Thesis Ref.No……

ISOLATION AND GENOTYPING CAPRIPOXVIRUS FROM CATTLE IN EAST SHAWA, CENTRAL ETHIOPIA

MSc THESIS

BY

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June 2019

BISHOFTU, ETHIOPIA

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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 KEDIR HIRPO **entitled Isolation And Genotyping Capri poxvirus From Cattle in East Shawa, Central Ethiopia** and recommend that it be accepted as fulfilling the thesis requirement for the degree of: Masters of Veterinary Science in Veterinary Microbiology**.**

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First, I declare that this thesis is my authentic 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 (MSc) 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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Date of Submission: 20/06/2019

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ACKNOWLEDGEMENTS

Acknowledgement First, I would like to thank my Almighty ALLAH for keeping me healthy and helping success of my life. I am greatly indebted to my advisor Dr. Bedaso Mamo for his close intellectual advice, and devotion of his time to correct and edit this research paper. Next, I want to thank deeply Dr. Esayas Gelaye, National Veterinary Institute Bishoftu, who work on the research with me, sharing me a lot of interesting scientific experience and help me on this thesis arrangement. I am also grateful to NVI Research and diagnostic laboratory professionals Mr. Alebachew Belay for his support in facilitating laboratory activities and works with me in every laboratory activities. I wish to convey my heartfelt thanks to livestock resource, development and animal health staff members of the four districts of study areas for their collaborations during data collections. Lastly but not least, I would also wish to thank my beloved family, especially my wife Sukare Menza for every support she gave me in my life and continuous motivation through this thesis preparation.

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LIST OF ABBREVIATIONS

AGID	Agar gel immune diffusion test
^0C	Degree Celsius
CaPV	Capri pox virus
Cm	Centimeter
CO_2	Carbon di oxide
CPE	Cytopathic effect
CSA,	Central Stastics Agency
DNA	Deoxyribonucleic acid
ETB	Ethiopian birr
EU	European Union
GDP	Gross domestic product
GMEM	Glasgow Minimum Essential Medium
GTP	Goat pox
GTPV	Goat poxvirus
HF	Holstein-Friesian
HRM	High Resolution Melting
IFAT	indirect fluorescent antibody test
KSGPV	Kenyan Sheep and Goat Pox Virus
LSD	Lumpy skin disease
LSDV	Lumpy skin disease virus
NVI	National Veterinary Institute
OIE	Office International des Epizooties
PBS	Phosphate-buffered saline
PCR	Polymerase Chain Reaction
pH	Potential Hydrogen
rpm	Revolution per minute
RT-PCR	Real Time Polymerase Chain Reaction
SNNP	Southern Nations Nationalities and People

SPPV	Sheep Pox Virus
SPSS	Statistical Package for the Social Sciences
TEM	Transmission electron microscopy
VNT	Virus neutralization test

ABSTRACT

Capri poxviruses (CaPVs) represent one of eight genera within the chordopoxvirus (ChPV) subfamily of the *Poxviridae*. These viruses are responsible for some of the most economically significant diseases of domestic ruminants in Africa and Asia. CaPV infections are generally host specific and they have specific geographic distributions CaPVs are, however, serologically indistinguishable from each other, able to induce heterologous cross-protection, and able in some instances to experimentally cross-infect. Lumpy skin disease (LSD) is an infectious disease of cattle, caused by a Lumpy Skin Disease Virus. LSD causes considerable economic losses. In Ethiopia, the disease is distributed almost in all regions and regarded as one of the most economically important livestock diseases in the country. The current study focused on the identification of CaPVs based on outbreak reports in East shawa of the central Ethiopia. Outbreak survey found that there were 6.1% morbidity, 2.2% mortality and 36.2% case fatality rates in the study area. Skin lesion samples were collected from clinically sick cattle and virus was isolated on cell culture and shown the characteristics CPE of the virus. Vero cell however found less sensitive for LSDV primary isolation but isolation can be achieved by using continuous passaging of the virus. Out of 20 tissues samples 12 were grown on Vero cells and all of them showed cytopathic effect due to virus infection and all samples were positive by Polymerase Chain Reaction (PCR).The virus DNA was identified by amplifying the 172bp DNA fragment with conventional PCR and further was subjected to real time PCR assay can differentiate between the three genotypes of Capri poxviruses (CaPV) strains based on differences in the melting point temperature (Tm) obtained after fluorescence melting curve analysis. Isolation and genotyping of the virus from these outbreak cases shows the occurrence of the disease. Preventing movement the diseased animals along with vector control, regular annual vaccinations and awareness creation for cattle owners play great role in disease prevention and control, finally genotyping of CaPVs from different areas of the country and testing potency and efficacy of the currently used vaccines are highly recommended.

Key Words: *Central Ethiopia ,East Shawa, genotyping , capripox virus, Real Time Polymerase Chain Reaction, Virus Isolation.*

1. INTRODUCTION

Ethiopia is believed to have the largest livestock population in Africa and livestock production constitutes a vital part of the agricultural system and it accounts about 40% of the agricultural gross domestic product (GDP) (CSA, 2017). Despite its large population size, the contributions of livestock production to agriculture and the overall economy of the country are low. This is associated with a number of complex and inter-related factors such as widespread diseases, limited genetic potential and husbandry standard (Nagassa *et al*., 2011).

Livestock diseases are the major production constraints in Ethiopia in addition to poor nutrition, low genetic potential of indigenous livestock, lack of marketing infrastructure and water shortages (GebreEgziabhare, 2010). Lumpy skin disease (LSD) is one of the most economically important viral diseases listed as notifiable trans-boundary animal diseases by the World Organization for Animal Health (OIE) and the second significantly important cattle disease in Ethiopia (Gelaye *et al*., 2015; OIE, 2017).

Capripoxviruses (CaPVs) represent one of eight genera within the chordopoxvirus (ChPV) subfamily of the Poxviridae. The capripoxvirus genus is currently comprised of lumpy skin disease virus (LSDV), sheeppox virus (ShPV), and goatpox virus (GPV) .Lumpy skin disease (LSD) which was occurred as new skin disease was first described in Zambia in 1929. Initially, it was considered to be the result of either poisoning or a hypersensitivity to insect bites (Tuppurainen and Oura, 2011). LSD was first found and diagnosed in East Africa (Kenya) in 1957, Sudan in 1972, and in West Africa in 1974. Tanzania, Kenya, Zimbabwe, Somalia and the Cameroon, also reported an outbreaks of epizootic LSD between 1981 and 1986 with mortality rates of 20% in affected cattle (Brenner *et al*., 2006). In Ethiopia LSD was first observed in the northwestern part of the country (southwest of Lake Tana) in 1983 (Mebratu *et al.,* 1984).

Lumpy skin disease is caused by LSD virus which is a member of Capri pox viruses (CaPVs) that are large double-stranded DNA viruses belonging to the family *Poxviridae*. The genus includes Sheep pox virus (SPPV), Goat pox virus (GTPV) and Lumpy skin disease virus (LSDV) (Facquet *et al.,* 2005; Murphy, 2012OIE, 2017).

These viruses have genome of approximately 150 kb and share a high degree of sequence homology, with 97% identity between LSDV and both GTPV and SPPV genomes (Tulman *et al.*, 2001*).*

Capri poxvirus infections are generally host specific and not reported on CaPV infecting all three species: sheep, goats and cattle (Bhanuprakash *et al.*, 2010; Tuppurainen *et al*., 2014). They have also specific geographic distributions in which diseases of GTP and SPP are prevalent in Africa above the equator, Asia, the Middle East, and occasional outbreaks occur in regions of Europe surrounding the Middle East. In contrast, LSD is endemic in Africa and outbreaks have been occurred in the Middle East countries surrounding Egypt and in some parts of Europe like Greece (Bhanuprakash *et al.*, 2011; Tuppurainen *et al*., 2015; Babuik *et al.*, 2008).

Experimental and field evidence indicate that LSDV is inefficiently transmitted between animals through direct contact (Weiss, 1968; Carn and Kitching, 1995).LSD is a vector borne disease transmitted by different biting and blood-feeding arthropods. Circulation of LSDV is often, but not necessarily, associated with warm and humid weather conditions and with a high density of biting insects (Tuppurainen and Oura, 2012). The disease is more prevalent in low-lying areas and along water courses (Weiss, 1968).

LSD is an acute, sub-acute or in apparent viral disease of cattle, characterized by fever, lacrimation and the sudden appearance of firm circumscribed skin nodules that undergo necrosis. Similar lesions may be present in the skeletal muscles and the mucosa of the digestive and respiratory tracts. Animals affected by capripoviruses (CaPVs) will eventually clear the infection and do not become carriers (Rao and Bandyopadhyay, 2000; Babuik *et al*., 2008; Gari *et al.*, 2015).

LSDV as a member of CaPVs, it has a single serotype, do not cause persistent infection, have a limited host range and vaccines are available that may provide long term immunity. These attributes increase the prospect of successfully implementing regional control programs, leading to the elimination of the virus and conceivably global eradication. Control and prevention of LSD in endemic countries like Ethiopia is mainly by vaccination. In Africa and the Middle East countries several live attenuated CaPV vaccine strains are currently used for cattle and small ruminants. These include LSDV Neethling strain, Kenyan sheep and goat pox virus (KSGPV) O-

240 and O-180 strains, Yugoslavian RM65 SPP strain, Romanian SPP and Gorgan GTP strains (Gari *et al.*, 2015; Babuik *et al.*, 2008). In Ethiopia there are problems related to lack of vaccine efficacy and continuous outbreaks in vaccinated animals (Tilahun *et al.*, 2014).

The disease is now the problem of almost all the regions and agro ecological zones of Ethiopia. Major outbreaks of LSD have been occurred in different regions of Ethiopia like Amhara and west Oromia Regions in 2000/2001, Oromia and Southern nations nationalities and people (SNNP) regions in 2003/2004 and Tigray, Amhara and Benishangul regions in 2006/2007 (Ayelet *et al*., 2013). LSD is an OIE listed disease because of considerable financial losses and in Ethiopia due to the endemic nature of LSD; the country is facing serious difficulties in exporting live cattle and their products. In addition, this situation contributes a negative impact on the national economic growth through the loss of meat and milk production and poor quality of skin and hides (Gelaye *et al.,* 2015). The disease outbreak was observed in the cattle regardless of previous vaccination with Kenyan sheep- and goat pox vaccine the occurrence of LSD outbreak scale, despite the use of a vaccination regime, is suggestive of vaccine failure. Apparent emerging vaccine failure is a serious problem for efficient control of LSD, as the disease has been manifested by high morbidity and mortality rates, regardless of vaccination status (Ayelet *et al.*, 2013). Consequently continuous surveillance on the status of the disease and genetic information on circulating field viruses is mandatory in order to take effective measures for the control disease in the country (Body *et al.*, 2011).

Therefore the objectives of the current study were:

- To investigate LSD outbreaks in the four districts of East Shawa Zone.
- To isolate the field infectious virus responsible for the disease by cell culture.
- To genotyping the virus isolates using by conventional Polymerase Chain Reaction and Real Time Polymerase Chain Reaction.

2. LITERATURE REVIEW

2.1. History

The first description of the clinical signs of LSD was reported in 1929 in Zambia (Morris 1931). Same clinical signs were occurred in Botswana, Zimbabwe and the Republic of South Africa between 1943 and 1945, where the infectious nature of the disease was recognized in these outbreaks (Davis, 1991).

In 1949 South Africa, LSD occurred as a panzootic, which affected eight million cattle. In1957, LSD was identified in East Africa in Kenya. In 1972, the disease was reported in Sudan (Ali and Obeid 1977) and West Africa in 1974. Nowadays, LSD occurs in most of African continent (Tuppurainen and Oura 2012).

In the Middle East, the outbreaks of the LSD were reported in Oman in 1984 and 2009 (House *et al.*, 1990; Kumar 2011; Tageldin 2014). Kuwait in 1986 and 1991, Egypt in 1988 and 2006 (Ali *et al.*, 1990; Ali and Amina 2013), Israel in 1989 and 2006 (APHIS 2006), Bahrain in 1993 and 2002-2003, Yemen, United Arab Emirates in 2000 and the West Bank also reported LSD invasion (Kumar 2011; Sherrylin *et al*., 2013). LSD is exotic to the European Union (EU), but incursions of LSD have occurred in EU neighboring areas (EFSA Journal, 2015).

2.2. Etiology

LSDV is grouped under the family of *poxviridae*. The family *Poxviridae* is subdivided into two subfamilies: *Chordopoxvirinae* (poxviruses of vertebrates) and *Entomopoxvirinae (*poxviruses of insects). Lumpy skin disease virus (LSDV) belongs to the genus Capripoxvirus and the subfamily Chordopoxvirinae. There is only one serotype of LSDV which is prototype strain of LSDV is the Neethling virus and it is closely related antigenetically to sheep and goat poxvirus and can be distinguished by routine virus neutralization or other serological tests. The LSDV primarily affects cattle but can affect sheep and goats, experimentally. Lumpy skin disease virus will grow in tissue culture of bovine, ovine or caprine origin; although maximum yield is obtained using lamb testis cells (Gelaye *et al*., 2015). The members of this family are among the largest of all viruses. It is an envelope, linear ovoid shape with a molecular brick shaped or ovoid virions measuring 220-450 nanometer (nm) by

140-266nm (Figure 1). LSDV has ds DNA genome of about 151kb (Yehuda *et al.*, 2012).

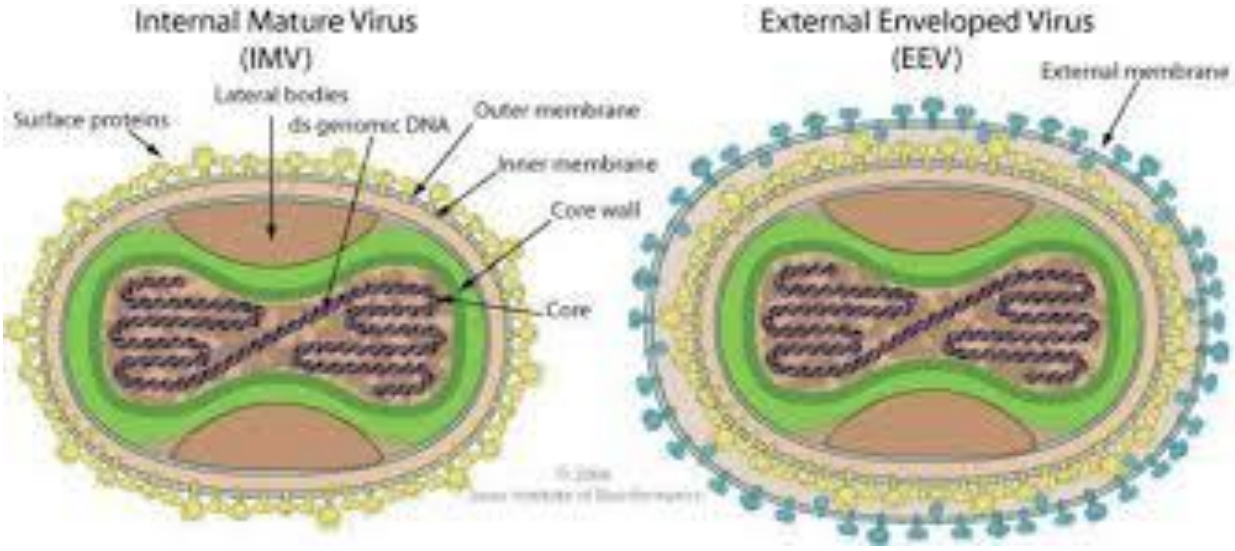

Figure 1: Schematic virion of lumpy skin diseases virus

Source: Yehuda *et al*., (2008).

2.3. Lumpy Skin Disease Virus Characteristics

2.3.1 *Viral genome*

LSDV is a double stranded DNA virus. The size of the LSDV genome is 151 kbp and it consists of a central coding region with identical 2.4 kbp inverted terminal repeats and 156 putative genes. The genes encoding host range, virulence and immune evasions are located at the terminal parts of the genome (Tulman *et al*., 2001).

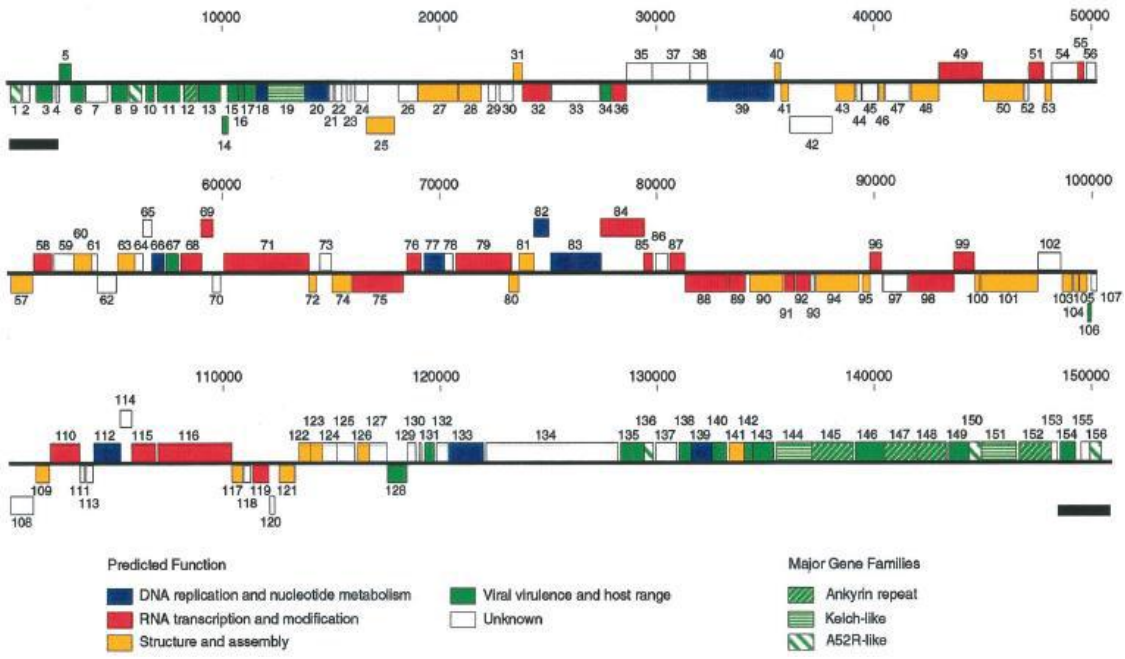

Figure 2: Linear map of the LSDV genome

Source: Tulman *et al.*, (2001)

2.3.2. Replication cycle

The replication of LSDV occurs in the cytoplasm of the host cell in intra cytoplasmic eosinophilic inclusion bodies which can be detected using microscopic examination of a haematoxylin and eosin-stained LSDV infected monolayer of cells (Weiss, 1968; Prozesky and Barnard, 1982).

2.3.3. Physico- chemical property

LSDV is remarkably stable for long periods at ambient temperature, especially in dried scabs. It can persist in necrotic skin nodules for up to 33 days or longer, desiccated crusts for up to 35 days, and at least 18 days in air-dried hides. It can remain viable for long periods in the environment. The virus is vulnerable to sunlight and detergents containing lipid solvents, but in dark environmental conditions, such as contaminated animal sheds, it can persist for several months. The virus can be inactivated at temperature of 55°C for 2 hours and 65°C for 30 minutes. In contrast it can be recovered from skin nodules kept at –80°C for 10 years and infected tissue culture fluid stored at 4°C for 6 months. It is susceptible to highly alkaline or acid pH but, no significant reduction in titer when held at pH 6.6-8.6 for 5 days at 37°C. The

virus is susceptible to ether (20%), chloroform, formalin (1%), phenol (2% for 15 minutes), sodium hypochlorite (2-3%), iodine compounds (1:33 dilution) and quaternary ammonium compounds (0.5%) (OIE, 2013)

2.4. Epidemiology of LSD

2.4.1. Occurrence of the disease

LSD is an endemic disease of most African countries particularly in those of the sub Saharan region. After 2012 it has spread rapidly through the Middle East, south-east Europe, the Balkans, Caucasus, Russia and Kazakhstan (OIE, 2017; Coezer and Tuppurainen, 2004). Mostly, field outbreaks can be severe and generalized infection with high morbidity and mortality rates, while in others there may be few affected animals and few or no deaths recorded but in general outbreaks are more severe with the initial introduction of the infection to a region and then will decrease, probably associated with the development of widespread immunity. Morbidity rates reach 80% during epizootics, but are nearer 20% in endemic areas (Radostits *et al*., 2006).

2.4.2. Hosts and susceptibility

Domestic cattle and Asian water buffalo are the animals affected by LSDV naturally during field outbreaks (El-Nahas *et al.,* 2011; Al-Salihi, 2014). Some strains may replicate in sheep and goats but to date no epidemiological studies have evidenced small ruminants as reservoirs for the virus (Tuppurainen, 2017).Very little is known about the susceptibility of wild ruminants to LSDV. The susceptibility of host animals mostly depends on immune status, age and breed rather than the virulence of the virus. European cattle breeds are generally more susceptible than indigenous African and Asian breeds (Tageldin *et al.*, 2014).

2.4.3. Sources of the virus

Lumpy skin diseases virus is present in cutaneous lesions and crusts. Virus is also present in blood, nasal and lachrymal secretions, milk, semen and saliva, which may be sources for transmission (Davies F.G, 1991). All secretions contain LSD virus when nodules on the mucous membranes of the eyes, nose, mouth, rectum udder and genitalia ulcerate. Shedding in semen may be prolonged since viral DNA has been found in the semen of some bulls for at least 5 months after infection.

Approximately 50% of infected animals are likely to show clinical signs; the majority of experimentally infected animals become viraemic and source of the virus. In experimentally infected cattle LSD virus was demonstrated in saliva for 11 days, semen for 22 days and in skin nodules for 33 days, but not in urine or faces (Tuppurainen *et al*., 2012).

2.4.4. Transmission

Different types of biting and blood feeding arthropods (including mosquitoes and other flies such *astabanids*, *Culicoides*, biting midges and Glossinaspecies) are likely responsible for the mechanical spread of the LSD virus (Chihota *et al*., 2003). Disease incidence is highest in wet/warm weather. Incidence decreases during the dry season, which is possibly linked to decreases in insect vector occurrence/numbers. Minor sources of infection could include direct and indirect contact (e.g. through infective-saliva contaminated feed and water). Pox viruses are highly resistant and can remain viable in infected tissue for more than 120 days or probably longer time. The virus is also found in blood, nasal discharge, lachrymal secretion, semen and saliva, which considered as main sources for LSD transmission (FAO, 2011). Other potential transmission routes include the milk of lactating cows and the semen of infected bulls, since the LSD virus can persist for extended periods of time in both (Irons *et al.*, 2005).

2.5. Pathogenesis and Clinical Signs

The actual incubation period of LSD under field conditions has not been reported, but following experimental inoculation of the virus is 6–9 days until the onset of fever. LSDV replicates inside the host cells such as macrophages, fibroblasts, pericytes and endothelial cells in the lymphatics and blood vessels walls leads to vasculitis and lymphangitis, in severe cases thrombosis and infarction may also develop (Al Salhi, 2014).

In the acutely infected animal, there is initial pyrexia, which may exceed 41°C and can persist for 1 week. The superficial lymph nodes become enlarged and lesions may develop over the body, particularly on the head, neck, udder, scrotum, vulva and perineum between 7 and 19 days and the first ones usually appearing in the perineum. In lactating cattle there is a marked reduction in milk yield (OIE, 2017; Radostits *et*

al., 2006). Lesions of LSD are round and firm, 1 to 4 cm in diameter, and are flattened and the hair on them stands on end. They vary in number from a few to hundreds; they are intradermal and, mostly confined to the skin area. Lacrimation, nasal discharge, salivation, and lameness can also be observed in association with the pyrexia. Lesions in the nostrils and on the turbinates, causing mucopurulent nasal discharge, respiratory obstruction and snoring; plaques and ulcers in the mouth causing salivation, nodules on the conjunctiva, causing severe lacrimation can be observed in severe cases. Lymph nodes draining the affected area become enlarged and cause local edema (Radostits *et al.,* 2006; Maclanchilan and Dubovi, 2011).

In experimental studies, the intravenous route develops severe generalized infection, while in the intradermal inoculation only 40-50% of animals may develop localized lesions or no apparent disease at all. A localized swelling at the site of inoculation after four to seven days and enlargement of the regional lymph nodes, develop after subcutaneous or intradermal inoculation of cattle with LSDV (Al-Salihi, 2014; Abdulqa *et al.,* 2016).

2.6. Diagnosis of LSD

There are no available commercial diagnostic test kits for LSD virus detection. Thus, the tentative diagnosis of LSD is usually based on the characteristic clinical signs, differential diagnosis, and confirmation is done by laboratory tests using molecular techniques of conventional or real time polymerase chain reaction (PCR) and cell culturing. LSD should be suspected clinically when there are characteristic skin nodules, fever and enlargement of superficial lymph nodes (Abdulqa *et al.,* 2016; Tuppurainen, 2017a; OIE, 2017).

2.6.1. Virus isolation

Confirmation of LSD in a new area requires virus isolation and identification. Virus isolation is the method used to investigate the viability of the virus in the samples (Tuppurainen, 2017a). LSDV will grow in tissue culture of bovine, ovine or caprine origin, although maximum yield is obtained using lamb testis or bovine dermis cells. In cell culture, LSDV causes a characteristic cytopathic effect (CPE) and intracytoplasmic inclusion bodies that is distinct from infection with Bovine

herpesvirus 2, which causes pseudo-lumpy skin disease and produces syncytia and intranuclear inclusion bodies in cell culture (Abdulqa *et al*., 2016; OIE, 2017).

2.6.2. Molecular detection methods

Laboratory confirmation of LSD virus can be done very rapidly using a PCR method specific for Capri poxviruses or by the demonstration of typical Capri pox virions in biopsy material or desiccated crusts using the transmission electron microscopy (TEM). Genome detection using Capri pox virus-specific primers for the attachment protein and fusion protein gene has been reported, and several conventional and real-time PCR methods have been established to be used on blood, tissue and semen specimens (Abera *et al*., 2015, Abdulqa *et al.*, 2016; OIE, 2017).

Recently, a capripox virus real-time PCR method using primers and a probe has been validated (Bouden *et al*., 2009; Tuppurainen and Oura, 2011). Molecular tests using loop mediated isothermal amplification to detect capripoxvirus genomes are also reported to provide sensitivity and specificity similar to real-time PCR with a simpler method and lower cost (Das *et al.*, 2012; Murray *et al.*, 2013).

2.6.3. Serological tests

Serological tests that can be used for LSDV include an indirect fluorescent antibody test (IFAT), virus neutralization, enzyme-linked immunosorbent assays (ELISA) and immune blotting (Western blotting) (Abera *et al*., 2015a). The virus neutralization test (VNT) is the only validated serological test available. The agar gel immune diffusion test (AGID) and IFAT are less specific than the VNT due to cross-reactions with antibody to other poxviruses. Western blotting using the reaction between the P32 antigens of LSDV with test sera is both sensitive and specific, but is difficult and expensive to carry out. Some antibody-detecting ELISAs have been described but none is sufficiently validated to be recommended for use (OIE, 2017; Babuik *et al*., 2008).

2.6.4. Differential diagnosis

The main differential diagnosis is pseudo-LSD caused by bovine herpesvirus 2 (BoHV-2). This is usually a milder clinical condition, characterized by superficial nodules, resembling only the early stage of LSD. Intra-nuclear inclusion bodies and

viral syncytia are histopathological characteristics of BoHV-2 infection not seen in LSD (OIE, 2017; Radostits *et al*., 2006). Other differential diagnoses include: dermatophilosis, dermatophytosis, bovine farcy, photosensitization, actinomycosis, actinobacilosis, urticaria, insect bites, besnoitiosis, nocardiasis, demodicosis, onchocerciasis, pseudo-cowpox, and cowpox. Differential diagnoses for mucosal lesions include: foot and mouth disease, bluetongue, bovine viral diarrhoea, malignant catarrhal fever, infectious bovine rhinotracheitis, and bovine popular stomatitis (OIE, 2017; Abera *et al*., 2015).

2.7. Economic Importance

LSD is an economically important disease of cattle, serious economic losses from outbreaks that have a high morbidity and can produce a chronic debility in infected cattle. There is a great loss of milk production since the disease is more severe in cows in the peak of lactation and causes a sharp drop in milk yield because of high fever caused by the viral infection itself and secondary bacterial mastitis predisposed by the development of lesions on the teats (Abera *et al.*, 2015; Radostits *et al*., 2006).

Even though the mortality rates of LSD are usually low, it is an economically important disease of cattle in Africa because of the prolonged loss of productivity of dairy and beef cattle, use of the animals for traction, decrease in body weight, mastitis, severe orchitis, which may result in temporary infertility and sometimes permanent sterility (Abera *et al*., 2015; OIE, 2017; Gari *et al*., 2011). A study done in Ethiopia has shown that the annual financial cost 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 was estimated to be United state dollar (USD) 6.43 (5.12–8) per head for local zebu and USD 58 (42–73) per head for HF/crossbred cattle (Gari *et al.*, 2011). Another study also showed that the average cost of a single ox dying from LSD was calculated as 9,000 Ethiopian birr (ETB), equivalent to US$477.7 (USD) = 18.84 ETB) (Ayelet *et al.*, 2014).

In addition to quality degradation of skin and hides skin LSD induces associated economic losses due to reduction of wool quality, meat, losses as a result of culling and mortalities and related with cost of treatment and prevention of the diseases. Even

though there are no specific antiviral treatments for LSD-infected cattle, there will be treatment cost for secondary bacterial infection. Treatment cost represents the expenses incurred by farmers for medication at the local public veterinary clinics when farmers bring their clinically sick animals for treatment (Abera *et al.,* 2015b). Emaciation and a long convalescence period can also significantly decrease the growth rate in beef cattle (Tuppurainen *et al.,* 2015).

LSD have been identified as one of the major impediments for genetic improvement of cattle populations and, consequently, for the development of intensive production units in Africa. It is well known that high producing dairy cattle, such as Holstein-Friesian (HF) and Jersey are more susceptible to CaPV infection than indigenous African and Asian cattle breeds (Bhanuprakash *et al.,* 2011; Tuppurainen and Oura, 2011). The susceptibility of European cattle breeds and challenges facing dairy-genetics improvement in LSDV-endemic settings in Ethiopia was recently highlighted (Gari *et al.,* 2011).

Currently live cattle export from Ethiopia is largely feedlot based. The introductions of LSD into feedlots certainly affect access to specific markets. For longer time, Middle East markets are the traditional destination of Ethiopian live bulls. However, the current health status of Borena bulls in market chain unquestionably becomes a challenge for the country's future live cattle export opportunities to those countries (Alemayehu *et al*., 2012). Costly control and eradication measures such as vaccination campaigns as well as the indirect costs because of the compulsory limitations in animal movements also cause significant financial losses on national level (Tuppurainen and Oura, 2011; Gari *et al*., 2011; Abera *et al.,* 2015b).

2.8. Control and Prevention

The OIE Terrestrial Animal Health Code Chapter 11.11, (2017) on lumpy skin disease (caused by group III virus, type Neethling), establishes the international standards on disease control and safe international trade. Each country has its own national legislation applied to LSD.

Successful control of LSD relies on early detection of the index case, followed by a rapid and widespread vaccination campaign. Immunity acquired from natural infection of the disease might be lifelong and vaccination has been successfully used.

LSD could be kept under control by vaccination of cattle every year (Thomas, 2002). 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 capripox virus 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. In countries where LSD is endemic, vaccination against this infection was successfully used by vaccinating animals every year.

The efficacy of total stamping-out (killing all clinically affected cattle and unaffected herd mates) and partial stamping-out (killing only clinically affected cattle) policies have been compared using mathematical modeling. The study found that total stamping-out and partial stamping-out resulted in a similar probability of eradicating the infection. The study also highlighted the importance of initiating vaccination campaigns ahead of virus entry (OIE, 2017).

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 but in those nations which experience the infection can limit the spread of the lumpy skin disease by restriction of the animal movement from one place to another, quarantine, keeping of sick animals well apart from the rest of the herd and must not share drinking or feeding troughs by making awareness creation of the farmers (Thomas, 2002).

2.9. Status in Ethiopia

In Ethiopia LSD is the one of the most economically important livestock diseases. After the first occurrence in 1983 it has spread widely throughout the country and now it is the problem of almost all the regions and agro ecological zones (Gari *et al*., 2010; Mebratu *et al.,* 1984). Its spread was mainly enhanced by cattle movements, communal grazing and watering, and pastoralist ways of life (Tuppurainen and Oura, 2011; Gari *et al.,* 2012).

In Ethiopia from 2007-2011 totals of 1352 disease outbreaks of LSD have been reported and highest frequency was documented in Oromia region and the least in Afar region (Gumbe, 2018). Another study also showed that a total 3811 LSD outbreaks reported in Ethiopia between 2000 and 2015. Most of these outbreaks were

from Oromia (54.5%), Amhara (27.9%), SNNP (10.1%) and Tigray regional states (3.6%) No out breaks were reported from Harari and Dire dawa. It also shows that LSD affects districts for one or two years and then spreads to other nearby areas with a susceptible cattle population with a trend of LSD outbreaks increased over time (Molla *et al.*, 2017a).

Since the country has no well-designed control strategy for this disease it is continuing to be a great problem. Even if the animal health authorities undertake vaccination campaigns when outbreak is reported, researches have shown that the different vaccines used in Ethiopia are not fully effective (Molla *et al*., 2017b; Ayelet *et al.,* 2013). There have been repeated concerns reported to NVI on the insufficient protection provided by the vaccine, for cattle against LSDV. In addition to this, lack of genetic information on the circulating isolates in the field and their relation to the vaccine strain in use, which is essential for better vaccine matching, is also a great problem in the country (Gelaye *et al.,* 2015)

3. MATERIALS AND METHODS

3.1. Study Area

The study was conducted from December 2018 to May 2019 in four selected areas of East Shawa, central Ethiopia. Samples were collected from Adama, Bosat, Adami Tulu Jido Kombolcha and Dugida districts based on LSD outbreak report.Where there is large population of cattle and the disease is supposed to be epizootic and Adama and Bosat out break occured in the vaccinated herd. Adama is special zone of Oromia and is city of East Shawa Zone. It is located at 8.54°N 39.27°E at an elevation of 1712 meters and 99 km Southeast of Addis Ababa.Olenchiti is a town in East-central Ethiopia located in the East Shewa Zone of the Oromia Region. This town has a longitude and latitude of 8°40′N 39°26′E and an elevation of 1436 meters above sea level. It is the administrative center of Bosat woreda 124km Southeast of Addis Ababa. Batu located on the road connecting Addis Ababa to Hawassa in the East Shewa Zone of the Oromia region of Ethiopia it has a latitude and longitude of 7°56′N 38°43′E with an elevation of 1643 meters above sea level, 163 km from Addis Ababa. Dugda is one of the woreda in the East Shawa Oromia region . The Administrative Center of Dugda is Meki town which 129 km from Addis Ababa("World Gazetteer Map," 2012).

3.2. Study Animals

Cattle that showed clinical signs of pox like skin lesion were targeted for this study. All cattle included in the study were local and cross breed from both sexes, no exception was put for the cattle age. Outbreak investigation were carried out to collect primary data from Veterinary clinic supervising Veterinarians and farm owners using semi-structured questionnaire regarding total number of cattle, number of affected cattle, number of cattle reported dead, age and, clinical findings. Clinical examination result was recorded in the prepared format in Annex 1.

3.3. Study design and sampling

3.3.1.Active disease investigation

The households under investigation were purposively selected on the basis of outbreak reports from the 4 Veterinary clinics of four districts of the study area. Animals were examined for characteristic clinical signs of LSD, such as visible skin nodules, enlarged lymph nodes, lameness and fever. In Addition, data like vaccination history, age, sex and breeds of animals were collected.

3.3.2. Sample collection

Purposive sampling strategy was used for sample collection from the LSD outbreak area. Sample was collected according to the OIE terrestrial manual (2017). Detailed physical examination was done on sick animals before collection. Samples were collected from clinically sick animals, based on typical signs of LSD. Two skin biopsies samples from cutaneous nodules from each representative cattle were taken aseptically by washing and cleaning the area with sterile scalpel blade. Tissue samples were placed in the sterilized universal bottle containing tryptose phosphate broth and transported to the National Veterinary Institute (NVI), Bishoftu maintaining cold chain system. Then, the tissue samples were stored at -20^{0}C until processing.

Table 1: List of collected samples from different study areas

Sample N^o	**District**	**Kebele**	**Sample type**
S1-S3	Bosat	Gari Nura Dera	Skin nodule
S4-S6	Bosat	Borichota	Skin nodule
S7-S9	Adama	Bati Dagaga	Skin nodule
S10 and S11	Adama	Kachama	Skin nodule
S12 and S13	Adama	Bati Garmama	Skin nodule
S14-S17	Adami tulu jido kombolcha	Washi Gula	Skin nodule
S18-S20	Dugida	Burika Dalacha	Skin nodule

3.4. Laboratory Investigation

3.4.1. Sample processing

The skin biopsy samples was thawed at room temperature and washed three times in sterile phosphate-buffered saline (PBS, pH 7.2). Approximately 1 g washed tissue sample was mixed with 9 ml sterile PBS and grounded using a sterile mortar and pestle. The tissue suspension was centrifuged at 4000rpm for 15 min and the supernatant filtered through a membrane of pore size 0.45 μm (OIE, 2017).

3.4.2. Virus isolation

Vero (African Green Monkey cells) cell line was used for isolation of the samples for LSDV. Vero cell was already available at the virology laboratory of the NVI with passage number 32. Thus, the laboratory work was started from sub culturing of this cell. Sub culturing was performed under aseptic condition in laminar airflow (Annex 2) and two cell cultures for each sample were used. Vero cell culture was prepared on 25cm^{2}tissue culture flasks. Another cell culture was kept as uninfected control.

The virus isolation procedure was performed according to the OIE, (2017). About 0.5 ml of the processed tissue suspension samples was inoculated onto confluent layer of Vero cell in 25cm2 tissue culture flasks and the cultures were incubated at 37^{0}C and allowed to adsorb for 1 hour. The culture was then washed with warm PBS (Annex 3) and covered with 10 ml of Glasgow Minimum Essential Medium (GMEM) containing streptomycin and 2% fetal calf serum (Annex 4).All the flasks, including the control flasks, were incubated at 37°C in a humidified incubator with 5% C^{O}2.The culture medium was replaced with fresh medium every 48hr or when it became acidic. The inoculated cell cultures were examined daily for evidence of cytopathic effects (CPE) microscopically and then virus was passaged three or four times.

3.4.3. DNA extraction

DNA was extracted from processed tissue samples suspension using DNeasy® Blood and Tissue kit (QIAGEN, Germany) following the manufacturer's instruction s. The tissue sample was cut in to pieces and grinded with sterile sand by adding PBS buffer and after centrifugation at 2000rpm for 2 minutes, the supernatant was collected in to new micro centrifuge tubes after that 200μl of it was taken and 20 μl of proteinase K

was added, mixed by vortexing, incubated at 56°C for 10 minutes and it was briefly centrifuged. Then 200µl ethanol (96-100%) was added and mixed thoroughly for 5 sec by vortex mixer and briefly centrifuged. This mixture was applied to the QIAamp mini spin column and centrifuged at 6000 xg (8000rpm) for 1 minute. The spin column was transferred in to 2 ml collection tube and 500 µl buffer AW1was added then centrifuged it at 8000rpm for 1 min. After that 500µl buffer AW2 was added and centrifuged at full speed of 14000 rpm for 3 min. after discarding the filtrate 200 µl of buffer AE was added and incubated at room temperature for1 minute and continued with centrifugation at8000rpm for 1 min and this step was repeated to get the finale extract.

3.4.4. DNA amplification with Conventional PCR and agarose gel electrophoresis

Conventional PCR DNA amplification was performed to detect the presence of *Capripox*virus genome using *Capripox* virus specific primers targeted the RPO30 gene that was designed by Lamien *et al.* (2011).

Table 2: Sequences of forward and reverse primers Specific to CaPV used for conventional PCR

Primer Name	**Sequence**	**PCR product length**
Forward	5'-TCTATGTTCTTGATATGTGGTGGTAG-3'	172 bp
Reverse	5' -AGTGATTAGGTGGTGTATTATTTTCC- 3'	

Conventional (classical) PCR reaction mix preparation and DNA amplification was done according to the adapted NVI master mix preparation and PCR work sheet protocol for CaPVs. Master Mix was prepared in a reaction volume of 20µl containing 3 µl RNase free water, 2µl forward primer, 2µl reverse primer, 10µl IQ Super mix and 3µl DNA template. The PCR tubes were transferred to a PCR machine The PCR protocol was performed with an initial denaturation at 95^{o}C for 4min followed by 40 cycles of denaturation at 95^{o}C for 30 sec, annealing 50^{o}C for 30s and extension at 72^{o}C 30, and final extension at 72^{o}C for 5 min.

PCR products were loaded and separated using electrophoresis apparatus (BIORAD) in 3% agarose gel, stained with 4 µlGelRed loading dye added with 10 µl PCR product and 10 µl Marker (ladder) and run at 120V for 1.20 hour. The gel was visualized through UV transilluminator gel documentation system and gel picture was captured using a Polaroid photograph camera (UVitec) and results were recorded.

3.4.5. Real-time PCR

Real-time PCR was performed using the amplified primers and PCR protocol as described by Gelaye *et al.,(*2013). The primers that were used are;
Snapbackprimer:5'-GGTGTAGTACGTATAAGATTATCGTATAGAAA CAAGCCTTTA- 3'

Reverse primer: 5'-AATTTCTTTCTCTGTTCCATTTG- 3'

The RT- PCR master mix was prepared in a reaction volume of 20µl containing 4.84µl of RNAase free water, 2µl of forward primer, 0.16µl of reverse primer, 10µl of SsoFast Eva Green Super mix (BioRad) and 3µl template DNA. The PCR reaction was run with initial denaturation at 95^{o}C for 3min, followed by 45 cycles of denaturation at 95^{o}C for 15sec, annealing and extension at 58^{o}C for 80 sec using Low Profile Hard-Shell® 96 well PCR plate (BioRad). To perform Melting curve analysis, the product was then denatured at 95^{o}C held for 1minute, cooled to 40^{o}C and heated continuously at 0.5^{o}C for 10 sec with fluorescence acquisition from 45^{o}C to 85^{o}C.
The melting temperatures were analyzed using the CFXTM Manager Software Version 2.0 (Bio-Rad) and the correspondent curves were displayed as negative first-derivative plots of fluorescence with respect to temperature. High-Resolution Melting (HRM) analysis, a post-PCR melting analysis method used to identify variations in nucleic acid sequences, was also used to plot the melting profile of the three genotypes using the Precision Melt Analysis TM Software (Bio-Rad). Normalized melt curves and difference in curves were acquired by selecting pre-and post-melt regions for amplicons separately (Gelaye *et al.,* 2013).

3.4.6. Virus genotyping

Samples were genotyped with a new molecular assay for CaPV that use unlabeled snapback primers and dsDNA intercalating dye, designed by Gelaye *et al.*, (2013),

and High-Resolution Melting (HRM) analysis. This PCR assay can differentiate CaPV, through the fluorescent melting curve of the products targeting the CaPV RPO30 gene and it is valuable in detecting and genotyping field isolates of CaPV (Gelaye *et al.*, 2013).

3.5. Data management and analysis

The collected data during sample collection and laboratory investigation were coded and stored into Microsoft office Excel spread sheet 2010. The data was thoroughly screened before subjecting to statistical analysis. The field survey data was analysed with SPSS 18. Descriptive statistics was used to summarize data of field survey and laboratory investigation.

4. RESULTS

4.1. Active Outbreak investigation

In this study four active outbreaks were investigated between December, 2018 and May 2019. The first outbreak was reported from Bosat District Gari Nura Hera Peasant association (PA) and the second was from Adama District Bati Dagaga Peasant association (PA), the third was reported from Dugida district Burka Dalcha Peasant association (PA) andthe last was reported from Adami Tullu Jido Kombolicha District of Washi Gula Peasant association (PA) .During these outbreaks, 105 cattle were affected and 38 animals died. The LSD affected all age groups and both local and cross breeds. Out of 105 suspected cattle with vaccination history 24 animals were affected by LSD. The most commonly observed clinical signs of LSD were initial fever, skin nodules on different body parts, enlarged peripheral lymph nodes, depression, lameness and lacrimation

Figure 3: Clinical signs of LSD affected cattle.

Table 3: Summary of outbreak data in the study areas

Area	No of cattle at risk	No. of affected cattle	Morbidity rate (%)	Number of death	Mortality rate (%)	Case fatality rate (%
Adama	380	28	7.4	7	1.8	25
Bosat	480	30	6.25	12	2.5	40
AdamiTulluJido Kombolcha	450	25	5.6	9	2	36
Dugida	420	22	5.2	10	2.4	45.5
Total	1730	105	6.1	38	2.2	36.2

Table 4: Mortality, morbidity and case fatality rates of LSD according to age, sex, and breed and vaccination status of animals

Variable	Number at risk	Number affected	Number of dead	Morbidity rate(%)	Mortality rate (%)	Case fatality rate (%)
Sex						
Female	815	45	17	6.13	2.09	37.7
Male	915	65	21	7.1	2.29	32.3
Age						
< 2	630	45	12	7.93	1.9	26.6
≥2	1100	65	26	5.9	2.36	34.11
Breed						
Local	1620	90	37	5.5	2.28	37
Cross	110	15	1	13.63	0.9	0.2
Vaccination status	870	22	6	2.52	0.69	27.27
vaccinated	860	83	32	9.65	3.7	38.55
Not Vaccinated						
Total	1730	105	38	2.2	6.1	36.2

4.2. Virus Isolation

From the total of 20 samples collected, 12of them were grown on Vero cells for virus isolation. Cytopathic effects (CPE) were observed on those plates starting from the 3rd day of culture. CPEs were characterized by rounding of single cells, aggregation of dead cells and destruction of monolayer. None of the negative control cultures showed any CPE.

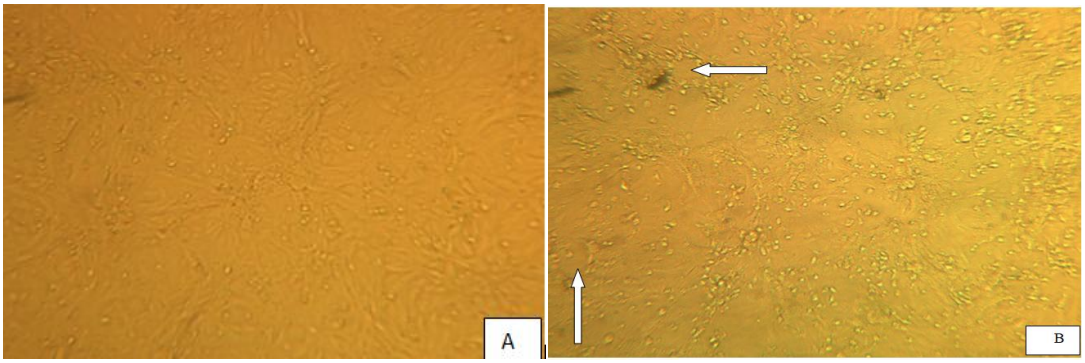

Figure 4: LSDV growth on Vero cell culture
(A) Vero cell culture after 24 hrs incubation; (B) LSDV CPE 3rd passage at 5th day

4.3. Viral DNA Identification by Conventional PCR

Conventional PCR was run targeting the RPO30 gene of the collected samples. Amplicons was analysed by 3% agarose gel electrophoresis. The specific primers set amplified a DNA fragment of 172 bp equal to the expected amplification product size from LSDV. The result showed that, the reference strain of the LSDV and the local isolate from skin nodules had the same size of RPO30 gene fragment 172 bp (Fig. 5).

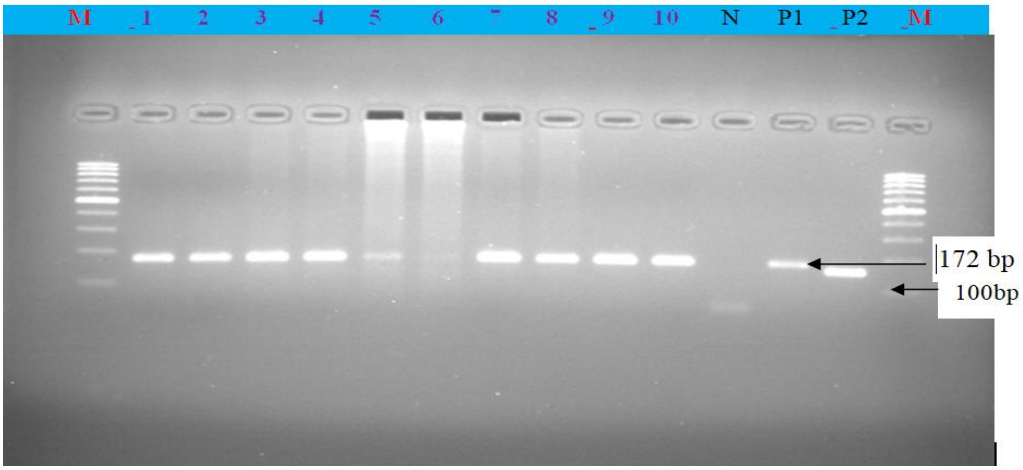

Figure 5: Classical PCR gel picture from skin nodule of LSDV infected cattle.

Lanes M: DNA ladder (100bpFermentas); Lanes 1, 2 and 3 represent positive samples from Bosat; Lanes 4, 5and 6represent positive sample from Adama; Lanes 7and 8 represent positive sample from Adam Tulu; Lane 9 and 10 represent positive sample from Dugida;Lane N: Negative Control without template; P1- Positive control for LSD and P2- Positive control for SPP

4.4. Real time Polymerase chain reaction

Real time polymerase chain reaction (RT- PCR) assay was used to detect the virus with Caprip oxvirus-specific primers used.

After amplification of the DNA template, the positive samples were noted by amplification fluorescence curves, melting curves (at 73 ℃), and cycle threshold (Ct) values from the assay which were used to describe the positive samples: Ct values with no or higher than 37 were indicated as negatives suggesting absence of the virus from the tissue specimens.

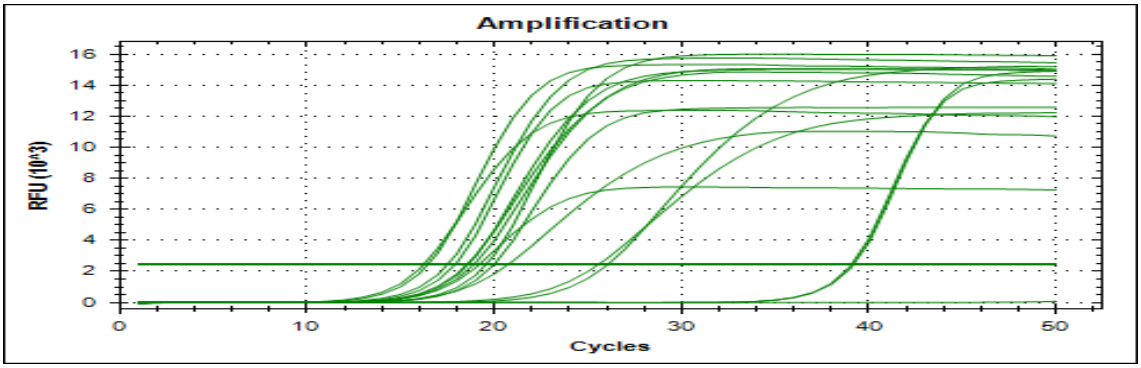

Figure 6: Real time PCR amplification results

Real time PCR results where the tested samples were positive showing amplification curve while the negative control stays horizontal. As indicated in (Table 5), positive samples have the Ct values lying between16-38.

Table 5: Real time PCR Ct values

Sample NO	Kebele	Ct value
S1	Gari Nura Dera	19.45
S2	Gari Nura Dera	18.65
S3	Gari Nura Dera	16.75
S4	Gari Nura Dera	16.45
S5	Borichota	17.40
S6	Borichota	19.35
S7	BatiDagaga	17.50
S8	BatiDagaga	18.70
S9	BatiDagaga	20.45
S10	Kachama	18.35
S11 positive control	----------	16.35
S12 negative control	----------	No
S13	Kachama	19.35
S14	BatiGarmama	20.49
S15	BatiGarmama	16.56
S16	Washi Gula	17.74
S17	Washi Gula	22.45
S18	Washi Gula	16.65
S19	Washi Gula	16.45
S20	Burika Dalacha	17.80
S21	Burika Dalacha	22.35
S22	Burika Dalacha	23.56
S23 Positive control	----------	18.60
S24 Negative control	----------	No

Cts< 29 are strong positive reactions indicative of abundant target nucleic acid in the sample

Cts of 30-37 are positive reactions indicative of moderate amounts of target nucleic acid

Cts of 38-40 are weak reactions indicative of minimal amounts of target nucleic acid which could represent an infection state or environmental contamination.

Cts of negative specimens were indicated very high Ct values (around 41).

4.4.1. Virus Genotyping

Melting curves are generated from the DNA of skin biopsy sample with respect to known LSDV, SPP and GTP controls. The normalized and overlaid fluorescence curves and melting pick are shown in Fig. 7 (A and B). The different samples trace each other very closely. As shown in the plots, the same melting profile as that of the LSDV reference strain, the amplicons 73.0^{o}C and the snapback 51.0^{o}C was obtained for all screened isolates. Whereas SPPV has 51.5ºC/72.5ºC and GTPV has 56.0ºC/72.5ºC.

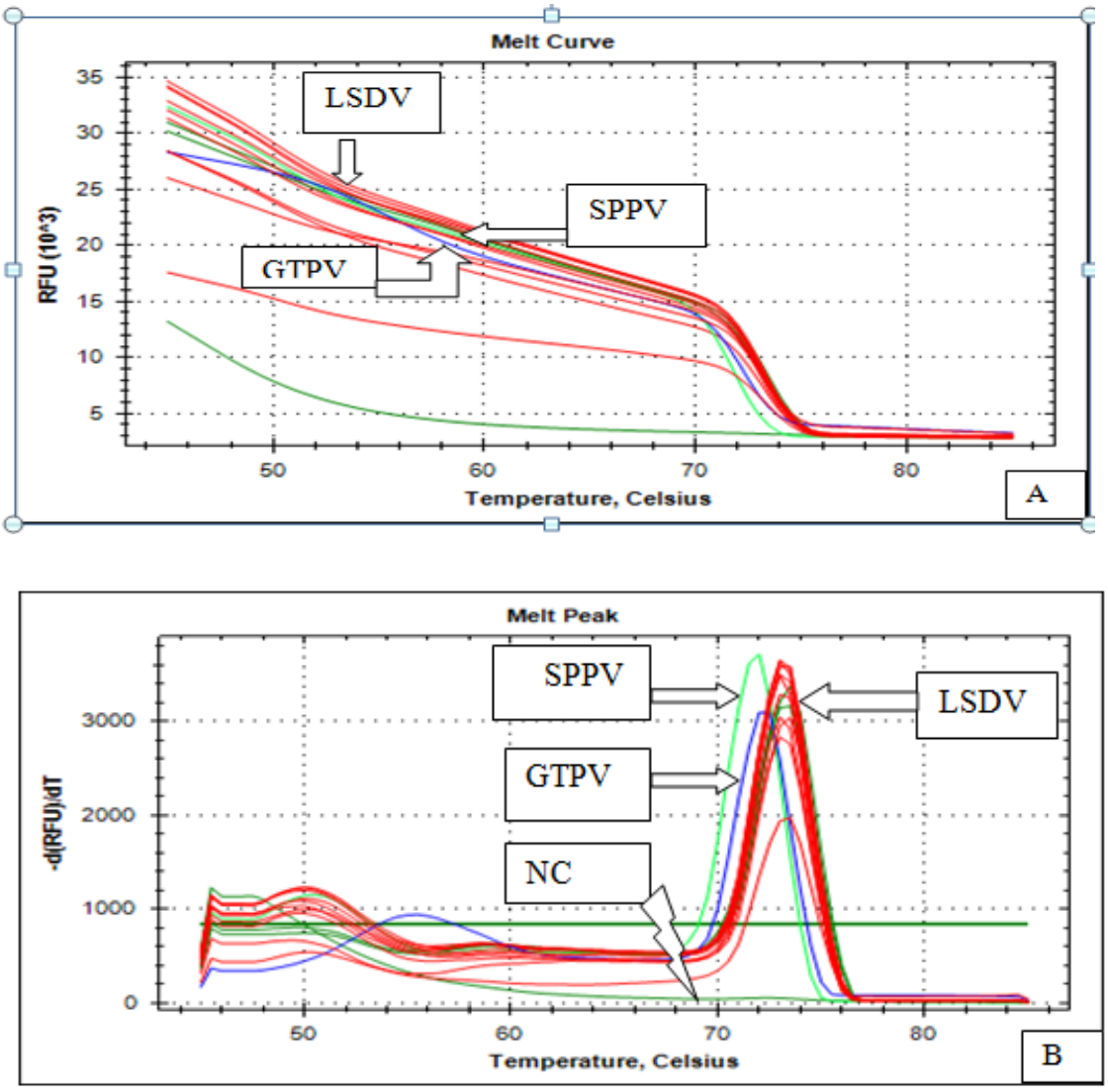

Figure 7: Melting curve analysis of Capri poxviruses of field isolates

5. DISCUSSION

LSD outbreak in central Ethiopia was reported at different time findings are in agreement with the findings, recently in central Ethiopia Mesay (2018) reported LSD with 8.77% morbidity, and 2.12 % mortality and 25.61 % case fatality rates and (Ayelet *et al.,* 2014) reported LSD with 13.61% morbidity, and 4.97% mortality and 36.94 % case fatality rates.

In the present study morbidity, mortality and case fatality rates of LSD were 6.1 %, 2.2 % and 36.2 % respectively, indicating an important impact posed by the disease in the area. This may be due to the farming and management system practiced in the area which favors the vector transmission and poor nourishment of diseased animals which will die due to secondary disease. The case fatality rate was closer to reported from the study in central Ethiopia with 36.94 % (Ayelet *et al.,* 2014). Higher morbidity (21.2%) and mortality rates (4.5%) than the current study were previously reported by (Molla *et al.*, 2017) from Ethiopia. In addition, higher morbidity rate (26%), but lower mortality rates (1.9%) were also recorded in Jordan (Abutarbush *et al.*, 2015).

Different morbidity, mortality and case fatality rates were recorded in different districts with the highest morbidity in Adama (7.4%) and lowest in Dugida (5.2%). This might be due to the proximity of Adama district near to Awash River which might be suitable for the replication of arthropod vectors.

In the present study, the morbidity rate of LSD indicated that calves were more susceptible to infection than adult cattle. This might have occurred due to the natural susceptibility of young animals which may also usually show more severe clinical disease (Coezer and Tuppurainen, 2004). This finding is in line with the reports of Ahmed and Zaher (2008) and Ayelet *et al.,* (2014). On the contrary, the low prevalence in calves in other study may be associated with lower susceptibility of calves to biting flies and keeping them where there is less insect activity (Troyo *et al.*, 2008). Another possibility may be the presence of passive maternal immunity which protects calves for about 6 months (Weiss 1968).

There were no significant differences between sex and LSD mortality in this study despite the slightly higher mortality of LSD in males (2.29) than females (2.09 %) while the case fatality was higher among female animals (34%) than male animals

(32.3%). Even if the physiological conditions of female animals (like pregnancy and lactation) might affect their susceptibilities, evidenced by increase in case fatality rates. The farmers in the area use oxen for ploughing their lands and stress and fatigue might have increased the mortality rates.

The study also shows that rates of morbidity and mortality were higher in cross breeds than local cattle with statistically significant differences. The genetic differences between breeds may have influenced susceptibility to the disease (Abera *et al.,* 2015; Davies, 1991). In contrast, high case fatality rates were recorded in local cattle. This might be due to lack of proper management of diseased local animals because of their lower market values. These variations could be from the differences in geographic location and climate; the management conditions, breed, immune status and condition of the animals, virulence of the virus, and the number and types of putative insect vectors (Tuppurainen and Oura, 2011).

In the area more number of cattle was vaccinated, the disease has been manifested by low morbidity and mortality rates consider of vaccination status findings are in agreement with findings Local strain of Kenyan sheep and goat pox virus has been shown to effectively immunize sheep, goats and cattle against infection with capripox virus with a remarkable success (OIE, 2010). In addition, problems in vaccine management, including transport, storage and vaccine route may have influenced vaccine efficacy. Introduction of animals already incubating the virus or newly vaccinated animals becoming infected before they develop protective immunity due to untimely vaccine campaigns (vaccination after the out breaks have already occurred in the area) might have also a great contribution for vaccine failure (Ayelet *et al.,* 2014).

LSDV can be propagated in a variety of primary cells or cell lines of bovine, ovine or caprine origin. The virus has been adapted to grow on the chorioallantoic membrane of embryonated chicken eggs and African green monkey kidney (Vero) cells (OIE, 2017). It grows slowly on cell cultures, and CPE can usually be detected four to six days after inoculation (Tuppurainen, 2017). A characteristic CPE consisting of retraction of the cell membrane from surrounding cells, and rounding of individual cells and margination of the nuclear chromatin can be seen (OIE, 2017). In these outbreak investigation the virus was isolated by growing on 32st passage Vero cells

and CPE 3rd passage at 5th day were characterized by aggregated cells, destruction of cell monolayers and rounding of cells. Similar CPE characteristics were recorded by other authors (Ayelet *et al*., 2014; Lamya *et al*., 2017, Mesay, 2018).

In presennt studythe total of 20 samples collected, 12 of them were grown on Vero cells for virus isolation cytopathic effects (CPE) were observed study indicated a fair agreement between results of the virus isolation (VI) and PCR, although virus could not be isolated from all PCR-positive samples. These results support those of other workers (Awad *et al*., 2010; Tuppurainen *et al*., 2005), who reported that the PCR was more sensitive and accurate. The large proportion of positive samples in PCR tests may be related to the assay's ability to detect LSDV DNA in blood and skin biopsies for a longer period than VI (Tuppurainen *et al*., 2005). Further, the PCR can detect low genome copies of the pathogen even in the dead state, its rapidity and relative ease of analysis and interpretation (Al-Salihi 2014; Awad *et al*., 2010).Samples were tested by conventional PCR for the presence of LSDV DNA amplifying the RPO30 gene with specific primer. The reference strain of the LSDV and all the local isolate from skin nodules had the same size of attachment protein gene fragment 172 bp.

Real-time PCR is a rapid, sensitive and specific method for confirmation of capripoxviruses including LSD (Balinsky *et al.,* 2008). Real-time PCR technique was used for identification of the virus responsible for the outbreaks and all samples were positive. The Ct values taken from the positive samples indicate lower numbers lying around the Ct values of positive controls. No or higher values were indicated as negatives in which lower or no loads of the virus are present. This is in agreement with the other finding which reported Ct values less than 37 as positives (Salnicove *et al*., 2018).

The samples were genotyped with real time PCR by using an assay developed by (Gelaye *et al*., 2013). This assay is able to genotype CaPVs based on the snapback and amplicon melting temperature. Genotyping was performed for the reason that LSD confusion with SPPV may occur, as it is rarely transmitted to cattle producing skin lesions (Kitching and Carn, 1995), also (Burdin and Prydie, 1959) reported that an experimental infection of cattle with SPPV can produce similar lesions to LSD.

The results come out Provide with melting temperature of 73.0^{o}C for the amplicons and 51.0^{o}C for the snapback confirming that the isolated samples are indeed LSDV Whereas SPPV has 51.5ºC/72.5ºC and GTPV has 56.0ºC/72.5ºC. This agreement with the other finding that real time PCR assay make a significant contribution to CaPVs diagnosis and to the better understanding of the epidemiology of CaPVs by enabling rapid genotyping and gene-based classification of viral strains and unequivocal identification of isolates(Charles *et al*., 2010).

6. CONCLUSION AND RECOMMENDATIONS

In the present study three LSD outbreaks in East shawa zone were investigated. The disease affected all age groups of cattle regardless of the differences in breed, sex and vaccination status and already caused great economic loss due to high mortality, morbidity and case fatality rates. Cell culture and conventional PCR has detected Capri pox virus in this study. Finally, real time PCR confirmed that the outbreaks were due to LSDV. Regular and timely vaccination strategy is the best choice available for effective control of the disease accompanied with early detection of the disease.

Therefore the following recommendations were forwarded from the above conclusions.

- Annual surveillances on the status of the disease.
- Regular annual vaccinations by effective and well managed vaccines,
- Awareness creation for cattle owners to vaccinate healthy animals for control and support the diseased ones to prevent death and disease transmission.
- Broader and detailed investigation including sequence characterization and determination of their evolutionary relationship of the viruses as compared to the vaccine strain in our country.

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8. ANNEXES

ANNEX 1: LSDV field outbreak assessment questionnaires form

Region: _____

Zone: _____

Type of disease suspected: _____

Date: _____

Farm (Herd) history

Production type: _____

Breed _____

Sex _____

Age _____

Total population of animal _____

No. Sick _____

No. death _____

Vaccination history _____

Clinical sign observed _____

ANNEX 2: Vero cell line culture preparation

1. First all the cell culture mediums were warmed at 37oC 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, 1 - 2 ml per 25 cm2 of flask surface, and was gently spread over the entire surface by tilting the vessel.
3. The cultures were placed in an incubator at 37^{o}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 was collected by pipetting culture medium over the surface of the vessel, i.e. 2 - 5 ml per 25 cm2 of flask surface then was mixed carefully to disperse the cells into a single-cell suspension.
5. The cell suspensions was transferred into a new 25cm2 tissue culture flask containing GMEM with FCS 10% and streptomycin antibiotics at 7.2-7.4 PH.

6. The cell cultures were incubated at 37oC temperature in 5% CO2 incubator and were checked regularly. The medium in each flask was changed after 3 days until monolayer of the cells were formed. The cell cultures were passaged continuously until they are well formed.

ANNEX 3: Phosphate Buffer Saline (PBS) solution.

No.	Ingredients	Amount required
1	Di- sodium hydrogen phosphate (Na2HPO4)	1.6 gms
2	Potassium di hydrogen phosphate (KH2PO4)	0.51 gms
3	Sodium chloride	7.3 gms
4	Double distilled water	Up to 1 liter

Adjust pH to 7.4 with NaHCO3 solution

ANNEX 4: Growth and maintenance media preparation for cell culture

Growth medium consist of GMEM supplemented with 10% tryptose phosphate broth (TPB), 0.63% of a 10% NaHCO3 solution, 1% of Antibiotic-Antimycotic Mixture 100X (Gibco, Grand Island, New York, USA), and 10 % fetal calf serum (FCS). For virus isolation, this medium was replaced before 24 h by the same medium but supplement with only 2% FBS.

ANNEX 5: Trypsin Solution (0.25%)

Ingredients	Amount
NaCl	8.0 gm
KCl	0.4 gm
Glucose 0.4 gm	1.0 ml
Phenol red (0.5% solution)	1.0 ml
Trypsin (1:0.25)	1.0 ml
$NaHCO_3$	0.35 gm
Purified H20 q.s.	1 liter

Adjust pH to 7.4 with $NaHCO_3$solutionn

ANNEX 6 : Virus inoculation on cell culture

1. First, disinfect all the medium and solution bottles and other materials outer surfaces with 70% Ethanol before setting to the safety cabinet.
2. Decant the culture medium from the flask forming monolayer of the ell.
3. Wash monolayer gently with 2-3 ml of pre warmed PBS 2-3 times.
4. Add 0.5-1ml sample inoculums to the cell culture the depending on the flask size. Rock each plate gently to distribute inoculums evenly over the cell monolayer.
5. Incubate inoculated cultures at 37 ^{O}C incubators for 1 hr to allow virus to adsorb.
6. Shake the inoculated flasks once or twice during incubation.
7. Add GMEM with 2% FCS maintenance medium to each flask and incubate at 37oC for about 14 days.
8. Check flasks daily for cytopathogenic effect (CPE) and condition of cells under inverted microscope.
9. To harvest samples, freeze-thaw 2-3 times.

ANNEX 7: DNA extraction (Qiagen, Germany)

1. The processed tissue sample is placed in a 1.5 ml microbe centrifuge tube. Add 180 µl buffer ATL, 20µl proteinase K, mix by vortexing, and incubate at 56oC until completely lysed. Vortex occasionally during incubation. Vortex 15 s directly before proceeding to step 2.
2. Add 200 µl buffer AL. Mix thoroughly by vortexing. Incubate samples at 56oC for 10 min.
3. Add 200µl ethanol (96-100%). Mix thoroughly by vortexing.
4. Pipet the mixture into a DNeasy Mini spin column placed in a 2 ml collection tube. Centrifuge at ≥ 6000 x g (8000 rpm) for 1 min. Discard the flow-through and collection tube.
5. Place the spin column in a new 2 ml collection tube. Add 500 µl Buffer AW1. Centrifuge for 1 min at ≥6000 x g. Discard the flow-through and collection tube.
6. Place the spin column in a new 2 ml collection tube. Add 500 µl Buffer AW2, and centrifuge for 3min at 20,000 x g (14,000 rpm). Discard the flow-through and collection tube.
7. Transfer the spin column to a new 1.5 ml or 2 ml micro centrifuge tube.
8. Elute the DNA by adding 50µl Buffer AE to the center of the spin column membrane. Incubate for 1 min at room temperature (15-25oC). Centrifuge for 1 min at ≥ 6000 x g.

ANNEX 8: Conventional PCR master mix preparation and PCR work protocol for RPO30 gene amplification

PCR was conducted in a reaction volume of 25 µl containing 4 µl forward and reverse primers, 2.5 µl dNTPs, 2.5 PCR Buffer (Qiagen), 10.75 RNAse free water, 2 MgCl2, 0.25 of Taq Polymerase (Qiagen) and 3 template DNA. The PCR was run with initial denaturation at 95 ^{o}C for 4 min, followed by 40 cycles of denaturation, annealing and elongation at 95 ^{o}C for 30 s, 55^{o}C for 30 s and 72 ^{o}C for 30 s, respectively then a final extension at 72 ^{o}C for 5 min. PCR products were checked using electrophoresis on a 1.5% agarose gel stained with GelRed (Biotium, inc.) for 1 h at 100 V.