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EVALUATION OF HUMORAL IMMUNE RESPONSE OF CATTLE BEFORE AND AFTER VACCINATION AGAINST LUMPY SKIN DISEASE IN ADA'A DISTRICT, CENTRAL OROMIA, ETHIOPIA

MVSc THESIS

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MVSc THESIS

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

BY

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Bishoftu, Ethiopia

College of Veterinary Medicine and Agriculture
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As MVSc research advisors, we here by certify that we have read and evaluated this Thesis prepared under our guidance by Nebyou Moje Hawas entitled: "*Evaluation of humoral immune response of cattle before and after vaccination against Lumpy Skin Disease in Ada'a district, Central Oromia, Ethiopia*" we recommend that it can be submitted as fulfilling the MVSc thesis requirement.

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As member of the Board of Examiners of the MVSc, open defence examination, we certify that we have read and evaluated the thesis prepared by ***Nebyou Moje*** and examined the candidate. We recommended that it can be accepted as fulfilling the thesis requirement for the degree of Master Science in Veterinary Microbiology.

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DEDICATION

I dedicated this thesis/dissertation manuscript to all mankind suffered and/or suffering from COVID-19 outbreak. Additionally, I would also dedicate this paper to my late grandmother, Bontu Badhadha that passed away in this June. I always remember you, Bontiyo!

I also dedicated this thesis to my favourite legend artist Hachalu Hundessa (Hacce) who was murdered on June 29 in the capital city of Oromia and Ethiopia. Rest in peace and you will stay in the memory of millions forever!

STATEMENT OF AUTHOR

First, I declare that this thesis is my authentic work and that all sources of materials used for this thesis have been properly acknowledged. This thesis has been submitted in partial fulfillment of the requirements for MVSc degree in Veterinary Microbiology at Addis Ababa University, College of Veterinary Medicine and Agriculture and it is deposited at the University/College library to be made available to borrowers under rules and regulations of the library. I solemnly state that this thesis is not submitted to any other institution anywhere for the award of any academic certificate, diploma or degree.

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LIST OF ABBREVIATIONS

AU-PANVAC	African Union Pan-African Veterinary Vaccine Centre
AU-IBAR	African Union Inter-African Bureau for Animal Resource
CaPVs	Capri pox Viruses
cm	Centimeter
CPE	Cytopathic Effect
CSA	Central Statistics Agency, Ethiopia
DIVA	Differentiating Infected from Vaccinated Animals
DNA	Deoxyribonucleic Acid
dpv	Days Post-vaccination
DMEM	Delbico minimum essential cell culture medium
ds	Double-strand
DZARC	Debre-Zeit Agricultural Research center
ELISA	Enzyme Linked Immunosorbant Assay
EMPRES-I	EMPRES Global Animal Disease Information System
EARI	Ethiopian Agricultural Research Institute
FAO	Food and Agriculture Organization
GMEM	Glasgow's minimum essential cell culture medium
Hr	Hour
IFAT	Indirect Fluorescent Antibody Test
Kbp	Kilo base pair
KD	Kilo Dalton
KSGP	Kenyan sheep and goat pox virus
LSD	Lumpy Skin Disease
LSDV	Lumpy Skin Disease Virus
nm	Nano meter
NVI	National Veterinary Institute
NZGGRC	New Zealand Agricultural Greenhouse Gas Research Centre
OD	Optical Density
OIE	World Organization for Animal Health
PBS	Phosphate Buffered Solution
PCR	Polymerase Chain Reaction
SNT	Serum Neutralisation Test
TPB	Tryptose Phosphate Broth
WAHID	World Animal Health Information Database
WCI	World Cattle Inventory

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ABSTRACT

Lumpy Skin disease (LSD) is an economically important viral disease of cattle affecting all ages and breeds that can be prevented by vaccination in endemic regions including Ethiopia. However, different disease outbreaks in vaccinated cattle raise a question on vaccine efficacy that needs a huge attention to look in to it. To understand why vaccinated cattle experience LSD; longitudinal study coupled with semi-structured interview was employed in Central Ethiopia from October 2019 to June 2020. Semi-structured interview of 30 household was employed to determine knowledge related to LSD and related issues. From these households, 36.67% identified LSD signs and from these households that have knowledge of LSD signs; 16.67% and 3.33% morbidity and mortality was recorded at herd level. The other study of longitudinal approach with 399 sera collected from 113 cattle showed lower proportion of protective antibody level (cut-off value of log titer ≥ 1.5) on pre-vaccination (7.08%) than post-vaccination (8.85% to 41.67% from 7 to 30 days post-vaccination (dpv) sampling. This is logical as immune system need time to build detectable amount of antibody after vaccination. In most of post-vaccination sampling, highest proportion was recorded on 30 dpv. This include, extensively managed (64% at 30dpv), local breed (64%) cows (52%) with the age of ≥ 5 years that could relate with a better immune system development as compared to their counterpart. However, there was no statistical association between those risk factors and their protective antibody appearance. Nonetheless, in both sampling (pre and post-vaccination except day 30th), significant proportions of cattle did not show LSD-specific protective antibody though this does not necessarily mean those cattle were not developed immunity taking cellular immunity in to account. In conclusion, the study showed less number of cattle owner with the awareness of LSD signs and vaccination. Additionally, serum neutralization technique (SNT) result showed a varied level of antibody status with overall less antibody detections that pose a question on a current LSD-vaccine efficacy. To this effect, further field challenge of circulating field strains should be done on vaccinated cattle and assessment of cellular immune response could also play its role to correlate it with vaccine protection level. Additionally, local isolates from outbreaks in the country could be compared with the vaccine strain and used for vaccine production.

***Key words**: Longitudinal study, LSD, Pre and Post-Vaccination, Protective antibody, Risk factors, Semi-structured interview, SNT, Vaccination*

1. INTRODUCTION

In sub-Sahara African countries including Ethiopia, livestock plays a multifunctional activity. In Ethiopia, livestock roles are irreplaceable in the livelihood of rural communities and country's economy that put cattle production at the center in its impact (FAO, 2009; FAO, 2019). There are more than 56 million heads of cattle in Ethiopia, providing over 3.8 billion litres of milk (FAO and New Zealand Agricultural Greenhouse Gas Research Centre (NZGGRC), 2017; Central Statistics Agency (CSA), 2018; FAO, 2019) and roughly one million tonnes of beef per year (Shapiro *et al*., 2015). These resources of livestock are backed by the fact that the country is leading in cattle population from Africa and fifth in the world (World Cattle Inventory (WCI), 2015). This livestock sector has been contributing considerable portion to the country's economy, and still promising to bring together the economic development of the country. In relation to this, 85% of country's population economy depends on farming and animal husbandry that rely on this population (Ayelet *et al*., 2014). Additionally, Ethiopian livestock plays an important role in providing export commodities, such as live animals, hides and skins to earn foreign exchanges to the country (CSA, 2018). However, livestock diseases in the country pose a huge problem on fundamental sector that plays a crucial role to lift the country from poverty. Lumpy skin disease (LSD) is one of the most common viral diseases mentioned in hampering livestock production and productivity with its higher morbidity rate (Tuppurainen and Oura, 2012; Molla *et al*., 2018).

Lumpy skin disease (LSD) is characterized by its signs including eruptive, infectious and occasionally causing death to affected animals (OIE, 2010). It is caused by the family *Poxviridea* and genus *Capripox* with a strain of Neethling virus, a double stranded DNA virus (King *et al*., 2012). LSD is considered as list A disease by OIE considering its huge impact on socio-economic status of the community. The economic implication of this disease on cattle industry usually related to debilitating chronic impacts on animal that causes production problems. These problems include reduction in milk production, abortion, temporary or permanent sterility and damaged hides (OIE, 2010; Tuppurainen and Oura, 2012). LSD presents itself in different forms including acute, sub-acute or in apparent diseases in which their severity is dependent on the virus strain and susceptible breed. As mentioned by Radostits *et al.* (2007), *Bos indicus* is known to be less susceptible to clinical disease than *Bos taurus.* Furthermore, Carn and Kitching, (1994) identified that even lactation status of a cow influence the severity of this disease and showed cows in lactation are more at risk. LSD is less contagious with low mortality (less than 10% in most reports) and varying morbidity rate (1-90% mostly and few reports of 100%) (Davis, 1991; Coetzer, 2004; Babiuk *et al.*, 2008a). Apart from factors related to animals, environmental factors such as season can have its own impact on LSD occurrence. As such, LSD case is expected to be high in wet than dry seasons which is related to a favourable condition of biting insects that taught to transmit the disease (Gari *et al*., 2010).

The first reported cases of LSD in the world was in Africa, Northern Rhodesia (now Zambia) in 1929 and spread to the rest of African countries as well as other parts of the world by different means. For example, introduction of the disease in to Middle East in 1991 was correlated with live animal importation from affected areas (Davis, 1991; Brenner *et al.*, 2006; El-Kholy *et al*., 2008). However, the way of LSD introduction in to Ethiopia is unknown, it was first observed in the North-western part of the country (Southwest of Lake Tana) in 1983 (Mebratu *et al*., 1984). It has been spread to almost all the regions and agro-ecological zones of the country. This is related to the existence of LSDV host, cattle in almost all agro-ecological parts of the country that mainly rely on cattle production. Due to the fact that almost all farmers depend on their cattle population as a means to accomplish their livelihood. These include food and agricultural processes, with a higher impact on country's economy as far as livestock disease is concerned (Gari *et al*., 2010).

The diagnosis of *Capripox* virus (CaPVs) diseases can be attained with clinical signs that proceed with a confirmation of laboratory; i.e. virological and/or serological methods. One of the major problems encountered in the CaPVs diagnosis is poor level of sero-conversion. For this reason, the confirmation of the disease is generally based on the detection of *capripox* virions or antigens through electron microscopy, virus isolation and Real-time PCR (OIE, 2017). The poor sero-conversion is expected to relate with the predominance of cell mediated immune response during CaPVs infection. Yet, the humoral immunity can also play its own role in fighting the antigen. The later types of immunity present serum antibody titration which can reflect the protection level of the individual animal. This mechanism of humoral immunity is used in the blood test for LSDV antibody detection (Bhanuprakash *et al*., 2006). Moreover, the lack of available diagnostic kits for cellular immune response to monitor vaccine efficacy made a higher preference to humoral immune response detection to monitor vaccine efficacy (Tuppurainen *et al*., 2017a).

There are different methods to detect LSD virus antibody from blood samples. These are serum neutralization test (SNT), indirect fluorescent antibody test (IFAT) and enzyme-linked immune-sorbent assay (ELISA) (Gari *et al*., 2008; OIE, 2017). From these tests, SNT are considered to be a reference and the unique validated serological test that has been used to evaluate immune status in individual animals or in post-vaccinated populations. It was also mentioned in OIE (2017) that, SNT is the only serological test validated by the OIE with a high specificity for detecting *Capripox* virus-specific antibodies. This is mostly due to its strong specificity that can reach 100% but less sensitivity between 70% and 96% for CaPVs. However, coupling with the use of standard viral strain and pre-vaccination antibodies control can improve this less sensitivity of the test (Bhanuprakash *et al*., 2006).

Ethiopia has been striving to control LSD using mass vaccination at a specified season as well as following a report of the case. Most research finding also characterise LSD virus following an outbreak. Yet few (Zenebe *et al.*, 2014) have checked for immune response of cattle after vaccination aiming mainly on different capripox vaccine comparison. Even the reported one mostly focused on stationed farm of crossbred cattle (Zenebe *et al.*, 2014). Additionally, different report has been coming out from animals' owner and in LSD outbreak areas of Ethiopia about a suspected vaccine failure (Ayelet *et al.*, 2013; Ayelet *et al.*, 2014; Molla *et al.*, 2017). Taking this in to account, further comparisons considering breeds, management system and other risk factors on immunological reaction have to be done to unlock the existing differences in immunological responses of vaccinated and unvaccinated animal state. Therefore, the objectives of the current research were designed:

- To gather information on community awareness on LSD signs and vaccination
- To measure and compare LSD-specific antibody responses of cattle before and after vaccination using serum neutralization test/SNT
- To monitor the persistence post-vaccination protective antibody with SNT and
- To evaluate whether protective level of antibody was related to risk factors (management, breed, age, sex, body condition score, lactation status and parity level) of the vaccinated animals.

2. LITERATURE REVIEW

2.1. Definition

Lumpy skin disease (LSD) is an infectious disease of cattle characterized by the eruption of cutaneous nodules on any part of the body together with generalized lymphadenitis (OIE, 2018). Additionally, Pseudourticaria, Exanthema Nodularis Bovis and Knopvelsiekte have been used as a synonym to LSD that is given after the observed clinical signs of this disease. However, Neethling Virus disease is another name given to this disease based on its causative agent, Neethling virus (King *et al.*, 2012). Another synonym of LSD was "Ngamiland cattle disease" which was given after its outbreak place of occurrence in 1943 in Ngamiland, Bechuanaland Protectorate (Botswana) (Von Backstrom, 1945).

2.2. History

LSD origin and wide spread in African cattle population used to make it animal diseases of African continent. The first disease was seen in northern Zambia (Previously Northern Rhodesia) in 1929 (Houston, 1945). At a time, the cause was associated with either allergic reaction to insect bites or plant poisoning and they called it "pseudo-urticaria" or "lumpy disease" (Weiss, 1968; Tuppurainen *et al*., 2005). After this, it seems to be disappeared and/or unreported from any other countries for almost two decades till it occurred in Ngamiland, Bechuanaland Protectorate (Botswana) in 1943 (Von Backstrom, 1945). However, from 1943 till 1947, the disease was reported almost every year in different countries of the same continent. For example, this disease was reported by South Africa in 1944 (Thomas and Mare, 1945), Southern Rhodesia (Zimbabwe) in 1945 (Houston, 1945), Mozambique and Swaziland in 1946 (De Sousa Dias and Limpo-Serra, 1956; Diesel, 1949), and Basutoland (Lesotho) in 1947 (Diesel, 1949). After some period of interruption, the disease still continue to spread and appeared in Madagascar in 1954 (Lalanne, 1956), and Belgian Congo (Democratic Republic of the Congo) in 1955 (Anonymous, 1955).

LSD continues to spread towards the Eastern African regions affecting cattle population in those countries. Accordingly, Kenya was the first country in the region that reported the disease in

1957 (Macowen, 1959), followed by Sudan in 1971 (Ali and Obeid, 1977), and Ethiopia in 1983 (Mebratu *et al.*, 1984). Furthermore, the disease continues to spread to the other parts of African countries that shared border with the one that reported the disease. Likewise, Chad and Niger reported the disease in 1973 and Nigeria in 1974 (Nawathe *et al.*, 1978). As time advances, the coverage increases to include Egypt in 1988, a country that links Northeast Africa with the Middle Eastern countries (Ali *et al.*, 1990).

The abovementioned timeline showed the endemic characteristic feature of LSD to African countries. However, with different factors including animal movement; the disease considerably expanded in its range reaching beyond African continent. Accordingly, the first reported case of LSD outside Africa came from Kuwait from 1986 to 1988 (Anonymous, 1988). Further spread continues to occur reaching different countries such as Israel in 1989 (Yeruham *et al.*, 1995). Furthermore, LSD expanded to other Middle East countries, Russia and even countries in European Union (Tasioudi *et al*., 2016; Biryuchenkova *et al*., 2015).

2.3. Etiology

Lumpy skin disease (LSD) is caused by lumpy skin disease virus (LSDV) that belongs to genus *Capripoxvirus* with the Prototype strain called Neethling Virus. This type of virus was isolated in tissue culture for the first time by Alexander *et al*., (1957) and then confirmed by Prydie and Coackley (1959). However, infective nature of the virus was demonstrated before six years of isolating the virus (Thomas and Maré, 1945; Von Backstrom, 1945). This isolated virus belongs to the family *Poxviridae* that retain the largest size of all animal viruses (Fenner *et al.*, 1987). In this family, there are two subfamilies, the one affecting insects (*Entomopoxvirinae* (pox viruses of insects)) and that of vertebrates (*Chordopoxvirinae* (pox viruses of vertebrates)) with several genera under it. The later subfamily have nine genera including *Capripox virus* which in turn contain three species namely Sheep pox (SPV), goat pox (GPV), LSDV affecting sheep, goat and cattle, respectively (MacLachlan and Dubovi, 2011). Though these three strains affect different hosts, they do have closely related genetic (about 95% homology) and antigenic properties (Alexander *et al*., 1957; Kara *et al*., 2003). However, SPV and GPV have additional nine non-functional genes. From these non-functional genes, some of them are responsible to infect cattle.

So, those genes are the one that mainly differ between LSDV with that of SPV and GPV that enables LSDV to infect cattle (Tulman *et al*., 2001).

LSDV is an enveloped double stranded (ds) DNA virus, ovoid shape with a known molecular size (350 x 300nm) and weight (73 to 91 kilo Dalton (KDa)). According to Kara *et al.* (2003), LSDV genome sequences were assembled into a contiguous sequence of 150.08kbp which is in accordance with previous size estimates of 145 to 152 kilo base-pair (kbp) by Tulman *et al.* (2001). In these genes, the terminal genomic sequences have at least 34 genes with a unique complement. Those 34 genes are responsible for different characteristic features of LSDV such as virulence, host range and/or immune evasion (Tulman *et al*., 2001; Kara *et al.*, 2003). Furthermore, LSDV have complement of genes such as IL-10, (IF-γ) receptor; IL-lR, IFN-α/β and IL-18 binding proteins are secreted. Those secretions are responsible for modulation or evasion of host immune response, inhibition of host cell apoptosis and in cell or tissue tropism (Barthold *et al*., 2011). Virion particles enter in to host cell through cell membrane penetration and then uncoated itself to independently replicate inside the host cell nucleus (Carn and Kitching, 1994).

LSDV is susceptible to sun light and detergents containing lipid solvents. Additionally, this virus could not resist heating for 1 hr at 55°C (Davies and Otema, 1981; Lefèvre and Gourreau, 2010). However, this virus is relatively resistant to physical and chemical agents. This is particularly true as it endure drying; pH between 6.6 and 8.6 and can survive for months in dark room. Furthermore, LSDV can persist in skin plugs for about 42 days (Babiuk *et al*., 2008b; Lefevre and Gourreau, 2010). This long persistence is expected to associate with the viral A type inclusion body protein in infected cells that may protect the virion after the scab has disintegrated, although this has not yet been proven (Babiuk *et al*., 2008a).

2.4. Epidemiology

2.4.1. Susceptible Hosts

Lumpy skin disease is host-specific, causing natural infection in cattle and Asian water buffalo (*Bubalus bubalis*) though the morbidity rate is significantly lower in buffalo (1.6%) than in cattle

(30.8%) (El-Nahas *et al.*, 2011). Both cattle and Buffalo are susceptible regardless of their breed and age differences. However, some specific breeds such as *Bos taurus* is particularly more susceptible to clinical disease than zebu cattle. Furthermore, fine skinned Channel Island breeds develop more severe disease among *Bos taurus*, (OIE, 2010). Production status is also incriminated to have an impact on susceptibility. Accordingly, lactating cows appeared to be severely affected with a consequence in a sharp drop of milk production. Most of these effects are related to high fever caused by LSDV infection and secondary bacterial mastitis. Even though mixed herds of cattle, sheep and goats are common, no epidemiological evidence on the role of small ruminants as a reservoir for LSDV has been reported yet (Tuppurainen *et al.*, 2017b).

Experimental infection done on impala (*Aepyceros melampus*) and giraffe (*Giraffa camelopardalis*) result in clinical signs of LSD. Additionally, the disease has also been reported in an Arabian oryx (*Oryx leucoryx*) and springbok (*Antidorcas marsupialis*). However, the susceptibility of wild ruminants or their possible role in the epidemiology of LSD is not known with this disease also unable to affect humans (Tuppurainen *et al*., 2017b).

2.4.2. Transmission

Cattle movements between farms, regions or countries can play a significant role in the introduction of LSD. In this regard, the first outbreak reported by Israel in 1989 was associated with cattle introduction from Egypt. Additionally, vectors were also incriminated to carry the virus supported by winds to even go that further (Yeruham *et al.*, 1995; Tuppurainen *et al*., 2017b). There are different vectors species that plays a greater role in LSD transmissions. These vectors are the common stable fly (*Stomoxys calcitrans*), mosquito (*Aedes aegypti*), and some African tick species (*Rhipicephalus* and *Amblyomma* spp.). These vectors could also transmit the virus from infected carcasses to naive live animals (Tuppurainen *et al*., 2017b). Even though vectors transmissions are playing the central role, transmission via animal products (milk), fomites (equipment and clothing) and personnel are also possible means of LSD transmission (Tuppurainen *et al*., 2005).

In Ethiopia, outbreaks were seen to highly correlate with the wet and warm weather that are related to the favourability to mechanical vectors (Gari *et al*., 2010). In addition, husbandry related issues can also have a role in this disease transmission. For example, sharing of the same feeding and watering troughs give an opportunity of contaminating feed and/or water with the saliva of infected animals (Haig, 1957). In support to this, Gari *et al.* (2010) find out the communal grazing and watering points in Ethiopia were found to be associated with the occurrence of LSD. Additionally, those points were a potential higher risk to existing herd with an introduction of new infected animals.

The virus persists in the semen of infected bulls so that natural mating or artificial insemination may be a source of infection for females (Tuppurainen *et al*., 2017b). Additionally, there was a report related to the transmission of LSDV to calves that suckle on infected milk as well as teats lesions. Another transmission way is related to iatrogenic intra- or inter-herd transmission. This is due to the use of contaminated needles during vaccination or other injections with a common use of needles between animals or herds. Eventually, affected animals clear the agent from their body which made impossible to stay cattle in their carrier state (Tuppurainen *et al*., 2017b). The following figure 1. shows the role of vectors and introduction of infected animal via different transportation in to another country for the spread of LSDV in its range.

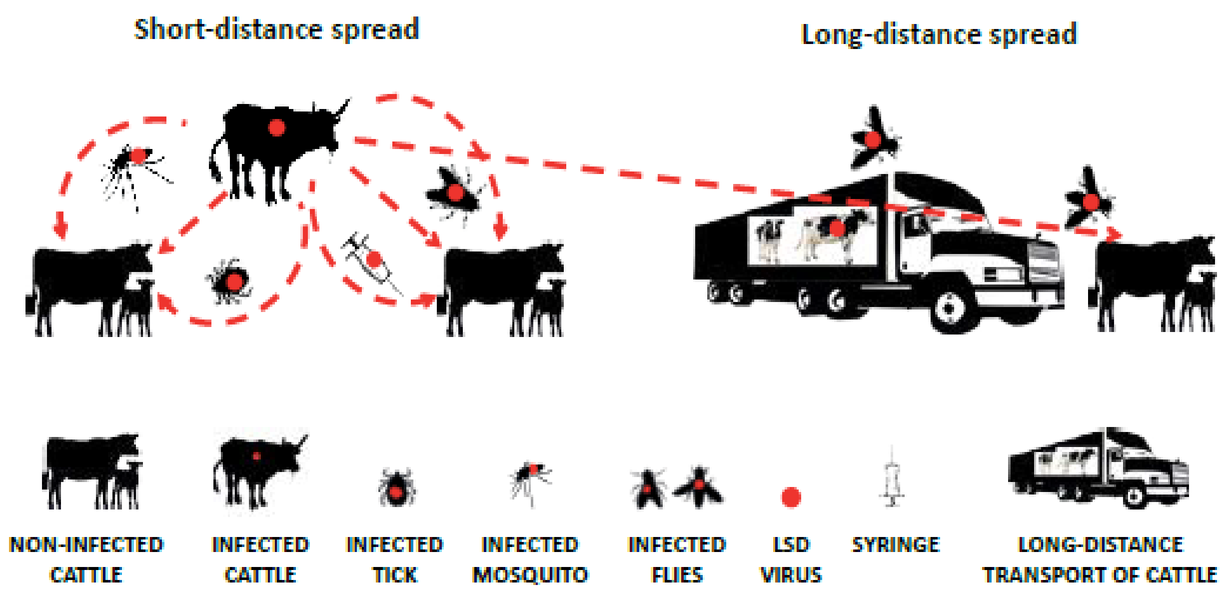


Figure 1: Transmission of LSDV (Source: Tuppurainen *et al*., 2017b)

2.4.3. Geographic distribution

Northern Rhodesia (Zambia) was the first country to report LSD in 1929 (MacDonald, 1930). This endemic disease remained till 1990 to sub-Saharan Africa including Ethiopia that reported in 1983 for the first time (Mebratu *et al.*, 1984). The spread continue into North Africa and then into the Middle East (Ali *et al*., 1990). Furthermore, the geographical range continues to include many countries of Middle Eastern countries mostly since 2013 (Tuppurainen *et al*., 2017b). More recently, LSD has spread into parts of southeast Europe, with outbreaks reported in Turkey and Russia amongst other countries (Artem, 2016; Standing Group of Experts (SGE), 2017). Accordingly, Tuppurainen and Oura, (2012) put LSD as the potential for global emergence affecting most of the countries and continents in the world (Figure 2).

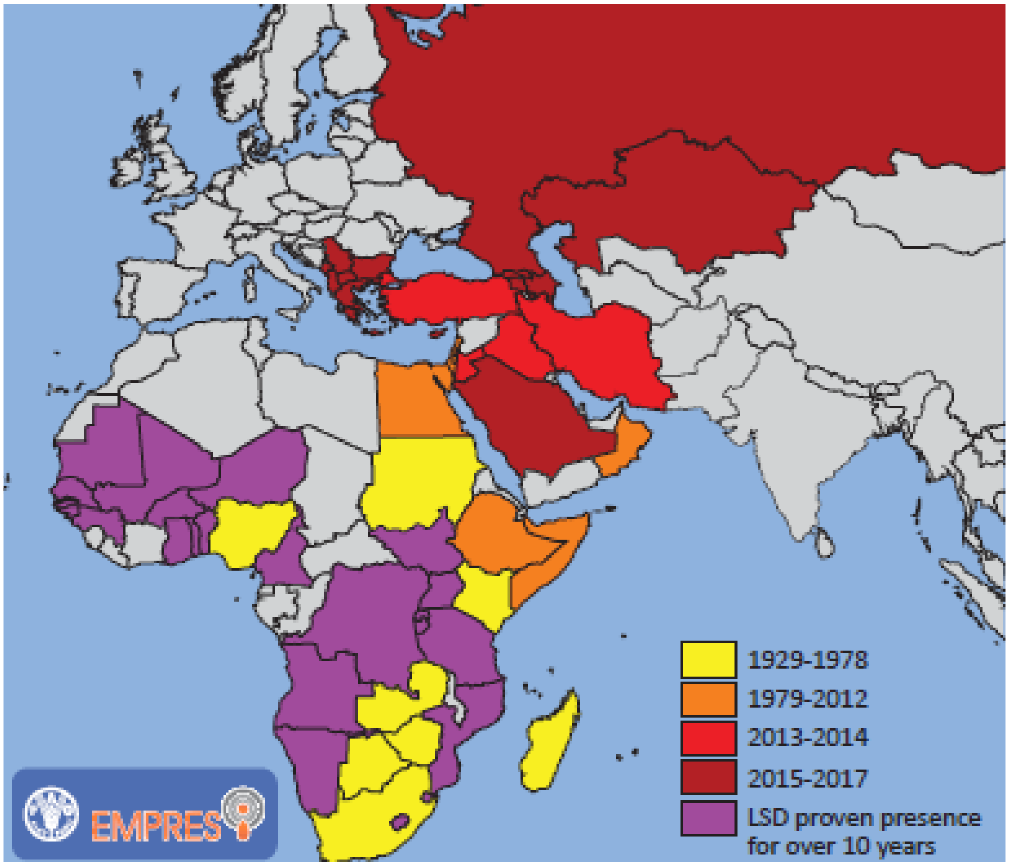


Figure 2: Geographical coverage of LSD in the world (Source: OIE World Animal Health Information Database (WAHID) and EMPRES-I, 2017)

LSD is endemic disease in Ethiopia with an increase in its range from its existence in 1983 from Southwest of Lake Tana (Mebratu *et al*., 1984). In current situation, the disease wide spread throughout the country regardless of the altitude as well as husbandry systems of the location and farming system, respectively. Taking this in to consideration, Ministry of Agriculture kept this disease as a reported disease that must be reported as outbreak notification to the National veterinary service office under the ministry that receive the specific information from the specified site. Different reports of outbreaks were reported from almost all parts of the country including Central parts of the country (Ayelet *et al.*, 2014). A map below (Figure 3) prepared by Ayelet *et al.* (2014) showed an outbreak occurrence in the country as example from 2007 to 2011. However, under-reporting of the cases could occur due to different factors including lack of professionals in some remote areas of the country.

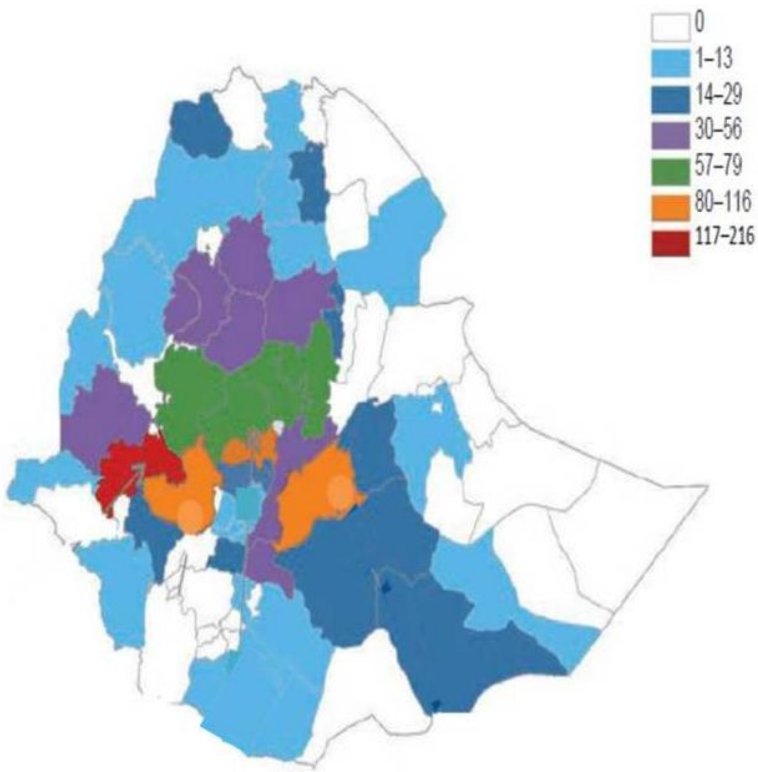


Figure 3: Map of Ethiopia showing the distribution of lumpy skin disease outbreaks, 2007–2011 (Source: Ayelet *et al.*, 2014)

2.4.4. Morbidity and Mortality rates

Morbidity of LSD varies greatly that ranges from 3 to 85% in different epizootic situations and even 100% in rare cases. The mortality rate of this disease mostly considered not very high from

1 to 2% and sometime up to 10%. However, mortality may reach up to 40% in severe outbreak cases (Coetzer, 2004; Fenner, 2011; Paolo *et al*., 2018). These broad ranges for morbidity and mortality are related to different factors including cattle breed, health status, viral isolates and insect vectors involved in the transmission. For instance in Africa, imported breeds from Europe or Australia has showed high susceptibility to LSD (Davies, 1991).

Other than breeds, host species can also have an impact on those rates. Accordingly, the morbidity rate is significantly lower in buffalo (1.6%) than in cattle (30.8%) (El-Nahas *et al.*, 2011). In the later animal (Cattle), Tuppurainen and Oura, (2012) reported LSD morbidity varies significantly depending on the immunity status and the abundance as well as the distribution of mechanical arthropod vectors. To this extent, African trypanosomosis contribute greatly to the severity of LSD infection there by to morbidity and mortality (Babiuk *et al*., 2008b).

2.5. Pathogenesis

Pathogenesis differs in field and experimental conditions due to a significant difference in their incubation period. Accordingly, a longer incubation period has been seen in natural (2 to 4 weeks) than experimental infections (about five days) (Barnard *et al.*, 1994; OIE, 2010). During natural infection of field condition, the most efficient ways of LSDV introduction is through the skin via the bite of arthropods, such as mosquitoes, ticks, *Culicoides* spp., or sand flies (Tuppurainen *et al*., 2017b). The introduction site of skin is expected to swell after a week followed by enlargement of regional lymph nodes. However, generalized eruption of skin nodules usually occurs between 7 to 19 days. In most cases of LSD, introduction of the virus in reaching blood vessel (intravenous) or limited to skin (intrademal/subcutaneous) determine the characteristic lesions. Accordingly, localized and generalized skin lesions are expected if the inoculation limited to skin surface and reaches blood vessels, respectively. Furthermore, this site of inoculation to reach intravenous circulation produces viremia that causes more severe diseases (Barnard *et al*., 1994).

Different cell are expected to be affected by LSDV in the course of disease development. From these various cells, pericytes, fibroblasts, epithelial and endothelial cells can be infected by the virus are the one infected with this virus. Consequently, the attack of those cells by this virus could lead to severe vasculitis, lymphangitis and even infarction in severe cases (Prozesky and Barnard, 1981). Furthermore, LSD inflammatory effect due to viral infection causes fever which is followed shortly by the development of nodular lesions in the skin that subsequently undergo necrosis. Generalized lymphadenitis and edema of the limbs are also common. Lacrimation, nasal discharge, and loss of appetite are reported to occur in the early stages of this disease. Additional signs are from the skin nodules involving the dermis and epidermis appeared to be raised and later ulcerated, and may even become infected with bacteria as a secondary complication (Barthold *et al*., 2011).

Experimental infection of cattle with LSDV showed the existence the virus in saliva after 11 days after fever development. On other specimens such as semen and skin nodules, the virus has been recovered after relatively longer period. Likewise, virus appeared after a double (22 days) and triple (33 days) folds of dates of the one seen in saliva when appeared in semen and skin nodules, respectively. However, the virus is not recovered from urine or fecal samples (Weiss, 1968).

2.6. Diagnosis

2.6.1. Clinical signs

All cattle breeds, both sexes and all ages groups are susceptible. LSDV also affect sheep and goats experimentally though natural infection is limited to cattle and Asian water Buffalo. This virus usually causes low mortality and relatively high morbidity. The course of the disease can exist in acute, subacute or chronic forms. In experimental infection, about 40% to 50% of animals develop generalised skin lesions while the rest appeared to be subclinical (Weiss, 1968). In experimental infection, incubation period has been seen to be shorter (about 5 days) than that of natural infection (i.e. 2 to 4 weeks) (Barnard *et al.*, 1994; OIE, 2010).

LSD clinical signs mainly observed on skin as nodules (0.5 to 5 cm in diameter) throughout the skin surface or subcutaneous tissue involving superficial lymph nodes (mainly sub-scapular and pre-crural lymph nodes) (Abdulqa *et al*., 2016). Furthermore, the nodules spread to the nasal, oral, ocular, and genital mucosa. These cutaneous lesions may resolve rapidly or may indurate and persist as hard lumps, or become sequestrated to leave deep ulcers partly filled with granulation tissue, which often suppurates (Wainwright *et al*., 2013).

Papules most easily observed in hairless areas of perineum, udder, inner ear, muzzle and eyelids (Babiuk *et al*., 2008b). Some cattle can develop edematous swelling of legs that causes lameness. Severity of infection is more in cows at peak lactation that can reflect it through a sharp drop in milk production. This drop in milk production is usually associated with the higher fever (i.e. 40 to 41 ^{o}C) and mastitis due to secondary bacterial infection (Abdulqa *et al*., 2016). In bulls, LSD could cause infertility temporarily though severe orchitis may cause a permanent sterility. This disease also has an effect on pregnant cows by causing either abortion or anoestrus for several months (Alexander *et al.*, 1957; Weiss, 1968; Barnard *et al*., 1994). Additionally, pneumonia may be observed as a result of secondary bacterial complications of upper respiratory tract that undergoes extensive necrosis. Even after recovery of this respiratory signs, stenosis of trachea has been reported following the healing of lesions by scar formation (Center for food security and public health/CFSPH, 2008).

Generalized clinical signs that are unspecific to LSD are also common. These are depression, disinclination to move, inappetence, salivation, lachrymation and a nasal discharge, which may be mucoid or muco-purulent (Weiss, 1968).

2.6.2. Histopathology

In acute stage of LSD, histpathological changes in skin lesions showed vasculitis, perivasculitis, lymphangitis, thrombosis, oedema, necrosis and infarction. Different cells infiltrate necrotic areas in orderly manner as neutrophils, macrophages and eosinophils (sometimes) exist immediately followed by lymphoblasts, lymphocytes, plasma cells, macrophages and fibroblasts. Additionally, eosinophilic intracytoplasmic inclusion bodies are seen mainly in macrophages, endothelial cells, pericytes and keratinocytes in the skin but also occur in epithelial cells associated with the hair follicles, skin glands and in smooth muscle cells (Barnard *et al*., 1994).

Histo-pathological methods using haematoxylin and eosin staining of skin lesions can visualise intra-cytoplasmic inclusion bodies of LSD virus-infected cells. Another method of immunohistochemical techniques with immunoperoxidase staining is used to mark viral antigens of skin lesion (Burdin, 1959; Haegemana et al., 2020). Histopathology can also be considered as an important tool to exclude different causes of skin nodules. This is specially a good tool to rule out viral, bacterial or fungal causes of nodular development in clinical cases and characteristic cytopathic effects (necrotised epidermis, ballooning degeneration of squamous epithelial cells and eosinophilic intracytoplasmic inclusion bodies) in cases of LSD (Ali *et al*., 1990; Brenner *et al*., 2006).

2.6.3. Virus isolation

Viral isolation and identification are methods of confirming LSDV. For isolation, primary or secondary pre-pubertal lamb testis cell cultures are most commonly used for this virus. Because these cell cultures are more sensitive than kidney, muscle, lung, skin, thymus or endothelial cells (Davies, 1991). In the culture, virus shows a characteristic features that can be used to differentiate LSDV from that of herpes virus that causes pseudo-LSD. This differentiating method is achieved through a staining of cultures infected with suspensions of early skin lesions between 48 to 56 hrs (Davies *et al.*, 1971). The resulting characteristic feature of cytopathic effect coupled with intra-cytoplasmic location of inclusion bodies is used to identify LSDV from herpes virus. The cytopathic effects resulting from the LSDV develop at 5 to 14 days in most primary cultures, but it is recommended if they have to be frozen and thawed with a possibility of one blind passage to be carried out. This process will detect LSDV strains that adapt slowly to the cultures or that were present only in very small amounts in the original sample (Davies, 1991).

2.6.4. Molecular detection methods

LSDV is possibly confirmed with molecular methods and transmission electron microscope. From molecular techniques both real-time and conventional polymerase chain reaction (PCR) through genome detection with primers specific to *capripox* virus from different samples including blood, tissue biopsy, tissue culture and semen (Abdulqa *et al*., 2016; OIE, 2017).

However, this method has its own limitation in differentiating between the strains of *Capripoxvirus* (LSDV, SPV and GPV). Tulman *et al*. (2001) justify this with the genome similarity between LSDV, GPV and SPV that ranges from 96% between viral species to 99% between isolates of the same species. Nevertheless, the use of primers for viral attachment protein gene and the viral fusion protein gene is specific for all the strains within the genus *Capripoxvirus* (Ireland and Binepal, 1998). Moreover, Bowden *et al*. (2009) stated the valid use of probe in real-time PCR method using primers for *Capripoxvirus* detection. On the other hand, molecular tests using loop-mediated isothermal amplification to detect *Capripoxvirus* genomes are also reported as a simple and cost effective method with a comparable sensitivity and specificity with real-time PCR (Murray *et al*., 2013).

2.6.5. Serological tests

Though immune response against *capripoxvirus* depends mainly on cell-mediated response, humoral immunity also plays its own important role in curing circulating antigen. This circulating antibody from humoral immunity remains mostly for seven to eight months (Lefèvre and Gourreau, 2010; OIE, 2010). However, immunity of newborn captured from dam provides protection for at least six months (Davies, 1991).

Serological tests are unable to distinguish LSDV, SPV and GPV because they share a common major antigen for neutralising antibodies (OIE, 2010). Even in the existing tests, interpretations are not easy as a result of lower antibody titre in animals mildly affected and the one that are vaccinated (Coetzer, 2004). Furthermore, Tuppurainen and Oura, (2012) stated the difficulty of serological assay that emanates from the nature of virions surface proteins. This is mainly due to the formation of different antibodies in the host for different surface proteins of non-enveloped and enveloped intracellular as well as extracellular virions. So, their antibodies proportion varied during the different stages of infection caused by the different virions (Tuppurainen and Oura, 2012). There are different serological techniques to detect LSDV antibodies; these serological tests include indirect fluorescent antibody test (IFAT) that used to determine LSD sero-prevalence (Gari *et al*., 2008; Abera *et al*., 2015), ELISA virus neutralization test (VNT), and immune-peroxidase monolayer assay (IPMA) (Haegeman *et al*., 2016).

2.6.5.1. Indirect fluorescent antibody test (IFAT)

IFAT is a method of detecting antibody against LSD. The timeline for antibodies to be detected mostly start from two days after the onset of clinical signs and reach at rise between twenty one and 42 days though the antibody continue to exist till seven months (OIE, 2010). The method of IFAT uses fluorescein isothiocyanate conjugated anti-bovine gamma-globuline (IgG) of rabbit diluted in lamb serum as a blocking buffer (Gari *et al.*, 2008). The end result is checked with Zeiss Fluorescent microscope under 40 x magnifications. In this observation, bright fluorescence foci showing the reaction between antibody and virus is seen as a positive test serum while dark field or dim gray foci in the absence of specific antibodies in the serum (Gari *et al.*, 2008; Gari *et al*., 2012; Abera *et al*., 2015). Though IFAT is used commonly due its good sensitivity, the test has limitation as it does not differentiate if antibodies of rarely occurring Parapox and Orthopox virus which affect its specificity at low serum dilution rates (Davies and Otema, 1981).

2.6.5.2. Enzyme linked immune-sorbent assay (ELISA)

Previously ELISA that mostly relies on recombinant CaPV proteins was developed. This ELISA used either mature virion envelope protein P32 or viral core protein 095 and 103 as coating antigen (Carn and Kitching, 1994; Bowden *et al*., 2009). Additionally, there is a difficulty to get sufficient quantities of heat inactivated antigen and instability of recombinant antigens (Bowden *et al*., 2009). However, this method of ELISA lacks sufficient sensitivity to be used specially for larger scale (Tuppurainen *et al.*, 2017b).

In recent times, commercially available ELISA (manufactured by IDVet) that is possibly used in both infected and vaccinated animals to detect antibodies (Ochwo *et al*., 2019). The one used by Ochwo *et al*. (2019) was ID Screen® Capripox double antigen multi-species ELISA and able to detect antibodies against *Capripox* viruses including LSDV, SPV and GPV. This ELISA has very high specificity (> 99.7%) and unable to cross-react with parapox viruses. Another important feature of this ELISA is its easiness to perform and allows for high throughput screening without requiring high-level containment facilities (Tuppurainen *et al*., 2017b).

2.6.5.3. Immunoperoxidase monolayer assay (IPMA)

The development of IPMA is commonly used for different diseases including swine influenza (Direksin *et al*., 2002), swine hepatitis E (Liang *et al*., 2014), porcine circovirus type 2 (PCV2) (Pileri *et al*., 2014), and African swine fever (Afayoa *et al*., 2014). This test is advantageous in its simplicity to perform, basic equipment requirements and the need for small amounts of purified antigen without affecting its sensitivity and specificity (Pileri *et al*., 2014). Recently, Haegeman *et al.* (2020) used IPMA for the detection of LSD antibodies.

2.6.5.4. Virus neutralization test (VNT),

In most cases serum (SNT) and virus (VNT) neutralisation test are used interchangeably. But it has minor differences in the use of a either known antigen (SNT) or antibody (VNT) (Coetzer *et al.*, 2018). During natural infection, animals that develop a neutralising antibody have a different titre at different time period. Accordingly, increment in antibody titre is expected in approximately one week after the onset of fever. The antibody continues to rise and reach its peak between two to three weeks. However, the titre starts to fall down after three weeks and become below detectable level during neutralisation (Kitching and Hamond, 1991).

The use of SNT as a serological method to detect antibody titre is very common regardless of the lengthy and tedious procedure. This method is the only validated test for detecting specific antibodies to *Capripoxviruses*. With regard to antibody testing in *Capripoxviruses*, it is most commonly and widely used antibody testing method (Davies and Otema, 1981; Babiuk *et al*., 2008a; OIE, 2018). However, this test cannot distinguish antibodies of different strains of *Capripoxviruses* (LSDV, SPV and GPV) as they share a common major antigen for neutralizing antibodies (OIE, 2010). In spite of this, SNT has a strong specificity (more than 97%) though sensitivity (70 to 96%) is relatively less (Bhanuprakash *et al*., 2006; Gari *et al*., 2008; Gari *et al*., 2012). Nevertheless, coupling with a reference antibody level before exposure of animal can be used for comparison and there by improves the less sensitivity of SNT (Bhanuprakash *et al*., 2006).

2.6.6. Differential diagnosis

Different skin lesions from pseudo lumpy skin disease (caused by bovine herpesvirus-2), insect bites, demodex infection, onchocercosis, besnoitiosis and dermatophilosis can mistakenly suspected as LSD. Additionally, diseases that cause lesions on mucosa like rinderpest, bovine viral diarrhoea/mucosal disease, foot and mouth disease and bovine malignant catarrhal fever can still confuse with LSD (Barnard *et al*., 1994).

The way to differentiate those diseases from LSD varied depending on the clinical signs and in some laboratory diagnosis might be an important tool for confirmation. In psudo-LSD, their clinical signs are milder with a characterized superficial nodule, which might only confuse early stage of LSD. Further laboratory techniques with observation of intra-nuclear inclusion bodies and viral syncytia in histo-pathological examination characterise bovine herpesvirus-2 as it is absent in LSD. For the rest of the diseases, assessing their clinical signs coupled with epidemiological evidence can play a greater role in identifying one from the other (OIE, 2017).

2.7. Economic impact

LSD is usually considered economically important diseases due to its impact on livestock productivity. This is usually related with the prolonged effect on productivity of dairy and beef cattle through decreased body weight, mastitis, infertility (can be temporary or permanent) and abortion (Weiss, 1968). Additionally, the lesions on animal skin can bring a permanent damage to the hides which affect the leather (Green, 1959).

In Ethiopia, the financial cost in infected herds was estimated to be USD 6.43 (5.12–8) per head for local zebu and USD 58 (42–73) per head for HF/crossbred cattle. This economic loss was the sum of the average production losses from morbidity and mortality rate. This can be observed through milk loss, beef loss, traction power loss, treatment, and vaccination costs. However, eeffective vaccination against LSD can reduce the expenses by 17% per head in local zebu herds and 31% per head in *Holstein Friesians*/crossbred herds (Gari *et al.*, 2011). The other observation in to direct economic losses from animal death and treatment cost was estimated to be 9,000 Ethiopian birr (ETB), (US$477.7) and 16.50 ETB per animal, respectively (Ayelet *et al*., 2014). Further study conducted by Abebe *et al*. (2017) indicated the existence of significant economic losses. In this cases, largest components of the loss at herd level was from mortality (1000 USD) accompanied by milk loss (120 USD) while control costs were the least losses recorded at heard level.

2.8. Immunity in cattle against lumpy skin disease

During the occurrence of infection with capripox virus including lumpy skin disease (LSD) virus; cell-mediated immune response plays a central role of protection (Bachh *et al*., 1997) though humoral immunity also play its own part. In cell-mediated immune response, different cells including lymphocytes appeared to be increased in their number to destroy the virus. Accordingly, Ahmed *et al.* (2007) found out the increased lymphocyte proliferation in the presence of LSD antigen. Furthermore, Abdelwahab *et al.* (2016) reported increment in lymphocyte in vaccinated cattle.

Cytokine, more specifically gamma interferon (IFN-γ) plays an important role in the defense mechanism from viral and microbial pathogens**.** It acts by the activation of the pathways that can directly inhibit virus (Strichman and Samuel 2001; Biron and Brossay 2001). In the finding of Charles *et al*. (2012), IFN-γ was reported its detection till seven days post-vaccination. Additionally, this same study detected serum IFN-γ, IL-12 and other pro inflammatory cytokines except IFN-α.

Viral spread from cell-to-cell usually occurs and countered by effector cells of cell mediated immune response. Accordingly, natural killer cells or cytotoxic T lymphocytes are responsible to prevent viral replication and spread by identifying and eliminating infected cells (Seet *et al.*, 2003). Furthermore, humoral immune response plays its own role orchestrating itself with cellular immunity for the complete protection. For example, a study on orthopox viruses showed the joint action of cellular and humoral immune response in the elimination of infection. Humoral immune response can stand and considered enough for the future re-infection (Tuppurainen *et al.*, 2017a).

2.9. LSD Vaccine types and vaccination in Ethiopia

Vaccines developed against LSD have different types including ‘live’ attenuated and inactivated vaccines. Comparatively, the inactivated one are considered to be less effective (Boumart *et al*., 2016). However, ‘live’ attenuated vaccines are relatively effective, cheap and available with recorded herd immunity (over 80%) through annual vaccination (Tuppurainen *et al*., 2017a). There are four CaPVs strains of 'live’ vaccines namely Yugoslavian RM 65 sheep pox strain, Kenyan sheep and goat pox (KSGP) virus, Romanian sheep pox strain and LSDV strain from South Africa, used to control the spread of LSD (OIE, 2017). Regardless of its availability and suggested efficacy, vaccine failure is reported in different areas that pose its effect on the effective control of LSD (Gari *et al*., 2015). Even in effective vaccination outcome, the existence of large local reaction at injection site was reported as a side effect (Davies, 1991). The skin reaction sometimes can affect the cattle owners’ attitude and discourage the use of vaccine though the loss to the production and productivity from the disease outbreak is enormous (OIE, 2017).

In general control and prevention methods include vaccination, animal control movement and slaughter of affected and in contact animals. However, some of these methods are not affordable practices especially in developing countries. Vaccination is considered to be a realistic approach to control the disease in endemic regions and considered cost-effective compared to the abovementioned one (Tuppurainen *et al*., 2012). LSD as endemic disease to Ethiopia is approached with annual vaccination scheme and following an outbreak (Molla *et al*., 2017). Kenyan sheep and goat pox (KSGP) virus is a vaccine produced by Ethiopian National Veterinary Institute (NVI) and available when needed with affordable price (NVI, 2019). However, there have been repeated complaints towards vaccine effectiveness due to the occurrence of outbreak in vaccinated cattle population (Ayelet *et al*., 2013; Molla *et al*., 2017)

3. MATERIAL AND METHODS

3.1. Study Area

The study was conducted in two purposely selected peasant associations (PAs) of Ada'a district and one city administration of East Shoa zone. The selection considers absence of previous outbreak of LSD in the sites and accessibility to reach both sites for sampling. The stationed farm was taken to look and compare the differences between the management systems. Dire and Bekejo were the two PAs while Bishoftu was a city administration where Debre-Zeit Agricultural Research Center (DZARC) dairy farm is located. Both PAs never reported LSD outbreak while the farm reported an outbreak in different times. East Shewa zone is located in the middle of Oromia, connecting the western regions to the eastern ones. One of the district in this zone is Ada'a, central Ethiopia that has a bimodal rainfall season: the long rainfall season from late June to late September and the short season from February to April, with a mean annual rainfall range of 450 mm to 1,000 mm and temperature range of 17°C to 30°C (Eastern Shewa Agricultural Bureau report, 2014). The total cattle population in Ada'a district was 172, 256 from which 7,595 and 3,604 cattle belongs to Dire and Bekejo, respectively (Ada'a district animal health office, 2020). In the research center, a total of around 95 cattle (including calves) were available, from which 38 crossbred and 57 Borena breed kept for dairy and research purposes (Personal communication).

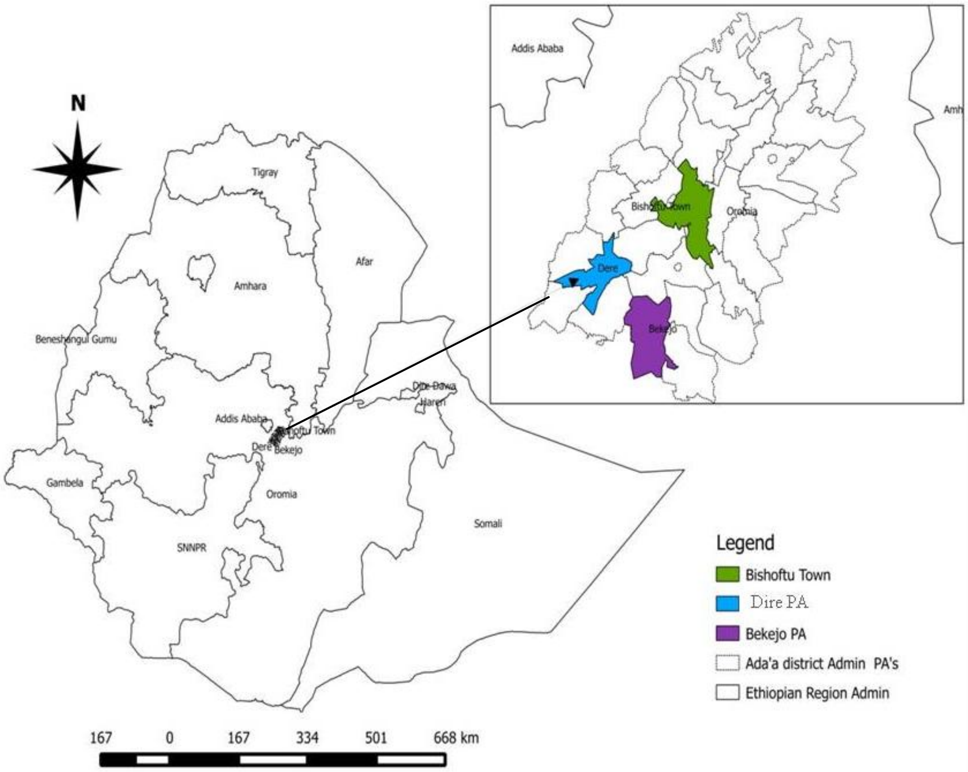


Figure 4: Map of study area

3.2. Study animals

Both male and female local and crossbred cattle that range from 6 months to 12 years old were included in this study. These cattle were sampled from Dire and Bekejo representing extensive management system while cattle at DZARC dairy farm Bishoftu, represent intensive management system. Those cattle in extensive managements were used mainly for agricultural purpose including ploughing while the one kept intensively were meant for dairy and research. A total of 113 cattle (30 from Dire, 30 from Bekejo and 53 from DZARC) were sampled at different day's during pre and post vaccination. Those animals were mostly zebu cattle (60 from Dire and Bekejo; 23 Borena cattle from DZARC) while the remaining from DZARC (i.e. 30 cattle) were crossbred cattle with a range of 50 to 98.7% blood levels.

3.3. Study design

Longitudinal study design coupled with semi-structured interview was designed to determine level of LSD antibody in cattle before and after vaccination with live attenuated LSD vaccine

(Kenyan sheep and goat pox strain virus (KSGPV), Batch no. 11-19) against the disease at the study sites.

3.4. Sampling Methods and Sample Collection

Purposive sampling was used to select the study sites by taking in to account the reported cases of LSD in selected districts and/or areas, and accessibility of the sites. Additionally, cattle selection per household took the following points in to considerations; owner closeness to veterinary clinic, and their willingness for repeated sampling. After taking these points in to considerations, cattle registration with their households was made on the first day. Cattle to be sold soon, calves below six months, diseased and pregnant ones were ruled out and not included in the study. Suspected LSD (through clinical assessment) or other diseased cattle from participating owners were not included as a study animal, but those cattle were treated with antibiotic (against secondary bacterial complication) for free to encourage owner's participation in the study.

3.4.1. Owners Selection

Questionnaire survey was undertaken from household with semi-structured interview after verbal consent and explanation of the objectives of survey before it started (Annex IV). Accordingly, 30-household (15 from Dire and 15 from Bekejo) were selected taking their interest to participate and herd size in to consideration (at least owner having minimum of 10 cattle were included in the study).

3.4.2. Animal selection

Cattle selection for humoral immune response assessment against LSD vaccine was made purposively. Accordingly, thirty cattle per site from Bekejo and Dire (total 60 cattle) were followed and taken blood sample for serum separation four times (one pre-vaccination (day zero) and three times post-vaccination on days 7, 15 and 30) that brought 240 serum samples. About 4 to 15 cattle were selected per owners and ear tagged to make the simple tracing of cattle for ease of repeated sampling. Additionally, 53 cattle (30 crossbred and 23 Borena (except 30dpv)) was included in this study from DZARC dairy farm and sampled similarly (day 0, 7, 15 and 30 days).

On 30 dpv, only 60 cattle sera samples were taken (30 from DZARc and 30 Bekejo) that brought 159 sera, giving a total of 399 sera collected from 113 cattle. Cattle identification including breed, age, sex, body condition score (BCS), parity, lactation status, and location (kebele or farm) was recorded with a format designed for this purpose (Annex I). BCS was measured according to Mishra *et al.* (2016) before sampling and scored on scale from 1-5 (Annex II). Additionally, age category was made based on dentition according to Johnson (1998) which was seen on restraining in preparation to sampling (Annex III).

3.4.2.1. Blood sample collection

Blood collection was made while the animals were restrained inside the crush. About 5 to 7 ml of blood was collected from each and every animal jugular vein with plain vaccutainer tube and needle. Each sample was labeled appropriately in a similar fashion to the data collection paper (Annex I) with permanent marker. Collection of sample was made at two stages; before vaccination and after vaccination. Samples were taken before cattle were vaccinated (day=0) followed by administering *Capripox*-LSD vaccine, via subcutaneous route with a dose of 1ml per animal. This vaccine is a homologous LSD vaccine produced from attenuated Kenyan sheep and goat pox strain virus (KSGPV) (Batch no. 11-19) bought from National Veterinary Institute (NVI). Samples taken before vaccination can give the information about antibody level against LSDV from natural infections. Moreover, the antibody from before vaccination is used to compare the antibody levels with that of after vaccinations. The second phase of sampling was done after vaccination on days 7, 15, and 30. Except at 30 dpv, only 60 cattle sera were sampled (30 crossbred from DZARC and 30 local breed from Bekejo) where as a total of 113 cattle were taken on day zero (pre-vaccination), and days 7, 15 and 30 post-vaccination.

3.4.2.2. Serum separation

Collected blood samples were transported carefully from field sites to DZARC microbiology laboratory and allowed to clot for about 16 to 24hrs at room temperature. In the next day, serum samples were separated in to labelled cryovials. Labelling was made to enable tracing back of the risk factors (management systems, breed, age, sex, BCS etc) with a similar coding with that of the one written on plain vaccutainer tubes (used for blood collection).

3.5. Laboratory diagnosis

3.5.1. Serum neutralisation test (SNT):

SNT, recommended by OIE (2010) as the only validated techniques to detect antibodies against CaPV, was used for assessment of cattle immune status from the collected sera. As NVI has adopted OIE (2010) procedure and guidelines for SNT test, it was used and followed in this study (Annex V).

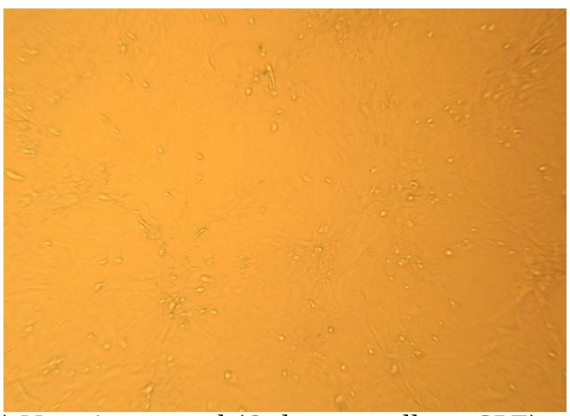
Laboratory process started by thawing the frozen (-20°C) sera samples at room temperature followed by serial dilutions of 1/5, 1/25, 1/125, 1/625 and 1/3125 was made in Glasgow's minimum essential cell culture medium (GMEM). The addition of GMEM was made with a volume of 75µl to columns of 1 and 7 for each of them. However, 100µl volume of GMEM was added individually to columns from 2 up to 6 and from 8 up to 12. As the procedure of SNT continues; a volume of 25µl tested sera was added in to 1, 2, 7 and 8 columns. Five-folds of serial dilutions were made and added in to 2 to 6 and 8 to 12 columns with multichannel pipette. But the initial dilution of 1/5 and 25 µl of suspension was discarded from the end point dilution, 1/3125 (that is from column 6 and 12) (Annex V- A and B).

A volume of 100µl of 1000TCID/ml (50% tissue culture infective dose) (38^{th} passage) viral suspension of vaccine strain (Kenyan sheep and goat pox strain/KSGPV) was added in each well from columns 2 to 6 and 8 to 12; for virus control. For the five wells in each row, different dilutions of antigen were added and the plates were sealed (with plate sealer), mixed (with plate mixer) and incubated at 37 °C in CO_2 incubator containing 5% carbon dioxide (CO_2) for 1hr. On the other hand, a cell control was prepared with in five well of the last row. After an hour of incubation, 50 µl of Vero cells was prepared at the concentrations of $4x10^5$ cells/ml and added in each well followed by checking for the cell concentration using microscope. The plates were again sealed and further incubated at 37°C (containing 5% CO_2) for 4-9 days (Annex V).

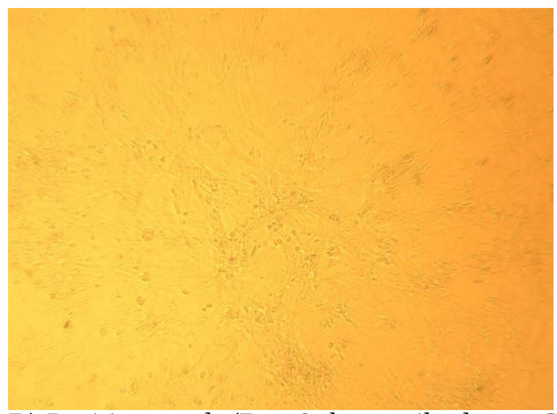
Starting from fourth day, the plates of monolayer cells were checked for cytopathic effect (CPE) under inverted microscope. The checking for CPE was continued and the final check was made on the ninth day. This last day examination was recorded from the highest dilution which inhibited CPE in both or either of the duplicate wells and recorded as the reciprocal of the log titration. The final interpretation was made by considering wells without CPE at dilution of ≥ 1/25 (index of ≥1.5) as positive, adapted from OIE (2010). This means the antibody against the LSDV has reacted with the vaccine strains and inhibited the growth of the virus thereby shows the absence of cytopathic effect on the cell culture (OIE, 2010; Gari *et al*., 2010).

3.5.2. Characterization of CPE on cell culture

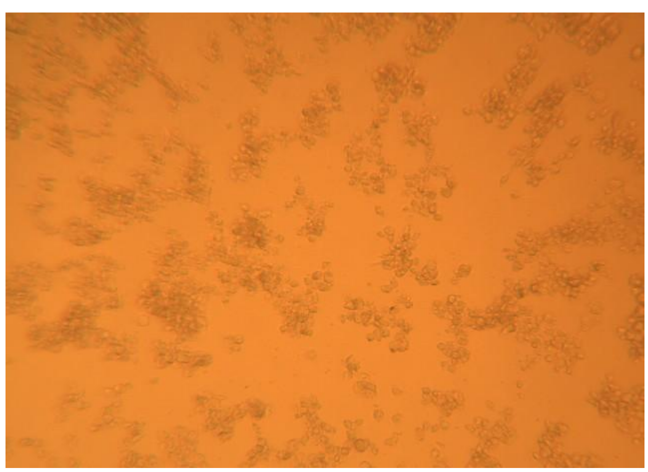
The presence and absence of cytopathic effect (CPE) was assessed after incubation of test sera on monolayer vero-cells injected with a vaccine strain between days 4 and 9. With CPE close look via inverted microscope, the final interpretation was made by considering wells without CPE at dilution of ≥1/25 (an index of ≥1.5) as positive. Therefore, the absence of CPE in cell culture indicate the presence of protective antibody in test sera that neutralise the LSD vaccine strain virus which in turn leave the cells intact without damage by the virus (i.e. absence of CPE; Figure 5).



A) Negative control (Only vero cell, no CPE)



B) Positive result (Day 9; has antibody, no CPE)



C) Negative result (Day 9; no antibody to neutralise LSDV in the sample, with CPE)

Figure 5: Microscopic observation of Vero cell culture monolayer during SNT (100x)

3.6. Data management and analysis

The collected data was entered and stored into Microsoft office Excel spread sheet 2010 and thoroughly screened before subjecting to statistical analysis. Accordingly, BCS were categorized into three as good (BCS 4 and 5), medium (BCS 3), and poor (BCS 1 and 2) (Annex II; Mishra *et al*., 2016). Descriptive analysis was made to determine proportion of cattle with its levels of SNT antibody titres across each sampling day (pre and post-vaccination sampling). SNT cut-off value was used also by Gari *et al.* (2010) and Zenebe *et al.* (2014) that referred from OIE (2010). This value is considered to be an effective antibody concentration that gives protection to LSD. Based on this value, vaccinated cattle were classified either protected or at risk group at each sampling day. Furthermore, the association between the development of LSD-specific antibody with different risk factors (BCS, age, lactation status, management system and parity) were assessed with STATA Corp. Version 13 (Stata Corp, 2013). Throughout the analysis, 95% of confidence intervals with 5% of precision were set and a $p < 0.05$ was considered as significance difference between the risk factors and antibody titre level was used.

3.7. Ethical Consideration

Ethical clearance for the study was given by animal research ethical review Committee of Ethiopian Institute of Agricultural Research Institute (EIAR). A detail of the project regarding the study approach on sampling cattle and precaution measures was submitted to the committee on September, 2019. The committee thoroughly undergone through the request and methods followed in the study which finally given a permit/clearance. Additionally, Ada’a district animal health office was communicated followed by the cattle owner consent for sampling their cattle repeatedly as well as for the interview session.

4. RESULTS

4.1. Questionnaire survey

A total of 30 households from Dire and Bekejo area were assessed for the knowledge of clinical signs of lumpy skin disease (LSD) and farmer's disease experience in their cattle. The analysis indicated that about 36.67% (11/30) of the household specify the signs LSD. On previous LSD occurrence in the herd, only 6.67% reported its previous occurrence before a year ago in their herd. The other aspects of the semi-structured interview focused on morbidity and mortality with 16.67% and 3.33% value, respectively. Those cattle that harbour LSD cases were recovered by themselves without any clinical intervention. Though annual vaccination scheme was designed, community awareness to vaccinate their cattle was less, as mentioned by respective area veterinary clinicians. On the other hand, farmers took this disease as a minor problem (if they know it is LSD) or they do not know about the disease that made them absent during vaccination. From the total households, only 16.67% (5/30) of house-hold vaccinated their cattle in the last year vaccination scheme (Table 1).

Table 1- Households responses on their knowledge and previous LSD occurrence in the study area (N=30)

Questions raised	**No. of households with positive response**	**Proportion of positive response**
Knowledge of LSD signs	11	36.67
Previous LSD occurrence in the herd (more than a year ago)	2	6.67
Previous vaccination against LSD	5	16.67
Previous visit of clinic with LSD suspected case	0	0
Case related responses (herd level)	**Morbidity rate**	**Mortality rate**
LSD case and death in the herd	16.67 (5/30)	3.33 (1/30)

4.2. Longitudinal study

4.2.1. Serum neutralization test

From a total of 399 sera collected from 113 cattle on each day of sampling except day 30 on which only 60 cattle were sampled. A total of 57 sera were found reactive to SNT on pre and post-vaccination sampling. Accordingly, higher number and proportion of cattle sera was observed during post-vaccination. From post-vaccination, 30 dpv showed the highest number and proportion of cattle sera with protective antibody (25/41.67%) (Table 2 and Figure 6).

Table 2- SNT result of cattle sera collected before and after vaccination

Days (Pre and post vaccination)	SNT reactive cattle
Pre-vaccination, Day=0 (n=113)	7.08% (8/113)
Post-vaccination Day=7 (n=113)	8.85% (10/113)
Day=15 (n=113)	12.39% (14/113)
Day=30 (n=60)	41.67%(25/60)

'n': number of cattle sampled at different time period

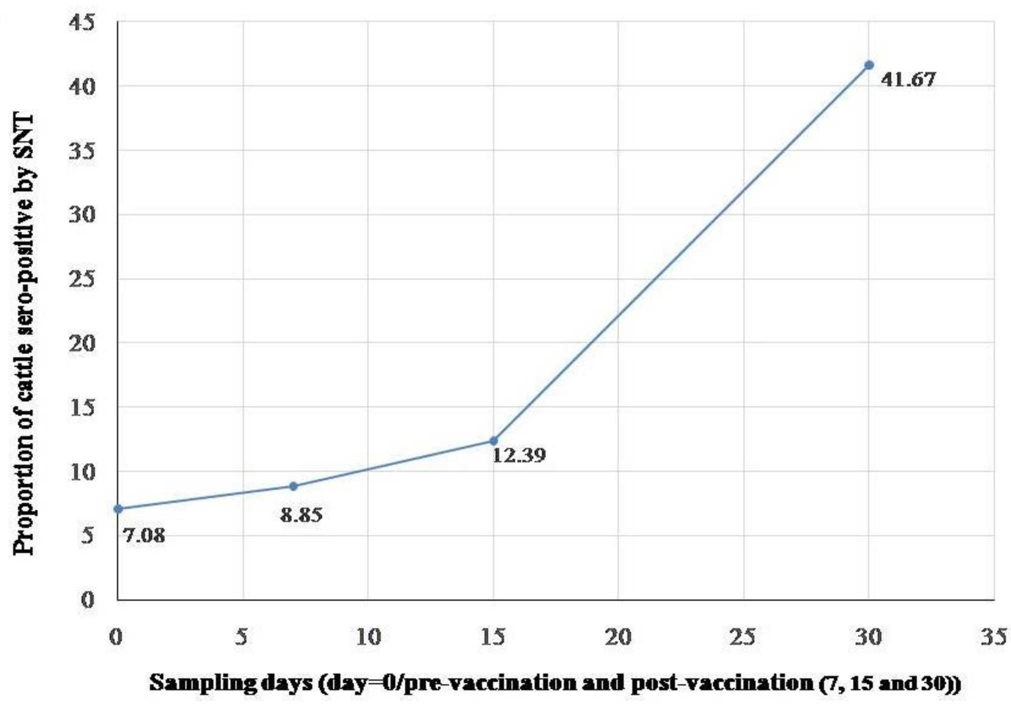


Figure 6- Percentage serum positivity across different sampling days

4.2.2. LSD protective antibody versus risk factors

Proportion of protective antibody titer differs across risk factors before and after vaccination. Accordingly, intensively managed cattle, local breed, medium BCS, ≥5 year's old and dry cows appeared with higher number (proportion) in having protective antibody. Comparisons of different days post-vaccination showed higher number cattle sera with protective antibody. Likewise, extensively managed local breeds (64%) of female (52%) cattle aged ≥5 years (64%) with medium BCS (52%) appeared to be in higher proportion in its protective antibody (Table 3).

Table 3- Risk factors associated with the responses of cattle immunity through LSD-specific antibody production till 30 days post-vaccination (dpv)

Risk Factors	Total Number of cattle (%)	Number of positive cattle sera (%)			
		Day=0 (n=8,N=113)	**PVD=7 (n=10,N=113)**	**PVD=15 (n=14,N=113)**	**PVD=30 (n=25, N= 60)**
Management					
Extensive	60 (53.10)	3 (37.5)	5 (50)	8 (57)	16 (64)
Intensive	53 (46.90)	5 (62.5)	5 (50)	6 (43)	9 (36)
Breed					
Local	83 (73.45)	4 (50)	8 (80)	11 (78.6)	16 (64)
Cross	30 (26.55)	4 (50)	2 (20)	3 (21)	9 (36)
Sex					
Female	69 (61.06)	6 (75)	7 (70)	8 (57)	13 (52)
Male	44 (38.94)	2 (25)	3 (30)	6 (43)	12 (48)
Age					
< 5 years	63(55.75)	2 (25)	3 (30)	3 (21)	9 (36)
≥5 years	50 (44.25)	6 (75)	7 (70)	11 (78.6)	16 (64)
BCS					
Poor	28 (24.78)	1 (12.5)	3 (30)	4 (28.6)	7 (28)
Medium	58 (51.33)	6 (75)	4 (40)	6 (43)	13 (52)
Good	27 (23.89)	1 (12.5)	3 (30)	4 (28.6)	5 (20)
Parity (n=62)					
NA (either male or calf)	51/113(45.13)	1 (12.5)	1 (10)	4 (28.6)	10 (40)
Heifer/Cow without calf	32 (51.61)	2 (25)	6 (60)	6 (43)	4 (16)
Cow with ≥1 calf	30 (48.39)	5 (62.5)	3 (30)	4 (28.6)	11 (44)
Lactation status;n=56/113)					
NA (Heifer, male or calf)	57/113(50.44)	1 (12.5)	2 (20)	5 (35.7)	12 (48)
No	34 (60.71)	3 (37.5)	6 (60)	6 (43)	4 (16)
Yes	22 (39.29)	4 (50)	2 (20)	3 (21)	9 (36)

'NA': not applicable, *'n'*: total number of cattle showing detectable amount of antibody, *'N'*: total number of sampled cattle during specific period

The regression analysis showed the absence of significant differences between different risk factors (management, breed (this one was omitted for 30dpv due to its collinearity with that of management systems), BCS, sex, age, lactation status and parity) ($P > 0.05$) (Table 4).

Table 4- Regression analysis of SNT results at pre-and post-vaccination of study cattle groups

Factors	Day=0 (n=8)		PVD=7 (n=10)		PVD=15 (n=14)		PVD=30 (n=25)	
	OR (95% CI)	p-value	OR (95% CI)	p-value	OR (95% CI)	p-value	OR (95% CI)	p-value
Manag.								
Intensive								
Extensive	2 (0.5, 8.7)	0.367	1.2 (0.3, 4.2)	0.837	1.2 (0.4, 3.7)	0.746	3 (0.6, 14.8)	0.171
Breed								
Cross								
Local	3 (0.7, 13)	0.134	1.5 (0.3, 7.5)	0.625	1.4 (0.4, 5.3)	0.644	★	★
Sex								
Female								
Male	2 (0.4, 10.4)	0.41	1.5 (0.4, 6.3)	0.546	1.2 (0.4, 3.7)	0.748	2.3 (0.8, 6.8)	0.127
Age (Years)								
< 5								
≥5	1.4 (0.3, 7.2)	0.706	1.1 (0.3, 4.3)	0.944	1.8 (0.5, 6.7)	0.414	1.1 (0.4, 3.1)	0.928
BCS								
Poor								
Medium	3.1 (0.4, 27.2)	0.304	1.6 (0.3, 7.8)	0.547	1.4 (0.4, 5.6)	0.595	1.02 (0.3, 3.4)	0.97
Good	1.03 (0.1, 17.5)	0.979	1.04 (0.2, 5.7)	0.962	1.04 (0.2, 4.7)	0.956	1.2 (0.3, 5.5)	0.823
Parity (n=62)								
Heifer/Cow								
Cow with ≥1 calf	3 (0.5, 16.8)	0.212	2 (0.5, 9.2)	0.335	1.5 (0.4, 6)	0.564	1.02 (0.2, 4.7)	0.982
Lac. Status (n=56)								
No								
Yes	2.3 (0.5, 11.4)	0.31	2.1 (0.4, 11.7)	0.38	1.4(0.3, 6.1)	0.691	1.2 (0.3, 6.3)	0.811

'n': total number of cattle with protective antibody, 'Ref': reference, '★'Dropped due to collinearity, Lac. Status: lactation status ('Yes': for milking cattle, 'No': for dry/non-milking)

5. DISCUSSION

In this study, significant portion of the farmers were unaware (63.33%) of lumpy skin disease (LSD) signs. From the one that identified signs (36.67%), smaller proportion took their cattle for vaccination against LSD (16.67%) indicating the absence of awareness about the disease and vaccination in the community. The disease existence was mentioned at herd level with a morbidity of 16.67% and 3.33% mortality. This finding was higher than the report of Molla *et al.* (2017) who reported 10.1% and 0.7% morbidity and mortality, respectively. However, Ayelet *et al.* (2014) reported a slightly lower morbidity (13.61%) and slightly higher mortality (4.97%) than the current study. Nevertheless, the current and previous result still remain in the range of morbidity (10 to 20%) and mortality (1 to 5%) suggested by OIE (2017).

Some farmers take their cattle for vaccination against the disease which recommended for LSD control. As it is known, vaccination is an effective disease control methods globally including Ethiopia. Likewise, vaccination is an important recommendation to effectively control lumpy skin disease (LSD) in endemic countries (Brenner *et al.*, 2006; OIE, 2010) like Ethiopia. Though vaccination is still an ongoing task against LSD in Ethiopia, vaccine failure was suspected due to the occurrence of LSD outbreak in vaccinated herds and complaints to NVI by the owner's and field veterinarians (field feedback report of the Ethiopian NVI; Ayelet *et al*., 2014). In the current study, Kenyan Sheep and Goat pox (KSGP) vaccine strain was used to vaccinate cattle for serum antibody production and assessment of neutralisation effect of the sera recovered after cattle vaccination against the vaccine strain. The observation of increment in serum antibody titre overtime confirms the well-established fact of increment in immune response overtime. Accordingly, serum antibody titre collected on day 30 post-vaccination gave best titre with more proportion of cattle had protective serum antibody titre as determined by SNT (Tuppurainen *et al*., 2005; OIE, 2017, 2018)

In the current study, as expected cattle with protective LSD specific antibody at pre-vaccination were lower in its proportion (7.08%) compared with cattle sera collected during post vaccination sampling (reach up to 41.67%). This is due to the fact that antibody level rises in vaccinated cattle compared with unvaccinated one (Kitching, and Hamond, 1991). From these 7.08% of cattle with protective antibody; dominant proportions were from intensively managed cattle (62.5%) that could be related to the previous vaccination held in the center (from personal communication). However, Samojlovic *et al*. (2019) correlate their similar finding of less cattle presentation with the specific protective antibody to the duration of detectable humoral immune response to be less than a year as vaccination was commonly done annually. In support to this, OIE (2017) put a time range of about 7 months of detectable antibodies from the time of vaccination and about 6 months from the time of infection (Weiss, 1968; Tuppurainen *et al*., 2018).

According to the product catalog of NVI, (2019), a KSGP strain of LSD vaccine has a potential to induce immunity starting from 8^{th} days post-vaccination (dpv) where specific LSD-antibody are expected at such specific day. However, not all sampled cattle showed the specific protective antibody with SNT during pre-vaccination and/or post-vaccination suggesting there could be non-responding cattle. These significant numbers of non-responding cattle without a protective antibody does not mean those cattle does not have LSD-specific antibody, rather their level was lower unless cellular immune response was exhausted. This is because of the fact that cellular immune response plays a central role during capripox viral infection (Bachh *et al*., 1997). Comparatively, more cattle sera with protective antibody were observed from post-vaccination (range from 8.85% to 41.67%) than pre-vaccination (7.08%) sampling. From post-vaccination cattle sera, maximum and minimum number of cattle sera with protective antibody titre was recorded on 30 (41.67%) and 7 (8.85%) days post vaccination, respectively. while fewer cattle sera was observed on 7 days post-vaccination (dpv). Similar fewer finding of LSD specific antibody was reported by Zenebe *et al*. (2014) on day 7 post-vaccination. However, the current proportion of cattle sera with protective antibody was less than that of Zenebe *et al.* (2014) that based their research only on stationed cattle.

Higher number of cattle showed protective antibody at 30 dpv (up to 41.67%). This report align with the previous finding Zenebe *et al.* (2014) and Samojlovic *et al.* (2019) who also reported highest protective antibody 30 days after vaccination. Such cases of antibody appearance during post vaccination is related and an indication of better immune response to vaccination (Milovanović *et al*., 2019). In those cattle, higher proportion was observed in extensively managed local breeds (64%) with better protection compared with crossbred cattle. This could be related with the susceptibility of those crossbreds that made their immune build up slower (Davies, 1991). Additionally, cattle with the age of ≥5 years appeared to be higher in their proportion (64%) with protective antibody. In line with this, Knopvelsiekte, (2008) put the importance of host related factors including age on the immune system reaction as early age may lead to low level of encounter to infection and/or vaccination. However, that risk factor does not show any statistical significance association with the appearance of protective antibody during pre and post-vaccination sampling ($P > 0.05$). This similar finding of absence of association was observed also seen with the finding of Zenebe *et al.* (2014).

6. CONCLUSION AND RECOMMENDATIONS

In this study, morbidity and mortality rates of 16.67% and 3.33%, respectively, were recorded. A higher proportion of farmers did not have knowledge about lumpy skin disease (LSD) clinical signs and much lesser proportion took their cattle for vaccination. Relatively in LSD vaccine respondent cattle, higher number of cattle with protective antibody was detected on 30 dpv suggesting the mounting of immune response over time. In those cattle, higher proportion was observed in extensively managed local breeds with better protection compared with crossbred cattle. Additionally, cattle with the age of ≥5 years appeared to be higher in their proportion with protective antibody. In general, higher proportion of cattle sera with protective antibody appeared at 30 days post-vaccination. However, statistical significance association was not observed between the appearance of protective antibody during pre and post-vaccination sampling with that of different risk factors. Some of this factor seems to overlap one on the other (such as management system and breeds), so their effects may need further study to underline with a conclusive remark on it. Regardless, in both sampling (pre and post-vaccination except day 30th), significant number of cattle did not show LSD-specific protective antibody though this does not necessarily mean those cattle were not developed immunity as cellular immunity is the dominant immune response against LSDV infection. However, serum neutralization/SNT antibody detection variation leads to question vaccine efficacy. This is due to the occurrence of significant number of cattle unable to show a detectable level of LSD-specific antibody till 30th dpv. Therefore, this study showed the existing of LSD cases and its effect (morbidity and mortality) as well as slower/less immunogenicity of the available vaccine that needs further attention. With this conclusive remark, the following recommendations are forwarded:

- Awareness should be created in the community regarding the clinical signs of LSD
- Further field challenge with circulating field strains should be undertaken on vaccinated cattle to understand the vaccine protection against the disease
- Extended period of follow up for post-vaccination should be exhausted to look when to fall down in the number of cattle with protective antibody
- Cellular immune response against LSD need to be assessed to understand the full aspects of immune response and correlate it with vaccine protection

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8. ANNEXES

ANNEX I: Data collection sheet

Cattle/Sample ID	Breed	Sex	Age	BCS	Parity	Lactation status	Previous Vaccination	Previous LSD clinical signs	SNT results (days in reference to vaccination)			
									0	7	15	30
1												
2												
3												
4												
5												
6												
7												
8												
9												
10												

ANNEX II: Body condition score of cattle

Category	Descriptions
Poor	Prominent spines with sharp process at tail head and deep cavity with no fatty tissue under skin at loin region
Medium	Shallow cavity with prominent pain bones at tail head and horizontal process can be identified individually with rounded end at loin region
Good	Fat covered with entire area but pelvic can be felt and the end of horizontal process can only be felt with pressure slight depression at loin region

Source: Mishra *et al*. (2016)

ANNEX III: Age determination of cattle based on dentition

	At birth to 1 month	Two or more of the temporary incisor teeth present. Within first month, entire 8 temporary incisors appear.
	2 years	As a long-yearling, the central pair of temporary incisor teeth or pinchers is replaced by the permanent pinchers. At 2 years, the central permanent incisors attain full development.
	2-1/2 years	Permanent first intermediates, one on each side of the pinchers, are cut. Usually these are fully developed at 3 years.
	3-1/2 years	The second intermediates or laterals are cut. They are on a level with the first intermediates and begin to wear at 4 years.
	4-1/2 years	The corner teeth are replaced. At 5 years the animal usually has the full complement of incisors with the corners fully developed.
	5 to 6 years	The permanent pinchers are leveled, both pairs of intermediates are partially leveled, and the corner incisors show wear.
	7 to 10 years	At 7 or 8 years the pinchers show noticeable wear; at 8 or 9 years the middle pairs show noticeable wear; and at 10 years, the corner teeth show noticeable wear.
	12 years	After the animal passed the 6th year, the arch gradually loses its rounded contour and becomes nearly straight by the 12th year. In the meantime, the teeth gradually become triangular in shape, distinctly separated, and show progressive wearing to stubs. These conditions become more marked with increasing age.

Source: Johnson (1998)

ANNEX IV: Questionnaire format

QUESTIONNAIRE FORMAT

Date__________________

Dear respondent,

The purpose of this survey is to collect data on LSD previous outbreak, vaccination and clinical signs knowledge at household level. Your answers will be kept confidential and you are also free to withdraw from the study if you feel the questions are uncomfortable for you to answer. But your response is very important for this study and appreciable if you will be part of this survey.

1) Background information:
   A) Owner's name__________
   B) Educational status___(i)Primary, ii)secondary, iii)Higher educat., iv) Illiterate v) Other_______)
   C) Address: District____________, Kebele__________
2) Number of cattle per house holds____________
3) Have you had skin diseases of cattle in your herd? ___________________________
4) Can you describe the lesions on your cattle skin? ___________________________
5) Have you had LSD in your cattle? Yes__ No___
6) Can you state LSD clinical signs?
   ____
   ____
   ____
   ____
7) If your answer was "yes" to Q.5; Onset of disease occurrence:
   A) When was clinical signs observed______________?
   B) When was the first death observed______________
8) Have you seen such outbreak in the area before this time,
   A) < 1yr__
   B) 1-2 Yrs___
   C) 2- 3Yrs___
   D) >3Years___
9) How frequent LSD reoccurs in the area?
   A) Don't Know ________
   B) Every 1yr________
   C) Every 2yrs________
   D) Others________
10) Herd information:
   A) No. of cattle in the herd________________,
   B) No. of suspected cattle,____________, C) No. Of Deaths____________________
11) Cattle ID (Affected):
   A) Origin__________,
   B) Age_________,
   C) Sex_____,
   D) Breed_____,

12) Production type and vaccination:
   A) Production type_________(Intensive/Semi-intensive/Extensive),
   B) Vaccination status of suspected cattle_________;
13) Have you encountered LSD outbreak in the area? A) Yes B)NO
14) If 'Yes' to Q.13; When did the last outbreak of LSD occurred in the area?___________________
15) Environmental assessment:
   A) Contacts with other herds___________________
   B) Use of communal grazing/watering______________;
   C) Contacts with wild ruminants___________
16) Cattle/Care staff movement records:
   A) New cattle recently introduced into a herd______
   B) Origin of newly introduced cattle/s__________;
   C) Cattle that have left the herd and their destination___________;
   D) Movements of cattle care staff and other visitors____________;
   E) Artificial inseminator visits and use of a breeding bull_________;
   F) Any farms visited before and after outbreak_________________;
   G) Recent veterinary treatments and cattle health records________________
17) Fomite and potential vector movement:
   A) Milk collection vehicle________________;
   B) Cattle trader/slaughterhouse transport vehicle visits________________:
   C) Potential vector activity__________________,
   D) Presence of vector breeding sites such as lakes, rivers_________________;
18) Did you vaccinate your cattle for LSD? Yes_____ No_____.
19) If yes when? Before LSD onset_____ After LSD occurrence_______
20) Vaccination status:
   A) Have you vaccinated your cattle against LSD? Yes_______ No ______
   B) When the vaccination performed? _____________
   C) Who gave the vaccine? AHA ___ CAHWs_____
   D) No. of cattle get sick from vaccinated (No sick/total No vaccinated) ____
   E) Opinion of the owner about the vaccine__________________________
21) Economic Impact of LSD:
   A) Do you milk LSD infected cows? Yes _____ No _____
   B) Do LSD infected pregnant cows abort? Yes____ No_____
22) How many pregnant females aborted, in number ________.
23) Estimated Cost for treatment for individual diseased cattle _________________
24) Estimated cost of dead cattle___________
25) Any additional comment/s on LSD disease and vaccine:
   ____
   ____
   ____
   ____

ANNEX V- Test Procedure for Sera neutralisation test/SNT

- Test sera stored at -20^{o}C was thawed at room temperature
- Test sera including a negative and a positive control were diluted with 1/5 GMEM or DMEM.
- A record sheet was filled in the layout of the 96 plate according to the samples to be tested.
- 100 µl of cell medium (DMEM, GMEM+2%TBP and calf sera +Antibiotic+Amphotracin) was added to each well.
    - 25 µl of test sample 1-16, added in well A1-H1 and A7-H7
    - 25 µl of test sample 1-16, added in well A2-H2 and A8-H8

  With the multichannel pipette, 5 fold of serial dilutions (from column 2-6 and 8-12) with initial dilution 1/5 was performed and 25 µl of suspension from the end point dilution 1/3125 was discarded; i.e. from column 6 and 12.
- 100 µl of 1000TCID$_{50}$/ml viral suspension of KSGP strain was added to each wells from 2-6 and 8-12 (add 75 µl of cell culture media in column 1 and 7) and incubated in 37^{o}C for 1 hr.
- 50 µl of vero cell prepared in the 4x10^{5} cells/ml was added to each well.
- The micro-titre plates were covered and incubated at 37°C for 9 days.
- Using an inverted microscope, the monolayers were examined daily from day 4 for the evidence of CPE. There should be no CPE in the cells of row H. Using the 0240 KSGP vaccine strain of capripoxvirus, the final reading is taken on day 9, and the titre of virus in each duplicate titration is calculated by the Kärber method. If left longer, there is invariably a ‘breakthrough’ of virus in which virus that was at first neutralised appears to disassociate from the antibody.
- ***Interpretation of the results:*** The neutralisation index is the log titre difference between the titre of the virus in the negative sera and in the test sera. An index of ≥1.5 is positive.

A) Test plate layout in single row for each test sera and 16 test sera plate

		1/5	1/25	1/125	1/625	1/3125		1/5	1/25	1/125	1/625	1/3125
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
A												
B												
C												
D												
E												
F												
G												
H												

Control plates were separately prepared in the following manner:

- ***Positive control***: 6 well in each 4 row were filled with different dilutions of KSGP virus ($100TCID_{50}$, $10TCID_{50}$, $1TCID_{50}$, $0.1TCID_{50}$ in 100 µl suspension) then plate was incubated for one hour similar to other plates.
- ***Negative control***: 6 wells in the last row were without virus and 50 µl of vero cell prepared in the $4x10^5$ cell/ml was added to each well and plate was incubated for one hour similar to other plates.

B) Control layout for the positive and negative controls. No sera added to control plate

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**Up to 12**
A									100TCID50, in 50µl
B									10TCID50, in 50µl
C									1TCID50, in 50µl
D									0.1TCID50, in 50µl
E									No virus, only cell
F									
G									
H									

ANNEX VI- Reviving and Subculture of vero cells preparation

Reviving of vero cells

- Selected cryovial was taken and placed directly on to warm water bath or bucket at 37^{o}C.
- The vial content was waited until the suspension was thawed completely.
- The vial was disinfected with 70% ethanol.
- The vial was opened in laminar air flow hood and transferred the content by means of a sterile pipette to universal bottles which contain 10ml 10% GMEM.
- About 200g of cell was centrifuged for 10 minutes at +4^{o}C.
- The supernatant with preservative was discarded and resuspension of the cell was made at the required concentration in fresh complete sterile medium (10% GMEM) inside the laminar air flow hood.
- The cell suspension was adjusted to the desired concentration 20x 10^{6} cell/ml for diploid cell lines, followed by labelling of type of cell line, date of incubation and passage. Then incubated at +37^{o}C

Subculture

- 0.05% trypsin solution was pre-warmed for sub-culturing working (trypsine) and complete media for cell growth at +37^{o}C
- Cell culture flask was taken with confluent monolayer from the incubator.
- Old media was decanted.
- The cells was washed with PBS (which must be free of Ca, Mg if version is to be used as dispersant) to remove residual sera (which would inactivate the trypsin) and residual bivalent ions (which would inactivate the versene).
- Enough amount of trypsine was added slowly on the opposite wall of attached cell monolayer culture flask and the action of trypsine was observed for two minutes.
- The trypsine was decanted, followed by placing the flask inverted until the cell detached from it inside the laminar flow hood.
- Growth medium (10% GMEM) (sterile new borne calf sera) was added and vigorously pipette with pipette, mechanically until monolayered became dispersed to a single cell.