**ADDIS ABABA UNIVERSITY COLLEGE OF VETERINARY MEDICINE AND AGRICULTURE**

**MVSc THESIS ON**

# PRODUCTION OF MONOCLONAL ANTIBODY FOR LUMPY SKIN DISEASE, SHEEP POX, AND GOAT POX VIRUSES TO DEVELOP ENZYME-LINKED IMMUNOSORBENT ASSAY

**BY:**

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**JUNE, 2024**

**BISHOFTU, ETHIOPIA**

# PRODUCTION OF MONOCLONAL ANTIBODY FOR LUMPY SKIN DISEASE, SHEEP POX, AND GOAT POX VIRUSES TO DEVELOP ENZYME-LINKED IMMUNOSORBENT ASSAY

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

**By**

**Kalkidan Asnake Mekuria**

**JUNE, 2024**

**BISHOFTU, ETHIOPIA**

**Addis Ababa University**

**College of Veterinary Medicine and Agriculture,**

**Department of Microbiology, Immunology and Veterinary Public Health**

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**PRODUCTION OF MONOCLONAL ANTIBODY FOR LUMPY SKIN, SHEEP POX, AND GOAT POX VIRUSES TO DEVELOP ENZYME-LINKED IMMUNOSORBENT ASSAY**

**Submitted by:** Kalkidan Asnake _____ 06-06-2024

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**Addis Ababa University**

**College of Veterinary Medicine and Agriculture,**

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As member of the Examining Board of the final MSc open defense, we clarify that we have read and evaluated the thesis prepared by: **Production of monoclonal antibody for lumpy skin disease, sheep pox, and goat pox viruses to develop Enzyme-linked immunosorbent assay**, and recommended that it be accepted as fulfilling the thesis requirement for the degree of Master of Science in Veterinary Microbiology.

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## ANIMAL USE ETHICAL CLEARANCE



This is to certify that the under-mentioned project proposal has been cleared and approved by the AU-PANVAC Animal Welfare and Ethics Committee, at its meeting held on November 01, 2023, at AU-PANVAC, Debre Zeit, Ethiopia.

**Project Title:** Production of monoclonal antibody for lumpy skin, sheep pox, and goat pox viruses to develop an enzyme-linked immunosorbent assay.

**Name of the investigator:** Kalkidan Asnake Mekuria

**Project Supervisor:** Dr. Jean de Dieu BAZIKI

**Species and No. of animals used:** 12 mice

**Name of the institution:** AU-PANVAC

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**STATEMENT OF AUTHOR**

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

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## LIST OF ABBREVIATIONS

| | |
|---|---|
| µg | Microgram |
| A | Adenine |
| AB | Antibiotic |
| AF | Antimycotic |
| Ag | Antigen |
| AGID | Agar Gel Immunodiffusion |
| ANK | Ankyrin |
| AU-PANVAC | African Union Pan African Veterinary Vaccine Centre |
| BB | Blocking Buffer |
| BCA | Bicinchoninic Acid |
| CaPV | Capripoxvirus |
| CFSPH | Center for Food Security and Public Health |
| $CO_2$ | Carbon dioxide |
| CPE | Cytopathic Effect |
| CSA | Central Statistical Agency |
| DNA | Deoxyribonucleic Acid |
| EDTA | Ethylene Diamine Tetra acetic Acid |
| EFSA | European Food Safety Authority |
| ELISA | Enzyme-Linked Immunosorbent Assay |
| FAT | Fluorescence Antibody Test |
| FBS | Fetal Bovine Serum |
| GMEM | Glasgow modified Eagle Medium |
| GPCR | G-Protein coupled Chemokine Receptor |
| GTP | Goatpox |
| GTPV | Goatpox virus |
| HCl | Hydrochloric acid |
| HF | Holstein Friesian |
| HGPRT | hypoxanthine-guanine phosphoribosyltransferase |

| | |
|---|---|
| hr | Hour |
| HRP | horseradish peroxidase |
| iELISA | Indirect ELISA |
| IFN-γ | Interferon gamma |
| IL | Interleukins |
| IL-1R | Interleukin-1 receptor |
| ISO | International Organization for Standardization |
| ITR | Inverted Terminal Region |
| Kbp | kilobase pair |
| KD | kilo Daltons |
| KSGPV | Kenyan sheep and goat pox virus |
| LSD | Lumpy skin disease |
| LSDV | Lumpy skin disease virus |
| LT | Lamb testicle |
| M | Molarity |
| mAbs | Monoclonal antibodies |
| MDBK | Madin-Darby bovine kidney |
| Min | Minute |
| ml | Milliliter |
| MOA | Ministry of Agriculture |
| mRNA | Messenger RNA |
| nm | Nanometer |
| OD | Optical-density |
| OIE | World Organisation for Animal Health |
| Orf | Contagious ecthyma |
| PBS | Phosphate Buffered Saline |
| PBS-T | PBS-Tween |
| PCR | polymerase chain reaction |
| PCR-RFLP | PCR-Restriction Fragment Length Polymorphism |
| PEG | Polyethylene glycol |
| pH | Potential of Hydrogen |

| | |
|---|---|
| QMS | Quality Management System |
| RNA | Ribonucleic acid |
| RPMI | Roswell Park Memorial Institute Medium |
| RT | Room temperature |
| SGP | Sheep and Goat Pox |
| SNNP | Southern Nations, Nationalities, and Peoples |
| SPP | Sheeppox |
| SPPV | Sheeppox virus |
| T | Thymine |
| TBE | Tris/Borate/EDTA |
| TEM | Transmission electron microscopic |
| TMB | Tetramethylbenzidine |
| UK | United Kingdom |
| USA | United States of America |
| USDA | United States Department of Agriculture |
| VNT | Virus Neutralization Test |
| WOAH | World Organisation for Animal Health |

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**ABSTRACT**

Lumpy skin disease (LSD), sheeppox (SPP), and goatpox (GTP) are economically significant pox disease of ruminants, caused by lumpy skin disease virus (LSDV), sheeppox virus (SPPV), goatpox virus (GTPV), respectively. The new emergence of disease caused by capripoxviruses and spreading outside of their endemic regions, stressing the urgent need to develop high-throughput serological surveillance tools. This experimental study was conducted to produce Monoclonal antibodies (mAbs) against LSDV, SPPV, and GTPV to development ELISA assay. The study was conducted in African Union Pan African Veterinary Vaccine Centre (AU-PANVAC), from October 2023 to May 2024. The mAbs were produced through immunization of BALB/C mice with purified antigen of LSD, SPP, and GTP, with consecutive booster injection. A hybridoma technology was used to produce the hybridoma clones (LC 5.14 and LC 5.4) which were subsequently mass-produced and tested. These LC 5.14 and LC 5.4 mAbs were precipitated and then quantified through the Bicinchoninic Acid protein assay kit. The isotype for the two mAbs were determined through Pierce™ Rapid Antibody Isotyping Kit, and both mAbs were IgG1. The cross-reaction of the two mAbs with GTPV and SPPV Ag were studied and those two mAbs were cross-reacted with the GTPV Ag but not with the SPPV Ag LC 5.14 and LC 5.4 mAbs were then conjugated with horseradish peroxidase enzyme; hence enzyme linked mAbs were used for ELISA development. The dot blot test was conducted by using of the LSDV, SPPV, and GTPV Ags with the two conjugated mAbs on the nitrocellulose membrane. Finally, the conjugated mAbs (LC 5.14 and LC 5.4) were titrated to determine the concentration which is required for ELISA test, and the titer of 1/10 for LC 5.14 and titer of 1/5 for LC 5.4 were determined to use for ELISA assay. Further repeated tests with the positive serum from immunized cattle, sheep, and goat and the negative serum from the same species of animals are required to produce the validate kit which can be used as a diagnostic purpose for the capripoxviruses.

**Keywords:** *ELISA, GTPV, LSDV, mAbs, SPPV*

# 1. INTRODUCTION

Lumpy skin disease virus (LSDV), sheeppox virus (SPPV) and goatpox virus (GTPV) are the three members of the genus *Capripoxvirus* (CaPV), one of the largest (170-260 nm by 300-450 nm), enveloped double stranded DNA viruses (Matthews, 1982), within the *poxviridae* family. Despite the high nucleotide sequence similarity (96–97%) that these three viruses share (Tulman *et al*., 2001; Tulman *et al*., 2002), they exhibit specific host preference (Lamien *et al*., 2011; Le Goff *et al*., 2009). SPPV and GTPV can infect sheep and goats, causing sheeppox (SPP) and goatpox (GTP), respectively. Some strains exhibit preferences for either one or the other species, even though the majority of them cause disease in both sheep and goats (OIE, 2017; Tulman *et al*., 2002). Likewise, LSDV causes lumpy skin disease (LSD) in cattle and buffaloes (Tuppurainen and Oura, 2012).

LSD, SPP, and GTP are categorized by the World Organization for Animal Health (WOAH) as notifiable diseases (OIE, 2020). All this three CaPVs species cause high consequence transboundary diseases in ruminants, resulting in substantial production losses (Bolajoko *et al*., 2019; Casal *et al*., 2018; Limon *et al*., 2020; Molla *et al*., 2017).

SPP and GTP diseases results in substantial loss in the production and productivity of sheep and goats in Ethiopia. Ethiopia is believed to have the largest livestock population in Africa and has an estimated 39.89 million sheep, 50.50 million goats, and 65.35 million cattle (CSA, 2020). Livestock production has massive potential to achieve several of Ethiopia's national and international assurances on poverty mitigation, food security, and improved nutrition. The economic contribution of the livestock subsector in the country is approximately 45% of the total worth of agricultural production (FAO, 2019). The introduction of foreign sheep and goat breeds as well as the growth of intensive livestock production are severely hampered by this Sheep and Goat pox diseases. The economic losses from SPP and GTP diseases result from decreased milk production, and damage to the quality of skins. Furthermore, the diseases presence negatively impact in the trade of

animals and animal products, which reduces revenue from exports earnings (Hurisa *et al*., 2018).

LSD is characterized by a high fever, swollen lymph nodes, firm and circumscribed nodules, is one of the most economically significant viral diseases (OIE, 2010). LSD has a high morbidity and low mortality rate. It causes significant economic losses as a result of reduced milk production, beef production loss, draft power loss, abortion, infertility, and damage to the hide (CFSPH, 2008).

Currently there are a number of molecular diagnostic assays like (conventional gel-based PCR, real-time PCR, *orf068* gene-based recombinase polymerase amplification assay (RPA) (Khalafalla, 2022), which are used for the identification and characterization of capripoxviruses (Balinsky *et al*., 2008; Chibssa *et al*., 2019; Gelaye and Lamien., 2019; Lamien, *et al*., 2011; Le Goff *et al*., 2009; Murray *et al*., 2013; Stram *et al*., 2008; Settypalli *et al*., 2016). In contrast, only a few serological assays have been described. All of them present several drawbacks. For instance, the virus neutralization test (VNT) for anti-CaPV antibody detection which is recommended by WOAH (OIE, 2018), do have lots of drawbacks like the test requires large amounts of sera, is not easy to implement, and its results can be difficult to read (Tuppurainen and Oura, 2012). In addition, VNT also requires the use of live viruses, which limits its use to BSL-3 facilities in capripox non-endemic countries or in well-equipped virological laboratories in endemic countries. The indirect fluorescent antibody test (IFAT), also on the WOAH list of available serological tests, cross-reacts with orf (contagious pustular dermatitis) in sheep and other poxviruses. Serological capripoxvirus antibody identification can also be accomplished using Western blot and agar gel immunodiffusion assays; however, the Western blot is more costly to perform and less practical for high-throughput testing, while the agar gel immunodiffusion assays cross-react with antibodies against parapoxviruses (OIE, 2018).

Antibodies are mainly produced for diagnostic and therapeutic applications. Hybridoma technology is one of the most common methods used to produce monoclonal antibodies (mAbs). In 1975, Kohler and Milstein discovered a technique called hybridoma technology

for the production of mAbs. In this procedure, after immunizing mice with a specific antigen, antibody-producing B lymphocytes are extracted from the animals and fused with immortal myeloma cell lines to generate hybrid cells, which are also known as hybridoma cell lines. In order to generate mAbs against a specific antigen, these hybridoma cells are cultured in a laboratory. It is preferred method to produce monoclonal antibodies because antibodies thus produced are of high purity and are highly sensitive and specific (Mitra and Tomar, 2021).

The production of the mAbs against LSDV, SPPV, and GTPV have an advantage of brooding the spectrum of serological tests for the CaPV through development of new assay kit by using of those highly purified, sensitive and specific mAbs. So, by using of those mAbs as serological diagnostic tool for LSD, SPP, and GTP diseases, the surveillance and control measures can implement easily, and at the same time mAbs be an additional tool for the study of the immune response against these three CaPVs.

Having all these facts in mind the objectives of the present study were:

- To produce and characterize mAbs against LSDV, SPPV, and GTPV
- To develop an ELISA test through conjugation of those produced mAbs with horseradish peroxidase (HRP) enzyme

# 2. LITERATURE REVIEW

## 2.1. Etiology

Lumpy skin disease, Sheep pox, Goat pox, and belongs to the genus CaPV, in the subfamily *Chordopoxvirinae* and *Poxviridae* family (King *et al*., 2011). The LSDV of cattle is closely related to the SPPV and GTPV, which primarily infect sheep and goats, respectively. The genome size of CaPV is relatively constant and it is around 150 kbp. Furthermore, the genome comprises large, complex, brick-shaped, double-stranded DNA (Biswas *et al*., 2020). CaPV have the highest A-T content which is about 73-75% of the genome and this gives the virus characteristics to undergo an extensive cross hybridization between the species of the virus (Santhamani *et al*., 2014).

The genome of CaPV contributes approximately 150 putative genes, together with conserved replicative and structural genes, as well as conserved virulence and host range-related genes. The majority of the genes in CaPV's central genomic region are involved in replicative processes, whereas the genes in the terminal area are involved in pathogenesis and host range functions (Zeng *et al*., 2014). The replication of CaPV occurs in the cytoplasm of infected cells rather than in the nucleus (Schramm and Locker, 2005). CaPV's DNA is surrounded by a false lipid envelope that is susceptible to damage from a variety of acids and disinfectants (Hosamani *et al*., 2004).

One of the variable genes within the CaPVs is the homolog of the G-protein-coupled chemokine receptor (GPCR) gene, which is utilized to genetically distinguish CaPV members (Tulman *et al*., 2001; Le Goff *et al*., 2009). The nucleotide sequences of CaPVs are extremely conserved, with over 95% homology found between LSDV, SPPV, and GTPV. LSDV is genetically and antigenically closely related to a strain of SPP and GTP virus (Kara *et al*., 2003). LSDV has an additional nine genes that are non-functional in SPP and GTP virus, some of which are likely responsible for their ability to infect cattle (Tulman *et al*., 2001).

There is only one serotype of LSDV which is the Neethling virus and it is closely related antigenically to SPP and GTP viruses. The LSDV primarily affects cattle but can affect sheep and goats, experimentally (Gelaye *et al*., 2015). LSDV is an envelope, linear ovoid shape with a molecular brick shaped or ovoid virions measuring 220-450 nanometer (nm) by 140-266nm and has double-stranded DNA genome of about 151kbp (Stram *et al*., 2008).

The LSDV full-genome sequencing has 156 putative genes inside a core coding region that is bounded by identical 2.4 kbp-inverted terminal repeats (ITR) (Tulman *et al*., 2001). These putative genes encode a number of pox viral proteins which are known to be structural or involved in virion morphogenesis and assembly. The terminal genomic sequences contain a unique complement of at least 34 genes which are responsible in virulence, host range and immune evasion (Johnston and McFadden, 2003; Kara *et al*., 2003). LSDV also have complemented of genes such as Interleukin-10 (IL-10), interferon gamma (IFN-γ) receptor; Interleukin-1 receptor (IL-1R), Interferon-alpha/beta receptor (IFN-α/β) binding protein and Interleukin-18 (IL-18) (binding protein) are secreted and responsible for modulation or evasion of host immune response, inhibition of host cell apoptosis and in cell or tissue tropism (Fleming *et al*., 2000; Lalani *et al*., 1999).

LSDV is susceptible to sun light and detergents containing lipid solvents. After heating the virus for an hour at 55°C, the virus could become inactive (Davies and Otema, 1981). However, it can withstand drying and pH variations up to extreme pH changes and survive for months in a dark area. This virus can also persist in skin plugs for about 42 days (Babiuk *et al*., 2008).

SPP and GTP viruses are large (170–260 nm by 300-450 nm), double stranded genomic DNA with about 150kbp size containing less variable central region bounded by two identical inverted terminal repeats (ITR) at the ends (Tulman *et al*., 2002). The genome of SPP and GTP viruses contains at least 147 putative genes (Muhaidi *et al*., 2018). Virons are brick shaped, enveloped with complex symmetry and about 300x270x200 nm in size. The 147 putative genes shared by viruses encode proteins ranging in size from 53 up to 2,027 amino acids, which are probably involved in structure, replication, pathogenicity,

and host range (Zhao *et al*., 2017). Over the course of their entire genomes, the SPPV, GTPV, and LSDV show 96% amino acid and nucleotide identity; nevertheless, the nine LSDV genes with probable virulence and host range function are disrupted in the genome of SPPV and GTPV. While it is likely that both SPPV and GTPV descended from an LSDV-like ancestor, they differ in specific nucleotides, and it suggests that both viruses are phylogenetically distinct (Madhavan *et al*., 2016).

Generally, SPP and GTP viruses will be inactivated at 56°C within two hour or at 65°C within thirty minutes. They can survive at a pH between 6.6 and 8.6 but, they are susceptible to highly acidic or alkaline pH, for example, 2% HCl or $H_2SO_4$ can completely destroy these viruses within 15 min (OIE, 2014). They can persist for long period of time in suitable environmental condition like in scab and hair or wool of the animals for 3 months. However, they are susceptible to sunlight (Hopker *et al*., 2019).

### 2.2. Epidemiology and Geographical Distribution

LSD has a different geographical distribution (OIE, 2010). The disease was first observed in 1929 in Zambia. At first it was thought to be the result of either poisoning or a hypersensitivity to insect bites because at that time was the year with the highest populations of biting insects (Bagla, 2005). LSD cases were reported in Botswana, Zimbabwe, and the Republic of South Africa between 1943 and 1945. The infectious nature of the disease was recognized at this time (OIE, 2008). From 1929 until 1986, LSD was restricted to countries in sub-Saharan Africa but after that it become endemic in the majority of African countries, including of Madagascar (Zeynalova *et al*., 2016). LSD was first diagnosed outside of Africa in Israel in 1989. Cases were then recorded in Bahrain, Kuwait, Oman, Yemen, Lebanon, and Jordan in subsequent years. In 2006, the disease was re-introduced into Egypt through imported cattle from East Africa, and subsequently emerged throughout the Middle East (Alkhamis and VanderWaal, 2016; Al□Salihi, and Hassan, 2015; Tuppurainen *et al*., 2017). This followed by reports of the virus in the Middle East since 2012 (Gumbe, 2018). LSD has been spreading on an unusually large scale throughout Middle Eastern countries as subsequently years was reported from Oman,

Yemen, Israel, Kuwait, Bahrain, Egypt, Iran, Saudi Arabia, Lebanon, Jordan, and in United Arab Emirate (Zeynalova *et al*., 2016). Since 2015 widespread LSDV outbreaks had occurred across several eastern European countries like Russia, Turkey, Greece, Albania, Bulgaria, Montenegro, Serbia, and Macedonia (Alkhamis and VanderWaal, 2016; Al Salihi and Hassan, 2015; Tuppurainen *et al*., 2017).

In Ethiopia, LSD was spread to almost all the regions and agro-ecological zones (Gari *et al*., 2010). According to data analysis conducted from the national disease outbreak report database between 2000 and 2009, there were significant LSD epidemic outbreaks in the Amhara and West Oromia regions of the country in 2000 and 2001. Then, in 2003 and 2004, it spread to the country's central and the southern parts, covering large parts of Oromia and the Southern Nation, Nationalities, and Peoples (SNNP) regions. In 2006 and 2007 another extensive outbreak reappeared in Tigray, Amhara and Benishangul regions in the northern and north-western parts of the country. Consequently, between 2007 and 2009, the number of outbreaks in the center Oromia Region steadily increases, whereas in the northern areas of Tigray, Amhara, and Benishangul, the outbreaks appeared to be gradually declining (Gari, 2011).

Distributions of the LSD vaccination increased year between 2014 and 2018. In 2018, the highest number of LSD vaccines was distributed within the country to control the disease; this trend has showed that LSD outbreaks are dramatically increasing in Oromia, SNNP, Amhara, and Tigray regions in every year. The majority of LSD vaccine sales in Ethiopia have been regularly associated with disease outbreaks (Zewdie, 2022).

Small ruminant CaPV infections are more widespread than LSD and have a global distribution. Numerous studies and reports suggest that SPP and GTP viruses are highly distributed in northern and central Africa, the Middle East, Europe and Asia (Tuppurainen *et al*., 2017). Sporadic outbreaks of SPP and GTP have been reported in Southern Europe (Rao and Bandyopadhyay, 2000). During 2013, the disease outbreaks were recorded in Bulgaria and Greece, Israel in 2014, and Russia and Mongolia in 2015 (Tuppurainen *et al*., 2017). In addition, the disease has been reported in the Caucasus region, Kazakhstan

and Kyrgyzstan, but American and Australia are free from CaPV virus infections (Babiuk *et al*., 2009; Gu *et al*., 2018).

In Ethiopia, SPPV and GTPV are highly distributed in all regions of the country and cause huge production losses and mortality (Yune and Abdela, 2017). But very limited research has been conducted on SPP and GTP viruses in selected parts of the country (Gari *et al*., 2015). However, the diseases have a widespread distribution in all regional states of the country and affect the production and productivity of the subsector in the country. In the Gondar veterinary clinic, the prevalence was 40% in sheep and 8.12% in goats (Molla *et al*., 2017a); in the Gamo Gofa zone of the country, the prevalence was 31.96% in sheep and 35.28% in goats (Kebede, 2018). Studies on the seroprevalence of SPP and GTP diseases have recently been conducted. However, the spatiotemporal clustering of SPP and GTP incidence rates has not been studied (Aregahagn *et al*., 2021).

### 2.3. Mode of Transmission

There was no clearly denied method for the transmission of LSD, but there were indications from circumstantial evidence that the disease could be spread by biting insects (Sameea *et al*., 2017). As of right now, it is widely acknowledged that arthropod vectors are the primary means of transmission of LSDV, although direct or indirect contact between infected and susceptible animals or using contaminated objects or contaminated feed and water is also another way for the transmission. The virus was then extracted from arthropod vectors and the role of vectors in transmission of the virus is experimentally confirmed (Tuppurainen, 2015). Three bloods sucking arthropods which are hard tick species, the mosquitoes *Aedes aegypti*, and the lice *Stomoxys calcitrans*, have been reported to involve in the transmission of LSDV in sub-Saharan Africa (Lubinga *et al*., 2014). In most of sub-Saharan Africa, LSD outbreaks are associated with the rainy season due to the abundance of arthropod vectors (Davies, 1991). However, the incidence drops during the dry and cold weather seasons. In Ethiopia, higher prevalence of LSD has been related with risk factors like warm, humid agro-climate and abundance of vector population (Gari *et al*., 2010). This three tick species *Rhipicephalus (Boophilus) decoloratus* (blue tick), *Rhipicephalus*

*appendiculatus* (brown ear tick) and *Amblyomma hebraeum* have been identified as vectors of the disease and also act as reservoirs for the virus (Lubinga *et al*., 2014). Secretions of the infected animals, like blood, milk, semen, saliva, and nasal and lachrymal secretions, which consists of the virus can also spread the LSD infection (Babiuk *et al*., 2008). Experimentally, LSDV has been isolated from infected semen (Irons *et al*., 2005). Similarly, it has been proven that LSDV is transmitted to heifers through semen (Annandale *et al*., 2014). LSD is also transmissible to suckling calves through infected milk (Gumbe, 2018).

Direct contact with contaminated respiratory droplets or indirect contact through contaminated environments and vectors are the main ways for transmission of SPPV and GTPV (Sprygin *et al*., 2019). Experimentally, sheep and goats have been infected with intradermal (Bowden *et al*., 2008) and intranasal inoculation of the respective viruses (Balinsky *et al*., 2007). According to Bhanuprakash *et al*., (2006) the SPP and GTP viruses are mechanically transmitted by stable flies. The high virus concentrations in the skin scab could potentially contribute to the spread of SPP and GTP through insect vectors. Although there is no transmission detected with biting lice (Mallophaga species) or sucking lice (Damalinia species), there is evidence that stable flies (Stomoxys calcitrans), Tsetse fly, and musca species can act as an effective mechanical vector of SPPV and GTPV. The virus is spread by flies to susceptible sheep and goats and some flies can carry the virus for up to four days (Ebissa and Waktole, 2020). High virus titers, resistance of the virus, vectors with large mouthparts and their frequent feeding habits are the basic factors favoring mechanical transmission of SPP and GTP (Rodistits *et al*., 2007; Tuppurainen *et al*., 2017). The virus can infect feed, water, wool, and the environment through saliva, ocular and nasal discharge, skin sores and scabs, urine, and feces (Ebissa and Waktole, 2020). This can result in an indirect transmission of the virus either oral or via cutaneous abrasions. The infectious virus is well-protected inside the scabs which are shed by infected animals. When scabs dissolve the virus may be released into the environment and this may continue for several months after the outbreak but, there is no report that have been published on survival of SPPV or GTPV in litter, fodder and feed (EFSA, 2014).

### 2.4. Morbidity and mortality rates

In outbreaks of LSD, morbidity and mortality of the disease varies extremely depending on the ages, breeds, geographic location, and climatic condition, and virus virulence, immunological status of the host and the abundance of arthropod vectors (Tuppurainen and Oura, 2012). Naturally, the European breed *Bos taurus* is more susceptible than the local Zebu breed *Bos indicus*, and cows at peak lactations are usually the most severely affected (Irons *et al*., 2005). The morbidity can reach as high as 100% in natural outbreaks while mortality rate rarely exceeds 5% (Babiuk *et al*., 2008).

SPPV and GTPV cause major economic impact because of the relatively high morbidity and mortality of the susceptible animals. Accordingly, depending on the virulence of the isolates, the morbidity and mortality in endemic areas range from 75% to 100% and 10% to 85%, respectively (Bhanuprakash *et al*., 2006). Additionally, the mortality rate can reach up to 100% in stressed and susceptible animals (Domenech *et al*., 2006). Species, stress, coexisting infection, breeds, age, host immunity, and virus isolates may all influence disease morbidity and mortality (Babiuk *et al*., 2009a; Tuppurainen *et al*., 2017). Local breeds are more resistant to CaPV than European breeds. Young animals are generally at greater risk than adults because of extensive interstitial viral pneumonia (Kitching *et al*., 1986).

### 2.5. Host Range

LSDV primarily affects cattle, although it has also been seen in domestic Asian water buffaloes (Elhaig *et al*., 2017; Sprygin *et al*., 2019). The European breed (*Bos taurus*) is usually more susceptible than Zebu or Sub-Saharan Africa breed (*Bos indicus*). Moreover, giraffe and impala have experimentally infected with LSDV. Compared to wild buffaloes, domesticated buffaloes are more susceptible to LSDV (Capstick and Coackley, 1961).

SPPV isolates cause disease mainly in sheep, and GTPV isolates cause disease primarily in goats (Bhanuprakash *et al*., 2010). However, both species of small ruminants may

acquire the disease from a single strain of the virus. Some sheep strains cause mild disease in goats and severe disease in sheep, whereas virulent goat strains can also infect sheep (Bhanuprakash *et al*., 2010; He *et al*., 2020). SPPV and GTPV can cause cross-infection either naturally or experimentally (Davies, 1982). However, no evidence has supported that LSDV can cause disease in small ruminants (USDA, 2016).

### 2.6. Pathogenesis

Poxviruses carry out both replication and transcription of their genome within the cytoplasm of infected cells and encode most of the proteins required for the synthesis of viral macro-molecules. The viral core has been released into the cytoplasm following endocytosis or virion fusion with the plasma membrane. Within minutes of infection, the release of transcriptase from the virion core aids in the synthesis of mRNA (Moss, 2013). The polypeptides produced by translation of these mRNAs complete the uncoating of the core within 1.5 to 6 hours of infection before the actual viral DNA synthesis begins. Two forms of virion have released from the infected cells (virion with one membrane, and virion with two membranes) and both types are infectious (Yang *et al*., 2011).

LSD has an incubation period of 2 to 4 weeks **(**Tuppurainen and Oura, 2012**).** Intravenous, intradermal and subcutaneous routes are used in experimental infection. The intravenous route develops severe generalized infection. Subcutaneous or intradermal inoculation of cattle with LSDV results in enlargement of the regional lymph nodes and a localized swelling at the site of inoculation after four to seven days (Vorster and Mapham, 2008). However, generalized eruption of skin nodules usually occurs seven to 19 days after inoculation. LSDV replicates inside the host cells such as macrophages, fibroblasts, pericytes and endothelial cell in the lymphatics and blood vessels walls lead to developing vasculitis and lymphangitis, while thrombosis and infarction may develop in severe cases. Viraemia occurred after the initial febrile reaction and persisted for two weeks. In a natural infection, newborn calves, nursing cows, and malnourished animals appear to experience more severe disease; this could be because of an impaired humoral immunity. A lifelong cell- mediated immunity is developed in most animals that recover from clinical disease.

Calves are born from the infected cow acquire maternal antibodies that may protect them from diseases for approximately six months. LSDV was demonstrated in saliva at least for 11 days after the development of fever, in semen for 42 days and in skin nodules for 39 days, from experimentally infected cattle (Al-Salihi, 2014).

Incubation period of SPP is 4-8 days and that of GTP is 4-15 days. SGP virus have tropism for epithelia tissue in which after the virus enter to the host through any route of infection it can replicate locally and infest epithelial tissue of the host. After 3-4 days, it causes primary viremia in which the viremia will spread to different parts of the body thus affecting spleen, lung, gastrointestinal tract and liver. After seven days following infection, the virus can replicate to a high titer and spread to the local lymph nodes, where it can further multiply and cause more infection. In skin, nodules develop from 7 to 14 days after inoculation where the virus titers persisted and within 24 hours of the appearance of generalized papules affected animal develop conjunctivitis, rhinitis and enlargement of all superficial lymph nodes particularly prescapular lymph nodes. Following infection, it causes excessive salivation but the virus titer can decrease with the development of serum antibodies (OIE, 2012).

### 2.7. Clinical Signs

There are five stages in the development of poxvirus infection namely roseola, papular, vesicular, pustular, and scab. The roseola stage is characterized by skin lesions that typically begin with small red spots within three days of infection and progress to papules which is accompanied by development of febrile state after three days of roseola stage. The vesicular stage is characterized by nodular skin lesions that are developed form from roseola stage (red spots), which are hard to palpate and transform into vesicles within 5-6 days. After the vesicular stage, the pustular stage appears within three days. This stage develops to the last stage of the pox lesion, known as scab formation, and it is characterized by having a high viral load (Bowden *et al*., 2008).

LSD is characterized by large skin nodules covering all parts of the body, high fever (>40.5 $^{0}$C), enlarged lymph nodes, loss of appetite, depression, reduction in milk production, nasal discharge and lachrymation. Compared to the adults, young calves often have more severe disease (Gumbe, 2018). The severity of clinical signs of LSD depends on the host immunity status, age, sex and breed type. Jersey and Holstein Friesian (HF) breeds are among the breeds that are more prone to contracting LSD infection (Kumar, 2011). Furthermore, the disease affects cattle and, due to mastitis, LSD tends to be more severe in milking cows during the peak of lactation (Gari *et al*., 2011). The nodules that developed on the skin range in size from 2 to 7 cm. They are firm and slightly raised from the surrounding skin, and they resemble well-defined, rounded patches. Short-haired animals tend to have more noticeable nodules. The nodules in cattle with long hair are frequently invisible unless the skin is palpated or wet. In most cases the nodules are particularly noticeable in the hairless areas of perineum, udder, inner ear, muzzle, eyelids and on the vulva. In addition, other frequently affected areas include the head and neck, genitalia, limbs, udder, and legs (Alemayehu *et al*., 2013).

SPP and GTP virus symptoms begin with nasal and ocular discharge, fever (40-42 °C), breathing difficulties because of the presence of blisters inside their respiratory tract and lungs (Bowden *et al*., 2008), depression and loss of appetite. Skin lesions are usually first noticed on the face around the lips and nares and on the eyelids (Mahmoud *et al*., 2016). Skin lesions develop in the following stages: roseola, papular, vesicular, and pustular, then scabs form. Although the lesions may cover the entire body, it is easier to detect them on the skin's hairless areas and the mammary glands (Chu *et al*., 2011). Clinically observation of the disease with lesions of papule development under the tail of sheep and goat is highly indicative and almost considered as pathognomonic signs of the diseases (EFSA, 2014). Ulcerative lesions can also appear on the mucous membranes of the mouth, nasal cavities and throughout the digestive and respiratory tracts (Bowden *et al*., 2008). The presence of nodules in the intestine leads to diarrhea (Rao and Bandyopadhyay 2000; Haller *et al*., 2014). The animal may recover in three to four weeks with permanent scars. The condition is typically severe in lambs, especially in suckling animals, causing lesions on

the oral mucosa, anterior nares, and the entire digestive tract. Pneumonia, enlargement of the udder and abortion may occur in severe cases (Zangana and Abdullah, 2013).

## 2.8. Diagnosis

CaPV infections have similar clinical manifestations (Rao and Bandyopadhyay, 2000). Because only a single serotype of CaPV exists, it is challenging to differentiate between LSDV, SPPV, and GTPV using serological (Babiuk *et al*., 2008; Bowden *et al*., 2008) and antigenic (Babiuk *et al*., 2008) tests. However, in order to distinguish between LSDV, SPPV, and GTPV, genetic sequencing and phylogenetic analysis of the GPCR (G-protein coupled receptor) and RPO30 genes encoding the 30 kD genes have been developed (Lamien *et al*., 2011). Among capripoxviruses, P32, GPCR, and RPO30 genes are highly conserved (Mahmoud and Khafagi, 2016; Venkatesan *et al*., 2012).

Laboratory investigations and identification of LSDV can be conducted through the following tests based on OIE Terrestrial Manual (OIE, 2010; WOAH, 2023).

### 2.8.1. Antigen detection

*i. Isolation of the virus*

Confirmation of LSDV requires virus isolation and identification. Before neutralizing antibodies develop, within the first week of the onset of clinical symptoms, samples should be obtained for viral isolation. Skin biopsies of early lesions, ones where necrosis has not occurred, provide samples that can be used for virus isolation. In addition, LSDV can be isolated from buffy coat from the blood sample collected into EDTA or heparin during the viremic stage of LSD. Samples aspirated from enlarged lymph nodes can be also used for virus isolation (OIE, 2010). LSDV grows in tissue culture of bovine, ovine or caprine origin. The most susceptible cells are thought to be those found in primary or secondary culture of bovine dermis cells or lamb testis (LT) cells. MDBK (Madin–Darby bovine kidney) cells are often used, as they support good growth of the virus and are well characterized (Fay *et al*., 2020). Primary cells, such as lamb testis (LT) cells, also support

viral growth (WOAH, 2023). LSDV have been also adapted to grow on the chorioallantois membrane of embryonated chicken eggs and African green monkey kidney (Vero) cells, which are not recommended for primary isolation (OIE, 2010).

*ii. Polymerase chain reaction (PCR)*

For the identification of the capripoxvirus genome in EDTA blood, semen, or tissue culture samples, the conventional gel-based PCR method provides an easy, quick, and sensitive technique (Tuppurainen *et al*., 2005).

Quantitative real-time PCR methods have been described that are reported to be faster and have higher sensitivity than conventional PCRs (Balinsky et al., 2008; Bowden *et al*., 2008). A real-time PCR technique that differentiates between LSDV, sheep pox virus and goat pox virus has been published (Lamien *et al*., 2011).

*iii. Transmission Electron Microscopy (TEM)*

Transmission electron microscopic (TEM) diagnosis of LSD can be confirmed within a few hours of receipt of specimens. TEM demonstration of virus in negatively stained preparations of biopsy specimens taken from affected skin or mucous membranes. Compared to orthopox virions, mature capripox virions are more oval in profile and have larger lateral bodies, with an average size of 320 x 260 nm (OIE, 2010).

*iv. Fluorescent Antibody Tests (FAT)*

Fluorescence antibody assays can also be used to detect the capripoxvirus antigen on the infected cover-slips or tissue culture slides. The indirect FAT using immune cattle sera is subject to high background colour and nonspecific reactions. However, a direct conjugate can be prepared from sera from convalescent cattle (or from sheep or goats convalescing from capripox) or from rabbits hyperimmunized with purified capripoxvirus. Since

antibodies to cellular components can be generated and cause issues, so uninfected tissue culture should be included as a negative control (WOAH, 2023).

*v. Immunohistochemistry*

Immunohistochemistry using F80G5 monoclonal antibody specific for capripoxvirus ORF 057 has been described for detection of LSDV antigen in the skin of experimentally infected cattle (Babiuk *et al*., 2008).

2.8.2. Serological tests

*i. Virus neutralization*

A test serum can either be titrated against a constant titer of capripoxvirus, TCID50 (50% tissue culture infective dose) or a standard virus strain can be titrated against a constant dilution of test serum in order to calculate a neutralization index. Because of the variable sensitivity of tissue culture to capripoxvirus, and the consequent difficulty of ensuring the accurate and repeatable seeding of TCID50/well, the neutralization index is the preferred method in most laboratories, although it does require a larger volume of test sera. The test is described using 96-well flat-bottomed tissue-culture grade microtiter plates (WOAH, 2023).

*ii. Enzyme-linked immunosorbent assay (ELISA)*

Enzyme-linked immunosorbent assays (ELISAs) for the detection of capripoxviral antibodies are widely used and are available in commercial kit form (Milovanovic *et al*., 2019; Samojlovic *et al*., 2019).

*iii. Indirect fluorescent antibody test*

Capripoxvirus-infected tissue culture grown on cover-slips or tissue culture microscope slides can be used for the indirect fluorescent antibody test. Uninfected tissue culture

control, and positive and negative control sera, should be included in the test. The infected and control cultures are fixed in acetone at –20°C for 10 minutes and stored at 4°C. Dilutions of test sera are made in PBS, starting at 1/20 or 1/40, and positive samples are identified using an anti-bovine gamma-globulin conjugated with fluorescein isothiocyanate. Antibody titres may exceed 1/1000 after infection. Sera may be screened at 1/50 and 1/500. Cross-reactions can occur with orf virus (contagious pustular dermatitis virus of sheep), bovine papular stomatitis virus and perhaps other poxviruses.

*iv. An agar gel immunodiffusion (AGID)*

AGID test has been used for detecting the precipitating antigen of capripoxvirus, but it has the disadvantage that this antigen is shared by parapoxvirus. Agar gel immunodiffusion tests may also give false-positive reactions due to cross reaction with bovine papular stomatitis virus and pseudocowpox virus (OIE 2010).

*v. Western blot analysis*

Western blotting of test sera against capripoxvirus-infected cell lysate provides a sensitive and specific system for the detection of antibody to capripoxvirus structural proteins, although the test is expensive and difficult to carry out (WOAH, 2023).

Clinically, based on symptoms, lesions, and postmortem abnormalities, it is difficult to differentiate GTP from SPP (Bowden *et al*., 2008). Additionally, due to differences in the host response, virus species, and virulence of the viral strains, a variety of clinical and pathological symptoms are identified (Sumana *et al*., 2020). Recently, strains of the GTPV and SPPV have been distinguished from field samples using PCR-RFLP based on the P32 gene, RPO30 gene, and GPCR gene sequencing and analysis (Khameis *et al*., 2018; Saminathan *et al*., 2016). One of the most significant distinctions amongst SGP viruses is the presence of aspartic acid in the SP virus at position 55 of the P32 gene, which is absent in the other viruses of the same genus.

## 2.9. Preparation of monoclonal antibodies using hybridoma technology

### 2.9.1. Immunization

In order to induce B cell differentiation into plasma B cells and memory B cells, the initial stage is injecting laboratory animals, such as mice or rabbits, with a particular antigen against which the antibodies are generated through a series of injections are given for several weeks. After a few weeks of vaccination, when the animal's serum contains sufficient number of antibodies, the animal is sacrificed (Ganguly and Wakchaure, 2016).

### 2.9.2. Isolation of B lymphocytes

In order to separate the activated B-cells, the spleen is removed under aseptic circumstances after sacrifice. Density gradient centrifugation is used in this process. (Ganguly and Wakchaure, 2016). The activated B lymphocytes are then fused with myeloma cells (Ganguly and Wakchaure, 2016; Mitra and Tomar, 2021).

### 2.9.3. Preparation of myeloma cell lines

Metastatic tumor cells are cultured in 8-azaguanine for a few weeks prior to cell fusion in order to obtain non-functional hypoxanthine-guanine phosphoribosyltransferase (HGPRT) genes in the myeloma cells. Non-functional HGPRT can stop the assembly of nucleotides from the salvage pathway and makes the metastatic tumor cells sensitive to HAT media as the preferred method in hybridoma technology (Ganguly and Wakchaure, 2016).

### 2.9.4. Cell fusion

Cell fusion is the process in which the activated B lymphocytes are fused with HAT-sensitive myeloma cells Polyethylene glycol (PEG) is used in this procedure (Ganguly and Wakchaure, 2016). PEG facilitates the fusing of cells by promoting the myeloma cells plasma membranes and the antibody-producing cells plasma membranes to fuse together. This results in the formation of heterokaryon, a multinucleated cell. Electrofusion is a

different fusion technique where cells are fused under the effect of an electric field (Buck *et al*., 1984; Ganguly and Wakchaure, 2016).

### 2.9.5. Hybridoma selection

Cells are united to produce hybridoma cells in the PEG-containing media, however only 1 to 2% of fused hybridoma cells can form, even using the most effective fusion technique. Moreover, around 1 out of every 100 cells will be viable hybrid cells. As a result, the medium contains some unfused cells (Mitra and Tomar, 2021). Selection the fused cells from among all the unfused cells is made possible by this stage. This is accomplished by culturing the cell mixture in HAT media (a selection media) for ten to fourteen days after it has been incubated. HAT medium contains hypoxanthine-aminopterin-thymidine. Aminopterin, which is found in HAT media, prevents cells from using the de novo synthesis pathway to synthesize nucleotides. Hypoxanthine and deoxythymidine allow the myeloma cells with functional hypoxanthine-guanine phosphoribosyltransferase (HGPRT) genes to survive through salvage pathways. Due to a limited life span, unfused B cells perish within a few days. Unfused malignant neoplastic cells die as a result of the lack of the hypoxanthine-guanine phosphoribosyltransferase (HGPRT) gene (Luckenbach, 1988). Therefore, the remaining viable cells left in the media are the hybrid cells; these hybrid cells have the ability to grow and divide on HAT media because they have functional HGPRT gene from the B lymphocytes, which make them HGPRT positive, and thus, they can grow in unlimited concentration on HAT media (Ganguly and Wakchaure, 2016).

### 2.9.6. Screening of hybridoma cells

The supernatant of HAT-selection hybridoma cells are transferred to ELISA plates, where each well houses a single hybridoma cell (Ganguly and Wakchaure, 2016). The genes of the B cell lineage present in the hybridoma cells produce a specific antibody with a specific epitope; this antibody is known as “monoclonal antibody.” There may be other hybridomas present in other wells producing antibodies specific to another epitope for the same antigen. After the separation and isolation of different hybridomas, screening is performed for

selecting hybridomas that produce the desired antibodies targeting specific epitopes for an antigen (Parray *et al*., 2020).

#### 2.9.7. Cloning and propagation of hybridoma cell

After selecting hybridomas that produce the desired antibodies, the cells are grown by using of cell culture flasks. These hybridoma cells can be maintained and preserved in the culture media for the production of monoclonal antibodies (Parray *et al*., 2020).

### 2.10. Economic importance of disease due to LSD, SPP, and GTP viruses

The economic importance of LSD is reflected with loss of milk production, temporary or permanent sterility in bulls and cows, damage to hides and deaths from secondary bacterial infections, expensive control and eradication measures, and enforced animal movement restrictions (Alemayehu *et al*., 2013; Alkhamis and VanderWaal, 2016; Tuppurainen and Oura, 2012). Moreover, LSD is one of the trans-boundary diseases that transcends national borders. Hence, movement restriction of live animals and animal products can significantly affect the global trade (Tuppurainen *et al*., 2017). In Ethiopia, the financial losses estimated based on milk, beef, draught power, mortality, treatment and vaccination costs for individual head of local zebu to be 6.43 USD and for the Holstein Friesian 58 USD (Gari *et al*., 2010), and the total loss of USD 667,786 in feedlots in and around Adama due to mortality and rejection (Alemayehu *et al*., 2013).

Within the endemic areas of small ruminant disease, infectious diseases, such SPP and GTP, are the most significant viral infections that result in significant production losses in sheep and goats. This disease also restricts international trade and results in economic losses (Hopker *et al*., 2019; Tuppurainen and Oura 2012). For this reason, the World Organization for Animal Health (OIE) has designated CaPV as a notifiable disease because of their rapid transboundary nature and extensive economic impact on the livestock industry (Hamdi *et al*., 2021; Tuppurainen and Oura 2012; Zeng *et al*., 2014). Globally, the disease causes a serious risk to small ruminant production and food security and jeopardizes international trade (Tuppurainen *et al*., 2015).

Furthermore, CaPV can cause significant economic losses due to control costs and trade restrictions. In an endemic area, the economic losses of SPP and GTP have been reflected by reduced milk and mutton production, decreased weight, abortion, significant damage to wool and hides, and vulnerability to pneumonia and fly strike. The disease's direct economic impacts are primarily due to higher morbidity rather than mortality in susceptible animals (Al-Salihi and Hassan 2015; Molla *et al*., 2017b).

Depending on the current scenario in Ethiopia, small ruminant production is a basic source of income and food for the small farm holder community in the country, and it has a high potential for foreign exchange earnings. Additionally, small ruminant production is considered as savings sector for smallholder farmers in addition to a source of income and food because it eliminates threats to disadvantaged communities in the absence of crop production due to natural disasters. Furthermore, other socioeconomic and cultural functions are involved for small-holder households. Small ruminants do have advantages through meat export values of 63 million USD and live animal export values of 148 million USD during 2010/11 (Haile *et al*., 2018). Similarly, it is anticipated that 75% of goat and 97% of sheep hides will be exploited currently, with estimated annual off-take rates for sheep and goats being 33% and 35%, respectively. The country supplies many finished and semi-finished small ruminant hides and skins to the global market and accounts for approximately 12-16% of the total value of its exports (Yacob *et al*., 2008; Zemene and Addis, 2012).

Additionally, as previously reported, the yearly economic losses due to mortality of sheep and goats vary from 12-14% and 11-13%, respectively (MOA, 2013). Therefore, these diseases have a substantial negative economic impact and pose considerable barriers to international trade and the introduction of improved animal breeds into endemic areas.

### 2.11. Treatment, control and prevention

There is no specific treatment for viral diseases, but secondary bacterial complications can be treated with antibiotics, and good nursing care is recommended to reduce morbidity and

other complications (Hajer *et al*., 1988). Additionally, better consideration of disease occurrence and its distribution would lead to improved control measures (Fentie *et al*., 2017; Limon *et al*., 2020).

Hence, LSD diseased animals may be removed from the herd and given supportive treatment such as antibiotics, anti-inflammatory drugs, and vitamin injections to treat secondary bacterial infections or to improve the animal's appetite. Since blood sucking insects and parasites are thought to be the main source of LSD transmission, movement restrictions and quarantines by themselves are not very efficient ways to control LSD unless it combined with mass vaccination (Al-Salihi, 2014). All strains of CaPVs from cattle, sheep and goat origin, share a common major antigen that makes it possible to use SPP or GTP vaccine against LSDV infection in cattle (Kitching and Mellor, 1986).

The effective means of controlling and preventing LSD in endemic countries is vaccination against LSD with live attenuated vaccines (Hunter and Wallace, 2001). Because immunity to CaPVs is mainly cell mediated and is better stimulated by the use of live attenuated vaccines. CaPV vaccine strains that have been used to control LSD include LSDV Neethling, Kenyan sheep and goat pox virus (KSGPV) O-240 and O-180 strains, Romanian SPP, Yugoslavian RM65 SPP strain, and Gorgan GTP strains (Brenner *et al*., 2009; Kitching and Mellor, 1986; Panel, 2015). Homologous live attenuated (Neethling strain) LSD vaccines are more successful than vaccines based on attenuated sheep pox viruses. A vaccination with a heterologous live attenuated virus may result in mild to severe side effects (Al-Salihi, 2014).

Effective control and eradication of CaPV of small ruminants in previously CaPV-free countries could be practiced by slaughtering all contaminated and in-contact animals (Tuppurainen and Galon, 2016), Considering that it might not be beneficial to administer live attenuated vaccinations in nonendemic places (Bowden *et al*., 2008). However, compared to inactivated vaccines, live attenuated vaccinations offer longer-lasting protective immunity in many endemic countries (Bhanuprakash *et al*., 2012; EFSA, 2014). Furthermore, live attenuated Capripox vaccines are a safe and effective to prevent these

diseases (Tuppurainen *et al*., 2017), but inactivated vaccines need two doses of injection and do not provide sustained immunity (Boumart *et al*., 2016). Despite the result of different studies which have shown the insufficient protection and short-liveness of inactivated Capripox vaccines, recent research strongly suggests that inactivated Capripox vaccinations are safe and effective against CaPV (Es-sadeqy *et al*., 2021; Wolff *et al*., 2020). Lifelong protection against CaPV infection is provided by both cellular immunity and antibodies (Bhanuprakash *et al*., 2006). However, cell-mediated immunity elicits long-term protection (Carn, 1993).

LSD, SPP, and GTP have a close antigenic relationship (which shares 97% sequence similarity), which theoretically allows for a single vaccine to protect against all these diseases (Brenner *et al*., 2009). It is possible to produce a CaPV vaccine because of the ANK gene of the CaPV virus, but recent reports have revealed mutations in the ankyrin (ANK) and kelch-like proteins of certain CaPV vaccine strains. As a result, a common feature of many live CaPV vaccines is that ANK genes have changed (Biswas *et al*., 2020). Vaccine failure may be linked to insufficient vaccination coverage and the production of low-performance local vaccines (Gelaye *et al*., 2015). When choosing vaccine strains to immunize cattle, sheep, and goats, it is important to consider the characteristics and attenuation properties of the virus strain, according to Liu *et al*. (2019).

About twenty-two distinct vaccine kinds have been manufactured in Ethiopia for both internal and export markets. Among these, the live attenuated KSGPV O-180 vaccine strain has been manufactured using continuous cell lines (Vero cells) and is a lyophilized vaccine with a stabilizer that is stored at -20 °C for 2 years. The KSGPV O-180 vaccine strain is now used to control LSD in cattle, despite that some cattle breeders doubting whether it provides adequate protection. However, if and only if appropriate handling, storage, transportation, and vaccination coverage are maintained, this strain of vaccine in Ethiopia is safe and successful in reducing Goat pox Sheep pox (Zewdie *et al*., 2021). Annual mass vaccination with the (KSGP) O-180 virus strains has been demonstrated to be a safe, effective and affordable method to control small ruminant pox virus (Fentie *et al*., 2017).

On the other hand, inadequate infrastructure might make it more difficult to put enough herd immunity into practice (Barua *et al*., 2017; Mirzaie *et al*., 2015).

## 3. MATERIALS AND METHODS

### 3.1. Study area

The study was conducted in African Union Pan African Veterinary Vaccine Centre (AU-PANVAC) which is found in Bishoftu (Ethiopia). The AU-PANVAC is mandated to provide international independent quality control services for veterinary vaccines produced in and imported to Africa and monitor the production and distribution of essential diagnostic reagents for animal disease surveillance and diagnosis. Launched on March 12th, 2004 in, Bishoftu, Ethiopia, the center strives to enhance customer satisfaction by fulfilling the requirements of ISO 9001:2015 Quality Management System (QMS) (Figure 1) (https://au.int/en/articles/aupanvac-plays-critical-roles-quality-control-vaccines-africa); accessed on 02/10/2023).

**Figure 1**: *Administration office of AU-PANVAC*

*Source: https://www.au-ibar.org/ accessed on 02/10/2023 at 3:30 pm*

### 3.2. Preparation of the LSD, SPP, and GTP virus crude Ag

#### 3.2.1. Cell culture

The adherent Madin-Darby bovine kidney cells (MDBK cells) (AU-PANVAC, Ethiopia) were cultivated in Glasgow modified Eagle medium (GMEM) (Gibco, UK) supplemented with 5% (*v/v*) fetal bovine serum (FBS) (FBS, South America) and 1% (*v/v*) antibiotic (AB) - antimycotic (AF) (Gibco, USA). Cells were maintained in a humidified incubator with 5% $CO_2$ at 37 °C until 70-80% confluence were reached (Wang *et al*., 2021).

The same as MDBK cells, African green monkey renal epithelial Vero cells (AU-PANVAC, Ethiopia) were cultured in a GMEM culture medium (Gibco, UK) containing 5% fetal bovine serum (FBS, South America), 1% (v/v) antibiotic (AB) - antimycotic (AF) (Gibco, USA) and incubated at 37 °C in a 5% $CO_2$ humidified incubator up to reaching 80%-90% confluent monolayer (Gan *et al*., 2016).

The Ag8 myeloma cell (STEMCELL Technologies; Catalog Number 29044) were cultivated in RPMI-1640 (Gibco, Invitrogen, USA) culture medium supplemented with 10% (*v/v*) fetal bovine serum (FBS) (FBS, South America). The cell lines were cultured at 37 ℃ in a humidified incubator supplied with 5% $CO_2$ atmosphere. After adapting to grew on RPMI-1640 medium then the myeloma cells were cultured in ClonaCell®-HY Pre-Fusion Medium (Medium A) (STEMCELL Technologies; Catalog Number 29044) for at least 1 week to ensure that they were well adapted to this medium prior to fusion (Shabani *et al*., 2010).

#### 3.2.2. Virus infection of cells

After the cells reaching 70-90% confluency the cells, both MDBK and Vero cells, were washed three times separately with phosphate buffered saline (PBS, pH 7.4) and then treated in 1 ml of 1% trypsin (Gibico, Waltham, MA, USA) followed by 3 min incubations at 37 °C (Wang *et al*., 2021). Then cells were blown gently after trypsin digestion to form cell suspension. Then the cells were cultured in a GMEM culture medium (Gibco, UK)

containing 5% fetal bovine serum (FBS, South America), 1% (v/v) antibiotic (AB) - antimycotic (AF) (Gibco, USA) and incubated at 37 °C in a 5% $CO_2$ humidified incubator for 4-6 hrs. (Gan *et al*., 2016).

Live attenuated LSD vaccine seed (AU-PANVAC, Ethiopia) was reconstituted with 2 ml of the incomplete GMEM media (media without FBS) and then diluted in 35 ml of incomplete GMEM media, and 5 ml of the diluted vaccine seed was inoculated in to each flask which consists of MDBK cells. Similarly, live attenuated SPP vaccine seed (AU-PANVAC, Ethiopia) and GTP vaccine seeds (AU-PANVAC, Ethiopia) were also reconstituted and diluted as LSD vaccine seed, then 5 ml of the diluted SPP and GTP vaccine seed was inoculated in to different flasks which consists of Vero cells. Then the flasks were incubated at 37°C for 1hr for the absorption of the virus into the cells. One hour later, the inoculum (the 5 ml) was discarded and 60 ml of GMEM culture medium (Gibco, UK) containing 5% fetal bovine serum (FBS, South America), 1% (v/v) antibiotic (AB) - antimycotic (AF) (Gibco, USA) was placed in each flask and incubated at 37 °C in a 5% $CO_2$ humidified incubator. When 80-90% cytopathic effect (CPE) was observed in cells (within 7-9 days), flasks were frozen at -20°C and thawed at room temperature (freeze-thawing process) which was about 3 times. Non-infected MDBK and Vero cells were also used as negative controls **(**Gülyaz *et al*., 2020).

After the third thaw, brief centrifugation was done at 400× *g* for 10 min, and 10 % of saturated ammonium sulfate (Fluka™, USA) was added in each of the Ag and the mixture was stirred over night at +4°C. Then the Ag were pelleted down by centrifugation at 12,000 × *g* for 30 min. The pellets were then resuspended in 25 ml of PBS (Batra *et al*., 2015).

### 3.3. Detection of LSD, SPP, and GTP Ag

#### 3.3.1. Genomic DNA extraction and gene amplification

The LSDV, SPPV, and GTPV genomic DNA was extracted using the QIAamp DNA mini kit (Qiagen; Catalog Number 201913) according to the manufacturer's instructions.

Conventional PCR was used for amplification of the RNA polymerase subunit (RPO30) gene of Capripoxviruses (LSDV, SPPV, and GTPV) by using specific gene primers SpGpRNAPol-F (forward primer): 5′-TCTATGTCTTGATATGTGGTGGTAG-3′ and SpGpRNAPol-R (reverse primer): 5′- AGTGATTAGGTGGTGTATTATTTTCC -3′. The composition of the PCR master mixes included 2.5 µl 1x Taq PCR buffer, 2.5 µl of dNTPs, 2.5 µl of forward primer (SpGpRNAPol-F), 2.5 µl of reverse primer (SpGpRNAPol-R), 0.125 µl of Taq polymerase, plus balance distilled water to a final volume of 25 µl. Then 5 µl of template (viral DNA) was added on the master mix (Das *et al*., 2022)**.** Amplification was conducted with the Taq DNA Polymerase , specifically conducted with 40 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s and extension at 72°C for 30 s. The 40 cycles were followed by a final extension at 72°C for 5 min (Ntombela *et al*., 2023).

#### 3.3.2. Gel-Electrophoresis

Ten microliters of each amplified product were analyzed by agarose gel electrophoresis on 2.5% of agarose, containing 1 µg/ml red gel in Tris-borate-EDTA (TBE) buffer and visualized under UV transilluminator (Mangana-Vougiouka *et al*., 2000).

### 3.4. Antigen and Serum Sample Testing

#### 3.4.1. Titration of LSD, GTP, and SPP Ag

LSD, SPP, and GTP Ag titer starting from the concentration of 1/2.5 were used to coat the ELISA plates (Thermo scientific, Denmark). In order to examine the background of the ELISA plate, the last two rows of the plate were coated with PBS. The coated plates were incubated overnight at room temperature (RT). In the following day, the remaining free protein binding sites of the plate wells were blocked by 200 µl of PBS-T containing 5% skimmed milk (PBS-T-Milk) for 30 minutes incubation at RT. After 30 minutes of incubation the blocking buffer was removed and 100 µl of the serum sample from immunized mice (after the second booster dose) diluted in 1:500 in blocking buffer of PBS-T containing 5% skimmed milk (PBS-T-Milk) was added into each well. The plates were incubated at 37°C for 1hr, and after 1 hr incubation the plates were washed three times with

the washing buffer: 0.002 M PBS containing 0.05% Tween 20 (PBS-T). Then 100 µl of conjugate anti-mouse immunoglobulin peroxidase-labelled (Invitrogen, USA) diluted 1:100 in PBS-T-Milk were distributed into each well. The plates were further incubated at 37°C for 45 minutes. After incubation the plates were washed three times again with PBS-T, and 50 µl of the chromogenic substrate 3,3′,5,5′-tetramethylbenzidine (TMB) were added in each well. After 15 minutes incubation at 37°C, the enzymatic reaction was stopped with 50 µl of sulphuric acid (1M). The optical densities (OD) of the samples were read through spectrophotometry at 450 nm wavelength (Bodjo *et al*., 2018).

### 3.4.2. Titration of anti-mouse

LSD antigen with the concentration of 1/200 were used to coat the ELISA plates (Thermo scientific, Denmark). In order to examine the background of the ELISA plate, the last two rows of the plate were coated with PBS. The coated plates were incubated overnight at RT. In the following day, the remaining free protein binding sites of the plate wells were blocked by 200 µl of PBS-T containing 5% skimmed milk (PBS-T-Milk) for 30 minutes incubation at RT. After 30 min of incubation the blocking buffer was removed and 100 µl of the serum sample from immunized mice (after the second booster dose) diluted in 1:500 in blocking buffer of PBS-T containing 5% skimmed milk (PBS-T-Milk) was added into each well. The plates were incubated at 37°C for 1hr, and after 1 hr of incubation the plates were washed three times with the washing buffer: 0.002 M PBS containing 0.05% Tween 20 (PBS-T). The stock solution of anti- mouse was prepared by reconstitute the stock powder by 1.5 ml of distilled water. The reconstituted stock solution was incubated for 30 minutes at RT prior to making further dilutions. Then the anti-mouse titer starting from 1/2500 diluted in blocking buffer of PBS-T containing 5% skimmed milk (PBS-T-Milk) was added into each well. The plates were further incubated at 37°C for 45 minutes. After incubation the plates were washed three times again with PBS-T, and 50 µl of the chromogenic substrate TMB were added in each well. After 15 minutes incubation at 37°C, the enzymatic reaction was stopped with 50 µl of sulphuric acid (1M). The OD of the samples were read through spectrophotometry at 450 nm wavelength (Bodjo *et al*., 2018).

### 3.5. Generation and characterization of mAbs against LSDV, SPPV, and GTPV

#### 3.5.1. Immunization of mice and collection of spleen

Three-four weeks old male BALB/c mice (4 mice for each of the antigen) were immunized by intra-peritoneal injection with 200 µl emulsion of the antigens (LSDV, SPPV, and GTPV Ag) and for the first injection each of the antigens were mixed in 1:1 ratio with Freund's complete adjuvant. Four booster injections were given at intervals of two weeks; the three consecutive boosts are given with incomplete Freund's adjuvant and the last boost was given without any of the adjuvants. Four days after the last boosting the mice was euthanized through dislocation of the cervical spine, and spleen cells were collected and fused with Ag8 myeloma cells (STEMCELL Technologies; Catalog Number 29044) (Bodjo *et al*., 2018).

#### 3.5.2. Laboratory animal management

The BALB/C mice were housed in temperature-controlled rooms with a 12-hr. light-dark cycle to stimulate natural condition and minimize stress. Bedding was regularly changed to maintain cleanness, and cages were equipped with all necessary maintenance. Throughout the experiment, all mice received a standard diet and free access to water in order to meet their nutritional requirements. All experimental procedures were approved by the animal care and use committee of PANVAC and conducted in accordance with established guidance.

#### 3.5.3. Testing for the seroconversion

LSD, SPP, and GTP antigen with the concentration of 1/200, 1/400, and 1/40 respectively, were used to coat the ELISA plates (Thermo scientific, Denmark). The coated plates were incubated overnight at RT. In the following day, the remaining free protein binding sites of the plate wells were blocked by 200 µl of PBS-T containing 5% skimmed milk (PBS-T-Milk) for 30 minutes incubation at RT. After 30 min of incubation the blocking buffer was removed and 20 µl of the serum sample from immunized mice (after the second boosting) with 80 µl of blocking buffer of PBS-T containing 5% skimmed milk (PBS-T-

Milk), and 20 µl of serum from day 0 (before immunization) with 80 µl of blocking buffer of PBS-T containing 5% skimmed milk (PBS-T-Milk) was added into different well. The plates were incubated at 37°C for 1hr and after 1 hr incubation the plates were washed three times with the washing buffer: 0.002 M PBS containing 0.05% Tween 20 (PBS-T). Then 100 µl of conjugate anti-mouse immunoglobulin peroxidase-labelled (Invitrogen, USA) diluted 1:100 in PBS-T-Milk were distributed into each well. Then the plates were further incubated at 37°C for 45 minutes. After incubation the plates were washed three times again with PBS-T, and 50 µl of the chromogenic substrate TMB were added in each well. After 15 minutes incubation at 37°C, the enzymatic reaction was stopped with 50 µl of sulphuric acid (1M). The optical densities of the samples were read through spectrophotometry at 450 nm wavelength (Bodjo *et al*., 2018).

### 3.5.4. Preparation of splenocytes

Preparation of splenocytes were conducted using the ClonaCell™-HY Hybridoma Kit (STEMCELL Technologies; Catalog Number 29044), following manufacturer's instructions. Briefly, after extraction of splenocytes, the cells were put in the tubes. Then splenocytes were washed 3 times in 30 ml of Medium B from ClonaCell™-HY Kit and centrifuged at 400 x *g* at RT for 10 min, each time the supernatant was removed carefully. After the final wash the cells were resuspended in 25 ml Medium B from ClonaCell™-HY Kit (Puckette *et al*., 2020).

### 3.5.5. Fusion of splenocytes and Ag8 myeloma cells

Splenocytes were fused with Ag8 myeloma cells by treatment with polyethylene glycol (PEG) using the ClonaCell™-HY Hybridoma Kit (STEMCELL Technologies; Catalog Number 29044), following manufacturer's instructions. In brief, Ag8 myeloma cells were cultured in Medium A (STEMCELL Technologies; Catalog Number 29044), pelleted and washed three times with 30 ml of ClonaCell®-HY Fusion Medium (Medium B) (STEMCELL Technologies; Catalog Number 29044). The Ag8 myeloma cells ($2.5 \times 10^8$ in 25 ml) and mice splenocytes (for LSD $6.2 \times 10^7$, for SPP $8.0 \times 10^7$, for GTP $2.5 \times 10^8$ in 25 ml) each of the splenocytes of LSDV, SPPV, and GTVP were mixed with

1.24 ml, 1.6 ml, 5 ml of myeloma cells respectively in a separated 50 ml conical tube and centrifuged at 400 x *g* for 10 min, the cell counting was done through cell counting machine. The supernatant was removed by aspiration, and cell pellets were disrupted by gentle tube tapping followed by the addition of 0.5 ml polyethylene glycol (PEG) drop-wise over the course of 1 min. Cells were gently stirred by pipette for 1 min, and 5 ml of Medium B was added while stirring. An additional 5 ml of Medium C was slowly added. Then the cells were transferred to a T-75 flask containing 40 ml of Medium C, final volume of 50 ml of Medium C, and incubated overnight at 37 °C, humidified incubator supplied with 5% CO2 atmosphere (Puckette *et al*., 2020).

### 3.5.6. Selection, subcloning and colony amplification

Fused cells were plated onto media provided with the ClonaCell™-HY Hybridoma Kit (STEMCELL Technologies; Catalog Number 29044), per manufacturer's instructions. Briefly, following incubation, fused cells were transferred from a T-75 flask to a 50 ml tube and pelleted at 400 x g for 10 min. The supernatant was removed, and the cell pellet was suspended in 10 ml ClonaCell®-HY Liquid HAT Medium (Medium D) (STEMCELL Technologies; Catalog Number 29044) and then transferred into 90 ml of ClonaCell®-HY Liquid HAT Medium (Medium D) (STEMCELL Technologies; Catalog Number 29044). 200 µl of cell suspension medium were putted into each well of 96-well tissue culture plates aseptically. Then plates were incubated for 10 to 14 days at 37 °C humidified incubator with 5% $CO_2$. Then the parental clones were screened and the positive clones were diluted (passaged) to 12 (16) well plates by using of 25 ml of ClonaCell®-HY Fusion Medium E (STEMCELL Technologies; Catalog Number 29044). The plates were incubated at 37 °C and 5% $CO_2$ for 4 to 5 days. Wells demonstrating hybridoma propagation, determined by observation under a microscope, were transferred to T-25 flasks containing 5 ml of Medium E and passaged continuously to ensure cell stability prior to cryopreservation at -196 °C (Puckette *et al*., 2020).

### 3.5.7. Screening of mAbs

Indirect ELISA (iELISA) tests were performed to screen positive clones producing mAbs using LSDV, SPP, and GTP crude antigens. Briefly, ELISA plates (Thermo scientific, Denmark) were coated with the antigen solution of LSD, SPP, GTP (100 µl/well) with the concentration of 1/200, 1/50, and 1/400 respectively, on the separate plates. The plates were incubated at RT overnight. In the following day, the remaining free protein binding sites of the plate wells were blocked by 200 µl of PBS-T containing 5% skimmed milk (PBS-T-Milk) and incubated for 30 minutes at RT. Blocking solution was removed and 50 µl of the supernatant from hybridoma cells culture was added with 50 µl of blocking buffer of PBS-T containing 5% skimmed milk (PBS-T-Milk) into each well. The plates were incubated at 37°C for 1hr and washed three times with the washing buffer: 0.002 M PBS containing 0.05% Tween 20 (PBS-T). Then 100 µl of conjugate anti-mouse immunoglobulin peroxidase-labelled (Invitrogen, USA) diluted 1:100 in PBS-T-Milk were distributed into each well. The plates were further incubated at 37°C for 45 minutes. After incubation the plates were washed three times again with PBS-T, and 50 µl of the chromogenic substrate TMB were added in each well. After 15 minutes incubation at 37°C, the enzymatic reaction was stopped with 50 µl of sulphuric acid (1M). The optical density of the samples was read with filter at 450 nm wavelength (Bodjo *et al*., 2018).

### 3.5.8. Freezing hybridoma cells

After demonstrating hybridoma propagation, the hybridoma cells were transferred to T-75 cell culture flasks containing of 50 ml of ClonaCell®-HY Fusion Medium E (STEMCELL Technologies; Catalog Number 29044) and passaged continuously to ensure cell stability prior to cryopreservation. After cells were collected from the flasks and put in the tube then centrifuged at 400 x g for 10 min, and resuspended with 5 ml of Fetal Bovine Serum (FBS) (FBS, South America). 20% of DMSO solution was prepared in 5 ml of FBS. Then FBS which consists of 20% of DMSO (which was about 1 ml of DMSO with 4 ml of FBS) was mixed with reconstituted cell by 5 ml of FBS (the final cell suspension had become 90% FBS: 10% DMSO). Then 1 ml of cells with FBS and DMSO were transfer in cryovial (sterile 2 ml cryovials). Then cryovials were immediately put in -20 °C freezer for 45 min.

After 45 min the vials were removed from -20 °C freezer and store at -196°C in liquid nitrogen (Bhunia *et al*., 1995).

### 3.6. Purification and quantification of mAbs

#### 3.6.1. Purification of mAbs

An ammonium sulfate precipitation method was carried out for mAb purification. The two mAbs (Lc 5.4 and Lc 5.14) were harvested through growth of the two hybridoma cells in the separate flasks. Then the supernatant was collected and putted in the separate sterile tubes. Then saturated 50 % ammonium sulfate (Fluka™, USA) was added to the supernatant and the mixture was stirred at +4°C for overnight and then centrifuged at 10,000 x g for 30 min. The supernatant was discarded and the immunoglobulin (the pellet) was reprecipitated by 2.5 ml of PBS (pH 7.4) (Hurisa *et al*., 2022). The mAbs were then cleaned on a PD Mini-Trap G10 desalting column (GE Healthcare, UK). The column was washed four times with PBS (pH 7.4) and then the reprecipitated samples in 2.5 ml of each of the mAbs were added in the different columns and the flow through discarded. Then the mAbs were eluted with 3.5 ml of PBS and the elute was collected in tube (Fischler and Orlando, 2019).

#### 3.6.2. Quantification of mAbs

Pierce™ BCA (Bicinchoninic Acid) Protein Assay Kit (Thermo scientific, USA) was used to determine mAbs concentration by comparing the assay response of a sample to that of a standard whose concentration was known. The mAbs (samples) and protein standards were processed in the same manner by mixing them with assay reagent and using a spectrophotometer to measure the absorbances. working reagent (WR) were prepared by mixing 50 parts of BCA Reagent A with 1 part of BCA Reagent B (50:1, Reagent A: B). Briefly, in ELISA plates (Thermo scientific, Denmark) 25 µl of each standard and 25 µl of the two mAbs were put separately in two replicates. Then 200 µl of the (WR) was added to each well and plate were mixed thoroughly (sample to WR ratio = 1:8). Then the plate

was incubated at 37°C for 30 minutes. Finally, the absorbance was measured at 492 nm on the spectrophotometer (Scientific, 2013).

### 3.7. Cross reactivity of the mAbs with SPPV and GTPV Ag

LSDV, SPPV, and GTPV antigen with the concentration of 1/200, 1/400 and 1/40 respectively, were used to coat the ELISA plate (Thermo scientific, Denmark). The coated plate was incubated overnight at RT. In the following day, the remaining free protein binding sites of the plate wells were blocked by 200 µl of PBS-T containing 10% skimmed milk (PBS-T-Milk) for 30 minutes incubation at RT. Blocking solution was removed and 50 µl of the two mAabs of LSD (LC 5.4 and LC 5.14) supernatant from hybridoma cells culture was added with 50 µl of blocking buffer of PBS-T containing 5% skimmed milk (PBS-T-Milk) into separate well. The plates were incubated at 37°C for 1hr and washed thrice with the washing buffer: 0.002 M PBS containing 0.05% Tween 20 (PBS-T). Then 100 µl of conjugate anti-mouse immunoglobulin peroxidase-labelled (Invitrogen, USA) diluted 1:100 in PBS-T-Milk were distributed into each well. The plates were further incubated at 37°C for 45 minutes. After incubation the plates were washed three times again with PBS-T, and 50 µl of the chromogenic substrate 3,3′,5,5′-tetramethylbenzidine (TMB) were added in each well. After 15 minutes incubation at 37°C, the enzymatic reaction was stopped with 50 µl of sulphuric acid (1M). The optical density of the samples was read with filter at 450 nm wavelength (Bodjo *et al*., 2018).

### 3.8. mAbs Isotype Identification

The two mAbs (LC 5.4 and LC 5.14) isotype was determined through Pierce™ Rapid Antibody Isotyping Kit (Thermo Fisher Scientific Inc, USA). Briefly, the pouch which contains the cassettes were removed from refrigeration and equilibrated fully to the room temperature for 1 hr After it equilibrated to the RT, the pouch was opened and the cassettes were removed, and then 150 µl of diluted samples (the mAbs were diluted in 1:100 with PBS, which was prepared by adding 5 µl of mAbs were added to 0.5 ml (500 µl) of PBS)

were added to the wells of each of the two cassettes. After 10 minutes the color bands were appeared and the results were immediately evaluated (Mroz, 2017).

### 3.9. mAbs Conjugation with HRP

The conjugation of the Lc 5.14 and Lc 5.4 mAbs to HRP and the subsequent purification of the conjugate were performed as per EZ-Link™ Plus Activated Peroxidase protocol (Catalog number 31489). Briefly, the kit provided a high activity HRP, which was periodate-treated, and aldehyde-activated, ready for conjugation to antibodies via primary amine sites (EZ-Link Plus Activated Peroxidase; Catalog number 31489). 1mg of mAbs were prepared in 1 ml, which is 667 µl of mAb LC 5.14 with 333 µl of PBS and 333 µl of LC 5.4 with 667 µl of PBS. Then 1mg of lyophilized EZ-Link Plus Activated Peroxidase was Reconstituted with 100 µL of PBS and added to the mAbs solution and the reaction was incubated for 1 hr at RT. After 1 hr of incubation 10 µl of Sodium Cyanoborohydride was added and incubated for overnight at +4°C. The reaction was stopped by 20 µl of 'Quenching buffer' (3-M ethanolamine, pH 9) with 15 min incubation at RT. Finally, adding 10 ml of Pierce Peroxidase Conjugate Stabilizer (Product No. 31503) was added and stored at -20°C (Voss *et al*., 2021).

### 3.10. Dot Blot Hybridization Technique

Dot Blot Hybridization (DBH) was applied according to Southern (1975) and Khandijan (1987) by adding 10µl of LSD, SPP, GTP Ag on the nitrocellulose membrane. Overnight incubation at 37 $^0$C incubator was done. In the following day, the remaining free protein binding sites on nitrocellulose membrane were blocked by 50 ml of PBS-T containing 5% skimmed milk (PBS-T-Milk) at 37°C for 30 minutes incubation. Blocking solution was removed and 50 ml of conjugate mAb (peroxidase-labelled) diluted 1:10 in PBS-T-Milk were distributed onto the surface of nitrocellulose membrane. The membrane was incubated at 37°C for 45 minutes and washed with the washing buffer: 0.002 M PBS containing 0.05% Tween 20 (PBS-T) before adding 50 ml of the chromogenic substrate TMB (ElHaig *et al*., 2013).

### 3.11. ELISA assay on titration of conjugated mAbs

The direct ELISA technique was used for conjugated mAbs titration. ELISA plate (Thermo scientific, Denmark) was coated with the LSD antigen with the concentration of 1/200 (100 µl/well). Plate was incubated at RT overnight. In the following day, the remaining free protein binding sites in the plate wells were blocked by 200 µl of PBS-T containing 10% skimmed milk (PBS-T-Milk) for 30 minutes incubation at RT. Blocking solution was removed and appropriate titration (dilution) of the conjugated mAbs starting from concentration of 1/2.5 was added into wells and the plate was incubated at 37°C for 45 minutes. Then the plate was washed three times again with PBS-T before adding in each well 50 µl of the chromogenic substrate TMB. After 15 minutes incubation at 37°C, the enzymatic reaction was stopped with sulfuric acid (1M, 50µl/well) and the plate was spectrophotometrically read at 450 nm wavelength (Hurisa *et al*., 2019).

### 3.12. Statistical Analysis

All of the data collected for this study was entered into Microsoft Excel and then analyzed through Excel graphs. The ELISA OD values were analyzed through Excel bar and line graphs.

# 4. RESULTS

## 4.1. Molecular detection of LSDV, SPPV, and GTPV Ag

Molecular detection of the SPPV, LSDV, and GTPV was conducted through conventional PCR amplification that resulted in amplification of 151 bp band for SPPV, and 172 bp band for LSDV and GTPV bp (Figure 2).

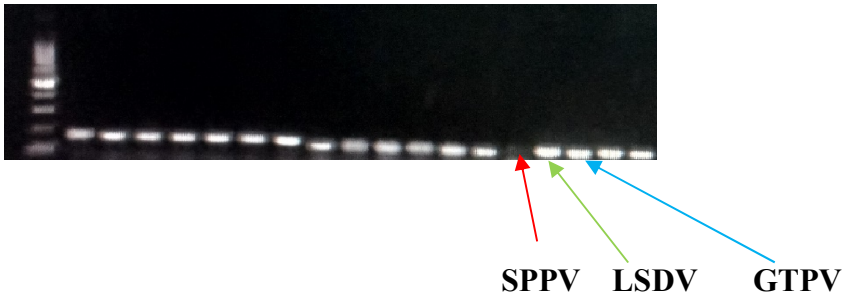


**Figure 2:** Gel electrophoresis result of SPPV, LSDV, and GTPV

## 4.2. GTPV, SPPV, and LSDV Ag titration

In order to determine the concentration of the Ag required to coat the plate, titration of each of the Ag was conducted starting from the concentration of 1/2.5 up to 1/5120, and two-times replication have been conducted for each of the concentration. The OD was determined through spectrophotometry at 450 nm of wavelength. The OD values above 0.7 were considered as acceptable values (AU-PANVAC) which then further used to determine the concentration of the Ag required to coat plates.

The figurative illustration of titration of GTPV Ag (Figure 3) indicates that the concentrations between 1/160 and 1/640 (OD value of above 0.7) were the accepted concentration to coat the plate for further tests.

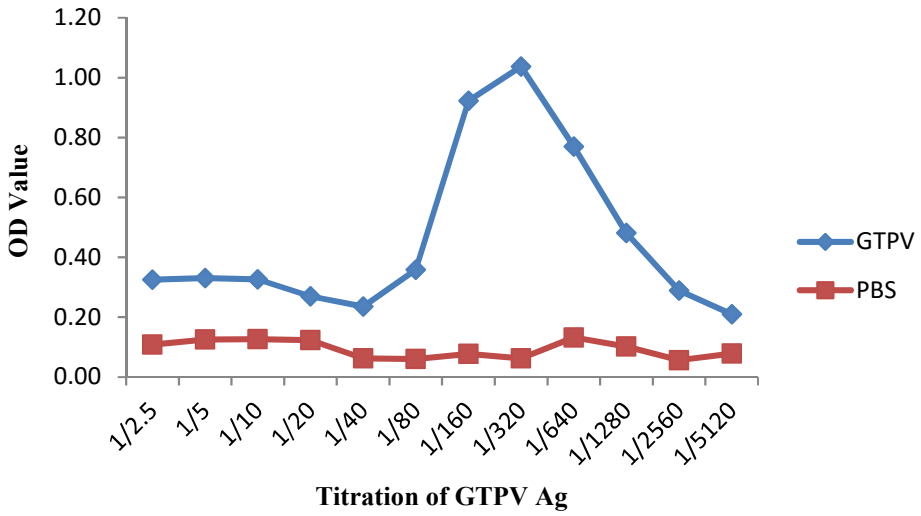


**Figure 3:** Titration of GTPV Ag as compared with PBS

The figurative illustration of titration of SPPV Ag (Figure 4) also indicates that the concentrations between 1/20 and 1/40 (OD value of above 0.7) were the accepted concentration to coat the plate for further tests.

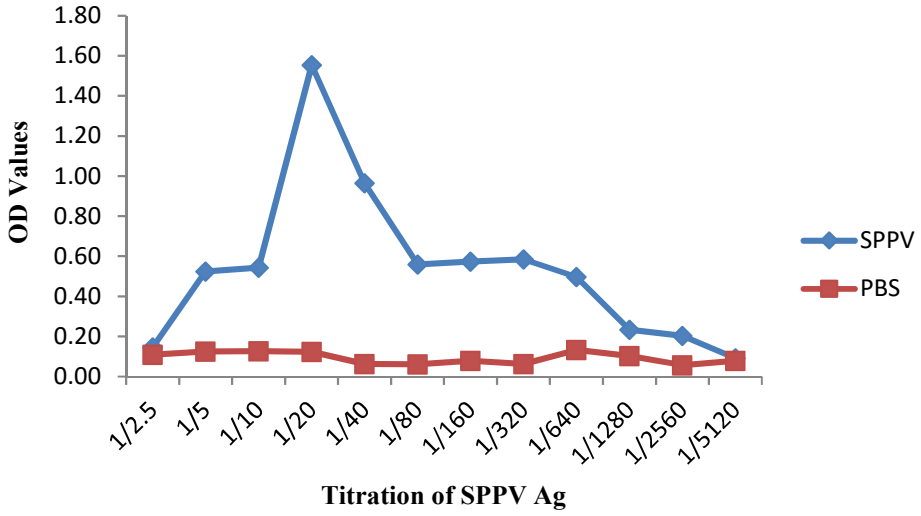


**Figure 4**: Titration of SPPV Ag as compared with PBS

The figurative illustration of titration of LSDV Ag (Figure 5) also indicates that the concentrations between 1/20 and 1/640 (OD value of above 0.7) were the accepted concentration to coat the plate for further tests.

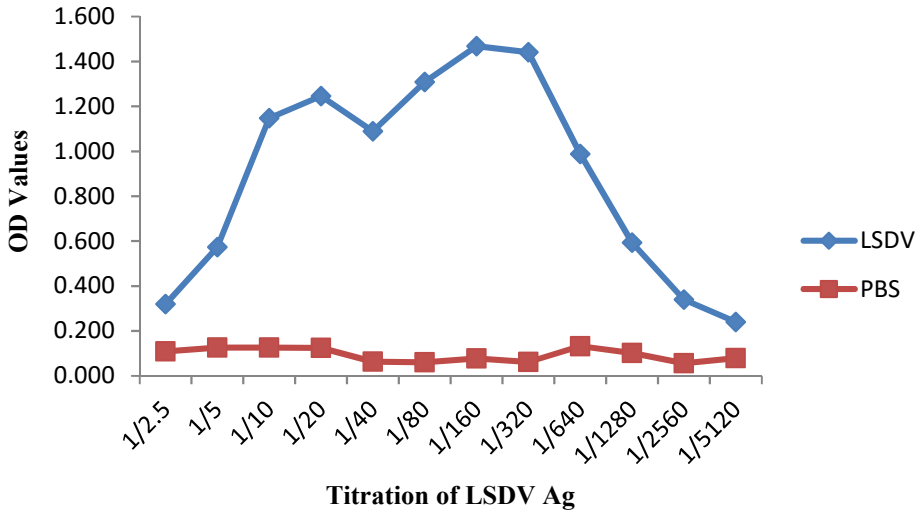


**Figure 5**: Titration of LSDV Ag as compared with PBS

### 4.3. Titration of anti-mouse

In order to determine the antibody production from the immunized mice the concentration of the anti-mouse (enzyme labeled anti-antibodies which were against mouse antibody) titer from stock solution need to be determined. The titration of the anti-mouse was conducted starting from the concentration of 1/2500 up to 1/5120000 and for each concentration six replication have been done. The titration was conducted on the plate which was coated with LSDV Ag with concentration of 1/200. The OD was determined through spectrophotometry. The OD values above 1 were considered as acceptable values (AU-PANVAC) which then further used to determine the titer (dilution) of anti-mouse required.

The figurative illustration of titration of anti-mouse (Figure 6) indicates that the titer between the concentration of 1/2500 up to 1/5000 (OD values of above 1) was acceptable titer (dilution).

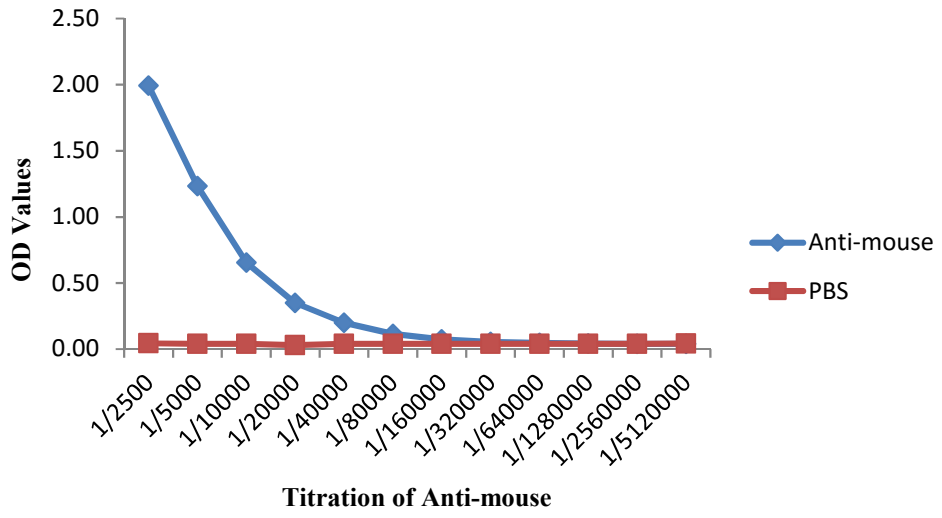


**Figure 6:** Titration of the anti-mouse as compared with PBS

### 4.4. Seroconversion Test

In order to determine the seroconversion of the immunized mice after the second booster injection the iELISA test was conducted on the serum samples which were collected before immunization (day 0) and after the second booster injection for each of the Ag. This test was conducted on the plate coated with SPPV, GTPV, and LSDV Ag with the concentration of 1/40, 1/400, and 1/200, respectively.

The average OD value of the serum after the second booster injection for each of the antigens (SPPV, GTPV, and LSDV) compared with serum sample at day 0 (before immunization) (Figure 7) indicated that there was evidence of the seroconversion.

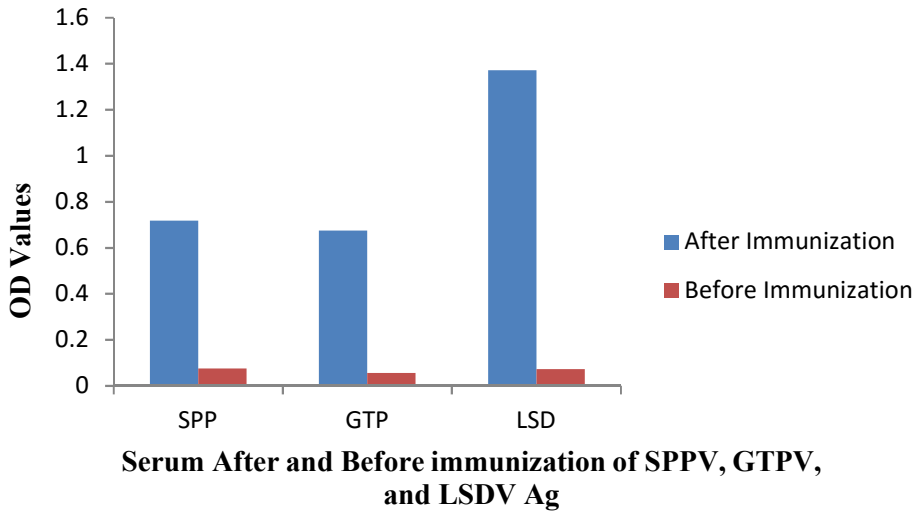


**Figure 7:** The comparation of OD values of serum before and after immunization

### 4.5. Cross-reaction of the two mAbs with SPPV, and GTPV Ag

In order to determine the cross reaction of the two LSD mAbs LC 5.14 and LC 5.4, iELISA test was conducted on the plate coated with LSDV, SPPV, and, GTPV Ag with the concentration of 1/200, 1/40, and 1/400, respectively. As positive and negative control the serum and the blocking buffer (BB) were used, respectively. Then the OD was determined through spectrophotometry. The OD values above 0.5 were considered as acceptable values (AU-PANVAC) which then further used to interpret the cross-reactivity of the two mAbs LC 5.14 and LC 5.4 with SPPV and GTPV Ag.

The average OD values indicates that the two mAbs LC 5.14 and LC 5.4 cross reacts with GTP Ag but not with SPP Ag (Figure 8).

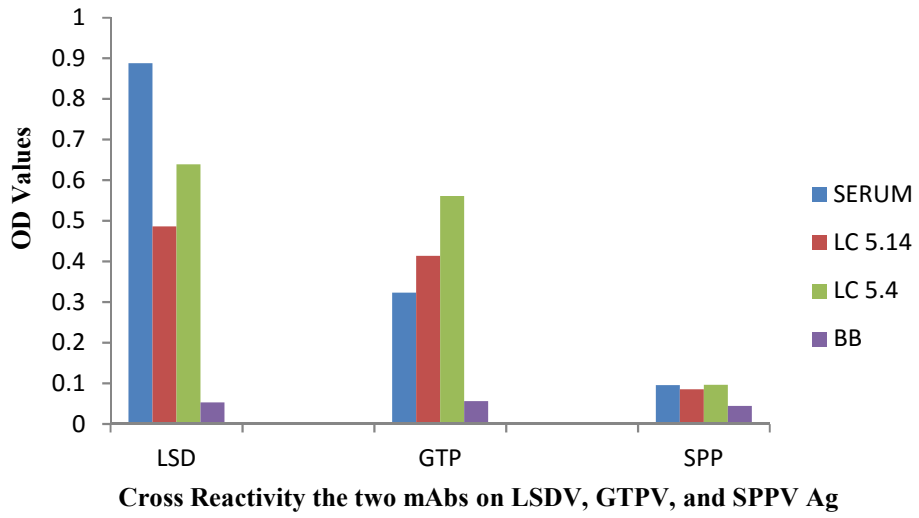


**Figure 8**: Comparation of the average OD value of each of the antigen with serum, the Mabs LC 5.14 and LC 5.4, and BB

### 4.6. BCA Protein Assay

The quantification of total amount of protein of the two LSD mAbs (LC 5.14 and LC 5.4) was determined by using of the OD value of the standard and the samples based on Pierce™ BCA Protein Assay Kit. The OD was determined through spectrophotometry.

The average OD value of the standard which represents the concentration (amount of protein) in the standard was calculated through excel and the expected result from Pierce™ BCA Protein Assay Kit were used to determine the correlation between the average OD value of standard (amount of protein of standard) with the expected values from the kit. Then by using of the correlation between the two the obtained protein concentration of the standard was calculated. The correlation was further used to determine the amount (concentration) of the sample (Table 1).

**Table 1**: Average of OD of standard (Std) with the expected and obtained results

| STD | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 |
|---|---|---|---|---|---|---|---|---|---|
| Average OD Std | 1.15 | 0.94 | 0.70 | 0.58 | 0.40 | 0.25 | 0.17 | 0.09 | 0.07 |
| mg/ml expected Std | 2 | 1.5 | 1 | 0.75 | 0.5 | 0.25 | 0.125 | 0.025 | 0 |
| mg/ml obtained Std | 1.9 | 1.5 | 1.1 | 0.86 | 0.5 | 0.27 | 0.119 | -0.03 | -0.1 |

The correlation between the average OD value of standard (amount of protein of standard) with the expected values from the kit was calculated by using of excel graph sheet (Figure 9).

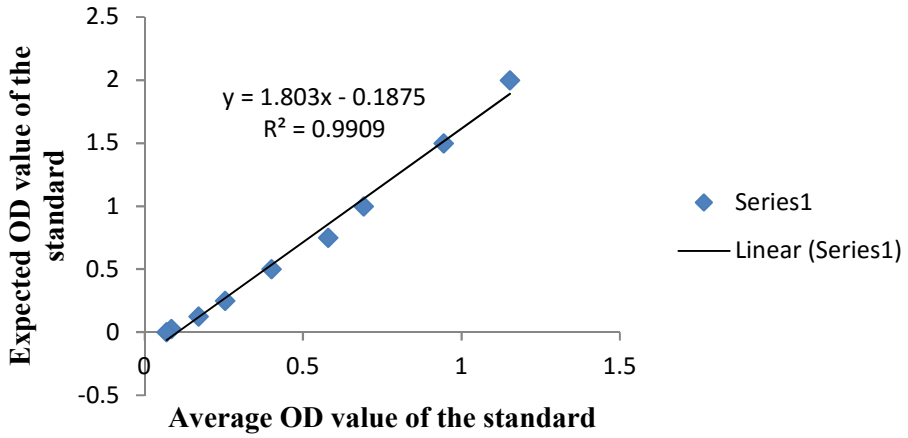


**Figure 9**: The correlation of the OD of the standard with expected

The above equation was used to determine the obtained sample concentration by using of the average OD value of the samples. The concentration of the mAb LC 5.14 and LC 5.4 was about 1.5 mg/ml and 3 mg/ml, respectively (Table 2).

**Table 2**: Average of OD of the two mAbs with the obtained result

| Samples | LC 5.14 | LC 5.4 |
|---|---|---|
| Average OD samples | 0.97 | 1.842 |
| mg/ml obtained sample | 1.56 | 3.13 |

### 4.7. Isotyping of mAbs

The isotyping result for the two mAbs LC 5.14 and LC 5.4 was determined by the Pierce™ Rapid Antibody Isotyping Kit and both of the mAbs (LC 5.14 and LC 5.4) were IgG1 (Figure 10).


A B C D

**Figure 10**: Isotyping of the two mAbs LC 5.14 and LC 5.4 as shown in A and C

### 4.8. Dot-blot Test

Dot blot was conducted for the conjugated LC 5.14 mAb with the concentration of 1/10 on the nitrocellulose membrane for LSDV, GTPV, and SPPV and as negative control, PBS was used. The interpretation was done through visual observation of the membranes and the positive samples were standardized to contain a strong bluish circle, surrounded by week staining background, and for the negative control PBS was without any staining as shown on the left and right corner (Figure 11).

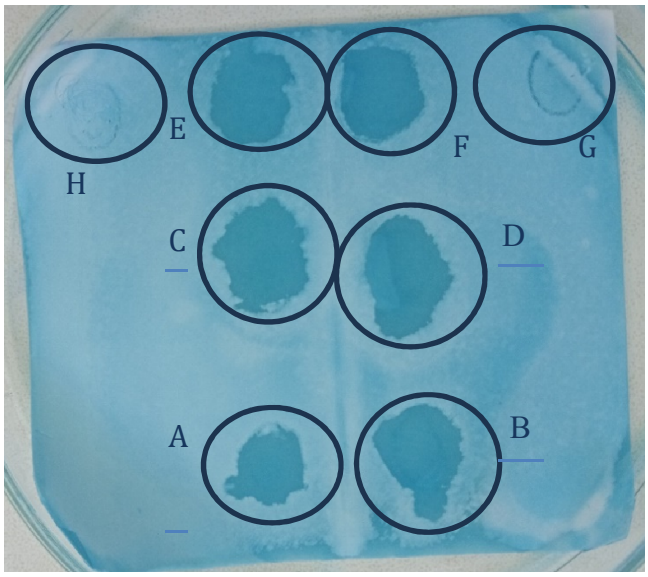


**Figure 11:** Dot blot of the mAb LC 5.14 on SPPV Ag A and B, on GTPV Ag C and D, on LSDV Ag E and F, and on PBS H and G.

Dot blot was conducted for the conjugated LC 5.4 mAb on the nitrocellulose membrane for LSDV, GTPV, and SPPV and as negative control PBS was used. The interpretation was done through visual observation of the membranes and the positive samples were standardized to contain a strong bluish circle, surrounded by week staining background, and for the negative control PBS was without any staining as shown in the middle (Figure 12).

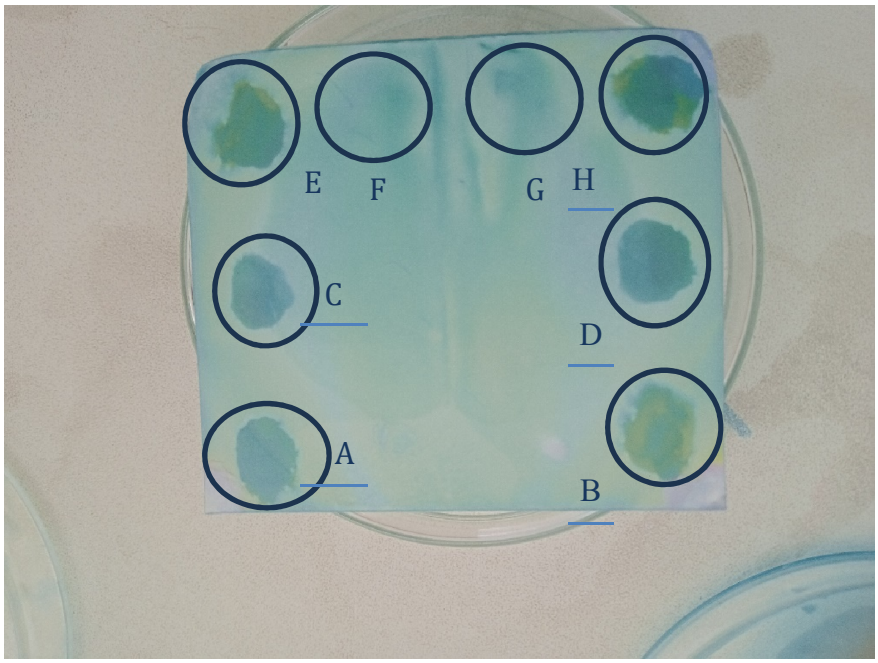


**Figure 12**: Dot blot of mAb LC 5.4 on SPPV Ag A and B, on GTPV Ag C and D, on LSDV Ag E and H, and on PBS F and G.

### 4.9. ELISA assay on titration of conjugated mAbs

In order to determine the titer of the conjugated mAbs (LC 5.14 and LC 5.4) required to test the coated plate with LSDV and GTPV Ags, titration of the two mAbs were conducted starting from the concentration of 1/2.5 up to 1/5120. The OD values above 0.7 were considered as acceptable values (AU-PANVAC) which then further used to test LSDV and GTPV Ag coated plates.

The figurative illustration of titration of LC 5.4 mAb (Figure 13) indicates that 1/2.5 and 1/5 (OD value of above 0.7) were the accepted titration (dilution) to use LC 5.4 mAb on LSDV and GTPV Ag coated plates.

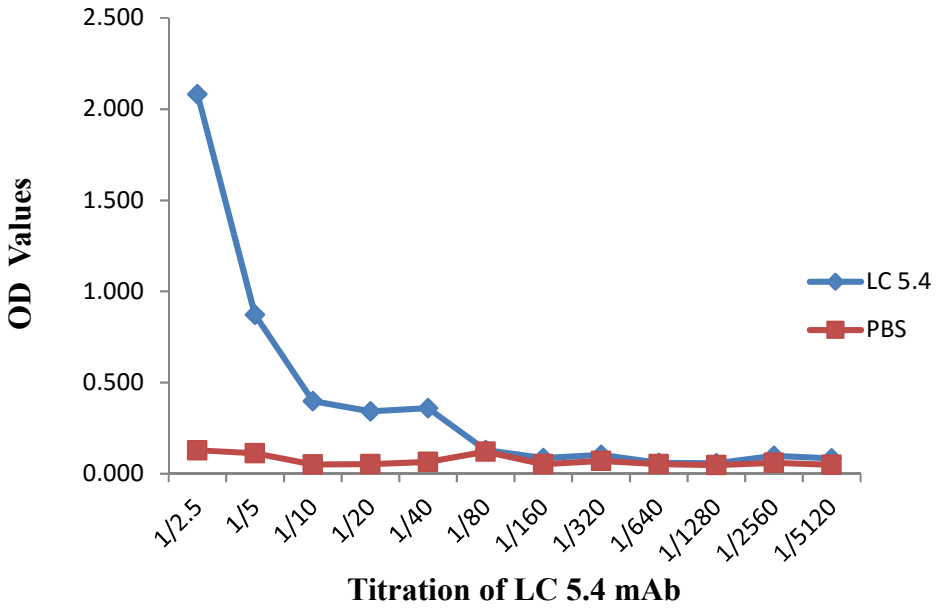


**Figure 13**: Comparation of titration of LC 5.4 with PBS

The figurative illustration of titration of LC 5.14 mAb (Figure 14) indicates that 1/2.5 and 1/10 (OD value of above 0.7) were the accepted titration (dilution) to use LC 5.14 mAb on LSDV and GTPV Ag coated plates.

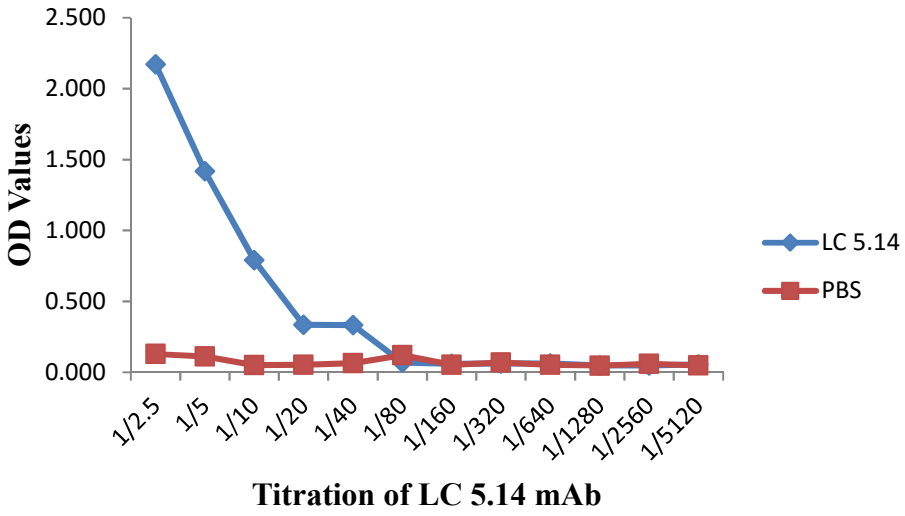


**Figure 14**: Comparation of titration of LC 5.14 with PBS

## 5. DISCUSSION

Antibodies are emerging as fundamental tools with great commercial and medical, and are important substances in diagnostic and medical research, and therapeutics (Harlow and Lane, 1988; Stavitsky and Jarchow, 1954). In the pharmaceutical industries antibody product category is thought to be expanding at the highest rate (Lipman *et al*., 2005; Maleki *et al*., 2013). Hybridoma technology is one of the most common methods used to produce monoclonal antibodies. Monoclonal antibodies are useful in diagnostic and therapeutic purposes and have a very high clinical significance. Once hybridoma cells become stable, these cell lines offer limitless production of homogenized antibodies. This method is also cost-effective and the antibodies produced by this method are highly sensitive and specific to the targeted antigen (Mitra and Tomar, 2021).

The recent expansion of capripox diseases into new geographical regions has stressed the need for high-throughput tools for their surveillance (Baselli *et al*., 2023). In this research paper, we produced and evaluated the mAb production against the virion of LSD, SPP, and GTP after intra-peritoneal immunization of BALB/c mice. Related studies showed that immunization with SPP, and GTP antigens was started in 1903 by Borrel (Bennett *et al*., 1944). The use BALB/c mice for the mAb production for different agents have been previously mentioned in lots of research studies (Alejandra *et al*., 2023; Baradaran *et al*., 2013; Sepehr *et al*., 2012).

In this study the molecular detection of capripoxvirus were performed by amplification of RPO30 gene. The RPO30 gene was used for detection and differentiation of the three capripoxviruses mainly of LSDV, SPPV, and GTPV (Zhou *et al*., 2012).

LSD, SPP, and, GTP viruses are similar, sharing 97% nucleotide identity, and studies have also revealed that SPP and GTP viruses share approximately 96% nucleotide identity over their entire length (Chand *et al*., 1994). Taking into consideration the genomic similarity of capripoxviruses, we used the LSDV, SPPV, and GTPV antigen to determine the seroconversion in the blood of immunized mice for the three Ags, and the result reveled

that high similarities in positive and negative OD for values in both SPPV and GTPV which share similar nucleotides, but higher OD of positive serum of LSDV (Figure 8). Similar study which was conducted with the titration of serum of immunized mice to check the seroconversion supports this result (Hurisa *et al*., 2021). Overall, the positive (after the second boost injection) and negative (before administration of the Ag) showed that there is variation between the positive and negative serum samples for all of the antigens (for LSD, SPP, and GTP).

After humanely scarification of the mice, the spleen was collected and fused with Ag8 myeloma cells. The fused cells were incubated for 14 days. At the first screening of the hybridoma cells culture after 14 days of the incubation period, the cells were performing well by producing the mAbs. But after the third passage and screening the viability and the production of the mAbs start to decline. Finally, the two mAbs (LC 5.14 and LC 5.4) for the LSDV have been produced, but the production of the mAbs for SPPV and GTPV were lost through the process. Other studies mentioned the same phenomena that the initial cell viability used to study the performance of the hybridoma cell cultures grown well in the first passage, about 80 % Corrêa *et al*. (2016). Also, other studies reported that reductions in the mAb productivity (Li *et al*., 2006) as well in cell growth (Vallejos *et al*., 2009) show that different cell passages can behave differently. Those changes in the hybridoma cell lines might be because of the cells might have suffered from harmful interferences, as passages run, for the production of mAbs (Corrêa *et al*., 2016).

In this study two different mAbs namely LC 5.4 and LC 5.14 were produced successfully in mice immunized with LSDV Ag. The two mAbs of LSD belongs to the IgG1 isotype of antibodies. The protein concentration for LC 5.14 and LC 5.4 was determined through BCA protein assay and it was about 1.5 mg/ml and 3 mg/ml, respectively. The use of BCA for protein assay was also done in other studies (Baselli *et al*., 2023). After quantification both MAbs were successfully conjugated to HRP enzyme.

The conjugated mAbs were used to develop the dot-blot assays successfully but needs further evaluation and standardization to be used as reagent for diagnostic use. In the dot-blot test the use of an established mAb concentration, and its simplicity, precision and speed demonstrate that the assay can be used for large scale diagnosis with further standardization using positive and negative serum samples, and in comparison, with the other in use diagnostic tests for the disease. Once standardized, it is an attractive test for routine diagnosis.

The two mAbs (LC 5.14 and LC 5.4) were further used to develop ELISA assay using the prepared viral antigen, which permits to display a broad spectrum of immunogenic epitopes to recognition. LSDV and GTPV antigens were detected by using of those produced mAbs. The concentration of the mAbs required for the ELISA assay has been determined through titration of conjugated mAbs.

## 6. CONCLUSION AND RECOMMONDATIONS

In the present study two mAbs against LSDV was successfully produced while the production against SPPV, and GTPV was not successful. The produced mAbs were conjugated with HRP to develop direct ELISA. This study also showed that which titer of the conjugated mAb can be used for ELISA test. The mAbs were produced through immunization of BALB/c mice. Nave BALB/c mice were immunized by each of the Ag intraperitonially with booster doses. After immunization the mice were humanely scarified and the spleen were taken and fused with Ag8 myeloma cells. Through enormous screening ELISA tests the fused hybridoma cells were selected. Those mAb producing hybridoma cells were cultured in the appropriate media to make sure on their sustainability and adaptability. Then the hybridoma cells can further be kept for longer period of time through storage in the liquid nitrogen. The supernatant from the hybridoma cell culture flask, which consists of the produced mAbs, were used for further examination after precipitation and purification process. Then the mAbs were subjected for protein quantification through BCA Protein Assay Kit. The isotype for the two mAbs were determined through Pierce™ Rapid Antibody Isotyping Kit. The mAbs were then conjugated with the HRP enzyme which was furtherly used for ELISA test.

Based on the above conclusion the following recommendation are forwarded;

- The produced mAb that was conjugated with horse-radish per oxidase can be further standardized using of known positive and known negative serum samples.

- Those produced and conjugated mAbs might be further used for production of diagnostic assay Kit after a proper standardization and validation tests.

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## 8. APPENDIXES

**Appendix I**: Cell Culture

**Materials and Reagents required**

1. GMEM
2. Fetal Calf Serum (FCS)
3. Antibiotic-Antifungal (Penicillin–streptomycin solution) (100X)
4. 70% Ethanol solution or isopropanol.

**Equipment**

1. 25 mL sterile serological pipettes
2. 10 mL sterile serological pipettes
3. 5 mL sterile serological pipettes
4. Cell culture flask
5. Incubator
6. Pipette-aid
7. Low speed bench centrifuge
8. Water bath
9. Biological safety cabinet
10. Liquid nitrogen
11. Inverted microscope
12. Glove

**Procedure**

1. In the biological safety cabinet, add 5% Fetal Bovine Serum (FBS) and 1% (v/v) antibiotic (AB) - antimycotic (AF) to Glasgow modified Eagle medium (GMEM)

to produce the complete GMEM medium and store at 4°C until it is required to use.

2. Prepare one tube containing 9 mL of complete GMEM medium warmed at 37°C in water bath.
3. Remove one vial of cells from the storage container (liquid nitrogen)
4. Put vial of cells in to 10 ml of warm water from 37°C water bath until the suspension is just thawed
5. In the biological safety cabinet, use a sterile glass pipet to transfer the contents of the vial slowly into the tube containing 9 mL of complete GMEM medium
6. Centrifuge the cells at 1200 rpm (400 x g) for 10 min to obtain a pellet
7. Discard the supernatant containing DMSO and suspend the cell pellet in 10 mL of GMEM growth medium
8. Transfer the cells to a 75 cm2 tissue culture flask which contains 25 ml of complete GMEM medium and incubate at 37°C humified incubator with 5% CO2
9. Examine cultures daily using an inverted microscope to ensure that the culture was not contaminated during the freeze-thaw process and to make sure that the cells are growing.

**Appendix II**: Cell Passaging

**Materials and Reagents required**

1. GMEM
2. Fetal Calf Serum (FCS)
3. Antibiotic-Antifungal (Penicillin–streptomycin solution) (100X)
4. Phosphate Buffered Saline (PBS)
5. 1% Trypsin solution
6. 70% Ethanol solution or isopropanol.

**Equipment**

1. 25 mL sterile serological pipettes
2. 10 mL sterile serological pipettes
3. 5 mL sterile serological pipettes
4. 15 mL sterile falcon tubes
5. Cell culture flask
6. 37°C water bath
7. Biological safety cabinet
8. Pipette-aid
9. Low speed bench centrifuge
10. Inverted microscope
11. Glove

**Procedure**

1. When the cells reach 80%–100% confluence, typically after 5-7 days of culture for the first passage after thawing a fresh tube of cells; later, the cells grow faster with an approximately 3-days passage time.

2. Aspirate the old culture medium from the culture flask using a serological pipette and discard the medium. Wash the cells three times by gently adding 10 mL Phosphate Buffered Saline (PBS) to the flask. Aspirate the PBS and discharge the solution.
3. Detach cells by adding 1 mL of 1%Trypsin to the flask and return the flaks to 37°C for 1 min. Deactivate trypsin by adding 10 mL of complete GMEM medium. Aspirate the cell suspension and transfer to a 15 mL falcon tube.
4. Centrifuge the cell suspension at 400 × *g* for 10 min.
5. Resuspend the cells in 10 mL of complete GMEM medium and then put the cells into two 75cm2 of new cell culture flasks each consists of 25 ml of complete GMEM medium

**Appendix III**: DNA Extraction

**Materials and Reagents required**

1. Normal saline
2. Ethanol (96-100%)
3. Buffer AVL
4. Buffer AW1
5. Buffer AW2
6. Buffer AVE

**Equipment**

1. 1-10 µl Micro pipettes
2. 10-100 µl Micro pipettes
3. 30-300 µl Micro pipettes
4. 100-1000 µl Micro pipettes
5. 1.5 ml micro centrifuge tube
6. Mini centrifuge
7. Vortex
8. Biosafety cabinet

**Procedure**

1. In a sterile tube pipet 560 µl of AVL buffer with 5.6 µl of carrier RNA. Then gently mix by inverting the tube 10 times.
2. To 560 µl buffer AVL buffer containing carrier RNA in 2 ml micro-centrifuge tube, add 140 µl of sample (antigen).
3. Then Add 560 µl ethanol (96–100%). Vortex for 15 s. Briefly centrifuge the tube to remove drops from the lid.

4. Carefully pipet the 650 µl of the mixture onto the QIAamp Mini spin column (in a 2 ml collection tube). Centrifuge at 6000 x g (8000 rpm) for 1 min. Discard the flow-through and collection tube.
5. Place the QIAamp Mini spin column in a new 2 ml collection tube and add 500 µl Buffer AW1. Centrifuge at 6000 x g (8000 rpm) for 1 min. Discard the flow-through and collection tube.
6. Place the QIAamp Mini spin column in a new 2 ml collection tube and add 500 µl Buffer AW2. Centrifuge at full speed (20,000 x g; 14,000 rpm) for 3 min. Discard the flow-through and collection tube.
7. Place the QIAamp Mini spin column in a new 1.5 ml microcentrifuge tube, add 80 µl Buffer AVE or distilled water and incubate at room temperature for 1 min. Centrifuge at 6000 x g (8000 rpm) for 1 min to elute the DNA.

**Appendix IV**: DNA Amplification

**Materials and Reagents required**

1. PCR kit: Qiagen Taq DNA polymerase kit with (Taq polymerase, PCR buffer)
2. Forward primer (SpGpRNAPol-F)
3. Reverse primer (SpGpRNAPol-R)
4. Template DNA
5. Positive reference sample
6. Negative reference sample

**Equipment**

1. Biosafety cabinate
2. Mini centrifuge
3. Vortex
4. Thermocycler
5. 1-10 µl Micro pipettes
6. 10-100 µl Micro pipettes
7. 30-300 µl Micro pipettes
8. 100-1000 µl Micro pipettes
9. PCR tube strips 0.2 ml with caps

**Procedure**

1. Thaw 10x PCR Buffer, dNTP mix, primer solutions, at room temperature. Keep the solutions on ice after complete thawing. Mix well before use to avoid localized differences in salt concentration.
2. Prepare a master mix. The master mix typically contains all of the components needed for PCR except the template DNA. Prepare a volume of master mix 10% greater than that required for the total number of PCR assays to be performed. A negative control (without template DNA) should also be included. Start with 2.5 µl

of PCR buffer, 2.5 µl of dNTPs, 2.5 µl of 2.5 µl of forward primer (SpGpRNAPol-F), 2.5 µl of reverse primer (SpGpRNAPol-R), 0.125 µl of Taq polymerase, and plus balance nuclease-free water ( 10 µl) to a final volume of 25 µl.

3. Mix the master mix thoroughly, and dispense 20 µl of the master mix into PCR tubes. Mix gently (by pipetting the master mix up and down a few times). It is recommended that PCR tubes are kept on ice before placing in the thermal cycler.
4. Add template DNA of 5 µl to the individual tubes containing the master mix.
5. Amplification starts with 40 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s and extension at 72°C for 30 s. The 40 cycles were followed by a final extension at 72°C for 5 min
6. Finally, the PCR product can be directly loaded onto an agarose gel with the addition of a PCR loading buffer and gel tracking dyes.

**Appendix V**: Immunization of BALB/c Mice

**Materials and Reagents**

1. Complete frauds adjuvants
2. Incomplete frauds adjuvants
3. Antigen
4. 3-4-week nave BALB/c mice

Equipment

1. Syringe
2. Capillary tube
3. Glove

**Procedure**

1. Inject 4 adult nave BALB/c mice with antigen. Typically, 20 - 100 µg of purified antigen is mixture with the adjuvant (Complete frauds adjuvant for the first injection and incomplete frauds adjuvant for the three consecutive booster injections) is injected intraperitoneally in a total volume of 200 µL (i.e. 200 µL of a 1:1 emulsion of antigen in adjuvant)
2. Repeat the booster injection 14 days later.
3. After the third booster injection, take 100 - 200 µL of blood by cutting 1 - 2 mm from the tip of the tail and collect the blood it into a capillary tube. Prepare serum from the blood sample and titrate in ELISA.
4. To obtain the highest antibody titers further boosting with antigen is required (at least four booster injections are required). Continue to give injections at 2 weeks intervals.

5. 1 - 4 days before the day of the fusion boost the BALB/c mice intraperitoneally with 200 µL of antigen without any of the adjuvants. Prepare to fuse spleen cells 3 - 4 days later

**Appendix VI**: Preparation of the Splenocyte Suspension

**Materials and Reagents required**

1. 95% Ethanol
2. 3% Acetic Acid with Methylene Blue
3. Medium A; Contains Dulbecco's Modified Eagle's Medium (DMEM), pre-selected serum, gentamycin, and supplements (ClonaCell™-HY Hybridoma Kit (STEMCELL Technologies; Catalog Number: 03800))
4. Medium B; Contains DMEM and gentamycin (ClonaCell™-HY Hybridoma Kit (STEMCELL Technologies; Catalog Number: 03800))

**Equipment**

1. 50 mL sterile conical tubes
2. 50 mL sterile serological pipettes
3. 25 mL sterile serological pipettes
4. 10 mL sterile serological pipettes
5. 5 mL sterile serological pipettes
6. 10 cm petri dish
7. 3 mL syringe
8. Forceps
9. Fine scissors
10. Fine-mesh metal screen (disposable cell strainer)
11. Biohazard safety cabinet certified for level II handling of biological materials
12. Low speed bench centrifuge
13. Pipette-aid
14. Hemacytometer
15. Inverted microscope
16. 37°C water bath

**Biologicals**

1. Primed mouse (4 days after final antigen boost)

**Procedure**

1. Sacrifice an immunized mouse through cervical dislocation and wash the fur with 95% ethanol. Clip fur, cut skin, and pull back to expose chest.
2. Remove the spleen and place in a sterile petri dish containing 5 mL of Medium A. Trim off any large pieces of fatty tissue.
3. Disaggregate the spleen into a single cell suspension. Then transfer the spleen to a fine mesh screen placed on top of a 50 mL conical centrifuge tube, and use the plunger of a 3 mL syringe to grind the cells through the screen.
4. Rinse the screen with Medium B to help all of the cells go through the screen. All that should be left in the screen is the spleen membrane. Gently pipette the cells up and down to disrupt clumps. Try not to cause the solution to foam.
5. Wash the splenocytes 3 times in 30 mL of Medium B, centrifuging at 400 x g at RT for 10 minutes each time and removing the supernatant by pipette. After the final wash resuspend the cells in 25 mL Medium B. It is important to remove all the serum adhering to the cells by washing with serum-free Medium B. If the serum is not removed, the PEG will not fuse the cell membranes and the fusion frequency will drop drastically.
6. Prepare a 1/10 dilution of cells in 3% acetic acid, e.g. by mixing 10 µL of the cell suspension with 90 µL of 3% Acetic Acid with Methylene Blue.
7. Count cells in this diluted sample using a hemacytometer. Calculate the volume of cell suspension that contains 1 x 108 cells.

**Appendix VII**: Myeloma Cells preparation

**Materials and Reagents required**

1. RPMI medium
2. Medium A (ClonaCell™-HY Hybridoma Kit (STEMCELL Technologies; Catalog Number: 03800))
3. Medium B (ClonaCell™-HY Hybridoma Kit (STEMCELL Technologies; Catalog Number: 03800))

**Equipment**

1. 50 mL sterile serological pipettes
2. 25 mL sterile serological pipettes
3. 10 mL sterile serological pipettes
4. 5 mL sterile serological pipettes
5. Biohazard safety cabinet certified for level II handling of biological materials
6. Low speed bench centrifuge
7. Pipette-aid
8. Hemacytometer
9. Inverted microscope
10. 37°C water bath

**Procedure**

1. Revive the parental myeloma cells (see Appendix I) and culture in RPMI medium, then culture in ClonaCell®-HY Pre-Fusion Medium (Medium A) for at least 1 week to ensure that they are well adapted to this medium prior to fusion. Seed cells at a density of approximately 5 x 104 cells/mL and passage every 2 days. If cells are allowed to grow beyond 8 x 105 cells/mL, passage at least 2 times to bring them back to early-mid log phase growth prior to fusion.

2. The day before fusion, count the viable cells and split so that at least 2 x 107parental myeloma cells are available the next day. The recommended cell density for fusion is 2 x 105 cells/mL. Only 100 mL of cells is needed, but 200 mL should be cultured to ensure sufficient cell numbers for fusion.
3. Harvest the parental myeloma cells in a 50 mL conical centrifuge tube by centrifuging at 400 x g for 10 min at room temperature (RT). Wash 3 times by adding 30 mL of ClonaCell®-HY Fusion Medium (Medium B), centrifuging again as before, and removing the supernatant. Resuspend the cell pellet in 25 mL of Medium B. This step may be performed simultaneously with, or after, the spleen cell preparation to ensure that the myeloma cells are not sitting for an extended period of time. It is important to remove all the serum adhering to the cells by washing with serum-free Medium B (If the serum is not removed, the PEG will not fuse the cell membranes and the fusion frequency will drop drastically).
4. Count live cells using a viability stain. Viability of parental myeloma cells should be greater than 95%.
5. Calculate the volume of cell suspension that contains 2 x 107 viable cells. Keep cells at RT until fusion.

**Appendix VIII**: Fusion

**Materials and Reagents required**

1. Medium B (ClonaCell™-HY Hybridoma Kit (STEMCELL Technologies; Catalog Number: 03800))
2. Medium C; Contains DMEM, pre-selected serum, gentamycin, and supplements (ClonaCell™-HY Hybridoma Kit (STEMCELL Technologies; Catalog Number: 03800))
3. Polyethylene glycol (PEG)

**Equipment**

1. 50 mL sterile serological pipettes
2. 25 mL sterile serological pipettes
3. 10 mL sterile serological pipettes
4. 5 mL sterile serological pipettes
5. Cell culture flask
6. Biohazard safety cabinet certified for level II handling of biological materials
7. Low speed bench centrifuge
8. 37°C incubator with humidity and gas control to maintain >95% humidity and an atmosphere of 5% CO2 in air
9. Pipette-aid
10. Hemacytometer
11. Inverted microscope
12. 37°C water bath

**Procedure**

1. Prewarm PEG and media (Medium B and C) in 37ºC water bath.
2. Add 2 x 107 parental myeloma cells and 1 x 108 viable splenocytes to a 50 mL conical centrifuge tube and centrifuge for 10 minutes at 400 x g (~1350 rpm). Aspirate off supernatant. Complete removal of the supernatant is essential to avoid dilution of PEG in the next step.
3. Disrupt the cell pellet obtained by gently tapping the bottom of the tube. Add 0.5 mL of ClonaCell®-HY PEG Solution (PEG) dropwise to the pellet using a 1 mL pipette. Centrifuge the mixture at 133 x g (~800 rpm) at RT or 37°C for 3 minutes. Aspirate off all the PEG.
4. It is important to completely break up the cell pellet prior to adding PEG in order to ensure efficient fusion of the cells. During this procedure, not all cells will form a pellet, as some will clump in the PEG. Do not aspirate the clumped cells. Work quickly since cells must not be exposed to PEG for too long or cell viability will drop. Carefully add 5 mL of Medium B dropwise to the pellet while gently swirling the tube to resuspend the cells.
5. Slowly add 5 mL of ClonaCell®-HY Hybridoma Recovery Medium (Medium C) to the solution. Continue to swirl the tube.
6. Transfer the cell suspension to a T-75 cm2 tissue culture flask containing 40 mL of Medium C (total culture volume = 50 mL). Incubate for 16 - 24 hours at 37°C in 5% CO2 atmosphere.
7. There will still be clumps of cells at this point which will dissolve overnight. Be gentle with these cells.

**Appendix IX**: Growth of Hybridomas (Selection of Hybridomas)

**Materials and Reagents required**

1. HAT selection medium (Medium D); Contains DMEM, pre-selected serum, HAT, gentamycin, and supplements (ClonaCell™-HY Hybridoma Kit (STEMCELL Technologies; Catalog Number: 03800))
2. Medium E Contains; DMEM, pre-selected serum, HT, gentamycin, and supplements (ClonaCell™-HY Hybridoma Kit (STEMCELL Technologies; Catalog Number: 03800))

**Equipment**

1. Biohazard safety cabinet certified for level II handling of biological materials
2. Low speed bench centrifuge
3. 37°C incubator with humidity and gas control to maintain >95% humidity and an atmosphere of 5% CO2 in air
4. Pipette-aid
5. Inverted microscope
6. 37°C water bath
7. 50 mL sterile conical tubes
8. 50 mL sterile serological pipettes
9. 10 mL sterile serological pipettes
10. 5 mL sterile serological pipettes
11. T-75 cm2 sterile cellculture flask
12. 96-well sterile tissue culture plates
13. Multi-channel pipettor, 12-channel, 20 - 200 µL

**Procedure**

1. On the day of the fusion, place ClonaCell®-HY Liquid HAT Hybridoma Selection Medium at 2 - 8°C and thaw overnight.
2. As only 100 mL of the ClonaCell®-HY Liquid HAT Medium is required for this procedure, this medium can be aliquoted and frozen for later use. Warm medium to 37°C before use.

3. Transfer the fused cell suspension into a 50 mL conical tube and centrifuge for 10 minutes at 400 x g (~1350 rpm) at RT or 37°C. Remove the supernatant by pipette. Resuspend the cells in ClonaCell®-HY Liquid HAT Medium to a total volume of 10 mL.
4. Transfer the 10 mL cell suspension into 90 mL of ClonaCell®-HY Liquid HAT Medium. Mix thoroughly by gently inverting the bottle.
5. Using a 12-channel pipettor, aseptically plate out 100 µL of cell suspension medium into each well of ten 96-well tissue culture plates. Incubate plates at 37°C in 5% CO2 atmosphere. 5 days after fusion, feed the plates with 100 µL/well of ClonaCell®-HY Hybridoma Growth Medium (Medium E). Continue feeding as required.