Isolation and Molecular Characterization of Lumpy Skin Disease Virus from Four Selected Area of Active Outbreaks in Oromia Region, Central Ethiopia

By Milki Mojo Tufa

A Thesis Submitted To the Department of Applied Biology

School of Applied Natural Sciences

Presented in Partial Fulfillment of the Requirement for the Degree of Master's in Applied Biology (Specialization in Biotechnology)

Office of Graduate Studies

Adama Science and Technology University

November, 2024

Adama, Ethiopia

Isolation and Molecular Characterization of Lumpy Skin Disease Virus from Four Selected Area of Active Outbreaks in Oromia Region, Central Ethiopia

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Declaration

I hereby declare that this Master Thesis entitled “Isolation and Molecular Characterization of Lumpy Skin Disease Virus from Four Selected Area of Active Outbreaks in Oromia Region, Central Ethiopia” is my original work. That is, it has not been submitted for the award of any academic degree, diploma or certificate in any other university. All sources of materials used in this thesis have been duly acknowledged through citation

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I/we, the advisor(s) of this thesis, hereby certify that I/we have read the revised version of the thesis entitled “Isolation and Molecular Characterization of Lumpy Skin Disease Virus from Four Selected Area of Active Outbreaks in Oromia Region, Central Ethiopia” prepared under my/our guidance by Milki Mojo submitted in partial fulfillment of the requirements for the degree of Mater’s of Science in biotechnology. Therefore, I/we recommend the submission of revised version of the thesis to the department following the applicable procedures.

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Co-advisor	*Signature*	*Date*

Approval Page

I/we, the advisors of the thesis entitled “Isolation and Molecular Characterization of Lumpy Skin Disease Virus from Four Selected Area of Active Outbreaks in Oromia Region, Central Ethiopia” and developed by Milki Mojo; hereby certify that the recommendation and suggestions made by the board of examiners are appropriately incorporated into the final version of the thesis.

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We, the undersigned, members of the Board of Examiners of the thesis by Milki Mojo have read and evaluated the thesis entitled “Isolation and Molecular Characterization of Lumpy Skin Disease Virus from Four Selected Area of Active Outbreaks in Oromia Region**,** Central Ethiopia” and examined the candidate during open defense. This is, therefore, to certify that the thesis is accepted for partial fulfillment of the requirement of the degree of Master of Science in biotechnology.

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ACKNOWLEDGEMENTS

First of all, I want to start by giving my gratitude to the Almighty God for providing me with health and strength at all times. Next, I would like to thank my major adviser, Prof. Hunduma Dinka, for his advice and follow-up during this thesis work. I would also want to express my heartfelt thanks to my co-advisor Dr. Esayas Gelaye for his guidance, critical evaluation, editing and comments throughout the work of this thesis. I am also glad to thank Mr. Adugna Geresu, and Mr. Eyob Asefa who helped me during data collection for this study. I want to give my deep gratitude for Mrs. Hawa Mohammad and Mr. Abdurahman Jewaro for helping me during virus isolation and Mr. Kenaw Birhanu for helping me all molecular work and editing of my thesis. My thanks also go to Mr. Getaw Deresse for his support and Mr. Debebe Shimekit for helping me on outbreak information gathering. I am also deeply thanks to Adama Science and Technology University for facilitating from the beginning up to the end of this thesis work, the National Veterinary Institute for providing laboratory facilities and reagents used in this thesis work and Oromia state University for supporting me logistics during sample collection from Batu area. Furthermore, I would like to thank my entire staffs who aren't listed here. Finally, I would like to give my special thanks to all my family members.

TABLE OF CONTENTS

CHAPTERS **PAGE**

LIST OF TABLES

TABLES PAGE

LIST OF FIGURES

FIGURES PAGE

ACRONMYS AND ABBREVIATIONS

ARERC	Animal Research Ethics and Review Committee
BHK-21	Baby Hamster Kidney Cells -21
BLAST	Basic Local Alignment Search Tools
CAPV	Capripox Virus
CO_2	Carbon Dioxide
CPE	Cytopathic Effect
DNA	Deoxyribonucleic Acid
ESH-L	Embryonic Sheep Kidney Cell
GIS	Geographic Information System
GMEM	Glasgow's Minimum Essential Medium
GPCR	G Protein-Coupled Receptors
GTPV	Goat Poxvirus
IFAT	Indirect Fluorescent Antibody Test
KSGP	Kenyan Sheep and Goat Pox
LSD	Lumpy Skin Disease
LSDV	Lumpy Skin Disease Virus
MDBK	Madin Darby Bovine Kidney

MM	Millimeter
mRNA	Messenger Ribonucleic Acid
NCBI	National Center of Biotechnology Institute
NVI	National Veterinary Institute
ORF	Open Reading Frame
PBS	Phosphate-Buffered Saline
PCR	Polymerase Chain Reaction
RPO30	RNA polymerase subunit 30 kDa
SNP	Single Nucleotide Polymorphism
SPPV	Sheep poxvirus
TPB	Tryptose Phosphate Broth
UV	Ultraviolet
VTM	Virus Transport Media
WOAH	World Organization for Animal Health

LIST OF ANNEXS

ABSTRACT

Lumpy skin disease (LSD) is an economically important notifiable viral disease of cattle caused by the Lumpy skin disease virus (LSDV). A live attenuated vaccine strain Kenya sheep and goat pox O-180 (KS -1) is used for immunization of cattle in Ethiopia. But, outbreaks of the disease have been repeatedly reported in different parts of the country. The cause of these outbreaks while animals were receiving the vaccine was not investigated and documented. Therefore, this study was aimed to explore the cause of LSD outbreaks, isolate and molecularly characterize the virus and the genetic diversity of the LSDV strains that caused the outbreaks in cattle in, four selected area of Oromia region, central Ethiopia. For these purpose, a cross-sectional study was conducted on active LSD outbreaks in four selected area namely; Batu, Bishoftu, Sululta, and Mojo Oromia region, central Ethiopia. Purposive sampling methods were used to collect skin nodules and swabs from animal with suspected LSD clinical signs such as skin nodules covering the entire body of the animal, high fever, nasal discharge, lacrimation, leg swelling, salivation, decreased appetite, reduced milk production, and weight loss. 8.49% of morbidity rate and 1.3% of mortality rate were recorded in this study. Total of 32 samples were collected in universal bottle with virus transport media and transported to National veterinary institute (NVI) for molecular detection, virus isolation and sequencing. Out of total collected sample 25 samples were detected using conventional polymerase chain reaction (PCR) and real- time PCR and nineteen (19) positive samples were obtained. Eight field samples and one KS-1 vaccine were used for virus isolation on embryonic sheep kidney cell line and six LSD field viruses were isolated. During virus isolation cytopathic effect(CPE) was observed started from passage one on day four for the field isolates, whereas the KS-1 revealed CPE on day three of passage one. Four isolated virus were sequenced, sequences were deposited in National center of biotechnology institute (NCBI) data basis and the following accession number were obtained PQ119823, PQ119824, PQ119825 and PQ119826. Sequence comparisons were done between the current isolates, KS-1 vaccine and previous Ethiopian isolates. Sequence variation were observed at nucleotide positions 41 A/C and 292 T/C, as well as amino acid sequence variations at 14N/P and 98S/P between current isolate and KS-1 vaccine ,whereas single genetic variations at nucleotide position 41 A/C and amino acid 14 N/P were observed between current isolate and previous isolates in Ethiopia. Phylogenetic tree analysis indicates current isolate grouped to LSDV and identical to each. The findings confirmed that the outbreaks were caused by LSDV. Targeting full-genome sequencing to capture the full genetic diversity of LSDV strains in Ethiopia were recommended.

Keywords: *Lumpy skin disease virus, outbreak, dairy farm, fattening farm, field isolates, vaccine strains.*

1. INTRODUCTION

1.1 Background of the study

Lumpy skin disease (LSD) is emerging viral disease that holds great economic significance due to its ability to cause a decline in milk production, weight loss, reduced fertility, and in severe cases, even death (Gumbe, 2018; WOAH, 2023). The disease shows either acute or sub-acute, exhibiting varying degrees of severity depending on the host breed, viral strain, immunity level and age of the animals with clinical signs including, skin nodules covering body of the animal, high fever, nasal discharge, lacrimation, leg swelling, salivation, decreased appetite, reduced milk production, and weight loss. This disease typically has a high morbidity rate but a low mortality rate (Gari *et al*., 2015; WOAH, 2023).

LSD is caused by the Lumpy skin disease virus (LSDV) and classified under the genus *Capripoxvirus* and family *Poxviridae*, with a genome length ~151 kbp (Sprygin *et al.,* 2018; Onyejekwe *et al*., 2019; WOAH, 2023). The Capripoxvirus consists of three members: LSDV, Sheeppox Virus (SPPV), and Goatpox Virus (GTPV). These three viruses have a high level of sequence homology, with a 96% identity (Tulman *et al.,* 2001; Tulman *et al*., 2002; Abutarbush and Tuppurainen, 2018). LSD was initially identified in Zambia in 1929 and quickly spread throughout Africa, such as in Zimbabwe, Botswana, South Africa, Egypt, Sudan, and Kenya (Molini *et al.,* 2017; Selim *et al.,* 2021), has more recently expanded its reach to various regions in Europe, the Middle East, South Asia, and Southeast Asia (Kenawy and Tholoth, 2011). The virus is a highly contagious double-stranded DNA virus that leads to a fatal skin disease in Bovines (*Bos indicus, Bos taurus*) and domestic water buffaloes (*Bubalus bubalis*) (Davies, 1991). Additionally, this disease can also affect other animal species in their natural habitats such as camels, giraffes, and wildebeests (Kumar *et al*., 2023).

Ethiopia is challenged with endemic diseases such as LSD, SPP, GTP, and other ruminant diseases, which makes difficult to export live animals and their products (Ayelet *et al.,* 2014). LSD cases were first reported in in 1983 in the northwestern area of the country near Lake Tana (Mebratu *et al*., 1984). LSD is a vector-borne disease, primarily transmitted through the biting and blood-feeding vectors such as mosquitoes, flies, and ticks (Ayelet *et al*., 2014; Gelaye and

Lamien, 2019; Kumar *et al*., 2021). LSD outbreaks related with seasonal variations, with the highest frequency recorded from September to December following the rainy season. This could be because of increase in the population of the vector. Infected animals can transmit the virus to uninfected ones through various bodily fluids such as milk, blood, nasal discharge, lachrymal secretion, saliva, sperm, and vaginal mucous membranes (Mulatu and Fayisa, 2018). Effective farm management strategies, such as implementing vaccination protocols, vector control measures, and strong biosecurity practices, good husbandry practices, play a pivotal role in preventing and managing the distributions of the LSD virus among cattle populations (Ayelet *et al.*, 2014).

1.2 Statements of the problem

Ethiopia has implemented disease prevention measures through producing vaccine for vaccination. Despite the utilization of the Live attenuated Kenya sheep and goat pox O-180 (KS-1) vaccine for disease prevention, the number of widespread disease outbreaks within the country has not been declined, even among vaccinated animals (Ayelet *et al*., 2014). Another study has documented outbreaks in vaccinated animals that have received the KS-1 vaccine. These outbreaks were observed in dairy farms between two weeks and two months after vaccination (Zewdie *et al*., 2019). Therefore, the underlining reasons for those outbreaks while animals were receiving the vaccine was not documented. Another possibility factor that needs to be further investigated is the identity of the vaccine strains, which requires additional studies to confirm the similarity between the vaccinal strain in use and the actual virus strain found in the field (Tuppurainen *et al*., 2014).

1.2 Objectives of the study

1.2.1 General objective

- To explore the cause of LSD outbreaks, isolate and molecularly characterize, and conduct genetic diversity of LSDV isolates responsible for outbreaks in four selected area of Oromia region, central Ethiopia.

1.2.2 Specific objectives

- To explore LSD outbreaks and isolate the virus responsible for the outbreaks
- To characterize the isolated LSDV using conventional PCR, real time PCR and DNA sequencing.
- To conduct comparative genomic analysis on the isolated LSDV with the previously circulating homologous virus sequences and the LSD vaccinal strain currently in-use in the country

1.3 Significance of the study

The present study yields scientific data on the cause of the outbreak as well as sequence information for the circulating LSD virus strains. The isolated virus will also be used for future vaccine development, evaluation and animal experimentation research. In general, the outbreak investigation and virus genetic data will assist researchers to better understand the disease situation.

1.4 Research hypothesis

µ1: There is genetic difference between the currently in-use vaccine strain and field strains of LSDV

1.5 Delimitation and limitation of the study

The study was carried out in four specific areas of central Ethiopia. The study was conducted from November 2023 to May 2024. Samples were collected only from cattle suspected with LSD. The limitations of this study sample were not collected from all area of Oromia region, which did not adequately represent central Ethiopia. Certain areas were inaccessible due to security concerns, where outbreaks were reported and challenging sample collection efforts. Additionally, another limitation not all collected samples processed for molecular detection, and representative viruses from the sampled areas were not isolated and the isolated viruses were not sequenced; this was happening by a shortage of reagents. These limitations were affect understanding genetic diversity of field virus circulate in central Ethiopia.

2. LITERATURE REVIEW

2.1 History of Lumpy skin diseases virus

LSD a viral disease in cattle that causes a major economic losses by affecting trade in cattle and their products from LSD endemic and epidemic areas through reducing milk yield, loss of fetus, infertility, loss of body weight, and loss of hide quality (Babiuk *et al*., 2008, Limon *et al.,* 2020; WOAH, 2023). LSD is identified as the list of notifiable animal diseases by the World Organization for Animal Health (WOAH, 2023). Serotype Nettling, otherwise known as LSDV, was initially identified in South African countries (Abdulqa *et al.,* 2016). The disease have been identified by the developments of high body temperature, the formation of skin nodules cover all over the body, enlargement of lymph nodes, body weight loss, skin edema, high morbidity rate, and low mortality rate (Wolff and Tuppurainen, 2021). LSD was reported for the first time as outbreak cases in Ethiopia in 1983 in the northwestern region of the country around southwest of Lake Tana (Mebratu *et al*., 1984). It has now spread to almost all regions and agro-ecological zones of the country (Ayelet *et al*., 2014). Based on the findings of Gumbe (2018), common grazing and watering, Agro-climate conditions, as well as introduction of infected animals significantly influence the distribution and prevalence of LSD in Ethiopia.

2.2 Etiology of Lumpy skin diseases

LSD is caused by LSDV and the virus belongs to the genus *Capripoxvirus* with a member of the *Poxviridae* family. LSDV is a linear enveloped virus, characterized by its double-stranded DNA structure, and possesses a genome length of approximately 151 kilobase pairs. The genome of LSDV consists of a central coding region flanked by identical 2.4 kbp inverted terminal repeats. The genome consists of 156 putative genes, commonly known as open reading frames (ORFs). These genes have a 95% coding density and are responsible for encoding proteins involved in various viral processes such as nucleic acid biogenesis, virion structure and assembly, DNA replication, and protein processing (Tulman *et al.,* 2001; Onyejekwe *et al.,* 2019).

Poxvirus genomes show genomic variation, such as sequence variation, rearrangements, gene gain and loss, these changes primarily happen in the flanking regions of the genome, whereas conserved genes is located in the central part of the genome (Brennan *et al*., 2023). The virus

contains 30 structural or nonstructural homologues of poxviral proteins and is closely related to SPPV and GTPV, sharing 96% nucleotide sequence identity between them; however, LSDV contains nine additional genes compared to the other two viruses (Tulman *et al*., 2000; Abutarbush and Tuppurainen, 2018). LSDV encodes genes such as IL-10, (IF-) receptor, IL-1R, IFN-/binding protein, and IL-18, that play a role in regulating or evading the host immune response, inhibiting host cell apoptosis, and tissue or cell tropism. Upon entering the host cell membrane, the virion particles go through penetration and uncoating processes to enable replication without relying on the host nucleus (Fleming *et al.*, 2000).

2.3 Lumpy skin disease virus replication

Poxviruses undergo replication and transcription of their genetic material in the cytoplasm of host cells, and they possess the genetic information required to produce viral macromolecules. The viral core is released into the cytoplasm once the virion fuses with the plasma membrane or through endocytosis. The transcriptase produced from the virion core facilitates the rapid synthesis of mRNA within minutes of infection (Moss, 2013). Prior to the initiation of viral DNA synthesis, which occurs between 1.5 to 6 hours following infection, the polypeptides produced through the translation of these mRNAs play a crucial role in assisting the viral core's uncoating process (Yang *et al*., 2011).

2.4 Lumpy skin disease virus physicochemical properties

The LSD virus has the ability to survive for an extended period in tissue samples. According to Salihi (2014), the virus can be detected in skin biopsies that have been stored at -80°C for ten years and in tissue culture suspensions kept at 4°C for six months. Furthermore, the authors reported that LSDV could be detected in the sperm of an infected bull after 42 days, and it can also be isolated from ocular and nasal discharges, saliva, and blood of infected cattle using the PCR technique (Gupta *et al*., 2005). The virus exhibits remarkable stability within the pH range of 6.6 to 8.6, with no significant reduction in viral load observed after five days of incubation at 37°C. Moreover, the virus may remain detectable in necrotic skin lesions for up to 33 days, desiccated crusts for a maximum of 35 days, and air-dried hides for at least 18 days (Pal and Gutama, 2023). The virus can be inactivated by exposure to sunlight and detergents containing

lipid solvents, but it can remain stable for extended periods at room temperature and in contaminated animal barns, especially in the absence of sunlight (Babiuk *et al*., 2008).

2.5 Lumpy skin disease epidemiology

The agro-ecological conditions are very important in the epidemiology of LSD. The distribution and transmission of disease are heavily influenced by several factors such as vectors, agent, and host, as well as their interactions. These predisposing factors have a great role in maintaining the arthropod vector and transmitting the virus to susceptible animals (Gari *et al*., 2010). The emergence of disease is significantly influenced by climate conditions, particularly rainfall, when the vector population is abundant (Molla *et al.*, 2017). The distribution and occurrence of LSD is significantly influenced by three key factors, such as the introduction of affected animal free areas, the sharing of grazing and watering together, and the impact of agro-climatic conditions (Tamire, 2022).

Capripox virus infections are often host specific as reported by Tuppurainen et al. (2014) and WOAH (2023). While cattle are the primary natural hosts for LSD viruses, other domestic animals such as water buffaloes and yaks are also susceptible to infection, as noted by Amin et al. (2021) and WOAH (2023). According to the recent reports from 2023, the disease may also affect other in their natural habitats, including camels, giraffes, and wildebeests (Kumar *et al*., 2023). There is no association between the occurrence of LSD in cattle age and sex (Elhaig *et al.*, 2017); however, crossbred cattle have been found to be more susceptible than to local breeds (Kiplagat *et al.*, 2020). Young calves, lactating cows, and malnourished animals are more susceptible to LSD (Mulatu and Feyisa, 2018).

LSDV can be transmitted by biological vector through biting and blood-feeding vectors such as mosquitoes, biting flies, and hard ticks (Gelaye and Lamien, 2019). The transmission of LSD has been recorded in three prevalent African hard tick species: the *Rhipicephalus Boophilus decoloratus* (blue tick), *Amblyomma hebraeum* (black tick), *and Rhipicephalus appendiculatus* (brown tick) (Tuppurainen *et al.*, 2017; Gelaye and Lamien, 2019). The disease can also be transmitted through direct contact with milk, saliva, lesions, and semen of infected cattle, when no vectors are present, the likelihood of direct transmission is reduced (Magori *et al*., 2012,

Sprygin *et al.*, 2019; Gubbins, 2019).

2.6 Diagnosis of Lumpy Skin Disease

2.6.1 Clinical sign

Early diagnosis of LSD often conducted based on clinical signs which help to suspect whether the animal is infected with the LSD virus or not. During the early stages, naturally affected cattle often show clinical signs such as high fever, skin nodules, lesions on the muzzle, reduction of milk yield, swelling on legs, and secondary bacterial infections (Zewdie, 2019). According to several authors, the clinical signs of LSD are similar to other different diseases such as demodicosis, bovine viral diarrhea/mucosal disease, bovine malignant catarrhal fever, and herpes virus infection (Sprygin *et al.*, 2018). The similarity of these diseases complicates the field diagnosis. Therefore, rapid laboratory confirmation is mandatory (Tupurainen, 2018).

2.6.2 Virus isolation

LSD is capable of replicating in various types of cells, including primary cells and cell lines such as Vero cells derived from African green monkeys, lamb kidney cells, testicular cells, embryonic sheep kidney cells, lung cells, and baby hamster kidney cells (BHK-21), and Madin Darby bovine kidney cells (MDBK) (Ayelet *et al.*, 2013; Gelaye *et al*., 2015; WOAH, 2023). The initial cytopathic effect (CPE) caused by LSDV commonly observed 4 to 6 days post inoculation into cell cultures (Tuppurainen *et al*., 2021). According to Mikhael et al. (2021), viral isolation is an important method for amplifying the virus, aiding in the investigation of virus pathogenicity and vaccine production. Rapid confirmation of the field LSDV is critical for effective LSD management and control in both endemic and non-endemic countries (Regulska *et al*., 2022).

2.6.3 Serological diagnosis

Serological diagnosis is inconclusive due to antigenic similarity between *capripoxvirus* and *parapoxvirus* members. As a result, the test cannot distinguish mild or natural infections, as well as antibodies from vaccinated animals. This limitation arises from the virus's ability to trigger cellular immunity, making it challenging to differentiate between LSDV, SPPV, or GTPV

(Tuppurainen *et al*., 2011; Tuppurainen *et al.,* 2021); however, out of the various serological diagnostic methods, virus neutralization test (VNT) is the gold standard technique for detecting antibodies produced against CaPVs. In addition to VNT, the indirect fluorescent antibody test (IFAT) is widely used; IFAT was tested for sensitivity and specificity and found to have a relatively high accuracy for the diagnosis and sero-surveillance analysis of LSD in the target group (Gari *et al.,* 2008).

2.6.4 Molecular identification

Confirmation of the LSD virus in the laboratory can be efficiently achieved through a Capripoxvirus-specific PCR by identifying Capripoxvirions in either biopsy samples or dried specimens. The genome has been identified using primers specific to Capripoxvirus that target the genes for the attachment and fusion proteins, and numerous conventional and real-time PCR methods have been established for analyzing blood, tissue, and sperm samples(Abdulqa *et al.,* 2016). Skin nodules are preferred over blood samples due to the higher viral load present in the lesions, which leads to more positive PCR results. Various sample types, such as saliva, nasal swabs, and whole blood can be used for molecular confirmation of the virus in infected cattle. Real-time PCR is recommended for LSD verification since it saves time and labor over conventional PCR methods (Tuppurainen *et al*., 2011; Elhaig *et al*., 2017).

Conventional and real-time PCR assays are useful for accurately and rapidly detecting the LSDV (Mikhael *et al*., 2021). A molecular method for genotyping CaPVs has been developed using unlabeled snapback primers and EvaGreen dye in the presence of dsDNA. Moreover, this method is cost-effective as it eliminates the need for fluorescent-labeled probes or high-resolution melting curve analysis tool (Gelaye *et al.*, 2013). Another real-time PCR method was developed for the simultaneous detection and genotyping of panpoxviruses, which have veterinary and public health importance (Gelaye *et al*., 2017).The real-time PCR method with primers and a probe was validated for Capripoxvirus(Bowden *et al.*,2009).

2.6.5 Genome sequencing

Molecular epidemiological investigations rely on specific regions of the viral genome, including P32, GPCR, RPO30, and EEV. Among these regions, GPCR and EEV have been the most commonly used markers for distinguishing between LSDV vaccines and LSDV field strains (Gelaye *et al*., 2015; Chibssa *et al.*, 2021). According to Tuppurainen et al. (2018), various genes have been employed to classify *Capripoxviruses*. One of these genes is the LSDV074 (P32) gene, which has been found to be indicative of sheep pox and goat pox viruses. Additionally, the RPO30 and GPCR genes have been identified as genes that can be used to differentiate between different species of *Capripoxviruses*, such as LSD, sheep pox, and goat pox virus (Gelaye *et al.*, 2015). These three genes are the common conserved gene in *Capri pox virus* (Zhou *et al*., 2012).

2.7 Treatment, Control and Prevention of Lumpy Skin Disease

Although there is no treatment for LSD, isolating affected cattle from the herd followed by antibiotics, anti-inflammatory drugs, and good management system has been beneficial in reducing the problems and saving lives. These medications are frequently used to prevent secondary bacterial infections, inflammation, and fever, hence boosting the animal's appetite (Tuppurainen and Oura, 2012). LSD is prevented and controlled through vaccination, quarantines, livestock movement, and insect vector control in the early stages of an outbreak to reduce mechanical transmission of the virus vector control, slaughter of infected and exposed animals, cleaning and disinfection of the premises, and an awareness campaign to encourage industry and community cooperation (Gumbe, 2018).

Currently, there are four strains of live attenuated capripoxvirus that are utilized for the production of vaccines. These strains include Kenyan sheep and Goat pox (KS1-O180), Yugoslavian RM 65, Romanian sheeppox, and South African Neethling LSDV strains (Brenner *et al*., 2006; WOAH, 2023). One distinguishing features of these capripoxvirus strains is the presence of a shared major neutralizing site among all three strains. This commonality enables their use in the vaccination of cattle against LSD infection in various regions worldwide (WOAH, 2023). While vaccination remains the most effective approach for disease control in endemic countries, the use of live attenuated vaccines is highly questionable in terms of safety

(Ayelet *et al.,* 2013; Tuppurainen, 2021). To date the only available vaccines for livestock have been live attenuated vaccines derived from various field strains. If a specific vaccine can provide immunity to over 80% of the herd, it is deemed to offer effective protection against LSD (Babiuk *et al*., 2008).

2.8 Challenge of Lumpy Skin Diseases Vaccine

LSD virus is highly similar to sheeppox virus (Bhanuprakash *et al.,* 2006). Consequently, several authors have suggested utilizing vaccines that are developed from the sheeppox virus. However, Ayelet et al. (2013) found that the Kenyan sheep and Goat pox virus strain vaccine failed to provide good protection for cattle against LSD. The failure of the vaccine can be attributed to several reasons, such as differences in strains between the vaccine and the field strain, low vaccine titer, vaccination of calves less than 6 months of age due to maternal antibody, administration of the vaccine to already infected cattle, differential diagnosis of 'pseudo lumpy skin' disease caused by herpes virus and improper handling of the vaccine during transportation and storage (Hunter and Wallace, 2001). The failure of the LSD vaccine and the subsequent re-infection of vaccinated cattle, pose significant challenges in controlling LSD in different countries, including Ethiopia. A 2015 study carried out in Ethiopia demonstrated that both the Neethling and KSGPO-180 vaccines were ineffective in protecting cattle against LSDV. This lack of efficacy was linked to the vaccines' inadequate immunogenicity, possibly due to over-attenuation leading to genetic modifications. These alterations ultimately resulted in the inability to induce the required protective immunity (Gari *et al*., 2015). Additionally, a study conducted confirmed that the commonly used Kenyan sheep and goat pox vaccine virus (KSGP) O-180 or O-240, which was believed to be SPPV, is genetically an LSDV (Tuppurainen *et al*., 2014; Gelaye *et al*., 2015).

2.9 Status of Lumpy Skin Disease in Ethiopia

LSD is an endemic disease in Ethiopia and causes significant financial losses; the country is experiencing serious difficulties in exporting live cattle and their products such as meat and milk production, as well as quality of skin and hides (Gelaye *et al.,* 2015). After the first appearance of LSD, a rapid, unexpected and uncontrolled epidemic spread from the north to the center to the southern parts of the country within three to five years, easily covering the wide territory of Ethiopia's highland and midland sections were recorded (Abera, 2019).LSD outbreaks have been documented in various regions of the country at different times periods. In particular, outbreaks were noted in the Amhara and Oromia regions in the years 2000/2001, followed by outbreaks in the Oromia and Southern Nation's Nationalities and People Regions in 2003/2004. Following this, the disease occurred in 2006/2007 in the Tigray, Amhara, and Benishangul regions (Ayelet *et al.,* 2014). From 2007 to 2011, a total of 1352 instances of LSD outbreaks were recorded in Ethiopia, with the highest incidence observed in the Oromia region and the lowest in the Afar region (Gumbe, 2018).

3. MATERIALS AND METHODS

3.1 Study Area

The study was conducted in four purposefully chosen areas of central Ethiopia (Figure 1), focusing on intensive dairy and fattening farms. The study took place between November 2023 and May 2024. Sululta, Batu, Mojo, and Bishoftu were selected for the study because of the active outbreak of LSD.

Sululta is located at 9º11′0″N latitude and 38º 45′0″E longitude, with an altitude of 2500 to 2700 meters above sea level (masl) in Oromia Regional State. The annual rainfall and mean temperature are 1119 mm and 14.7^{o}C, respectively. The area is known for its commercial livestock production, particularly dairy and poultry. According to the data obtained from 2024 annual report of Sululta Agricultural Office there is 185,695 cattle, 1,112,150 poultry, 81,145 small ruminant and 9,187 equine population in Sululta town.

Mojo town is located in the East Shewa zone of central Ethiopia, 77 km from Addis Ababa, at latitude 8°39′N and longitude of 39°5″E, with elevations ranges from 1788 to 1825 meters above sea level. The average temperature in Mojo is approximately 23°C. Throughout the month, the maximum and minimum temperatures at Mojo station normally vary from 25.6°C to 30.8°C and 8.5°C to 13.5°C, respectively. Data obtained from 2024 annual report of Lume District Agricultural Office there are 95,975 Cattle, 178,298 Small ruminants, 27,438 Equine and 175,505 Poultry population in and around Mojo town.

Batu town is situated in the East Shewa Zone of Oromia Regional State. It is 222 km away from Addis Ababa, located at latitude 7°56′N and longitude 38°43′E, with an elevation of 1,643 meters above sea level. The climatic conditions have 85% low land and 15% semi-high land. It receives mean annual rainfall of 760to 1000 mm. Batu is a town which is in Adami Tulu Jiddo kombolcha districts in central Ethiopia. The average annual temperature ranges from 22 to 28ºC. The data obtained from Batu town Agricultural Office annual report in 2024 indicates that there are 38,969 cattle, 39,999 small ruminants, 13,728 Equines, and 704,000 poultry population in Batu town.

Bishoftu, a town located in central Ethiopia in the Oromia Region, positioned at a latitude of 8.7525° N and longitude of 38.9785° E, with an elevation of 1,920 meters above sea level. The town is divided into three subcities (Dibayu, Calalaka, and Dukem). Again the town the sub-city divided into Woreda and kebele. The town situated at about 47 km southeast of the capital city, Addis Ababa, Bishoftu is recognized for its distinctive characteristics, particularly the presence of crater lakes like Lake Bishoftu, Lake Hora, Lake Bishoftu Guda, and Lake Koriftu. According to the data obtained from Bishoftu town Agricultural Office annual report in 2024 there are 98,570 cattle, 944,867 poultry, 43,073 small ruminants and 59,808 equine populations in Bishoftu town.

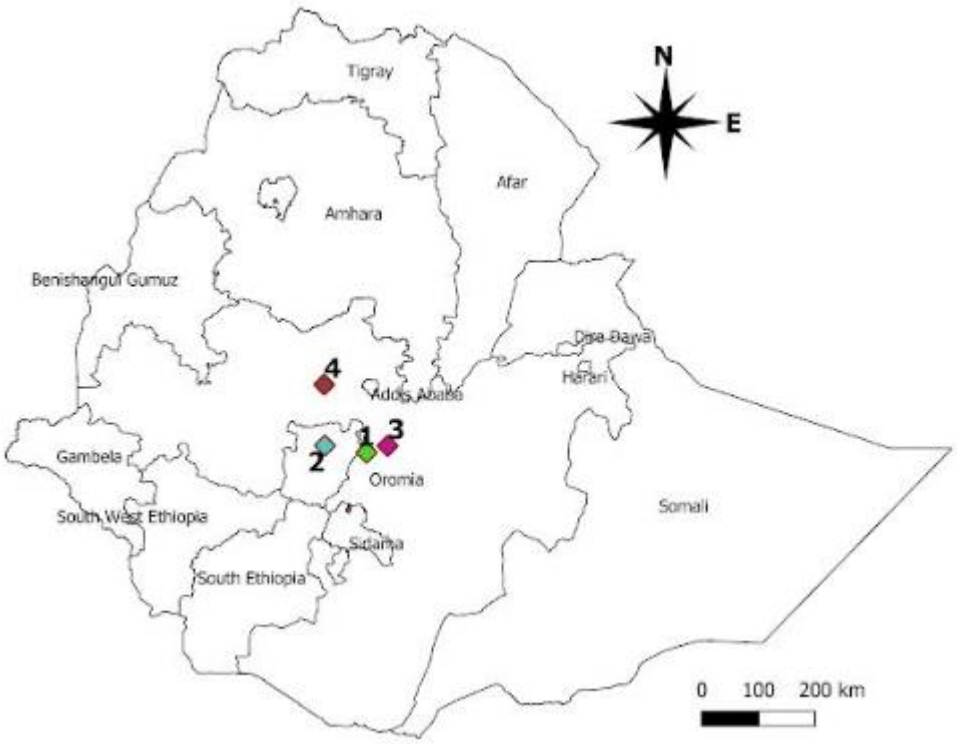

Figure 1: Location of Sample Collection Area. Rectangles marked in different color indicate the area where samples have been collected during the study period. Each number indicates area of outbreak: (1) Batu, (2) Bishoftu, (3) Mojo and (4) Sululta, (GIS software).

3.1 Study Animals

All cattle included in the study were managed in an intensive farming system. During the study period, a total of 377 cattle were assessed from two private farms (one fattening and one dairy farm) and two Governments owned dairy farms. Animals with active clinical signs of LSD were sampled. Fattening farms have male local breed whereas dairy farms hold female cross-bred.

3.2 Study Design

Cross-sectional study design and purposive sampling technique was implemented based on the reports of suspected LSD outbreaks in the study areas in central Ethiopia. The areas were selected based on the information provided by the farm owners and animal health professionals who were actively working in the areas. These outbreak reports were received by NVI through mobile phone communications. The LSD outbreak was investigated based on clinical signs demonstrated by diseased animals. The type of the farm, date of sampling, clinical sign observed, the animal's physiological status, age, and breed of each sampled cattle were recorded on the proper recording format (Annex 1).

3.3 Sample Collection

Skin nodule and nasal swab specimens were collected aseptically from LSD suspected Cattle after physically and clinically inspected. Total of 32 (twelve skin nodules and twenty nasal swabs) samples were collected. These samples were obtained from both vaccinated and non-vaccinated animals. Out of total collected samples, 17 samples (six skin nodules and eleven nasal swabs) were from fattening farm whereas 15 (six skin nodules and nine nasal swabs) were collected from dairy farms. The samples collected for virus isolation and molecular detection were done in accordance with WOAH (2023). Briefly, tissue samples were collected aseptically by cleaning and disinfecting the area with 70% ethanol and removing the hairs with sterile scalpel blade. About 2-3gm of skin nodules and nasal swabs were collected and placed in a universal bottle containing freshly prepared Virus Transport Medium (VTM) containing antibiotics and antifungals. All collected samples were labeled, placed in cold chains, and transported to the NVI for laboratory analysis. After arrival the sample were stored at -20°C until processed.

3.4 Laboratory Analysis

3. 4.1 Sample Processing

The skin nodule and swab samples stored at -20°C were thawed at room temperature and washed three times using sterile phosphate buffer saline (PBS) containing antibiotics and antifungal at pH 7.2 under class II biosafety cabinet. Approximately 1gm of the samples were cut and grinded into small pieces using sterile scissor, pestle and mortar, then 9 ml of PBS was added to grinded sample and transferred to a test tube and swabs samples from the field were directly vortexed. Then processed sample were centrifuged at 3500 rpm for 10 minutes. Finally supernatant from tissue and nasal swab suspension were collected for molecular detection. The remaining supernatant was filtered using a 0.45μm pore size filter pad (Millipore, USA) and stored at -20°C until used for virus isolation (WOAH, 2023).

3.4.2 Cell culture preparation

Embryonic sheep kidney cell (ESH-L) was obtained from AU-PANVAC. The cryovial with ESH-L frozen cell was placed in water bath until the cell was thawed. After the cell was thawed, outside cryovial was disinfected with 70% alcohol. The suspension was pippeted into cell culture flask containing complete Glasgow's Minimum Essential Medium (GMEM) culture media (Sigma-Aldrich) supplemented with 10% (v/v) tryptose phosphate broth (TPB) and 10% (v/v) fetal calf serum (Gibco). Then culture was incubated at 37°C with 5% CO_2. The medium was changed after 24 hours. After obtaining 70% confluent monolayer cell, the cell was sub-cultured by discarding the old medium, washed three times using sterile PBS in aseptic conditions under biosafety cabinet label II. Then, 0.5 ml of warm trypsin was added and incubated at 37°C for 3 minutes to make the cell detached from the flasks. After the cells detached, 1ml of serum containing media was added to stop the trypsin's activity and gently pipetted to disperse the clumped cells. The mix was transferred to centrifuge test tube and centrifuged at 2500rpm for 10 minutes after which the supernatant was discarded. Then, remaining of the sediment cell was sub-cultured into two new 25cm^2 tissue culture flasks containing complete medium and incubated at 37°C with 5% CO_2 and monitored daily for cell confluence (DeliveReD, 2012).

3.4.3 Virus Isolation

Virus isolation was conducted from eight field samples and one KS-1 vaccine by inoculating 1 ml of filtered supernatant into confluent monolayer ESH-L cell culture in 25 cm^2 tissue culture flasks. After inoculation, the tissue culture flasks were incubated at 37°C for 1 hour for virus adsorption onto the cell. The monolayer formation suspension was discarded, and 9 ml of maintenance GMEM media supplemented with 2% fetal calf serum and 0.1% Pen-strep was added. Finally, the flasks were incubated at 37°C with 5% carbon dioxides (CO_2). After 48 hours, the media was changed, and cells were monitored daily for 12 days using an inverted microscope to determine the development of virus induced CPE, which were characterized by rounding of single cell, aggregate of died cell and detachments of mono layer (Nahas *et al.*, 2011, Tassew *et al.*, 2018). After passage one, the tissue culture was harvested, frozen and thawed three times to realize the virus from the cell, and inoculated again into a new cell culture flask. A sample was considered negative when no CPE is observed after three blind passages. The cells with CPE were frozen at –20°C until processed for virus DNA extraction.

3.4.4 DNA Extraction

Out of 32 collected samples from field outbreaks, only 25 samples were processed at the molecular biology laboratory for the extraction of viral DNA. Among these samples, 12 were skin nodule suspensions and 13 were nasal swabs collected from the field and one vaccine batch LSD 09/2023 was extracted. The extraction process was carried out using the commercial kits of DNeasy® Blood and Tissue kit (Qiagen, Germany) following the manufacturer's protocols (Annex 2). According to Sambrook and Russell (2000), the DNA extraction was performed by transferring 200µl of the field-collected and cell culture suspension into a 1.5ml Eppendorf tube. To lyse the cells 200µl of lysis buffer (AL) was added along with 20µl of Proteinase K to digest the proteins. The mixture was thoroughly mixed using a wagtech (vibro-Gene SA4, UK) vortex and then incubated at 56°C for 10 minutes in a dry block thermostat (biosan SIA, Lativia). Following incubation, 200µl of 96% ethanol was added to precipitate the DNA and the mixture was mixed by overtaxing. Subsequently, the mixture was transferred from the Eppendorf tube to a 2ml DNeasy min spin column collection tube and centrifuged at 8000rpm for 1 minute, after which the Eppendorf tube was discarded. The first wash was performed using 500µl of washing

buffer (AW1) on the mini spin column in a new collection tube, which was then centrifuged at 8000 rpm for 1 minute, and the collection tube was discarded again. The second wash was carried out using 500µl of washing buffer (AW2), followed by centrifugation at 14000rpm for 3 minutes. To dry the membrane column the mini spin column was inserted into a new collection tube and centrifuged at 14000rpm for 1 minute and discard the collection tube at the end. The min spin column was carefully transferred to a new labeled 1.5ml micro centrifuge tube. Finally, 50µl of elution buffer was added and incubated for 5 minutes at room temperature and centrifuged at 8000rpm for 1 minute to recover the extracted DNA. The spin column was then discarded, and the eluted DNA was stored at -20°C until amplification was carried out.

3.4.5 Conventional polymerase chain reaction

A total of 12 DNA extracted samples were amplified to detect the presence of the RPO30 gene of the virus using conventional PCR with Capripox virus-specific primers previously designed by Lamien *et al.* (2011) with an expected amplification size of 172bp. Master mix protocols carried out for the PCR reaction is presented below in Table 1.

Table 1: Master mix preparations, PCR primer sequences and amplification conditions used for RPO30 gene

Types of reagents/conditions		For one reaction (µl)	Total 12 reaction
RNase free water		3	36
SPGPRNApol-Fow-5pm/µl-5′-TCTATGTTCTTGATATGTGGTGGTTAG-3′		2	24
SPGPRNApol-REV-5pm/µl-5′-AGTGATTAGGTGGTGTATTATTTTCC-3′		2	24
IQ super mix		10	120
Add Template (DNA)		5	60
Total volume		22	264
	Temperature	Time	Cycle
Initial denaturation	95°C	5 minutes	1-Cycles
Denaturation	95°C	30 second	
Annealing	50^{o}C	30 second	40 Cycles
Elongation	72°C	30 second	
Final Elongation	72°C	7 minutes	1-Cycles

Following amplification, the PCR products were separated based on fragment size using agarose gel electrophoresis. An agarose gel was prepared by combining 3% agarose and 1% Tris/Acetate/EDTA (TAE) buffer solution. The solution was homogenized and boiled in a microwave oven and then poured onto gel electrophoresis casting dish with a comb placed to make a well and keep for 20 minutes solidify. The presence of viral DNA was confirmed by analyzing the PCR results according to (Mangana-Vougiouka *et al*., 1999). Five µl PCR product was mixed with 1µl loading dye containing GelRed® (Biotium, Inc.) and then loaded onto wells on pre-prepared gel wells. Electrophoresis was conducted at 120 volts for 1 hour. A DNA ladder (GeneRuler 100bp DNA Ladder, Thermo Scientific) was utilized as a molecular marker. The

DNA bands were visualized using a UV transilluminator and the presence of positive result was confirmed when bands were observed at 172bp size.

3.4.6 Real-Time Polymerase Chain Reaction

Real-time polymerase chain reaction (qPCR) was conducted as previously designed by (Gelaye *et al.*, 2013) in the NVI Molecular Biology Laboratory. Briefly, the qPCR master mix was prepared in a total volume of 20µL where 4.84 µl of nuclease free water, 2µL of forward primer, 0.16µL of reverse primer, 10µL of Ssofest™ EvaGreen® Supermix (Bio-Rad) and 3 µL template DNA samples for the amplification of the CaPV RPO30 gene (Annex 3). The primer sequences: CPHRMSb-Fow-5pm/µl 5′-GGTGTAGTACGTATAAGATTATCGTATAGAAA CAAGCCTTTA-3′ and CPHRM1Sb-Rev-5pm/µl 5′-AATTTCTTTCTCTGTTCCATTTG-3′ were used. The qPCR amplification was carried out using the following steps: initial denaturing at 95°C for 3 minutes, followed by denaturation at 95°C for 15sec and annealing and extension at 58°C for 1.20 minutes for 40 cycles using Low Profile Hard-Shell® 8-well PCR strips (Bio-Rad). The PCR products was then heated at 95°C for 1minute for denaturation, cooled at 40°C for 1minute, and then heated again from 45°C to 85°C for 5/10 seconds for fluorescence acquisition. The CFX Manager software Version 2.0 (Bio-Rad) was used to analyze the melting temperature and melting curve analysis. After amplification, positive samples were identified by the presence of amplification fluorescence curves at 73.5°C cycle threshold (Ct) values from the assay, which was described as positive samples when Ct value is less than 40, and negative samples when Ct value is greater than 40 (Gelaye *et al*., 2013).

3.4.7 Sequencing

For sequencing of LSD virus, RPO30 gene was amplified by using capripoxvirus RPO30 gene overlapping one and overlapping two primers. According to Gelaye et al. (2015), primers were used to amplify the full length of the RPO30 gene. The first primer pair: CPRPO30-OL1-Fow 5′-CAGCTGTTTGTTTACATTTGATTTTT-3′ and CPRPO30-OL1-Rev 5′-TCGTATAGAAACAAGCCTTTAATAGA-3′, with an expected amplification size of 554bp. The second primer pair: CPRPO30-OL2-Fow 5′-TTTGAACACATTTTATTCCAAAAAG-3′ and CPRPO30-OL2-Rev 5′-AACCTACATGCATAAACAGAAGC-3′, resulting in an expected

amplification size of 520 bp. These primers were specifically designed to target overlapping regions within the RPO30 gene in order to create the full-length gene sequence. PCR was performed in a reaction volume of 50 µL, consisting of 4µL forward primer (5pm/µl) and 4µL reverse primer (5pm/µl), 12µL RNase-free water, 20 µL IQ super mix (Bio-Rad), and 10 µL template DNA. The process began with an initial denaturation at 95ºC for 4 minutes, followed by 40 cycles at 95°C for 30 seconds, 55°C for 30 seconds, and 72°C for 30 seconds, and a final elongation at 72°C for 5 minutes. Aliquots of the PCR product were checked by electrophoresis on a 1.5% agarose gel containing GelRed® (Biotium, Inc.), run at 100 volts for 1 hour. A 100bp molecular marker was also used.

The positive PCR products of the amplified RPO30 gene with strong band on gel electrophoresis were selected and purified for sequencing using the Wizard® SV Gel and PCR clean-up system kit (Promega, Germany) protocol. The concentrations of the purified PCR product were quantified using the NanoDrop 2000c Spectrometer (Thermo Scientific, USA) (Annex 4). Concentration of each purified product were adjusted and prepared for Sanger sequencing according to the instruction recommended by the sequencing company. The PCR product mixed with sequencing primer (overlapping 1 and overlapping 2) and four field samples were submitted for sequencing to LGC Genomics (Germany).

3.5 Ethical Clearance

Ethical clearance for this study was obtained from institutional review board of the National Veterinary Institute's Animal Research Ethics and Review Committee (ARERC) with reference number NVI/2217(Annex 5), which was in accordance with the International Guidelines for Animal Welfare.

3.6 Data Management and Analysis

Data collected during sample collection, virus isolation in cell culture, and virus detection using conventional PCR, and real-time PCR were recorded and saved in Microsoft Office Excel Spreadsheet 2010. The raw DNA sequence fragment of each isolate produced by overlapping primers targeting the RPO30 gene were assembled to get the full length of RPO30 gene (606bps) using Staden package software. Sequence of four LSDV isolate were submitted into NCBI data basis and the following accession number (PQ119823, PQ119824, PQ119825, and PQ119826) were obtained. Comparative genomics by multiple sequence alignment were done using BioEdit sequence alignment editor (Hall, 1999), using the ClustalW algorithm for sequence similarity search between isolates in this study, vaccine strain currently in use and previous Ethiopian viral isolates retrieved from NCBI GenBank. The Basic Local Alignment Search Tool (BLAST) in NCBI was used for sequence similarity search between isolates in this study with sequences of other isolates available in the GenBank database. MEGA 11 software was used for phylogenetic tree construction to study the genetic relationship of the RPO30 gene of the outbreak LSDV isolates with vaccine strain and other LSDV isolates retrieved from NCBI data base. The Neighbor-Joining algorithm was used with the maximum composite likelihood nucleotide substitution model and 1000 bootstrap for confidence interval were used for tree construction (Kumar *et al*., 2016).

4. RESULTS

4. 1 Clinical examination of LSD outbreaks

During the outbreak investigations, 377 animals were thoroughly examined of which 99 dairy cows were from three dairy farms and 278 fattening cattle from one fattening farm, all of which were kept in an intensive farming system. Among the animals observed, 17 cattle's showed clinical signs of LSD and four were died in fattening farm and 15 cattles were observed with active clinical sign and one dairy Heifer was died in Batu dairy farm (Tables 2). During the study period, LSD outbreaks resulted morbidity and mortality rates of 8.49% and 1.3%, respectively. In this study the highest morbidity were recorded in dairy farms (15.15 %) whereas 6.11 % of morbidity recorded in fattening farm.

Table 2: LSD outbreaks results based on study area, farm type, sample types, and LSD morbidity and mortality rates

Study Area	Farm type	Breed	No of cattle examined	Sample type		Affected cattle (Morbidity (%))	Death (Mortality (%))	Vaccination
				Skin nodule	Swab			
Mojo	Fattening	L	278	6	11	17 (6.11)	4 (1.44)	NV
Batu	Dairy	E	30	3	5	8 (26.67)	1 (3.33)	NV
Bishoftu	Dairy	E	11	1	1	2 (18.18)	-	V
Sululta	Dairy	E	58	2	3	5 (8.62)	-	V
Total			377	12	20	32 (8.49)	5 (1.33)	

L: local breed, E: Exotic-breed, NV: non-vaccinated, V: vaccinated

According to the information collected from animal owners and animal health professionals, samples from Mojo and Batu study areas were from non-vaccinated animals, whereas samples from Bishoftu and Sululta dairy farms were from vaccinated animals where the disease occurred two months after vaccination. The status of one dairy cow sampled from Bishoftu dairy farm, was pregnant and without abortion. The common clinical signs observed in this study included skin nodules covering the entire body of the animal (Figure 2), an open wound on the leg, high fever, nasal discharge, lacrimation, leg swelling, salivation, decreased appetite, reduced milk production in dairy farms, and weight loss in fattening farms.

Figure 2: photos taken during LSD outbreaks investigations from Sululta (A), Batu (B), and Mojo (C&D) area.

Clinically LSD suspected cattle were successfully sampled which is important for virus isolation.

4.2 Virus isolation using cell culture

Out of the total samples (32) collected and molecularly screened (25 samples) for the LSD virus, only 76 % (19/25) showed positive results. Among these positive samples, 42% (8/19) were field samples and one was vaccine strain (used for control) were inoculated onto embryonic sheep kidney cell (ESH-L) culture, where only 50% (4/8) induced CPE starting from the fourth day, despite no blind passage. In contrast to the field samples, the LSD vaccines strain (KS-1) showed CPE starting from third day. The remaining samples 25% (2/8) displayed CPE after one blind passage on the third day, while strong CEP were observed on the third passage after 48 hours. However, 25% (2/8) did not show any CEP until the third passage, leading to their result as negative. The observed CEP included cell rounding, dead cell aggregation, and destruction of the ESH-L cell monolayer. On the other hand, the control cells did not show any morphological change (Figure 3).The viruses were successfully isolated which is critical for further molecular characterizations.

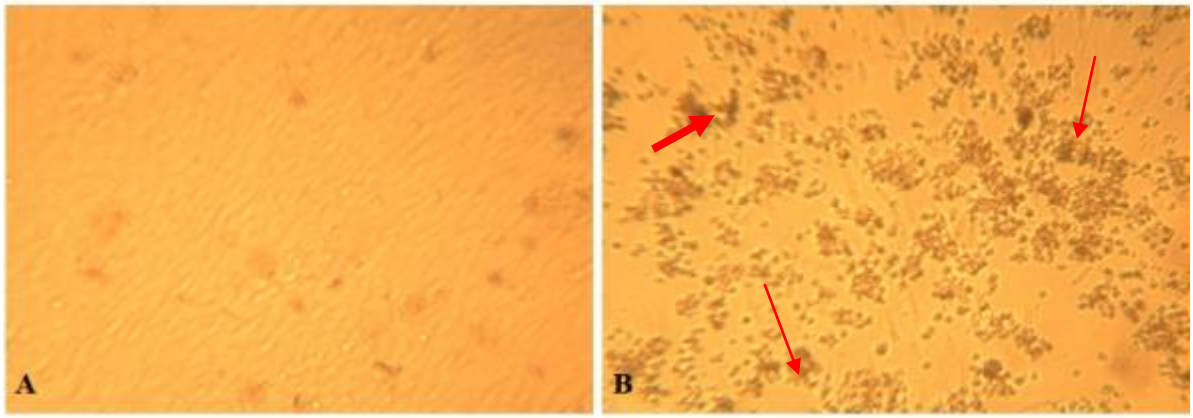

Figure 3: Photo Taken Using an Inverted Microscope onto Esh-L Cell. ESH-L cells used as a control day three without virus inoculation (A), and ESH-L cells showing LSDV induced cytopathic affect (CPE),passage two day three (B). Red Arrows indicate observed CPE

4.3 Molecular detection of LSD virus

4.3.1 Conventional PCR results

Virus DNA extracted from 12 skin nodules samples were amplified using conventional PCR method. Out of 12 amplified samples only 8 LSD clinical samples tested positive for LSDV, while the remaining four samples were negative (Table 3).

Table 3: Description of LSD test results from skin nodule samples using conventional PCR

Study Area	sample Type	Tested sample	Positive samples
Batu	SN	3	2
Bishoftu	SN	1	1
Sululta	SN	2	1
Mojo	SN	6	4
Total		12	8

Note: SN- Skin Nodules.

There was no DNA band identified in nucleases-free water used as a negative control. Known LSDV positive sample was used as a positive control. The result indicated that the field LSD virus samples and LSDV positive control were amplified at 172bp band size, as shown in Figure 4.

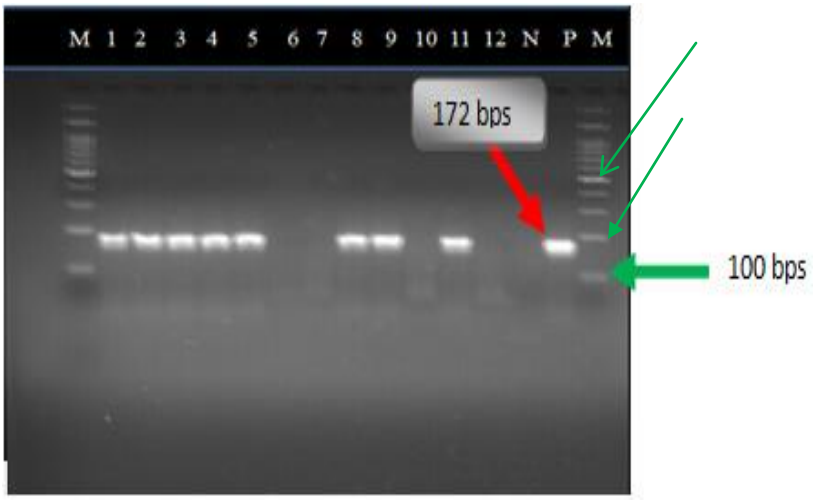

Figure 4: Gel picture of the conventional PCR showing amplification of RPO30 gene with 172 band size.M: molecular ladder (GeneRuler™ 100bp plus DNA Ladder, Thermo Scientific), (1, 2,3,4,5, 8, 9 and 11) are positive samples; whereas 6, 7, 10, and 12 are negative samples: N: negative control and P: positive control. Red arrow indicate amplified field sample, whereas green arrow indicate molecular ladder.

4.3.2 Real-time PCR results

A total of twenty-two samples were tested using real-time PCR including thirteen direct swab samples, eight cultured samples and one vaccine strain of cell culture suspension. The test involved a positive control, an LSDV positive sample and a negative control (which was nuclease free water). Out of total tested samples 72.72 %(16/22) tested positive, while the remaining 27.27 %(6/22) samples were negative. The positive samples, 68.75 % (11/16) were from swab samples and the other 31.25 %(5/16) samples were from cultured viral suspension. The melting temperatures of the sample were observed 73.5°C (Figure 5), with Ct values ranged from 20.56 to 39.61(Table 4).

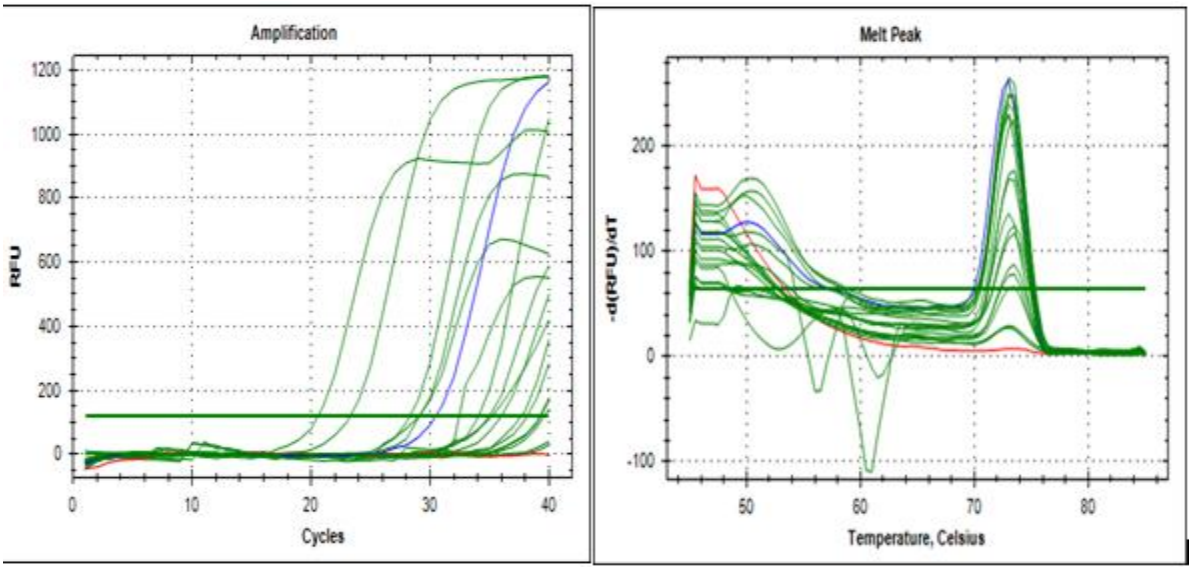

Figure 5: Real-Time PCR Picture Showing Amplification and Melting Peak Of RPO30 Gene of LSDV. Green color indicates positive samples, blue color indicates positive control, and red color indicates negative control.

The virus was successfully characterized using conventional PCR and qPCR which were very important for selected isolated virus for further characterization by sequencing.

Table 4: Real time PCR Ct values

Sample no	Area	Types of sample	Ct values
1	NVI	Cultured -vaccine	32.45
2	Batu	Cell culture	36.00
3	Batu	Cell culture	29.10
4	Batu	swab	38.16
5	Batu	Swabs	35.19
6	Batu	Swab	30.57
7	Batu	Swab	20.56
8	Mojo	Cell culture	N/A
9	Mojo	Cell culture	N/A
10	Mojo	swab	39.13
11	Mojo	swab	39.22
12	Mojo	Swab	34.96
13	Mojo	Swab	N/A
14	Mojo	swab	N/A
15	Bishoftu	Cell culture	N/A
16	Bishoftu	Cell culture	37.66
17	Bishoftu	Swab	28.28
18	Sululta	Cell culture	23.27
19	Sululta	Cell culture	N/A
20	Sululta	Swab	34.07
21	Sululta	Swab	39.33
22	Sululta	Swab	39.61
23		Positive control	25.34
24		Negative control	N/A

N/A –Not Applicable

4.4 Result of sequence analysis

Similarity analyses between recently field isolates in this study and vaccinal strains with accession number KP663685 scored that 99.67% identity, whereas 99.835% of identity score were observed between newly isolate and previously isolated in Ethiopia retrieved from NCBI databases. Multiple sequences alignment was performed for similarity searching by using nucleotide sequences. The results demonstrated that there were genetic differences observed between isolate in this study and KS-1 vaccine used in the Ethiopia at nucleotide 41, where adenine replaced cytosine and at nucleotide 292, where thymine replaced cytosine. But only single nucleotide differences were observed between new isolate and other previous isolate stored in gene bank at position 41 cytosine in the previous isolate was replaced by thymine in the new isolate (Annex 6).In order to search for amino acid similarity the RPO30 full gene sequence was translated into an amino acid sequence between the four isolates in this study, one vaccine strain used in Ethiopia, and 21 previous isolates available in the GenBank. The results indicated that variations were observed at amino acid positions 14 and 98. Asparagine (N) replaced threonine (T) in other LSDV isolates including vaccine strain, at residue position 14 (N14T). Only the vaccine strain changed serine (S) with proline (P) at residue position 98 (S98P) (Annex 7). Raw sequence was successfully edited and analyzed which is critical for further phylogenetic analysis.

4 .5 Phylogenetic analysis

Phylogenic tree was constructed based on the RP30 gene nucleotide sequences of the newly sequenced isolates in this study, KS-1 vaccine strain, previously isolates from Ethiopia and from other African country's isolates retrieved from NCBI GenBank. The three members of *capripoxviruses* are clustered into three groups, SPPV, GTPV, and LSDV (Figure 7). The results of current four Ethiopian isolates in this investigation were identical to each other based on RPO30 gene sequence analysis and phylogenetic tree constructed. The result revealed that the current LSDV isolated are closely related to GU119938/LSDV Sudan Obied isolate with 100% sequence similarity than previous Ethiopian isolate and also KS-1 vaccine grouped to LSDV. The genetic mean distance within the four Ethiopian LSDV isolates is zero (0), within the GenBank retrieved LSDV isolates is 0.0005 and between the two groups (the current four Ethiopian LSDV

isolates and the GenBank retrieved sequences) is 0.005, whereas overall mean distance among the LSD virus isolates is 0.002.

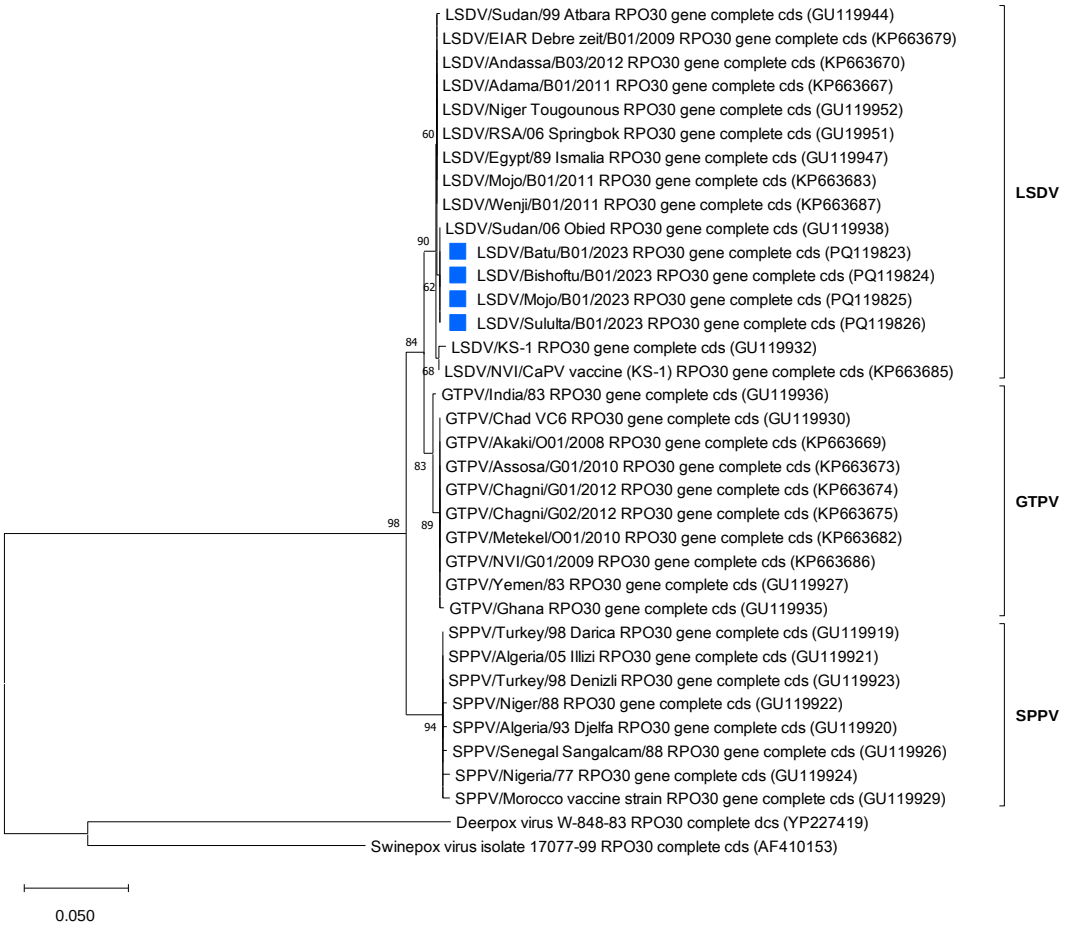

Figure 6: Phylogenetic analysis of Capripoxviruses using nucleotide sequences of the RNA polymerase 30 kD subunit (RPO30) gene. The analysis included 36 nucleotide sequences. Evolutionary analyses were conducted using MEGA11. Four Ethiopian current outbreak isolates (PQ119823-PQ119826, labeled with a rectangle), Two KS-1 vaccine strains (local vaccine strain KP663685 and one from GenBank GU119932), Five old Ethiopian isolate (KP663667, KP663670, KP663679, KP663683 and KP663687), other four old African isolate (GU119938, GU119944, GU119951 and GU119952), ten GTPV (GU119927, GU119930, GU119935 , KP663669, KP663673, KP663682 and KP663685) and eight SPPV (GU119919-GU119924, GU119926 and GU119929) sequences obtained from GenBank were used. The homologous

gene sequences of Deer poxvirus (YP2227419) and Swine poxvirus (AF410153) were used as an out group. The evolutionary history was inferred using the Neighbor-Joining method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches (greater than 50%). The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site.

Phylogenetic tree was successfully constructed which was important to conclude new isolate related with previous isolated stored in Gene bank.

5. DISCUSSION

In the present LSD outbreak investigation, infected animals showed clinical signs such as skin nodules covering the entire body, high fever, nasal discharge, lacrimation, and swelling of the leg, an open wound on the leg, salivation, and appetite loss, milk reduction, and weight loss. These findings were in agreement with the previous report from Wolaita Zone, Southern part of Ethiopia by Mathewos *et al.* (2022). According to the findings of Gelagay *et al.* (2014), cases of abortion were documented in exotic dairy cows, although these signs were not observed in the current study.

In the current study, the morbidity and mortality rates were 8.49% and 1.3%, respectively. The average morbidity rate recorded in this study was lower than that reported by Leliso *et al*. (2021), 18% morbidity rate, but higher than that reported by Tassew *et al.* (2018), 5.695 morbidity rate, whereas the results were consistent with LSD morbidity and mortality rates ranging from 3% to 85% and 1 and 40%, respectively (Babiuk *et al*., 2008; Tuppurainen and Oura, 2012; WOAH, 2023). In this study, Batu dairy farm had the highest morbidity (26.67%), while Mojo fattening farm had the lowest (6.11%). The highest mortality rate of 3% was also observed in Batu dairy farm when compared with Mojo fattening farm, this was due to genetic difference between exotic dairy cows and local breed in the fattening farm. These findings are in agreement with the observation that morbidity and mortality vary based on genetic differences in animal breeds (Babiuk *et al*., 2008; Tuppurainen *et al.*, 2015). High morbidity and mortality rates recorded in Batu dairy farms were due to animal in Batu dairy farm was exotic breed and not vaccinated and mortality was not recorded at the other two dairy farms in Sululta and Bishoftu. This was may be due to animal in Sululta and Bishoftu dairy farms were vaccinated. The finding are in agreement with the report of other researchers (Radostits *et al.*, 2007), indicating that morbidity and mortality from LSD vary depending on breed and immunity level of the animal population.

The virus genome was analyzed molecularly using both conventional PCR and real-time PCR. The Ct values ranged from 20.56 to 39.61 and the measured melting temperature was 73.5°C.Lower Ct values in positive samples indicated a higher amount of viral DNA present in the DNA samples. The melting temperatures indicated that the virus detected was LSDV. These findings were in agreement with the report of Gelaye *et al*. (2013), which stated that the melting

temperature of the amplicon at 73.5°C was used to differentiate LSDV from other Capripoxviruses such as sheep poxvirus and goat poxvirus. These diagnostic approaches proved to be useful in detecting and genotyping LSDV in collected samples quickly and accurately, which is essential for early disease outbreaks detection and control.

According to previous studies, the LSDV was isolated through the use of the Vero cells (Gelagay *et al.,* 2014; Gelaye *et a*l., 2015). In this study LSDV was isolated from samples obtained during active outbreaks and inoculated into ESH-L cell cultures , where 75% (6/8) field samples showed CEP on ESH-L cells beginning on day 4 post-inoculation from passage one to passage three, whereas the remaining 25%(2/8) samples could not be isolated. ESH-L cell cultures inoculated with vaccine seed showed CEP on day three without blind passage, indicating that the vaccine strain had already been adapted to the cells. LSDV CPE observed on ESH–L cells during the outbreak investigation included rounding single cells, aggregated dead cells, and detachment of monolayer from tissue flasks. This indicates the presence of the virus inside the cells and the result was also consistent with the CPE characteristics observed by Tassew *et al.* (2018).

According to the results of this investigation, the isolates in the present study and the KS-1 vaccine (KP663685) had 99.67% similarity of the RPO30 gene sequence with nucleotide sequence and Amino Acid variation at two positions and at one position with previous isolate in Ethiopia with 99.835% sequence identity score. These results support alternative hypothesis that there is genetic difference between new field isolate and the currents vaccine in use in the country. These the genetic difference observed were the main reason for KS-1 vaccine not fully protect animals from the diseases. These results are consistent with the findings of Tassew *et al.* (2018), who reported similar sequence variations from East Hararghe and East shoa zone, Oromia Region. The finding of sequence analysis indicates that the isolates in this investigation is more closely related to previous Ethiopian isolates rather than the KS-1 vaccine strain and there is a limited genetic variation among the field circulating LSDV strains over time. Additionally, the findings supported the report that field circulating viruses differed from the vaccine strains (Gelaye *et al*., 2015). In contrast, no genetic variation was identified among the current four LSDV isolates.

The samples used in this study were also collected from vaccinated cattle. The present findings are in agreement with the report of outbreaks occurred in cattle vaccinated with KS-1 vaccine following vaccination on smallholder dairy farms (Zewdie *et al*., 2019). Yet the results indicated that the sequence of virus isolated from vaccinated animal had no difference from the virus isolated from non-vaccinated animal. This indicates that vaccine is not the cause of the disease outbreak. The report is forward that vaccine should be handled carefully during transportation, storage, and vaccination. The tree result revealed that the four LSD viruses are more closely clustered to GU119938/LSDV Sudan Obied isolate. New isolate in this study also clustered with KS-1 vaccine strain (GU119932 and KP663685) and previously sequenced Ethiopian LSDV isolates. This was also supported by the overall mean genetic distance(0.002), observed among the LSD virus isolates indicating that the current Ethiopian and GenBank retrieved LSDV isolates are genetically closely related with over all sequence similarity of 99.83%. Furthermore, the phylogenetic tree results indicate that the NVI KS-1 vaccine is more closely related to LSDV than the homologous sheep and goat pox virus sequences, which is in agreement with previous studies (Tuppurainen *et al*., 2014, Gelaye *et al*., 2015), indicating that the sheep and goat pox vaccine (KS-1) is genetically an LSDV. Clinical signs, virus isolation, molecular detection, sequencing, and phylogenetic tree analysis collectively indicate that the disease outbreak was caused by LSD virus.

6. CONCLUSIONS AND RECOMMENDATIONS

In the present study, skin nodules and swabs were collected from clinically affected cattle. LSDV, the cause of the disease outbreaks was isolated on ESH-L cells and characterized using molecular tools such as conventional PCR, real-time PCR, and DNA sequencing. Sequence differences were observed between the isolate in this study, a vaccine produced at NVI and previous isolates in the country. The phylogenetic tree result indicates that isolates in this investigation are more closely related to Sudan isolates than the previous Ethiopian LSDV isolates. Clinical signs, virus isolation, molecular detection, sequencing, and phylogenetic tree results show that the cause of the disease outbreak was LSDV. The current LSD field cell culture isolates were labeled properly and stored in the NVI biological bank.

Based on the findings, the following recommendations were forwarded:

- Target full-genome sequencing to capture the full genetic diversity of LSDV strains in Ethiopia.
- Study covering bigger areas of the country is necessary to evaluate the genetic variation that exists across the animal breed, geographical location, and vaccination history.
- NVI recommended developing vaccine from field circulating strains of new isolate. These help to solve the problem of disease outbreak after vaccination due to sequence difference between vaccine and field circulating strain.

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8. APPENDICES

Annex 1: Information recorded during data collection

Area	Sex	Types of farms	Date of sampling	Breed	Age (years)	Clinical sign observed	Physiological status of Animal's
Batu	F	DF	3/11/2023	Exotic	>2 years	High fever 40.9^{o}C , milk reduction ,appetite loss, skin nodules on the body , swelling of leg ,nasal discharge	1 heifer ,1 dry cow and other are lactating cows
Bishoftu	F	DF	28/12/2023	Exotic	>3 years	Swelling of the leg ,skin nodule dispersed on the body, nasal discharge ,open wound on the leg	One pregnant cow and one lactating cow
Mojo	M	FF	9/10/2023	Local	>4years	loss of body weight ,loss of appetite, depression ,lameness .nasal discharge ,lacrimation and skin nodules	All are matured bull
Sululta	F	DF	15/11/2023	Exotic	>3 years	Milk reduction ,appetite loss , nasal discharge, skin nodules cover the body of the animal's, swelling of leg	All are lactating cows

Note: F-female, M-male, DF- dairy farm, FF- fattening Farm

Annex 2: DNA extraction procedure

- Put necessary equipment and reagents in laminar flow
- Add 200µl of tissue/cell suspension to labeled PCR tube and keep the remaining original sample in -20^{o}c refrigerator
- Add 20µl proteinase K enzyme
- Add 200 µl lysing buffer
- Add 200µl 96% ethanol , vortex , spine and centrifuge for 1min at 8000rpm
- Warm at 56^{o}c for 10 minutes in water bath
- Transfer the lysate to a labeled DNeasy mini spin column containing 2ml collection tube, centrifuge for 1min at 8000rpm, discard the tube containing the sediment and Place spine column into new 2ml collection tube
- Add 500µl washing buffer one (AW1), centrifuge for 1min at 8000rpm and discard the flow through and collection tube
- Place spine column in new 2ml collection and add 500µl washing buffer two(AW2), centrifuge for 3 min at 14000rpm then discard the flow through and collection tube.
- Transfer the spine column to new 1.5ml or 2ml micro centrifuge tubes.
- Elute DNA by adding 200 µl buffer AE to the center of the spine column membrane
- Incubate for 1 minutes at room temperature
- Centrifuge for 1 minute at 8000rpm.

Annex 3: Master mix prepared for real-time and amplification temperatures PCR

#	Types of reagents	For one reaction (µl)	Total reaction for 24 (µl)
1	RNase free water	4.84	116.16
2	CPHRMSb-Fow-5pm/µl 5′-GGTGTAGTACGTATAAGATTATCGTATAGAAA CAAGCCTTTA-3′	2	48

3	CPHRM1Sb-Rev-5pm/µl 5′ - AATTTCTTTCTCTGTTCCATTTG-3′	0.16	3.84
4	Safest Evagreen IQ super mix	10	240
5	Add Template(DNA)	3	72
	Total volume	20	

1. PCR Reaction

	Temperatures	Times	Cycles
Initial denaturation	95^{o}C	3 minutes	1 cycles
Denaturation	95^{o}C	15 seconds	40 cycles
Annealing and extension	58^{o}C	1.2 minutes	
Melting curve analysis			
	Temperatures	Time	
Denaturation	95^{o}C	1 minutes	
Annealing	40^{o}C,	1 minute	
	45^{O}C/0. up to 85^{o}C	5 upto 10 seconds	

Annex 4: Concentration of purified PCR product selected for sequencing

Sample name and code	Initial concentration (ng/µL)	Purified PCR DNA volume (µL)	Water volume (µL)	Total volume (µL)
RPO30-OP1-103	30	20	10	30
RPO30-OP1-104	31	19	11	30
RPO30-OP1-105	30	20	10	30
RPO30-OP1-106	34	17	13	30
RPO30-OP1-107	36	16	14	30
RPO30-OP2-108	28	21	9	30
RPO30-OP2-109	28	21	9	30
RPO30-OP2-110	24	25	5	30
RPO30-OP2-111	29	20	10	30
RPO30-OP2-112	15	30	0	30

Annex 5: Ethical clearance approval sheet

NATIONAL VETERINARY INSTITUTE

Ref.No: NVI/1217

Date: 04/01/2024

To: Milki Mojo

Subject: Approval for Ethical Clearance

The research ethical committee of the National Veterinary Institute reviewed and discussed your research project entitled "Isolation and Molecular Characterization of Lumpy Skin Diseases Virus From Active Outbreak Cases in Central Ethiopia" on December, 2023. After review and discussion of your project proposal it is scientifically and ethically sound from relevance, originality and technical competence of view. Hence the project allowed to be executed provided that:

1. All procedure, condition stipulated in the proposal are respected and any deviation, variation or change may be made only in consultation between reported to the committee.
2. All comments given by the committee should be considered and fulfilled by the researchers
3. The project activity is open for occupational supervision by the committee whenever this is deemed necessary.

The committee expects to be informed about the progress of study with any changes in protocol.

Sincerely,

Birtneh Akalu(DR.)
R&D Directorate
Director

Cc

Director General

Deputy Director General

FAX: 011 433 83 00

Annex 6: LSDV RPO30 gene nucleotide sequence alignment results

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                                        10        20        30        40        50        60
                                ....|....|....|....|....|....|....|....|....|....|....|....|
LSDV/Batu/01/2023               ATGGATGATGATAATACTAATTCATATAGTGATAATACTAACCCCACATATCAAGACATA
LSDV/Bishoftu/01/2023           ............................................................
LSDV/Mojo/01/2023               ............................................................
LSDV/Sululta/01/2023            ............................................................
KP663685/LSDV/KS-1/Vaccine      ..........................................C.................
KP663667/LSDV/Adama/01/11       ..........................................C.................
KP663668/LSDV/Adama/02/11       ..........................................C.................
KP663670/LSDV/Andasa/03/12      ..........................................C.................
KP663671/LSDV/Andasa/04/12      ..........................................C.................
KP663672/LSDV/Andasa/05/12      ..........................................C.................
KP663678/LSDV/EDMTI-DZ/09       ..........................................C.................
KP663679/LSDV/EIAR-DZ/09        ..........................................C.................
KP663680/LSDV/F-Field/09        ..........................................C.................
KP663681/LSDV/Kajima/09         ..........................................C.................
KP663683/LSDV/Mojo/01/11        ..........................................C.................
KP663684/LSDV/Mojo/02/11        ..........................................C.................
KP663687/LSDV/Wenji/01/11       ..........................................C.................
KP663688/LSDV/Wenji/02/11       ..........................................C.................
KP663689/LSDV/Wenji/03/11       ..........................................C.................
GU119943/LSDV/RSA/08            ..........................................C.................
GU119945/LSDV/RSA/07            ..........................................C.................
GU119946/LSDV/Burkina           ..........................................C.................
GU119947/LSDV/Egypt/89          ..........................................C.................
GU119948/LSDV/RSA/00            ..........................................C.................
GU119951/LSDV/RSA/06            ..........................................C.................
GU119952/LSDV/Niger             ..........................................C.................

                                        70        80        90       100       110       120
                                ....|....|....|....|....|....|....|....|....|....|....|....|
LSDV/Batu/01/2023               GAAGATATAATTTATAAATATGTAAAAGAAAAATCAAAGGTAAAAGAAATATTAAAATGG
LSDV/Bishoftu/01/2023           ............................................................
LSDV/Mojo/01/2023               ............................................................
LSDV/Sululta/01/2023            ............................................................
KP663685/LSDV/KS-1/Vaccine      ............................................................
KP663667/LSDV/Adama/01/11       ............................................................
KP663668/LSDV/Adama/02/11       ............................................................
KP663670/LSDV/Andasa/03/12      ............................................................
KP663671/LSDV/Andasa/04/12      ............................................................
KP663672/LSDV/Andasa/05/12      ............................................................
KP663678/LSDV/EDMTI-DZ/09       ............................................................
KP663679/LSDV/EIAR-DZ/09        ............................................................
KP663680/LSDV/F-Field/09        ............................................................
KP663681/LSDV/Kajima/09         ............................................................
KP663683/LSDV/Mojo/01/11        ............................................................
KP663684/LSDV/Mojo/02/11        ............................................................
KP663687/LSDV/Wenji/01/11       ............................................................
KP663688/LSDV/Wenji/02/11       ............................................................
KP663689/LSDV/Wenji/03/11       ............................................................
GU119943/LSDV/RSA/08            ............................................................
GU119945/LSDV/RSA/07            ............................................................
GU119946/LSDV/Burkina           ............................................................
GU119947/LSDV/Egypt/89          ............................................................
GU119948/LSDV/RSA/00            ............................................................
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GU119951/LSDV/RSA/06      ..................................................
GU119952/LSDV/Niger       ..................................................
```

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                                 130       140       150       160       170       180
                          ....|....|....|....|....|....|....|....|....|....|....|....|
LSDV/Batu/01/2023         GCAACAGACAAAGCTTCCAAGTTTTATATAAGAAATATTATTAATACAAAGTCAAATATA
LSDV/Bishoftu/01/2023     ..................................................
LSDV/Mojo/01/2023         ..................................................
LSDV/Sululta/01/2023      ..................................................
KP663685/LSDV/KS-1/Vaccine..................................................
KP663667/LSDV/Adama/01/11 ..................................................
KP663668/LSDV/Adama/02/11 ..................................................
KP663670/LSDV/Andasa/03/12..................................................
KP663671/LSDV/Andasa/04/12..................................................
KP663672/LSDV/Andasa/05/12..................................................
KP663678/LSDV/EDMTI-DZ/09 ..................................................
KP663679/LSDV/EIAR-DZ/09  ..................................................
KP663680/LSDV/F-Field/09  ..................................................
KP663681/LSDV/Kajima/09   ..................................................
KP663683/LSDV/Mojo/01/11  ..................................................
KP663684/LSDV/Mojo/02/11  ..................................................
KP663687/LSDV/Wenji/01/11 ..................................................
KP663688/LSDV/Wenji/02/11 ..................................................
KP663689/LSDV/Wenji/03/11 ..................................................
GU119943/LSDV/RSA/08      ..................................................
GU119945/LSDV/RSA/07      ..................................................
GU119946/LSDV/Burkina     ..................................................
GU119947/LSDV/Egypt/89    ..................................................
GU119948/LSDV/RSA/00      ..................................................
GU119951/LSDV/RSA/06      ..................................................
GU119952/LSDV/Niger       ..................................................
```

```
                                 190       200       210       220       230       240
                          ....|....|....|....|....|....|....|....|....|....|....|....|
LSDV/Batu/01/2023         GAAGAAACAAAATTTGAACCAAGAAACAACATAGGTATTGAATACTCAAAAGATTCAAAA
LSDV/Bishoftu/01/2023     ..................................................
LSDV/Mojo/01/2023         ..................................................
LSDV/Sululta/01/2023      ..................................................
KP663685/LSDV/KS-1/Vaccine..................................................
KP663667/LSDV/Adama/01/11 ..................................................
KP663668/LSDV/Adama/02/11 ..................................................
KP663670/LSDV/Andasa/03/12..................................................
KP663671/LSDV/Andasa/04/12..................................................
KP663672/LSDV/Andasa/05/12..................................................
KP663678/LSDV/EDMTI-DZ/09 ..................................................
KP663679/LSDV/EIAR-DZ/09  ..................................................
KP663680/LSDV/F-Field/09  ..................................................
KP663681/LSDV/Kajima/09   ..................................................
KP663683/LSDV/Mojo/01/11  ..................................................
KP663684/LSDV/Mojo/02/11  ..................................................
KP663687/LSDV/Wenji/01/11 ..................................................
KP663688/LSDV/Wenji/02/11 ..................................................
KP663689/LSDV/Wenji/03/11 ..................................................
GU119943/LSDV/RSA/08      ..................................................
GU119945/LSDV/RSA/07      ..................................................
```

```
GU119946/LSDV/Burkina     ..............................................................
GU119947/LSDV/Egypt/89    ..............................................................
GU119948/LSDV/RSA/00      ..............................................................
GU119951/LSDV/RSA/06      ..............................................................
GU119952/LSDV/Niger       ..............................................................
```

```
                                  250       260       270       280       290       300
                          ....|....|....|....|....|....|....|....|....|....|....|....|
LSDV/Batu/01/2023         AACAAATTATCGTATAGAAACAAGCCTTTAATAGAGACAAATAAAGATTATTCTGACATA
LSDV/Bishoftu/01/2023     ..............................................................
LSDV/Mojo/01/2023         ..............................................................
LSDV/Sululta/01/2023      ..............................................................
KP663685/LSDV/KS-1/Vaccine..............................................C.....
KP663667/LSDV/Adama/01/11 ..............................................................
KP663668/LSDV/Adama/02/11 ..............................................................
KP663670/LSDV/Andasa/03/12..............................................................
KP663671/LSDV/Andasa/04/12..............................................................
KP663672/LSDV/Andasa/05/12..............................................................
KP663678/LSDV/EDMTI-DZ/09 ..............................................................
KP663679/LSDV/EIAR-DZ/09  ..............................................................
KP663680/LSDV/F-Field/09  ..............................................................
KP663681/LSDV/Kajima/09   ..............................................................
KP663683/LSDV/Mojo/01/11  ..............................................................
KP663684/LSDV/Mojo/02/11  ..............................................................
KP663687/LSDV/Wenji/01/11 ..............................................................
KP663688/LSDV/Wenji/02/11 ..............................................................
KP663689/LSDV/Wenji/03/11 ..............................................................
GU119943/LSDV/RSA/08     ..............................................................
GU119945/LSDV/RSA/07     ..............................................................
GU119946/LSDV/Burkina    ..............................................................
GU119947/LSDV/Egypt/89   ..............................................................
GU119948/LSDV/RSA/00     ..............................................................
GU119951/LSDV/RSA/06     ..............................................................
GU119952/LSDV/Niger      ..............................................................
```

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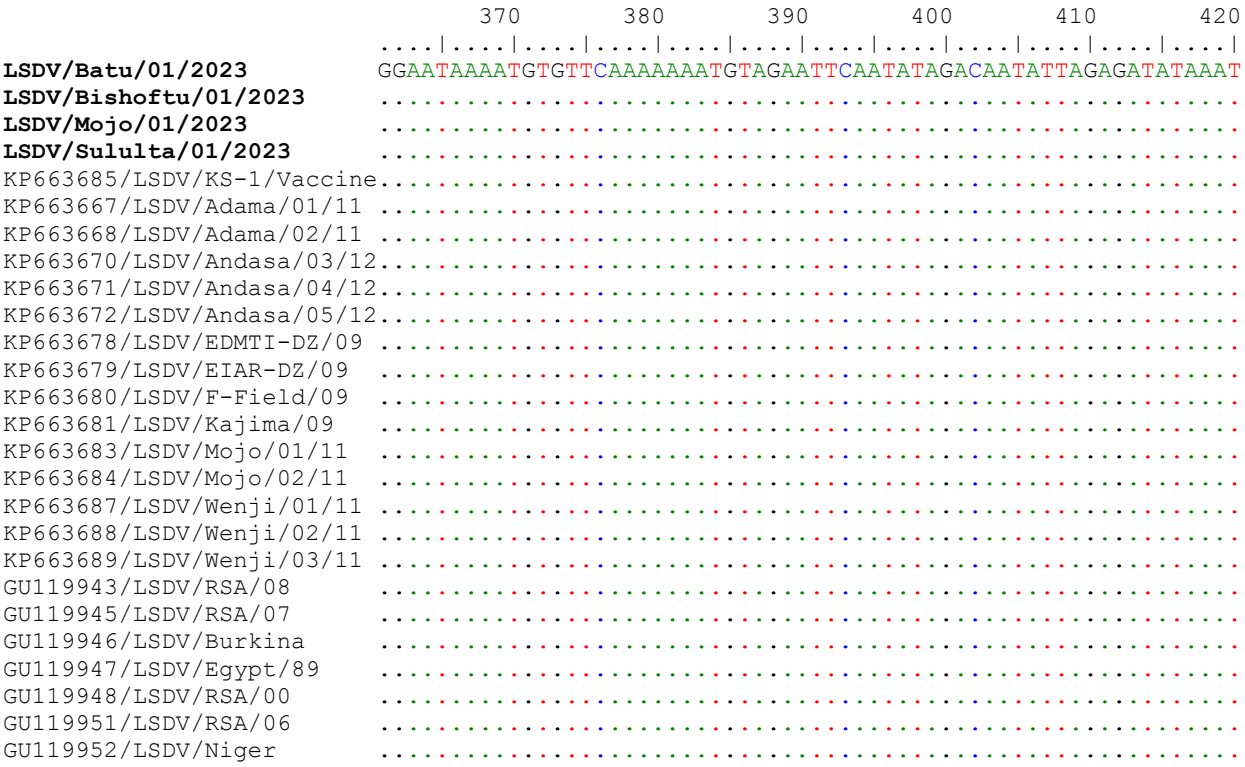
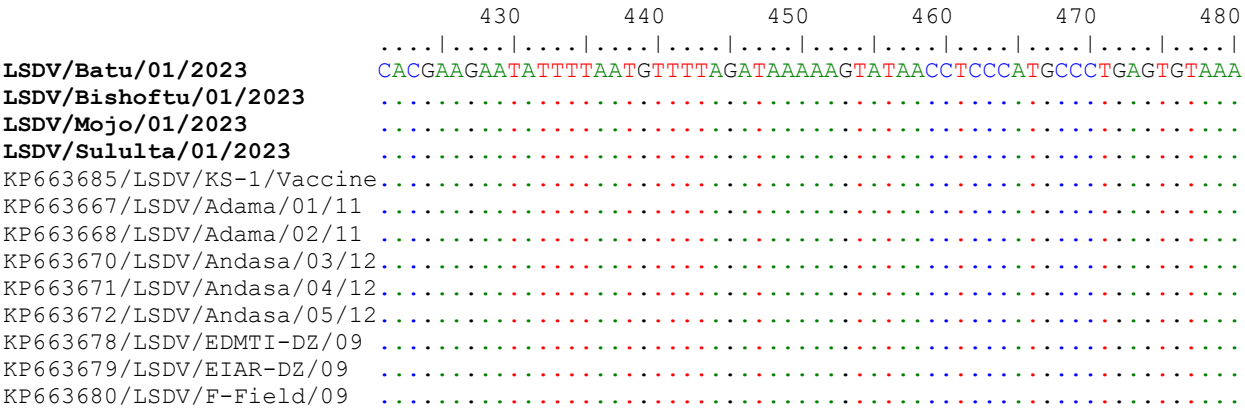
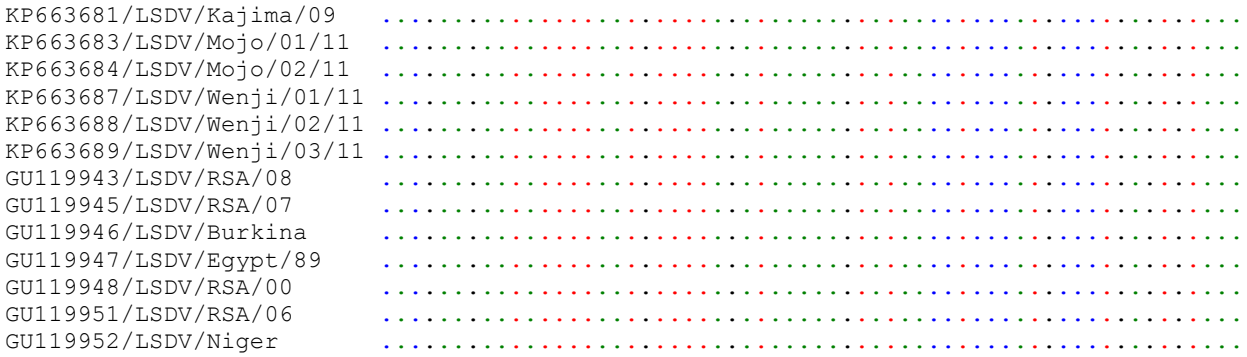
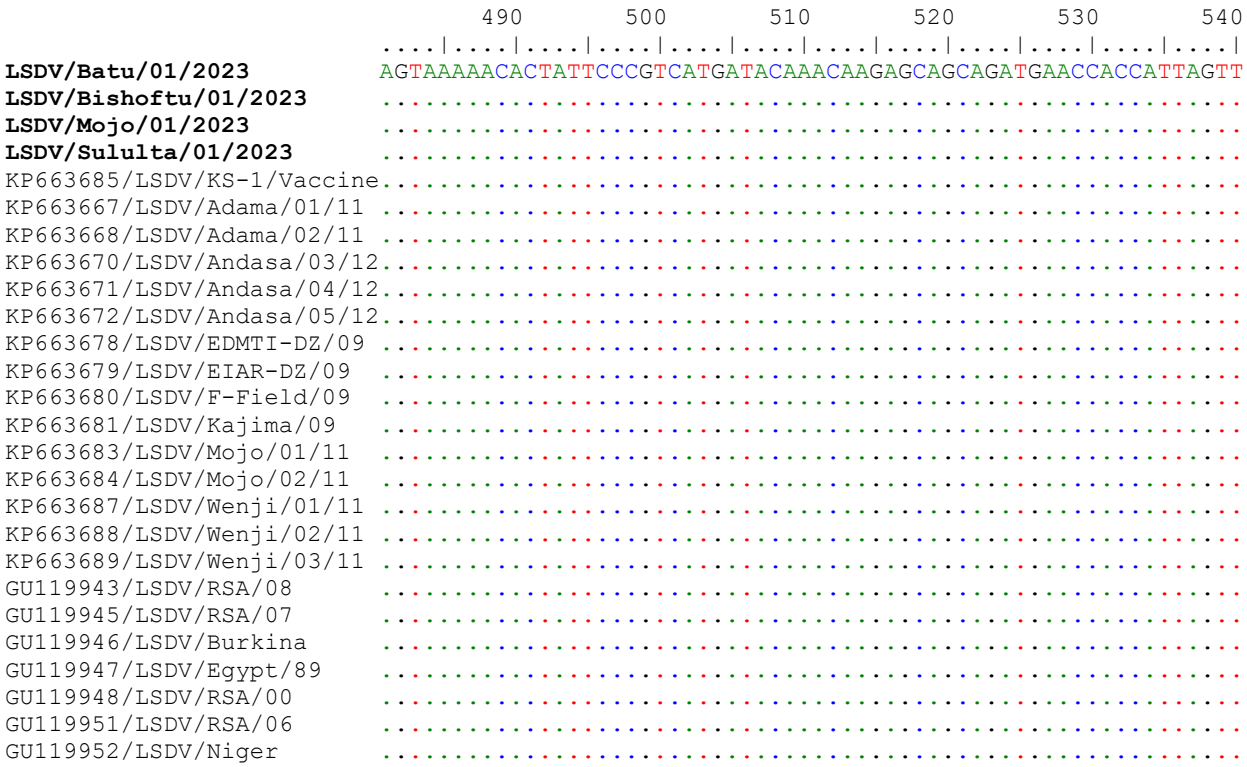
                                  310       320       330       340       350       360
                          ....|....|....|....|....|....|....|....|....|....|....|....|
LSDV/Batu/01/2023         TGTGATCTTATACGTACGACAAATGGAACAGAGAAAGAAATTTTAAGATATATACTTTTT
LSDV/Bishoftu/01/2023     ..............................................................
LSDV/Mojo/01/2023         ..............................................................
LSDV/Sululta/01/2023      ..............................................................
KP663685/LSDV/KS-1/Vaccine..............................................................
KP663667/LSDV/Adama/01/11 ..............................................................
KP663668/LSDV/Adama/02/11 ..............................................................
KP663670/LSDV/Andasa/03/12..............................................................
KP663671/LSDV/Andasa/04/12..............................................................
KP663672/LSDV/Andasa/05/12..............................................................
KP663678/LSDV/EDMTI-DZ/09 ..............................................................
KP663679/LSDV/EIAR-DZ/09  ..............................................................
KP663680/LSDV/F-Field/09  ..............................................................
KP663681/LSDV/Kajima/09   ..............................................................
KP663683/LSDV/Mojo/01/11  ..............................................................
KP663684/LSDV/Mojo/02/11  ..............................................................
KP663687/LSDV/Wenji/01/11 ..............................................................
KP663688/LSDV/Wenji/02/11 ..............................................................
```

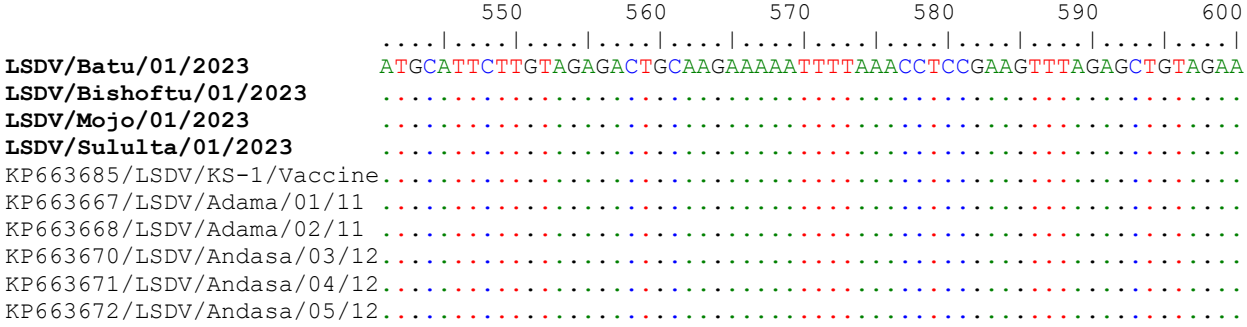

KP663689/LSDV/Wenji/03/11
GU119943/LSDV/RSA/08
GU119945/LSDV/RSA/07
GU119946/LSDV/Burkina
GU119947/LSDV/Egypt/89
GU119948/LSDV/RSA/00
GU119951/LSDV/RSA/06
GU119952/LSDV/Niger


370
380
390
400
410
420
LSDV/Batu/01/2023
GGAATAAAATGTGTTCAAAAAAATGTAGAATTCAATATAGACAATATTAGAGATATAAAT
LSDV/Bishoftu/01/2023
LSDV/Mojo/01/2023
LSDV/Sululta/01/2023
KP663685/LSDV/KS-1/Vaccine
KP663667/LSDV/Adama/01/11
KP663668/LSDV/Adama/02/11
KP663670/LSDV/Andasa/03/12
KP663671/LSDV/Andasa/04/12
KP663672/LSDV/Andasa/05/12
KP663678/LSDV/EDMTI-DZ/09
KP663679/LSDV/EIAR-DZ/09
KP663680/LSDV/F-Field/09
KP663681/LSDV/Kajima/09
KP663683/LSDV/Mojo/01/11
KP663684/LSDV/Mojo/02/11
KP663687/LSDV/Wenji/01/11
KP663688/LSDV/Wenji/02/11
KP663689/LSDV/Wenji/03/11
GU119943/LSDV/RSA/08
GU119945/LSDV/RSA/07
GU119946/LSDV/Burkina
GU119947/LSDV/Egypt/89
GU119948/LSDV/RSA/00
GU119951/LSDV/RSA/06
GU119952/LSDV/Niger


430
440
450
460
470
480
LSDV/Batu/01/2023
CACGAAGAATATTTTAATGTTTTAGATAAAAAGTATAACCTCCCATGCCCTGAGTGTAAA
LSDV/Bishoftu/01/2023
LSDV/Mojo/01/2023
LSDV/Sululta/01/2023
KP663685/LSDV/KS-1/Vaccine
KP663667/LSDV/Adama/01/11
KP663668/LSDV/Adama/02/11
KP663670/LSDV/Andasa/03/12
KP663671/LSDV/Andasa/04/12
KP663672/LSDV/Andasa/05/12
KP663678/LSDV/EDMTI-DZ/09
KP663679/LSDV/EIAR-DZ/09
KP663680/LSDV/F-Field/09


KP663681/LSDV/Kajima/09
KP663683/LSDV/Mojo/01/11
KP663684/LSDV/Mojo/02/11
KP663687/LSDV/Wenji/01/11
KP663688/LSDV/Wenji/02/11
KP663689/LSDV/Wenji/03/11
GU119943/LSDV/RSA/08
GU119945/LSDV/RSA/07
GU119946/LSDV/Burkina
GU119947/LSDV/Egypt/89
GU119948/LSDV/RSA/00
GU119951/LSDV/RSA/06
GU119952/LSDV/Niger


490 500 510 520 530 540
LSDV/Batu/01/2023 AGTAAAAACACTATTCCCGTCATGATACAAACAAGAGCAGCAGATGAACCACCATTAGTT
LSDV/Bishoftu/01/2023
LSDV/Mojo/01/2023
LSDV/Sululta/01/2023
KP663685/LSDV/KS-1/Vaccine
KP663667/LSDV/Adama/01/11
KP663668/LSDV/Adama/02/11
KP663670/LSDV/Andasa/03/12
KP663671/LSDV/Andasa/04/12
KP663672/LSDV/Andasa/05/12
KP663678/LSDV/EDMTI-DZ/09
KP663679/LSDV/EIAR-DZ/09
KP663680/LSDV/F-Field/09
KP663681/LSDV/Kajima/09
KP663683/LSDV/Mojo/01/11
KP663684/LSDV/Mojo/02/11
KP663687/LSDV/Wenji/01/11
KP663688/LSDV/Wenji/02/11
KP663689/LSDV/Wenji/03/11
GU119943/LSDV/RSA/08
GU119945/LSDV/RSA/07
GU119946/LSDV/Burkina
GU119947/LSDV/Egypt/89
GU119948/LSDV/RSA/00
GU119951/LSDV/RSA/06
GU119952/LSDV/Niger


550 560 570 580 590 600
LSDV/Batu/01/2023 ATGCATTCTTGTAGAGACTGCAAGAAAAATTTTAAACCTCCGAAGTTTAGAGCTGTAGAA
LSDV/Bishoftu/01/2023
LSDV/Mojo/01/2023
LSDV/Sululta/01/2023
KP663685/LSDV/KS-1/Vaccine
KP663667/LSDV/Adama/01/11
KP663668/LSDV/Adama/02/11
KP663670/LSDV/Andasa/03/12
KP663671/LSDV/Andasa/04/12
KP663672/LSDV/Andasa/05/12

```
KP663678/LSDV/EDMTI-DZ/09 ..................................................
KP663679/LSDV/EIAR-DZ/09  ..................................................
KP663680/LSDV/F-Field/09  ..................................................
KP663681/LSDV/Kajima/09   ..................................................
KP663683/LSDV/Mojo/01/11  ..................................................
KP663684/LSDV/Mojo/02/11  ..................................................
KP663687/LSDV/Wenji/01/11 ..................................................
KP663688/LSDV/Wenji/02/11 ..................................................
KP663689/LSDV/Wenji/03/11 ..................................................
GU119943/LSDV/RSA/08     ..................................................
GU119945/LSDV/RSA/07     ..................................................
GU119946/LSDV/Burkina    ..................................................
GU119947/LSDV/Egypt/89   ..................................................
GU119948/LSDV/RSA/00     ..................................................
GU119951/LSDV/RSA/06     ..................................................
GU119952/LSDV/Niger      ..................................................
```

```
                          605
                         ....|.
LSDV/Batu/01/2023        AAATAA
LSDV/Bishoftu/01/2023    ......
LSDV/Mojo/01/2023        ......
LSDV/Sululta/01/2023     ......
KP663685/LSDV/KS-1/Vaccine......
KP663667/LSDV/Adama/01/11 ......
KP663668/LSDV/Adama/02/11 ......
KP663670/LSDV/Andasa/03/12......
KP663671/LSDV/Andasa/04/12......
KP663672/LSDV/Andasa/05/12......
KP663678/LSDV/EDMTI-DZ/09 ......
KP663679/LSDV/EIAR-DZ/09  ......
KP663680/LSDV/F-Field/09  ......
KP663681/LSDV/Kajima/09   ......
KP663683/LSDV/Mojo/01/11  ......
KP663684/LSDV/Mojo/02/11  ......
KP663687/LSDV/Wenji/01/11 ......
KP663688/LSDV/Wenji/02/11 ......
KP663689/LSDV/Wenji/03/11 ......
GU119943/LSDV/RSA/08      ......
GU119945/LSDV/RSA/07      ......
GU119946/LSDV/Burkina     ......
GU119947/LSDV/Egypt/89    ......
GU119948/LSDV/RSA/00      ......
GU119951/LSDV/RSA/06      ......
GU119952/LSDV/Niger       ......
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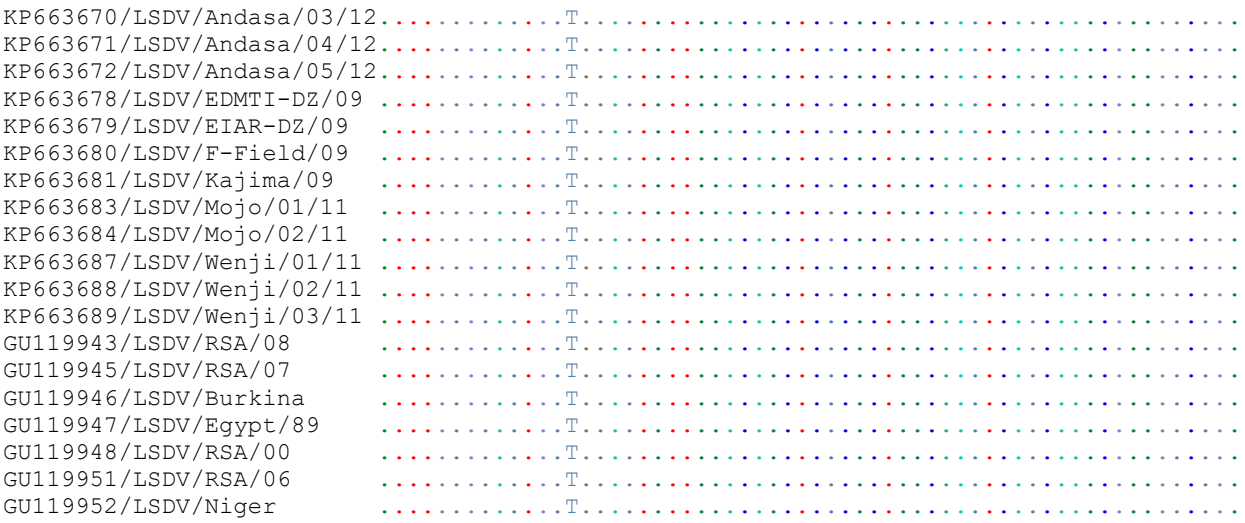
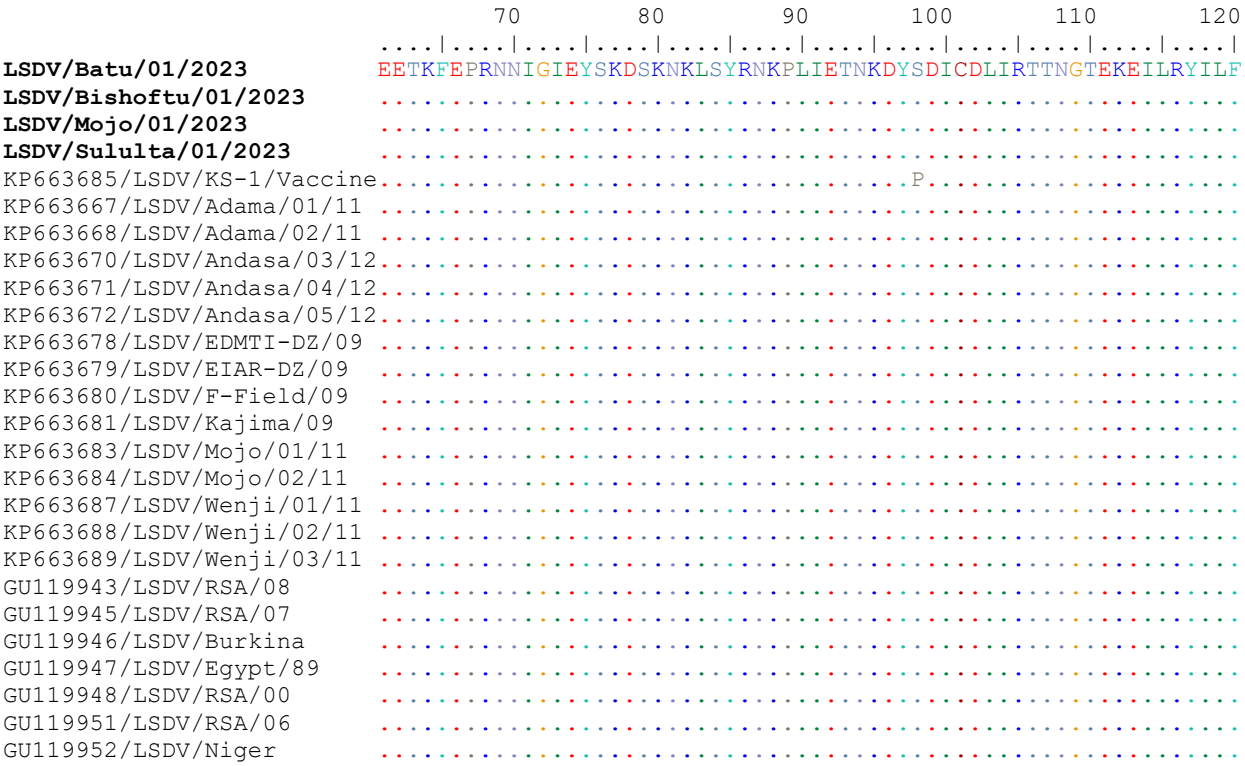
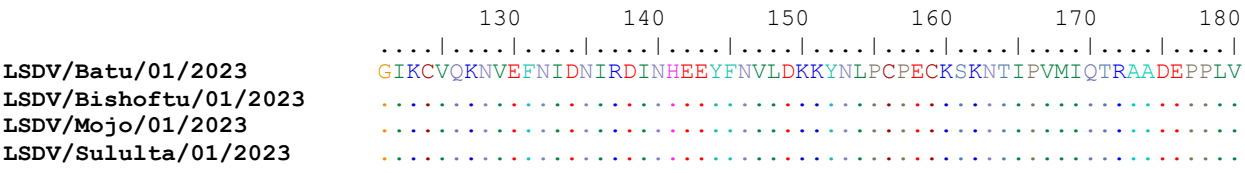
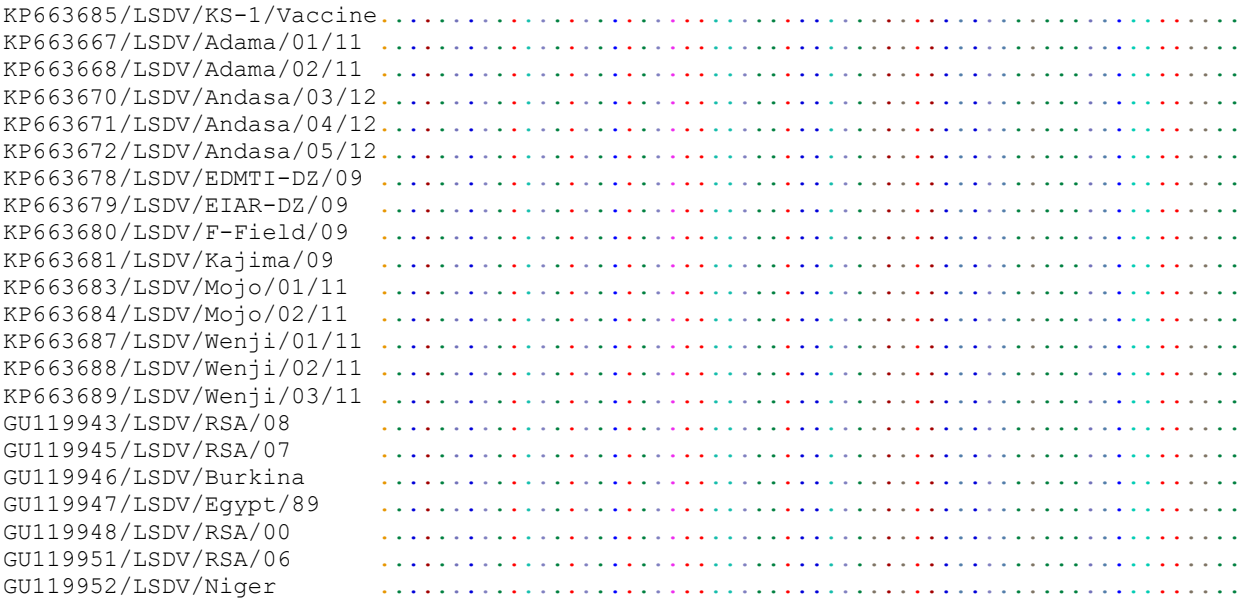
Annex 7: LSDV RPO30 gene amino acid sequence alignment results

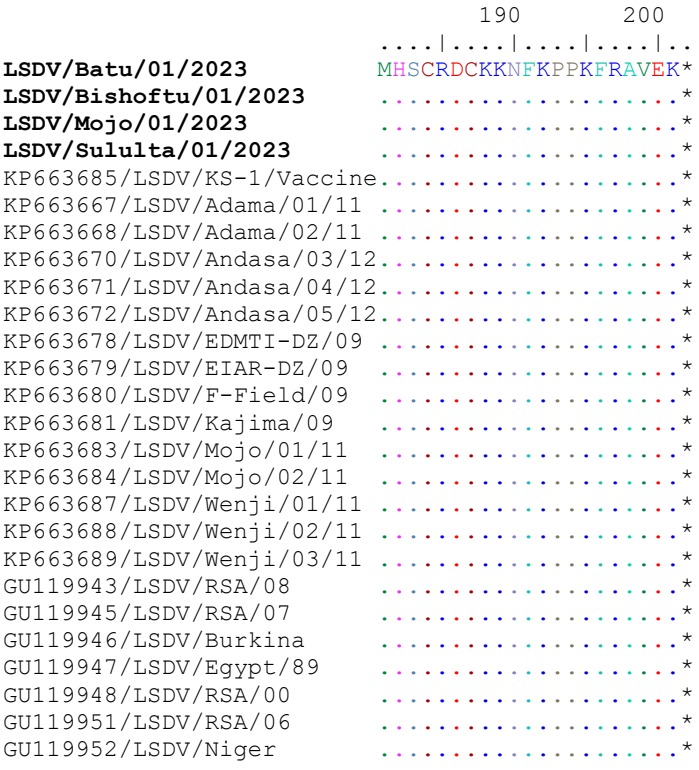
```
                                  10        20        30        40        50        60
                         ....|....|....|....|....|....|....|....|....|....|....|....|
LSDV/Batu/01/2023        MDDDNTNSYSDNTNPTYQDIEDIIYKYVKEKSKVKEILKWATDKASKFYIRNIINTKSNI
LSDV/Bishoftu/01/2023    ............................................................
LSDV/Mojo/01/2023        ............................................................
LSDV/Sululta/01/2023     ............................................................
KP663685/LSDV/KS-1/Vaccine............T...........................................
KP663667/LSDV/Adama/01/11 ............T...........................................
KP663668/LSDV/Adama/02/11 ............T...........................................
```


KP663670/LSDV/Andasa/03/12
KP663671/LSDV/Andasa/04/12
KP663672/LSDV/Andasa/05/12
KP663678/LSDV/EDMTI-DZ/09
KP663679/LSDV/EIAR-DZ/09
KP663680/LSDV/F-Field/09
KP663681/LSDV/Kajima/09
KP663683/LSDV/Mojo/01/11
KP663684/LSDV/Mojo/02/11
KP663687/LSDV/Wenji/01/11
KP663688/LSDV/Wenji/02/11
KP663689/LSDV/Wenji/03/11
GU119943/LSDV/RSA/08
GU119945/LSDV/RSA/07
GU119946/LSDV/Burkina
GU119947/LSDV/Egypt/89
GU119948/LSDV/RSA/00
GU119951/LSDV/RSA/06
GU119952/LSDV/Niger


70
80
90
100
110
120
LSDV/Batu/01/2023
EETKFEPRNNIGIEYSKDSKNKLSYRNKPLIETNKDYSDICDLIRTTNGTEKEILRYILF
LSDV/Bishoftu/01/2023
LSDV/Mojo/01/2023
LSDV/Sululta/01/2023
KP663685/LSDV/KS-1/Vaccine
KP663667/LSDV/Adama/01/11
KP663668/LSDV/Adama/02/11
KP663670/LSDV/Andasa/03/12
KP663671/LSDV/Andasa/04/12
KP663672/LSDV/Andasa/05/12
KP663678/LSDV/EDMTI-DZ/09
KP663679/LSDV/EIAR-DZ/09
KP663680/LSDV/F-Field/09
KP663681/LSDV/Kajima/09
KP663683/LSDV/Mojo/01/11
KP663684/LSDV/Mojo/02/11
KP663687/LSDV/Wenji/01/11
KP663688/LSDV/Wenji/02/11
KP663689/LSDV/Wenji/03/11
GU119943/LSDV/RSA/08
GU119945/LSDV/RSA/07
GU119946/LSDV/Burkina
GU119947/LSDV/Egypt/89
GU119948/LSDV/RSA/00
GU119951/LSDV/RSA/06
GU119952/LSDV/Niger


130
140
150
160
170
180
LSDV/Batu/01/2023
GIKCVQKNVEFNIDNIRDINHEEYFNVLDKKYNLPCPECKSKNTIPVMIQTRAADEPPLV
LSDV/Bishoftu/01/2023
LSDV/Mojo/01/2023
LSDV/Sululta/01/2023


KP663685/LSDV/KS-1/Vaccine
KP663667/LSDV/Adama/01/11
KP663668/LSDV/Adama/02/11
KP663670/LSDV/Andasa/03/12
KP663671/LSDV/Andasa/04/12
KP663672/LSDV/Andasa/05/12
KP663678/LSDV/EDMTI-DZ/09
KP663679/LSDV/EIAR-DZ/09
KP663680/LSDV/F-Field/09
KP663681/LSDV/Kajima/09
KP663683/LSDV/Mojo/01/11
KP663684/LSDV/Mojo/02/11
KP663687/LSDV/Wenji/01/11
KP663688/LSDV/Wenji/02/11
KP663689/LSDV/Wenji/03/11
GU119943/LSDV/RSA/08
GU119945/LSDV/RSA/07
GU119946/LSDV/Burkina
GU119947/LSDV/Egypt/89
GU119948/LSDV/RSA/00
GU119951/LSDV/RSA/06
GU119952/LSDV/Niger


190
200
LSDV/Batu/01/2023
LSDV/Bishoftu/01/2023
LSDV/Mojo/01/2023
LSDV/Sululta/01/2023
KP663685/LSDV/KS-1/Vaccine
KP663667/LSDV/Adama/01/11
KP663668/LSDV/Adama/02/11
KP663670/LSDV/Andasa/03/12
KP663671/LSDV/Andasa/04/12
KP663672/LSDV/Andasa/05/12
KP663678/LSDV/EDMTI-DZ/09
KP663679/LSDV/EIAR-DZ/09
KP663680/LSDV/F-Field/09
KP663681/LSDV/Kajima/09
KP663683/LSDV/Mojo/01/11
KP663684/LSDV/Mojo/02/11
KP663687/LSDV/Wenji/01/11
KP663688/LSDV/Wenji/02/11
KP663689/LSDV/Wenji/03/11
GU119943/LSDV/RSA/08
GU119945/LSDV/RSA/07
GU119946/LSDV/Burkina
GU119947/LSDV/Egypt/89
GU119948/LSDV/RSA/00
GU119951/LSDV/RSA/06
GU119952/LSDV/Niger