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EVALUATION OF POTENCY AND IMMUNOGENECITY OF INACTIVATED CELL CULTURE RABIES VACCINE, PREPARED AT NATIONAL VETERINARY INSTITUTE, ETHIOPIA

MVSc THESIS

BY
ENDALKACHEW BESHAH

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

June, 2018
Bishoftu, Ethiopia

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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 Veterinary Science in Veterinary Microbiology

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ENDALKACHEW BESHAH

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Department of Veterinary Microbiology, Immunology and
Veterinary Public Health

As members of the Examining Board of the final MVSc open defense, we certify that we have read and evaluate the thesis prepared by: Endalkachew Beshah Werdofa entitled "Evaluation of Potency and Immunogenicity of National Veterinary Institute Inactivated Cell Culture Rabies Vaccine" and recommend that it be accepted as fulfilling the thesis requirement for the degree of: Masters of Veterinary Science in Veterinary Microbiology.

Takele Beyene (BSc, MSc)	_____	_____
Chairman	Signature	Date
Hannah Joan (DVM, MSc, PhD)	_____	_____
External Examiner	Signature	Date
Addisu Demeke (DVM, MSc)	_____	_____
Internal Examiner	Signature	Date

Advisors

Bedaso Mamo (DVM, MSc, PhD Fellow)	_____	_____
Main advisor	Signature	Date
Berecha Bayissa (DVM, MSc, PhD Fellow)	_____	_____
Co-advisor	Signature	Date
Gezahegne Mamo (DVM, MSc PhD, Assoc. Prof)	_____	_____
Dep. Head	Signature	Date

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Name: Endalkachew Beshah Signature_____

Addis Ababa University College of Veterinary Medicine and Agriculture, Bishoftu

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LIST OF ABBREVIATIONS

BEI	Binary Ethylene Imine
BHK	Baby Hamster Kidney
BPL	Beta Propio Lactone
CCV	Cell Cultured Vaccine
CD	Cluster of Differentiation
CDC	Centers for Disease Control
CNS	Central Nervous System
CVS	Challenge Virus Standard
DMEM	Dulbecco's Modified Eagle Medium
DNA	Deoxy Ribonucleic Acid
EPHI	Ethiopian Public Health Institute
ERA	Evlelyn-Rokitnicki-Abelseth
FAT	Fluorescent Antibody Test
FAVN	Fluorescent Antibody Virus Neutralization
FITC	Fluorescein isothiocyanate
HEP	High Egg Passage
IU	International Unit
LD	Lethal Dose
LEP	Low Egg Passage
MEM	Modified Eagle Medium
MHC	Major Histocompatibility Complex
MLV	Modified Live Vaccine
NIH	National Institute of Health
NTV	Nerve Tissue Vaccine
NVI	National Veterinary Institute
OIE	Office International des Epizootics
PEP	post exposure prophylaxis
PrEP	Pre-Exposure Vaccination
PV	Pasteur Virus

LIST OF ABBREVIATIONS (continued)

RABV	Rabies Virus
RIG	Rabies Immuno Globulin
RNA	Ribonucleic acid
RNP	Ribonucleic Protein
RVNA	Rabies Virus Neutralizing Antibody
SAD	Street Alabama Dufferin
SAG	Street Alabama Guifn
SOP	Standard Operational procedure
TCID	Tissue Culture Infective Dose
VN	Virus Neutralization
VNA	Virus Neutralization Antibody
VRG	Vaccina Rabies Glycoprotein
VSV	Vesicular Stomatitis Virus
WHO	World Health Organization

ABSTRACT

Rabies is the first prioritized zoonotic disease threatening public health in Ethiopia. Mass vaccination is the best existing strategy to control canine rabies. The objective of the present research was to evaluate potency and immunogenicity of Evlelyn-Rokitnicki-Abelseth (ERA) inactivated cell culture anti-rabies vaccine producing in National Veterinary Institute (NVI). The vaccine was produced as per the institute's production standard operation procedure (SOP). Potency test of the vaccine conducted in Swiss Albino mice according to National Institute of Health, America (NIH) potency test procedure and Verorab rabies vaccine was used as reference vaccine. However, the immunological experiment undertaken in three to four months old puppies. Randomly assigned eight puppies to the vaccinated group were subcutaneously administered 1ml of NVI's rabies vaccine while other eight were kept as control. Their antibody response was monitored on 0, 7, 14, 21, 30 and 60 days post vaccination. With regards to potency, the median effective dose (ED_{50}) of the test vaccine was 1.47 while the reference vaccine was 1.3. Therefore, relative potency (RP) of the test vaccine found to be 1.45IU/ml in 1ml of single recommended dose. On the other hand, all dogs recruited for immunological experiment had low antibody titer and the titer of control dogs remained at lower level over experiment period. However, the mean antibody titer of vaccinated puppies statistically rose to 1.556IU/ml on 7^{th} day of post vaccination. Similarly, the mean titer increased for the next consecutive two weeks. The antibody titer reached peak of 3.585IU/ml on 30^{th} day of post vaccination. Despite of its reduction, it stayed at higher level until the end of experiment. In general, the test vaccine is potent enough to meet requirement of veterinary inactivated rabies vaccine and is effective and immunogenic for two months of post vaccination. However, immunological experiment in dogs needs to be undertaken for longer period and complimented with protective efficacy clinical trial.

Key words: *Dogs, Immunogenicity, Mice, Potency, Rabies, Rabies Vaccine*

1. INTRODUCTION

Rabies is an acute encephalitis illness caused by rabies virus. Rabies virus is the prototype species of the genus *Lyssavirus* in the family of *Rhabdoviridae* (Botvinkin *et al*., 2003) The virus affects virtually all mammals and infected species invariably die from the disease once clinical signs are manifested (Jackson and Wunner, 2007). The disease is endemic in developing countries of Africa and Asia and most human deaths from the disease occur in these countries (Yousaf *et al*., 2012). There is an estimation of 59,000 people death caused by rabies mostly in Africa and Asia annually but these numbers are assumed to be substantial underestimates by experts due to lack of systemic surveillance and reporting by developing countries (Hampson *et al*., 2015).

The domestic dogs are the main reservoir and source of infection for humans in developing countries and accounts for more than 99% of all human rabies case (WHO, 2005). Empirical observations and models of the transmission of canine rabies indicate that rabies can be eradicated if 70% of the dog population is vaccinated repeatedly to achieve herd immunity (WHO, 1987; Coleman and Dye, 1996). Hence, a better control of rabies could be achieved through vaccination of dogs (OIE, 2013). As the result, canine rabies was successfully eliminated from most developed countries. In contrary, it is still widespread and threatens the public health in developing countries (WHO, 2005).

Ethiopia, being developing country, is highly endemic for rabies, which is the top prioritized zoonotic disease in the country (Yimer, 2002). Rabies has been believed to result in a significant loss of human lives annually (Fikadu, 1997; Hurisa *et al*., 2015). In the country, more than 98% of rabies infection for humans and livestock is caused by rabid dogs (Deressa *et al*., 2010). However, many households own dogs usually for guarding property, but dog management is often poor (Deressa *et al*., 2010). Efforts to control the disease have also limited to urban centers through vaccination and killing of stray dogs (Deressa *et al*., 2010). Nevertheless, the vaccination coverage is low and killing of dogs may negatively influence the herd immunity of the dog population. The existing canine rabies control in the country has been worsened by shortage of quality rabies vaccine for animals use.

In effect, the government had taken the initiative for commenced the production of rabies vaccine for veterinary use. Thus, National Veterinary Institute (NVI) as governmental institute has started producing inactivated cell culture rabies vaccine. The institute obtained the vaccine seed, *Evlelyn-Rokitnicki-Abelseth (ERA*) strain, from Ethiopian Public Health Institute (EPHI) and their *SOP* was adopted for producing inactivated cell culture rabies vaccine. EPHI was already produced inactivated cell culture vaccine on Vero and Baby Hamster kidney (BHK-21*)* cell lines (Hurisa *et al*., 2013a). The safety and immunogenicity of this trial vaccine was evaluated in mice and dogs and the results confirmed that their vaccine confers protective immunity for six months (Hurisa *et al.*, 2013b; Hurisa *et al*., 2015). From this successful trials, NVI had developed its own production Standard Operation Procedure (SOP) based on findings of the experiment of ERA virus growth on Baby Hamster kidney (BHK-21) and Vero cell lines on different hours (Hurisa *et al*., 2013a). However, changes in the SOP were made by replacement of formalin by β-propiolactone and addition of Aluminum Hydroxide ($Al(OH)_3$) gel adjuvant in formulation. In ISO 9001: 2008 guideline, clause 7, indicates that any change in production SOP and inputs requires carrying out potency and immunogenicity experiments on laboratory animals and target population.

Therefore, the objectives of this study were:

- To determine the potency of rabies vaccines of Evlelyn-Rokitnicki-Abelseth strain produced in the settings of NVI's rabies laboratory; and
- To evaluate immune response induced by NVI Dog's rabies vaccine

1. LITERATURE REVIEW

1.1.Etiology

Rabies virus is a member of the *Lyssavirus* genus of the *Rhabdoviridae* family (Botvinkin *et al*., 2003).*Lyssavirus* is one of the seven genera that form the family *Rhabdoviridae,* within the order Mononegavirale. It comprises classical rabies virus (RABV; genotype 1), Lagos bat virus (LBV; genotype 2), Mokola virus (MKV; genotype 3), Duvenhage virus (DV; genotype 4), European bat lyssavirus 1 (EBLV-1; genotype 5), European bat lyssavirus 2 (EBLV-2; genotype 6), and Australian bat lyssavirus (ABLV; genotype 7) (Paweska *et al*., 2006). Recently, four additional viruses, isolated from insectivorous bats, have been proposed as new members of *Lyssavirus* genus: Aravan virus (AV), Khujand virus (KV), Irkut virus (IV), and West Caucasian bat virus (Botvinkin *et al*., 2003). Even though the *lyssaviruses* differ antigenically, they are morphologically similar and neurotropic (Bleck and Ruprecht, 2002).

1.1.1. Morphology of Viruses

Rabies virus is an enveloped bullet-shaped virus, 180nm long and 75nm wide, composed of five structural proteins. Rabies virus contains one copy of a single-stranded, non-segmented, negative (noncoding) RNA of approximately 12,000 nucleotides (Madhusudana *et al.,* 2012). The virus envelope contains glycosylated G-protein spikes that bind to cells. This protein induces the production of rabies virus-neutralizing antibodies (RVNA) that are the major immune effectors in protecting against infection with rabies virus (Wandeler, 2006; Wunner, 2007). The matrix (M) protein is located on the inner virus envelope, inside which the virus nucleoprotein (N) tightly binds the viral RNA to form the nucleocapsid core. This core, along with a large transcriptase protein (L) and a phosphorylated protein (P), is the rabies virus nucleocapsid (RNP). The ribonucleoprotein complex consisting of the N, P, L, and negative-strand genomic RNA has been reported to potentially play a role in the establishment of immunologic memory and long-lasting immunity (Dietzschold, 2008).

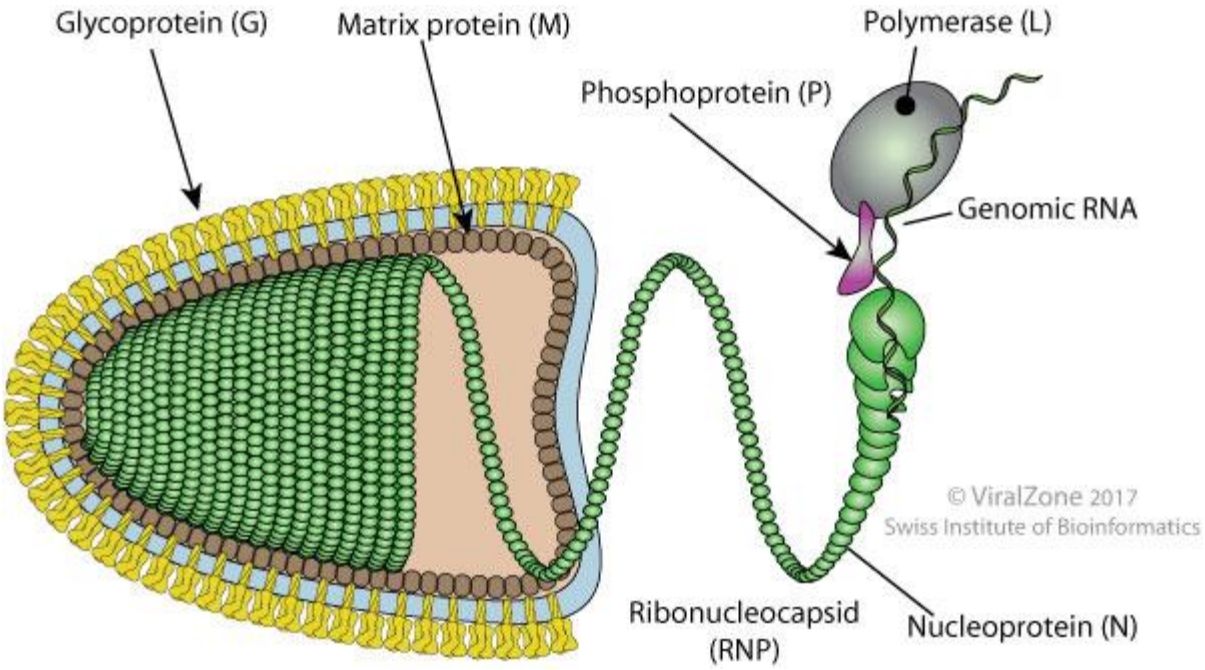

Figure 1: Structure of Rabies virion

Source: Viral Zone (2017)

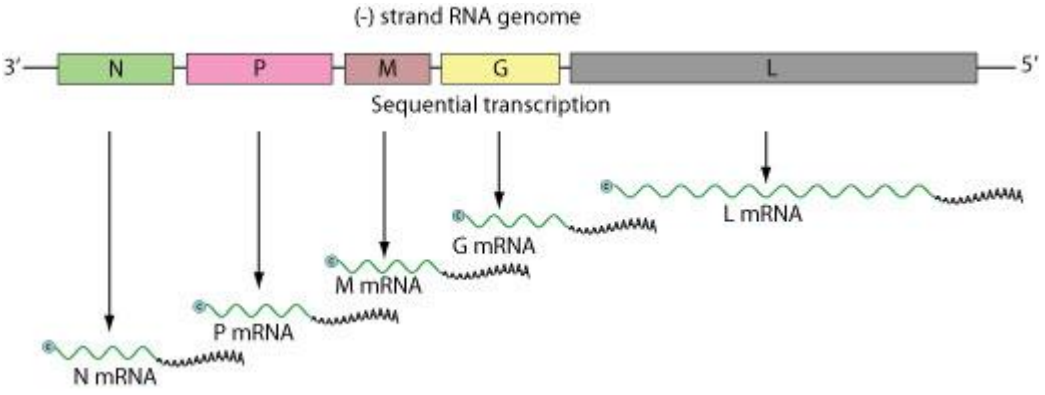

Figure2: Genome Arrangement of Rabies virion

Source: Viral Zone (2017)

Rabies virus can be inactivated by sodium hypochlorite, 45-75% ethanol, iodine preparations, quaternary ammonium compounds, formaldehyde, phenol, ether, trypsin, β-propiolactone, and some other detergents. It is also inactivated by a very low pH (below

3) or very high pH (greater than 11). This virus is susceptible to ultraviolet radiation. It is rapidly inactivated by sunlight and drying, and (in dried blood and secretions) it does not survive for long periods in the environment (CFSPH, 2012).

1.1.2. Transcription and Replication

After receptor binding, rabies virus enters its host cells through the endosomal transport pathway (Le Blanc *et al*., 2005) via a low-pH-induced membrane fusion process catalyzed by the glycoprotein (Finke and Conzelmann, 2005). All transcription and replication events take place in the cytoplasm inside a specialized 'virus factory', the Negri body, as reviewed by Albertini *et al*, (2008). According to Albertini *et al*, (2011) cited from Wagner, (1991) much of the understanding of rabies virus transcription and replication comes from studies on vesicular stomatitis virus (VSV). The first step of the replication cycle is transcription of the viral genome. The L-P polymerase complex enters the nucleocapsid at the 3' end and starts transcription with the productions of short RNA molecules, the leader RNA, that is neither capped nor polyadenylated. After the transcription stop, the polymerase restarts with the transcription of nucleoprotein mRNA, which is capped and polyadenylated by the viral polymerase complex as shown for VSV (Ogino and Banerjee, 2007). Later in infection, the activity of L switches to replication in order to produce full length positive-strand RNA copies without caps or poly (A) tails. These complementary or cRNAs are also encapsidated by N and bind the L-P complex. They are used as templates to make new negative strand RNA genomes for encapsidation by N in order to form new nucleocapsids (Arnheiter *et al.,* 1985).

2.2. Epidemiology

2.2.1. History of rabies

Rabies is the ancient disease and great dreaded infection of human and animals. It was first recognized in Egypt around 2300 BC and in Greece, where it was well described by Aristotle (Singh *et al*., 2017). Rabies originated from the word 'rabha' meaning violence (Takayama, 2008). Rabies is one of the oldest documented diseases in the world (Bleck

and Ruprecht, 2002). Canine rabies was also described in sixth century BC, in the Avesta (Persia), first century in the Susrutasamhita (India). The infectious nature of saliva from infected dogs was recognized by Zinke in 1804. No effective prevention or curative treatment in animals was available before Pasteur's discovery in 1885. On the other hand, described by Yousaf *et al*, (2012), in Africa, an unconfirmed epidemic of rabies in dogs occurred in western Zambia in 1901 and its existence was confirmed in South Africa in 1928. Similarly, In Ethiopia, the first and only recorded of rabies case in Addis Ababa occurred in August 1903 as described by Richard, (1990).

2.2.2. Geographical Distribution

Rabies is prevalent throughout the world except in Islands. Many of the countries are endemic for rabies, except Australia and Antarctica. Some countries such as the United Kingdom, Ireland, Sweden, Norway, Iceland, Japan, Australia, New Zealand, and Singapore, most of Malaysia, Papua New Guinea, the Pacific Islands and some Indonesian islands have been free of this virus for many years (OIE, 2013). As per the definition of World Health Organization (WHO), a country that has no record of indigenously acquired case of human or animal rabies within two years period due to surveillance and import regulations can claim rabies free status. But susceptibility to reintroduction from neighboring countries does exist in spite of undertaking vaccination programs in wildlife (Dutta and Dutta, 1994).

According to Raux *et al*, (2000) cited from WHO global vaccines research forum, over 30,000 people die every year due to rabies in Asia while it causes at least 24,000 deaths per year in Africa. The high death rates reported in poor rural communities and children. The major cause of spread of rabies in this region is urbanization (Niloufer, 2003). Rabies is predominant in Bangladesh and India followed by Nepal, Myanmar, Bhutan, Thailand and Indonesia (Yousaf *et al*., 2012). In Africa, maximum mortality rates are documented in children and underprivileged agrarian people. The most important reason for transmission of rabies in Africa is dog population and urbanization (Hemachudha, 1994). Several an unconfirmed epidemic of rabies in dogs occurred in African countries (Yousaf

et al., 2012). On the other hands, in European continent, though rabies is still exist, but rabies cases in human have been vanished from most of the European nations most likely due to the enforcement of policies regarding vaccination in animals especially in dogs (De Benedictis *et al*., 2010)

2.2.3. Host range

Rabies primarily is an animal infection. Humans become infected through exposure to infected animals. All mammals are susceptible to rabies, but only a limited number of species also act as reservoir hosts. They include members of the families *Canidae* (dogs, jackals, coyotes, wolves, foxes and raccoon dogs), *Mustelidae* (e.g., skunks), *Viverridae* (e.g., mongooses), and *Procyonidae* (raccoons), and the order *Chiroptera* (bats) (OIE, 2008).

Rabies reservoirs are generally grouped into terrestrial (i.e., land-dwelling) species and bat species. Rabies can occur sporadically in individuals or can exist in an enzootic or epizootic state in animal populations. In enzootic state rabies is indigenous to a reservoir species in a locality and occurs with a relatively stable incidence rate. An epizootic occurs when the incidence of disease increases markedly in the reservoir species. Rabies that is transmitted sporadically from reservoir to non-reservoir species is said to "spillover". These reservoir species are: raccoon (*Procyon* lotor), striped skunk (*Mephitis* mephitis), coyote (*Canislatrans*, infected with the dog variant), gray fox (*Urocyon cinereoargenteus*), and Arctic fox (*Alopex lagopus*) and red fox (*Vulpes vulpes*) (Bruce and Margaret, 2001). As Singh referred from WHO (1998), prevalence of rabies in different species have been documented as 48% in dogs, 21.9% in cats, 61.4% in cattle and buffalo, 48.7% in goats and 45% in horses .

2.2.4. Rabies transmission

The commonest way of rabies transmission is by the bite of an infected mammal bites by rabid animals generally inoculate virus-laden saliva through the skin into muscle and subcutaneous tissues (Warrell and Warrell, 2004). Other inoculation routes are rare

(Gibbons, 2002). Rabies virus entry occurs through wounds or direct contact with mucosal surfaces. Otherwise, the virus cannot cross intact skin. The risk of rabies infection by a bite (5%-80%) is at least 50 times greater than that by a scratch (0.1%-1%) (Hemachudha, *et al.,* 2013) Mortality after untreated bites by rabid dogs ranges from 38% to 57% and depends on the severity and location of the wound as well as on the presumed virus concentration in the saliva (Hemachudha *et al.,* 2002; Warrel and Warrel, 2004).

However, bat virus might be more infectious when inoculated superficially into the epidermis since it replicates more rapidly in non-neuronal cells and at lower temperatures than do dog rabies viruses. Percutaneous infection probably occurs during unnoticed skin contact, which may result in a minute bite (Gibbons, 2002). The route of viral entry into epithelial nerves and eventually into the central nervous system (CNS) is unknown (Messenger *et al*., 2002)

Inhalation of aerosolized RV occurred accidentally in laboratories of vaccine production (Helen brand, 2005) or in caves inhabited by numerous infected bats (Gibbons, 2002). Infection through the digestive tract has also been reported (CDC, 1999). Contact with animal vaccines may be significant when attenuated vaccine is used. In these situations, rabies prophylaxis is necessary. The handling and skinning of infected carcasses can be of risk for workers in refrigeration plants and butchers' shops, and veterinarians (Consales and Bolzan, 2007). As Singh referred from Fekadu *et al* (1992), the contact with infected people could be a potential risk for their relatives and health workers when unprotected direct contact with secretions from a patient containing viable virus occurs. There are many reports of organ transplantation involved in the transmission of rabies. The most frequent cases have been observed in corneal transplantation. The most recent case reported was of a German patient and occurred in 2005 (Hellenbrand *et al*., 2005)

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2.3. Rabies Immunity

2.3.1. Response to immunization

The kinetics of the immune responses to rabies virus have been widely studied in experimentally infected laboratory mice, in vaccinated dogs and in humans given post-exposure vaccination, but there is very little information regarding possible immune responses or immune modulation in naturally infected animals and there is no explanation to date as to how the virus persists in the site of inoculation, without stimulating the host's immune response (Woldehiwet, 2002). Immune response and possible immune suppression are largely influenced by strain, dose and route of inoculation (Lodmell *et al*., 2004). Immune systems responsible for protection against rabies virus infection have proved that inactivated rabies vaccines can induce the production of cytotoxic T-cells (Dietzschold, 2008).

Additional experimental evidence investigating the role of cell-mediated immunity in mice confirmed the fact that cytotoxic T- cells alone do not protect against rabies, as the depletion of CD8+ T-cells had no effect on the resistance to disease nor on the survival rate of vaccinated animals (Lafon, 2007). Research indicates that inactivated rabies vaccines stimulate B-cells as well as CD4+ cells using major histocompatibility complex class II (MHCII) mechanisms and confer protection through the induction of an immune response including the activation of lymphocytes, CD4+ antibody-secreting plasmocytes and neutralizing antibodies that migrate into the nervous system parenchyma (Dietzschold, 2008). The activation of CD4+ T-lymphocytes ultimately results in the production of RVNAs that target and destroy rabies virus, thus playing a major role in protecting victims exposed to rabies against developing the disease (Moore *et al*., 2006). Plant-derived antigens induced strong mucosal and humeral immune responses after administration via either an oral or an intramuscular route in mice (Loza-Rubio *et al*., 2012). DNA-based vaccine should provide efficient ways to induce a cell-mediated cytolytic CD8+ T cell response, CD4+ T cells, and VNA (Perrin *et al*., 2000).

2.3.2. Immunopathology

Immunopathology is the almost complete lack of an inflammatory response within the CNS characterized by perivascular cuffing with mononuclear cells, local gliosis and neuronophagia. Lesions occur in most areas of the CNS but are frequently more severe in the brainstem. This contrasts with other viral diseases of the CNS, in which inflammation is the major pathological characteristic. These observations suggest that neuronal dysfunction, rather than neuronal death, is probably responsible for the fatal outcome of rabies under normal conditions (Hooper *et al*., 1998). Viral glycoprotein is the target for most RV-neutralizing antibodies and has been a strong inducer of apoptosis in infected cells, which is evidently an immunogenic process in rabies (Préhaud *et al*., 2003).

2.4. Rabies vaccines

Mass vaccination of dogs remains the main strategy for controlling urban rabies in endemic areas as almost all human rabies deaths worldwide result from dog bites (Franka *et al*., 2013). However, in most countries, the major prophylactic measures for controlling rabies in reservoirs comprise culling free-ranging dogs using various methods. A decrease in the vector population did not prevent the spread of rabies because the decrease was insufficient (WHO, 2005). Furthermore, dogs' mass vaccination must be complimented with baits vaccination of wild animals to prevent the spill over the disease from wild animals. An efficient method of controlling wildlife rabies is distributing bait rabies vaccine to risk regions (Yang *et al*., 2012). In general, vaccination coverage of approximately 70% of the vector population is estimated to be sufficient to block rabies transmission (Ertl, 2009). However, the existing rabies vaccines for human and animals have inherent drawbacks (Yang *et al*., 2012), thus efforts have been made subsequent improving of the traditional rabies vaccines in light of the advancement of technology.

2.4.1. Nerve-tissue rabies vaccine

The Pasteur's vaccine is the first nerve-tissue rabies vaccine (Hicks *et al*., 2012) which was desiccated rabies virus infected rabbit spinal cord (Johnson *et al*., 2010). Empirically, the spinal cord dried for progressively shorter periods contained increasingly virulent rabies virus. Obviously, the infectious nature of Pasteur's vaccine received severe criticism in his time, and some patients died of rabies following infected spinal cord suspension vaccination (Gelfand, 2002). Despite of criticism, Pasteur's method was used for more than half a century (Wu *et al*., 2011). Pasteur's vaccine was modified by adding phenol to partially or completely inactivated live rabies viruses. Unfortunately, phenol distorts protein structure and disrupts rabies virus antigenicity. For more than a decade, the WHO disapproves the production of NTVs in 2005 as these vaccines are weak antigens and short immune response (Yang *et al*., 2012) and are associated with neurological reactions (Warrell, 2012).

There are still three types of NTVs being produced globally in spite of WHO recommendation. The Fermi vaccine was a 5% aqueous suspension of rabies virus-infected sheep or goat brain, treated with phenol (Aga *et al*., 2016). Semple was totally inactivated Fermi vaccine (Wu *et al*., 2011) which contains a combination of live and inactivated virus. The Fermi vaccine is still used in at least Ethiopia today (Aga *et al*., 2016) while Semple vaccine in Asia countries such as Mangolia, Myanmar, and Pakistan (Banyard *et al*., 2013). Pasteur, Fermi and Semple vaccines contained high levels of myelin that causes sensitization in some vaccine recipients and, in extreme cases, fatal encephalitis (Hicks *et al*., 2012). The suckling mouse brain vaccine (SMBV) is the third NTV developed to minimize myelin related adverse effects. However, this vaccine was not fully free of myelin and other undesirable components as claimed. The SMBV is still produced in Argentina, Bolivia, Ecuador, El Salvador, Honduras, Peru, Uruguay, and Algeria (Wu *et al*., 2011).

2.4.2. Inactivated cell culture rabies vaccine

Modern rabies vaccines for human and animals use are produced in vitro, bypassing the need to infect live animals. The rabies virus was first adapted on grow in hamster kidney cell, which led to considerable progress in cell culture technologies (Hicks *et al*., 2012). Cell culture technology allows for standardized, safe, effective, economical, and large-scale vaccine production (Wu *et al*., 2011). The cell culture vaccines (CCVs) represent a significant advance, particularly over the first crude NTVs (Briggs, 2007). Several different cell substrates have been used for the production of cell culture rabies vaccines, including Syrian baby hamster kidney cells, human diploid cells, primary cell lines produced from embryonated chicken and duck eggs, and continuous cell lines produced from Vero cells and baby hamster kidney (Briggs, 2007). These cell cultures have used for large-scale production of antigen for both attenuated and inactivated vaccines for human and animal use (Yang *et al*., 2011).

Worldwide, many rabies virus strains, such as Challenge Virus Standard (CVS), Flury Low Egg Passage (LEP), Flury High Egg Passage (HEP), Kelev, Evelyn Rokitniki Abelseth (ERA), Vnukovo-32, Street Alabama Dufferin (SAD), Pasteur Virus (PV), and Pitmann Moore (PM) have all been used for production of inactivated animal rabies vaccines (Yang *et al*., 2012; OIE, 2013). These strains confer uniform protection against member of the *lyssavirus* genotype 1 rabies virus (OIE, 2013). However, the Flury LEP strain is widely used for making rabies vaccines for humans and animals because it can achieve high titers when grown in cell culture and because of its ability to elicit high immunogenicity (Nandi and Kumar, 2010).

These inactivated cell culture vaccine are inactivated with beta propiolactone (BPL), ultraviolet light, acetyl ethylamine, or binary ethylenimine (BEI) (OIE, 2013). Commonly used adjuvant are aluminum hydroxide, aluminum phosphate, and saponin for animal immunization (Johnson *et al*., 2010).These inactivated has permitted to be used for dogs, cats, cattle, goats, and fox for prevention of animal rabies (Yang *et al*., 2012). Inactivated rabies vaccine has been shown to be a safe and efficient means to control rabies in dogs

(Tao *et al*., 2011). However, the vaccination rate of dogs in many developing countries is low, especially in rural areas, mainly due to low economic development and the high cost of vaccination (Knoble *et al*., 2005). Once, the inactivated rabies vaccine was considered for use as an oral immunogen in wild carnivores but, the vaccine needed large amounts of inactivated protein to induced rabies-specific virus neutralization antibodies and revealed partial protection against lethal rabies infection (Rupprecht *et al*., 1992).

2.4.3. *Modified live vaccine (MLV*)

The minimum requirements for live rabies vaccine are non-pathogenic in animals, ability to propagate high virus titers in cells, ability to induce protective immunity after administration, and thermal and genetic stability (Yang *et al*., 2011). To ensure the safety of candidate vaccines, most researchers modified the virus by serial passage in various cells. These traditionally attenuated rabies viruses have been used successfully for oral immunization of animals in the wild that consume baits containing the vaccine (Ertl, 2009). Many attenuated derivatives of the SAD, such as ERA, SAD-B19, Street Alabama Dufferin (SAG-1) and SAG-2, are widely used in live rabies vaccines for oral vaccination in wildlife, or parental injection in dogs and other animals (Wu *et al*., 2011). Traditional rabies virus attenuation, in general, is linked to the amino acid sequence of the viral glycoprotein (G). For example, a mutation in position 333 of the glycoprotein where arginine is replaced by glutamic acids generally attenuates the virus and provides the basis for the apathogenic SAD strains (Yang *et al*., 2014). Attenuation by a single amino acid exchange, however, may not guarantee a stable nonpathogenic phenotype of a rapidly mutating RNA virus.

2.4.4. DNA based rabies vaccine

One approach for developing new generation rabies vaccines is to use a Deoxy ribonuclotide acid (DNA) based or plasmid vaccine encoding the rabies glycoprotein gene. Advanced recombinant DNA technology has made it possible to generate a variety of DNA vaccines against infectious agents. DNA based vaccines developed to induce a broad-spectrum immune response when delivered to the host have several advantages,

such as action in the presence of maternal antibodies, strong stability, mass production, and cost effectiveness (Dhama *et al*., 2008). However, the antibody level is very low to undetectable after the first DNA injection but increases both with the number of injections and the amount of injected DNA (Perrin *et al*., 2000). Numerous studies have demonstrated the relative effectiveness of DNA based rabies vaccines at inducing RABV specific Virus Neutralizing Antibody (VNA) based on various parameters, including the plasmid dosage and inoculation route. However, DNA-based rabies vaccines were not successful at protecting non-human primates following pre- and post-exposure vaccination (Lodmell *et al*., 2001).

2.4.5. Vaccinia rabies glycoprotein (V-RG)

V-RG vaccine was the first recombinant rabies vaccine to be constructed, field tested, and considered for regulation in Europe and North America for wildlife rabies control. This vaccine has been extensively reviewed to ensure safety (tested in >40 species of mammals and birds) and efficacy (proved against severe rabies challenge in target species). Following the success of the V-RG vaccine against fox rabies in Belgium and France, preliminary field trials suggest its potential utility for rabies control in raccoons, foxes, and coyotes in the U.S. (Lyles and Rupprecht, 2007).

2.4.6. Oral rabies vaccines derived from plants

Plants have provided new systems for the large-scale production of recombinant proteins at low cost, simplifying the production process. A variety of genetically engineered vaccines using tobacco mosaic virus and tomato bushy stunt virus have been developed for expressing foreign antigens in plants (yusibov *et al*., 2002). Rabies antigen expressed in plant tissue was immunogenic and protective in mice immunized intramuscularly and orally. One of the more advanced approaches for expressing foreign antigens in plants is to construct transgenic plants (Sugiyamaa and Ito, 2007). To produce a plant-derived rabies antigen, the native signal peptide within the rabies glycoprotein gene was replaced with that of the pathogenesis-related protein of *Nicotiana tabacum.* Codon optimization of the rabies glycoprotein gene is necessary for providing plant-preferred codons. These

antigens have several advantages, including post-translational modifications, stability for storage, and ease of delivery (yusibov *et al*., 2002). The rabies glycoprotein has been expressed in several plants, including tobacco, tomato, spinach, carrot, and maize. Although plant-derived antigens have many advantages, some problems should be solved before oral rabies vaccines originating from plants are given to domestic animals. The most important are the need to improve the expression in raw transgenic plants and to reduce the substantial variability in the level of protein expression among different lines and subsequent generations. Additionally, it is necessary to shorten the time needed to obtain antigens from different plants (Loza-Rubio *et al*., 2012).

2.4.7. Recombinant rabies virus-vectored vaccines

Many scientists have been searching for new vaccine strains that can induce protective antibodies and effectively protect animals from rabies without raising any safety issues. Most live attenuated vaccine strains can cause rabies in wild animals, although the incidence is low. Reverse genetics can provide more stable variants of rabies vaccine strains and generate homologous virus vectors expressing a variety of foreign genes (yusibov *et al*., 2002). The most important modification to reduce the pathogenicity of RABV is to replace the codon for arginine at position 333 in the glycoprotein gene sequence with another amino acid codon, such as for glutamic acid, glycine, isoleucine, leucine, methionine, or cysteine. Changing the arginine codon converts the pathogenic virus into a non-pathogenic phenotype (Loza-Rubio *et al*., 2012).

2.4.8. Recombinant poxvirus-vectored vaccines

In the mid-1980s, a MLV using the SAD B19 strain was used in bait to immunize wild foxes in Europe. However, the vaccine strain raised serious concerns about safety in certain wild animals. A recombinant Vaccinia-rabies vaccine has been used successfully for oral bait vaccination in several species. Nonetheless, the lack of efficacy in important rabies reservoirs such as skunks and safety concerns over the use of live virus vaccine as a vector have impaired the expansion of V-RG bait to new target species and new areas (Weyer *et al*., 2009). *Poxviruses* have merit as vaccine carriers. First, *poxviruses* have

large DNA genomes ranging from 139,000 base pairs for open reading frame virus to 379,000 base pairs for Avipox virus, which allows the insertion of up to 30,000 base pairs of foreign DNA. Second, poxviruses are potent inducers of both arms of the immune response. Manipulation of the poxvirus is relatively easy in the laboratory, resulting in the development of new recombinant vaccine strains. Another advantage of using recombinant poxviruses as a vaccine is that poxviruses are thermally stable at environmental temperatures. Although recombinant poxvirus expressing rabies glycoprotein induced protective immunity in foxes, it was less effective in other animals. As a result, the canary pox virus expression vector (ALVAC) was developed as a highly host-restricted virus with interrupted replication in mammalian cells. Rabies recombinant ALVAC vector expressing a RABV glycoprotein gene has been proven safe and efficacious (Poulet *et al*., 2007).

2.5. Diagnosis of Rabies

Laboratory diagnosis and surveillance for animal and human rabies are severely constrained in much of the developing world where rabies is endemic (Lembo *et al*., 2006) But it is essential for timely post-exposure prophylaxis (Hemachudha *et al.,* 2002). Rabies diagnosis may be carried out either *in vivo* or postmortem (Warrell and Warrell, 2004). Serological tests may help but RV antibodies have been detected in only 20% of unvaccinated rabies patients tested 1-26 days after the onset of the disease. Antibodies appear in the cerebrospinal fluid later (Hooper *et al.,* 1998; Hemachudha *et al*., 2004).

A definitive diagnosis of rabies can be made only with the appropriate laboratory methods. The basic techniques are described in the WHO publication Laboratory Techniques in Rabies (Meslin *et al.,* 1996) and the OIE Manual of Diagnostic Tests and Vaccines for Terrestrial Animals (OIE, 2012).

Brain biopsy is not practicable, nor is it recommended for the diagnosis of rabies, but it could be of high sensitivity (Hemachudha *et al*., 2004). False negative results may occur

when biopsy of the frontal and temporal regions is carried out on the first day of the disease.

2.5.1. Identification of the Agent

Clinical observation may only lead to a suspicion of rabies because signs of the disease are not characteristic and may vary greatly from one animal to another (WHO, 1992). The only way to perform a reliable diagnosis of rabies is to identify the virus or some of its specific components using laboratory tests. As rabies virus is rapidly inactivated, refrigerated diagnostic specimens should be sent to the laboratory by the fastest means available. Shipment conditions must be considered to be part of the 'rabies diagnostic chain' (WHO, 1996).

A. Virus Isolation

Virus isolation is required for confirmatory diagnosis, especially when FAT gives an uncertain result and more importantly for molecular characterization of viruses in a geographical location and for tracing the origin of the virus if rabies occurs in a rabies-free area. Two techniques can be employed for this purpose: the mice inoculation technique (MIT) and rapid tissue culture infection test (RTCT) (Koprowsky, 1996; Webster and Casey, 1996).

B. Mouse Inoculation Test

Three-to-ten mice, 3-4 weeks old (12–14 g), or a litter of 2-day-old newborn mice, are inoculated intracerebrally with the clarified supernatant of a 10–20% (w/v) homogenate of brain material in an isotonic buffered solution containing antibiotics (Meslin *et al.,* 1996). The inoculated mice are observed daily for 28 days; they develop typical signs and symptoms of rabies any time after 5–7 days depending on the incubation period (OIE, 2008). These consist of initial ruffling of hair, hunch back, and dragging hind limbs followed by paralysis of hind- and forelimbs. Further confirmation of the diagnosis can be made by extracting the brain of the diseased mouse and subjecting this to FAT.

The disadvantage of MIT is the long interval before a diagnosis can be made since the inoculated mice need to be kept under observation for 28 days as some wild viruses may have a very long incubation period. If cell culture facilities exist in the laboratory, consideration should be given to replacing the mouse inoculation test with cell culture whenever possible as it avoids the use of live animals, is less expensive, and gives more rapid results. However, advantages of MIT are that when the test is positive, a large amount of virus can be isolated from a single mouse brain for strain identification purposes and that it can be easily and practicably applied in situations where skills and facilities for other tests (e.g., cell culture) are not available (OIE, 2012).

2.5.2. Rapid Rabies Enzyme Immunodiagnosis (RREID)

The rabies N antigen can also be detected by applying immunohistochemical techniques as well as enzyme immunoassays. An ELISA-based technique was developed in 1986 which is known as rapid rabies enzyme immunodiagnosis (RREID) (Perrin *et al*, 1986). This technique is based on capturing rabies N protein in a brain homogenate by a polyclonal or monoclonal anti-N antibody coated on the solid phase. Subsequently, the captured antigen is detected by adding peroxidase conjugated monoclonal or polyclonal antibody rose in a different species or even better by the addition of biotinylated N antibody followed by streptavidin peroxidase and colour development with o-phenylenediamine dihydrochloride (OPD) and hydrogen peroxide. In various studies, the test is found to be as sensitive and specific as FAT (Saxena *et al,* 1989; Miranda and Robels, 1991).

2.5.3. Fluorescent Antibody Technique (FAT)

The most widely used test for postmortem rabies diagnosis is the fluorescent antibody test (FAT), which is recommended by both World Health Organization (WHO) and World Organization for Animal Health (OIE). Developed by Goldwasser and Kissling in 1957, this test is still the gold standard for rabies diagnosis (Goldwasser and Kissling 1958; Dean *et al.,*1996; Shankar, 2009).The FAT is based on antibodies specific for nucleoprotein but, being conjugated to fluorescein isothiocyanate, requires a fluorescent

microscope to visualize any specific antibody bound to viral protein within the test sample (Dean and Abelseth, 1973). It is routinely used to detect virus antigen in badly decomposed sample material for the purpose of testing samples in the developing world where suitable cold storage for samples is often unavailable (Barrat, 1996).

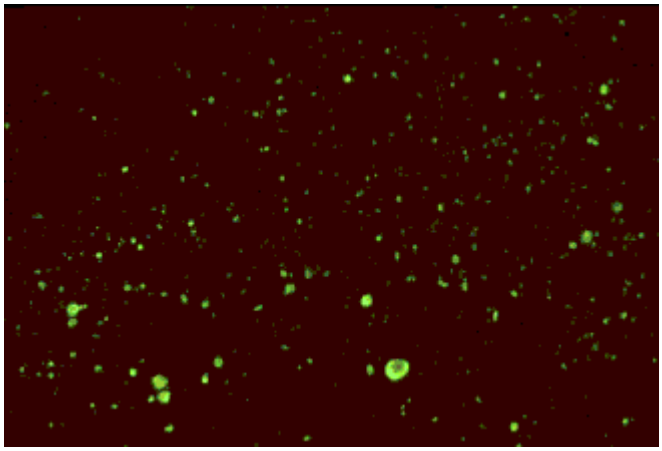

Figure 3: Direct fluorescent antibody test of rabies

Source: CDC, 2011

2.5.4. Nucleic Acid Detection Techniques

Nucleic acid amplification and detection techniques have revolutionized the diagnosis of rabies in recent years and have an important role in the ante mortem diagnosis of human rabies. Currently, several polymerase chain reaction (PCR) based assays have been evaluated as an adjunct to conventional tests for antemortem and postmortem rabies diagnosis. Most assays target the highly conserved rabies viral nucleoprotein gene for amplification. Several conventional gel-based reverse transcriptase PCR (RT-PCR) assays with nested/ hemi nested protocols for detection of rabies viral RNA on clinical samples have been described (Coertse *et al.,* 2010; Biswal *et al.,* 2012). The amplicons generated in these assays can be sequenced for further virus characterization and phylogenetic analysis (Johnson *et al*., 2002). However a major drawback of these assays is the risk of cross-contamination, which precludes their routine use for diagnosis of human and animal rabies (Belak and Thoren, 2001).

Real-time PCR based assays allow for the detection and quantification of genome copies and a considerable reduction in cross-contamination is achieved due to the "closed-tube" nature of these assays (Gibson *et al.,* 1996). Real-time PCR using the SYBR Green chemistry has been evaluated on human saliva samples for antemortem rabies diagnosis (Nagaraj *et al*., 2006) and as a universal real-time assay for the detection of Lyssaviruses (Hayman *et al.,* 2011). Though these assays are promising, extreme care is needed to ensure specificity (Nadin-Davis *et al.,* 2009). Real-time PCR assays using the TaqMan fluorogenic probes, however, ensure a high specificity because of the intrinsic hybridization reaction (Wacharapluesadee *et al.,* 2008;Mani *et al*., 2013) have a wide range of detection, and are 10–1000 times more sensitive than traditional nested RT PCR. A generic real-time TaqMan-PCR for the detection and differentiation of lyssavirus genotypes 1, 5, and 6 has also been developed (Wakeley *et al*., 2005). This assay utilizes a pan-lyssavirus primer set, which has been shown to amplify a large panel of representative Lyssaviruses, with probes specifically designed to discriminate between classical rabies virus and the European Bat Lyssaviruses type-1 and -2 (EBLV-1 and EBLV-2) (Hughes *et al*., 2004).

2.6. Prophylaxis and control

There is no specific treatment for rabies, which is a fatal disease that implies, incurable but preventable (Blackmore, 2014). There is lack of information on rabies treatment and prevention both in humans and animals. People have clear understanding on the danger of the disease but believe to cure with different traditional and religious treatment rather than seeking effective post exposure prophylaxis. Most people use wide variety of traditional treatment in cases of bite by animals (mostly dogs) believed to be rabid. The significance of the disease is evident from the continued existence of traditional specialists in rabies treatment within the community (Knobel *et al*., 2005). Control of rabies in developing countries can be very successful based on appropriate planning, health education, 70% vaccine coverage for dog populations, and epidemiological surveillance (Reisner and Taheripour, 2007). The most practical and cost effective way to end *Canine* rabies is mass dog vaccination, which saves lives of both dogs and humans.

During mass vaccination campaigns, all dogs should be vaccinated regardless of Age, Weight or state of health (WHO, 2013). Primary vaccination can be a single injection (live attenuated vaccines) or two inoculations of 1 month apart. After that vaccines are given annually, biannually or triennially to boost their immunity depending on the efficacy of the vaccine (Hanlon *et al*., 2003). The first successful example of a mass vaccination program in a dog population occurred in the city of Memphis and Shelby County, Tennessee in the United States in 1948 which reduced both animals and humans cases to zero (Tierkel *et al.,* 1950). Dogs, cats and ferrets that have never vaccinated and are exposed to rabid animal should be euthanized immediately. If the owner is unwilling to have this done, the animals should be placed in strict isolation for 6 month. Rabies vaccine should be administered up on entry in to isolation or up to 28 days before release to comply with pre-exposure vaccination recommendation (Hanlon *et al.,* 2002).

Human rabies can be prevented by a) eliminating exposure to rabies virus, b) providing appropriate rabies pre exposure prophylaxis, and c) prompt local treatment of bite wounds combined with appropriate rabies post exposure prophylaxis (CDPH, 2012). Inactivated human vaccines are available for at risk veterinary staff, other animal handlers, wildlife officers, laboratory workers and others at high risk of exposure (MMWR, 2012).

3. MATERIAL AND METHODS

3.1. Study Location

The experiment was conducted from February, 2018 to May, 2018 at NVI, Ethiopia. NVI was established at Debrezeit/Bishoftu in 1964 with 40 persons under the Ministry of Agriculture, getting technical assistance from the French Government through the French Veterinary Mission in Ethiopia. The infrastructure of NVI is well developed and playing an important role in the attraction of international institution such as Pan African Veterinary Vaccine Control Center. It has been given the responsibility to produce and supply enough vaccines for PTA (Preferential Trade Area) countries of Eastern, Western and Southern Africa. The institute has reached the level of using state of the art equipments and technologies for biological production and vaccine related research. The Institute produces more than 21 different viral and bacterial vaccines for animal use, inactivated cell culture rabies vaccine is one of them (NVI, 2018).

3.2. Study Animals

Potency test was conducted on Swiss white mice. The mice (male) were two to three weeks of age and weighting 6 – 8gm. All mice were obtained from NVI animal breeding center. On the other hand, dogs were used for immunological response experiment. Male dogs without considering their breed were recruited at the age of three to four month and purchased from Bishoftu towns. The animals were quarantined for three weeks for checking their health status. Their seronegativitye for anti-rabies antibody were confirmed by Fluorescent Antibody Virus Neutralization (FAVN) Test. Only puppies with no detectable rabies antibody were included in the study. Identification was given to all recruited dogs by writing their identification number on their cages. All experimental puppies were dewormed and kept for 21 days prior to introducing to the experiment.

3.3. Study Design

3.3.1. Potency test

Potency test of inactivated rabies vaccine was performed on manufactured date to determine the optimum dose for vaccination of dogs. The potency test was carried out according to National Institute of Health procedure. Verorab, (Lot N^{o}. P1B461M, Sanofi Pasteur, France) containing 2.5 IU/vial was used as reference vaccine. Briefly, the test vaccine was serially diluted using phosphate buffered saline in five-folds (1:5, 1:25, 1:125, 1:625, and 1:3125) in comparison with a standard reference vaccine, given in four five-fold dilutions (1:10, 1:50, 1:250 and 1:1250). Sixteen mice were randomly assigned to each dilution. Swiss mice were immunized intra-peritoneal at 0.5ml of tested vaccine using 26 gauge needles on days 0 and 7. After 14 days, all vaccinated mice were challenged intra-cerebrally with 30µl of Challenge Virus Standard (CVS-11) containing 25MLD$_{50}$. The CVS-l1 titration was executed in parallel. Intracranial titration of the challenge virus (original and three 10-fold dilutions) with 10 mice per group was undertaken to evaluate the virus dose used in the test. Animals were monitored for 21 days and clinical signs were recorded at each observation. Direct Fluorescent Antibody Test (DFAT) was performed for confirmation the death of mice specific to challenge rabies viruses (Blancou, 2003).

3.3.2. Immunogenicity experiment

Random clinical trial design type was implemented and experimental puppies were randomly divided into two treatment groups using lottery method. Eight puppies were allocated to ERA strain vaccine group and eight puppies were used as control. Therefore, a total of sixteen dogs were required for this clinical immunological experiment. One ml of rabies vaccine was administered subcutaneously for vaccinated group after blood samples were collected from all experimental animals at day 0. Following vaccination, blood samples were collected on day 7, 14, 21, 30, and 60 days. Rabies virus neutralization antibody levels in the sera were measured using FAVN test (Cliquent *et al*., 1998).

3.4. Experimental Animals Management

The potency test was undertaken in laboratory animals' room of NVI rabies vaccine production facilities. Sixteen mice belongs to similar treatment group were kept in cage. Mouse' feed palate and water was given to them *adlibitum*. Mice were quarantined for one week before the day of trial started to adopt the environment. On the other hand, dogs used for immunological experiment were housed in a separate cage over the experimental period. The puppies were fed twice a day and water was provided *adlibitum*. One attendant was assigned to provide feeding and watering and cleaning of the dogs' houses after he received a pre-exposure rabies vaccination.

3.5. Blood Sampling and Sera Collection

Blood samples were aseptically collected from dogs' cephalic vein using plain vaccutainer which was labeled immediately prior to sampling. Five ml of blood was drawn from each animal during each sampling time. The blood was allowed to clot at room temperature for about 20 minutes. Sera were separated by centrifugation at 3,000 rpm for 20 minutes. The sera were decanted into coded crayovials and stored at -20^0C until further use.

3.6. Laboratory Techniques

3.6.1. Cells and Viruses

Inactivated cell culture rabies vaccine was produced on Vero cell lines as per standard operation procedure of the Institute while CVS-11 strain (CDC/Atlanta) was propagated on BHK-21 cell line. The cell lines were maintained in Dulbecco's Modified Eagle Medium (DMEM) with 2mM L-glutamine, 0.1mM non-essential amino acid, 10% heat inactivated calf serum, and antibiotic solution of 100IU/ml penicillin and 10µg streptomycin. The rabies vaccine was produced using ERA seed vaccinal strains.

3.6.2. Rabies Virus Titration

Virus titer was determined using BHK-21 cell line in 96 wells micro plate. The virus suspension was initially serially diluted in ten-folds using Dulbecco's Modified Eagle Medium (DMEM). One hundred µl of each virus dilution was dispensed into five wells of a row starting highest dilution and 100µl of DMEM was added into control wells. Then 50µl cell suspension containing 5×10^4 cells were distributed into each well of micro plate. Each dilution was limited to five replicate wells for economic reason. After 72 hours of incubation at 37^0C, the micro plate was washed with Phosphate buffer saline (PBS) without Mg+2, Ca+2, fixed with 80% cold acetone and then stained with fluorescein-labeled anti rabies nucleocapsid immunoglobulin. The virus titration ($TCID_{50}$) was calculated according to Spearman–Karber method (WHO, 1996). Finally, the virus MLD_{50} was determined by *in vivo* titration and a concentration sufficient to kill 80% of controls were administrated.

3.6.3. Fluorescent Antibody Virus Neutralization (FAVN)

Rabies virus neutralizing antibody levels in the sera were measured using FAVN test. In brief, a positive WHO reference serum adjusted to 0.5 IU/ ml was used as the positive control. Serum samples and the positive and negative controls were distributed in four consecutive wells and serially diluted. Then, Rabies virus (CVS-11 strain) containing about 100 $TCID_{50}$/50µl was added to each well. A 50µl aliquot of BHK-21 cells containing 4×10^5 cells/ ml was added to each well after 60 min of incubation at 37^0C, and the micro plates were incubated in a humidified incubator with 5% CO_2 at 37^0C for 48 hours. After discarding cell culture medium, monolayer on micro plate well was fixed in cold acetone for 20 min. After three successive washes with PBS, the micro plates were stained with anti rabies fluorescein isothiocyanate conjugate for 45 min at 37^0C. After rinsing with PBS, the micro plates were air-dried and examined at 10× under a fluorescence microscope. The comparison of the measured titer of the tested sera with that of the OIE positive standard serum of a known Virus Neutralization (VN) titer allowed determination of the VN titer of the tested sera in IU/ ml. The comparison to IU/ ml was made by using the mean value of the OIE standard serum (OIE, 2008).

3.6.4. Direct Fluorescent Antibody Test (FAT)

Direct Fluorescent Antibody Test (DFAT) was performed for confirmation of the death of mice specific to challenge rabies viruses. Smear was prepared from a composite sample of brain tissue that includes the brain stem. The smears were fixed in 100% high-grade cold acetone for 20 min, air dried and then stained with a drop of rabies specific conjugate for 30 min at 37°C. The rabies virus antigens were detected in brain samples' smear with a fluorescein-isothiocyanate (FITC) conjugated anti-rabies virus monoclonal antibody (Blancou, 2003; OIE, 2008; Abera *et al*., 2015).

3.7. Statistical Analysis

The collected data was entered and stored into Microsoft office Excel spread sheet 2007 and thoroughly screened before subjecting to statistical analysis. Median Lethal dose (MLD) and the effective dose (ED) of challenge virus strain were calculated by Spearman-Karber formula. Similarly, the 50% endpoint of antibody content of the sera and virus titrations ($TCID_{50}$) was calculated according to Spearman-Karber formula. A volumetric method of calculation was used for calculating test vaccine (TV) potency by comparing its dilution protecting 50% of mice with Verorab (reference) vaccine (RV). The relative potency (RP) of the test vaccine was determined by formula: RP = (Reciprocal of ED_{50} of TV/Reciprocal of ED_{50} of) x (dose of TV/dose of RV). The median effective dose (DE_{50}) of the reference and test vaccines was determined, based on the number of survivors. The relative potency of the test vaccine was then calculated by comparing the DE_{50} of the test vaccine with that of the reference vaccine. The potency is expressed in International Unit per ml (IU/ml).Mean and standard deviation was used to summarize neutralizing antibody titer across sampling times. STAT version 13 was used to conduct the statistical analysis. Neutralizing antibody titer over sampling times was subjected to analysis of variance (ANOVA) of repeated measurement followed by pair-wise Bonferroni's *t* test. P-value of less than 0.05 was considered statistically significant.

4. RESULTS

4.1. Rabies Vaccine Potency Test on Mice

Out of 174 experimental mice, only two assigned to 1:125 and 1:3125 dilutions of the test vaccine were died within five days after challenge. Both mice did not show any clinical sign of rabies such as ruffling of hair coat, hunch back, dragging of hind limbs and paralysis. They were also negative for direct fluorescent antibody test; thus, they were recorded as not due to rabies virus and excluded from the potency test calculation.

Table1: Summary of the NIH potency test of NVI rabies vaccine

Vaccine	***Dilution***	***No. of mice assigned***	***Result***			***Effective Dose 50***
			No of died		***No. of survived***	
			Specific	***Non-specific***		
Test (NVI) vaccine	1:5	16	3		13	1.47
	1:25	16	5		11	
	1:125	16	5	1	10	
	1:625	16	10		6	
	1:3125	16	13	1	2	
Reference (Verorab) vaccine	1:10	16	3		13	1.31
	1:50	16	5		11	
	1:250	16	8		8	
	1:1250	16	10		6	
Control	10^{-1}	10	9		1	
	10^{-2}	10	7		3	
	10^{-3}	10	3		7	

In the present potency test, thirty control mice were used to estimate the LD_{50} of the working challenge virus. The working challenge virus was diluted in tenfold for consecutive three first dilutions. Out of ten mice in each dilution, nine, seven and three mice were died from the first (10^{-1}), the second (10^{-2}) and the highest (10^{-3}) dilutions, respectively. The Spearman-Karber method calculation of these values indicated that the working challenge virus had about $25MLD_{50}$/0.03ml.

With regards to potency of NVI rabies vaccine, from the lowest to the highest five dilutions of the test rabies vaccine, 13 (n=16), 11 (n=16), 10 (n=15), six (n=16) and two (n=15) mice, respectively, were survived from CVS-11 virus challenge (Tab 1). The Spearman-Karber calculation of those values indicates that the test vaccine had 1.47 ED_{50}. On the other hand, 13 (n=16) from lowest dilution of the reference vaccine (Verorab) were survived and 25 mice from other three dilutions were not died on 21 days after challenge (Table 1). The calculation of those values discloses that the reference vaccine resulted in 1.31 ED_{50}. Therefore, relative potency of the test vaccine was found to be 1.45 IU/ml in 1ml of the recommended single dose based on the volumetric method calculation.

4.2. Antibody Response of Dogs after Vaccination

All dogs recruited for immunological experiment had low antibody titer ($\leq$ 0.25IU/ml) and the titer of control dogs was remained at lower level over experimental period (Fig 4). Nevertheless, the mean neutralizing antibody titer of vaccinated puppies rose to 1.556 IU/ml on7thday of post vaccination (Table 2). Their mean of antibody titer was continued its increment to 1.815 IU/ml on 14thday and 2.311 IU/ml on 21st day of post vaccination. Then after the highest mean antibody titer of 3.585 IU/ml was observed 30th day post vaccination (Table 2). However, their mean was relatively lowered to 3.402IU/ml on the end of the experiment (Table 2).

Table2: Mean and standard deviation of neutralizing antibody response to rabies vaccine over sampling period

	Days of post vaccination					
	0	**7**	**14**	**21**	**30**	**60**
Mean antibody titer (IU/ml)	0.090	1.556	1.815	2.311	3.585	3.402
Standard deviation	0.064	0.597	0.499	0.698	1.188	1.117

The repeated measurement ANOVA analysis showed that there was statistically significant ($F_{(5,35)}$=33.68, $P\leq$ 0.001) difference among antibody titer means of vaccinated dogs over sampling days. Likewise, the pair-wise *t* test revealed that all mean antibody titers of post vaccination was statistically (P<0.001) higher than the mean titer of pre-vaccination. However, despite of antibody titer increment, there was no statistical significant (P> 0.05) difference between the mean titers of day 7th and 14th of post vaccination while the 7th day mean antibody titer was statistically lower than mean titers of 21st day (P<0.05), 30th day ($P\leq$ 0.001) and 60th day ($P\leq$ 0.001) of post vaccination. On the other hand, 14th day mean titer was no varied from 21st day mean antibody titer whereas it was statistically inferior to mean titers of 30th and 60th days of post vaccination. Similarly, antibody titer mean of 21st day was statistically ($P\leq$ 0.001) lower than those of mean titers of 30thday and 60thday.The mean antibody response by the vaccinated group became peak on 30th day of post vaccination. Even though antibody titer went down on 60th day of post vaccination, the reduction was statistically not significant (P>0.05).

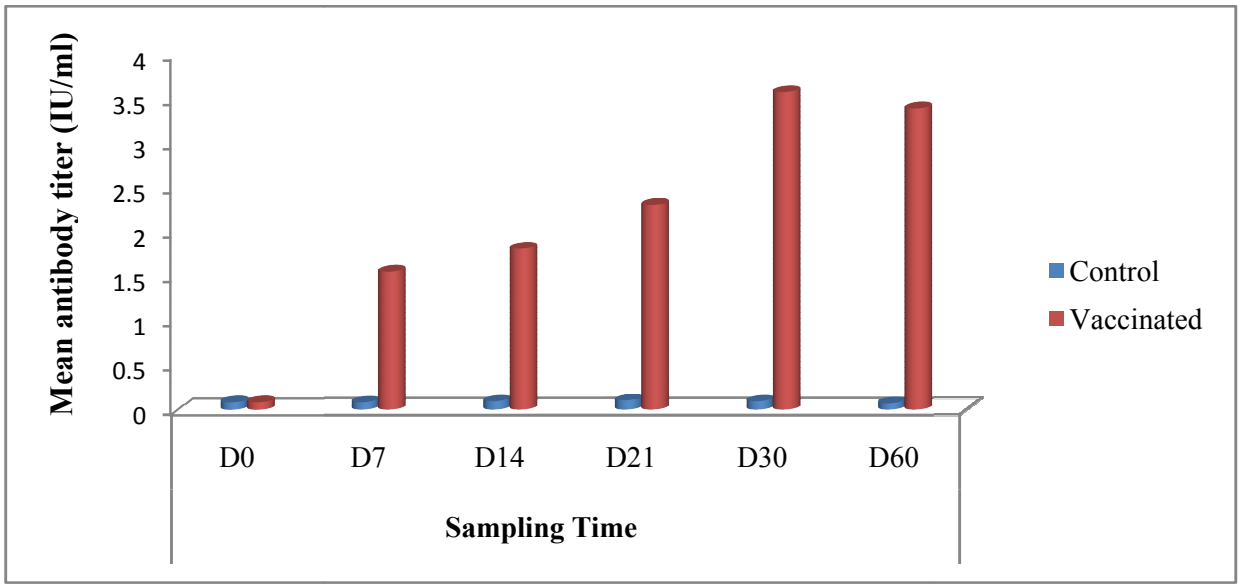

Figure 4: Mean neutralizing antibody titer (IU/ml) of control and vaccinated groups over experiment period

5. DISCUSSION

The potency test was undertaken using the NIH procedure and thirty unvaccinated mice were included to evaluate the effective lethal dose of the working challenge virus. Out of ten mice that received each dilution; nine, seven and three mice were died from 10^{-1}, 10^{-2} and 10^{-3} dilutions of working CVS-11, respectively. Hence, the challenge virus can cause death with $25LD_{50}$/0.03ml. In favor of the present result, OIE recommended that the challenge dose needs to have 12–$50LD_{50}$ for a valid potency test (OIE, 2013). Therefore, the working challenge virus was effective and strong enough to conduct a valid test.

In the present experimental study, two mice from test vaccine were died nonspecifically during the potency as the nonspecific deaths were confirmed by absence of specific clinical sign and direct fluorescent test. Hence, these mice were excluded from the calculation of ED_{50}. Mice were died from all dilutions of test and reference rabies vaccine because of the challenge virus. From the lowest to the highest five dilutions of the test rabies vaccine, 13 (n=16), 11 (n=16), 10 (n=15), six (n=16) and two (n=15) mice were survived from the challenge. In similar way; 13, 11, eight and six mice did out of 16 mice from each consecutive four fivefold dilutions of the reference vaccine. These figures suggested that the number of survived mice from each group was decreasing as dilution of both vaccines became higher. Moreover, Spearman-Karber calculation of those values confirmed that test vaccine had 1.47 ED_{50}while the reference vaccine had 1.3. Therefore, the RP of NVI inactivated cell culture rabies vaccine was 1.45 IU/ml antigen concentration in 1ml of the recommended single dose. However, the RP of NVI rabies vaccine was comparatively lower than Pasteur and ERA inactivated cell culture rabies vaccines conducted by Mengesha *et al*. (2014). This difference might be attributed to the difference in formulation the final vaccine because they were attempting for producing human rabies vaccine, which is required a minimum of 2.5IU/ml antigen concentration per dose (WHO, 2005). In the contrary, inactivated rabies vaccines for veterinary use are required to have more than 1.0 IU/ml in a single dose based on potency test on mice

(OIE, 2013). Therefore, 1ml of the tested batch of NVI rabies vaccine containing 1.45IU/ml meets the OIE requirement for single dose.

Vaccination of dogs against rabies effectively controls canine rabies and prevents its transmission to human (WHO, 2005).Mass vaccination of dogs remains the most efficient control measure of endemic canine rabies (Belloto, 1998). The measurement of neutralizing antibodies to rabies virus is commonly used to assess the level of immunological responses to rabies in animals. Induction of these antibodies by vaccine is a key determinant of viral neutralization and animal protection against disease development (Cliquet *et al.*, 1998).

In support of previous literature, all vaccinated dogs became sero-converted and rapidly developed higher rabies virus neutralizing antibodies in a week of post vaccination. However, the mean antibody titers of all control dogs stayed at a level less than 0.18 IU/ml over the experimental period. Hence, the pre-vaccination mean neutralizing antibody titer was highly significant lower than those of post-vaccination as the result of rabies vaccine administration. Similar scenarios were reported by Hurisa *et al*. (2013b) and Hurisa *et al*. (2015) from Ethiopia, by Kallel *et al*. (2006) from Tunisia and by Darkaoui *et al*. (2016) from Morocco.

On seventh day of post vaccination, the mean antibody response by vaccinated dogs reached 1.556 IU/ml, demonstrating that the test vaccine was immunogenic and effective. The present experiment is in agreement with the mean antibody titer of 1.55 IU/ml (Hurisa *et al*., 2013b), 1.59 IU/ml (Hurisa *et al*., 2015) and 1.41 IU/ml (Darkaoui *et al*., 2016) on 7^{th} day of post vaccination. Moreover, the least antibody titer recorded on the 7^{th} day was 0.59IU/ml in this experiment and the recommended threshold protective rabies virus neutralizing antibody titer for animal is 0.50 IU/ml (OIE, 2013). Therefore, all dogs vaccinated with 1ml of NVI rabies vaccine developed protective antibody within a week of post vaccination.

Within two weeks of post vaccination, the mean antibody titer increased to 1.815 IU/ml, but it was not statistical higher that of 7th day mean antibody titer. This figure is comparable to that of 1.73 IU/ml on 14th day reported by Hurisa *et al*. (2013b) and Hurisa *et al*. (2015). Nevertheless, mean antibody titer recorded in the present trial was lower than those of 6.1 IU/ml (Kallel *et al*., 2006) and 3.32 IU/ml (Darkaoui *et al*., 2016) on 15th day. Then after, the mean titers statistically remained its rising to 2.311 IU/ml on 21st day and 3.585 IU/ml on 30th day. These findings are in accords to Hurisa *et al*. (2013b) and Hurisa *et al*. (2015) while Darkaoui *et al*. (2016) and Kallel *et al*. (2006) reported higher titer of 5.81 IU/ml on 21st day and 5.0 IU/ml on 30th day of post vaccination, respectively. In this experiment, the antibody levels peaked at 30th day and then slightly declined to 3.402 IU/ml on 60thdayof post vaccination. However, the mean titer of day 30 and 60 was not statistically significant (P>0.05). Such trend also observed by Hurisa *et al*. (2013b), but Hurisa *et al*. (2015) indicated the antibody titer continued its increment up to 60th day. On the other hand, Sage *et al*. (1993), Kallel *et al*. (2006) and Darkaoui *et al*. (2016) informed that antibody titer reached a pick as early as at 15 - 21 day of post vaccination and then progressive declined. Such variation could be resulted from difference in vaccine quality, vaccine seed and breed of dogs used in experiments.

Shortly, the NVI vaccine was effective and immunogenic over two months study period. However, the inactivated cell culture vaccine can induce protective antibody for at least one year (OIE, 2013). Consequently, WHO and OIE recommended annual mass vaccination of dogs to control canine rabies in endemic countries. Therefore, the two months immunological study was too short to assess antibody dynamic over a year and it was difficult to determine time when to booster animals to keep their antibody to effective protective level.

6. CONCLUSION AND RECOMMENDATION

The recommended single dose of NVI inactivated cell culture rabies vaccine is potent enough to meet the requirement of inactivated rabies vaccine for veterinary use. The vaccine can induce a protective level of rabies virus neutralizing antibody in a week of post-vaccination. The protective antibody progressively increased and reached a peak on 30^{th}dayof post vaccination. The vaccine is effective and immunogenic for at least two months. However, the study period was too short to assess immunogenicity of the vaccine for the recommended period of inactivated rabies vaccine for animal use.

Therefore, based on the above conclusion following are forwarded:

- Further immunological experiment in target animals needs to undertaken for one year;
- The immunological study requires to be supported by protective efficacy clinical trial in target animals and
- The potency test should be multiple.

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8. ANNEXES

Annex 1.Essential equipments and Titration of virus in TCID50 (50% tissue culture infective dose)

Essential equipments

Humidified incubator at37^{o}c with 5% CO_2 Bio safety cabinet, Fluorescence microscope suitable for FITC fluorescence equipped with x10 eye-piece and x10 objective

Biological and reagents

Modified Eagles medium (MEM) +10%heat inactivated fetal calf serum
PBS, PH 7.2, without Ca2+ and Mg2+ stored at 4^{o}c
Acetone 80% (diluted with deionized water)
Cells: BHK-21 with 10% FCS and Antibiotics
DMEM with 10% FCS and Antibiotics
Vaccines : NVI rabies vaccine, manufactured by National Veterinary Institute, which was Vero cell cultured base ERA fixed rabies strain containing 1IU/ml in a single dose, was used in this study.
Standard serum**:** OIE Standard Serum of dog origin (OIE reference laboratory for rabies, Nancy, France) stored at + 4°C and diluted to 0.5 IU/ml with PBS according to the titer of the batch was used to the internal control which was used for FAVN testing.
Virus*:* CVS-11 (ATCC VR 959) strain, which is available from the ATCC or the OIE Reference Laboratory for Rabies, Nancy, France Vials are stored at –80°C;

Titration of virus in TCID50 (50% tissue culture infective dose)

This titration method uses BHK-21 C13 cells (ATCC CCL-10) in micro titer plates.

i) *Cell suspension:* the day before titration, a cell suspension containing 10^5 cells/ml is prepared in cell culture medium containing 10% heat-inactivated FCS, and is distributed,

200 µl per well, into 96-wellmicrotitre plates. The plates are then incubated for 24 hours at 35.5°C–37°C with 5% CO2.

ii) *Dilution of the virus:* the serial dilutions are performed in 5 ml tubes using a cell culture medium without FCS as diluents. Tenfold dilution from 10^{-1} to 10^{-12} is prepared (0.9 ml of diluents with 0.1 ml of the previous dilution).

iii) *Infection of the cells:* the medium in the micro titre plates is discarded using an aspiration system. Fifty µl of each virus dilution is distributed per well. Six replicates are used per dilution. The micro titre plate is then incubated for 1 hour at 35.5–37°C with 5% CO_2. Then 200 µl of cell culture medium, containing5% FCS, is added.

iv) *Incubation:* incubate for 3 days at 35.5–37°C in 5% CO_2

v) *Staining and calculation of titre:* The cells are stained using the FAT, as detailed below. Reading is qualitative, every well that shows specific fluorescence is considered to be positive. The titre calculation is made using either the neoprobit graphic method (2) or the Spearman–Karber formula.

vi) The CVS titration must be performed by FAVN test to establish the infective dose in $TCID_{50}$

Test procedure

i) The micro plates are used for the titration of CVS (rows 1 to 4), and for the controls, standard sera and naive dog serum are used. All other plates are used for the sera to be tested.

ii) Medium is added to the wells as follows: plate 1, rows 1 to 4 and cells A9 to A12: add 150 µl per well; in the other plates, rows 6 and 12: add 200 µl per well; all other wells: add 100 µl.

iii) Sera to be tested are heat inactivated for 30 minutes at 56°C 50 µl of each undiluted serum to be tested is added to four adjacent wells.

iv) Dilutions of sera are conducted in the micro plates:

Addition of challenge virus standard

i) Stock CVS is stored in 1 ml micro tubes at –80°C. One tube is thawed rapidly under cold running water, and placed in melting ice.
ii) One dilution from this tube is prepared in order to obtain 100 TCID50 in 50 µl. Of this dilution, 50 µl is added to each serum-filled well.
iii) Incubate the micro plates at 35–37°C in a humid incubator with 5% CO2 for 1hour.
iv) Addition of cells*:* trypsinise a sub confluent culture of 3-day-old BHK-21 cells. Re suspend the cells to obtain a 4×10^5 cells/ml suspension in DMEM supplemented with 10% heat-inactivated FCS. Add 50 µl of the cell suspension to each well.
v) Incubate the micro plates for 48 hours at 35–37°C in a humid incubator with 5% CO_2.

Fixation and staining

i) After the 48-hour incubation period, the medium is discarded, and the micro plates are rinsed once in PBS, pH 7.2, and once in 80% acetone. The micro plates are then fixed in 80% acetone at room temperature for 30 minutes, and are dried at room temperature for at least 30 minutes.
ii) Add 50 µl of the FITC anti-rabies conjugate, at the working dilution, to each well, gently rock the micro plates and incubate at 35–37°C for 30 minutes. Discard the fluorescent conjugate and rinse the micro plates twice with PBS. Excess PBS is removed by briefly inverting the micro plates on absorbent paper.

Reading and interpreting the results

i) The total surface of each well is observed. The reading evaluation is qualitative (plus or minus): no fluorescent cell – a minus score is recorded for the well; fluorescent cells (one cell or more) – a plus score is recorded for the well.
ii) Cell and virus controls are read first. For titration of CVS, naïve serum, and OIE standard serum, titers are calculated according to the Spearman–Karber method or the neoprobit graphic method (2).

iii) Results of titration of CVS (TCID50), naive serum (D50 [median dose]) and positive standard (D50) are reported on a control card for each of these three controls. The control results of the current test are compared with the accumulated control test results from previous tests using the same batch of control. The test is validated if the values obtained for the three controls in the current test are not statistically different from the mean (± 2 SD) of all the values obtained in the tests conducted previously according to this technique.

iv) The result of the test corresponds to the non-neutralized virus after incubation with the reference serum or with the serum to be tested. These titres are calculated with the neo-probit graphic method (2) or with the Spearman–Karber formula. The comparison of the measured titre of the tested sera with that of the OIE positive standard serum of a known neutralizing titre allows determination of the neutralizing titre of the tested sera in IU/ml. The conversion to IU/ml can be made by using either the log D50 value of the day or the mean value of the OIE standard serum.

Annex 2: Mice Inoculation Record Form

Day	Dilution											
	10^{-1}						10^{-2}					
	No. of mice						No. of mice					
	H	RF	DP	PP	P	D	H	RF	DP	PP	P	D
0												
1												
2												
3												
4												
5												
6												
7												
8												
9												
10												
11												
12												
13												
14												
15												
16												
17												
18												
19												
20												
21												
22												
23												
24												
25												
26												
27												
28												
total												

Where: H= Health; RF: ruffled fur; DP=Depression; PP= Partial Paralysis; P= Paralysis; and D= death

Annex 3. Photos captured during vaccination and sample collection