ADDIS ABABA UNIVERSITY

COLLEGE OF NATURAL AND COMPUTATIONAL SCIENCES

DEPARTMENT OF MICROBIAL, CELLULAR AND MOLECULAR BIOLOGY

Sequential Adaptation of Vero Cell Lines in Serum free Medium for fixed Rabies virus Propagation

By

Jemal Mohammed

March, 2020

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A Thesis Submitted to the School of Post Graduate Studies of Addis Ababa University, in Partial Fulfillment of the Requirements of Degree of Master of Science in Biology (Applied Microbiology)

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Declaration

I hereby declare that this MSc thesis entitled “Sequential Adaptation of Vero Cell lines in Serum free Medium for fixed Rabies Virus Propagation” is my original work and has not been presented for a degree in any other University and all source of materials used for the thesis have been duly acknowledged.

Jemal Mohammed Kabeto	_____	_____
Name of the Designate	Signature	Date

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List of Acronyms and Abbreviations

ATCC	American Type Culture Collection
BHK	Baby Hamster Kidney
BSE	Bovine Spongiform Encephalitis
CVS	Challenge Virus Standard
MEME	Minimal Essential Medium Eagle
DMSO	Dimethyl Sulfoxide
DRIT	Direct Rapid Immune Histochemistry Test
ECACC	European Collection of Animal Cell Culture
ERA	Evenly Rockitniki Abelseth
EDTA	Ethylene diamine Tetra acetic Acid
EMEM	Eagle's Minimal Essential Medium
FAT	Fluorescent Antibody Test
FANV	Fluorescent Antibody Neutralization Test
FBS	Fetal Bovine Serum
FCS	Fetal Calf Serum
FMD	Foot and Mouth Disease
FITC	Fluorescein Isothiocyanate
FFD	Fluorescent Focus Dose
MOI	Multiplicity of Infection
NTV	Nerve Tissue Vaccine
OIE	Office International des Epizooties
PBS	Phosphate Buffer Saline
PEP	Post Exposure Phrophylaxis
PV	Pasteur Virus
RABV	Rabies Virus
Rpm	Revolution per minutes
SFM	Serum Free Medium
TCID	Tissue Culture Infection Dose
VP-SFM	Virus Propagation Serum Free Medium
WHO	World Health Organization

Abstract

Culturing cell is a process of growing cells under physically controlled and aseptic environment in artificial medium. There are several cell lines including vero cell lines propagated using this method. Vero cell lines are derived from kidney of the African green monkey (*Cercopithecus aethiops)*. They are continuous and anchorage based cells that need sufficient surface to proliferate in growth medium. Vero cells are most commonly used to detect bacterial toxins and production of different vaccines. It has also used to grow different strains of rabies viruses to produce cell culture based vaccine for human and animal use; however vero cells propagated in serum supplemented medium that is very expensive and source of different contaminant agents. The aim of this research was therefore to adapt Vero cell lines in serum free medium sequentially for propagation of Pasteur virus (PV) and Evelyn Rokitnicki Abelseth (ERA) rabies virus strains. Vero cells were adapted sequentially in serum supplemented media by gradually reducing from 10% to 0% of serum concentration. Viable cells were counted until passage seven in each serum concentrations. The maximum viable cell density of Vero cells at each serum supplemented medium (0%, 1%, 2.5%, 5%, 7.5% and 10%) was $2.86x10^6$ cells/ml, $2.75x10^6$ cells/ml, $2.75x10^6$ cells/ml, $2.70x10^6$ cells/ml, $2.92x10^6$ cells/ml and $2.75x10^6$ cells/ml, respectively. The preferable incubation times to obtain those maximum viable cells were 96 hours and 144 hours for vero cells proliferated in 2.5% - 10% and 0% - 1% supplemented medium respectively. The incubation time increased as serum concentration in growth medium decreased to reach the confluence stage. The yield of viable cells grown in serum free medium were similar to cells proliferated in a serum supplemented medium. The virus titration was carried out at each serum concentration proliferated cells to determine the virus titer of both viruses. The maximum recorded virus titers for PV was $10^{5.36}TCID_{50}$/ml with 0.01 Multiplicity of infection after 96 hours incubation on serum free grown cells propagated virus and the maximum virus titer for ERA was $10^{5.61}TCID_{50}$/ml with 0.001 Multiplicity of infection on serum free media propagated viruses after 120 hours incubation. The incubation times were also increased as serum concentration was decreased to obtain the higher titer in both virus strains. From the results of this study, it can be concluded that cells that were adapted to serum free conditions is suitable for use in the rabies vaccine production.

Key words: Adaptation, fetal bovine serum, rabies, titer, vero cells

1. INTRODUCTION

1.1. Background

Cell culturing is an advanced process by which cells are grown under controlled and aseptic conditions, generally outside of their natural environment (WHO, 2018). In practice, the term 'cell culture' refers to the culturing of cells originated from multi-cellular eukaryotes, mostly animal cells. Vero cell lines were derived from African green monkey (*Cercopithecus aethiops)* kidney in Chiba University, Japan and sub-cultured several times before taken to culture collections. Vero cells are continuous adherent cell lines that need attachment to grow on surface as monolayer (Yasumura and Kawakita, 1963). Vero cell lines proliferate on flasks, roller bottle and micro-carrier for different scale biopharmaceuticals production. The quality of initial vero cells adhesion is affected by various factors, among those the amount of cells inoculated during subculture have great influence, and constraints of surface area for anchorage of cells also limit the growth of vero cells in culture (Mohamad and Taher, 2016).

As vero cells reach their confluence, they stop further proliferation and lift off from culture surface or start to die; therefore, it is extremely important to monitor them and subculture as they form confluent monolayer. In serum supplemented medium actively growing vero cell cultures double approximately every 24 hours and passage required based on initial cell number and flask size. Normal cell proliferation and survival in culture can be affected by accumulation of metabolic end products that inhibit or stimulate growth (Ara *et al.,* 1986). Continuous cell lines of mammalian origins are very important for the production of biological pharmaceuticals. This anchorage dependent cell lines are used extensively in virology and other applications including study of intracellular bacteria. For instance, vero cells are used for production of number of viruses such as rabies virus, rotavirus, measles virus, rubella virus, polioviruses and influenza viruses. They are also susceptible to bacterial toxins including diphtheria toxin and Shiga-like toxins (Posung *et al.,* 2010).

There are different rabies viruses strains propagate well in Vero cell lines for production of valuable biological products. Several vaccine manufactured by various pharmaceutical companies used Vero cells as main substrate due to its important characteristics for propagation of the desired virus on specified medium that allows proliferation of cells as well as extensive pathogens growth. Serum due to its composition supports the cells as additional nutrition.

However, serum containing culture medium is becoming undesirable for production of vaccines (WHO, 2013). There are a number of disadvantages of serum supplementation including batch-to-batch variation in composition, the high protein content which affects product purification and the potential for viral, mycoplasma, or prion contamination (Butler *et al.*, 2000). Continuous passages of viruses on many cell lines resulted in accumulation of a mutational variant which was responsible for reduced infectivity. Vero cell lines are the candidate of choice for viral vaccine production, due to their: efficiency of primary virus isolation and replication to high infectivity titers; genetic stability while maintaining the antigenic properties of human derived viruses, similarities in the pattern of protein synthesis and morphological changes between virus-infected Vero cells (Khadang *et al.*, 2012).

Serum originates from different animals; like horse, cows and sheep. It is one of the most expensive components in culture medium. Use of serum in cell culture is important because of nutritional factors, culture stimulating factors (growth factors, hormones and proteins) and protecting agents, both for biological protection (antitoxin, antioxidant, anti-protease) and for prevention of mechanical damage. However, using animal serum is potentially risky for health, due to the possible presence of adventitious agents, such as virus and prions (Chen *et al.*, 2011). In addition, serum can contain contaminants such as bacteria, fungi and Mycoplasmas, which can negatively affect cell culture. Another strong limitation to the use of sera is its variability among different lots and suppliers, which hinders the standardization of the culture medium and the reproducibility of culture performance (Ahmad *et al.*, 2010). The threat to human health caused by the undefined agents of bovine spongiform encephalopathy is likely to limit the continued use of bovine serum in culture processes used for the synthesis of health care products such as viral vaccines (Merten, 2002).

Vaccine producing industry, primary drivers in process development and materials optimization include economic considerations such as production capacity and cost analysis, as well as the recent popularity of outsourcing elements of process development to experienced suppliers with deep bio-manufacturing experience (William and Fairbank, 2011). Previously in Ethiopia rabies viruses were propagated in serum supplemented Vero cell lines for anti-rabies vaccine production (Birhanu Hurisa *et al.*, 2013).

1.2. Statement of the problem

Serum derived from various animals such as calf, bovine, horse and other domestic or wild animals that affect animal welfare. It could be the source of mycoplasma; that can pass through the pore of filter paper due their small size and may causes zoonotic diseases. Additionally the use of serum in cell culture medium needs high cost that holds greater costs of all culture media components. The suppliers provide different sources of serum which may vary in composition that affects the uniformity of the product that causes lot-to-lot variation in large scale production of vaccines (WHO, 1999). In Ethiopia cell culture based rabies vaccine for human use have been produced from Vero cell lines proliferated in serum supplemented media without consideration of the above mentioned problems. These challenges were not explored before in Ethiopia through adaptation of Vero cells in serum free medium that can reduce or eliminate the risk of serum in the culture medium. Owing to the above challenges, this research was initiated to explore a valuable mechanism that helps the production of vaccine with low cost and minimal risks for human health. This study aimed to adapt the Vero cell line in serum free medium and propagate rabies virus vaccine strains to minimize the above mentioned risks of serum supplemented culture.

1.3. Research question

- Do Vero cells grow in serum free media?
- Are cells population density of serum free adapted and serum supplemented cells equal?
- Is the viral titer of the virus propagated in Vero cells grown in serum free adapted and serum supplemented media comparable/ differ significantly?

1.4. Objectives of study

1.4.1. General objective

- To develop serum free medium through sequential adaptation of vero cell lines and fixed rabies virus propagation

1.4.2. Specific objectives

- To grow Vero cells in serum free media.
- To compare density of cells proliferated in serum supplemented and serum free media.

- To compare titers of virus propagated in Vero cells grown in serum supplemented and serum free media.

1.5. Significance of the study

Vero cell lines are used as substrate for production of different vaccine production. The result of this study is important to grow of Vero cells with limited cost and avoid the serum from medium components that hinders the quality of vaccines. Cells were adapted in serum free medium with great maximum viable cells that was sufficient for virus proliferation for anti-rabies vaccine production. The maximum virus titer obtained in this study was in serum free medium that is important to proliferate vero cells for consistent batch production of vaccines cause by different in source of serum suppliers. This also minimizes the risk of human health caused by serum in culture media; due to free of infectious agents. Recently to prevent and control rabies, Ethiopia Public Health Institute has produced cell culture based anti-rabies vaccine for human use. This vaccine from fixed rabies virus strain on Vero cell lines proliferated in serum supplemented media. Therefore, this study provides the means of adaptation of Vero cell line in serum free media and virus propagation to avoid risk aspects.

2. LITERATURE REVIEW

2.1. Vero cell lines

Vero cells were originally isolated from the kidney of a normal (healthy) adult African green monkey on March 27, 1962 at the Chiba University in Chiba, Japan (Yasumura and Kawakita, 1963). The cell line was brought to the National Institute of Allergy and Infectious Diseases at the National Institutes of Health in the United States and was taken to the American Type Culture Collection. Currently the suppliers of Vero cells were ATCC and the European Collection of Animal Cell Cultures (ECACC) reservoir sites. Commonly used Vero cell lines from both companies have little code differences for different isolates includes; Vero, Vero E6, Vero C1008, Vero 76 and Vero-WHO. All of them require a similar nutritional source to proliferate (Tyler *et al.*, 2015).

The Vero cell lines are continuous and aneuploid, meaning that they have an abnormal number of chromosomes. A continuous cell lines can be propagated through many cycles of division and not become mortal. Those cells are interferon deficient; unlike normal mammalian cells and they do not secrete interferon alpha or beta when infected by viruses. However, they still have those interferons type receptor, therefore, they respond normally when interferon from another source is in culture media (Abiko *et al.*, 2007). Elimination of serum from culture media provides rapid and efficient purification of final products due to avoiding of high protein content of serum. Therefore, considering those side effect of serum, several companies generally recognized that serum-free media are now the essential alternate for various biological products (Lednicky and Wyatt, 2012).

2.2. Basic systems of growing cell culture

There are two basic systems for growing cells in culture, as monolayer on an artificial substrate (i.e., adherent culture) or free-floating in the culture medium (suspension culture). The majority of cells derived from vertebrates, with the exception of hematopoietic cell lines and a few others are anchorage-dependent and have to be cultured on a suitable substrate that is specifically treated to allow adhesion of the cells and spreading (i.e., tissue-culture treated). However, many types of cell lines can also be adapted for suspension culture. Cell line like Vero and BHK-21 are adherent cells that need anchorage surface for proliferation (White and Ades, 1990).

2.3. Vero cells production

Cell culture systems are mostly advanced in virus vaccine development. The primary and diploid cell culture techniques are now replaced by the use of continuous cell lines. These substrates are getting increasing acceptance from regulatory authorities as improved technologies to avoid risk of their potential oncogenic properties. Vero cell line is the most widely accepted continuous cell lines by regulatory authorities and has been used for over three decades for the production of polio and rabies virus vaccines. Recently most advanced, viral vaccines produced from pathogens propagated in Vero cell lines. These developments indicate the value of this cell culture platform in the rapid development of vaccines against a range of virus diseases. Vero cell lines are attachment based cells that requires large surface to proliferate in maximum density for different application such as vaccine production; it requires the scaling-up of Vero cell cultures (Ammerman *et al.,* 2008). There are two growth techniques used for the scaling-up of anchorage-dependent cell lines: roller bottles and micro-carriers (Han and Sha, 2017).

2.3.1. Roller bottles

Roller bottles are cylindrical vessels where the cells grow on the inner surface of the tube. The bottles slowly rotate to continually distribute the cells in growth medium. The cells are cultured on stacked surfaces and oxygen transfer might pose a potential problem (Toriniwa and Komiya, 2007). The recommended speed of roller is 0.1 to 0.3 rpm during the first 24 hours of attachment and a maximum of 2 rpm afterward. The optimal speed for a particular cell type may vary and this must be determined experimentally. The formation of well-dispersed homogeneous cultures is dependent upon the rate at which the cells settle and attach to the glass. The more rapidly they attach the more even the distribution of cells on the glass surface (Tian and Keping, 2009). The rate of attachment is dependent on the temperature of the medium, rotational velocity of the bottle and cells density (Fayaz *et al.,* 1997).Fresh media are replaced based on the color change within two days. Addition of cold media affects the cells viability and attachments, therefore replaced media should be pre warmed before dispensing into the culture. The cells in roller bottle cultures can tolerate a density nearly two times higher than that of stationary cultures (Kaneatsu *et al*., 1966).

2.3.2. Micro-carrier

Vero cell cultures can be performed on glass or plastic micro carriers at room temperature in the presence or absence of fetal calf serum. The higher rate of cell attachment occurred to plastic micro carriers and a lower one to plastic surface, the presence of FCS in the culture medium having an inhibitory influence on the cells attachment to glass or plastic surface but no influence on the cell attachment to Micro-carriers. The cells spreading were highly dependent on the presence of FCS, were comparable on glass or plastic surface and slower on Micro carriers. The rates of cell spreading were higher at 37 °C than at the room temperature, with exception for the plastic surface where the temperature did not influence the cell spreading. The ability of vero cells to spread over micro-carriers was fully dependent on the presence of FCS and decreases progressively with a delayed addition of FCS into the culture medium (Mendon *et al.,* 1999). The surface area available for cell attachment can be even further increased by growing the cells on micro-carrier beads. The beads usually are around 0.2 mm, can be made of dextran, cellulose, gelatin, glass or silica and can considerably increase the surface area available for Vero cell growth. Vero cells growth using roller bottles and micro-carriers are used for the production of viral vaccines (Kniel, 2008).

2.4. The use of serum in culture medium for growth of cells

Previously human and horse serum collected aseptically through venipuncture was the source of serum for tissue culture. This was later replaced by less expensive bovine serum sourced from blood taken from slaughterhouse of bovines. The bovine blood is collected using somewhat crude methodology; the blood clotted serum is separated and then usually filtered using 0.1 µm filters. Both calf and fetal bovine serum (FBS) are used for cell culture media (Tian and Keping, 2009). Serum is the clear portion of blood obtained after removing cells, platelets and clotting factors. It is one of the most important components of cell culture medium. Animal serum such as FCS and FBS are routinely added to culture media as a source of nutrients and other defined factors. It contains amino acids, proteins, growth factors, adhesion factors, hormones, vitamins, inorganic substances, nutrients and metabolites. Serum is a complex mixture that provides the following advantage for cell culture; it is able to bind and neutralize toxins, hormonal factors for stimulating cell growth and proliferation (El-Dakhly *et al*., 2015).

Additionally serum promotes differentiation functions, transport proteins carrying hormones (e.g. transcortin), minerals, trace elements and lipids, attachment and spreading factors, stabilizing and detoxifying factors needed to maintain pH or to inhibit proteases either directly (e.g., α-antitrypsin inhibitor in serum is an important inhibitor of the protease (trypsin) or indirectly by acting as an unspecific sink for proteases and other toxic molecules (Fayaz *et al*., 1997).

2.5. Drawback of using serum in vero cells culturing

The use of serum in culture medium has a number of disadvantages such as batch-to-batch variation that makes standardization of product difficult, risk of contaminants (Mycoplasmas, bacteria, fungi and viruses) and unwanted effects such as stimulation or inhibition of growth condition. Fetal bovine serum (FBS) carries the risk of transmission of zoonotic infections and other adverse factors. As a byproduct of beef industry, FBS supply is complicated by health concern such as BSE (bovine spongiform encephalitis), FMD (foot and mouth disease) and rinderpest (Posung *et al.,* 2010). While of course we don't advocate cruelty to any living being, there are also many compelling scientific reasons as to why fetal calf serum should no longer be used in research. There are several strong arguments that many substances present in FCS have not yet been identified and the substances responsible for the functions of the cultured cells are not always clear (Carlo *et al.,* 2002).

Fetal calf serum can interfere with genotypic and phenotypic cell stability, which can also influence experimental outcome. Serum can suppress cell spreading, attachment and embryonal tissue differentiation, which is the process by which embryonic cells develop into specialized cells for particular functions. Critically, this can actually prevent an objective of cell growth research especially when we talk about growing new organs and limbs (Fayaz *et al.,* 1997). Fetal bovine serum (FBS) is a common component of animal cell culture media that serve as nourishment. Fetal calf serum is prepared from blood extracted from fetuses removed from cows found pregnant at slaughter. The fetus is removed during evisceration and blood extracted via cardiac puncture without any anesthesia that is unethical activity (Josef, 2005). It is harvested from bovine fetuses taken from pregnant cows during slaughter by means of a cardiac puncture without any form of an aesthesia within 5-30 minutes after the mother's death. Blood pumped out by injection of needle into the fetus' heart and pumped through

vacuum or cardiac massage. The serum is usually separated by filtration using 0.1µm filter papers. The minimum age of fetus is three months to ensure that their heart is large enough to puncture. Fetuses are likely exposed to pain or discomfort and therefore current practice of fetal blood harvest is inhumane apart from moral concerns, scientific and animal welfare (Hernández and Fischer, 2006).

Today there is an increased awareness of fetal sensitivity to pain and growing evidence of resistance to anoxia in mammalian fetuses. Consequently calf fetuses are likely to be alive and have normal brain function during blood collection and can be expected to experience suffering until death actually occurs. It is estimated that one to two million bovine fetuses are subjected each year to this inhumane process, yet many scientists regularly using FCS for cell culture remain unaware of the animal suffering involved in its collection (Clark, 1980).

Typically, nine month bovine fetuses only provide approximately 550 ml raw fetal bovine serum. Therefore, for one year supply of raw FBS 500, 000 liter, around 1-2 million of nine-month old fetuses are required. Serum is most commonly used in mammalian cell culture research practice small scale, but it is not a choice at large scale production since presence of serum often affects the purification of biological products. Recently the use of serum is also moving towards use of serum-free media or serum alternatives due to animal welfare and ethical issues in serum processing and production. The demand of FBS has been increasing dramatically, but the supply is not sufficient as needed (Gstraunthaler and Valk, 2013). Among the many varieties, fetal bovine serum is the serum of choice in mammalian cell culture due to the presence of growth promoting components, specifically most portion of bovine serum are albumin and fetuin (serum protein abundant in fetus) because of its rich content of growth factors and its low γ-globulin (antibody) content. Fetal bovine serum has been adopted as the standard supplement of cell culture media. But production of FBS from bovine fetuses posed an ethical issue. The upstream processing is seen as very essential and against animal welfare (Hashim *et al.,* 2011). Serum and other bovine-derived materials should be tested for adventitious agents such as bacteria, fungi, mycoplasmas and viruses prior to use in the production of manufacture of biological products. Particular consideration should be given to those viruses that could be introduced from bovine-derived materials and that could be zoonotic or oncogenic (e.g. bovine viral diarrhoea virus, bovine circoviruses, rabies virus, bovine adenoviruses, bovine parvovirus, bovine respiratory

syncytial virus, infectious bovine rhinotracheitis virus, bovine parainfluenza virus, Cache Valley virus, bluetongue virus are some disease that may be caused as a result of serum usage in growth media (Mohammad *et al.*, 2015). Cell cultures have already proved immensely valuable in replacing procedures on living animals. Their value to animal welfare would be further enhanced by the removal of serum, in particular FCS, from culture media (El-Dakhly *et al*., 2015).

2.6. Adaptation of cell line in serum free medium

The growth of cell is achieved using defined basal medium supplemented with high serum or protein, but it is important to avoid the serum and other animal derived proteins due to problems in biopharmaceuticals product quality. Serum free and protein free cell culture media offer batch to batch consistence and reduce production cost. Therefore, it is very essential to replace the medium designed to use in cell culture with chemically defined media. As the culture condition is changed the cell physiology respond to stimuli and becomes ready to adapt to a new culture condition or medium. Two general strategies are used to adapt cells to serum-free media. The simplest one is direct adaptation, in which cells are converted directly from serum-supplemented medium into a serum free alternative (Endler *et al.*, 2002).

The second method, sequential adaptation or weaning, is the alternate approach that switches cells from serum-supplemented to a serum-free medium through several sequential steps. Weaning tends to be less harsh on cells than direct adaptation, so it can be beneficial for adaptation of less robust cell lines. There is no single adaptation technique applicable in all circumstances. But to maximize the chance for success, a number of points should be considered before beginning adaptation (John *et al*., 1972).

2.7. Strains of rabies viruses

2.7.1. Fixed rabies virus

The fixed viruses are passaged several times in laboratory and attenuated virus with known properties, in terms of incubation period, pathological and clinical effects (Kaneatsu *et al.*, 1966). These viruses used for production of vaccine and other bio-pharmaceuticals by producing them in large scale. Pasteur developed the first vaccine in 1885, many rabies vaccines were also developed using different cell substrates such as; animal brains, avian embryos, primary mammalian cells, human diploid cell and Vero cells. For many years (over 100 years) rabies

vaccine was also produced in mammalian neural tissues. The vaccines that were produced in cell culture and embryonated eggs have decreased human deaths.

Using neural tissue vaccine (NTV) was very common in the past and this type of crude vaccine was improved over the years, but remained associated with serious adverse events and severe neurological reactions, because of the production of antibodies against myelin or other substances of the animal nervous tissue present in the product. Hence, the use of NTV phased out slowly as recommended by the World Health Organization, but few countries including Ethiopia produce and utilizing this vaccine till now. Therefore Ethiopia exists on the right track to replace this old type of vaccine by cell culture based vaccine that is recommended by world health organization to minimize risk of efficacy and safety (Birhanu Hurisa *et al*., 2013). The viral titer of many fixed rabies virus strain propagated in serum supplemented cell lines like Vero and BHK-21 vary with multiplication of infection. These vaccine strains are used for rabies vaccine production (Perez and Paolazzi, 1997b).

2.7.2. Street rabies viruses

Street rabies viruses are isolated from naturally infected either domestic or wild animals. The street virus may have incubation periods varying from 2 weeks to more than 1 year and variable clinical effects. These rabies viruses are more pathogenic than fixed rabies virus strains based on their genotype. Characterization of these virus is needed as it may be used for vaccine production for human and animals use. The pathogenicity of street rabies virus different based on the reservoir hosts it originated. The street rabies virus isolated from insectivorous bats is less pathogenic than other hosts (Fuocoa *et al.,* 2018). Adaptation of street rabies virus in different cell line was used to detect the rabies specific neutralizing activity of monoclonal antibody (Peng-Cheng *et al*., 2018). The antigenic differences of street rabies virus isolated from various sources also affect the vaccine effectiveness due to the variation in glycoprotein between street viruses (Wang *et al.,* 2019).

2.8. Rabies antigen detection

The rabies sign observed in both animals and human should be confirmed by laboratory diagnostic methods approved by WHO and OIE. The most widely used primary diagnostic test for rabies diagnostic is fluorescent antibody test (FAT). Impression smears prepared from a

composite sample of brain tissue are treated with anti-rabies serum or globulin labeled with fluorescein isothiocyanate (FITC). Specific aggregates of rabies virus antigen are detected by their fluorescence using a reflected light (incident light) of fluorescence microscope. The FAT is accurate, sensitive and rapid, i.e. results can often be obtained within 1 to 2 hours of receipt of the specimen. Alternatively, particularly where fluorescence microscopy is not available, a direct rapid immunohistochemistry test (dRIT) was developed with a similar sensitivity and specificity as the FAT. The principle is similar to the FAT except that the dRIT uses streptavidin–biotin peroxidase staining (Duong *et al.*, 2016). Lateral flow devices for rapid detection of rabies virus antigen under field conditions have been developed, however only partly meeting expectations in terms of test characteristics (sensitivity and specificity). Adequate validation according to international standards is still required so that they can be useful in surveillance situations in which the resources for the use of recommended testing are lacking (Gupta, 2005).

2.9. Epidemiology of rabies

2.9.1. Epidemiology of rabies in the world

Rabies is neglected zoonotic disease caused by rabies virus. It present in more than 150 countries and on all continents except Antarctica (WHO, 2004). It is endemic in Africa, Asia and Latin America. Domestic animal rabies recorded in the United States and Europe represents less than 10% of all rabies types. Rabies is important public health problem throughout the world and cause of more than 60,000 human deaths annually among these, Africa and Asia holds 36.4% and 59.6%, respectively. Although urban canine vaccination in developing countries is a major economic burden to those countries, it seems to be the only effective means of controlling rabies. Vaccination coverage of 70% has been sufficient to control canine rabies in desired region, but the exact level of coverage required is likely to vary according to the demographic and spatial characteristics of the dog population (WHO, 2004). In many counties like, Cambodia, Myanmar, Nepal, Pakistan, Bangladesh, India, Sri Lanka, Bolivia, Chile, Colombia, Ecuador, Peru, Venezuela, Algeria, Morocco, Botswana and Ethiopia, rabies cases are significantly underreported. Proper rabies case reports provides public health priorities, economic impact, monitor the use of vaccine, cost effectiveness of interventions allocate resources for disease prevention and control (WHO, 2018).

Rabies affects mammals and most often transmitted through the bite and scratch of rabid animals. The virus affects the central nervous system in mammals, travels within the nerves and multiplies in brain rapidly (WHO, 2018b). Wild animals are common hosts for rabies and the major source of transmission to humans and domestic animals. Before the onset of specific clinical symptoms of human's rabies are similar to many other illnesses, such as fever, headache and general weakness or discomfort. As the disease rise, more specific symptoms appear those includes insomnia, anxiety, confusion, slight or partial paralysis, excitation, hallucinations, agitation, hyper salivation (increase in saliva), difficulty swallowing, and hydrophobia (fear of water). Death usually occurs within days of the onset of these symptoms (Tekki *et al.*, 2013).

2.9.2. Epidemiology of rabies in Africa

More than 99% of human rabies burden occurs in Africa, this is due to poor prevention and control methods. Rabies is a neglected zoonotic viral disease with limited case report in many African countries (WHO, 2004). Annual dog vaccination data is not available in many countries, but the estimated average national dog vaccination submitted during 1996-2000 was 10.3% in Africa that is too far from threshold designed by world health organization (WHO, 2018b). 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. The estimated annual dog-mediated human rabies death in Africa was 21, 476 (WHO, 2018). Human nerve tissue vaccines remain in production in two countries in Africa such as Algeria and Ethiopia. Africa pay limited estimated cost to spend the least on PEP (3.28% of the global non-human mortality cost) and have the highest cost of human mortality (45%), indicating that many lives could be saved if access to PEP was improved or the prevalence of dog-mediated rabies is reduced (WHO, 2018b).

2.9.3. Epidemiology of rabies in Ethiopia

Surveillance reports usually underestimate incidence and are poor indicator of the status of the disease in developing countries like Ethiopia where human, domestic and animal's health status systems are inadequate. There is limited recorded information on rabies both in humans and animals. Many people have insufficient awareness to treat rabies when individuals are exposed. Rabies in Ethiopia is considered as disease of dogs; because the main reservoirs of the rabies virus are dogs. Canine vaccination was carried out without destruction of stray dogs, rabies in

owned dogs and the number of post-exposure treatments decreased. However, the numbers of human rabies cases due to bites by rabid stray dogs remained high. The main challenge is effective delivery of vaccines to ensure adequate vaccination coverage in the reservoir dog population. Risk of rabies exposure increased since man-dog relation is very common (Semayat Oyda and Bekele Megersa, 2017).

Many people are infected with rabies in Ethiopia each year. For instance, according to Center of Disease Control and Prevention an estimated 2,700 people died of rabies in 2017 which is one of the highest death rates in the world but the accurate number of deaths caused by rabies is unknown because the disease is underreported and rabies diagnostic laboratories are not established. Furthermore, few places in Ethiopia offer lifesaving human rabies post-exposure prophylaxis and most people don't have the means to make it to a major hospital to get treated. Besides to this; people's awareness about rabies treatment after bitten by dogs is low, and people often do not seek medical help when bitten. However, like other countries with high human rabies death rate, dog's vaccination program is very low in Ethiopia that is below the 70 % recommended by WHO to prevent the transmission of canine rabies. In 2017 there was training on organizing mass dog vaccination campaigns, building rabies laboratory diagnostic capacity and expertise, manufacturing a safe vaccine, and creating rabies awareness materials for the general public. During this moment more than 7,000 dogs have been vaccinated in Addis Ababa as part of the mass vaccination trainings. Three states of the art rabies diagnostic laboratories across the country were established and had been assisting with mass vaccination campaigns outside of Addis Ababa. In Ethiopia more than 82% of post exposure treatment of rabies is Nervous Tissue Vaccine which is produced from nervous tissue composed of rabies virus-infected sheep brain inactivated with phenol. This type of vaccine is less immunogenic than the cell culture vaccine and known for its fatality and disability rate due to the occurrence of severe adverse reactions. Ethiopia emphasizes to replace this NTV with cell culture based anti-rabies vaccine production for human use that meets the WHO requirement (Birhanu Hurisa *et al.*, 2013).

In Ethiopia among the estimated number of persons exposed to rabies cases dogs were responsible for more than 98% of all post-exposure vaccinations (Fikadu Makonnen, 1982). The reported cases of animals and humans rabies by regions in Ethiopia showed that the disease is

enzootic throughout the country. However, there is no legislation enforced for registering, licensing and vaccinating pets in the country. Anti-rabies vaccination services are offered for dogs at government veterinary clinics and laboratories in some parts of the country, but these services are usually not affordable for most people to use and therefore the vaccinated dog population is very limited. In the beginning of 1965, when the incidence of rabies in Addis Ababa cases recorded the government allowed some free vaccination service for two months. During this period 54,000 dogs were vaccinated that represent only about 20-30 % of the city's estimated dog population but the rabies cases due to owned dogs substantially decreased (Moges Nibret, 2015).

3. MATERIALS AND METHODS

3.1. Study setting and design

The study was carried out in Ethiopian Public Health Institute (EPHI), Vaccine and Diagnostic Production Directorate, cell culture based anti-rabies vaccine production laboratory and involves laboratory based experimental study with quantitative and descriptive methods.

3.2. Biologicals

3.2.1. Cell lines

The cell lines used in this study was Vero cell; it was donated by Ethiopian National veterinary institute (NVI) and originally purchased from American Type Culture Collection (ATCC).

3.2.2. Virus strains

Rabies Virus strains; Pasteur Virus (PV) and Evelyn Rokitnicki Abelseth (ERA) provided by Pasteur Institute (Paris, France), available in vaccine and diagnostic production laboratory used to propagate into the Vero cell lines proliferated in serum supplemented and serum free medium.

3.3. Media preparation

All media components used in this study were prepared in aseptic condition under biosafety cabinet; the Minimal Essential Medium Eagle (Batch # 021M8316) bought from Sigma life science was dissolved in pure distilled water following manufacturer's instruction. The mixture was agitated to homogenize the components. The media were sterilized by filtering with microbiological filter system with pore size of 0.22µm. The sterility test was performed by taking sample from the medium and incubating it for 3 days at 37°C. Medium contamination was detected based on the color change of phenol red present in the medium, which gives a yellow-orange color due to pH change to acidic value as a function of microbial growth. A sample was examined microscopically to detect any microbial growth. After passing the sterility test, the medium was stored in refrigerator at (2 - 8 ^{O}C) until use. The fetal bovine serum (F7524, Lot: BCBR0718V) from Sigma life science, used in media preparation was preserved at -20°C until use. The fetal bovine serum was thawed overnight at 4°C and then incubated for 30 min prior to use at room temperature (John *et al*., 1972).

3.4. Reviving Vero cell lines

Minimum Essential Medium Eagle supplemented with 10 % Fetal Bovine Serum (FBS), penicillin and L-glutamine were used in biosafety cabinet and other glass wares sanitized with 70 % alcohol. The cells had drawn from deep freezer, defrosted in water bath and transferred to 75cm^2 T-flasks aseptically. Then 20 ml of fresh media dropped slowly on wall of the flasks and incubated at 37^{o}C for 72 and 96 hours until it reach the confluence stage. The confluence was determined through observation of adherent cell occupied surfaces proportion. Finally, the cells were ready for further subculture (Moura *et al*., 2008).

3.5. Splitting and sub culturing of Vero cells

Cells splitting was carried out when the cells reached approximately 80% confluent stage. The flasks was removed from incubator and sanitized with 70% alcohol before placing into the biosafety cabinet to prevent the cross contamination. Exhausted medium was poured off carefully into waste pot/ discarding jar. Then the flasks contained attached/ anchored cells were washed twice by pouring/pipetting 15 ml of Phosphate Buffer Saline (NaCl: 8 g/L, KCl: 0.2 g/L, Na_2HPO_4: 1.44 g/L and KH_2PO_4: 0.24 g/L) into the flask, rinsed gently and removed out into the discarding jar. Using sterile pipette, 2 ml of 0.05% trypsin EDTA was dropped in (10 %, 7.5% and 5%) serum supplemented media grown cells, 2 ml of 3:1 diluted 0.05% trypsin EDTA in (2.5% and 1%) serum supplemented medium and 2 ml of 1:1 diluted 0.05% trypsin EDTA in 0% serum supplemented grown cells and allowed to cover the bottom of the flask gently to ensure trypsin contact with all cells (Ammerman *et al.*, 2008).

The flasks were incubated at 37^{o}C for 3-4 minutes (El-ensahsy *et al.*, 2009). As soon as cells were detached the flasks were gently tapped to the hands to facilitate the trypsin penetration and enhance detachment. The flasks were seen under inverted microscope to confirm if the cells were fully detached from the surface of the flask. Then the flasks were sanitized and taken under biosafety cabinet, 10ml of each culture media of serum concentration were added to their respective flasks to inhibit trypsin activity. Serum free adapted Vero cell lines were splitted with diluted trypsin, to minimize cells damage due to trypsin activity; previously added medium was centrifuged at 800 rpm for 5 minute and removed off gently. The flasks were labeled, ready to use for subculture and detail information of culture was recorded in separate log book with the

cell line, passage number and date. cells suspension were transferred into new flasks based on decided split ratio and incubated at 37°C until it reached the confluence for next passage (Marta and Freire, 2005).

3.6. Vero cells enumeration and preservation

Cells were routinely counted in a Neubauer chamber/hemocytometer after staining with 0.4% of trypan blue for identification of viable and dead cells. The cells counting was carried out by adding 50 µL cell sample, 50 µL trypan blue and 400 µL PBS together in single micro titer plate well and mixing gently. Then 10 µL of sample was loaded into each side of a hemocytometer slide (Petterson, 1979). Then cells counting were done by using inverted phase contrast microscope. The cell density was calculated and recorded in the specified log book for cell culture (Essam *et al*., 2018).

For freezing and preservation, mono layers carried out as the cells reach confluence stage. The cells were detached, counted and viable cells were adjusted to 1 x 10^7/ ml with fresh medium supplemented with 10% fetal bovine serum of each serum concentration and 10% dimethyl Sulfoxide (DMSO). The preparation was dispersed into sterile cryotubes (Nunc) and frozen for 1 hour at -20^{o}C, then placed in -70^{o}C for further preservation for serum supplemented medium and Preservation of serum free adapted cells were carried out with 7% DMSO, 46.5% serum free media and 46.5% media supernatant of centrifuged cells at 800 rpm for 5 minutes (Perez and Paolazzi, 1997a).

3.7. Adaptation of Vero cell lines in serum free medium

Previous cells were grown on serum supplemented medium (10% FBS), to adapt the cell in serum free medium sequential approach was followed by decreasing the serum content in growth medium in subsequent subcultures. The serum content in the medium was expressed in percent such as 10 % FBS, 7.5 % FBS, 5 % FBS, 2.5 % FBS, 1 % FBS and 0 % FBS.

The adaptation process was started with density of 1.50x10^6 cells. Cells were splitted every 72 and 96 hours for cells propagated in 10% FBS, 7.5% FBS, 5% FBS and 2.5% FBS to compare the better incubation that yield the maximum viable cells density. Those cells proliferated in 1% FBS and 0 % FBS supplemented medium and passaged every 120 hours and 144 hours incubation to check the preferable time that resulted in higher viable cells.

The term ‘passage’ in the adaptation process refers to the moment in which the cells have been

Replaced/ splitted allowed to grow with respective incubation times. Throughout the adaptation process cultures were cultivated for seven passages to check the consistency of adaptation and then evaluated regarding cell growth and viability (Biaggio *et al.,* 2015). All serum concentration were required at least three passages to adapt into their respective serum supplemented medium (Merten *et al.,* 1997). The decision to transfer the cells into the next serum concentration was based on growth and viability at each passage (El-ensahsy *et al*., 2009). Cells viability was enhanced by supplementing 20 ml fresh medium per 75cm^2 T-flask based on cells growth and color change appearance in exhausted medium (Tian and Keping, 2009). Through adaptation in serum-free medium, the cells were preserved, at 1 x 10^7cells /ml per adapted serum concentration (Majoul *et al.*, 1999).

3.8. Cells infection and virus production

Cells infection was carried out on cultures with cells density of more than 2x10^6cells/ ml. Virus with multiplicity of infection (MOI) was 0.01and 0.001 for PV and ERA respectively (Birhanu Hurisa *et al.,* 2013). The desired volume of respective viruses were inoculated in 50 ml test tube which contain Vero cells suspension, incubated at 37^{o}C with 5 % CO_2 for 30 minutes and agitated properly in the 5 minute interval (Yu-chen and Joseph, 2000). Then transferred to T-flask contained respective growth medium and incubated at 37^{o}C with 5 % CO_2 for 72 and 96 hours in cells propagated in 2.5% - 10% and 0% - 1% serum supplemented for PV virus production respectively. For ERA rabies virus production it was incubated for 96 and 120 hours in cells propagated in 2.5% - 10% and 0% - 1% serum concentration grown respectively. Finally the virus culture was removed from incubator and kept in deepfreeze for a minimum of 24 hours. Freeze thawing was proceeded to disrupt the cells and to remove the viruses into the supernatant; 1ml sample was taken from each serum concentration virus culture for the virus titration test and the remaining part of virus suspension was stored in proper temperature condition (El-ensahsy *et al.,* 2009).

3.9. Virus titration

For determination of rabies virus titer, the cells was adjusted at 5x10^5 and 50 µl/wells suspension mono-layers of Vero cells was distributed on 96 wells micro titer plates were infected with virus sample serially dilution process. All dilutions of both viruses were carried out in four replica and incubated at 37°C with 5% CO_2 for 72 hours (Sannat *et al*., 2014). The cells were fixed with

50µl/well of 80 % cold acetone, incubated at room temperature for 30 minutes. The plates were washed with 100 µl/ well of phosphate buffered saline (PBS) twice and then 100 µl/ well florescent- labeled anti rabies immunoglobulin was added and incubated for 1 hour at 37°C with 5 % CO_2. The micro titer plate was washed with PBS twice and dried at room temperature. Finally the plate was observed under fluorescence microscope and virus titer was expressed as tissue culture infectivity doses fifty ($TCID_{50}$) as calculated by Spearman-Kärber method.

3.10. Data analysis

Data were analyzed by using SPSS statistical software version 20 and expressed in graphs and tables. One-way analysis of variance was used to test the difference between both virus titer and viable cells density proliferated in each serum concentration. To determine statistical difference, P-value < 0.05 was regarded as statistically significant.

4. RESULTS AND DISCUSSION

4.1. Adaptation Vero cell lines

4.1.1. Propagation of cells in 10% serum concentration

The incubation period considered in this study to proliferate cells in 10% serum concentration was 72 and 96 hours in separate 75 cm^2 T- flasks. At both incubation periods the viable cell density was higher than the density of cells inoculated at the start of the experiment in all the passages. The relative preferable incubation was 96 hours compared to 72 hours incubation due to its high cell density yield throughout the passages as shown in (Table 1) below. Beyond this incubation time the cells were detached from the surface and their viability decreased. The minimum and maximum viable cell density obtained were counted to be $2.35x10^6$ cells/ml at 72 hours and $2.75x10^6$ cells/ ml at 96 hours incubation time respectively.

Table 1: Viable cells /ml and incubation time of Vero cells in 10 % serum supplemented medium before adaptation

Passages	Incubation (Hours)	Viable cells $x10^6$cells/ml
1	72	2.46
2	72	2.35
3	72	2.46
4	72	2.48
5	72	2.65
6	72	2.47
7	72	2.50
1	96	2.48
2	96	2.53
3	96	2.46
4	96	2.50
5	96	2.75
6	96	2.50
7	96	2.55

The result of this study is slightly close to a study done by Ahmad *et al.* (2010), who obtained the maximum viable cells of $2.50x10^6$ cells/ml with 96 hours incubation period. The initial inoculants play role to determine the length of incubation periods and cells density harvested on each passage levels based on the surface available for cell culture. Therefore the more initial inoculants during subculture of Vero cell lines the limited time required to reach the confluence stage to be harvested. In a study carried by Trabelsi *et al.* (2005), cells were grown in serum supplemented medium on micro carrier bioreactors. The authors reported the maximum viable cells of 4.73 $x10^6$cells/ ml after 96 hours incubation. This difference attributed between the result of the current study and the study carried by Trabelsi *et al*. (2005), might be due to the difference in the methodologies used where the authors used advanced and well controlled bioreactors. In the current study, significantly different variations were not observed ($p > 0.05$) in the yield of viable cells at 10% serum concentrations.

The study conducted by Butler *et al*. (2000) and Yokomizo *et al.* (2004), reported that the maximum viable cells of 1.0×10^6 cells/ml and $2.10x10^6$ cells/ml at 96 hours incubation time respectively in 10% serum supplemented media in micro-carriers culture. The maximum viable cells counted were greater in current study; indicates that the adaptation/handling technique carried was preferable for proliferation of the cells. The studies done by Mohd *et al.* (2010), record the maximum yield of Vero cells proliferated in micro-carrier stirred tank bioreactor was $1.93x10^6$ cells/ ml after 56 hours incubation period. The reason of these limited viable cells result, even though the growth mode is advanced and controlled condition might be higher initial inoculant cells used to proliferate the cells that reach confluence within two days and start detaching from the surface that decrease their viability. Other investigation done by Souza *et al.* (2005), obtained the maximum viable cells $2.70x10^6$ cells/ ml after 144 hours incubation. The longer incubation time reported might be due to lower initial inoculants used to proliferate in this serum concentration. The differences in the mode of culturing might bring differences in the result of the current study and other researches.

4.1.2. Adaptation of cells in 7.5% serum concentration

The adapted cells were grown well and the minimum recorded cells were on first passage that was $2.33x10^6$ cells/ml. The maximum cell density was counted at passage six at both incubation times. The preferable incubation was 96 hours to reach the maximum viable cells density. In this serum concentration the minimum and the maximum viable cells at 96 hours incubation were $2.45x10^6$ cells/ml and $2.92x10^6$ cells/ml that was higher than 10% serum supplemented medium proliferated cells. The viable cells counted at 96 hours incubation were greater in all passages as shown in (Table 2), compared to the 72 hour incubation, which indicates that the preferable incubation time to harvest higher viable cells. After the cells were adapted to this serum concentration their growth pattern were observed through proceeding up to passage seven. Reduction of serum in growth medium is important technique to avoid its side effects in biological products. The morphology of Vero cells was similar to the 10% serum supplemented in every aspect except the variation in serum concentration.

Table 2: Viable cells and Incubation time of Vero cells in 7.5 % serum supplemented medium before adaptation

Passages	Incubation (Hours)	Viable cells $x10^6$cells/ml
1	72	2.33
2	72	2.50
3	72	2.37
4	72	2.42
5	72	2.40
6	72	2.90
7	72	2.52
1	96	2.55
2	96	2.55
3	96	2.65
4	96	2.59
5	96	2.45
6	96	2.92
7	96	2.57

The cells culture aggregates formation at confluence stage was not observed and the viable cells counted were higher than previous serum supplemented medium in all passages. This serum concentration provides sufficient viable Vero cell density used various biological purposes. This result is very close with the data reported by Rourou *et al*. (2009), who recorded the maximum viable cells $2.90x10^6$cells/ ml after 96 hours incubation. Other study carried out by Majoul *et al.* (1999), who got the maximum cells density of $2.30x10^6$ cells /ml after 96 hours incubation. In contrast the study carried out by White and Ades, (1990) grown the cells in micro-carriers bioreactors and obtained the maximum viable cells $2.10x10^6$cells/ml after 96 hours incubation. These variations of viable cells density in current study and other investigators might be difference in cells number used at the beginning of the experiment and techniques of growing the cells. Therefore the viable counted cell in the current study is preferable and the technique of adaptation is important to proceed for further serum reduction.

4.1.3. Adaptation of cells in 5% serum concentration

The minimum and the maximum growth recorded during this experiment was $2.38x10^6$ cells/ml and $2.70x10^6$ cells/ ml at 72 and 96 hours incubation time respectively. The cells morphology was similar with cells proliferated in higher serum concentration supplemented media. The preferable incubation time to obtain maximum cells proliferation was 96 hours and higher cell yield was obtained on the passage five. As shown in (Table 3) below, the cells growth showed slight variation in viability throughout the experiment, at both incubation times. The viable cell count showed slight increment until the passage five and slightly declined on the last two passages. This serum concentration supplemented cells required the same concentration of trypsin as higher serum supplemented media grown cells to detach from the surface during sub culturing. This indicated that, gradually adapted cells were not affected by trypsin at desired incubation period to remove the cells from the surface. The result indicated that viable cells harvested through adaptation were statistically similar in cells density ($p > 0.05$), with serum supplemented cells proliferated in serum supplemented media. This result correlate with the study done by Khaled *et al.* (2005), who obtained the maximum cell density 2.85×10^6 cells/ml after 144 hours incubation, which were longer incubation time with higher cells density. In contrast in another study carried by LeFloch *et al.* (2006), the maximum viable cells found was $1.80x10^6$ cells/ml at 80 hours incubation times**,** this result was lower in cells density and

incubation time compared to the result of the current study. This variation might be due to the culture modes, cells density at start of experiment and medium composition used to grow the cells. Therefore the result obtained in current study is preferable in terms of incubation time and maximum viable cells harvested used to proliferate the rabies virus.

Table 3: Viable cells and incubation time of Vero cells proliferated in 5% serum supplemented medium before adaptation.

Passages	incubation	viable cells $x10^6$/ml
1	72	2.38
2	72	2.56
3	72	2.50
4	72	2.47
5	72	2.43
6	72	2.50
7	72	2.47
1	96	2.43
2	96	2.60
3	96	2.51
4	96	2.64
5	96	2.70
6	96	2.51
7	96	2.52

The maximum viable cells recorded in the current study is also slightly close to the result reported by Frazatti-gallina *et al.* (2004) and Fountain, (2003) who found the maximum viable cell of $2.50x10^6$ cells/ ml at 96 hours incubation time. In another investigation carried out by Merten *et al. (*1999), a maximum cell density of 3.10×10^6 cells/ ml was found for Vero cells proliferated in micro carrier stirred tank reactor after 116 hours of incubation.

The study done by Majoul *et al*. (1999), obtained the maximum viable cells of $3.25x10^6$ cells/ ml after 144 hours incubation time. These longer incubation time and higher viable cells might be due to the greater initial inoculants and availability of larger surface for cell growth.

4.1.4. Adaptation of cells in 2.5% serum concentration

Cells proliferation without any morphological changes was observed on cell lines as indicated in Appendix 2. The incubation time taken to subculture the cells was compared between 72 and 96 hours to select the better viable cells yield harvested during the experiment. Among those two incubation times 96 hours was the preferable time that resulted in maximum cells density count throughout the passages performed during incubation times. The yield of viable cells increased at first two passages and declined on passage three as shown in (Table 4), then increased until passage five to reach the maximum growth and decline gradually on last passages.

Table 4: Viable cells and incubation time of Vero cells proliferated in 2.5% serum supplemented medium before adaptation.

Passages	Incubation	viable cells $x10^6$/ml
1	72	2.25
2	72	2.41
3	72	2.58
4	72	2.52
5	72	2.65
6	72	2.28
7	72	2.33
1	96	2.50
2	96	2.63
3	96	2.38
4	96	2.52
5	96	2.75
6	96	2.44
7	96	2.42

The minimum and maximum viable cells at this serum concentration after 96 hours incubation period were $2.38x10^6$cells/ml and $2.75x10^6$cells/ml respectively. The cells were adapted through three consecutive passages and showed sufficient viable cells count as above serum concentration supplemented media. The cells proliferated in 2.5% serum supplemented medium

counted by Chen *et al.* (2011) and obtained the maximum viable cells of $2.70x10^6$ cells/ml at 96 hours incubation time. The study done by White and Ades, (1990) who grown the cells in 2% serum supplemented medium in micro-carriers bioreactors and obtained the maximum viable cells $2.80x10^6$cells/ml after 96 hours incubation. The growth condition of current study was T-flask that was not advanced for Vero cells proliferation, but the viable cells counted were greater than those grown in micro-carriers bioreactor. This indicated that growing of the cells adapted in this serum concentration in advanced growth mode might be resulted in higher viable cells. The viable cells counted in this serum concentration supplemented medium grown were not statistically significant ($P > 0.05$) in all passages.

4.1.5. Adaptation of cells in 1% serum concentration

This serum supplemented medium was the minimum concentration of serum to proliferate Vero cell lines in serum free medium that limit the side effect of this component of animal origin and allow the cells without serum to attain high viability. The incubation times of 144 hours resulted in greater viable Vero cells counted during this study. As showed in (Table 5) bellow, the viable cells counted increased until passage five and decreased gradually in the last passages. The use of 1% serum supplemented medium to proliferate Vero cells required special care because it was ready to transit to relatively new condition in growth medium in the absence of nutrient component of serum that facilitate to anchorage to surface. A small amount of serum is necessary for proper attachment of the cells before adaptation in serum free medium but after adaptation they anchored to the surface without the presence of serum in the growth medium. The minimum and maximum viable cells counted at preferred 144 incubation time were 2.29×10^6 cells/ml and $2.75x10^6$ cells/ ml respectively. The previous study carried out by Chen *et al*. (2011), obtained the maximum Vero cells number of $2.80x10^6$ at 168 hours incubation time. The results from current study and the previously reported by Butler *et al.* (2000), suggested that extending incubation time is important to obtain the maximum viable cells density in lower serum concentration. In the study done by Frazetti-Gallina and his coworkers record the maximum cells density of $1.60x10^6$ cells/ ml, through proliferation of the cells in 1% of Serum concentration supplemented medium Frazetti-Gallina *et al.,* (2001). This difference in result might be due to a lack of adaptation from serum containing medium without gradual adaptation and direct transfer of the cells into Serum free supplemented medium. This suggests that a gradual adaptation of

Vero cells in serum free medium is preferable to get the high yield of viable cells used for virus propagation.

Table 5: Viable cells and incubation time of Vero cells proliferated in 1% serum supplemented medium before adapted in T-flasks.

passages	incubation	viable cells $x10^6$/ml
1	120	2.23
2	120	2.35
3	120	2.33
4	120	2.56
5	120	2.60
6	120	2.42
7	120	2.29
1	144	2.35
2	144	2.53
3	144	2.67
4	144	2.63
5	144	2.75
6	144	2.43
7	144	2.38

4.1.6. Adaptation of cells in 0% serum concentration

The cells were fully adapted sequentially at zero serum concentration/serum free medium. In this 0% serum concentration incubation time was longer to reach the confluence stage relative to higher serum concentration supplemented medium. The viable cells counted in this serum free medium was increased up to the passages five and decreased gradually at both incubation times. To prevent the error due to initial inoculants at each subculture, cells were counted and homogenized cells density was transferred to undergo subculture. Adapted cells in this medium were very sensitive to any internal and external changes in their growth conditions such as changes in pH and temperature that affects their proliferation. Therefore, they need careful handling to reach confluence stage to be harvested. The proper proliferation stages of Vero cell

lines adapted in serum free medium to maximum cell yield were indicated on (Table 6) below, which was $2.86x10^6$ cells/ml after 144 hours incubation. The cell density and morphology did not changed in this sequential adaptation of Vero cell lines. Therefore, adapting and preserving Vero cells under proper conditions is very important for various biological usages. Relatively small difference in the maximal and minimal cell densities was obtained in both incubation times during the experiment. Among those incubation periods 144 hours was more preferable to harvest higher cells density with mentioned initial inoculants.

The minimum and the maximum cell densities obtained in this study were during passage seven of 120 hours incubation and passage five of 144 hours incubation time, which were $2.23x10^6$ cells/ml and $2.86x10^6$ cells/ml, respectively.

Table 6: Viable Vero cells adapted in 0% serum supplemented medium at different incubation time.

Passage	Incubation	viable cells cells $x10^6$/ml
1	120	2.25
2	120	2.54
3	120	2.45
4	120	2.49
5	120	2.75
6	120	2.44
7	120	2.23
1	144	2.35
2	144	2.55
3	144	2.56
4	144	2.56
5	144	2.86
6	144	2.52
7	144	2.35

The adapted viable cells recorded by LeFloch *et al.* (2006), was 1.80 $x10^6$ cells/ml after 110 hours incubation time this was slightly short incubation time and limited viable cells were

obtained as compared to current study. The result of current study showed slight variation with the finding obtained by Carter and Granchelli, (2012) who obtained maximum viable cell count of $2.58x10^6$ cells/ml for cells adapted to serum free medium. In contrast to other studies indicate the maximum viable cells count of $2.00x10^6$ cells/ml after 144 hours incubation periods reported by (Biaggio *et al.*, 2015). The study done by Rourou *et al.* (2007), obtained the maximum cells density of $2.23x10^6$ cells/ ml in VP-SFM after 120 hours incubation in bioreactor. Another finding by Litwin, (1992) record the maximum viable cells count of 2.50×10^6 cells/ml after 144 hours incubation in serum free condition. The investigation carried out by Chen *et al.* (2011), proliferated Vero cells in different serum free medium and got the highest viable cells $2.60x10^6$ cells/ml after 168 hours incubation in spinner flask micro-carrier cultures.

Important explanation for the observed change in cell morphology between serum free propagated and serum supplemented grown cells might be differences in cell handling during adaptation. The viable cells counted in current serum free adapted Vero cells compared with serum supplemented medium were not significantly different ($p > 0.05$), even though their incubation time were extended to get confluence. The cells proliferated in different serum free media gave growth results above 10^6 cells/ml after 96 hours incubation time (Litwin, 1992). In current study the cells grown in serum free media showed higher viable cells yield through seven consecutive passages. In contrast of the current study, Frazetti gallina and his colleagues (2000) indicated that cells propagated in serum free medium resulted in lower cell density, altered cells morphology and were detached from micro carrier.

4.1.7. Viable cells count in different serum supplemented medium

Proliferation of Vero cells line in various concentration of serum supplemented medium showed slight increment viable cells count at initial passages and decreased at higher passage levels. In all serum levels the viable cells counted were not statistically significant ($p > 0.05$). As shown in (Figure 1) below, growth of cells at different incubation period showed parallel pattern from initial to end passages, this indicated that they resulted in approximately similar pattern of viable cells yield. In this aspect the two lower and two higher incubation periods flow similar trends that make the preferable incubation time among both. Therefore, by observing this graph 96 and 144 hours incubation periods were very important to obtain higher yield viable Vero cell lines in their respective serum supplemented medium. The result of this study is in line with the result

reported by Rourou *et al.* (2009), who obtained the maximum viable cells after 144 hours incubation time in serum free medium on sixth passage. Rourou and his colleague, (2019) obtained the maximum viable cells after 96 hours incubation on eighth passage of serum supplemented medium. In contrast the study carried out by Kallel *et al.* (2002), got the maximum viable cells on 80 hours, which is lower than the result of the current study. The variation of incubation time and passages at which the maximum viable cells obtained might be the difference in initial inoculants and conditions used to grow the cells.

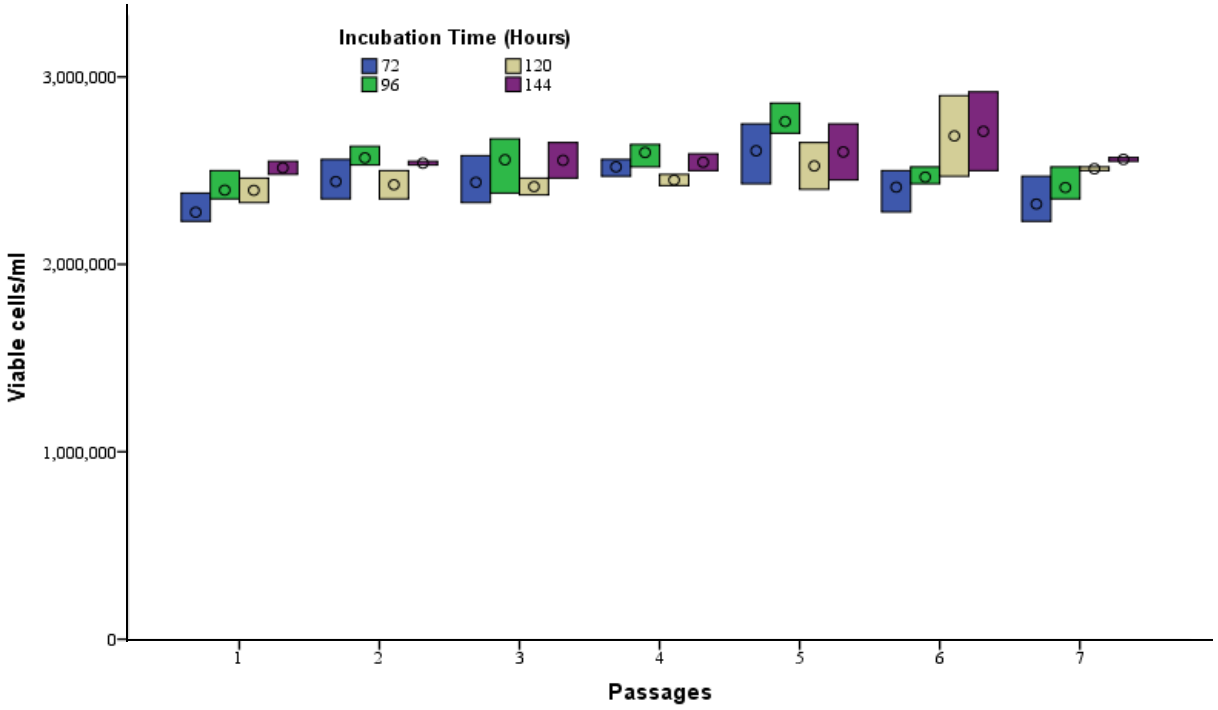

Figure 1: Viable Vero cells counted in different serum concentration supplemented medium.

4.2. Virus titer in cells grown in different serum concentration supplemented media

4.2.1. Titer of PV virus strains in Vero cell lines

Titration of PV virus rabies strains was carried out with various serum supplemented medium propagated Vero cell line as shown in (Table 7) below. The multiplicity of infection (MOI) used in this study was 0.01 to infect the cells for 72 hours in higher serum supplemented grown cells and 96 hours for lower serum concentrations. As indicated in table below the maximum virus titer at 72 and 96 hours incubation time was $10^{5.11}TCID_{50}$/ml and $10^{5.36}TCID_{50}$/ml respectively, this indicated that Vero cells proliferated in serum free medium slightly produced higher virus

titer than serum supplemented medium grown cells. Virus titer decrease with serum concentration until 5% serum and increased to serum free grown cells, this showed that rabies virus strain prefer the cells proliferate in serum free medium to propagate at higher titer and presence of serum in growth medium at higher concentration also hinders the virus titer. The cells used for infection in this study after they were adapted in respective serum concentration. The difference in the cell density used for virus infection didn't affect the titer since it was adjusted based on multiplicity of infection ratio. Therefore, Vero cells adapted in serum free medium produced the maximum PV virus strain titer compared to cells grown in serum supplemented medium, this indicated that virus prefer the cells grown in serum free medium to reach its optimum titer. The maximum titer of PV virus rabies strains obtained was $10^{5.36}$ $TCID_{50}$ /ml from serum free adapted cell line at 96 hours incubation. The virus titer obtained in this study is not significant ($p > 0.05$) among the virus propagated in serum supplemented and serum free medium grown cells, Even though the incubation time was longer, virus titer obtained in Vero cells proliferated in serum free medium was higher than the cells grown on serum supplemented medium. Previously study done by Birhanu Hurisa *et al.* (2013), obtained the maximum titer of $10^{7.5}TCID_{50}$ /ml after 72 hour incubation, through growing of the cells in serum supplemented medium in roller bottle growth technique, this might be the reason for the variation titer obtained in current study and these researchers.

Table 7: Titration of PV viruses in different serum supplemented medium grown Vero cell lines

Virus	Medium	Serum conc. (%)	Infected (cells/ml x10^6)	Incubation time (Hours)	Virus titer($TCID_{50}$/ml)
PV	MEME	10	2.42	72	$10^{5.11}$
		7.5	2.60	72	$10^{4.61}$
		5	2.33	72	$10^{4.46}$
		2.5	2.67	72	$10^{4.61}$
		1	2.56	96	$10^{5.11}$
		0	2.35	96	$10^{5.36}$

MEME- Minimal Essential Medium Eagle, ml- milliliter, PV- Pasteur Virus, MOI-Multiplicity of Infection, TCID-Tissue Culture Infectivity Dose

The result of current study is similar with the investigation carried out by Moura *et al.* (2008), who report the maximum virus titer of $10^{5.35}TCID_{50}$ /ml from the cells grown in serum free medium after 120 hours incubation time, that was the long incubation time considered to obtain high virus titer as compared to current study. Other investigations carried out by Carlos, (2013) and Kolell *et al.* (2007), obtained the virus titer of $10^{5.37}$ $TCID_{50}$/ml at 120 hours incubation in serum free adapted cells. The result obtained in current study showed that the higher virus titer record in serum free adapted cells which met the goal of harvesting sufficient virus for various biological products. The virus titer recorded in this study was also slightly close with the result obtained by Frazatti-gallina, (2015) that was $10^{5.5}$ $TCID_{50}$/ml for cell line grown on serum free media after 96 hours incubation. The result of this study used to propagate the PV rabies virus that is essential for human vaccine production with limited cost and great quality.

The study carried out by Perrin *et al.* (1995), PV virus harvested after the 120 hours incubation time in serum free medium was $10^{8}TCID_{50}$/ml in bioreactors for the production of experimental rabies vaccines. This variation might be due to advanced method to produce the virus and the extended incubation time to harvest the virus. The amount of rabies virus titer obtained based the cells density available for infection Trabelsi *et al.* (2005), the cells density achieved in all serum concentration, in current study was sufficient for virus infection and adjusted based on virus multiplicity of infection.

4.2.2. Titer of ERA rabies virus strains in Vero cell lines

As shown in (Table 8) below Vero cells grown in different serum supplemented medium were infected by the ERA rabies virus strains with 0.001 multiplicity of infection. In this study the maximum titer obtained was $10^{5.61}TCID_{50}$ /ml in serum free medium adapted Vero cells after 120 hours incubation, this suggest that virus prefer the cells grown in serum free to proliferate to reach its optimal titer. This indicated that ERA rabies strains virus infectivity is enhanced as the cells are grown in serum free medium. In previous study carried out by Birhanu Hurisa *et al.* (2013), the maximum titer of ERA strain rabies viruses was $10^{7.25}$ TCID50/ml after 96 hours incubation in serum supplemented medium grown cells propagated virus. The result obtained in this study showed slightly close with the result of a study done by Carlos, (2013) who got the maximum virus titer of $10^{5.32}TCID_{50}$/ml after 72 hours incubation. Other investigation done by Peng-Cheng *et al.* (2018), who record the maximum rabies virus titer of $10^{5}TCID_{50}$/ml after 120

hours incubation. Therefore multiplication of rabies virus in this medium for vaccine and different biological production is very important to increase the quality of the products. At the lower and serum free adapted cells incubation time was extended due to slow growing of the cells. The cells used for infection were taken after fully adapted in their respective serum supplemented medium and adjusted based on the MOI ratio and the respective medium. These results indicated that Vero cells proliferated in serum free medium produced the higher titer that can be used to solve the problems faced due to serum and rabies virus availability used to produce vaccines. Titration of ERA virus in different serum supplemented medium grown Vero cell lines.

Table 8: Titration of ERA viruses in different serum supplemented medium grown Vero

Virus	Medium	Serum conc. (%)	Infected (cells/ml x10^6)	Incubation time(Hours)	Virus titer($TCID_{50}$)/ml
ERA	MEME	10	2.55	96	$10^{5.11}$
		7.5	2.51	96	$10^{4.36}$
		5	2.50	96	$10^{4.61}$
		2.5	2.42	96	$10^{5.11}$
		1	2.56	120	$10^{4.86}$
		0	2.55	120	$10^{5.61}$

DMEME- Minimal Essential Medium Eagle, ERA-Evenly Roktincki Abelseth, ml – milliliter, MOI – Multiplicity of infection, TCID – Tissue culture infectivity dose, %-Percent

5. CONCLUSIONS AND RECOMMENDATION

5.1. Conclusions

Adaptation of Vero cell lines through reduction of serum in growth medium showed normal cells morphology and viability in all stages. The virus titers in each serum concentration medium grown Vero cells were little variation as compared to serum supplemented cells. Both rabies virus strains (ERA and PV) showed the maximum virus titer in serum free grown Vero cells that was higher than serum supplemented medium, this indicated that the serum free adapted Vero cells sensitivity to these virus strains was increased as serum concentration decreased in growth medium. Gradual reduction of serum concentration in growth medium for Vero cell lines didn't affect the cells density and virus titer yielded in different serum supplemented medium. Except for the serum concentration of 7.5% all serum supplemented media proliferated Vero cells maximum viability was obtained at passage five. There were two incubation times considered in this study to grow the cells in each serum concentrations, the preferable incubation times were 96 and 144 hours for serum concentrations of 10% to 2.5% and 1% to 0% respectively that resulted in higher viable cells. Therefore, the time required to the cells to reach the confluence stage was higher in serum free medium adapted Vero cells to harvest the cells for rabies virus production. Generally it is concluded that through gradual adaptation the viable counted Vero cells in serum free medium and rabies virus strains propagated were sufficient for vaccine production for human as well as animals use.

5.2. Recommendations

In recent years, the use of serum supplemented medium is switched to serum free medium to produce safe, effective and affordable vaccines; therefore the possible area for further research would be the production of rabies vaccines from virus propagated on adapted cells and its quality control tests. Besides to this it is important to investigate further improvements required to enhance the both strains of rabies virus titers through several consecutive passages on serum free medium. This recommended and low cost method used for rabies vaccine production could be suitable for countries like Ethiopia where rabies is an important health problem and lacks sufficient modern cell culture based vaccine to prevent the disease. Additionally rabies vaccines produced from this adapted Vero cells immunogenicity should be checked and compared with standard vaccine. The cells metabolic activities like; glucose, lactate and ammonia levels of the adapted cells should be relevant to investigate to determine the utilization of the nutrients and physiological variability of the cells.

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7. APPENDIX

Spearman-Kärber formula

Titer = $\left[Xo\text{-}d/2+d\sum ri/ni \right]$

Where:

x_0= ($\log_{10}$ 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.

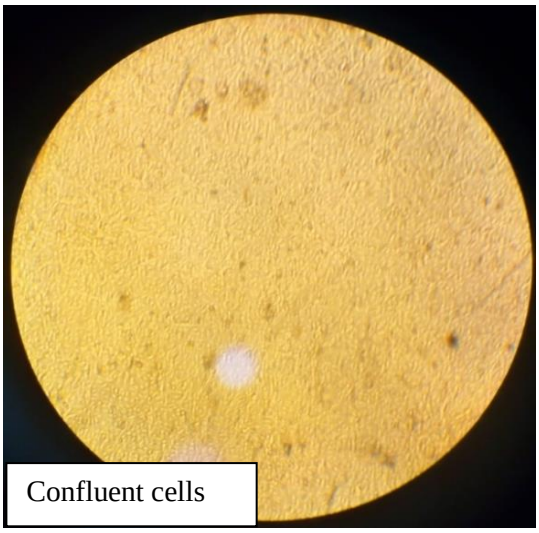
Titration of virus in $TCID_{50}$ (50% tissue culture infective dose)

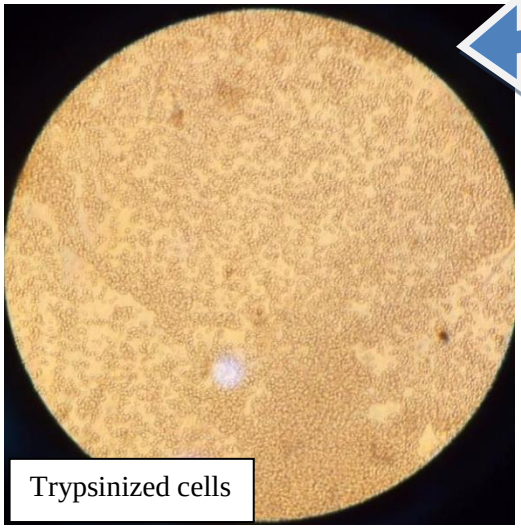
This titration method uses Vero cell lines in microtiter plates.

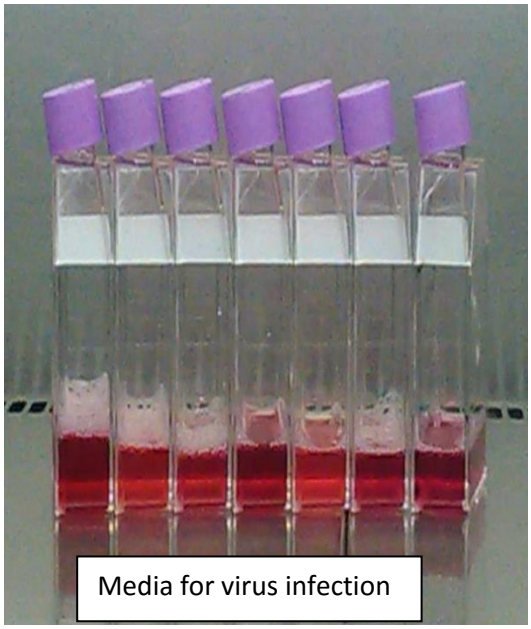
- ✓ *Cell suspension:* the day before titration, a cell suspension containing 10^5 cells/ml is prepared in cell culture medium containing 10% heat-inactivated FCS, and is distributed, 200 µl per well, into 96-well microtiter plates. The plates are then incubated for 24 hours at 35.5°C–37°C with 5% CO_2.
- ✓ *Dilution of the virus:* the serial dilutions are performed in 5 ml tubes using a cell culture medium without FCS as diluent. Ten-fold dilutions from 10^{-1} to 10^{-12} are prepared (0.9 ml of diluent with 0.1 ml of the previous dilution).
- ✓ *Infection of the cells:* the medium in the micro titer plates is discarded using an aspiration system. Fifty µl of each virus dilution is distributed per well. Six replicates are used per dilution. The microtiter plate is then incubated for 1 hour at 35.5–37°C with 5% CO2. Then 200 µl of cell culture medium, containing 5% FCS, is added.
- ✓ *Incubation: incubate for 3 days at 35.5–37°C in 5% CO_2.*

- ✓ *Staining and calculation of titer:* 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 titer calculation is made using the Spearman–Kärber formula (WHO, 1996).

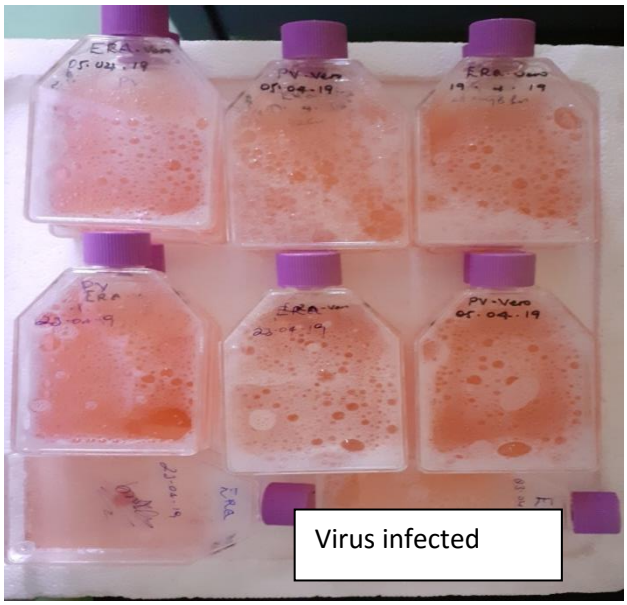
Pictorial presentations





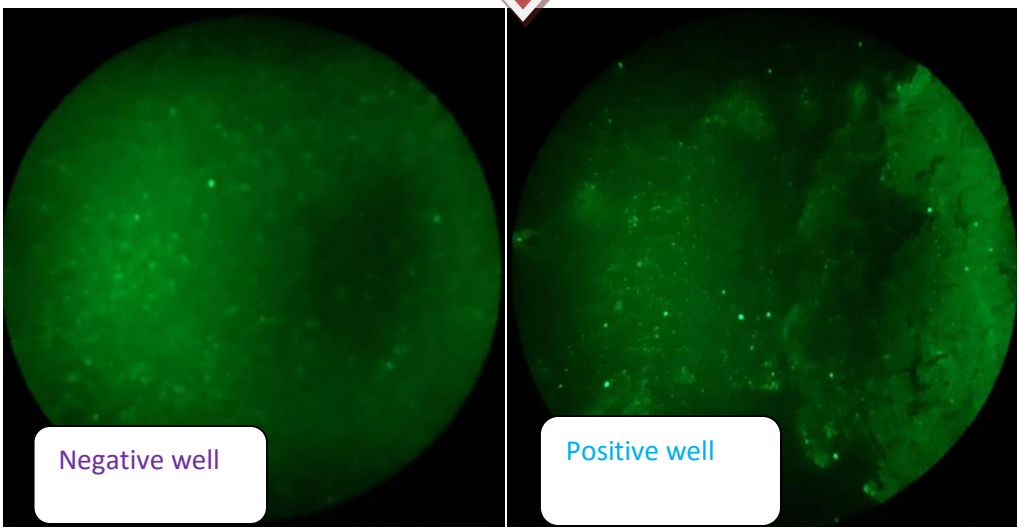


Media for virus infection



Virus infected

Virus titration test


Negative well
Positive well