ISOLATION AND MOLECULAR DETECTION OF LUMPY SKIN DISEASE VIRUS FROM OUTBREAK CASES IN BALE ZONE, OROMIA REGION, ETHIOPIA

MSc THESIS

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Isolation and Molecular Detection of Lumpy Skin Disease Virus from Outbreak Cases in Bale Zone, Oromia Region, Ethiopia

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I hereby certify that I have read and evaluated this thesis entitled "**Isolation and Molecular Detection of Lumpy Skin Disease Virus from Outbreaks Cases in Bale Zone, Oromia Region, Ethiopia"** prepared under my guidance by Muzeyen Mohammadnur Biltu. I recommend that it be submitted as fulfilling the thesis requirement.

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DEDICATION

To my mother Zamu Hassein, wife Naima Mohammed, and father Mohammadnur Biltu, who motivate me to strive for more, this thesis is dedicated.

STATEMENT OF THE AUTHOR

By my signature below, I declare and affirm that this thesis is my own work. I have followed all ethical and technical principle of scholarship in the preparation, data collection, data analysis and compilation of this thesis. Any Scholarly matter that is included in the thesis has been given recognition through citation.

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BIOGRAPHICAL SKETCH

The author was born in 1981 E.C. in Ilani Godage Kebele, Agarfa District, within the Bale Zone of the Oromia Regional State. He is the son of Mohammadnur Biltu Haro and Zamu Hasen Tilmo. His educational journey began at Ilani Godage Elementary School in 1991 E.C., followed by Agarfa Senior High School in 1999 E.C., and Agarfa Preparatory School in 2001 E.C. He then enrolled at Haramaya University, where he pursued a degree in Veterinary Medicine, graduating with a Doctor of Veterinary Medicine (DVM) in 2008 E.C.

After graduation, he was employed by the Sinana District Agricultural Office from 2009 to 2011 E.C. He then joined Bash Pharmaceutical Company of Sudan from 2011 to 2014 E.C. In 2014, he returned to the Sinana District Agricultural Office, serving as the district Veterinary Epidemiologist. In 2015 E.C., he resumed his academic pursuits at Haramaya University, enrolling in the College of Veterinary Medicine, Department of Veterinary Microbiology, to pursue his MSc in Veterinary Microbiology. His diverse experiences reflect a deep commitment to veterinary medicine and public health in his community.

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LIST OF ACRONYMS

AHI	Animal Health Institute
AOR	Adjusted Odd Ratio
AUSVETPLAN	Australia Veterinary Emergency Plan
CAM	ChorioAllantoic Membrane
CaPV	Capripox Virus
CIRAD	Center for International Cooperation in Agricultural Research for Development
COR	Crude Odds Ratio
CPE	Cytopathic effect
Ct	Cycle threshold
DOVAR	Date of Verification of the Animal Report
EFSA	European Food Safety Authority
ELISA	Enzyme-Linked Immunosorbent Assay
ESH-L	Epithelial Sheep Skin origin cell line
GMEM	Glasgow's Modified Eagles's Medium
GTPV	Goat Pox Virus
HRM	High Resolution Melting
IFAT	Indirect Fluorescent Antibody Technique
LSD	Lumpy skin disease
LSDV	Lumpy Skin Disease Virus
OR	Odd Ratio
qPCR	Real Time Polymerase Chain Reaction
PBS	Phosphate buffer saline
PCR	Polymerase Chain Reaction
SPPV	Sheep Pox Virus
TCID	Tissue Culture Infectious Dose
Tm	Melting temperature
VNT	Virus Neutralization Test
WOAH	World Organization for Animal Health

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ABSTRACT

Lumpy Skin Disease (LSD) is a transboundary viral disease of cattle that has rapidly spread from Africa to Europe and Asia, causing significant economic losses. This study aimed to isolate and molecularly detect the Lumpy Skin Disease Virus (LSDV) in three selected districts of the Bale zone, Ethiopia, and assess associated risk factors. A cross-sectional study was conducted from January to July 2024 in purposively selected districts. A total of 227 cattle from outbreak areas were examined, of which 13 showed clinical signs of LSD. Additionally, 100 house-holds were surveyed using semi-structured questionnaires to assess knowledge, disease history, and risk factors. Samples collected included skin nodules (n=10), nasal swabs (n=1), and saliva swabs (n=2). Virus isolation was performed using the Vero cell line (P-40), followed by molecular detection via real-time polymerase chain reaction (PCR). Data were analyzed using SPSS 20, employing descriptive statistics and logistic regression. All 13 samples exhibited cytopathic effects (CPE) consistent with LSDV, and real-time PCR confirmed their positivity. The study revealed morbidity, mortality, and case fatality rates of 5.7%, 1.32%, and 23.08%, respectively. Logistic regression identified key risk factors, including lack of vaccination (AOR: 7.46, $p = 0.001$), introduction of new animals (AOR: 6.146, $p = 0.003$), and communal watering points (AOR: 6.394, $p = 0.027$). The study confirmed the presence of LSDV with high case fatality rates in the Bale zone, driven by factors such as unvaccinated herds, new animal introductions, and shared watering sources. Effective disease management, continuous surveillance, and advanced molecular research, such as genome sequencing, are crucial to mitigate the economic impact of LSD, improve outbreak control, and enhance vaccine development.

Keywords: *Bale, Isolation, LSD, LSDV, Molecular detection, Risk factors*

1. INTRODUCTION

1.1. Background Information and Rationale of the Study

Livestock plays a crucial role in Ethiopia's economy, providing essential products such as milk, meat, and hides, while also serving as a source of animal protein, agricultural power, transportation, and household energy (Asenso-Okyere *et al*., 2013; World Bank, 2017).In 2017, the livestock sector accounted for 40% of agricultural GDP, 20% of total GDP, and 20% of foreign exchange earnings (World Bank, 2017). Ethiopia has an estimated cattle population of 70 million, with 56% females and 44% males, of which 60% are aged 3 years or younger. Local breeds dominate (97.4%), with hybrids and exotics making up 2.3% and 0.31%, respectively (CSA, 2021). In the highlands, oxen are used for farming, while in arid and semiarid areas, cattle, sheep, goats, and camels are managed through migratory pastoral systems (Asenso-Okyere *et al*., 2013). Despite its importance, the sector faces challenges such as low productivity due to diseases, with lumpy skin disease being particularly damaging (Sileshi *et al*., 2001; Solomon *et al*., 2020).

Lumpy skin disease (LSD) is an infectious viral disease of cattle characterized by fever, skin nodules, and lesions in the mouth, pharynx, and respiratory system, the lesions may cover the entire animal's body in its severest forms, as well as emaciation, swollen lymph nodes, skin edema, and occasionally mortality (WOAH, 2022). The irreversible harm to hides makes the disease among the most significant viral diseases of cattle. The disease also shows some systemic effects like pyrexia, anorexia, dysgalactia, lameness, and pneumonia. Although many cattle exhibit severe emaciation and loss of production for several months, there is a considerable breed difference (Davies, 1991).

Lumpy skin disease is caused by a virus called Lumpy skin disease virus (LSDV), which is part of the *Capripox Viruses* family. *Capripox Viruses* have two sub-families, 22 genera, and 83 species. Capripoxvirus includes three types of poxviruses: SPPV, GTPV, and LSDV. The genome of LSD is 156 kbp long and is very similar to other poxviruses (Tulman *et al*.,

2001). Because of this high genetic identity, it's hard to tell it apart from the other Capripoxviruses in serological tests (MacLachlan and Dubovi, 2017).

Lumpy Skin Disease (LSD), has a distinct geographical distribution and host specificity compared to sheep and goat pox, as cattle strains of capripoxvirus do not infect or transmit between sheep and goats (Ahmed and Zaher, 2008; WOAH, 2022). First identified in Zambia in 1929, LSD has since spread across sub-Saharan Africa, including Ethiopia, and expanded to the Middle East (e.g., Egypt, Israel) and Southeast Europe (e.g., Turkey, Greece) (Tuppurainen *et al*., 2011; Beard, 2016; Moudgil *et al*., 2024). The virus is now widespread in most of Africa, including Madagascar, and poses a significant risk to neighboring regions in Europe and Asia due to its rapid transboundary spread (Akther *et al*., 2023). LSD causes substantial economic losses, with morbidity rates ranging from 3% to 85% and mortality rates around 3%, particularly impacting cattle-dependent economies like South Africa (Boshra *et al*., 2015). Due to its severe economic impact and rapid spread, LSD is classified as a notifiable disease by the World Organization for Animal Health (WOAH) (Tuppurainen and Klement, 2018).

Incidence of LSDV is associated with high temperature and humidity, and vector burden. It is usually more prevalent during the wet summer and autumn months, 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 (Hanshaw, 1968).

Lumpy skin disease-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 and Klement, 2018).

In Ethiopia, LSD was first observed in 1981 in the northwestern part of the country (Yoseph *et al*., 1984). Nearly every region and agro-ecological zone in the nation is currently affected by the disease. Because of the wide distribution of the disease and the size and structure of

the cattle population in Ethiopia, LSD likely is one of the most economically important livestock diseases in the country (Getachew *et al*., 2011).

The prevalence of Lumpy Skin Disease (LSD) in Ethiopia reveals a wide range of morbidity rates across various regions, indicating significant variability in the disease's impact. For instance, a study conducted by Mesfin (2022) reported a notably high morbidity rate of 36.2% in Wollaita Zone. Other significant findings include a 22.9% morbidity rate in Bishoftu (Gelagay *et al*., 2013), 18% in Bale Zone reported by (Shubisa *et al*., 2021), and 21.2% in central Ethiopia as reported by Wassie *et al*. (2017). In addition to morbidity, mortality rates also reflect the severity of LSD's impact on cattle populations. Mortality rates in Ethiopia range from 1.92% in West Hararghe (Umer *et al*., 2024), and 2.12% in central Ethiopia (Mesay, 2018). This variability emphasizes the need for localized studies to better understand the dynamics of LSD in different environments and to formulate effective control measures. As such, the data suggest that while LSD remains a critical threat across Ethiopia and beyond, targeted interventions are crucial to mitigate its economic and health impacts on livestock.

Lumpy Skin Disease Virus (LSDV) can grow in bovine, ovine, and caprine tissue cultures, with bovine dermal and lamb testis cells being the most susceptible. While capripoxvirus strains can also be cultured on embryonated chicken egg membranes and Vero cells, Vero cells are preferred for prolonged experiments due to their stability and longevity (WOAH, 2017; Kumar *et al*., 2021). Polymerase chain reaction (PCR) is effective for detecting LSDV in nodular tissue, with real-time PCR providing quick and reliable confirmation of field isolates. Additionally, PCR methods can differentiate between SPP vaccine strains and field isolates, offering a valuable diagnostic tool (Tesfaye *et al*., 2018; Mikhael *et al*., 2023).

A significant obstacle to the export of live cattle and their products is Ethiopia's extensive prevalence of the disease. Moreover, the reduction in the production of meat and milk, along with the poor quality of hides, negatively impacts the growth of the national economy (Esayas *et al*., 2015). Since the nation lacks a well-thought-out plan for controlling this disease, it remains a major issue. The Neethling vaccine effectively reduces LSDV outbreaks in Ethiopia but requires better delivery systems, while sheep pox/goat pox

vaccines offer partial protection (Gelagey *et al*., 2013; Esayas *et al*., 2015. Live attenuated vaccines, though effective, risk causing disease in immunosuppressed animals and recombination with field strains, and poor implementation limits their impact (Tuppurainen *et al*., 2017; Wassie *et al*., 2017). Recombinant vaccines are a promising alternative for safer, long-lasting immunity (Babiuk *et al*., 2008). Another significant issue is the paucity of genetic data on in-field circulating viruses and their correlation with the vaccine currently in use, which is crucial for improving vaccine matching (Esayas *et al*., 2015). Serological diagnosis for lumpy skin disease (LSD) in Ethiopia also faces significant challenges due to inadequate diagnosis kits (Nebyou *et al*., 2024).

Isolation circulating field strains of Lumpy Skin Disease (LSD) using molecular techniques is crucial for developing effective vaccines. While LSD is typically diagnosed using blood or tissue samples, non-invasive samples like nasal and saliva swabs remain underexplored. Despite extensive research on LSD outbreaks in Ethiopia, the highland of Bale Zone has been unexplored, representing a critical gap. Investigating this area could provide insights into local environmental factors, livestock management, and vaccination history, which impact disease dynamics. Such studies are essential for improving cattle health and supporting the nation's livestock-dependent economy. Molecular detection combined with epidemiological understanding is key to achieving these goals.

1.2. Study Objectives

The general objective of this study was to detect the LSDV circulating in outbreaks from Bale Zone, Oromia Regional State of Ethiopia by using different diagnostic techniques. The specific objectives were:

- To isolate LSD virus from skin nodules, and nasal and saliva samples using cell culture.
- To conduct molecular detection of LSD virus circulation among outbreak cases in the study areas.
- To assess the risk factors associated with the history of LSD occurrences in the study areas.

2. LITERATURE REVIEW

2.1. History

Lumpy skin disease was first identified in Africa in 1929, and it was referred to as 'Pseudo-urticaria'. People thought it was some poisoning or hypersensitivity reaction to insect bites due to the huge amount of bugs at that time of year. It later became known as 'Ngamiland Cattle Disease' and spread to the Ngamiland, Bechuanal, and Protectorate (now Botswana) in 1943 (Khafagi *et al*., 2022). In 1957, Kenya was the first to report the disease's arrival in East Africa, with high mosquito numbers and an unknown source of infection. It mainly affected calves and spread to other farms even with quarantine and sanitation measures in place (Salib and Osman, 2011). Then in 1971, Sudan reported the first sighting of LSD in the west, before it spread to the east (Hussien *et al*., 2022). Ethiopia had its first reports in 1981 and 1983 in the northwest, west, and center, which were diagnosed through clinical observation, virus isolation, and electron microscopy (Yoseph *et al*., 1984).

Since it was identified, LSD was known as *Pseudo uretrica* in Zambia (MacDonald, 1931). It spread to Egypt and Israel and more recently, other Middle Eastern countries and South East Asia, with the first outbreak recorded in Bangladesh in 2019 and it is traveling to other places in Europe. It is not present in the Americas (Das *et al*., 2021; Maclachlan and Dubovi, 2010). For the majority of the last century, LSD was mainly a sub-Saharan African disease, and it was thought that if it did spread, it would be contained (Tuppurainen *et al*., 2017). But it's since spread to Africa, the Middle East, Southeastern Europe, Central Asia, South Asia, and China. It is now endemic in various countries in Africa, some parts of the Middle East, and Turkey (FAO, 2017). Plus, it's spread to the Middle East (Tuppurainen *et al*., 2017). It could soon reach Central Asia, Western Europe, and Central-Eastern Europe. This fast spread of LSDV outside of Africa has highlighted its economic importance, especially when it emerged as a major epizootic pathogen in 2012 (Shubisa *et al*., 2021).

2.2. Etiology

Lumpy skin disease virus belongs to the family *Poxviridae*, which is divided into two categories; *poxviruses* that affect insects and poxviruses that affect vertebrates. CaPVs are one of eight genera 5 within the Chordopox virus subfamily, and include Lumpy Skin Disease Virus, *Sheep Pox Virus*, and *Goat Pox Virus*. The Neethling strain, which is the prototype of LSDV, was first identified in South Africa (Alexander *et al*., 2020). Although they are the same serologically, these CaPV infections are usually host-specific and found in certain geographic regions (Tulman *et al*., 2001).

2.3. Lumpy Skin Disease Virus Characteristics

2.3.1. Viral genome

Lumpy skin disease virus is a double-stranded DNA virus that's pretty stable - its 151 kbp genome has 156 putative genes, 95% of which code for proteins responsible for things like nucleic acid biogenesis, virion assembly, and more. The similarities between LSDV and other *Capripoxviruses* are found mainly in the middle part of the genome, but the two ends can be quite distinct. All in all, this virus is pretty stable and has minor genetic variations (Kumar *et al*., 2021). Transmission electron microscopy shows that Capripoxviruses have a biconcave core with the genome in a triple-folded tube or coil. The core and two lateral bodies are surrounded by the capsid according to Figure 1 (Tuppurainen *et al*., 2018).

In addition, LSDV has a bunch of genes associated with its host range; these genes can affect the immune response, stop or alter apoptosis, and give the virus a preference for certain cells and tissues. It has a unique gene compared to other viruses that give its specificity for certain hosts. Some of the genes are responsible for interfering with the host's immune system, and it has also four proteins that can modulate the immune system. The virus also has proteins that control its virulence and how it grows in cells, as well as influencing apoptosis (Tulman *et al*., 2001). The virus is shaped like a brick (300x270x200nm) and has a core, two lateral bodies, a membrane, and an envelope according to Figure 2. The envelope is made of two layers of cellular lipids and some virus-

specific proteins. Upon exiting the host cell, the virus is enveloped. The virus is not enveloped in the host cell when it ruptures (Tuppurainen *et al*., 2005).

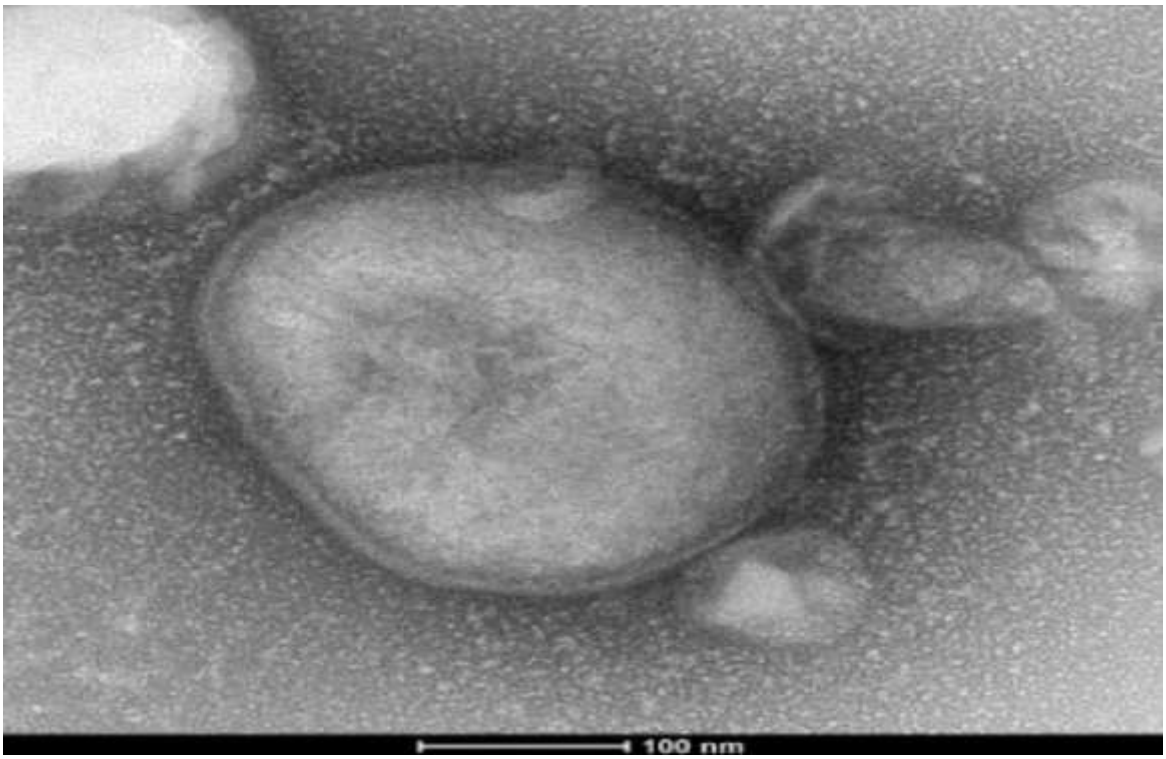

Figure 1. Electron micrograph of a lumpy skin disease virus particle prepared by conventional negative staining (Tuppurainen, 2015).

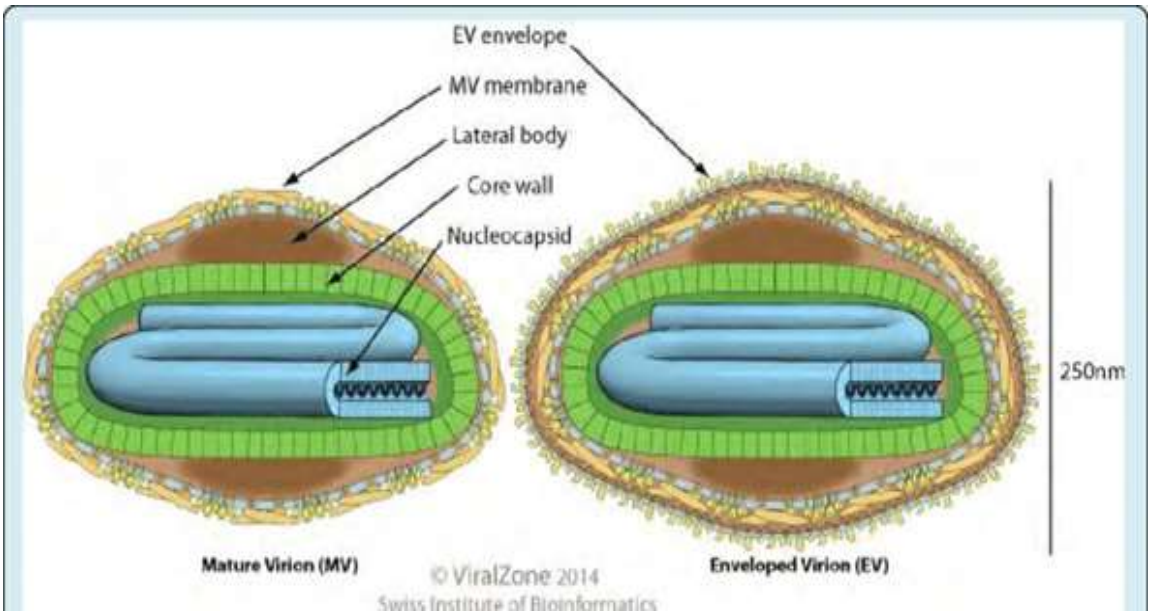

Figure 2. The General structure of *Capripoxviruses* (Chala *et al*., 2022)**.**

2.3.2. Replication cycle

Viral replication generally happens in the cytoplasm, which is different from other DNA viruses. The process of the virus entering the cells includes actin dynamics, cell signaling, and ten viral proteins. The mature virion (MV) fuses with the cell membrane, which allows the core to enter the cytoplasm and start gene expression. A set of viral proteins on the MV membrane aids the fusion process, allowing the virus to spread from cell to cell and for the membrane wrapper of the extracellular virions to break down (Maclachlan and Dubovi, 2010).

After the virus membrane and host cell membrane fuse, a series of genes get transcribed in the Early, intermediate, and late phases. These genes are triggered by the viral transcriptase and other factors in the core. When the core is uncoated, the viral genome is released into the cytoplasm (Maclachlan and Dubovi, 2010). Intermediate genes enable DNA replication and the late genes encode virion proteins and early transcription factors. The progeny virions assemble in cytoplasmic factories, forming a spherical immature particle. This matures into the brick-shaped intracellular mature virion (IMV), which can be released by lysing the cell or through exocytosis. These mature virions do not have an additional membrane (Maclachlan and Dubovi, 2010; Tuppurainen *et al*., 2018)

2.3.3. Physicochemical property

Lumpy skin disease virus is susceptible to temperature at 55 ^{0}C for 2 hours and 65 ^{0}C for 30 minutes(WOAH, 2016), and is stable in the pH range of 6.6 to 8.6 for 5 days at 37°C(Weiss, 1968). It can also be inactivated by ether (20%), chloroform (1%), formalin (1%), and some detergents, like sodium dodecyl sulfate. It is even more resilient in dried scabs, necrotic skin nodules (up to 33 days), and air-dried hides (up to 18 days). It can survive in the environment for extended periods of time. However, it is susceptible to sunlight and detergents with lipid solvents, so if it's left in a dark and contaminated environment, it can last for many months (WOAH, 2017).

2.4. Geographical Distribution

Lumpy skin disease was documented in a wide geographical range, in Africa, the Middle East, Central Asia, and Europe covering a diversity of climates. Using the presence-only maximum entropy ecological niche modeling technique to characterize the geographical risk factors for diseases 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 belief 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 C° (Tuppurainen *et al*., 2018).

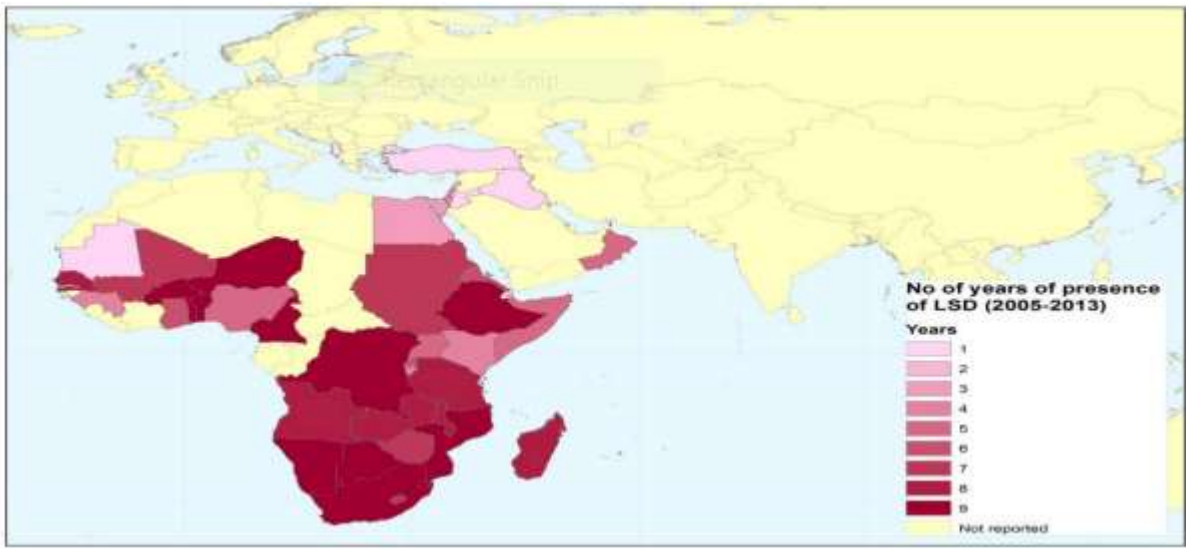

Figure 3. Geographical distribution of Lumpy skin disease in different countries as reported to WOAH (2005–2013); Source :(EFSA, 2015).

Between 2007 and 2011, a total of 1,675 outbreaks occurred in Ethiopia, resulting in 62,176 cases and 4,372 deaths. The highest number of outbreaks was seen in 2010, with the most frequent reports in October and November. From 2000 to 2015, research conducted in Ethiopia showed that LSD appeared in every region of the country (Wassei *et al*., 2017) with the majority of outbreaks in Oromia, Amhara, and SNNPR but the administration of Dire Dawa City and Harari Regional State did not record any outbreaks during the last

retrospective assessment, which covered the period from January 2007 to December 2011 (Gelagay *et al*., 2014). The majority of outbreaks were in the central and southwestern parts of the country. The most affected zones were Illubabor, Jimma, South-West Shoa, and Arsi according to Figure 4 (Gelagay *et al*., 2014).

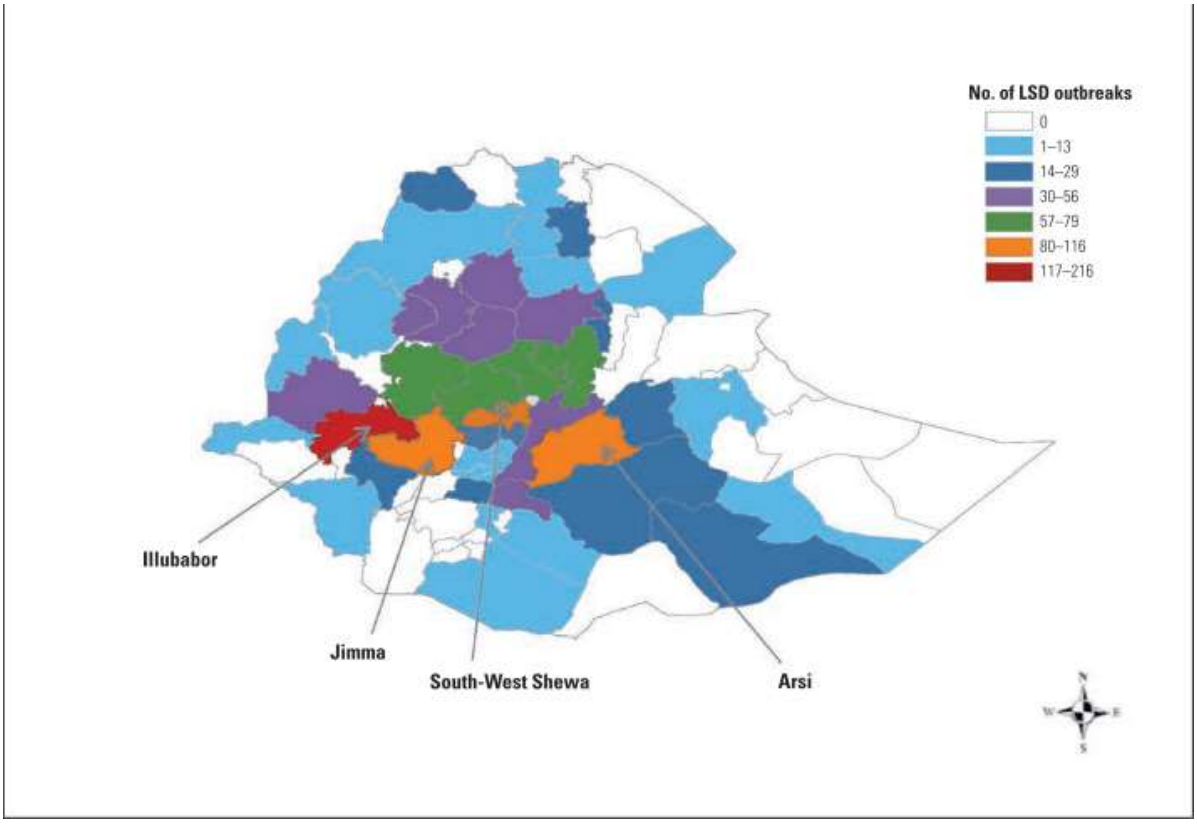

Figure 4. Map of Ethiopia showing the distribution of Lumpy skin disease outbreaks (2007-2011). Source: (Gelagay *et al*., 2014).

2.5. Host Range and Transmission Mechanism

Lumpy skin disease virus with a few exceptions only causes clinical disease in cattle. However, it has also been seen in giraffes, water buffalo, and impalas. *Bos taurus* (Fine skinned) cattle breeds such as Holstein-Friesian and Jersey are known to be the most susceptible to the disease. On the other hand, *Bos indicus* (Thick-skinned) breeds including the Afrikaner, and their cross-breed show tolerance towards the disease. This can be explained that *Bos indicus* relative resistance to ectoparasites. Young calves and cows at

peak lactation show more severe clinical symptoms, but all age groups are susceptible to the disease (Hanshaw *et al*., 1968).

Infection in cattle with lumpy skin disease virus can occur by mechanical transmission through insect or tick vectors. The virus can also be transmitted through blood, nasal discharge, lacrimal secretions, semen, and saliva. Additionally, LSDV can spread to nursing calves through contaminated milk (Kanokwan *et al*., 2022). In experimentally infected cattle, LSDV was found after 22 days in semen suggesting that there is a possibility of transmission sexually *(*Annandale *et al.*, 2010). Similarly, the virus was detected in saliva 11 days post-infection and after 33 days in skin nodules. However, the transmission of LSDV by direct contact is considered to be inefficient. The virus is not detected in urine or feces. Similar to other pox viruses, which are known to be highly resistant, LSDV can remain functional in infected tissue for more than 120 days (Awad *et al*., 2010).

Mechanical transmission of LSDV through blood-feeding vectors remains the most important mode of transmission. This hypothesis is based on the observation that LSD outbreaks are correlated with warm and wet seasons (Hanshaw *et al*., 1968). A single species vector that is responsible for transmitting the virus has not been identified. Instead, the LSDV has been isolated from *Stomoxys*, *Biomyia fasciata*, *Glossina*, *Tabanidae,* and *Culicoides* species. Different studies have demonstrated that LSDV can be transmitted by *Aedes aegypti*, female mosquitoes, from infected to susceptible cattle (Tuppurainen *et al.*, 2013, 2018).

Research done by Tuppurainen in 2013, shows that different tick types like that of Hard (ixodid) tick *Rhipicephalus appendiculatus* and *Rhipicephalus decoloratus* could mechanically transmit the virus. Another type of tick, *Amblyomma hebraeum*, was also proven to possess the transmission ability of LSD in an experiment done by Lubinga *et al*. (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 (Tuppurainen *et al*., 2013, 2018).

2.6. Risk Factors for LSD Outbreaks

Lumpy skin disease can spread in a few ways. The research done during outbreaks of LSD from 2021-2022 in Thailand showed that it is probably due to insects found around since cattle do not move between farms much and there were outbreaks in multiple farms close together (Punyapornwithaya *et al.*, 2022). This was backed up by other studies in Albania and Kazakhstan. In China, it's believed to be spread by mechanical vectors such as insect and arthropod vectors (including mosquitoes, the biting midges, stable flies, and hard ticks) and maybe even illegal animal movement and transport, that is illegal transport of animal products such as hides and skins (Tuppurainen *et al*., 2011; Tasioudi *et al*., 2016). In addition, many farm owners do not know about LSD as an emerging disease, so cases go unreported (Lu *et al*., 2021).

Lumpy skin disease was first reported in the EU, Greece, in August 2015 until the end of December 2015 showed that the outbreak was due to illegal movement of Bovine than small ruminants around the territory. The enormous number of vectors around there also affected disease transmission. Mild weather conditions during autumn are easily favorable for LSD spread in northern Greece. According to the Hellenic National Meteorological Service, the average daily temperature was 18.6°C from 20 August to 30 November 2015 and 22.7°C during the last outbreak that occurred in South Evros (Tasioudi *et al*., 2016).

In the Midland zone of Ethiopia, the prevalence of LSD was reported to be higher than in the Highland and Lowland zones. It also showed that the populations of biting flies begin to increase and peak within a similar period. From April onwards, both epidemic diseases and biting flies increase simultaneously with a peak in September (Getachow *et al*., 2010). In Ethiopia, cattle movement for trade, seasonal grazing migrations, and cultural event participation are all major factors contributing to the Lumpy Skin Disease. All these activities lead to more contact between infected animals and susceptible ones thus increasing chances for transmission (Tuppurainen and Oura, 2012). It is also at higher risk in areas with high cattle density especially those characterized by extensive pastoralism like the Somali and Afar regions due to close contact among animals which is conducive to virus transmission (Getachow *et al*., 2012). Factors such as limited access to veterinary services coupled with irregular vaccination programs experienced in rural areas tend to make it easier

for the disease to persist and repeat its cycle after it has been contained lumpy skin disease (Getachow *et al*., 2011). These low biosecurity, livestock management practices, grazing lands, and watering points make conditions favorable; they render rural Ethiopian cattle highly vulnerable to Lumpy Skin Disease infection (Esayas *et al*., 2019).

2.7. LSD Morbidity and Mortality

Morbidity and mortality vary considerably depending on the breed of cattle, the immunological status of the population, the insect vectors involved, and isolates of the virus. Naturally, *Bos Taurus* is more susceptible than *Bos Indicus,* and lactating cows are believed to be at the most risk. The distribution and relative abundance of insect vectors have also proven to be a factor in morbidity rates. There are contradicting results regarding age and sex-related susceptibility to LSD. In some studies, higher morbidity was related to calves while others claim they are more resistant to the disease. The same inconsistency was reported regarding sex as well (Davies, 1991; Wassie *et al*., 2018).

Furthermore, stress factors such as trypanosomiasis compromise the immune status of the animal contributing to the severity of LSD infection. Thus, in an outbreak of the disease morbidity ranges from 3% to 85% and mortality at about 3%. However, morbidity is usually estimated at least 10% in endemic areas and mortality rate is generally low (1–3%) but may sometimes reach 40 % (Tuppurainen *et al*., 2018).

2.8. Economic Importance of the Disease

Lumpy skin disease is a major problem in Africa and the Middle East, causing big losses for cattle owners. The WOAH lists it as a notifiable disease due to the economic damage it does. It does not usually kill the cattle, but the amount of illness it causes makes it expensive for herd owners, consumers, and those who use the product and by-products from the livestock (Tuppurainen *et al*., 2011; Tuppurainen and Klement, 2018). Lumpy skin Disease have a significant financial impact on Ethiopia because they lower the quality of the hides, reduce milk output, increase treatment expenses, decrease the traction power of oxen, and cause animal deaths (Mitiku, 2022).

Lumpy skin disease in cattle can cause some major damage up to a 60% loss in production as seen in intensive farming (Tuppurainen *et al*., 2011). Developing countries are especially affected, with the poorest small-scale farmers bearing the brunt of the disease. Not only does it make cattle sick, but it also reduces their ability to work, causes abortions in pregnant cows, and lowers milk production. Hides are damaged, and there is sterility and infertility in both sexes of cattle, leading to a permanent loss of draught power and traction (Tuppurainen *et al*., 2011; Abdulqa *et al*., 2016).

The number of cattle that get sick and die from Lumpy skin disease differs a lot in different places that have it. If it started in a country that never had it before, it would likely have a high rate of cattle getting sick. If this were to become a regular occurrence, it could result in significant economic losses and decreased productivity. The deaths of numerous animals, the expenses related to vaccinations, and a decline in consumer purchases from the country would all negatively impact the economy, leading to job losses (Tuppurainen *et al*., 2011).

According to a study done in Ethiopia, the yearly financial cost was estimated to be USD 6.43 (5.12–8) per head for local zebu and USD 58 (42–73) per head for HF/crossbred cattle. This was calculated as the sum of the average production losses due to morbidity and mortality arising from milk loss, beef loss, traction power loss, and treatment and vaccination costs at the herd level (Getachew *et al*., 2011). According to another study the average cost of a single ox dying from LSD was estimated to be 9,000 Ethiopian birr (ETB), or US$477.7 (USD1 = 18.84 ETB). However, the typical price may rise to more these days (Gelagay *et al*., 2014).

2.9. Pathogenesis

Lumpy skin disease is a virus that affects cattle and is generally more severe for milking cows in the peak of lactation and young animals (Getachew *et al*., 2011). It has an incubation period of two to four weeks and causes hyperplasia and ballooning degeneration of stratum spinosum keratinocytes, development of epidermal microvesicles, and infiltration of inflammatory cells into the dermis (Limon *et al*., 2020). The virus replicates in macrophages, fibroblasts, pericytes, and endothelial cells in the lymphatics and blood vessel walls leading to developing vasculitis and lymphangitis. It also produces eosinophilic,

intracytoplasmic pox inclusion bodies in cells of epithelioid, hair follicles, and cells of muscles and skin glands (AUSVETPLAN, 2009; Victor, 2005).

When a virus enters the body, it can cause disease if it is able to replicate enough and cause harm to essential cells, either directly, or by triggering an immune response to the virus proteins. This includes planting the virus, multiplying it in the body, spreading it to other organs, and releasing it into the environment (Baron, 1996).

The incubation period of LSD under field conditions is two to four weeks (Davies *et al*., 1971). The virus can be injected into cattle through the intravenous, intradermal, or subcutaneous routes. When it comes to symptoms, it has a localized swelling at the site of inoculation after four to seven days, enlargement of the regional lymph nodes, and a generalized eruption of skin nodules seven to 19 days after inoculation. Viremia occurred after the initial febrile reaction and persisted for two weeks (Al-Salihi, 2014).

2.10. Clinical Signs

Cattle can get lumpy skin disease, an infectious and sometimes deadly condition. It causes skin nodules in the skin and other areas of the body. It can get worse from bacterial infections (Merck Veterinary Manual, 2011). Animals with LSD get a high fever that can last from 4 to 14 days. They can also become depressed, not wanting to move, lose appetite, salivate, cry, and have a mucus or pus-like nasal discharge. The lymph nodes near the shoulder, leg, and neck tend to swell too (Carn and Kitching, 1995).

The symptoms of LSD in cattle can start to show up seven days after they are infected, by day 6(six), the virus in the blood can be detected. The most common signs are fever, nodular skin lesions, swollen dewlap, and lymphadenitis (Brenner *et al*., 2009). They may also have lacrimation, nasal discharge, loss of appetite, and necrotic wounds. Nodules often show up on their legs, perineum, scrotum, and ears mostly on the back and neck (Gupta *et al*., 2021). In one study, feverish cattle were confirmed to have LSD (Endalu and Abdi, 2018). Cattle can carry the virus without showing any symptoms, which may facilitate its spread (Sprygin *et al*., 2020a).

The cattle infected with LSD cause some serious issues. Changes in the immuno-biochemical components happen, like an increase in some elements and a decrease in protein and others (Yanni *et al*., 2021). After 48 hours, nodular skin lesions erupt that cause febrile pain. These nodules show up in the head, neck, perineum, genitalia, udder, and limbs. They are 5 to 50 mm, firm, round, and raised. They can even get down to the muscles (Tuppurainen *et al*., 2013). This is causing an economic loss for sure (Yanni *et al*., 2021).

2.11. Diagnosis

Diagnosing LSD is usually made based on the common signs it causes. Symptoms like excessive tears, snot, swollen lymph nodes, soaring fever (>40.5 ^{0}C), low milk production, skin nodules, necrotic sores around the nose and muzzle, and skin issues on the legs plus secondary bacterial infections can all be seen in infected animals (Girma, 2021). However, even mild or no symptoms can still need to be tested in the lab to find out for sure. To do this, samples of tissue from the skin nodules, lungs, or lymph nodes can be taken during post-mortem within the first week of symptoms occurring before the body starts to make antibodies to fight it off (Awad *et al*., 2010).

Testing for LSD involves either identifying the virus through electron microscopy, egg inoculation, isolation in cell cultures, a fluorescent antibody test, or detecting its specific antibody through serological tests. Polymerase chain reaction tests are the most accurate and quick way to detect LSDV, and they are also more sensitive and reliable than other methods (Heine *et al*., 1999; Stram *et al*., 2008). El-Kholy *et al*. (2008) and WOAH (2010) both agreed that PCR is the best approach for diagnosing LSD.

2.11.1. Virus isolation

Capripoxviruses, such as lumpy skin disease, sheep pox, and goat pox, have a tissue tropism for epithelial cells. This means they can be propagated in a range of primary cells or cell lines from bovines, sheep, and goats, with a virus titer of up to 10^{6} $TCID_{50}$ per ml (Tuppurainen and Klement, 2018). These cells come from organs such as the kidney, testes, adrenal, thyroid, skin, and muscle (Tuppurainen *et al*., 2018). The most common cells for *Capripoxviruses* are primary lamb kidney and primary lamb testis cells (Plowright and

Witcomb, 1959). The lumpy skin disease virus can also be isolated on the Chorioallantoic Membrane (CAM) of Embryonated Chicken Eggs (ECE), where pock lesions indicate the presence of the virus (El-Bagoury *et al*., 2023).

Lumpy Skin Disease Virus is a slow-growing virus; it takes 4-6 days to see the first effects in cell cultures. The CPE includes membrane retraction from the surrounding cells and cell rounding (WOAH, 2021). Isolation is essential to study its infectivity and to develop vaccines. It also helps to retrieve the virus population (Tassew *et al*., 2018).

2.11.2. Molecular detection

It is possible to detect LSDV from nodular tissue using a standard polymerase chain reaction. A real-time PCR assay can be used to quickly and accurately confirm field isolation (Mikhael *et al*., 2023). A molecular assay for CaPV genotyping was designed with unlabeled snapback primers of dsDNA in the presence of intercalating EvaGreen dye, which detected and genotyped CaPVs in samples with 100% sensitivity and specificity. It is also less expensive because it does not need fluorescently labeled probes or high-resolution melting curve analysis software (Gelagay *et al*., 2013). There's also a PCR method that differentiates SPP vaccine strains from field isolates and other CaPVs, which has good sensitivity and specificity (Tesfaye *et al*., 2018).

Researchers came up with a way to test for the SPPV vaccines that have two deletions of 21 and 27 nucleotides within the capripoxvirus homolog of the Variola virus B22R gene. This High-Resolution Melting (HRM) assay can generate four different melting peaks, making it possible to differentiate between SPPV vaccines, SPPV field isolates, GTPV, and LSDV. It's a super sensitive, specific test that's also super cost-effective (Tesfaye, 2019).

Researchers recently created a new test using recombinase polymerase Amplification (RPA) Cas12a-fluorescence that can quickly detect Lumpy Skin Disease Virus (LSDV). They used a commonly found LSDV gene, called poly (A) polymerase small subunit (ORF068), as the target for their CRISPR/Cas-based detection method (Jiang *et al*., 2022).

2.12. Prevention and Control Strategies

The WOAH's (2017) Terrestrial Animal Health Code Chapter 11.11 has global standards for controlling and trading lumpy skin disease (Neethling virus type III). Every country has their laws in place when it comes to LSD. Early detection of the index case is key to controlling and eradicating Lumpy skin disease. Vaccination campaigns can be used to acquire immunity that can last a lifetime. Vaccinating cattle every year is a successful way to keep the disease under control (Birhanu *et al*., 2015). Live, attenuated vaccines are available commercially. Homologous and heterologous vaccines have been developed from wild-field isolates (Sprygin *et al*., 2020b). These vaccines, along with import restrictions, vector control, and quarantine stations are effective ways to control the disease. Culling infected animals can also be an option (Gumbe, 2018). Substantial vaccinations with safe and effective vaccines are the most effective method of controlling LSD (Hamdi *et al*., 2020).

Researchers compared the effectiveness of killing all clinically affected cattle and their herd mates (total stamping-out) versus killing just the sick cattle (partial stamping-out) using math modeling. The results showed that both methods had similar chances of wiping out the infection. They also said how important it is to start vaccinating before the virus enters (WOAH, 2017). There are four vaccines used worldwide against LSDV, and they all have common neutralizing sites, so one can protect another host from the disease (Gelagay *et al*., 2014). They often use the Kenyan KSGP 0240 strain for vaccinating ruminants, but it's not great. Experiments have shown that an LSDV strain can protect cattle from LSDV, so they should use similar strains for the vaccine (Hamdi *et al*., 2020). All the current vaccines are live attenuated viruses (Tuppurainen *et al*., 2021).

A perfect vaccine should offer wide protection against contagious types to stop potential spread, be able to tell the difference between infected and vaccinated animals, not mix with field types, give a powerful, long-lasting immune response, and be low-cost to make and administer (Trinh *et al*., 2022). Vaccinating animals against LSD with homologous vaccines is the most effective method of control. Still, data suggests that the LSD virus is still in some

areas of Europe and that unprotected cattle are still in danger, even in places with a good level of vaccine coverage (Calistri *et al*., 2019).

Trying to stop the spread of LSD and vaccinate against it, limiting animal movement, and taking out those who are sick or exposed can help. But it takes a lot of money, people, and resources, plus good information systems, and Ethiopia hasn't had enough to do all that. So for a while now, vaccination has been the main way to manage LSD (Gelagay *et al*., 2014)

2.13. Status of Lumpy Skin Disease in Ethiopia

Ethiopia first detected Lumpy Skin Disease between 1981 and 1983 in the northwestern, western, and central regions of the country (Yoseph *et al*., 1984). After its first appearance, an explosive sudden and uncontrolled epidemic spread from the north through the central to the southern part of the country. In the subsequent three to five years, it had easily covered the vast area of the highland and midland parts of Ethiopia (Nebyou *et al*., 2023).

Data investigations from the national disease outbreak report database during the period 2000-2009 showed that major epidemic outbreaks of LSD occurred in 2000/2001 in the northern parts of the country in Amhara and West Oromia regions. 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 up to 2009 the outbreak number progressively increased in the Oromia Region situated in the central part of the country while it seemed to be gradually decreasing in the northern part of the country including Tigray, Amhara, and Benishangul regions. The national disease outbreak report during these 10 years showed that LSD has spread virtually to all the regions in the country and different agro-climatic zones (Getachew *et al*., 2011; Nebyou *et al*., 2023).

A cross-sectional study in Ethiopia found 8.1% animal-level prevalence (Getachow, *et al*., 2010) and 2.12% mortality for LSD (Mesay, 2018). The disease remains a major concern due to the lack of a structured control plan. Vaccines used in Ethiopia have shown limited effectiveness, even during outbreak response campaigns (Wassei *et al*., 2017).

Table 1. Summary of the disease prevalence in Ethiopia.

Study Area	Sample Type	Diagnostic Technique Used	Prevalence	Reference
East Wollega (Nekemte)	-	Questionnaire Survey	7.02%	Reggasa, 2003
Ethiopia	-	Questionnaire Survey Retrospective Data	8.1%	Getachow *et al*., 2010
Central Ethiopia(Bishoftu)	Skin Nodules	Questionnaire Survey, Isolation and Molecular Techniques	22.9%	Gelagay *et al*.,2013
Borena Zone	Clinical Signs	Questionnaire Survey	6.1%	Gezahegn *et al*., 2013
North Eastern Ethiopia	-	Questionnaire Survey	7.4%	Birhanu *et al* 2014
Ethiopia	Skin Nodules	Retrospective Data	13.61%	Gelagay *et al*., 2014
Ethiopia	Skin Nodules	Questionnaire Survey Isolation RT-PCR Gene Sequencing		Easayas *et al*., 2015
Eastern Wollega zone	Clinical Signs	Questionnaire Survey	17.91%	Fedhessa *et al*., 2015
Ethiopia(at district level)	-	Outbreak Report Data	5.58% / 16 years	Wassie *et al*.,2017
Western Amhara	Blood	Retrospective Data VNT	14.9%	Tesfa,2017
Kombolcha And Dessie	Skin Nodules	Questionnaire Survey, Isolation and Molecular Techniques	7.22%	Yasin *et al*.,2017
South Wollo Zone	Skin Lesions	Questionnaire Survey Isolation and Molecular Detection	15.71%	Bethlehem,2018
Central Ethiopia	Skin Lesion	RPO30 Gene Sequence Isolation PCR	8.77%	Mesay,2018
Ethiopia	Clinical Signs	Questionnaire Survey	21.1%	Wassie, 2018
Ethiopia	Blood	Questionnaire Survey VNT	25.4%	Wassie *et al*.,2018
East Hararghe and East Shoa Zone	Skin Nodules	Questionnaire Survey, Isolation and Molecular Techniques	5.69%	Asmelash *et al*., 2018
Central Ethiopia	Skin Nodules	Isolation, Molecular charact.	12.2%	Girma *et al*.,2019

Continued

Bale Zone(Sawena District)	Skin Nodules Nasal Swabs	Questionnaire Survey, Isolation and Molecular Techniques	18%	Shubisa *et al*., 2021
Illubabor Zone	Skin Lesions	Questionnaire Survey Isolation and Molecular Detection	15.49%	Chala *et al*.,2022
Wolayita Zone	Skin Tissue	RT- PCR and Histopathological Techniques	36.2%	Mesfin *et al*.,2022
Central Ethiopia	Skin Nodules	Isolation Molecular Techniques RPO30 Gene Sequencing Analysis VNT	7.8%	Mihiret, 2022
Sidama Regional State	Blood	Questionnaire Survey VNT	40.8%	Nebyou *et al*.,2024
West Hararghe Zone	Skin Nodules	Molecular Detection Isolation	11.68%	Umer *et al*., 2024
Northwest Oromia	Skin Lesion	Questionnaire Isolation and Molecular Detection	6.5%	Workisa *et al*.,2024

3. MATERIALS AND METHODS

3.1. Study Area

The study was conducted in three districts of Bale Zone Administration of Oromia regional state, located approximately 430 km southeast of the capital city, Addis Ababa. These were Agarfa, Goba and Sinana. Agarfa woreda is found at approximate latitude of 7.06° N and longitude of 39.83° E, with an altitude ranging from about 2,000 to 3,500 meters above sea level. Goba woreda lies at approximately 7.0° N latitude and 39.98° E longitude with elevations between 2,400 and 4,377 meters that include some of the high peaks of the Bale Mountains. Sinana woreda is also found approximately at latitude of 7.12° N and a longitude of 40.21° E, with altitudes mostly varying from 2,500 to 3,700 meters above sea level. The study area was purposely selected for the study based on the LSD outbreak report from Agarfa, Goba, and Sinana district/woreda to the Zone Animal Health Team and Asella Veterinary Regional Laboratory through DOVAR (Date of Verification of the Animal Report). The temperature in the Agarfa and Goba districts ranges between 4°C and 25°C, while rainfall ranges from 400 mm to 1400 mm according to elevation (Feyissa and Gebbisa 2021; GDSEP 2015). On the other hand, the temperature of Sinana Woreda annually ranges between 15–18°C, with a rainfall value of 900–1150 mm, while the maximum temperature range is 25°C and the minimum temperature range is 10°C (Addisu *et al*., 2015). According to a CSA (2021) Bale zone between 2019 and 2021, there had 961,215 cattle. The study area showed by map according to figure 5.

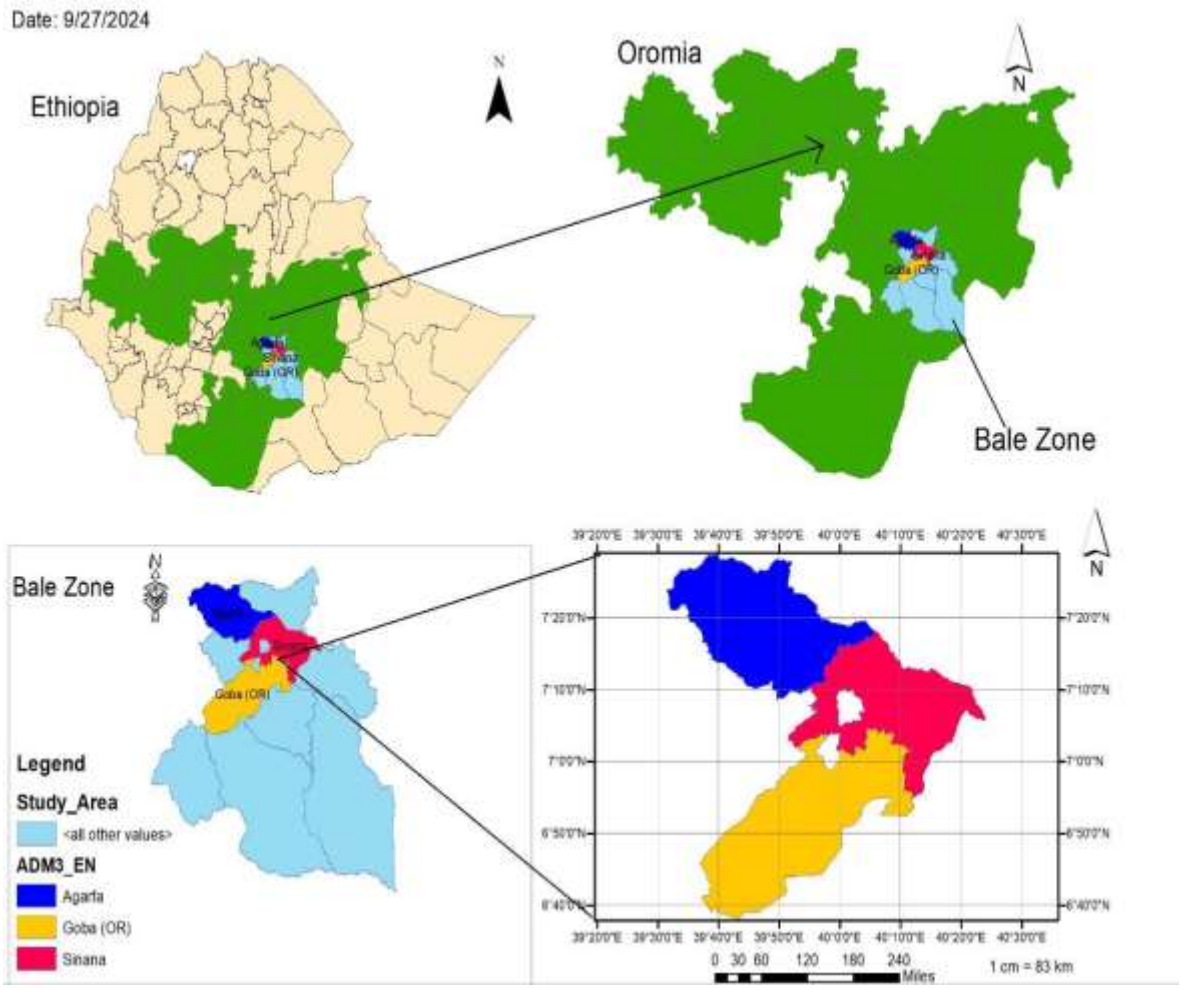

Figure 5. Map of Bale Zone of Oromia Regional State.

3.2. Study Population and Animals

The study population was cattle found in the three woredas of the Bale Zone of Oromia Regional State of Ethiopia. The study animals were cattle of all age groups and both sexes that showed signs and symptoms of LSD, such as fever (41°C), swollen lymph nodes, and skin nodules that were firm and measured up to 5 cm in diameter.

3.3. Study Design and Sampling Strategy

A cross-sectional study was used to isolate and conduct molecular detection of LSDV from January 2024 to July 2024. Active outbreaks were assessed together with veterinary professionals who have been working in the district veterinary clinics. For sampling,

unvaccinated cattle of all age groups, breeds, both sexes, all herd sizes, and animal production systems were considered.

A thorough physical examination was conducted on sick animals before sample collection. Samples were taken from animals exhibiting clinical signs typical of Lumpy Skin Disease (LSD). Tissue samples (skin nodules), and nasal and saliva swabs from visibly affected cases were collected for analysis. Additionally, animal owners were randomly selected for interviews.

3.4. Sample Size Determination

To determine the sample size for a study on Lumpy Skin Disease (LSD) in the Bale Zone, Ethiopia, based on a previous prevalence of 18% (from the Sawena District, now part of East Bale Zone), according to Thrusfield's formula for cross-sectional studies (Thrusfield, 2018);

$$n = \frac{Z^2 * p(1-p)}{d^2} = \frac{(1.96^2) * 0.18(1-0.18)}{(0.05)^2}$$

$$= \frac{0.567}{0.0025}$$

$$= 226.8 \sim 227$$

n = required sample size

Z = Z-value for the desired confidence level (1.96 for 95% confidence)

P = expected prevalence (18% or 0.18, based on previous data from Sawena District, now part of East Bale Zone)

d = desired absolute precision (5% or 0.05)

3.5. Sample Collection and Transportation

Samples were collected according to the WOAH terrestrial manual (2017) after an animal was safely restrained. The skin biopsy samples from cutaneous nodules from each representative cattle were taken aseptically after washing and cleaning the area with 70% alcohol, and then the skin nodules were taken by sterile scalpel blade. A sterile nasal swab

was collected by inserting swab into one nostril and rotating for 5-10 seconds, while a new sterile swab for saliva was placed under the tongue for 30 seconds. About 2–3 g of tissue samples were collected, and placed in a bottle with a 50% phosphate buffer saline (PBS) with glycerol enriched at a PH of 7.2–7.6 with Anti-Anti (Antibiotic-Antimycotic). Then samples were labeled, placed in a cold box, and immediately transported to the Animal Health Institute maintaining a cold chain system. Then, the tissue samples were stored at -20 ^{0}C until processing.

3.6. Questionnaire Survey

Cattle owners were randomly selected and interviewed using a semi-structured questionnaire to reveal information regarding cattle size, health status, grazing management, introduction of new animals, mixing different species of livestock, clinical signs of disease encountered, frequency of LSD outbreaks, number of diseased and dead animals and the knowledge of the herders of cattle on vaccination and the effect of animal movements on disease spread according to Appendix 2. The number of participants for the knowledge assessment was calculated using the formula described by Arsham (2020). By assuming the standard error of 5%, precision level of 0.05, confidence interval of 95%.

$$N = \frac{0.25}{SE^2} = \frac{0.25}{(0.05)^2} = 100$$

3.7. Laboratory Investigation

3.7.1. Preparation of glassware

For cultivation and maintenance of the Vero cell line, glassware, reagents, and media were prepared and sterilized according to standard operating procedures (Smith and Jones, 2020). The used glasswares were dipped in surf detergent, brushed thoroughly washed in running tap water for 20 minutes, and washed ten times with deionized water. The washed glassware was kept inverted on a clean surface table top to drain out the water content and dry. The glassware was wrapped in wrapping papers and aluminum foils. All the glassware including fresh ones was placed in a hot air oven at 180 ^{0}C for 30 min (Berhanu, 2006).

3.7.2. Sample processing

For skin nodules a sterile scalpel blade and forceps were used to mince the lesion material for virus isolation. Next, macerate the mixture in a sterile steel ball-bearing mixer mill or grind the material with a pestle in a sterile mortar with sterile sand and an equal volume of sterile phosphate-buffered saline (PBS, pH 7.2) or serum-free modified Eagle's medium (DMEM) Lot number 2672044 with Anti-Anti 100x (Antibiotic-Antimycotic 3%) Lot number 2585892 (source: gibco, UK) in the Biosafety Cabinet Level 2 (BIOBAN 130). The preparation of nasal and saliva swabs for isolating the Lumpy Skin Disease Virus (LSDV) involves a series of carefully executed steps to ensure the integrity and viability of the viral sample. The swab was placed in a sterile tube containing 1-2 ml of phosphate-buffered saline (PBS) or culture medium for both types of sample. The tube was then vortexed vigorously for 30 seconds to release the virus from the swab into the solution. This step was essential for maximizing the recovery of viral particles, ensuring that the sample was suitable for further processing (Tuppurainen *et al*., 2017). After three freeze-thaw cycles, the suspension was centrifuged for ten minutes at 600xg in a bench centrifuge. When a virus was isolated from skin samples, nasal, and saliva swabs or when bacterial contamination of the sample was anticipated, the supernatant was filtered through a 0.45 µm filter pore size filter during the process of centrifugation according to appendix 3 (WOAH, 2023).

3.7.3. Virus isolation

Vero (African green monkey cells) cell line (P-40 source: CIRAD, France) was used for isolation of the samples for LSDV. Thus, the work was started from the sub-culturing of this cell. Sub culturing was performed under aseptic conditions in laminar airflow and two cell cultures for each sample were used. Vero cell culture was prepared on $25cm^2$ tissue culture flasks appendix 4. Another cell culture was kept as negative control (Binepal *et al*., 2001).

The virus isolation procedure was performed according to the WOAH terrestrial manual 2017, chapter 2.4.13 appendix 5. Briefly, about 0.5 ml of the processed suspension samples were inoculated onto a confluent layer of Vero cell in $25cm^2$ tissue culture flasks, and the cultures were incubated at 37^0C and allowed to adsorb for 1 hour. The culture was then washed with warm PBS and covered with 10ml of Glasgow Eagle’s minimal essential

medium (GMEM) Lot number 2672044 containing Antibiotic-Antimycotic 3% and 2% fetal calf serum (source: gibco, UK). All the flasks, including the control flasks, were incubated at 37°C in a humidified incubator with 5% CO2. The culture medium was replaced with a fresh medium every 48 hr or when it became acidic. The inoculated cell cultures were examined daily for evidence of cytopathic effects (CPE) (such as cell rounding, detachment, and the formation of cell aggregations) microscopically and then the virus was passed three times.

The virus was passed three times for those samples that showed no or slight CPE. A numerical system of scoring when reading the cell cultures for cytopathic changes was adopted. The severity of LSDV effect on Vero cell was scored on a scale from 0 to 4. At one end of the scale, 0 represents a normal culture without CPE, while at the other extreme; a completely degenerated culture is scored 4+ (Mesay 2018; Ahmad *et al*., 2020).

3.8. Molecular Detection

3.8.1. DNA extraction

DNA was extracted from skin nodules, nasal and saliva swabs by Qiagen kit (from Germany), according to the manufacturer's instructions (QIAamp D. 2012) according to Appendix 6. DNA extracts were kept at -20°C before proceed to amplification by real-time PCR.

3.8.2. PCR techniques

The field isolates of LSDV collected from sick animals and adopted on Vero cell lines were tested by polymerase chain reaction (PCR) as described by WOAH (2010).

3.8.2.1. Real-time PCR amplification

The detection of LSDV was accomplished through the use of Reverse and Forward *Capripoxviruses* specific primers, which have the following sequences:
Forward Primer- 5′TATGGATTTAGGAGTAGA3′ and
Reverse Primer- 5′GCTTTACTTTAATATCATTG 3′.

The RT-PCR amplification (from APPLIED BIOSYSTEMS which is 7500 Fast System V1.4.0) was carried out in a 20 µl reaction system, utilizing 18 µl of the prepared master mix and 2 µl of extracted sample nucleic acid or template controls. The PCR Master Mix (Evagreen Super Mix® from Bio-Rad with Lot number L000915) consists of 10µl, 2µl of forward Primer (CP-HRM SbF), 2 µl of reverse primer (CP-HRM SbR), and 4 µl RNase-free H_2O (USA product with Lot number 1807287). Utilizing the subsequent amplification protocol, the polymerase chain reaction was conducted: Using MicroAmp®Optical 96-Well Reaction Plate (applied biosystem) by life technologies from China. The PCR reaction ran initial denaturation at 95°C for 3 minutes. Then 45 cycles at 95°C for 15 seconds, 58°C for 80 seconds and 72 °C for 30 seconds. After PCR products denatured the last cycles at 95°C for 1 minute and cooled to 45°C for 1 minute, 65°C for 15 seconds, and 60°C for 15 seconds for melting curve analysis. Positive samples were identified using cycle threshold (Ct) values, melting curves (73°C), and fluorescence curves. A Ct value greater than 40 or less than 0 indicated no virus in samples (Esayas *et al*., 2013). Higher Ct values correlated with lower LSDV DNA quantities, while lower Ct values indicated higher LSDV DNA levels (Parvin *et al*., 2022).

3.9. Data Management and Analysis

Data from sample collections and laboratory investigations were coded, stored in Microsoft Excel 2010, and screened before analysis. Descriptive statistics summarized field survey and laboratory data. Morbidity, mortality, and case fatality rates were calculated as: morbidity = (sick cattle/total at-risk) × 100; mortality = (deaths/total at-risk) × 100; case fatality = (deaths/affected animals) × 100. Chi-square (χ^2) tested associations between variables such as vaccination, herd mixing, grazing/watering points, and breeds and history of LSDV occurrence at $P \leq 0.05$. Multivariable logistic regression analyzed LSDV history with covariates (district, herd size, grazing/watering points, new animals, herd mixing, vaccination) that had $P < 0.25$ in univariable analysis.

4. RESULTS

4.1. Clinical Signs Observed

The most frequently observed clinical signs during the LSD outbreak investigation in the study area were skin lesions (skin nodules), swelling of the limbs and dewlap, enlarged lymph nodes, lameness, and fever. Less common signs included excessive salivation, lesions on mucous membranes, respiratory distress, and discharge from the nose and eyes. Out of 227 cattle that were inspected, 13 of them showed signs of pyrexia, salivation, nasal discharge, swelling of the limbs, and nodular lesions on the skin on the different parts of the body according to figure (6, 7, 8). In the study area, there were 23.08% case fatality rates, 1.32% mortality rates, and 5.7% morbidity rates related to the LSD outbreak, as per the findings. According to this study, the mortality rates, morbidity rate, and case fatality rates were relatively lower in the Sinana district, while relatively higher mortality and morbidity rates were recorded in the Goba, and Agarfa districts, respectively according to Table 2.

Table 2. Morbidity, mortality, and case fatality rate of affected cattle.

Districts	**№ of Cattle at Risk**	**№ of Affected Cattle**	**№ of Cattle Died**	**Morbidity Rate (%)**	**Mortality Rate (%)**	**Case Fatality Rate (%)**
Agarfa	105	7	1	6.67	0.95	14.3
Goba	74	4	2	5.4	2.7	50
Sinana	48	2	0	4.17	0	0
Total	227	13	3	5.7	1.32	23.08

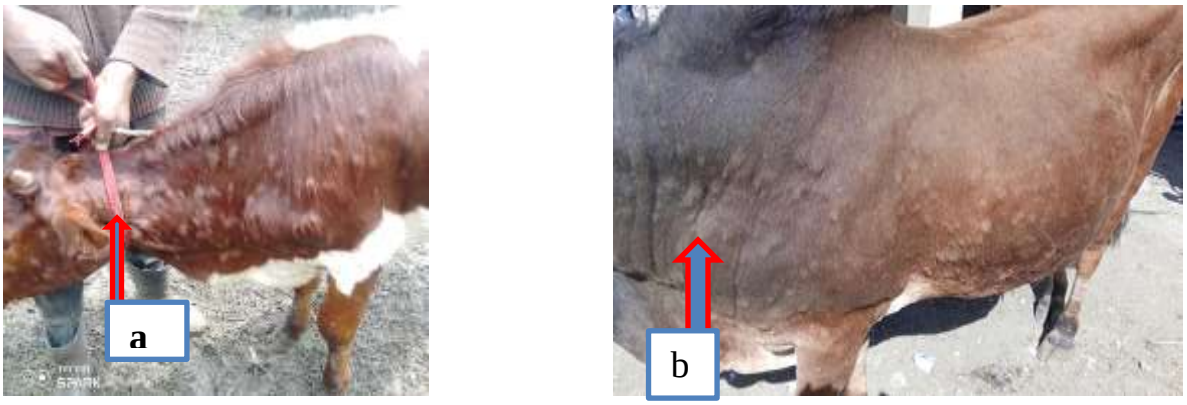

Figure 6. Skin nodules; a) found on the neck b) Skin Nodules found over the body

Figure 7. Lacrimation

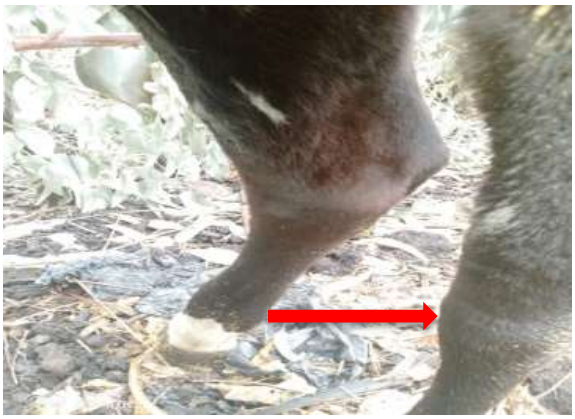

Figure 8. Swelling of hind limbs

4.2. Virus Isolation

All 13 of the samples that were inoculated to the Vero cell line (P-40) able to cause CPE, as evidenced by the rounding of individual cells, the destruction of monolayers, and the formation of cell aggregations according to figure (11,12). Six days after inoculation, the first cytopathic effect (CPE) was observed during the second passage. The virus-induced CPE was observed in the inoculated samples until day 14 and all infected Vero cells were shown CPE. After three freeze-thaw cycles, the negative control samples exhibited no CPE. Following the second or third passage, the negative control sample exhibited no CPE on the cell according to Figure 10. The scoring system used to assess damage in Vero cell cultures infected with LSDV ranges from 0 to 4. A score of ’0’ indicated no damage (cells are healthy) while scores of ’1’ to ’3’ signified escalating levels of infection severity, and a score of ’4’ indicated total cell degeneration. The majority of samples and the positive control exhibited a score of ’4’ indicating CPE presence; in contrast, to this result, the control showed no signs of CPE development solidifying that uninfected cells remain undamaged according to Table 3.

Table 3. The CPE level Score.

Sample ID	Score				
	0	**1**	**2**	**3**	**4**
8486	-	-	-	-	++++
8487	-	-	++	-	-
8488	-	-	-	-	++++
8489	-	-	-	+++	-
8490	-	-	-	-	++++
8491	-	-	-	+++	-
8492	-	-	-	-	++++
8493	-	-	-	-	++++
8494	-	-	-	-	++++
8495	-	-	-	+++	-
8496	-	-	-	-	++++
8497	-	-	-	-	++++
8498	-	-	-	+++	-
Pos. cntrl	-	-	-	-	++++
Neg. cntrl-1	-	-	-	-	-

'++++' for completely degenerative cell, '+++' for 50-75% CPE, '++' 10% of infection CPE, and '-'for culture without CPE.

Figure 9. Growing Vero cell after 18 hrs

Figure 10. Negative control LSDV

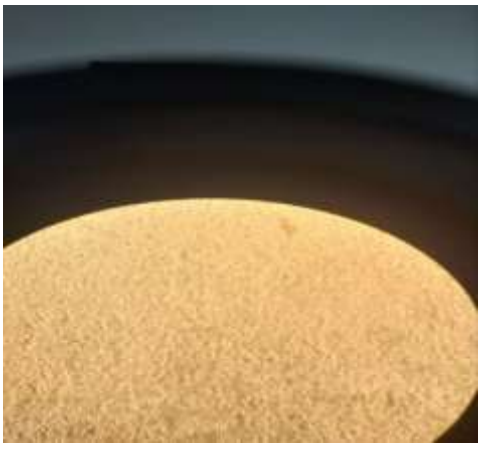

Figure 11. CPE of LSDV on Vero cells

Figure 12. Highly affected Vero cell by LSDV

4.3. Molecular Detection of LSDV

Lumpy Skin disease virus DNA was found in all 10 tissue samples, 1 nasal swab, and 2 saliva swabs when real-time PCR used. The increase in DNA amplification over PCR cycles, as shown by the rise in fluorescence signal (Delta Rn) for each sample is illustrated in figure 13. As PCR products were heated, a single melting temperature (Tm) peak at 73.9°C confirms the amplification of a specific DNA sequence. The presence of a single, well-defined peak further indicated product specificity as illustrated by figure 14.

The melting characteristics of PCR products, showed a prominent peak around 73.9°C, characteristic of the amplified LSDV DNA as seen in Figure 15. A tall red peak likely represented positive samples, while smaller or absent peaks suggested negative or non-specific amplification. In the amplification curve, a steep rise in fluorescence around cycle 20 in positive samples confirms successful LSDV DNA amplification as illustrated by Figure 16. Cycle threshold (Ct) values for positive samples ranged from 17.25 to 34.72, indicating high DNA levels and confirming all samples as positive for LSDV. These values, higher than the positive control's Ct of 15.43, suggested substantial virus concentrations, while negative samples had Ct values above 40 or were undetectable. The melting curves, closely matching known LSDV controls, further confirmed the presence of LSDV DNA in the samples according to Table 4.

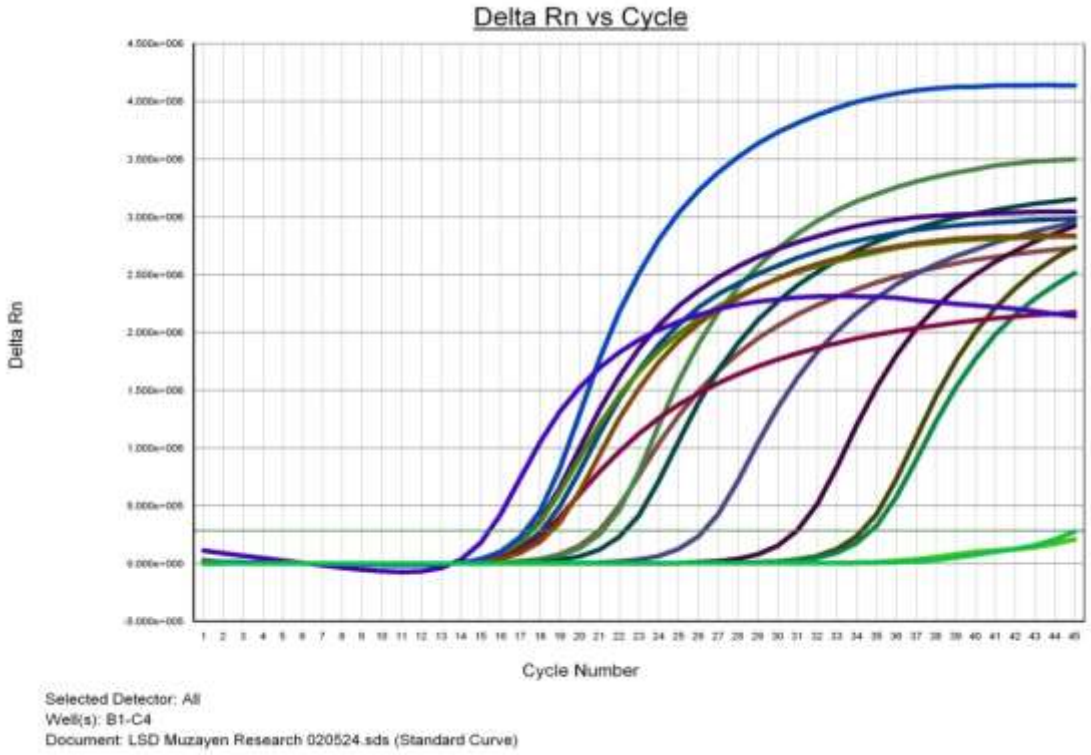

Figure 13. Amplification Plot of LSDV

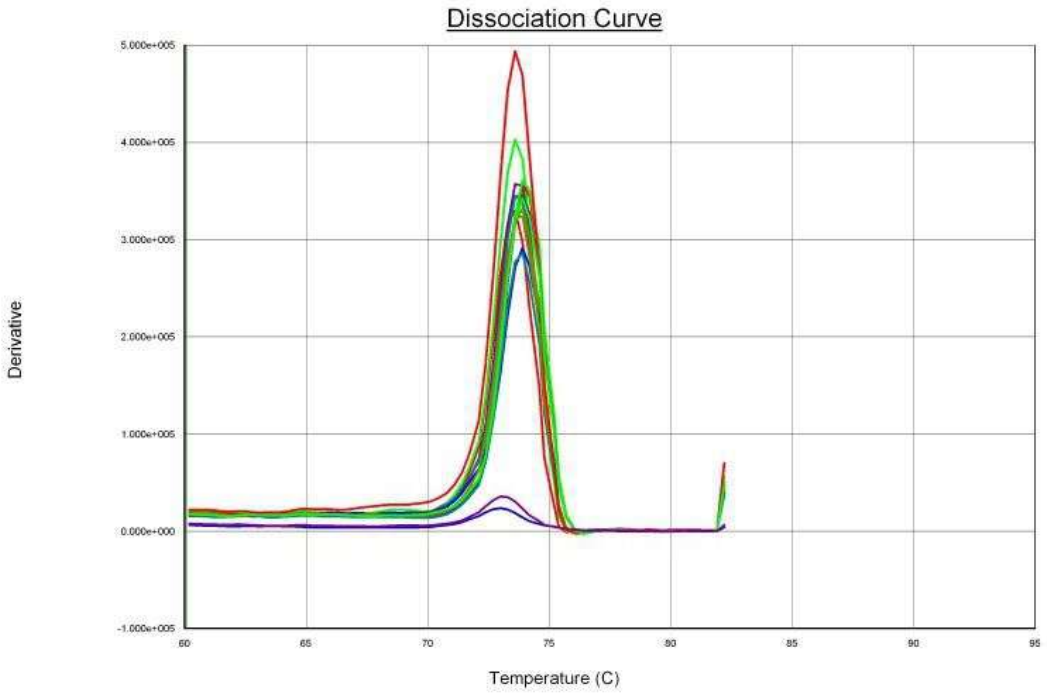

Figure 14. Dissociation curve (Wells: B1-C4)

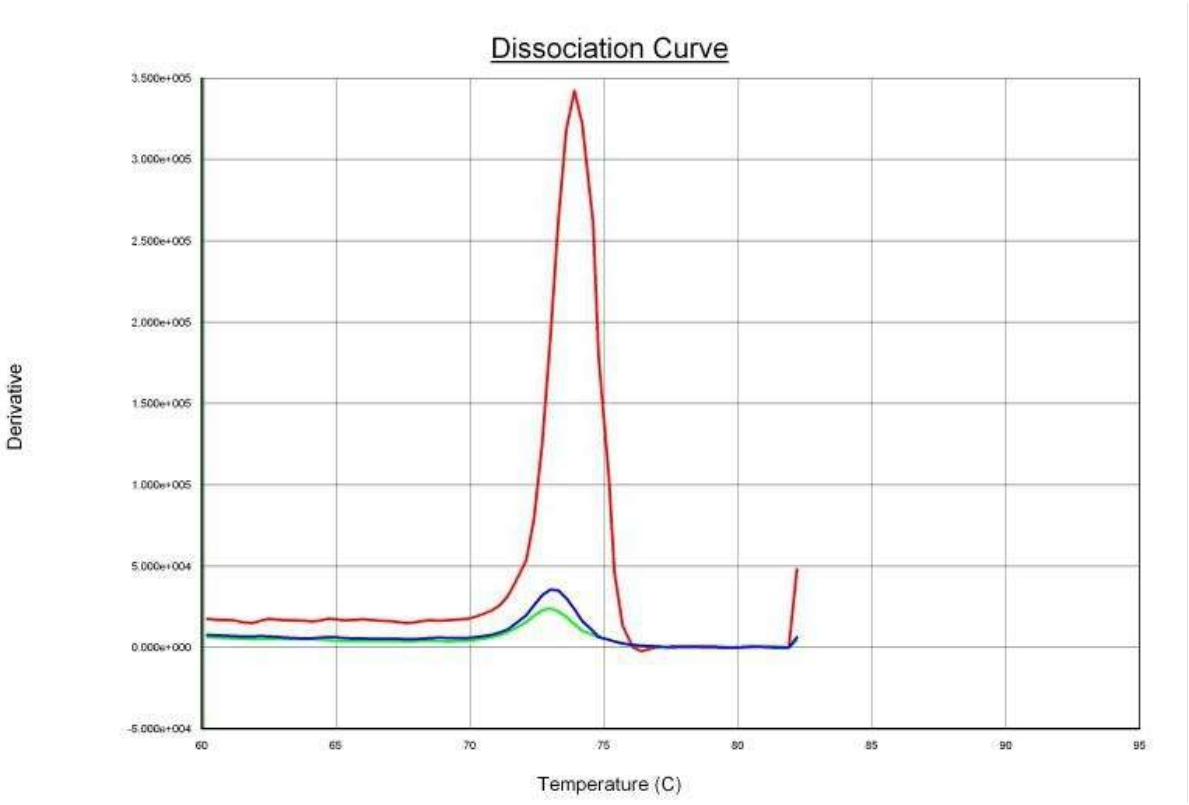

Figure 15. Positive and Negative Dissociation Curve (Wells: C2-C4).

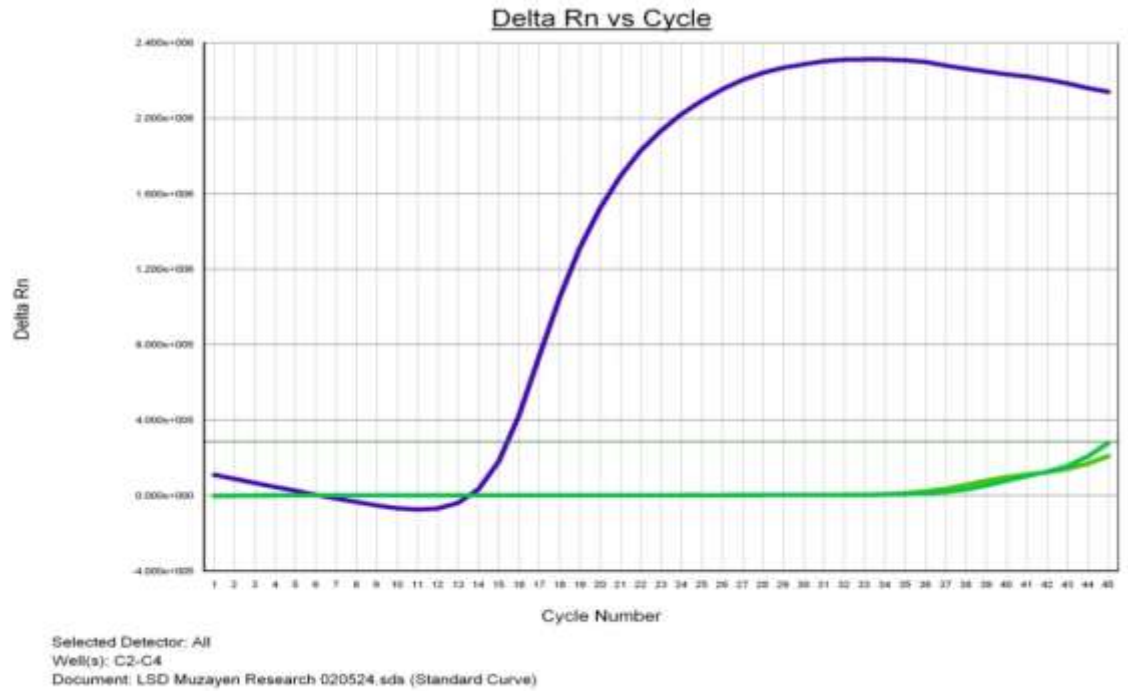

Figure 16. Positive plot of LSDV (Wells: C2-C4).

Table 4. Real-time PCR Ct Values and Tm (°C) of the Collected Samples.

Well	Sample ID	Ct Value	Tm(^{o}c)
B1	8486	21.0032	73.6
B2	8487	21.158	73.6
B3	8488	26.2617	73.6
B4	8489	22.2983	73.6
B5	8490	30.9976	73.9
B6	8491	34.2634	73.9
B7	8492	18.0782	73.9
B8	8493	17.5377	73.9
B9	8494	34.7187	73.9
B10	8495	17.5978	73.9
B11	8496	18.3286	73.9
B12	8497	18.5885	73.9
C1	8498	17.2538	73.6
C2	Pos.cntrl	15.4323	73.9
C3	Neg.cntrl -1	Undet.	73.0
C4	Neg.cntrl-2	Undet.	73.0

Pos. Cntrl for positive Control, Neg. Cntrl for negative control, Undet. for undetected

4.4. Risk Factors and History of LSD Occurrence

According to herders, the local names for LSD were reported as follows: 44% identified it as "Doolaa Loonii," 8% as "Dhitessa," while others were unsure. Among 100 respondents surveyed about LSD, 58% indicated that the disease frequently occurs every year after the rainy season during the dry months. In this study various risk factors that might play a role in history of lumpy skin disease occurrence such as vaccination status, introduction of new animals, watering point, grazing point, mixing herds with neighbor herds and breed were considered.

The statistical analysis revealed there was a very strong association between vaccination practice and history of LSDV occurrence with a Chi-Square Value: of 21.423 and a p-value was =0.001. The introduction of new animals was significantly associated with the history of LSDV occurrence with a Chi-Square Value: of 19.268 and a p-value was =0.001. There was a highly significant association between the watering point and history of LSDV occurrence with a Chi-Square Value: of 17.242 and a p-value was =0.001. There was a statistically significant association between the grazing point and history of LSDV occurrence, with a

Chi-Square Value: of 6.357 and a p-value was 0.012, indicating that the type of grazing point was linked to the history of the disease according to Table 5.

The logistic regression analysis indicated that lack of vaccination practice strong associated with (AOR = 7.467, 95% CI: 2.191–25.440, p = 0.001) the history of LSD occurrence. Unvaccinated herds being highly exposed to Lumpy skin diseases, highlighting the importance of robust vaccination programs. Introducing new animals into herds significantly increased risk (AOR = 6.146, 95% CI: 1.873–20.165, p = 0.003), as they may carry pathogens from different regions. Similarly, the use of protectable ponds as water sources posed a significant risk (AOR = 6.394, 95% CI: 1.239–32.988, p = 0.027), particularly in communal settings. Other factors were not statistically significant in the multivariable indicated in Table 6.

In summary, vaccination practice, introduction of new animals, and certain watering points were the most influential factors, significantly associated with the history of the LSD occurrence. The presence of new animals increased the risk, while vaccination substantially lowered it. Other variables, such as specific watering points (Protectable pond), showed potential effects. Whereas, districts, mixing herds with others, and grazing point were not statistically significant predictors in this model.

Table 5. Potential risk factors associated for history of LSD occurrence.

Factors	**History LSD Occurrence (Yes)**	**History LSD Occurrence (No)**	**Total**	**Chi-Square (χ^2)**	**P- value**
Mixing Herds with Others	Yes: 24, No: 18	Yes: 18, No: 40	100	6.816	0.009*
Vaccination History	Yes: 32, No: 10	Yes: 17, No: 41	100	21.423	0.001*
Introduction of New Animals	Yes: 16, No: 26	Yes: 47, No: 11	100	19.268	0.001*
Watering Point	river: 12, potable water: 23, protectable pond: 7	river: 31, potable water: 9, protectable pond:18	100	17.242	0.001*
Grazing Point	communal: 20, private: 22	communal: 42, private: 16	100	6.357	0.012*
Breeding	Local:24 Cross:18	Local:18 Cross:40	100	1.066	0.302

N.B. * significant at $p<0.05$

Table 6. Logistic regression summary: Risk factors for the history of LSD occurrence.

History of LSD Occurrence	**Univariable**			**Multivariable**		
	P-Value	**C. Odd Ratio (COR)**	**95% CI**	**P-Value**	**A. Odd Ratio (AOR)**	**95% CI**
District						
(Goba)	0.089	2.352	0.878- 6.297	0.188	3.235	0.564-8.555
(Sinana)	0.318	1.644	0.620-4.360	0.344	2.137	0.443-10.314
Herd Size	0.186	1.741	0.764-3.968	0.122	2.731	0.765-9.746
Grazing Point						
(Communal)	0.013*	2.887	1.252- 6.659	0.734	1.338	0.248-7.207
Watering Point						
(River)	0.0001*	6.60	2.383-18.285	0.161	3.039	0.643-14.354
(protectable pond)	0.002*	6.571	2.050-21.056	0.027*	6.394	1.239-32.988
Mixing Herds with Others						
(Yes)	0.010*	2.962	1.296-6.771	0.270	2.346	0.516-10.660
Vaccination Practice						
(No)	0.0001*	7.717	3.113-19.128	0.001*	7.467	2.191-25.440
Introduction of New Animals						
(Yes)	0.000*	6.943	2.809-17.160	0.003*	6.146	1.873-20.165
Cons	0.147	2.263	0.805-6.454	0.001	0.006	0.000- 0.112

N.B. * significant at $p<0.05$, Cons for constant, COR for crude odd ratio, AOR for Adjusted odd ratio, CI for Confidence interval

5. DISCUSSIONS

The current study attempts to detect a lumpy skin disease outbreak in Bale Zone of Ethiopia, using clinical diagnosis, PCR, and virus isolation. The application of different diagnostic methods helps to quickly and accurately detect the disease in the samples studied, which is important for detecting early and controlling it. The existing knowledge provided a strong background to study the LSD occurrence in the study area. The current study expanded knowledge of LSD epidemiology as has been previously examined by several authors on LSD outbreaks (Chala *et al*.; 2022; Mihiret 2022). It also agreed with the works of Mesfin (2022) and Umar *et al*., (2024) focused on molecular detection but in different areas of study. This study was in agreement with Mesay (2018), Workisa *et al*. (2024), and Shubisa *et al*., (2021, who emphasized virus isolation and characterization.

The lumpy skin disease outbreaks occurred in Bale Zone in early January 2024. The signs shown by the infected cattle in this study area, like nodules on the skin, necrotic nodules, swollen lymph nodes, nasal, saliva, and lacrimation, which is consistent with previous studies conducted in different parts of the country (Esayas *et al*., 2015; Girma *et al*., 2019; Workisa *et al*., 2024). The severity of the disease can be influenced by factors like the virulence of the virus strain, physiological status, secondary infections, and route and dose of infection (AUSVETPLAN, 2009).

The results of the current study indicated a morbidity rate of 5.7% and a mortality rate of 1.32%. When compared to findings from other regions, this morbidity rate was lower than several reports from Ethiopia, including 7.02% (Regassa, 2003), 8.1% (Getachow *et al*., 2010), 7.4% (Birhanu *et al*., 2014), 7.22% (Yasin *et al*., 2017), 8.77% by Mesay, (2018) from the farms in central Ethiopia, 11.68% in west Hararghe, Eastern Ethiopia by Umer *et al*. (2024) from the herds, 13.61% in Central Ethiopia by Gelagey *et al*. (2014) from the feedlots, 18% in Bale Zone by Shubisa *et al*. (2021) from the herds, 36.2% (Mesfin *et al*., 2022) in the Wollaita Zone, and from abroad 8.7% in Greece by Tasioudi *et al*. (2016), 17.9% in Northwestern Iran by Yousefi *et al*. (2017), 26% in Egypt by Abutarbush *et al*. (2015). The lower morbidity rate in the study area likely reflects a combination of favorable

environmental conditions, stronger animal immunity, and effective management practices that reduce LSD transmission and severity.

The morbidity rate aligned more closely with some lower rates reported from other areas, such as 5.69% in East Hararghe and East Shoa Zone (Asmelash *et al*., 2018), 5.9% in Mongolia (Odonchimeg *et al*., 2022), 6.1% in Borena (Gezahegn *et al*., 2013) from the feedlots, 6.5% in North West Oromia (Workisa *et al*., 2024), 6% in Kingdom of Saudi Arabia by Kasem *et al*. (2017), and 6.5% in Azerbaijan by Zeynalova *et al*. (2016). For instance, Tuppurainen and Klement, (2018) indicated a morbidity rate spectrum of 3-85% and 1-5% is usually observed. The morbidity rate in the study area reflects positively on management strategies and control measures implemented.

The mortality rate of 1.32% reported in this study was consistent with slightly lower mortality figures documented in the different areas, such as 1.8% in Borena, 2.12% in central Ethiopia, and 1.92% in West Hararghe (Gezahegn *et al*., 2013; Mesay, 2018; Umer *et al*., 2024), respectively. Even though the mortality rate in the study area was smaller than others, it is closely aligned with the 1.34% mortality rate reported in Sawena district of Bale Zone (now it's part of East Bale Zone) by Shubisa *et al*. (2021) and 1.4% in Illubabor Zone by Chala *et al*. (2022). The mortality in the current study area was slightly higher than the 0.34% reported from East Hararghe and East Shoa by Asmelash *et al*. (2018) and 0.50% mortality rate revealed by Workisa *et al*. (2024). This consistency underscores the success of preventive measures, early treatment, and overall veterinary care, contributing to reduced mortality across comparable environments.

The case fatality rates observed in the present study area is 23.08% which slightly align with higher rates reported by Gelagay *et al*. (2014) and Gezahegn *et al*. (2013), which indicated rates of 36.49% and 30%, respectively. This finding raises questions about the factors contributing to higher case fatality rates in specific districts, such as Goba, which exhibited an alarming 50% case fatality rate despite having a morbidity rate of only 5.4%. In high-altitude or colder regions, variations in cattle immune response can lead to higher mortality rates (Getachow *et al*., 2010). Poor vaccination coverage exacerbates disease severity, often resulting in higher case fatality rates (Girma *et al*., 2019). Additionally, limited resources for

veterinary care may delay treatment, increasing mortality even when disease prevalence is low (Wassie *et al*., 2017).

Moreover, the purposively selected districts in Bale Zone showed variability in morbidity and mortality rates. For instance, Agarfa reported 6.67% morbidity and 14.29% case fatality, whereas Goba's statistics highlight the need for targeted interventions to address the severe outcomes observed there. Sinana's lower morbidity rate of 4.17% may indicate a more effective management strategy. Previous studies have shown that the morbidity and mortality rates of LSD differ based on geographic location, season, climate conditions, management practices, animal movement, virus virulence, and the population of insect vectors (Ahmed and Zaher, 2008; Tuppurainen *et al*., 2011; Gelagay *et al*., 2014; Brenner *et al*., 2009; Hasib *et al*., 2021; Kasem *et al*., 2017). This study compared its findings on prevalence with those of previous studies, but it focused specifically on herds rather than feedlots and farms. Unlike other studies that were conducted in feedlots or farms, this study based its findings on clinical diagnosis.

The present study demonstrated that Vero cells are effective for *Capripoxviruses* adaptation, showing characteristic cytopathic effects (CPE) such as cell clustering by the fourth passage, consistent with Kumar *et al*., (2021). While Umer *et al*., (2024) reported observing CPE by the third passage, differences in viral strain or inoculum concentration could account for the variations. Observations of CPE development aligned with WOAH (2017) reports, which noted gradual CPE progression starting at 2 days post-infection, and were similar to Shubisa *et al*., (2021) regarding cell aggregation and monolayer destruction. Prabhu *et al*., (2024) found delayed CPE emergence, suggesting strain or inoculation differences, while Ansary *et al*., (2022) highlighted LSDV's adaptability by showing pock lesions on the Chorioallantoic membrane, underscoring the value of using various cell types in virus studies.

The current real-time PCR result has shown that the Ct Value is between 17.2538 and 34.7187. These results were in agreement with Mihiret, (2022) with a Ct value between 16.39 and 30.92, Hodhod *et al*., (2020) with a Ct value between 11 and 30, Mesfin *et al*., (2022) Ct values between 14.82 and 23.25, Parvin *et al*., (2022) with Ct values between 18 and 25 that positive samples consistently exhibit Ct values indicative of significant viral presence. These results agreed with Parvin *et al*., (2022) that the LSD viral DNA load was

greatest in the skin lesions and lowest in saliva and nasal discharge, as indicated by Ct values. Furthermore, it also agreed with Parvin *et al*., (2022) idea that samples with higher Ct values contained a smaller quantity of LSDV DNA, while those with lower Ct values had the highest levels of LSDV DNA. This quantification capability underscores the utility of real-time PCR in clinical settings for rapid diagnosis and subsequent control measures.

This study identified a significant relationship between vaccination practice, introduction of new animals, watering points (protectable pond), and the occurrence of Lumpy Skin Disease Virus (LSDV). A notable similarity with Getachow *et al*., (2010) was the emphasis on environmental factors and their impact on disease prevalence, particularly in varying agro-climatic zones. Both studies highlighted that the introduction of new animals increases the risk of LSDV transmission.

Live attenuated LSDV vaccines have shown effectiveness in reducing morbidity and controlling outbreaks, as demonstrated by Wassie *et al*. (2017) and Gelagay *et al*. (2020) in Ethiopia, where vaccinated herds experienced lower morbidity rates and reduced cases in outbreak-prone areas. Proper vaccine handling and storage likely enhance effectiveness. However, challenges remain, as Girma *et al*. (2019) and others reported vaccine failures, including the Ethiopian Neethling vaccine's inability to protect against local LSDV strains, reinfections in vaccinated animals, and mild clinical reactions like nodules, which complicate diagnosis and farmer acceptance. These failures are attributed to factors such as poor vaccine performance, inadequate coverage, and high viral loads in endemic areas. The current study aligns with positive outcomes but disagrees with Girma *et al*. (2019), suggesting that proper storage, handling, and partial protection may explain the discrepancy.

This study revealed that the communal watering point (protectable pond) increases the risk of lumpy skin disease occurrence. Furthermore, Shubisa *et al*. (2021) indicated that communal contact points account for a significant source of infection, aligning with our findings on watering points (protectable pond). This suggested a common understanding of the risks associated with communal resources. Gezahegn *et al.* (2013) further support this by noting the high likelihood of LSDV introduction through market practices. In summary, all studies underscored the importance of vaccination and management practices in controlling LSDV.

This study faced several challenges, including budget and time constraints that limited year round disease observation, hindering the understanding of seasonal patterns. The complexity of disease transmission and vector behavior added to the difficulty of control efforts. Inadequate advanced diagnostic infrastructure, especially for genetic sequencing, restricted detailed pathogen analysis. Overcoming these limitations could enhance future research and disease management strategies.

6. CONCLUSION AND RECOMMENDATIONS

Lumpy Skin Disease (LSD) is a significant global economic threat, particularly impacting livestock productivity and trade. In Ethiopia, it causes severe agricultural losses, including permanent hide damage, reduced milk production, infertility, and abortion. This study investigated LSD in three districts of the Bale Zone, focusing on clinical manifestations, epidemiological characteristics, and laboratory findings. LSD outbreaks were consistently reported after the rainy season, with a relatively low morbidity rate but a high case fatality rate. Laboratory confirmation of LSDV infection was achieved through cytopathic effects (CPE) in Vero cells and real-time PCR, with all samples testing positive and showing high viral loads (indicated by low Ct values and specific dissociation curves at 73.9°C). Epidemiological surveys identified key risk factors, including lack of vaccination, introduction of unvaccinated animals, and reliance on communal watering points. Vaccination significantly reduced LSD incidence, highlighting its importance in disease control. These findings underscore the need for comprehensive vaccination programs, strict biosecurity measures, and improved animal management practices to mitigate LSD outbreaks. Targeted strategies, such as vaccination campaigns and restrictions on the movement of unvaccinated or newly introduced animals, are essential for effective disease control and prevention in the study areas.

Based on the study findings the following are recommended to effectively manage Lumpy Skin Disease (LSD) in endemic regions like Ethiopia;

- ✓ Effective management strategies, including vaccination, biosecurity measures, and regular monitoring, are crucial for controlling and eradicating LSD.
- ✓ The government should allocate consistent funding for long-term surveillance programs and research projects.
- ✓ Implement comprehensive studies on LSDV transmission dynamics, focusing on direct contact and vector-borne spread.
- ✓ Develop educational programs and training sessions for herders to increase awareness of LSDV symptoms, transmission routes, and prevention methods.
- ✓ Future studies should focus on molecular analyses of isolates, genetic differences between field and vaccine strains, and developing new vaccines from local isolates.

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8. APPENDICES

Appendix 1. Participant Information Sheet.

Haramaya University

Postgraduate Program Directorate

College of Veterinary Medicine

Department of Veterinary Microbiology

Dear respondent,

Let me start by introducing myself. My name is Muzeyen Mohammadnur Biltu, and I am a Master of Science in Veterinary Microbiology student at Haramaya University. I am conducting research on the topic of "**Isolation and Molecular Detection of Lumpy Skin Disease Virus from Outbreaks Cases in the Bale Zone, Oromia Region, Ethiopia**" as one of the requirements for the project. This disease is rapidly spreading throughout the nation and severely damaging the economy of the state. I guarantee that any response you offer and any information you supply in this regard will be kept completely private and used only for academic study. I sincerely hope you will take a moment of your precious time to freely and candidly respond to the following query. As a result, the information you submit will decide the quality of the research; so, I respectfully ask that you answer each question with accurate and factual information.

Note:

- ✓ The data you provide will only be utilized for academic purposes.

Thank You

Muzeyen Mohammadnur Biltu

Appendix 2. Questionnaire Format for LSDV Field Outbreak Assessment

Questionnaire ID.No._____ Date/season of data collection: _____

I. General Information

1. Address: District: _____:Zone_____, Farm/ owner Name: _____

2. Agroecological Category: Highland ☐ Midland ☐ Lowland ☐

3. **Temperature/Humidity Index** of the district: _____

II. Information about Herd

1. Size of the Herd: 1-5 ☐ 6-10 ☐ 11-15 ☐ >15 ☐

2. Breed Type: Exotic ☐ Cross ☐ Local ☐

3. Other animals contact with the herd: Sheep ☐ Goats ☐ Poultry ☐

4. Overcrowding of animals (average no per 100ha): Yes ☐ No ☐

5. What type of reproductive disorders have you encountered in the Herd?: Anestrus:☐ Abortion ☐ Weak Calf ☐ FM Retention ☐ Metritis ☐ Repeated breeding ☐ No disorder ☐

Biosecurity Issues

6. **Breeding system**: AI ☐ Bull ☐ Both ☐

7. **Animal Grazing**: Private ☐ Communal ☐

8. **Water Source**: River ☐ potable water ☐ Protected Well/ Pond ☐

9. **Mixing of animals** with the nearest dairy HERD: Yes ☐ No ☐

10. Have you heard about the LSDV **diseases** in the nearest Herds? Yes ☐ No ☐

11. If yes, would you mention the disease symptoms?_____

12. **Source of animals** for **replacement**: Purchases ☐ Farm-grown ☐

13. Have the herd Vaccinated against **LSDV**: Yes ☐ No ☐

14. Do you know the local name of LSD_____

III. Information Related with Sampled Animals from the herd

Sample Code	**Sex**	**Age**	**Breed**	**Body condition**	**Skin lesion**	**Other clinical sign**

Appendix 3. Preparation of Skin Nodules, Nasal and Saliva Swabs for Inoculation

1. Skin Nodules:
    a) Cut the nodules into small pieces using sterile scissors or a scalpel.
    b) Homogenize the tissue in a sterile mortar and pestle or using a homogenizer with 1–2 mL of transport medium (e.g., PBS or VTM).
    c) Centrifuge the homogenate at 600 × g for 10 minutes to pellet debris.
    d) Collect the supernatant, which contains the viral particles.
    e) Filter the supernatant through a 0.45 µm filter to remove bacteria and large debris.
    f) Add antibiotics anti-anti (antibiotic-antimycotic 3%) to the filtered supernatant to prevent bacterial contamination during inoculation.
    g) Collect the supernatant for inoculation
2. Nasal and Saliva Swabs:
    a) Vortex the swabs in the transport medium to release viral particles.
    b) Centrifuge the swab medium at 600 × g for 10 minutes to pellet debris.
    c) Collect the supernatant.
    d) Filter the supernatant through a 0.45 µm filter.
    e) Add anti-anti (antibiotic-antimycotic 3%) to the filtered supernatant to prevent bacterial contamination during inoculation.
    f) Collect the supernatant for inoculation

Appendix 4. Vero Cell Line Culture Preparation

1. First all the cell culture mediums were warmed at 37°C in a water bath and the trypsin solution allowed reaching room temperature. The culture medium from the cells was decanted.
2. The cells were washed twice with PBS. Trypsin was added to the cells, 2 ml per 25 cm^2 of flask surface, and was gently spread over the entire surface by tilting the vessel.
3. The culture was placed in an incubator at 37°C temperature for about 5 minutes. The culture flask was microscopically examined to determine if all the cells had detached and was re incubated when necessary.
4. The cells were collected by pipetting culture medium over the surface of the vessel, i.e. 7 ml per 25 cm^2 of flask surface then were mixed carefully to disperse the cells into a single-cell suspension.
5. The cell suspension was transferred into a new 25cm^2 tissue culture flask containing GMEM with FCS (10% for Vero) and Anti-Anti (Antibiotic-antimycotic 3%) which is known as gibco of UK by its trade name.
6. The cell cultures were incubated at 37^0C temperature in a 5% CO_2 incubator and were checked regularly. The medium in each flask was changed after 3 days until the monolayer of the cells were formed. The cell cultures were passed continuously until they were well formed.

Appendix 5. Virus Inoculation on Cell Culture

1. First, disinfect all the medium and solution bottles and other materials outer surfaces with 70% Ethanol before setting it to the safety cabinet.
2. Decant the culture medium from the flask forming monolayer of the cell.
3. Wash monolayer gently with 2ml of pre warmed PBS 3 times.
4. Add 0.5ml sample inoculum to the cell culture depending on the flask size. Rock each plate gently to distribute inoculum evenly over the cell monolayer.
5. Incubate inoculated cultures at 37°C incubators for 1 hour to allow virus to adsorb.
6. Shake the inoculated flasks once or twice during incubation.
7. Add GMEM with 10% FCS maintenance medium to each flask and incubate at 37°C with +5% CO_2 and humidity 55% for about 14 days.

8. Check flasks daily for cytopathogenic effect (CPE) and condition of cells under inverted microscope (Olympus CKX41).
9. To harvest samples, freeze-thaw 3 times.

Appendix 6. DNA Extraction (Qiagen, Germany**)**

Skin Nodule Sample

1. The processed tissue sample is placed in a 1.5 ml microcentrifuge tube. Add 200 µl Lysis Buffer (AL), 20µl proteinase K, and 200 µl Sample with a total 420 µl per tube mixed by vortexing, and put it in a water bath with 56°C for 10 minutes. After 10 minutes 15 s directly before proceeding to step 2.
2. After 10 minutes 200µl ethanol (Absolute Alcohol) (from Belgium) was added to make 620 µl per tube. Mixed thoroughly by vortexing again.
3. Pipet the mixture into a DNeasy Mini spin column (silica based) placed in a 2 ml collection tube. Centrifuge at ≥ 6000 x g (8000 rpm) for 1 min. DNA binds to silica membranes. Discard the flow-through and collection tube.
4. Place the spin column in a new 2 ml collection tube. First the concentrate of the AW1 was prepared by adding 130 ml Ethanol. Add 500 µl Washing Buffer 1(AW1) (Concentrated). Centrifuge for 1 min at ≥6000 x g (8000rpm). Discard the flow-through and collection tube.
5. Place the spin column in a new 2 ml collection tube. First the concentrate of the AW2 was prepared by adding 160 ml Ethanol. Add 500 µl Washing Buffer 2 (AW2), and centrifuge for 3 min at 20,000 x g (14,000 rpm). Discard the flow-through and collection tube.
6. Transfer the spin column to a new 1.5 ml or 2 ml microcentrifuge tube.
7. Elute the DNA by adding 100µl Elution Buffer AE to the center of the spin column membrane. Incubate for 3 min at room temperature (15-25°C). Centrifuge for 1 min at ≥ 6000 x g (8000 rpm).

Nasal and Saliva Swabs Sample

DNA Extraction from Nasal and Saliva Swabs for LSDV Using a Qiagen Kit

1. Sample Collection - Collect nasal and saliva swabs using sterile swabs and place them in transport medium (e.g., viral transport medium or PBS).

2. Lysis of Viral Particles - Transfer swabs to a tube with lysis buffer (e.g., ATL buffer), mix thoroughly, and incubate at 56°C for 10–15 minutes.

3. Proteinase K Digestion - Add Proteinase K, mix gently, and incubate at 56°C for 10–15 minutes.

4. DNA Binding - Add binding buffer (e.g., AL buffer) and ethanol, and then mix thoroughly.

5. Spin Column Loading - Transfer the mixture to a Qiagen spin column, centrifuge at 6,000–8,000 × g for 1 minute, and discard the flow-through.

6. Washing - Add wash buffers (e.g., AW1 and AW2), centrifuge, and discard the flow-through after each step.

7. Elution - Transfer the column to a clean tube, add elution buffer (e.g., AE buffer), incubate for 1–5 minutes, and centrifuge to collect purified viral DNA.

8. Storage - Store DNA at -20°C or -80°C for long-term use or use immediately.

Appendix 3. Different field and laboratory pictures during disease investigation, Sample Collection, Laboratory materials and laboratory procedures.

Figure 1. During travelling for sample collections.

Figure 2. During investigating for clinical Sign of the LSD.

Figure 3. During taking skin nodules from infected cattle.

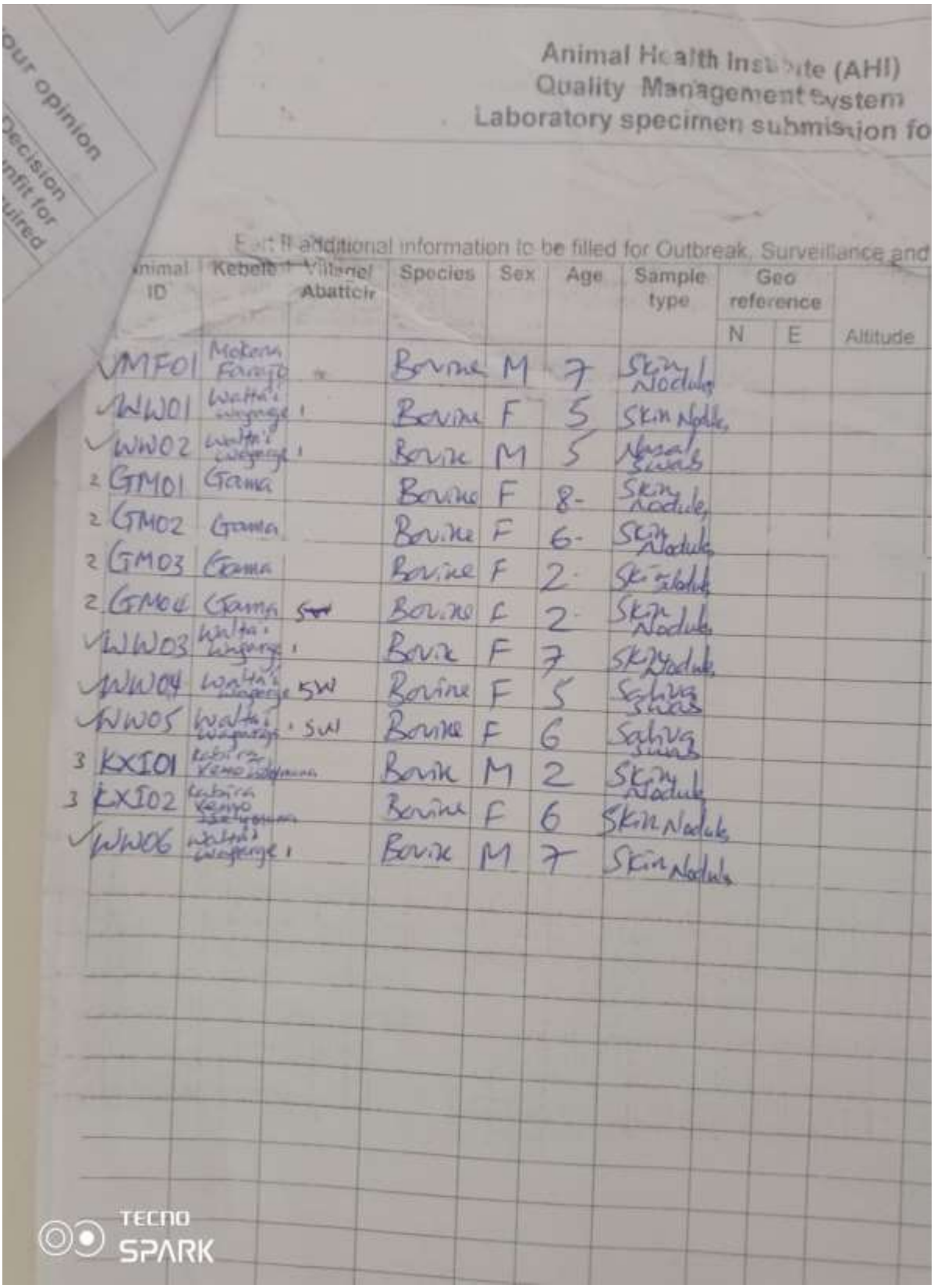


Figure 4. Sample Submission format at Animal Health Institute (AHI).

Figure 5. Different chemicals used during DNA extraction.

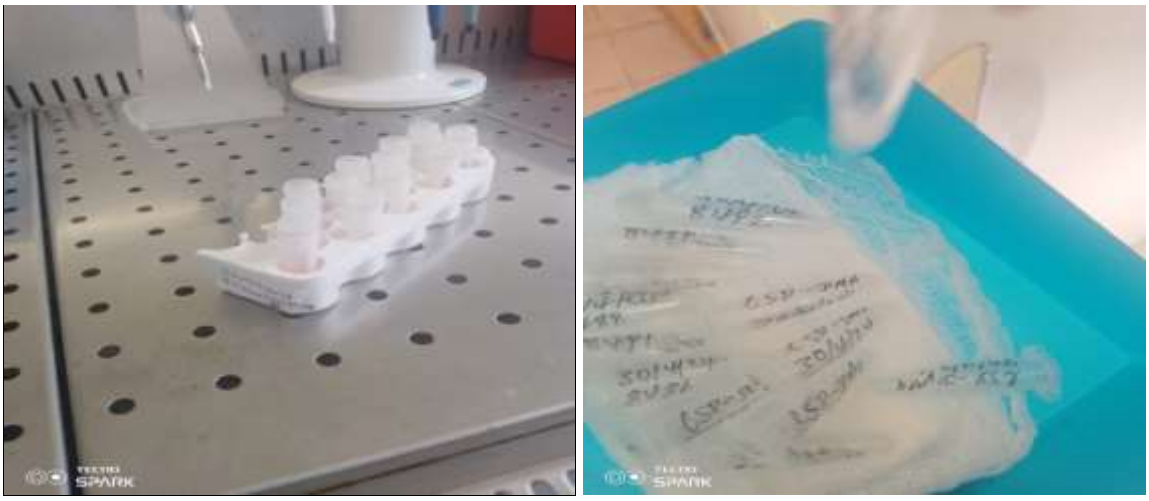


Figure 6. Extracted DNA from Skin nodules, saliva and nasal swabs.

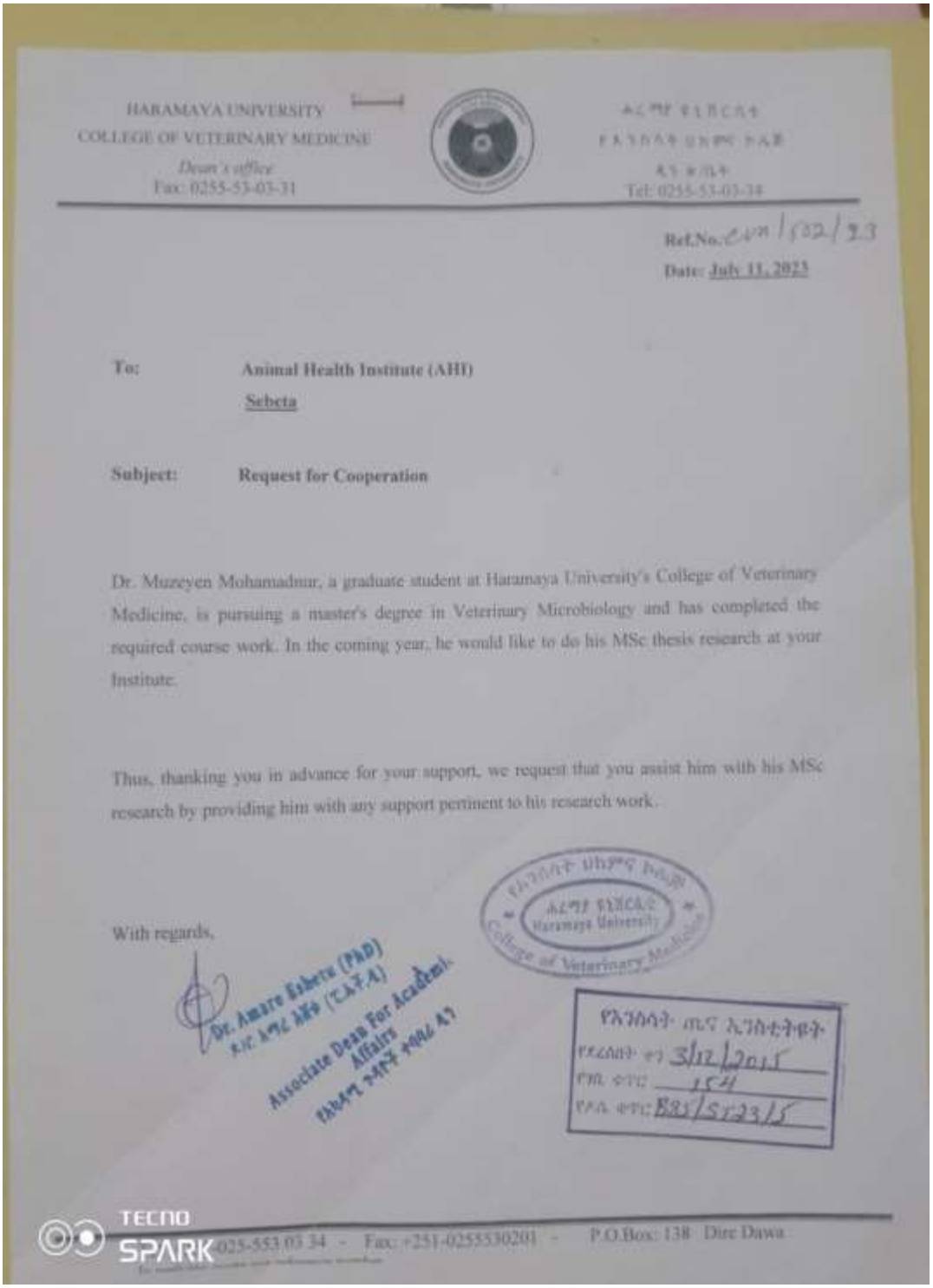
Figure 7. During preparation of extracted DNA for RT-PCR.

Figure 8. Media and antimicrobial used for cell culture.

Figure 9. Vero cell line inoculated with DNA Extracted Supernatant.

Figure 10. During checking of the Vero cell line for CPE.

Appendix 4. Different letters.



HARAMAYA UNIVERSITY
COLLEGE OF VETERINARY MEDICINE
Dean's office
Fax: 0255-53-03-31

Tel: 0255-53-03-34

Ref.No. CVM/502/23
Date: July 11, 2023

To: Animal Health Institute (AHI)
Sebeta

Subject: Request for Cooperation

Dr. Muzeyen Mohamadnur, a graduate student at Haramaya University's College of Veterinary Medicine, is pursuing a master's degree in Veterinary Microbiology and has completed the required course work. In the coming year, he would like to do his MSc thesis research at your Institute.

Thus, thanking you in advance for your support, we request that you assist him with his MSc research by providing him with any support pertinent to his research work.

With regards,

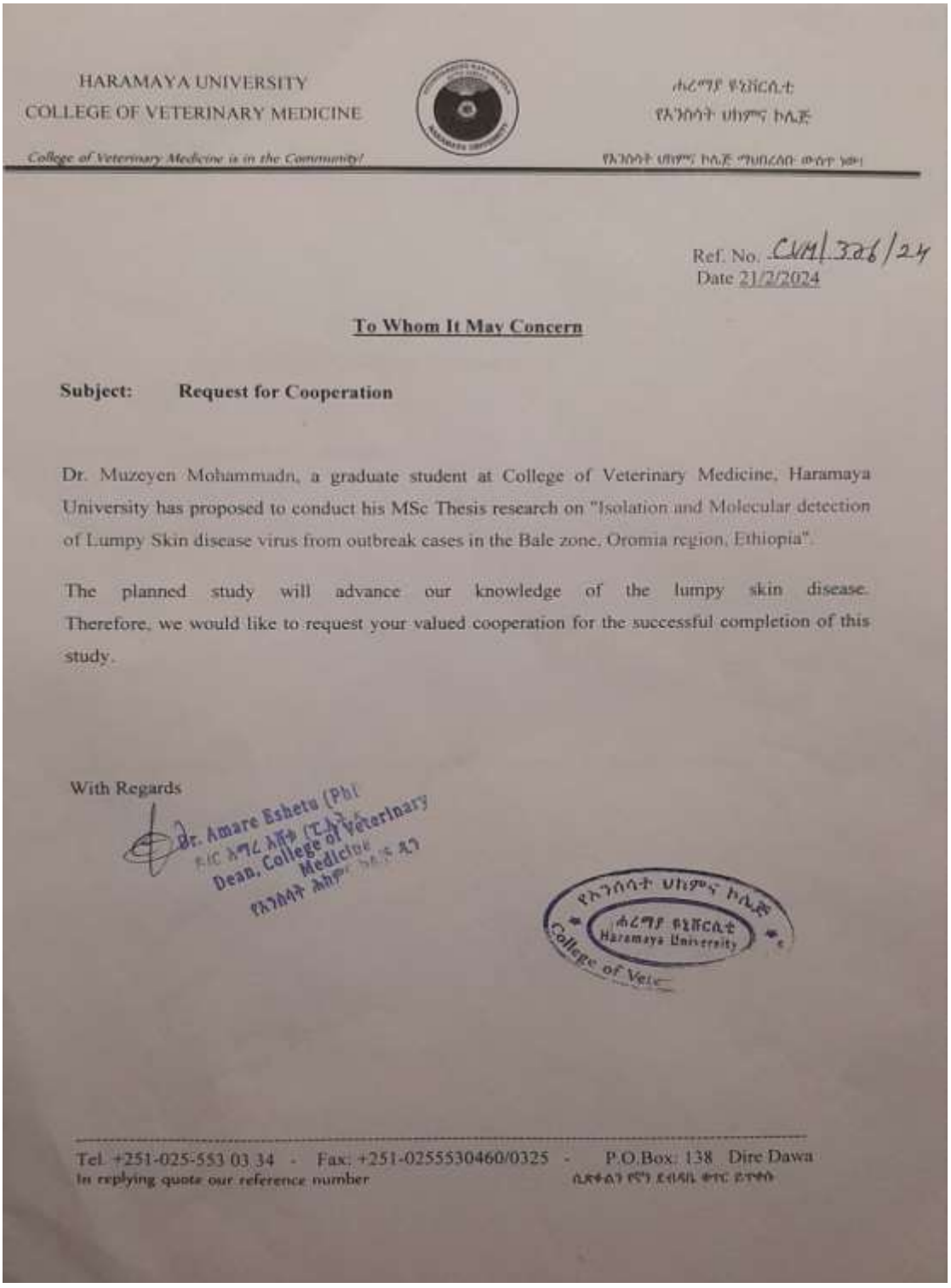
Dr. Amare Eshetu (PhD)
Associate Dean For Academic Affairs

Haramaya University
College of Veterinary Medicine

TECNO SPARK

025-553 03 34 - Fax: +251-0255530201 - P.O.Box: 138 Dire Dawa

Letter written for Animal health Institute



HARAMAYA UNIVERSITY
COLLEGE OF VETERINARY MEDICINE

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College of Veterinary Medicine is in the Community!

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Ref. No. CVM/326/24
Date 21/2/2024

To Whom It May Concern

Subject: Request for Cooperation

Dr. Muzeyen Mohammadn, a graduate student at College of Veterinary Medicine, Haramaya University has proposed to conduct his MSc Thesis research on "Isolation and Molecular detection of Lumpy Skin disease virus from outbreak cases in the Bale zone, Oromia region, Ethiopia".

The planned study will advance our knowledge of the lumpy skin disease. Therefore, we would like to request your valued cooperation for the successful completion of this study.

With Regards

Dr. Amare Eshetu (Phl
Dean, College of Veterinary Medicine

Tel. +251-025-553 03 34 - Fax: +251-0255530460/0325 - P.O.Box: 138 Dire Dawa
In replying quote our reference number

Letter’s written for cooperation to different government organizations during sample collection