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ISOLATION AND MOLECULAR CHARACHTERIZATION OF LUMPY SKIN DISEASE VIRUS IN CENTRAL PART OF ETHIOPIA

MSc. THESIS

ADDIS ABABA UNIVERSITY COLLEGE OF VETERINARY MEDICINE AND AGRICULTURE DEPARTMENT OF MICROBIOLOGY, IMMUNOLOGY AND VETERINARY PUBLIC HEALTH

BY

MIHIRET SHIMELIS

JUN, 2022

BISHOFTU, ETHIOPIA

ISOLATION AND MOLECULAR CHARACHTERIZATION OF LUMPY SKIN DISEASE VIRUS IN CENTRAL PART OF ETHIOPIA

MSc. THESIS

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

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Addis Ababa University

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As member of the Examining Board of the final MSc open defense, we certify that we have read and evaluated the Thesis entitled: "**Isolation and Molecular Characterization of Lumpy Skin Disease Virus in Central Part of Ethiopia**" we recommended that it be accepted as fulfilling the thesis requirement for the degree of Master of Science in Veterinary Microbiology.

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LIST OF ABBREVIATIONS

CaPV	Capripox virus
CO_2	Carbon dioxide
CPE	Cytopathic effect
DMEM	Dulbecco's Modified Eagle Medium
DNA	Deoxyribonucleic acid
ELISA	Enzyme linked immunosorbent assay
FAO	Food and Agriculture organization
FCS	Fetal calf serum
GMEM	Glasgow Minimum Essential Media
GPCR	G- protein coupled receptor
GTPV	Goat pox virus
IPMA	Immunoperoxidase monolayer assay
Kbp	Kilobase pair
KS-1	Kenyan sheep pox virus strain
LSD	Lumpy skin disease
LK	Lamb kidney
LT	Lamb testis
LSDV	Lumpy skin disease virus
MEGA	Molecular Evolutionary Genetics Analysis
OIE	Office International des Epizooties
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
RM 65	Ramyar strain
SNPs	Single nucleotide polymorphisms
SNT	Serum Neutralization Test
SPF-ECE	Specific pathogen free embryonated chicken egg
SPPV	Sheep pox virus
USA	United states of America
VNT	Virus neutralization test
VTM	Virus transport medium

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ABSTRACT

Lumpy skin disease is economically important disease that is currently being reported as an emerging disease in various countries. Since, its inception in Ethiopia, LSD has spread in all the geographic locations affecting the livestock production system. Lumpy skin disease which cause lumpy skin disease is of the *capripoxviruses*, which is classified under the family *poxviridae*. Despite, efforts to contain by vaccination there have been several outbreaks of the disease in the country. These elucidate the importance of continual studies emphasizing on the viral genetics and associated factors. Thus, in this study lumpy skin disease virus was studied aimed at the virus isolation and molecular characterization from the outbreak reported areas of central Ethiopia. The study took place between October 2021 and May 2022. Purposive sampling technique was used when collecting Skin nodular samples and whole blood for serological assay. The virus was isolated using lamb kidney cells and molecularly characterized by amplifying the DNA fragment using polymerase chain reaction (PCR) and sequence analysis using the complete RPO30 gene 606bp analysis. Multiple Sequence alignment indicated the nucleotide and amino acid variations among the capripoxvirus and to other viral isolates basically with the vaccine isolate. The alignment result indicated the absence of unique variation between the current field isolates and previous isolates. However, a single nucleotide variation was found between the outbreak isolates and the vaccine strain T/C at nucleotide position 292. All in all, the study in line with the veterinary professional's vaccination practices gave an emphasis that outbreaks of the disease have continued to be a problem in different areas and the need of continual advanced studies.

Keywords: *Capripoxviruses, LSDV, Molecular characterization, RPO30 gene*

1. INTRODUCTION

Livestock production accounts for nearly 19% of Ethiopia's GDP. Ruminants, in particular, make up the majority of the national herd and provide an important source of income for smallholder farmers (Tsouloufi *et al.*, 2020). The size of the country's livestock herd, one of Africa's largest, makes it a resource with the potential to significantly contribute to national development, including poverty reduction (Shapiro *et al.*, 2017). The total cattle population is estimated in Ethiopia to be about 65.35 million (CSA, 2020). However, infectious diseases impede livestock production, having a significant impact on productivity, food security, and international trade provided by the livestock sector (Gizaw *et al.*, 2021). Livestock diseases are known to be the major production constraint in Ethiopia in addition to other contributing factors. Among the economic important diseases that are known to pose a risk in the livestock sector, lumpy skin disease is one of the most important viral diseases in cattle.

Lumpy skin disease has been listed as a notifiable disease by World Organization for Animal Health (OIE) due to its rapid transboundary spread and ability to cause significant cattle production losses (Tuppurainen *et al.*, 2018a). It originated in Africa, Zambia in1929 and has spread over the Middle East, Eastern Europe, and Asia. It is economically important, transboundary, and an emerging disease of cattle with the highest effect in Africa's livestock sector (Sprygin *et al.*, 2019; Leliso *et al.*, 2021; Mikhael *et al.*, 2021). LSD and the two *Capripoxviruses* are the most serious of all domestic animal pox diseases resulting in significant economic loss and high mortality in a young and immunologically naive animal.

LSD is caused by the LSD virus a member of *Capripoxviruses* (CaPVs) having a large double-stranded DNA virus classified under the family poxviridae. Recent taxonomic classification of the poxviridae tells that there are two sub-family that are the *Chorodapoxvirinae* and *Entomopoxvirinae,* 22 genera and 83 species of the virus. Genus *Capripoxvirus* comprises three highly related poxviruses namely, SPPV, GTPV and LSDV. The genome of LSD is 156 kbp long which shares genomic similarities with other poxviruses (Tulman *et al*., 2001). LSDV shares 96-97% genome similarity with the other *Capripoxviruses*. Because this high genetic identity to the other *Capripoxviruses,* it is indistinguishable from them in serological tests (Maclachlan and Dubovi, 2017). CaPVs are also particularly different in their geographic distribution SPPV and GTPV are found in parts of Africa north of the Equator, as well as parts of the Middle East and Asia, including India and China. On the other hand, LSD was initially

recognized in an outbreak in Zambia in 1929 and it expanded to other areas including Egypt, Israel and different regions of the middle east beyond its endemic occurrence in sub-Saharan (Maclachlan and Dubovi, 2017).

The transmission of LSDV, SPV, and GPV is not fully understood, especially in endemic settings (Limon *et al.*, 2020). Blood-feeding insect vectors feeding on cattle and switching hosts induce the virus in the other host. When naive cattle are transferred to LSDV-infected properties after the virus has been stamped out, they get infected within a week or two, demonstrating that the virus lingers in vectors, the environment, or both (Tuppurainen *et al.*, 2017). In an outbreak, the LSDV DNA was detected by PCR in insects and ticks *Dermacentor marginatus* and *Hyalomma asiaticum*, as well as horseflies (*Tabanus promise)* biting flies *Stomoxys calcitrans* (Orynbayev *et al.*, 2021). This makes mechanical transmission to be the most common route of infection. The primary vector might be altered depending on climate, season, temperature, humidity, and vegetation (FAO, 2017). However, for the first time, indirect contact-mode transmission for a naturally occurring recombinant LSDV isolated from the field was detected. This was performed in an insect-proof facility and provided that there is strong evidence that the novel LSDV strain can pass to in-contact animals (sprying *et al.*, 2020a).

In Ethiopia, the first outbreak of LSD dates back to the year between 1981 and 1983 in the northwestern, western, and central regions of the country (Mebratu *et al.*, 1984). Since then the disease has spread to almost all regions and agro-ecological zones of the country(Ayelet *et al.*, 2014). Agro-climate, communal grazing/watering management, and animal importation are all likely to have an impact on the distribution and prevalence of LSD in Ethiopia (Gumbe, 2018).

In the Ethiopian context where other disease transmission controls like the stamping out are not applied, controlling LSD through substantial vaccinations with safe and effective vaccines is the best strategy. Unfortunately, incursions have been reported despite vaccinations in different parts of areas where the disease became endemic (Bamouh *et al.*, 2021). Regardless of KS-1O-180 immunization status, the disease was observed, which may be due to a lack of cross-protection of the KS-1 vaccine strain against circulating virulent field strains, this vaccine failure is a barrier to effective control of LSD (Ayelet *et al.*, 2014; Gari *et al.*, 2015). In addition, in a field pattern, the KS1 O-180 couldn’t be successful both in direct clinical

protection and reducing transmission because transmission of the virus was detected both in vaccinated and non-vaccinated animals (Molla *et al.*, 2017).

Although LSD is an endemic disease in East Africa, reports on the detailed understanding of the virus at the molecular level are still yet (Chibssa *et al.*, 2021). In general, the disease's recurrence, reappearance and expansion in various parts of the world highlighted the importance of re-evaluating disease biology, viral transmission mechanisms, and updated preventive and adaptive control techniques (Das *et al.*, 2021).

Vaccine failure, inability to protect cattle from LSD outbreak, the virulence effect of the live vaccine, and the recombinant virus of vaccine strain with the wild strain that is genetically distinct from the vaccine or the virus strain are all recent breakthrough reports contributing to vaccine failure. Thus, these all give an implication of a study required to investigate the viral strain responsible for the disease outbreak happening during various time.

The above facts are also true for Ethiopian contexts as a result, subsequent surveillance and genetic studies of this virus are required to get a robust vaccine and diagnostics for Lumpy skin disease. Therefore, this study's general objective is to investigate the lumpy skin disease virus through genotyping of the viral gene (RPO30) of virulent field strains and antibody detection from expected outbreaks in the central part of Ethiopia.

The objectives of the study were:

- To isolate LSDV from affected cattle
- To detect and Molecularly characterize the isolated LSDV isolates based on RPO30 gene
- To explore the efficacy of LSDV vaccine and vaccination practices of animal health professionals in the study area

1. LITERATURE REVIEW

1.1. History

LSD being first discovered in Africa was distinct as an African disease and it was annotated by different terms associated with the clinical symptoms. When LSD was first discovered in a region known as Northern Rhodesia in 1929, the etiology of the disease was known as "Pseudo-urticaria"(Weiss, 1968). The disease was considered a case of poisoning or hypersensitivity reaction to insect bites as per the abundance of biting insects at that time of year (Das *et al.*, 2021). Later on, a skin disease with clinical symptoms of skin nodules and lymphadenitis was called Ngamiland cattle disease for short time. The disease was spread to the Ngamiland, Bechuanal and Protectorate in 1943 (Botswana) (Backstrom, 1944).

Furthermore, the introduction of the disease in East Africa was first reported in Kenya in 1957 in Aonarm. Apart from the presence of high mosquito infestation, the source of the infection was unknown. Moreover, the disease was confined to calves and was fair frequently occurring on different farms despite quarantine and sanitary management taken (Macowen, 1959). The disease was reported in other countries in East Africa including Ethiopia. In 1971 Sudan reported the first incursion of the disease in the western part which then spread to the eastern part of Sudan. The cattle population where the disease was observed had heavy tick infestations (Ali and Obeid, 1977). The first record of LSD in Ethiopia was in 1981 and 1983 in the northwestern, western, and central regions of the country. Clinical observation, virus isolation and identification through electron microscopy were used the diagnosis the disease (Mebratu *et al.*, 1984).

Beginning from its discovery the first LSD was recognized as Pseudo uretrica in Zambia (MacDonald, 1931). However, it spread into Egypt and Israel, with recent incursions into other Middle Eastern countries and South East Asia, which was free of LSD until the first outbreak recorded in Bangladesh in 2019 and the transboundary spread to various places and some parts of Europe made clear how much it grabbed attention. And To this day Capripoxviruses are not present in the Americas (Maclachlan, 2017; Das *et al.*, 2021).

Since, the first observation of the disease in Zambia in 1929, LSD was largely a sub-Saharan endemic disease in the past century, and was thought to be restricted to Africa or if it spreads it was assumed to be easily contained (Tuppurainen *et al.*, 2018a). However, it has spread

progressively and extensively throughout Africa, the Middle East, Southeastern Europe, Central Asia, and more recently South Asia and China. Currently, the disease is endemic in several countries across Africa, parts of the Middle East (Iraq, Saudi Arabia, Syrian Arab Republic), and Turkey (FAO, 2020). In addition, the disease has spread to the middle east (Tuppurainen *et al.*, 2017). Currently, there is an increased risk of LSD reaching Central Asia, Western Europe and Central-Eastern Europe. And this rapid spread of LSDV into new geographical locations outside of Africa where it was restricted had implied its high economic importance. The disease emerged as a significant epizootic pathogen in 2012 (Chibssa *et al.*, 2021).

1.2. Lumpy skin disease virus

The LSDV belongs to the genus *Capripoxvirus (*CaPV) within the *Poxvirida*e family and shares high antigenic similarities with the sheeppox virus (SPPV) and the goatpox virus (GTPV). In general, *Capripoxvirus (*CaPV) are closely related to each other but phylogenetically distinct. The genome identity between LSDV and other groups of the poxvirus lies in the central part of the whole viral genome whereas their similarity disrupts at the two (right and left) ends. The large, double-stranded DNA virus is very stable, with minor genetic variation (Tulman *et al*., 2001; Kara *et al.*, 2003; Tuppurainen *et al.*, 2017). The genome of LSD is 156 kbp long having 156 putative genes in which it shares genomic similarities to the other *Capripoxviruses*. These putative genes in LSD are 156 ORF which represents a 95% coding density encoding proteins of 53 to 2025. The genes encode proteins responsible for the viral Nucleic acid biogenesis, virion structure, and virion assembly. They are involved in rudimentary replicative mechanisms, including genes encoding RNA polymerase subunits, mRNA transcription, initiation, elongation, and termination factors, and enzymes that direct post-transcriptional processing of viral mRNA. Other genes encode for proteins involved in virion morphogenesis and assembly. These include proteins found in the virion core, intracellular mature virus (IMV) proteins, and associated membranes.

In addition, LSDV comprises several potential host range genes that are likely to be involved in host immune response modulation or evasion, host cell apoptosis modulation or inhibition, and cell and/or tissue tropism. Though the LSDV gene shares a gene similar to that of another gene in the host range, they encode a unique complement gene which implies its specific host range properties. Others encode for proteins responsible for disruption or modulation of host immune

responses. The virus also contains four potentially membranes localized, immunomodulatory proteins. Furthermore, LSDV encodes proteins that influence virus virulence, virus growth in specific cell types, and/or cellular apoptosis (Tulman *et al.*, 2001).

The *Capripoxvirus* virions are brick-shaped with a size of 300×270×200 nm. The virions contain over 100 polypeptides that are organized into a core, two lateral bodies, a membrane, and an envelope. The membrane and envelope are important structures for the interaction with the host cell. Mature virions that exit the cell without causing cell disruption are enveloped. The envelope is made up of two layers of cellular lipids and several virus-specific polypeptides. Whereas, the virions released by the rupture of the host cell are not enveloped (Tuppurainen, 2005).

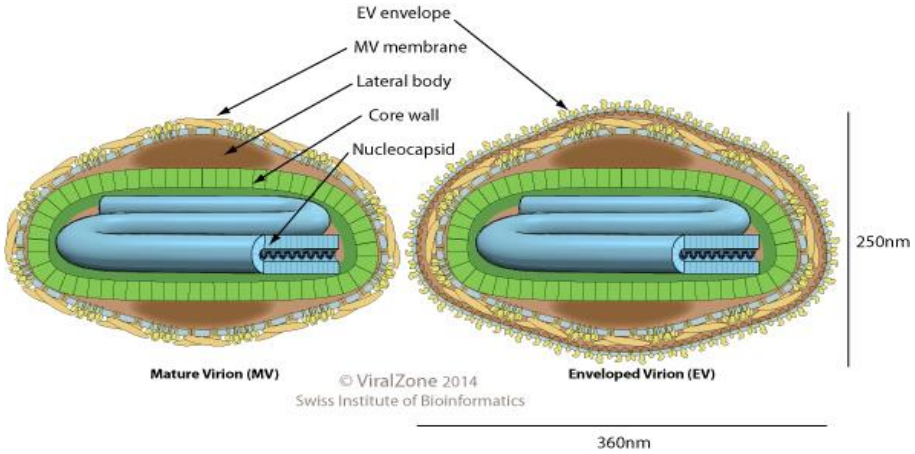

Figure 1: General Structure of Capripoxviruses;

Source: *https://viralzone.expasy.org/152(accessed 20/Jan/ 2022)*

1.3. LSDV virus replication

The principal viral replication takes place in the cytoplasm, unlike other DNA viruses. Poxvirus entry into cells occurs by fusion of the viral membrane(s) with plasma or endosomal membrane, in a process involving actin dynamics, cell signaling, and at least ten viral proteins. The mature virion (MV) membrane fuses with the cell membrane, enabling the core to enter the cytoplasm and start gene expression. Then, a newly identified group of viral protein components of the MV membrane mediates fusion. MV entry fusion proteins are required for a virus-induced cell-to-cell spread compelling the disruption of the membrane wrapper of

extracellular virions before fusion. In addition, the same group of MV entry/fusion proteins are required for the virus-induced cell-cell fusion(Moss, 2006; Maclachlan and Dubovi, 2017).

Following the fusion of the virus membrane to the surface of the host cell, cascades of the temporal patterns take place for the transcription of the genes. These genes are transcribed in the Early, intermediate and late phases of the temporal pattern. The genes are also called early, intermediate and late. Minutes post-infection early genes are transcribed in the cytoplasm by viral RNA polymerase as they are instigated by the viral transcriptase and other factors carried in the virion core that mediates RNA production. And the core is uncoated at the end of the early gene expression letting the viral genome free in the cytoplasm (Maclachlan and Dubovi, 2017).

Expression of the intermediate genes triggers genomic DNA replication following the release of the genome from the core and progeny DNA serves as the template for intermediate- and late-phase genes transcription. Intermediate genes encode transcription factors that are required for late gene transcription. Late genes encode virion proteins as well as early transcription factors that are packaged in virions and used early in subsequent rounds of infection producing all the structural proteins. Progeny virions are assembled in cytoplasmic viral factories, resulting in a spherical immature particle. This virus particle develops into a brick-shaped intracellular mature virion (IMV). The intracellular mature virion (IMV) virion can be released when a cell is lysed, or it can acquire a second double membrane from the trans-Golgi and bud as an external enveloped virion (EEV), Enveloped virions are released by exocytosis and the ones released by budding or cell lysis are the mature virions which don't have an additional membrane (MVs) (Maclachlan, 2017; Tuppurainen *et al.*, 2018a).

1.4. Physicochemical properties of LSDV

Lumpy skin disease virus is remarkably stable between pH 6.6 and 8.6 and will show no significant reduction in titer after exposure for 5 days at 37°C within the pH range mentioned above (Weiss, 1968). The virus is susceptible to ether (20%), chloroform (1%), formalin (1%), and some detergents, such as sodium dodecyl sulphate. The virus in dried scabs, on the other hand, can survive for long periods at room temperature. It can be found in necrotic skin lesions for up to 33 days or longer, desiccated crusts for up to 35 days, and air-dried hides for at least 18 days due to its resistance to inactivation. It can survive in the environment for a long time.

The virus is sensitive to sunlight and detergents containing lipid solvents, and it persists in exposed areas (OIE, 2017).

1.5. Epidemiology of LSD

Environmental determinants (Risk factors) play a great role in the epidemiology of LSD. The various determinants including agent, host, and vectors as well as the interaction between them play a pivotal role in the distribution and spread of the viral disease. These predisposing factors have a great role in the maintenance of the arthropod vector and transmission of the virus to susceptible animals (Degu, 2020).

The reoccurrence of the disease is consistently associated with high rainfall, the emergence of large numbers of vectors and a low level of herd immunity (Molla *et al.*, 2017). The distribution and occurrence of LSD in Ethiopia are considered to be influenced by three major factors, these are, the effect of agro-climate, communal grazing/watering management and the introduction of new animals (Tamire, 2022). In Midland agro-climate, herd-level LSD prevalence was higher than in highland and lowland agro-climate zones (Gari *et al.*, 2010). The spatial pattern of the incidence of the disease is sporadic and its endemicity is dependent on the management and immunological status of the area (Molla *et al.*, 2017).

2.5.1 Occurence of Lumpy skin disease

Northern Rhodesia was the first to report the disease in 1929 (MacDonald, 1931). This was followed by Southern Rhodesia and It had been recognized in East Africa since its declaration in Sudan in 1971, in Niger and Chad in 1973, and in Nigeria in 1974 (Mebratu *et al.*, 1984). Then the disease was identified in Egypt and Israel with the assumption to be carried to Israel through merchants. The disease was confined to Africa for quite some time, however, it had spread and became endemic to the Middle East, the Balkans, the Caucasus and the southern Russian Federation in 2015 (Tuppurainen *et al.*, 2017). The outbreaks of the disease have been associated with the distribution of biting flies that play a role as vectors in the mechanical transmission of the disease.

Following the initial introduction in the north-west (Gojjam and Gondar) in 1981 and the west in 1982, LSD spread to the country's east, followed by all other regions and agro-climatic areas in Ethiopia (Mebratu *et al.,* 1984). Agro-climate, communal grazing/watering management,

and animal importation are all likely to have an impact on the distribution and prevalence of LSD in Ethiopia (Gumbe, 2018). Outbreaks of the disease reach their highest after the annual wet season and occur throughout the country, with the midland agro-ecological zone having the highest prevalence (Ayelet *et al*., 2014).

2.5.2. Hosts

LSDV is highly host-specific causing diseases only in cattle (*Bos indicus* and *B. taurus*) and water buffalo (*Bubalus bubalis*), even though the susceptibility varies among the breeds and buffalo have lower mortality and morbidity than cattle. LSDV was able to be isolated from naturally infected giraffes (Dao *et al.*, 2022). There is not much evidence of an LSDV wildlife reservoir; a wildlife case in a single eland was found for the first time where LSDV DNA was detected, however neutralizing antibodies to the virus have been evaluated to be low in various wild animal species (Molini *et al.*, 2021). Therefore, from the generally negative results obtained it was disclosed that wildlife in Africa probably does not play a very important part in the preservation and dissemination of LSDV, the antibody tested animals included kudu (*Tragelaphus strepsiceros*), waterbuck (*Kobus ellipsiprymnus*), Reedbuck and Springbok although in Giraffe and Reedbuck (*Antidorcas marsupialis*) virus neutralizing titers were of a similar status to that of the convalescent cattle and was assumed to be indicative of past infection (OIE, 2017).

Several studies in Ethiopian regions revealed differences in immunity among affected animals of different breeds, sexes, and ages. Which, cross breeds had significantly higher seroprevalence than local zebu cattle, and there was a significant difference between age groups (Abera *et al.*, 2015). Moreover, the production system and the type of breed play a role in determining the susceptible host. In this regard, Exotic and cross-breed cattle have been observed to be susceptible to the disease in which intensive farms become severely affected in an outbreak when compared to the production system in crop-livestock(mixed farming) system (Molla *et al.*, 2017b). The different geographical distribution of LSD between sheep and goat pox implies that cattle strains of *Capripoxvirus* do not infect and transmit between sheep and goats (OIE, 2021). Susceptibility of the host to CaPVs hangs on several factors, including the virulence of the virus and the immune status, age, and, breed of the host. Still, some LSDV strains may replicate in sheep and goats. Although diverse herds of cattle, sheep, and, goats are

common, no epidemiological evidence on the role of small ruminants as a reservoir for LSDV has been reported (Tuppurainen *et al.*, 2017).

Local zebu cattle, on the other hand, were significantly susceptible to LSD in a study, which could be due to the stress on the animal as they are used for draft power in Agricultural practices. The fact that they are unable to protect themselves from biting flies attracted to the skin scratches due to the harness yokes exposes them to the biting flies (Gari *et al.*, 2011). The disease also has morbidity higher in young cattle of less than two years than in other ages and female animals showed significant Susceptibility to male animals (Leliso *et al.*, 2021).

1.5.1. Sources of the virus

The main Sources of LSD virus are skin nodules, scabs and crusts which contain relatively high amounts of LSDV. The virus can be isolated from ocular and nasal discharges, saliva, and semen (Babiuk *et al.*, 2008; OIE, 2017). LSDV is very stable in the clinical sample and survives well on farm premises.

Infectious LSDV is well-sheltered within crusts, especially when they fall away from skin lesions. Contaminated natural or farm environments will likely remain contaminated for a long time if they are not thoroughly cleaned and disinfected. When naive cattle are transferred to LSDV-infected properties after the virus has been stamped out, they get infected within a week or two, demonstrating that the virus lingers in vectors, the environment, or both (Tuppurainen *et al.*, 2017).

1.5.2. Transmission

Due to cattle migrations, the virus may be able to spread over large areas. Furthermore, short-distance jumps are induced by a large number of local blood-feeding insect vectors feeding on cattle and switching hosts frequently between feeds, equating to how far insects can fly (usually 50 km) (Tuppurainen *et al.*, 2017). This makes Mechanical transmission to be the most common route of infection. Moreover, in different regions, climate, season, temperature, humidity, and vegetation are likely to alter the primary vector (FAO, 2017).

The transmission dynamics also can be accompanied by the farm status if the farm had a history of the disease outbreak and if not cleaned thoroughly, the contaminated environment

remains contaminated for a long time. And will be a premise for a new outbreak, when naïve animals are transferred to it (Tuppurainen *et al.*, 2017).

Arthropod vectors are thought to be the primary mode of transmission. Mosquitoes, *Culex mirificens* and *Aedes natrionus*, biting flies, *Stomoxys calcitrans* and *Biomyia fasciata*, and male ticks, *Riphicephalus appendiculatus* and *Amblyomma hebraeum*, may also be involved in the virus's transmission. The importance of different arthropod vectors is likely to vary in different areas depending on the abundance and feeding behavior of the vector (OIE, 2017). Vector abundance due to seasonal changes is the predominant cause of seasonality of vector-borne diseases (Molla *et al.*, 2017)

However, for the first time, indirect contact-mode transmission for a naturally occurring recombinant LSDV isolated from the field was detected. This was done in an insect-proof facility and provided that there is strong evidence that the novel LSDV strain can pass to in-contact animals (Sprygin *et al.*, 2020a).

1.6. Pathogenesis and clinical signs

CaPVs have a specific keratinocyte tropism. Hyperplasia and ballooning degeneration of stratum spinosum keratinocytes, development of epidermal microvesicles, and infiltration of inflammatory cells into the dermis are all characteristics of skin lesions. In lumpy skin disease, epidermal microvesicles coalesce into large vesicles that quickly ulcerate and may become nodules between 0.5 and 3 cm in diameter (Maclachlan and Dubovi, 2017; Limon *et al.*, 2020).

In an experiment where cattle infected with the LSDV developed a localized swelling at the site of inoculation and enlargement of the regional lymph nodes appeared after four to seven days, while generalized skin nodules occurred after seven to nineteen days after inoculation(Yimer, 2021). Various changes occur following the viral disease occurrence in infected cattle. From which, biochemical alterations occurred in LSD-affected calves where the mean leukocyte count; lymphocyte and monocyte count in the infected calves were recorded when compared to the normal non-diseased cattle. Total protein, total creatinine kinase, aspartate aminotransferase, blood urea nitrogen, creatinine and potassium levels were also recorded higher while cholesterol levels were lower. These indicated that there is a severe inflammatory process associated with a natural cause of the disease (Rouby, 2021).

In infected cattle the clinical observation started seven days post-inoculation; measurable viremia was found on day six post-inoculation as confirmed with PCR. The clinical manifestation of LSD in cattle is characterized by fever, followed by the development of circumscribed nodular lesions in the skin that can cover the entire body, deep scab formation, swelling of the dewlap and oedema of the limbs and generalized lymphadenitis are prevalent (Brenner *et al.*, 2009). In the early stages, affected cattle have lacrimation, nasal discharge, and loss of appetite. Ruptured necrotic wounds may result from a complication of secondary infection (Maclachlan and Dubovi, 2017; Leliso *et al.*, 2021). Also, the affected cattle have multiple urticarial lesions and the nodules are distributed on the legs, perineum, scrotum and ears and mostly on the back and neck (Pandey *et al.*, 2022). A state of the disease was investigated in a case study where a febrile state of the cattle was confirmed to be LSD (Feyisa, 2018). On the other hand, Carriers of LSDV can be asymptomatic and may contribute to the spread of the disease(Sprygin *et al.*, 2020b).

Changes in the immuno-biochemical components have been observed with an increased level of some of the biochemical elements and a decrease in protein and some other contents. Inferring that the LSD infection in cattle prompts severe effects on immune-biochemical, antioxidant parameters, and inflammatory markers causing great economic loss (Yanni *et al.*, 2021).

1.7. Economic Importance

The economic impact of disease stems from direct losses caused by disease-related mortality and morbidity. However, there are indirect losses as a result of the disease, such as decreased productivity and changes in cow fertility. Furthermore, the disease may result in a restriction on international livestock trade as well as significant economic damage (Degu, 2020; Kiplagat *et al*., 2020). Because the effect of LSD threatens international trade, the economic significance of the disease is of great concern (Namazi and Khodakaram Tafti, 2021).

The substantial economic importance includes reduced milk production, increased abortion rates, decreased weight gain, increased susceptibility to secondary bacterial infections, and high mortality has contributed LSD to being considered the most important poxviral disease of livestock (Maclachlan and Dubovi, 2017). LSD is one of the most important viral diseases of cattle, causing loss of condition in infected animals and permanent damage to hides (Ayelet *et al*., 2014). Furthermore, it has an impact on food security due to decreased animal production

output and high disease control costs, which have a significant impact on livestock and their products (Gumbe, 2018; Degu, 2020).

1.8. Genetic alterations in LSDV

The genetic comparison of four gene loci (RPO30, GPCR, EEV, and BRR2) for LSDV strain and as well as LSDV field isolate with LSDV vaccine- strains are the major genotyping method used. In those genotyping studies, while some of the gene mutations were to be point mutations resulting in some amino acid changes without changing the status of the virus gene others caused changes in amino acid sequences (Chibssa *et al.*, 2021).

More recently breakthrough reports are indicating that vaccine failure, the virulence effect of the live vaccine, and the recombinant virus of vaccine strain with the wild strain that is genetically different from the vaccine or the virus strain. Thus, giving an implication of a study required to investigate the viral strain responsible for the disease outbreak happening during the various time. Especially, the strains found to be recombinants between the vaccine strain and the field strain indicating using a live virus for vaccination leading to the recombination to that of the live field virus highlighted the potential for new strains of LSDV emerging (Sprygin *et al.*, 2018; Tuppurainen *et al.*, 2021).

Molecular characterization of LSDV strains circulating in Nigeria between 2000 and 2016 also revealed that there was a change in LSDV strains in Nigeria (Wolff *et al.*, 2022). Also, molecular characterization of the LSDV occurring at different times in Iran has indicated genetically distinct LSDV which assumed that there could be two strains of LSDV(Hedayati *et al.*, 2021). Interestingly, another vaccine-like LSDV was detected in non-biting flies in China. The sequenced gene implied the genetic closeness between the newly identified vaccine-like LSDV to that of the vaccine-like recombinant virus in Saratov/Russia although there is no evidence to support the cross-border transmission of the virus (Wang *et al.*, 2021).

1.9. Diagnosis of LSD

LSD is usually diagnosed based on typical clinical characteristics. In naturally infected animals, lacrimation, nasal discharge, swollen lymph nodes, high fever (>40.5^{0}C), decreased milk production, generalized skin nodules, necrotic pox lesions around the muzzle, skin lesions in the legs and secondary bacterial infections, and the deep scab has all been recorded (Zewdie,

2021). In addition, the lumpy skin disease virus can be identified and characterized using a variety of virological, molecular, and serological diagnostic methods. Off-course, diagnostic methods should be accredited by relevant assurance systems so that sample processing, testing, and reporting are by good laboratory practice (Sprygin *et al.*, 2020).

2.9.1. Virus isolation

Capripoxviruses including lumpy skin disease, Sheep pox, and goat pox have tissue tropism for epithelial cells. This tissue affinity enables *Capripoxviruses* to be propagated in a wide variety of primary cells or cell lines of bovine, ovine, or caprine origin with a virus titer up to 10^6 TCID50 per ml (Tuppurainen, 2005; Tuppurainen *et al.*, 2018a; OIE, 2021). These cell types include cells from tissues, including the kidney, testes, adrenal, thyroid, skin and muscle (Tuppurainen *et al.*, 2018a). Among these, the most commonly used cells for the propagation of *Capripoxviruses* are primary lamb kidney or primary lamb testis cells (Ferris and Plowright, 1958; Plowright and Witcomb, 1959). The isolation of lumpy skin disease virus is also applicable to the Chorioallantoic Membrane (CAM) of Embryonated chicken Egg (ECE), where the LSDV isolated on the CAM of ECE produces characteristics of pock lesion as an indication of the presence of the virus (El-Bagoury *et al.*, 2009).

Regarding the characteristics of the virus, LSDV grows slowly on cell cultures, and the first cytopathic effect can usually be detected within four to six days after inoculation (Tuppurainen *et al.*, 2018a; OIE, 2021). The CPE has characteristics of retraction of the cell membrane from surrounding cells and eventually rounding of cells (OIE, 2021). LSD is a slow-growing virus and takes a few days in the propagation process in different cell culture materials whether be primary cells or secondary cells. Virus isolation is a necessary method for virus propagation, which is used in the study of viral infectivity and vaccine development. Virus isolation also greatly improves virus population retrieval (Tassew *et al.*, 2018; Mikhael *et al.*, 2021).

1.9.1. Molecular detection

LSDV can be detected from nodular tissue samples using a standard polymerase chain reaction. Moreover, a real-time PCR assay can be used to confirm the LSD virus field isolation quickly and accurately (Mikhael *et al.*, 2021).

A molecular assay for CaPV genotyping was developed and its analytical performance was described using unlabeled snapback primers in the presence of dsDNA intercalating EvaGreen dye. The assay detected and genotyped CaPVs in samples with a sensitivity and specificity of 100%. The genotyping was done by observing the melting temperatures of hairpin snapback stems and full-length amplicons, which revealed high pathogen specificity and multiplatform capability (scalable). In addition, it is less expensive because it does not require fluorescent-labelled probes or high-resolution melting curve analysis software (Gelaye *et al.*, 2013). Also, a PCR method that differentiates SPP vaccine strains from field isolate and from other CaPVs was developed which showed good sensitivity and specificity (Chibssa *et al.*, 2018).

Another recent assay was developed that works with a High Resolution Melting (HRM) based on a unique region in the SPPV vaccines with two deletions of 21 and 27 nucleotides within the capripoxvirus (CaPV) homolog of the vriola virus B22R gene. The developed assay generates four distinct melting peaks, allowing differentiation of SPPV vaccines, SPPV field isolates, GTPV, and LSDV. This HRM assay is sensitive, specific, and provides a cost-effective method for detecting and classifying CaPVs as well as distinguishing SPPV vaccines from SPPV field isolates (Chibssa *et al.*, 2019).

A very recent assay based on recombinase polymerase Amplification (RPA)-Cas12a-fluorescence assay for the rapid on-site detection of LSDV was developed. This was accomplished by employing a highly conserved LSDV gene, the poly(A) polymerase small subunit (ORF068) gene, as the target for developing the CRISPR/Cas-based detection method (Jiang *et al.*, 2022).

1.9.2. Genome sequencing

Because of the repeated outbreaks reported, there are increased complete genome sequences from field isolate and single nucleotide polymorphisms (SNPs) that differentiate the phenotypically diverse vaccine-associated strains genetically (Schalkwyk *et al.*, 2020). Genetic studies are done using sequencing of the different genes found in the virus genome regions which are responsible for the virus's several activities. Also, molecular epidemiological studies rely on several regions of the viral genome, such as P32, GPCR, RPO30, and EEV. GPCR and EEV were dominantly used to distinguish LSDV vaccines from LSDV field strains (Chibssa *et al.*, 2021). In the same manner, P32(LSDV074)gene has been demonstrated to be able to characterize sheep pox and goat pox viruses. And also, the RPO30

and GPCR genes have been recognized as genes to classify *Capripoxviruses* into their respective species such as LSD, sheep pox, and goat pox virus (Tuppurainen *et al.*, 2018a). RPO30 gene is a CaPV homologue of the Vaccinia virus E4L gene which encodes the 30 kDa DNA-dependent RNA polymerase subunit (Lamien *et al.*, 2011a). RPO30 gene sequencing has been used as a gene of interest as the gene has markers on which the CaPVs differ from each other (Zewdie *et al.*, 2019). GPCR sequence comparison can also be performed, providing insight into improved control strategies at the regional, national, and global levels.

In a single or repeated outbreak of LSD, Sequencing enables to know the specific diversity of LSDV strains responsible for the outbreak. In addition, it makes possible to trace back to the ancestral origin of the LSDV which will let to identify trans boundary circulation and transmission to adjacent regions (Saltykov *et al.*, 2021).

1.9.3. Serological tests

Virus neutralization is the gold standard test for the detection of antibodies raised against CaPVs. In addition to VNT indirect fluorescent antibody test (IFAT) is commonly used (Degu, 2020). IFAT has been evaluated for its sensitivity and specificity and has been discovered that it has a reasonable high accuracy for the diagnosis and sero-surveillance analysis of LSD in the target population(Gari *et al.*, 2008). VNT/MDBK had shown suitability of VNT/MDBK for the detection LSD virus specific neutralizing antibodies (Krešic *et al.*, 2020). Virus neutralization is a subset of immunoassay that does not detect all antigen–antibody interactions. It only detects antibodies capable of preventing virus replication (Payne, 2017).

Western blot can also be used which is highly sensitive and specific method but expensive and difficult to perform (OIE, 2021). New commercial kits for detecting C*apripoxviruses* antibodies are being developed and released to the market (OIE, 2017). *Capripoxviral* antibodies can be detected using enzyme-linked immunosorbent assays (ELISAs), which are available as commercial kits (Milovanovic *et al.*, 2019; Samojlovic *et al.*, 2019; OIE, 2021).

2.9.3. Other tests

Furthermore, immunoperoxidase Monolayer Assay (IPMA) was developed for the detection of antibodies against lumpy skin disease virus in a simple and minimized tech setting. Using two dilutions (1:50 and 1:300) in a duplicate format, the test was shown to be highly sensitive,

specific, and repeatable. In comparison to the VNT and a commercial ELISA, the LSDV-IPMA was able to detect the LSDV antibodies earlier in infected, vaccinated, and vaccinated/infected animals (Haegeman *et al.*, 2019). As a confirmatory test of the LSDV antigen in nodular tissues of infected cattle, immunohistochemistry can be performed by using specific anti-LSDV antibodies. The viral antigen can be observed in the cytoplasm of epidermal basal cells layer and prickle cells, as well as the epithelial outer and inner roots of hair follicles(Amin *et al.*, 2021).

1.9.4. Differential diagnosis

Initial cases of infection may be difficult to differentiate especially with other skin diseases that confuse with LSD, but severe cases of LSD are highly characteristic to recognize. Differential diagnoses include pseudo-lumpy skin disease caused by bovine herpesvirus 2 (BoHV-2), this is usually a milder clinical condition, with characteristics of superficial nodules, similar to the early stage of LSD. BoHV-2 (a disease with lesions generally confined to the teats and udder) which is caused by bovine herps virus 2 has similar clinical symptoms which can be identified by the microscopic appearance of the viruses. Also, dermatophilosis, ringworm, insect or tick bites, besnoitiosis, rinderpest, demodicosis, *Hypoderma bovis* infestation, photosensitization, bovine papular stomatitis, urticaria, cutaneous tuberculosis, and onchocercosis are the differential diagnosis of lumpy skin disease (Tuppurainen *et al.*, 2018b; OIE, 2021). Mucosal lesion causing diseases that confuse with LSD are rinderpest, bovine viral diarrhea, and bovine malignant catarrhal fever (Barnanad, 1994).

2.9.6. Status of LSD in Ethiopia

Once its first incursion in Ethiopia, LSD has spread all over the parts of Ethiopia including the different agro-ecological zones (Ayelet *et al.*, 2014: Gelaye *et al.*, 2015). The disease has become endemic in the various agro-climatic zones and it has been widely distributed across a variety of agro-climatic zones, with substantial differences between districts due to variations in agro-ecological zones and farming practices (Gari *et al.*, 2010). An epidemiological study in central Ethiopia between 2007 and 2011 showed the highest LSD outbreak in Oromia followed by Amhara regional state and the Southern Nations, Nationalities and People's Region. In addition, the highest outbreak accounted for the year 2010 (Ayelet *et al.*, 2014).

The genetic studies using the specific gene loci specifically, G-protein coupled chemokine receptor (GPCR) and RNA polymerase subunit 30 KD (RPO30) genes of CaPVs isolates collected from different geographical locations in Ethiopia between the years 2008 and 2012 revealed that only a few distinct LSDV and GTPV isolates are circulating in the country (Gelaye *et al.*, 2015).

2.10. Prevention and control

Effective disease control measures against lumpy skin disease include mass vaccination, import restrictions on livestock and their products, vector control, and quarantine stations. Furthermore, culling infected animals is an optional method (Gumbe, 2018). However, substantial vaccinations with safe and effective vaccines are the most effective method of controlling LSD (Hamdi *et al.*, 2020; Bamouh *et al.*, 2021). There are Live attenuated homologous and heterologous vaccines against LSD based on attenuated strains of CaPVs, which originated from wild field isolates (Saltykov *et al.*, 2021). Homologous vaccines are consisting of live attenuated LSD virus (LSDV) whereas heterologous vaccines are which contain live attenuated sheeppox or goatpox virus (SPPV/GPPV) and both can be used for the control of LSDV(Sprygin *et al.*, 2020).

Currently, at least four vaccines are used globally against LSDV. These vaccines are live attenuated strains of capripoxvirus, Kenyan sheep and goat pox strain (KS-1), the Yugoslavian RM 65 sheep pox strain, the Romanian sheep-pox strain and the south African onderstepoort LSDV strain (Brenner *et al.*, 2009; Ayelet *et al.*, 2014). All the capripox viruses examined share major neutralizing sites, thus one vaccine can cross protect another host from the disease. Vaccination of ruminants against Capripox infections is commonly done with the Kenyan strain KSGP 0240 but the protection provided is still questionable (Bamouh *et al.*, 2021). In experimental work, LSDV strain protected cattle against LSDV and this suggested that vaccination against LSDV should be carried out with homologues strain (Hamdi *et al.*, 2020). To date, available LSDV vaccines are live attenuated viruses (Tuppurainen *et al.*, 2021).

In an attempt to control LSD distribution and Vaccination, limiting animal mobility and removing diseased and exposed animals can all help to manage LSD. However, sufficient financial, physical, and human resources, as well as information systems, are required and in the case of Ethiopia, it has not been possible to follow the strategies. Thus vaccination has been

adopted as the most important practical approach to LSD control for many years (Ayelet *et al.*, 2014).

After vaccination immunity to CaPVs is both cell-mediated and humoral. Antibodies appear 15 days after vaccination or natural infection and peak 21–30 days after infection. However, viruses may travel in the skin without being released into the cell, rendering them to be hidden from humoral activities (Tuppurainen *et al.*, 2021). All those vaccination schedules and seasonality should underscore the immune activation period versus outbreak season.

On the contrary, incursions have been reported despite vaccinations in different countries. According to Brenner *et al.* (2009) study report, the live attenuated Yugoslavian vaccine strain was unable to protect the animals from the clinical symptom affected by the natural LSD outbreak, emphasizing the need to assess the potency of the vaccine. In addition, another outbreak occurred in Egypt where the animals were vaccinated before the outbreak (Salib and Osman, 2011; Rouby *et al.*, 2021). Cattle vaccinated with the Romanian vaccine have been unable to be protected from the incursion of the virus in Saudi Arabia and Egypt (Kasem *et al.*, 2018; Rouby *et al.*, 2021). And also Vaccine failure has been observed in different outbreaks in vaccinated cattle in Ethiopia (Ayelet *et al.*, 2013; Molla *et al.*, 2017b; Abera, 2019). All those need further studies on new vaccine development and improvements.

An ideal vaccine is characterized by broad-spectrum protection against infectious species to limit potential transmission, the ability to differentiate between infected and vaccinated animals, no recombination between vaccines and field strains, a strong and long-lasting immune response, and low manufacturing and administration costs (Le *et al.*, 2022).
Mass vaccination of animals against LSD with live homologous vaccines is the most effective control technique. Despite this, data suggests that the LSD virus is still present in some parts of Europe and that non-immune cattle are still at risk, even in areas where vaccine coverage is rather high (Calistri *et al.*, 2019).

2. MATERIALS AND METHODS

2.1. Study area

The study was carried out in the central part of Ethiopia which includes in and around Addis Ababa, the capital city and under the Oromia region state includes special zones of Fenfine zuria, Adaá district including Bishoftu town, Bekejo, Dire, Adulala, Hidi and Katila and Arsi zone, Dodota district (Fig.1).Those areas were selected based on their active case reports rather than randomization.

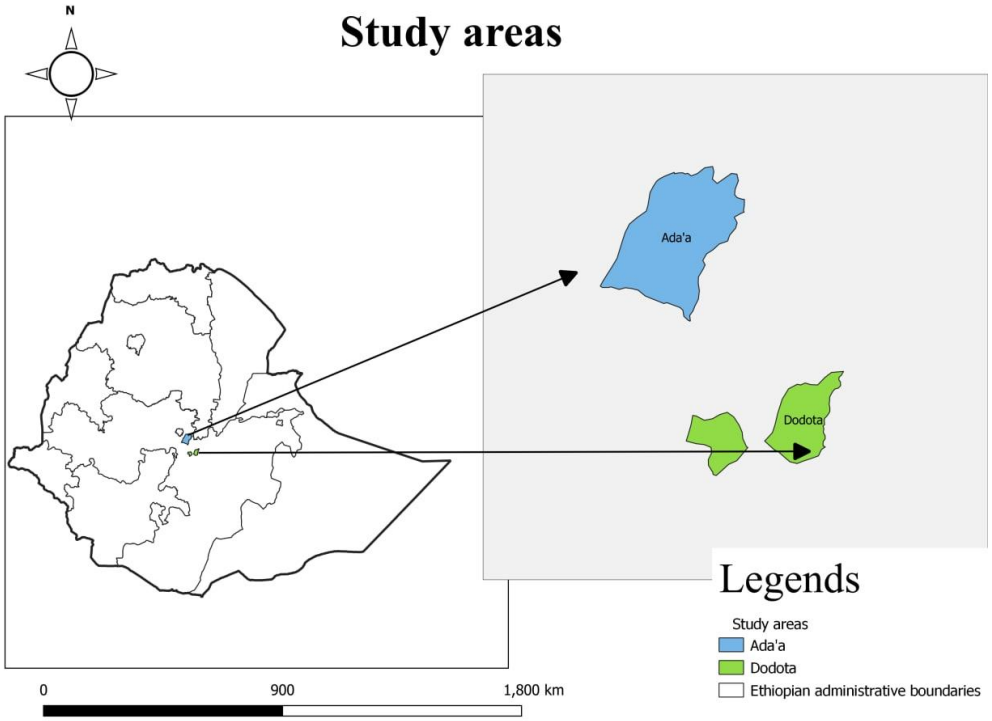

Figure 2: Map of study area, sampling area with sampling points using QGIS version 3.16.8-Hannover

2.2. Study population

The study population comprised cattle population including local and crossbreeds in any age group following the live outbreak of lumpy skin disease virus. Active disease outbreak was followed by communicating with district veterinary officials to address the different clinics in different localities under the district. Cattles of any breed and age were managed to be investigated for LSD.

2.3. Study design and sampling

A cross-sectional study design was followed from October 2021 to May 2022. The study was done purposively based on the active outbreak reports in the study areas. Samples required for this study were nodular skin lesions from acutely sick cattle and serum sample from both clinically sick and asymptomatic cattle. Based on that the case investigation for sampling was carried out by going through the case history and clinical observation of the animal. These included observing the nodules and nodular lesions, lameness and pyrexia. The clinical observations were also supported by taking baseline information about the breed type, sex, vaccination history and other parameters. The biopsy samples were collected from positive clinical lesions, especially skin nodules, and scabs manifestation. In addition to tissue samples, serological samples were also collected based on the herd level reported for an outbreak. Whole blood samples (serum) were collected from both clinically positive and healthy cattle for antibodies against LSDV detection.

2.4. Sample collection

The tissue and whole blood sample collection was performed during the weeks of reported outbreaks. And about 2-5 grams of nodular skin lesions were collected by using a sterile scalpel blade and an incision was made to reach the epidermis and the dermis of the skin. The skin biopsy was placed in the Virus transport media (VTM) which contains 10% antibiotics. whole blood samples were collected for the serum with a plain tube (Tuppurainen *et al*, 2017; OIE, 2017). The tissue samples with VTM in a falcon tube and the blood sample were transported within an ice box to the laboratory (AHI) and stored at -20^0C until used for the next laboratory processes.

Questionnaires were prepared for animal health professionals incorporating questions with a major emphasis on LSD vaccine and vaccination practices which include issues on the management, storage, transportation, administration of the vaccine and related questions. Additionally, background data were also collected in each locality clinics and farms where the outbreak occurred, including the date of data collection, age, sex, and previous history of vaccination using these questionnaires.

2.5. Laboratory Diagnosis

2.5.1. Virus isolation

The nodular tissue samples were thawed at room temperature and washed three times in sterile phosphate-buffered saline (PBS, pH 7.2). With 9 ml sterile PBS containing antibiotic (0.1% gentamicin, Sigma-Aldrich, Germany) and about 1 g of washed tissue sample was mixed and ground using a sterile mortar and pestle. Following this tissue suspension was centrifuged at 3000rpm for 15 min and the supernatant was filtered through a membrane of pore size 0.45 µm (Millipore, United States of America). Approximately 100 µL of filtered supernatant was inoculated onto a monolayer of (Lamb kidney cells). Primary lamb kidney cells were established and propagated in a Dulbecco's Modified Eagle Medium (DMEM) (Sigma-Aldrich, Germany) supplemented with antibiotics and 10% fetal bovine serum. And then the cells were aliquoted into different flasks and used when required by adding trypsin and incubating for ten minutes. When detachment takes much time the cells were then manually washed from the flask using the pipette. Then the cells were transferred to the 24 micro well plates. The cells were then reconstituted with the growth maintenance medium (DMEM) supplemented with 1% fetal bovine serum and cultured incubated at 37°C in a humidified incubator with 5% CO2. The cells were then checked for the 80% confluence. After checking the confluence of the cell, DMEM was added to the cells by removing the first media then, virus suspension was added to the cells. The whole process of the cell culture was performed under the biosafety cabinet level 2.

Virus suspension was inoculated on the cell after removing the media from the cell and washing the cell using 100µl phosphate buffers. The inoculation was done on a 24-well plate and incubated for 1 hour at 37°C for the adsorption time**.** Maintenance media was added and the culture was followed for up to 14 days for the presence of CPE. The negative samples were then made to continue for the consecutive 2nd to 3rd blind passages until the CPE was observed. This was done by freeze-thawing so that virus-cell particles are released from the culture cell. Then the collected suspension was vortexed and transferred to a new fresh cell, and monitored daily using an inverted microscope for the evidence of virus-induced CPEs.

2.5.2. DNA extraction

DNA of LSDV from tissue samples was extracted by QIAamp DNA Mini Extraction Kit (Qiagen, Germany) for blood and tissue based on the protocol set for DNA extraction in its manual for user. The skin nodular samples were processed by grinding using a sterile pestle and mortem and by adding sterile coarse sand. Following grinding the suspensions were centrifuged at 2000 rpm for 2 minutes and the 200µl of supernatant was transferred into a sterile microcentrifuge tube and 20 µl proteinase k was added to digest the cell. Afterwards, 200 µl of AL (lysis buffer) was added, mixed by vortex and incubated at 56°C for ten minutes in a water bath. Then 200µl of 95% ethanol was added to it and mixed thoroughly, this was to ensure the binding process. The mixture was transferred to the mini spin column in a 2ml collection tube and centrifuged at 6000 xg (8000 rpm) for 1 minute. The first wash was done using washing buffer AW1 with an amount of 200 µl on a mini-spin column in a new collection tube. That was then centrifuged at 8000 rpm for 1 minute. The next washing was done using AW2 (washing buffer 2) with 200 µl and centrifuged at 14000 rpm for 3 minutes. Finally, the collection tube was discarded, and the min-spin column was carefully transferred into a new 1.5ml microcentrifuge tube with 200 µl elution buffers and incubated for 1 minute at room temperature before centrifuging at 14000rpm for 3 minutes until all the liquid has completely passed through. The eluted DNA was labelled and stored in a -20C freezer until downstream activities start.

2.5.3. Molecular detection and characterization

Real time-PCR

LSDV detection using the RT-PCR was performed in reaction nuclease-free water (Invitrogen, USA) in a reaction volume of 20 µL. The resulting cycle threshold value implies the cycle number at which the amplification curve crosses the fluorescence threshold set at 0.1 in the rotar Gene Q thermal cycler software (Qiagen, Germany).
Forward: 5″-GGTGTAGTACGTATAAGATTATCGTATAGAAACAAGCCTTTA-3″;
Reverse: 5″-AATTTCTTTCTCTGTTCCATTTG-3″ .

DNA was amplified in a final volume of 20 µl that contained 10 µl evergreen super mix, 2µl forward and 2µl reverse primers, 4µl RNase-free water, and 2 µl Template DNA. The master mix was poured into all of the wells, including the positive and negative control wells. The

negative control implies a reaction well without the template. The amplification program was used for the analysis was with the initial denaturation at 95°C for 3 minutes, followed by 45 cycles at 95°C for 15 seconds, 58°C for 80 seconds, and last cycles at 95°C for 1 minute, 40°C for 1 minute, and 40–85°C for 5–10 seconds. Positive samples were identified using the assay's amplification fluorescence curves (at 73°C), and cycle threshold (Ct) values after amplification of the DNA template. A greater than 0.1 increase in the value of the (fluorescence threshold) combined with a Ct value less than 40 were considered positive for the PCR to detect DNA from the sample. If the samples had a fluorescence threshold of less than 0.1 and a Ct greater than 40, the RT-PCR result was read as negative.

Polymerase chain reaction

A conventional polymerase chain reaction was performed to detect a 30 kDa RNA polymerase subunit (RPO30) gene using two primers (forward and reverse) designed based on the lumpy skin disease NI-2490, complete genome (Acc no=NC_003027) (Tulman *et al*, 2001), retrieved from NCBI data bank. The primers were designed by using a snapGene viewer software to generate the subunit of the RPO30 gene. The primers were expected to amplify the RPO30 gene with a band length of 606bp. The following are the two primers:
Forward: 5'-ATGGATGATGATAATACTAATTCAT-3' and RPO30 subunit and Reverse: 5'-TATTTTTCTACAGCTCTAAACTTC-3'.

PCR was conducted in a reaction volume of 25 µL containing; 0.5µL of F primer, 0.5µL of REV primer, 0.5µL of dNTPs, 2.5µL of 10x PCR Buffer (Qiagen), 0.125 µL Taq polymerase (Qiagen), 17.875µL Milli-Q water and 3 µL template DNA. The PCR run set was with the first denaturation at 95°C for 2 minutes, followed by 40 cycles of elongation at 95°C for 30 seconds, 50°C for 30 seconds, and 72°C for 30 seconds, followed by a final extension at 72°C for 5 minutes. Aliquots of PCR products were checked using electrophoresis on a 1% agarose used Midori Green Advance (NIPPON Genetics EUROPE, inc.) in gel staining for 40 minutes at 100v.

2.5.4. Virus Neutralization test

A virus neutralization test for LSDV antibody detection was performed based on the VNT procedure (OIE, 2017; Krešic *et al.*, 2020). This test was performed to detect the presence of serum-specific antibodies against the Lumpy skin disease virus by neutralizing the viral

cytopathic effect on the cell culture in vitro. And it relies upon the virus reacting with a specific antibody in the test serum.

This test was used to detect the presence of serum-specific antibodies against the Lumpy skin disease virus by neutralizing the viral cytopathic effect on cell culture in vitro. The test was performed on a primary cell of a lamb kidney to determine the antibody titer in clinically symptomatic and asymptomatic cattle against LSDV. The primary lamb kidney cells were grown in DEMEM containing 10% foetal calf serum and 1% antibiotic and antimitotic. To decomplement heat-labile components, serum samples were inactivated by heat at 56^0c for 30 minutes before the test. A vaccine virus strain with a known virus titer of $10^{4.5}$, which is approximately 31622 TCID50, was diluted in a media with 2% antibiotics antimycotic and no FCS to give a 10^3TCID50/ml. Then, 100 µl of the DEMEM media was added to each well. Subsequently, 25 µl serum samples were added to wells A1-H1 and A7-H7. Furthermore, the same test serum was added to wells A2-H2 and A8-H8. A fivefold serial dilution was performed by a multichannel pipette from columns 2-6 and columns 8-12 starting from the initial dilution1/5 and 25 µl of the suspension from the end point dilution 1/3125 was discarded(from the 6^{th} and 12^{th} well)(Annex 2)

Amount of 100 µl of 1000 TCID 50/ml vial suspension of vaccine strain (Kenyan sheep and goat pox strain/2KSGPV) was added to each well from columns 2-6 and 8-12. Finally, 75 µl of cell culture media without serum and with antibiotic and antimitotic was added to columns 1 and 7 while, the rest had 100 µl each. After an hour of incubation, 50 µl of lamb kidney cell was prepared at the concentrations of $4x10^5$cells/ml and added to each well followed by checking for the cell concentration using a microscope. The plates were sealed and incubated at 37°C (5% CO2), with CPE being checked every four days until the final reading on day nine. The control plate was prepared in another plate containing positive and negative controls. The control plates also were prepared accordingly (Annex 2)

Final reading was made by comparing with the control tests. And the end dilution at which the CPE was observed was used as the highest dilution at which the antibody neutralized the virus, thus showing virus-induced CPE.

3.7. RPO30 sequencing and analysis

Following the amplification of the RPO30 subunit gene and observation of a clear band through the gel, the run was confirmed for the expected band length. Then, the remaining PCR product was cleaned by using the Wizard™ SV Gel and PCR Clean-Up kit (Promega, Germany) based on the instruction set in the protocol. Then the concentration of the cleaned elute was measured by the simple-NanoDrop 2000c spectrophotometer (Thermo Scientific, USA). Each purified product's concentration which was above10 ng/µl was prepared for Sanger sequencing according to the instruction of the sequencing service provider of the company. The purified PCR products were mixed with the amplification/sequencing primers and submitted for sequencing to Eurofins Scientific SE (Luxembourg).

3.8. Data management and analysis

Questionnaire data collected *were* entered in MS- excel, edit well and transferred to R software package with version (R.0.s) for descriptive analysis.

Molecular sequence data received from Eurofins Scientific SE were edited and cleaned to get the consensus sequence, and then all the sequence data was analyzed using a bioinformatics tool. The comparative sequence analysis of the RPO30 gene subunit of, Ethiopian Lumpy skin disease virus isolates was carried out along with those of other Capripoxviruses available in the NCBI database (https://www.ncbi.nlm.nih.gov/). The nucleotide sequences of the RPO30 gene subunit generated by the Sanger sequence and other publicly available sequences retrieved from NCBI data bank were transported to BioEdit (version 7.2) software package (https://bioedit.software.informer.com/7.2/), and were edited, joined together for each of the isolates. Finally, multiple sequence alignments were performed using the ClustalW algorithm implemented in **BioEdi**t software package to compare the RPO30 genes sequences of the outbreak isolates and the reference strain retrieved from the gene NCBI database, and to that of the vaccine strain. Moreover, the sequences obtained from the RPO30 gene amplicons were translated into corresponding amino acid sequences and were checked if any of the unique signatures associated with LSDV happened.

4. RESULT

Veterinary professionals in the study area have given their responses to the Questionnaires regarding the vaccination practices they often apply. According to the respondents, all sources of LSD vaccine were from the national vaccine Institute (NVI), Ethiopia. In addition, they all use the annual vaccination program in the seasons from July to August. The vaccine for LSD after arriving at the clinics was stored in refrigerators at +4 or in the freezers for some vaccines like LSDV. Even though most veterinary clinics have refrigerators, the power supply in the clinics is poor. Based on this study area, 71.4% of the respondents said they have very poor and interruptive electricity whereas only 14.3% of them said there was a good electric power supply. Of those who have an electric supply, 85.7% of them stated that the electric power is not stable and there is no alternative to the direct electric supply. While 14.3% of them said to have an alternative to direct power supply.

Concerning the effectiveness of the vaccine, 42.3 % of the respondents have responded to the current in-use vaccine effectiveness while the same number of the respondents replied that the vaccine could be moderately effective and the rest 14.3% of the respondents said the vaccine failed to protect the animals from LSD. Of those respondents who believe the LSD vaccine failed nearly 54% of them concluded that the vaccine's failure to protect the animals was due to the vaccine's efficacy issues. And 28.6% of them believe the problems are due to cold chain breaks during transportation and storage. Furthermore, farmers' lack of vaccination awareness was also cited by 14.3 per cent of respondents as a reason for vaccination failure.

The respondents also annotated that they use an ice box during transporting the vaccine from the site where they store it until use. And they all responded that they use an ice box for the transportation of the vaccine. Moreover, regarding the storage of the vaccine, 42.9% of them said that they store it in the deep freeze (-20) and among the other professionals 28.6% of them store the vaccine in a +4 refrigerator and others said they purchase it on the day of vaccination.

4.1. Clinical Observation

During clinical examination, the investigated cattle had typical clinical manifestations which showed circumscribed nodular lesions on the skin, anorexia, lacrimation, enlarged lymph nodes (prescapular and prefemoral), depression and salivation. Among the approximately 328 cattle examined, some cattle displayed typical skin nodular tissues, pyrexia, salivation, and limping.

However, only eleven of them were taken for the skin nodular sample. These observed clinical symptoms were pathognomic signs (Fig.3)

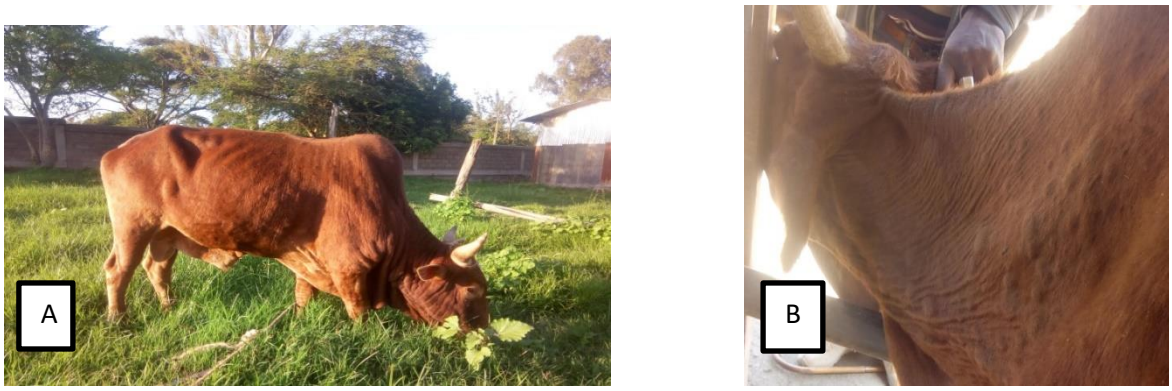

Figure 3: Clinical signs of LSD affected cattle A) swelling of prefemoral lymph nodes B) Skin nodules on the neck of cattle.

Table 1: Districts and localities where LSD outbreak reported during the study period

Outbreak area	**Date of sample collection**	**Purpose of rearing**	**Vaccination history**
Adea district			
Hidi kebele	8/11/2021	Mixed farming	Non-vaccinated
Bekejo	7/12/2021	Mixed farming	Non-vaccinated
Kality	25/11/2021	Mixed farming	Non-vaccinated
Adulala	29/11/2021	Mixed farming	Non-vaccinated
Katila	8/11/2021	Mixed farming	Non-vaccinated
Dodota district	25/11/2021	Dairy Farm	Non-vaccinated

4.2. Virus isolation

From the inoculated samples on the primary cell (Lamb Kidney), four of the eleven skin biopsy samples, were able to induce CPE with cells forming an aggregation and characteristics of an agglomeration. The first CPE was observed on the second passage at the third day after inoculation. The negative samples showing no CPE were then freeze thawed three times and the suspensions were transferred to the third passage by inoculated on a new cell. The newly inoculated samples showed virus induced cytopathic effect five days post inoculation.

The inoculated samples were followed until day 10-11 to observe virus induced CPE. The virus induced cytopathic effect observed had characteristics of rounding of single cells and aggregation of dead cells. After infection CPE of the first two cultures were observed on day seven and the rest of the viral suspension were then transferred to the third blind passage. On the other hand, after the second or third passage none of the negative samples showed virus induced CPE.

Table 2: Pattern and day at which CPE was observed

Passage No	**Day at which CPE was observed**	**Observed CPE**
1	No observed CPE within the 14 days	No CPE
2	Third day post infection	Cell aggregation
3	Fifth day post infection	Cell aggregation, detachment, cell death

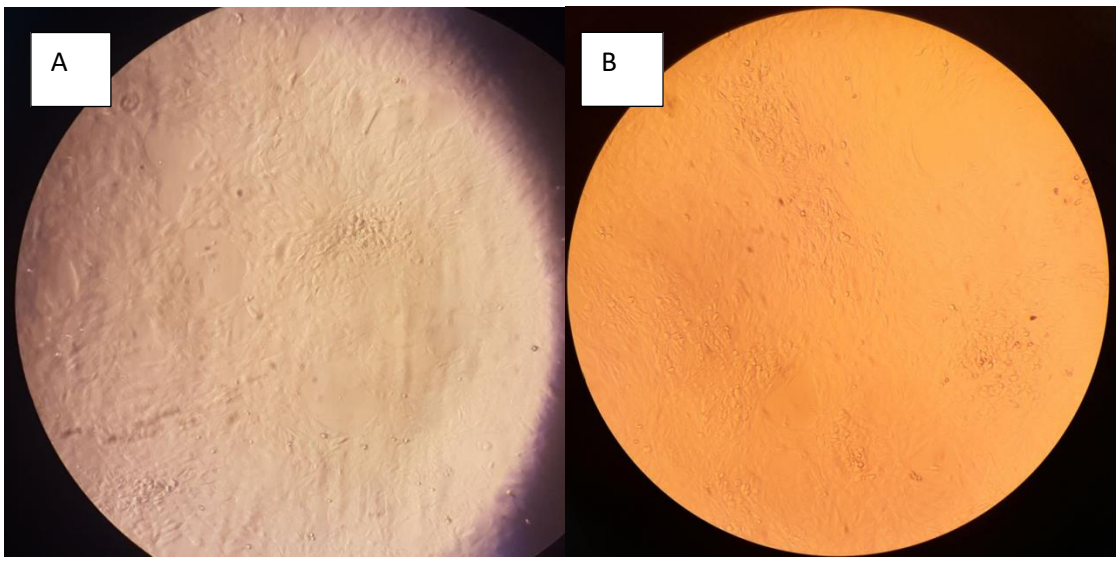

Figure 4: A) Third day of the second passage isolates, B) fifth day of the second passage

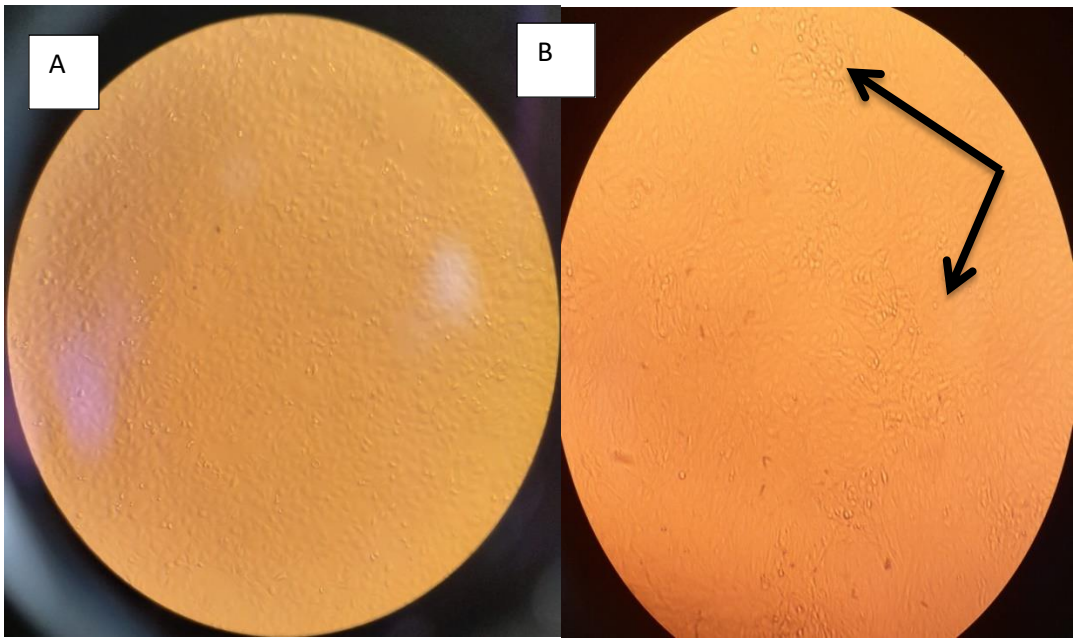

Figure 4: A:Normal lamb kidney cell as a Negative control and B: CPE of the viral isolates

4.3. Real time-PCR

From 11 tissue samples extracted DNA amplified by the RT-PCR, all of them (11) detected positive for LSDV DNA. Here indicated are the amplification curves of the real time PCR.

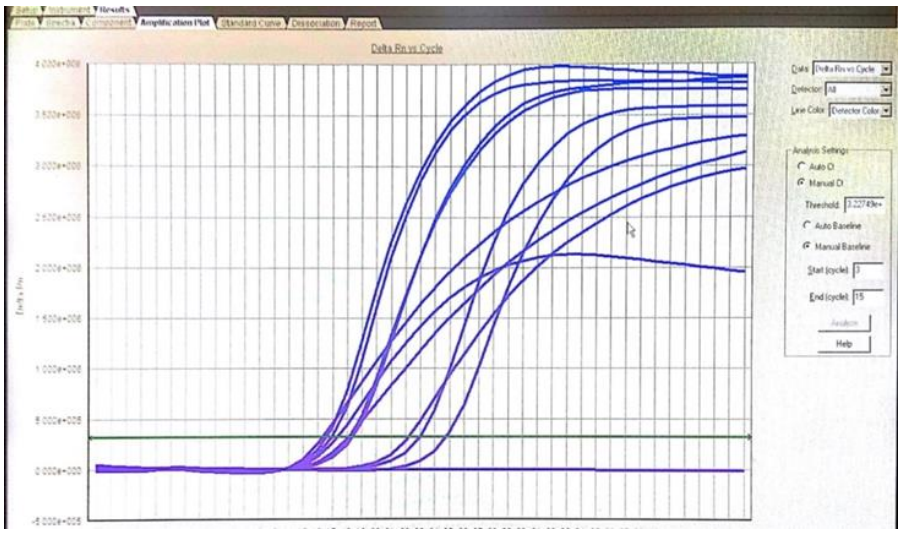

Figure 5: Real time PCR result

The cycle threshold of the amplified samples was between 16.39 and 30.92. This is indicative of the level of the nucleic acid that contains the fluorescence emitted in the cycles. Therefore, the shorter cycle on which the fluorescent was absorbed tells the high amount of nucleic acids

present in a specimen. The Ct values indicated here lie between the values of the fluorescent threshold values, indicating that they are positive.

Table 3: Real time PCR Ct values

Sample no	**Area**	**Ct values**
1	Adea	20.06
2	Adea	18.12
3	Adea	16.39
4	Adea	16.59
5	Adea	24.82
6	Adea	23.02
7	Adea	17.00
8	Dodota	30.92
9	Dodota	17.35
10	Dodota	18.19
11	Dodota	22.64
NTC	……	Undet
PTC	……	18.36
PTC	……	22.22

Note: Undet. denotes undetermined Ct values.

4.4. Polymerase chain reaction

LSD virus isolates from the primary lamb kidney cell were harvested and the supernatant taken for amplification using the RPO30 gene primer designed specifically for that gene. The expected amplicon size was detected here on all the samples. The result obtained was according to the primer designed band of approximately 606bp (Fig6). This was later on presented by the RPO30 gene sequenced result.

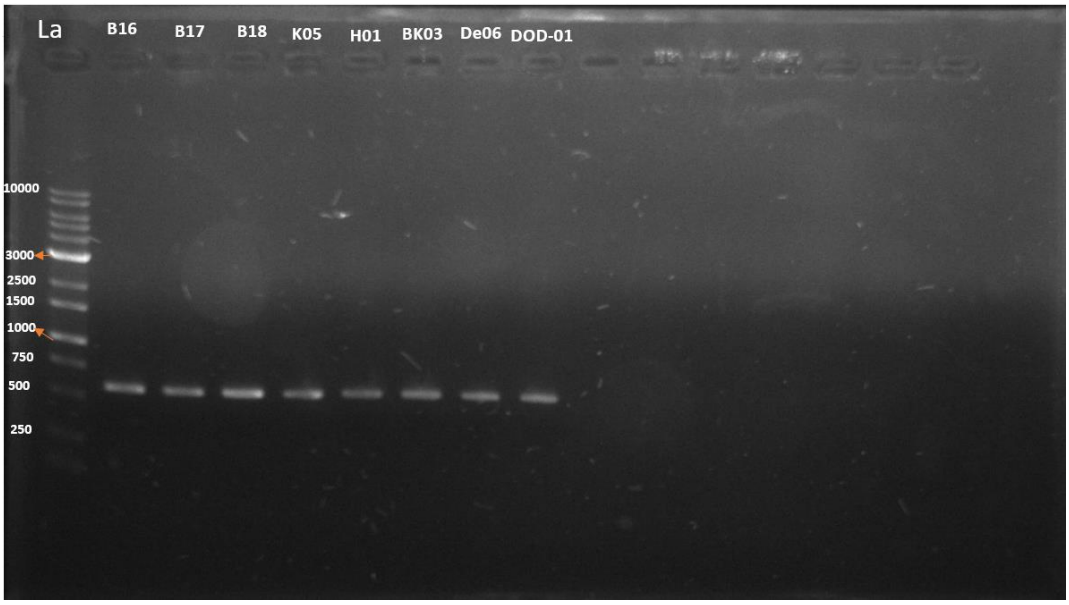

Figure 6: Conventional PCR gel result for the RPO30 subunit gene

The Lane La on the left side denotes DNA ladder of a 1kp (1000bp). And Lane B16-18 represents isolates collected from Bishoftu town, k05 stands for Kality and others from lane 5 to 8 are for isolates collected from Hora, Bekejo, Dembi and Dodota (Arsi) respectively.

4.5. RPO30 gene sequencing analysis result

These outbreak isolates in addition to the local vaccine strain (KS-1) and the sequences retrieved from the GenBank were used for the sequence alignment comparison. RPO30 gene sequence of four isolates from the current outbreak was analysed. The sequence result showed that the field isolates and vaccine differed in a single nucleotide position, indicating that the field isolates are distinct from the vaccinal strain.The RPO30 gene sequencing result displayed the current isolates from the outbreak area have a similar sequence to other previous field isolate sequences. A unique nucleotide variation was observed in the current and previous field LSDV isolates in contrast to the vaccine strain at nucleotide position 292 T/C, while identical nucleotides are indicated with dots (Fig7).


10 20 30 40 50 60 70 80
MK302112.1LSDV Galesa/B12/2008 RPO30 gene
KP663684.1 LSDV Mojo/B02/2011(RPO30)
LSDV/Ethiopia/Bishoftu/B16-2022 RPO30 gene,
LSDV/Ethiopia/Kality/K05-2022 RPO30
LSDV/Ethiopia/Hora/Ho1-2022 RPO30 gene
LSDV/Ethiopia/Dembi/De06-2022 RPO30 gene
KP663685.1LSD/Vaccine/NVI/CaPV Vaccine(KS-1)(RPO30)ge
LSD-RPO30/Holota/B9/-2008
KP663680.1LSDV FairField Debre zeit/B01/2009 RPO30
MK302112.1 LSDV Galesa/B12/2008 RPO30gene
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MK302096.1LSDV Asella/B2/2011 RPO30 gene,
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JQ310674.1Goatpox virus GTPV/HuB/2009/China (RPO30)
KF495229.1Goatpoxvirus/Jammu Kashmir/Sheep/27 (RPO30)
GU119929.1Sheeppoxvirus/Morocco/vaccine strain (RPO
MG201832.1Sheeppox virus/MAR/KEN2014 (RPO30) gene,
KT964234.1Sheeppox virus/Tunisia 14/15 (RPO30) gene,
MG458362.1Sheeppox virusShanX-YL/2015/China(RPO30)ge
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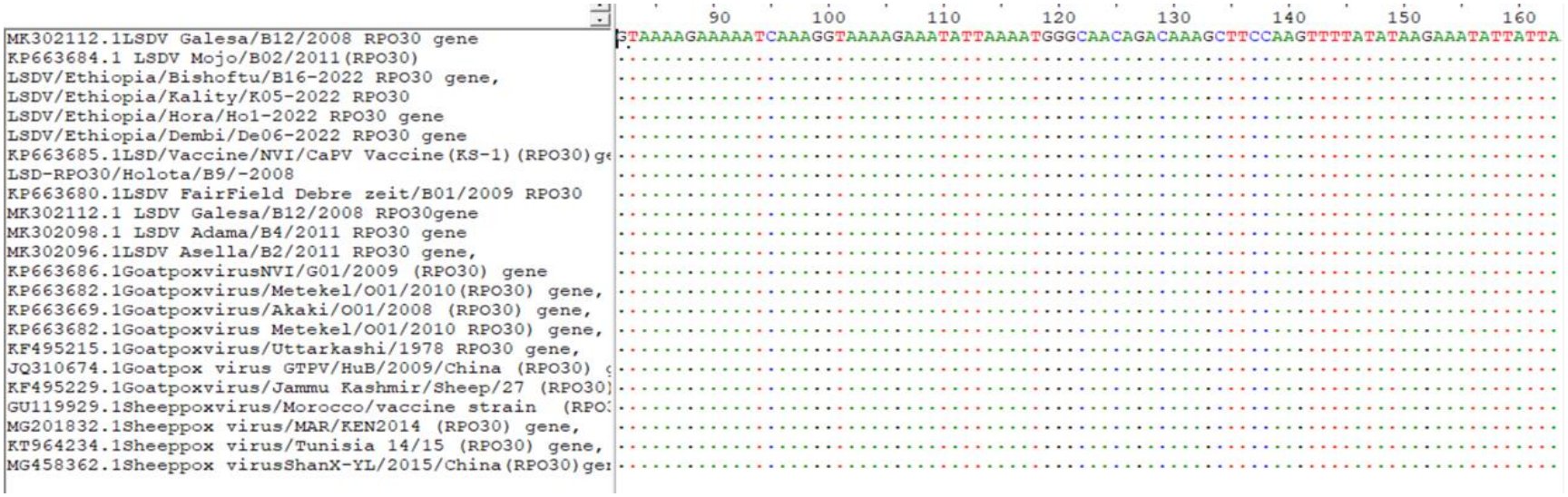
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GU119929.1Sheeppoxvirus/Morocco/vaccine strain (RPO
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KT964234.1Sheeppox virus/Tunisia 14/15 (RPO30) gene,
MG458362.1Sheeppox virusShanX-YL/2015/China(RPO30)ge
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MK302112.1LSDV Galesa/B12/2008 RPO30 gene
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MG458362.1Sheeppox virusShanX-YL/2015/China(RPO30)ge
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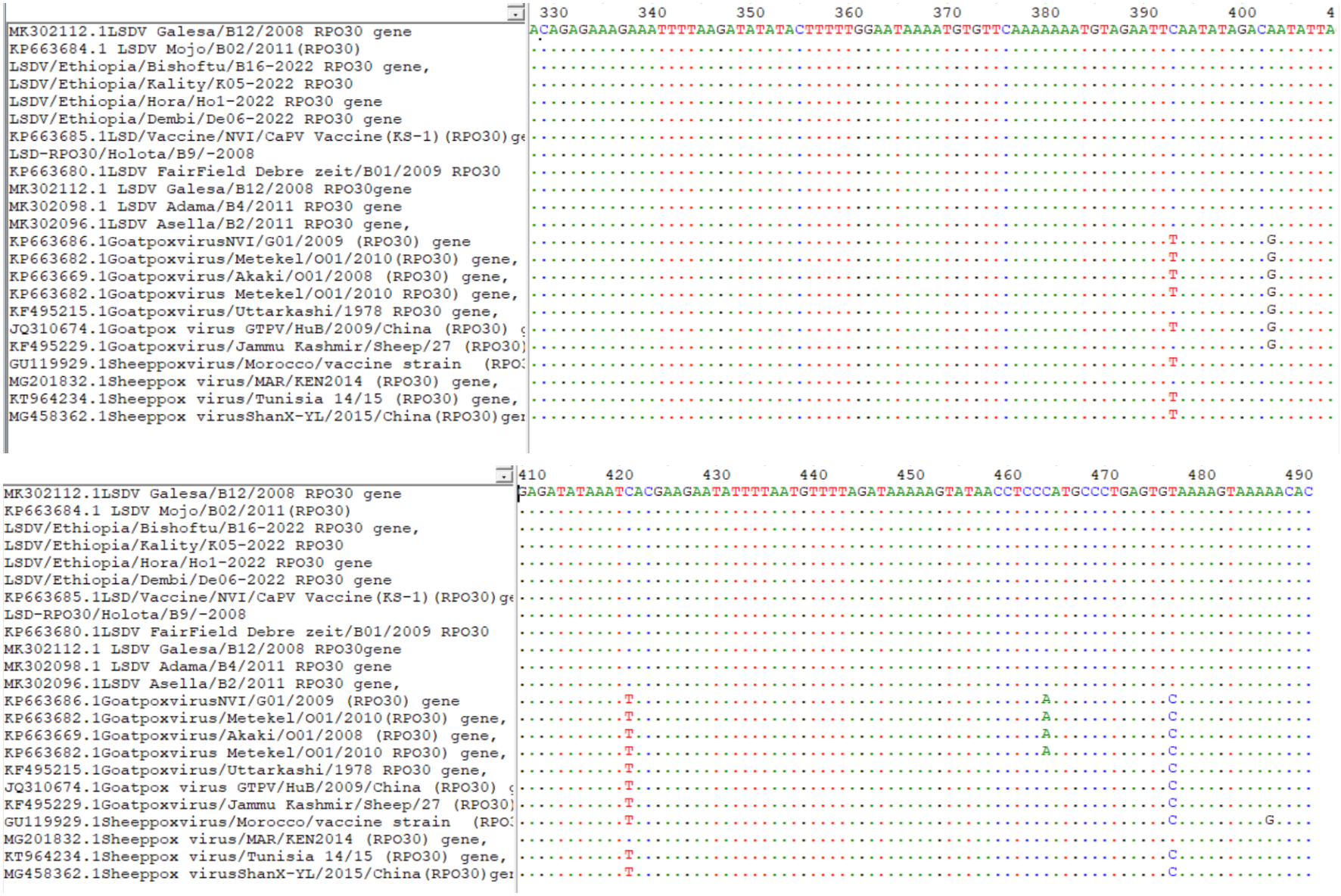
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MG458362.1Sheeppox virusShanX-YL/2015/China(RPO30)ger
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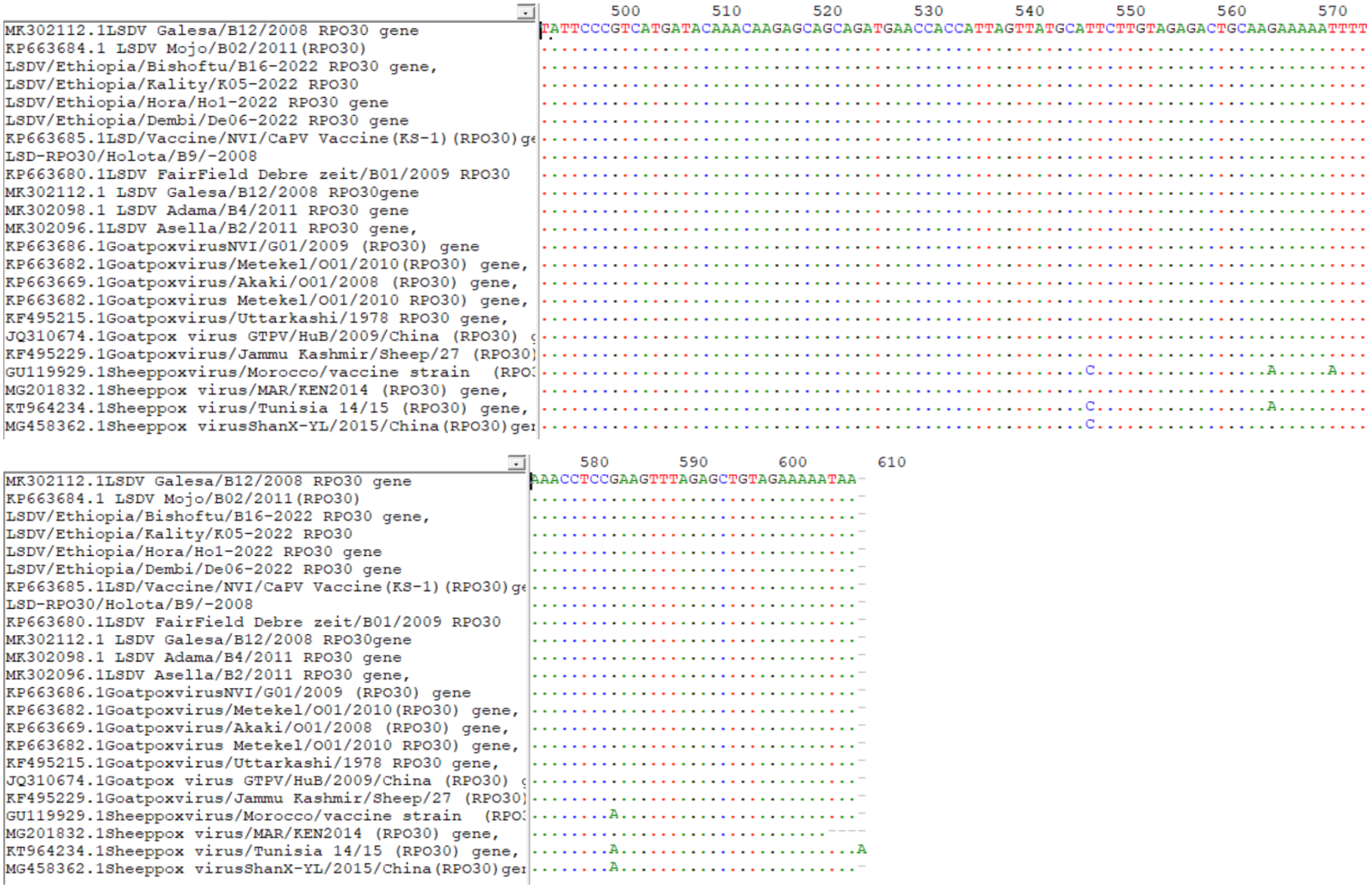
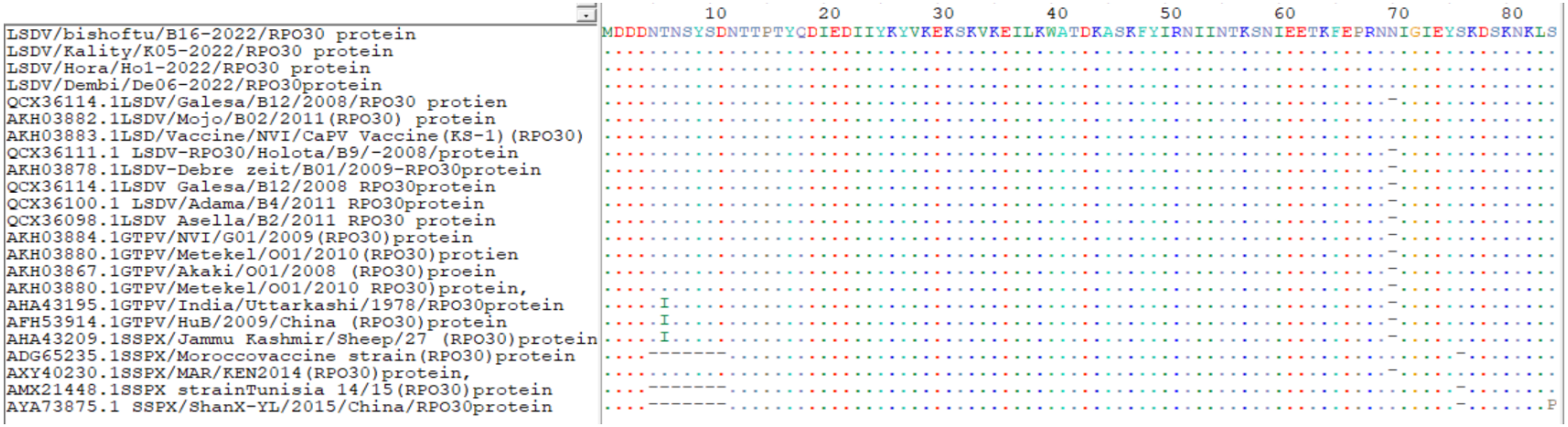
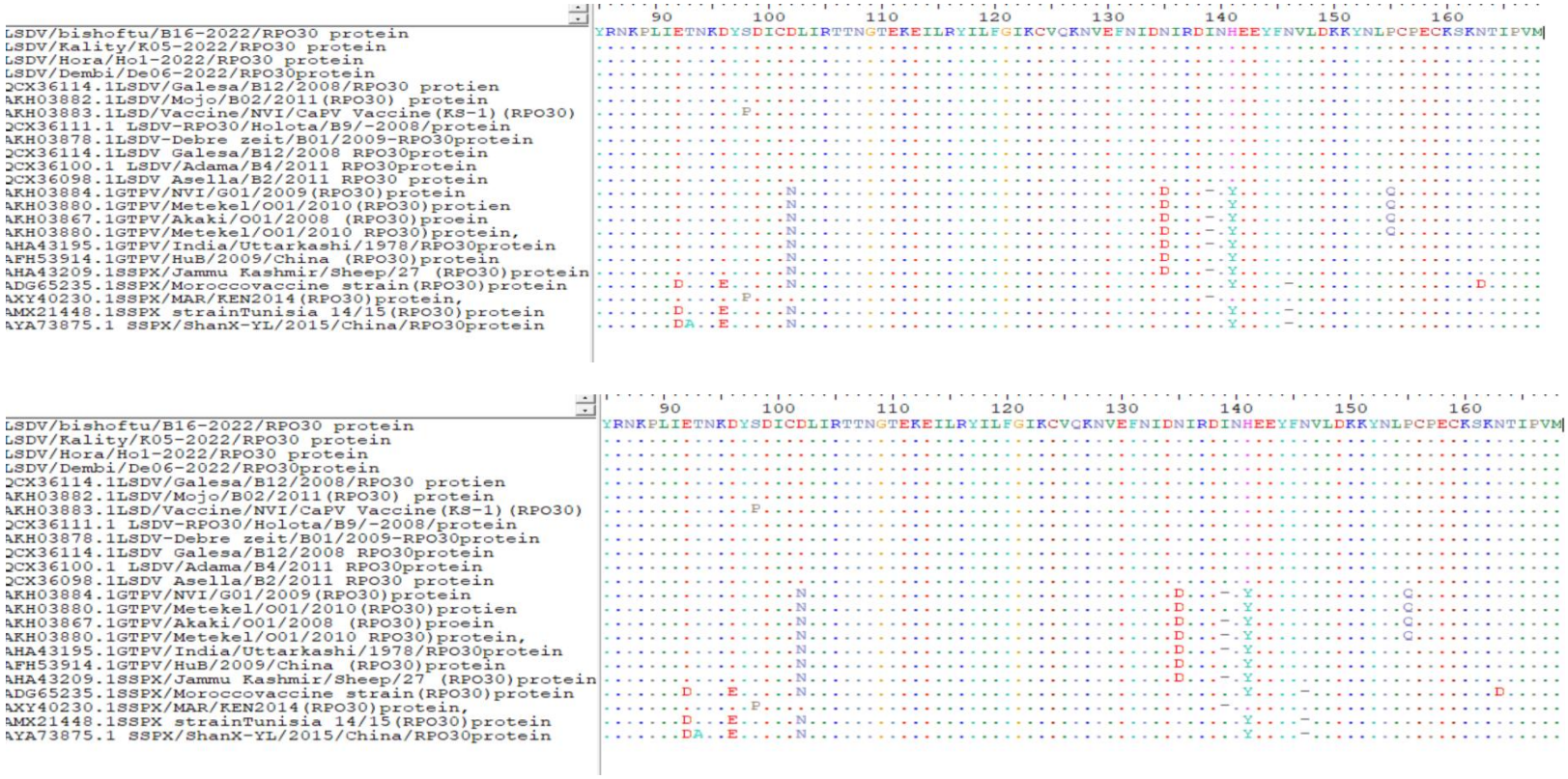


Figure 7: Plot identity alignment of the RPO30 606(nts) gene of Lumpy skin disease virus isolates(LSDVs) and those of capripoxvirus retrieved from the Gene bank, LSDV isolates(n=4), Previous Ethiopian isolates(n=6), KS-1vaccine strain(n=1), SPPV isolates (n=4), GTPV isolates(n=7).

4.6. RPO30 gene sequencing analysis

All LSDV RPO30 gene sequences were translated into amino acid sequences (a.as) by using an online tool Transeq (EMBOSS) (https://www.ebi.ac.uk/Tools/st/. For other sequences retrieved from Genebank data base , the translated a.a sequences were retrieved for multiple protein analysis. Then , the result of multiple amino acid sequence analysis has shown that they have 7 a.a residues additional compared to sheep pox virus isolates retrieved from genbank just like LSDV and Goat pox virus isolates. In addition to that all of the current isolates (4) serine (S) amino acid residue whereas the vaccine strain (KS-1)from NVI has proline at a position of 98 in the a.a sequence.

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LSDV/Kality/K05-2022/RPO30 protein
LSDV/Hora/Ho1-2022/RPO30 protein
LSDV/Dembi/De06-2022/RPO30protein
QCX36114.1LSDV/Galesa/B12/2008/RPO30 protien
AKH03882.1LSDV/Mojo/B02/2011(RPO30) protein
AKH03883.1LSD/Vaccine/NVI/CaPV Vaccine(KS-1)(RPO30)
QCX36111.1 LSDV-RPO30/Holota/B9/-2008/protein
AKH03878.1LSDV-Debre zeit/B01/2009-RPO30protein
QCX36114.1LSDV Galesa/B12/2008 RPO30protein
QCX36100.1 LSDV/Adama/B4/2011 RPO30protein
QCX36098.1LSDV Asella/B2/2011 RPO30 protein
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AFH53914.1GTPV/HuB/2009/China (RPO30)protein
AHA43209.1SSPX/Jammu Kashmir/Sheep/27 (RPO30)protein
ADG65235.1SSPX/Moroccovaccine strain(RPO30)protein
AXY40230.1SSPX/MAR/KEN2014(RPO30)protein,
AMX21448.1SSPX strainTunisia 14/15(RPO30)protein
AYA73875.1 SSPX/ShanX-YL/2015/China/RPO30protein

90 100 110 120 130 140 150 160
LSDV/bishoftu/B16-2022/RPO30 protein
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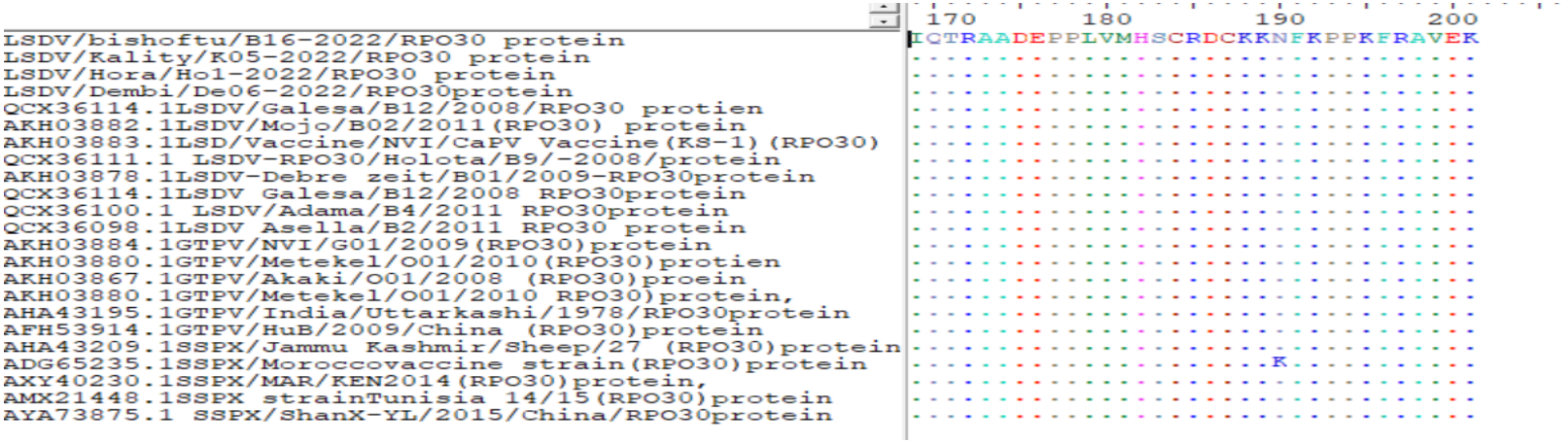

Figure 8: Plot identity of the deduced amino acid sequences of RPO30 gene of LSDV and those of capripoxvirus retrieved from the Gene bank, LSDV isolates(n=4), Previous Ethiopian isolates(n=6), KS-1vaccine strain(n=1), SPPV isolates(n=4), GTPV isolates(n=7).

4.6. Phylogenetic tree analysis

The test for phylogeny for construction of phylogenetic tree was bootstrap method with number of replication 1000. The percentage bootstrap scores are shown next to the branches. Genetic relationship between the new four isolates and previously reported LSDV isolates from different region in Ethiopia were compared to see new variations. And, multiple genetic comparison were done to see the relation of LSDV isolates with other LSDV circulating in other countries of Africa, Eastern Europe and the middle east. Representative sequences from the new field isolates were used together with the vaccine strains of LSDV and along with the strains of Sheeppox virus and goatpox virus. The homologue RPO30 subunit gene sequence from one Swine poxvirus and one Deer poxvirus isolates were also retrieved from NCBI data base and used as an out group strains. The overall nucleotide sequences in used in this analysis were 25 nucleotide sequences.

All the current Ethiopian LSDV isolates are identical, according to the RPO30 gene sequence analysis and phylogenic tree illustration. Furthermore, the new LSDV sequence clustered with LSDV field strains but not with the LSDV vaccine strain. The outbreaks were caused by field LSDV strains, according to the results of nucleotide sequence analysis.

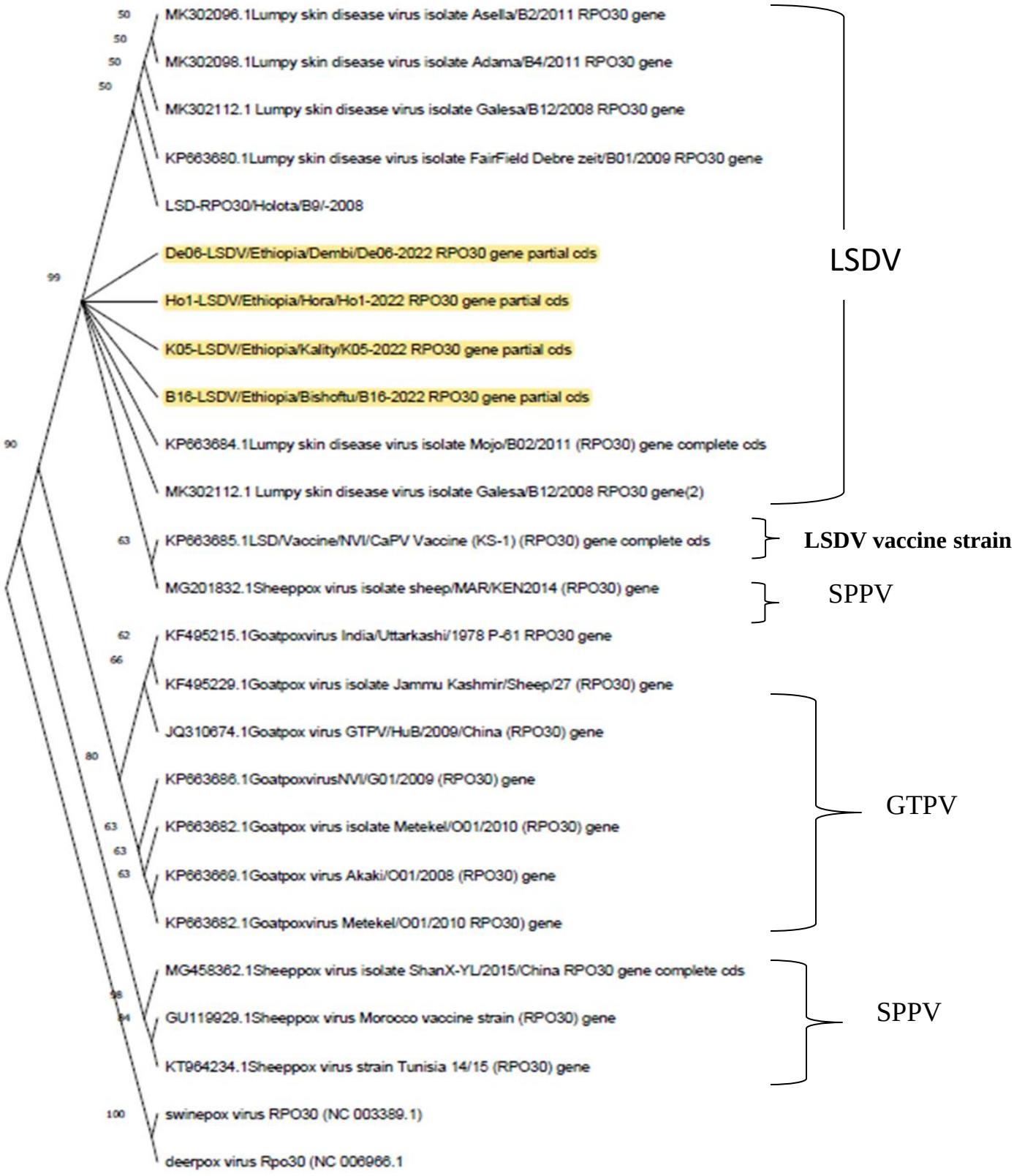

Figure 9: Phylogenic tree analysis, four of LSDV isolates sequenced in the present study

4.6. Virus neutralization test result

The virus neutralization test revealed that out of the sampled cattle three of them tested positive which accounted for 7.8% of the sample. Whereas, the negative samples on which CPE was observed and not the virus neutralized was 92.1%. The virus was neutralized on the first titer which was 1:25 titers. The neutralizing effect was observed until the ninth day of reading after inoculation. The result gives a few briefs of the association between diseased and in-contact animals.

Table: VNT Result

	VNT positive	VNT negative
Symptomatic	18.2 %	81%
Asymptomatic	9.096%	90.9%

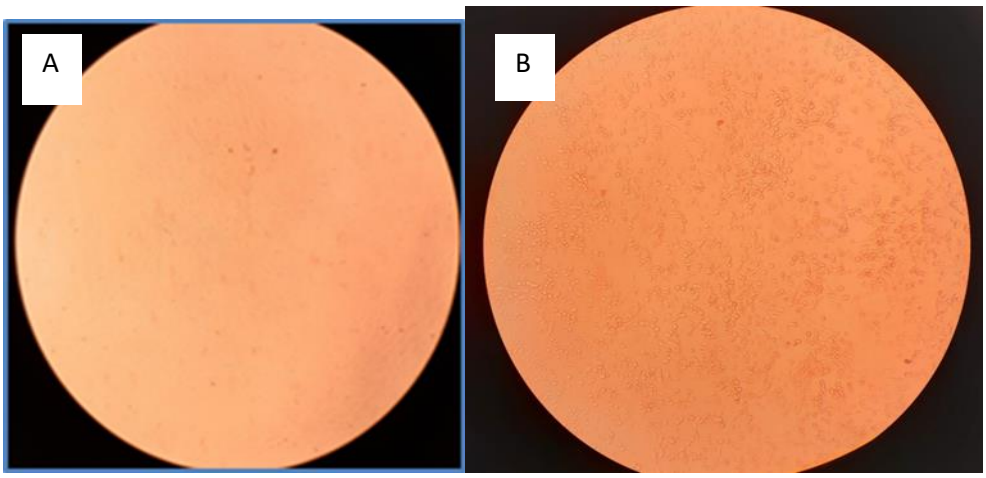

Figure 10: VNT test showing A) VNT Positive, no CPE B) VNT Negative showing CPE

5. DISCUSSION

In recent years LSD has spread unprecedentedly to new areas as an emerging disease. This, along with the significant economic impact, has sparked interest in studying the disease's dynamics to the point of understanding the genetics and evolutionary status. The disease has a multitude of impacts on the livestock sector resulting in production loss and setting a risk in the international trade. The LSD is recognized in Ethiopia since 1929 and has spread over the wide areas of the country. As various outbreak investigations indicate most of the outbreaks occur in midland agro-ecological zones of the country. In addition, LSD is species-specific and it has a significant effect on exotic or cross breeds animals of young age have been observed to be affected more than the other groups of animals.

This study was done with the aim of a general understanding of lumpy skin disease dynamics in the central part of Ethiopia through outbreak investigation, virus isolation, genotyping of the virus, and a survey on vaccine and vaccination practices. Thus, the questionnaire survey-based study on animal health professionals provided insightful information about the vaccination practices in the study area. Based on the information gained from the respondents, the only source of the vaccine was the National Vaccine Institute (NVI), Ethiopia which provides a KS-1 vaccine strain (Ayelet *et al.*, 2014). Most of the respondents had speculated the vaccine efficacy problem as the reason for the vaccine's failure which is in agreement with vaccine efficacy failure reported by Ayelet *et al.*, (2013). On the other side, the respondents have said that farmers are willing to vaccinate their cattle; which disproved the claims reported by some studies that had depicted farmers as unwilling to vaccinate their cattle.

During this study, the major clinical signs observed on the target animals and used for outbreak investigations were enlarged superficial lymph nodes, Skin nodules, discharge from the eyes and nose, lymphadenitis and oedema in the limbs, which are mentioned by OIE, 2017. And used similarly by other authors like Ayelet *et al.*, (2014); Molla *et al.*, (2017b).

In this study for the identification of the virus, skin nodular and scab samples were used as the main source of sampling. As Sudhakar *et al.*, (2020) have reported, Skin nodules or skin scabs acquire a higher virus population than other sources of the virus. We have isolated the virus

from skin nodular lesions by inoculating and culturing it in Primary lamb kidney cells (PLK). The isolated viruses on the PLK showed virus-induced cytopathic effects on the second and third passages, between three to five days post-inoculation. The characteristic CPE observed were cell rounding and aggregation that are scattered through the monolayer followed by detachment and finally cell death. Those observations were in agreement with Kononova *et al.*, (2021) isolated LSDV in lamb kidney and testis cells resulting in virus growth characterized by the cell rounding up, detachment and agglomeration.

In our study, from the antibody samples tested by VNT, 7.8% of them were positive for the presence of neutralizing antibodies. Among these, the VNT-positive cattle were higher in the clinically affected cattle than asymptomatic in contact cattle. Moje, (2020) has also detected neutralizing antibodies only in 7.08% of the cattle tested without vaccination. The detected neutralizing antibody was found in a low number of cattle which could be because of the early sampling from the required time for antibody production, which needs at least twenty days.

In the current study, all molecularly detected samples were positive by Real-time PCR with a Ct value between 16.39 and 30.92. This is in agreement with the report by Tassew *et al.*, (2018); Leliso *et al.*, (2021). Real time-PCR is a very fast molecular detection method with high specificity and sensitivity used in an outbreak and disease diagnosis (Babiuk *et al.*, 2008).

The expected amplicon size was detected by the conventional PCR using the specific primer according to the primer-designed band of the RPO30 gene 600bp. The 30 kDa DNA-dependent RNA polymerase subunit (RPO30) gene provides genetic information that can be used to characterize and compare genetic data with other related genetic information (Lamien *et al.*, 2011a).

Here in this study, molecular characterization was performed by sequencing the RPO30 (606nt) full gene sequence of the LSDV using sanger sequencing. This work has similarities with Gelaye *et al.*, (2015) in which the RPO30 gene was used for the specific gene analysis. In support of our result, RPO30 gene sequences have been targeted as useful genetic markers in the molecular study of the field of virus (Sudhakar *et al.*, 2020). Multiple sequence alignment analysis of the RPO30 gene sequence of the current LSDV sequences revealed nucleotide

variation between the current field strains. This goes to Tassew *et al.*, (2018) in which the nucleotide variations didn't occur. On the other hand, in this study, the multiple sequence alignment which compared the current sequence with the other LSDV strains retrieved from the Genbank have presented no nucleotide variation as they denoted nucleotide similarity. However, the current study result has revealed a single nucleotide mutation between the isolated and the previously characterized field Ethiopian LSDV isolates causing A/C change at nucleotide position 41.

Our result insights, a sequence alignment comparison of nucleotide and amino acid variation between LSDV, GTPV, and SPPV, as well as a comparison of the new and previous Ethiopian isolates and some isolates from other countries. In the sequence alignment, the field and previous LSDV isolates have nucleotide substitutions to that of the sequence of SPPV and GTPV retrieved from the Genbank. In addition, various single nucleotide substitutions were found between the LSDV clade in which our current strains are grouped, to that of the GTPV and SPPV. The LSDV sequences showed C/T, T/A, C/T, A/G, T/G, T/G, A/G and G/A nucleotide substitution at positions 17, 22 to 25 and at nucleotide positions 28 and 29 respectively in the GTPV isolates. Also, LSDV isolates showed nucleotide substitution in both SPPV and GTPV isolates at the same nucleotide positions. In addition to that, the 21 nucleotide deletion seen on sheep pox when compared to our four virulent field isolates differentiates them from SPPV strains.

Similar to nucleotide, amino acids (a.as) multiple sequence alignment was also done to see if there were any structural changes. And the result of multiple amino acid sequence analysis has shown that they have 7 a.a residues additional compared to sheep pox virus isolates retrieved from GenBank just like LSDV and Goat pox virus isolates which are in agreement with Lamien *et al*. (2011a) and Gelaye *et al.*(2015). The alignment of the amino acid indicated a unique GTPV signature of nucleotide substitution N/D(Aspargine to Aspartate) at position 136, the rest of the sequences are shared by LSDV and SPPV, which is as illustrated by Lamien *et al.*, (2011a).

Here in this study, the aligned sequences depicted the difference between the current field LSDV strains and KS-1 vaccine strain. This sequence difference was a single nucleotide

substitution of T/C at position 292. This sequence variation was also observed in the deduced amino acid sequence alignment, where variation existed at the position of residue 98 with S/P(serine to proline) between the current virulent field isolates versus vaccine strain (KS-1) from NVI. This was also indicated by a multiple sequence alignment done in central Ethiopia (Gelaye *et al.*, 2015; Tassew *et al.*, 2018).

The phylogenetic tree clustered the new LSDV isolates in a closer relationship with the viral isolates from Galesa/B02/2008 and mojo/B12/2011. The phylogenic tree constructed in this study allowed for an understanding of the evolutionary relationship between the current isolates previously studied isolates in Ethiopia and isolates from other countries. In agreement with some other studies implemented in Ethiopia, the Ethiopian isolates were found to be identical and clustered in the same group. In addition, phylogenic analysis of the RPO30 gene sequence indicated the grouping of the field LSDV and the vaccine strains into two clusters while both being grouped in the same clade. This goes to the results of Agianniotaki *et al.*, (2017). The genetic characterization of circulating LSDV strains provides useful information for molecular epidemiology, outbreak tracing, vaccine design, and vaccine selection for LSD control.

6. CONCLUSION AND RECOMMENDATION

The current study has examined the outbreak of LSD in central part of Ethiopia based on the reported cases. In the study period the disease was observed at the start of October/2021 and continued appearing at different places until December. The disease dynamics in this study period has indicated the end of the rainy season a time where the incursions occur. From the affected cattle skin nodular lesions were collected and the viruses isolated by primary cell culture. The viral DNA was detected by Conventional and Real time PCR. Furthermore, the LSDV was characterized by Sanger sequencing using the RPO30 gene. Serum sample also collected for virus neutralization test as support data. A molecular detection using both Real time-PCR and conventional PCR has revealed that all the collected samples were LSDV positive. For the isolates multiple realignments were done to see single nucleotide polymorphism (SNPS). And it was found no SNPS among the current isolates. The phylogenic sequence analysis based on RPO30 subunit gene stipulate that the LSDV field isolates fall in the same group and varied from the vaccine strain while being under the same LSDV clade.

With the emphasis viewed in this study, the subsequent recommendations are provided, for the success of prevention and control of the disease.

- Advanced molecular characterization based on the various gene of LSDV should be performed to examine the genetic dynamics and variations that might occur.
- Extensive geographical coverage is needed to really understand molecular epidemiology of LSDV since this study limited to specific outbreak areas.
- Intensive trainings and scientific communications should be shared with Animal health professionals emphasizing on the vaccine and vaccination practices.

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8. ANNEXES

Annex 1: Primary Lamb kidney cell culture preparation

1. Thaw the biopsy samples at room temperature and wash three times in sterile phosphate-buffered saline (PBS, pH 7.2).
2. Take approximately 1 g washed tissue sample mix with 9 ml sterile PBS containing antibiotic (0.1% gentamicin, Sigma-Aldrich, Germany) and ground using a sterile mortar and pestle.
3. Centrifuge the tissue suspension at 600 x g for 15 min and filter the supernatant through a membrane of pore size 0.45 μm (Millipore, United States of America [USA]).
4. Inoculate approximately 0.4 ml filtered supernatant onto a monolayer of PLK cells in a 6 well plate.
5. Incubate at 37°C for an hour for adsorption, and then add 9 ml Dulbecco's Modified Eagle Medium (DMEM, Sigma-Aldrich), containing 0.1% gentamicin and 2% fetal calf serum (Sigma- Aldrich).
6. Incubate the inoculated flasks at 37°C in a humidified incubator with 5% CO2.
7. Monitor the cells daily for 14 days for evidence of CPE

Annex 2: Virus Neutralization test procedure

Principle: to detect the presence of serum specific antibodies against Lumpy skin disease virus by *in vitro* neutralization of the viral cytopathic effect on cell culture. This method is based on a reaction between the virus and specific antibody in the test serum. un-neutralized virus is detected by cytopathic effect. A loss of infectivity of the virus is caused by interference by the bound antibody with any of the steps leading to the release of the viral genome from the host cells including attachment, infection, or viral release.

Procedures

- Trypsinize the lamb kidney cells and prepare suspension of $4X10^5$ cells per ml in complete MEM medium. The cells are then kept appropriately diluted at 37^0C in the incubator.
- Dilute the viral suspension to be titrated in sterile tubes 10 fold from 10-1 to 10-7 (0.5ml viral suspension in 4.5 ml of MEM without serum) and place it in ice rack.
- With multichannel pipette, dispense 100µl of this cell suspension in all the wells ($4x10^5$ cells/well)
- In the wells of the last row, which serve as a negative control, dispense 100µl of the MEM without serum
- Dispense there after different viral dilutions on to cells in a way to have ten replicates for each dilution in 100µl volumes.
- Fill the periphery wells with 200µl media so as to make barrier against desiccation
- Incubate at 37^0C under 5% CO2 and humidity. In case of no CO2 supply, it is possible to wrap the cells in paraffin (making an impermeable envelope). The humidity supplied by the water tank/rack is a requirement against desiccation.
- The test serum from -80 is thawed by incubating 56^0c for 30 minutes
- A working record format is prepared in a layout of the 96 plate according to the samples to be tested, each sample must be recorded individually, the stock virus, the sera dilution(inverse log), and all the information required
- Prepare a sufficient volume of medium: MEM + 2% antibiotic/antimycotic + 1% L-Glutamine of 200 mM with and without serum.
- Take out the viral suspension from -80 right before use.

- The virus stock with known titer should be diluted to give a 10^3 $TCID_{50}$ /ml. Use MEM without serum but with 2% antibiotics & antimycotics and 1% glutamine.
- 100 µl (10^2 $TCID_{50}$) of this viral dilution will be added to each well, therefore 10ml of the viral suspension is required for one plate.
  - Addition of medium: 100 µl of medium without serum is distributed in all wells.
  - Take a 96 well tissue culture plate. One plate allows testing 8 sera in duplicate in serial dilutions (1:5).

25 µl of test sample 1-16, added in well A1-H1 and A7-H7

25 µl of test sample 1-16, added in well A2-H2 and A8-H8

With the multichannel pipette set at 25 µl, perform 5 fold serial dilutions (from colomn1-6, and Colomn7-12) with initial dilution 1/5 and discard 25 µl of suspension from the end point dilution 1/15625 i.e. from column 6 and 12.

- Addition of virus: Add 100µl of 1000 TCID50/ml viral suspension to each wells.
- **Control plate**: The plate should contain positive and negative controls and prepare separately

Negative control: 6 wells in the last row are without virus.

Positive control: 6 wells in each 4 rows are filled with different dilutions of LSDV

Viruses (100$TCID_{50}$, 10$TCID_{50}$, 1$TCID_{50}$ and 0.1$TCID_{50}$ in 100 µl suspension) then incubate in 37°c for 1 hour.

- Addition of cells: After 1 hour add in each well of the plate 50 µl of lamb kidney cells suspension of ($4x10^5$ cell/ml**).** Then the plates are incubated at 37°c with 5% CO2.
- Microscope reading: Plate reading to monitor for CPE formation at 8,10 and 14 days
- The test result is valid only if CPE does not occur in the Negative control wells and
- For the positive controls the CPE formation should be according to the virus titer in each four rows and expected standard protocol would be (++++, +++, +- +-, ----).

100$TCID_{50}$/100µl: + + + + +

10$TCID_{50}$/100µl: + + + + +

1$TCID_{50}$/100µl: + - + - +

0.1$TCID_{50}$/100µl: - - - - -

Annex 3: DNA extraction (Qiagen)

1. Cut the tissue sample in to pieces and grind it with sand by adding PBS buffer.

2. After centrifugation at 2000 rpm for 2 minutes, collect the supernatant in to new micro centrifuge tubes after that

3. Take 200µl of it put in to new micro centrifuge tube and add20 µl of proteinase K and mix by vortex. To ensure efficiency of lysis add 200 µl of AL buffer (lysis buffer) and mix it with pulse vortexing for 15 sec.

4. Incubate it at 56°C for 10 minutes then briefly centrifuge

5. Add 200µl ethanol (96-100%) and mix thoroughly for 5 sec by vortex mixer and briefly centrifuge it.

6. Apply this mixture to the QIAamp mini spin column and centrifuge at 6000 x g (8000rpm) for 1 minute.

7. Transfer the spin column in to 2 ml collection tube and add 500µl buffer AW1and centrifuge it at 8000rpm for 1 min.

8. Discard the collection tube, transfer the spin column in to new 2 ml collection tube and add 500µlbuffer AW2 and centrifuge at full speed 14000 rpm for 3 min.

9. Carefully transfer the spin column in to a new 2 ml collection tube and discard it.

10. Centrifuge the old collection tube with the filtrate for 1 min and add200 µl of buffer AE and incubate at room temperature for 1 minute and continue with centrifugation at 8000rpm for 1 min and this step was repeated to get the finale extract.

11. Collect the extracted DNA and store at -20°C until use

Annex 4 : Pictures of sick animals and sample collection

Annex 5: Ethical Clearance

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ADDIS ABABA UNIVERSITY
College of Veterinary Medicine
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Animal Research Ethical Review Committee

Ethical clearance certificate

Certificate Ref. No: VM/ERC/06/02/14/2022

Name of Applicant: **Mihiret Shimelis Haile (BSc in Vet. Lab. Tech, MSc fellow)**

Address: Department of Microbiology, Immunology and Vet. Public health, College of Veterinary Medicine and Agriculture, Addis Ababa University

Title of the project: *Lumpy skin disease serological detection and molecular characterization in Central Ethiopia*

Date of application:	**December, 2021**
Nature of the project:	**Mildly invasive (little stress)**
Target animal species:	**Cattle**
Number of animals involved:	**38**
Study area:	**Central Ethiopia**

Minutes No. and date of review: **VM/ERC/02/14/022, 01/03/2022**

The above indicated research project is acceptable from ethical perspective, relevance, originality and technical competence points of view. Hence the project is ethically sound to be executed provided that:

1. All procedures and conditions stipulated in the proposal are respected, minor comments are corrected and any deviation or changes be reported to the committee
2. The project activities be open for occasional supervision by the committee when deemed necessary

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

Professor Getachew Terefe (DVM, PhD)
Chairman

Signature

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