Addis Ababa University

College of Natural Sciences

Department of Microbial, Cellular and Molecular Biology

Biomedical Science Stream

Pathogenicity and cross protection studies of local rabies virus isolates with cell culture anti-rabies vaccine produced in Ethiopia

By

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List of Acronyms/Abbreviations

ABLV	Australian bat Lyssavirus
ATCC	American Type Culture Collection
BHK	Baby Hamster Kidney
CDC	Center for Disease Control and Prevention
CFSPH	Center for Food Security and Public Health
CNS	Central Nervous System
CO	Cow Origin
CVS	Challenge Virus Standard
DNA	Deoxyribonucleic Acid
DO	Dog Origin
EBLV	European bat Lyssavirus
EPHI	Ethiopian Public Health Institute
ERA	Evenyl Roktincki Abelseth
FAT	Fluorescent Antibody Test
FAVN	Fluorescent Antibody Virus Neutralization
HDCV	Human Diploid Cell Vaccine
HOS	Human Origin Sululta
HOW	Human Origin Wollega
IC	Intracerebral
IM	Intramuscular
IU	International Unit
LEP	Low Egg Passage
MEM	Minimum Essential Medium
MICLD	Mice Intracerebral Lethal Dose

NIH	National Institute of Health
OIE	Office International des Epizooties
PBS	Phosphate Buffered Saline
PCEC	Purified Chick Embryo Cell vaccine
PCR	Polymerase Chain Reaction
PDEV	Purified Duck Embryo Vaccine
PM	Pitman Moore
PV	Pasteur Virus
PVCV	Purified Vero Cell Vaccine
RABV	Rabies Virus
TCID	Tissue Culture Infectivity Dose
TCSN	Tissue Culture Supernatant
USA	United State of America
UV	Ultra Violet
VDPD	Vaccine and Diagnostics Production Directorate
WHO	World Health Organization

ABSTRACT

Rabies is a worldwide problem, and the case is most severe in developing countries where cell culture derived anti-rabies vaccines are unaffordable or the available nervous tissue-derived vaccines are of questionable immunogenicity and may cause neurological complications. The aim of this research was to study pathogenicity and cross protection of local rabies virus isolates with Evenyl Roktincki Abelseth (ERA) based cell culture anti-rabies vaccine produced in Ethiopia. The viruses were isolated from rabid dogs' brains and human saliva, and adapted on Swiss albino mice brain and cell lines. By titration, a minimum of $10^{6.5}TCID_{50}$/ml (in vitro) and $10^{4.5}MICLD_{50}$/0.03ml (in vivo) virus titer were obtained. For pathogenicity study, mice were inoculated intramuscularly with 250MICLD50/0.1ml of each adapted virus isolate and observed for 45 days. Only two virus isolates, human origin sululta (HOS) and dog origin (DO) caused 12.5% death. Cross protection of local isolates with ERA vaccinal strain was studied by in vivo and in vitro methods. For in vivo method, a group of mice were immunized on day zero and seven with 0.5ml (1:5 dilutions) of ERA based cell culture anti-rabies vaccine produced locally. On day fourteenth, mice were challenged with working dilution of each local isolate and one group with challenge virus standard (CVS-11), and observed for further 14 days. Based on the survival rate, high protection was recorded in CVS-11 challenged mice and low protection in all isolates; protection to HOS challenged mice was very low. In vitro test was done by fluorescent antibody virus neutralization (FAVN) test on BHK-21 cell lines. Sera from dog immunized with locally produced vaccine and OIE standard serum were incubated with working dilution of local virus isolates and CVS-11 for 48 hours in the presence of cell lines. Maximum antibody titer (2.74IU/ml) was obtained with CVS-11 challenge virus and minimum antibody titer (1.55IU/ml) was obtained with cow origin (CO) virus isolate. All locally isolated rabies virus show low antibody titer when compared to CVS-11 and PV-12. From the results of this study, it can be concluded that local isolates have some genetic variation from fixed virus strain which can affect efficacy of the candidate vaccine and potency value should be set in-terms of local virus isolates as challenge virus. Generally, the exact genetic relationship should be studied by molecular techniques and canine anti-rabies vaccine should be developed from locally isolated virus.

Key Words: *Cell Culture Vaccine, Cross protection, Local Isolate, Pathogenicity, Rabies*

1. Introduction

1.1 Background

Rabies is a viral disease that affects the central nervous system (CNS) of mammals and has an extremely high case fatality rate. In developing countries, with limited access to high-quality anti-rabies biologics, approximately 55,000 people and millions of animals die every year due to rabies (WHO, 2005). Rabies is endemic in Africa and Asia, and most human deaths occur in these endemic countries (WHO, 2010). The annual cost of rabies in Africa and Asia was estimated at US 583.5 million dollar, most of which is due to cost of post-exposure prophylaxis (Wudu *et al*., 2013). Ethiopia, being one of the developing countries, is highly endemic for rabies. Approximately 10, 000 people were estimated to die of rabies annually in Ethiopia which makes it to be one of the most affected countries in the world (Fekadu, 1997).

In Ethiopia, rabies is an important disease that has been recognized for many centuries. The first rabies case in Addis Ababa was detected in August 1903 (Teshome *et al*., 1992). The incidence of human post-exposure treatments and human rabies cases per million population of Ethiopia were 73.6 and 12.6, respectively (Bogel and Motschwiller, 1986). In Africa, the highest recorded human death due to rabies was for the year 1998 which was 43 reported from Ethiopia (Asefa *et al*., 2010). Surveillance reports usually underestimate incidence and are poor indicator of the status of the disease in countries like Ethiopia where human and animal health information systems are inadequate. There is lack of accurate and quantitative information on rabies both in humans and animals, and little is known about the awareness of the people about the disease to apply effective control measures in Ethiopia.

The dissemination of the rabies virus within the body immediately after infection makes it unique from other viruses in its movement and CNS invasion. The virus enters an eclipse phase during which it is not easily detected. During this phase, it replicates in non-nervous tissue, such as muscle. It does not usually stimulate an immune response at the time, but it is susceptible to neutralization if antibodies are present. After several days or months, the virus enters the peripheral nerves and is transported to the central nervous system by retrograde flow in the axons. After dissemination within the CNS, where clinical signs develop as the neurons are infected, the virus is distributed to highly innervated tissues via the peripheral nerves. Most of the virus is found in nervous tissue, salivary glands, saliva and cerebrospinal fluid (CSF), and all should be handled with extreme caution. The virus can also been detected in small amount in other tissues and organs, including the lungs, adrenal glands, kidneys, bladder, heart, ovaries, testes, prostate, pancreas, intestinal tract, cornea, germinal cells of hair follicles in the skin, sebaceous glands, tongue papillae and the brown fat of bats (CFSPH, 2009). The rabies virus is contained within the neurons, and handling most body fluids or intact organs is thought to carry a low risk of infection. Organ transplants pose a rare risk, if the donor is not known to have been infected with rabies. Blood, urine and feces are not thought to be infectious; however, a few studies have suggested that viremia might occur at some point during the infection. A recent study in mice, using a polymerase chain reaction (PCR) assay, found viral RNA in mice when they were clinically ill, but not during the asymptomatic stage when virus was migrating to the CNS (Elenice *et al*., 2010).

Illness due to rabies can be prevented by administering anti-rabies antibodies and a series of vaccination, provided exposure is recognized before the symptoms appear. Closely related lyssaviruses circulate among bats, and can cause an illness identical to rabies in human and domesticated animals (Smith *et al*., 1978). Rabies virus and the rabies-related lyssaviruses have been classified into two or more phylogroups, based on their genetic relatedness. Rabies vaccines and post-exposure prophylaxis are thought to provide some protection against some of these viruses (Phylogroup I), but not for some of phylogroup II species (Badrane *et al*., 2001). Rabies-related lyssaviruses can be found even in countries classified as rabies-free. The virus has a non-segmented and negative-stranded RNA genome with about 12,000 nucleotides. Lagos bat virus, Duvenhage virus, European bat lyssavirus (EBLV) 1, EBLV 2, Australian bat lyssavirus (ABLV), Mokola virus and Irkut virus have caused clinical cases in humans or domesticated animals (Smith *et al*., 1978). Phylogroup I contains rabies virus, Duvenhage virus, EBLV 1, EBLV 2 and Australian bat lyssavirus, and Phylogroup II consists of Lagos bat virus, Mokola virus and Shimoni bat virus (Leslie *et al*., 2006). Viruses that are more closely related to rabies virus can be neutralized, at least to some extent, by antibodies to rabies virus (Wright *et al*., 2008). Viruses that are far from rabies virus in their glycoprotein genetic makeup cannot be neutralized by antibody raised to classical rabies virus and can affect efficacy of the vaccine (Leslie *et al*., 2006; Sarcamento *et al.*, 1992).

1.2 Statement of the problem

In Ethiopia, many people receive anti-rabies post exposure treatments annually due to wide-spread of dog bites. The isolation of two rabies related viruses, Mokola and Lagos bat viruses from domestic animals in Ethiopia (Teshome *et al*., 1992) is of public and veterinary concern due to lack of effective vaccines against these agents which cannot be protected by vaccine from fixed rabies virus strains. Studies done in France confirm that 14.7% divergence between wild type rabies virus and vaccinal strain which can strongly affect efficacy and potency of anti-rabies vaccine (Sacramento *et al*., 1992). Dogs are responsible in maintaining the continuous persistence as well as dissemination of rabies in Ethiopia (Bethelhem *et al*., 2004). The problem is most severe in Ethiopia due to unaffordable imported cell culture-derived anti-rabies vaccines and the available nervous tissue-derived vaccines are of questionable immunogenicity and may produce neurological complications. Since the 1940's, Ethiopia produces and uses Fermi-type anti-rabies vaccine. Currently EPHI has successfully produced cell culture anti-rabies vaccine from PV and ERA fixed rabies virus strain (Birhanu *et al*., 2013). The efficacy of this vaccine should be tested with local rabies virus isolates for cross protection with fixed virus strain. Regular intervention targeted at vaccinating stray dogs with standardized anti-rabies vaccine evaluated in-terms of local rabies virus isolate is strongly recommended for effective disease prevention and control.

1.3 Significance of the study

In developing countries, with limited access to high-quality anti-rabies biologics, approximately 55,000 people and millions of animals die every year due to rabies. Ethiopia Public Health Institute has produced high quality cell culture-based anti-rabies vaccine. This vaccine was produced from fixed rabies virus vaccinal strain (ERA) on Vero cell lines and should be tested for its efficacy with locally circulating rabies viruses. Therefore, this study evaluate pathogenicity of local rabies virus isolates and compare cross protection with cell culture-based anti-rabies vaccine produced from ERA fixed rabies virus strain.

1.4 Objective

1.4.1 General objective

The aim of this study was to adapt clinical rabies virus isolates on mice brain and BHK-21 cell lines, and to study pathogenicity and cross protection with ERA based cell culture anti-rabies vaccine produced locally.

1.4.2 Specific objectives

- To isolate local rabies virus from clinical samples and adapt on mice brain (in vivo) and BHK-21 cell lines (in vitro)
- To determine lethal dose (LD_{50}) of the isolated rabies virus by titration on BHK-21 cell lines (in vitro) and Swiss albino mice brain (in vivo)
- To study pathogenicity characteristics and cross protection of local rabies virus isolates with ERA based cell culture anti-rabies vaccine produced locally
- To compare genetic relationship between local rabies virus isolates and ERA fixed virus strain based on the pathogenicity and cross protection

2. Literature review

2.1 Over all description of rabies virus

Rabies is a zoonotic viral disease which causes acute encephalitis in humans and animals. The disease affects domestic and wild animals, and spread to humans through close contact with infectious material, usually saliva, via bites or scratches. As the virus are transmissible to humans, all suspected infected material must be handled under the appropriate safety conditions specified by the World Health Organization (WHO[a], 2007). Once symptoms of the disease develop, it is 100% fatal to both humans and other animals. Rabies virus causes an acute infection of the central nervous system. Five general stages are recognized in humans; incubation, excitation, acute neurologic period, coma, and death. The incubation period is exceptionally variable, ranging from fewer than 10 days to longer than 2 years, but is usually one to three months (Ruppretcht *et al*., 2001). The only step that makes rabies control possible is not by cure but by prevention. The integral part of prevention is by pre- and post-exposure vaccination (Toovey, 2007).

The five proteins that compose the RABV are responsible not only for the virus activities on a molecular level, but their effects are far-reaching and can be observed in its interactions with the host's immune system and the physiological symptoms of the host. The matrix protein in the RABV is essential in the release of virus particles from the cell via ribosomes in exocytosis. The nucleocapsid protein previously discussed is believed to act as an exogenous super antigen, and is unique in this respect given that it is the only identified viral super antigen (Wunner, 2007). The nucleocapsid activities are responsible for a variety of responses in the host including its activation of the peripheral blood lymphocytes in human vaccines, its ability to produce a more rapid and heightened response upon injection of inactivated rabies vaccines. Its induction of early T cell activation steps and expansion and mobilization of CD4+ T cells to trigger and support production of viral neutralizing antibody (VNA) and ability to bind to HLA class II antigens expressed on the surface of cells (Wunner, 2007).

2.2 Virus structure and phylogenetic variations

Based on the phylogenetic analysis, the seven genotypes of Lyssavirus can be divided into two major phylogroups. Phylogroup I comprises the worldwide genotype 1 (classic rabies virus), the European bat lyssavirus (EBL) genotypes 5 (EBL1) and 6 (EBL2), the African genotype 4 (Duvenhage virus), and the Australian bat lyssavirus genotype 7. Phylogroup II comprises the divergent African genotypes 2 (Lagos bat virus) and 3 (Mokola virus) (Smith *et al*., 1978; Baer *et al*., 1990). Viruses from phylogroup I (Rabies virus and EBL1) were found to be pathogenic for mice when injected by the intracerebral or the intramuscular route, whereas viruses from phylogroup II (Mokola and Lagos bat viruses) were only pathogenic by the intracerebral route (Badrane *et al*., 2001). Within each phylogroup, the amino acid sequence of the glycoprotein ectodomain was at least 74% identical, and anti-glycoprotein virus-neutralizing antibodies displayed cross-neutralization. Between phylogroups, the identity was less than 64.5% and cross-neutralization was absent (Wright *et al*., 2008; Badrane *et al*., 2001). This indicate that the classical rabies vaccines (phylogroup I) cannot protect against lyssaviruses from phylogroup II.

The majority of rabies in terrestrial animals and humans is caused by classical rabies virus (RABV). Phylogenetic evidence suggests that one or more host-switching events from bats into terrestrial mammals were originally responsible for the ongoing global epidemic of terrestrial RABV (Smith *et al*., 1978). There is sufficient antigenic variation within the genus to cause variable vaccine efficacy, but this variation is difficult to characterize quantitatively. Antigenic neutralization data can be used as high-resolution robust interpretation. Pre- or post-exposure prophylaxis, using vaccination and passive immunoglobulin administration according to World Health Organization (WHO) guidelines is the only effective way to prevent rabies after infection with a lyssavirus (Horton *et al*., 2010). The efficacy of both active and passive immunization is likely to be affected by antigenic differences between viruses (Dietzshold *et al*., 1983; Sarcamento *et al*., 1992). The lyssavirus trimeric glycoprotein is the primary surface antigen, the major target for neutralizing antibodies (Baer *et al*., 1988; Benmansour *et al*., 1991; Leslie *et al*., 2006), and is involved in cell binding and entry (Figure 1).

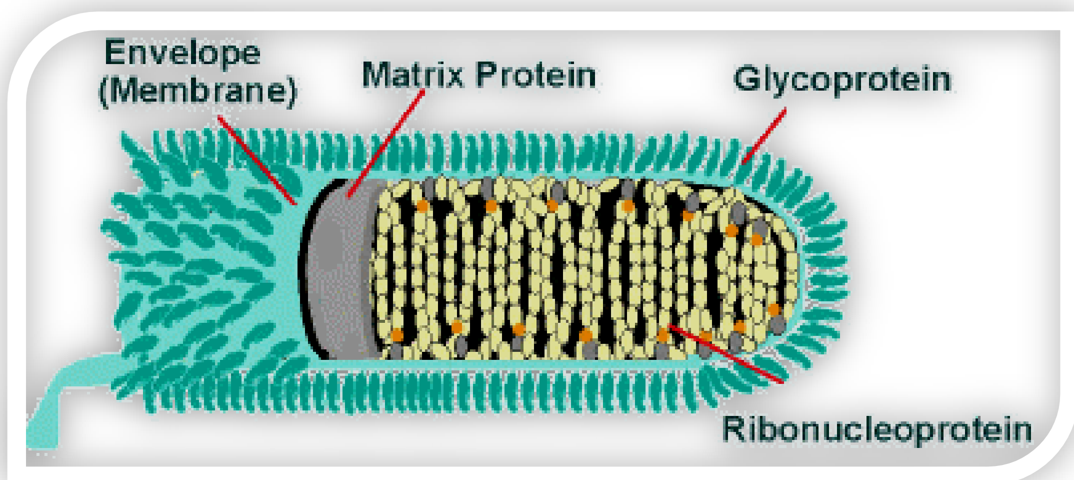


Figure 1. Rabies virus structure: Glycoprotein (the most antigenic determinant), Ribonucleoprotein, Matrix protein and Envelope (membrane) (Source: dokuwiki.noctrl.edu)

Antigenic sites on the glycoprotein have been described using monoclonal antibody escape mutants (Benmansour *et al*., 1991, Dietzschold *et al*., 1988; Prehaud *et al*., 1988). The phosphoprotein within the riboneucleioprotein core of the virus is critical in preventing an infected cell from creating an interferon response to the virus (Rupppretcht *et al*., 2001). The large protein is involved in three activities regarding the binding and utilization of ATP, which are the transcriptional activity that require binding to substrate ribonucleic triphosphates, polyadenylation, and protein kinase activity for specific phosphorylation of the phosphoprotein transcriptional activation. Thus, using these proteins, the RABV exhibits a number of virulence factors that typically result in the death of an infected host.

2.3 Disease burden and host ranges

Almost all human rabies is caused by the bite of a rabid animal. The risk of rabies is highest in countries with hyper-endemic canine rabies, including most of Asia, Africa, and Latin America (Lembo *et al*., 2006). In the United States and Europe, domestic animal rabies was largely controlled during the 1940–50s and now represents less than 10% of all animal rabies recorded. Wildlife rabies in the United States occurs primarily among wild terrestrial carnivores, such as raccoons, skunks, foxes, and coyotes, and in insectivorous bats (Rupprecht *et. al*., 2001).

Rabies is one of the most severe infectious diseases in Ethiopia, with many cases of the disease diagnosed in various parts of the country (Fekadu, 1982). Most of the cases are due to bite by stray dogs. The dog is the species most responsible for human exposure, with over 94.01% of the total positive animals (Tefera *et al*., 2002). As a result, vaccination of domestic animals (mostly dogs) and wildlife (mostly foxes) is the only way of reducing or elimination of rabies (Bethelihem *et al*., 2004). In Ethiopia, brain samples collected from domestic animals, two rabies-related viruses were isolated (Teshome *et al*., 1992). According to their reactivity pattern with anti-nucleocapsid monoclonal antibodies, they were characterized as Lagos bat virus and Mokola virus. But, little is known about the biological characteristics of these isolates and about the ability of current rabies vaccines to elicit immune responses which would provide cross-protection.

2.4 Diagnostic techniques

Several techniques used to detect positive rabies cases. 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. The only way to undertake 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.

Several laboratory techniques may be used, and the methods vary in their efficiency, specificity and reliability. They are classically applied to brain tissue, but they can also be applied with variable sensitivity and specificity to other organs like salivary glands. In the brain, rabies virus antigen is particularly abundant in the thalamus, cerebellum and medulla. It is recommended that a pool of brain tissues that includes the brain stem should be collected and tested (Bingham and Van der Merwe, 2002). The most widely used test for rabies diagnosis is the fluorescent antibody test (FAT), which is recommended by both WHO and OIE, and is sensitive, specific and cheap. Brain is removed following the opening of the skull in a necropsy room, and the appropriate samples are collected preferably Ammon’s horn, thalamus, cerebral cortex and medulla oblongata.

2.4.1 Fluorescent antibody test (FAT)

The most widely used test for rabies diagnosis, FAT is recommended by both WHO and OIE due to its sensitivity, specificity and low cost to perform the test. This may be used directly on a smear, and can also be used to confirm the presence of rabies antigen in cell culture or in brain tissue of mice that have been inoculated for diagnosis. The FAT gives reliable results on fresh specimens within a few hours in more than 95–99% of cases. The FAT is sensitive, specific and cheap. The sensitivity of the FAT depends on the specimen, the degree of autolysis and how comprehensively the brain is sampled.

For direct rabies diagnosis, smears prepared from a composite sample of brain tissue, that includes the brain stem, are fixed in 100% high-grade cold acetone for at least 20 minutes, air dried and then stained with a drop of specific conjugate for 30 minutes at 37°C. Anti-rabies fluorescent conjugates available commercially are either polyclonal or monoclonal antibodies (MAbs), specific to the entire virus or to the rabies nucleocapsid protein, conjugated to a fluorophore such as fluorescein isothiocyanate (FITC). FAT slides then examined for specific fluorescence using a fluorescent microscope and filter appropriate for the wavelength of the fluorescent conjugate used, for instance FITC is excited at 490 nm and re-emits at 510 nm. Aggregates of nucleocapsid protein are identified by specific fluorescence of bound conjugate. Fluorescent antibody conjugates made locally should be fully validated for specificity and sensitivity before use, including its ability to detect lyssaviruses other than rabies.

The FAT can be applied to glycerol-preserved specimens after a washing step. If the specimen has been preserved in a formalin solution, the FAT may be used only after the specimen has been treated with a proteolytic enzyme (Warner *et al.*, 1997). However, the FAT on formalin-fixed and digested samples is always less reliable and more cumbersome than when performed on fresh tissue (Barrat, 1992). In cases of inconclusive results from FAT, in all cases of human exposure, further tests on the same sample or repeat FAT on other samples are recommended. This is particularly important where sample autolysis is confirmed or suspected.

2.4.2 Immunochemical tests

Immunoperoxidase methods can be used as an alternative to FAT with the same sensitivity (Lembo *et al*., 2006). It must emphasised that this technique needs one incubation step more than the FAT. Peroxidase conjugate can be used on fresh brain tissue or sections of formalin-fixed tissue for immunohistochemical tests.

2.4.3 Enzyme-linked immunosorbent assay (ELISA)

An ELISA that detects rabies antigen is a variation of the immunochemical test. It is useful for large epidemiological surveys (Xu *et al*., 2007). The specificity and sensitivity of such tests for locally predominant virus variants should be checked before use. In case of human contact, these tests should be used in combination with confirmatory tests such as FAT or virus isolation.

2.4.4 Rapid immunodiagnostic test (RIDT)

A rapid immunodiagnostic test (RIDT) was developed recently to overcome the challenge of sample transportation during field condition and availability of detection methods (Kang *et al*., 2007). This test can be used under field conditions and in developing countries with limited diagnostic resources. This test detects the infectivity of a tissue suspension in cell cultures or in laboratory animals. They should be used if the FAT gives an uncertain result or when the FAT is negative in the case of known human exposure. Wherever possible, virus isolation on cell culture should be considered in preference to the mouse inoculation test (MIT). Cell culture tests are as sensitive as MIT (Rudd and Trimarchi, 1989) but are less expensive, give more rapid results and avoid the use of animals.

2.4.5 Cell culture inoculation

Neuroblastoma cells (BHK-21, Vero, CCL-13) in the American Type Culture Collection (ATCC) are highly susceptible to infection with lyssaviruses. The cells are grown in Dulbecco's modified Eagle's medium (DMEM) with 5% fetal calf serum (FCS), incubated at 37°C with 5% CO_2. Baby hamster kidney (BHK-21) cells are also sensitive to most street isolates without any adaptation step, but should be checked for susceptibility to locally predominant virus variants before use. Cell culture tests can be undertaken in multi-well plastic plates, multi-chambered glass slides or on glass cover-slips. The use of 4-day passage in four wells of a 96-well micro-titration plate has been shown to have comparable sensitivity to MIT for rabies strains (Rudd and Trimarchi, 1989). However additional passages could be considered to increase sensitivity of virus detection.

2.4.6 Mouse inoculation test

A mice, 3-4 weeks old (8-12g), or a litter of 2-day-old newborn suckling mice, are inoculated intracerebrally. The inoculum can be from clarified supernatant of a 10–20% (w/v) homogenate of brain material including brainstem (cortex, Ammon's horn, thalamus, and medulla oblongata) in an isotonic buffered solution containing antibiotics. The mice are observed daily for 28 days, and every dead mouse is examined for rabies using the FAT. For faster results in newborn mice, it is possible to check one baby mouse by FAT on days 5, 7, 9 and 11 post inoculations. Any deaths occurring during the first 4 days are recorded as nonspecific due to stress or bacterial infections.

2.4.7 Molecular techniques

Various molecular diagnostic tests, like detection of viral RNA by reverse transcription PCR (RT-PCR), PCR-ELISA, hybridisation in situ and real-time PCR are used as rapid and sensitive additional techniques for rabies diagnosis (Fooks *et al*., 2009). The principle of lyssavirus-specific PCRs is a reverse transcription of the target RNA (usually parts of the N gene) into complementary DNA followed by the amplification of the cDNA by PCR. Although those molecular tests have the highest level of sensitivity, their use is currently not recommended for routine post-mortem diagnosis of rabies (WHO, 2005) due to high levels of false positive or false negative results without standardisation and very stringent quality control. Nevertheless, they are useful for confirmatory diagnosis, as a first step in virus typing.

2.4.8 Histological identification of characteristic cell lesions

Negri bodies correspond to the aggregation of viral proteins, but the classical staining techniques detect only an affinity of these structures for acidophilic stains. Techniques that stain sections of paraffin embedded brain tissues are time consuming, less sensitive and more expensive than FAT. Seller's method on unfixed tissue smears has a very low sensitivity is only suitable for perfectly fresh specimens. These methods are no longer recommended for routine diagnosis. Immuno-histochemical tests are the only histological methods specific to rabies.

The histological identification describes methods to accurately diagnose rabies and to isolate and identify the virus. Typing of the virus can provide useful epidemiological information. These techniques would include the use of MAbs, nucleic acid probes, or the PCR, followed by DNA sequencing of genomic areas for typing the virus (Bourhy *et al*., 1993). These characterisations enable, for instance, a distinction to be made between vaccinal strain virus and a field strain of virus, and possibly identify the geographical origin of the latter.

2.4.9 Serological tests

The main application of serology for classical rabies is to determine responses to vaccination, either in domestic animals prior to international travel, or in wildlife populations following oral immunization. As neutralizing antibodies are considered a key component of the adaptive immune response against rabies virus (Hooper *et al*., 1998), the gold standard tests are virus neutralization (VN) tests. However, indirect ELISAs have been developed that do not require high-containment facilities and produce rapid results. Care should be taken when correlating results between virus neutralization tests and ELISAs owing to the inherent differences between them. Multiple publications demonstrate a variable sensitivity and specificity for ELISAs in both humans and animals.

2.5 Prevention and control

Illness due to rabies can be prevented by administering anti-rabies antibodies and a series of vaccinations, provided exposure is recognized before the symptoms appear. The wound should be immediately and thoroughly washed with soap and water, and then treated with 40-70% ethyl alcohol or an antiseptic such as benzyl ammonium chloride. The risk of exposure to rabies and whether prophylactic treatment should be given are determined after consultation (Smith *et al*., 1978). If the animal is available, the brain should be examined for rabies virus antigen by fluorescent antibody test. In some cases, if the bite was from a domesticated cat or dog, the animal may be kept under close observation for ten days.

There are two types of anti-rabies vaccines; the nerve tissue-based vaccine and the non-nerve tissue vaccine. The currently available non-nerve tissue vaccines are Purified Chick Embryo Cell (PCEC) vaccine, Human Diploid Cell Vaccine (HDCV), Purified Vero Cell Vaccine (PVRV) and Purified Duck Embryo Vaccine (PDEV). Similarly, there are two types of rabies virus strains; the fixed type of rabies virus which is used for vaccine production and the wild type virus which is street rabies virus strain.

The vaccine is administered as a series of injections over a 4-week period. HRIG (human rabies immunoglobulin) should also be given for the third type exposure. HRIG is prepared from the plasma of hyperimmune donors. Up to half of the recommended dose is infiltrated into the wound area if required. The remainder is given as an intramuscular injection. A separate syringe and a separate site are used for the HRIG and the vaccine so that the HRIG does not neutralize the vaccine.

People at risk for rabies infection may be vaccinated as a preventive measure. Such individuals include: rabies-laboratory workers, certain people in areas with enzootic rabies who are at risk for exposure to rabid animals, veterinarians and their staff, wildlife control workers, spelunkers (mainly those cave explorers who go into undeveloped caves with bat colonies); travelers who will be spending more than a month in areas with enzootic rabies. People at high risk for exposure to rabid animals should have regular serologic testing and booster vaccinations when necessary. If a vaccinated person is exposed to rabies, they still need to get post-exposure prophylaxis, but the number of post-exposure vaccination shots is reduced and HRIG is not used.

2.6 The effect of phylogenetic difference on vaccine efficacy

Closely related lyssaviruses circulate among bats, and can cause an illness identical to rabies in people and domesticated animals (Smith *et al*., 1978), which can not protected by vaccine produced from fixed strain of rabies virus. The rabies vaccine currently produced in Ethiopia (Fermi-type) was no more recommended by WHO because of its serious side effect and questioned immunogenicity (Leslie *et al*., 2006; WHO, 2007). Eventhough the Fermi type vaccine has side effects; it’s still distributed throughout the country to save lives of thousands of peoples and animals. Currently Ethiopia produced cell culture-based anti-rabies vaccine which can be used for human and animal immunization from two fixed virus strain; Pasteur Virus (PV) and Evenyl Roktincki Abelseth (ERA) (Birhanu *et al.*, 2013) and working to avail for the market.

The inactivated commercial rabies virus vaccines for human and animal use, such as the Pitman Moore (PM), PV, ERA and Flury Lep low egg passage (LEP), all belong to genotype 1 (rabies virus). These vaccines induce immunity against viruses of the phylogroup I but fail to protect against viruses of the genotype 2 (Elenice *et al*., 2010). Therefore, phylogenic relationship between local isolate and strain used for vaccine production should be studied before release of a candidate vaccine.

Street rabies virus can be isolated either by mouse inoculation methods or cell culture techniques. Intracerebral inoculation of Swiss albino mice for isolation of rabies virus was developed for the first time by Webster and Dawson (Webster and Dawson, 1935). The test, in spite of its simplicity, depends on the accuracy of its performance for dependable results. In mid 1970's another technique for isolation of street rabies virus was developed by Larghi and colleagues (Larghi *et al*., 1975; Smith *et al*., 1978; Smith *et al*., 1983). The technique was performed on cell lines (Baby Hamster Kidney and Chicken Embryo).

Several studies conducted and provided useful information on the value and potential use of the *in vitro* assays for measurement of the antigen content in vaccines. The potency test for rabies vaccine was originally developed at the National Institute of Health (NIH), Bethesda, USA. This test measures the degree of protection conferred by inactivated vaccines in immunized mice challenged with rabies virus (Branche, 1996). There appears to be three important considerations in assessing a potency test before the release of the vaccine (Sacramento *et al*., 1992). From these, the third requirement states that standardization of different vaccines will be comparable in relation to challenge virus strain used from circulating street rabies virus from the area of vaccine to be applied. The protection of candidate vaccine against local virus isolate using as a challenge virus should be evaluated.

Standard rabies vaccine consists of inactivated viral particles which induce humoral response, has the potency of 2.5IU/ml (for human use) and 1IU/ml (for animal use) which can be measured by monoclonal antibody assays (Wilbur and Aubert, 1996). The older attenuated vaccine such as Fermi-type vaccine produce both humoral and cell mediated immunity which could be reverted to virulent forms and produce undesirable side effects.

A vaccine strain of a particular country used for vaccine production should be studied with rabies virus isolates from that particular country before release of the vaccine (WHO, 2007). All the available anti-rabies vaccines produced from rabies virus strains are effective for rabies infection but, not for rabies related viral infections (Horton *et al*., 2010; Fekadu *et al*., 1988). Rabies virus strains Pitman-Moore and Pasteur virus strains show no protection for Mokola and Lagos Bat viruses isolated from Ethiopia (Mebatsion *et al*., 1992). Rabies vaccines are all based on rabies virus, and seem to provide little or no protection from rabies-related lyssaviruses in phylogroup II or those provisionally classified in phylogroup III (CFSPH, 2012). Limited vaccination and challenge studies with local isolate suggest that they may provide cross-protection against rabies-related Lyssaviruses in Phylogroup I. But still within Phylogroup, amount of protection vary with specific virus and should be supported via challenge test with local virus isolate.

3. Materials and Methods

3.1 Study design

This study involves laboratory based experimental study with quantitative and descriptive methods.

3.2 Study setting

This study was carried out at the Ethiopian Public Health Institute (EPHI), Vaccine and Diagnostics Production Directorate (VDPD), cell culture anti-rabies vaccine production laboratory.

3.3 Biologicals

3.3.1 Laboratory animals

Swiss albino mice were used for clinical isolation, blind passage, in vivo virus titer determination and mice protection study. Mice, 3-4 weeks old, weighing 6-8 grams with identical sex were used. All mice were obtained from EPHI, Laboratory Animal Breading Center.

3.3.2 Cell lines

A Baby Hamster Kidney (BHK-21) cell line purchased and supplied from American Type Culture Collection (ATCC) was used for the adaptation of the virus isolates, in vitro cross neutralization test and titer determinations.

3.3.3 Virus used

Samples were collected from rabid animal brain and human saliva for this study. A total of five samples were used; which were originally collected from rabid cow brain, from human saliva (human clinical case) and from rabid dog brain. Four local virus isolates; two from human saliva (human origin Wollega (HOW) and human origin Sululta (HOS)), one from rabid dog brain (dog origin (DO) from Gojjam) and one from rabid cow brain (cow origin (CO) from Butajira) were used. Pasteur Virus (PV-12) currently used for Fermi type vaccine production was used to compare neutralization of anti-rabies sera raised with ERA fixed virus strain based cell culture anti-rabies vaccine. Challenge virus standard (CVS-11) was used as standard for comparison of protection in comparison to local virus isolates.

Table 1. Type of virus used and their origin

No.	Type of Virus	Origin	Location	Remarks
1.	HOW	Human Saliva	Wollega	Mice inoculation positive
2.	HOS	Human saliva	Sululta	Mice inoculation positive
3.	DO	Dog brain	Gojam	FAT positive
4.	CO	Cow brain	Butajira	FAT positive
5.	PV-12	Cow brain	EPHI	Pre adapted to brain
6.	ERA	Dog brain	CDC	Pre adapted to cell lines
7.	CVS-11	Cow brain	CDC	Pre adapted to cell lines and mice brain

The first four viruses are isolated from locally circulating and the other three are fixed rabies virus strains originally isolated from rabid cow in France, Paris (PV-12 and CVS-11) and adapted to mice brain and cell lines several times (Table 1). ERA fixed rabies vaccinal strain was originally isolated from USA, Florida and adapted to in vivo and in vitro titer growth on cell lines and laboratory animals.

3.4 Methods

3.4.1 Fluorescent antibody test

Fluorescent antibody test (FAT) is based on applying a suspension of commercially available anti-rabies monoclonal antibody on tissue impression smears. Antigens reacting with antibody labeled with fluorescent appear under UV light as fluorescent apple green color. After collection of the samples, FAT test was done to confirm the presence of viruses. Brain sample was applied on slide and fixed with acetone at -20°C for 5 minute. After the acetone dried out, samples were covered with anti-rabies antibody and incubated at 37^{o}C for 30min. Slides were rinsed with PBS twice and examined under 40x objective fluorescent microscope. This test was used throughout the experiment to confirm collected samples and to identify specific and non-specific death of mice inoculated with the rabies virus during the experiment.

3.4.2 Isolation of the samples

All positive brain samples were homogenized and prepared as 10% weight by volume (w/v) brain suspension. Homogenization was done by suspending the sample in 10%w/v phosphate buffered saline (PBS), stored at -80^{o}C for 1-2hrs and by shaking vigorously. Brain suspensions were centrifuged at 3,000 x g under refrigeration for 10min. The supernatant fluid containing rabies virus was used as original inoculum (without dilution), for mice inoculation and adaptation on cell lines after purification by filtration (purification with filter pore size 0.22µm).

3.4.3 Virus adaptation

To adapt the local virus isolates to cell lines, it is required to adapt first to mice brain to increase infectivity titer. The virus was adapted to mice brain by passing five times on mice brain without finding virus titer. Mice handled during experiment according to the guide for the care and use of laboratory animals. After five passages, the last brain samples were homogenized in 10%w/v PBS, and purified by centrifugation (at 3,000 x g) and filtration with 0.22µm pore size sterile filter. BHK-21 cell lines were cultured in $25cm^2$ tissue culture flask using Eagle Minimum Essential Medium (EMEM) supplemented with 10% fetal bovine serum. After 72 hours, when monolayer (cell confluence) was reached, cells were trypsinized. The cell concentration was determined and inoculated with brain supernatant at 0.1virus/cells. Infected cells were incubated at 37^oC for 30 minutes with mixing every 5 minutes. After 30 minutes, cells were centrifuged at 2500rpm for 10 minutes at 4^oC, supernatant removed and cell pellet re-suspended in 5ml complete medium (contain 10% fetal bovine serum, 3.5% $NaHCO_3$ and 1% L-glutamine). The cell suspensions were transferred to 25cm tissue culture flask and incubated for 72 hours at 37°C in a humid incubator with 5% CO_2. Viruses were passaged twenty times on cell lines with titration every five passage. At each passage, tissue culture supernatant (TCSN) were harvested, aliquoted, and stored at −80°C.

3.4.4 Virus titration

Titration of the virus was used to determine growth and multiplicity infection of the rabies virus during passage on mice brain and cell lines. Rabies virus was titrated on cell lines at each five blind passage to control the level of virus adaptation on cell lines. A serial tenfold dilution of the virus was prepared in incomplete media (media without fetal bovine serum) and distributed on 96 well micro-titration plates. Concentration of BHK-21 cell lines adjusted to $6x10^5$ cells/ml and 30µl/well was added to the plate. After 48 hours of incubation period at 37°C with 5% CO_2, plates were stained with anti-rabies conjugate and reading was done under fluorescent microscope. Based on the positive/negative readings, titer of the virus was calculated using Spearman-Kärber formula. This was expressed as tissue culture infectivity dose per milliliter

($TCID_{50}$/ml) of virus suspension. In addition, titration of the virus was done on mice brain after twenty passages on cell lines. Tenfold serial dilution of original cell lines adapted viral suspension was prepared up to 10^{-8} using PBS as diluents. A group of eight mice were inoculated with 0.03ml of each virus dilution intracerebrally. Mice were observed for twenty days and death and survival rate recorded separately. Based on the number of specific deaths, mice intracerebral lethal doses ($MICLD_{50}$/0.03) were calculated using Spearman-Kärber formula.

$$\log_{10} \text{(end-point dilution)} = \left(x0 - \frac{d}{2} + d\sum \frac{ri}{ni}\right)$$

Where:

$x0$ = ($\log_{10}$ of the lowest dilution with all wells positive)

d = $\log_{10}$ of the dilution step, one in this case

ni = number of replicates

ri = number of positive wells.

3.4.5 Anti-sera

Blood collected and sera were drawn from two dogs previously immunized with ERA fixed rabies virus based cell culture anti-rabies vaccine produced locally (potency value greater than 2.5IU/ml). The serum was heat inactivated at 56°C for 30 minutes and stored at -20°C until use. For positive control serum, canine based standard serum supplied by OIE was prepared in PBS solution and stored at -20°C. Sterile fetal bovine serum used for cell culture media was used as negative control serum.

3.4.6 Cross neutralization

The neutralizing abilities of the sera raised to ERA fixed rabies virus strain based vaccine were assessed using fluorescent antibody virus neutralization (FAVN) tests on BHK-21 cell lines. Volumes of viruses' tissue culture infectivity dose ($50TCID_{50}/50\mu l$) were added to serial three fold dilutions of test and reference serum on 96 well micro-titration plates. The plate was incubated for 48 hours at $37^{o}C$ with 5% CO_2 in the presence of BHK-21 cell lines. After 48 hours, the plate was stained with anti-rabies antibody labeled containing fluorescent conjugate. The reading was done as positive/negative fluorescent apple green under fluorescent microscope. The 50% endpoint dilution, where neutralization of the virus ceased, was calculated using Spearman-Kärber methods as shown below.

$$\text{Serum titer (IU/ml)} = \frac{[(10\ (\text{serum log } D_{50} \text{ value})) \times \text{theoretical titer of OIE serum 0.5 IU/ml}]}{(10\ (\log D_{50} \text{ of OIE serum 0.5 IU/ml}))}$$

3.4.7 Mouse protection study

Mice protection test is an in vivo method used to measure protection indices of mice immunized with a candidate vaccine when challenged with challenge virus. For the immunization of mice, locally produced anti-rabies vaccine (ERA strain based), propagated in Vero cell lines and chemically inactivated with formalin was used. The vaccine potency was determined previously using NIH potency test protocol and greater than 2.5IU/ml potency value obtained. In mice protection test, five groups of mice containing 16 in each group were immunized with 0.5ml (1:5 dilutions) of ERA based vaccine twice on day zero and day seven. The mouse intracerebral lethal dose ($MICLD_{50}/0.03ml$) was determined by injecting 0.03ml of all virus isolates and challenge virus standard (CVS-11) on four week-old mice by the IC route before challenge day.

On day 14 after first immunization, all group of mice and control groups were challenged with working dilution of local rabies virus isolates and CVS-11 intracerebrally. The mice were observed for 20 days after challenge inoculation. All specific and non-specific death was recorded separately according to the protocol. Mice dead after five days without showing any sign of rabies was confirmed by FAT test on brain sample. The degree of protection (protection index) of candidate vaccine in mice challenged by local virus isolates was determined based on the survival rate of the challenged mice when compared to challenge virus standard.

3.4.8 Determination of pathogenicity

After twenty passages of local rabies virus isolates on cell lines, the mice intracerebral lethal dose viral titer was determined on mice brain. The titers of the viral isolates were determined by intracerebral (IC) inoculations of tenfold virus dilutions into 4-week-old mice, and 50% mouse IC lethal dose was calculated using the Spearman-Karber formula before the day of pathogenicity test. In pathogenicity test, one group of mice (n = 16), were injected through intramuscular (IM) route on the thigh with 0.1mL (250MICLD$_{50}$/0.1mL) of each virus isolates. Control mice were injected with PBS. All test and control groups of mice were observed for 45 days. Brain of mice died during the incubation period was removed and FAT was performed to confirm the presence of rabies virus.

3.5 Data analysis

Data analysis was performed using SPSS version 20, and expressed graphically and in table form. 95% confidence intervals with 5% error were used during analysis to express statistical significance difference. One-way analysis of variance was used to test the difference between virus titer at each blind passage and between virus isolates at specific point. Fisher's exact test was used to test significance difference between in vivo and in vitro cross protection due to virus differences.

4. Results

4.1 Results of virus adaptation

The virus was passaged on mice brain five times and twenty times on cell lines to adapt and increase multiplicity of infection. In each blind passage, the virus shows different multiplicity of infection by in vitro titration methods at each blind passage. A one-way analysis of variance was conducted to test if there were difference in virus titer among blind passages (passage 5, 10, 15 and 20). According to these results, the rabies viruses showed increase infectivity titer throughout the passage. Adaptation of the virus expressed as a titer and the result of titration both in vivo and in vitro methods was performed (Figure 2). Data analysis was performed using one-way analysis of variance. The independent nominal variable was blind passage and the dependent interval variable was virus titer. There was significant difference in growth of virus titer between each blind passage for each viruses, $F(1, 4467) = 2528.9$, $p = 0.000$. But, there was no significant difference in infectivity titer between viruses at each blind passage, $F(1, 4467) = 0.697$, $p = 0.451$.

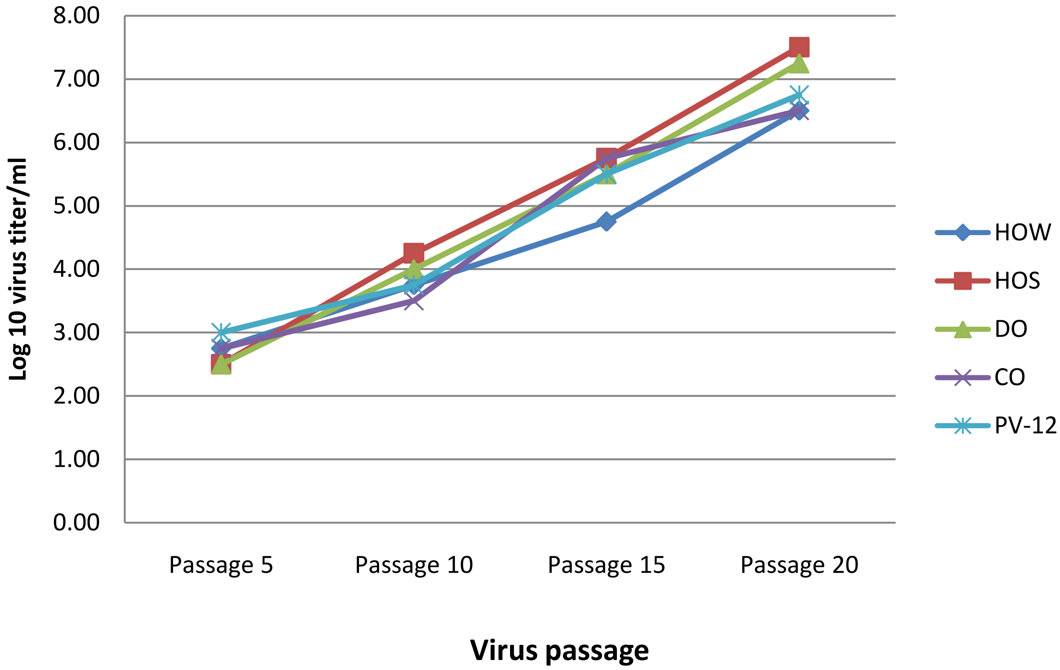


Figure 2. *In vitro* virus titer result at each five blind passages up to twenty passages on BHK-21 cell lines which can be used for further cross neutralization and pathogenicity test.

According to this result, the titer of the virus increased throughout the blind passages reaching maximum and minimum of virus titer $10^{7.5}$/ml (HOS) and $10^{6.5}$/ml (HOW and CO) in terms of tissue culture infectivity dose per milliliter. Similar study done by Guo et al (2014) support the idea of increase in virus titer through passage but after pick point ($10^{7.9}$ FFU/ml), the titer start to drop (Guo *et al*., 2014). These results show that at these titers, it can be used for studies of cross neutralization with locally produced anti-rabies vaccine and other tests. In vivo method of titer determination was done on Swiss albino mice brain after twenty passages on cell lines to determine mice intracerebral lethal dose (LD_{50}) (Table 2).

Table 2. Virus titration result (*in vivo*)

No.	Virus origin	$MICLD_{50}$/0.03ml (P_{20})
1.	HOW	$10^{4.5}$
2.	HOS	$10^{5.9}$
3.	DO	$10^{5.5}$
4.	CO	$10^{4.5}$
5.	PV-12	$10^{5.25}$

These were used for calculation of working virus dilution used during mice protection study. The result shows that, maximum and minimum of $10^{5.9}$/0.03ml (HOS) and $10^{4.5}$/0.03ml (HOW and CO) mice intracerebral lethal dose ($MICLD_{50}$) were obtained (Table 2). This result was high enough to use the viruses during the challenge test; protection of vaccinated mice when challenged with local virus isolates and challenge virus standard to compare protection of immunized mice. These results were used to determine working dilution of the specific virus according to its titer for challenge test.

4.2 Mice protection test

Mice protection study was used to evaluate protection of mice immunized with ERA fixed virus strain based vaccine when challenged with local isolates by in vivo method. In this test, mice showed different incubation period when compared to fixed challenge virus strain. A minimum of four days difference was observed with five days additional incubation period to complete the test in addition to fourteen days follow up. The result shown in figure 3 summarizes death/survival rate of mice after challenged with local rabies virus isolates and challenge virus standards.

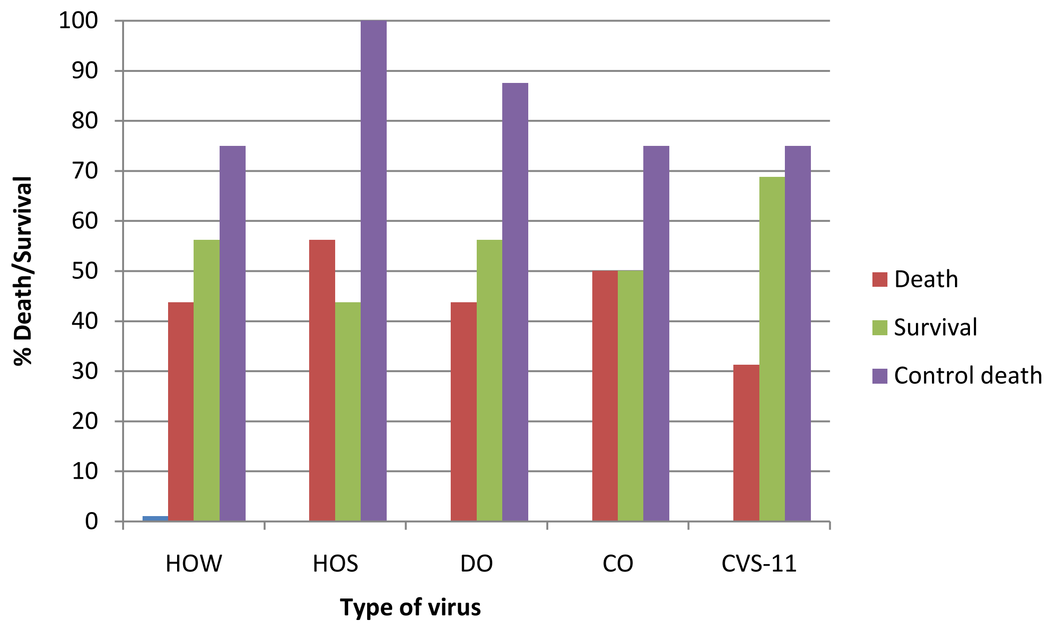


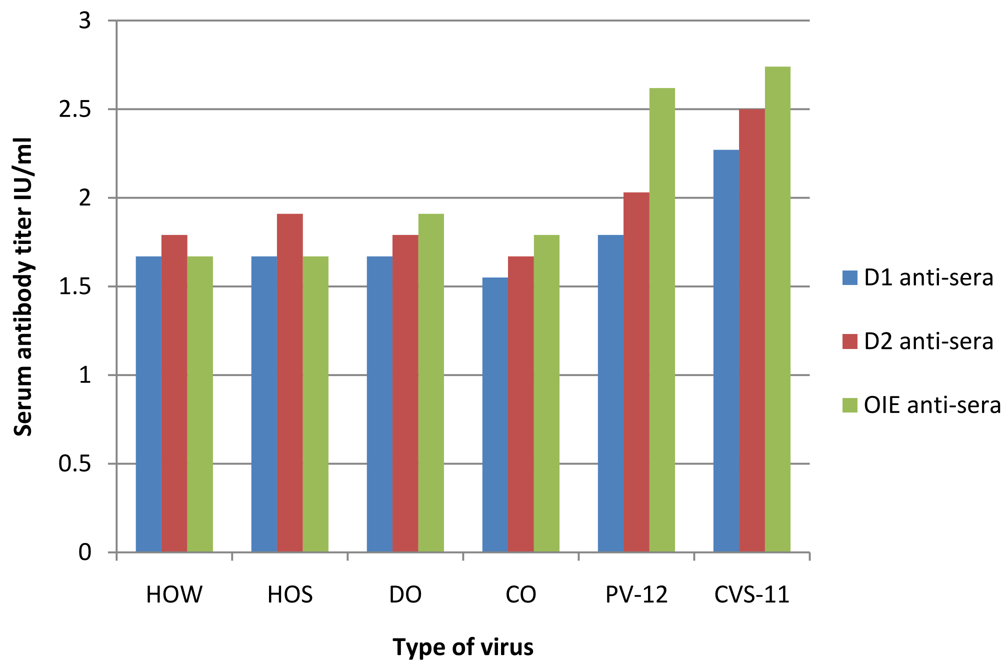
Figure 3. *In vivo* mice protection study results showing survival and death rate of immunized mice when challenged with challenge virus standard and local virus isolates.

According to the results, locally produced ERA based anti-rabies vaccine showed different level of protection against each local virus isolates and challenge virus standard. Eventhough the viral concentration of each virus was adjusted to $50MICLD_{50}/0.03ml$ before challenge, the number of mice protected by locally produced ERR based vaccine was different. The highest protection was observed for CVS-11 challenge virus standard with eleven mice survived out of sixteen. For all control groups, eight mice inoculated per virus and minimum of six death (75%) were recorded which was greater than 50%. The minimum protection index was observed with human origin Sululta (HOS) local virus isolate with nine deaths out of sixteen mice challenged. Data analysis performed using Fisher's exact test and linear by linear association showed significance difference (p = 0.046) for CVS-11 challenged mice and local virus isolates. But there was no significance difference in protection between local isolates. The other local virus isolates (HOW, DO and CO) showed low protection when compared to challenge virus standard.

During determination of infectivity titer and cross neutralization test on mice brain, unexpected incubation period and sign of paralysis observed for inoculated mice. According to the protocol and work experience, IC inoculation requires only fourteen days to complete the test, but local virus isolates showed death after twelve days and death recorded up to nineteen days. The last death for CVS-11 inoculated mice was on 12th day.

4.3 Cross neutralization test

Cross neutralization test was performed to evaluate neutralizing level of local virus isolates with antibody raised to ERA fixed rabies virus strain. Anti-sera of two dogs (D1 and D2) previously immunized with ERA based anti-rabies vaccine produced locally were used to neutralize the viruses of local origin (HOW, HOS, DO and CO) and CVS-11 challenge virus standard. PV-12 vaccinal strain currently used for Fermi vaccine production was used to compare neutralizing capacity between fixed virus strain (PV-12 and CVS-11) and clinical rabies virus isolates. Based on the FAVN test results, different titers of dogs (D1 and D2) and OIE anti-rabies antibody were obtained for each local virus isolate and CVS-11 challenge virus standard (Figure 4).



D1= dog 1 sera; D2= dog 2 sera

Figure 4. *In vitro* cross neutralization test result of challenge virus standard and local virus isolates with antibody raised to locally produced vaccine.

According to the data analysis performed using Fisher's exact test, cross tabulation showed significance difference in antibody titer between challenge virus standard and local rabies virus isolates (p = 0.000). But, there was no statistical significance difference between sera at each virus isolate. The results indicate that neutralization of local virus isolates with dogs (D1 and D2) and OIE anti-sera was lower when compared to standard challenge virus strain and PV-12 fixed rabies virus strain. Antibody titer was highest for all sera when fixed virus strain (PV-12 and CVS-11) used for neutralization compared to local isolates. The difference in serum antibody titer when local virus isolate used were very low (0.36IU/ml). But, the difference in antibody titer between local isolates and fixed virus standard was very high (1.19IU/ml). Each dog sera also showed some difference in serum antibody titer for the same local virus isolate, challenge virus standard and PV-12 vaccinal strain, but the difference was very low (0.25IU/ml). Antibody titers for dogs as well as OIE were highest when PV-12 was used as challenge virus in comparison to local isolates. Based on the history of fixed virus strain development, PV-12 and CVS-11 originated from the same virus origin (France, Paris 1882). The only difference was the number of passages and host used for adaptation mechanisms (Baer, 1991). Several passages may bring genetic mutation but virulence of the virus other than cell lines and intracerebral inoculation almost declined.

4.4 Pathogenicity test

The pathogenicity of local virus isolate was evaluated after five passages on mice brain and twenty passages on BHK-21 cell lines. According to the results obtained, all virus isolates almost lost their virulence to intramuscular inoculation except two virus isolates (HOS and DO) that killed two mice related to rabies virus out of 16 inoculated mice (Figure 5). This shows that only 12.5% death occurred from inoculated mice which may indicate the decline of pathogenicity of the virus for IM route of inoculation.

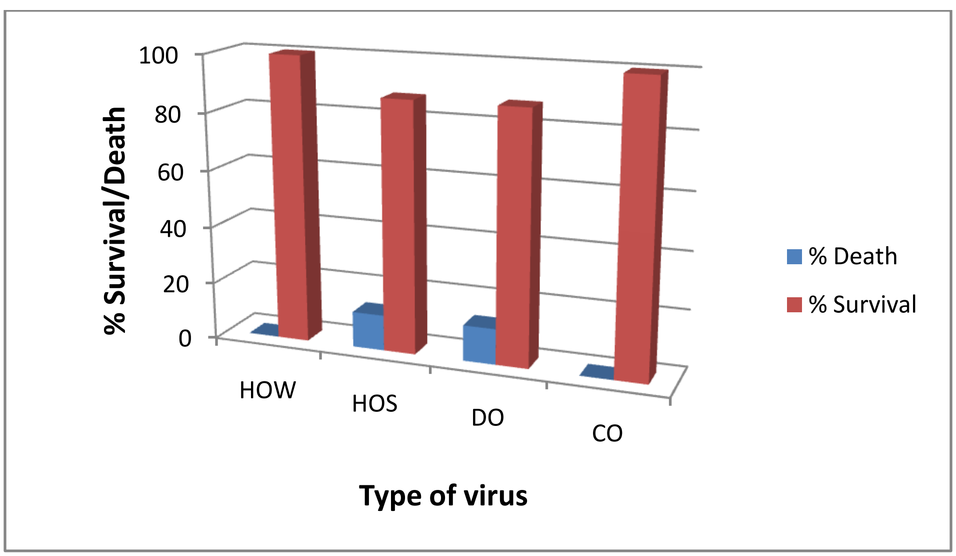


Figure 5. Pathogenicity of the virus isolates when inoculated intramuscularly in to mice after five passages on mice brain and twenty passages on BHK-21 cell lines.

5. Discussion

The present study was designed to evaluate the efficacy of cell culture based anti-rabies vaccine produced locally (ERA based) with local virus isolates in terms of cross protection and pathogenicity with intramuscular route of inoculation. The local isolates of rabies virus were adapted to mice brain (five passages) and cell lines (twenty passages) to increase infectivity titer of the viruses. All virus isolates grew well and showed increase in infectivity titer throughout the passages reaching a minimum titer of $10^{6.5}$ $TCID_{50}$/ml and $10^{4.5}$$MICLD_{50}$/ml in vitro and in vivo virus titer, respectively. These differences in virus titer reflect the adaptation nature of the virus to both mice brain and cell lines. According to WHO recommendation, the virus titer should be greater than or equal to 100$TCID_{50}$/100µl and 25$MICLD_{50}$/0.03ml to be used for a given study (WHO, 2005). Therefore, the titers of the viruses were high and can be used for further cross neutralization and pathogenicity test.

Cross neutralization of the viruses with antibody raised to locally produced cell culture vaccine was carried out in two ways, mice immunization and challenging (in vivo) and FAVN test (in vitro) on dog sera immunized with the locally produced ERA based cell culture vaccine. In vivo method showed different protection of immunized mice for each virus isolates and challenge virus standard (CVS-11). Eventhough the virus titer adjusted to 25$ICLD_{50}$/0.03ml for all virus isolates and challenge virus standard, CVS-11 challenged mice showed high protection; whereas local rabies virus isolates (HOS) showed the lowest protection. For HOS local virus isolate, only seven mice survived the challenge (43.75%) out of sixteen which showed low protection but, CVS-11 challenge virus standard show eleven survival (68.75%) with highest protection. The difference in protection of challenge virus standard was at least 12.50% greater than local rabies virus isolates. This support the result obtained by Wright and his co-worker which state that cross-neutralization tests using sera from RABV-vaccinated humans and animals on pseudotypes with CVS-11, EBLV-1 and EBLV-2 envelopes showed that the relative neutralization titers correlated broadly with the degree of G-protein diversity (Wright *et al*., 2008). According to the calculation of relative potency for a given vaccine, such difference in death/survival rate strongly affects potency result and should be carefully evaluated with local isolates to prevent the possible failure of the vaccine. These differences in protection index indicate the effect of challenge virus on potency of a given vaccine in terms of local isolates which correlate with studies done by Badrane et al. (2001) indicating that different phylogroups shows varying level of protection (Badrane *et al*., 2001). Studies done in USA also support this finding that neither pre-exposure vaccination nor conventional post-exposure prophylaxis using classical rabies based vaccine provided significant protection to rabies related viruses (Hanlon *et al*., 2005). But, still the locally produced ERA based anti-rabies vaccine was protective against the local rabies virus isolates although the protection was lower when compared to CVS-11 standard challenge virus. This low protection index causes low potency value for the locally produced ERA based anti-rabies vaccine which can result in failure of protection.

During in vitro cross neutralization test, the two dog sera and OIE sera showed different antibody titer for each virus. The highest antibody titer was recorded for OIE anti-sera (2.74IU/ml and 2.62IU/ml) when CVS-11 and PV-12 (same origin with ERA) was used as challenge virus, respectively. The lowest antibody titer was recorded for dog one (D1) (1.55IU/ml) when cow origin virus isolate was used as challenge virus. The levels of antibody titers for all test sera were above WHO recommendation (0.5IU/ml), but the vaccine used for dog immunization have potency value of greater than 2.5IU/ml which may improve efficacy of the vaccine resulting in high antibody production. For the three different anti-sera, the values of antibody titers were highest when CVS-11 challenge virus standard and PV-12 vaccinal strains were used compared to the local virus isolates. Studies done by Wright and his colleague support this idea that human serum samples (fixed virus strain based) neutralized CVS-11 to the greatest degree, compared to EBLV-2 and EBLV-1 and the same neutralizing profile was observed (Wright *et al*., 2008; Fekadu *et al*., 1988). Based on the history of fixed virus strain development, PV-12 and CVS-11 originated from the same virus origin (France, Paris 1882). The only difference was the number of passages and host used for adaptation mechanisms (Baer, 1991). Studies done by Guo and his coworkers also support the idea of growth in virus titer bring genetic mutation and increase efficacy of the vaccine related to the adapted virus (Guo *et al*., 2014). This may indicate genetic compatibility between PV-12 and ERA fixed vaccinal strain due to their origin and several passages on different route of inoculation. Similar mechanism was stated by Sacramento and his co-workers that the wild isolate showed marked divergence from those of fixed vaccinal strains (Sacramento *et al.*, 1992). In vivo protection test also showed similar result with in vitro test which can predict the effect of challenge virus on the potency of locally produced cell culture anti-rabies vaccine. Since small numbers of mice survived local virus isolate challenge (25% difference) compared to CVS-11, this has significant effect on the potency of the vaccine. Therefore, genetic origin and several passages between fixed virus strains may be the reason for compatibility in neutralization of the virus with antibody raised to ERA vaccinal strain.

The difference in incubation period between fixed virus strain and local isolate may be due to difference in genetic structure and host adaptation. Different signs observed on mice between fixed virus strains; type of paralysis (only two legs paralyzed for four days) and fixed virus strain (hair erection and then full paralysis) which agreed with protocol and related research papers may indicate genetic difference between local virus isolate and fixed virus strain which can only be detected quantitatively by molecular techniques. Protection of immunized mice depends on type of challenge virus inoculated. According to the result, low protection for HOS challenged mice showed low potency of the ERA based anti-rabies vaccine which can lead to failure. High protection in CVS-11 challenged mice also may lead to false potency value resulting in loss of full protection with locally circulating virus. Therefore, potency should be set in terms of local virus isolates to ensure full protection.

Pathogenicity test of the local virus isolates was performed by inoculating a group of mice with 250MICLD$_{50}$/0.1ml of local virus isolates. Studies done in China also support that virulence of rabies virus declined with change in adaptation of the virus to check embryo cells (Guo *et al*., 2014). The rest of the virus isolates (HOW and CO) have no pathogenicity at these numbers of passages. The virulence of the lyssavirus isolates declined during several passages on different route of inoculation. The study done in South Africa confirmed that inoculation with 10% suspension of the original brain material resulted in an increase in percentage mortality and a decrease in mean incubation period compared to inoculation with a dose of 10^5TCID$_{50}$ (Kgaladi *et al*., 2013). This several passages affect the pathogenicity of the virus on IM route of inoculation resulting only pathogenic to cell lines and intracerebral route of inoculation. In studies done by Koraka and his colleague, pathogenicity of lyssaviruses known to be invasive when isolated from wild and inoculated on mice before adaptation by several passages to cell lines and mice brains (Koraka *et al*., 2012). Therefore, decline in pathogenicity of the virus to intramuscular route of inoculation may indicate that the viruses have adapted to grow on mice brain and cell lines. Therefore, this test was expected to determine pathogenicity through IM route of inoculation after several passages of the virus to assess decline in virulence. Only two local virus isolates (HOS and DO) of the four viruses isolates showed virulence (12.5%) where as HOW and CO were non-pathogenic to IM inoculation. These results indicate complete adaptation of the virus. It can also predict the phylogroup origin of the viruses to some extent indicating phylogroup I origin of the virus isolates with decline in virulence due to several passages. This decline in pathogenicity may be due to adaptation of the viruses on different hosts (mice brain and cell lines) during increasing their infectivity titer.

6. Conclusion and recommendation

Adaptation of street rabies virus isolates in cell culture stated previously (Smith *et al*., 1983). The study of pathogenicity and cross neutralization of local rabies virus isolates were done to evaluate protection by ERA based cell culture anti-rabies vaccine produced in Ethiopia. Adaptation of local virus isolates to mice brain and cell lines were done until the virus titer reaches high to be used for the tests. Continuous growth of the virus titer through the passages observed reaching more than the titer required for the test after twenty passages on cell lines. All virus isolates showed high multiplicity of infection on cell lines and mice brain which was used for cross neutralization and pathogenicity test. From the pathogenicity test of the viruses, it can be concluded that after several passages, pathogenicity show 12.5% death only for two virus isolates (HOS and DO) showing adaption of the viruses to cell lines and mice brain. Since some pathogenicity observed for HOS and DO, these viruses can be grouped under phylogroup I viruses which related to classical rabies virus. The other two virus isolates (HOW and CO) shows no death which may be due to the several passages of the virus resulting complete decline in pathogenicity, but activity of the viruses to antibody raised by locally produced vaccine were similar in all local virus isolates. This support the assumption of all local virus isolates originates from phylogroup I lyssaviruses. Eventhough the virus may originate from the same phylogroup

(phylogroup I), protection by classical rabies virus based vaccine can vary due to genetic difference with in the phylogroup.

Cross neutralization of local virus isolates with sera raised to ERA based anti-rabies vaccine showed different protection intensity. All virus isolates show the lowest antibody response (low cross protection) when compared to challenge virus standard. Since challenge virus standard originate from fixed virus strain, high antibody titer (2.74IU/ml) support the idea of genetic compatibility with fixed virus strain (ERA) but less protection with local rabies virus isolates. The lowest antibody titer detected can protect from the specific virus isolate as it was greater than WHO recommendation (0.5IU/ml) but potency value of vaccine during immunization was greater than 2.5IU/ml and may be responsible for the high antibody titer recorded.

This finding support WHO recommendation that anti-rabies vaccines must protect from all lyssavirus genotypes and variants in area vaccine applied should be tested for its efficacy before vaccine licensing (WHO, 2005). Generally, from the result it can be concluded that the vaccine under trial can protect from the local virus, but potency of the vaccine should be set appropriately in relation to the local virus isolate to ensure efficacy of the vaccine. To determine the exact genetic difference between vaccinal strains and local virus isolates, further molecular techniques should be used which can differentiate phylogroup origin of the viruses. Finally, it is recommended that developing vaccine from locally circulating rabies virus isolate is the best option for mass vaccination to prevent and control the disease. Therefore, developing vaccine from local virus isolates for canine vaccination helps to control the disease and overcome the possible failure of the fixed virus strain based ant- rabies vaccine. In addition, genetic distance between local rabies virus isolate and fixed rabies virus vaccinal strain should also be studied using modern molecular techniques for further decision making.

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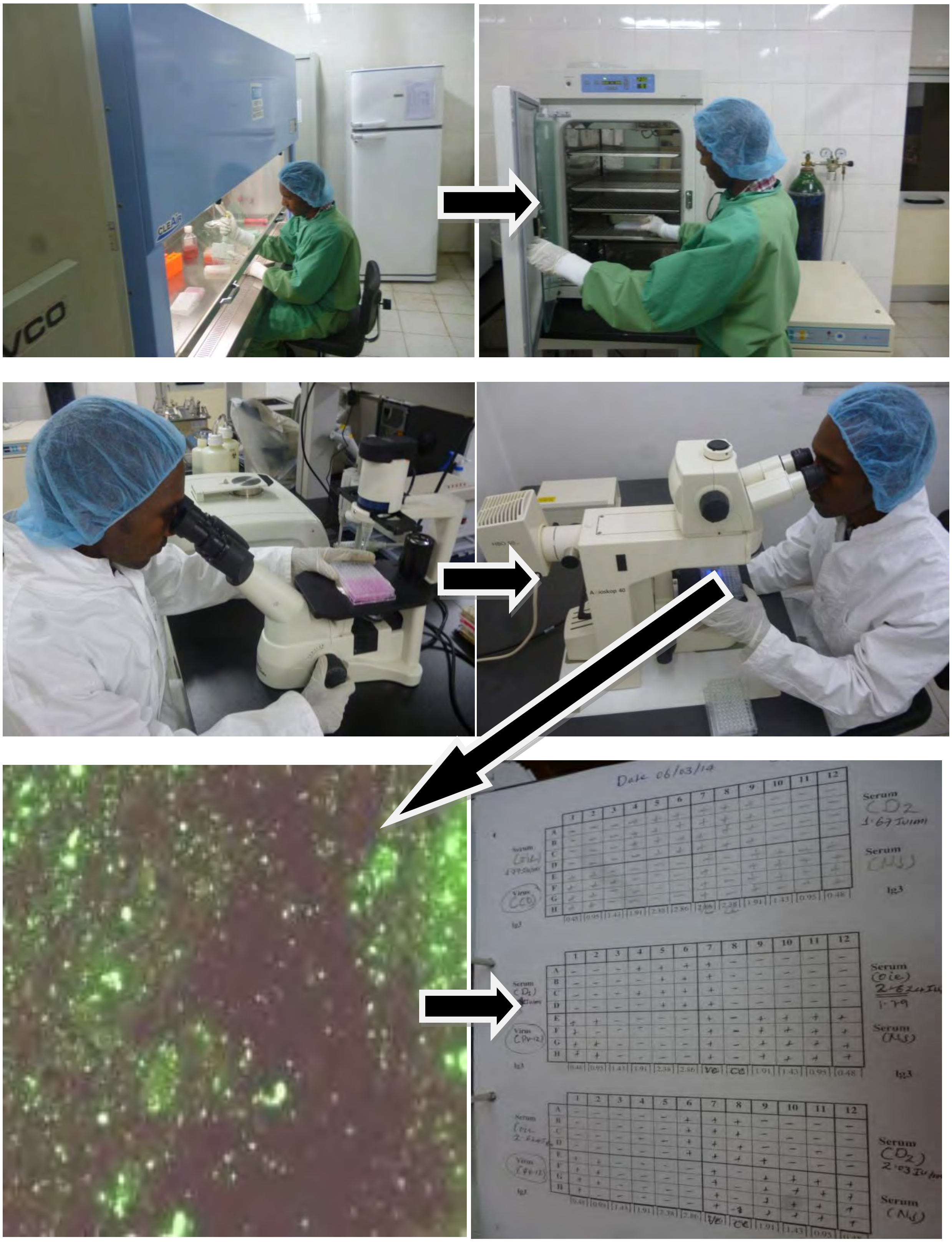
8. Appendix

Pictorial presentation of different laboratory activities undertaken during the study.

1. Virus adaptation

2. In vivo test

3. In vitro test



Declaration

I declare that the research paper titled "Pathogenicity and cross protection studies of local rabies virus isolates with ERA based cell culture anti-rabies vaccine produced in Ethiopia" submitted for partial fulfillment of the degrees of masters is my actual and original work. Any reference to work done by other person or institution have been duly cited and referenced. I further certify that this research paper has not been published or submitted for publication anywhere.

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