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MOLECULAR CHARACTERIZATION OF LUMPY SKIN DISEASE VIRUS ISOLATES FROM OUTBREAK CASES IN CATTLE FROM SAWENA DISTRICT OF BALE ZONE, OROMIA, ETHIOPIA

MVSc THESIS

BY

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BISHOFTU, ETHIOPIA

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A Thesis submitted to the College of Veterinary Medicine and Agriculture of Addis Ababa University in Partial Fulfillment of the Requirements for the Degree of Master of Science in Veterinary Microbiology

By

Shubisa Abera

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As members of the Examining Board of the final MVSc open defense, we certify that we have read and evaluated the thesis prepared by Shubisa Abera entitled: **"Molecular Characterization of Lumpy Skin Disease Virus (LSDV) Isolates from Outbreak Cases in Cattle from Sawena District of Bale Zone, Oromia, Ethiopia"** and recommended that it be accepted as fulfilling the thesis requirement for the Degree of: Master of Science in Veterinary Microbiology**.**

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SIGNED DECLARATION SHEET

First, I declare that this thesis is my original work and that all sources of material used for this thesis have been duly acknowledged. This thesis has been submitted in partial fulfillment of the requirements for an advanced (MVSc) degree at Addis Ababa University, College of Veterinary Medicine and Agriculture and is deposited at the University/College library to be made available to borrowers under rules of the Library. I solemnly declare that this thesis is not submitted to any other institution anywhere for the award of any academic degree, diploma, or certificate.

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LIST OF ABBREVIATIONS

Bp	base pair
CaPV	Capripoxvirus
CI	Confidence interval
CPE	Cytopathic effect
CSA	Central Statistical Authority
DNA	Deoxyribonucleic acid
dsDNA	Double stranded deoxyribonucleic acid
EDTA	Ethylene diamine tetra acetic acid
EMV	Extracellular mature virus
GMEM	Glasgow Eagle minimal essential medium
GTPV	Goat pox virus
IMV	Intracellular mature virus
Kbp	Kilo base pair
LAMP	Loop-Mediated Isothermal Amplification
LSD	Lumpy skin disease
LSDV	Lumpy skin disease virus
Mm	millimeter
mRNA	Messenger ribonucleic acid
NAHDIC	National Animal Health Diagnosis and Investigation Center
Nm	nanometer
OA3.Ts	Lamb testis cell line
OIE	Office International des Epizooties
PBS	Prophet-Buffered Saline
PCR	Polymerase Chain Reaction
SNNPR	Southern Nation Nationalities and Peoples Region
SPPV	Sheep pox virus
Vero-cell	African Green Monkey Kidney Cells
VTM	Virus transporting media
X^2	Chi- square

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ABSTRACT

Lumpy skin disease (LSD) is a viral disease caused by LSD virus and is one of the most economically significant transboundary and emerging diseases of cattle. An outbreak investigation of the disease was monitored from October 2016 to April 2017 in southern pastoral areas of Bale Zone Oromia, Ethiopia. In December 2016, LSD outbreak occured in Sewena district of Bale Zone, from which necessary biopsy samples were collected from actively infected animals for the purpose of virus isolation, and characterization using different molecular techniques at Animal Health and Diagnostic Investigation Center (NAHDIC) of Sebeta, Ethiopia. In addition clinical examination of infected and in contact animals were carried out together with questionnaire survey. A questionnaire survey was designed to identify potential risk factors of the disease. Based on the clinical manifestations, LSD was recorded in 18% (94/522) of examined animals, whereas biopsy samples from 20 clinically positive animals were collected for further laboratory process. The morbidity rate was higher in animals less than two years 29.97% (31/107) than other ages and showed statistically significant difference with ($P<0.05$). Female animals showed high morbidity rate of 20.59% (76/369) than male animals 11.76% (18/153) with significant difference at ($P= 0.003$). Mortality rate and case fatality were also significantly higher in young animals than other age groups. Viruses were isolated from both skin samples and nasal swabs on Vero cell line. Conventional PCR and Real-Time PCR analysis confirmed that DNA extracts from both skin biopsies and nasal swabs of all virus isolates were positive for LSDV. The questionnaire survey revealed that the common sources of infections were communal points (like markets, watering and grazing areas) and introduction of sick animals to the herd. In conclusion there were huge losses from occurrence of LSD as the isolates were confirmed by characterizations in the laboratory and its economic losses estimated from single outbreak in the present study. Providing adequate diagnostic facilities, establishing strategic policies for effective control and eradication, awareness creations for communities for early identification/or reporting were recommendations made to minimize economic losses of the disease.

Key words*: Bale Zone, Ethiopia, LSD, Outbreak Investigation, PCR, Virus Isolation*

1. INTRODUCTION

In the Greater Horn of Africa (GHA), pastoralists occupy large parts of arid and semi-arid lands of Ethiopia, Kenya, Somali, Djibouti, Eritrea, Sudan, Uganda, and Tanzania (FEWS NET, 2004). Together with agro-pastoralists, they comprise significant proportions of national populations in each of these countries. (Mekonnen *et al*., 2001). Ethiopia has the largest livestock population in Africa, possessing more than 57.8 million cattle, 56.6 million small ruminants, 1.2 million camels and 10.4 million equines and 60.5 million chickens. This livestock sector has contributed considerable portion to the economy of the country, and still promising to rally round the economic development of the country. Livestock production remains crucial and represents a major asset among resource-poor small holder farmers by providing milk, meat, skin, and manure and traction force (CSA, 2016). The contribution of livestock to the national economy particularly with regard to foreign currency earnings is through exploration of live animal, meat and skin and hides (MoARD, 2008).

In the highlands, livestock are kept under settled or transhumant systems utilizing common pastures, many of which have a high clover content and crop residues. Such livestock includes some 9.3 million oxen providing draught power, for the mixed farming system that prevails. In the arid and semi-arid extensive grazing areas of the eastern, western, and southern lowlands, cattle, sheep, goats, and camels are managed in migratory pastoral production systems (FAO/WFP, 2005).

The Bale pastoralists' livelihoods depend predominantly on livestock and their products. They practice a transhumance nomadic system, which had been their traditional and primary survival strategy. However, physical infrastructure is poorly developed in areas where pastoralists live. Poor health and productivity of animal due to disease has considerably become the major stumbling block to the potential of livestock industry (Mekonnen *et al*., 2001).

Lumpy skin disease is one of the most economically significant transboundary, emerging viral diseases. It is currently endemic in most Africa countries and expanded to Middle East region (Tuppurinen and Oura, 2012). It is a disease with a high morbidity and low mortality rate and affects cattle of all ages and breeds. It causes significant economic problems as a result of reduced milk production, beef loss and draft animals, abortion, infertility, loss of condition and damage to the hide (CFSPH, 2008). It becomes an important threat to livestock and dairy industry in the Middle East and Africa (Kumar, 2011).

LSD is an acute infectious disease characterized by fever, nodules on the skin, mucous membranes and internal organs, emaciation, enlarged lymph nodes, edema of the skin, and sometimes death (Radostitis *et al*., 2006). It is caused by the virus classified in *Capripoxvirus* of family *Poxviridae*. Various strains of *Capripoxvirus* are responsible for the disease and these are antigenically and serologically indistinguishable from strains causing sheep pox and goat pox but distinct at the genetic level (Babiuk *et al.*, 2008a). LSD has a partially different geographical distribution from sheep and goat pox, suggesting that cattle strains of *Capripoxvirus* do not infect and transmit between sheep and goats (OIE, 2010). The disease occurs in different ecological and climatic zones and extends its boundaries to different areas (Davies, 1991).

LSD enters a new area by the introduction of infected animals. Biting insects that have fed on infected cattle may travel and be blown for substantial distances. The movement of contaminated hides represents another potential means for this resistant virus to move (Davies, 1991).Vaccination is the only effective method to control the disease in endemic countries like Ethiopia. The experience in the major parts of the country showed that the vaccination approach is commonly chosen and is often that of ring vaccination around a local foci outbreak when it occurs. Animals that recover from virulent LSD infection generate lifelong immunity consisting both of a humoral and cell mediated protective immunity (Kitching *et al*., 1987).Maternal immunity provides protection from LSD in calves at least for 6 months (Davies 1991).

Consequently, LSD brought high economic pressure on subsistence of the poor farmers particularly pastoralists at which their central economy relay on the production of livestock and mixed farming system (Buller *et al*., 2005). Lumpy skin disease causes international ban on the trade of livestock and their products (Merck Veterinary manual, 2011). LSD was spread to East Africa in 1957 in Kenya and disease was extensively expanded to the rest of the region in subsequent years (Davies, 1991). Determination of seroprevalence of LSD has a time limitation for the presence of detectable antibodies in the serum for more than seven months of post infection. Serological tests such as virus neutralization are less sensitivity and time consuming to detect the low level antibody titres following the infection of the animals (Vorster, 2008; OIE, 2010).

In Ethiopia limited works has been done on LSD so far and few reports have been made on risk factors assessments, epidemiological aspects, seroprevalence and financial impacts in selected areas of the country, Amhara Region (North Wello, South Wello), Oromia Region (Borena and Guji) administrative zones in southern part of Ethiopia and in northern part of Ethiopia (Getachew *et al.*, 2010, 2011 and Alemayehu *et al.*, 2013). A report on seroprevalence of disease using virus neutralization and indirect fluorescents antibody test indicated that the disease is widely distributed across the country and increases its impacts (Getachew *et al.*, 2012). Hence, it is important to generate information on isolation of LSD from outbreak reported and its molecular characterization.

Therefore, the objectives of this study are to investigate LSD outbreak in pastoral areas and identify potential risk factors with major emphasis on isolation and characterization of LSDV circulating in study area.

2. LITERATURE REVIEW

2.1. History of LSD

For the first time in 1929, skin disease with new clinical symptoms occurred in Zambia. At that time it was considered that it was caused by either plant poisoning or an allergic response of insect bite (Weise, 1968; Bagla, 2005). After fourteen years, in October 1943, another outbreak of the disease occurred in Botswana and it was named provisionally as "Ngamiland cattle disease" as the case occurred for the first time in Ngamiland. After two years, 1945 the disease spreads to Zimbabwe and South Africa where the disease was named lumpy skin disease and demonstration of transmission of the infectious agent by inoculation of cattle with suspension of the skin nodules was determined (Davies, 1991).

The disease was diagnosed in Kenya in 1957; Sudan in 1971; Chad and Niger in 1973; Nigeria in 1974 and Somalia in 1983 (Tuppuraninen, 2005). In Ethiopia, LSD was first observed in 1983 in the western part of the country (southwest of Lake Tana) (Mebratu *et al.,* 1984). In 1988, the first outbreak was occurred in Egypt in Ismailia and although control and eradication measures had been taken place the disease remains endemic in these areas (Ali *et al*., 1990). It was also observed clinically in Israel in herds of dairy farms in 1989 which was suggested as it was spread from Egyptian outbreaks by insect vectors carried by wind (Yeruham *et al.*, 1995). The disease was primarily considered as an endemic disease to Africa and Middle East and other areas. According to annual disease information released by the OIE, outbreak cases were reported from Bahrain in 1993/94, 2002 Iran in 1996,2001 and other similar cases have been reported in United Arab Emerate, Kuwait and Oman (OIE, 2010).

2.2. Etiology

LSD is caused by lumpy skin disease virus (LSDV) within the genus *Capripoxvirus* and the Prototype strain is Neethling Virus. It is an enveloped DNA virus, ovoid shape with a molecular size of 350*300nm and a molecular weight that ranges from 73 to 91 (Kilodalton) KDa. LSDV genome sequences were assembled into a contiguous sequence of 150.8 kilobase pair (kbp) which is in accordance with previous size estimates of 145 to 152 kbp (Tulman *et al.*, 2002; Kara *et al.*, 2003). These genes encode several poxvirus proteins known to be structural or involved in virion morphogenesis and assembly. The terminal genomic sequences contain a unique complement of at least 34 genes which are responsible in virulence, host range and/or immune evasion (Tulman *et al.*, 2002; Kara *et al.*, 2003). LSDV is genetically and antigenically closely related to a strain of sheep and goat pox virus (Alexander *et al*., 1957). Comparison of LSDV genome with published restriction fragment analysis of the SPPV and GTPV genome indicates that there may be additional terminal sequences of less than 200 bp present (Gershon and Black, 1987; Kitching *et al*., 1989; Tulman *et al.*, 2002).

LSDV is susceptible to sun light and detergents containing lipid solvents. The virus could be inactivated after heating for 1 hour at 55°C (Davies and Otema, 1981; Lefèvre and Gourreau, 2010). However, it withstands drying, pH changes if not an extreme pH and can remain viable for months in dark room such as infected animal shade off its host. LSDV can persist in skin plugs for about 42 days (Babiuk *et al*., 2008b; Lefèvre and Gourreau, 2010). It is likely that the viral A type inclusion body protein in infected cells may protect the virion after the scab has disintegrated, although this has not yet been proven (Babiuk *et al*., 2008a).

2.2.1. Viral genome

The LSDV genome is about 151-kbp long double-stranded DNA covalently cross-linked at the ends, similarly to other pox viruses. The genomic sequence of LSD consists of a central coding region bounded by identical 24 kbp-inverted terminal repeats and contains

156 putative genes (Tulman *et al*., 2002). These genes encode several pox viral proteins known to be structural or involved in virion morphogenesis and assembly. The terminal genomic sequences contain a unique complement of at least 34 genes which are responsible in virulence, host range and/or immune evasion (Tulman *et al*., 2002; Kara *et al.*, 2003).

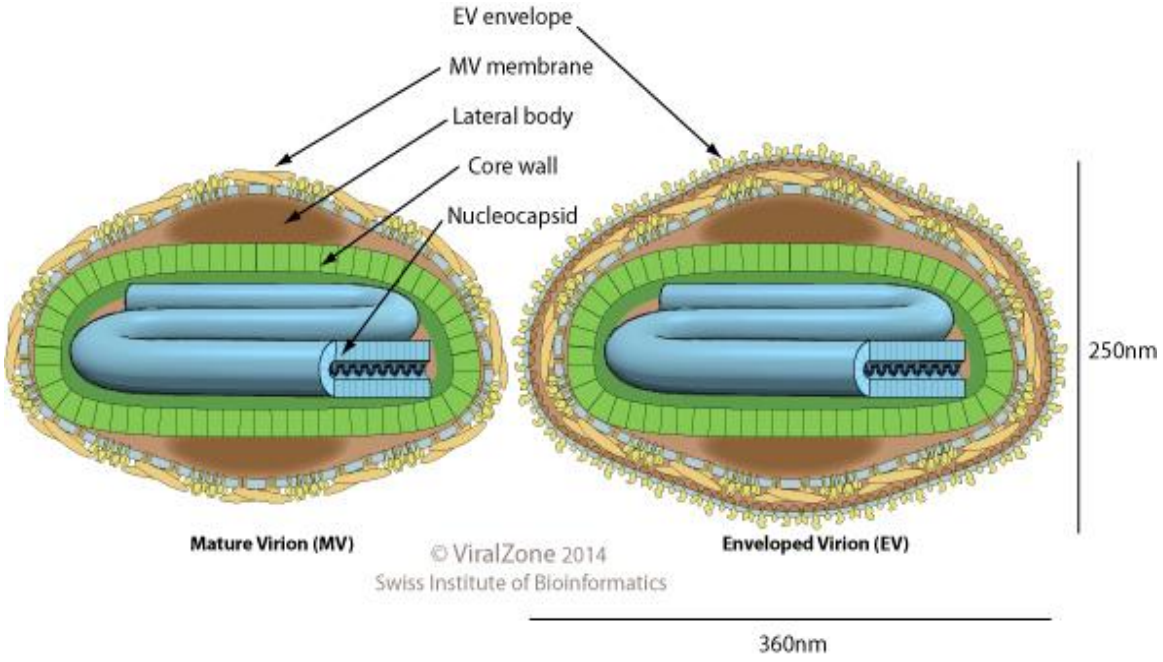

Figure 1: General structure of *capripoxviruses*

Enveloped, brick-shaped, with 300 *270*200nm in size. The surface membrane displays surface tubules or surface filaments. Two distinct infectious virus particles exist: the intracellular mature virus (IMV) the extracellular enveloped virus (EEV). http://viralzone.expasy.org/all by species/ 152.html (accessed Nov, 2016).

Capripoxvirus genomes sequences are highly conserved and there is more than 95% homology amongst LSDV, SPPV, and GTPV (Kara *et al*., 2003). LSDV is genetically closely related to a strain of Sheep and Goat pox virus (Alexander *et al*., 1957). LSDV has an additional nine genes that are non-functional in Sheep pox and Goat poxviruses, some of which are likely responsible for their ability to infect cattle (Tulman *et al*., 2002). Comparative sequence analysis of the two field isolates of LSDV with the genome of the

South Africa Onderstepoort vaccine strain suggests that *Capripoxvirus* virulence are linked to a number of genes putatively involved in host immune-modulation (Kara *et al*., 2003).

LSDV have complement of genes such as IL-10, (IF-γ) receptor; IL-lR, IFN-α/β binding protein and IL-18(binding protein) are secreted and responsible for modulation or evasion of host immune response, inhibition of host cell apoptosis and in cell or tissue tropism (Lalani *et el*., 1999). Entrance of the virion particles in to the host cell membrane undergoes penetration and uncoating to carry out its replication independently to the host nucleus (Carn, 1994).

2.2.2. Viral replication

Replication of poxvirus occurs in the cytoplasm. After fusion of the virion with the plasma membrane or via endocytosis, the viral core is released into the cytoplasm. Transcription is initiated by viral transcriptase and functional capped and polyadenylated messenger Ribonucleic Acid (mRNAs) are produced within minutes after infection. The polypeptides produced by translation of these mRNAs complete the uncoating of the core and about half of the viral genome is transcribed prior to replication, comprising genes encoding proteins involved in host interactions, viral DNA synthesis, and intermediate gene expression. With the onset of DNA replication 1.5 to 6 hours after infection, there is a dramatic shift in the gene expression and almost the entire genome is transcribed, but transcripts from the early genes (i.e. those transcribed before DNA replication begins) are not translated. Two forms of virions are released from the infected cells (virions with one membrane, and virions with two membranes) and both types are infectious (Fenner *et al.*, 1987; Bertagnoli and Séverac, 2010).

2.3. Epidemiology

2.3.1. Transmission

Though there was no clearly defined method of transmission of LSD, circumstantial evidences suggestions that the disease might be transmitted by biting insects. Later on the virus was isolated from arthropod vectors and the role of vectors in transmission of the virus was experimentally confirmed. Epidemiological evidence suggests that, outbreaks of LSD were highly associated with prevalence of high insect vector population and with upcoming rainy season (Weise, 1968). Biting insects play major role in transmission of LSDV. Epidemics of LSD are associated with rainy seasons, river basins and ponds during which cattle grazed in and humid areas conducive to insect multiplication. These biting insects transmit the virus mechanically during their blood meals Chihota *et al.* (2001).

LSDV is transmitted mainly by arthropod vectors. This vector-related transmission is apparently mechanical, rather than biological. This distinction is important because infectious organisms do not generally survive in vectors for long periods for multiplication or over-wintering in these insects (Chihota *et al.,* 2001). The virus can survive 2-6 days post feeding from infected cattle and transfers these to susceptible cattle by female mosquito, *Aedesegypti* during experimental infection. The virus can survive only for about average four days and this can't permit for recurrence of disease in the coming season. It was thought that infected vectors can transmit the disease some distance kilometers from the foci of infection as the occurrence of outbreak in 1989 in Israel following aerial movement of infected insect vectors from Egypt (Yeruham *et al.*, 1995).

Mosquitoes and other flies such *tabanids, culicoides*, biting midges and *Glossina* species like tsetse fly are among the other arthropod vectors that play a great role in the transmission of the virus. The participation of these flies in the spread of LSDV have been confirmed by isolation of the virus from the stable flies feed on infected cattle and

this indicated that these flies are efficient vectors of *Capripoxviruses* (Bruce *et al*., 2004). Flies, including housefly, bush fly and blowflies are also very commonly associated with infected cattle possible to siphon off infected lachrymal, nasal or other secretions and transfer the virus to another susceptible animal. Vermin, predators and wild birds might also act as mechanical carriers of the virus (Kitching and Mellor, 1986).

Outbreaks of LSD are highly associated with seasonal peak of mechanical vectors in wet and warm weather conditions in Ethiopia (Getachew *et al*., 2010). The molecular evidence of the potential viral transmission by hard ticks. The virus could be transmitted through transstadial and transovarian in *Boophilus decoloratus* and mechanical transmission by *Repicephalus appendiculatus* and *Ambyloma hebraeum*. Transmission of LSD is also possible by sharing of the same feeding and watering troughs which may be contaminated by the viruses in the saliva of the infected animals or ingestion of the already contaminated food or by iatrogenic agents (Haig, 1957). Suckling calves may be infected through infected milk (Thomas, 2002).

Transmission by contact in the absence of the arthropod vectors was not efficient (Carn and Kitching, 1995). A study in Ethiopia also showed that communal grazing and watering points were found to be associated with the occurrence of LSD and introduction of new animals to a herd had a strong association with an increased risk of disease in the herd (Getachew *et al*., 2010). Excretion of LSDV in semen was detecting using PCR from experimentally challenged bulls (Osuagwuh, 2006). Great risks are imposed that semen or movement of semen from countries where the disease is endemic can transmit the disease, but no standard procedures were present to detect the presence of LSDV in semen (Irons *et al.*, 2005). Information was unavailable on transmission of LSD virus via semen or embryos. The virus excretes in the semen for up to 22 days in clinically affected bulls and about 12 days in sub clinically affected bulls (Weiss, 1968). There were also assumptions that virus also secreted in vaginal secretions. The extremely resistant nature of the virus to the environment would therefore make venereal transmission very likely (Committee on Managing Global Genetic Resources, 1993).

Virus can be transmitted by animal products such as milk, fomites such as equipment and clothing as well as personnel. Though most infection is thought to be the result of insect transmission, field observations have demonstrated that the spread of the virus from farm to farm and district to district might be due to the absence of complete restriction of all animal movements (Tuppurainen, 2005).

2.3.2. Host susceptibility

All breeds and age group are susceptible; *Bos Taurus* is particularly more susceptible to clinical disease than zebu cattle. Among *Bos Taurus,* fine skinned Channel Island breeds develop more severe disease (OIE, 2010). Lactating cows appearing to be severely affected and result in a sharp drop in milk production because of high fever caused by viral infection itself and secondary bacterial mastitis (Tuppurainen and Oura, 2011). Young animals are severely affected and clinical symptoms are rapid to appear. Apart from these animals, few cases have been reported in Asian water buffalo (*Bubalusbubalis*). Clinical cases or antibodies have been reported in other species such as Oryx, but may have been caused by closely related poxviruses. Generally clinical severity of disease depends on susceptibility and immunological status of the host population (CFSPH, 2008).

2.3.3. Morbidity and mortality rates

In outbreaks of the disease, the morbidity of LSD varies enormously depending on the immunity status and the abundance of mechanical arthropod vectors (Tuppurainen and Oura, 2012). By natural predisposition, *Bos Taurus* is more susceptible than *Bos Indicus* and lactating cows appearing to be at most risk (Davis, 1982; Kitching and smale, 1986a). The distribution and relative abundance of insect vectors were also thought to reflect the differences in morbidity rates in the various habitats. Mechanical insect vectors, which are capable to pierce deep in to the tissue feeding from intravenous blood, are assumed to cause severe clinical LSD (Kitching and Mellor, 1986b; Carn and Kitching, 1995a; Chibota *et al*., 2001). Furthermore, stress factors and trypanosomosis

compromise the immune status of the animal might contribute to the severity of LSD infection. Thus, the morbidity can reach as 100% in natural outbreaks while morbidity rate rarely exceeds 5% (Babiuk *et al*., 2008c; Irons *et al*., 2008).

2.3.4. Geographic distribution

LSD distribution has extended its distribution and currently not only found in sub-Saharan countries but also in Egypt, Western Africa, Middle East countries like Israel (Brenner et al., 2006) and Europe. Epidemiological trend of LSD suggests that there could also be a considerable potential risk of the disease spreading further into North Africa, into the Middle East countries and to Mediterranean regions because of global climatic changes and trade movement in animals and animal products (Davies, 1991; Babiuk *et al*., 2008a). The current global situation of LSD was reported recently by Tuppurainen *et al*. (2015) and presented in Figure 2**.**

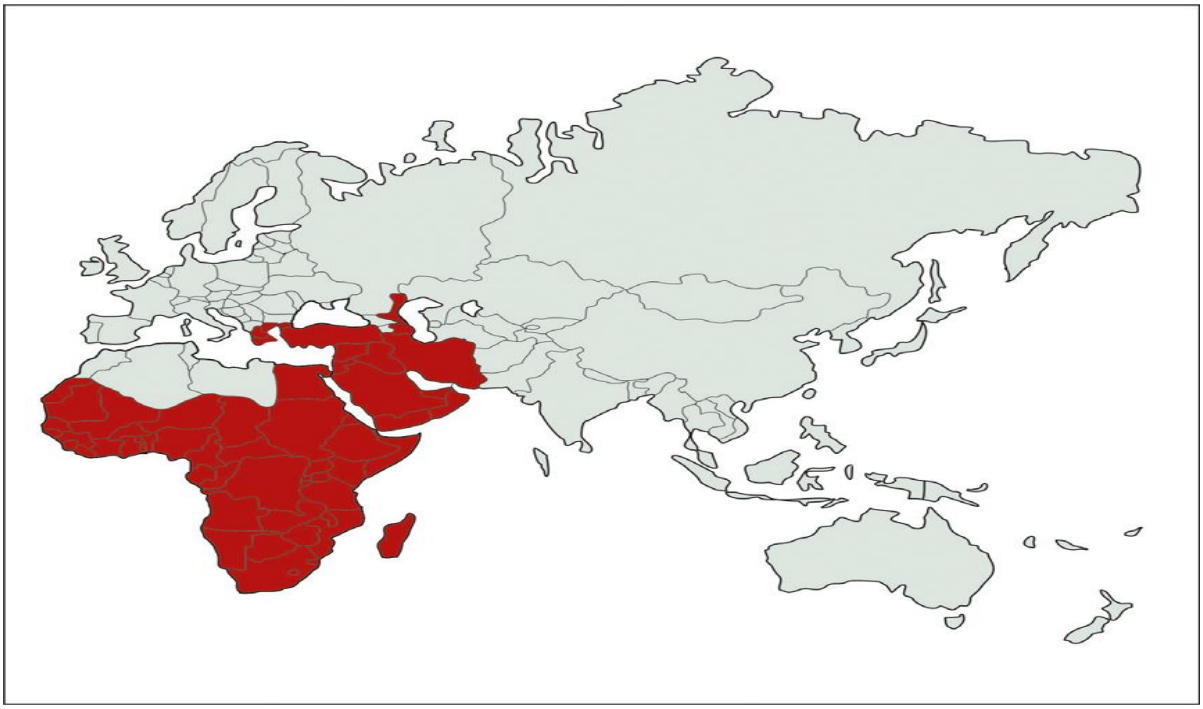

Figure 2: Geographical distribution of lumpy skin disease
(Source: Tuppurainen *et al*., 2015).

2.3.5. Status of the lumpy skin disease in Ethiopia

In Ethiopia, LSD was first observed in 1983 in the western part of the country (southwest of Lake Tana) (Mebratu *et al*., 1984). After its first appearance, an explosive sudden epidemic spread from the north through the central to the southern part of the country. In the subsequent three to five years, it had covered the vast area of the highland and midland parts of the country. LSD is one of reported the diseases in Ethiopia which deserves outbreak notification to the National veterinary services. Studies based on clinical disease observation done around Nekemte town, Wolliso town and in Southern rangeland in Ethiopia have reported different prevalence of LSD ranging from 7 to 28% (Asegid, 1991; Beshahwured, 1991; Regassa, 2003).

However, a variable degree of under-reporting of the outbreak cases could exist from different parts of the country. 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 had 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. Other extensive outbreak were reported in 2006/07 from Tigray, Amhara and Benishangul regions and between 2007 and 2009 the outbreak number progressively increased in central part Oromia region. This showed that an epidemic reoccurs after an interval of 5-6 years cycle in unvaccinated cattle population. The national disease outbreak report during these 10 years showed that LSD has spread virtually to all the regions in the country and in different agro-climatic zones (Gari, 2011).

2.4. Pathogenesis

LSD is developed by infectious LSDV and accompanied with febrile reaction (Vorster and Mapham 2008). Mechanism by which the virus was observed to cause skin lesions was due to replication of the virus in specific cells such as pericytes and endothelial cells

of lymphatic and blood vessels walls. LSD is generalized and epithelia trophic disease that cause localized and systemic reaction and results in vasculitis and lymphadenitis. In some severe cases thrombosis and other symptoms were observed (Radostitis *et al*., 2006; Merck Veterinary manual, 2011). Incubation period of LSD can vary under field condition and experimental conditions vary from 5 days in experimentally inoculated animals and 2-4 weeks in naturally infected animals, gives a maximum incubation period, for regulatory purposes, of 28 days (Wood, 1990; Barnard *et al.*, 1994; OIE, 2010).

Nodules of LSD may be found on subcutaneous tissues, muscle fascia and musculature, which are grey-pink with caseous necrotic cores. A gross lesion of LSD is congested, hemorrhagic, edematous and necrotic and involves all layers of skin, epidermis, and dermis, subcutaneous and underlying musculature (Haig, 1957; and Barnard *et al*., 1994). Circumscribed necrotic lesions may appear in muzzle, mucous membrane of mouth, respiratory tract, trachea, vulva and prepuce which may ulcerate (Bagla, 2005, Radostitis *et al*., 2006). Histopathological sections of early skin lesions of epidermis show an epithelioid cells, lymphocytes, macrophages, plasma cells and fibroblast proliferation appear in later stages and if secondary infection occurs, necrosis, polymorph nuclear and red cells seen. Typical eosinophilic, intracytoplasmic pox inclusion bodies may be seen in cells of epithelioid, hair follicles and cells of muscles and skin glands (Bagla, 2005).

2.5. Clinical Signs

Lumpy skin disease is infectious, eruptive and occasionally fatal disease of cattle. It is an acute to chronic viral disease characterized by skin nodules in the skin and other body parts. It might be exacerbated by secondary bacterial complication (Merck Veterinary Manual, 2011). It is an acute to in apparent cattle disease caused by LSDV. It is characterized by fever, nodule in the skin, mucous membrane and internal organs and swelling of superficial lymph nodes (OIE, 2010; Tuppurinen and Oura, 2011).

Course of lumpy skin disease may be acute, sub-acute and chronic and infection of LSDV may occur both experimentally and under natural condition. The virus causes from

in apparent infection to severe clinical symptoms and those animals which develop clinical disease may have a biphasic febrile reaction. Some of the visible clinical signs are; fever of 40-41.5^{o}C which may last 6-72 hours, lacrimation, increased nasal and pharyngeal secretion, loss of appetite, reduced milk production, some depression and movement reluctance. Severity of clinical signs depends on strain of *Capripoxvirus* and breed of the host and in case of experimental infection route of transmission and dose of the virus also has determinant factor (Carn and Kitching, 1995; OIE, 2010).

LSD infection of cattle under field condition may develop generalized skin lesions after one to two days of febrile, nodular cutaneous lesions appear which may cover whole body ranging from a few to multiple nodules but in majority of the cases, initial evidences of symptoms are lacrimation and fever but some cases are non-febrile. Pre-scapular and precrural lymph nodes are some of the superficial lymph nodes which commonly seen during clinical manifestation of the disease. The most common sites are head and neck, perineum, genitalia, limb and udder; involve skin, cutaneous tissues and sometime underlying part of the muscle (Tuppurinen and Oura, 2011).

Diameter of nodular lesion may be up to 1-7cm diameter appears as round, circumscribed areas of erected hair. In severe cases, ulcerative lesions may develop in mucous membrane of mouth, trachea, and larynx and esophagus (Radostitis *et al*., 2006). Such ulcerative lesion also develops in conjunctiva, muzzle, nostrils and small nodules may resolve spontaneously without any consequence. Secondary bacterial complication and infestation of fly worms may be occurred (CFSPH, 2008). Nasal discharge and salivation may be developed in to mucoid or mucopurulent, lacrimation to conjunctivitis, superficial lymph nodes markedly enlarged and inflammatory and edematous lesions in limbs, brisket and genitalia may develop and skin lesion may be necrotic and ulcerative lesions may become fibrotic. Some of the scabbed lesion remains there and other sloughed leaving a hole full of skin thickness which becomes infected by pus-forming bacteria and large areas of skin may slough (Barnard, 1994). Rapid deterioration in body condition results and animals that recover may remain in extremely poor condition for up to 6 months. Pneumonia is a common bacterial complication and usually fatal disease and

absence of estrus cycle and abortion are common consequences observed in female animals and painful genitalia may prevent bulls from serving (Bagla, 2005).

2.6. Diagnosis of LSD

Lumpy skin disease can be clinically diagnosed by its pathognomonic nodular lesions on the skin, mucous membranes, swelling of the superficial lymph nodes and systemic involved symptoms by experienced practitioners. However, LSD could be confused with other diseases causing skin lesions such as pseudo-lumpy skin disease (bovine herpesvirus-2 infection), insect bites, demodecosis, and dermatophilosis. Moreover, diseases causing mucosal lesions, such as rinderpest, bovine viral diarrhea/ mucosal disease, and bovine malignant catarrhal fever, also complicate field diagnosis (Barnard *et al*., 1994). Although skin and visceral pox lesions are strong indicative of the diseases in question, a definitive diagnosis requires laboratory confirmation. Above all, the effective control or eradication of LSD in endemic and non-endemic areas requires rapid and accurate diagnosis methods to confirm a presumptive diagnosis (Tuppurainen *et al*., 2005).

Field diagnosis of LSD is often based on characteristic clinical signs of the disease. These may base usually the presence of fever, nodules on the skin, mucous membranes, enlargement of superficial lymph nodes and edema of the skin in livestock, but it is a presumptive diagnosis that must be confirmed by laboratory methods. However, mild and subclinical forms require rapid and reliable laboratory testing to confirm diagnosis (Alaa *et al*., 2008; Knopvelsiekte, 2008).

2.6.1. Differential diagnosis

The skin lesions of pseudo-lumpy skin disease (caused by herpes virus-2) lesions involve only the epidermis and leave a scab after sloughing, and systemic signs do not develop (Geering *et al*., 1995), skin tuberculosis urticarial, bovine lymphangitis, cowpox, mycotic dermatitis, photosensitization, severe infections with demodectic mange could also be

confused with LSD. Differentiations depend on isolation of the animal and identification of the virus. Histopathological and ultrastructural examination of nodules may be helpful (Barnard *et al*., 1994).

2.6.2. Histopathology

Histopathological examination shows that the epidermis is extensively necrotic. While in the intact areas, some ballooning degeneration of squamous epithelial cells with occasional intracytoplasmic inclusion is observed. Prominent lesions of vasculitic necrosis with cell debris and severe diffuse infiltration with inflammatory cells mainly neutrophils, have been seen in the superficial and deep dermis (Prozesky and Barnard, 1982). Histophalogy of skin lesions provides a method to recognize the intracytoplasmic inclusion bodies of LSDV infected cells by using hematoxylin and eosin staining. Immunohistochemically methods e.g. immuneperxidase staining can be used to identify the antigen in the skin lesions (Haines and Chelack, 1991; Haines and Clark, 1991). Histopathology can also be an important tool to exclude viral, bacterial or fungal causes of nodular development in clinical cases and characteristic cytopathic effects (necrosed epidermis, ballooning degeneration of squamous epithelial cells and eosinophilic intracytoplasmic inclusion bodies) in cases of LSD are well documented (Ali *et al*., 1990; Brenner *et al*., 2006).

2.6.3. Electron Microscopy

Electron microscopy can be used for rapid detection of *Capripoxvirus* virions in skin lesions. The large brick-shaped *Capripoxvirus* particles are readily seen in most lesion material from day 9 to 21 (Davis *et al*., 1971). At least three different sets of biopsy material from different affected animals should be diagnosed. The examination can be done using negative staining on fresh biopsy material or in 5 µm sections formalin fixed material processed for histopathology and positively stained with uranyl acetate and lead citrate (Woods, 1988). As the author indicated, the later method is applicable to LSD examination. However, electron microscopy cannot differentiate between SPPV, GTPV

and LSD (Kitching and Smale, 1986a). Neither can electron microscopy distinguish *Capripoxviruses* from *Orthopoxviruses* except by the application of specific immunological staining. *Orthopoxviruses* infections are documented in buffalo and cattle in India (Singh *et al*., 2007) and Cattle in Brazile (Damaso *et al*., 2000).

2.6.4. Virus Isolation on cell culture

Virus isolation is essential in the confirmation of clinical disease and determining the isolate. LSDV can be isolated using a wide variety of sheep kidney cell, goat and calf kidney and rabbit kidney cells (Binepal *et al*., 2001). However, virus isolation is best carried out in primary lamb kidney cell or lamb testis cell cultures (Babiuk *et al*., 2007). Secondary lamb testis cell line (OA3.Ts) has been proven to replace the primary cell cultures for better efficiency and easily managed to grow *Capripoxvirus* (Babiuk *et al*., 2007).

The development of cytopathic effect (CPE) may take seven up to 14 days after inoculation. The development of CPE is characterized by rounding, shrinking and detachment of cells. Strains of *Capripoxvirus* that cause LSD have been adapted to grow on the Chorioallantoic membrane of embryonated chicken eggs and African green monkey kidney (Vero) cells. *Capripoxvirus* isolation can be confirmed by immunostaining using anti-*capripoxvirus* serum (Babiuk *et al*., 2007) but it is not yet possible to differentiate between SPPV, GTPV and LSDV, as there is only a single *Capripoxvirus* serotype (Kitching and Smale, 1986a). Immunostaining also allows easier visualization of C*apripoxvirus* plaques (Babiuk *et al*., 2007).

2.6.5. Virus isolation on primary cell

The use of cell cultures has replaced the use of live animals in the diagnostics of LSDV. Thin-skinned susceptible calves can be inoculated by intradermal route with dilutions of the virus. Intradermal inoculation of a calf may be made with 0.1 ml aliquots of a 10% homogenate of the biopsy in transport medium. This should be diluted further to

1/100,000, and inoculated at 4 sites per dilution on the shaved side of the calf (Weiss, 1968). The lesions will appear after 5-9 days and show the characteristic form of LSD skin reaction. Further biopsy material can then be taken. In experimentally produced LSD, only 40-50% of the infected cattle developed generalized skin lesions (Weiss, 1968).

2.6.6. Serological methods

Neutralizing antibody appears 3-4 days after the onset of the clinical signs and reaches the peak titer level in 2-3 weeks. Both complement fixing and precipitating antibodies are present in the serum of infected and recovered animals. Immunological defense against *capripoxvirus* relies mainly on cell-mediated immune response and humoral immunity would remain in the circulation for a short period within the time range of mostly seven to eight months (Capstick and Coackley, 1962; Lefèvre and Gourreau, 2010; OIE, 2010). The maternal immunity provides protection from LSD in cattle for at least 6 months (Davies, 1991).

All *Capripoxviruses* share a common major antigen for neutralizing antibodies, hence; not possible to distinguish strains of *Capripoxvirus* from cattle, sheep or goats using serological techniques (OIE, 2010). The interpretation of serological results may sometimes be difficult due to low antibody titers in vaccinated animals and some individuals following mild infection (Coetzer, 2004). Moreover, all serological assays face the same problem that different surface proteins of non-enveloped intracellular mature virions and intracellular and extracellular enveloped virions induce the formation of different antibodies in the host, and their relative proportions may vary during different stages of infection (Tuppurainen and Oura, 2012). The gold standard for detecting specific antibodies to *Capripoxviruses* is the virus neutralization test. It is the most common widely used serological test for C*apripox* antibody detection (Davies and Otema, 1981; Babiuk *et al*., 2008a). Although, it has high specificity and excludes cross-reaction with cowpox and *Parapoxvirus* antibodies, it is not sensitive enough to identify

animals that have in contact with the viruses but have developed only low levels of neutralizing antibodies (Davies and Otema, 1981).

2.6.7. Molecular diagnosis techniques

Molecular diagnostic tests play an important role in monitoring the spread of these viruses and controlling outbreaks in susceptible livestock. Polymerase chain reaction (PCR) does provide a rapid and sensitive diagnostic technique for *Capripoxvirus* genome detection. Furthermore, the nucleic acid detection results confirmed our conclusions pertaining to the relatively low VI isolation rates because PCR testing was able to detect viral RNA in 100% of skin and blood samples collected from clinically infected cows. Several groups have reported using conventional PCR (Heine *et al*., 1999; Mangana-Vougiouka *et al*., 2000) or real time PCR for detection of *Capripoxvirus* genetic material (Balinsky *et al*., 2008; Bowden *et al*., 2008).

LSDV belongs to *Capripoxvirus* (CaPV) genus of the *Poxviridea* family of viruses also comprises GTPV, and sheep pox viruses, members of the CaPV genus are closely related, with genomic identities ranging from 96% between viral species to 99% between isolates of the same species (Tulman *et al*., 2002). Since PCR is simple, fast and sensitive method, possible to detect *Capripoxvirus* genome in EDTA blood, biopsy, semen or tissue culture samples (Ireland and Binepal, 1998). However, it does not allow differentiation between LSD and sheep and goat poxviruses. Primers for viral attachment protein gene and the viral fusion protein gene (Ireland and Binepal, 1998) are specific for all the strains within the genus *Capripoxvirus*. By the use of sequence and phylogenetic analysis, strains of virus can be identified. It is impossible to distinguish between strains of *Capripoxvirus* from cattle, sheep or goats using serological techniques. The only rapid method available so far for a routine genotyping of the three *Capripoxviruses* is a real-time PCR assay based on dual hybridization probe technology (LeGoff *et al*., 2009). The strengths of real-time PCR are its speed, its quantitative nature and the ability to include controls for detection of reaction inhibitors (Babiuk *et al*., 2008c).

Capripoxvirusvirus genotyping using a cost-effective, rapid, highly sensitive, specific and easy method to perform urgently needed by diagnostic laboratories in countries endemic for LSD, SPP, and GTP. The accurate and rapid identification of the virus will assist in the appropriate vaccine selection and will improve the prospects for the control and eradication of the disease (Wittwer *et al*., 2003; Liew *et al*., 2004; Dames et al., 2007, Wu *et al*., 2011). Real-time PCR assays can also be used to differentiate LSDV, SPPV, and GTPV from each other (Lamien *et al*., 2011b).

However, poorly equipped field laboratories face difficulties accessing these molecular techniques that are reliant upon expensive and relatively fragile equipment. A new group of nucleic acid detection assays for rapid detection of *Capripoxviruses* has been shown to be highly specific with no apparent cross-reactivity to other related viruses that cause similar clinical signs. The assay formats include Loop-mediated isothermal amplification (LAMP) (Notomi *et al*., 2000), which is a novel method of nucleic acid amplification that is catalyzed by a DNA polymerase with standard displacement activity and occurs under isothermal conditions at temperatures between 60 and 65 ^{o}C (Mori and Notomi, 2009; Notomi *et al*., 2000). It is simple to use, inexpensive, and particularly well suited for the diagnosis of *capripox* in less well equipped laboratories and in rural settings where resources are limited (Notomi *et al*., 2000).

2.7. Economic Importance of the Disease

Capripoxviruses are becoming an emerging worldwide threat to sheep, goats and cattle (Babiuk *et al.*, 2008a). Lumpy skin disease is one of the economically significant diseases in Africa and the Middle East countries that cause severe production loss in cattle. The world organization for animal health (OIE) categorizes the disease as notifiable diseases because of its severe economic losses. The economic importance of the disease was mainly due to having high morbidity rate rather than mortality (Tuppurainen and Oura, 2011). The financial implication of these losses is greatly significant to the herd owners, consumers and the industrial sectors which can process the livestock products and by products. In intensive farming of cattle, the direct and indirect production losses caused

by LSD were estimated to be as high as 45-60% (Tuppurainen and Oura, 2011). It was reflected that the severity of the disease was much more in developing countries where the poorest small scale farmers was found. Reports from Ethiopia indicated that the financial loss estimated based on milk , beef, draught power, mortality, treatment and vaccination costs in individual head of local zebu were lost 6.43 USD and for the Holstein Friesian 58 USD (Getachew *et al.*, 2010). The disease was mainly affects cattle with subsequent effects on production through the morbidity and reduces productivity (CFSPH, 2008).

Major consequences of the disease are retarded genetic improvement, limits the ability of the animal to work, draught power and traction loss, abortion in pregnant cows, marked reduction of milk yield during the active case of the disease, sterility and infertility in both sexes of cattle, permanent damage to hide and chronic debility in beef cattle (Tuppurainen, 2005; OIE, 2010).

Overall, LSD is considered as a disease of high economic pressure because of its ability to compromise food security through protein loss, draft power, reduced output of animal production, increase production costs due to increased costs of disease control, disrupt livestock and their product trade, result of reduced milk yield, weight loss, abortion, infertility in cows, mastitis and infertility in lactating cows, infertility in bulls (Weiss, 1968; Kumar, 2009). Permanent damage to the skin and hide greatly affect leather industry. It causes ban on international trade of livestock and causes prolonged economic loss as it became endemic and brought serious stock loss (Getachew*et al.*, 2010).

2.8. Control and Prevention

2.8.1. Vaccination in endemic areas

LSD control and prevention in endemic countries like Ethiopia relies mainly on vaccination. The experience in the major parts of the country showed that the vaccination approach is commonly chosen and is often that of ring vaccination around a local foci

outbreak when it occurs. Animals that recover from virulent LSD infection generate lifelong immunity consisting of both a humoral and cell mediated protective immunity (Kitching *et al*., 1987).

Immunity acquired from natural infection of the disease might be life-long and vaccination has been successfully used. LSD could be kept under control by vaccination of cattle every year (Thomas, 2002). All strains of *Capripoxvirus* examined so far, whether of bovine, ovine or caprine origin, share a major neutralizing site, so that animals that have recovered from infection with one of the strains are resistant to infection with any other strain. Consequently, it is possible to protect cattle against LSD using strains of *Capripoxvirus* derived from either of the sheep or goats as used in Egypt by Romanian sheep pox strain (OIE, 2010).

Live, attenuated vaccines against LSD are commercially available. These have antigenic homology and there is cross protection among them. Local strain of Kenyan sheep and goat pox virus has been shown to effectively immunize sheep, goats and cattle against infection with *Capripoxvirus* with a remarkable success. The next one is attenuated South African LSD virus (Neethling strain) vaccine derived from cattle, freeze dried product is also available (OIE, 2010). In countries where LSD is endemic, vaccination against this infection was successfully used by vaccinating animals every year. LSDV has been used as a recombinant *Capripoxvirus*, combined with rinderpest or rabies virus and *Capripoxvirus* is an excellent vector for recombinant vaccines because of its narrow host range (Shen *et al*., 2011).

2.8.2. Vaccination in new areas

Maternal immunity provides protection from LSD in calves at least for 6 months. Risks of introduction of the disease in to the new areas are by the introduction of infected animals and contaminated materials. If the occurrence of LSD is reported or confirmed in new areas, before the spread of the disease to other areas extensively, quarantine of the area and slaughtering of the diseased and in contact animals are used to control the disease. When equipment contacted it must be cleaned and disinfected (Davies, 1991).

Ring vaccination of cattle within the foci of infection with a radius of 25-50 km , quarantine and restriction of animal movement should be applied to eradicate the disease from infected area, but if the area coverage of the disease is large, the most convenient techniques for the control of the disease is mass vaccination (Yeruham *et al*., 1995).

2.8.3. Other control techniques

For countries free of the disease, the introduction of the disease can be prevented by restriction of the importation of the animals and their products. In those nations which experience the infection the spread of the LSD can be limited by restriction of the animal movement from one place to another, quarantine or keeping of sick animals well apart from the rest of the herd and such animal must not share drinking or feeding troughs and also by awareness creation of the farmers (Thomas, 2002).

Animals older than six months must be vaccinated against LSD during spring. It is safe to vaccinate pregnant cows. All animals must be vaccinated once a year. When vaccinating the animals during a disease outbreak, it is important to use one needle per animal so that the virus is not spread from sick to healthy animals but the practicality and economic feasibility of use of one needle per cattle need to be carefully considered. Professional help and recommendation on vaccines must be carefully followed and practiced. Broad spectrum antibiotics are also given to prevent the secondary bacterial complication as the defense mechanism of the body weakened which can prolong the complete recovery of the diseased animals (CSFPH, 2008).

2. MATERIALS AND METHODS

3.1. Study Area

The outbreak investigation was conducted from November 2016 to April 2017 in Bale Administrative Zone of Oromiya Regional State, which is located 430 km southeast from the capital city, Addis Ababa. Bale zone is bounded by four Oromiya Zones and Somali Regional State: West Arsi zone in the north, Guji zone in the south, Somali Regional State and West Hararge zone in the east and West Arsi Zone in the West. Bale zone is mostly pastoral and covers a vast lowland area in southeast Ethiopia. The town of the zone Robe is geographically found at 7°7′N 40°0′E with an elevation of 2,492 meters above sea level (Bale zonal pastoral are office, 2016). Bale zone of Oromia regional state was purposively selected for the study based on LSD outbreak report to Yabello Regional Veterinary Laboratory and Zonal pastoral office. Sawena district found in Bale administrative zone, Oromia regional state in which LSD outbreak was investigated.

The altitude of the study area ranges from 850 to 2800 m.a.s.l, where the lowland area predominates with a narrow strip of high land area in the Northern part of Sawena district. The area experiences a bimodal rainfall occurring from September to November and March to June. An average annual temperature of 20-25 °C and rainfall of 200 mm are recorded in Sawena district (Bale zonal pastoral office, 2016). Surface water is a serious problem in Sawena district, where only seasonal streams, ephemeral ponds and shallow temporary wells are sources of water in the rainy season and these usually dry out after a few days during the dry season. Sawena district has a pastoral vocation with livestock rearing being the dominant economic activity of the district.

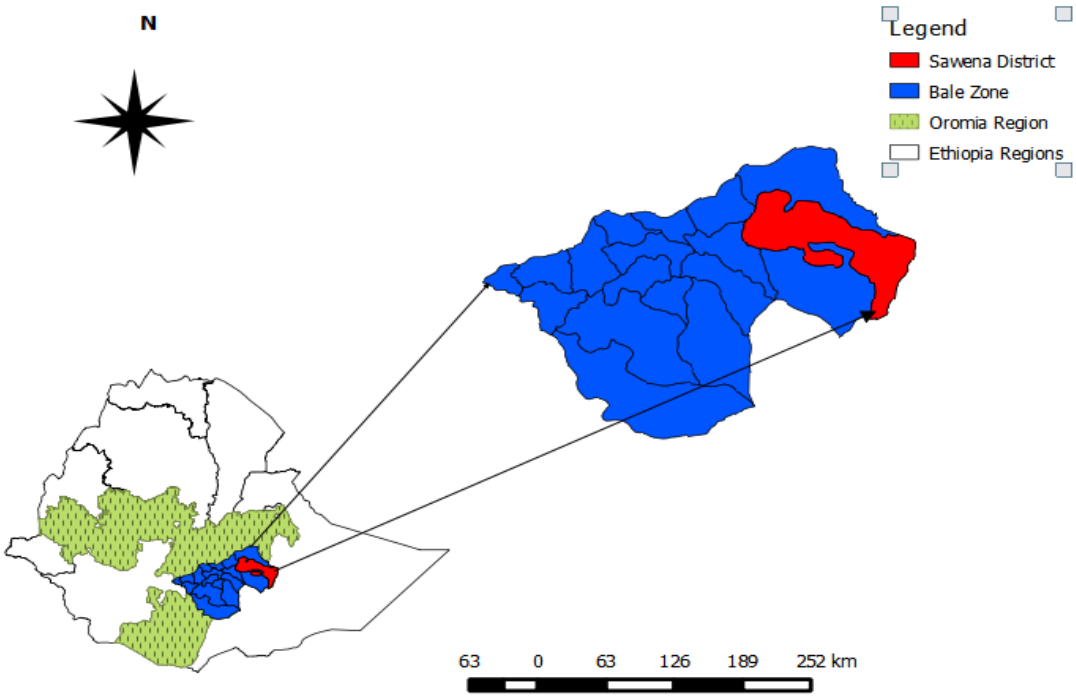

Figure 3: Map of the study area

3.2. Study Population

The active outbreak investigation was conducted in extensive management system in local cattle. All age and sex groups which are reared under two different production and management systems (small number of animals specially milking cows and calves that are kept around the home and the majority of animals which are driven long distances in search of good pasture and surface water) were involved in the outbreak investigation in Sawena district of Bale zone.

3.3. Study Design

The study areas were selected purposively based on the reports of LSD outbreaks to Yabello Regional Veterinary Laboratory. Active outbreaks were assessed together with veterinary professionals who are working in the Regional veterinary Laboratories, zonal veterinary office and district veterinary clinics. Clinical and epidemiological data were

recorded and samples were collected for virus characterization isolation, and identification that were conducted at animal health and diagnostic investigation center (NAHDIC) at Sebeta, Ethiopia

3.3.1. Questionnaire survey and epidemiological data collection

Additionally structured questionnaires were used to interview 20 pastoralists on LSD occurrence and its associated impacts. In the questionnaire, the vaccination history, clinical sign, age and sex of affected cattle and mortality and source of the disease was gathered (Annex). The cattle owners expressed their views and shared their practical knowledge's about the prevailing situations regarding LSD using their native language (Afan Oromo) among the questions included were awareness about clinical signs of LSD, age affected, sex affected, seasons of LSD outbreak, livestock movement, milk reduction, abortion in pregnant cows, presence of death due to the disease, status of vaccination and water sources for the livestock. The pastoralist called the local name of the diseases "Itesa''. The questionnaires were used to interview individual owners of cattle at each outbreak or study sites. Relevant data was gathered by observing clinically sick animals and interviewing cattle owners and animal health workers working at the field. Information was carefully recorded on a designed format.

3.4. Sample Size and Sampling Technique

During the study period, the total animal examined was 522. A field investigation was conducted purposively at the specific site of the outbreak within Bale zone. Animals with the clear signs, symptoms and suspected to be diseased with LSD were selected to be sampled. Skin nodules from cattle which were with severe clinical signs of the disease were taken aseptically by washing and cleaning the area and removing the hairs with the help of sterile scalpel blade. Nasal swabs were collected with sterile swabs from infected animals.

3.5. Data and Sample Collection Methodology

3.5.1. Clinical examination

After arriving at the specific outbreak sites during disease outbreak investigation, the animals were examined for presence of nodules on the head and neck, perineum, genitalia, udder and limbs and markedly enlarged regional lymph nodes, watering eyes, increased nasal and pharyngeal secretions in each individual animal. The temperature of animals was checked whether it is fevered or not.

3.5.2. Sample collection

According to the procedures of OIE (2010), samples for virus isolation and molecular characterization were collected from clinically sick animals. From total of 20 samples, 7 skin nodules and 13 nasal swabs were collected. The representative samples were aseptically collected from infected cattle with typical develops severe clinical sign of the disease. Skin nodules were taken aseptically by washing and cleaning the area and removing the hairs with the help of sterile scalpel blade and nasal swab were taken aseptically by cleaning the external part of noses. Tissue samples were placed in the sterilized universal bottle and nasal swab were placed in the sterilized cryovial tubes kept at -20C until transported to virology laboratory of National Animal Health Diagnostic and Investigation Center, Sebeta Ethiopia.

3.6. Laboratory Techniques

3.6.1. Sample processing

The skin biopsy and nasal swab samples were thawed at room temperature in Biosafety cabinet class II. The biopsy tissue samples were minced using sterile scissors and forceps. About 1gm of the sample was grounded using sterile pestle and mortar by adding 5 ml of the virus transporting media (VTM). The tissue suspension was centrifuged at 1,500 rpm for 10 min; the supernatant fluid was collected and labeled for the extraction of DNA of the virus.

3.6.2. Extraction of viral DNA

DNA extraction was performed using DNeasy (from blood and tissue) extraction kit (QIAGEN, ROCM and HAAS Company, USA) according to the procedures of the manufacturer (Qiagen). A 20 µl Qiagen protease (proteinase K) dispensed in to a 1.5 ml of micro centrifuge tube, and 200 µl of the supernatant DNA from specimens was added to the micro centrifuge tube. In addition to ensure efficiency of lysis 200 µl of AL buffer (lysis buffer) was added to the sample and mixed by pulse-vortexing for 15 sec. The mixed sample was then incubated at 56 ^{0}C for 10 min. and briefly centrifuged. A 200 µl of ethanol (96-100%) was added to the sample and mixed thoroughly with the help of vortex mixer for 5 sec. and briefly centrifuged. The mixture was transferred in to QIAamp Min spin column 2 ml collection tube and centrifuged at 8,000 rpm for 1 min. The spin column was transferred in to a new 2 ml collection tube and 500 µl of buffer AW1 was added and centrifuged for 1 min at 8000 rpm. Then, the collection tube was discarded and the Min spin column placed in a new 2 ml collection tube and 500 µl buffer AW2 was added and centrifuged for 3 min. at 14,000 rpm. The mini spin column was transferred carefully in to a new 2 ml collection tube and discarded. The old collection tube with filtrates was centrifuged for 1 min. and 200 µl buffer AE (distilled water) was added and incubated for 1 min at room temperature (15-25^{o}C), then centrifuged for 1 min at 8000 rpm. The extracted DNA was collected and stored at -20°C until used for subsequent experiment.

3.6.3. Polymerase chain reaction (PCR)

The extracted DNA was amplified by polymerase chain reaction (PCR) assay using Capripoxvirus-specific primers: (SpGpRNAPol F) 5″-TCTATGTCTTGATATGTGGTG GTAG-3″ and (SpGpRNAPol R) 5″-AGTGATTAGGTGGTGTATTATTTTCC-3″, (Lamien *et al.*, 2011b). DNA was amplified in a final volume of 50 µl containing the following: 5 µl PCR buffer (10 mM), 1.5 µl MgCl2 (25 mM), 1 µl dNTP mixture (10 mM), 1 µl forward primer (50 mM), 1 µl reverse primer (50 mM), 5 µl DNA template, 0.5 µl Taq DNA polymerase (5 U/µl) (Invitrogen) and 35 µl of RNAse-free water. The

PCR was run in a thermocycler (Applied Biosystems® 2720, USA) using the following amplification program: initial denaturation at 95°C for 1 min, followed by 40 cycles of denaturation at 95°C for 30 sec. annealing at 55°C for 30 sec. and elongation at 72°C for 1 min. An Additional elongation step was performed at 72°C for 5 min. and PCR products were stored at +4 ^{o}C until analysis. The amplified products were analyzed by running the PCR product on 2% gel electrophoresis and positive results were confirmed based on the size (172 base pairs [bp]) of the bands.

3.6.4. Agaros gel electrophoresis

The amplified DNA from extracted specimens was analyzed by Agaros gel electrophoresis as described by (Mangana-Vougiouka *et.al*, (1997) with some modification to confirm the presence of DNA. Amplified products was analyzed using a gen ruler^{TM} 100bp DNA ladder (Fermentes, Germeny) as a molecular marker on 2% agarose gels prepared in Tris/Acetate/EDTA (TAE) buffer and 10mg/ml ETDM-bromide strain, then 20µl of PCR product was mixed with 4 µl loading buffer and loaded to wells in gel and run at 100 volt for about 60 minutes in parallel with DNA molecular weight marker in electrophoresis apparatus until the DNA samples have migrated a sufficient distance through the gel. DNA bands were visualized using an UV transilluminator at a wavelength of 590 nm, and positive results were confirmed according to the size of the bands formed on agarose gel. The PCR results were considered positive for LSDV and GTPV DNA when a 172bp band was observed and for SPPV the band size is 151bp.

3.6.5. Virus isolation

The supernatant of skin biopsy sample were thawed at room temperature. Approximately 1 ml was inoculated on Vero cell lines according to the method of Elzein *et al*., (2003) and Balinsky *et al*., (2008) to ascertain the presence of infectious virus. The Vero cell line was propagated in Glasgow Eagles minimal essential medium (GMEM) supplemented with 10% calf serum. Vero cell line was grown in 25 cm^2 tissue culture flask and was inoculated with 5% CO2 at 37^{o}C until the cell became confluent monolayer washed three times using sterile warm PBS pH of 7.2 in Bio-safety cabinet level II. One ml of tissue

homogenate was inoculated onto the confluent monolayer and incubated at 37^{o}C and allowed to absorb for 1 hour. Then, to the infected monolayer, 10 ml of GMEM, containing antibiotics and 2% fetal calf serum was added into the flask and incubated at 37^{o}C. All the flasks, including the control flasks were inoculated at 37^{o}C in a humidified incubator with 5% CO_2. The medium was changed every 48 hours. Cells were monitored daily using an inverted microscope for evidence of virus induced cytopathic effects (CPEs) for 7-14 days post-inoculation. Three more blind passages were carried out for samples that were initially negative for CPE. Infected cells developed a characteristic CPE consisting of retraction of the cell membrane from surrounding cells; eventually rounding and aggregation of cells. When 80% CPE was observed, virus was frozen overnight at -20^{o}C and harvested. The harvested cell culture was thawed twice at room temperature to release the virus particles.

3.7. Data Management and Analysis

The collected data was coded, entered and stored into Microsoft Excel spread sheet 2010. The data was thoroughly screened and properly coded before subjecting to statistical analysis. The data was imported from the Microsoft Excel and analyzed using Statistical Package for Social Sciences (SPSS) software version 20. The morbidity and mortality were estimated in accordance with sex and different age categories. Confidence Interval was also used to describe morbidity and mortality across different variables. Chi-square (X^2) was employed to test the presence of association among different categorized variables. In all the analyses, confidence levels at 95% were calculated, and a $P \leq 0.05$ was used for statistical significance level (Moulton *el al*., 1995). Descriptive statistics was also used to quantify the results on awareness of community on importance of the disease; age and sex of affected and died cattle during the outbreaks.

3.8. Ethics approval

Sampling from Animals were carried out according to the experimental practice and standard approved by the Animal welfare and Research Ethics committee at Addis Ababa University College of Veterinary Medicine and Agriculture, Bishoft Campus that is accordance with the International guidelines for Animal welfare, with verification number VM/ERC/25/06/09/2017.

4. RESULTS

4.1. Observed Clinical Signs

Out of 522 animals examined, clinical manifestation relevant with LSD was recorded in 18% (94/522) animals. The common clinical signs observed in cattle affected by LSD were fever, development of different sizes of circumscribed nodules on the skin, necrotic nodules, deep scab formation, swelling of dewlap, and enlargement of superficial lymph nodes. Lacrimation, dewlap and superficial lymph nodules enlargement were very prominent. Burst necrotic wounds were often complicated with secondary infection (Fig. 4 and 5).

Figure 4: Characteristic signs of LSD with generalized circumscribed skin nodules covering the entire body. Encircled area showed developed circumscribed nodules on the skin.

Figure 5: An inflammation and whiteness of the eye of cattle infected with LSD. Encircled area showed lacrimating with probably due to secondary bacterial complication**.**

Of the kebeles where outbreak of LSD was investigated, slightly higher morbidity rate was observed in Arda galma kebele but the mortality and case fatality is relatively lower (Table 1). The clinical cases of LSD was significantly highest in younger cattle (p=0.001) and in male animals (p=0.003) as compared with the other age and sex categories respectively (Table 2).

Table 1: Morbidity rate, mortality rate and case fatality rates of LSD outbreaks in study area.

Name of Kebeles	**No. of Susceptible cattle**	**No. of affected cattle**	**No. of death**	**Morbidity rate (%)**	**Mortality rate (%)**	**Case fatality**
Arda galma	186	39	2	20.96	1.08	5.13
Kore korme	336	55	5	16.36	1.49	9.09
Total	**522**	**94**	**7**	**18.00**	**1.34**	**7.44**

Table 2: LSD morbidity rate in different age and sex groups

Risk factor	No. of cattle at risk	No. of cattle affected	Morbidity rate (%)	X^2	P-value
Age					
<2	107	31	29.71	14.562	0.001
≥2-4	314	41	13.05		
>4	101	22	21.78		
Total	**522**	**94**	**18.00**		
Sex					
F	369	76	20.59	11.554	0.003
M	153	18	11.76		
Total	**522**	**94**	**18.00**		

The mortality observed among the different age groups was significantly significant ($P<0.05$) which was higher in young animals less than 2 years of age (Table 3).

Table 3: Mortality rate in association with risk factors of age and sex

Risk factors	No. of cattle at risk	No. of cattle affected	No. of death	Mortality rate (%)	X^2	P-value	Case fatality
Age							
<2	107	31	5	4.67	11.55	0.003	16.12
>2-4	314	41	1	0.31			7.14
>4	101	22	1	0.99			4.54
Total	**522**	**94**	**7**	**1.34**			**7.44**
Sex							
F	369	76	4	1.08	0.62	0.428	5.2
M	153	18	3	1.96			16.66
Total	**522**	**94**	**7**	**1.34**			**7.44**

4.2. Molecular Characterization

4.2.1. Polymerase Chain Reaction

The extracted DNA of 20 specimens was amplified using *Capripoxvirus*-specific primers. The amplicon size of PCR product had molecular weight of 172 bp (Fig. 6), which is the expected amplicon size for the LSDV genomic region targeted. The resulting PCR products of LSDV uniformly aligned in on line suggesting they have the sample amplicons size.

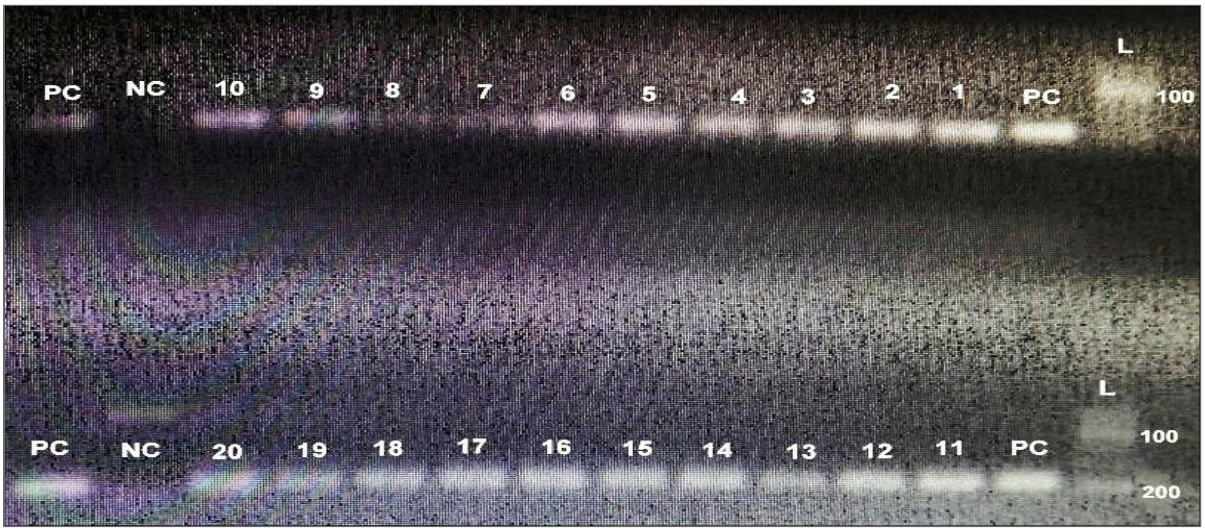

Figure 6: PCR based detection of LSDV in specimens of infected animals. L=DNA ladder; PC=Positive control; NC=negative control.

4.2.2. Real–Time PCR

The extracted DNA from both skin biopsies and nasal swabs were directly tested by PCR and LSDV differentiated from SPPV. The peak melting curve of the real time revealed that, all 20 virus isolates were characterized as LSDV since the negative control for LSDV was 59.9 ^{o}C while the positive control was 73.9 ^{o}C and the range of samples melting temperature was 73.2 ^{o}C-73.9 ^{o}C (Fig. 7).

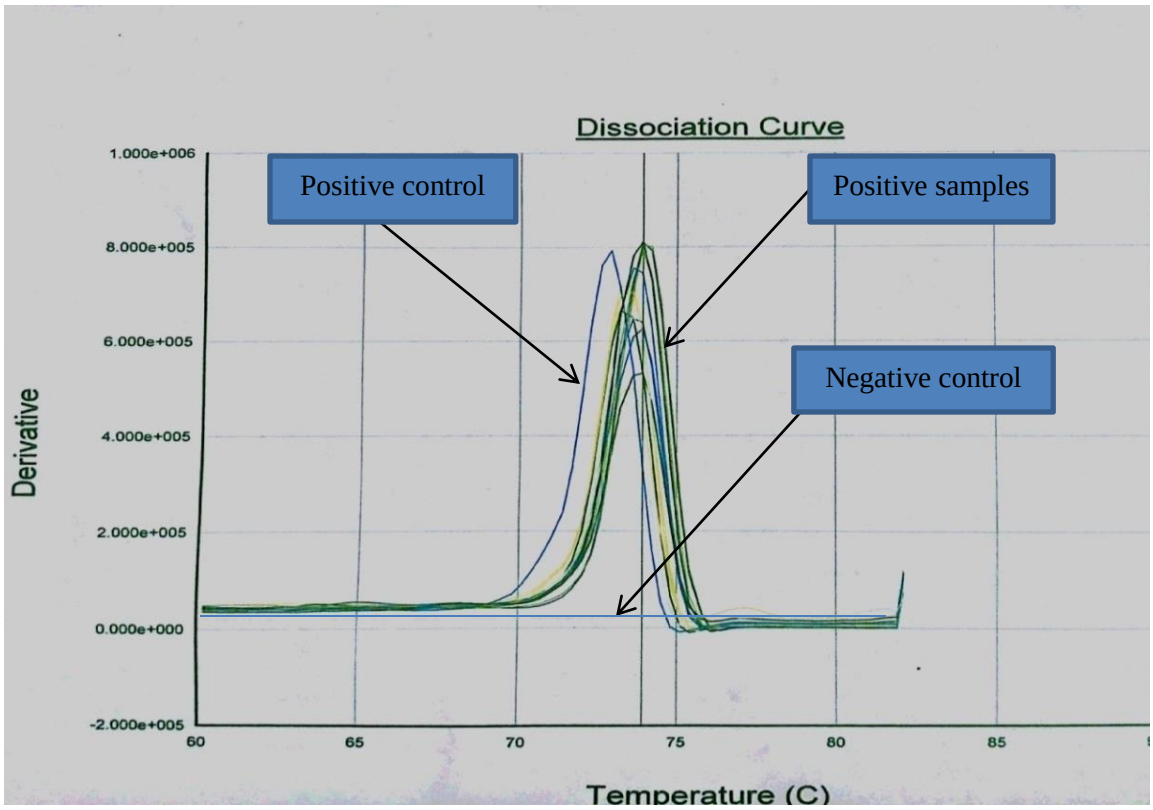

Figure 7: Real time PCR dissociation curve showing temperature values of the melting peaks obtained from clinical samples.

The known CaPV positive samples were tasted for comparison. Real-time PCR assay detected differences in the melting point temperatures for SPPV and LSDV after fluorescence melting curve analysis from each other (Table 4).

Table 4: Ct values and sample type for characterized 20 samples

Type of samples	RT-PCR Ct value	Kebele name sampled
Swab	37.9	Arda galama
Swab	45.4	Kore korme
Tissue	21.5	Arda galma
Swab	40	Arda galama
Swab	36.1	Kore korme
Tissue	45.8	Kore korme
Swab	41	Arda galma
Swab	38.2	Kore korme
Tissue	35.5	Kore korme
Swab	40.2	Kore korme
Swab	40.1	Kore korme
Tissue	19	Kore korme
Swab	38.6	Kore korme
Swab	38.7	Kore korme
Swab	37.7	Kore korme
Tissue	24.6	Arda galma
Swab	40.4	Kore korme
Tissue	25.8	Arda galama
Swab	39.3	Kore korme
Tissue	34.5	Arda galma

4.3. Virus Isolation

Out of the 11 samples (5 skin biopsies and 6 nasal swabs), characteristic of poxvirus CPE was observed in infected Vero cells in 6 samples within 5-11 days post-inoculation following one to three times blind passages while the virus was not isolated from the remaining 5 samples. The CPEs were characterized by rounding of single cells,

aggregation of dead cells and destruction of monolayers (Fig. 9). None of the negative control cultures showed any CPE (Fig. 8)

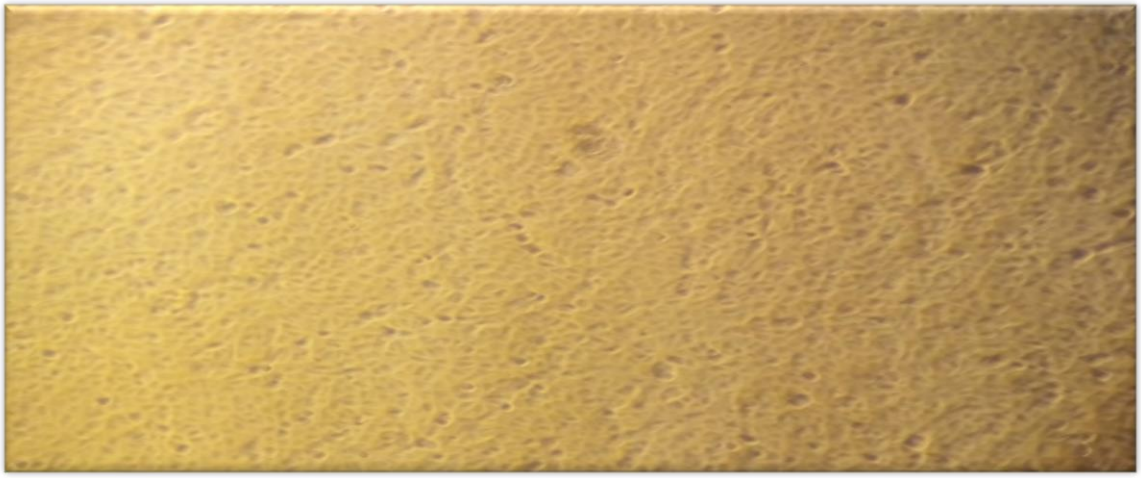

Figure 8: Un-inoculated Vero cell (negative control)

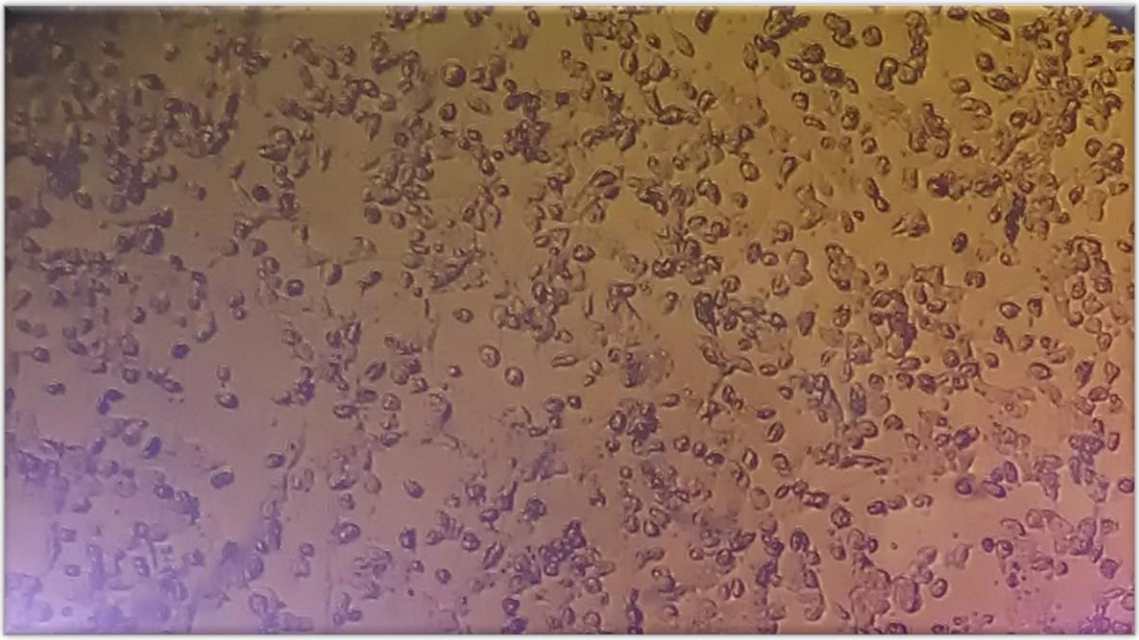

Figure 9: Infected Vero cells showing typical CPE

4.4. Questionnaire Survey

According to the pastoralist community the local name for LSD was mentioned as "*itesa"*. About 17 of the 20 respondents said that the LSD outbreak re-occurs in the frequency of three years; while 3 individuals said the disease occur an interval of two years. They also reported that the incidence of the disease increased during the rainy season. Concerning the possible source of infection, the pastoralist responded as the source could be different in that contact at communal points (like marketing, watering and grazing) accounted for 45% (9/20), introduction of sick animals to the herd accounted 30% (6/20) while the other 25% (5/20) pastoralists did not know of any source.

The survey enabled an estimation of the direct economic losses resulting from animals dying from LSD. Production losses were estimated from the weighted average price of each animal that died. An average cost of a single ox died from this disease is 7,000 of Ethiopian birr. For the one active outbreak investigated in two villages, the total economic losses from the deaths of 7 animals were 50,000 ETB (US$ 2174). The estimated total expense incurred for the treatment of LSD was also assessed. An average of 21 birr/animal was incurred for treatment of LSD with frequency of treatment of once per month. A high economic loss was incurred for supportive veterinary treatment of LSD during outbreak which estimated as 2286 ETB (US$ 99.42).

As pastoralist said 0.6% (3/74) pregnant cow has aborted due to the diseases. Further, animals that recovered were no longer fit for export purposes and were therefore sold at local markets at a lower price. Lastly, the survey found that animals that had recovered from LSD produced less milk and suffered a loss in draught power.

Figure 10: Picture showing during questionnaire survey in the study areas.

5. DISCUSSION

The present study reported an outbreak of lumpy skin disease occurred at the end of November 2016. As far as the objective is to characterize LSDV from an outbreak cases, the occurrence of LSD was examined using clinical diagnosis, PCR and virus isolation. Generation of sequence data ongoing and the sequences will be analyzed and released up on completion. Accordingly all (20) typical clinical cases sampled and tested in the present study were confirmed as positive for LSD using PCR.

The clinical manifestations observed in the present finding, such as fever, circumscribed nodules on the skin, necrotic nodules, enlargement of superficial lymph nodes and lacrimation are in agreement with those documented by (Ayelet *et al*., 2014); (Gari *et al*., 2010) and Alemayehu *et al.* (2012) in different areas of the country. Host susceptibility, age, immunological status of the animal, dose and route of virus inoculation affects the severity of disease (Knopvelsiekte, 2008).

In the present study, the observed morbidity rate (18%) is slightly higher than that reported by Alemayehu *et al*., (2012); Ayelet *et al*. (2014), who recorded 6.1% and 13.61%, respectively in different parts of the country. Other authors reported wide ranges of morbidity rates ranged from 3% up to 85% (Bennett and Jpelaar, 2005; Tuppurainen *et al*., 2012). Moreover, it is far higher than reported by Davies (1991), who indicated that the usual morbidity rate is within a range of 1 up to 5%. In outbreaks of the disease, the morbidity rate varies depending on host susceptibility and the abundance of mechanical arthropod vectors.

With regard to mortality rate, the present finding report (1.34%) is slightly lower than reported by Ayelet *et al*. (2014), who reported 4.97%. The current finding agreed with the report of Alemayehu *et al.* (2012) recorded 1.8% in feedlot. Woods (1988) also reported higher mortality rates above 5%. In the present study, the observed case fatality rate (7.44%) is also lower than reported by Ayelet (2014) and Alemayehu *et al*. (2012), who

reported 36.48% and 30%, respectively at different parts of the country. However, the latter was conducted on feedlot cattle and result is based on clinical diagnosis, which may not be suitable for direct comparison.

In the present study LSDV was isolated from samples collected from naturally infected cattle by inoculation on Vero cell. Characteristic pock lesions were observed after 1st passage and become clear after 3rd passage, this finding agrees with Hasan *et al.* (1999); House *et al* (1990), who successfully cultivated LSDV to detected the characteristic pock lesions.

The CPEs characterized by rounding of cells, aggregation of dead cells and destruction of monolayers is in line with the reports made by Ayelet *et al*. (2014). PCR technique is highly suggested by different authors as a means to confirm of LSD from clinical specimens (Ayelet *et al*., 2014; Zeynalova *et al*., 2016).

In agreement with the present finding, Zeynalova *et al.* (2016) indicated that, on average, skin nodule samples exhibited higher concentration of virus than other samples, as evidenced by the lower average Ct values observed in PCR testing.

PCR was the test of choice for rapid detection and identification of the LSD outbreak causative agent. The PCR assay used in this work showed high specificity as a unique band of the expected size (172bp) was obtained for DNA samples derived from skin biopsies, nasal swab and Neethling reference strain of LSDV.

The present study outbreak was reported at the end of November 2016 which is after the end of the main rainy season in most parts of the lowland and some highland agro-ecological zones. The seasonality of the outbreaks was also substantiated by questionnaire respondents who provided information on active disease surveillance. This is in agreement with the report of Ayelet (2014) who indicated that, the disease is higher during rainy season and decreases in the dry season.

Others environmental risk factors associated with spread of LSD were found to be worm humid agro-climate, communal grazing/watering and introduction of new animals in a herd. The incidence of LSD occurrence is high during wet seasons when biting-fly populations are abundant and it decreases or ceases during the dry season (Gari *et al.*, 2010).

The findings associated with LSD mortality and veterinary expenses for treating sick animals are suggestive of heavy losses in the sector. In line with current finding, various studies indicated that, lumpy skin disease causes severe economic losses as a result of the prolonged debilitating clinical course of the illness, reduced weight gain, temporary or permanent loss of milk production, infertility problems or even sterility in bulls, abortions in pregnant cows and permanent damage to hides (Ayelet., 2014).

6. CONCLUSION AND RECOMMENDATIONS

LSD was found to be the major cattle health problem causes severe economic loss due to permanent damage to hides, a prolonged debilitating clinical course, reduced weight gain, temporary or permanent loss of milk production, temporary or permanent infertility or even sterility in bulls, and abortion of pregnant cows. In the present study LSD cause significant effect from morbidity and mortality of animals that leads lead to economic loss due to abortion of pregnant cows, milk yield reduction, cost of dead animals, and cost of treatment. From outbreak investigated of the present study all age group of animals were infected with significantly higher morbidity and mortality rate in young animals than other group. Extracted DNA from skin biopsies and nasal swabs tested by using PCR revealed that all tested samples were LSDV. This implies failure of LSD vaccines, due to studied group of animals were annually vaccinated against the disease. Lumpy skin disease is considered as transboundary and trade band disease which has significant impendent on livestock market and animal products. Based on the above conclusions, the following recommendations are forwarded.

- Increasing appropriate handling facilities of vaccine in addition to providing quality vaccine and good administration skills should be the major considerations due to vaccination is the only effective method to control the LSD in endemic countries like Ethiopia.
- The government should establish strategic policies for effective control and eradication of the disease, i.e. strategic vaccination program, restriction of livestock movement.
- Availability of simple diagnostic test that can help confirm the cases at field level is important to take control measures early during its occurrence.
- More investigations should be carried out about the economic impact of LSD and the methods of spread particularly the involvement of vectors.
- Detailed molecular analysis of different isolates with in the country need to be carried out for further confirmation.

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8. ANNEXES

Annex 1: Cell culture procedures

1. Thawed the tissue sample at room temperature and wash three times using sterile washing buffer
2. Take enough tissue samples and grounded by sterile mortar and pestle by adding washing buffer containing antibiotic.
3. Put the grounded tissue suspension in to test tube and centrifuged three times at 1500 rpm for 15 min.
4. Collect and filter the supernatant in to sterile container
5. Inoculate filtered tissue suspension in to Vero monolayer cells and then flashed with suitable medium
6. Incubate it at 37^{o}C and 5% CO2 in a humidified incubator for 48 hrs. Cells monitored daily for 14 days for evidence of CPE

Annex 2: **Procedure of Agaros Gel Electrophoresis**

1. Prepare 100 ml of 2% agarose in 1 x TBE buffer.
2. Either heat in microwave for 2 min on full power or place in a beaker of boiling water until melted.
3. Allow to cool to about 45 0C and add 10 µl of ethidium bromide (stock=mg/µl) per 10 ml, giving a final concentration of 0.5µg/ml. this can be increased to 15µg/ml if no ethidium bromide is added to the buffer
4. Pour the gel and insert well former (comb). Allow to set on a flat surface for about 15min.
5. Pour buffer 1 x TBE (containing 0.55µg/ml ethidium bromide, i.e. 1 µl of 5 mg/ml stock to every 10 ml of buffer) in to tank and remove comb from gel.
6. Prepare sample in tubes, a multi well plate or on para film. 1 µl of loading buffer 5 µl of PCR product
7. Prepare molecular weight marker. 0.5 µl molecular weight marker 1 µl loading buffer 4.5 µl H2O
8. Loading sample in to the wells formed in the gel. It is often useful to load the molecular weight marker in both the first and the last lanes.
9. Electrophorese at 120 volts for 60 min.
10. View and photograph the gel on an UV-trans illuminator.

Annex 3: Questionnaire format

1. Background information:
   A. Owner's name_____
   B. District_____
   C. Kebele_____
2. Animal description
   Animal name or tag. No_____
3. Number of cattle per house holds_____
4. Have you had skin diseases of cattle in your herd? _____
5. Have you had LSD (Itesa) in your cattle? Yes_____ No_____
6. When did the disease commence in the area (Kebele)? Season __mon____ year____
7. Have you seen such outbreak in the area before this time, < 1yr__ 1-2 Yrs___2-3Yrs___ >3Years___

8. How frequent LSD reoccurs in the area? Don't Know ___Every 1yr____every 2yrs__ >3yrs____

9. Total herd size of the farmer before onset of LSD_____: Herd structure Ox____ Bull_____Lactating cow_____ Dry cow_____ Heifer_____ Calf_____

11. Occurrence of LSD infection
    a. Occurrence
       First time _____
       Commonly occurred _____
    b. Season of occurrence
       Dry season _____
       Short rainy season _____
       Long rainy season_____
       Any season _____
    c. The last outbreak of LSD in the village occurred in
       Before 2005_____

In between 2006-2007_____

Last year _____

Not yet _____

12. Do you consider LSD as an important disease and how do you score it?

V.severe____ Severe____ Moderate_____ Low _____

13. Animals at risk

Breed	Sex	Age group		
		< 2 yrs	2-4 yrs	>4 yrs
	M			
	F			

14. Animals affected

Breed	Sex	Age group		
		< 2 yrs	2-4 yrs	>4 yrs
	M			
	F			

15. Total number of animal dead

Breed	Sex	Age group		
		< 2 yrs	2-4 yrs	>4 yrs
	M			
	F			

16. Likely source of outbreaks

16.1. Introduction of infected animal's _____

16.2.Contact at communal points _____

16.3.Movement of infected animals_____

17. Did you vaccinate your cattle for LSD? Yes_____ No_____.

If yes when? Before LSD onset_____After LSD _____

18. Vaccination status

18.1. Have you vaccinated your animals against LSD? Yes_____ No _____

18.2. When the vaccination performed? _____

18.3. Who gave the vaccine? AHA _____Community animal health Workers (CAHWs)_____

18.4. Total No of animals get sick (No sick/total No of cattle) _____

18.5. No of animals get sick from vaccinated (No sick/total No vaccinated) ____

18.6. Opinion of the owner about the vaccine_____

19. Economic Impact of LSD

19.1. Do you milk LSD infected cows? Yes _____ No _____

19.2. Do LSD infected pregnant cows abort? Yes_____No_____

20. How many pregnant females aborted, in number _____.

21. Estimated Cost for treatment for individual diseased animal _____

22. No Dead (No died/total No of cattle) _____

23. Estimated cost of dead animals_____