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

MSc. THESIS

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BISHOFTU, ETHIOPIA

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

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

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As Msc research advisors, we here by certify that we have read and evaluated this thesis prepared under our guidance by entitled: "**Isolation And Molecular Characterization Of Lumpy Skin Disease Virus in Central Ethiopia**" we recommend that it can be submmited as fulfilling the Msc thesis requirement.

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STATEMENT OF AUTHOR

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LIST OF ABBREVIATIONS

CAM	Chorio Allantoic Membrane
CaPV	CapriPox Virus
CPE	Cytophatic effect
ESH-L	Epithelial Sheep Skin origin cell line
FAT	Fluorescent Antibody Technique
FCS	Fetal Calf Serum
GMEM	Glasgow's Modified Eagles's Medium
GTPV	Goat Pox Virus
HRM	High Resolution Melting
LSD	Lumpy skin disease
LSDV	Lumpy Skin Disease Virus
LT	Lamb Testes
MDBK	Madin-Darby bovine kidney
MOA	Ministry of Agriculture
NVI	National Veterinary Institute
OA3.Ts	Ovine Testis Cell Line
OIE	Office International des Epizooties
PBS	Phosphate buffer saline
PCR	Polymerase Chain Reaction
RT-PCR	Real Time Polymerase Chain Reaction
SPPV	Sheep Pox Virus

ABSTRACT

Lumpy skin disease (LSD) is an infectious disease of cattle, caused by a Lumpy Skin Disease Virus. LSD Causes considerable economic losses due to emaciation, damage to hides, infertility and, loss of milk production. In Ethiopia the disease is distributed almost all regions and is regarded as one of the most economically important livestock diseases in the country. Outbreak investigations have been carried out at different regions of the country on different times. The current study focused on the identification of LSDV based on outbreak reports central Ethiopia. Outbreak survey found 8.77% morbidity, 2.12% mortality and 25.61% case fatality rates in the region. Skin lesion samples were collected from clinically sick cattle and virus isolated on cell culture and shown the characteristics CPE of the virus. The virus DNA was identified by amplifying the 172bp DNA fragment using real time and conventional PCR. Phylogenetic analysis of the RPO30 gene sequence revealed that, the present isolates are form matching with previously identified isolates from Ethiopia and also with the KS-1 vaccine strains. The present study also attempt to isolate virus on Vero, ESH-L and LT primary cell cultures and comparing their susceptibility. The study found that lamb testes primary cell was best suited for primary isolation of LSDV. Vero cell however was found to be less sensitive for LSDV primary isolation but isolation can be achieved through continuous passage of the virus.

Keywords: Cattle, Cell Culture, Ethiopia, LSD, LSDV

1. INTRODUCTION

Lumpy skin disease (LSD) is an infectious disease of cattle, caused by a Lumpy Skin Disease Virus (LSDV), which is found in the family Poxviridae, genus Capripoxvirus. It is closely related antigenically to sheep and goat poxvirus (Alexander *et al*., 1957). LSD Causes considerable economic losses due to emaciation, damage to hides, infertility, mastitis, loss of milk production, and mortality of up to 20% (Al- Salihi, 2014).

Experimental and field evidence indicates 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 (Ali *et al*., 2012; Tuppurainen and Oura, 2012). The disease is more prevalent in low-lying areas and along water courses (Weiss, 1968).

Fever is the initial sign that is followed within two days by the development of nodules on the skin and mucous membranes (Brenner *et al*., 2006; Tuppurainen and Oura 2012). A presumptive diagnosis of the disease can be made based on highly characteristic clinical signs of LSD. However, mild and asymptomatic disease may be difficult to diagnose and rapid laboratory methods are needed to confirm the diagnosis. Different molecular tests are the preferred diagnostic tools (Kumar, 2011).

Immunization of the susceptible animals is the effective methods to control the disease. The most commonly used live LSDV vaccines are derived either from the South-African LSDV Neethling strain or an attenuated LSDV field strain and are manufactured in South Africa (Ben *et al*., 2015). In Ethiopia vaccine against LSD has been producing at the National Veterinary Institute (NVI), Bishoftu. The attenuated SPPV Kenya O-180 vaccine strain (referred to as KS-1) of Kenyan origin is used for the production of capripoxvirus vaccine, for small ruminants and cattle, by the NVI (Ayelet *et al*., 2013).

There have been repeated concerned reports to NVI on the insufficient protection provided by the vaccine, for cattle against LSDV. A preliminary assessment had ruled out the possibility that the vaccination failure could be due to a low titer of the vaccine, the presence of maternal antibodies, improper vaccine storage and handling, or the possibility that animals were incubating the disease prior to vaccination (Ayelet *et al*., 2013). The study conducted by Gelaye *et al*., (2015) showed that, analysis of the GPCR and RPO30 genes showed major sequence differences between the vaccine strain and the field isolates, a 12-nucleotide deletion observed on the GPCR gene sequence of the field isolates.

According to the OIE terrestrial 2017 manual the efficacy of LSDV vaccine must be demonstrated in statistically valid vaccination challenge experiments under laboratory conditions using sero negative young LSDV susceptible dairy cattle breeds challenged with a known virulent capripoxvirus strain. Trials are made at NVI to develop a new vaccine strain, challenge virus and to study pathogenicity of the virus with experimental inoculation.

LSDV will grow in tissue culture of bovine, ovine or caprine origin, although primary or secondary culture of bovine dermis cells or lamb testis (LT) cells are considered to be the most susceptible. 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 however is not recommended for primary isolation (OIE Terrestrial Manual, 2017). Ovine testis secondary cell line (OA3.Ts) has been evaluated for the propagation of capripoxvirus isolates (Babiuk *et al.,* 2007). Different researches proved the favorable use of LT cells for growing LSDV for diagnostic and vaccine production purposes.

Therefore, this research was undertaken with the following objectives:

General Objective

- ✓ To investigate the occurrence of LSD in central Ethiopia using different diagnostic techniques

Specific Objectives

- ✓ To investigate the occurrence of LSD in central Ethiopia
- ✓ To isolate the infectious LSDV from skin lesion samples in different cell cultures
- ✓ To detect and genotype the virus using molecular methods
- ✓ To sequence the virus DNA and see the genetic relation with other LSD viruses

2. LITRATURE 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, 1999).

In 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; House *et al*., 1990; Davies 1991; Fayez and Ahmed 2011; Ali and Amina 2013), Israel in 1989 and 2006 (Shimshony 1989; 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

LSD virus (LSDV) belongs to the family Poxviridae that is divided into two subfamilies, poxviruses affecting insects (Entomopoxvirinae) and vertebrates (Chordopoxvirinae). CaPVs represent one of eight genera within the Chordopox virus (ChPV) subfamily. The capripox virus genus consists of *Lumpy skin Disease Virus*, as well as *sheeppox virus*, and *goatpox virus*. The prototype of LSDV, Neethling strain, was isolated in South Africa

(Alexander *et al*., 1957). CaPV infections are usually host specific within specific geographic distributions even though they are serologically indistinguishable from one another (Tulman *et al*., 2001).

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).

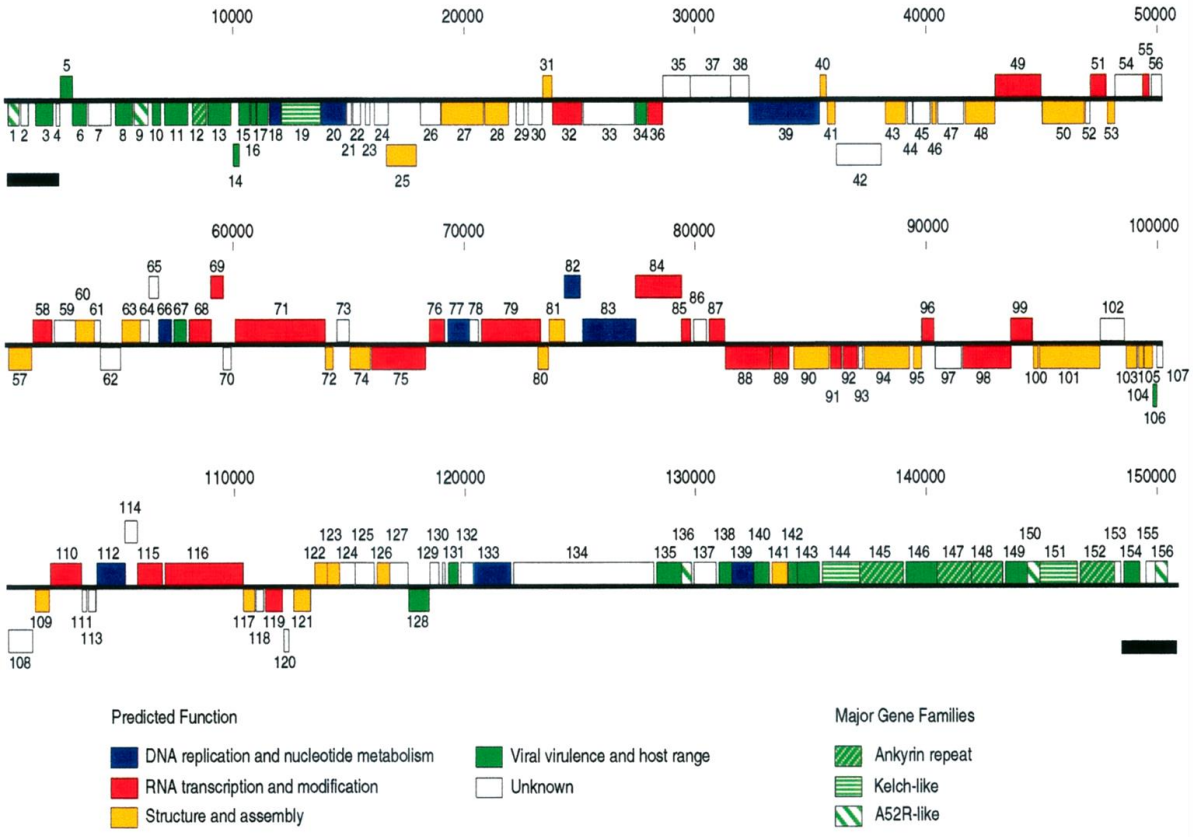

Figure 1: Linear map of the LSDV genome (Tulman *et al.*, 2001)

2.3.2. Replication cycle

The replication of LSDV occurs in the cytoplasm of the host cell in intracytoplasmic 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 susceptible to temperature at 55^{o}C for 2 hours and 65^{o}C for 30 minutes. The virus is susceptible to highly alkaline or acid pH. LSDV has remarkably stable, surviving for long periods at ambient temperature, especially in dried scabs. LSDV is very resistant to inactivation. It is surviving 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. Meanwhile, the virus is susceptible to sunlight and detergents containing lipid solvents, while, in dark environmental conditions, such as contaminated animal sheds, it can persist for many months (OIE, 2014).

2.4. Pathogenesis

LSD is caused by infectious LSDV and accompanied with febrile reaction (Vorster and Mapham, 2008). The disease affects cattle and tends to be more severe in milking cows in the peak of lactation and in young animals (Gari *et al*., 2011). LSDV replicates inside the host cells such as macrophages, fibroblasts, pericytes and endothelial cell in the lymphatics and blood vessels walls lead to developing vasculitis and lymphangitis, while thrombosis and infarction may develop in severe cases. Mechanism by which the virus observed to cause skin lesions is through to replication of the virus in specific cells such as pericytes and endothelial cells of lymphatic and blood vessel walls. Histopathological sections of early skin lesions of epidermis show an epitheloid 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; AUSVETPLAN, 2009).

Pathogenic mechanism of viral disease involves implantation of virus at the portal of entry, replication at that site, spread to target organs, and spread to sites of shedding of virus into the environment. Viral disease occurs if the virus replicates in essential cells sufficiently and destroys them directly or damages organ function indirectly as a result of the host immune response to the presence of virus proteins (Baron *et al*., 1996).

The incubation period of LSD under field conditions is two to four weeks (Haig, 1957). Intravenous, intradermal and subcutaneous routes are used in experimental infection. 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. However, generalized eruption of skin nodules usually occurs seven to 19 days after inoculation. Viraemia occurred after the initial febrile reaction and persisted for two weeks (Vorster and Mapham 2008).

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). In animals that develop clinical disease, there is a biphasic febrile reaction that may exceed 40^{o}C. They remain febrile for 4 to 14 days. This is accompanied by depression, disinclination to move, in appetence, salivation, lachrymation and a nasal discharge, which may be mucoid or mucopurulent. The superficial lymph nodes, especially prescapular, precrural and subparotid, are usually markedly enlarged (Haig, 1957; Weiss, 1968; Prozesky and Barnard, 1982; Carn and Kitching, 1995).

The eruption of nodular skin lesions usually occurs within 48 hours of onset of the febrile reaction. Predilection sites are the skin of the head, neck, perineum, genitalia, udder and limbs. Nodules are 5 to 50 mm in diameter, circumscribed, firm, round raised, and involve the skin, subcutaneous tissue and sometimes even the underlying muscles (Tuppurainen and Aoura, 2012).

Figure 2: Nodules on neck and abdominal area.

Some acutely affected animals may develop severe subcutaneous oedema of the ventral parts of the body such as the dewlap, brisket, limbs, udder, scrotum and vulva. Oedematous and necrotic lesions in the udder may result in mastitis. In some animals, necrotic lesions in the trachea and lungs may lead to pneumonia. Bulls usually become temporarily infertile, but sometimes because of severe orchitis they may become permanently infertile. Pregnant cows may abort and be in anoestrus for several months (Alexander *et al*., 1957; Haig, 1957; Weiss, 1968; Prozesky and Barnard, 1982; Davies, 1991; Carn and Kitching, 1995).

2.6. Diagnosis

Diagnosis of LSD is often based on characteristic clinical signs. However, mild and subclinical forms require rapid and reliable laboratory testing to confirm diagnosis. Isolation of virus can be made from collected biopsy or at post-mortem from skin nodules, lung lesions or lymph nodes within the first week of the occurrence of clinical signs, before the development of neutralizing antibodies (House, 1990; OIE, 2010; Davies, 1991; CFSPH, 2008).

Laboratory diagnosis of LSD comprised either identification of the virus using: electron microscopy, egg inoculation, isolation in cell cultures, fluorescent antibody test; or detection of its specific antibody using serological tests. Several polymerase chain reaction (PCR) assays have been developed for more accurate and rapid detection of LSDV in suitable specimens (Heine *et al*., 1999 and Stram *et al*., 2008). PCR for the diagnosis of LSD is with a greater sensitivity and good specificity and it is most appropriate technique (Kholy *et al*., 2008; OIE, 2010).

2.6.1. LSD virus isolation on different cell cultures

Diagnosis of CaPV is based upon clinical signs with laboratory confirmation by virus isolation, polymerase chain reaction (PCR) and electron microscopy. Although CPV will grow in a variety of cell types from cattle, goat, and sheep origin (Binepal *et al*., 2001); primary lamb kidney or primary lamb testis are the most commonly used cell systems for isolation (Ferris and Plowright, 1958). Use of these cells is preferred mainly because of their ability to support the replication of a variety of CPV isolates (Kalra and Sharma, 1981; Zhou *et al*., 2004) and the ability to obtain sufficient numbers of cells from the respective organs. Weiss in 1968 used primary lamb testes (LT) to grow CPV. However, the reduced availability of lambs, mainly owing to efforts to minimize animal use, has necessitated investigating the suitability of continuous cell lines.

Different studies are conducted on the search to find continuous cell lines suitable for the primary isolation of the CPV. Babiuk *et al*. (2007) evaluated the utility of OA3.Ts cells for CPV propagation, titration, and serological detection while comparing with primary lamb kidney (LK) cell. The study found that OA3.Ts cells are a suitable ovine cell line for the propagation of CPV (both laboratory strains as well as field isolates). Unlike other primary and secondary ovine cells, the OA3.Ts cells provide reproducible virus growth and are susceptible to infection by a variety of CPV strains, without culture adaptation. Other continuous cell lines, vero, ESH-L, Madin-Darby bovine kidney (MDBK) are also used for isolation of LSDV.

2.7. Lumpy Skin Disease Virus with Other CapriPox Viruses

Capripoxviruses comprise 1 of 8 genera within the subfamily Chordopoxvirinae, family Poxviridae. Members of the genus are sheeppox, goatpox, and lumpy skin disease viruses that cause disease in sheep, goats, and cattle respectively. Lumpy skin disease virus (LSDV) is endemic in Africa, whereas goatpox and sheeppox are endemic in Africa, the Middle East, and Asia and are the most economically significant pox diseases of ruminants (Carn, 1993; Kitching, 2003).

CaPVs are double-stranded DNA viruses with genomes approximately 150 kbp in size. Goat and SPP share at least 147 putative genes. LSDV has an additional 9 genes likely involved in the ability to infect cattle. DNA analysis using restriction endonucleases on field samples and vaccine strains showed 80 % homology between strains of CaPVs (Black *et al*., 1986).

CaPVs are enveloped or non-enveloped, brick or oval shaped viruses (Fenner *et al*., 1987) with similar morphology (except parapoxviruses). CaPV virions are different than orthopox virus virions in that they have a more oval profile, as well as larger lateral bodies. The average size of CaPVsis 320 nm by 260 nm.

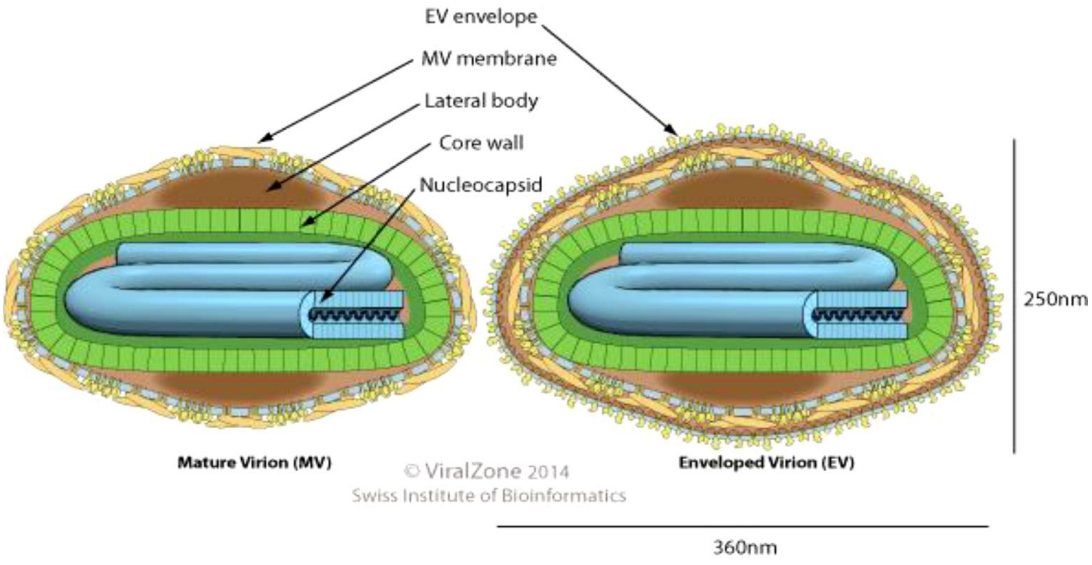

Figure 3: General structures of Capri poxviruses: Enveloped, brick-shaped with average size of 260-320 nm.

Source**:** (Viral Zone, 2018)

It is not possible to distinguish between different strains of CaPV using serological assays (Kitching, 2003). Molecular studies have demonstrated that LSDV, SPPV and GTPV are phylogenetically distinct (Tulman *et al*., 2001; Tulman *et al*., 2002) and, recently, by sequencing the host specific Gprotein coupled chemokine receptor (GPCR), or RNA polymerase (RPO30) genes, species specific molecular assays have been developed for differentiation of CaPVs, enabling the phylogenetic grouping of CaPVs (Le Goff *et al*., 2009; Lamien *et al*., 2011a; Lamien *et al*., 2011b). Gelaye and his colleagues (2013) developed a new molecular assay for CaPV genotyping using unlabelled snapback primers in the presence of dsDNA intercalating dye. This assay is able to simultaneously detect and genotype CaPVs.

2.8. Prevention and Control

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 and eradication 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 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. 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 modelling. 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. Economic Importance of the Disease

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.

The disease was mainly affects cattle with subsequent effects on production through the morbidity and reduced 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).

The morbidity and mortality rates for LSD vary greatly in different endemic areas depending on the severity of strain, prevalence of insect vectors and susceptibility of the host (Getachew *et al.*, 2010). An outbreak in a previously free country could be expected to result in a high morbidity rate. If LSD became endemic, continuing economic loss and

poor productivity would occur due to stock losses, reduced production in cattle industries and cost of preventative vaccination. Permanent loss of some markets would also be expected, with associated downturn in rural economy and increased rural unemployment (Tuppurainen and Oura, 2011).

2.10 Lumpy Skin Disease in Ethiopia

LSD was first reported in 1983 in the northwestern part of the country near Lake Tana(Mebratu *et al.,* 1984). There were frequent outbreak reports of LSD in the county that are highly associated with seasonal peak of mechanical vectors in wet and warm weather conditions (Getachew *et al*., 2010).The disease has spread to almost all regions and agro-ecological zones of the country. Because of the wide distribution of the disease and the size and structure of the cattle population in Ethiopia, it is likely that LSD is one of the most economically important livestock diseases in the country.

One of the recent outbreaks of LSD was occurred in central Ethiopia in 2007 to 2011. These outbreaks were described as active. It was investigated in four districts: Adama, Wenji, Mojo and Welenchiti. The totally 1,675 outbreaks were reported over 5 years period from 2007 to 2011, with 62,176 cases and 4,372 deaths. The Oromia represented the highest numbers of outbreaks (1,066), followed by Amhara (365) and the Southern Nations, Nationalities and People's Region (123). The 2010 were reported the highest number of outbreaks that were frequently seen between September and December. The morbidity and mortality rates were 13.61% (296) and 4.97% respectively (Ayelet *et al.*, 2014).

3. MATERIAL AND METHODS

3.1. Study Area

The study was conducted from October 2017 to April 2018 in four selected areas of central Ethiopia, based on LSD outbreak report. Samples were collected from Asela, Bishoftu, Akaki and Holeta Genet towns of central Ethiopia. Asela is a town and separate woreda in central Ethiopia, situated in the Arsi Zone of the Oromia Region, about 175 kilometers from Addis Ababa. This city has a latitude and longitude of 7°57′N 39°7′E, with an elevation of 2,430 meters. Bishoftu, formerly known as Debre Zeit, is a town located 47.9 km southeast of Addis Ababa. The site is found at an altitude of 1920 m.a.s.l and has a latitude and longitude of 8°45′N 38°59′E. Akaki is one of the woredas in the Oromia Region of Ethiopia, located to northwest of Addis Ababa. The altitude of this woreda ranges from 1500 to 2300 m.a.s.l. Holeta Genet has a latitude and longitude of 9°3′N 38°30′E and an altitude of 2391 meters above sea level, located 22km from Addis Ababa ("World Gazetteer Map," 2012).

The laboratory investigation was conducted at the Debre Zeit, National Veterinary Institute (NVI) located 47.9km southeast of Addis Ababa.

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 farm supervising veterinarians and farm owners using semi-structured questions 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 (Annex1).

3.3. Sample Collection and Transportation

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 was placed in the sterilized universal bottle containing tryptose phosphate brothand transported to NVI maintaining cold chain system. Then, the tissue samples were stored at -20^0C until processing.

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 (source NVI) was already available with passage number 72. 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 terrestrial manual 2017, chapter 2.4.13. Lumpy Skin Disease. About 0.5 ml of the processed tissue suspension samples was inoculated onto confluent layer of vero cell in 25cm^2 tissue culture flasks and the cultures were incubated at 37^{0}C and allowed to adsorb for 1 hour. The culture is then washed with warm PBS(Annex 3) and covered with 10 ml of 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% CO2.The culture medium was replacedwith fresh mediumevery 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 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 at NVI molecular biology laboratory (Annex 6).

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).

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Table 1: Specific primers used for conventional PCR

Primer Name	Sequence	PCR product length	Reference
Forward	5'-TCTATGTTCTTGATATGTGGTGGTAG-3'		Lamien *et al.*
reverse	5' -AGTGATTAGGTGGTGTATTATTTTCC- 3'	172 bp	(2011)

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 thermal cycler (Applied Bio Systems). 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 µl Gel red 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 trans-illuminator gel documentation system and gel picture was captured using a Polaroid photograph camera (UVitec) and results were recorded.

3.4.5. Virus genotyping

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

Real-time PCR was performed using the amplification primers and PCR protocol as described by Gelaye_*et al.*(2013). The primers that were used are;

Snapback primer: 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. RPO30 gene amplification and sequencing

The RNA polymerase subunit (RPO30) 30 kDa gene of the samples was amplified and purified for sequencing. Two sets of primers for the RPO30 gene were used for the amplification.As described by Gelaye_*et al*. (2015).

CaPVRPO30-OL2Fow: 5'-TTTGAACACATTTTATTCCAAAAAG-3'
CaPVRPO30-OL2Rev: 5'-AACCTACATGCATAAACAGAAGC-3'

Convectional PCR master mix preparation and work protocol for RPO30 gene amplification is available at Annex 7.
The positive PCR products of the amplified RPO30 gene were purified using the Wizard SV Gel and PCR clean-up system kit (Promega, Germany) and sequenced commercially by LGC Genomics (Germany).

3.4.7. Sequence analysis and phylogenetic tree reconstruction

The sequence data were edited and fragments were assembled using Vector NTI Advance™ 11.5 software (Invitrogen, Carlsbad, CA, USA). Multiple sequence alignments were performed using the ClustalW algorithm implemented in BioEdit software package to compare the RPO30 gene of the outbreak isolates and the reference strain. For comparative studies, blastn was used to collect additional CaPV RPO30 gene sequences from GenBank for inclusion in the data set. For construction of phylogenetic tree, multiple sequence alignments were performed to align the sequences as codons using the Muscle algorithm in MEGA6 (Tamura *et al*., 2013). The Neighbor-Joining algorithm was used with the maximum composite likelihood nucleotide substitution model with the pair wise deletion option was used. For construction of phylogenetic tree, 1000 bootstrap replicate was used.

3.5. Cell cultures comparison for isolation of LSDV

3.5.1. Cells and Viruses

According to the OIE (2017) manual on LSD, LSDV will grow in tissue culture of bovine, ovine or caprine origin, although maximum yield is obtained using lamb testis or bovine dermis cells. Three cell cultures, Vero cell that is originated from African green monkey, ESH-L cell from Epithelial Bovine Skin and lamb testes (LT) primary cell were compared for the isolation of the virus.

Vero and ESH-l cell cultures were prepared according to the procedure indicated at annex 2. LT primary cell was grown from 3-7 day old lambs. Steps, materials and reagents used for the preparation of LT primary cell culture is stated at annex 8.

For comparison of these cell cultures, a reference virus, the attenuated SPPV Kenya O-180 vaccine strain (referred as KS-1) and wild virus that was obtained from skin lesion samples of three cattle, selected randomly from the collected samples after confirming as positive with real time PCR were used. The field samples were assigned as 1, 2 and 3.

3.5.2. Virus isolation

Virus isolation was performed following the same procedure indicated in section 3.5.2 and the virus was passaged three times for those samples that show no or slight CPE. A numerical system of scoring when reading the cell cultures for cytopathic changes was adapted. The extent of involvement was scored on a scale from 0 to 4. At one end of the scale, 0 represents a normal culture without CPE, while at the other extreme; a completely degenerated culture is scored 4+ (E. Venter, 2014).

3.6. 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 analysedwithSPSS 18. Descriptive statistics was used to summarize data of field survey and laboratoryinvestigation.

4. RESULT

4.1.Outbreak Investigation

During field investigation on the study areas where LSD outbreak were reported, about 992 cattle where investigated according to the clinical signs of LSDV and some were found showing the characteristics signs of the disease such as fever, nasal discharges, depression and skin nodules in different parts of their body (Fig. 3).

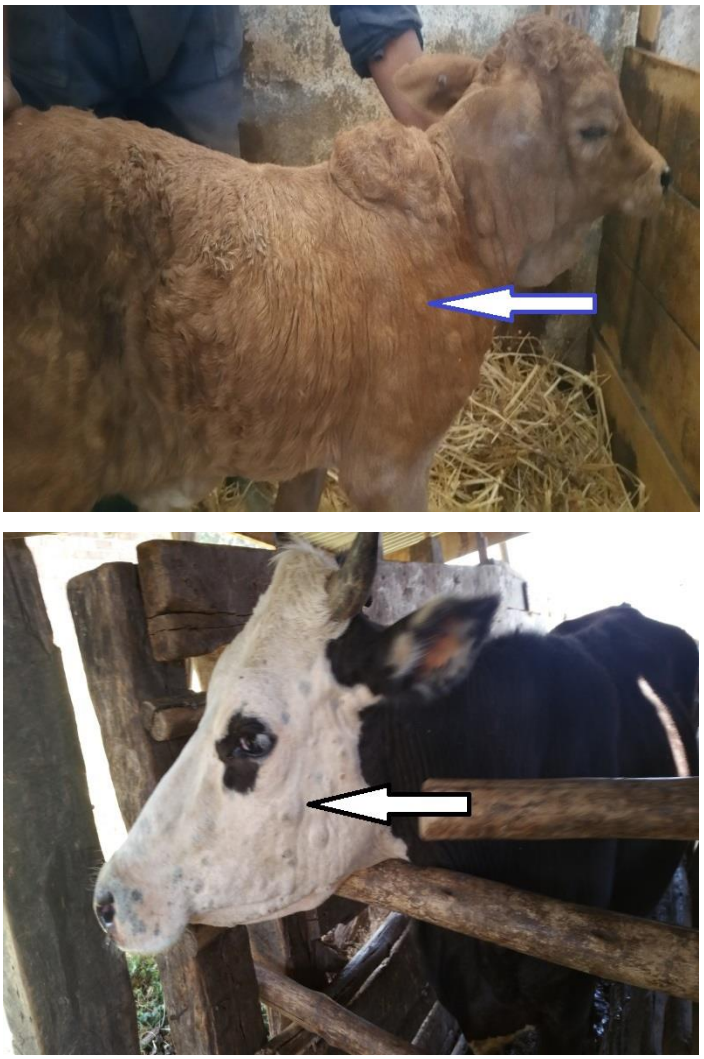


Figure 4: Characteristic of LSD with generalized circumscribed skin nodules covering the entire body. Pictures were taken during field investigation

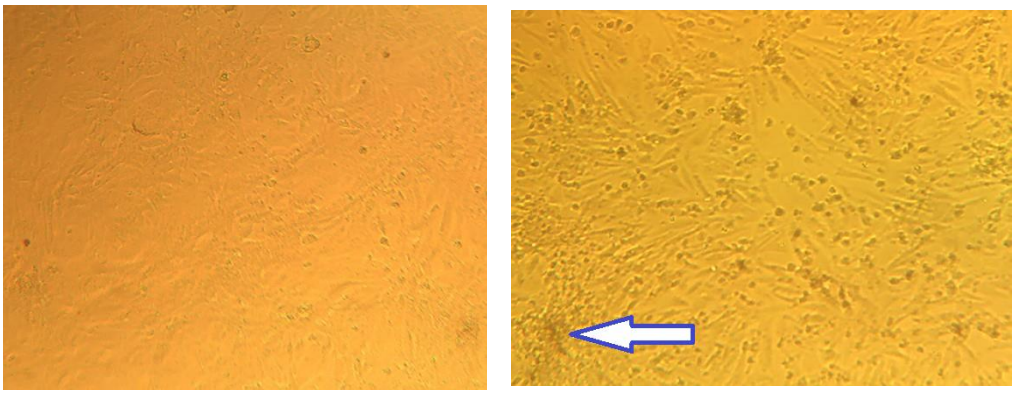
According to the result (Table 2), there was 8.77% morbidity, 2.12% mortality and 25.61% case fatality rates.

Table 2: 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 (%)
Bishoftu	65	12	18.46	2	3.07	16.67
Akaki	107	16	14.95	6	5.61	37.5
Asela	320	27	8.4	5	1.56	18.52
Holeta	500	32	6.4	8	1.6	25.0
Total	**992**	**87**	**8.77**	**21**	**2.12**	**25.61**

4.2.Virus Isolation

All the collected skin biopsies samples were inoculated in vero culture and the virus was passaged three times. Although, it was not be able to isolate all samples, the characteristic LSDV CPE was perceived in most infected cell cultures (Fig 5). The CPEs were characterized by rounding of single cells, aggregation of dead cells and destruction of monolayers.



(A) Vero monolayer cell after 24 hrs incubation; (B) LSD CPE, 3rdpassage at 11th day

Figure 5: LSDV growth on vero cell culture

(A) Confluent monolayer of vero grown in GMEM with 10% FCS growth medium after 24 hours of culture; (B) Characteristic cytopathic effect (CPE) of LSDV on vero developed after 11th days post-infection of passage 3.

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. 6).

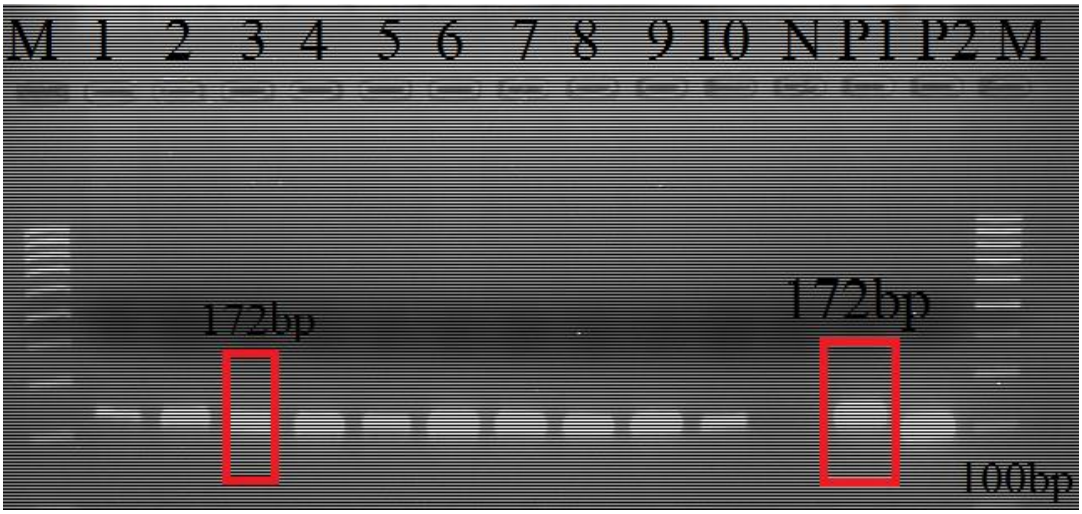


Figure 6: Classical PCR gel picture from skin nodule of LSDV infected cattle

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

4.4.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.

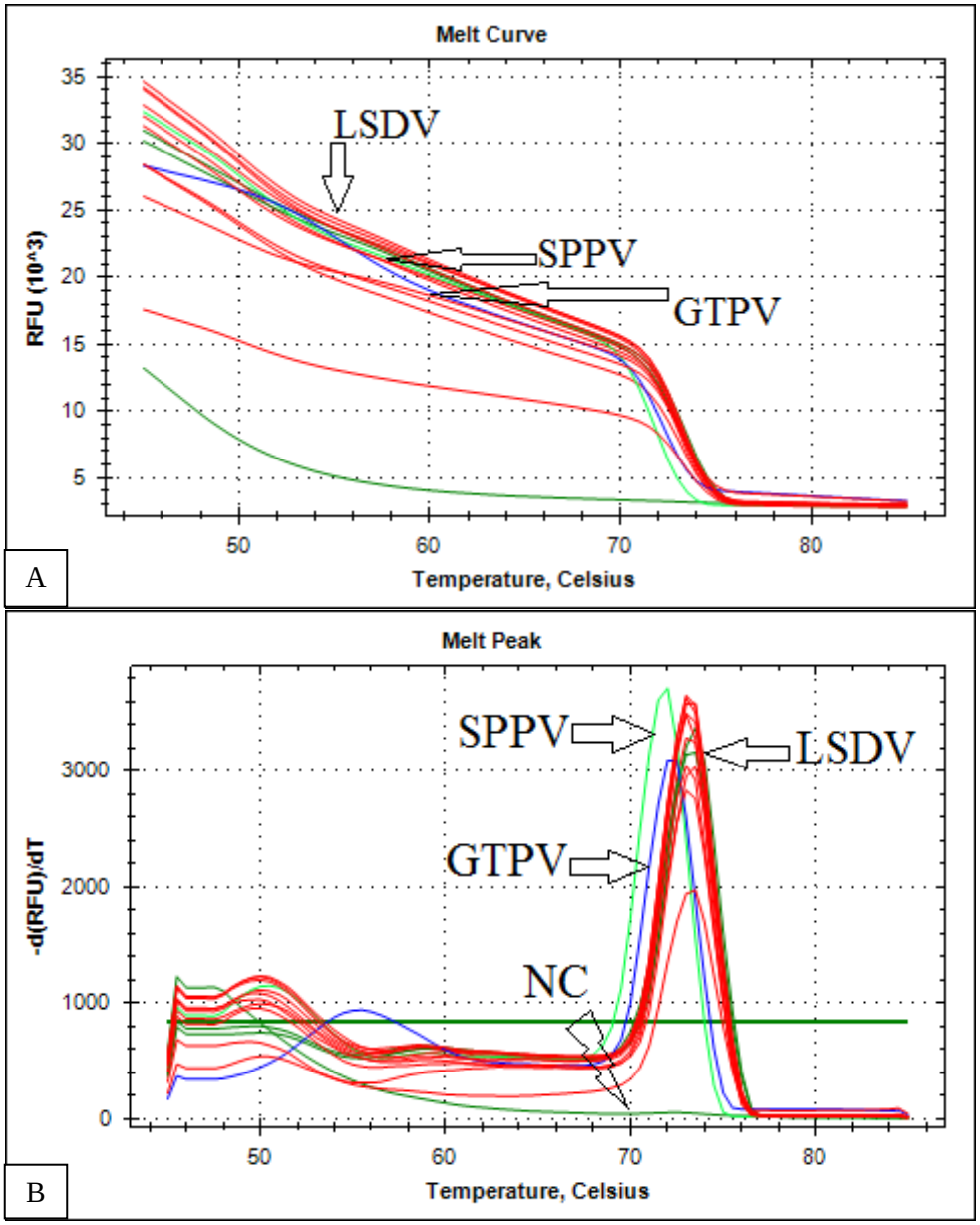


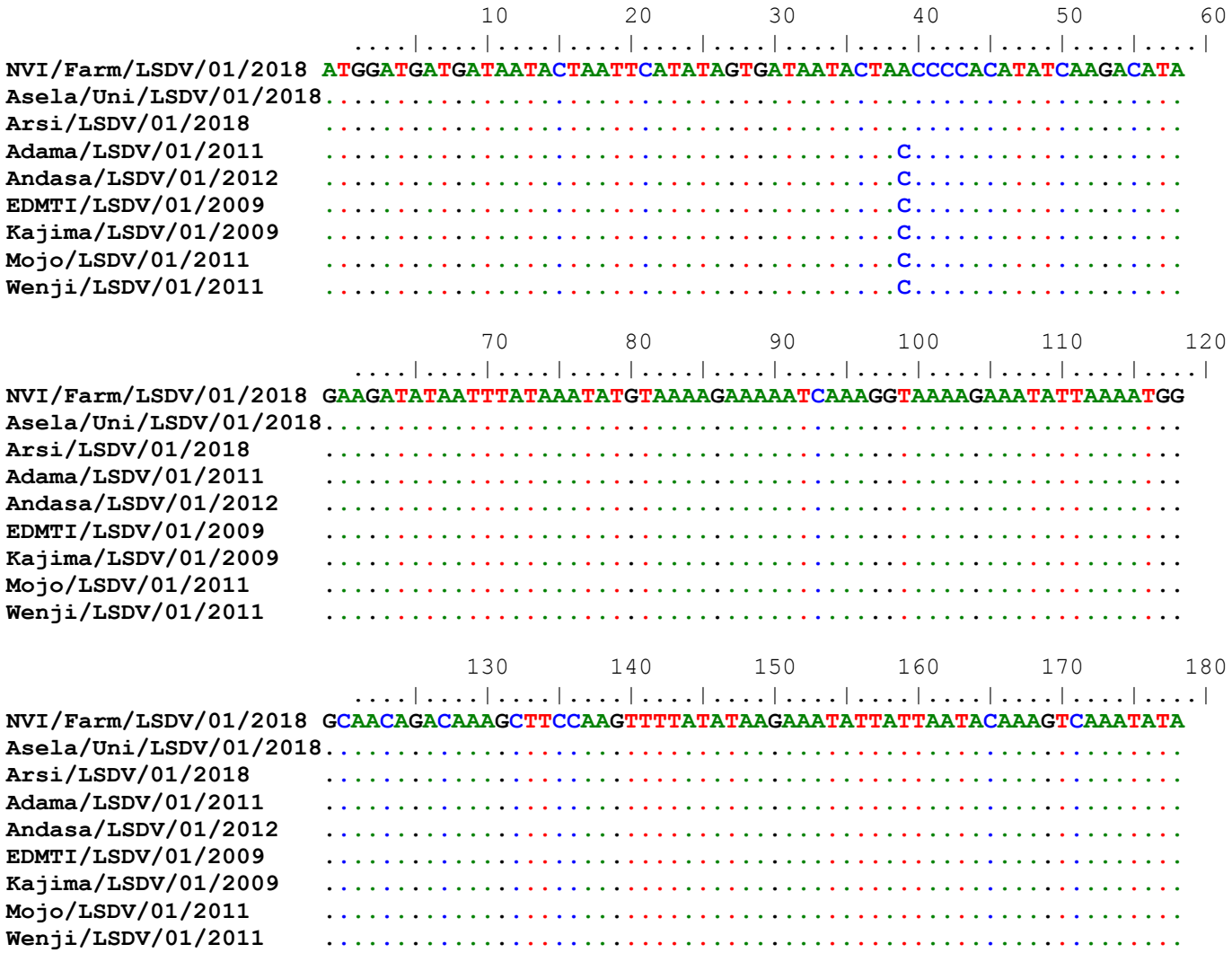
Figure 7: Melting curve analysis of field isolates of LSDV

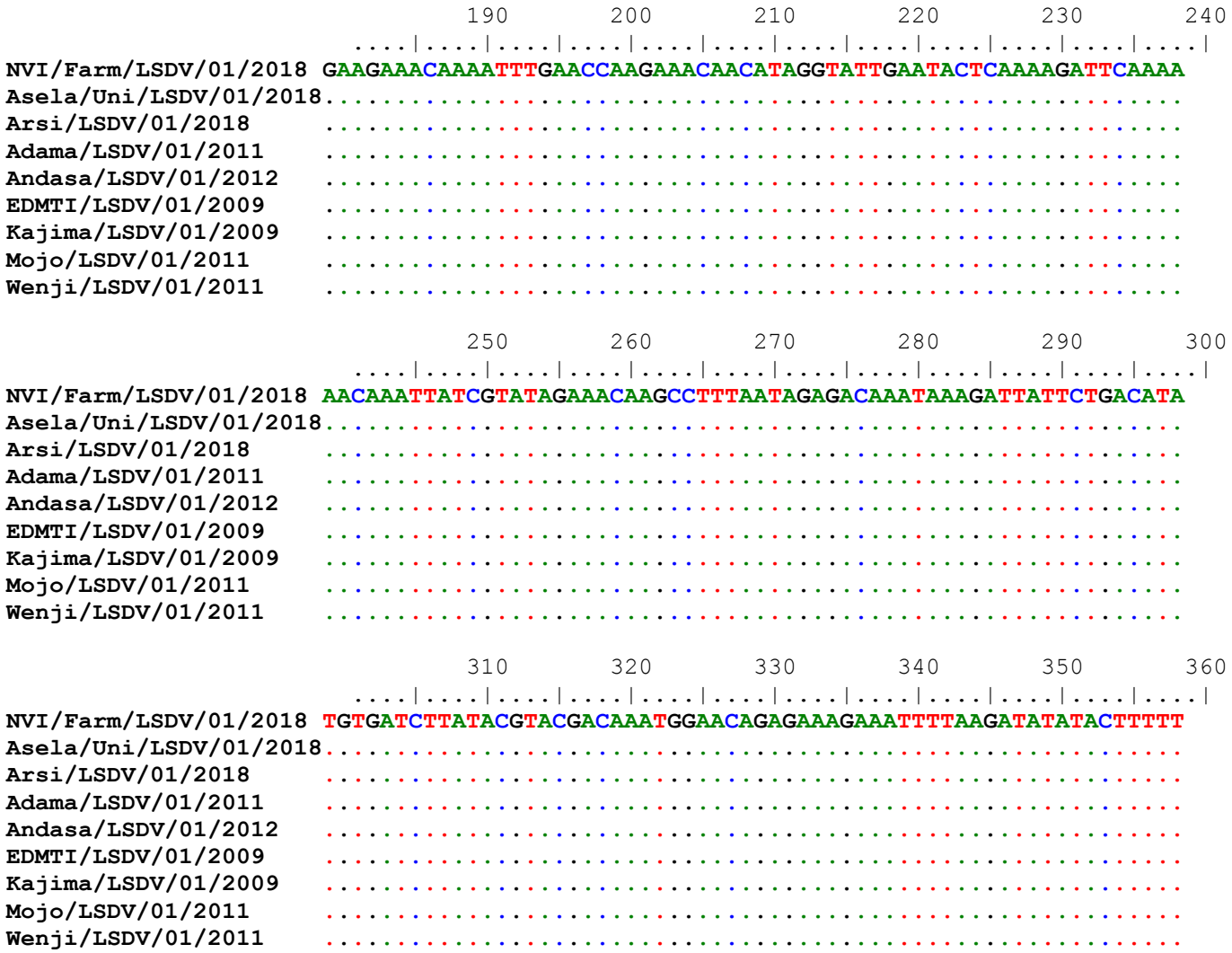
Figure 7(a) Graph showing the difference melting curve profiles after PCR amplification of the RPO30 gene of the tested samples and CaPV controls. (b) Shown the Melt Peak for field isolates of LSDVs and controls. Complete melting curves were displayed, including both hairpin and amplicon melting transitions that the curves were normalized and the negative first derivative of fluorescence with respect to temperature is displayed.

The snapback primer includes a 5' tail that is complementary to its own extension product. After asymmetric PCR, both intramolecular snapback hairpins and intermolecular duplexes of full-length amplicons were formed. In the presence of a saturating fluorescent DNA dye (Eva Green), the melting of both snapback and amplicon duplexes were observed as peaks on negative first-derivative plots of fluorescence with respect to temperature. Pair of melting temperature each for snapback tail and full amplicon was recorded as LSDV at $51^{o}C/73.5^{o}C$, GTPV at $56^{o}C/72.5^{o}C$, and SPPV at $52^{o}C/72.5^{o}C$ for genotyping of the tested isolate.

4.5. Sequence Analysis

The RPO30 gene multiple sequence alignment shown that the current virus strain had almost 100% identity with the virus isolated eight years before, however, sequence analysis of the current field and the previous isolates in Central Ethiopia revealed that, there was a mutation at nucleotide position 41 on the current isolates that cytosine is substituted by adenine (Fig. 8).


10 20 30 40 50 60
NVI/Farm/LSDV/01/2018 ATGGATGATGATAATACTAATTCATATAGTGATAATACTAACCCCACATATCAAGACATA
Asela/Uni/LSDV/01/2018
Arsi/LSDV/01/2018
Adama/LSDV/01/2011
Andasa/LSDV/01/2012
EDMTI/LSDV/01/2009
Kajima/LSDV/01/2009
Mojo/LSDV/01/2011
Wenji/LSDV/01/2011
70 80 90 100 110 120
NVI/Farm/LSDV/01/2018 GAAGATATAATTTATAAATATGTAAAAGAAAAATCAAAGGTAAAAGAAATATTAAAATGG
Asela/Uni/LSDV/01/2018
Arsi/LSDV/01/2018
Adama/LSDV/01/2011
Andasa/LSDV/01/2012
EDMTI/LSDV/01/2009
Kajima/LSDV/01/2009
Mojo/LSDV/01/2011
Wenji/LSDV/01/2011
130 140 150 160 170 180
NVI/Farm/LSDV/01/2018 GCAACAGACAAAGCTTCCAAGTTTTATATAAGAAATATTATTAATACAAAGTCAAATATA
Asela/Uni/LSDV/01/2018
Arsi/LSDV/01/2018
Adama/LSDV/01/2011
Andasa/LSDV/01/2012
EDMTI/LSDV/01/2009
Kajima/LSDV/01/2009
Mojo/LSDV/01/2011
Wenji/LSDV/01/2011


190 200 210 220 230 240
NVI/Farm/LSDV/01/2018 GAAGAAACAAAATTTGAACCAAGAAACAACATAGGTATTGAATACTCAAAAGATTCAAAA
Asela/Uni/LSDV/01/2018
Arsi/LSDV/01/2018
Adama/LSDV/01/2011
Andasa/LSDV/01/2012
EDMTI/LSDV/01/2009
Kajima/LSDV/01/2009
Mojo/LSDV/01/2011
Wenji/LSDV/01/2011
250 260 270 280 290 300
NVI/Farm/LSDV/01/2018 AACAAATTATCGTATAGAAACAAGCCTTTAATAGAGACAAATAAAGATTATTCTGACATA
Asela/Uni/LSDV/01/2018
Arsi/LSDV/01/2018
Adama/LSDV/01/2011
Andasa/LSDV/01/2012
EDMTI/LSDV/01/2009
Kajima/LSDV/01/2009
Mojo/LSDV/01/2011
Wenji/LSDV/01/2011
310 320 330 340 350 360
NVI/Farm/LSDV/01/2018 TGTGATCTTATACGTACGACAAATGGAACAGAGAAAGAAATTTTAAGATATATACTTTTT
Asela/Uni/LSDV/01/2018
Arsi/LSDV/01/2018
Adama/LSDV/01/2011
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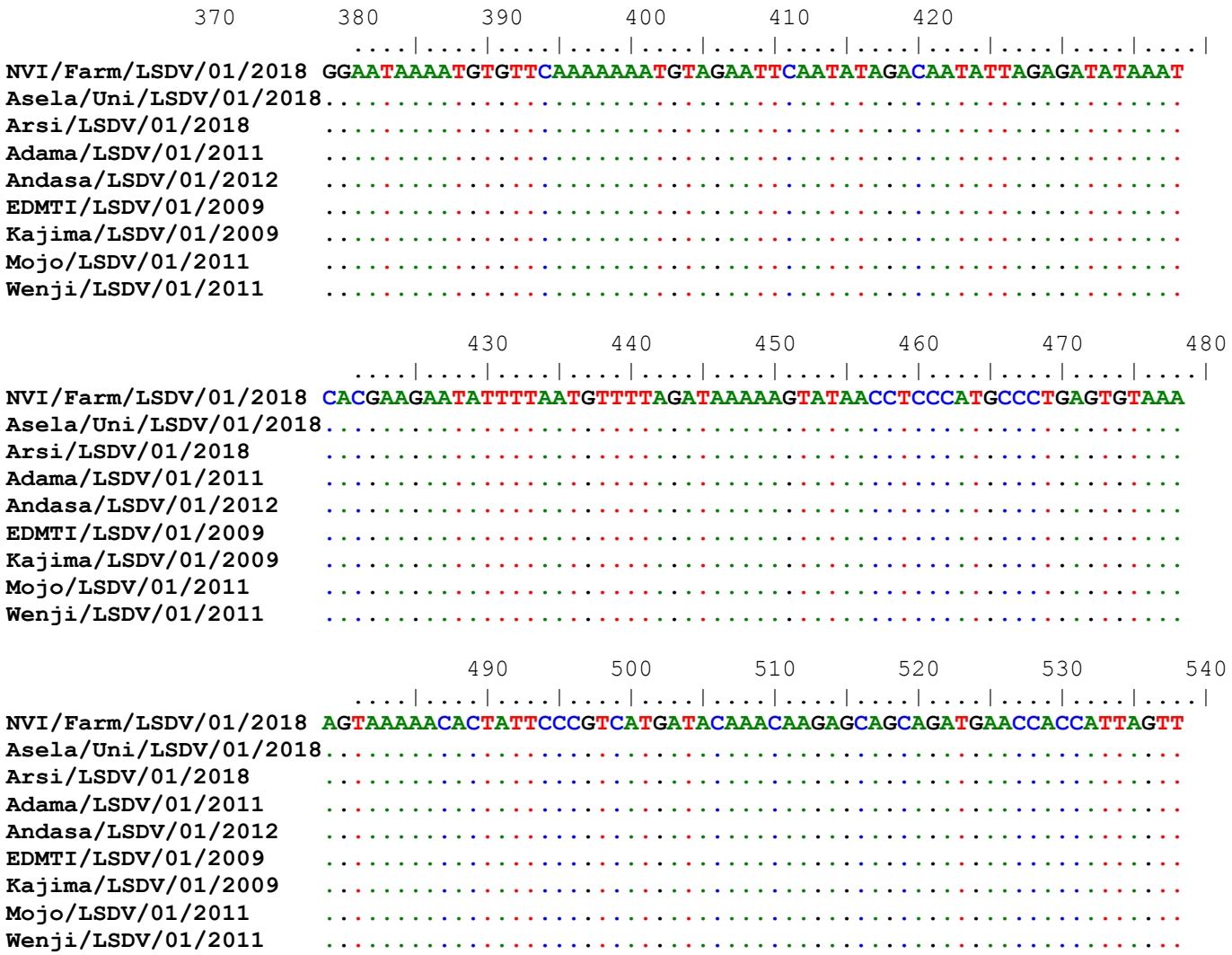
Wenji/LSDV/01/2011


370 380 390 400 410 420
NVI/Farm/LSDV/01/2018 GGAATAAAATGTGTTCAAAAAAATGTAGAATTCAATATAGACAATATTAGAGATATAAAT
Asela/Uni/LSDV/01/2018
Arsi/LSDV/01/2018
Adama/LSDV/01/2011
Andasa/LSDV/01/2012
EDMTI/LSDV/01/2009
Kajima/LSDV/01/2009
Mojo/LSDV/01/2011
Wenji/LSDV/01/2011
430 440 450 460 470 480
NVI/Farm/LSDV/01/2018 CACGAAGAATATTTTAATGTTTTAGATAAAAAGTATAACCTCCCATGCCCTGAGTGTAAA
Asela/Uni/LSDV/01/2018
Arsi/LSDV/01/2018
Adama/LSDV/01/2011
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EDMTI/LSDV/01/2009
Kajima/LSDV/01/2009
Mojo/LSDV/01/2011
Wenji/LSDV/01/2011
490 500 510 520 530 540
NVI/Farm/LSDV/01/2018 AGTAAAAACACTATTCCCGTCATGATACAAACAAGAGCAGCAGATGAACCACCATTAGTT
Asela/Uni/LSDV/01/2018
Arsi/LSDV/01/2018
Adama/LSDV/01/2011
Andasa/LSDV/01/2012
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Kajima/LSDV/01/2009
Mojo/LSDV/01/2011
Wenji/LSDV/01/2011

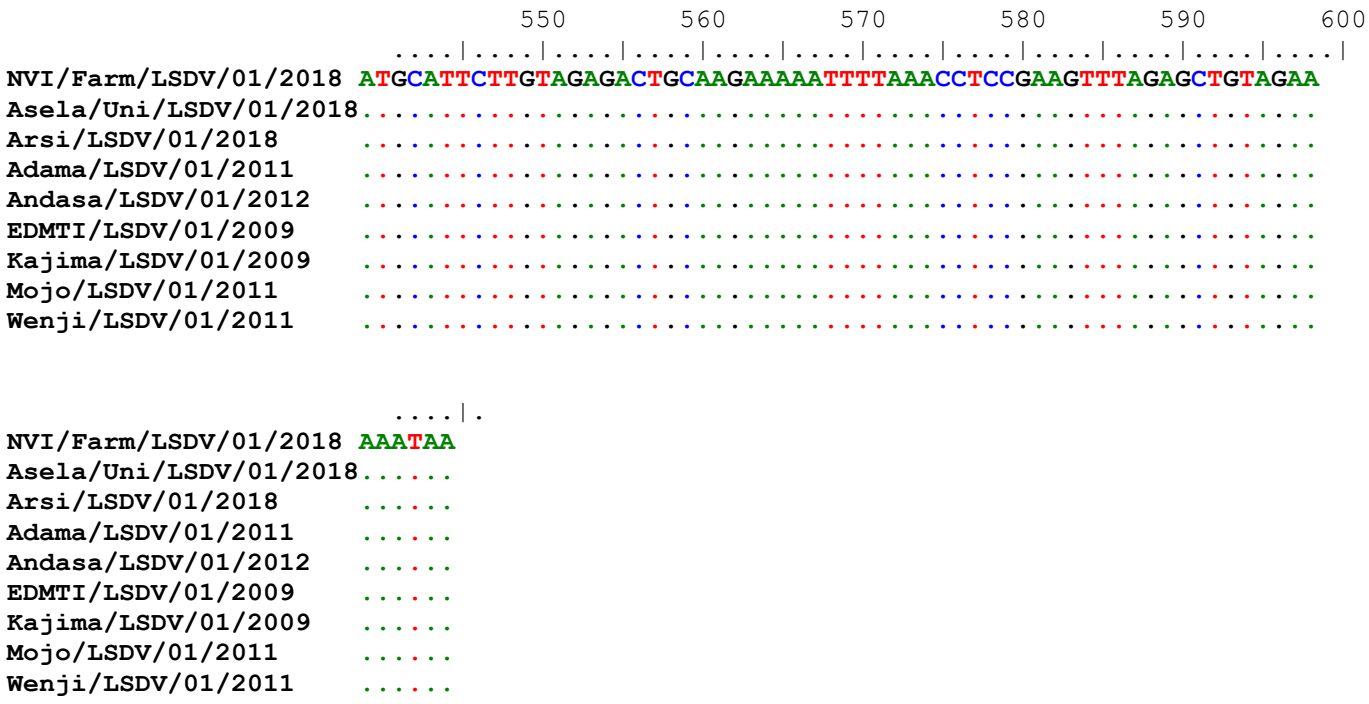

Figure 8: Nucleotide sequence alignment of RPO30 gene for current field isolates and previously identified Ethiopian isolates

4.6. Phylogenetic Analysis

From the phylogenetic analysis of the RPO30 gene, it is clear that all of the skin biopsy samples collected in Ethiopia contained LSDV DNA and that the sequences were identical to each other and related to the reference of the Sudan isolate (Fig. 9). In addition, they were matching to previously identified LSDVs from Ethiopia and also the vaccine strain, KS-1.

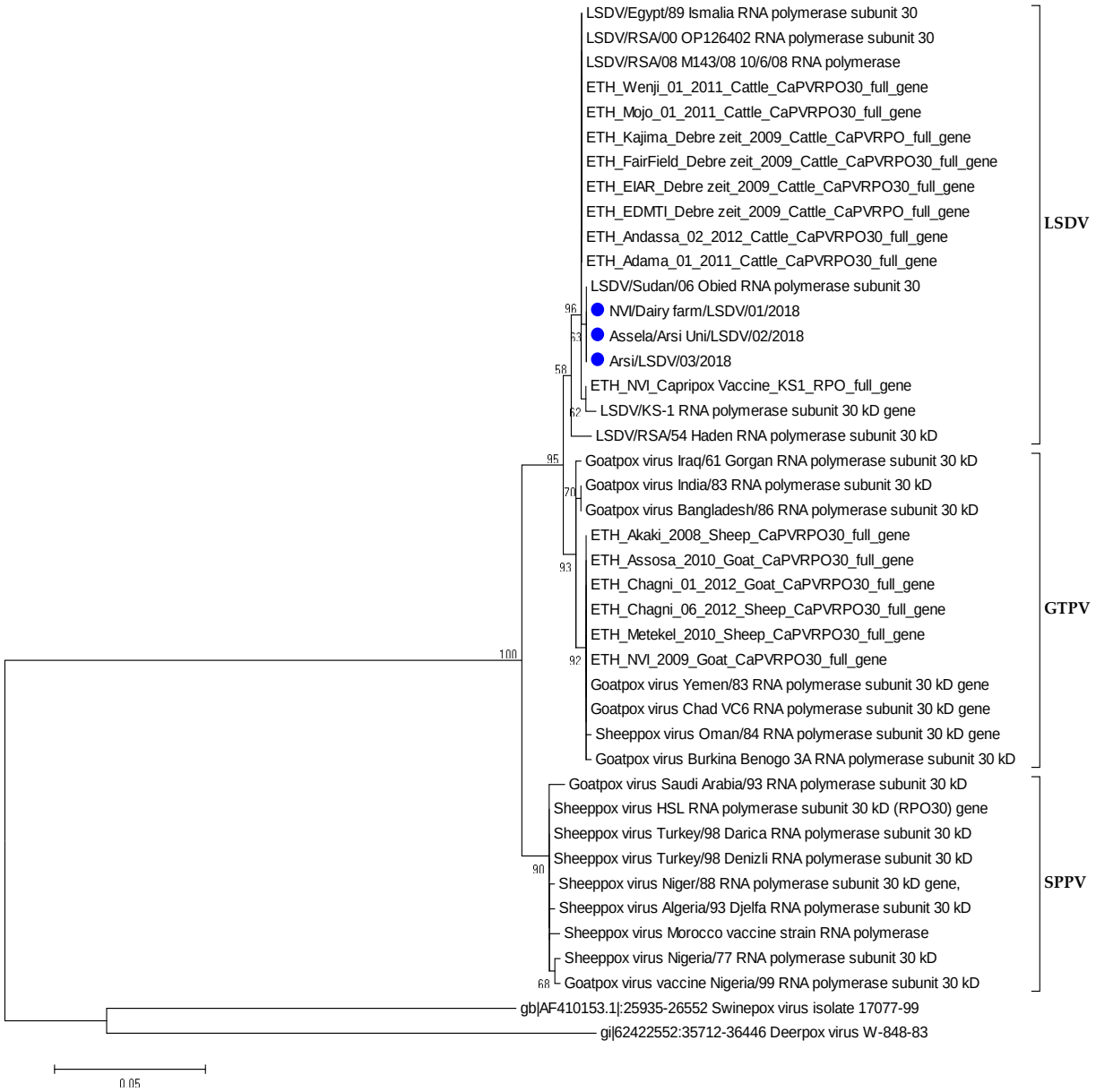

Figure 9: Phylogenetic analysis of local LSDV isolates.

Phylogenetic analysis of 42 capripoxviruses based on nucleotide sequences of the RPO30 gene. Three current LSDV outbreak isolates and the sequences retrieved from the GenBank were used. The Neighbor-Joining method with the maximum composite likelihood nucleotide substitution model and the pair wise deletion option was compute using MEGA6. The percentage bootstrap scores above 50% (out of 1000 replicates) are shown next to the branches. The homologue gene sequence from one Deer poxvirus and one Swine poxvirus isolates retrieved from the GenBank were used as out-group. The isolates sequenced in this study are indicated plain circle.

4.7.Cell cultures comparison for primary isolation of LSDV

Three cell cultures, Vero cell that is originated from African green monkey, ESH-L cell from Epithelial Bovine Skin and LT primary cell were prepared. Attempt was made to isolate three skin lesions samples selected randomly from the collected samples assigned as 1, 2 and 3 in table, after confirming as positive for LSDV with PCR and the reference strain, O-180.

The result of the attempts to isolate the virus on the three cell types is given in table.

Table 3: passage number required to isolate LSDV different cell cultures from skin lesion samples and vaccine strain.

Virus isolate	Cell culture		
	LT	Vero	ESH-l
O-180 vaccine strain	1^{3+}	1^{4+}	1^{3+}
1	1^{4+}	3^{3+}	2^{3+}
2	1^{3+}	3^{0}	3^{2+}
3	1^{3+}	3^{2+}	3^{3+}

Numerical systems of scoring when reading the cell cultures for CPE were given. ^{0}culture without CPE; $^{1+}$one or a few foci of infection; $^{2+}$ 10% of infection; $^{3+}$50% -75% CPE and $^{4+}$ completely degenerated culture.

Table 2 shows the attempts to isolate LSD virus in three different cell cultures from recent outbreak samples, and indicates the number of passages needed in each system before virus could be demonstrated. For LT primary cell culture only one passage is required to show the characteristics LSDV CPE in all samples while, ESH-l cell culture isolate all samples even after the first and second passage. Vero cell can isolate two samples on the third passage found less sensitive for primary isolation of the virus.

5. DISCUSSION

LSD is an infectious disease of cattle characterized by rapid spread and sudden appearance of lumps in skin after fever. The importance of this disease increase gradually as the way of eradication and control is very difficult. The present study concerned with isolation and characterization of LSDV in central Ethiopia. During field survey LSDV characteristics signs; fever, nasal discharges and skin nodules in different parts of their body were observed. Clinical signs of the disease are such as nodular cutaneous eruptions, lymphadenitis, and edema in one or more limbs are mentioned on OIE, 2017 manual. LSD outbreak in central Ethiopia was reported at different time, recently Ayelet *et al.*, (2014) reported LSD with 13.61% mortality, 4.97% mortality and 36.94 % case fatality rates. In the present study there was 8.77% morbidity, 2.12% mortality and 25.61% case fatality rates.

The virus was isolated from skin lesion samples collected from infected cattle by inoculation on vero cell culture. CPE was detected after second or third passages of the samples on the cell culture and CPE was characterized by cell rounding, cell aggregation, coalesce together to form clusters that scattered over the monolayer as mentioned in the OIE, 2017 LSD manual. 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.

The samples were genotype 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, 2008), also (Burdin and Prydie, 1959, Capstick, 1959) reported that an experimental infection of cattle with SPPV can produce similar lesions to LSD. The results come out

provide with melting temperature of73.0^{o}C for the amplicons and 51.0^{o}C for the snapback confirming that the isolated samples are indeed LSDV.

The RPO30 gene sequence shown that, one LSD virus strain caused the current outbreak in the central Ethiopia. Although there is a single nucleotide mutation, the same strain was isolated in the pass years. Moreover, the current virus strain found to be identicalto the virus strain isolated from East HarargeandEast ShoaZone, Ethiopia in 2017 (Aster, 2017).The lineage specificity was confirmed by phylogenetic reconstruction. In the phylogenetic tree analysis of RPO30 gene for three selected samples, all samples were clustered under LSDV together with KS-1 vaccine strain, previously isolated LSDVs in Ethiopia and in Sudan. The phylogenetic analyses also revealed that local isolates of LSDV are highly related to not only other LSDV strains but also other CaPVs (sheep and goat pox). These results coincide with the theory of that all CaPVs are genetically related and originated from one ancestor lineage **(**Tulman *et al*., 2001**).**

The control of the LSD is dependent on fast and effective diagnostic techniques. Many researchers are working on the development of better diagnostic method for LSDV and other CaPVs such as; Binepal *et al*., (2001) found alternative cell lines for propagation of LSDV, Babiuk *et al*., (2007) evaluate ovine testis cell line (OA3.Ts) for propagation of LSDV, Gelaye *et al*., (2013) develop a new molecular assay for rapid detection of CaPVs, Mahmoud *et al*., (2013) compare Polymerase chain reaction and Dot blots hybridization techniques for diagnosis of LSD,Lamya*et al*., (2017*)* Isolate LSD virus on CAM and MDBK cell culture and identification of isolated virus by FAT and PCR. In the present study an attempt was made to compare vero cell line, ESH-l cell line and LT primary cell for better isolation of LSDV. Infection of the three cells with LSD wild and reference virus resulted in CPE characterized by viral plaque formation but with different passage number and degree of CPE on the cells monolayer. LT cell isolated all the samples at the first passage showing high CPE. ESH-l cell was able to isolate all the samples at second or third passage, although this cell is not as suitable as LT primary cell for isolation of LSDV, it was found better than vero cell for primary isolation. Vero cell was less sensitive for primary isolation, requiring three to four blind passages until CPE appreciated. The

vaccine strain, KS-1 is well adapted to grow on vero cell, ESH-l and LT cells can also grow the vaccine strain well. This result is in agreement with OIE, 2017 manual and many other researches findings that strongly recommend the use of primary cell from ovine or bovine origin for primary isolation of LSDV. African green monkey kidney (Vero) cell has been used for vaccine production and virus isolation such as CaPVs. Although this cell is not recommended for primary isolation of LSDV by OIE 2017 manual, CPE can be achieved by continuous passaging of the virus. ESH-l cell has been usedfor the propagation of LSDV at different laboratories such as PANVAC and it was reported conferring better susceptibility to LSDV. LT primary cell has been utilized for LSDV isolation since 1968 when Weiss isolated CaPV using this cell for the first time. This primary cell is regarded as the most susceptible for LSDV by OIE, 2017 manual.

6. CONCLUSION AND RECOMMENDATIONS

LSD is an important disease in cattle causing considerable economic loses. In Ethiopia, the disease is continuing to appear every year. The present study found that the disease is highly prevalent in central Ethiopia. All the collected samples were identified as LSDV with molecular techniques and DNA sequencing. The phylogenetic tree indicated that, the present isolates are related to the previously isolated LSDVs in the country. Regular and timely vaccination strategy is the best choice available for effective control of the disease accompaniedwith early detection of the disease. Different diagnostic techniques are available for early detection of the disease. The disease was first confined in Africa, as the disease continues to spread to other continents, it is now many countries from Europe and other continents began to concern.

Based on the findings of this study the following points are recommended to enhance disease prevention and controlling strategies:

- Disease prevention and control measures such as vaccination must be regularly applied.
- Suitable cell lines for fast isolation of the virus need to be identified.
- Study on the genetic similarity/difference among the vaccine strain with the field isolates should be continuing.
- Pathogenicity and immunological study is necessary to know more about the disease

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8. ANNNEX

Annex 1: LSDV field outbreak assessment questionnaires form

Region: _____

Zone: _____

Type of disease suspected: _____

Date: _____

Farm history

Production type: _____

Breed _____

Sex _____

Age _____

Total population of animal _____

No. Sick _____

No. death _____

Vaccination history _____

Clinical sign observed _____

Annex 2: Vero and ESH-L cell line culture preparation

1. First all the cell culture mediums were warmed at 37^{o}C in a water bath and the trypsin solution allowed reaching room temperature. The culture medium from the cells was decanted.

2. The cells were washed twice with PBS. Trypsin was added to the cells, 1 - 2 ml per 25 cm^2 of flask surface, and was gently spread over the entire surface by tilting the vessel.
3. The cultures was Placed in an incubator at 37^oC 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 cm^2 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 25cm^2 tissue culture flask containing GMEM with FCS (10% for vero and 5% for ESH-l) and streptomycin antibiotics at 7.2-7.4 PH.
6. The cell cultures were incubated at 37^oC temperature in 5% CO_2 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) water Preparation

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

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 % foetal calf serum (FCS). For virus isolation, this medium will be replaced before 24 h by the same medium but supplement with only 2% FBS.

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
NaHCO3	0.35 gm
Purified H20 q.s.	1 liter

Adjust pH to 7.4 with NaHCO3 solution

Annex 6: 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 56^{o}C 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 56^{o}C 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 3 min 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 microcentrifuge 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 (15-25^{o}C). Centrifuge for 1 min at ≥ 6000 x g.

Annex 7: Convectional 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 $MgCl_2$, 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.

Annex 8: Lamb Testes Primary cell culture preparation

1. Lambs, 3-7 days were Castrated under aseptic condition. The testicles were collected on ice in a sterile PBS antibiotic solution. The solution contains antibiotics comprising of penicillin 100U/ml streptomycine 0.1mg/ml and gentamycin 90.1mg/ml.

2. The testicles were socked the in the PBSA antibiotic solution for 2 hr at 4^{o}C. the epididymides were removed aseptically remove from the testicles using sterile forceps and scissors inside safety cabinet.
3. The caput (head) and caudal (tail) of each epididymis were incised longitudinally with a scalpel to remove the connective tissue, the intertubular tissue were kept moist in the PBS antibiotics solution.
4. The epididymis caput and caudal were cut into small tissue pieces of approximately 2 to 3 mm squares and placed the tissue pieces into a 50ml tube and centrifuge at 1000x g for 5 minutes at room temperature. The supernatant were Discarded.
5. The tissue pieces were transferred into a screw-capped trypsinizing flask for dissociation.
6. 50ml of trypsin (0.25%) solution was placed for every 5g of wet tissue pieces into a trypsinizing flask and Incubated at 37^{O}c for 30 minutes while mixing slowly with magnetic stirrer.
7. The trypsin solution was decanted into a sterile funnel wrapped in fine mesh gauze and the filtrate was collected in a 50ml centrifuge tube. The filtrate was immediately centrifuged at 100Xg for 5 min and the supernatant was discarded. The pellets were re suspended in 4ml of culture medium for later pooling with collagenase digests.
8. The tissue pieces that were trapped on the gauze were transferred back into the trypsinizing flask. 50ml of collagenase solution was added and incubated at 37^{O}c for 30 minutes while mixing slowly on a magnetic stirrer and the above step was repeated.
9. With serum free DMEM culture medium the pellets were as rinsed twice.
10. The primary cells was collected by pipetting the pellets with culture medium and transferred into a $25cm^2$ tissue culture flasks containing GMEM with 10% (v/v) FCS.
11. Finally the flasks were incubated at 37oC with 5% CO2 for 48hrs until they show LT primary cell monolayer.

Annex 9: 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 fladk 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 inoculum to the cell culture the depending on the flask size. Rock each plate gently to distribute inoculum 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 37^{o}C 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.