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Name: Bezawit Tesfaye

Signature _____ Date of submission: _____

College of Veterinary Medicine and Agriculture, Bishoftu, Ethiopia

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LIST OF ABBREVIATIONS

ADCC	Antibody Dependent Cell-Mediated Cytotoxicity
ATG13	Autophagy Related Protein
BCR	B Cell Receptor
BLRI	Bangladesh Livestock Research Institute
CD150	Cluster Of Differentiation
CDV	Canine Distemper Virus
C-ELISA	Competitive Enzyme-linked Immunosorbent Assay
CeMV	Cetacean Morbilliviruses
CPE	Cytopathic Effect
CPV	Recombinant Capripoxvirus
C-RNA	Complementary RNA
CSA	Central Statistic Authority
CTL	Cytotoxic t-Lymphocytes
DIVA	Differentiating Infected from Vaccinated Animals
DNA	Deoxyribonucleic Acid
F	Fusion
FAO	Food and Agriculture organization
FBM	Fetal Bovine Serum
FN-α/β	Interferon-Alpha/Beta
FRCVM	Federal Research Center for Virology and Microbiology
ARRIAH	Federal Centre for Animal Health
GE	Gene End
GMEM	Glasgow's Minimum Essential Medium
GP	Goat Pox
H	Hemagglutinin
HEK293T	Human Embryonic kidney 293T Cell
HRA	Heptad repeats A
HRB	Heptad repeats B
IFN-γ	Interferon-Gamma
IgM	Immunoglobulin M
ISGs	Interferon-Stimulated Genes

LIST OF ABBREVIATION (continued)

KS1-O18	Kenya O-180 strain of the sheep pox virus
LSD	Lumpy Skin Disease
M	Matrix
M.O.I	Multiplicity Of Infection
MHC	Major Histocompatibility Complex
mRNA	Messenger RNA
MV	Measles Virus
N	Nucleocapsid
NF-Kb	Necrosis Factor kappa B
NVI	National Veterinary Institute
ORFs	Open Reading Frames
PAMPs	Pathogen Associated Molecular Patterns
PBS	Phosphate Buffered-Saline
PBSA	PBS with antibiotics
PMV	Porcine Morbilliviruses
PPR- GCES	Peste Des Petitis Ruminants -Global Control And Eradiation Strategy
PPR	Peste Des Petitis Ruminants
PPRV	Peste Des Petits Ruminant Virus
RBVs	Recombinant Baculoviruses
RdRp	RNA Dependent RNA Polymerase
RIG-I	Retinioc Acid - Inducible Gene I
RNA	Ribonucleic Acid
RNP	Ribonucleoprotein Complex
RPV	Rinderpest Virus
SLAM	Signaling Lymphocyte Activation Molecule
SNPs	Single-Nucleotide Polymorphisms
SNPs	Single-Nucleotide Polymorphisms
SNT	Serum Neutralization Test
SP	Sheep Pox
TCID	Tissue Culture Infective Dose
TIL-1	Toll-Interleukin 1

LIST OF ABBREVIATION (continued)

TNF-α	Tumor Necrosis Factor Alpha
VLP	Virus Like Particle
VNAs	Neutralizing Antibodies
VTM	Viral Transport Medium
WOAH	World Organization for Animal Health

ABSTRACT

Peste des petits ruminants (PPR), sheeppox (SP) and goatpox (GP) are economically important diseases in Ethiopia. These diseases are commonly distributed throughout Ethiopia, hindering productivity and leading to substantial economic losses. Given Ethiopia's ambitious goal to eradicate PPR by 2027, this research aimed to develop and evaluate the efficacy of a combined vaccine by starting with the preparation of monovalent PPR and SP/GP live attenuated vaccines separately using Vero cell culture and with the determined titer 4.8 $\log_{10}$ $TCID_{50}$ per ml and 4.6 $\log_{10}$ $TCID_{50}$ per ml respectively, before being combined. Quality control tests, including sterility, moisture content, and safety in guinea pigs, were performed. A total of 10 sheep and goats were immunized, and their immune responses were monitored through daily body temperature readings that was maintained in the normal range between 37.38^{o}C-39.32^{o}C, C- ELISA test result for antibody detection of PPR read its peak on 10.59% for group A and 10.58% for group B, while Group C remained with no antibody, heightened that Group A and B animals confers protection up on natural infection. The SNT test results also revealed that vaccinated animals in each group had strong antibody titers against SP/GP, defined as values greater than 1.5 $\log_{10}$. Each of these animals challenged by PPR and GP viruses isolated on Verodog-SLAM for PPRV and on Vero and ESH-L cells for GPV. Finally, the challenge experiment discloses that vaccinated animals stayed in their normal behavior while control animals unequivocally manifested disease-specific clinical signs, which was confirmed by molecular detection of nucleic acids of both viruses. These findings underscore the vaccine's robust efficacy in preventing overt disease and secured immunogenicity of both vaccines. Further researches on the deployment of this live attenuated combined vaccine on a field basis have of utmost importance to completely asses its efficacy and effectiveness.

Key words: *Antibody titers, combined vaccine, challenge test, efficacy, goat pox, peste des petits ruminants, quality control, Sheep pox.*

1. INTRODUCTION

Ethiopia is home to a vast population of small ruminants with 42 million sheep and 52 million goats (CSA, 2021/22). This accounts for about 10% of Africa's and 4% of the world's small ruminant population (FAO, 2019). Ethiopia's agricultural sector contributes 40% of the country's gross domestic product and 20% of its export earnings, while 80% of the nation relies on it for income, nutrition and livelihood (Shapiro *et al*., 2017). There is increased demand both domestically and worldwide for Ethiopian sheep and goat meat which contributes to large foreign currency as well as main source of income for farmers (Mijena and Getiso, 2022). As a result, the country the ranked the 9th largest exporter of sheep and goat meat globally, earning $92.6M in 2022 alone. This export market has grown markedly in the Middle Eastern Gulf States and some African countries (OEC World, 2020).

Nonetheless, the generated economic proceeds are discordant with the country's potential, with part of the reason being disease leading to poor animal productivity (Kasye *et al*., 2016). Infectious diseases having a serious impact include PPR, SP, and GP, which are commonly distributed throughout the regions, particularly PPR is deemed the foremost livestock disease in Ethiopia (Babiuk *et al*., 2008; Jemberu *et al*., 2022).

The morbidity and mortality rates concerning PPR can reach up to 100% and 90%, respectively. In particular areas where PPR is endemic, the mortality rate may be lower, but it leaves underlying detrimental effect on flock productivity. Consequently, the economic loss globally due to PPR is estimated to be 1.2 to 1.7 billion USD each year, related to animal deaths, deteriorated production, and the expenses spent to fight against the disease (WOAH, 2024).

Pest des petits ruminant (PPR) is a severe infectious disease affecting both domestic as well as wild small ruminants. It also leads to socio-economic consequences in humans. To address this issue the PPR Global Control and Eradiation Etrategy (PPR- GCES) was launched in Côte d'Ivoire in 2016 by the Food and Agriculture Organization of the United Nations (FAO) and the World Organization for Animal Health (WOAH) aiming to eradicate PPR by 2030. PPR GCES relies on a four-stage stepwise approach: in Stage 1, countries are recommended to prioritize appraising the local epidemiological situation; Stage 2 is dedicated to controlling

PPR infection, mainly through vaccination campaigns informed by surveillance; in Stage 3, eradication should be sought by strengthening surveillance and preventive measures; Finally in Stage 4, vaccination must be halted and data supporting that PPRV is no longer circulating must be collected (Benfield *et al*., 2023).

PPR monitoring and assessment tool is a companion to PPR-GCES functions to determine the country's stage and provide guidance. It has five technical elements described by GCES (Diagnostics, Surveillance, PPR Prevention and Control, Legal framework, and Stakeholder involvement). Based on the lessons learned from the global eradication of rinderpest and the availability of effective PPR vaccine, vaccination has been issued as a cornerstone for prevention and control measures required for stage 2 and stage 3 (Jones *et al*., 2016; Zhao *et al*., 2021). This is particularly relevant for countries like Ethiopia, which has set an ambitious goal to eradicate PPR by 2027 (Kumbe *et al*., 2024).

The other infectious diseases called SP and GP are wide spread in Ethiopia, leading to a significant production loss. The death rate accounts for 75-100% in endemic areas and local breeds have a mortality rate that declines to 5-10% (Zewdie *et al*., 2021). All PPR, SP and GP are transboundary animal diseases prioritized by the World Organization for Animal Health (WOAH). The combined vaccine prepared for PPR, SP and GP showing no immunogenicity interference with each other confirms its feasibility (Rajak *et al*., 2005).

Statement of the problem

Currently, the Ethiopian government is conducting mass vaccination of sheep and goats against PPR as part of the global campaign to eradicate the disease. Integrating protection against Sheep Pox (SP) and Goat Pox (GP) into the same vaccination program using a combined vaccine would be a strategic approach. This is particularly relevant because the major costs of vaccination programs are typically tied to delivery logistics, such as storage, transportation, and personnel, which remain relatively stable whether one or multiple diseases are targeted simultaneously. Therefore, it is advantageous to explore opportunities to address additional diseases like SP and GP alongside PPR. These diseases not only share similar geographic distribution with PPR but rely on live attenuated viral vaccines cultures on cells, making them suitable candidates for combination (Diallo and Singh, 2021).

This study highlights the importance of developing a potent combined vaccine for the prevention of mixed infections, especially in endemic areas like Ethiopia. A single injection that protects against two or more infections could significantly enhance vaccination coverage, which is currently hindered by poor infrastructure and limited access to flocks. This approach presents a promising solution to address these challenges and enhance vaccination efforts to achieve adequate herd immunity.

1.2. Objectives

General Objective:-

The general objective of this research is to reduce morbidity and mortality in sheep and goats populations by preparing and evaluating the efficacy of a potent combined vaccine against PPR, SP and GP diseases.

Specific objectives:-

- To prepare a potent combined vaccine against PPR, SP and GP diseases.
- To evaluate the safety in guinea pigs, and the immunogenicity and efficacy in experimental sheep and goats, of the prepared combined vaccine.

2. LITERATURE REVIEW

2.1. Peste des petits ruminant virus

The peste des petits ruminant virus (PPRV) is an enveloped, pleomorphic, linear, single-strand, non-segmented, negative-sense RNA (Ribonucleic Acid) virus responsible for causing a disease called PPR in sheep and goats. It can also infrequently affect cattle and African buffaloes (*Syncerus caffer*), which are typically asymptomatic. Additionally, deer and gazelles, are affected during PPR outbreaks, while camels may exhibit clinical signs and experience mortality (Intizar *et al*., 2009; Balamurugan *et al*., 2012a; Saeed *et al*., 2015; Abubakar *et al*., 2019; Ahmed *et al*., 2021).

From its taxonomy, PPRV is the only member of the newly renamed Morbillivirus *caprinae* species within the genus Morbillivirus, which is part of the subfamily Paramyxovirinae, the family Paramyxoviridae, and the order Mononegavirales (ICTV, 2023; Courcelle *et al*., 2024). This genus encompasses a group of antigenically related viruses with a diverse host range. Members include the human Measles virus (MV), Rinderpest virus (RPV), Canine distemper virus (CDV) which affects a broad range of canid and mustelid species, Feline morbillivirus (FeMV), as well as Porcine Morbilliviruses (PMV) and Cetacean Morbilliviruses (CeMV) which are uniquely adapted to infect marine mammals (Arruda *et al*., 2021; Libbey and Fujinami, 2023).

2.2. The genome of Peste des petits ruminants virus

The size of PPRV measures specifically from 150 nanometers to 700 nanometers (Martín *et al*., 2025). It has a genome containing 15,948 nucleotides in length (Zhang *et al*., 2024). Its genes arranged in the sequential order of 3’ N–P/C/V–M–F–H–L 5’ (Kinimi *et al*., 2022). It codes for several structural and non-structural proteins that work in tandem for successful infection to occur in the host. The structural proteins include nucleocapsid (N), phosphoprotein (P), fusion (F), hemagglutinin (H) matrix (M) and large polymerase (L). The two non-structural proteins are C and V proteins (Gaur *et al*., 2024).

A negative staining in an electron microscope showed that the thickness of PPRV ranges between 8-15nm while the length of the spikes from 8.5-15.5nm. The ribonucleoprotein

strands appear as a herring bone carrying a thickness of approximately 14- 23 nm. All genus morbillivirus genomes features rule of six meaning that their length is polyhexameric, which is essential for the efficient viral genome replication. On the contrary, the PPRV obeys a rule of six but carries a degree of flexibility (Bailey *et al*., 2007). By still unknown mechanism transcription and replication of PPRV can accommodate some deviation in genome length +1, +2, +3 nucleotides (Munir *et al*., 2013).

2.2.1. Functions of peste des petits ruminants virus structural proteins

The nucleocapsid (N), phosphoprotein (P), and large polymerase protein (L) combine together with the PPR viral RNA to create the ribonucleoprotein complex (RNP complex), which encapsulates the PPRV genome (Ansari, 2018). These ribonucleoprotein complexes safeguard the RNA from host RNAses, to keep the virus's genome never found free RNA (Parida, *et al*., 2015).

The N protein with a molecular weight of approximately 60 kDa is the main structural component of morbilliviruses, its amino acid sequence identity within the entire genus founds between 67% and 74% (Gebreeziabher *et al*., 2006). It plays an important role in the creation of immunosuppression by morbilliviruses, while having a principal function in virus transcription, replication, and the inhibition of interferon β (INF) production. This inhibition occurs through its specific interaction with interferon regulatory transcription factor 3 (IRF3) that ultimately blocking its activation (Zhu *et al*., 2019).

The P protein acts as a reaction intermediary to connect components of ribonucleoprotein (RNP) when virus replication and transcription processes occur. The P gene encodes for the phosphoprotein (P) and two nonstructural proteins (V and C) via overlapping open reading frames (ORFs) (Chinnakannan *et al*., 2013). It grouped under the least conserved among morbillivirus proteins (Madhuchhanda *et al*., 2003).

The M protein is extremely important for the formation of viral particles, by anchoring the RNP complex in to plasma membrane via N protein interaction. This process encompasses the plasma membrane, glycoprotein tails, and RNP complexes typically for assembling new virions. It also suppresses transcription and membrane fusion to promote budding (Nizamani, 2010).

F protein is initially produced as a precursor form of F0. Which undergoes cleavage proteolytically using cytosolic enzymes, then results the formation of F1 and F2 fragments connected by a disulfide bond (Von Messling *et al*., 2004). The F1 membrane-bound fragments have an important conserved motif with the family paramyxoviruses. The fusion peptide (FP) for membrane insertion, heptad repeats A and B (HRA and HRB) forming a six-helix bundle essential for fusion, and the transmembrane domain (TM) (Gardner *et al*., 2007). In the pre-fusion conformation this protein had a high activation energy threshold that helps to limit a fusion activity, with a conserved leucine residue in the intervening sequence playing a key role in regulating the process (Plattet *et al*., 2009).

The H protein typified by a spike that stand out from the surface of the virus, plays a major role in attaching the virus to the host receptor. This involves cleavage of sialic acid residues on the carbohydrate component of host glycoproteins, facilitating virus adsorption and entry, while contributing to the virus's pathogenic capabilities (Rojas *et al*., 2021). This protein has hemagglutinin and neuraminidase activity thus also referred to as the HN protein (Riaz *et al*., 2023). Mutations in the H protein's receptor-binding region allow morbilliviruses to expand their host range (Yadav *et al*., 2019).

The L protein is essential for viral transcription, replication, massager RNA (mRNA) capping, methylation, and polyadenylation. Although the specific study is limited, the presence of three conserved motifs suggests its underscored role across the genus morbillivirus as RNA-dependent RNA polymerases due to the similarity (Munir *et al*., 2013). As an example it has high similarity about 70.7% between RPV and PPRV L proteins (Bailey *et al*., 2005). The domain residue spanning from 1-606 is functioning as RNA-binding motif. The second domain residues from 650 and 1694 is considered the core site for RdRp activity bordered by hydrophobic regions, The third domain, covering residues 1717 to 2183, is linked to kinase activity (Barrett *et al*., 2006).

2.2.2. Function of non-structural proteins of Peste des Petits Ruminants virus

The paramyxovirus P genes due to efficiently produce non-structural proteins C and V, along with P, through overlapping ORFs and co-transcriptional editing, expanding their functional repertoire (Mahapatra *et al*., 2003; Wu XiaoDong *et al*., 2016). These proteins are crucial for viral replication by suppressing host immunity, though their specific roles in PPRV

replication and pathogenicity are not yet well understood (Alfred *et al*., 2021). In PPRV C binds the transactivation domain, while V attaches to the Rel homology domain (RHD) both inhibiting necrosis factor kappa B (NF-κB) transcriptional activity and reducing the expression of pro-inflammatory cytokines. V protein blocks NF-κB p65 nuclear translocation in response to tumor necrosis factor-alpha (TNF-α), whereas C does not (Jain *et al*., 2023).

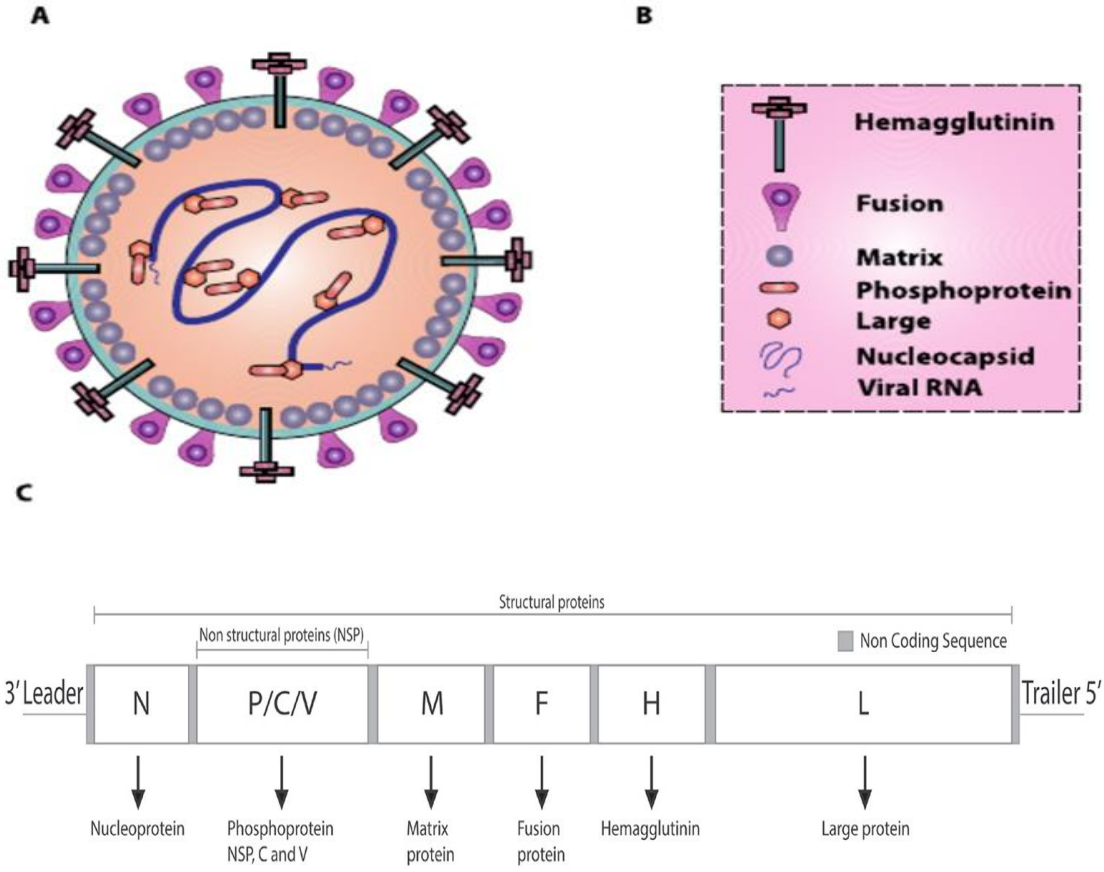


Figure 1: Schematic representation of PPRV morphology and its genome

Structure (A) illustrates the arrangement of viral proteins, with their names listed in box (B). The gene layout of PPRV and the arrangement of its proteins are shown in (C).

Source: (Munir *et al*., 2012 (A and B); Libeau *et al*., 2014 (C)).

2.3. Peste des petits ruminants viral pathogenesis

2.3.1. Cluster of Differentiation 150 as a receptor for Peste des Petits Ruminants Virus

Peste des petits ruminants virus majorly bind to the cluster of differentiation (CD150) or signaling lymphocyte activation molecule (SLAM) receptor, which is a 70 kDa glycoprotein in the immunoglobulin (Ig) superfamily, belonging to the CD2 subset and expressed on B cells (B95a), activated T cells, memory T cells, T cell clones, and immature thymocytes, facilitating entry and infection (Pawar *et al*., 2008).

SLAM is highly glycosylated, structurally it has a transmembrane region, cytoplasmic tail with three tyrosines, and two extracellular Ig domains (V and C2) and binds to SLAM-associated protein via SH2 domains (Ohno *et al*., 2003). During PPRV infection its expression includes post-transcriptional regulation. It has distinct amino acid sites within V structural domain, including N58-R85, F119-F131, I210, A211, S226, and R227, which are essential for binding with viral proteins and making key interactions (Fan *et al*., 2024).

The SLAM genes are quite comparable between different species of animals including goats, sheep, cattle and buffalo with amino acid and nucleotide identities range from 92.9% - 96.8% and 96.3% - 98.5%, respectively. Regarding amino acid sequence caprine SLAM is highly similar to sheep SLAM (96.8%), followed by cattle (93.5%), buffalo (92.9%), dogs (70.2%), and humans (62.8%). Basically SLAM helps to create species barrier, but its conserved amino acid occurrence across different animal species may be connected to morbilivirus' capacity to adopt new species (Sarkar *et al*., 2009; Prajapati *et al*., 2019).

During early infection period of PPRV, typically in the respiratory tract, it is trafficked to lymphoid tissues by a coordinated action of lymphocytes, monocytes, and dendritic cells. Specifically, macrophages and dendritic cells, encountering the virus at the entry site, internalize and transport it to regional lymph nodes, where initial viral replication occurs extensively. The primary cellular receptor mediating this crucial step is SLAM, facilitating viral entry and subsequent amplification within the lymph nodes (Pope *et al*., 2013).

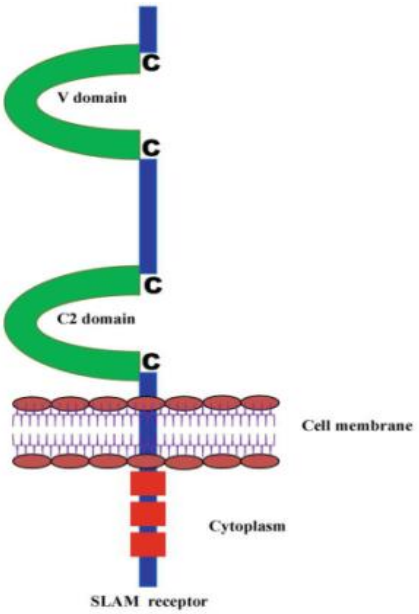
2.3.2. Nectin 4 as a receptor for Peste des Petits Ruminants Virus

Nectines are a group of immunoglobulin-like ectodomains with V and two C2 domains. They have a single transmembrane helix, and an intracellular domain (Mühlebach *et al*., 2011). There is 1-4 nectin family that mediates mediate cell-cell adhesion. Each of them has extracellular region, three Ig-like loops, a transmembrane segment and a cytoplasmic region.

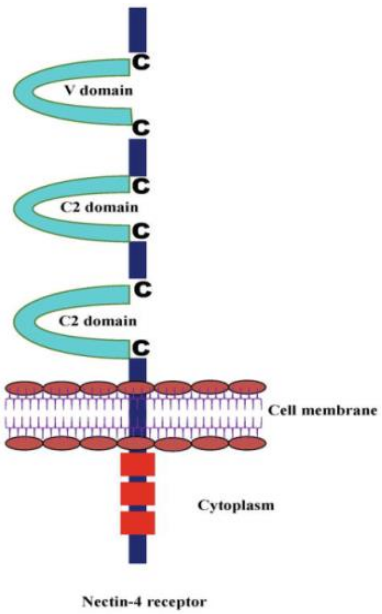
The extracellular domain make hemophilic or heterophilic interaction in-*trans* fashion to enable adhesion. This domain also forms diverse cell junctions alone or with cadherins, claudins, and junctional adhesion molecules. They recruit cadherins, forming adherens junctions in epithelial cells, fibroblasts, and neuronal synapses (Samanta and Almo, 2015).

Nectines are important for making apical-base polarity at cell-cell adhesion sites. The cell movement, intracellular communications, proliferation, polarization, differentiation of cells, survival and immune recognition are under the regulation of nectines. Nectines form junctions between spermatids and sertoli cells in the testis and between commissural axons and floor plate cells in the developing nueral tube unlike cadherin-dependent mechanisms (Takai *et al*., 2008b; Kurita *et al*., 2011; Zhang *et al*., 2013).

The ectodomains of Nectin4 and Nectin1 interact majorly through the V domain of Nectin1. This soluble V domain, lacking the C domains, can bind both Nectin4 and Nectin3, targeting a specific conformational region on it which is defined by the C-C'-C"-D β-strands. Nectin2 establishes a bond with Nectin3. The V domains are essential for both homotypic and heterotypic interactions of Nectin1, with the C2 domains enhancing binding affinity in these trans-interactions (Fabre *et al*., 2002). Notably, these heterophilic trans-interactions between nectin-1 and nectin-3 is about 3- fold higher than the forces of the homophilic trans-interactions of nectin-1 and nectin-3 (Martinez-Rico *et al*., 2005).



(A)



(B)

Figure 2: Schematic diagram of SLAM receptor (A) and Nectin-4 molecules (B).

Source: (Kumar *et al*., 2025).

Nectin-4 can be called poliovirus receptor-like protein 4 (PVRL4) is the epithelial receptor for PPRV. It acts as epithelial receptor for PPRV in sheep with breed-specific haplotypes affected expression. PPR outbreak often cause abortion in pregnant goats, but the pathogenic mechanisms remain unclear. The endometrial epithelial cells affected by PPRV, showed rapid gene dysregulation: 146 genes altered at 1 hour post-infection (85 upregulated, 61 downregulated), expanding to 568 genes by 24 hours (307 upregulated, 261 downregulated). Resulting peaked nectin-4 expression at 24 hours post-infection in endometrial epithelial cells (Yang *et al*., 2018).

In ovine, nectine-4 is receptor for PPRV, with high mRNA expression in respiratory epithelial tissues, suggesting a role in PPRV-related epithelial pathogenesis. With slow SLAM expression PPRV causes lesions in the mouth, nose, and upper respiratory tract, suggesting Nectin-4 aids spread in these areas post-viremia. However, Nectin-4 doesn't fully explain PPRV pathogenesis, as intestinal damage occurs despite low Nectin-4/SLAM levels. Higher Nectin-4 expression in the stomach correlates with less pathology, tells other factors drive intestinal issues. Diseases characteristics of morbillivirus may be influenced by the expression of SLAM/Nectin-4 like PPRV induced pneumonia. Mapping SLAM/Nectin-4 mRNA expression in the lung could clarify PPRV-induced pneumonia; SLAM mRNA in epithelial tissues likely comes from infiltrating immune cells, like macrophages and dendritic cells not the epithelial cells themselves (Birch *et al*., 2013).

2.4. Entry and replication of peste des petits ruminants virus in host cell

2.4.1. Entry of peste des petits ruminants virus

The most likely entry point for PPRV is the respiratory pathway, after entry it allocates itself in the tonsil and lymph nodes of throat and mandible where consequently multiplication takes place (Misbah *et al*., 2009). Attachment involves the binding of host cell membrane and the conserved domains of the F1 subunits (HRA and HRB), to bring the virus and host closer together, causing fusion. However, the F protein can independently induce cell-cell fusion

(Lee *et al*., 2007; Mühlebach *et al*., 2008). Crucially, F and H proteins initially reach at the cell surface independently. Briefly, the H protein interacts with Nectine-4 receptor which automatically send an activation signal to F protein and leading cell membrane fusion accompanied by the conformational change of F protein, where its HRA and HRB regions associate to form stable six-helix bundle (6-HB) (Meng *et al*., 2018; Marcink *et al*., 2020).

After initial binding and fusion, caveolae with viral components are internalized into vesicles. Dynein, a motor protein, forms a spiral ring around these vesicles, contracting and distorting them, which detaches them from the membrane to form early endosomes in the cytoplasm then successful internalization occurs which depends on the acidic pH within these vesicles (Jimah and Hinshaw 2019).

2.4.2. Peste des petitis ruminants viral replication

Peste des petitis ruminants virus primarily replicates in draining lymphoid tissues rather than respiratory epithelial cells. By 8 days post-infection, PPRV antigens are found in alveolar macrophages highlighting macrophages' key role in pathogenesis but the specific macrophage subtypes involved, including M1 and M2 (M2a, M2b, M2c, M2d), remain largely unexplored. Notably, the M2c subtype, or deactivated macrophages, shows high SLAM expression (Gautam *et al*., 2021). Replication begins when PPR viral genomic RNA, N proteins, and RNA-dependent RNA polymerase (RdRp) combined for transcription and translation. After entry, the viral polymerase transcribes the negative-strand viral RNA into mRNA or short leader RNA, which serves as a template for viral protein synthesis. The viral RNA then produces full-length complementary RNA (cRNA), which serves as a template to generate new negative-strand viral RNA (Wang *et al*., 2024).

Similar to all viruses in the Mononegavirales order, the PPRV RdRP must operate in two distinct modes: mRNA transcription mode and replication mode. The polymerase starts the synthesis of distinct capped and polyadenylated mRNAs from each gene when it is in mRNA transcription mode after recognizing the signals for the start and end of the gene. These signals are ignored when the RdRP is in replication mode, and transcription proceeds throughout the entire genome (or antigenome) (Baron *et al*., 2014).

The L protein first produces a short, uncapped leader RNA, released at the first intergenic region which then locates the first gene start (GS) signal to resume RNA synthesis, with the new RNA capped and methylated by L protein. Upon reaching the gene end (GE) signal, the polymerase adds a poly(A) tail, releases the mRNA, and scans for the next GS signal. Transcription re-initiation is inefficient thus leading to decreasing mRNA levels for downstream genes (Bloyet, 2021). Once N protein accumulates sufficiently, it binds nascent RNA from the 3' genomic promoter, causing polymerase to bypass termination signals and synthesize full-length positive-sense antigenome. This encapsidated antigenome serves as template for synthesis of new negative-sense genomic RNA, which then initiates a secondary mRNA synthesis phase, highly amplifying the production of viral proteins (MacLachlan and Dubovi, 2017).

The infectious particles formed only after viral glycoproteins and RNPs assembled at specific plasma membrane sites. Viral M proteins coordinate assembly by binding membranes and linking RNP cores with glycoproteins via their cytoplasmic tails. Acting as adapters, M proteins ensuring selective glycoprotein incorporation and often enabling efficient budding (Harrison *et al*., 2010). Finally, PPRV enhances infection by using its H protein to upregulate SLAM in nearby uninfected cells by suppressing microRNA called miR-218 that normally inhibits SLAM expression. This miR-218 suppression stabilizes or increases SLAM mRNA, allowing infected cells to remodel the environment and boost viral spread by increasing SLAM receptor availability on uninfected neighboring cells (Qi *et al*., 2019).

2.5. Peste des petitis ruminants virus and the host: A constant struggle for supremacy

2.5.1. Innate immune response

The Toll like receptors (TLRs) from innate immunity, have intracellular Toll-interleukin 1 (IL-1) receptor (TIR) domains necessary for downstream signal transduction, transmembrane domains, and ecto domains with leucine-rich repeats that mediate the recognition of pathogen associated molecular patterns (PAMPs) (Akira *et al*., 2006). When activation of TLR occurs there would be downstream activation of type I interferons with other antiviral cytokines (Hodgson, 2020).

The host defends its self against viral infection by activating interferon type I (Interferon-alpha/beta or IFN-α/β) and type II (Interferon-gamma or IFN-γ) responses leading to the induction of interferon-stimulated genes (ISGs) that inhibit viral replication (Li *et al*., 2019). Specifically goats infected with PPRV have significantly increased INF- γ, while level of IL-4 and IL-10 doesn't notably rise. Toll-like receptors 3, 7, and 8, demonstrated increased expression in goats infected with PPRV (Das *et al*. (2017).

The infection caused by PPRV induces autophagy related protein (ATG13) expression, which act as antiviral weapon in concert with IFN production thus curbing viral infection. When ATG13 is knocked down, PPRV replication surges, revealed its crucial role in viral restriction. Additionally, the retinioc acid - inducible gene I (RIG-I)-like receptors uses their tandem recruitment domains at the amino-terminus that are important for down signaling of IFN- β promoter (Platanias *et al*., 2005; Randall and Goodbourn *et al*., 2008). This dramatic drop in IFN-β transcription highlights ATG13's essential role in enhancing the interferon response and reveals a complex interaction between autophagy and innate immunity (Ma *et al*., 2020).

In human embryonic kidney 293T (HEK293T) cells, PPRV infection strongly induces IFN-λ3 transcription, while IFN-β and IFN-λ2 show only slight increases. Although type I and III IFN genes are activated, no functional IFN proteins are detected. Importantly, treatment with exogenous IFNs (IFN-α-2b, IFN-β, and IFN-λ3) significantly inhibits PPRV replication in these cells (Chang *et al*., 2019)

2.5.2. Adaptive immunity

The cell mediated immunity is mostly elicited by T-helper-1/type-1 cytokines, while TH-2/type-2 cytokines elicit the humoral immunity (Patel *et al*., 2012). N protein of PPRV has a greater role in stimulating cell mediated immunity specifically, the CD8+ and CD4+ T-cell response. At early infection PPRV the activated T- cells recognize the non-structural proteins through IFN- γ production or major histocompatibility complex (MHC)-linked cytotoxicity. However the high level of non- neutralizing N- specific antibodies indicates that there is ongoing T-cell immunity rather than clearing the virus. In contrary it is the T-cell response stimulated by H and F proteins of PPPRV that essentially clears the infection (Milovanović *et al*., 2023).

The H and F protein targeting antibody dependent cell mediated cytotoxicity (ADCC) helps to clear infected cells. The immune sera from infected sheep trigger ADCC against PPRV infected cells, demonstrated ADCC's role in controlling infection (Rojas *et al*., 2014); Rojas *et al*., 2019).

Experimental study revealed that, kid's maternal antibody are detectable up to 6 month, afterwards it start to decline and fall extremely below the protection level by the fourth month. 21 days post vaccination kid's with serum neutralization titre of 1:8 at the time of immunization demonstrated a substancial PPRV specific antibody response (percentage inhibition of 76; SN titers >1:16) and was fully immune to infection when exposed to a virulent PPRV challenge. Similarly, the kid with 1:8 SN titers was completely protected from PPR infection on active challenge (Balamurugan *et al*., 2012b). A study by Herbert *et al*., (2014) showed that both naïve and vaccinated goats showed a small rise in the percentage of CD8+ T-cells seven days after challenged by PPRV, indicating that the infection induces cytotoxic t-lymphocytes (CTL) responses. However, four days after the challenge, naïve animals may show a drop in the percentage of circulating CD4+ cells, whereas vaccinated animals do not.

2.5.3. Immunosuppression by peste des peitis ruminants virus

PPRV after its entry to cytoplasm it encapsulates its RNA with nucleoproteins to evade RIG-1 detection. Despite partial activation of RIG-1 and MDA-5, the morbillivirus V protein binds MDA-5's cysteine-rich C-terminus leading to signal blocking and suppression of immune response (Torsson, 2019). In severe PPRV infection there is reduction of type I IFN response and an enhancement of anti-inflammatory interleukin (IL)-10 production as a consequence contributing to immunosuppression and facilitating virus spread (Rojas *et al*., 2021). The PPRV V protein, though weakly binding RIG-I, powerfully suppresses IFN-β production by targeting MDA-5 and blocking both induction pathways. In contrast, the C protein does not bind these sensors but is critical for early immunosuppression, as PPRV lacking C protein unable to inhibit IFN-β activation, while the V-deficient virus still effectively suppresses IFN induction, underscoring a dominant role for C protein in early immune evasion (Sanz *et al*., 2017).

2.6. Peste des petitis ruminants vaccine: Different strains and alternatives

2.6.1. Live attenuated vaccine strains

Nigeria 75/1, the first homologous PPR vaccine, was developed using attenuated PPRV. By using a sick Nigerian goat afflicted with PPR as the source of the virus Nigeria 75/1 in 1975. This vaccine has been widely used in the production of live attenuated vaccines to protect small ruminants. It was able to confer further an additional protection for small ruminants against RPV when rinderpest epidemics occurred by the time. Four goats inoculated with the PPR attenuated vaccine resisted the challenge with a highly virulent rinderpest virus completely (Taylor *et al*., 1979; Couacy-Hymann *et al*., 1995).

For prophylaxis of PPR in India a homologous Vero cell-based live attenuated PPR vaccine was developed using an Indian virus isolate number ‘‘PPRV-Sungri/96’’. A goat in Himachal Pradesh, India, yielded the Sungri isolate (lineage IV) in 1994. This virus was first adapted in Marmoset lymphoblastoid (B95a) cells for up to 10 passages. Up to 59 passages the virus was then administered in Vero cells. This isolate has been widely studied with regard to virus characterization, potency, safety and thermostability. It was also been used for mass immunization campaigns after being produced both by privet industries and government institution. Apart from this, another research on development of vaccine was performed using the isolate number ‘‘PPRV-ARASUR/87’’ emanate from southern part of India (Sreenivasa *et al*., 2002; Singh *et al*., 2010).

Additionally, a vaccine based in India called PPRV/Coimbatore/97 (developed from an isolate from an infected goat), was attenuated following 75 passages in Vero cells. Both laboratory and field trials confirmed its complete attenuation and effectiveness in the southern states of India while used to protect the sheep and goat population form PPR (Singh *et al*., 2015).

In Russia, two institutions, the Federal Research Center for Virology and Microbiology (FRCVM) and the Federal Centre for Animal Health (ARRIAH), are authorized to produce PPR vaccines. Both institutions utilize attenuated virus strains derived from the "45G35" strain, attenuated in Russia during the 1980s. FRCVM employs the "45G37/35-k" strain, obtained through serial passage of the "45G35" strain in lamb kidney, lamb testes, and Saiga

kidney primary cells. ARRIAH utilizes the "ARRIAH" strain, but the specific passage history following its derivation from "45G35" remains unknown (Kwiatek *et al*., 2022).

When ARRIAH vaccine given to Zaannen and Nubian goat breeds that were later challenged with a virulent, circulating lineage IV Mongolia/2021 isolate, the ARRIAH vaccination showed high effectiveness, no side effects or viremia compared to the Nigeria75-1 vaccines. These results are a first step toward the ARRIAH strain vaccine's universal recognition (Byadovskaya *et al*., 2024).

Bangladesh Livestock Research Institute (BLRI) developed a tissue culture adapted vaccine against PPRV in 2001. This vaccine required a good cold chain facility, which was difficult to maintain in field conditions in Bangladesh and led to BLRI to develop a thermostable vaccine, named Titu (lineage IV, goat origin (EFSA-AHAW-Panel (Panel on Animal health and welfare), 2015). However, Veterinarians working in the field and goat farmers both complain about the PPR infection observed post immunization (Rahman *et al*., 2011).

The live attenuated PPRV vaccines were shown to be fully attenuated, safe, and effective both in laboratory and field tests. Challenge studies were conducted according to OIE guidelines in sheep and goats to test the potency of live attenuated PPR vaccines of Indian origin, including Sungri 96 (IVRI), Arasur 87 (Tamil Nadu-1, TN-1), Coimbatore 97 (Tamil Nadu-2, TN-2), and Institute of Animal Health and Veterinary Biologicals, Bangalore (IAH&VB) vaccine (made from Arasur 87 seed). The effectiveness of these vaccines was assessed using PPR competitive Enzyme-linked immunosorbent assay (C-ELISA) then, the first three of the vaccines were found to be effective in sheep and goats (Saravana *et al*., 2010).

Table 1: Some of alternative vaccines for PPR

S/N	Vaccine type	Strains/proteins/ chemicals used	Effectiveness and/ efficacy	Status	References
1	Recombinant vaccine	Plasmid of rabies virus, pD-SRV9-PM-LASV as	Exhibit good proliferative activity and	Prospective inactivated bivalent	(Wang *et al*., 2022)

		backbone, PPRV F and H proteins	stability	vaccine candidate	
	Vectored vaccine	Recombinant capripoxvirus (CPV) expressing PPRV H or F antigen	Good titers and duration, and the pre-existing immunity to CPV could be overcome completely, significantly enhanced immune responses, protected against the PPR challenge	A practical candidate DIVA vaccination for nations with recently emerging PPR/without stamp-out strategies	(Chen *et al*., 2010)
2	In-activated vaccine	Moroccan strain (Maroc/2008) inactivated with binary ethyleneimine	Safe and efficient to protect goats	Larger field research should be conducted to further assess it.	(Cosseddu *et al*., 2016)
	In-activated vaccine	PPR virus (Nigeria 75/1)strain inactivated by H_2O_2	A strong neutralizing antibody and effective T cell responses	Additional research is needed on the target animals.	(Akbarian *et a l*., 2021)
3	Multiepitope vaccine	H, M, F, and N Proteins	Predicted peptides can indeed elicit the desired immunogenic response and T cell activation.	It remains in-*vivo* validation	(Gaafar *et al*., 2019)

4	Combined vaccine	ZH501 Rift Valley Fever Virus strain and Nigerian PPR virus (N75/1) strain	Safe and potent without antagonizing the effect on target sheep	Can serve as a replacement for individual immunizations against PPR and RVF	(Yehia *et al*., 2024)
5	Virus like particle (VLP) -based vaccines	Recombinant baculoviruses (rBVs) expressing M, F, and H proteins of Lineage IV Tibet/30 virulent strain and PPRV Nigeria 75/1	Both VLPs induced high titers of virus neutralizing antibodies (VNAs), as well as H- and F-specific antibodies	Constitute a viable option to prevent and eradicate PPR.	Yan *et al*., 2020

2.7. Regional patterns in the distribution of peste des petits ruminants lineages

The molecular epidemiology of PPRV has demonstrated four separate lineages (I–IV) by exploring sequence variations at variable region of C- terminus of nucleoprotein gene (positions 1360 to 1614, including 255 nucleotides) and fusion protein gene from 256 to 575, comprising 319 nucleotides) (Clarke *et al*., 2017).

Following PPR first identification in Côte d'Ivoire, additional reports heard from different neighboring countries, particularly from Senegal, Chad, Togo, Benin, Ghana, Nigeria, Oman, Sudan, Saudi Arabia, India, Jordan, Israel, Ethiopia, Kenya, Uganda, and Pakistan in sequential order (Parida *et al*., 2016). The emerging sequence evidence suggests that PPRV lineages I and II originated in West Africa, specifically senegal for lineage I, Nigeria for lineages II), while lineage III likely emerged in East Africa (Ethiopia) or the Arabian Peninsula (Oman/UAE) though definitive conclusion is hampered by limited data (Mahapatra *et al*., 2021). Genotyping data demonstrated that these lineages exhibit distinct land coverage suggesting active viral spread and potential pre-existing presence in these regions: Lineage I predominantly circulate in West and Central Africa, Lineage II in West Africa, Lineage III in

East Africa and the Middle East, and Lineage IV in the Arabian Peninsula, the Middle East, and South Asia (Kamal *et al*., 2024).

Lineage IV becoming the predominant lineage in many areas across East, North, West and Central Africa since from its discovery in Cameron in 1977, which afterwards displacing previously prevalent lineages and spreads to Sudan in 2000 and Eritrea in 2002 (Muniraju *et al*., 2016). This is supported a study by Baazizi *et al*. (2017) which links the 2008 Moroccan outbreak to Lineage IV originating from the Sudan 2000 outbreak. The continued presence of Lineage IV PPRV in the Maghreb region, integrate with inadequate regional control measures, that poses a risk to European countries given the virus's proximity to the North African coast. The full genome sequences phylogenetic analysis of Muniraju *et al*. (2014) showed that the PPRV isolates from Ethiopia 1994, Oman 1983, UAE 1986, and Uganda 2012 are belonged to lineage III and the isolates Sungri 1996, Morocco 2008, and Ethiopia 2010 belongs to lineage IV.

Ethiopia reported the first case of PPR in 1977, following studies on genetic characterization revealed the residence of lineage III. Even so, Shahriari *et al*. (2019) and Mohammed *et al* et al. (2022) disclose that lineage IV is the dominant, replacing Lineage III. Similarly Veronica *et al*. (2019) arrive to a conclusion that only Lineage IV, specifically sub-clade II of clade I, indicating its widespread presence and displacement of other lineages by studying in multiple regions like (Amhara, Benshangul-Gumaz, Afar, Oromia, Dire Dawa). To a greater extent, the initial outbreak of lineage IV in Ethiopia was traced back to 2010 in goats purchased from the Bishoftu market, and subsequent samples were collected from locations approximately 400 km from the original outbreak site (Bishoftu), highlighting its persistence and geographical spread (Alemu *et al*., 2019).

2.8. Poxviruses of Sheep and Goats: genomic insights

Sheep and goats contract the diseases called sheep pox and goat pox caused by sheep pox virus and goat pox virus, respectively (Gelaye and Lamien, 2019). These viruses are large, double-stranded, deoxyribonucleic acid (DNA) viruses having 147 ORFs (Teffera *et al*., 2025). They belong to Poxviridae family under chordopoxvirinae subfamily within the genus Capripoxvirus. Their size measures 294 × 273 nm (Manjunatha *et al*., 2024).

The genome of the poxvirus is rich in A+T and has about 100 polypeptides organized in the core, lateral body, membrane, and envelope. A core and lateral bodies are encircled by a lipoprotein bilayer membrane. Randomly distributed surface tubule components dotted the outside surface. The nucleoprotein core and lateral bodies of the intracellular mature virion are surrounded by inner and outer membranes (Kamal, 2022).

The central genomic region surrounded by two identical inverted terminal repeats (ITRs) at the ends of SGPV (ORFs 024 to 123) comprises homologues of conserved genes involved in basic replication systems as well as in viral DNA replication, transcription, RNA modification, and structure and assembly of intracellular mature and extracellular enveloped virions. While the terminal open reading-frame (ORF) (001 to 023 and 124 to 156) contain genes involving with virulence, host immune evasion and host range functions. Mature virion cell attachment (P32) comprises the main antigenic determinants which are important for the pathogenicity and diagnosis of the virus (Kenubih *et al*., 2021).

Sheep pox and goat pox viruses share 97% of their nucleotide identities with lumpy skin disease virus (LSDV), another member of the *Capripoxvirus genus* (Mummadisetty, 2024). A study by hajjou *et al*. (2019) demonstrates how highly conserved the P32 gene is among capripoxviruses (CaPVs). Phylogenetic analysis using the P32 gene demonstrates that SPPV, GTPV, and LSDV are phylogenetically distinct. While a whole-genome phylogenetic tree supports the grouping of GTPV, SPPV, and LSDV into a separate branch, it also reveals a closer genetic affinity between GTPV and LSDV (Zeng *et al*., 2014). Furthermore, Sheep poxvirus (SPPV) has approximately 323 residues, while LSDV has only 322. This difference is due to the insertion of an extra aspartate residue at position 55 in SPPV, which is absent in both LSDV and GTPV (Sayed, *et al*., 2023).

Notably, both the SPPV and GTPV genomes exhibit disruptions in nine LSDV genes that are likely involved in virulence and host range activities. These comprise the two copies of the myxoma virus M003.2 and M004.1 genes, the vaccinia virus F11L, N2L, and K7L genes, a gene specific to LSDV (LSDV132), and genes that resemble those coding for the interleukin-1 receptor (Tulman *et al*., 2002).

Apoptosis of virus-infected cells, cytokine binding protein homolog, IL-10, IL-18, and epidermal growth factor (EGF) like proteins, protein kinase R (PKR) inhibitors, and host

immune evasion are all facilitated by the terminal regions of the genomes of the sheep pox and goat pox viruses, which are 2.1 to 2.3 kbp in size on each side. Due to their lack of conservation, these terminal areas may experience genetic recombination and assimilation with novel genes, resulting in varying levels of virulence among isolates (Sarkar, 2021).

Genes linked to virulence in SGP are varied. Of these, SPPV encodes three proteins that resemble kelch: SPPV-019, SPPV-144, and SPPV-15. When SPPV-019 is administered intradermally or intranasally, it significantly alters SPPV virulence. The kelch-like protein has an impact on several physiological functions, such as protein ubiquitination, ion channel modulation, transcriptional regulation, and cytoskeleton dynamics and organization (Balinsky *et al*., 2007).

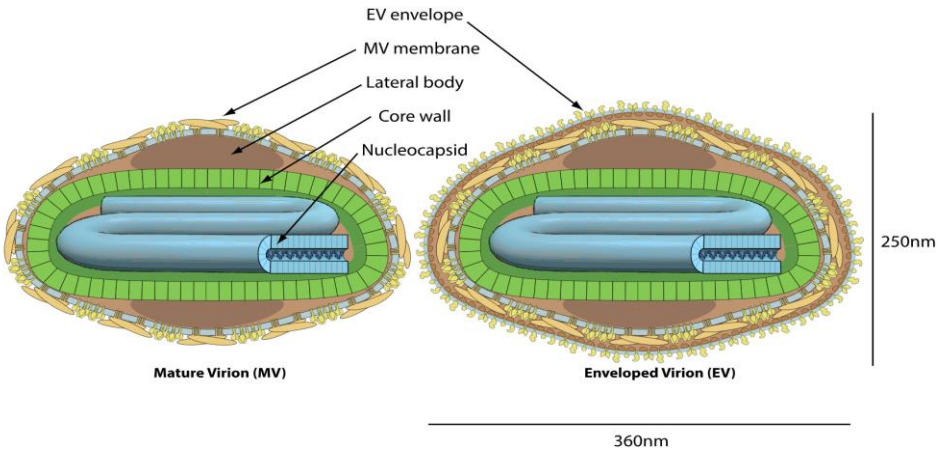
2.9. Sheep pox and goat pox: The interplay between viral replication and host defenses

2.9.1 Entry of sheep pox and goat pox viruses in host cell

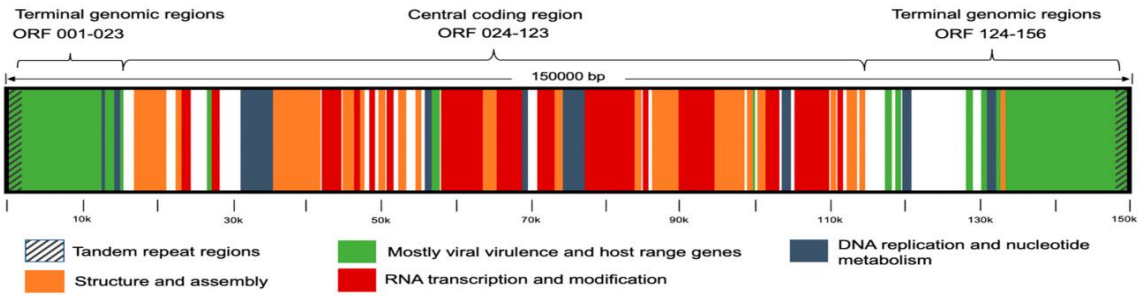
Sheep pox virus modulates the expression of several important host proteins such as CD40, CD14, Fas cell surface death receptor (FAS), Integrin-linked kinase (ILK) leading to viral attachment and entry (Sonowal *et al*., 2021). The virus uses the glycosaminoglycans to attach to a particular host cell receptor. Poxvirus differs from other DNA viruses in that its life cycle is finished inside the cytoplasm of the host cell. Within minutes of viral entrance, the production of the early gene mRNAs begins. Early gene transcripts are translated in the cytoplasm of the host cell after being extruded through the viral core's pores. These early genes, which number about 100 and make up almost half of the viral genome, encode the proteins required for uncoating, transcription factors (which are necessary for the later expression of late genes), and the entire set of enzymes and proteins required for replication. Following early gene expression, the viral core goes through a difficult uncoating phase where the DNA is liberated and the viral core wall dissolves. After the genome is released from the core, DNA replication and transcription of late genes take place. The viral particles are assembled, encased in the intracellular membrane, and discharged in the last stage of their life cycle (Palanivelu, 2025).

Poxviruses produce two main infectious forms: intracellular mature virion (IMV) and extracellular enveloped virion (EEV). IMVs, assembled in the cytoplasm, consist of a core with the genome and enzymes, surrounded by an envelope containing at least eleven proteins.

IMVs accumulate in the cytoplasm and are released either by cell lysis or, after acquiring additional membranes from the trans-Golgi, as EEVs through budding (Kushwaha *et al*., 2019).



(A)



(B)

Figure 3: Capripox virus morphology and genome structure

Source: (https://viralzone.expasy.org/152, 2014) (A); Eltom *et al*., (2021) (B))

2.9.2. Innate immune response

The acute phase response (APR) is part of the early-defense which is triggered in this case by infection and inflammation. Serum albumin is the major negative APP, and its synthesis may be markedly reduced during the APR. The study result of Oreiby *et al*. (2022) revealed a significant decrease in the absolute values of albumin in sheep pox infected animals when compared to the healthy animals. The synthesis of positive acute-phase proteins is markedly increased during the acute inflammatory processes.

The inflammatory cytokines are upregulated in response to virus infections; TNFα, IL-15, and IL-10 are significantly upregulated and crucial in SPPV infection. Antigen-presenting cells are subsequently drawn to the infection site by elevated IL-15 and TNFα, which triggers an adaptive immune response (Chibssa *et al*., 2021). According to a different study by Karakurt *et al*. (2023), pro-inflammatory cytokine levels, including TNF-α, IFN-γ, IL-1β, IL-2, IL-8, and IL12 (Th1), are significantly higher than those of anti-inflammatory cytokines, such as IL-10 and IL-6 (Th2), indicating that the cellular immune response is far more effective in sheeppox than the humoral immune response.

2.9.3. Adaptive immune response

An important defense against sheep pox is humoral immunity, more especially neutralizing antibodies ≥1. When colostrum is given to lambs from immunized dams, maternal antibodies can offer protection for as long as 60 days, as long as the lambs' blood contains significant amounts of IgG. However, when lambs are challenged with virulent SPPV, the antibodies frequently fail to totally stop virus multiplication at the injection site, despite the maternal protection. Clinical symptoms including fever and swelling of the prescapular lymph nodes close to the inoculation site are caused by this localized viral replication (Ebrahimi-Jam *et al*., 2022). After researching the durability of mother antibodies in kids, Abdollahi *et al*. (2024) came to the sound conclusion that the GTP vaccine should be administered between 100 and 120 days of age, which is a reasonable window of time given that goat offspring from immunized does obtain enough colostrum.

In the cell-mediated immune response against the Capri poxvirus, T lymphocytes are the most paramount element. T cells can differentiate into helper (LTh) cells (CD4+) and cytotoxic T lymphocyte (LT) cells (CD8+) that are specific to poxviruses, but their function in defense against capripoxviruses is still unclear (Tuppurainen *et al*., 2017). Antibodies can attach directly to the virus and enable complement-mediated lysis or opsonization, which leads to phagacytosis via the Fc receptor. Typically, the poxvirus-specific cytotoxic T-lymphocytes (CTLs) regulate poxvirus infection by neutralization, preventing virus uncoating. Additionally, antibodies have the ability to attach to the infected host cell, resulting in antibody-dependent cell mediated cytotoxicity (ADCC), which stops the virus from adhering to the cell and internalizing (Smith and Kotwa, 2002).

2.9.4. Immune modulation by sheep pox virus and goat pox virus

The goatpox virus's greater affinity for binding to its corresponding receptor is what causes the infection to be more severe; nevertheless, the SGP P32-encoded viral envelope protein changes genetically to evade the host's strong defenses against invasion (Alwan *et al*., 2023). In order to evade the immune system, poxviruses also downregulate cell surface receptors by impacting antigen presentation and immune cell recognition. This includes the downregulation of class I MHC molecules, observed after infection with several poxviruses, which normally present endogenous antigens to circulating CD8+ CTL (Seet *et al*., 2003).

Genome wise, the 135 gene of GTPV encodes an immunomodulatory protein that functioned as an inhibitor of necrosis factor (NF-B) activation and apoptosis, similar to the N1L protein of vaccinia virus (Zhang *et al*., 2018). More precisely, depending on how effectively targeting develops, poxviruses can either delay or quiet pro-inflammatory and interferon responses by blocking transcription factor activation with both discrete and multifunctional inhibitors (Lawler and Brady, 2020). Finally, Sheep pox and goat pox viruses use infected monocytes and macrophages to disseminate through the systemic circulation with localization to skin and other tissues as detected by the expression of capripoxvirus antigen in CD68+ cells (Embury-Hyatt *et al*., 2012).

2.10. Sheep pox and goat pox vaccine: Strains and types

2.10.1. Live attenuated vaccine strains

Vaccines against the Capripox virus that are live attenuated usually produce humoral and cellular immune responses. The RM-65 strain attenuated on sheep kidney cells, the SPV Romanian strain attenuated on a green monkey kidney cell line, and the Kenyan live-attenuated sheeppox strains are among the commonly used vaccines against sheeppox. After vaccination, protective antibodies take time to develop (Oreiby *et al*., 2022).

The Indian Veterinary Research Institute (IVRI) provided the attenuated Srinagar 38/00 strain of SPV, which was attenuated by passing in Vero cells for 38 passages. Sero-conversion and challenge studies in sheep as well as safety and immunogenicity studies in experimental animals administered 1 ml of sheep pox vaccine ($10^{3.75}$ CCID /50 dose) by intramuscular and

subcutaneous route, respectively, demonstrated that the vaccine is safe, immunogenic, and potent (Rao and Reddy, 2020).

Infecting confluent monolayers of kidney cells from kid less than two months of age with a different live attenuated goat pox virus vaccine (Gorgan 55, Iranian) strain produced an 80% cytopathic effect (CPE) on day five of virus injection. The pool virus's estimated $TCID_{50}$ was $10^{5.5}$/ml. After vaccination, it is deemed safe in all mice and guinea pigs as confirmed to be healthy. After being challenged with 1 milliliter of virulent goat pox virus ($TCID_{50}$ $10^{4.5}$/ml), vaccinated goats showed no unintended reactions, but controls treated with 1 milliliter of virulent goat pox virus (TCID50 $10^{3.5}$/ml) had pox lesions and an increase in body temperature (Abbas *et al*., 2010).

Attenuation of a virulent field isolate through 20 serial passages in LK cells resulted in the G20-LKV strain, which was then used to create a GTP vaccine. This vaccine provided greater protection to vaccinated animals than the SPP Niskhi strain vaccine. Vaccination of goats with the G20-LKV-derived vaccine resulted in immunity within five days, lasting for at least a year; these vaccines also protect cattle from a field strain of a virulent lumpy skin disease (Zhugunissov *et al*., 2020).

In Ethiopia, the live attenuated Kenyan sheep pox virus strain vaccine (KSGPV) O 180 vaccine strain is a lyophilized vaccine with a stabilizer that is kept at -20°C for two years. It is produced utilizing continuous cell lines (Vero cells). If and only if the vaccines are handled, stored, transported, and adequately vaccinated, this strain in Ethiopia is safe and effective in preventing sheep and goat pox. The KSGP vaccination strain is used to control LSD in cattle, despite some cattle breeders doubting whether it offers sufficient protection (Zewdie *et al*., 2021).

It the sole means of LSD control in Ethiopia and the results from Molla *et al*. (2017) showed that KS1O-180 strain vaccine reduces susceptibility of cattle to LSD but it also increases infectiousness by about the same amount. However, a recent study by Abulaiti *et al*. (2025) in China demonstrated that the attenuated GTP Gorgan vaccine strain is generally used as a heterologous vaccine known for its cross-protective efficacy against LSDV and goat pox. It induces a significant antibody titer without affecting general health or productive parameters, making it a safe and cost-effective way to prevent LSD in buffaloes.

Table 2: Types of vaccines for sheep pox and goat pox

S/n	Vaccine type for sheep and goat poxes	Strains and/proteins used	Efficacy and effectiveness	Vaccine status	REFERENCES
1	Multi-epitope subunit vaccine	Merging immune-dominant CTL, Helper T-lymphocyte (HTL) and B-cell epitopes	High levels of antigenicity and immunogenicity	Promising vaccine candidate	(Long *et al*., 2024)
2	Recombinant vaccine	SPPV NISKHI strain, capsid protein (VP1) of FMD and outer membrane protein 25 (OMP25) of *Brucella* spp.	Elicited specific antibodies to both SPPV and foreign genes	Potent vector for multivalent vaccines not commercialized yet	(Chervyakova *et al*., 2021)
3	Chimeric trivalent vaccine	B5R protein of GPV, glycoprotein of SPPV, P32 of LSDV	Economic, effective and aimed to robust activation of both arms of immune system	Designed but lefts with in*vivo* trial	(Pashupathi *et al*., 2022)
4	Inactivated vaccine	SPPV Romanian strain inactivated by β propiolactone	No risk of reversion, potent as live vaccine, induced immunity lasts for 9months	Has a potential to replace attenuated vaccine	(Boumart *et al*., 2016)

2.11. Factors for vaccine failure

Animal producers rely on vaccination as a vital strategy for disease prevention and economic stability. This approach is especially valuable in animal farming, offering a reliable, effective, and affordable means of protecting flock. The quality and nature of the vaccine, how it is handled, the utilization of local antigens and the host's immunogenic response, as well as adherence to the manufacturer's recommendations, are all important components of a successful vaccination program otherwise with vaccinated flocks, the possibility of disease outbreaks cannot be totally eliminated because of vaccine failures (Toka and Geinoro, 2023).

2.11.1 Host related conditions

Immunosuppression, which can be caused by harm to immune cells and organs, abnormal cytokine release, and weakening of overall immune system, can drastically lower vaccine efficacy (Tian *et al*., 2022). The second host-related factor is maternal immunization, which protects lambs from immunized mothers against sheep pox for up to 60 days after birth, as previously stated. Consequently, early vaccination may interfere with the vaccinal virus and maternal antibodies obtained through colostrum, leading to immunization failure or decreased effectiveness (Ebrahimi-Jam *et al*., 2022).

A key insight into the inhibitory effect of maternal antibodies is their ability to suppress antibody production, even when T cell responses remain intact. This inhibition occurs because vaccine-antibody complexes can cross-link the B cell receptor (BCR) with the Fcγ-receptor IIB, effectively deactivating B cells. However, this suppression can be partially overcome, as demonstrated in animal studies, by administering a monoclonal IgM antibody specific to the vaccine. Unlike IgG, IgM can directly activate B cells by cross-linking the BCR with the complement protein C3d and the antigen, thus engaging the complement receptor 2 (CR2) signaling complex. Moreover, it has been shown that interferon alpha, through simultaneous binding to the interferon receptor and the CD21 chain of CR2, can drive B cell responses even in the presence of maternal antibodies (Niewiesk, 2014).

Third factor, as with all vaccinations, it is imperative to confirm that the animals to be vaccinated are healthy; thus, they would elicit a protective immune response (Vasileiou *et al*., 2022). If animals are so ill that vaccination is considered unsafe, they should not remain in

the shelter unless appropriate care and treatment can be provided in a physically separate isolation area where they will not be exposed to disease (Larson and Schultz, 2021).

Genetic variation within a population is also another factor that leads to inconsistencies in the duration and intensity of vaccine-induced immunity. The same individual encountering the same immunological challenge (by vaccination or virus exposure) may show a range of results, from full protection to partial protection or even no protection at all (Valdés-Fernández *et al*., 2021). Polymorphisms in MHC genes, as well as in genes encoding Toll-like receptors (TLR) and RIG-I-like receptors (RLR), grant to differences in humoral and cellular responses to vaccinations. Additionally, various single-nucleotide polymorphisms (SNPs) related to cytokine production, virus recognition, and vitamin utilization also affect vaccination responses. Moreover, variations in gene expression patterns influence vaccine reactions; individuals with higher expression of genes involved in antigen processing, presentation, and early interferon signaling mainly unveil stronger antibody responses (Zimmermann and Curtis, 2019). Therefore, vaccine design is guided by population genetic analysis of vaccine candidate antigens, which provides insight into the magnitude and dynamics of genetic polymorphisms (Ajibaye *et al*., 2020).

2.11.2. Logistical and procedural errors

Failures can also stem from improper handling and delays in the cold chain, which are frequently cited as major contributors to vaccine potency loss. A deficiency in effective cold chain management, such as the absence of continuous temperature loggers, hinders the ability to monitor vaccine temperatures outside of regular working hours or when personnel are unavailable. Undetected temperature excursions during these periods, even if transient, can compromise vaccine integrity, particularly in frozen vaccines. Thawing and refreezing often leads to hairline fractures which allow bacteria to compromise the vaccine's sterilization. The lack of fuel for standby generators exacerbates this loss of potency due to the accelerated thawing of vaccines (Dairo and Osizimete, 2016). The result of inadequate cold storage is not only vaccine deterioration, but also an increased risk of disease outbreaks and compromised preventative efforts (Griffith *et al*., 2023).

The other factor related with farmers, who are discouraged from using vaccination to protect their livestock herds against priority infectious diseases due to a lack of timely access to

vaccines, a lack of knowledge about the effectiveness and profitability of vaccines, and the high cost of vaccination services. However, vaccination is positively associated with increased resilience to adversity and the experience of diseases in herds (Nuvey *et al*., 2023). Likewise, gaps in health professionals' knowledge can lead to medical errors with potential consequences for patients. Vaccine-related medication errors, in particular, can result in vaccine ineffectiveness or adverse effects, and may also contribute to decreased public confidence in vaccination (Poiraud *et al*., 2023).

2.11.3. Vaccine related factors

Several factors can limit vaccine effectiveness, even when the intended disease is preventable. These include: (1), incomplete coverage of all circulating strains, serotypes, genotypes, or antigenic variants of the pathogen; (2) imperfect protection against included antigens, and (3) antigenic interference or other interactions when vaccines are co-administered. Furthermore, manufacturing flaws and batch-to-batch variability can also contribute to vaccine failure (Heininger *et al*., 2012).

Manufacturing high-quality veterinary vaccines is complex, and failures can stem from weak infrastructure, unreliable equipment, unclear processes, or poorly trained staff. Inadequate facility design can lead to cross-contamination, while hard-to-clean materials in production and testing areas may harbor pathogens. Deficient air handling systems that fail to ensure clean air can result in contamination, especially from zoonotic agents, reducing vaccine efficacy. Improper pressure differentials between rooms further jeopardize product integrity (Yeary *et al*., 2021).

3. MATERIALS AND METHODS

3.1. Experimental site and experimental design

This experimental study was carried out at the National Veterinary Institute (NVI) from November 2024 to May 2025, Bishoftu. The main experimental activities performed include vaccine preparation, vaccine quality control tests, screening and immunization of target animals, outbreak investigation, and conventional molecular detection of the challenge virus, evaluation of humoral immune responses in target animals as well as clinical sign scoring.

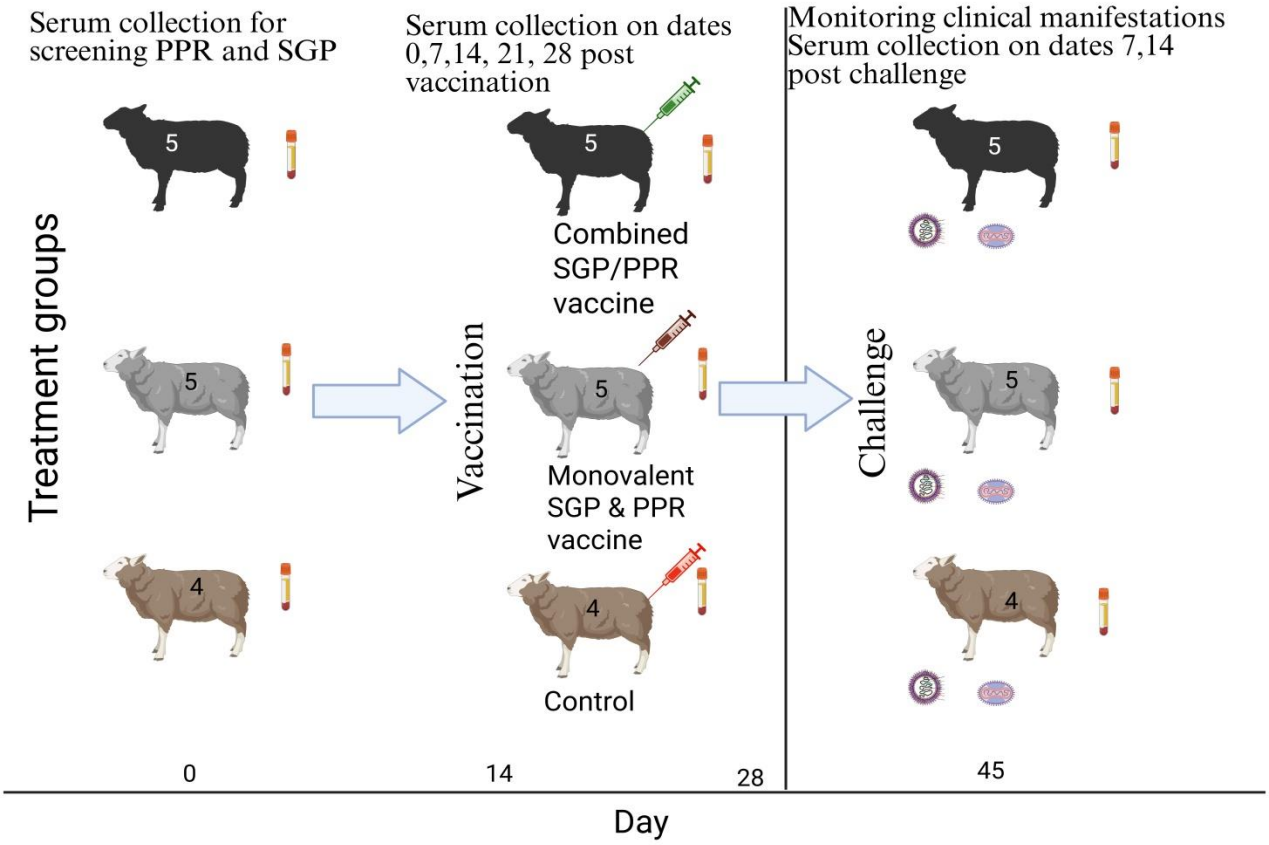


Figure 4: Illustration of experimental workflow for vaccine efficacy testing.

There were seronegative three groups of animals that took combined vaccine, the co-administration of PPR and SGP vaccines and there were unvaccinated control animals. During the challenge experiment, all groups of animals were challenged by virulent field isolated PPRV and GPV.

Source: Created by using https://app.biorender.com/ (Version 30).

3.2. Vaccine strains

The African Union Pan-African Vaccine Center (AU-PANVAC) provided the lineage II Nigerian 75/1 with the thirty-third passage number, which was attenuated on African green monkey kidney (Vero) cell that served as the PPR virus's master seed. Additionally, the African Union Pan-African Vaccine Center (AU-PANVAC) provided the Kenya O-180 strain of the sheep pox virus (SPPV) KS1-O180 with the thirtieth passage number, which was attenuated on an African green monkey kidney cell that served as the master seed. NVI verified both master seeds' purity and identity tests using polymerase chain reaction test.

3.3. Monovalent live attenuated vaccine preparation

3.3.1. The cell

Vero cells were seeded into ample amounts of cell culture roux flasks measuring 25cm^2 and 75cm^2 cell culture plastic flasks and then transferred to 175cm^2 cell culture plastic flaks for vaccine production.

3.3.2. Monovalent peste des petitis ruminants, Sheep pox and goat pox vaccines production

The PPR and sheep pox vaccines were produced separately as per, NVI live vaccine production protocol. The Vero cell culture was used to cultivate the master seeds, which was then passed through 40 and 45 passages for PPR and SGP respectively, until the viruses produced a cytopathic effect (CPE) of greater than 80%. In detail, the Vero cell was revived from liquid nitrogen at -196^{0}C and then thawed at -2^0 C then + 4^{0}C, and transferred into 25 cm^2 roux flask. Glasgow's minimum essential medium (GMEM) (base media having 10% fetal bovine serum (FBM)) was then added. The growth of the confluent monolayer cells was observed after an overnight incubation period. Here, the cell was then given two phosphate-buffered saline (PBS) washes after the media was discarded. Trypsin was then added and incubated to detach the cells from the base of the flask; which was subsequently discarded after 3 minutes. Next, the flask was pipetted with 10 ml of 10% GMEM media to obtain cell suspension. An additional 30 ml of base media was then added, and equal volumes were transferred to two more 75 cm² roux flasks (each receiving 15 ml of base media), while the original flask retained 10 ml of base media along with the cells.

All of the flasks were then incubated for a full night. After verifying that cells had grown to a monolayer in all flasks under an inverted microscope, the media was discarded and the cells were washed twice with PBS. Then, 1 ml of PPR diluted vaccine virus was used to infect the cells, while another 25 cm² Roux flask was labeled as a control. This procedure was repeated for Sheep/goat pox diluted vaccine as well. The virus was given an hour to attach to the virus after which all cell culture flasks were replenished with GMEM containing 2% FBS or maintenance media. The corresponding infected as well as control cell culture flasks were then cultured inside an incubator set to 37^0C and 5% carbon dioxide (CO2).

On alternate days, the cell culture flasks are refilled with maintenance media after being examined daily under a microscope for CPE to avoid dead cells that mislead to interpretation. When more than 75% CPE was detected in the working seed, it was collected and stored in a freezer at -80°C. To prepare a bulk of the first batch vaccine, cell culture was made on $175cm^2$ large roux flasks and infected with a virus that has passed about 40 and 45 more series of passages for PPR and sheep pox respectively. Finally, the virus was then pooled in 800 ml glass bottles that were maintained at -80^0C.

3.4. Preparation of live attenuated combined vaccine

A combined PPR and SPP vaccine was developed following a previously described method by Amanova *et al*. (2021). Briefly, attenuated Nigeria 75/1 PPRV and Kenya O-180 SPPV strains, propagated in Vero cells separately, were combined in 1 to 1 ratio and lyophilized as a single vaccine for the first time in Ethiopia. This combined vaccine was produced by mixing suspensions of the two vaccine strains, each with a titer of at least (2.5 $\log_{10}$ $TCID_{50}$/mL), in a 1:1 ratio. A peptone-sucrose stabilizer (3% peptone and 2% sucrose final concentration) was then added in a 1:1 ratio to the combined virus suspension. Finally, the vaccine liquid was aliquoted into 2.0 ml ampoules and lyophilized for storage.

3.5. Quality control tests

3.5.1. Titration of monovalent vaccines

After live attenuated PPR vaccine and sheep pox and goat pox vaccines were produced, their virus concentration was calculated following virus titration procedure in liquid form prior to combining and lyophilizing as a single combined vaccine, using Spearman-Karber's method

as described on NVI PPRV isolation protocol, (NVI VPS-RD-PR-64) Accordingly, the minimum single vaccinal dose titre was determined to be $1\times10^{2.5}$ $TCID_{50}$ both for PPR and sheep pox and goat pox vaccines by using the formula $\log_{10}TCID50=\log_{10}d$ (lowest dilution at which 100% CPE was observed) – ($\sum$p(positive units in subsequent dilutions)−0.5/ n(total number of replicate wells per dilution) $\times\log_{10}d$ (Log of dilution factor (1 for 10-fold). Briefly, 100µl of GMEM media were added to the first 12 columns of microplate and raw H1-12 except for the 11th column which was for cell control. A-E rows also received the media. 100µl of Vero cells were added to each well of the microplate having media except the 11th column. The ten-fold serial dilutions of PPR vaccine and sheep/goat pox vaccine samples were prepared in GMEM separately. For each dilution, 100 µl of virus suspension were added from the 7th universal tube to H raw up to the first raw having the 1st universal tube virus dilution. All the plates were incubated at 37^0C in the presence of 5% CO_2 after being sealed by plate sealer and checked for CPE under an inverted microscope for 15 days. Control (uninfected) wells were also kept for each dilution.

3.5.2. Sterility test

Separately pooled working seed, chilled, and freeze-dried vaccines for PPR, sheep pox/goat pox as well as, a combined vaccine were tested for sterility using randomly selected sample vials. These samples were inoculated into Sabouraud's agar, Soyabean casein digest media (SCDM) and thioglycolate media to detect any aerobic and anaerobic bacterial, and fungal growth. All Medias used were sterilized using an autoclave at 121^0C for 15minutes before being inoculated with the vaccines, some of them left without inoculation and incubated for 24 hours to check no contamination existed. In detail, thioglycolate and SCDM were prepared by dissolving 29.75 g of powder in 1000ml of sterile water, and the resulting medium were dispensed into six and three test tubes, respectively. Each test tube was then inoculated with 0.01 ml of a 1× field dose of the diluted vaccine. Three of thioglycolate and SCDM test tubes were incubated at 37°C with 5% CO_2, while the remaining three test tubes of thioglycolate media were kept at room temperature. Separately, Sabouraud's agar was prepared by dissolving approximately 30 g of Sabouraud's powder in 1000 ml of sterile water. This was then distributed into three petri dishes and incubated at 37°C with 5% CO_2. All petri dishes were observed daily for signs of contamination over a period of seven days.

3.5.3. Residual moisture content of lyophilized vaccine

The recommended residual moisture content of a vaccine is below 3.5% as described by Diallo *et al*. (2024). The minimum and maximum residual moisture content of lyophilized vaccine is 0.5% and 3.5%, respectively as per NVI vaccine production protocol.

3.5.4. Safety test in guinea pigs

The safety testing of monovalent and combined PPR, sheep pox and goat pox vaccines was performed after freeze drying, following OIE, (2019) guidelines, which involved laboratory animals to assess the nonspecific toxicity associated with vaccine injection. A total of 14 guinea pigs (200–250 g) were used. Initially, 4 guinea pigs were assigned to the first groups (received PPR vaccine), to assess the safety of the individual monovalent vaccines: Sheep pox and goat pox inoculated in 3 guinea pigs. Following confirmation of their safety, a third group of 5 guinea pigs received the combined vaccine to evaluate its safety profile. A ten-fold concentrated dose (10 x field doses) of each representative vaccine, designated code A and B, was formulated for safety evaluation. This involved reconstituting a PPR vaccine vial with 2 ml of sterile saline water, followed by dilution with the remaining 98ml of sterile saline water to get a final volume of 100 ml, this procedure was repeated for the SGP vaccine as well, then 10ml of each prepared PPR and SGP vaccines were given, half of the groups received 5 mL of the diluted vaccine intramuscularly in the hind limb and 5 ml intraperitoneally, while un-inoculated guinea pigs were observed as controls. All were monitored for 3 weeks. Vaccinated guinea pigs showed no adverse local or systemic reactions.

3.6. Monitoring of target animals

Fourteen apparently healthy Horro breeds of sheep and goats, aged between 1–2.5 years as estimated by their dentition according to Hamito, (2009), exhibiting normal growth were purchased from Godino market in Bishoftu. Up on arrival at NVI, the animals were treated in a controlled environment and placed in one experiment room for the first two weeks. They were housed under similar, consistent and hygienic conditions with balanced feeding that included roughage and concentrate as well as adequate watering. Then blood was aseptically collected from each animal for screening tests, by puncturing the jugular vein using venoject

needles and 10 ml plain vacutainer tubes. Each sample was labeled with an individual identification code and carefully transported to the virology laboratory at NVI and kept overnight at room temperature to allow clotting and subsequent separation of serum, after 24 hours the serum was collected by using a pipette in sterile Eppendorf tubes inside a level 2 biosafety cabinet and finally stored at -20^{O}C until further tests performed (Gelana *et al*., 2020). Then after doing all screening tests, the sero-negative animals were treated with oral administration of a broad spectrum anthelmintics (Albendazole 300mg, 3.5mg/kg body weight (BW) manufactured by Ashi Life Science, Mumbai, India) using bulling gun steel and subcutaneous administration of Ivermectin (H- IVER 1%, 0.5ml per 25kg BW) for sheep and goat manufactured by Hebei hope harmony pharmaceutical, Herbei province, China). Thereafter, all of them were followed strictly for the detection of any deviations from the normal animal's behavior.

3.6.1. Competitive enzyme-linked immunosorbent assay for screening of antibody for PPRV

The obtained blood sera were analyzed in a competitive ELISA (c-ELISA) by using (ID Screen®PPR Competition, ID.vet, Montpellier, France) kit for the presence or absence of antibodies specific to the PPRV, by following instructions provided by the manufacturer. Subsequently, optical density at 450 nm was read using a spectrophotometer. The test validity was determined by the mean value of the negative control (OD_{NC}) > 0.700. The mean value of the positive control is less than 30% of the OD_{NC} ($0D_{PC}/OD_{NC} < 0.3$). Finally the sample-to-negative control (S/N) ratio was considered the determination of whether or not the given serum is positive; doubtful or negative for PPR. Briefly, when S/N ratio read ≤50%, then it was considered positive, when it was between 50% and 60%, it was considered doubtful; when it was >60%, it was considered negative for the presence of antibodies against PPRV. All these results were recorded as a group means.

3.6.2. Serum neutralization test for screening of sheeppox and goat pox antibody

The collected blood sera of quarantined sheep and goats were tested for GP and SP neutralizing antibodies by using a serum neutralization test (SNT) in Vero cell line, according to NVI serum neutralization test protocol for lumpy skin disease, sheep pox and goat pox diseases, (NVI−VPS-RD PR-43). Briefly, for sheep pox and goat pox, twofold serial dilutions of serum samples were prepared from 1/5, 1/25, 1/125, 1/625 and 1/3125 dilution,

and mixed with 100µl of vaccine strain virus. Following incubation at 37°C, for 1 hour, Vero cell suspension 400,000/ml to each well was added to a 96-well tissue culture plate. The plate was then incubated at 37°C with 5% CO_2 until the cytopathic effect (CPE) in the virus control wells became evident (4–9days).

3.7. Immunization of target animals

Animals whose test results been sero-negative for PPR, SP, and GP were used for the experiment. Those found sero-positive were replaced with another group of sero-negative animals purchased for a different purpose at NVI, then randomly assigned to one of the three groups: (Group A (n=5) and Group B (n=5), designated as treatment group) and Group C (n=4 assigned as control group). Group A: received a single dose administration of reconstituted combined PPR and sheep/goat pox freeze-dried vaccine in 100ml sterile water, with each animal receiving a precise dose of 1ml subcutaneously. Group B:co-administered with a mixture of PPR and SPG monovalent vaccines purchased from NVI with a minimum titer of 2.5 $\log_{10}$ $TCID_{50}$, reconstituted in sterile water separately. Each animal was given a total volume of 2ml: 1 ml of the diluted monovalent PPR vaccine and 1 ml of the diluted monovalent SGP vaccine. The Group C animals stay unvaccinated. The vaccines reconstitution and dilution was done using 100 ml of cool and sterile saline water as mentioned in product catalog (NVI - QMS - QF – 64, 2016).

3.7.1. Post-immunization evaluation of target animal's body responses

A dedicated session was conducted to meticulously record the daily body temperature of each and every animal in the morning and late afternoon using a calibrated thermometer, aimed at detecting any potential systemic reactions or febrile responses indicative of adverse vaccine effects.

There was also a scheduled serum sample collection where, samples were systematically collected from each animal during the afternoon session on specific designated days: 0 (pre-vaccination baseline), 7, 14, 21 and 28 post-vaccination. These serum samples served as the basis for subsequent tests, enabling the detection of antibody responses and offering valuable insights into the development of vaccine-induced immunity over time (Amanova *et al*., 2023).

3.8. Outbreak investigation of challenge viruses

A field outbreak of PPR in the previously named Sefera peasant association (now included under Kebele – 03) at Welenchiti (72.6 km from Bishoftu), provided samples for viral isolation. Two sheep and one goat, raised under backyard conditions, exhibited mild clinical signs consistent with PPR: pyrexia (40.9^0C-41.2^0C), mucopurulent nasal discharge, dyspnea, sneezing, diarrhea and notable dehydration as noted by Jones *et al*. (2020). One sheep succumbed to the illness the day before sampling. Following the owner's full consent, samples were collected promptly for virus isolation.

For sheep pox and goat pox virus isolation, the samples were obtained in November, 2024 from three sheep at the Addis Ababa University College of Veterinary Medicine and Agriculture Professor Fiseha Gebre/ab Teaching Hospital (AAU-CVMA, Bishoftu) displaying skin nodules under their tail and groin area (Gomes *et al*., 2023). The animals were brought to the hospital five days after the owner first noticed signs of illness and, the tissue sample was taken on their first appearance at the hospital with the owner's full consent for the procedure.

3.9. Sampling and sample transportation

Nasal swabs were collected aseptically from representative cases of PPRV infection as described by Rahman *et al*. (2023). Briefly, the sample collection process involved the careful use of sterile, absorbent cotton-wool swabs. These swabs were gently inserted into the nasal passages of the selected animals to collect nasal secretions containing potentially viable virus particles. Following collection, the stick portion of the swab was then cut off using sterile forceps and scissors. The remaining portion of each swab sample was thoroughly suspended within cryovials containing 1.5ml of viral transport medium (VTM). This VTM, composed of phosphate-buffered saline (PBS) with antibiotics at a pH of 7.4, was specifically formulated to maintain viral integrity and prevent degradation during transport. The cryovials were maintained on ice during collection and transported to the Virology laboratory at NVI for virus isolation.

To facilitate both the molecular detection and isolation of sheep pox virus and goat pox virus, the skin biopsies were aseptically collected as mentioned by Sharma *et al*. (2021) with minor

modifications. Briefly, the skin biopsies were incised from nodular lesions under the tai land at groin area of the patient using a sterile blade and placed in sterile screw-capped universal bottles containing 10mL PBS at PH range of 7.2-7.6 and placed immediately on an icebox and sent to the department of Virology laboratory in NVI and placed at in – 20°C until use.

3.9.1. Nasal swap sample processing

The contents of the cotton swap were extracted thoroughly in 6ml of 1x PBS and were processed as mentioned by Pandey *et al*. (2022). Briefly, the fluid of nasal swabs was transferred to sterile three test tubes filled with PBS until the mixture measured 9ml and centrifuged together with other three balancing test tubes filled with 9ml PBS at 3500 rpm for 10 minutes. Then, the supernatant was filtered using a nano-pore filter and poured into 2 ml graduated plastic cryovials, divided into two parts: one part was stored at -20°C for further molecular detection of RNA, while the other part was supplemented with 1 ml of penstrep 10,000 UI/ml and 100µg/ml streptomycin and 1 ml of 2.5 µg/ml amphotericin B, then stored at -20°C for virus isolation.

3.9.2. Skin biopsy processing

To prepare samples for analysis, one gram of each thawed tissue specimen was aseptically transferred to a sterilized Petri dish using thumb forceps as described by Wondimu *et al*. (2021). Briefly, the tissue was then ground in a sterile mortar and pestle after adding 9 mL of sterile 50% PBSA (PBS with antibiotics). The resulting homogenate was transferred to three sterile vacutainer tubes. The tubes were centrifuged at 3500 rpm for 10 minutes to clarify the solution. Approximately 1 mL of the supernatant was carefully collected, filtered and transferred to graduated plastic cryovial tubes, which were then capped, wrapped in aluminum foil, and labeled as SPX code A, code B and Code C. These prepared samples were stored at −20°C until dispatch to the molecular biology laboratory. The remaining homogenate was also stored at −20°C for virus isolation purposes.

3.10. Peste des petitis ruminants virus isolation

PPRV was propagated using Verodog-SLAM (VDS) cells that consistently express the canine SLAM receptor. VDS cells were cultivated in GMEM supplemented with 10% FBS, 1

mg/mL Zeocin (Invitrogen, Carlsbad, CA, USA), NaHCO3, Na-Pyruvate, and non-essential amino acids. Cells were cultured at 37°C with 5% CO_2, as explained by Murr *et al*. (2020). After VDS cells reached 80% confluency, they were infected with 500 microliters of supernatant from the respective processed nasal swab samples. Following this initial step, viral adsorption was allowed to occur for 1 hour at 37°C under 5% CO_2. The infected cells were then cultured in maintenance media, undergoing 3-4 blind passages until CPE became apparent.

3.11. Sheep pox and goat pox virus isolation

Vero and embryonic skin of sheep (ESH-L) cells were seeded in duplicate on 25 cm² cell culture flasks (Corning, U.S.) using GMEM supplemented with 10% FBS and incubated at 37°C with 5% CO_2 until reaching 80-90% confluency as mentioned by Rhazi *et al*. (2021) with minor modifications. Briefly, the cell monolayer was then washed twice with PBS, and 500 µL of viral inoculum was added to the flasks for virus adsorption. After a 1-hour incubation, 2% GMEM supplemented with antibiotics was added to the infected flasks. Uninfected flasks with 90% confluent cells were considered as control. The infected cells were examined daily until CPE was evident. Generally, two up to four subsequent blind passages were performed on ESH-L cells and Vero cells. The virus isolates were preserved at -20°C.

3.12. Molecular detection of the challenge peste des petitis ruminants virus

Peste des petits ruminants viral RNA was detected from a viral transport medium that contain swab samples using a one-step conventional reverse transcriptase polymerase chain reaction (RT-PCR) following nucleic acid extraction and master mix steps, as described by Adeoye *et al*. (2022). Briefly, viral RNA extraction was performed using the Qiagen RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions using the forward and reverse primer sequences of Primer- PPRNP3-flow-5pm/ µl 5'-TCTCGGAAATCGCCTCACTAC-3' and Primer PPRNP4-REV-5pm µl5'CCTCCTCCTCGTCCTCCAAGACTG-3', respectively. At the end of polymerase chain reaction, gel electrophoresis will be run on a 1.5% agarose gel containing ethidium bromide. Finally the band images were then captured using a gel documentation system (Bio-Rad, USA) (Ali *et al*., 2021).

3.13. Molecular detection of challenge sheeppox virus or goatpox virus

Sheeppox or goat pox virus DNA was extracted from tissue suspensions using the NucleoSpin® RNA Virus Kit (Macherey-Nagel, Germany), following the manufacturer's protocol. For the amplification 30 kDa RNA polymerase subunit (RPO30) gene, a conventional PCR assay was conducted. Each 25 µL reaction contained 10 µL of IQ super mix and 5 µL of template DNA. Once the PCR stopped the amplicons were stored at +4 ^{0}C, Gel electrophoresis of the PCR products was then performed. After PCR, the amplicons were stored at +4°C. Gel electrophoresis was performed by loading the samples and amplicons into their respective cassettes and applying an electric field of 120 volts for 1 hour and 20 minutes. Gel imaging was then conducted using a Bio-Rad gel documentation system.

3.14. Challenge experiment

This challenge experiment was conducted 31 days post-vaccination for 14 days. It was performed after determining the concentration of each virus in infected cells by isolated PPR, SP and GP virus titration protocol of NVI (NVI VPS-RD-PR-14). Then randomly selected animals from each group were challenged by either PPR or SP or GP viruses whose virulence was judged by their titer result and CPE on infected cells. The following table summarizes the general workflow of the challenge experiment including determined titer of viruses.

Table 3: Animal groups, doses, and infection routes for PPRV and SGPV challenges

Challenge viruses	Groups	Number of Animals	Dose and method of infection
PPRV	Group A	3	$TCID_{50}$ 1 × $10^{4.4}$/ml, intranasal and intravenous, 5ml/animal
	Group B	3	
	Group C	2	
SGPV	Group A	2	$TCID_{50}$ 1 × $10^{3.6}$/ml, intravenous and intradermal, 5ml/animal
	Group B	2	
	Group C	2	

3.15. Post-challenge monitoring of animal's body response

All animals from each group were monitored daily for their rectal temperature starting from day zero (post-challenge) up to 14 days. Clinical monitoring was performed based on their severity categorized as mild, moderated, and severe or corresponding rating scale of "0-3" in ascending order of severity. Score of "0" indicated absence of symptoms, score of "1" represent moderate clinical signs, score of "2" denoted high temperature and moderate clinical signs; and a score of "3", indicated severe clinical signs as indicated by El Harrak *et al*., (2012).

3.16. Data analysis

Data including serological responses and daily mean rectal temperatures during the post-vaccination and post-challenge periods was entered into an Excel spreadsheet and analyzed using Excel 2010 for Windows 10, version 2017. The statistical significance of the SNT antibody titer comparison between groups (Group A Vs Group B and Group A Vs Group C) was assessed using an independent t-test. A p-value of less than 0.05 was deemed statistically significant, indicating a meaningful difference between the groups, whereas a p-value greater than 0.05 was regarded as statistically insignificant, suggesting no noteworthy difference.

3.17. Ethical approval and consideration

Ethics approval for the study was obtained from the Institutional Review Board of Addis Ababa University College of Veterinary Medicine and Agriculture (Reference number: VM/ERC/23/04/15/2023) (Appendix VI). All participants in the study provided written informed consent, and their names were kept confidential (Annex 12).

4. RESULTS

4.1. Quality control test results of monovalent and combined vaccines

4.1.1. Sterility

The monovalent, as well as combined vaccines produced, are free from gram negative, gram positive bacterial, and fungal contamination. It is suggested by the clear, sterile and transparent appearance of all inoculated thioglycolate, SCDM and sabouraud dextrose media.

4.1.2. Vaccine titration

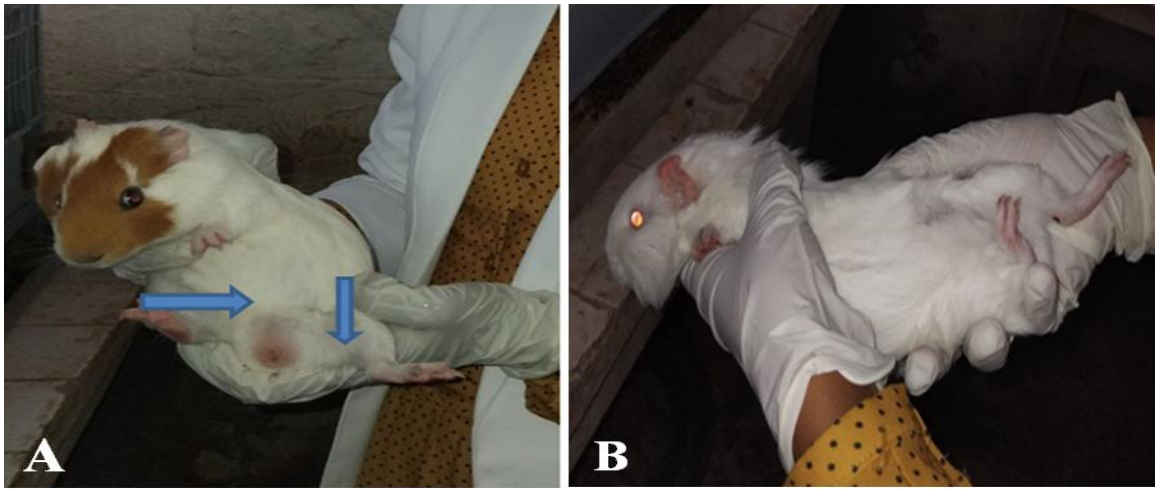
Titration was read on day 10 by starting from non-infected cell control wells and infected wells counted in all respective rows under an inverted microscope. The exact titer results of for SGP vaccine and PPR vaccine with batch number of 4/25 and 13/25 was determined to be 4.6 $\log_{10}$ $TCID_{50}$ per ml and 4.8 $\log_{10}$ $TCID_{50}$ per ml, respectively.

4.1.3. The moisture content of monovalent and combined vaccines

The residual moisture content of the developed combined vaccine was 1.7%, while the monovalent PPR and SGP vaccines had moisture contents of 1.09% and 1.3%, respectively. These recorded values were found within the recommended residual moisture range.

4.1.4. Monovalent and combined vaccines safety

No adverse reactions were observed at the site of vaccine inoculation in guinea pigs vaccinated with either monovalent or combined vaccines. It was confirmed through visual observation and palpation of the inoculation site, as well as by monitoring for any deviations from normal behavior.



A. Combined vaccine inoculated guinea pig **B.** Control guinea pig from group A

Figure 5: Guinea pigs from the combined vaccine safety test group

4.2. Peste des petits ruminants virus isolation

Verodog SLAM cells infected with PPR virus samples presented in (Figure 6) showing CPE deviated from the normal monolayer of cells. The virus was harvested on the 4th blind passages.

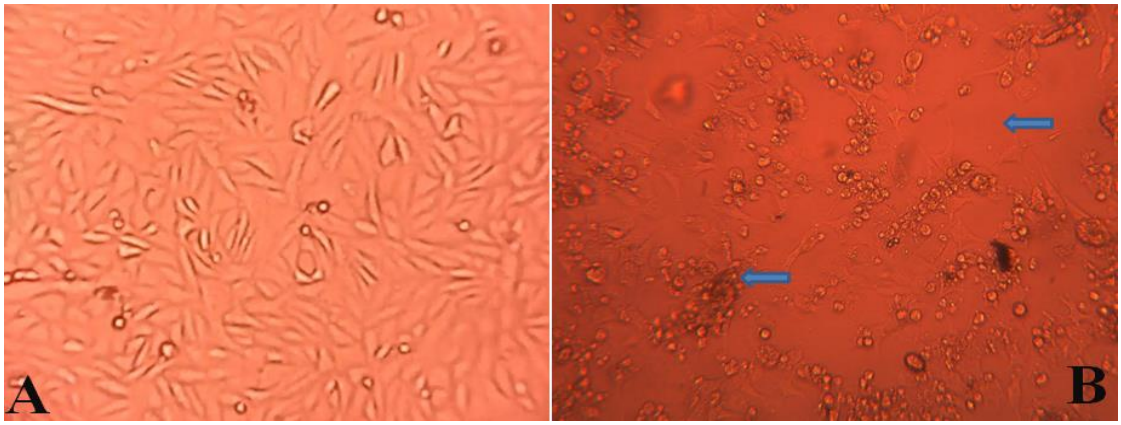


Figure 6: CPE at day 5 post inoculation of the supernatant harvest of the 4th blind passage

(A) Non-inoculated 24-hour aged confluent monolayer VDS-cell (100X) at passage 22. (B) Cell aggregation, detachment and gapping of cells from the cell sheet as indicated by arrows appreciated under an inverted digital microscope equipped with a camera.

4.3. Sheeppox and goatpox virus isolation

The isolated SP and GP virus had exhibited CPE within 7-16 days post inoculation on ESH-L and vero cell lines. The resulting cell morphology differs from its control cell by having cell aggregation and floating of cells originally adhered to the base of the flask.

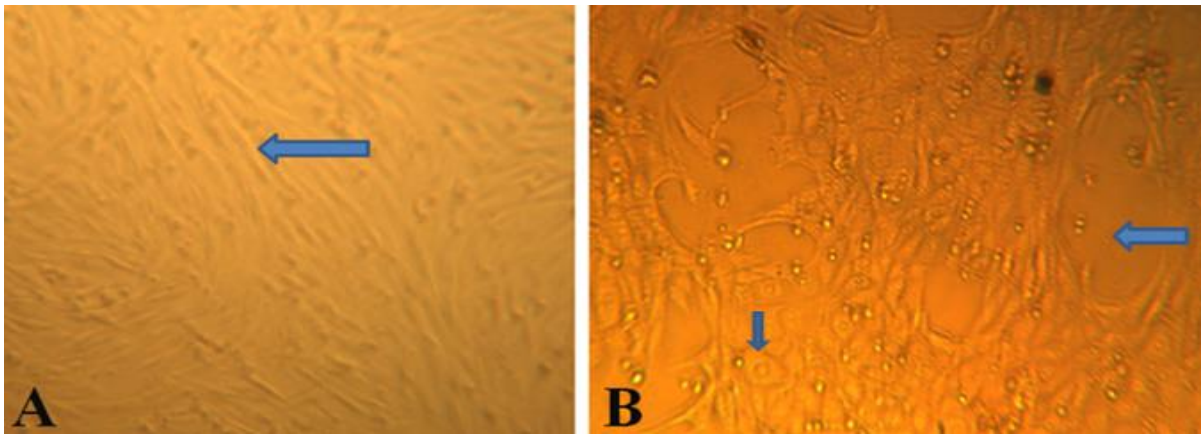


Figure 7: CPE of SP/GP virus isolated on ESH-L cell line after 4th day of 2nd blind passage.

(**A**) Shows the normal monolayer of the ESH-L cell line having fibroblast-like morphology as indicated by the arrow with 32 passages. (**B**) ESH-L cells monolayer is destroyed with infected cells detached from the surface on the 6th day of inoculation and loss of normal morphology of ESH-L cells through bulging.

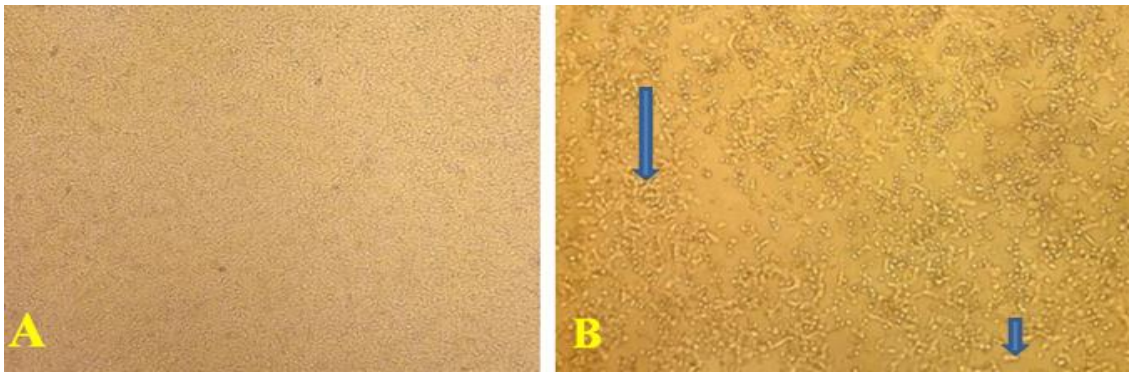


Figure 8: CPE developed by SP and GP virus on Vero cell line after the 3rd blind passage.

(**A**) The normal Vero cell monolayer with 44 passages. (**B**) CPE developed on approximately 8 days of inoculation characterized by clumping and rounding of cells.

4.4. Conventional polymerase chain reaction detection of challenge viruses

The three field samples of PPRV, as well as the three samples of SGP virus, tested positive on conventional PCR. In picture (a), the reference band length for PPRV is 350 bp, while in picture (b), the reference band length for goatpox virus is 172 bp.

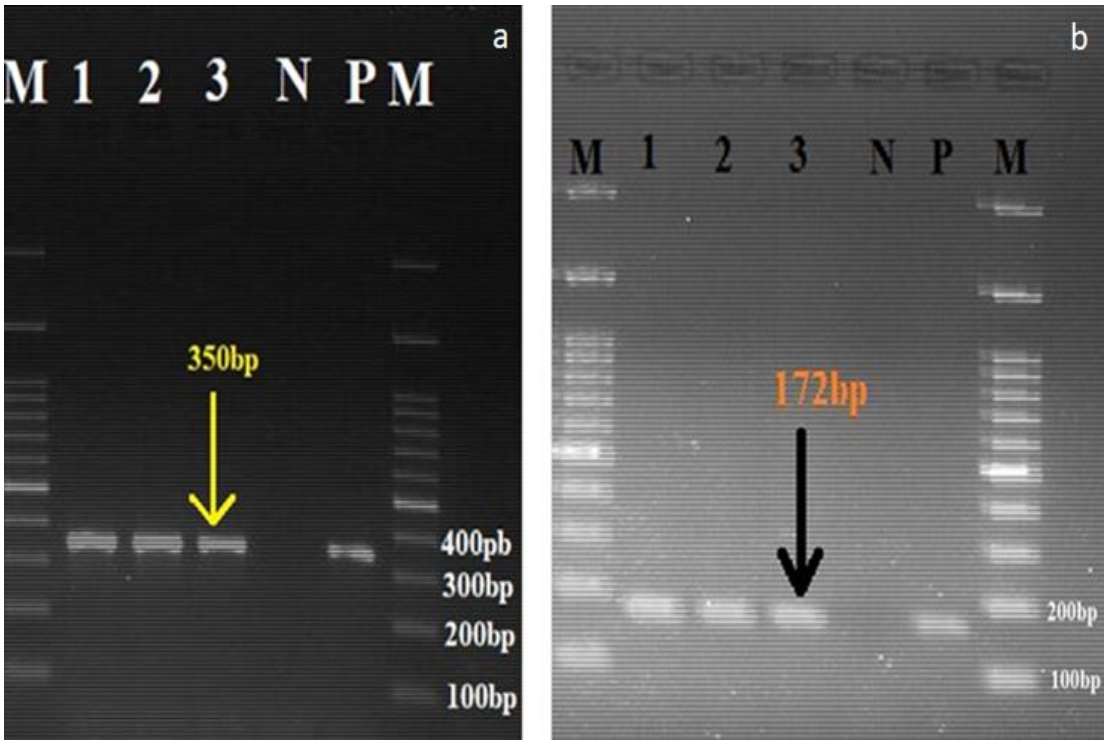


Figure 9: Detection of PPRV and SGPV using conventional PCR (M: Ladder, field sample designation (1-3), N: negative control and P: positive control).

4.5. Target animal's body response

4.5.1. The mean rectal body temperature on post-vaccination and post-challenge periods

The recorded post-vaccination and post-challenge rectal temperature patterns of the three groups followed for 45 days except the three days left before the challenge experiment began. The rectal temperature of Group A mean, Group B mean and Group C mean had no deviation from the normal range in the post vaccination period. However, a sharp rise was noted on the 5^{th} day of post-challenge period, which returned back to the normal range on the following days.

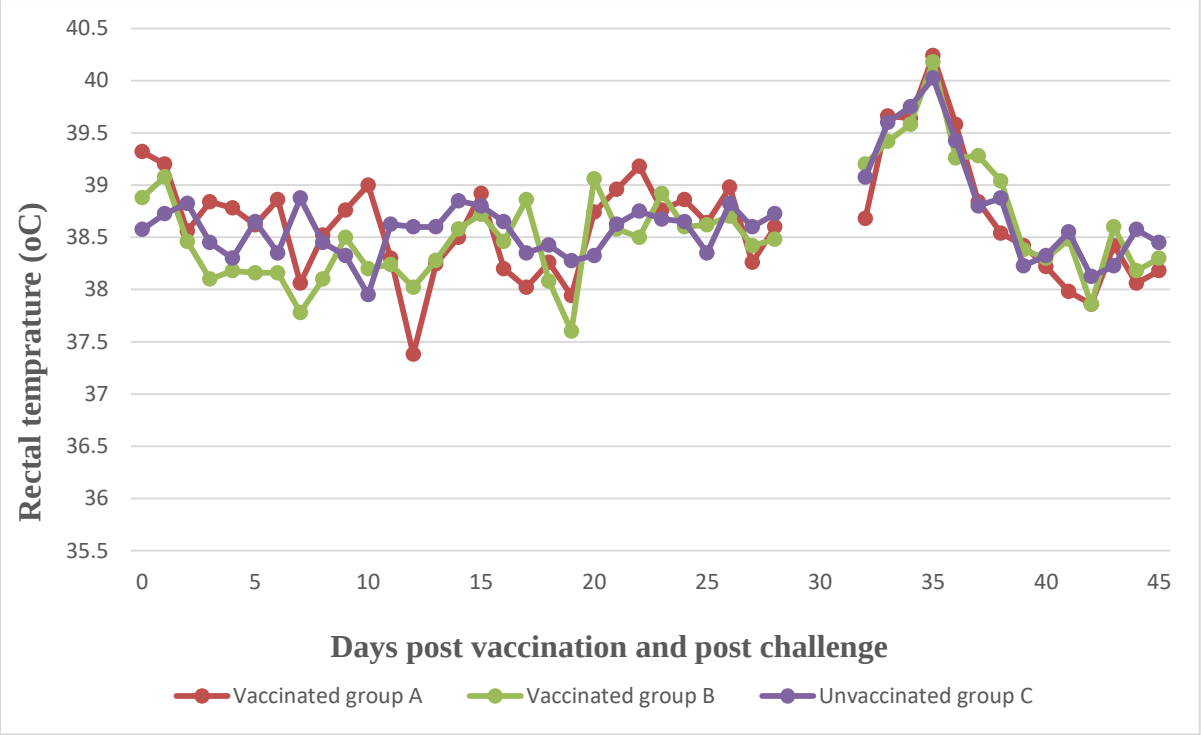


Figure 10: The group's post-vaccination and post-challenge rectal temperature pattern

4.5.2. Screening and post-immunization result of peste des petitis ruminants virus

The pre-vaccination, post-vaccination and post-challenge antibody response of target animals against PPRV showed in the graph below. It was demonstrated by using the percentage of average group mean (sample) to negative control. The Control animal's S/N ratio stayed above the threshold (50%) throughout the course of this experiment. The statistical analysis of Group A antibody tier mean and Group B antibody titer mean showed that there was no significant difference among them, whose P value was determined to be P=0.9122. The Group C antibody titer mean showed a significant difference with Group A animal's antibody titer mean with a P value of 0.0001.

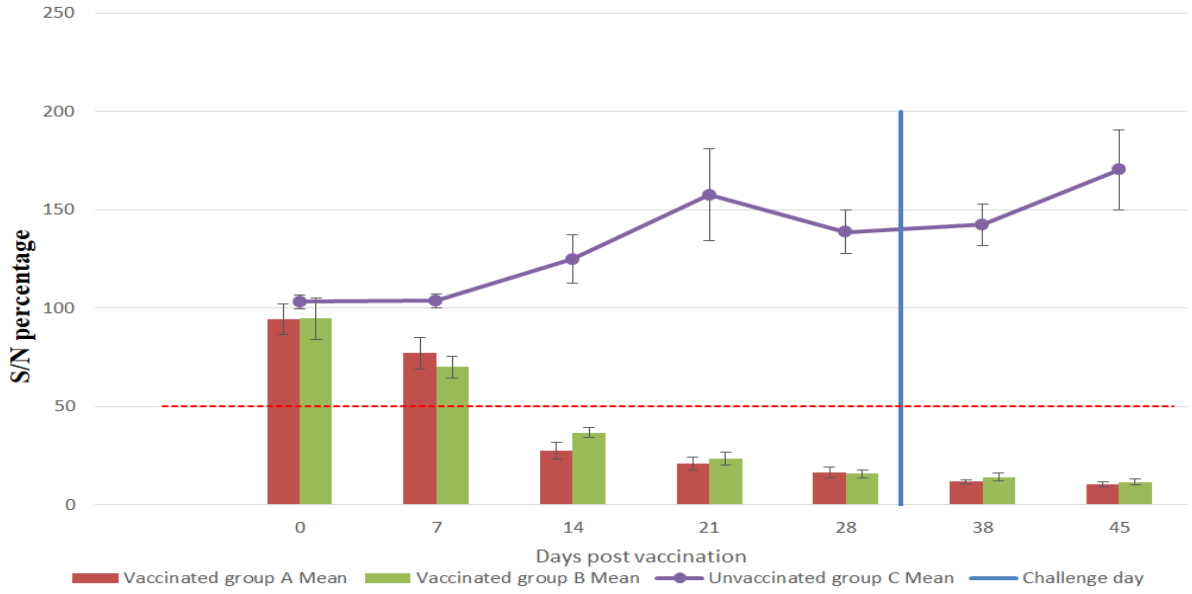


Figure 11: The group's mean antibody response against PPRV using C- ELISA

4.5.3. Screening and post immunization results of sheep pox and goat pox virus

The screening as well as post-vaccination and post-challenge mean antibody titer of the groups shown in the graph below. The control animal's antibody titer from the beginning of this experiment until its end was zero, while Group A and Group B mean antibody titer showed variable values on different days with increasing pattern.

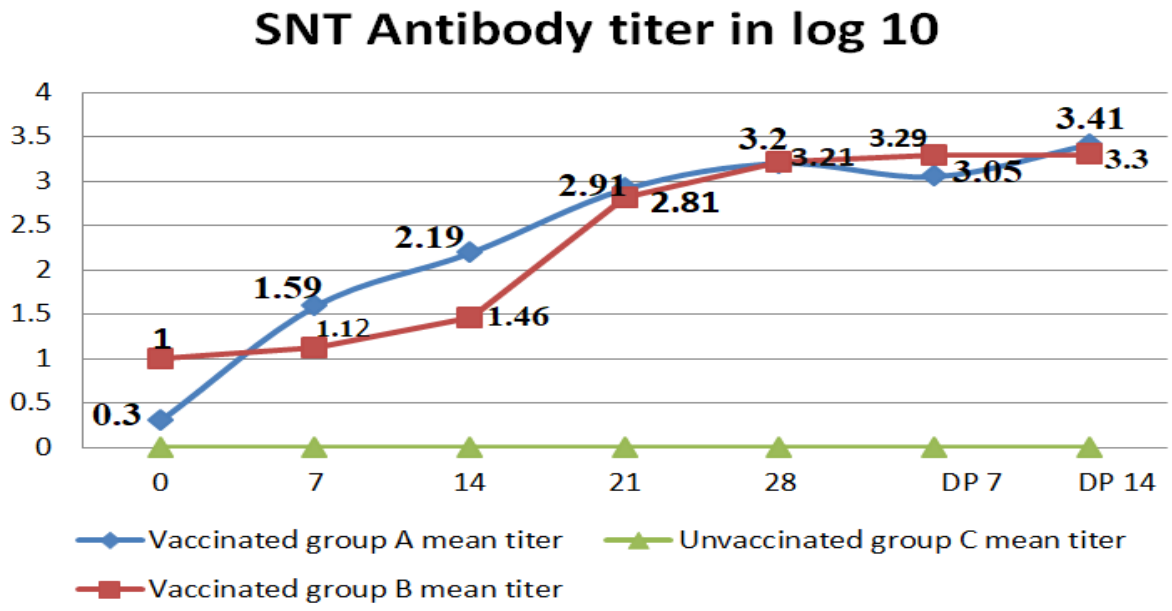


Figure 12: SNT result of group A, B and C animals

4.6. Post-challenge clinical sign lesion scoring result

The following table shows the daily monitoring of the target animal's body response following a challenge experiment for a cohort of 14 days. Only 50% of the unvaccinated group showed a specific PPR clinical signs such as sneezing, mucopurulent nasal discharge, dyspnea, accompanied by profuse diarrhea and finally one goat died due to anorexia and serious fluid loss, while the other recover shortly. Similarly, only a goat showed skin nodule very typical to that of goat pox, while the rest of affected control animals showed no skin nodule.

Animal ID	Pyrexia	Diarrhea	Lachrymal discharge	Nasal Discharge	Cough	Nodular pox lesion	Emaciation	In-appetence
FG 01	2	0	0	0	0	0	0	0
FG 02	2	0	0	0	0	0	0	0
FG 03	1	0	0	0	0	0	0	0
FG 04	2	0	0	0	0	0	0	0
G 10	2	0	0	0	0	0	0	0
FS 01	2	0	0	0	0	0	0	0
FS 02	1	0	0	0	0	0	0	0
FS 03	3	0	0	0	0	0	0	0
FS 04	1	0	0	0	0	0	0	0
S 10	1	0	0	0	0	0	0	0
G 64	3	3	1	3	3	0	3	3
G 62	3	0	0	0	0	3	2	1
SH 49	2	2	0	0	0	0	1	1
SH 51	3	0	0	0	1	0	0	0

4.7. Post-challenge molecular detection of nucleic acids from infected animals

The following conventional PCR Gel picture shows the positive bands for PPRV (on the left) and GP Virus (on the right) from the infected control animals following the sampling of nasal swap and tissue samples.

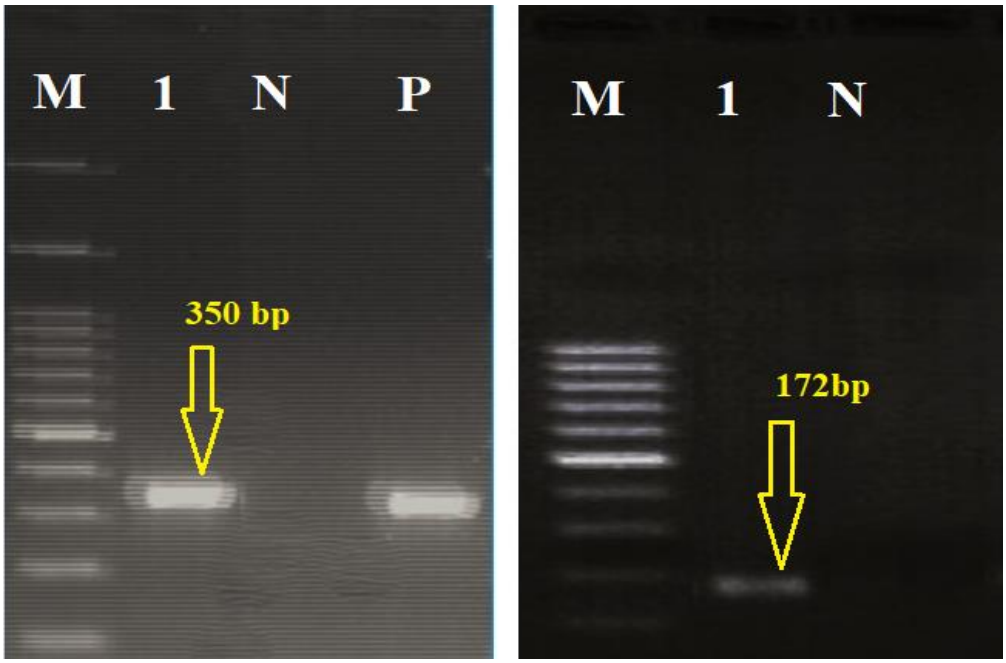


Figure 13: Gel picture for definitive diagnosis of infected control animals for PPR and GP viruses.

5. DISCUSSION

Globally, PPR triggers a significant socio-economic crisis, leading to anticipated financial losses of USD 1.2-1.7 billion and threatening the pecuniary wellbeing and nutritional stability of over 330 million families in endemic nations. In spite of the fact that, there is a substantial impact, the global investment directed toward PPR vaccination ranges between USD 270 and 380 million (Njeumi *et al*., 2015). Consequently, in Ethiopia, a study conducted in the Afar region found that economic loss due to PPR-caused mortality reached approximately $43,478.3 USD, highlighting vaccination gaps in the area as one of contributing factors (Gizaw *et al*., 2018). Additionally, considering the absence of specific treatment for PPR, SP and GP diseases (Bukar and Mabu, 2023), this study addressed the efficaciousness of the developed combined vaccine against these diseases as a very important and economically feasible approach for prevention.

In this study, the quality control test result of the developed monovalent and combined vaccines revealed a sterile appearance as free from bacterial and fungal contaminants similar result was reported by Zeidan *et al*. (2016). Furthermore, the PPR monovalent vaccine infectious virus concentration at post lyophilization step was expressed by having a minimum of $1\times10^{2.5}$ $TCID_{50}$ specifically, this developed vaccine had 4.8 $\log_{10}$ $TCID_{50}$ per ml, emphasizing that its virus concentration is expressed in 0.1ml of inoculum or per field dose, this result is comparable with the titer finding from monovalent vaccine preparation evidenced by Youssef *et al*. (2024) that was 5.5 $\log_{10}$ $TCID_{50}$ per ml using the same vaccine strain with the current study. Additionally the SP and GP monovalent vaccine's titer was determined to have 4.6 $\log_{10}$ $TCID_{50}$ per ml. This finding doesn't align with the titer result reported by Bhanuprakash *et al*. (2004) which was a titer of $1\times10^{9.63}$ TCID/ml, the reason for the huge gap with the present study could arise from the materials used for development, such as the secondary lamb testicular cells, which differ from the Vero cells used in the current study. Additionally, the vaccine strains varied; the prior study used live attenuated sheep pox Ranipet strain-R, while the current investigation employed KS1-O180 (SPPV). Furthermore, the method used for calculating vaccine's tissue culture infective dose was Reed and Muench calculation, whereas the Spearman-Kärber calculation was applied in the current study.

The moisture content of the developed combined, monovalent PPR and SGP vaccines was found to be 1.7%, 1.09% and 1.3% respectively, which is between 0.5% and 3.5%. If the post-lyophilization residual moisture content falls below the recommended minimum, this can lead to over-drying, affecting the vaccine's efficacy and stability. When it is above the maximum range, then it will deteriorate before the expiration. Accordingly the result of Diallo *et al*. (2024) confirms moisture content to be less than 3.5%.

The developed combined as well as the monovalent vaccines were passed for safety test in laboratory animals (Guinea pigs). None of these were found to have complications at the site of vaccine administration. The vaccines were considered safe without inducing any abnormal clinical reactions and no confirmation of pathology as outlined by OIE, (2023).

The efficacy test was measured using the S/N ratio of C-ELISA for PPR. In this study, animals that received the combined vaccine showed a highly increment of antibody titer following day 14 post-vaccination compared to simultaneous administration of PPR, SP/GP vaccine freeze-dried individually. However the antibody titer for control group remains above the threshold 50%, throughout the post-vaccination and post-challenge periods. This implies that control animals could get infected with PPR easily due to low antibody titer. This aligns with Amanova *et al*. (2021) who reported antibody titer for a combined vaccine remains uniformly higher till day 30 post-vaccination with the typical increase starting around day 14 post-vaccination. Another study conducted by Hurisa *et al*. (2024) illustrated that in a group of animals receiving simultaneous administration of PPR, SP and GP vaccines the S/N ratio declined sharply from its peak of 70% on day 7 to 40%, then gradually decreased until day 28, with consistency observed until day 35, thus marking a protective antibody titer.

The test result of neutralizing antibody titer for PPR, SP and GP vaccine in Group A as well as in group B detected by SNT revealed that the titer remain higher and higher starting from day 7 of post-vaccination period and reach its peak log3.41. This finding is in consistent with similar study done by Saad *et al*. (2024), where the neutralizing antibody index reaches its peak of approximately 2.8 by week 4 post-vaccination for a group of animals vaccinated with a combined vaccine of PPR and SGP. On the other side control group remained negative throughout the course of both experimental studies. Most importantly, there was no significant difference in mean antibody titers between Group A and Group B animals during the post-vaccination and post-challenge studies ($p=0.9122$, where $p>0.005$). In contrast, the

antibody titers of Group C animals exhibited a significant difference compared to those of Group A, with a p value of 0.0001. This result is deviated from those of Zhang *et al*. (2021), who demonstrated that administering these two vaccines simultaneously in sheep not only enhanced the immune response to the PPR vaccine but also suppressed the immune efficacy of the GP vaccine in vivo. Such discrepancies could potentially stem from differences in the interpretation of SNT results. An antibody titer of ≥1:10 was considered positive, while the current research adopted a more stringent threshold of ≥1:1.5 for positivity accompanied by the use of only one species of animal in the study, while in the current experimental study sheep and goats were both utilized, more importantly virus challenge study on vaccinated animals was not conducted that contradicts with the exclusive conclusion given on the research paper.

While maintaining optimal residual moisture content ensures vaccine stability, it was equally important to confirm that the lyophilization process does not compromise the vaccine's ability to elicit a protective immune response. The vaccine's efficacy was tested by challenging all groups with field isolates of PPR and GP viruses. The result showed these viruses showed CPE comparative to uninfected monolayer cells. In case of PPR, isolated on Vero Dog SLAM cell the CPE development is in agreement with what was described by Sannat *et al*. (2014) where there is the formation of cell rounding, aggregation and cell lysis. However CPE indicative of PPR viral replication was demonstrated by Aklilu *et al*. (2024) with large syncytium at the central part and also surrounded by rounded and fibroblast like cells. This typical CPE development by PPRV could be justified by several possible reasons for example, the PPRV used was known with its strain called the PPRV/Ethiopia/ Habru/2014 isolate from Habru area in Ethiopia from a PPR patient of goat, thus this virus was adapted to continuous cells and grow by living a characteristic CPE. Unlike to the present study whose source of the virus was directly from the field and unknown strain. Additionally, the nasal swap sample processing differs from this study in that it was homogenized and centrifuged at 1650 g for 20minutes, also monolayer cells with 70% confluency treated with intermittent agitation every 15 minutes to facilitate virus adsorption, which differs from NVI sample processing and cell infection protocol.

In the present study the GP and SP virus isolated on Vero cells demonstrated CPE such as clumping, rounding of cells and detachment of cell sheet, A similar CPE was reported by Mahmoud *et al*. (2016). On the contrary Hamdi *et al*. (2021) demonstrated the same kind of

CPE however the control monolayer cell as well as the infected cell used were not Vero cells instead it was primary testis cells.

Immunization of groups A and B animals showed no adverse reaction at the site of injection as well as no deviation from the normal behavior of shoat in the 28 days of post-vaccination period. However, a rise in a rectal temperature for all groups of animals with the highest (40.3^0C) was seen in the vaccinated group A animals after challenge virus administration through intravenous and intradermal routes for GP and intravenous and intranasal routes for PPR virus. This suggests that the vaccine was qualified at the level it can't pose any risk of seroconversion at all. Only 50% of the unvaccinated group showed a specific PPR clinical signs such as sneezing, mucopurulent nasal discharge, dyspnea, accompanied by profuse diarrhea and finally one goat died due to anorexia and serious fluid loss, while the other recover shortly. It was confirmed by detecting the PPRV in nasal swab samples. This observation was similar to the finding of Fakri *et al*. (2015) however a post-mortem diagnosis was performed for further investigation of gross pathological findings of PPR in affected control animals in contrast to the present study.

In this study the prepared combined vaccine and simultaneously administered monovalent vaccines did not interfere with the immune response mounted. In the PPR vaccine both forms of administration mimic PPR-specific C- ELISA antibody response in an increasing tendency like SP/GP vaccine does, evidenced by the strong immune response for both diseases for vaccinated groups. Similarly a study carried out by Khalil *et al*. (2019) proved the vaccines potency given for group B animals in the current study, each of the immunogens does not interfere immunogenesis pathway of the others instead induces effective immuno-prophylaxis against both diseases simultaneously indicated by the parallel administration of PPR vaccine with FMD and CCPP. Unlike to this experimental study Hurisa *et al*. (2024) reported that there was no immune response to SP when it was administered alongside other vaccines. The presence of one vaccine may inhibit the immune response to another. Alternatively, the concurrent administration of other vaccines with SGP could have stimulated the immune system in such a way that it suppressed the response to SGP, potentially due to issues related to the vaccine formulation or administration.

With respect to the challenge study, all groups of animals showed an increment of rectal temperature with a mean peak of 40.3 ^{o}C in group A following infection with both challenge

viruses compared to the pre immunization period. In comparison, unvaccinated animals recorded rectal temperature of 40^0C, in agreement with the finding of Chaudhary *et al*. (2009). The rise of rectal temperature was noted by all groups of animals' post-challenge period, with an average of 40.86 ^{O}C.

This research has several gaps, including the inability to perform molecular characterization of challenge viruses and challenge experiment conducted with isolated viruses were not tested its virulence on a sheep or a goat before proceeding to target animal safety tests. This was due to lack of sufficient budget. In addition the combined vaccine titration was not conducted, which requires specific and known monoclonal antibodies. Additionally, the efficacy tests performed on target animals lasted only for 2 months, including the acclimatization period; therefore, the exact time needed for the booster dose remains to be determined through further study. Beside the antibody titer for SP and GP had to be determined through cell-mediated immune response measurements like flow cytometer and total leukocyte count. Furthermore, the field trial which was also necessary for complete recognition of vaccine effectiveness remained unstudied. As a result, in the next investigation, other researchers should have to fill these gaps when performing the development and efficacy evaluation of combined vaccine against PPR, SP and GP.

6. CONCLUSION AND RECOMMENDATIONS

This study conclusively demonstrates that the mixed formulation and freeze-dried live attenuated combined vaccines, that contain antigens for PPR, SP/GP, elicit a robust immune response without any observable deleterious effects in vaccinated animal groups. Notably, upon challenging both vaccinated and unvaccinated groups, a stark contrast in their physiological responses was evident among them, where vaccinated animals maintained normal species-specific behaviors, indicative of effective protection and also the absence of clinical manifestations of the diseases. Conversely, unvaccinated or controlled animals unequivocally manifested disease-specific clinical signs, and its presence was evidenced by molecular detection, thereby underscoring the vaccine's efficacy in preventing overt disease. More importantly, these findings hold significant implications for Ethiopia's ambitious PPR eradication plan, slated for completion by 2027 GC, hence the deployment of the live attenuated combined vaccine would significantly contribute to the mass vaccination campaigns which encompass addressing very remote areas where often haphazard infrastructure is, thus a single shot at a time will accelerate disease control as well as decreases distress due to multiple injections to the animals.

Therefore, in light of the foregoing conclusion, the following recommendations are put forth:

- Detailed research must be conducted to assess the long-term efficacy and effectiveness of the live attenuated combined vaccine in the field.
- The experimental trial for efficacy evaluation of combined vaccine should have to be conducted on animals of different physiological states (e.g., pregnant, lactating) to ensure generalizability.
- Vaccination programs should be integrated with active surveillance systems to monitor disease prevalence and vaccination coverage to enhance the campaign's effectiveness.
- Different shoat diseases have to be integrated in the preparation of combined vaccine to address multiple diseases in a single shot.

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Annex 1: Cell subculture

- Pre-warm both Trypsin 0.05%/EDTA0.02 solution
- Subculture working (trypsin) and complete media for cell growth at $+37^0$C.
- Take cell culture flask with confluent monolayer from the incubator
- Decant old media and wash the cells with PBS to remove residual serum (which would inactivate the trypsin) and residual bivalent Ions.
- Add slowly enough amount of trypsine on the opposite wall of the attached cell monolayer culture flask and observe the action of the trypsin for two minutes in an incubator.
- Decant the trypsin, wait by the placing the flask inverted until the cell detached from it inside the laminar flow hood. Then, add growth medium (10% GMEM) with pipette Bulbs PCV, mechanically until monolayer become dispensed to a single cell.
- Determination of viable cell using inverted microscope, cells that pass light through them are live, while dead cells can't and appear in black color.

Annex 2: Conventional molecular detection of PPR virus

1. RNA Extraction of PPR virus

- Viral suspension of 350µl added to the labelled Eppendorf tubes.
- 350µl of RLT lysis buffer added and homogenized gently by pipetting. A total of 350µl 70% ethanol will be added and homogenized by pipetting. Then, all 1050µl suspension transferred to mini spin column, and centrifuged at 13,000 rpm for 30 seconds, the flow through removed and 500µl RPE buffer added and centrifuged at 13,000 rpm for 30 seconds.
- The flow through discarded and 500µl RPE wash Buffer added and centrifuged at 14000 rpm for 2 minutes.
- The mini spin column placed in the 2 ml collection tube and centrifuged again for 1 minute at 14000 rpm to dry the column matrix. Then, the mini spin column was placed in the labelled Eppendorf tubes and eluted the RNA by adding 40µl elution buffer by centrifuging at 8000 rpm for 1 minute.
- The eluted RNA will be stored at -20˚C until processing.

2. One step RT-PCR Master mix preparation

Type of reagent	For one reaction	For three reactions
RNase free water	4µl	12 µl
5X PCR buffer	5 µl	15 µl
Q- solution	5 µl	15 µl
Primer- PPRNP3-flow-5pm/ µl 5'-TCTCGGAAATCGCCTCACTAC-3'	2 µl	6 µl
PrimerPPRNP4-REV-5pm /µl 5'CCTCCTCCTCGTCCTCCAAGACTG-3'	2µl	6 µl
10mM dNTPs	1 µl	3 µl
One step RT-PCR enzyme Mix	1 µl	3 µl
Add template (RNA)	5 µl	15 µl
Total volume	25 µl	

3. Run PCR

Steps	Temperature	Time	Cycle
cDNA synthesis	50^{o}c	30 minutes	1-Cycle
Initial Denaturation	95^{o}c	30 Sec	1- Cycle
Annealing	55^{o}c	30 Sec	35 cycles
Elongation	72^{o}c	30 Sec	
Final Elongation	72^{o}c	5 mints	1-Cycle
Put at	4^{o}c	Until machine off	–

4. Gel Electrophoresis

- Prepare 2 % Agarose gel
- Add 4 µl Gel red with loading dye, then loading PCR product 10 µl in each well and add 10 µl molecular marker (Ladder) started 100base pairs.
- Run Electrophoresis for 1hour at 120V.

- Read the result by using UV-light.
- A positive result for PPRV is around 350 base pairs.

Annex 3: Conventional molecular detection of sheep pox/goat pox virus

1. DNA Extraction (Extraction of sheep pox/goat pox virus)

DNA lysis

- Mix 200 µL of sheep pox virus/goat pox virus suspension with 20 µL of proteinase K and 200 µL of lysis buffer (AL buffer).
- Homogenize by vortexing for 15 seconds.
- Incubate the mixture at 56 °C for 10 minutes.

Addition of ethanol

- Add 200 µL of 96% ethanol to the clear lysis solution and homogenize again.
- Transfer the 620 µL suspension to a mini spin column.
- Centrifuge at 8,000 rpm for 1 minute.

Wash #1:

- Add 500 µL of AW1 buffer to the column.
- Centrifuge at 8,000 rpm for 1 minute.
- Discard the flow-through.

Wash #2:

- Add 500 µL of AW2 wash buffer.
- Centrifuge at 14,000 rpm for 3 minutes.
- Place the column in a clean 2 mL collection tube.
- Centrifuge at 14,000 rpm for 1 minute with the rotor cap open to dry the matrix.

Elution buffer

- Transfer the column to a labeled Eppendorf tube.
- Add 50 µL of elution buffer.
- Centrifuge at 8,000 rpm for 1 minute.
- Store the eluted DNA at −20 °C until further use.

2. Conventional PCR master mix and gel electrophoresis

Master mix preparation

Reaction components	For one reaction	For three reactions
RNase free water	6µl	18µl
primerSPGPRNApol-Fow-5pm/µl	2 µl	6 µl
Primer-SPGPRNApol-REV-5pm/µl	2 µl	6µl
IQ Super mix	10 µl	30 µl
Add Template (DN A)	5 µl	15 µl
Total volume	25 µl	

3. <u>Run conventional PCR</u>

Steps	**Temperature**	**Time**	**Cycle**
Initial Denaturation	95^{o}c	5 mints	1-Cycle
Denaturation	95^{o}c	30 Sec	40 cycles
Annealing	50^{o}c	30 Sec	
Elongation	72^{o}c	30 Sec	
Final Elongation	72^{o}c	7 mints	1-Cycle
Put at	4^{o}c	Until machine off	–

4. <u>Gel electrophoresis</u>

Making the gel (for a 2% gel)

- Weigh out 0.5g of agarose into a 250mL conical flask.
- Add 50mL of 1xTAE, swirl to mix.
- Microwave for about 1 minute to dissolve the agarose.
- Leave it to cool on the bench for 5 minutes down to about 60°C (just too hot to keep holding in bare hands).
- Add 2µL of ethidium bromide (10mg/mL) and swirl to mix
- Pour the gel slowly into the tank. Push any bubbles away to the side using a disposable tip. Insert the comb and double check that it is correctly positioned.
- Leave to set for at least 30 minutes, with the lid on if possible.

- Pour 1x TAE buffer into the gel tank to submerge the gel to 2–5mm depth. This is the running buffer.
- In the first lane load 5 µl 1kbp DNA ladder, while in remaining lanes load 5 µl sample after mixing with 2 µl DNA 6x loading dye in each well by using micropipettes.
- Connect the gel-running tank to the power supply.
- Observe the gel image under the UV trans-illuminator gel documentation system.

Annex 4: Quantification of virus particles using titration method

This method is used to determine the mass of virus particles and the infection dose of specific virus to the target cells.

- Check the cells growth under inverted microscope, if the growth is confluent, calculate the number of cells and take the required volume of cell suspension.
- Warm GMEM media, calf serum, and trypsin in water bath at 37^{o}C using water bath.
- Put on the light, clean and disinfect the working surface of the laminar flow hood with 70% alcohol & tissue paper. Then, take out the bottles from water bath and disinfect and dry them by tissue paper and take into the hood. Discard the media from the cell culture.
- Trypsinization: add 5 ml of trypsin and make gentle movement on the side of cell layer and discard the trypsin by using pipette, and repeat the procedure once again, then add 3 ml of trypsin close the stopper and put it in incubator for 3-5 minutes, meanwhile prepare the complete media (45 ml GMEM + 5ml calf serum, then, take out the flask from incubator and add 10 ml of complete media and mix thoroughly by pipetting.
- Centrifuge the cell suspension tube with similar tube for keeping the balance and adjust the time for 10 minutes, 1500rpm and temperature 20^{o}C
- Clean and adhere the cover slip on counting chamber. Take out from centrifuge and remove the media carefully using pipette (10ml). Don't mix the cells deposit. Re-suspend the cells with 1 ml of complete media and make 10% dilution of the cells (1:10).
- Take 50µl of the re-suspended cells and drop under the margin of cover slip and allow the suspension fill the inner space. Count the cells found in small grills (squares), cells counted only in one opposite margin (border of the square) not both.
- 10 squares counted as one group and similar other 30 squares, in three groups (10/each) and calculate the sum and mean of the cells counted in 40 squares (4 groups).
- Calculate the required volume of cell suspension. Then, Prepare serial dilution of the virus: Take 9 tubes and add 4.5 ml of Bulk media in all tubes, then add 0.5 ml of virus

suspension and mix well transfer 0.5 ml of the suspension from tube 1st to the 2nd tube and from 2nd tube to the 3rd up-to the 7th tube.

- Prepare microplate- make vertical lines between 1st & last column (1 & 12). Transfer virus titration from higher dilution to the lower. Transfer 50µl from the 7th tube in all 10 wells of H row, from 6th to G row up to row keep their sequential titration order. If necessary 1 & 2 titration tubes can be ignored. Leave11th Column blank and add in column 12thwells 50µl media and row H 1-12 except 11th column for cell control, add 50 µl cell suspension (100,000 cells/well in wells except Column 11. Cover the plate with plate sealer and incubate in 5% CO_2 incubator and keep it for 15 days.
- Check the CPE in virus infected cells under microscope. Start from the non-infected cell control. Count the infected wells number in all respective rows.
- Calculate the number of virus particles using the formula – t =Xo-d (P-0.5)

Annex 5: Serum neutralization test for detection of antibody for GP virus

- Test sera stored at -20^{0}C is thawed at room temperature.
- Test sera including a negative and positive control are diluted in 1/5 GMEM.
- Take a record sheet and fill in the layout of the 96 well plate according to the samples to be tested then add in each well 100microlitter of cell medium (2% GMEM + TBP + Antibiotics).
- Add in each in well A1-H1 and A7-H7, 25 µl of test serum sample 1-16
- Add in each in well A2-H2 and A8-H8, 25 µl of test serum sample 1-16, with multichannel pipette, perform 5fold serial dilution (from column 2-6 and column 8-12) with initial dilution 1/5 and discarded 25 µl of suspension from the end point. From column 6 and 12 then add 100 µl of 1000 µl TCID50/ml vial suspension of SGP to each wells from 2-6 and 8-12 (add 75 µl of cell culture media in column 1 and 7) and incubate in 37^{0}C for 1hr.
- Add 50 µl of Vero cell prepared in to each well.
- No virus added in the yellow columns, but adds 25 µl serum +75 µl cell media to each well.
- The positive control: In 6 wells in each 4 raw are filled with different dilution of SGP virus (100 $TCID_{50}$, 10 $TCID_{50}$, 1$TCID_{50}$, 0.1$TCID_{50}$ in 100 µl suspension) then, plate is incubated for 1 hour similar to other plates.

- The negative control: 6 wells in the last raw are without virus and add 50 µl of vero cell incubated for one hour like other plates. Then, Plates reading to monitor for CPE formation start on day 4^{th} and final readying is made on the 9^{th}.
- Interpretation: If CPE is observed when the serum is negative, in the contrary, no CPE or >80% inhibition shows the serum has antibody against SGPV. The neutralization index is the log titre difference between the titre of the virus in the negative serum and in the test serum. A neutralization index of ≥1.5 is positive.

Annex 6: Test procedure of Competitive ELISA for PPRV antibody detection

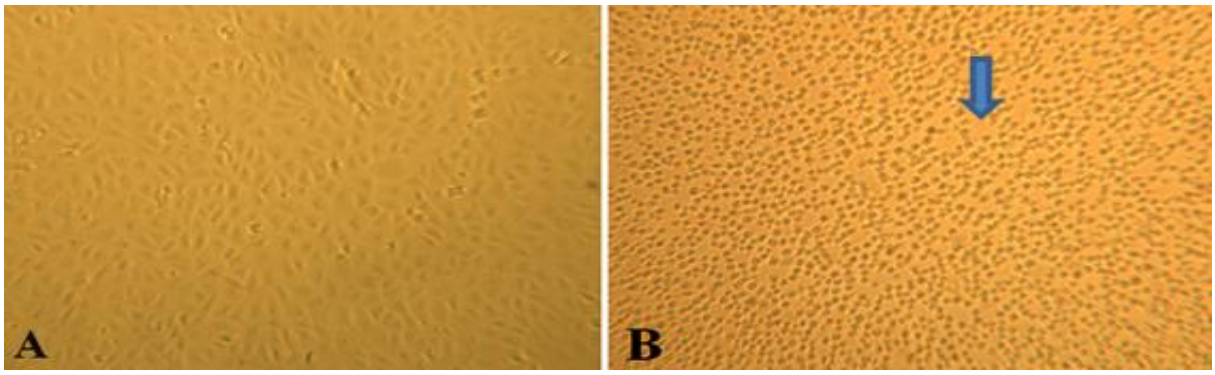
The material used includes Mono or multi-channelpipettors capable of delivering volumes of 10µl, 100µl and 200µl, Disposable tips, 96=well microplate reader, distilled or deionized water and manual wash system.

The sample preparation includes, bring the wash solution (20×) to room temperature (21^0C ± 5^0C) and mix thoroughly to ensure the wash concentrate is completely solubilized.

Prepare the wash solution (1×) by diluting the wash concentrate in distilled/deionized water. Here, we use $C_1V_1=C_2V_2$ formula. Procedures started by homogenizing all reagents by inversion or vortex.

- Add 25 µl of **Dilution Buffer13** to each well
- Add 25 µl of **Positive Control** to wells A_1 and B_1.
- Add 25 µl the **Negative Control** to wells C_1 and D_1.
- Add 25 µl of each sample to be tested to the remaining wells.
- Incubate **45min ± 4 minutes** at 37^oC (± 3^0C).
- Wash each well 3 times with approximately 300 µl of the **wash solution**. Avoid drying of the wells between washings.
- Prepare the conjugate (**1×**) by diluting the **Conjugate10×** to 1/10 **Dilution Buffer 4**.
- Add 100 µl of the **Conjugate 1×** to each well.
- Incubate **30 minutes ± 3 minutes** at 21^0C (± 5^0C).
- Wash each well 3 times with approximately 300 µl of the **Wash Solution**. Avoid drying of the wells between washings.
- Add 100 µl of the **Substrate Solution** to each well.
- Incubate 15minutes ± **2 minutes** at 21^0C (± 5^0C) in the dark.
- Add 100 µl of Stop Solution to each well in order to stop the reaction.
- Read and record the O.D. at 450nm.

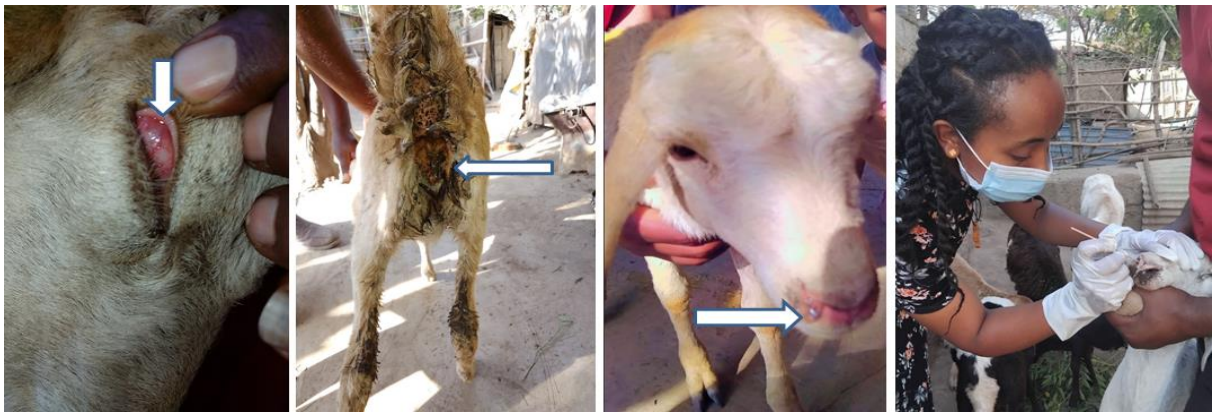
Annex 7: CPE development by SP and GP monovalent vaccine before being harvested.



The normal monolayer of Vero cell (A) SP and GP Vaccine with 100 %CPE (B), harvested at the third free thawing cycle.

PPR monovalent vaccine that was harvested before being mixed with SP and GP vaccine.

Annex 8: Sampling of nasal swap and Tissue from infected animals at field



One of the presentative PPR case from a patient in welnchiti displaying diarrhea, fever, depression, purulent nasal discharge.

Annex 9: The monovalent and combined vaccine injected Guinea pigs

Annex 10: Pictures taken during laboratory works

Performing trypsinization technique during cell subculturing and serum neutralization test

Annex 11: Pre and post challenge condition of target animals

Annex 12: Informed consent request form

Title of the Study: The development and efficacy evaluation of combined vaccine for PPR, Sheep pox and goat pox

Principal Investigator: Bezawit Tesfaye

Institution: MSC student in veterinary Microbiology at Addis Ababa University- CVMA

Address: Bishoftu, Oromia, Phone number: 0946038071

Email: bezaatat@gmail.com

INTRODUCTION:

You are being invited to participate in a research study. The purpose of this research is to assess the efficacy of developed combined vaccine for PPR, sheep pox and goat pox, through filed investigation of challenge PPR and SGP virus from recent outbreaks.

Study Procedures:

If you agree to participate, you will be asked to:

- ✓ Provide a patient animal suffering from Sheep pox and PPR.
- ✓ Allow me to collect samples like nasal swab and subcutaneous skin tissue for the study,

Risks and Benefits:

Risks: There will be invasive methods for collection this samples thus the animal may suffer from the sample collection which involves minor surgical procedure for skin tissue sample.

Benefits: Participation in this study may contribute to better understanding of the combined vaccine efficaciousness by challenging group of animals who took the vaccine in comparison with unvaccinated control groups.

Confidentiality:

Samples will be kept confidential. Identifiable information will not be shared with anyone outside the research team. Data will be stored securely and used solely for research purposes.

Voluntary Participation: Your participation in this study is entirely voluntary. You may decline to participate or withdraw at any time without any consequences.

Consent to Participate: By signing below, you indicate that you have read and understood the information provided above and consent to participate in this study. You will be given a copy of this consent form for your records.

Participant #2; Signature: _____

Date: _____

Researcher's Signature: _____ Date: _____

Annex 13: Ethical Consideration

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ADDIS ABABA UNIVERSITY
College of Veterinary Medicine
and Agriculture
Bishoftu

Animal Research Ethics Review Committee

Ethical clearance certificate

Certificate Ref. No: VM/ERC/23/04/15/2023

Name of Applicant: Dr Samson Leta (MSc, Associate Professor)

Address: Department of Biomedical Sciences, College of Veterinary Medicine and Agriculture (Addis Ababa University)

Title of the project: *Advancing animal health through development of field-deployable diagnostic assay, bivalent vaccine and promotion of indigenous knowledge for peste des petits ruminants (PPR) and Sheep and Goat Pox (SGP): to promote early detection and progressive control of major small ruminant diseases – DABV-project*

Date of application:	**December, 2022**
Nature of the project:	**Field investigation and experimental vaccine trial**
Target animal species:	**Small ruminants**
Number of animals involved:	**2000**
Study area:	**Different parts of Ethiopia**

Minutes No. and date of review: **VM/ERC/04/15/022, 15/02/2023**

The Animal Research Ethical Review Committee of the College of Veterinary Medicine and Agriculture of Addis Ababa University has reviewed the above research project and unanimously approved the application of Dr Samson Leta.

Professor Getachew Terefe (DVM, PhD)
Chairman

Signature

Federal Democratic Republic of Ethiopia * Addis Ababa University College of Veterinary Medicine and Agriculture

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ፋክስ} Fax 251-11-4339933
ስልክ } Tel. +251 114338450
ፖ.ሣ.ቁ P.o.x. Box) 34
ቢሾፍቱ፤ ኢትዮጵያ Bishoftu, Ethiopia

Annex 14: Plagiarism report