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ISOLATION AND MOLECULAR CHARACTERIZATION OF LUMPY SKIN DISEASE VIRUS IN CENTRAL ETHIOPIA

BY:

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ISOLATION AND MOLECULAR CHARACTERIZATION OF LUMPY SKIN DISEASE VIRUS IN CENTRAL ETHIOPIA

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 Masters of Science in Veterinary Microbiology

By

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As members of the Examining Board of the final MSc open defense, we certify that we have read and evaluated the thesis prepared by Mariamawit Zekarias titled "**Isolation and Molecular Characterization of Lumpy Skin Disease Virus in Central Ethiopia"** and recommend that it be accepted as fulfilling the thesis requirements for the degree of: Masters of Science in Veterinary Microbiology

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STATEMENT OF AUTHOR

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LIST OF ABBREVIATIONS

AGID	Agar gel immunodiffusion
CaPV	*Capripoxvirus*
CO_2	Carbon dioxide
CPE	Cytopathic effect
DNA	Deoxyribonucleic acid
ESH-L	Embryonic skin of sheep
FCS	Fetal calf serum
GMEM	Glasgow Eagle Minimal Essential Medium
GTPV	Goat poxvirus
IFAT	Indirect fluorescent antibody test
Kbp	Kilo base pair
KSGP	Kenyan sheep and goat pox
LSD	Lumpy skin disease
LSDV	Lumpy skin disease virus
MEGA	Molecular evolutionary genetics analysis
Mm	Millimeter
mRNA	Messenger ribonucleic acid
OIE	Office International des Epizooties
PBS	Phosphate-buffered saline
PCR	Polymerase Chain Reaction
rpm	Revolution per minute
SGPV	Sheep and Goat poxvirus
SNNPR	Southern Nation Nationalities and Peoples Region
SPPV	Sheep poxvirus
TCID50	Tissue culture infective dose 50%
VNT	Virus neutralization test

ABSTRACT

Lumpy skin disease (LSD) is high impact viral disease affecting cattle in various parts of Ethiopia and considerable number of countries worldwide. Taxonomically, LSD virus is classified in the family *Poxviridae* of the genus *Capripoxvirus*. The disease is caused by LSD virus and is characterized by nodules on the skin that cause permanent damage to hides and skins. LSD a serious disease that has been impossible to control in Ethiopia which is made clear by the outbreak that occurs year in and year out. Even though a continuous work has been done to understand the disease, tackling it prove to be unrealizable, urging more work to be done as much as possible that can assist as a building block to bring this devastating disease to its annihilation. The current work hopes to do that by isolating and characterizing of the virus that cause this disease from the most recent outbreak incident. A purposive sampling technique was implemented in the town of Mojo, Ejere, Ejersa and koka. Cell line originated from sheep skin (Embryonic skin of sheep/ESH-L), highly sensitive to *Capripoxvirus*, was used for the isolation of the infectious virus. The isolates were further processed for classical and real time PCR in order to genotype. The virus that was detected as LSDV, have been further characterized through the RPO30 gene amplification for sequencing and phylogenetic tree construction in relation to different viral isolates from a previous work done though out the country and beyond. Out of 15 samples collected 10 of them were found to be positive for LSD. Further sequencing shows there was a two nucleotide position variation when comparing the present study isolates and the vaccine (KS-1) resulting in a single nonsense amino acid mutation. Constant outbreak investigation and full gene sequencing are the major suggestion of the study.

Keywords: *Lumpy skin disease, LSDV, RPO30, molecular characterization, virus isolation*

1. INTRODUCTION

In Ethiopia, the agricultural sector is a back-bone of the economic and social life of the people. Ethiopia holds the largest livestock population in Africa estimated at about 55.2 million heads of cattle (Shapiro *et al*., 2017). The contribution of live animals and their products to the agricultural economy accounts for 42% and this sector is estimated to account for19% of the GDP (Shapiro *et al*., 2017) and provides employment to over 80% of the agricultural labor force (Asresie, 2015) without factoring in traction power, fertilizing and means of transport.

Even though livestock provides huge contribution to national GDP; it is hampered by various livestock diseases. One of the major livestock diseases is Lumpy skin disease (LS D). LSD cause high financial loss with morbidity ranging from 3%-85% and mortality rates at about 3% (Boshra *et al*., 2015b). Due to its capability of rapid trans-boundary spread and substantial cattle production losses (Tuppurainen *et al*., 2018) LSD is on the list of diseases required by law to be reported when encountered (OIE, 2019).

Lumpy skin disease virus belong to the genus *Capripoxvirus* virus, which is one of the eight members of the subfamily *Chordopoxvirinae*, along with Goatpox virus (GTPV) and Sheeppox virus (SPPV) which only infect ungulates (Morgan, 2019).

Lumpy skin disease is an infectious bovine viral acute or subacute disease characterized by the eruption of nodules on the skin, mouth and upper respiratory tract. Lesions may cover the entire animal's body causing permanent damage to the hide. Typically, infected cattle show clearly enlarged superficial lymph nodes. Systemic infections include pyrexia , anorexia, dysgalactia, lameness, and pneumonia. Although affected cattle suffer severe e maciation and production loss for considerable amount of time, there is a breed difference on the severity of the clinical signs (Davies, 1991).

Although LSDV was originally restricted to sub-Saharan Africa, it is now widely spread in most African countries including Madagascar. Since 1990, outbreaks have been reported in the Middle East and between 2013 and 2015 in Iraq, Iran, Turkey, Cyprus, Greece, Southeast Europe and the northern Caucasus region. This leaves the neighboring countries in Europe and Asia at risk of incursion of LSD into their territories (Efficacy *et al*., 2019; Tuppurainen *et al.*, 2018). LSD affected countries face severe losses directly and indirectly in all sections of the cattle farming industry. A considerable reduction in milk and meat production, abortions, fertility problems, damaged skins and hides as well as death or culling of sick cattle constitute the direct losses. Indirect losses follow the cattle movement and trade restrictions (Tuppurainen *et al*., 2018).

Outbreaks of LSDV are associated with high temperature and high humidity. It is usually more prevalent during the rainy season and fall, especially in low-lying areas or near bodies of water, however, outbreaks can also occur during the dry season. Blood-feeding insects such as mosquitos and flies act as mechanical vectors to spread the disease (Weiss ,1968).

Reports show the northern part of Ethiopia as the first one to report the disease of lumpy skin, this was in the year 1983 around southwest of Lake Tana (Mebratu *et al*., 1984). The disease is now reported and isolated form almost all regions of the country despite the difference agroecological zones. Considering the disease's vast prevalence and the considerable amount of Ethiopia's cattle population, it's extremely easy to comprehend that LSD affects the country's economy tremendously (Gari *et al*., 2010). Finding a means of controlling or even stopping this particular disease would potentially economically assist our agricultural sector and country in general.

Therefore, the objectives of this study were:

- To investigate LSD outbreak in the central parts of Ethiopia;
- To isolate and characterize virulent field isolates of LSDV circulating in the area.

2. LITERATURE REVIEW

2.1. Background of Lumpy skin disease and the virus

In 1929 a new disease comes in to advertence in the territory then known as Northern Rhodesia that baffles the scientists. It masquerades as bites of insects by the name of "pseudo-urticaria". A similar disease that manifested itself by the appearance of skin nodules persisted to occur in the ensuing years continuously being misapprehended as pla nt poisoning of some sort (Weiss, 1968). For 16 years the etiology of the this disease remained unknown until Von Backstom recognized it as an infectious malady that is capable of transmission at a higher rate (Von Backstrom, 1945). However, Morgan (2019) clams that LSD or LSD-like disease have been ignored for the many centuries due to the fact that LSDV genes are absent or fragmented in SPPV and GTPV genomes. Suggesting that the latter viruses are derived from an LSDV-like ancestor that became adapted to sheep and/or goats.

Despite heavy control measures the disease spread into Botswana and South Africa by 1943, resulting in significant economic loss as a result of the nearly eight million cattle that have died. By the year 1957 and 1970 it entered Kenya and Sudan, with some sort of link with sheep pox outbreak in the previous one, with in four years it had spread far west affecting Nigeria. A report from Mauritania, Mali, Ghana and Liberia come in the year 1977. Between the year 1981 and 1986 Tanzania, Kenya, Zimbabwe, Somalia and the Cameroon were affected, another LSD epizootic with high mortality rate of 20% (OIE, 2019). For the first time, Egypt and Israel reported the sickness in 1988 and 1989, proving the presence of LSD far north of Ethiopia and beyond the continent of Africa (OIE, 2019).

After the year of 2012 all the way to 2020 however, an explosion of the disease is being noted in a different countries including Turkey, Iraq, Cyprus, Greece, Bulgaria, N. Macedonia, Serbia, Albania, Montenegro, Armenia, Azerbaijan, Kazakhstan, Russia,

Namibia, S. Arabia, and Mozambique Bangladesh, China, India, and Syria, Bhutan, Nepa l, Djibouti, Vietnam, Hong Kong, Myanmar, and Sri Lanka, making the disease an international interest (Vidanović *et al*., 2021)

The first occurrence of Lumpy skin disease in Ethiopia was documented in 1983, in the western region of the nation, precisely southwest of Lake Tana. Despite agro-ecological zone differences, the illness spreads rapidly in practically all locations in the subsequent decades (Gari *et al*., 2010; Mebratu *et al*., 1984).

2.1.1. Lumpy skin disease virus

Lumpy Skin Disease Virus, also known as Neethling Virus, causes LSD. It is an enveloped double stranded DNA virus, ovoid in shape with a molecular weight that ranges from 73 to 91 KDa (Kilodalton) (Kara *et al*., 2003; Tulman *et al*., 2002). Lumpy skin disease virus belongs to the genus *Capripoxvirus* with in the family *Poxviridae* which is made up of two subfamilies: the *Chordopoxvirinae* which infect vertebrates and the *Entomopoxvirinae* which infect insects and the family contain several genera. The ver tebrate *poxviruses* share a group specific antigen (NP antigen) (Maclachlan and Dubovi, 2010; Tuppurainen *et al*., 2018).

Based on electron microscopic examination, Poxviruses have a unique morphology which is a kind of brick-shaped (Tuppurainen, 2018). Poxviruses are the largest of all animal viruses. The average size of lumpy skin disease virus (LSDV) particle is 294 ± 20 nm in length and 262 ± 22 nm in width. Within the virion there are over 100 polypeptides, which are arranged in a core, two lateral bodies, a membrane and an envelope. The membrane and envelope are important structures for the interaction with the host cell (Fenner *et al*., 1987).

Mature virions that are released from the cell without cell disruption are enveloped. The envelope contains two layers of cellular lipids and several virus-specific polypeptides. Most of the virions released by the rupture of the host cell are therefore not enveloped.

Both enveloped and non-enveloped virions are infectious (Fenner *et al*., 1987). Using transmission electron microscopy, *capripoxviruses* display a biconcave core consisting of the genome which is in a triple-folded coil or tube. The core and two lateral bodies are surrounded by the capsid (Tuppurainen *et al*., 2018).

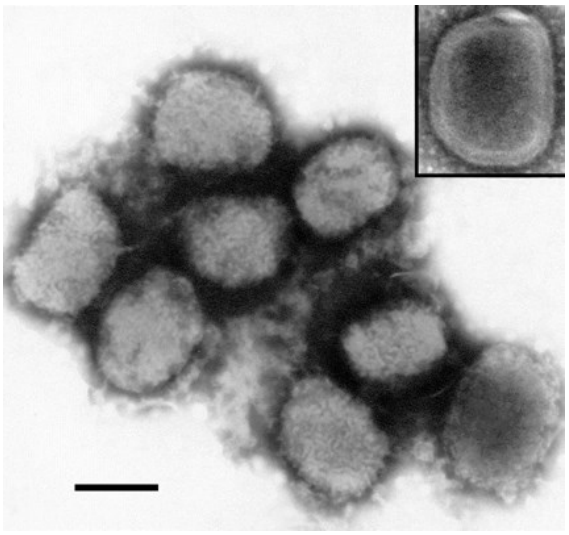


Figure 1: Electron micrograph of brick-shaped virus particles (scale bar = 200 nm)
Source: https://www.cabi.org/isc/datasheet/76779#toPictures (CABI, July 2020)

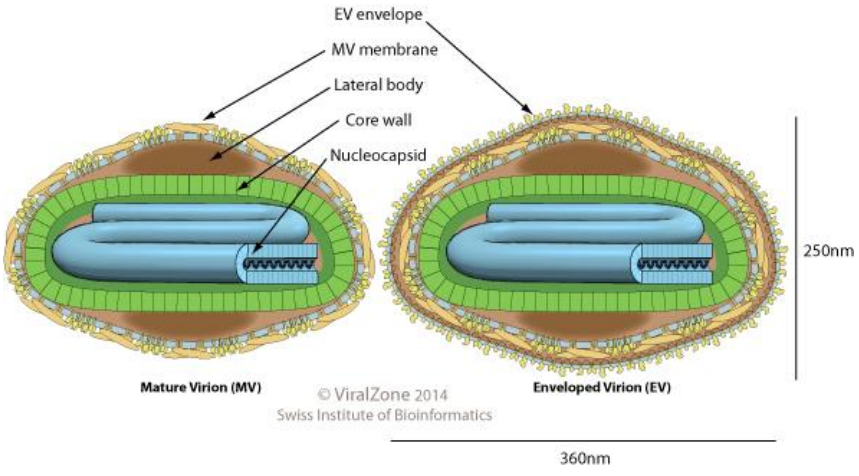


Figure 2: General structure of *capripoxviruses*
Source: http://viralzone.expasy.org/all_by_species/152.html

The LSDV genome is about 151-kbp long double-stranded DNA, covalently cross-linked at the ends(Kara *et al*., 2003). The genome comprises 156 putative genes, with a core coding region flanked by identical 2.4 kbp-inverted terminal repeats. LSDV has 146 conserved genes when Compared with *chordopoxviruses* of other genera which encode proteins involved in transcription and mRNA biogenesis, DNA replication, protein processing, nucleotide metabolism, virion structure and assembly, viral pathogenicity and host range (Tulman *et al*., 2001).

These genes encode several pox viral proteins known to be structural or involved in virion morphogenesis and assembly. Genomes have the capacity to encode about 200 proteins, as many as 100 of which are contained in virions. Unlike other DNA viruses, poxviruses encode all the enzymes required for transcription and replication, many of which are carried in the virion (MacLachlan *et al*., 2016). At least 34 genes are found in the terminal genomic sequences that are involved in virulence, host range, and/or immune evasion (Gari *et al*., 2011; Kara *et al*., 2003; Tulman *et al*., 2002).

The genome sequences of *capripoxviruses* are remarkably conserved, with more than 95% homology between Lumpy skin disease virus, Sheep pox virus, and Goat pox virus (Kara *et al*., 2003). A strain of sheep and goat pox virus is genetically and antigenically similar to LSDV. However LSDV includes nine non-functional genes in sheep and goat poxviruses, some of which are likely responsible for their capacity to infect cattle (Tulman *et al*., 2001). comparative sequence analysis of the two field isolates of LSDV with the genome of the South African Onderstepoort vaccine strain suggests that *capripoxvirus* virulence are linked to a number of genes putatively involved in host immune-modulation (Kara *et al*., 2003).

2.1.2. Virus replication

Poxviruses replicate mostly, if not completely, in the cytoplasm, in contrast to most other DNA viruses. Poxviruses establish a distinct region in the cytoplasm of cells, referred to as the “virosome” or “DNA factory”, where viral DNA replication occurs. Poxviruses enter cells by fusing their membranes with either the plasma membrane or the endosomal

membrane. After fusing with the plasma membrane or entering through endocytosis, the viral core is discharged into the cytoplasm (Maclachlan and Dubovi, 2010).

Transcriptase released from the core of the virion facilitates formation of mRNA within minutes after infection. Transcription is characterized by a cascade in which the transcription of each temporal class of gene ("early," "intermediate," and "late" genes) requires the presence of specific transcription factors that are transcribed from the preceding temporal class of genes. The polypeptides produced by translation of these mRNAs complete the uncoating of the core before the actual viral DNA synthesis begins 1.5 to 6 hours of infection (MacLachlan *et al*., 2016).

Poxvirus DNA replication can usually be detected within 2 hours after infection of a susceptible cell. It is initiated near the genome termini and involves the synthesis of long concatameric intermediates, which are subsequently cut into unit-length genomes that are ultimately incorporated into immature cores before their closure. Because poxvirus virions are composed of a very large number of proteins, virus assembly is a complex process and requires several hours to be completed (Maclachlan and Dubovi, 2010).

2.1.3. Host range and transmission mechanism

The host range of *capripoxvirus* includes sheep, goats and cattle breeds of all age and sexes, some wildlife have also been implicated. However, LSDV with a few exceptions only causes clinical disease in cattle. LSDV mainly affects cattle, but has also been seen in giraffes, water buffalo, and impalas. Fine- skinned Bos taurus cattle breeds such as Holstein-Friesian and Jersey are the most susceptible to the disease. Thick-skinned Bos indicus breeds including the Afrikaner and Afrikaner cross-breeds show less severe signs of the disease. This is probably due to the decreased susceptibility to *ectoparasites* that Bos indicus breeds exhibit relative to Bos Taurus breeds. Young calves and cows at peak lactation show more severe clinical symptoms, but all age groups are susceptible to the di sease (Hanshaw *et al*., 1968).

Infection in cattle with lumpy skin disease virus (LSDV) can occur by mechanical transmission of the virus by insect or tick vectors. The virus can also be transmitted through blood, nasal discharge, lacrimal secretions, semen and saliva. The disease can also be transmitted through infected milk to suckling calves. In experimentally infected cattle, LSDV was discovered in saliva 11 days after the onset of fever, in skin nodules after 33 days and in semen 22 days later, indicating that semen harboring lumpy skin disease virus might be transmitted sexually (Annandale *et al*., 2010). However, the transmission of LSDV by direct contact is considered to be inefficient. The virus is not found in urine or stool. Like other pox viruses, which are known to be highly resistant, LSDV can remain viable in an infected tissue for more than 120 days (Awad *et al*., 2010; Hanshaw *et al*., 1968).

It is widely agreed that the most important mode of transmission of LSDV is likely to be through mechanical transmission of the virus by a variety of blood feeding vectors. This hypothesis is supported by the observation that LSD outbreaks are correlated with warm and wet seasons (Hanshaw *et al*., 1968). A single species vector has not been identified. Instead, the virus has been isolated from *Stomoxys*, *Biomyia fasciata*, *Tabanidae*, *Glossina*, and *Culicoides* species. Different studies have demonstrated that LSDV can be transmitted by *Aedes aegypti*, female mosquitoes, within 2–6 days after infective feeding (Ga, 2013; Tuppurainen *et al*., 2018).

On a research done by Tuppurainen in 2013, shows that different tick types like that of Hard (ixodid) ticks *Rhipicephalus appendiculatus* and *Rhipicephalus decoloratus* could mechanically transmit LSD virus. Other type of tick, *Amblyomma hebraeum*, was also proven to possess transmission ability of LSD on experiment done by Lubinga in 2015. Even though ticks and mosquitos play a crucial role in transmitting the virus from infected to naive cattle there is no evidence that LSDV can replicate in any insect or tick vector (Ga, 2013; Tuppurainen *et al*., 2018).

2.1.4. LSD Morbidity and Mortality

Morbidity and mortality differ significantly depending on the breed of cattle, the population's immunological state, insect vectors involved in mechanical transmission, and virus isolates. By natural predisposition, Bos Taurus is more susceptible than Bos Indicus and lactating cows appearing to be at most risk. Insect vector distribution and relative abundance are hypothesized to reflect disparities in morbidity rates in different ecosystems. There are contradicting results regarding age and sex related susceptibility to LSD. In some studies higher morbidity was related with calves while other claim they are more resistant to the disease. Same inconsistency was reported regarding sex as well (Davies, 1991; Molla *et al*., 2018; Molla *et al*., 2017).

Furthermore, stress factors such as the prevalence of mechanical arthropod vectors and *trypanosomosis* wreak havoc on the animal's immune system, making LSD infection more severe. As a result, the disease's morbidity ranges from 3% to 85%, with mortality never exceeding 3% in an outbreak. In endemic areas, however, morbidity is frequently approximated around 10%. The mortality rate is normally low (1–3%), but it can occasionally approach 40% (Tuppurainen *et al*., 2018, 2014; Tuppurainen and Oura, 2012).

2.1.5. Geographical distribution

LSD was documented in a wide geographical range, in Africa, the Middle East, Central Asia and Europe and in a diversity of climates. Using the presence-only maximum entropy ecological niche modeling technique in order to characterize the geographical risk factors for disease occurring in the Middle East (Alkhamis and VanderWaal, 2016), it was shown that annual precipitation (positive association) or/and mean diurnal temperature range (negative association) were the most significant environmental factors to be associated with LSD outbreak distribution. This supports the notion that humid and warm regions are most appropriate for the development of LSD outbreaks as they support

the vector population. Despite this, LSD outbreaks can occur in moderate temperatures of 18–22℃ (Tuppurainen *et al*., 2018).

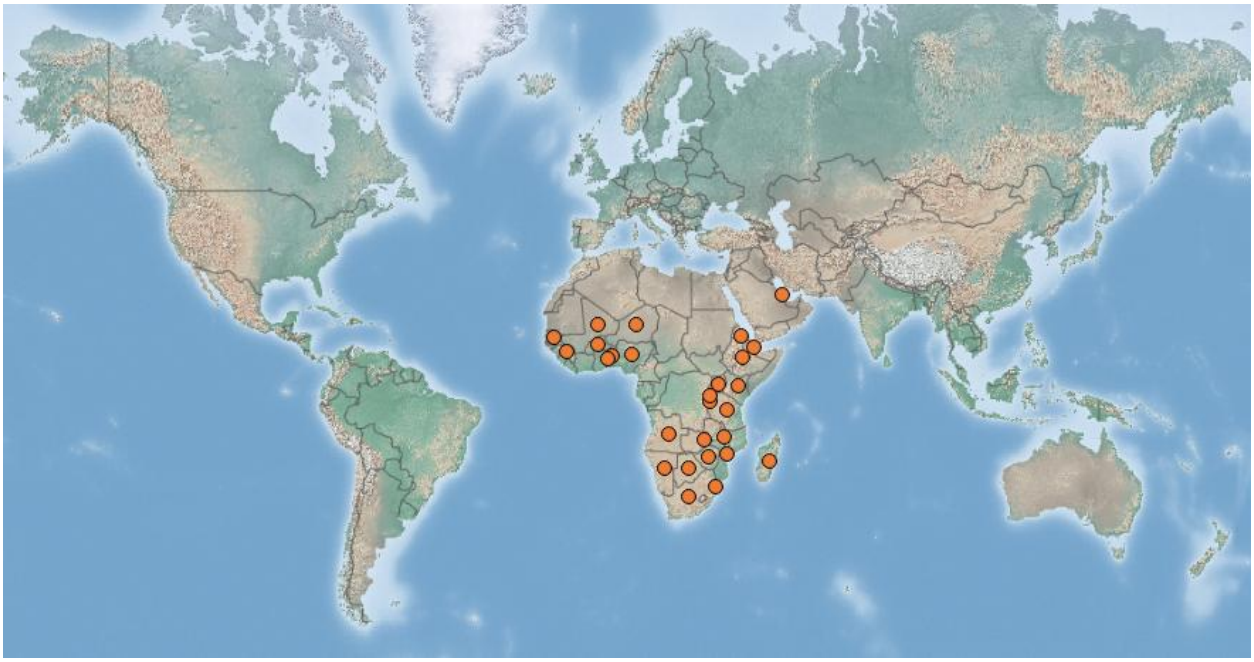


Figure 3: Geographical distribution of LSD
Source: https://www.cabi.org/isc/datasheet/76780#toDistributionMaps (CABI, July 2020)

2.2. Economic Importance of the Disease

Animal disease outbreaks constitute a substantial threat to livestock industries around the world, both in terms of the disease's direct economic impact and the steps taken to reduce the likelihood of disease introduction, which both coast hefty amount to deal with the disease (Rich and Perry, 2011). Lumpy skin disease is a cattle disease that is economically destructive across Africa and the Middle East. LSD inflicts serious constraints on livestock production in its endemic range, but poor rural communities without proper access to veterinary services are disproportionately affected during outbreaks of the disease (Ga, 2013).

Even though the morbidity and mortality rates of LSD are usually low, it is an economically important disease of cattle in Africa because of the prolonged loss of

productivity of dairy and beef cattle, decrease in body weight, mastitis, severe orchitis, which may result in temporary infertility and sometimes permanent sterility. Pregnant cows may abort and infertility of cows can last for several months. In severely affected animals damage to hides is permanent and the value of these for leather industry is greatly reduced. This affects individuals and the country as whole by hindering growth and attribute to significant economic losses in most endemic countries (Tuppurainen, 2018).

2.3. Lumpy Skin Disease Diagnosis

Nodular lesions on the skin and mucosal membranes, as well as swelling of the superficial lymph nodes, are pathognomonic for LSD (Jimolu, 2011). Although clinical indicators can be used to make a preliminary diagnosis, mild and inapparent disease can be difficult to identify, necessitating the use of quick laboratory tests to confirm the diagnosis (Tuppurainen, 2018). Confirmation of the diagnosis through laboratory techniques can be done using various methods (Jimolu, 2011).

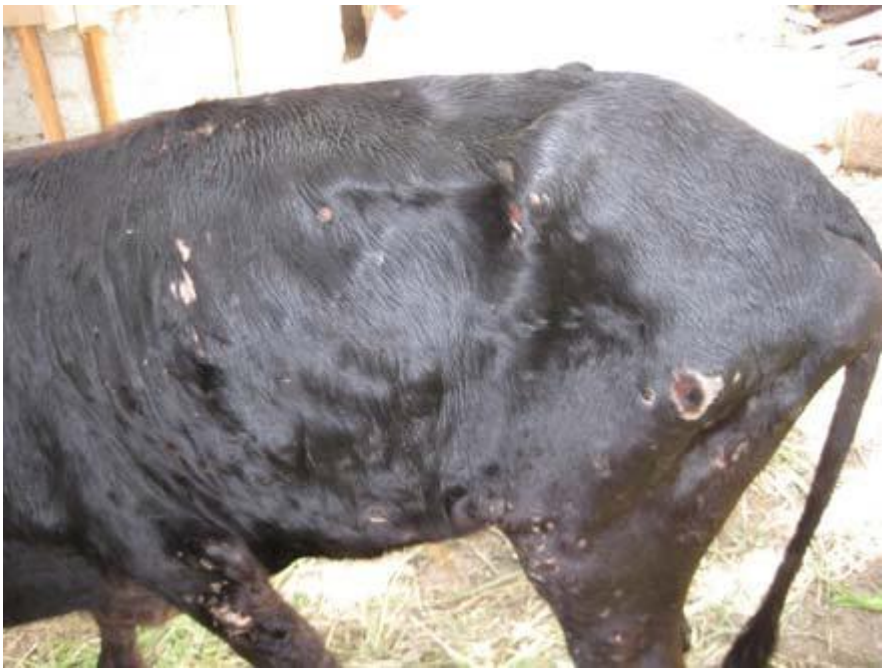


Figure 4: Lumpy skin disease: necrotic nodules and deep scab formation

There are a number of differential diagnoses that can be mistaken for LSD. This includes the following: Pseudo-lumpy skin disease (caused by bovine herpesvirus-2) can be confused with LSD, as can rinderpest, insect bites, Demodex infection, onchocerciosis, besnoitiosis, and dermatophilosis (Davies, 1991; Fenner *et al*., 1987). Field diagnosis is particularly complicated by diseases that cause mucosal lesions, such as cattle viral diarrhea/mucosal illness and bovine malignant catarrhal fever (Fenner *et al*., 1987).

Although cutaneous and visceral pox lesions are very suggestive of the diseases in issue, laboratory confirmation is required for a conclusive diagnosis. Above all, to effectively control or eradicate LSD in both endemic and non-endemic areas, swift and precise testing technologies are required to confirm a presumptive diagnosis (Awad *et al*., 2010; Body *et al*., 2012; Gari *et al*., 2008; Tuppurainen, 2018).

Laboratory diagnosis of LSD can be done either by identification of the agent using transmission electron microscopy (TEM), by its isolation in cell cultures, by direct fluorescent antibody test (FAT) or by detection of antibody using conventional serological tests such as the serum virus neutralization test (SNT), indirect fluorescent ant ibody test (IFAT) and agargel immunodiffusion test (AGID). Polymerase chain reaction (PCR) have been developed for detecting LSD antibodies and antigen (Carn, 1993; Carn and Kitching, 1995; Modrow, 2013; Prozesky and Barnard, 1982).

2.3.1. Virus isolation and identification

LSDV is separate from pseudo-LSD (Allotron-Herpes mammilitis), a herpesvirus that induces syncytia and intranuclear inclusion bodies, But LSD causes a cytopathic effect and intracytoplasmic inclusion bodies (Hanshaw *et al*,. 1968).

Lamb and calf kidney cells, lamb and calf testis cells, fetal lamb and calf muscle cells, lamb and calf adrenal and thyroid cultures, sheep embryonic kidney and lung cells, chicken embryo fibroblasts, rabbit fetal kidney and skin cells, adult vervet monkey

kidney cell line, and baby hamster kidney cells can all be used to culture the Lumpy skin disease virus. The virus can also be cultured in primary cell cultures of bovine dermis and equine lung cells. The development of cytopathic effects (CPE) may take up to 14 days during primary isolation (Tuppurainen, 2018).

Although LSDV can thrive in bovine, ovine, or caprine tissue cultures, primary or secondary cultures of bovine dermis cells or lamb testis cells are thought to be the most sensitive. One ml of filtered supernatant or buffy coat is injected into a confluent monolayer in a 25 cm^2 culture flask and left to adsorb for one hour at 37°C. The culture is then washed in warm PBS before being covered with 10 ml of an appropriate medium, such as GMEM, which contains antibiotics and 2% fetal calf serum (Balinsky and Ph, 2007; Kara *et al*., 2003; Massung *et al*., 1993).

The flasks are monitored daily for symptoms of cytopathic effects for 7–14 days (CPE). Cell rounding, retraction of the cell membrane from surrounding cells, and nuclear chromatin margination are all characteristics of infected cells. Small patches of CPE can be visible as early as 2 days after infection in certain cases; these develop larger during the next 4–6 days, finally encompassing the entire cell sheet. By day 14, if no CPE is apparent, the culture should be frozen and thawed three times, with the cleared supernatant transferred onto a new culture (Tuppurainen *et al*., 2018).

The chorioallantoic membrane of embryonated chicken eggs and Vero cells from African green monkeys have been modified to host LSD-causing *capripoxvirus* strains. However, this is not recommended for primary isolation (Hope and Ca, 2016).

2.3.2. Serological Diagnosis

Serology has been developed to characterize the immune response following infection with LSDV. Serological methods may be used retrospectively to confirm recent infections, but are not recommended for primary diagnosis (Davies, 1991). Neutralizing antibody appears 3-4 days after the onset of the clinical signs and reaches the peak titre

level in 2-3 weeks. Both complement fixing and precipitating antibodies are present in the serum of infected and recovered animals. Immunological defense against *capripoxvirus* relies mainly on cell-mediated immune response and humoral immunity would remain in the circulation for a short period within the time range of mostly seven to eight months (Capstick and Coackley, 1962).

Serologic testing cannot identify *capripoxvirus* strains from cattle, sheep, or goats since all *Capripoxviruses* have the same main antigen for neutralizing antibodies. The interpretation of serological test results may sometimes be difficult due to low antibody titres in vaccinated animals and in some individuals following mild infection. Moreover, all serological assays face the same problem that different surface proteins of non-enveloped intracellular mature virions and extracellular enveloped virions induce the formation of different antibodies in the host, and their relative proportions may vary during different stages of infection (Knipe and Howley, 2013; Tuppurainen *et al*., 2018).

Neutralization test

The gold standard for detecting specific antibodies to *capripoxviruses* is the virus neutralization test. It is the most common widely used serological test for *capripox* antibody detection. Although it has high specificity and excludes cross- reaction with *cowpox* and *Parapoxvirus* antibodies, it is not sensitive enough to identify animals that have been in contact with the viruses but have developed only low levels of neutralizing antibodies (Davies, 1991).

To calculate a neutralization index, a test serum can be titrated with a titre of capripoxvirus that is constant or a constant virus can be titrated and the test serum kept at a constant dilution. The neutralization index is the preferable method due to the varied sensitivity of tissue culture to *capripoxvirus* and the difficulties of ensuring the use of 100 $TCID_{50}$. It does, however, necessitate a bigger volume of test sera (OIE, 2019).

Indirect Fluorescence Antibody Test (IFAT)

The indirect fluorescent antibody test can be performed on *capripoxvirus*-infected tissue culture cultured on cover-slips or tissue culture microscope slides. The test should include an uninfected tissue culture control, as well as positive and negative control sera. Infected and control cultures are fixed in acetone for 10 minutes at –20°C before being kept at 4°C. Positive samples are recognized using an anti-bovine gamma-globulin conjugated with fluorescein isothiocyanate and dilutions of test sera in PBS commencing at 1/20 or 1/40. After infection, antibody titres may approach 1/1000. Sera can be tested at 1/50 and 1/500 scales. With the infectious pustular dermatitis virus, cross-reactions can arise (Hope and Ca, 2016).

Agar gel immunodiffision

The Agar gel Immunodiffusion test (AGID) has been used to detect the Precipitating antigen of *capripoxvirus*. However, due to cross-reaction with antibodies to bovine papular stomatitis and pseudo cowpox virus, this test is not recommended as a serological diagnostic for the diagnosis of LSD. As a result of this cross-reaction, false-positive results will be discovered. A test's lack of sensitivity might also result in false-negative results (Tuppurainen, 2018).

2.3.3. Molecular Diagnosis Techniques

Molecular diagnostic techniques are critical for tracking the transmission of these viruses and preventing epidemics in susceptible animals. Where the disease is endemic, polymerase chain reaction (PCR) is the most accessible diagnostic method for *capripoxvirus* genome detection since it is both quick and sensitive (Hamdi *et al*., 2020; MacLachlan *et al*., 2016; Tuppurainen *et al*., 2018).

Polymerase chain reaction (PCR)

Multiple versions of PCR are being developed with the intention of detecting a subtle genetical difference that the sheep pox, goat pox, and LSDV exhibit. A multiplex PCR-based species-specific primer to differentiate between *capripoxvirus* species has been developed. Another type of PCR called duplex PRC assay was developed to detect both *capripoxvirus* and contagious pustular dermatitis virus using the A29L gene region of *capripoxvirus*. Although this assay was only evaluated on sheeppox and goatpox viruses, the *capripoxvirus* primers in the assay will also amplify LSDV based on sequence homology (Weiss, 1968).

A more efficient and cost-effective Capripoxvirus genotyping approach based on snapback primer and dsDNA intercalating dye was developed. This method is able to differentiate lumpy skin disease virus, sheep pox virus and goat pox virus using the melting temperature of snapback stems of the hairpins and those of the full-length amplicons (Gelaye *et al*., 2013). Another multiplex PCR that uses somewhat the same mechanism allows the simultaneous detection and differentiation of eight poxviruses (Gel aye *et al.*, 2017).

2.4. Status of Lumpy Skin Disease in Ethiopia

In the years between 1981 and 1983, Ethiopia has first observed Lumpy skin disease in north western, western and central regions of Ethiopia (Mebratu *et al*., 1984). After its first appearance, an explosive rapid outbreak swept from the north across the center and southern parts of the country. In the subsequent three to five years, it had covered the vast area of the highland and midland parts of Ethiopia (Abera, 2019).

Data analysis of the national disease outbreak report database from 2000 to 2009 revealed that substantial LSD epidemic outbreaks occurred in the northern sections of the country in the Amhara and West Oromia areas in 2000/2001. Then it extended to the central and the southern parts of the country in 2003/04 covering large parts of Oromia and Southern

Nation, Nationalities and Peoples (SNNP) regions. In 2006/07 another extensive outbreak reappeared in Tigray, Amhara and Benishangul regions in the northern and north-western parts of the country. From 2007 to 2009, the frequency of outbreaks in Oromia Region, in the country's central region, steadily climbed, while it appeared to be steadily reducing in the northern regions of Tigray, Amhara, and Benishangul (Animal *et al*., 2010).

During these ten years, the national disease outbreak report revealed that LSD had spread to essentially all of the country's regions and agro-climatic zones (Abera, 2019; Gari *et al*., 2011; Jimolu, 2011).A cross-sectional study across different agro-ecological zones in Ethiopia showed an overall observed LSD animal-level prevalence of 8.1% and a mortality of 2.12% (Animal *et al*., 2010).

2.5. LSD prevention and control strategies

The most likely mode of entry of LSD into a new area is by the introduction of infected animals and contaminated materials. If LSD is confirmed in a new area before extensive spread occurs, the area should be quarantined, the infected and in contact animals slaughtered, and the premises cleaned and disinfected as an attempt to eradicate the disease from the country (Davies, 1991). Thus, restrictions on the importation of live animal and animal products from affected areas can prevent the introduction of the disease. However, eradication of LSD is the ultimate and optimal goal when it is reported in disease free countries. In those countries, slaughter of infected and in-contact, ring vaccination of cattle with quarantine in radius of 25-50 km, strict movement restriction of animals and destruction of contaminated hides should usually be sufficient to eradicate the disease (Boshra, *et al*., 2015; mekonnes, 2007).

Unfortunately, if the disease has spread over a large area, the most effective means of controlling losses from LSD is mass vaccination. Moreover, consideration should still be given to eliminating infected and exposed herds by slaughter, proper disposal of animals and contaminated material, and by cleaning and disinfecting contaminated premises, equipment, and facilities (Boshra, *et al*., 2015; Carn & Kitching, 1995b).

In endemic countries, vaccination against LSD is the only effective method to control and prevent the disease. The experience showed that the vaccination approach is commonly chosen and is often that of ring vaccination around a local foci outbreak when it occurs. The control of insects was not effective in preventing the spread of LSD in South Africa, but current insecticides together with repellents might help to reduce the spread of LSD. There is no specific treatment for LSD, but early stage antipyretic and antibiotic treatment could reduce secondary bacterial complications to improve recovery process (Carn, 1993; Kitching *et al*., 1986).

2.5.1. Immunization

Animals that recover from a virulent *capripoxvirus* infection generate lifelong immunity consisting of both antibody and cellular immunity, which protects the animals from all *capripoxvirus* isolates (Asfaw and Ameni, 2012). LSD infection can be prevented by the administration of *anti-capripoxvirus* serum, but cell-mediated immunity is likely the most significant component in recovery from infection and in long-term protection, evidenced by the protection afforded by vaccination where the presence of specific antibodies cannot be detected using existing serological tests (Asfaw and Ameni, 2012).

Until now, only live attenuated vaccines originating from field isolates have been commercially available (Vidanović *et al*., 2021). Attenuation of these vaccines was achieved by multiple passages in cell culture and in the chorioallantoic membranes of embryonated chicken eggs. If a particular vaccine is able to accomplish over 80% of immunity on the herd the vaccine is considered to have a good protection over LSD (Babiuk *et al*., 2008).

2.5.2. Types of available vaccines

There are three licensed vaccines for LSD: lumpy skin disease virus (LSDV) Neethling vaccine, Kenyan sheep and goat pox (KSGP) O-180 strain vaccines and Gorgan goat pox

(GTP) vaccine. However there are many more vaccine isolated from different outbreaks currently in use like that of Yugoslavian RM65 sheep pox strain vaccine and Romanian sheep-pox strain (Brenner *et al*., 2009).

LSDV Neethling Vaccine

The Neethling strain (Onderstepoort Biological Products, South Africa) developed by passing 60 times in lamb kidney cells and 20 times on the chorioallantoic membrane of embryonated chicken eggs (Asfaw and Ameni, 2012) does not cause systemic infection or serious disease in cattle. It produces a local response to a granuloma of 1-2 cm in diameter at the inoculation site in 50% of the vaccinated animals, and the temporary decrease in milk production in dairy cattle has a negative impact on the use of this vaccine. In recent decades in Africa and the Middle East, the use of Neethling vaccines has successfully controlled the spread of LSDV with high levels of vaccine coverage. A study done in 2020 showed that adverse effects due to Neethling vaccination are negligible (Morgenstern and Klement, 2020).

KSGP O-180 Vaccine

The Kenyan sheep and goat pox (KSGP) O-180 strain vaccines (KSGP O-180) and the KSGP O-240 strain was isolated from a sheep during the same epizootics and has previously been utilized successfully as a vaccine against SPPV, GTPV, and LSDV. A strain of Kenya sheep and goat pox virus was made by passing 18 times in fetal muscle cells (Asfaw and Ameni, 2012). The KSGP O-180 vaccine reduces the severity of LSD infection and susceptibility to LSD virus but increases the infectivity of vaccinated infected cattle (Vandenbussche *et al*., 2016).

Gorgan GTP Vaccine

The live attenuated Gorgan goat pox vaccine is produced to use against goat pox virus (GTPV) and lumpy skin disease virus in the Middle East. Compared to the LSD Neethling and KSGP O-180 vaccines, the GTP vaccine elicits a stronger immune

response in the herd. A higher Delayed Type Hypersensitivity (DTH) response is observed in vaccinated cattle, indicating that the Gorgan GTP vaccine is more immunogenic. The close correlation between GTPV genomic sequence and LSDV may be the reason why GTP vaccine shows better protection against LSDV. A recent study shows that Gorgan GTP strain vaccine does effectively protect all calves from the challenge of virulent LSDV wild-type strains, suggesting that the Gorgan GTP strain may be the preferred candidate vaccine for controlling LSD infection (Milovanović *et al*., 201 9; Vandenbussche *et al.*, 2016).

RM65 strain vaccine

The RM65 (Ramyar strain) this virus originated from a Yugoslavian outbreak of sheep pox and was kept in Germany, and was adapted for sheep pox vaccination by Ramyar. It subsequently became the vaccine strain RM65 produced by different country like Israel for use against lumpy skin disease (Brenner *et al*., 2009).

2.5.3. Challenges LSD vaccine presents globally

Vaccines are given with the goal of protecting vaccinated animals from re-exposure to the same infection and in hopes of providing sterile immunity. In the case of LSD vaccine the most desired criteria would be stopping an ongoing infection by a means of rigorous vaccination (Brenner *et al*., 2009). It is strongly believed and demonstrated that vaccination is a current way of controlling and eliminating viral diseases. Unfortunately, in the case of LSD, usage of live vaccines can result in various adverse events, which are similar to clinical signs induced by LSDV, named “Neethling disease”. Moreover, adverse and local reactions in the whole or at the injection site have been observed after application of particular vaccines (Babiuk *et al*., 2008).

In Jordan, research utilizing two types of LSD vaccinations, one produced from the RM65 isolate [Jovivacâ, manufactured by Jordan Bioindustries Centre (JOVAC)] and the other an unlabeled one that is later identified as a strain of lumpy skin disease virus

(LSDV), revealed adverse clinical signs identical to those seen in natural cases of lumpy skin disease (Abutarbush *et al*., 2016). In 2014 a data collected from 101 vaccinated and unvaccinated farms in Jordan, shows overall morbidity rate of 4.7%, mortality rate 1% and case fatality rate 22.9%. Decreased feed intake was seen in 23.8% of the population, decreased milk production and fever were seen in 21.4% and 23.8% of the cattle in the unvaccinated holdings, respectively. Percentage of decrease in milk production ranged from 0 to 100 per cent. This shows a considerable reduction compared to the unvaccinated population. However, the vaccine did not provide a complete protection against the disease and some sort of production loss and treatment cost are still a big concern (Abutarbush, 2014).

A study conducted in Israel on Four thousand six hundred and seven vaccinated cows, a cutaneous clinical manifestation appeared on 11% of the study population, when animals who had been vaccinated with the RM65 strain were re-exposed. The appearance of lesions in a considerable proportion of vaccinated animals hampered its practical use, especially with regard to statutory decision making. These results show that vaccines are not effective in protecting cattle from LSD, either through direct clinical protection or by reducing transmission. Therefore, there is an urgent need to of LSD vaccine development to control LSD in affected countries and prevent it from spreading to other countries (Brenner *et al*., 2009).

2.5.4. Challenges LSD vaccine presents in Ethiopia

LSD vaccine failure and re-infection of vaccinated cattle have become a bottle neck problem for the control of LSD in many countries endemic for LSDV including Ethiopia (Gari *et al.*, 2012). According to a study conducted in Ethiopia, the Kenyan sheep pox vaccine strain employed to reduce LSD did not provide the required protection. In fact, in the zebu cattle morbidity rate among vaccinated cattle was found to be more than four times higher than among non-vaccinated, while in the cross breeds, vaccine did not show any protective effect (Ayelet & Gelaye, 2013).

In 2015 study conducted in Sebeta shows that The Ethiopian Neethling and KSGPO-180 vaccines failed to provide protection in cattle against LSDV which is attributed to poor immunogenicity of the vaccines, which might be due to over attenuation resulting in genetic alteration leading to a failure in the generation of protective immunity (Gari *et al*., 2015). In this study the Gorgan GTP vaccine exhibits far more superior result with better immunity and protected all the calves from clinical signs (Gari *et al*., 2015).

Surprisingly, a study in 2014 confirms that the real identity of the commonly used Kenyan sheep and goat pox vaccine virus is not SPPV but is actually LSDV. This can possibly explain the adverse effect that comes after vaccination. The virus's low level of attenuation is likely insufficient for safe usage in cattle, resulting in clinical illness in vaccinated animals (Tuppurainen *et al*., 2014).

3. MATERIALS AND METHODS

3.1. Study Area

LSD outbreak investigation was undertaken from October 2020 to April 2021 in central Ethiopia, East Shewa Zone, Lome woreda of Oromia Regional State, in the towns of Mojo, Ejere, Ejersa, Koka. Mojo (also transliterated as Modjo) is a town in central Ethiopia, named after the nearby Mojo River and located at a distance of about 73 km south of Addis Ababa. The zone has 12 districts, most of which are situated in the Great East African Rift Valley lakes region that crosses the country. It has latitude of 8°35′12N and longitude of 39°7′15E with an elevation between 1788 and 1825 meters above sea level.

Figure 5: The location of the study area

Source: https://www.infomap.org/isc/datasheet/76754#tolocalMaps

3.2. Study animal

Cattle that showed pathognomonic clinical signs of pox like skin lesion were purposively selected for this study. No exception was put on cattle in regards to sex, breed, rearing style or purpose. All the cattle that are believed to be affected by this particular disease were taken as an animal of interest.

3.3. Sample Collection and Transportation

During visit, which are made based on the reports that come to NVI, visual inspection was made to observe health status of the entire animals in the farms and a detailed physical examination was done for sick animals suspected of having LSD. A total of 15 skin nodules were taken from representative sick animals in different farms for the purpose of identification and isolation of the causative agents. According to the procedures of OIE (OIE, 2019), sample collection from clinically sick animals were carried out for the purpose of virus isolation. Skin nodules from 15 representative cattle that had developed visible clinical sign of the disease were taken by cleaning the area and removing the hairs with the help of sterile scalpel blade. Tissue samples were placed in the sterilized universal bottle with PBS and antibiotic and transported to NVI virology laboratory with the help of icebox maintaining the cold-chain and kept at -20℃ until processed.

3.4. Laboratory investigation

3.4.1. Sample processing

The skin biopsy samples that were preserved in deep freezer (-20℃) was thawed at room temperature and washed three times with sterile phosphate buffered saline (PH 7.2) and a ntibiotic under the Biosafety Cabinet Class-II. Approximately one gram of tissue sample was cut to a fine detail with scissor and gradually mixed with 9 ml sterile PBS while bein g triturated with the help of sterile pestle and mortar. The tissue suspension was then cent rifuged at 3500rpm for 10 min and the supernatant filtered through a 0.45 μm pore size m embrane (OIE, 2019).

3.4.2. Virus isolation

The ESH-L cell line was used for the isolation of LSDV. ESH-L cells are an effective and better suited alternative to primary cells for growing Capripoxviruses (Rouby, 2016).

Cell line of passage 27 was already available at NVI (acquired from PANVAC). Ergo, su b culturing (Appendix 1) the cell took place prior to any work. ESH-L cell culture was propagated in Glasgow Eagle minimal essential medium (GMEM) supplemented with 10 % calf serum. The cell was incubated at 37^{o}C in humidified and 5% CO_2 supplemented in cubator and was grown in 25 cm^2 tissue culture flask until the cells became confluent mo nolayer. When a complete monolayer was evident the cell was considered to be suitable f or inoculation so the cell culture medium was decanted in aseptic conditions on to a beak er and the cell that formed monolayer washed three times using sterile warm PBS with pH of 7.2 in Bio-safety cabinet level II.

One ml of tissue homogenate was then inoculated onto the confluent monolayer and incubated at 37 ^{0}C and allowed to absorb for 1 hour. Then the inoculated monolayer was covered with 10 ml of GMEM, containing antibiotics and 2% fetal calf serum into the flask and placed into incubator. All the flasks, including the control flasks, were incubated at 37°C in a humidified incubator with 5% CO_2. The medium was changed every 48 hours. Cells were monitored daily using an inverted microscope for evidence of virus induced cytopathic effects (CPEs) for 7- 14 days post-inoculation. Three more blind passages were carried out for samples that were initially CPE free. Infected cells developed a distinctive CPE consisting of retraction of the cell membrane from surrounding cells followed by rounding of cells and interruption of the confluences of the cell were detected. When around 80% CPE was observed, Virus inoculated flasks were harvested and put in a freezer for the night at –20°C. For the purpose of releasing the virus particles, the cell cultures were freeze-thawed twice at room temperature which then stored at -20°C until processed for viral DNA detection.

3.4.3. DNA Extraction

At the NVI molecular biology laboratory, DNA was extracted from infected ESH-L cell supernatant using the DNeasy®, Blood and Tissue kit (QIAGEN, Germany) according to the manufacturer's instructions.

DNA was extracted from 10 specimens; 20µl QIAGEN proteinase K solution was pipetted on to 1.5ml of microcentrifuge tube. 200µl sample virus were added along with 200µl buffer AL (lysis buffer) was then mixed by vortexing and clarified by centrifugation at 8,000 rpm for 15 seconds after incubation time of 56°C for 10 min. 200 µl of ethanol (100%) was added and mixed thoroughly with the help of vortex mixer. The mixture was transferred in to spin column in 2ml collection tube and centrifuged at 8,000 rpm for 1 min. The spin column was transferred in to a new 2 ml collection tube and 500 µl of AW_1 was added and centrifuged at 8,000 rpm for 1 min. Then, the collection tube was discarded and the spin column placed in a new 2 ml collection tube and 500 µl of AW_2 was added and centrifuged for 3 min at 14,000 rpm, afterwards, the spin column was transferred carefully in to a new 1.5 ml of microcentrifuge tube and 200 µl of elution buffer (AE buffer) added and incubated for 5 min at room temperature and centrifuged for 1 min at 8,000 rpm. The final extracted product was transferred to PCR room for amplification.

3.4.4. Polymerase Chain Reaction (PCR)

The presence of the virus was detected using *capripoxvirus* specific primer sequences in a polymerase chain reaction (PCR).
primer SpGpRNAPol-FOW-
5pm/ µl 5' TCTATGTCTTGATATGTGGTGGTAG 3' and
primer SpGpRNAPol-REV-5pm/ µl 5' AGTGATTAGGTGGTGTATTATTTTCC-3',
were used (Lamien *et al*., 2011). 255µl of master mix was prepared for DNA amplification and 17µl of that master mix was pipetted on to 10 wells for the samples, 1 well for negative control and 3 wells for positive control. Each well containing of 3µl nuclease free water, 2µl of forward and backward primes each and 10µl of IQ super mix. The lid was then put on lightly and transferred to a PCR room where it was mixed with 3µl of template DNA. Finally the mix was incubated in a thermal cycler using the following amplification program: initial denaturation at 95^{o}C for 5 minute; 40 cycles of denaturation at 95^{o}C for 30 second, annealing at 50^{o}C for 30 sec and elongation at 72^{o}C

for 30 sec. An additional elongation step was performed at 72°C for 7 minute and the PCR products were stored at 4°C until analysis.

3.4.5. Agarose gel electrophoresis

To validate the presence of LSD DNA, the amplified DNA samples were examined using agarose gel electrophoresis as described by (Mahmoud and Khafagi, 2016) with certain modifications. Amplified products were analyzed using a Generuler 100bp DNA ladder (thermoscientific, Lithuania) as a molecular marker on 3% agarose gels prepared in Tris-Acetate-EDTA (TAE) buffer and 10mg/ml ETDM- bromide stain, Then 20µl of PCR product was mixed with 4 µl loading buffer and loaded to wells in previously prepared gel and run at 120 volt for about 80 minutes in parallel with DNA molecular weight marker in electrophoresis apparatus until the DNA samples have migrated a sufficient distance through the gel. DNA bands were visualized using UV transilluminator at a wave length of 590 nm, and the size of the bands generated on the agarose gel that align with the ladder were used to interpret the results. The PCR results for LSDV and GTPV DNA were declared positive when the bands aligned with 172 bp on a ladder, and for SPPV DNA when the bands aligned with 151 bp on a ladder, confirming a 21 nucleotide deletion for the latter (Lamien *et al*., 2011).

3.4.6. Real-time PCR

The PCR was set up in a 20 µL reaction volume. A total of 150 µl of the master mix was prepared for 10 samples. 17 µl of the master mix was dispensed onto each micro-well of PCR Streep (Bio-Rad). The master mix in each well was composed of 4.84 µl of nuclease free water, 2µl of forward primer (PrimerCP-HRMSb-Fow-5pm/µl 5'GGTGTAGTACGTATAAGATTATCGTATAGAAACAAGCCTTTA 3'), 0.16 µL of reverse primer (Primer- CP-HRM l1REV-5pm/ µl 5'-AATTTCTTTCTCTGTTCCATTTG-3'), and 10 µL of soFast™ EvaGreen® IQ Super mix. Then 3 µL of sample extracted DNA was added on all but the negative and positive controls which contain nuclease free water for the negative control and GTPV, LSDV and SPPV for the positive controls then the lid was sealed.

PCR was performed in a CFX96^{TM} real-time PCR detection system with an initial denaturation step at 95℃ for 3 minutes, followed by 40 cycles of 95℃ for 15 seconds and 58℃ for 80 second using a PCR Strip (Bio-Rad). The product was then denatured for 1 minute at 95°C, cooled to 40°C for 1 minute, then heated at 0.5°C/10 seconds with fluorescence acquisition from 40°C to 85°C. The CFX^{TM} Manager Software Version 2.0 (Bio-Rad) was used to examine the melting temperatures.

3.4.7. RPO30 gene amplification and sequencing

For sequencing, the 30 kDa RNA polymerase subunit (RPO30) gene was amplified and purified from the samples. In order to generate the full length RPO30 gene, two sets of overlapping primers for the RPO30 gene were employed for the amplification as described by (Gelaye *et al*., 2015). The primers were developed to amplify two overlapping segments of 554bp and 520bp in size. PCR was conducted in reaction volume of 50 µL containing;
4µL of first sets of primers
(gene sequence CPRPO30-
OL1F5'CAGCTGTTTGTTTACATTTGATTTTT3',CPRPO30-
OL1R5'TCGTATAGAAACAAGCCTTTAATAGA3', each
4µL of second sets of primers
(gene sequence CPRPO30-
OL2F5'TTTGAACACATTTTATTCCAAAAAG3'CPRPO30-
OL2R5'AACCTACATGCAT AAACAGAAGC 3') each,
5µL dNTPs, 5 µL 10x PCR Buffer (Qiagen), 0.5 µL Taq polymerase (Qiagen), 21.5µL RNase free water and 6 µL template DNA. The first denaturation was kept for 4 minutes at 95°C, followed by 40 cycles of elongation at 95°C for 30 seconds, 55°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 1.5% agarose gel stained with GelRed (Biotium, inc.) for 1h at 100v.

3.4.8. Sequencing

The Wizard®™ SV Gel and PCR Clean-Up System (Promega, Germany) was used to purify the positive PCR products of the amplified RPO30 gene (Appendix 4). The NanoDrop 2000c spectrophotometer (Thermo Scientific, USA) was used to measure the DNA concentration of the purified PCR product. Each purified product's concentration was prepared according to the instruction of the sequencing service provider company. The purified PCR products were mixed with the amplification/sequencing primers and submitted for sequencing to LGC Genomics (Berlin, Germany).

3.5. Data analysis

The information gathered from all investigations was coded and saved in an Excel spreadsheet 2010, where it was rigorously inspected before statistical analysis. R statistical program was used to do descriptive statistical analysis.

By the use of free Staden Package software (Pregap4 and Gap4), the raw sequence data were edited and fragments were assembled. The fragments of the RPO30 gene generated with both sets of overlapping primers were edited and joined together for each of the isolate, and the clean gene sequence was obtained. Multiple sequence alignments were performed using the ClustalW algorithm implemented in BioEdit software package to compare the RPO30 gene and align the amino acid (aa) of the outbreak isolates and the reference strain. For comparative studies, blastn was used to collect additional *Capripoxvirus* RPO30 gene sequences from GenBank for inclusion in the data set.

For construction of phylogenetic tree, multiple sequence alignments were performed to align the sequences as codons using the Muscle algorithm in MEGA6 (Tamura *et al*., 2013). The Neighbor-Joining algorithm was used with the maximum composite likelihood nucleotide substitution model with the pair wise deletion option was used. For construction of phylogenetic tree, 1000 bootstrap replicate was used using three current outbreak isolates, vaccine strain and sequences retrieved from the GenBank. The

percentage bootstrap scores are shown next to the branches. The homologue gene sequence from Swine poxvirus and Deer poxvirus isolates were used as an out group. By the use of phylogenetic tree genetic relationship among Ethiopian isolates as well as other CaPV isolates were established.

4. RESULTS

4.1. Observed Clinical Signs

During field investigation a total of 469 cattle were observed, which were suspected for LSDV infection. Most common signs observed were fever, nasal discharges, enlargement of superficial lymph nodes, reduction in body weight, depression and the pathognomonic sign of the disease skin nodules in different parts of their body.

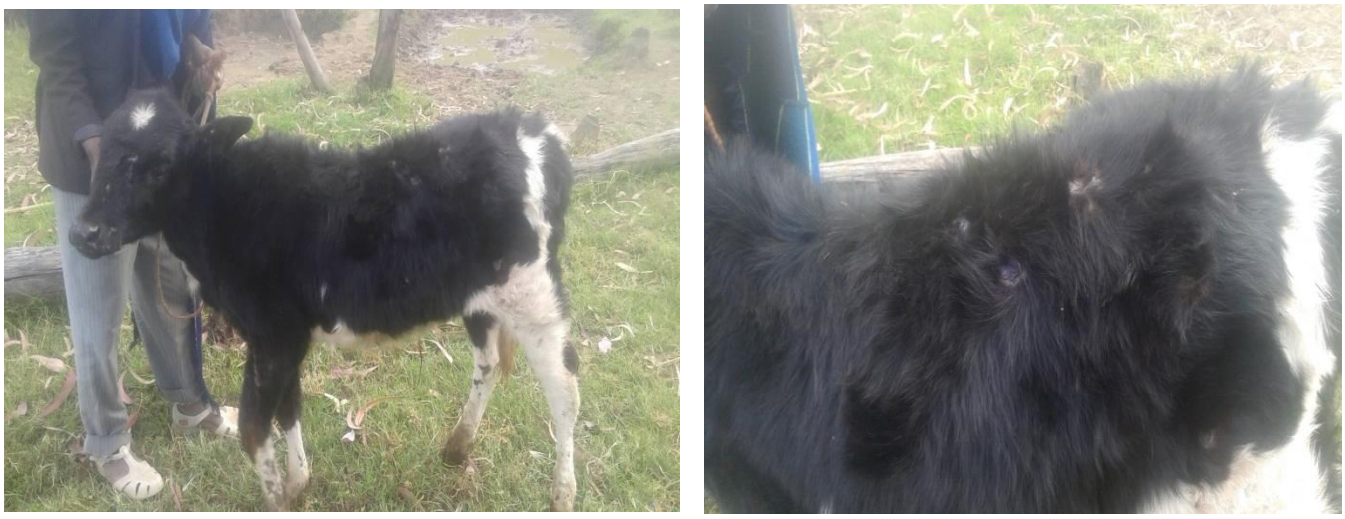


Figure 6: A pathognomonical LSD nodule on the skin of a calf

From observed animals 86 (22.45%) of them were showing clinical sign, 7 (1.8%) of them were dead of LSD and the case fatality rate was 8.13%.

Table 1: Summary of outbreak data in the study areas

Area	No. of affected cattle	No. of death	No. of susceptible cattle	Morbidity rate (%)	Mortality rate (%)	Case fatality (%)
Mojo	53	5	301	17.6	1.66	9.43
Ejere	15	1	39	38.46	2.56	6.66
Ejersa	9	1	24	37.5	4.16	11.11
Koka	9	0	19	47.37	0	0
Total	**86**	**7**	**383**	**22.45**	**1.8**	**8.13**

4.2. Virus Isolation

Out of the 15 skin biopsies, 10 field samples showed a characteristic poxvirus CPE in infected EHS-L cells within ten days of post- inoculation or after one or two blind passages. At first, cytotopathic alterations manifested as discrete foci or clumps of spherical, refractile cells. These foci grew larger over time, encircling cells in their immediate vicinity, while fresh foci developed throughout the cell sheet. Some of the affected cells eventually disengaged leaving irregular holes in the cell sheet, after three blind passages on the remaining five samples however the CPE were not detected. None of the negative control cultures showed any CPE after two or three passages.

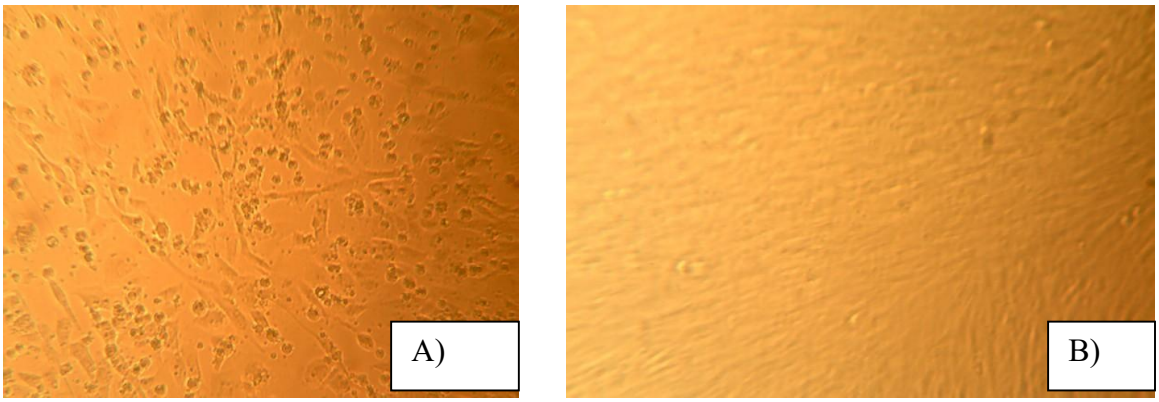


Figure 7: EHS-L cell line: A) CPE on ESH-L cell after eleven days post inoculation; B) control of cell culture

4.3. Polymerase Chain Reaction

EHS-L cell line supernatants that shows positive results were sent to molecular laboratory and amplified using poxvirus specific PCR as described in OIE manual (OIE, 2019). The PCR product's amplicon size was 172 bp (Fig.8), which was the expected amplicon size for the LSDV genomic region targeted. The resulting PCR products of GTPV/LSDV differed in length by 21 nucleotides produced from SPPV genomes.

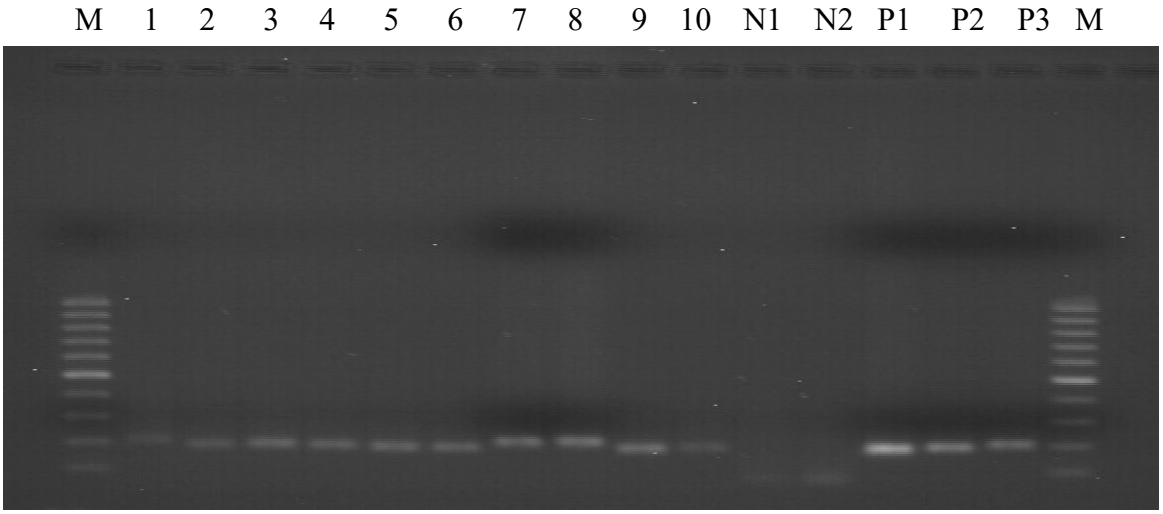


Figure 8: Conventional PCR gel result

Lanes M on both sides represents a DNA ladder of a 100bp. Lane N1 represent a negative extraction control and N2 indicate a negative control without template. Lanes 1 and 2 represent a sample from Ejere and Ejersa respectively. Lane 3 through 10 represents positive samples of Mojo region. P_1 is Positive control for Sheep pox of 151bp and Goat pox is represented by P3 which is around 172bp similar to P2 which is a Positive control for LSD.

4.4. Real-time PCR

To identify LSDV from GTPV, the DNA extracts that generated 172 bands on an agarose gel were treated to real-time PCR once again. The real-time peak melting curve indicated

that all 10 viral isolates were LSDV since the snapback melting temperature peaks were 51°C and the amplicon melting temperature peaks were 73.50°C shown in green below (Fig 9). Known CaPV positive samples were tested for comparison as positive control. After fluorescence melting curve analysis, a real-time PCR assay identified differences in melting point temperatures for SPPV, GTPV, and LSDV.

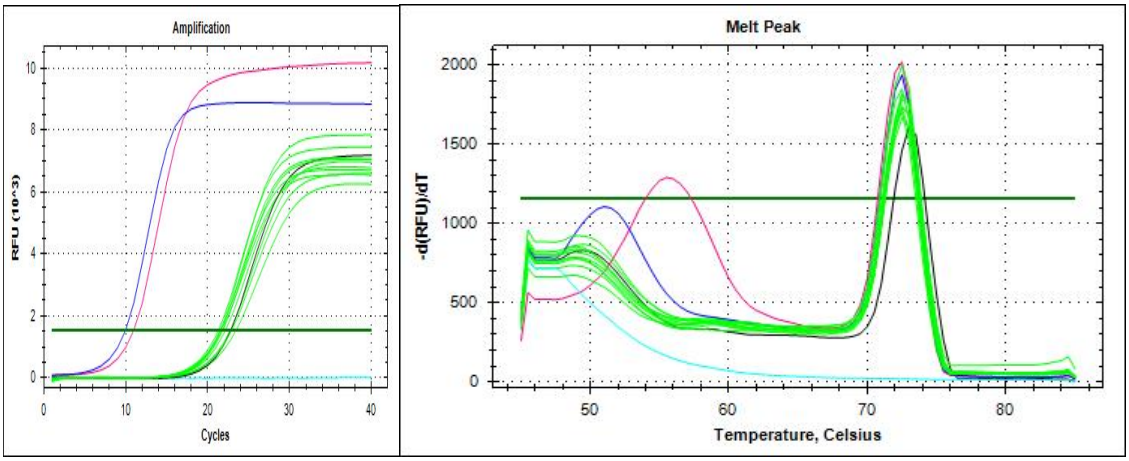
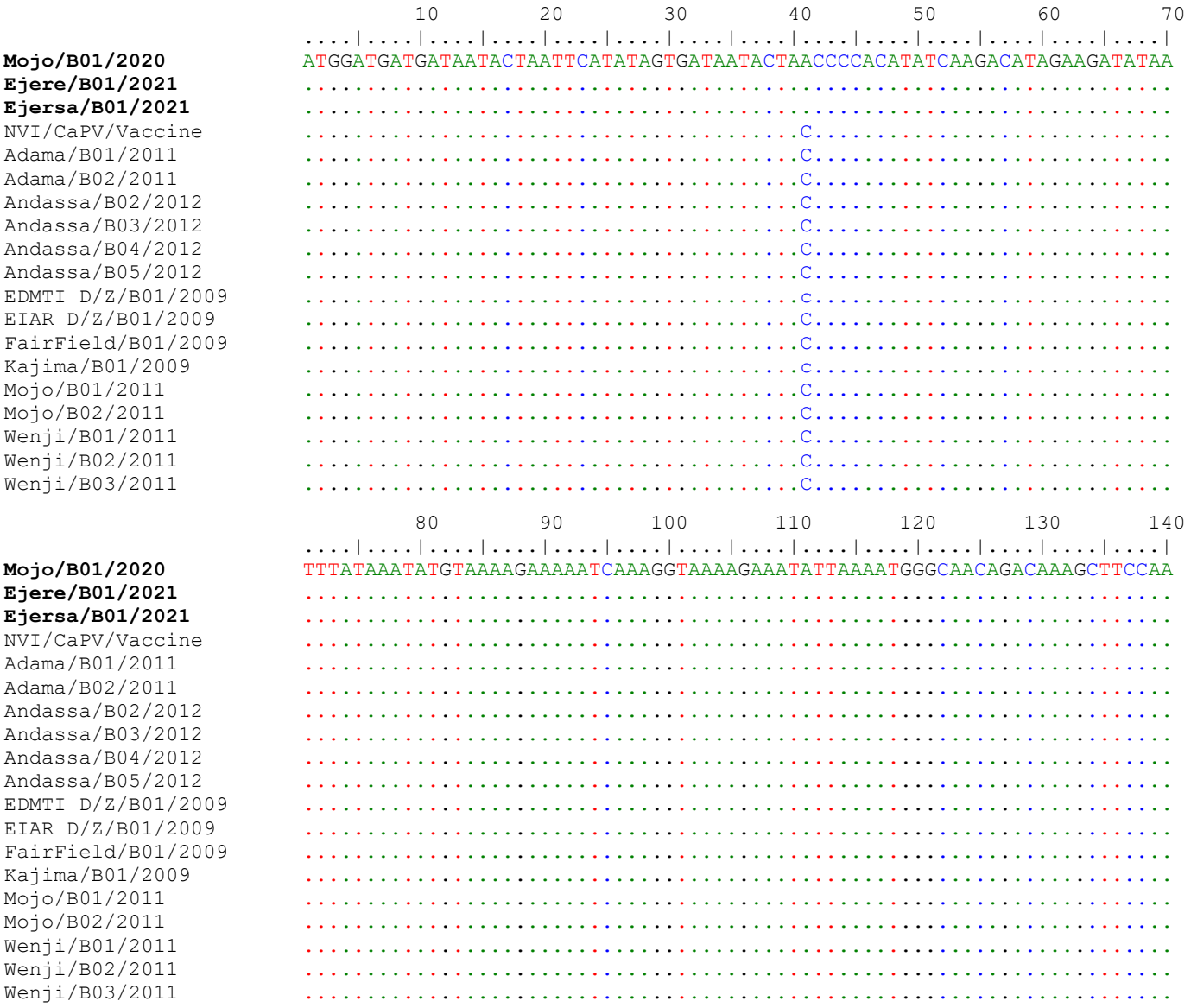


Figure 9: Melting curves of PCR products (Pink=GTPV, Blue=SPPV, Black=LSDV)

4.5. Sequence alignment analysis

Multiple sequence alignment analysis of complete RPO30 gene revealed that the nucleotide substitutions in all the filed virulent strains sequenced. The virulent isolates, Mojo/B01/2020, Ejere/B01/2021 and Ejersa/B01/2021 has a non-synonymous mutation at C41A nucleotide position (fig 10) compared to previous isolates of the country and of the previous isolates of the same study area. When comparing the vaccine (KS-1) with the present study filed virulent isolates, another two non-synonymous mutation are apparent in all the sequenced isolates at C41A and C292T nucleotide which lead to amino acid substitution of Proline (P) with Serine (S) at position 98 (P98S) (fig 11).


10 20 30 40 50 60 70
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Ejersa/B01/2021
NVI/CaPV/Vaccine
Adama/B01/2011
Adama/B02/2011
Andassa/B02/2012
Andassa/B03/2012
Andassa/B04/2012
Andassa/B05/2012
EDMTI D/Z/B01/2009
EIAR D/Z/B01/2009
FairField/B01/2009
Kajima/B01/2009
Mojo/B01/2011
Mojo/B02/2011
Wenji/B01/2011
Wenji/B02/2011
Wenji/B03/2011
80 90 100 110 120 130 140
Mojo/B01/2020 TTTATAAATATGTAAAAGAAAAATCAAAGGTAAAAGAAATATTAAAATGGGCAACAGACAAAGCTTCCAA
Ejere/B01/2021
Ejersa/B01/2021
NVI/CaPV/Vaccine
Adama/B01/2011
Adama/B02/2011
Andassa/B02/2012
Andassa/B03/2012
Andassa/B04/2012
Andassa/B05/2012
EDMTI D/Z/B01/2009
EIAR D/Z/B01/2009
FairField/B01/2009
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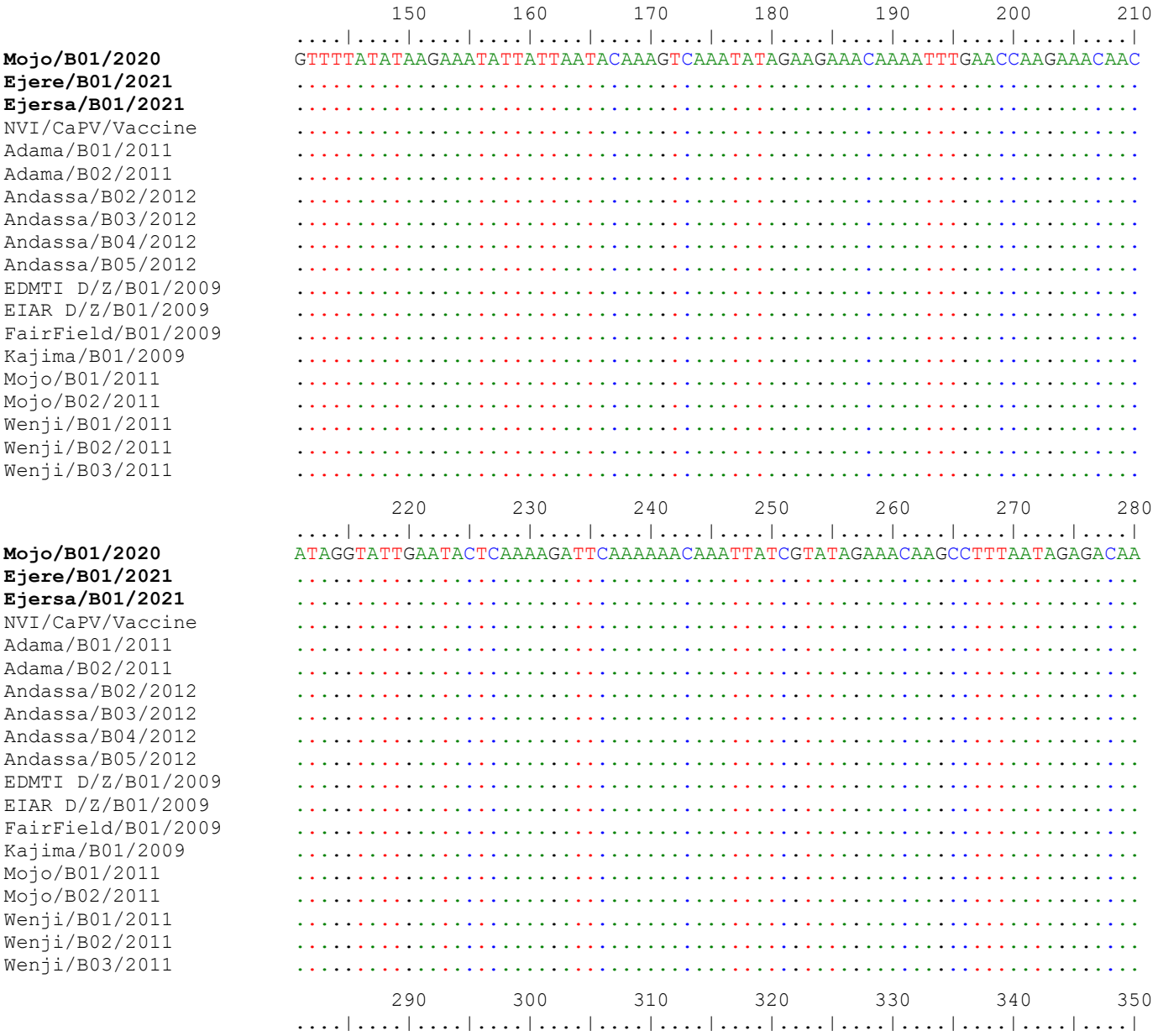
Wenji/B01/2011
Wenji/B02/2011
Wenji/B03/2011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Ejersa/B01/2021
NVI/CaPV/Vaccine
Adama/B01/2011
Adama/B02/2011
Andassa/B02/2012
Andassa/B03/2012
Andassa/B04/2012
Andassa/B05/2012
EDMTI D/Z/B01/2009
EIAR D/Z/B01/2009
FairField/B01/2009
Kajima/B01/2009
Mojo/B01/2011
Mojo/B02/2011
Wenji/B01/2011
Wenji/B02/2011
Wenji/B03/2011
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Ejersa/B01/2021
NVI/CaPV/Vaccine
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Andassa/B05/2012
EDMTI D/Z/B01/2009
EIAR D/Z/B01/2009
FairField/B01/2009
Kajima/B01/2009
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Wenji/B02/2011
Wenji/B03/2011
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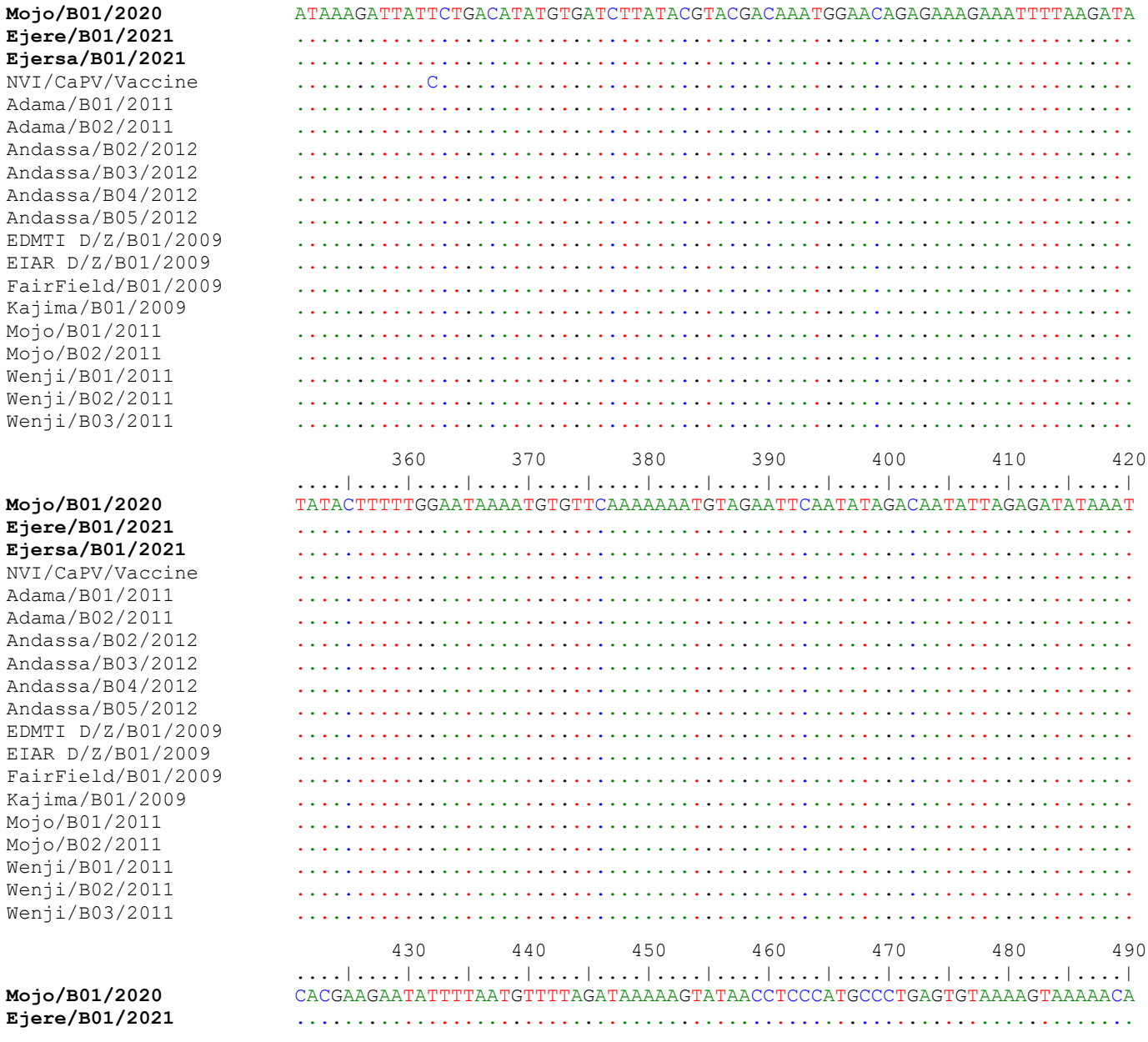
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Ejersa/B01/2021
NVI/CaPV/Vaccine .....C.....
Adama/B01/2011
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Andassa/B02/2012
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Wenji/B03/2011
360 370 380 390 400 410 420
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Ejere/B01/2021
Ejersa/B01/2021
NVI/CaPV/Vaccine
Adama/B01/2011
Adama/B02/2011
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Andassa/B03/2012
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FairField/B01/2009
Kajima/B01/2009
Mojo/B01/2011
Mojo/B02/2011
Wenji/B01/2011
Wenji/B02/2011
Wenji/B03/2011
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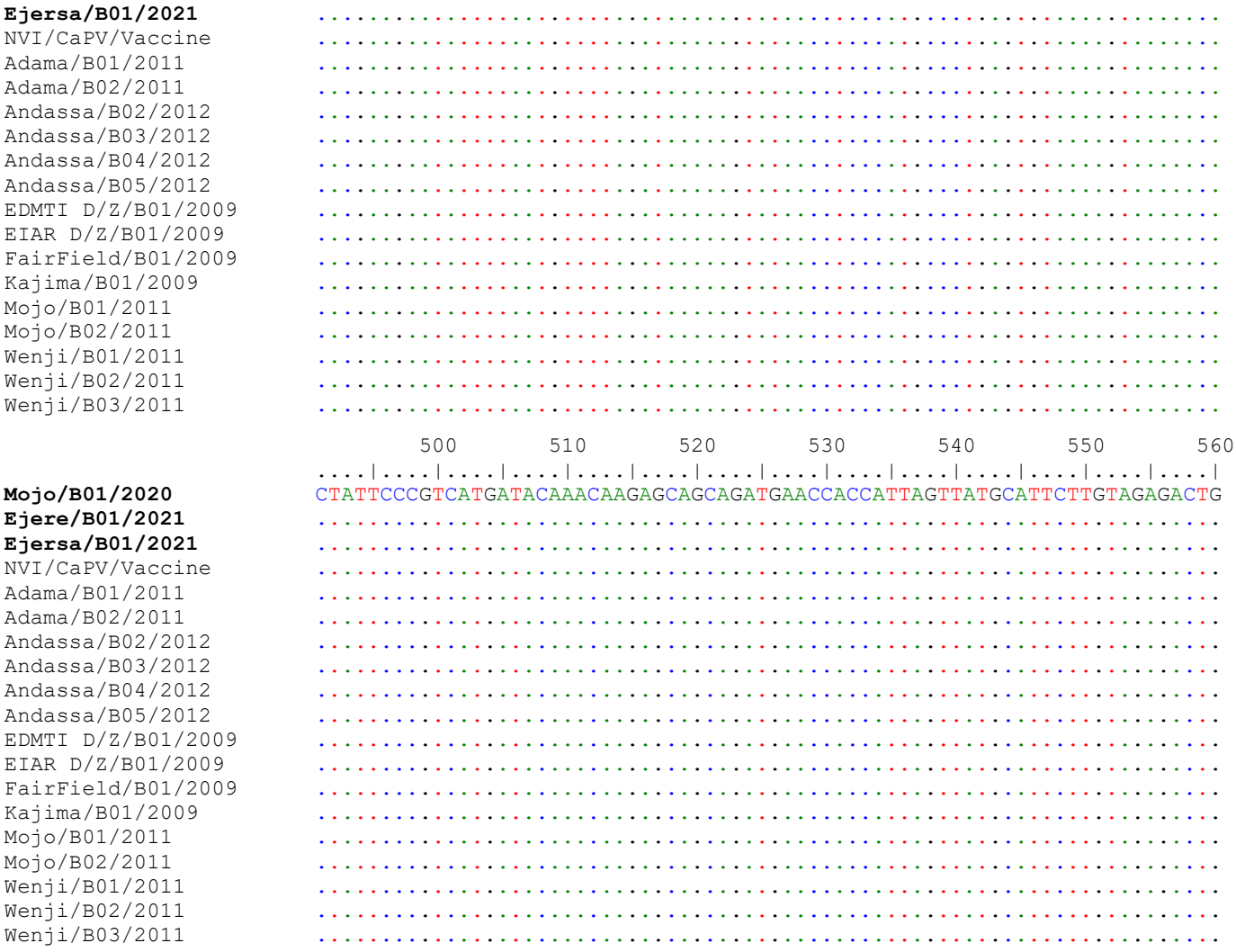
Ejersa/B01/2021
NVI/CaPV/Vaccine
Adama/B01/2011
Adama/B02/2011
Andassa/B02/2012
Andassa/B03/2012
Andassa/B04/2012
Andassa/B05/2012
EDMTI D/Z/B01/2009
EIAR D/Z/B01/2009
FairField/B01/2009
Kajima/B01/2009
Mojo/B01/2011
Mojo/B02/2011
Wenji/B01/2011
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Mojo/B01/2020 CTATTCCCGTCATGATACAAACAAGAGCAGCAGATGAACCACCATTAGTTATGCATTCTTGTAGAGACTG
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Ejersa/B01/2021
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FairField/B01/2009
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Wenji/B03/2011

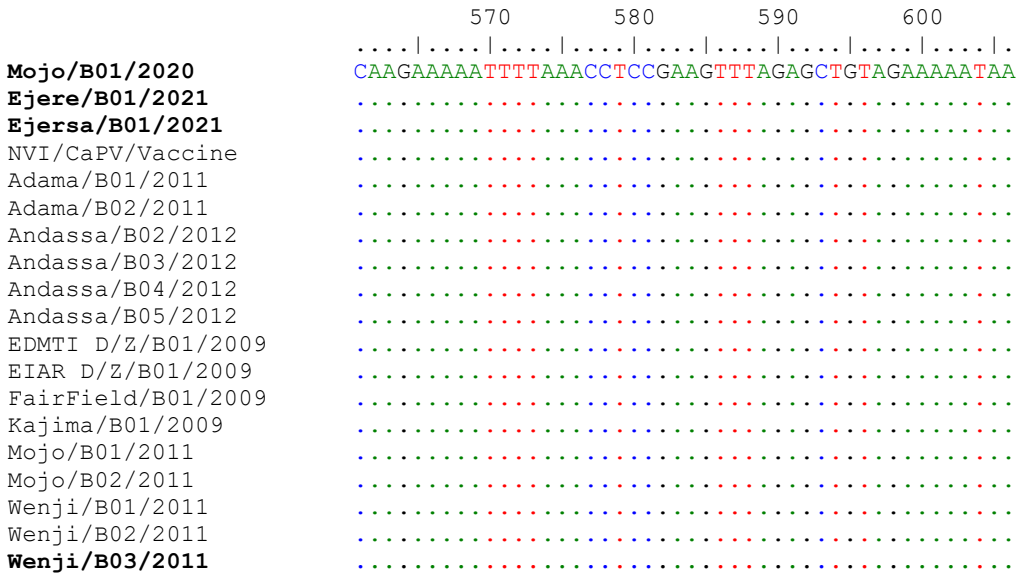
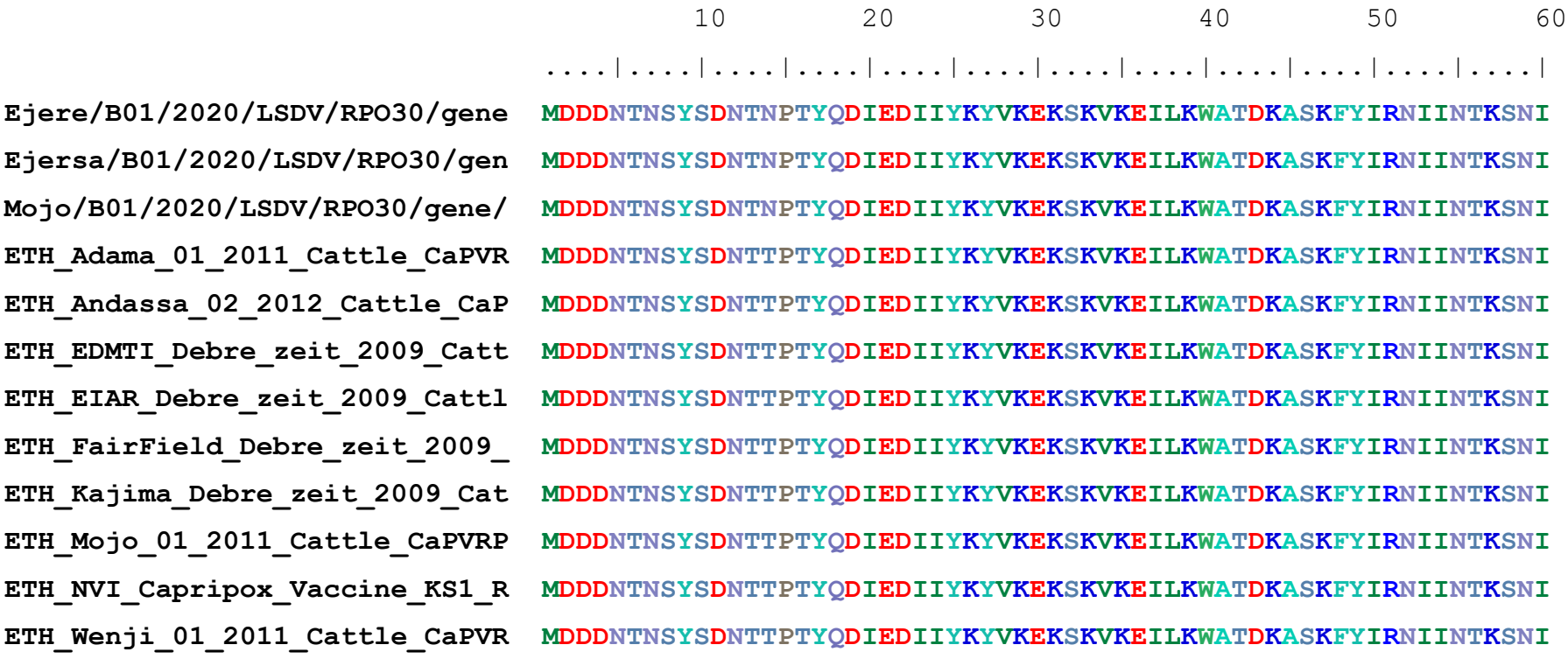


Figure 10: Plot identity of the RPO30 full gene nucleotide sequences (606bp) of Lumpy skin disease virus of the current isolates (n=3, indicated in bold) in comparison with the previously sequenced Ethiopian LSDV strains (n=15) and vaccine strain (n=1). Identical nucleotides are indicated as dot.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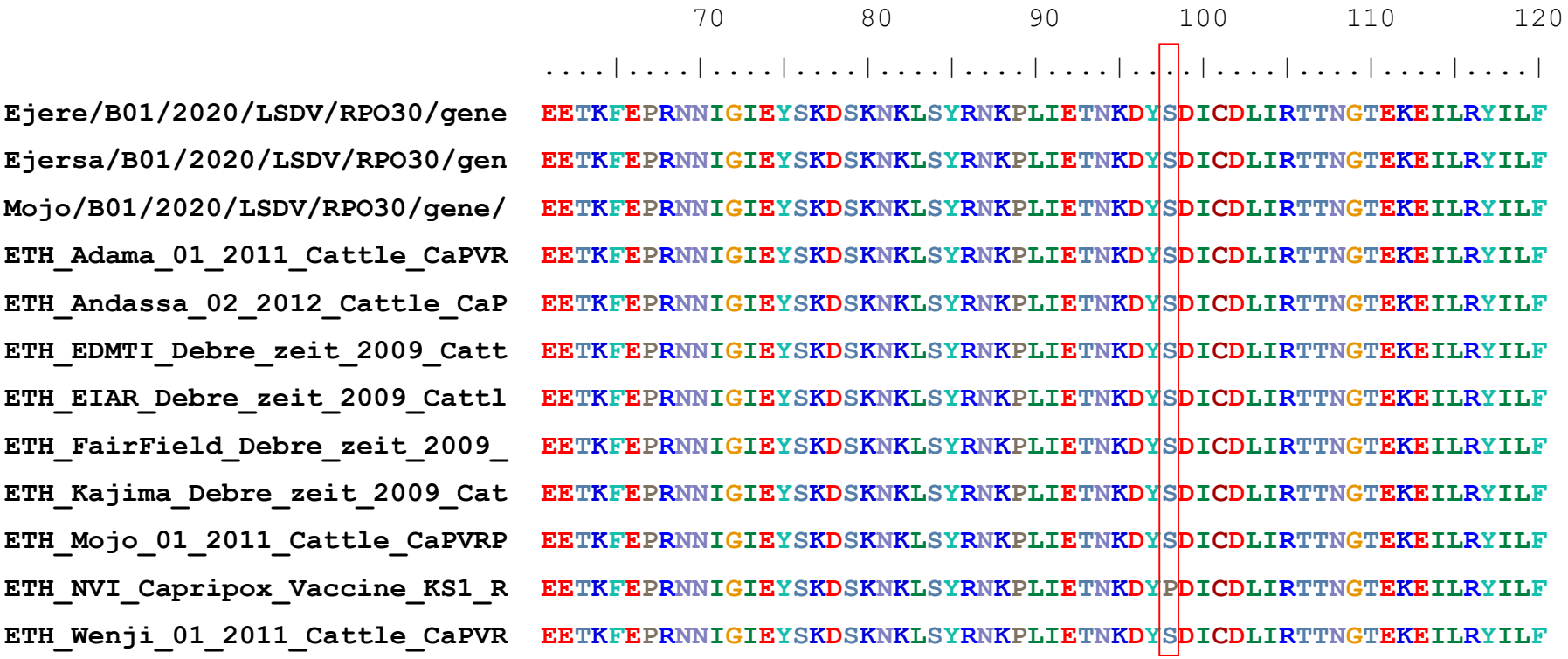
Ejersa/B01/2020/LSDV/RPO30/gen MDDDNTNSYSDNTNPTYQDIEDIIYKYVKEKSKVKEILKWATDKASKFYIRNIINTKSNI
Mojo/B01/2020/LSDV/RPO30/gene/ MDDDNTNSYSDNTNPTYQDIEDIIYKYVKEKSKVKEILKWATDKASKFYIRNIINTKSNI
ETH_Adama_01_2011_Cattle_CaPVR MDDDNTNSYSDNTTPTYQDIEDIIYKYVKEKSKVKEILKWATDKASKFYIRNIINTKSNI
ETH_Andassa_02_2012_Cattle_CaP MDDDNTNSYSDNTTPTYQDIEDIIYKYVKEKSKVKEILKWATDKASKFYIRNIINTKSNI
ETH_EDMTI_Debre_zeit_2009_Catt MDDDNTNSYSDNTTPTYQDIEDIIYKYVKEKSKVKEILKWATDKASKFYIRNIINTKSNI
ETH_EIAR_Debre_zeit_2009_Cattl MDDDNTNSYSDNTTPTYQDIEDIIYKYVKEKSKVKEILKWATDKASKFYIRNIINTKSNI
ETH_FairField_Debre_zeit_2009_ MDDDNTNSYSDNTTPTYQDIEDIIYKYVKEKSKVKEILKWATDKASKFYIRNIINTKSNI
ETH_Kajima_Debre_zeit_2009_Cat MDDDNTNSYSDNTTPTYQDIEDIIYKYVKEKSKVKEILKWATDKASKFYIRNIINTKSNI
ETH_Mojo_01_2011_Cattle_CaPVRP MDDDNTNSYSDNTTPTYQDIEDIIYKYVKEKSKVKEILKWATDKASKFYIRNIINTKSNI
ETH_NVI_Capripox_Vaccine_KS1_R MDDDNTNSYSDNTTPTYQDIEDIIYKYVKEKSKVKEILKWATDKASKFYIRNIINTKSNI
ETH_Wenji_01_2011_Cattle_CaPVR MDDDNTNSYSDNTTPTYQDIEDIIYKYVKEKSKVKEILKWATDKASKFYIRNIINTKSNI


70 80 90 100 110 120
Ejere/B01/2020/LSDV/RPO30/gene EETKFEPRNNIGIEYSKDSKNKLSYRNKPLIETNKDYSDICDLIRTTNGTEKEILRYILF
Ejersa/B01/2020/LSDV/RPO30/gen EETKFEPRNNIGIEYSKDSKNKLSYRNKPLIETNKDYSDICDLIRTTNGTEKEILRYILF
Mojo/B01/2020/LSDV/RPO30/gene/ EETKFEPRNNIGIEYSKDSKNKLSYRNKPLIETNKDYSDICDLIRTTNGTEKEILRYILF
ETH_Adama_01_2011_Cattle_CaPVR EETKFEPRNNIGIEYSKDSKNKLSYRNKPLIETNKDYSDICDLIRTTNGTEKEILRYILF
ETH_Andassa_02_2012_Cattle_CaP EETKFEPRNNIGIEYSKDSKNKLSYRNKPLIETNKDYSDICDLIRTTNGTEKEILRYILF
ETH_EDMTI_Debre_zeit_2009_Catt EETKFEPRNNIGIEYSKDSKNKLSYRNKPLIETNKDYSDICDLIRTTNGTEKEILRYILF
ETH_EIAR_Debre_zeit_2009_Cattl EETKFEPRNNIGIEYSKDSKNKLSYRNKPLIETNKDYSDICDLIRTTNGTEKEILRYILF
ETH_FairField_Debre_zeit_2009_ EETKFEPRNNIGIEYSKDSKNKLSYRNKPLIETNKDYSDICDLIRTTNGTEKEILRYILF
ETH_Kajima_Debre_zeit_2009_Cat EETKFEPRNNIGIEYSKDSKNKLSYRNKPLIETNKDYSDICDLIRTTNGTEKEILRYILF
ETH_Mojo_01_2011_Cattle_CaPVRP EETKFEPRNNIGIEYSKDSKNKLSYRNKPLIETNKDYSDICDLIRTTNGTEKEILRYILF
ETH_NVI_Capripox_Vaccine_KS1_R EETKFEPRNNIGIEYSKDSKNKLSYRNKPLIETNKDYPDICDLIRTTNGTEKEILRYILF
ETH_Wenji_01_2011_Cattle_CaPVR EETKFEPRNNIGIEYSKDSKNKLSYRNKPLIETNKDYSDICDLIRTTNGTEKEILRYILF
130 140 150 160 170 180
Ejere/B01/2020/LSDV/RPO30/gene GIKCVQKNVEFNIDNIRDINHEEYFNVLDKKYNLPCPECKSKNTIPVMIQTRAADEPPLV
Ejersa/B01/2020/LSDV/RPO30/gen GIKCVQKNVEFNIDNIRDINHEEYFNVLDKKYNLPCPECKSKNTIPVMIQTRAADEPPLV
Mojo/B01/2020/LSDV/RPO30/gene/ GIKCVQKNVEFNIDNIRDINHEEYFNVLDKKYNLPCPECKSKNTIPVMIQTRAADEPPLV
ETH_Adama_01_2011_Cattle_CaPVR GIKCVQKNVEFNIDNIRDINHEEYFNVLDKKYNLPCPECKSKNTIPVMIQTRAADEPPLV
ETH_Andassa_02_2012_Cattle_CaP GIKCVQKNVEFNIDNIRDINHEEYFNVLDKKYNLPCPECKSKNTIPVMIQTRAADEPPLV
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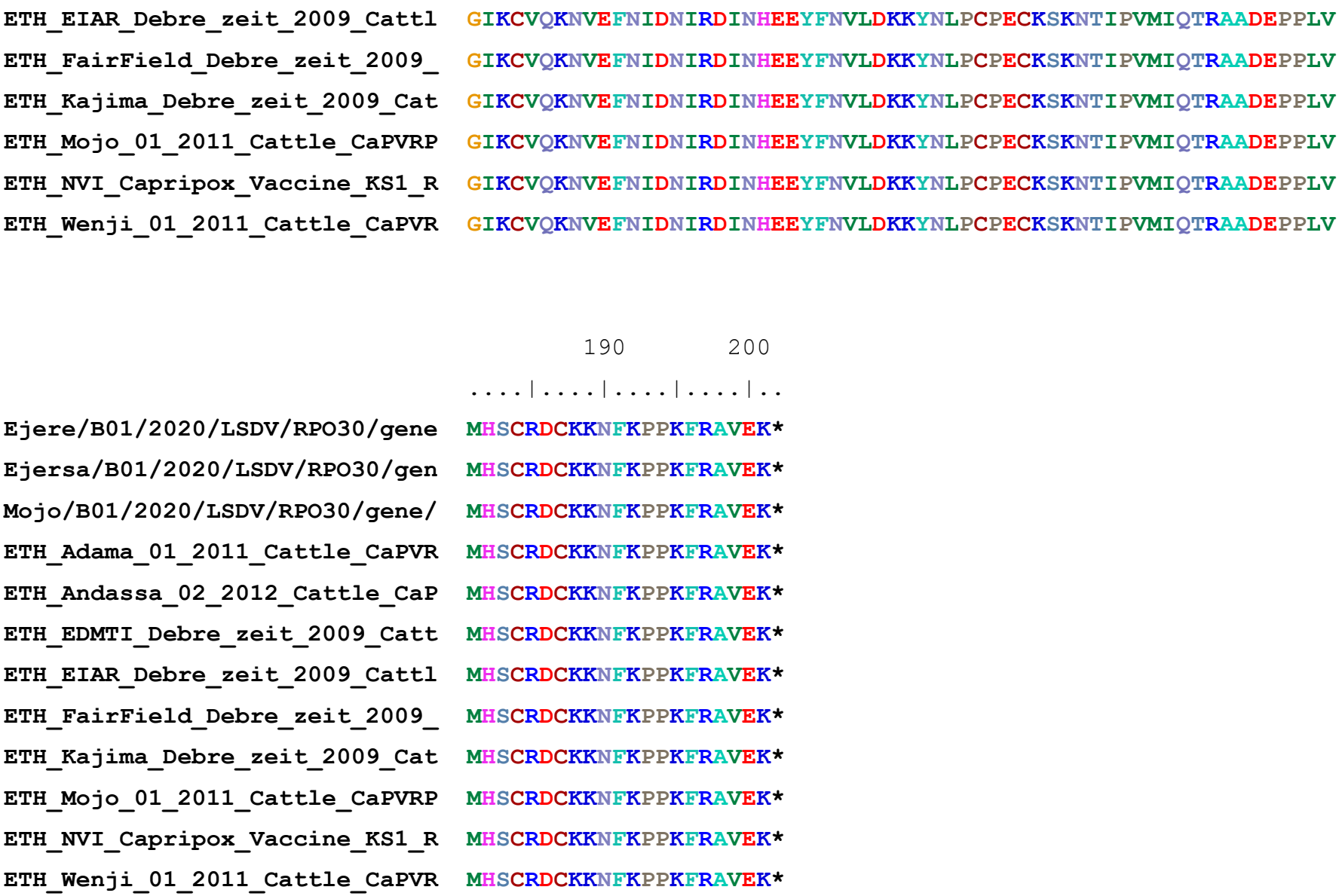

Figure 11: Amino acid sequence alignment of RPO30 gene of Ethiopian field isolates and vaccine strain

In phylogenetic analysis members of the *Capripoxvirus* are delineated into three distinct clusters of GTPV, SPPV and LSDV. Based on the RPO30 gene sequence, all current study representative isolates (n=3), that are indicated in red dots, were clustered to LSDV and have a close relation to Sudan, Mojo, Bishoftu, Bahir dar, Wenji and the vaccine strain that is used in Ethiopia.

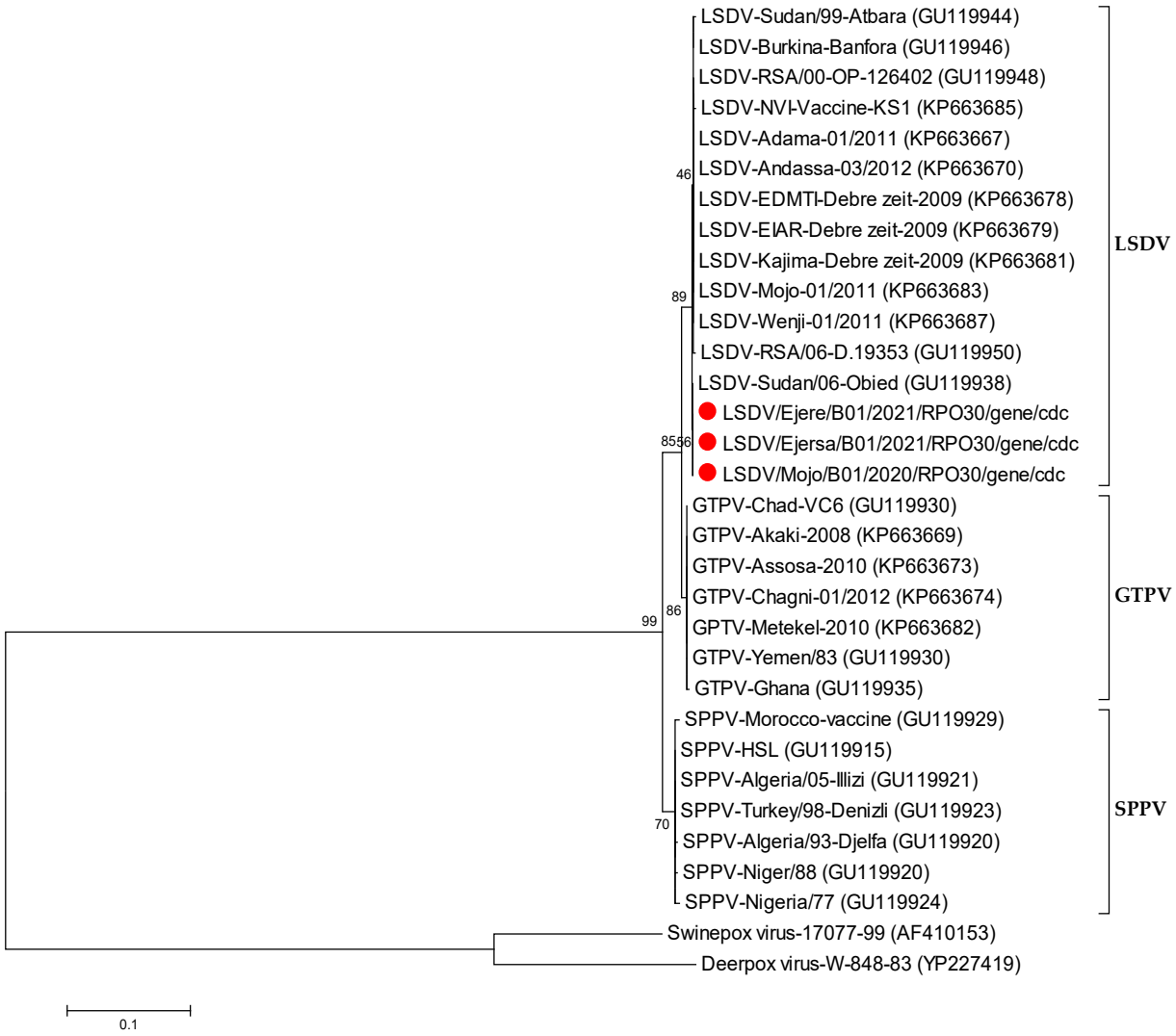

Figure 12: Phylogenetic tree analysis of 32 *capripoxviruses* based on the nucleotide sequences of the full RPO30 gene. The three LSDV isolates sequenced in the present study are indicated red color circle.

5. DISCUSSION

A considerable amount of LSD outbreak occur yearly in Ethiopia with no sign of declining as time progresses (Gelaye *et al*., 2015). The constant outbreak phenomenon affects the country's economy and the livelihood of individuals' tremendously (Jong, 2017). These losses are mitigated by vaccination campaign across the country. The availability of vaccine and wild virus strain at a same location can contribute to the divergence of the virus (Eclercy *et al*., 2019). This can only be evident with continuous molecular and genetic information of the virulent virus that causes the outbreak across the country.

In this study a current outbreak was appraised in accordance with the clinical symptoms, cell culture isolation, PCR results, genomic sequence and phylogenetic tree construction. This brings the deduction that the outbreak was in fact caused by LSDV. Previous research reports indicated that LSD occur in different parts of Ethiopia at different period in time (Abera, 2019; Animal *et al*., 2010; Asfaw and Ameni, 2012; Asresie, 2015; Ayelet *et al*., 2013; Gari *et al*., 2015, 2012, 2011; Gelaye *et al*., 2017; Jimolu, 2011; Mebratu *et al*., 1984; Molla *et al*., 2018; W Molla *et al*., 2017; Wassie Molla *et al*., 2017; Molla and Frankena, 2017).

Clinicaly, Fever, nasal discharges,lacrimation, enlargement of superficial lymph nodes, re duction in body weight, depression, milk yield reduction, circumscribed skin nodules in different parts of the body were the most common clinical manifestation that were encountered during the field investigation of the current study. OIE, (2019) documented this clinical signs as the most characteristic features of LSD along with edematous swelling of legs and lameness, which didn't come across during the time frame and area of the study. However, in a study conducted in Adama district in 2011 the latter two signs were the most common sign of the disease that were encountered (Alemayehu *et al*., 2015). The severity of disease is influenced by the host's susceptibility, age, immunological condition, dose, and mode of virus injection (Strategy, 2009).

Out of 15 skin biopsies, characteristic of *Capripoxvirus* CPE was observed only on 10 skin samples following inoculation on ESH-L cell line. Whereas, virus isolates could not be identified from the remaining five skin samples. Only 27 virus isolates were obtained from 31 skin samples, according to a comparable finding (Mammo, 2019). After sequential testing, conventional PCR and real-time PCR, all isolates were diagnosed as LSDV.

When it comes to the Lumpy skin disease, the vulnerable animal's morbidity can approach 100%, but death is much lower, at around 3%, which can be greater in a naive population (Maclachlan and Dubovi, 2010). On the current study 22.45% morbidity rate was recorded, this is in complete agreement with (Ayelet *et al*., 2013) who reported a similar value of 22.9% morbidity. However, a relatively lower morbidity at 5.69% and 18% were also reported (Leliso *et al*., 2021; Tassew *et al*., 2018). The difference in this figures might be due to geographical location, the climate and management conditions, breed, immune status, condition of the animals, virus virulence and types of putative inse ct vectors (Ahmed and Zaher, 2008; Virusforschung and Einzeldarstellungen, 1968).

Regarding mortality rate, the highest mortality rates were reported in Ejersa (4.16%) and Ejere (2.56%). These rates are in agreement with findings of other authors who have reported a slightly higher mortality rate (compared to the 3% mark) of 5.89% in different part of the country (Ayelet *et al.*, 2013). On the other hand, on a work done in east shewa zone, bale zone and around debre zeit the mortality rate were shown to never exceed 3% (Ayelet *et al*., 2013; Leliso *et al*., 2021; Tassew *et al*., 2018).

In addition, out of 10 samples 3 representative samples have the RPO30 full gene nucleotide amplified and sequenced (606bp). Based on a single gene comparison, the current outbreak viruses have a single Nucleotide (A/C) variation observed at a nucleotide position 41 from the previously isolated virulent virus (n=15) strains and the vaccine strain. These newly isolated viruses also have a (C/T) nucleotide variation at the position of 292 from the vaccinal strain. This two non-synonymous mutations (C41A and C292T) observed on multiple sequence alignment analysis were found to be distantly

located in the primary nucleotide sequence. This could explain why only one of the mutations resulted in amino acid substitution.

The resulting amino acid variation at a 98 position in which S (Serine) substitute P (Proline) (S98P) create a mutation in the RPO30 gene. Serine is a polar, neutral, hydrophilic, and fairly small in size amino acid that regulates the biochemical activity of many proteins. It has structural (capable of residing both within the interior of a protein or on the surface of protein) and biochemical functions (Points, 2015). On the contrary Proline is a nonpolar and hydrophobic compound that has a rotationally constrained rigid-ring structure. As a result, Proline residues in a polypeptide introduce restrictions on the folding of chains (B.Hughes, 2012; Points, 2015). This could potentially have an effect on the structure of polypeptide (RNA polymerase) that is resulted from RPO30 gene. A variation in a single nucleotide/amino acid between the vaccine strain and field isolates doesn't clearly say the vaccine is not protecting. It needs further study on full genome sequence analysis, proteomics study on potential immunodominant genes and vaccination-challenge experiments.

Furthermore, molecular phylogenetic analysis confirmed the identifications made using real-time PCR. The isolates made clusters with other LSD virus having the closest relation with multiple Ethiopian and African isolates. This further confirms that the conclusion made earlier using cell culture isolation, PCR results and RPO30 gene sequence result.

6. CONCLUSIONS AND RECOMMENDATIONS

Lumpy skin disease is one of economically important diseases, which affect cattle. Although the mortality rate is low, the morbidity related sequels are attributed to significant economic losses in Ethiopia. The current outbreaks investigated were confirmed to be caused by LSDV. The isolated viruses were further analyzed by PCR and gene sequencing. There was a two nucleotide position variation when comparing the present study isolates and the vaccine (KS-1) resulting in a single nonsense amino acid mutation. Furthermore, all the isolate have a single nucleotide substitution when compared to other isolates of the country and of the previous isolates of the same study area. The following points are suggested based on the study's findings:

- Continuous LSD outbreaks investigation need to be carried out
- Full genome analysis should be done to identify relationship of circulating virulent virus to the existing vaccines and find out new strain or different variations and location of the virus.
- Significance of the amino acid changes found in the filed isolate mutant need to be explored
- Vaccination and challenge study should be conducted under experimental control animals
- Awareness should be given to the community for early identification or reporting of the disease.

7. REFERENCES

Abera, Z.(2019): Survey on Distribution, Associated factors of Lumpy Skin Disease Occurrence and Its Vaccine Efficacy in selected Districts of East Wollega Zone, Western Oromiya. *Biomed. J. Sci. Tech. Res.* **13**: 9897–9906.

Abutarbush, S.M.(2014): Efficacy of vaccination against lumpy skin disease in Jordanian cattle. *Vet. Rec.* **175**.

Abutarbush, S.M., Hananeh, W.M., Ramadan, W., Al Sheyab, O.M., Alnajjar, A.R., Al Zoubi, I.G., Knowles, N.J., Bachanek-Bankowska, K., Tuppurainen, E.S.M.(2016) Adverse Reactions to Field Vaccination Against Lumpy Skin Disease in Jordan. Transbound. *Emerg. Dis*. **63**: e213–e219.

Ahmed, W.M., Zaher, K.S.(2008): Observations on lumpy skin disease in local Egyptian cows with emphasis on its impact on ovarian function 252–257.

Alemayehu, G., Leta, S., Eshetu, E., Mandefro, A.(2015): Incidence of lumpy skin disease and associated risk factors among export-oriented cattle feedlots at Adama District, Central Ethiopia **7**: 117–121.

Alkhamis, M.A., VanderWaal, K.(2016): Spatial and temporal epidemiology of lumpy skin disease in the Middle East, 2012-2015. *Front. Vet. Sci.* **3**: 1–12.

Animal, N., Diagnostic, H., Baillarguet, C.I. De, Epidemiology, V., Group, P.H.(2010): Risk factors associated with observed clinical lumpy skin disease in Ethiopia 1657–1666.

Asfaw, R., Ameni, M.(2012): Prevalence of Epizootic Lymphangitis in Cart Horses in Southwest Shewa of Oromia Region, Ethiopia. *Int. J. Livest. Res.* **2**: 146.

Asresie, A.(2015): Contribution of Livestock Sector in Ethiopian Economy : A Review **29**: 79–91.

Awad, W.S., Ibrahim, A.K., Mahran, K., Fararh, K.M., Moniem, M.I.A.(2010):

Evaluation of different diagnostic methods for diagnosis of Lumpy skin disease in cows. *Trop. Anim. Health Prod.* **42**: 777–783.

Ayelet, G., Abate, Y., Sisay, T., Nigussie, H., Gelaye, E.(2013): Antivira l Research Lumpy skin disease : Preliminary vaccine efficacy assessment and overview on outbreak impact in dairy cattle at Debre Zeit , central Ethiopia. *Antiviral Res.* **98**: 261–265.

B.Hughes, A.(2012): Amino Acids, Peptides and Proteins in Organic Chemistry.

Babiuk, S., Bowden, T.R., Boyle, D.B., Wallace, D.B., Kitching, R.P.(2008): Capripoxviruses: An emerging worldwide threat to sheep, goats and cattle. *Transbound. Emerg. Dis.* **55**: 263–272.

Balinsky, C.A., Ph, D.(2007): The Effect of Kelch-like protein S P P V 19 on Virulence of Sheeppox Virus Corey Andrew Balinsky, Ph.D. University of Connecticut, 2007 Sheeppox virus (SPPV) is classified in the.

Body, M., Singh, P.K., Hussain, H.M., Al-rawahi, A., Al-maawali, M., Al-lamki, K., Al-habsy, S.(2012): Clinico-histopathological findings and PCR based diagnosis of lumpy skin disease in the Sultanate of Oman. *Pak. Vet. J.* **32**: 206–210.

Boshra, H., Truong, T., Nfon, C., Bowden, T.R., Gerdts, V., Tikoo, S., Babiuk, L.A., Kara, P., Mather, A., David, B., Babiuk, S.(2015a): CSIRO Biosecurity Flagship , Australian Animal Health Laboratory , Geelong , Australia School of Public Health , University of Saskatchewan , Saskatoon , SK , Canada.

Boshra, H., Truong, T., Nfon, C., Bowden, T.R., Gerdts, V., Tikoo, S., Babiuk, L.A., Kara, P., Mather, A., Wallace, D.B., Babiuk, S.(2015b): A lumpy skin disease virus deficient of an IL-10 gene homologue provides protective immunity against virulent capripoxvirus challenge in sheep and goats. *Antiviral Res.* **123**: 39–49.

Brenner, J., Bellaiche, M., Gross, E., Elad, D., Oved, Z., Haimovitz, M., Wasserman, A., Friedgut, O., Stram, Y., Bumbarov, V., Yadin, H.(2009): Appearance of skin lesions in cattle populations vaccinated against lumpy skin disease: Statutory

challenge. *Vaccine*, **27**: 1500–1503.

C. H. Annandale1, D. E. Holm1, K.E. and E.H.V.(2010): Seminal transmission of Lumpy Skin Disease Virus in heifers 63–63. Capstick and Coackley, n.d. capstick1962.pdf.

Carn, V.M.(1993): Control of capripoxvirus infections. *Vaccine*, **11**: 1275–1279.

Carn, V.M., Kitching, R.P.(1995): An investigation of possible routes of transmission of lumpy skin disease virus (Neethling). *Epidemiol. Infect*. **114**: 219–226.

Davies.(1991): Special Review Series Lumpy Skin Disease , An African Capripox Virus Disease Of Cattle.

Eclercy, J., Renson, P., Lebret, A., Hirchaud, E., Andraud, M., Blanchard, Y.(2019): Virus (PRRSV-1) Modified Live Vaccines Shows Increased Viremia and Transmission in SPF Pigs 1–13.

Efficacy, V., Antibody, M., Reaction, P.C., Beard, P.M.(2019): Other Poxviruses of Ruminants Diseases of the Skin , Eye , Conjunctiva , and External Ear Capripoxvirus Diseases : Current Up- dates and Developed Strategies for Control.

Fenner, F., Bachmann, P.A., Paul, E., Gibbs, I., Murphy, F.A., Studdert, M.J., White, D.O., Jovanovich, H.B., San, O., New, D., Austin, Y., London, B., Toronto, S.T. (1987): *Veterinary Virology Academic Press*, Inc.

Ga, J.C.L.U.B.I.N.(2013): Short Report Mechanical transmission of lumpy skin disease virus by Rhipicephalus appendiculatus male ticks 425–430.

Gari, G., Abie, G., Gizaw, D., Wubete, A., Kidane, M., Asgedom, H., Bayissa, B., Ayelet, G., Oura, C.A.L., Roger, F., Tuppurainen, E.S.M.(2015): Evaluation of the safety, immunogenicity and efficacy of three capripoxvirus vaccine strains against lumpy skin disease virus. *Vaccine*, **33**: 3256–3261.

Gari, G., Biteau-Coroller, F., LeGoff, C., Caufour, P., Roger, F.(2008): Evaluation of indirect fluorescent antibody test (IFAT) for the diagnosis and screening of lumpy skin disease using Bayesian method. *Vet. Microbiol*. **129**: 269–280.

Gari, G., Bonnet, P., Roger, F., Waret-szkuta, A.(2011): Epidemiological aspects and financial impact of lumpy skin disease in Ethiopia. *Prev. Vet. Med.* **102**: 274–283.

Gari, G., Grosbois, V., Waret-Szkuta, A., Babiuk, S., Jacquiet, P., Roger, F.(2012): Lumpy skin disease in Ethiopia: Seroprevalence study across different agro-climate zones. *Acta Trop*. **123**: 101–106.

Gari, G., Waret-Szkuta, A., Grosbois, V., Jacquiet, P., Roger, F.(2010): Risk factors associated with observed clinical lumpy skin disease in Ethiopia. *Epidemiol. Infect*. **138**: 1657–1666.

Gelaye, E., Belay, A., Ayelet, G., Jenberie, S., Yami, M., Loitsch, A., Tuppurainen, E., Grabherr, R., Diallo, A., Euloge, C.(2015): Capripox disease in Ethiopia : Genetic differences between field isolates and vaccine strain , and implications for vaccination failure. *Antiviral Res.* **119**: 28–35.

Gelaye, E., Lamien, C.E., Silber, R., Tuppurainen, E.S.M., Grabherr, R., Diallo, A.(2013): Development of a Cost-Effective Method for Capripoxvirus Genotyping Using Snapback Primer and dsDNA Intercalating Dye. *PLoS One* **8**: 1–10.

Gelaye, E., Mach, L., Kolodziejek, J., Grabherr, R., Loitsch, A., Achenbach, J.E., Nowotny, N., Diallo, A., Lamien, C.E.(2017): A novel HRM assay for the simultaneous detection and differentiation of eight poxviruses of medical and veterinary importance. *Sci. Rep*. **7**: 1–11.

Hamdi, J., Bamouh, Z., Jazouli, M., Boumart, Z., Tadlaoui, K.O., Fihri, O.F., EL Harrak, M.(2020): Experimental evaluation of the cross-protection between Sheeppox and bovine Lumpy skin vaccines. *Sci. Rep*. **10**: 1–9.

Hope, R., Ca, B.(2016): Lumpy skin disease 1–10.

Jimolu, G.G.(2011): Impact in Ethiopia. *Sci. York* 0–161.

Jong, D.(2017): Economic impact of lumpy skin disease and cost effectiveness of vaccination for the control of outbreaks in Ethiopia Economic impact of lumpy skin disease and cost effectiveness of vaccination for the control of outbreaks in Ethiopia

Quantitative Veterina 100–107.

Kara, P.D., Afonso, C.L., Wallace, D.B., Kutish, G.F., Abolnik, C., Lu, Z., Vreede, F.T., Taljaard, L.C.F., Zsak, A., Viljoen, G.J., Rock, D.L.(2003): Comparative sequence analysis of the South African vaccine strain and two virulent field isolates of Lumpy skin disease virus. *Arch. Virol.* **148**: 1335–1356.

Kitching, R.P., Hammond, J.M., Black, D.N.(1986): Studies on the major common precipitating antigen of capripoxvirus. *J. Gen. Virol.* **67**: 139–148.

Knipe, D.M., Howley, P.M.(2013): Fields Virology 6 Edition, Journal of Chemical Information and Modeling.

Kuastros mekonnes. (2007): Major Animal Health Problems of Market Oriented Livestock 1–39.

Lamien, C.E., Le Goff, C., Silber, R., Wallace, D.B., Gulyaz, V., Tuppurainen, E., Madani, H., Caufour, P., Adam, T., Harrak, M. El, Luckins, A.G., Albina, E., Diallo, A.(2011): Use of the Capripoxvirus homologue of Vaccinia virus 30kDa RNA polymerase subunit (RPO30) gene as a novel diagnostic and genotyping target: Development of a classical PCR method to differentiate Goat poxvirus from Sheep poxvirus. *Vet. Microbiol.* **149**: 30–39.

Leliso, S.A., Bari, F.D., Chibssa, T.R.(2021): Molecular Characterization of Lumpy Skin Disease Virus Isolates from Outbreak Cases in Cattle from Sawena District of Bale Zone, Oromia, Ethiopia. *Vet. Med. Int.* 2021.

Maclachlan, N.J., Dubovi, E.J.(2010): Fenner's Veterinary Virology: Fourth Edition, Fenner's Veterinary Virology: Fourth Edition.

MacLachlan, N.J., Dubovi, E.J., Barthold, S.W., Swayne, D.E., Winton, J.R.(2016): Fenner's veterinary virology: Fifth edition, Fenner's Veterinary Virology: Fifth Edition.

Mahmoud, M.A., Khafagi, M.H.(2016): Detection, identification, and differentiation of sheep pox virus and goat pox virus from clinical cases in Giza Governorate, Egypt.

Vet. World **9**: 1445–1449.

Mammo, B.(2019): Isolation , Molecular Characterisation and Vaccine Effectiveness Study of Lumpy Skin Disease Virus in Selected Diary Farms of Central Ethiopia 1–13.

Massung, R.F., Jayarama, V., Moyer, R.W.(1993): DNA sequence analysis of conserved and unique regions of swinepox virus: Identification of genetic elements supporting phenotypic observations including a novel G protein-coupled receptor homologue. Virology.

Mebratu, G.Y., Kassa, B., Fikre, Y., Berhanu, B.(1984): Observation on the outbreak of lumpy skin disease in Ethiopia. Rev. d"elevage *Med. Vet. des pays Trop.* **37**: 395–399.

Milovanović, M., Dietze, K., Milicévić, V., Radojičić, S., Valčić, M., Moritz, T., Hoffmann, B.(2019): Humoral immune response to repeated lumpy skin disease virus vaccination and performance of serological tests. *BMC Vet. Res*. **15**: 1–9.

Modrow, S.(2013): Molecular Virology(BookZZ.org).pdf.

Molla, W., Frankena, K.(2017): Transmission dynamics of lumpy skin disease in Ethiopia.

Molla, W., Frankena, K., Gari, G., Kidane, M., Shegu, D., Jong, M.C.M. De.(2018): Seroprevalence and risk factors of lumpy skin disease in Ethiopia. *Prev. Vet. Med.*

Molla, W, Jong, M.C.M. De, Frankena, K.(2017): Temporal and spatial distribution of lumpy skin disease outbreaks in Ethiopia in the period 2000 to 2015 1–9.

Molla, Wassie, Jong, M.C.M. De, Gari, G., Frankena, K.(2017): Economic impact of lumpy skin disease and cost e ff ectiveness of vaccination for the control of outbreaks in Ethiopia. *Prev. Vet. Med.* **147**: 100–107.

Morgan.(2019): Poxviruses, Journal of Chemical Information and Modeling.

Morgenstern, M., Klement, E.(2020): The effect of vaccination with live attenuated

neethling lumpy skin disease vaccine on milk production and mortality—An analysis of 77 dairy farms in Israel. Vaccines **8**: 1–12.

OIE.(2019): Chapter 3 . 4 . 12 -Lumpy Skin Disease. Man. Diagnostic Tests Vaccines Terr. Anim. 2019 *Office Int. des Epizoot.* 1158–1171.

Points, K.(2015): Amino Acids 21–29.

Prozesky, L., Barnard, B.J.(1982): A study of the pathology of lumpy skin disease in cattle. Onderstepoort *J. Vet. Res.* **49**: 167–175.

Rich, K.M., Perry, B.D.(2011): The economic and poverty impacts of animal diseases in developing countries: New roles, new demands for economics and epidemiology. *Prev. Vet. Med.* **101**: 133–147.

Rouby, S.R.(2016): Reserch Article RPO30 Gene based PCR for Detection and Differentiation of Lumpy Skin Disease Virus and Sheep Poxvirus Field and Vaccinal Strains.

Shapiro, B.I., Gebru, G., Desta, S., Negassa, A., Nigussie, K., Gezahegn, A., Henok, M. (2017): Ethiopia livestock sector analysis: livestock sector analyses. ILRI Proj. Rep. **127**.

Strategy, D.(2009): Disease Strategy.

Tassew, A., Assefa, A., Gelaye, E., Bayisa, B., Ftiwi, M.(2018): Identification and Molecular Characterization of Lumpy Skin Disease Virus in East Hararghe and East Shoa Zone, Oromia Regional State. *ARC J. Anim. Vet. Sci.* **4**: 2455–2518.

Tulman, E.R., Afonso, C.L., Lu, Z., Zsak, L., Kutish, G.F., Rock, D.L.(2001): Genome of Lumpy Skin Disease Virus. *J. Virol.* **75**: 7122–7130.

Tulman, E.R., Afonso, C.L., Lu, Z., Zsak, L., Sur, J.-H., Sandybaev, N.T., Kerembekova, U.Z., Zaitsev, V.L., Kutish, G.F., Rock, D.L.(2002): The Genomes of Sheeppox and Goatpox Viruses. *J. Virol.* **76**: 6054–6061.

Tuppurainen, E.(2018): The detection of lumpy skin disease virus in samples of experimentally infected cattle using different diagnostic techniques University of Pretoriaetd – Tuppurainen , SM (2 0 0 5) The detection of lumpy skin disease virus.

Tuppurainen, E.S.M., Babiuk, S., Klement, E.(2018): Lumpy skin disease, Lumpy Skin Disease.

Tuppurainen, E.S.M., Oura, C.A.L.(2012): Review: Lumpy Skin Disease: An Emerging Threat to Europe, the Middle East and Asia. *Transbound. Emerg. Dis.* **59**: 40–48.

Tuppurainen, E.S.M., Pearson, C.R., Bachanek-Bankowska, K., Knowles, N.J., Amareen, S., Frost, L., Henstock, M.R., Lamien, C.E., Diallo, A., Mertens, P.P.C.(2014): Characterization of sheep pox virus vaccine for cattle against lumpy skin disease virus. *Antiviral Res*. **109**: 1–6.

Vandenbussche, F., Mathijs, E., Haegeman, A., Al-Majali, A., Van Borm, S., De Clercq, K.(2016): Complete genome sequence of Capripoxvirus strain KSGP 0240 from a commercial live attenuated vaccine. Genome Announc. **4**.

Vidanović, D., Tešović, B., Šekler, M., Debeljak, Z., Vasković, N., Matović, K., Koltsov, A., Krstevski, K., Petrović, T., De Leeuw, I., Haegeman, A.(2021): Validation of taqman-based assays for specific detection and differentiation of wild-type and neethling vaccine strains of lsdv. Microorganisms **9**: 6–11.

Virusforschung, D.I.E., Einzeldarstellungen, I.N.(1968): Virology Monographs Die Virusforschung.

Von Backstrom, U.(1945): Ngamiland cattle disease: preliminary report on a new disease, the aetiological agent being probably of an infectious nature. J. South African *Vet. Med. Assoc.* **16**: 29–35.

8. APPENDIX

Appendix 1: ESH-L cell line culture preparation

1. To begin, all of the required cell culture media were warmed in a water bath to 37°C, and the trypsin solution was allowed to cool to room temperature. The cells' culture medium was removed and decanted.

2. The cells were rinsed twice with PBS, then trypsin was injected at a volume of 1-2 ml per 25 cm^2 of flask surface, tilted gently to cover the entire surface.

3. The flask was then placed in an incubator at 37°C for around 5 minutes, after which it was microscopically checked to see if all of the cells had detached, and if required, it was re-incubated.

4. Pipetting culture medium over the vessel's surface, i.e. 2-5 ml per 25 cm2 of flask surface, collected the cells, which were then carefully mixed to disperse the cells into a single-cell suspension.

5. At 7.2-7.4 PH, the cell suspensions were transferred to a fresh 25cm^2 tissue culture flasks containing GMEM with 10% FCS and streptomycin antibiotics.

6. The cell cultures were grown at 37°C in a 5% CO2 incubator and were examined on a regular basis. After 3 days, the media in each flask was replaced until the cells formed a monolayer. The cell cultures were passed through several times until they were fully developed.

Appendix 2: Growth and maintenance media preparation for cell culture growth medium consist of GMEM supplemented with 10% tryptose phosphate broth (TPB), 0.63% of a 10% NaHCO3 solution, 1% of Antibiotic-Antimycotic Mixture 100X (Gibco, Grand Island, New York, USA), and 10 % foetal calf serum (FCS). For virus isolation, this medium will be replaced before 24 h by the same medium but supplement with only 2% FBS.

Appendix 3: virus inoculation on cell culture

1. Before putting the medium and solution bottles, as well as other components into the safety cabinet, disinfect all of their outer surfaces with 70% ethanol.

2. Remove the growth medium from the flask where the cell has formed a full monolayer.

3. Wash the monolayer 2-3 times with 2-3 ml of pre-warmed PBS.

4. Add 1ml sample inoculum to the cell culture and Rock each plate gently to distribute inoculum evenly over the cell monolayer.

5. Incubate inoculated cultures at 37℃ incubators for 1 hr to allow virus to adsorb.

6. During the incubation period, shake the infected flasks once or twice.

7. In each flask, add GMEM with 2% FCS (maintenance medium) and incubate for about14 days at 37°C.

8. Under an inverted microscope, check flasks daily for cytopathogenic effect (CPE) and cell condition.

9. Harvest samples to a deep freezer and freeze-thaw 2-3 times to release the viral particles.

Appendix 4: DNA Purification by Centrifugation

1. Add an equal volume of Membrane Binding Solution to the PCR amplification.

2. Insert SV Minicolumn into Collection Tube.

3. Transfer dissolved gel mixture or prepared PCR product to the Minicolumn assembly. Incubate at room temperature for 1 minute.

4. Centrifuge at 16,000 × g for 1 minute. Discard flowthrough and reinsert Minicolumn into Collection Tube.

5. Add 700µl Membrane Wash Solution (ethanol added). Centrifuge at 16,000 × g for 1 minute. Discard flowthrough and reinsert Minicolumn into Collection Tube.

6. Repeat Step 5 with 500µl Membrane Wash Solution. Centrifuge at 16,000 × g for 5 minutes.

7. Empty the Collection Tube and recentrifuge the column assembly for 1 minute with the micro-centrifuge lid open (or off) to allow evaporation of any residual ethanol.