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COLLEGE OF VETERINARY MEDICINE AND AGRICULTURE

DEPARTMENT OF MICROBIOLOGY, PARASITOLOGY, AND POULTRY HEALTH MANAGEMENT

DETECTION, ISOLATION, MOLECULAR CHARACTERIZATION AND PHYLOGENETIC ANALYSIS OF PESTE DES PETITS RUMINANTS VIRUS (PPRV) CIRCULATING IN AFAR REGION, NORTH EASTERN ETHIOPIA.

BY: KEBEDE DEBEBE

ADIVISOR: HIKA WAKTOLE (BSc, MSc, PHD. ASSOC. PROF)

CO-ADVISOR: OLANA MERERA (DVM, MSc, ASSIS. PROF)

JUNE, 12- 2025

BISHOFTU, ETHIOPIA

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CO-ADVISOR: OLANA MERERA (DVM, MSC, ASSIS. PROF)

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

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BISHOFTU, ETHIOPIA

APPROVAL

ADDIS ABABA UNIVERSITY
COLLEGE OF VETERINARY MEDICINE AND AGRICULTURE
DEPARTMENT OF VETERINARY MICROBIOLOGY, PARASITOLOGY, AND POULTRY HEALTH

As MSc research advisors, we certify that we have read and evaluated the thesis prepared under our guidance by **Kebede Debebe Sedessa** entitled: " **Detection, Isolation, Molecular Characterization, And Phylogenetic Analysis Of Peste Des Petits Ruminants Virus (PPRV) Circulating In Afar Region, North Eastern Ethiopia: Its Implication for Veterinary Microbiology**" We recommend that it be accepted as fulfilling the thesis requirement for the degree of Master of Science in Veterinary Microbiology.

Submitted by: Kebede Debebe Sedessa _____ _____
Name of Student Signature Date

Approved *for submittal to a* thesis assessment committee

1. Dr. Hika Waktole (Msc, PhD, Asso. Prof)_ _____ _____
   Advisor Signature Date

2. Dr. Olana Merere (DVM, Msc, Assis. Prof**)** _____ _____
   Co-Advisor Signature Date

3. Dr. Hailegebrael Bedada **(**DVM, MSc, Asso.Prof**)** _____ _____
   Department Chairperson Signature Date

APPROVAL

ADDIS ABABA UNIVERSITY
COLLEGE OF VETERINARY MEDICINE AND AGRICULTURE
DEPARTMENT OF VETERINARY MICROBIOLOGY,
PARASITOLOGY, AND POULTRY HEALTH

As members of the examining board of the final MSc open defense, we certify that we have read and evaluated the thesis prepared by **Kebede Debebe Sedessa** entitled: "**Detection, Isolation, Molecular Characterization, And Phylogenetic Analysis Of Peste Des Petits Ruminants Virus (PPRV) Circulating In Afar Region, North Eastern Ethiopia: Its Implication for Veterinary Microbiology**." And recommend that it be accepted as fulfilling the thesis requirement for the degree of Master of Science in Veterinary Microbiology.

Dr. Haileleul Nugusse (DVM, MSC,PhD,Asso.Prof)	_____	_____
Chairperson	Signature	Date
Dr.Berecha Beyissa (DVM, MSc, PhD)	_____	_____
External Examiner	Signature	Date
Dr.Addisu Demeke (DVM,MSc,PhD,Asso.Prof)	_____	_____
Internal Examiner	Signature	Date

Final approval and acceptance of the thesis dissertation is contingent upon submitting its final copy to the CGS/FGC through the departmental graduate committee (DGC) of the candidate's major department.

I hereby certify that I have read the revised version of this thesis prepared under my direction and recommend that it be accepted as fulfilling the thesis/dissertation requirement.

STATEMENT OF THE AUTHOR

I, Kebede Debebe, hereby declare that the research study titled "Detection, Isolation, Molecular Characterization, and Phylogenetic Analysis of Peste des Petits Ruminants Virus (PPRV) Circulating in the Afar Region of Northeastern Ethiopia" is entirely my own work. This study was carried out under the guidance of Hika Waktole (BVSC, MSC, PhD, Associate Professor), with the supervision of Olana Merera (DVM, MSC, Assistant Professor) at the College of Veterinary Medicine and Agriculture, within the Department of Microbiology, at Addis Ababa University, Bishoftu, Ethiopia, and Fasil Aklilu (DVM, MSc, PHD Candidate) from Animal Health Institute. I affirm that the findings, analyses, and conclusions presented in this document stem from my original research, supported by the referenced materials, and have not been submitted elsewhere for any degree or certification. Any contributions from other individuals or organizations have been properly acknowledged.

Signed: Kebede Debebe

Date: June, 202

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LIST OF ABBREVIATIONS

AHI	Animal Health Inistitute
CPE	Cytopathic Effect
CT	Cycle Threshold
DIVA	Differentiating Infected from Vaccinated Animals
DMEM	Dulbecco's Modified Eagle Medium
ELISA	Enzyme Linked Immunosorbent Assay
FAO	Food and Agriculture Organization
FBS	Fetal Bovine Serum
HKY	Hasegawa-Kishino-Yano (Phylogenetic model)
IAEA	International Atomic Energy Agency
Ic-ELISA	Immunocapture ELISA
ML	Maximum Likelihood (Phylogenetic model)
OD	Optical Density (in ELISA)
PBS	Phosphate Buffered Saline
PPR	Pest des petites Ruminants
PPRV	Pest des petites Ruminants virus
RNA	Ribonucleic Acid
RT-PCR	Reverse Transcription Polymerase Chain Reaction
TMB	Tetramethylbenzidine (substrate in ELISA)
VTM	Viral Transport Medium
WOAH	World Organization for Animal Health (formerly OIE)

ABSTRACT

Peste des Petits Ruminants (PPR) is a highly contagious viral disease of small ruminants, causing significant economic losses in endemic regions like Ethiopia. This study aimed to detect, isolate, and molecularly characterize *PPR* virus (*PPRV*) in suspected outbreaks across six districts of the Afar region. A total of 64 clinical samples were collected from ocular and nasal swabs, and 2 tissue samples were collected from suspected cases in goats and sheep. Immunocapture ELISA (Ic-ELISA) revealed an overall antigen positivity rate of 31.25% (20/64), all from goats (46.5%, 20/43), while sheep samples were uniformly negative. Virus isolation on Vero cell lines confirmed active viral replication in 7/20 swab and 2 tissue samples, as evidenced by characteristic cytopathic effects (CPE). RT-qPCR further validated *PPRV* in 80% (16/20) of the ELISA-positive samples, with Ct values ranging from 21.06 to 33.51, indicating variable viral loads. Twelve *RNA* extracts with low Ct values were submitted for partial nucleocapsid (N) gene sequencing; four yielded high-quality amplicons. Phylogenetic analysis of a 278 bp N gene fragment revealed that three isolates (ETH-7, ETH-8, ETH-12) clustered within Lineage IV, closely related to East African strains (e.g., ETH-*PPRV*2019, Tanzania-*PPRV* 2018). One isolate (ETH-11), however, was genetically indistinguishable from the Nigeria 75/1 vaccine strain (Lineage II), suggesting potential vaccine virus shedding or exposure. These findings confirm the endemic circulation of *PPRV* predominantly Lineage IV in goats in Afar, with occasional detection of vaccine-like strains. The study highlights the need for DIVA compatible diagnostics, thermo stable vaccine formulations, and enhanced molecular characterization to support Ethiopia's *PPR* control and eradication efforts.

Key words: *PPRV*, Small ruminants, ELISA, Isolation, RT-qPCR, Phylogenetic analysis, Nigeria 75/1 vaccine, Afar region.

1. INTRODUCTION

Peste des Petits Ruminants (*PPR*) is a highly contagious viral disease affecting small ruminants, particularly goats and sheep (Wassif *et al.,* 2024). The causative agent, *Peste des Petits Ruminants* Virus (*PPRV*), belongs to the genus Morbillivirus within the family Paramyxoviridae. Clinically, the disease is characterized by high fever, oral erosions, diarrhea, respiratory distress, and elevated case fatality rates (Kumar *et al.,* 2014). *PPRV* is an enveloped virus with a non-segmented, negative-sense, single-stranded RNA genome of approximately 15.9 kb. This genome encodes six structural proteins (N, P, M, F, H, L) and two non-structural proteins (C and V) (Etibor *et al.,* 2021).

Peste des Petits Ruminants (*PPR*) poses a significant threat to livestock-dependent communities, where small ruminants are vital for food security, income generation, and socio-economic stability (Udahemuka, 2023). The disease is prevalent in regions such as sub-Saharan Africa, the Middle East, and Asia, where it diminishes livestock productivity and exacerbates poverty among pastoralist populations (Kardjadj and Luka, 2016).

In Ethiopia where livestock farming forms the cornerstone of rural livelihoods *PPR* is among the most economically devastating trans boundary animal diseases (Mulatu *et al.,* 2021). The disease poses an even greater risk in areas with high animal mobility, limited veterinary infrastructure, and inadequate biosecurity, creating favorable conditions for virus survival and transmission (Lelenguyah *et al.,* 2023). Understanding the virus's epidemiology and genetic diversity within Ethiopia is essential to designing and implementing effective control and eradication strategies (OIE and FAO, 2015). Transmission occurs primarily through direct contact between infected and susceptible animals, making pastoral and agro-pastoral systems particularly vulnerable (Kardjadj and Luka, 2016).

Peste des Petits Ruminants virus (*PPRV*) is shed through respiratory secretions, feces, and other bodily fluids, enabling widespread transmission within flocks (Verma *et al.,* 2024). Contributing factors to its spread include high stocking densities, communal grazing practices, and inadequate biosecurity. Moreover, the absence of carrier animals, combined with suboptimal vaccination coverage, further perpetuates viral circulation (Kumar *et al.,*

2014). Effective control of *PPR*, therefore, necessitates a comprehensive understanding of viral genetics, transmission dynamics, and the socio-economic context of affected communities (OIE, 2015; Mulatu *et al.*, 2021).

Recent advances in molecular diagnostics and phylogenetic analysis have significantly improved our ability to detect and characterize *PPRV*. Tools such as enzyme-linked immunosorbent assays (ELISA), virus isolation in cell culture, and molecular techniques like polymerase chain reaction (PCR) have revolutionized the detection of the virus (Halecker, 2021). Additionally, phylogenetic analysis based on genomic sequencing allows researchers to trace the evolutionary origins of circulating *PPRV* strains and identify potential sources of outbreaks (Courcelle *et al.*, 2024).

Bridging the current knowledge gap on *PPRV* dynamics is critical for developing effective, evidence-based interventions (Pomeroy-Arthur, 2023). This study aims to investigate the genetic diversity of *PPRV* in Ethiopia's Afar region by utilizing antigen detection, virus isolation, molecular diagnostics, and phylogenetic analysis. Through this integrated approach, the study seeks to characterize circulating viral strains, assess their phylogenetic relationships with global lineages, and contribute to the broader understanding of *PPRV* epidemiology. The findings are expected to support national and regional efforts aimed at controlling and ultimately eradicating the disease.

1.1. Statement of the Problem

Peste des Petits Ruminants (PPR), caused by the *PPRV*, remains a serious and economically significant disease among small ruminants especially sheep and goats in Ethiopia’s North-Eastern Afar region (Mulatu *et al.*, 2021). In this region, pastoralist livelihoods heavily depend on small ruminants for food, income, and cultural practices. Despite this, the recurring outbreaks of *PPR* result in mortality rates reaching 50% up to 80%, and morbidity ranges between 80–100%, leading to substantial livestock losses, reduced productivity, and severe socio-economic consequences for the affected communities (Kardjadj and Luka, 2016).

Although national and international efforts such as the Global Eradication Programme aim to eliminate *PPR* by 2030, there remains a critical shortage of data on the epidemiology, molecular characteristics, and phylogenetic relationships of *PPRV* strains circulating in the Afar region. This lack of localized information is largely due to limited diagnostic infrastructure and insufficient disease diagnostic data. The absence of data on antigen detection, virus isolation, and molecular characterization hinders the development of effective disease eradicationprogram, including vaccination planning and outbreak containment.

Moreover, the potential for viral spread to neighboring regions and the risk of infecting wild small ruminants or other atypical hosts complicate the management of *PPR* and threaten broader livestock dependent economies (Abera., 2023). As such, there is an urgent need to document the presence of *PPRV* in the region, identify circulating strains, and generate evidence-based insights that can inform strategic disease control and eradication efforts in the Afar region and beyond.

1.2 Objectives

1.2.1 General Objective

- To detection, isolation, molecular characterization, and phylogenetic analysis of *PPRV* circulating in Afar region.

1.2.2 Specific Objective

- To detect the presence of *PPRV* antigen in small ruminants using antigen Ic-ELISA methods.
- To isolate the *PPRV* from clinical samples collected from suspected animals.
- To confirm the presence of *PPRV* using RT-PCR.
- To conduct phylogenetic to perform sequencing analysis of the isolated *PPRV* strains to understand their genetic relationships.

2. LITERATURE REVIEW

2.1 Overview on *PPRV*

The highly contagious *PPRV* mostly affects small ruminants, such as sheep and goats. Communities in Africa, the Middle East, and Asia that depend on livestock are at high risk from *PPR* diseases. High morbidity and mortality are the outcome of the clinical symptoms, which include diarrhea, pneumonia, nasal and ocular discharge, oral erosions, and a high temperature. Direct contact with contaminated secretions is the main way that epizootics are spread, and communal grazing, poor animal mobility, and inadequate biosecurity measures are all known to increase the disease. The World Organization for Animal Health (WOAH) has classified *PPRV* as a modifiable trans boundary disease due to its rapid spread and economic importance (OIE & FAO, 2015; Banyard *et al*., 2020).

The *Peste des Petits Ruminants Virus* is a member of the Paramyxoviridae family, notably the genus Morbillivirus. It is an enclosed virus with a single-stranded, negative-sense, non-segmented genome that is roughly 15.9 kilo bases in size. In addition to two non-structural proteins, C and V, which are generated from the P gene by RNA editing, it contains six structural proteins: nucleocapsid (N), phosphor protein (P), matrix (M), fusion (F), hemagglutinin (H), and large polymerase (L). Based to the "rule of six," the length of the genome is a multiple of six, which is a feature that is important to optimal replication. Such fundamental structure is characteristic of morbilliviruses and allows the virus to replicate, transcribe, and be pathogenic in small ruminants (Munir *et al.,* 2013; Batten *et al.,* 2011).

Phylogenetic characterisation and molecular diagnostics have significantly improved *PPRV* strain categorization and detection. Four genetic lineages (I–IV) of the virus have been identified; Lineage IV is the most widely distributed worldwide, with its circulation spreading into East Africa and Asia. In beyond helping respond to outbreaks, these technologies encourage vaccine research, especially in support of the WOAH and FAO worldwide eradication campaign, which aims toward eliminating *PPR* by 2030 (Courcelle *et al.,* 2024; Banyard *et al*., 2020).

Epidemiological

In pastoralist traditions like Afar, where goats and sheep are vital to people's socioeconomic and nutritional status, Peste des Petits Ruminants Virus (*PPRV*) remains a significant epidemic problem (Jones *et al.,* 2020). Direct contact with the secretions of infected animals can quickly spread the virus, which has a high rate of morbidity and mortality. In Afar, a number of factors have contributed to the issue, including low vaccination rates, communal grazing and watering systems, inadequate veterinary care, and ongoing animal migration based on by season migration (transhumance) (Jones *et al.,* 2020. The unfortunate fact that the majority of Afar's districts including Amibara and Gelealo are inaccessible makes it more difficult to diagnose and treat illnesses.

New virus strains are also introduced through unregistered cross-border trade with nearby nations, making control measures more difficult. These epidemiologic issues maintain the endemic spread of *PPRV* in the Afar region, endangering not only the health and production of animals but also food security and the fight against poverty. Targeted immunization campaigns, improved community-based disease reporting, and reinforced veterinary infrastructure that is tailored to the pastoral lifestyle can all help to solve these issues (Gizaw *et al.*, 2019).

2.2. Clinical and Pathological Manifestations of *PPR*

2.2.1 Clinical

A highly contagious and economically devastating morbillivirus, *Peste des Petits Ruminants Virus* (*PPRV*) selectively causes morbidity in small ruminants, particularly sheep and goats. The host species, the pathogenicity of the virus strain, and the animal's immunological condition are some of the variables that affect the clinical symptoms of PPRV infection (Fine *et al.,* 2020).

Usually, the sickness begins with a sudden, high temperature that exceeds at 41°C. Later, lethargy, anorexia, and sadness are systemic clinical signs. Crusting of the eyes and nose results from nasal and ocular discharges that start off serous but eventually turn

mucopurulent (Fine *et al.,* 2020). Coughing, dyspnea (breathing difficulties), and pneumonia are common respiratory involvements that are frequently made worse by subsequent bacterial infections. The morbidity seen during epidemics is greatly influenced by those factors (Clemmons *et al.,* 2021).

Another characteristic of the illness is oral lesions. With erosions and ulcerations affecting the gums, inner lips, tongue, and palate, they frequently manifest as necrotic stomatitis. Coughing and discomfort feeding can result from the necrosis of the oral mucosa in some situations, which creates a noticeable "checkerboard" pattern (Kothiwala & Vashisht, 2023).

Both acute and chronic diarrhea in young animals can result in severe weight loss, emaciation, and dehydration. Usually, there are conjunctivitis (eyelid irritation) and enlarged lymph nodes. The disease's financial toll on communities that depend on livestock is increased when pregnant animals abort (Wassif *et al.,* 2024). Compared to sheep, goats have more severe clinical illness. With mortality rates ranging from 50 to 80% during acute outbreaks, goats are susceptible to severe and deadly forms of the disease. Sheep may become asymptomatic reservoirs for viral shedding due to mild or subclinical infection (Clemmons *et al.,* 2021).

2.2.2 Pathological Manifestations

The process of *Peste des Petits Ruminants Virus (PPRV)* pathogenesis in goats is the virus replication within the lymphoid tissues first upon entry via the respiratory route. It then continues to include systemic spread through viremia, which results in the infection of epithelial tissues widely, especially of the respiratory and gastrointestinal tracts (Gautam *et al.,* 2021). Necrotic and hemorrhagic lesions of the lung tissue and fibrinous deposition reveal massive tissue destruction and secondary bacterial infections exacerbated by the immunosuppression caused by the virus (Niyogi *et al.,* 2023).

Peste des Petits Ruminants Virus (PPRV), a Morbillivirus, causes a highly contagious and devastating disease in small ruminants, primarily sheep and goats, with severe pathological manifestations across multiple organ systems (Abera, M., 2023). The

respiratory system is heavily affected, exhibiting bronchointerstitial pneumonia with epithelial necrosis, syncytial cell formation, and peribronchiolar lymphocytic infiltration, often progressing to hemorrhagic consolidation (Liu, J. and Shi, H., 2023). Secondary bacterial infections, such as pasteurellosis, exacerbate lung lesions due to *PPRV*-induced immunosuppression. The digestive tract suffers from erosive stomatitis, ulcerative mucositis, and severe hemorrhagic enteritis, with characteristic "zebra-striped" lesions in the cecum and colon, leading to profuse diarrhea, dehydration, and weight loss. These lesions reflect the virus's tropism for epithelial cells, with goats typically showing more severe clinical signs than sheep, influenced by breed and viral strain virulence (Eloiflin *et al.*, 2022).

Lymphoid tissues are primary targets, with *PPRV* causing profound lymphopenia and necrosis in lymph nodes, spleen, and mucosa-associated lymphoid tissues, impairing immune responses and facilitating systemic viral dissemination via viremia (Rossi Schenone, T., 2019). Systemic manifestations include high fever (40–41.3°C), mucopurulent ocular and nasal discharges, and mucosal petechiae, with hyperacute cases potentially leading to sudden death with hemorrhagic nasal discharge. Recent studies highlight the virus's early replication in respiratory and lymphoid tissues, detectable within days of infection, and its ability to infect atypical hosts like camels and wildlife, though with less clear epidemiological roles. The high morbidity (up to 90%) and mortality (up to 80–100%) underscore the socioeconomic impact, particularly in endemic regions like Africa and Asia, necessitating robust diagnostic and control measures (Banyard *et al.*, 2020; Bataille *et al.*, 2021).

2.2.3 Gastrointestinal Lesions

The intense congestion and hemorrhages in the intestinal tract indicate vascular injury, a characteristic of *PPRV* because of its specificity for endothelial cells (Hodgson, S., 2020). Also observed is the cytopathic effect of the virus on the gastrointestinal mucosa indicated by the formation of necrotic lesions and ulcerations, especially in lymphoid tissues like Peyer's patches. Edema and swelling of the intestine noted are typical signs of inflammation and fluid retention, typical in *PPRV* infection. Additionally, fibrinous exudations on the intestinal surfaces indicate the severity of the inflammatory response

and tissue damage resulting from the virus (Mantip *et al.*, 2021). Such lesions are in agreement with *PPRV* pathogenesis, involving epithelial and lymphoid tissue and resulting in severe enteritis, diarrhea, and death in infected goats.

Control

The implementation of a live attenuated vaccine, which produces strong and durable protection, is an essential to controlling the *Peste des Petits Ruminants Virus* (*PPRV*). In high-risk areas like the Afar region, where animal migration, pastoralism, and communal grazing all contribute to the spread of disease, organized mass vaccination efforts are especially necessary. Achieving effective control also requires early warning and quick action, which includes early widespread investigation, laboratory confirmation, and coordination at the field level. During epidemics, travel restrictions such as market inspection, quarantine, and animal health certification limit the spread of disease (Yadav *et al.*, 2020).

Prevention

Enhancing the availability of veterinary services and raising community awareness of *PPR* symptoms and transmission pathways are important aspects of preventive efforts. Regularly doing both active and passive surveillance offers the chance to identify cases early. In remote, in-transit locations like Afar with few official veterinary facilities, capacity building through community-based training of animal health workers is crucial. Community awareness initiatives must encourage the reporting of possible outbreaks and discourage high-risk practices like releasing new animals without a quarantine. Viral transmission is also prevented by improved biosecurity of animal housing, feeding areas, and watering locations (Leibler *et al.*, 2017).

Eradication

The Global *PPR* Eradication Programme (GEP-*PPR*), which aims to eradicate this disease by 2030, is in line with long-term, sustained *PPR* eradication (Fine *et al.*, 2020). This is attainable for Ethiopia through risk-based planning, regionally coordinated planning, and

the incorporation of epidemiological data into decision-making. The risk of reinfection will be reduced if viruses are jointly controlled across borders, especially between adjacent states. Government support, financial structures, and laboratory capacity building are essential for providing long-term improvement. Withdrawal depends on participatory strategies that involve residents in immunization and surveillance in pastoral areas, such as Afar.

2.3 Diagnostic Methods

2.3.1 Antigen Detection

Detection of the *PPRV* antigen was done with the Enzyme-Linked Immunosorbent Assay (ELISA), which is a frequently used technique to detect viral antigens from clinical specimens. ELISA has been specifically designed to quantify *PPRV* antigenic constituents, where the viral antigens are trapped on a solid support like a microtiter plate coated with *PPRV* antibodies. This is then followed by the addition of an enzyme-conjugated second antibody that binds to the trapped antigen to produce a colorimetric signal whose spectrophotometric quantitation can be achieved. The commercially available ID vet *PPR* Antigen ELISA Kit is utilized for the detection of *PPRV* antigens in clinical specimens such as nasal swabs, oral secretions, and tissue homogenates of infected animals (Halecker, 2021). Although lot number I06 is stated, it is better to verify this from ID vet directly or as given in the supporting documents accompanying the kit. The procedure is critical in early diagnosis and prompt treatment and has high sensitivity and specificity for the identification of *PPRV* antigens in clinical samples (Santhamani *et al.*, 2016).

2.3.2 Isolation

In the current study, the *Peste des Petits Ruminants Virus* (*PPRV*) was isolated using Vero cells, which are derived from African green monkey kidney epithelial cells (Chlorocebus sabaeus). Since vero cells lack the ability to make interferon, they are ideal for the growth of viruses from clinical samples, making them very vulnerable to Morbilliviruses like *PPRV*. Vero cells (ATCC CCL-81) are the preferred cell line for *PPRV* isolation,

according to the World Organization for Animal Health (WOAH, 2023), since they all exhibit pronounced cytopathic effects (CPE), such as rounding, detachment, and syncytia formation.

Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal bovine serum (FBS), 100 units/mL penicillin, and 100 µg/mL streptomycin was used to cultivate the cells for this study. After the samples were inoculated on Vero cell monolayers, the cultures were kept at 37°C in an incubator with 5% CO_2 and were examined every day for CPE using an inverted microscope. Usually lasting three to seven days, the effects confirmed that the infectious PPRV was actively replicating in the field samples. In addition to confirming the presence of infectious *PPRV*, successful detection of virus-induced CPE provides a valuable source for further molecular investigation such as RT-qPCR, sequencing, and phylogenetic analysis. Therefore, using Vero cells in line with WOAH protocols is a dependable and efficient way to isolate *PPRV* and perform diagnostic laboratory work.

2.3.3 Molecular Characterization

Molecular *PPRV* typing via real-time reverse transcription PCR (RT-qPCR) is carried out with a standard procedure to ensure high sensitivity and specificity. Clinical samples (nasal/oral swabs, tissue) are first processed for extracting RNA using silica-membrane kits (e.g., QIAamp Viral RNA Mini Kit), and the quality of the extracted RNA was determined by spectrophotometry (A260/A280 ratio ≥1.8). For RT-qPCR, primers and a FAM-labeled probe hybridize to the conserved N gene (forward: 5′-ATGGCAGTGAGTGATGG-3′; reverse: 5′-TTAGCTGCATCATGCCA-3′), with TaqMan Fast Virus Master Mix in a 40 cycle protocol (95°C, 3 sec; 60°C, 30 sec). Positive samples have Ct ≤35, confirmed against reference controls (Nigeria 75/1 strain). This assay detects ≤10 RNA copies/µL with no cross-reactivity to related morbilliviruses (OIE, 2023), allowing for diagnostics and viral load measurement. Confirmatory positive samples are sequenced Sanger and phylogenetic analysis examined on MEGA6 to identify lineage (I-IV), with the entire process from extraction through lineage determination done within 24 hours for rapid response to outbreaks (OIE., 2023).

2.3.4 Phylogenetic Analysis

During phylogenetic analysis, RT-PCR amplified products of *PPRV* genes, i.e., N (nucleoprotein) and F (fusion) genes, were cleaned by a PCR purification kit. PCR products were cleaned and sequenced by the Sanger sequencing technique. Sequencing reactions were performed by forward and reverse primers for precision and completeness of the genetic sequence (Crossley *et al.*, 2020).

The raw sequences obtained from retrieval were edited, assembled, and aligned by bioinformatics software like BioEdit and MEGA. ClustalW algorithm was implemented for multiple sequence alignment in order to match the obtained sequences with available reference sequences in the Gen Bank database. As per the guideline, phylogenetic trees were built on the maximum likelihood or neighbor-joining criterion under 1,000 bootstrap replicates to evaluate the stability of the tree topology (Zou *et al.*, 2024). Phylogenetic analysis yielded information about genetic association between Afar region *PPRV* isolates and other worldwide *PPRV* strains, thus assisting in the detection of circulating lineages and infection sources (Courcelle *et al.*, 2024).

3. MATERIALS AND METHODS

3.1 Study Area

The study was conducted in the Afar Region of Ethiopia, specifically within Zone 03, with a primary focus on Amibara Woreda. Elfora Agro-Industry, located in Amibara woreda, has been collecting and exporting small ruminant animals from different woredas. Amibara is located approximately 279 kilometers North-East of Addis Ababa, the capital of Ethiopia, and about 398 kilometers South_West of Samara, the administrative center of the Afar Region. Geographically, it lies at 9.279290°N latitude and 40.298870°E longitude, at an elevation of 827 meters above sea level.

Elfora Agro-Industry sources sheep and goats from various local markets across several woredas, including Amibara, Dulecha, Gewane, and Asebot. Following procurement, animals are transported to the Elfora facility in Amibara, where they are housed in purpose built structures. For traceability and compliance with export regulations, animals are segregated by their woreda of origin, with each group maintained in separate housing units for quarantine and export preparation. Additionally, small ruminants suspected of Peste des Petits Ruminants Virus (*PPRV*) infection were investigated in the villages of Awash Fentale and Gele'alo woredas.

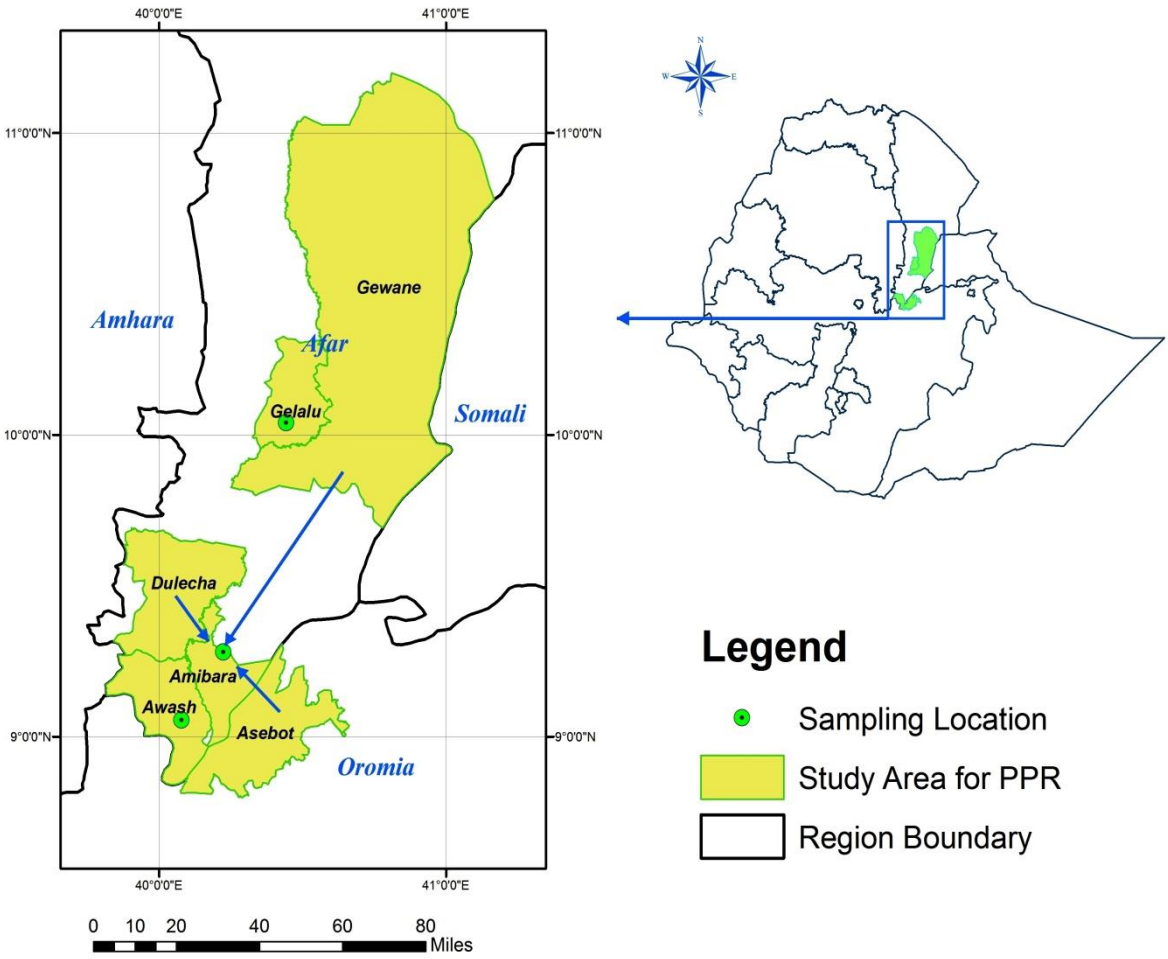


FIGURE 1: Map of Study Area and Location

(This Map constructed Software ArcGIS 10.7 (ESRI®) were used)Map of Amibara, Dulecha, Gewane, Asebot, Awash Fentale, and Gelealo Districts Showing the Sampling Locations in the Study Area

3.2 Study Design

This study employs a cross-sectional study design to investigate the nature, and genetic diversity of *Peste des Petits Ruminants Virus* (*PPRV*) in small ruminants (sheep and goats).

3.3 Study Animals

Goats and sheep ranging in age from three months to one and a half years provided 66 samples, all of which showed the clinical symptoms of *Peste des Petits Ruminants (PPR)*, including fever, diarrhea, eye discharge, and nasal discharge. The animals were bought by exporters from different pastoralist villages, and it was unclear to trace their immunization history, therefore it was uncertain whether vaccinated they were. 41 samples (39 oral/nasal swabs and 2 tissue samples from lymph nodes, spleen, and lungs) from clinically suspected sheep and goats in the Awash Fentale and Gelealo districts and 25 oral/nasal swabs from export sheep and goats were collected during two sampling sessions on July 20–25, 2024, at the Amibara Elfora Exporter Quarantine Facility.

3.4 Sample Collection

Clinically suspected animals were sampled for nasal and eye swabs, oral and nasal discharge swabs, and tissue samples using Vacutainer tubes and sterile swabs. These samples were placed in viral transport medium (VTM) or antibiotic-phosphate-buffered saline (PBS) and stored at ice during transportation and at −20°C before analysis. This was done to preserve sample integrity and viral viability, which is very important for diagnostic validity. Sample preservation and protection from microbial contamination during storage and transport were achieved through the use of VTM and PBS with antibiotics (Kumar *et al.,* 2022).

Postmortem tissues collected from two goats that showed clinical signs and were in a coma were examined. Some of the essential tissues that were collected included the lung, kidney, liver, mesenteric and bronchial lymph nodes, and intestinal mucosa. 2–3 cm of each piece of such organs, was put in viral transport media (VTM), tagged, and transported under cold chain conditions. The tissue samples were submitted to the Animal Health Institute (AHI) cell culture laboratory for virus isolation purpose.

3.5 Antigen Detection

Sandwich Enzyme-Linked Immunosorbent Assay (ELISA) reference standard for detecting Peste des Petits Ruminants Virus (*PPRV*) antigens in samples through the utilization of its superior sensitivity and specificity for quantitation of viral antigen (Halecker., 2021). The assay employed a commercial kit, e.g., the ID Screen® PPR Antigen Capture ELISA (ID Vet, France).

For antigen assay against *PPRV* antigen, the ID Screen® *PPR* Antigen Capture ELISA kit was used. The samples (nasal swabs, tears, tissue homogenate, and gum debris) were diluted in sample buffer, centrifuged, and added to pre-coated microplate with *PPRV*-specific capture antibodies. The plate was incubated at 37°C for 45 minutes, washed six times in PBS-Tween buffer, and incubated with HRP-labeled anti-nucleoprotein conjugate for 30 minutes at 21–25°C. Six additional washes were then followed by the addition of TMB substrate. The reaction was stopped with 1M sulfuric acid, making the solution yellow. Absorbance at 450 nm was measured. Results were confirmed by comparing the sample OD with a cut-off (mean negative control OD plus two or three standard deviations) or signal-to-noise ratio, where positivity was suggestive of *PPRV* antigen presence (Wolf, M.C., 2009).

3.6 Virus Isolation

For cultivation of *PPRV* from swabs, seed the swabs in viral transport medium and refrigerate at 4°C for immediate processing or store at -80°C for later use. Seed the Vero cells by trypsinization and suspend in a density of 2×10^5 cells/ml in complete MEM medium. Maintain the cells in 37°C CO_2 incubator. Dilute the swab sample 10-fold in MEM free of serum to prepare dilutions from 10^{-1} to 10^{-7}, and store the dilutions on ice. Seed 100 µl of the suspension of Vero cells (2×10^5 cells/well) into each well of a 96-well microplate in duplicate using a multichannel pipette. Use negative controls by adding 100 µl of MEM lacking serum into the bottom row of wells. Infect the cells with 100 µl of each viral dilution with 10 repeats per dilution. Overfill the periphery wells with 200 µl of MEM to avoid desiccation. Incubate the plate at 37°C in a CO_2 incubator with 5%

humidity; in the absence of CO_2, cover the plate with paraffin to conserve humidity. Observe the plate under an inverted microscope for cytopathic effect (CPE) at 10, 12, and 14 days. CPE-bearing wells are employed to confirm the presence of *PPRV*. This technique offers isolation of *PPRV* from swab samples by demonstrating viral replication through CPE in Vero cells (WOAH, 2023).

3.7 Polymerase Chain Reaction (PCR) Tests

3.7.1 RNA Extraction.

RNA from the swab tests was extricated utilizing the QIAamp Viral RNA Scaled down Pack (Qiagen, Hilden, Germany) taking after the manufacturer's convention, and eluted RNA was kept at -80°C for encourage RT-qPCR examination. Separated RNA was in this way utilized in a 20 µl RT-qPCR response on a Connected Biosystems 7500 warm cycler that intensified the N quality utilizing particular preliminaries and a test depicted in (Batten *et al.*, 2011). The response contained Express Widespread superscript ground works, test, RNAse-free water, and 3 µl RNA format, and enhancement at 50 °C for 15 min, 95°C for 20 s, and 45 cycles of 95°C for 3 s and 60°C for 30 s, with a test with Ct esteem <35 considered positive.

3.7.2 Real-Time PCR (RT-qPCR)

According to (Batten *et al*., 2011), all extracted viral RNA was subjected to the RT-qPCR experiment on an Applied Biosystems 7500 thermal cycler using particular primers and a N gene probe. In summary, the RT-qPCR was carried out in a final reaction volume of 20 µl that included the following: 10 µl of Invitrogen's Express Universal superscript, 0.5 µl of superscript enzyme, 0.5 µl of passive reference Rox, 1.5 µl of each of the following primers: PPRV forward primer (5′AGA GTT CAA TAT GTT RTT AGC CTC CAT 3′), PPRV reverse primer (5′TTC CCCARTCAC TCT YCT TTGT 3′), 1.0 µl of PPR probe (FAM-CAC CGG AYA CKG CAG CTG ACT CAG AAQSY), and 3 µl of RNA template. 50°C for 15 minutes, 95°C for 20 seconds, and 45 cycles of denaturation and annealing at 95°C for 3 seconds and extension at 60°C for 30 seconds were used for the amplification. Samples are considered positive if their Ct value was less than 35 (Batten *et al*., 2011).

3.7.3 Sequencing Samples

Out of twelve good cycle threshold (Ct) values of *Peste des Petits Ruminants Virus (PPRV)* samples, obtained from RT-PCR tests performed at the AHI molecular laboratory, were chosen for downstream analysis and sent to the Seibersdorf International Atomic Energy Agency (IAEA) Laboratories in Austria during September 2024. Despite efforts to sequence the partial N gene from all samples, amplicons were successfully amplified and sequenced from only four samples (#7, #8, #11, and #12) out of the total analyzed, as illustrated in Figure 4.

3.8 Partial N Gene Sequencing and Sequence Analysis

In this study, a 284 base pair (bp) region of the viral nucleocapsid (N) gene was amplified and sequenced to analyze phylogenetic relationships among different viral strains. The resulting sequences were analyzed using the Maximum Likelihood (ML) method implemented in MEGA 6 software. The Hasegawa-Kishino-Yano (HKY) model was chosen for tree construction because it accounts for different rates of transitions and trans versions, making it suitable for accurate modeling of nucleotide substitutions in viral genes. To assess the robustness of the inferred phylogenetic relationships, 1000 bootstrap replicates were performed. Only bootstrap values greater than 70% were shown to represent well-supported clades, indicating strong evidence for the grouping of those sequences.

4. RESULTS

4.1. Antigen Detection Using Ic-ELISA

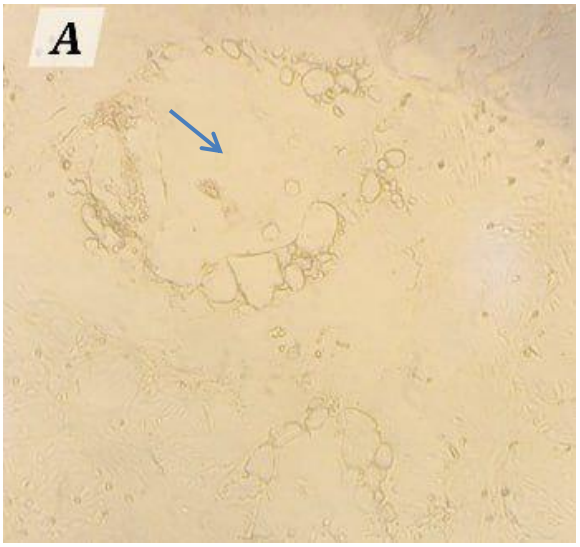
Antigen detection of acquired Peste des Petits Ruminants Virus (*PPRV*) suspected samples was performed using the ID Vet ELISA kit on 64 swabs collected from suspected cases. The result showed that 20/64 samples (31.25%) were positive, while the other 44/64 samples (68.75%) were negative for PPRV virus antigen.

TABLE 1: ELISA Results of *PPRV* in Small Ruminants

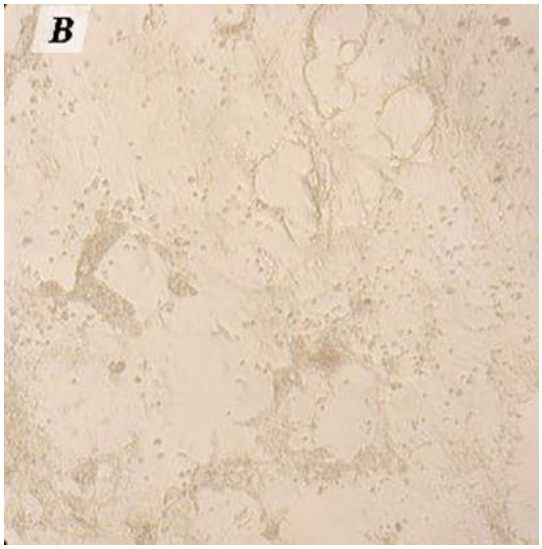
Species	Total Samples	Positive Samples	Negative Samples	Positive Rate (%)
Sheep	21	0	21	0.00%
Goats	43	20	23	46.51%
Total	64	20	44	31.25%

4.2 Virus Isolation

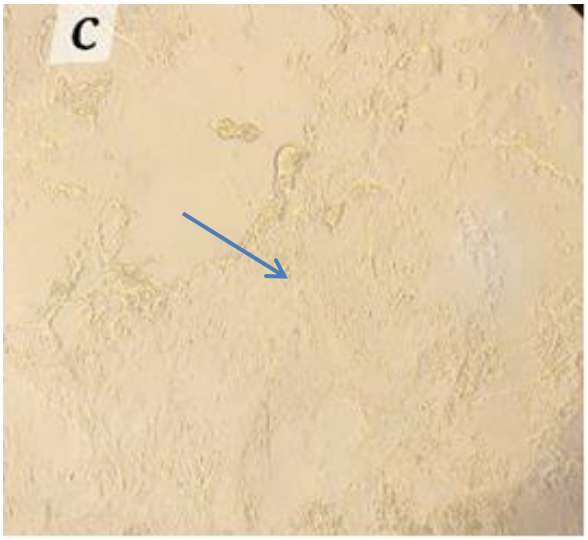
All 20 swabs positive with antigen Ic-ELISA and 2 tissue samples cultured, 7 swabs and two tissue samples in virus transport media underwent freeze-thaw cycles before homogenization and centrifugation at 3000 rpm for 10 minutes at 4°C. The supernatant was collected; 1:10 diluted with phosphate buffered saline (PBS) and inoculated onto Vero cells for virus isolation, incubated at 37°C with 5% CO2. After a one hour adsorption time, the plates were washed and maintained in Dulbecco's Modified Eagle Medium (DMEM) with 2% serum. Cytopathic effects indicating virus replication were monitored for five days. Cytopathic effects (CPE) like cell rounding, aggregation, syncytial formation and cell disruption were observed from the 3rd day onwards (figure 2).



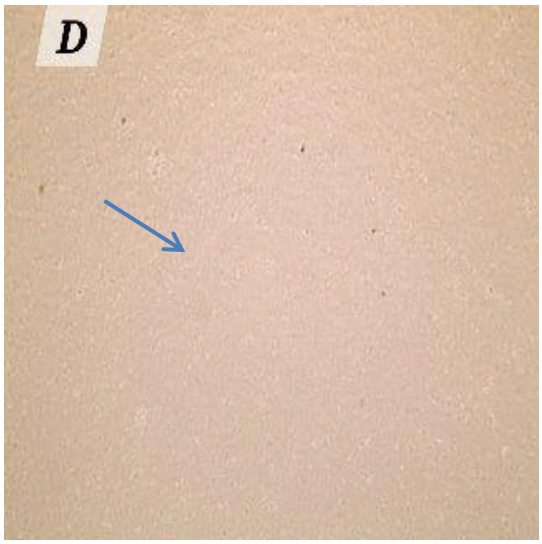
Early to intermediate infection stage with center clustering, bubble-like shape, and single cell rounding.



Mid-stage infection with more cell clumping and rounding in the upper-right and middle areas



Severe infection with syncytia, central lower area abnormalities, and extensive damage



A monolayer of uniform cells with little rounding or separation indicates a negative or very early infection.

Figure 2: Cytopathic Effects (CPE) Observed in Cells Infected with *PPRV.*

The cytopathic effect (CPE) demonstrates the course of infection of Peste des Petits Ruminants Virus (PPRV).

4.3 Molecular Detection Results (RT-PCR)

Molecular tests like RT-qPCR were utilized to characterize the positive samples of IC-ELISA tests. This was done in an attempt to make the tests more specific and identify the lineage. As summarized in table 3 below, the quantitative polymerase chain reaction (qPCR) results from a study on *Peste des Petits Ruminants Virus (PPRV)* in the Afar region, analyzed using the Applied Biosystems 7500 Fast System with SDS Software, detailing cycle threshold (Ct) values, sample names, well locations, and interpretations for 20 test samples and four control samples. Out of 20 test samples, 16 tested positive for RT-PCR, with Ct values ranging from 21.0596 (sample 15029, indicating a high viral load) to 33.5127 (sample 15026, near the positivity threshold of Ct < 35), suggesting variability in viral load likely due to differences in infection stage, sample quality, or disease severity. Four samples (15019, 15023, 15027, 15031) were undetermined (Undet.), interpreted as negative, indicating either no *PPRV* RNA or levels below the assay's detection limit, which could result from early infection, RNA degradation, or sampling issues. Samples collected from Gelealo and Awash Fentale woredas were not included in table 2 because all samples were negative in the antigen ELISA test and didn't test for RT-PCR.

TABLE 2: Summary of CT Values for Test Samples in RT-PCR Analysis.

Well	District	Ct Value	Interpretation
A1	Gewane	31.2823	Positive
A2	Gewane	29.0343	Positive
A3	Gewane	27.4262	Positive
A4	Gewane	28.25	Positive
A5	Gewane	Undet.	Negative
A6	Gewane	29.827	Positive
A7	Gewane	24.064	Positive
A8	Gewane	Undet.	Negative
A9	Dulecha	25.9218	Positive
A10	Dulecha	33.5127	Positive
A11	Dulecha	Undet.	Negative
A12	Dulecha	29.9447	Positive
B1	Dulecha	21.0586	Positive
B2	Amibara	26.6974	Positive
B3	Amibara	Undet	Negative
B4	Amibara	21.0596	Positive
B5	Amibara	27.046	Positive
B6	Asebot	29.8295	Positive
B7	Asebot	26.2845	Positive
B8	Asebot	24.2226	Positive

* Undet ; Undetected

TABLE 3: Molecular Detection of *PPRV* Using RT-PCR

Species	Total Samples	Positive Samples	Negative Samples	Positive Rate (%)
Goats	20	16	4	80%
Total	20	16	4	80%

The high positivity rate (16/20, 80%) corroborates the study's finding of *PPRV's* endemicity in Afar, with the range of Ct values providing insights into the viral burden across samples, where lower Ct values.

Often correlated with clinically severe cases. This robust qPCR dataset, supported by valid controls, underpins the molecular epidemiological analysis, highlighting the need for targeted control measures to address PPRV's prevalence and its socioeconomic impact on pastoralist communities.

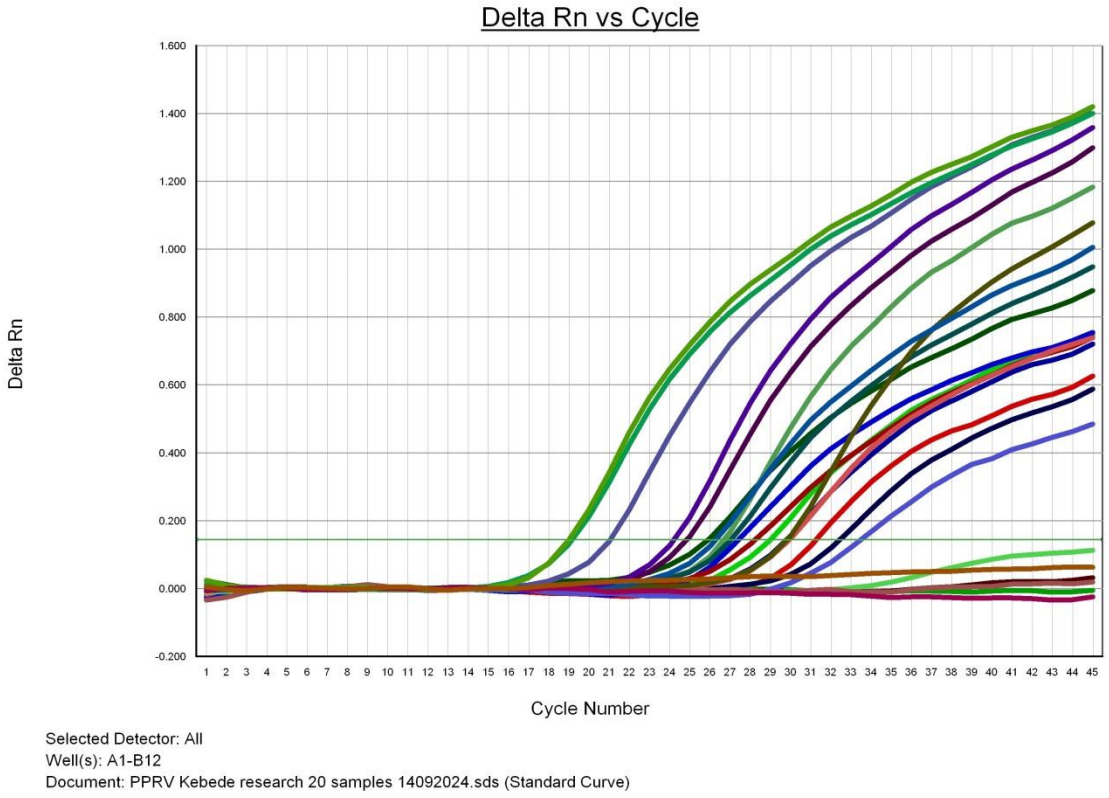


FIGURE 3: RT-PCR Detection Results of *PPRV* RNA

Fluorescence intensity (Delta Rn) versus cycle number for many samples in a real-time PCR amplification plot. Each curve in the plot represents a distinct sample or reaction

well, and it illustrates the exponential phase of amplification in each of the several distinct reactions between cycles 20 and 30. The cycle number is on the x-axis, and the Delta Rn is on the y-axis.

The amplification curves are required to be directly aligned to their Cycle Threshold (Ct), which is the cycle at which fluorescence exceeds background (usually at the inflection point of each curve), in order to accurately annotate each one on the Delta Rn vs. Cycle qPCR graph with its corresponding sample DI number and Ct value.

Figure 4: Assuming DI numbers from 1 to 20 listed the Ct values from lowest to highest:

DI #	Ct Value	Curve Order (Early to Late)
13	21.0586	1st
16	21.0596	2nd
7	24.064	3rd
20	24.2226	4th
9	25.9218	5th
19	26.2845	6th
14	26.6974	7th
17	27.046	8th
3	27.4262	9th
4	28.25	10th
2	29.0343	11th
6	29.827	12th
18	29.8295	13th
12	29.9447	14th
1	31.2823	15th
10	33.5127	16th
5, 8, 11, 15	Undet.	Flat lines (no amplification)

* Undet; Undetected

TABLE 4: RNA Samples with Low CT Values Sent to the IAEA Laboratory for Sequencing.

No	Ct value	Lab. Code	SPP
1	29.0343	Eth/*PPRV*2024/15016	*PPRV*
2	27.4262	Eth/*PPRV*2024/15017	*PPRV*
3	28.25	Eth/*PPRV*2024/15018	*PPRV*
4	24.064	Eth/*PPRV*2024/15021	*PPRV*
5	25.9218	Eth/*PPRV*2024/15025	*PPRV*
6	29.9447	Eth/*PPRV*2024/15028	*PPRV*
7	26.6974	Eth/*PPRV*2024/15030	*PPRV*
8	21.0596	Eth/*PPRV*2024/15032	*PPRV*
9	27.046	Eth/*PPRV*2024/15034	*PPRV*
10	29.8295	Eth/*PPRV*2024/15035	*PPRV*
11	26.2845	Eth/*PPRV*2024/15037	*PPRV*
12	24.2226	Eth/*PPRV*2024/15038	*PPRV*

Among 16 RT-PCR positive swab samples, 12 RNA samples with low Ct values (the highest viral load) were chosen and sent to the IAEA laboratory for partial genome sequencing. These were chosen as their high viral RNA content provides a better chance of successful amplification and quality sequence data. These sequences were useful in molecular characterization and genetic diversity of circulating strains of *PPRV*.

4.4 Phylogenetic analysis

A Maximum Likelihood (ML) phylogenetic tree was constructed using partial N gene sequences (278 base pairs) based on the Hasegawa-Kishino-Yano (HKY) substitution model, implemented in MEGA 6 software. The HKY model is suitable for this analysis because it accounts for different rates of transitions and trans versions, which improves the accuracy of evolutionary inference in nucleotide sequences. To assess the reliability of the tree topology, bootstrap analysis with 1000 replications was performed. Only bootstrap values greater than 70% are shown in the tree, indicating strong support for those clades.

Of the 12 *PPRV* samples which send to Seibersdorf in September, only four samples (#7, #8, #11, and #12) produced successful amplicons for sequencing. The resulting phylogenetic tree (attached) shows that three of these samples #7, #8, and #12 cluster within Lineage IV, grouping closely with other *PPRV* isolates previously characterized in our laboratory (indicated by blue and green triangles). In contrast, sample #11 falls into Lineage II and closely resembles the Nigeria 75/1 vaccine strain.

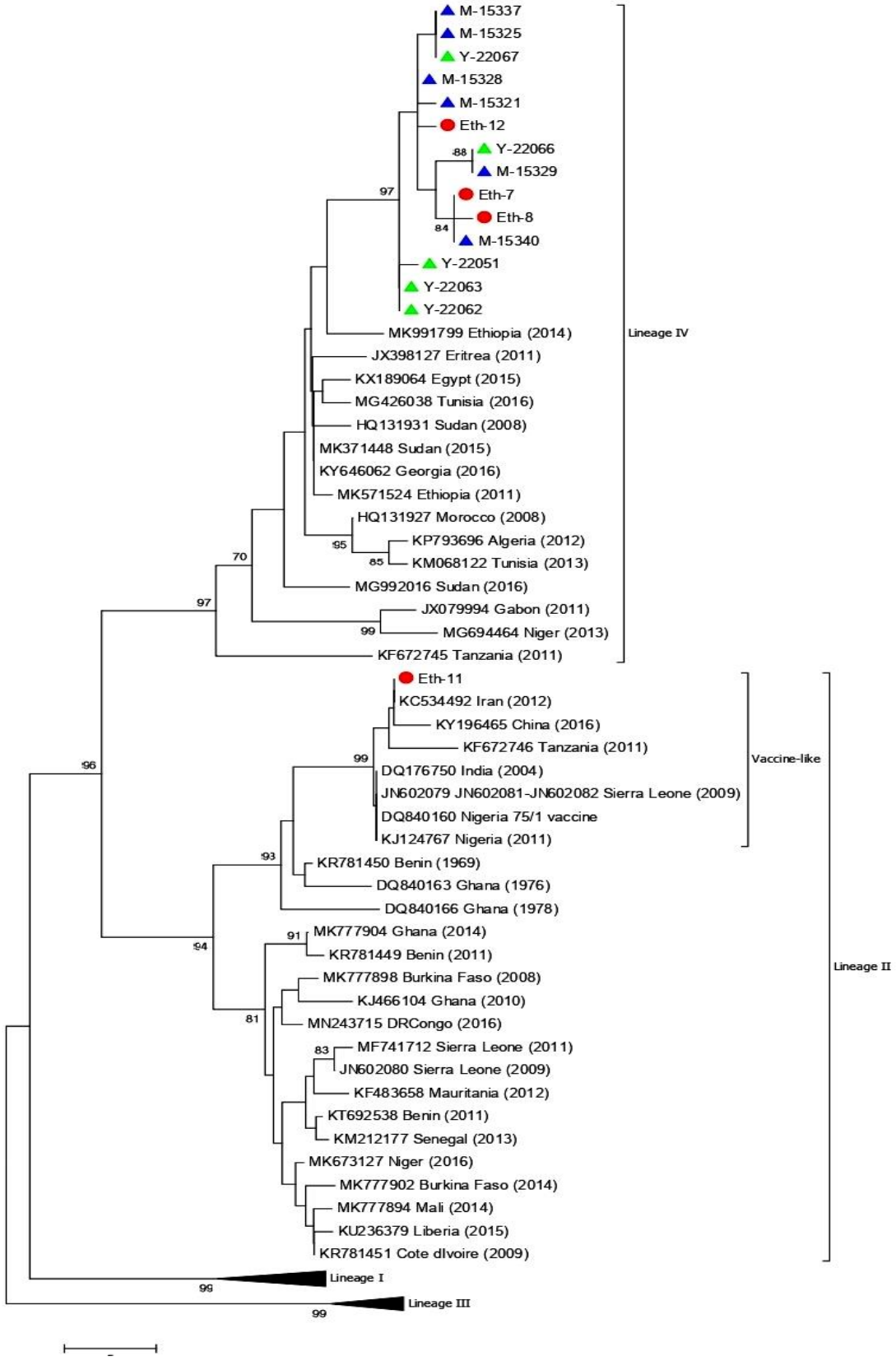


Figure 5*:* Phylogenetic Tree (N) Gene Gequenced (278 bp) of *PPRV*.

TABLE 5: Sequenced (N) Gene Comparison from Gene Bank

Isolate ID	Origin	Genotype	Closest Relative (N) gene	Nucleotide Identity
ETH-7	Amibara	Lineage IV	ETH-*PPRV*2019	98%
ETH-8	Amibara	Lineage IV	Tanzania-*PPRV*2018	98%
ETH-11	Asebot	Lineage II	Nigeria 75/1 vaccine	100%
ETH-12	Asebot	Lineage IV	ETH-*PPRV* 2019	99%

Three isolates from the Afar region (ETH7, ETH8, ETH12) are of Lineage IV with 98-99% identity to East African strains (ETH-*PPRV* 2019 and Tanzania-*PPRV* 2018), which establishes regional dissemination of the lineage. ETH-11 is of Lineage II (100% identical with Nigeria 75/1 vaccine strain).

The query sequence's nucleotides of isolation ETH-11 is 100% identical to the Nigeria 75/1 vaccination strain of the *Peste-des-petits-ruminants virus (PPRV).* The alignment shows a perfect match between locations 1355 and 1638 of the viral genome (Gen Bank ID: HQ197753), free of gaps and mismatches. As confirmed by the extremely low E-value of 1e-144. This clearly shows that the sample contains viral RNA originating from vaccines.

A total of four isolates of the *PPRV* ETH-7, ETH-11, ETH-12, and ETH-08 were submitted to the Gen Bank nucleotide sequence database. With the accession codes PV759141, PV759142, PV759143, and PV759144, the sequences are now publicly accessible in the public domain following curation and acceptance on board.

Table 6: Summary of Diagnostic Test Results across all Study Areas.

Sample Collection Area	No. of Samples	No. of Species		Sandwich ELISA	Virus Isolation	RT-PCR	Partial N Gene Sequencing
		Goats	Sheep				
Amibara	4	4	-	4	2	2	2
Asebot	8	8	-	7	2	7	2
Gewane	8	8	-	6	1	5	-
Dulecha	5	5	-	3	2	2	-
Awash Fentale	18	13	5	-	2	-	-
Gelealo	23	7	16	-	-	-	-
Total	**66**	**45**	**21**	**20**	**9**	**16**	4

Table 7: Comparison of IC-ELISA S/P%, RT-PCR Ct Values

, and *PPRV* Genotypes from Different Origins in the Afar Region"

Lab. cod	**Origen**	**IC-ELISA S/p%**	**RT-PCR (Ct)**	**Genotype**
15030	Amibara	558.06	26.6974	Lineage IV
15032	Amibara	511.47	21.0596	Lineage IV
15037	Asebot	334.76	26.2845	Nigeria 75/1 vaccine strain
15038	Asebot	578.9	24.2226	Lineage IV

Diagnostic and molecular typing of Asebot and Amibara *PPRV*-positive samples are illustrated in table 7. All of the samples were typed as having low RT-PCR Ct values and high IC-ELISA S/P% values, which reflect high antigen titers and active viral infection. Three samples (15030, 15032, and 15038) were genotypically typed as Lineage IV, which is the prevailing prevalent strain in East Africa. Sample 15037 from Asebot contained Nigeria 75/1 vaccine strain that can be from an immunized animal or a recent vaccination. These results confirm active spread of *PPRV* in the region, as indicated both by field and vaccine strain.

5. DISCUSSION

A 46.5% (20/43) ELISA-based antigen detection positivity rate in this study indicated a high incidence of *Peste des Petits Ruminants Virus (PPRV)* infection in goats with all the sheep tested (0/21) being negative. This is indicative of species vulnerability where the goats are more likely to be infected with *PPRV,* an aspect that has been greatly reported locally and internationally. Afera *et al.,* (2014) also cited the same finding in Northern Ethiopia where the virus showed a preference for caprine host and ELISA positivity rate was 43.8% in goats while 8.3% in sheep. Mohammed *et al.,* (2022) also employed Ic-ELISA to determine significantly higher antigen prevalence among goats in Southern Ethiopia. They assured it out by associating with more intense clinical manifestations and higher virus loads in goats than in sheep. These results are in agreement with antigen detection that showed that 46.5% of the caprine samples were positive for *PPRV* by capture ELISA. Failure to detect *PPRV* antigen in sheep by ELISA may indicate other latent infections with complications undetectable by *PPRV* Ag ELISA. The results call for species-specific management control interventions and monitoring in the context of the goat herds of Ethiopia's high-risk pastoral areas.

Following virus isolation onto Vero cells, a range of continuously changing cytopathic effects (CPE) were noted, including syncytia formation, rounding, and detachment, which were indicative of continuous *PPRV* replication. The results are consistent with other isolation studies that discovered similar CPE patterns, like those from Nigeria (Woma *et al.,* 2015) and Southern Ethiopia (Mohammed *et al.,* 2022). Figure 3 A and B make it evident that the variation in CPE intensity observed in this study is reflected in different viral stages of infection. This pattern has been documented in earlier PPRV isolations. This successful isolation establishes the virus's virulence in field samples and offers valuable material for additional virological and pathogenesis research.

With values for Ct from 21.06 to 33.51, the RT-qPCR revealed a rate of 80% positivity in goat samples, suggestive of a range of viral loads. Munir *et al.,* (2013) and El Arbi *et al.,* (2021) infected goats yielded such high positive rates and an equivalent range of Ct values, and this is suggestive of the efficiency of qPCR in detecting acute and subacute

infections. The trend for reduced susceptibility or potentially advanced-stage sampling, where viral RNA is no longer test sample negative with ELISA, and the sheep is confirmed by being negative on the ELISA, there is no further requirement for PCR due to the high sensitivity and specificity of the antigen ELISA. Aside from pointing to absolute stages of infection, the Ct value range enhances the speculation that qPCR has a central position in identifying the level of infection and the potential transmissibility of *PPRV* in a population. The positive results show significant presence of *PPRV* antigen among the sampled population and alert to their circulation in the population being studied. These results indicate active transmission and presence of *PPRV* in goats, which indicates the requirement for specific control measures.

The presence of the *PPRV* Lineage IV in three of the sequenced samples (#7, #8, and #12) is in keeping with a body of studies throughout Ethiopia and across the broader Horn of Africa that further confirms its presence in the region. Mohammed *et al.,* (2022) have earlier noted Lineage IV prevalence as common in Southern Ethiopia, and also Ayalew *et al.,* (2021) and Aklilu *et al.,* (2024) have noted its common circulation among pastoralist and agro-pastoral communities. Increased density levels of small ruminants, large animal movement systems, and collective grazing systems with high capacity for effective virus transmission would be preferred by the endemicity of Lineage IV. Lineage IV isolates were also clumped with strains typed earlier in our laboratory, which had likely localized re-emergence and transmission within the Afar region in this study. That such close genetic relatedness among Lineage IV isolates is significant and indicates limited evolutionary diversification among Lineage IV, as would be expected for endemicity and supporting the utility of regionally targeted vaccination.

In contrast, isolation of a Lineage II isolate (#11) is rare in Ethiopia and also has significant epidemiological implications. Lineage II is also reported in West Africa and more closely related to the Nigeria 75/1 vaccine strain that has been used in most areas for control of PPRV. The positioning of sample #11 within the vaccine-derived cluster suggests highly probable either laboratory contamination or sampling from a recently vaccinated animal. The same questions were also raised by (Mohammed *et al.,* 2022), who highlighted the lack of discrimination between vaccine and field strains in the absence of DIVA compatible tools. Likewise, (Luka *et al.,* 2020) also discussed sporadic

detection of vaccine-like strains in laboratory diagnosis with emphasis on precise history of vaccination and an unyielding sample handling process. Identification of Lineage II within the present research, while presumptive to be art factual, highlights an overarching need for expanded genetic monitoring and creation of diagnostic tests having discriminatory capability to distinguish between vaccinated and naturally exposed animals. Resolving these matters is pertinent to proper interpretation of molecular information as well as to national and regional *PPRV* eradication programs.

6. CONCLUSION AND RECOMMENDATIONS

This study provides molecular and virological evidence of active *Peste des Petits Ruminants Virus (PPRV)* infection among goats in the Afar region of northeastern Ethiopia. The findings confirmed a 31.25% antigen positivity rate via immunocapture ELISA (Ic-ELISA), with all positive cases originating from goats, highlighting species-specific susceptibility or exposure dynamics in the region. Successful virus isolation in Vero cells and RT-qPCR detection in 80% of ELISA-positive samples further validated the presence of replicating PPRV. Partial sequencing of the nucleocapsid (N) gene and subsequent phylogenetic analysis revealed that most isolates clustered within Lineage IV, consistent with previously reported East African field strains. However, the identification of one isolate (ETH-11) matching the Nigeria 75/1 vaccine strain (Lineage II) raises important questions regarding vaccine virus circulation, potential shedding, or misidentification of vaccine-derived viruses. Overall, the results underscore the endemic circulation of *PPRV* primarily of Lineage IV in goats within Afar's pastoral production systems and provide preliminary evidence of possible co-circulation or exposure to vaccine-related strains. These findings emphasize the need for improved diagnostic tools and targeted control interventions to prevent outbreaks and support national eradication goals.

Accordingly the following recommendations were forwarded:-

- Strengthen molecular surveillance systems
- DIVA-compatible diagnostics are essential.
- Thermo stable vaccine formulations
- Implement targeted vaccination campaigns
- Integrate findings into national and regional eradication strategies
- Establish regular and coordinated *PPR* surveillance in the Afar Region to detect. and respond rapidly to new outbreak.
- Enhance vaccination strategies based on circulating strain.
- Increase awareness and capacity building among Afar pastoralists on *PPR*.

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APPENDIX

Appendix 1: Protocol ID Screen® *PPR* Ag ELISA

Kit components/ reagents

- Microplates coated with *PPR* recombinant nucleoprotein
- Anti-nucleoprotein-HRP concentrated conjugate (10×)
- Positive and negative controls
- Dilution buffers 19 and 13
- Wash concentrates (20×)
- Stop solution (H2SO4 0.5 Mole)
- Substrate solution (TMB)

2. Sample preparation

Vortex the tube gently for about 10-15 seconds to ensure that any viral particles will released from the swab into the buffer. The sample tube containing the swab sample will agitate and remove the swab from the sample tube and discard it.

3. Wash solution preparation

The wash solutionwill prepared by diluting the concentrated wash solution (20×) in Deionized or distilled H2O after mixing gently.

4. Testing procedure

PPRV antigen ELISA test procedure entails a series of operations meant to accurately detect viral antigens. As a prerequisite to their uniform distribution, all the reagents are mixed well after room temperature storage for 30 minutes. Then 25 µl of DB 13 is added into all the wells, followed by 25 µl of Positive Control (PC) added in wells A1 and B1 and 25 µl of the Negative Control (NC) in wells C1 and D1. All the remaining wells are filled with 25 µl of all the test samples. The plate is incubated at 37 ± 3°C for 45 ± 4 minutes. Following incubation, the wells are washed three times using 300 µl preprepared wash solution to eliminate the unbound material. Then, 100 µl of the diluted HRP conjugate (1×) is put into wells and incubated in 21°C for 30 minutes. The wells are again washed three times using 300 µl wash solution. Next, 100 µl of substrate solution is put into each well, and the plate is incubated for 15 ± 2 minutes at 21 ± 5°C in darkness to

allow the colorimetric reaction. Lastly, 100 µl of H_2SO_4 are added to the wells to stop the reaction and the optical density (OD) is measured at 450 nm.

Validation

The test ito be valid when the OD mean value of the Positive Control (ODPC) is higher than 0.50 and the ratio between the mean ODPC and the mean OD of the Negative Control (ODNC) is higher than 3.

Interpretation

Competition percentage for every sample is computed by the following formula:
[Competition Percentage = (OD Sample - OD NC) / (OD PC - OD NC) × 100]
Those samples with S/N% values above 20% are deemed positive, while those with an S/N% value below 20% are deemed negative.

Appendix 2: Isolation Procedure of *PPR*

The isolation of the *PPR* (*Peste des Petits Ruminants*) virus from clinical samples such as nasal swabs, or tissues typically will involves several steps.

1. Nasal swabs, or tissues (such as lung or lymph nodes) will be collected from suspected animals showing clinical signs of *PPR*.
2. The collected samples will processed to extract the viral RNA or proteins using techniques like differential centrifugation, filtration, or chemical extraction methods.
3. The extracted material is then inoculated onto susceptible cell cultures, such as Vero cells or primary lamb kidney cells.
4. The cultures are incubated at 37°C in a CO_2 incubator for several days while monitoring for cytopathic effects (CPE), which indicate viral replication.
5. After incubating the cell cultures, the presence of the *PPR* virus is detected by observing characteristic cytopathic effects (CPE) such as cell rounding, fusion, and cell death.

6. The isolated virus can be further characterized through techniques like sequencing to confirm its identity as the *PPR* virus strain.

Appendix 3: RT-PCR into cDNA and Amplification.

Reverse transcription polymerase chain reaction (RT-PCR) is a laboratory technique that combines reverse transcription of RNA into complementary DNA (cDNA) and amplification.

Steps

1. Pipet 560 µl of prepared Buffer AVL containing carrier RNA into a 1.5 ml microcentrifuge tube.
2. Add 140 µl plasma, serum, urine, cell-culture supernatant, or cell-free body fluid to the Buffer AVL–carrier RNA in the microcentrifuge tube. Mix by pulse-vortexing for 15 s.
3. Incubate at room temperature (15–25°C) for 10 min.
4. Briefly centrifuge the tube to remove drops from the inside of the lid.
5. Add 560 µl of ethanol (96–100%) to the sample, and mix by pulse-vortexing for 15 s. After mixing, briefly centrifuge the tube to remove drops from inside the lid.
6. Carefully apply 630 µl of the solution from step 5 to the QIAamp Mini column (in a 2 ml collection tube) without wetting the rim. Close the cap, and centrifuge at 6000 x g (8000 rpm) for 1 min. Place the QIAamp Mini column into a clean 2 ml collection tube, and discard the tube containing the filtrate.
7. Carefully open the QIAamp Mini column, and repeat step 6.
8. Carefully open the QIAamp Mini column, and add 500 µl of Buffer AW1. Close the cap, and centrifuge at 6000 x g (8000 rpm) for 1 min. Place the QIAamp Mini column in a clean 2 ml collection tube (provided), and discard the tube containing the filtrate.
9. Carefully open the QIAamp Mini column, and add 500 µl of Buffer AW2. Close the cap and centrifuge at full speed (20,000 x g; 14,000 rpm) for 3 min. Continue directly with step 11, or to eliminate any chance of possible Buffer AW2 carryover, perform step 10, and then continue with step 11.
10. Recommended: Place the QIAamp Mini column in a new 2 ml collection tube (not provided), and discard the old collection tube with the filtrate. Centrifuge at full speed for 1 min.

11. Place the QIAamp Mini column in a clean 1.5 ml microcentrifuge tube (not provided). Discard the old collection tube containing the filtrate. Carefully open the QIAamp Mini column and add 60 µl of Buffer AVE equilibrated to room temperature. Close the cap, and incubate at room temperature for 1 min. Centrifuge at 6000 x g (8000 rpm) for 1 min.

Appendix 4: Shows the Clinical Symptoms of Goats Infected with *PPRV*

Appendix 5: Infected Lung and Pathological Lesions Observed During Postmortem.

Appendix 7: Pictures During Sampling at Various Pastoral Sites.

Appendix 8: Pictures During Postmortem at Awash Fentalle Sites.

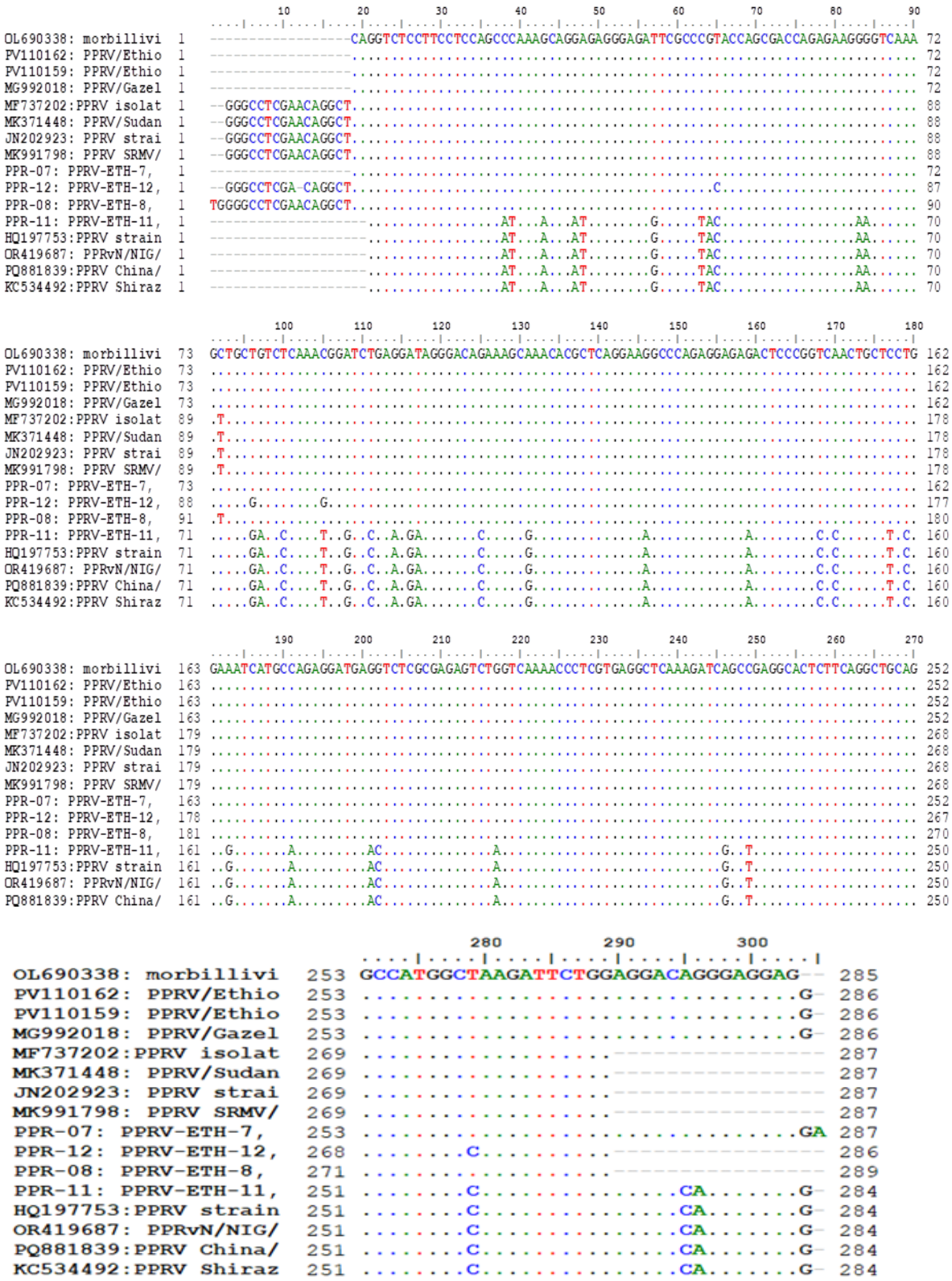
Peste-des-petits-ruminants virus isolate *PPRV*/Ethiopia/38920/2019, complete genome

Sequence ID: PV110162Length: 15948

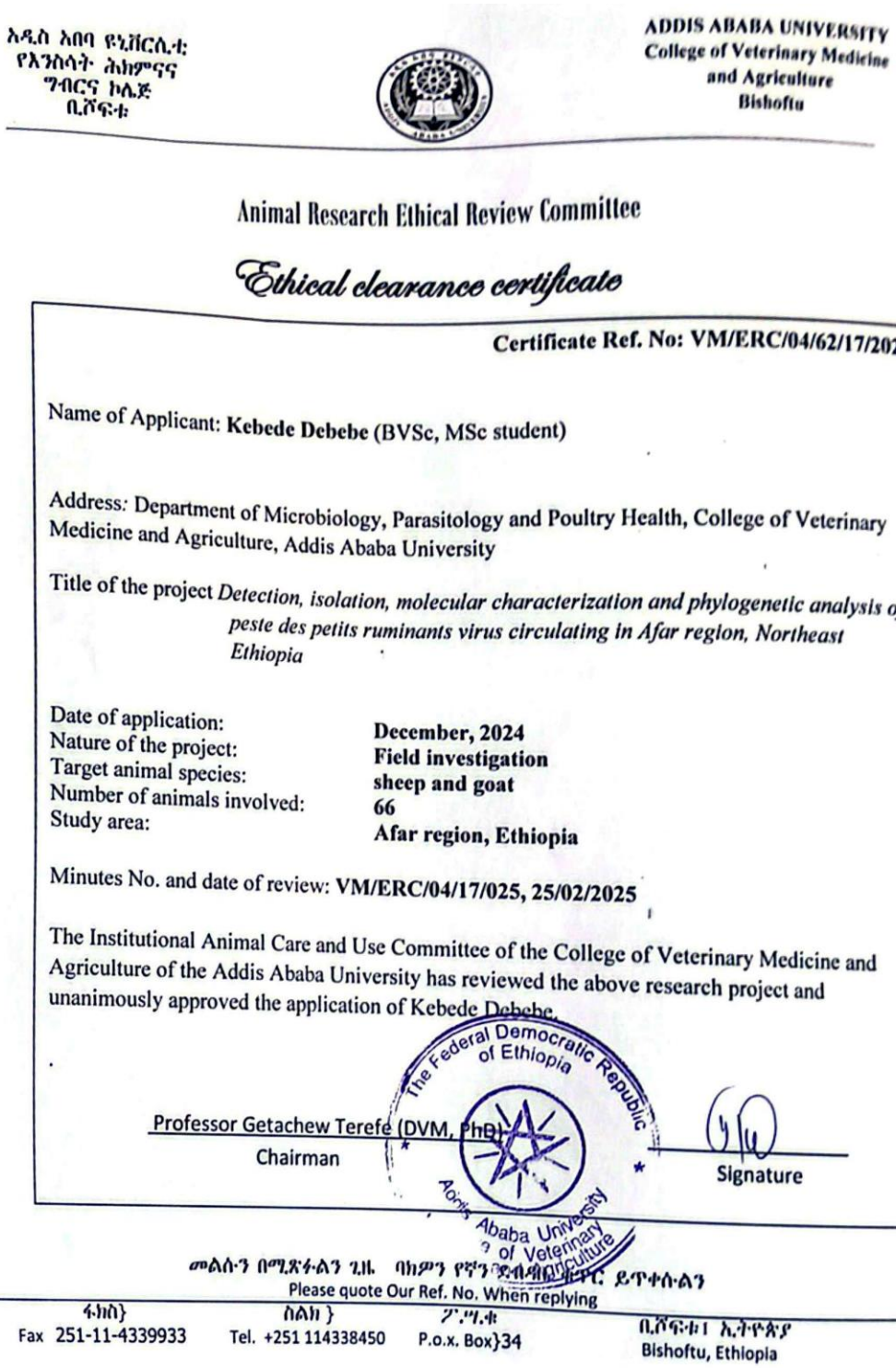
Range 1: 1353 to 1638 GenBank / GenPept

Score	Expect	Identities	Gaps	Strand
496.0 bits (268)	1e-135	280/286 (98%)	0/286 (0%	Plus/Plus

```
Query    1 CAGGTCTCCTTCCTCCAGCCCAAAGCAGGAGAGGGAGATTCGCCCGTACCAGCGACCAGA   60
           ||||||||||||||||||||||||||||||||||||||||||||||| ||||||||||||
Sbjct 1353 CAGGTCTCCTTCCTCCAGCCCAAAGCAGGAGAGGGAGATTCGCCCGCACCAGCGACCAGA 1412
Query   61 GAAGGGGTCAAAGCTGCTGTCTCAAACGGATCTGAGGATAGGGACAGAAAGCAAACACGC  120
           |||||||||||||||| ||||||||||||||||||||||||||||||||||||||||||||
Sbjct 1413 GAAGGGGTCAAAGCTGCGGTCTCAAACGGATCTGAGGATAGGGCAGCAGAAAGCAAACACGC 1472
Query  121 TCAGGAAGGCCCAGAGGAGAGACTCCCGGTCAACTGCTCCTGGAAATCATGCCAGAGGAT  180
           |||||||||| ||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 1473 TCAGGAAGGCTCAGAGGAGAGACTCCCGGTCAACTGCTCCTGGAAATCATGCCAGAGGAT 1532
Query  181 GA GGTCTCGCGAGAGTCTGGTCAAAACCCTCGTGAGGCTCAAAGATCAGCCGAGGCACTC  240
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
```



Appendix 13: Multiple Sequences Alignment of *PPRV* Nucleic Acid Sequences Compare to Reference Strain



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ADDIS ABABA UNIVERSITY
College of Veterinary Medicine
and Agriculture
Bishoftu

Animal Research Ethical Review Committee

Ethical clearance certificate

Certificate Ref. No: VM/ERC/04/62/17/2025

Name of Applicant: **Kebede Debebe** (BVSc, MSc student)

Address: Department of Microbiology, Parasitology and Poultry Health, College of Veterinary Medicine and Agriculture, Addis Ababa University

Title of the project *Detection, isolation, molecular characterization and phylogenetic analysis of peste des petits ruminants virus circulating in Afar region, Northeast Ethiopia*

Date of application:	**December, 2024**
Nature of the project:	**Field investigation**
Target animal species:	**sheep and goat**
Number of animals involved:	**66**
Study area:	**Afar region, Ethiopia**

Minutes No. and date of review: **VM/ERC/04/17/025, 25/02/2025**

The Institutional Animal Care and Use Committee of the College of Veterinary Medicine and Agriculture of the Addis Ababa University has reviewed the above research project and unanimously approved the application of Kebede Debebe.

The Federal Democratic Republic of Ethiopia

Addis Ababa University College of Veterinary Medicine and Agriculture

Professor Getachew Terefe (DVM, PhD)
Chairman

Signature

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ቢሾፍቱ፣ ኢትዮጵያ Bishoftu, Ethiopia

Appendix 14: Ethical clearance certificate

AHI

TEST RESULT REPORT FORM

Customer name: Kebede Debebe
Contact: phone : , email :
Sampling Place: Region: Afar, District: Amibara
Kebele: Amibara

Reference No. SR/Ou/325/2024
Test Report No. 325/2024

Date reported: 09-08-2024

Lab Code	Sample Type	Species	Sampling Date	Date Received	Date Analyzed
15015-15039	SWAB	Caprine	24/07/2024	02/08/2024	06/08/2024
					06/08/2024

Testing Lab	No.	Positive	Negative	Doubtful	Test	Method	SOP
AHI	25	20	05	0	PPR: Antigen detection	Capture ELISA	-

*Accredited Test (AHI is accredited for tests marked *)

Opinions and Interpretations

-Of 25 swab samples tested for PPR Ag capture ELISA, 20 were positive and the rest 05 samples were negative for the disease circulation.

Verified by:
Dr Demeke Zewde

Approved by:

Dr.Getnet Abie Mekonnen
Deputy Director General

Appendix 15: Antigen Ic-ELISA compiled test result

ATTACHMENT

Samples type: SWAB- Species: Caprine

List of Positive Samples: PPR: Antigen detection / Capture ELISA

SR No	Lab Code	Sample's Id	Result	Location
1	15015	1	Positive	Gewane
2	15016	2	Positive	Gewane
3	15017	3	Positive	Gewane
4	15018	4	Positive	Gewane
5	15019	5	Positive	Gewane
6	15021	7	Positive	Gewane
7	15025	11	Positive	Dulecha
8	15026	12	Positive	Dulecha
9	15027	13	Positive	Dulecha
10	15028	14	Positive	Amibara
11	15029	15	Positive	Amibara
12	15030	16	Positive	Amibara
13	15031	17	Positive	Amibara
14	15032	18	Positive	Asebot
15	15033	19	Positive	Asebot
16	15034	20	Positive	Asebot
17	15035	21	Positive	Asebot
18	15037	23	Positive	Asebot
19	15038	24	Positive	Asebot
20	15039	25	Positive	Asebot

List of Negative Samples: PPR: Antigen detection / Capture ELISA

SR No	Lab Code	Sample's Id	Result	Location
1	15020	6	Negative	Gewane
2	15022	8	Negative	Gewane
3	15023	9	Negative	Dulecha
4	15024	10	Negative	Dulecha
5	15036	22	Negative	Asebot

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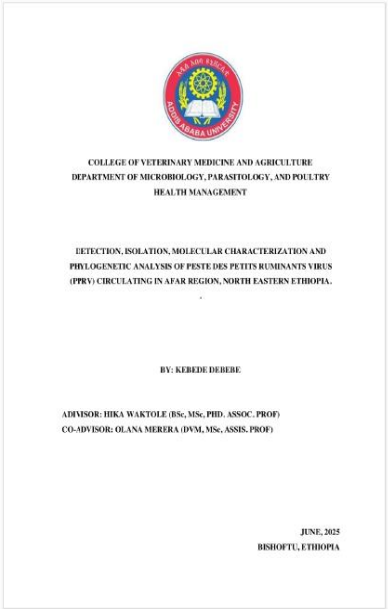

COLLEGE OF VETERINARY MEDICINE AND AGRICULTURE
DEPARTMENT OF MICROBIOLOGY, PARASITOLOGY, AND POULTRY HEALTH MANAGEMENT

DETECTION, ISOLATION, MOLECULAR CHARACTERIZATION AND PHYLOGENETIC ANALYSIS OF PESTE DES PETITS RUMINANTS VIRUS (PPRV) CIRCULATING IN AFAR REGION, NORTH EASTERN ETHIOPIA.

BY: KEBEDE DEBEBE

ADVISOR: HIKA WAKTOLE (BSc, MSc, PHD. ASSOC. PROF)
CO-ADVISOR: OLANA MERERA (DVM, MSc, ASSIS. PROF)

JUNE, 2025
BISHOFTU, ETHIOPIA

Appendix 16: Plagiarism checkup

DETECTION, ISOLATION, MOLECULAR CHARACTERIATIONAND PHYLOGENETIC ANALYSIS OF PESTE DES PETITS RUMINANTS VIRUS (PPRV) CIRCULATING IN AFAR REGION, NORTH EASTERN ETHIOPIA

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