Rabies and its Folk Drugs Remedies in Ethiopia: A Review. *European Journal of Biological Sciences* 6 (4): 104-109.
- Albertini AA, Schoehn G, Weissenhorn W, Ruigrok RW. (2008) Structural aspects of rabies virus replication. *Cell Mol Life Sci*; 65:282-294.
- Baer G. (1991) Vampire bat and bovine paralytic rabies. In *The Natural History of Rabies*. CRC Press; 389-403.
- Barecha CB, Girzaw F, Kand RV, Pal M. (2017) Epidemiology and Public Health Significance of Rabies. (5):55-57
- Barnard BJ, Voges SF. (1982) A simple technique for the rapid diagnosis of rabies in formalin-preserved brain. *Onderstepoort Journal of Veterinary Research*; 49; 193--194.
- Beigh AB, Sandhu BS, Singh CK, Gupta K, Sood NK. (2016) diagnosis of rabies in buffaloes: comparison of clinico-pathological, immunohistochemical and immunofluorescent techniques. *Buffalo Bulletin*; 35:2057-2059
- Blancou J. (2003) History of surveillance and control of transmissible animal diseases. *Office international des epizootics*; 193-219.
- Blanton JD, Dyer J, McBrayer J, Rupprecht CE. (2012) Rabies surveillance in the United States during 2011. *J. Am. Vet. Med. Assoc*; 241 (6): 712–722.
- Bourhy H, Kissi B, Audry L, Smreczak M, Sadkowska-Todys M, Kulonen K, Tordo N, Zmudzinski JF, Holmes EC. (1999) Ecology and evolution of rabies virus in Europe. *J. Gen. Virol.*; 80:2545–2557.
- Burki T. (2008) The global fight against rabies: *Lancet* 372 (9644): 1135–1136
- Centers for Disease Control and Prevention. (2004) Investigation of rabies infections in organ donor and transplant recipients--Alabama, Arkansas, Oklahoma, and Texas. *Morb Mortal Wkly Rep*; 53(26):586-9.

- CDC (2007). US declared canine-rabies free. Available from: <http://www.cdc.gov/news/2007/09/canine-rabies.html>.
- Center for disease control and prevention. (2011) National center for emerging and zoonotic diseases. Available at: <http://www.cdc.gov/rabies/location/world/index.html>
- Cleaveland S, Fèvre EM, Kaare M, Coleman PG. (2002) Estimating human rabies mortality in the United Republic of Tanzania from dog bite injuries. *Bull World Health Organ*; 80(4): 304–310.
- Coleman PG, Dye. (1996) Immunization coverage required to prevent outbreaks of dog rabies. *JAMA*; 276(3):185–186.
- Cox JH, Dietzschold B, Weiland F, Schneider LG (1980). Preparation and characterization of rabies virus hemagglutinin. *Infect Immun.*;30(2):572-577.
- Dacheux L, Wacharapluesadee S, Hemachudha T, Meslin FX, Buchy P, Reynes JM, Bourhy H. (2010). More accurate insight into the incidence of human rabies in developing countries through validated laboratory techniques. *PLoS Neglected Tropical Diseases*; 4(11):e765.
- Dean D, Ableseth M. (1996) Laboratory techniques in rabies: the fluorescent antibody test. *Monogr Ser World Health Organ*; (23):73-84
- Deressa A, Ali A, Beyene M, Newaye Selassie B, Yimer E. (2010) The Status of rabies in Ethiopia: A retrospective record review. *Ethiopian Journal of Health Development*; 24:127–132.
- Dietzschold B, Li J, Faber M, Schnell M (2008). Concepts in the pathogenesis of Rabies. *Future Virol.*;3:481–490
- Dodet B. (2008) Fighting rabies in Africa: the african rabies expert bureau (AfroREB). *Vaccine*; 26, 6295–6298.
- Fabio A, Kanitzi J, Felipetto C, Rudi W, Helena C, Batista E, Furtado F. (2001) Virus isolation in cell culture for confirmatory diagnostic of rabies in bovine specimens. *J Clin Microbiol*; 45:2193-2196
- Fekadu M. (1982) Rabies in Ethiopia. *Am. J. Epidemiol.*; 115 (2):266-273.
- Fekadu, M. (1988) Pathogenesis of rabies virus infection in dogs. *Rev. Infect. Dis.*; 10 (Suppl. 4): 5678–5683
- Finke S, Conzelmann KK. (2005) Replication strategies of rabies virus. *Virus Res.*; 111:120–131.
- Fogelman V, Fischman HR, Horman JT, Grigor JK. (1993) Epidemiologic and clinical characteristics of rabies in cats. *J Am Vet Med Assoc.*; 202:1829-1838

- Fooks A. (2005) Rabies and Wildlife Zoonoses Group, Veterinary Laboratories Agency, WHO Collaborating Centre for the Characterization of Rabies and Rabies-Related Viruses. United Kingdom 10(11):211-2
- Gadre G, Satishchandra P, Mahadevan A, Suja MS, Madhusudana SN, Sundaram C, Shankar SK. (2010) Rabies viral encephalitis: clinical determinants in diagnosis with special reference to paralytic form. *J Neurol Neurosurg Psychiatry*;81(7):812-820.
- Goldwasser RA, Kissling RE. (1958) Fluorescent antibody staining of street and fixed rabies virus antigen. *Proc Soc Exp Biol Med.* ;98(2):219-223
- Hellenbrand W, Meyer C, Rasch G, Steffens I, Ammon A.(2005) Cases of rabies in Germany following organ transplantation. *Euro Surveill.*;10(2):E050224.6
- Hemachudha T, Laothamatas J, Rupprecht CE.(2012) Human rabies: a disease of complex neuropathogenetic mechanisms and diagnostic challenges. *Lancet Neurol.*; **1**(2):101-9
- Hemachudha T, Wacharapluesadee S, Mitrabhakdi E, Wilde H, Morimoto K, Lewis RA. (2005) Pathophysiology of human paralytic rabies. *Journal of Neuro Virology* ; 11(1):93–100.
- Hurisa B, Tegbaru B, Nolkes D, Mengesha A, Kebede G, et al. (2013) Safety and Immunogenicity of ETHIORAB Rabies Vaccine. *J Vaccines Vaccin* 4:195.
- Jackson A. (1999) Case 21-1998: rabies. *N Engl J Med.*; 340(1):65
- Jemberu WT, Molla W, Almaw G, Alemu S (2013). Incidence of Rabies in Humans and Domestic Animals and People’s Awareness in North Gondar Zone, Ethiopia. *PLoS Negl Trop Dis.*;7(5): e2216.
- John TJ, Dandona L, Sharma VP, Kakkar M.(2011) Continuing challenge of infectious diseases in India. *Lancet*; 377 (9761): 252–269.
- Johnson KP, Swoveland PT, Emmons RW. (1980) Diagnosis of rabies by immunofluorescence in trypsin- treated histologic sections. *J. Am. Med. Assoc.*; 244:41-43.
- Kaare M, Lembo T, Hampson K, Ernest E, Estes A, Mentzel C, Cleaveland S. (2009) Rabies control in rural Africa: evaluating strategies for effective domestic dog vaccination. *Vaccine*; 27 (1); 152–160.
- Kawai A. (1977) Transcriptase activity associated with rabies virion. *J. Virol.*; 24:826-835.
- Kissling RE (1975). The FAT in rabies. The natural history of rabies New York. *Academic Press*;401-416.

- Knobel DL, Cleaveland S, Coleman PG, Fèvre EM, Meltzer MI, Miranda ME, Shaw A, Zinsstag J, Meslin FX.(2005) Re-evaluating the burden of rabies in Africa and Asia.*Bull. World Health Organ.*;83 (5): 360–368.
- Koprowski, H. (1996) The mouse inoculation test. In: Kaplan, M.M; Koprowski. H (Eds), *Laboratory Techniques in Rabies*, 4th ed. WHO, Geneva Switzerland, 80-97.
- Kuzmin IV, Hughes GJ, Botvinkin AD, Orciari LA, Rupprecht CE. (2005) Phylogenetic relationships of Irkut and West Caucasian bat viruses within the Lyssavirus genus and suggested quantitative criteria based on the N gene sequence for lyssavirus genotype definition.*Virus Res.*; 111: 28–43.
- Kuzmin IV, Mayer AE, Niezgodna M, Markotter W, Agwanda B, Breiman RF, Rupprecht CE. (2010) Shimoni bat virus, a new representative of the Lyssavirus genus.*Virus Research*; 149: 197–210
- Leighton FA, Williams ES. (1983) Intracytoplasmic neuronal inclusions in the hippocampus of non-rabid moose *Alces alces* (L). *J. Wildl. Dis.*; 19:285–288
- Li XQ, Sarmiento L, Fu ZF. (2005) Degeneration of neuronal processes after infection with pathogenic, but not attenuated, rabies viruses. *J. Neurovirol.*;79(15):10063-8.
- Markson LM. (1969) The laboratory diagnosis of rabies. *Trop. Anita. Hlth. Prod.*:1, 65-68
- Madhusudana SN, Paul JP, Abhilash VK, Suja MS.(2004) Rapid diagnosis of rabies in humans and animals by a dot blot enzyme immunoassay. *International Journal of Infectious Diseases*;8:339-345.
- Mani RS, Madhusudana SN.(2013) Laboratory Diagnosis of Human Rabies: Recent Advances. *Scientific World Journal*; 569:712.
- Mausner j, Bahn K. (1974) *Epidemiology: an introductory text*. Philadelphia, London, Toronto. *W. B. Saunders Co*; 245.
- Meslin FX, Kaplan MM, Koprowski H. (1996) An overview of lab. techniques in the diagnosis prevention of rabies and rabies research. In *Rabies* 4th ed. Geneva, WHO, 9-16.
- Müller T, Bätza HJ, Freuling C, Kliemt A, Kliemt J, Heuser R, Schlüter H, Selhorst T, Vos A, Mettenleiter TC (2012) Elimination of terrestrial rabies in Germany using oral vaccination of foxes. *Berl. Munch. Tierarztl. Wochenschr*;125 (5–6):178–190.
- Nel L, Markotter W. (2007) Lyssaviruses: Critical Reviews in Microbiology. 33: 301–324

- Newayeselassie B, Yimer E, Abebe P, Bekele A, Zewde B, Beyene M. (2004) Occurrence of Rabies in Humans and Animals in Addis Ababa in 2001-2002. *Ethiop. J. Biol. Sci.*; 3 (1): 59-67.
- Noordhuizen J PTM, Frankena K, Vander Hoofd CM, Graat EAM. (1997) Application of Quantitative Method in Veterinary Epidemiology. *Wagen Pers.*; 81-82.
- Office International Des Epizootics. (2000) OIE Manual of Standards for Diagnostic Test and Vaccine. 277-291.
- Pankhurst R. (1990) An introduction to the medical history of Ethiopia: the Red sea press. *Inc. Trenton New Jersey*; 93-101.
- Peggy T, Swoveland J, Kenneth P. (1979) Enhancement of Fluorescent Antibody Staining of Viral Antigens in Formalin-Fixed Tissues by Trypsin Digestion. *The Journal of Infectious Diseases*; 140:758-764
- Rahman SAE, Elbeskawy M, Hamed MF. (2015) Identification and genetic characterization of rabies virus from Egyptian water buffaloes (*Bubalus bubalis*) bitten by a fox. *Virus disease*; 26(3):141-6
- Ramos JM, Melendez N, Reyes F, Gudiso G, Biru D, Fano G, Aberra G, Tessema D, Tesfamariam A, Balcha S, Gutiérrez F. (2015). Epidemiology of animal bites and other potential rabies exposures and anti-rabies vaccine utilization in a rural area in Southern Ethiopia: *Ann Agric Environ Med.*; 22(1): 76–79.
- Reid FL, Hall NH, Smith JS, Baer GM. (1983) Increased Immunofluorescent Staining of Rabies-Infected, Formalin-Fixed Brain Tissue After Pepsin and Trypsin Digestion. *Journal of clinical microbiology*; 18(4):968-971
- Reta T, Teshale S, Deresa A, Ali A, Mengistu F, Sifer D, Freuling CM. (2014) Rabies in animals and humans in and around Addis Ababa, the capital city of Ethiopia: A retrospective and questionnaire based study. *Journal of Veterinary Medicine and Animal Health*; 178-186
- Rupprecht C, Hanlon C, Slate D. (2006a) Control and prevention of rabies in animals: paradigm shifts. *Dev. Biol. (Basel)*; 125:103–111.
- Rupprecht C, Barrett J, Briggs D, Cliquet F, Fooks A, Lumlertdacha B, Meslin F, Muller T, Nel L, Schneider C, Tordo N, Wandeler A. (2008) Can Rabies be eradicated. *Dev. Biol. (Basel)*; 131: 95–121.

- Rudd RJ, Trimarchi CV. (1987) Comparison of sensitivity of BHK-21 and murine neuroblastoma cells in the isolation of a street strain rabies virus. *J. Clin. Microbiol.*; 25:1456–1458.
- Schnell MJ, McGettigan JP, Wirblich C, Papaneri A. (2010) The cell biology of rabies virus: Using stealth to reach the brain. *Nat.Rev.Microbiol.*;8:51–61
- Senthilkumaran S, Jena NN, Gore SB, Manikam R, Thirumalaikolundusubramanian P.(2015).Factors for early death in rabies- the bitter truth.*Indian J Crit Care Med*; 19 (9):566-567.
- Shankar B. (2009) Advances in diagnosis of rabies.*Veterinary World*; 2(2): 74-78.
- Smith J,Baron P,Pfaller P,Tenover E,Yolken R.(1999) Rabies virus, Manual of Medical Microbiology seventh ed. *American Society for Microbiology*;1099–1106
- Soria B, Blancou J,Artois M. (1988) Study of rabies virus isolated from a European bat *Eptesius serotinum*: pathogenicity for sheep and red fox.*Revue de Médecine Veterinaire*;139 :615-621
- Srinivasan A, Burton EC, Kuehnert MJ, Rupprecht C, Sutker WL, Ksiazek TG, Paddock CD, Guarner J, Shieh WJ, Goldsmith C, Hanlon CA, Zoretic J,Fischbach B, Niezgoda M, El-Feky WH, Orciari L, Sanchez EQ, Likos A, Klintmalm GB, Cardo D, LeDuc J, Chamberland ME, Jernigan DB, Zaki SR.(2005) Rabies in Transplant Recipients Investigation Team. Transmission of rabies virus from an organ donor to four transplant recipients. *N Engl J Med*; 352 (11): 1103–1111.
- Srinunthapanth S, Triwanatham N,Khoonchan P.(1985) Rabies in cattle and buffalo, Case report.*Thai Journal of Veterinary Medicine*;15:279-285
- Steele JH,Fernandez, PJ. (1991) History of rabies and global aspects. In Baer GM (ed), The natural history of rabies, 2nd ed. *CRC Press,Boca Raton*;1–24.
- Susilawathi NM, Darwinata AE, Dwija IB, Budayanti NS, Wirasandhi GA, Subrata K, Susilarini NK, Sudewi RA, Wignall FS, Mahardika GN. (2012) Epidemiological and clinical features of human rabies cases in Bali. *BMC Infectious Disease*;12:81
- Théodoridès J. (1986) Histoire de la rage:Paris *Cave canem. Masson*;289.
- Tierkel ES. Atanasiu P D'antona D, Atanasiu P, Bequignon R, Falchetti E, Habel K, Hottle GA, Johnson HN, Kaplan MM, Komarov A, Koprowski H, Lepine P,Sellers TF, Vialat C.(1996) Laboratory Techniques in Rabies.*World Health Organization*; 55–65.

- Trimarchi C, Nadin D. (2007) Diagnostic evaluation. In: Jackson AC, Wunner WH (eds.) Rabies. 2nd ed. *Academic press*;411-462
- Tschopp R, Bekele S, Aseffa A.(2016) Dog Demography, Animal Bite Management and Rabies Knowledge-Attitude and Practices in the Awash Basin, Eastern Ethiopia.*PLoS Negl Trop Dis*; 10(2) :e0004471
- Udawat H, Chaudhary HR, Goyal RK, Chaudhary VK, Mathur R.(2001) Guillain-Barre syndrome following antirabies semple vaccine,a report of six cases. *Journal of Association of Physicians of India* ; 49:384–385.
- Umoh JU, Ezeokoli CD, Okoh AE.(1985) Immunofluorescent staining of trypsinized formalin-fixed brain smears for rabies antigen: results compared with those obtained by standard methods for 221 suspect animal cases in Nigeria. *J. Hyg.*; 94: 129-134
- Warner CK, Whitfield SG, Fekadu M, Ho H. (1997). Procedures for reproducible detection of rabies virus antigen mRNA and genome in situ in formalin-fixed tissues. *J.Virol. Methods*; 67: 5-12.
- Warner C, Fekadu M, Whitfield S, Shaddock J. (1999) Use of anti-glycoprotein monoclonal antibodies to characterize rabies in formalin-fixed tissues. *Journal of Virological Methods*; 77: 69–74
- Webster WA (1987).A tissue culture infection test in routine rabies diagnosis. *Can J Vet Res.*; 51(3): 367–369
- Whitfield SG, Fekadu M, Shaddock JH, Niezgodá M, Warner CK, Messenger SL; Rabies Working Group.(2001)A comparative study of the fluorescent antibody test for rabies diagnosis in fresh and formalin-fixed brain tissue specimens. *J. Virol.Methods*;95:145– 151.
- WHO. (2005) WHO Expert Consultation on rabies WHO Technical Report Series; 931: 1–88 (Accessed on February 4th 2013).
- World Health Organization (1992). WHO expert committee on Rabies. World Health Organ Tech Rep Ser;824:1–84
- Wilson JM, Hettiarachchi J, Wijesuriya LM. (1975) Presenting features and diagnosis of rabies. *Lancet*; 2(7945):1139-1140.
- Woldehiwet, Z. (2005).Clinical laboratory advances in the detection of rabies virus. *Clinica.Chimica. Acta*; 351:49–63

- Wu X, Gong X, Foley HD, Schnell MJ, Fu ZF. (2002) Both viral transcription and replication are reduced when the rabies virus nucleoprotein is not phosphorylated. *J Virol*;76(9):4153-4161
- Wyatt J. (2007) Rabies-Update on a Global Disease. *The Pediatric Infectious Disease Journal*; 26(4):351-352
- Yang DK, Shin EK, Oh YI, Lee KW, Lee CS, Kim SY, Lee JA, Song JY. (2012) Comparison of four diagnostic methods for detecting rabies viruses circulating in Korea. *J.Vet. Sci.*; 13(1):43-48
- Yimer E, Neway B, Girma T, Mekonnen Y, Yoseph B, Badeg Z, Mekoro B, Abebe B. (2002) Situation of rabies in Ethiopia: a retrospective study 1990-2000. *Ethiop J Health Dev*; 16(1):105-112
- Muhammad Zubair Yousaf MZ, Qasim M, Zia S, Khan MR, Ashfaq UA, Khan S. (2012) Rabies molecular virology, diagnosis, prevention and treatment. *Virology Journal* ; 9:50
- Zinsstag J, Dürr S, Penny MA, Mindekem R, Roth F, Menendez GS, Naissengar S, Hattendorf J. (2009) Transmission dynamics and economics of rabies control in dogs and humans in an African city. *PNAS*; 106 (35): 14996–15001

ANNEXS

ANNEX I: DEMOGRAPHIC CHARACTERISTIC OF SUSPECTED RAPID ANIMALS

No.	Questions	Response
1	Animal Species	A. Dog B. Feline C. Bovine D. Donkey E. Other
2	Sex of Animal	A. Male B. Female
3	Age of animals	A.<1 year B.>1 year
4	Ownership status	A. Owned B. Street
5	Vaccination status	A. Yes B. No
6	Animal presentation	A. Dead B. Alive
7	Geographical location for suspected animals	A. Addis Ababa B. Oromia C. SNNP D. Amhara E. Other

ANNEX II: CLINICAL CHARACTERISTICS SUSPECTED RAPID ANIMALS

No.	Question	Response
1	Clinical symptoms observed	A. Anorexia B. Behavioral change C. Hyper salivation D. Paralysis E. Frequent micturition F. Nervous sign G. No visible symptoms

ANNEX III: LABORATORY PROCEDURES

FAT Technique Fresh/Frozen Brain Specimens

- Make an impression smear from the brain tissue on a slide
- Impression of smears must air dry prior to fixation(15-20 minutes)
- Fresh, frozen thin sections of brain tissue on slides will be fixed in cold acetone (-20°C) for 1 hour to overnight
- After the fixation is air dried at room temperature, cover the smears with sufficient quantity(0.45 µl) of fluorescein isothiocyanate (FITC) conjugate
- Put the slides in a humidified staining chamber and incubate for 30 min at 37°C
- Staining excess conjugate is drained from the slides
- Slides rinsed in 1st tank containing PBS for 5 min

- 2nd tank containing PBS for 5 min
- Rinsing with distilled water
- The slides carefully blotted to remove excess liquid and air dry
- Apply few drops of mounting buffered glycerin(50% buffered glycerol)
- The slides will be examined under cover slips at magnification of 400x using a fluorescent microscope

FAT in formalin-fixed tissue

- The tissues will be held in buffered formalin (10% formalin) for a minimum of 96 hr.
- The FAT of formalin-fixed tissues will be performed as previously described (Warner et al., 1997)
- Approximately 2- 3 g of formalin-preserved brain, including portions of hippocampus, brain stem and cerebellum, will be emulsified in 15 *ml* of phosphate-buffered saline (PBS).
- The emulsion will be centrifuged at 1 000 rpm for 5 min
- The supernatant discarded,
- The pellet resuspended in 15 *ml* of 0.25% trypsin solution (mlv)
- Trypsin digestion for 90 minutes at 37 °C,
- The suspension will be centrifuged and the pellet washed once in PBS.
- Smears made & will be air-dried and fixed in acetone for 45-60 minutes at - 20 °C.
- After the fixation is air dried at room temperature,cover the smears with sufficient quantity(0.45 µl) of fluorescein isothiocyanate (FITC) conjugate
- Rinsing with distilled water
- The slides carefully blotted to remove excess liquid and air dry
- Apply few drops of mounting buffered glycerin(50% buffered glycerol)
- The slides will be examined under cover slips at magnification of 400x using a fluorescent microscope

Cell culture preparation

- Preparation of medium and solution
- Remove spent medium
- wash the cell monolayer with 1-2 ml of PBS
- add trypsin onto the washed cell monolayer, rotate flask to cover the monolayer with trypsin; discard excess trypsin; incubate (optional)
- add growth complete DMEM
- pipetting cell suspension up and down repeatedly until cell dissociated
- cell count,distribute the cell suspension
- resuspend into adequate volume medium; label; incubate.
- BHK-21 cell line incubated for 2 days
- Trypsinize the 2 old cell culture
- Collect concentrated cell suspension
- A 10% suspension of each of the brain samples were made in phosphate buffered saline with 2.5% FBS. The brain suspensions were centrifuged at 1500 rpm for 5 min and the supernatant used to infect the BHK cells
- Fixation and staining
- It was done out of bio-safety cabinet.
- The suspect clinical specimen or the brain homogenate is inoculated onto the cells(50 μ l) grown in a 96-well plates, incubated for 48 hr
- The cell cultures were fixed with cool 80% acetone solution (100 μ l) 45-60 minutes at - 20 $^{\circ}$ C.
- Tap water was used to rinse the acetone
- The staining was done using conjugated fluorescent anti-rabies antibody for one hour (45 μ l)
- The read was carried out using fluorescent microscope;
- Presence of rabies virus in the cells was revealed by the FAT.

ANNEX IV: Interpretation of results based on quantity and intensity of fluorescence in the smears examined.

Result (Grade)	Interpretation
++++	Massive infiltration of large and small brilliant apple green shinning inclusions/dots/particles at varying sizes and shapes in almost every area of the smear (only the + control show this grade).
+++	Brilliant apple green shinning inclusions /dots/particles of varying sizes and shapes are found in almost every microscopic field, the number of inclusions per field varies, but inclusions are numerous on most fields.
++	Brilliant apple green shinning inclusions/dots/particles of varying sizes and shapes are present in 10% to 50% of the microscopic field and most fields contain only few inclusions.
+	Brilliant apple green shinning inclusions/dots/particles of varying sizes and shapes are present in < 10% of the microscopic field and only few inclusions are found per field
-	Negative when there is no brilliant apple green shinning inclusions/dots/particles in any microscopic field.

DECLARATION

I, the undersigned, declare that this M. Sc Research thesis is my original work, has not been presented for a degree in other university and that all sources of materials used for the thesis have been duly acknowledged.

M.Sc. candidate

Tihitina Tesfaye , B. Sc

Signature

Date and place of submission

Addis Ababa, Ethiopia

Supervisor

Dr. Yimtubezenash Woldeamanuel, MD, M.Sc, PhD

Signature

Date and place of submission

Addis Ababa, Ethiopia

Supervisor

Dr. Daniel Asrat, MD, M. Sc PhD

Signature

Date and place of submission

Addis Ababa, Ethiopia