Isolation and Molecular Characterisation of
Lumpy Skin Disease Virus from Diary Farms of Central Ethiopia

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Abstract: Lumpy skin disease (LSD) is economically important viral disease of cattle mainly in Africa and Middle East. Mass vaccination is a better strategy to control the disease in endemic countries. However, there is an increasing field report of vaccine failure. The study was conducted in central parts of Ethiopia to isolate; characterize LSDV and for vaccine effectiveness. A total of 191 dairy cattle from smallholder and 1551 from big dairy farms were included for the study. Out of 1742 dairy cattle, 213 (12.2%) showed characteristic clinical signs of LSD and 27 cattle (1.5%) were died as the result of the disease. Thirty-one Skin biopsies were collected from outbreak cases. Twenty-six skin samples were developed a characteristic CPE on Vero cell line. Viruses were isolated and confirmed to be LSDV using conventional PCR and real-time PCR. The complete sequence of RPO30 gene of the isolates showed that all isolates substituted at least one nucleotide in respect to vaccine strains. The vaccine effectiveness of annual vaccination was estimated to be 67.1% (95% CI = 57.3 – 74.7) in contrast to vaccination during outbreaks. Thus, it's the time to direct efforts towards to the development of new effective vaccine.

Key words: Dairy Cattle · Lsd · Lsdv · Outbreak · Vaccine Effectiveness

INTRODUCTION

Lumpy skin disease (LSD) is a viral disease of cattle which is characterized by high fever, multiple circumscribed firm skin nodules, enlargement of superficial lymph nodes, nasal discharge and lachrymation; but in severity, clinical signs are highly variable. It is caused by Lumpy skin disease virus which is a member of the *Capripoxvirus* genus in the *Poxviridae* family. The virus is antigenically indistinguishable from other capripox viruses such as sheep pox virus (SPPV) and goat pox (GTPV) [1, 2]. LSDV mainly affects cattle and the disease is more severe in lactating and pregnant cows [3].

Transmission has mostly been associated with blood-feeding insects with frequent feeding habits [4, 5]. Ticks have recently been implicated in the transmission of LSDV [6]. The morbidity and mortality rates of LSD vary considerably, depending on host susceptibility and the abundance of insect vectors involved in transmission. The morbidity rates are generally between 1% and 20%, but in few outbreaks report the morbidity rates exceeded 50%. On the other hand, mortality of 10 to 40% and even higher have been reported on occasion but the lower range of 1 to 5% is more usual [7].

Lumpy skin disease was first observed in 1929 in northern Rhodesia (currently Zambia) and rapidly spread north and south of Africa [3]. The disease was then reported in out of Africa in different countries in the Middle East in late 1980s [8]. It now appears to be spreading to new territories such as turkey and Iraq and threatens the entire European countries [9].

LSD was first observed in Ethiopia in 1983 in the northwestern part of the country [10]. It has now spread to almost all regions and agro-ecological zones and became endemic. Epidemic reoccurs after an interval of 5-6 years cycle in cattle population [11]. The disease causes high economic losses through decreased milk

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production, emaciation and poor growth in infected animals, permanent damage to hides, abortion, temporary or permanent infertility and sometimes death [3, 7, 12].

Moreover, it adversely affects the trade of livestock and livestock products. LSD is categorized by the World Organization for animal Health (OIE) as notifiable diseases due to their potential for rapid spread and substantial economic impact. On the other hand, the livelihood of more than 80% of population of Ethiopia is dependent on livestock. Therefore, LSD is one of the most economically important livestock diseases in the country.

Vaccination is the effective method to control the disease in endemic countries like Ethiopia. The attenuated Kenyan sheep-and-goat pox vaccine strain KS-1 has been used to protect cattle against LSD in the country because of its advantage of conferring cross-protection to LSD [13]. In contrary, field reports of LSD outbreak in vaccinated animals has been increasing during past years [14-17].

Similar phenomenon has also reported from other countries [14, 16]. Furthermore, similar reports also came from different smallholder and big dairy farms following 2014/15 LSD outbreak eruption in Ethiopia. As the result, the vaccine production companies and scholars are motivated to rule out the cause behind the current LSD vaccination failure. There is also a knowledge gap in the country to fully understand the existing incomplete protection of the vaccine. In addition, there are few studies on the type of the virus circulating in Ethiopia.

Therefore, the objectives of this study were:

- To isolate and characterize virulent field isolates of LSDV circulating in the area;
- To assess the current LSD vaccine effectiveness based on the existing vaccination strategy

MATERIALS AND METHODS

Study Animals and Farms: All animals in the study were crossbreds with different exotic blood level and were kept for dairy purposes. The farms included in the study were categorized as 17 smallholder and 4 big dairy farms based on herd size and management system.

Sample Processing: The skin biopsy samples were thawed at room temperature and washed three times using sterile phosphate buffer saline (PBS) at a pH of 7.2 in Bio-safety cabinet Class II. The biopsy tissue was minced using sterile scissors and forceps. About 1 gm of the sample was ground using sterile pestle and mortar by

adding 9 ml of the sterile PBS at a pH of 7.2 containing 0.1% gentamycin. The tissue suspension was centrifuged at 1, 500 rpm for 15 min. The supernatant was collected, filtered through 0.45 µm pore size (Millipore, USA), labeled and preserved at - 20°C until use.

Isolation and Identification of the Virus: The field isolated *poxvirus* was inoculated on Vero cell lines according to the method of Balinsky *et al.* [18] to ascertain the presence of infectious virus. The Vero cell line was propagated in Glasgow Eagle minimal essential medium (GMEM) supplemented with 10% calf serum. Vero cell line was grown in 25 cm² tissue culture flask and was incubated with 5% CO_2 at 37°C until the cells became confluent monolayer. The tissue culture medium was then removed in aseptic conditions and the monolayer washed three times using sterile warm PBS pH of 7.2 in Bio-safety cabinet level II. One ml of tissue homogenate was inoculated onto the confluent monolayer and incubated at 37°C and allowed to absorb for 1 hour. Then after covered the monolayer with 10 ml of GMEM, containing antibiotics and 2% fetal calf serum into the flask and placed into incubator. All the flasks, including the control flasks, were incubated at 37°C in a humidified incubator with 5% CO_2. The medium was changed every 48 hours. Cells were monitored daily using an inverted microscope for evidence of virus induced cytopathic effects (CPEs) for 7-14 days post-inoculation. Three more blind passages were carried out for samples that were initially CPE. Infected cells developed a characteristic CPE consisting of retraction of the cell membrane from surrounding cells; eventually rounding and aggregation of cells were observed. When 80% CPE was observed, Virus inoculated flasks were harvested and frozen overnight at –20°C. The harvested cell culture was thawed twice at room temperature to release the virus particles. Finally, virus suspensions were stored at -20°C until processed for viral DNA detection.

DNA Extraction: DNA extraction was performed using DNeasy Blood and tissue kit (Qiagen, Rocm and Haas Company, USA) according to the procedures of the manufacturer. DNA was extracted from 26 specimens; 180 µl crude virus in culture supernatant from the LSDV infected cells were pipette in to 2 ml of micro centrifuge tube, clarified by centrifugation at 8, 000 rpm for 5 min. 200 µl of AL buffer (lysis buffer) was added in to the centrifuged virus suspension and mixed by vortexing and incubated at 56°c for 10 min. 200 µl of ethanol (100%) was added and mixed thoroughly with the help of vortex mixer. The mixture was transferred in to DNeasy minispin column

in 2 ml collection tube and centrifuged at 8, 000 rpm for 5 min. The spin column was transferred in to a new 2 ml collection tube and 500 µl of AW$_1$ centrifuged at 8, 000 rpm for 1 min. Then, the collection tube was discarded and the min spin column placed in a new 2 ml collection tube and 500 µl of AW2 was added and centrifuged for 3 min at 14, 000 rpm, min spin column transferred carefully in to a new 2 ml of micro centrifuge tube and 200 µl of elution buffer (AE buffer) added and incubated for 1 min at room temperature centrifuged for 1 min at 8, 000 rpm.

Polymerase Chain Reaction (PCR): A polymerase chain reaction (PCR) was carried out to detect the virus using Capripoxvirus-specific primer: Forward and reverse had the sequences (SpGpRNAPol-F) 5' TCTATGTCTTGATATGTGGTGGATAG-3' and (SpGpRNAPol-R) 5' AGTGATTAGGTGGTGTATTATTTTCC-3', respectively DNA amplification was carried out in a final volume of 50µl of 10X PCR buffer, 1.5 µl of MgCl2 (50Mm), 1 µl of dNTP(10Mm), 1µl of forward primer, 1µl of reverse primer, 1 µl of DNA template, 0.5 1µl of Taq DNA polymerase and 39 µl of nuclease free water. Finally the mix was incubated in a thermal cycler: first cycle: All PCR experiments performed in a PTC-100 thermal cycler (MJ-Research, Inc., Waltham, Massachusetts, USA) using the following amplification program: initial denaturation at 95°C for 1 min; 40 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 30s and elongation at 72°C for 1 min. An additional elongation step was performed at 72°C for 5 min and the PCR products were stored at 4°C until analysis.

Agarose Gel Electrophoresis: The isolated DNA from cell culture was analyzed by agarose gel electrophoresis as described by Mangana-Vougiouka *et al.* [19] with some modification to confirm the presence of DNA. Amplified products were analyzed using a gen ruler ™ 100bp DNA ladder (Fermentes, Germany) as a molecular marker on 3% agarose gels prepared in Tris/Acetate/EDTA (TAE) buffer and 10mg/ml ETDM- bromide stain, then 20µl of PCR product was mixed with 4 µl loading buffer and loaded to wells in previously prepared gel and run at 100 volt for about 60 minutes in parallel with DNA molecular weight marker in electrophoresis apparatus until the DNA samples have migrated a sufficient distance through the gel. DNA bands were visualized using UV transilluminator at a wave length of 590 nm and positive results were confirmed according to the size of the bands formed on agarose gel. The PCR results were considered positive for LSDV and GTPV DNA when a 172 bp and SPPV 151 bp product were observed.

Real-Time PCR: The real-time PCR was executed at Molecular Biology Laboratory, NVI. Each sample was tested in duplicate and every PCR run included no-template and positive GTPV, LSDV and SPPV controls. 5'GGTGTAGTACGTATAAGATTATCGTATATA-3' as forward primer and 5'AATTTCTTTCTCTGTTCCATTTG-3' as reverse primer were used for Real-time PCR analysis. The PCR was set up in a 20 µL reaction volume. A total of 1430 µl of the master mix was prepared to 26 samples. 17 µl of the master mix was dispensed into each micro-well of Low-Profile Hard-Shell H 96-well PCR plate (Bio-Rad). The master mix in each well was composed of 4.84 µl of RNAase free water, 2 µl of forward primer (CHFRsb), 0.16 µL of reverse primer (CHFR1) and 10 µL of 1x SsoFast Eva Green Super mix. 3 µL of sample extracted DNA; no-template (RNAase free water); and positive GTPV, LSDV and SPPV controls was added in duplicate fashion and sealed. PCR was performed in a CFX96™ real-time PCR detection system with an initial denaturation step at 95°C for 3 minutes, followed by 45 cycles of 95°C for 15 seconds and 58°C for 80 second using a Low-Profile Hard-Shell H 96-well PCR plate (Bio-Rad). The product was then denatured at 95°C (held for 1 minute), cooled to 40°C (held for 1 minute) and heated continuously at 0.5°C/10 seconds with fluorescence acquisition from 40°C to 85°C. The melting temperatures were analyzed using the CFX™ 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™ Software (Bio-Rad). Normalized melt curves and difference in curves were acquired by selecting pre-and post-melt regions for amplicons separately [20].

RPO30 Gene Sequencing: The RPO30 gene sequence was done for only seven PCR positives representative samples in the study areas. The DNA extracts were sent to Vienna, Austria, for gene sequencing. The PCR was also conducted to amplify the DNAs from the extracts. PCR products were also checked using electrophoresis on a 1.5% agarose gel for 1 h at 100 V. The positive DNA products were purified using Wizard SV Gel and PCR clean-up system kit (Promega) and sequenced commercially by LGC Genomics (Germany).

For genomic analysis, the sequence data were edited and fragments were assembled using Vector NTI Advance™ 11.5 software (Invitrogen, Carlsbad, CA, USA). Phylogenetic tree analysis was included 35 capripoxviruses based on nucleotide sequences of RPO30 gene complete sequence (606bp). LSDV field isolates, attenuated vaccine strain (KS-1 and Gorgon) and reference sequences retrieved from the Genbank database were considered in the analysis. The Neighbor-Joining method with the maximum composite likelihood nucleotide substitution with the pairwise deletion option was computed using the Kimura 2-parameter method of MEGA6 software [21]. The percentages of bootstrap scores above 50% (out of 1000 replicates) are shown next to the branches.

RESULTS

Observed Clinical Signs: The common clinical signs observed in our study were fever, circumscribed nodules on the skin with different sizes, necrotic nodules and deep scab formation, edematous swelling of one or two legs, enlargement of superficial lymph nodes and decrease in body weight (Fig. 1). Lameness and superficial lymph node enlargement were very prominent. The nodules on the limbs gradually burst and left necrotic wounds that were frequently seen to be complicated with secondary infection (Fig. 2). Dysentery and recombency were also seen.

Virus Isolation: Out of the 31 skin biopsies, characteristic poxvirus CPE was observed in infected Vero cells with 26 samples within ten days of post- inoculation or after one- or two-blind passages while the virus was not isolated from the remaining five samples. None of the negative control cultures showed any CPE after two- or three-blind passages.

Polymerase Chain Reaction: The DNAs of 26 isolates on Vero cell line were amplified using capripoxvirus-specific primers. The amplicon size of PCR product had molecular weight of 172 bp (Fig. 7), the expected amplicon size for the LSDV genomic region targeted. The resulting PCR products of GTPV/LSDV differed in length by 21 nucleotides produced from SPPV genomes. After electrophoresis migration of the PCR products on a 3% high-resolution agarose gel, all amplicons from the SPPV group were shorter (151 bp) and easily distinguishable, relative to the GTPV/LSDV amplicons 172 bp (Fig. 7).

Real-Time PCR: The DNA extracts forming 172 bands on agarose gel was again subjected to real-time PCR and LSDV differentiated from GTPV. The peak melting curve of the real time revealed that all 26 virus isolates were characterized as LSDV since their snapback of melting peaks were at 51°C while the second peaks were at 73.5°C (Fig. 8). Known CaPV positive samples were tasted for comparison. Real-time PCR assay detected differences in the melting point temperatures for SPPV, GTPV and LSDV after fluorescence melting curve analysis from each other (Fig. 9).

The RPO30 genes sequence analysis of the current Ethiopian field isolates and the previous LSDV isolates showed that substitution of some nucleotides (1-5) with respect to the current NVI vaccine strain (KS-1).

The 30 KDa DNA-dependent RNA polymerase subunit (RPO30) genes, Lamien *et al*. [22] of capripoxvirus were analyzed in order to characterize and compare local isolates and the local vaccine strain. Based on multiple sequence alignment of the present isolates' RPO30 gene complete sequences of 606 bp with KS-1 vaccine, all the present isolates, C nucleotide substituted by T (at nucleotide position 292) while Ambo, Ginchi, Holeta, Quarit and Selale isolates differed by additional one nucleotide substitution (at nucleotide position 41, C substituted by A) from KS-1 vaccine strain. These isolates were similar to previous isolate from East Shoa Zone of Oromia Regional State, Ethiopia.

Phylogenetic Analysis: Finally, phylogenetic reconstructions were performed to determine the genetic relationship among Ethiopian isolates as well as other CaPV isolates. In general, phylogenetic analysis indicates that all representative isolates (n=7) were identical to LSDV and related to SPPV and GTPV.

Phylogenic tree analysis categorized the present field virulent isolates into two clusters. The isolates obtained from Bishoftu and Kaliti were found to be 99% similar to the previously isolated LSDV strains from Adama andassa, Bishoftu, Modjo and Wenji. These isolates were also closely related to the isolates of Burkina Faso, Egypt and Niger. However, the isolates from Ambo, Ginchi, Holeta, Quarit (West Gojam) and Selale separately formed other cluster which was closely analogous to Sudan isolates (Fig. 8).

Vaccination Strategy Against LSD: Vaccination against LSD was usually done during outbreak eruptions in the study areas. All visited smallholder dairy farm owners

Fig. 1: Characteristic of LSD with generalized circumscribed skin nodules covering the entire body. Photo by: author

Fig. 2: An inflammatory swelling of the hind leg following complicated wound. Photo by: author
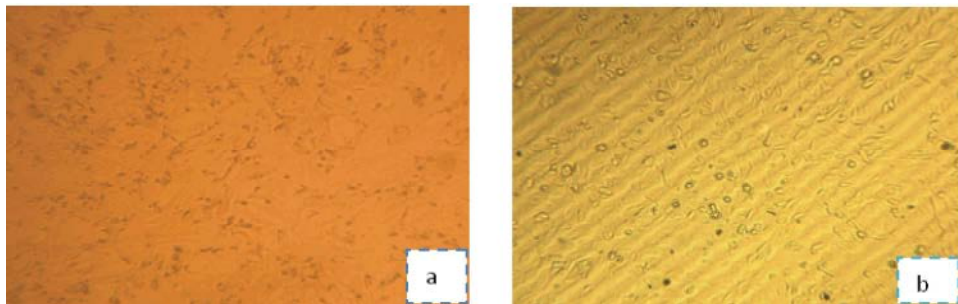

Fig. 3: Photo of Vero cell: a) CPE positive on Vero cell after seven days post inoculation; b) control of cell culture
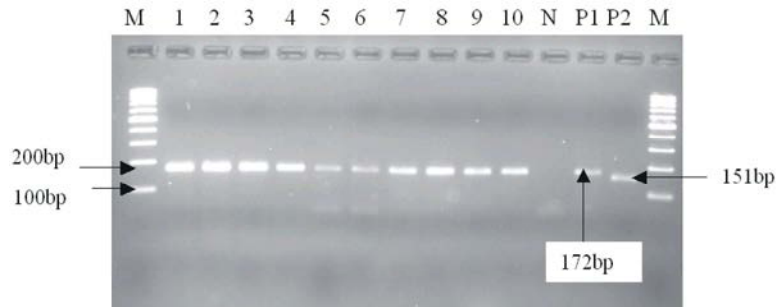

Fig. 4: PCR based detection of LSDV in samples taken from skin nodules of infected animals
Lanes M: DNA ladder; lane N: Negative Control without template; Lanes 1, 2 and 3 represent positive samples from Bishoftu; Lanes 4 and 5 represent positive samples from Holeta; Lanes 6 and 7 represent positive sample from Ginchi; Lane 8 and 9 is a positive sample from Selale; Lane 10 is a positive sample from Akaki; P_1- Positive control for LSD and Goat pox- (size 172bp); P2- Positive control for Sheep pox- (size 151bp)
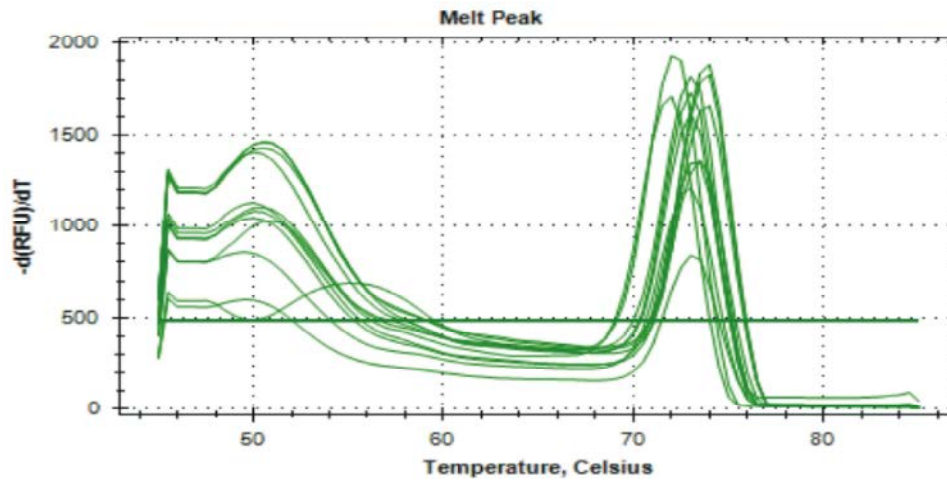
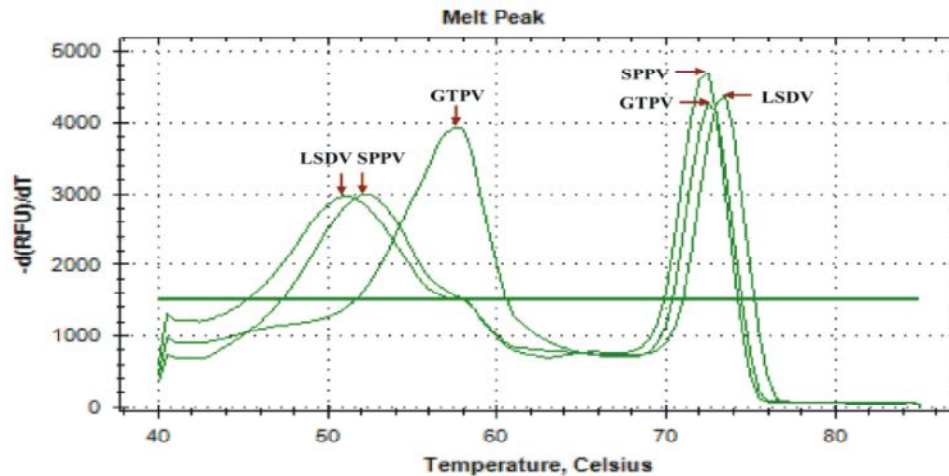Fig. 5: Differences in the melting curve for field isolates of LSDVs

Fig. 6: Differences in the melting curve for known positive isolates of CaPVs for comparison

responded that they did not have regular vaccination program against the disease. Out of 17 smallholder dairy farms, six farm owners vaccinated their animals and disease observed in their farms. However, the remaining 11 farm owners were (64.7%) vaccinated their animals prior to the occurrence of the first case in their farms. In such vaccination strategy, drastic increments of the number of new cases were a big concern for the farm owners and animal health workers. In big dairy farms do have their own regular vaccination schedule and vaccinate their animal annually against LSD before the beginning of the main rainy season. However, LSD outbreak was occurred in assessed big dairy farms after six to ten months of the last vaccination.

Outbreak Investigation and LSD Vaccine Effectiveness:
Out of 1742 dairy cattle observed, 213 animals (12.2%) were developed LSD clinical signs with physical examination. Low morbidity rate of 10% (95% CI = 0.08 - 0.11) was recorded in regularly (annually) vaccinated cattle, whereas high morbidity of 30.4% (95% CI = 0.24 - 0.37) was found in dairy cattle vaccinated during outbreaks. The 95% CIs of morbidity rates elicits that regularly vaccinated dairy cattle and dairy cattle vaccinated during outbreak were statically different. LSD vaccination strategy was also significantly associated (P<0.05, χ^2= 65.768) with morbidity rate (Table 2).

On the other hand, only 27 dairy cattle (1.5%) were dead from all visited dairy animals. Mortality rate of 0.6% (95% CI = 0.00 - 0.01) in regularly vaccinated dairy cattle was lower than mortality of dairy cattle vaccinated during outbreak 9.4% (95% CI = 0.05 - 0.14). The 95% CIs of the groups illustrate absence of statistical difference between the two dairy cattle populations. Statistical significant difference (χ^2 = 87.165, *P*< 0.05) was revealed between LSD mortality rate and vaccination history.
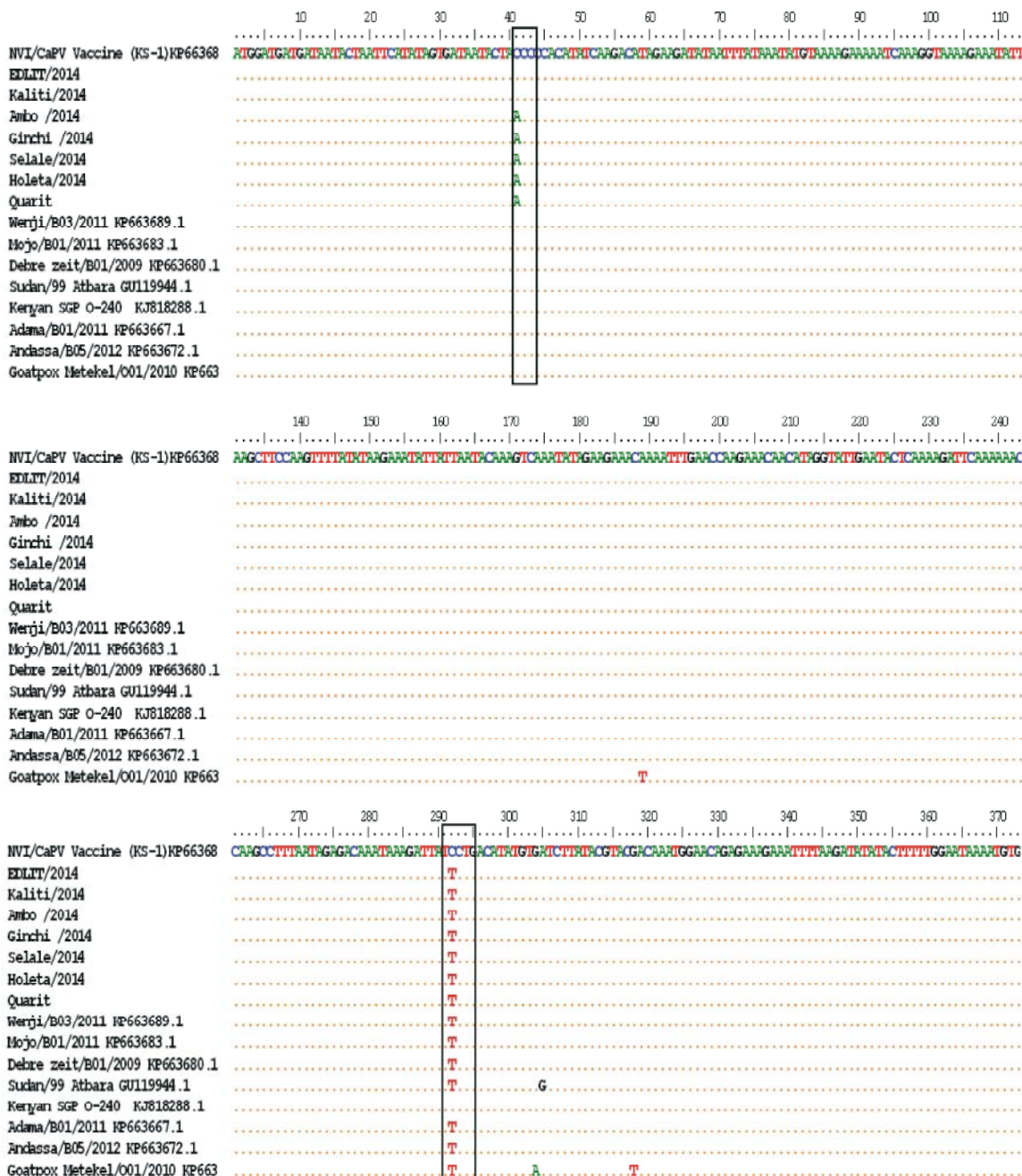
Fig. 7: Multiple nucleotide sequence alignment of RPO30 gene of present and previous Ethiopian LSDV field isolates, NVI vaccine strain (KS-1), Kenyan SGP O-240 and Ethiopian field GTPV. Sequence analysis of the Ethiopian field LSDV isolates showed substitution of at least one nucleotide as compared to the vaccine strain (KS-1)

In the context of the existing vaccination strategy, the relative risk of having LSD in annually vaccinated dairy cattle was 32.9% (95% CI = 0.253 – 0.427) lower than in dairy cattle vaccinated during outbreaks. Therefore, the effectiveness of regular annually vaccination against LSD was estimated to be 67.1% (95% CI = 57.3% - 74.7%) as compared to vaccination during outbreak occurrences. The relative risk of death in regularly vaccinated dairy cattle was calculated to be 6.2% (95% CI = 0.028 – 0.135) and thus, 93.8% mortality reduction was observed in regularly vaccinated dairy cattle.
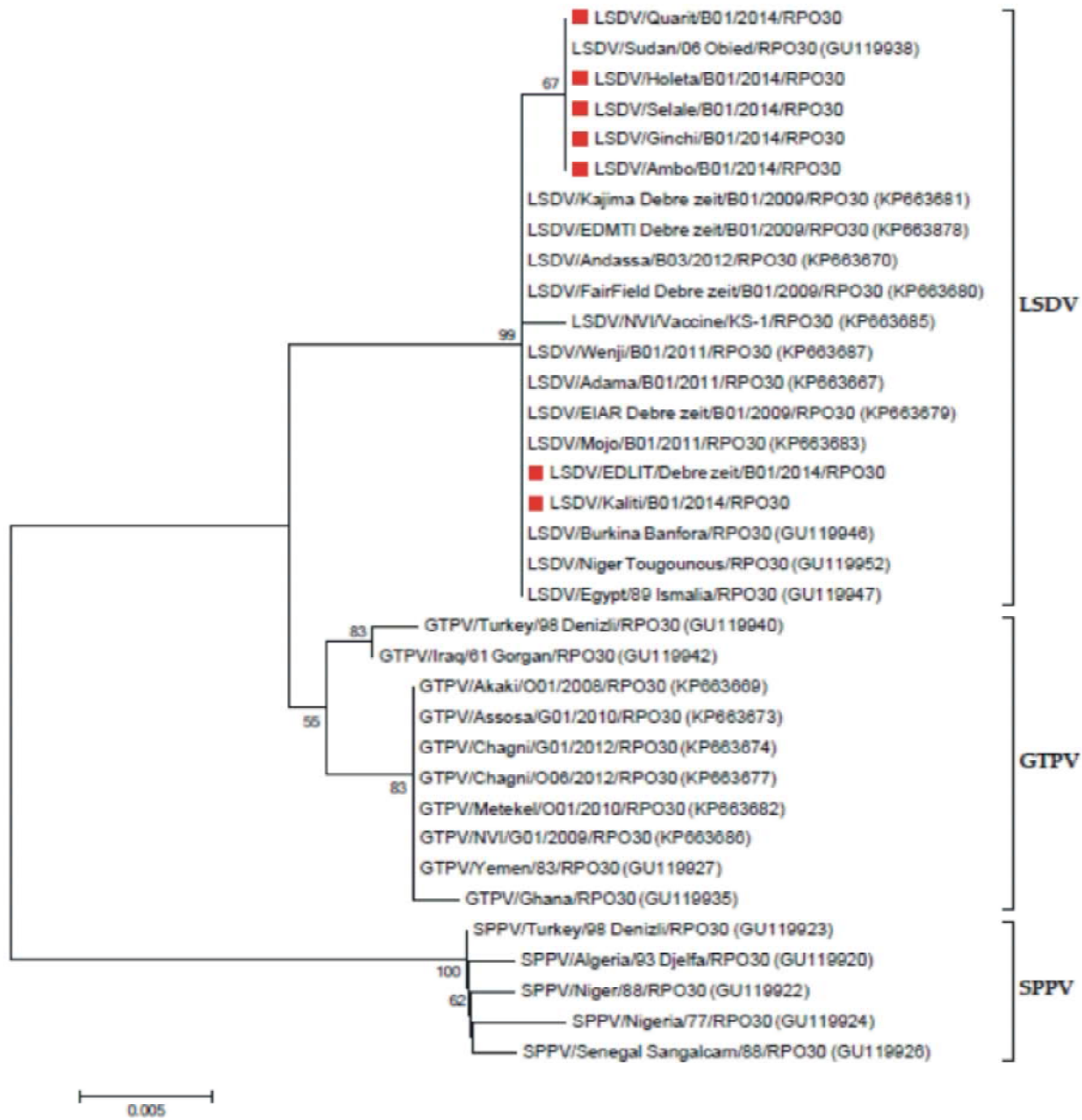
Fig. 8: Phylogenetic tree analysis of 35 capripox viruses based on nucleotide sequences of RPO30 gene complete sequence (606bp). The current isolates are marked with black square box.

Table 1: Morbidity of LSD in dairy cattle having regular vaccination and vaccination during outbreak

Regular vaccination	Total animals	No. of sick animals	Morbidity	95% CI	χ^2	P- value
Present	1551	155	0.100	0.08 - 0.11	65.768	0.000
Absent	191	58	0.304	0.24 - 0.37		
Total	1742	213	0.122	0.11 - 0.14		

Table 2: Mortality of LSD in dairy cattle with different vaccination history

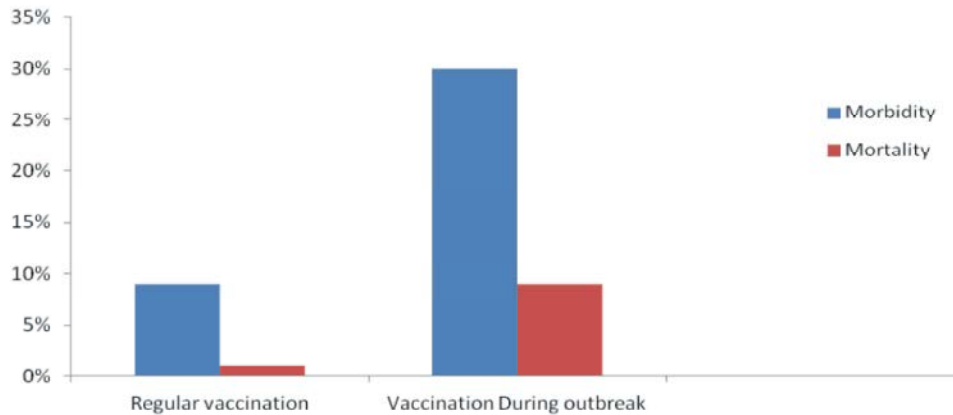| Regular vaccination	Total animals	No. of dead animals	Mortality	95% CI	χ^2	P- value
Present	1551	9	0.006	0.00 - 0.01	87.165	0.000
Absent	191	18	0.094	0.05 - 0.14		
Total	1742	27	0.015	0.01 - 0.02		

Fig. 9: Morbidity and mortality rates in dairy cattle with different vaccination strategy

Table 3: Morbidity of LSD in regularly vaccinated three big dairy cattle by age category

Age category	Total animals	No. of sick animals	Morbidity	95% CI	χ^2	P-value
< 2 years	389	121	0.31	0.26 - 0.36	2.434	0.000
≥ 2 years	1047	29	0.03	0.24 - 0.04		
Total	1436	150	0.10	0.09 - 0.12		

Morbidity was done in three big dairy farms and it included a total of 1436 cattle. Affected animals in regularly vaccinated farms were divided into two age groups: young (<2 years) and adult (≥2 years). There was higher morbidity of 31% (95% CI = 0.26 - 0.36) observed in young cattle group than 3% (95% CI = 0.02 - 0.04) morbidity rate in the adults. This difference was highly significant since the two groups of 95% CIs didn't overlap. The age specific morbidity was statistically significant association (χ^2 = 2.434, P< 0.05) to their age group in big dairy cattle.

DISCUSSION

The findings of clinical signs, virus isolation and PCR diagnosis confirmed that the outbreak was caused by LSDV. Clinical signs observed on LSD infected dairy cattle were fever, circumscribed skin nodules, enlargement of lymph nodes, inappetence, lacrymation, salivation, reduction of milk production and death. These signs have been documented as characteristic clinical features of LSD [13, 23, 24]. Edematous swelling of legs and lameness were very common signs. Similar situation was recorded during Oman LSD outbreak. The complications such as dysentery and recombency were also reported from Egypt and Oman [8]. Out of 31 skin biopsies, characteristic of *Capripoxvirus* CPE was observed only on 26 skin samples following post-inoculation on Vero cell line. Whereas, virus isolates could not be identified from the remaining five skin samples. This might show the absence

of infectious virus particles from scarified skin tissue. All isolates were identified as LSDV after sequential diagnosis using convention PCR and real-time PCR.

Furthermore, molecular phylogenetic analysis confirmed the identifications made using real-time PCR. Multiple alignment analysis of complete sequences of RPO30 gene 606 bp of present field virulent isolates, KS-1 vaccine strain and past isolates revealed that there was high homology percentage (99%) of the nucleotide sequence among the current Bishoftu and Kaliti isolates and previous isolates from Adama andassa, Bishoftu, Modjo and Wenji. This result rules out that these virulent LSDV field strains are still circulating in the eastern central part of the country. Other present isolates (Ambo, Ginchi, Holeta, Quarit and Selale) were slightly different from the former isolates by replacing A nucleotide by C (nucleotide position 41) and formed a separate cluster which is related to Sudan strain. Interestingly, all the current and previous field isolates have substituted T nucleotide by C (nucleotide position 292) in contrast to vaccine strains. Even though these findings might not show any change in amino acid substitutions, Gelaye *et al*. [17] revealed 12 nucleotides deletion from the previous isolates of GPCR genes as compared to KS-1 vaccine strain.

Attenuated KS-1 vaccine strain has been produced by NVI and distributed across the country to control LSD. The vaccine is usually used to control outbreaks by mass vaccination campaign, except in big dairy farms for annual vaccination. This vaccine strain was thought to be SPPV in the past years, but it has confirmed to be

LSDV in recent years [17, 25]. The Yugoslavian RM 65 sheep-pox virus strain, the Romanian sheep-pox virus strain and the South African Onderstepoort LSDV strain are recommended vaccines to control LSD [13, 26, 27]. All these strains of capripoxvirus were predicted that they could protect cattle against LSDV as they share a major neutralizing epitope. Moreover, it is claimed that vaccinated animals will develop protective immunity from 10 to 21 days post-vaccination and then required an annual booster dose [16, 28].

In contrary, outbreaks were appeared in vaccinated herds after five to ten months of the last vaccination date and two weeks to two months of post vaccination in smallholder dairy farms. The incomplete protection following vaccination discouraged dairy farmers to vaccinate their animals, consequently rendering more dairy cattle at risk. Abutarbush [29] has also reported the occurrence of outbreak after 5 to 20 days post vaccination.

Post-vaccination LSD outbreak occurrence may be indicative of incomplete protection. This finding is in support of incomplete protection of KS-1 vaccine strains against circulating virulent field strains in Ethiopia [15] and in Oman [8]. Such situation of other vaccine strains has been reported in Egypt and Israel [16] and Jordan [29]. Furthermore, there was increasing new cases following mass vaccination during outbreak eruptions. This might be result of vaccination of incubatory cattle accompanied by unhygienic vaccination.

In the present study, morbidity (12.2%) and mortality (1.5%) were recorded in the dairy cattle regardless of vaccination history. These findings are in agreement with 13.5 % of morbidity rate [16] and low mortality rates 1.26% [11]. However; several literatures reported that the morbidity and mortality rates of LSD vary. The morbidity rate of the disease ranges from 5 to 100% [2]. While occasional mortality rates from 10 to 40% have been reported [9], but the rate of 1 to 5% is usually observed [2]. These values vary with geography, climate, management conditions and immune status of the animals, breed and strain of virus involved [30, 3].

In the present study, the morbidity in dairy cattle vaccinated during outbreak was more than three times with that of annually vaccinated dairy cattle. The morbidity in annually vaccinated herds (10%) is in agreement with morbidity rate of 9.5% [29] and 11.1% [16] in vaccinated cattle population. A morbidity rate of 30.4% in dairy farms vaccinated after outbreak eruption is in comparable with the report of 22.5% morbidity in cattle vaccinated after infection [29].

On the other hand, the present mortality in dairy cattle vaccinated during outbreaks was recorded to be more than 15 times higher than those of dairy cattle annually vaccinated. The mortality of 0.6% in regularly vaccinated dairy cattle is relatively lower than 2.4% morality observed vaccinated Jordanian cattle [29]. Two third of dead dairy cattle as result of the LSD outbreak were reported in lactating and pregnant cows. Stressful physiological status of the animals makes them prone to the disease. The average price of a cow during study period was estimated to 35, 000 Eth birr while 25, 000 Eth Birr for a heifer. Thus, although economic losses mostly from morbidity [2], a mortality of 9.4% causes a heaviest economical burden on poor smallholder dairy farm owners whose livelihood mainly relies on.

Attempt was also made to compare KS-1 vaccine effectiveness between the existing vaccination strategies. In the present study, the relative risk of having LSD infection in regularly vaccinated dairy cattle was 0.329 times lowering than the risk of vaccinated during outbreaks. Thus, the vaccine effectiveness using annual vaccination was estimated to be 67.1% as compared to vaccination when outbreak occurrences. On the other hand, the implementation of annual vaccination in smallholder dairy farms could also reduce their mortality by 93.8%. Most of cattle used for dairy purpose were cross bred with Holstein-Friesian breed which are relatively susceptible to LSD infection [7]. Therefore, annual vaccination using KS-1 vaccine is still the existing alternative to reduce the impact of the disease in susceptible diary animals. However, the observation of a morbidity of 10% in annually vaccinated dairy cattle is frustrating and urges the development of better, effective and safe LSD vaccine. Similar to this finding, Zelalem *et al*. [31] reported that currently available live attenuated vaccine does not provide each individual with solid protection.

In regularly vaccinated dairy cattle, morbidity of young animals were ten times higher than adult cattle. This age specific susceptibility is in accordance to the findings of Ayelet *et al*. [15]. Calves less than six months of age were not received the vaccine during vaccination and the number of calves exhausting their maternal immunity increases as the last vaccination date is advancing. On the other hand, adult herd could build strong immunity against infection due to repeated vaccination or natural infection. Thus, herd immunity variation possibly explains morbidity difference between the two age categories in the present study.

CONCLUSIONS AND RECOMMENDATIONS

Lumpy skin disease (LSD) outbreak was occurred in vaccinated dairy cattle. Such phenomenon is an indicative of KS-1 vaccine incomplete protection. This study shows that there is nucleotide missing among the circulating isolates of LSDV in relation to the vaccine strain (KS-1). Thus, detailed molecular studies should be conducted to discover the variation of circulating field strains; developing effective vaccines by targeting genes specifically involved in virulence and host immune system modulation.

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