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MOLECULAR DETECTION AND GENOTYPING OF CAPRIPOXVIRUSES IN ETHIOPIA

MVSc THESIS

BY

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BISHOFTU, ETHIOPIA

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MOLECULAR DETECTION AND GENOTYPING OF CAPRIPOXVIRUSES IN ETHIOPIA

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TABLE OF CONTENT

LIST OF TABLES

LIST OF FIGURES

LISTS OF ANNEXES

LIST OF ABBEREVATIONS

CaPVs	Capri Pox Viruses
dsDNA	Double stranded Deoxyribose Nucleic Acid
GTPV	Goat Pox Virus
HRM	High resolution melt
ITRs	Inverted Terminal Repeats
LSDV	Lumpy Skin Diseases Virus
OIE	Organization of International Epizootics
PCR	Polymerase Chain Reaction
RPM	Revolution Per Minute
RT-PCR	Real Time PCR
SGPV	Sheep and Goat Pox Virus
SPPV	Sheep Pox Virus
Tm	Melting temperature

ABSTRACT

Capripoxviruses cause a severe problem and great economic losses in sheep, goats and cattle rearing countries of the world. In endemic regions of Africa, Middle East and in large parts of Asia, *Capripoxviruses* causes' severe problem to domestic ruminants with a devastating effect. This disease was endemic in Ethiopia and cause significant economic loss through damage to hide and skin, decreased milk yield, weight loss and exposure to other diseases. The effective and rapid laboratory diagnostic techniques needed to detect tentative clinical cases of *Capripoxviruses*.

In this study, 45 skin scrap, 27 buffy coat and 14 nasal swabs samples were collected from Oromia, Amhara, SNNPR, Tigray and Addis Ababa region with active outbreak cases of suspected sheep, goats and cattle to detect and to genotype the *Capripoxviruses* circulating in Ethiopia.

A total of 86 field samples were collected from sheep, goats and cattle. The overall percentages of positive samples using conventional PCR from skin scrap, nasal swab and buffycoat in cattle were 85.36% (35/41), 69.23% (9/13) and (0/27) respectively. The conventional PCR results in cattle also indicated that detection of LSDV is high in the skin scrap than in nasal swab. In real time PCR detection for skin scrap, nasal swab and buffycoat were 92.68% (38/41), 69.23% (9/13), and 70.37% (19/27) positive for LSDV respectively. Real time PCR was more sensitive to detect buffycoat samples than conventional PCR. In small ruminants all samples were 100% positive by conventional and real time PCR in skin scrap and nasal swab samples.

In current study, high resolution melting (HRM) assay also used for genotyping *Capripoxvirus* samples, in which LSDV differentiated from cattle and GTPV from sheep and goats.

Therefore, early detection and genotyping method for *Capripoxviruses* will contribute significantly for designing control strategy.

Keywords: buffycoat, *Capripoxviruses*, nasal swab, PCR, ruminant, skin scrap

1. INTRODUCTION

Capripoxviruses (CaPVs) are the cause of diseases in domestic ruminants and classified in the subfamily *Chordopoxvirinae* of the family *Poxviridae*. It is composed of three virus species of closely related and is highly contagious viral diseases of domesticated ruminants. Sheep poxvirus (SPPV), which infects sheep, Goat poxvirus (GTPV), which infect goats and both were prevalent in north equatorial. LSDV is restricted to Africa and the Middle East and naturally infects cattle by a Neethling strain (Tuppurainen and Oura, 2012).

Poxviruses contain a large linear double-stranded deoxyribonucleic acid (dsDNA) genome that contains genes encoding all the proteins necessary for their unique cytoplasmic replication. Poxviruses replicate almost independently from the infected cell nucleus (Burgers *et al., 2014)*. The most important of the viral proteins are required for transcription, replication, and virion assembly. Besides, several non-essential proteins are playing an important role in modulating the host's immune response to infection (Armson *et al.,* 2017).

Species distinction made according to the infected host species from which the virus was isolated and complemented by sequencing and phylogenetic analyses. In general, Capripox viruses show preference for their respective hosts, but some SPPV and GTPV isolates can infect both sheep and goats and the genomes of SPPV (150 kb), GTPV (150 kb) and LSDV (151 kb) have been sequenced and display approximately 96 % nucleotide sequence identity with one another (Haller *et al.,* 2014).

SPPV and GTPV strains can overtake between sheep and goats, although most cause more severe clinical disease in either sheep or goats. These transboundary animal diseases can spread into bordering, non-endemic areas (Zhou *et al.,* 2012).

The incubation period of the virus is between 8 and 13 days following contact between susceptible and infected animals. Following experimental infection, it may be as short as

four days by mechanical transmission due to insects or inoculation through intradermal. Some breeds of sheep, such as Soay, may die due to acute infection before progress of the skin nodules (Zro *et al.,* 2014).

The diagnosis of CaPVs diseases achieved by identifying the specific clinical signs and then confirmed in the laboratory by standard virological and/or serological methods. One of the major problems encountered in the CaPVs diagnosis is poor seroconversion, for this reason, the confirmation of the disease generally based on the detection of Capri pox virions or antigens through electron microscopy, virus isolation and/or Real-time PCR. Despite the fact that immune response against CaPVs is predominantly cell-mediated, the humoral immunity also plays a role (Tuppurainen *et al*., 2017).

The movement of animals without proper health checks has been associated with the current spread of SPPV, GTPV and LSDV in the Middle and Near East (Tuppurainen *et al.,* 2017). In the war torn areas or conflict regions vaccination of cattle and small ruminants is difficult to perform. Therefore, these regions will continue to serve as a source of infection, until basic infrastructure is constructed. Even though culling of all infected and in-contact animals is not an affordable or feasible to control the disease. To control CaPVs using movement restrictions or quarantine, total or modified stamping out is extremely difficult. However, the experiences obtained from Israel and the northern part of Cyprus show that LSDV outbreaks can be successfully controlled by using sufficient coverage and effective vaccines with well-organized vaccination campaign (Chen *et al.,* 2010).

Ethiopia has believed to have 124.43 million domestic ruminants, which was the largest population within the region of Africa (CSA, 2018). Although the benefit obtained from livestock plays, a significant role in national economy of the country but different constrains held back. Among the important technical constraints are livestock diseases, that have slow down the development of the sector by decreasing production and hampering trade in animal and animal products.

In this study molecular detection and genotyping of *Capripoxviruses* circulating in Ethiopia is conducted, using molecular diagnostic methods to confirm the occurrence of the disease and for subsequent designing of control strategy. Therefore, the main objective of this study is:

- To detect and to genotype the three Capri poxviruses circulating in Ethiopia from sheep, goats and cattle.

2. LITERATURE REVIEW

2.1.Etiology

Capri poxvirus (CaPV) genus belongs to sub-family *Chordopoxvirinae* and family *Poxviridae*. It is double stranded DNA virus comprises of Sheep poxvirus (SPPV), Goat poxvirus (GTPV), and Lumpy Skin Disease Virus (LSDV), which causes disease in sheep, goats and cattle, respectively. These viruses are considered as reportable disease to World Organization for Animal Health (WOAH) due to their potential for vital economic impact on livestock production (Buller *et al*., 2005).

2.2.Genomic Organization

CaPVs are double stranded genomic DNA and brick shaped, enveloped with complex symmetry and about 300×270×200 nm in size. *Poxviridae* are among the largest members of complex DNA viruses encoding a plethora of viral factors to manipulate host defense mechanisms in addition to a more conserved set of essential genes necessary for the viral cytoplasmic life cycle (Figure1). Host range factors are a group of virus-encoded proteins that are essential for the biologic tropism feature (Madhavan *et al.,* 2016).

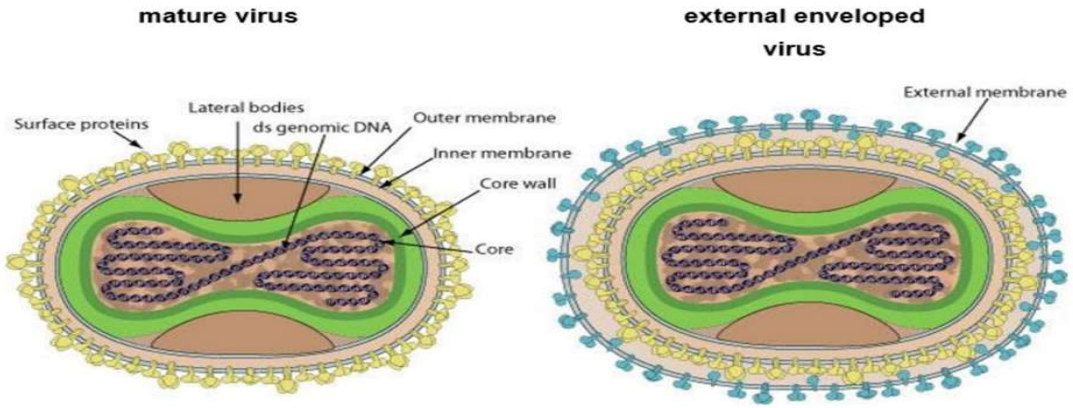


Figure 1: Poxvirus infectious particles

Source: www.poxvirus.org), accessed on September 26/2019

The essential genes such as those required for the transcription of viral genes, genome replication, and virion assembly, located in central position of the viral genome, are highly conserved among the various poxviruses (Norian *et al.,* 2019).

Poxvirus genome contains variable lengths of inverted terminal repetitions (ITRs), consisting of identical but oppositely oriented sequences at both ends of the genome (Gershon and Black, 1989). Next to the ends of the ITRs there are short tandem repeated sequences involved in recombination events (Gubser *et al.,* 2002).

The virion contains numerous antigens most of which are shared by all the members of the same genus even though every species have their own specific polypeptides (Heine *et al.,* 1999a).

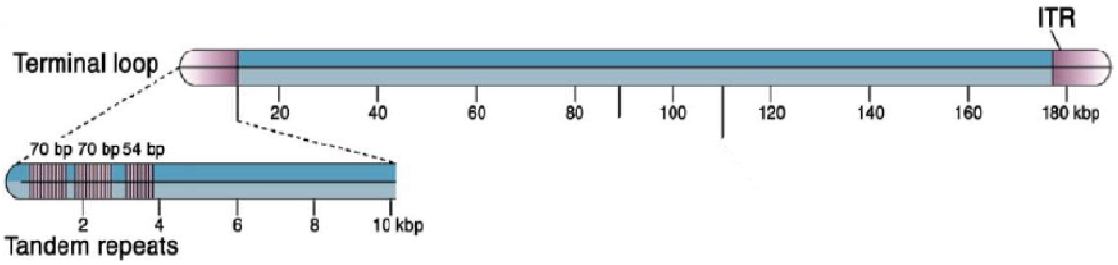


Figure 2: Poxvirus genome.

Source*: Principles of molecular Virology* (Cann Alan., 2012).

The double stranded DNA with the central core region containing highly conserved genes enclosed by terminal hairpin loops. The enlarged left ITR indicates the typical tandem repeats (Figure 2).

2.3. Antigenic and Physicochemical Properties

The poxviruses of goats and sheep are closely related antigenically and it is unable to distinguish poxviruses from each other with serological techniques (including serum neutralization), and were once consideration to be strains of a single virus. SGPV viruses are usually species specific; on the other hand, strains do exist that can infect both goats and sheep. Genetic sequencing has now definite that between these viruses recombination can occur, and Kenya sheep pox, goat poxvirus, infect both sheep and goats, these showed that some *Capripoxviruses* are not host specific. *Capripoxvirus* can survive for prolonged time with or without susceptible animals and highly stable in normal environment (Biswas *et al.,* 2019).

Capri pox viruses have lipid-containing envelopes and susceptible to a range of disinfectants containing detergents such as trypsin, formalin (1%), chloroform, ether (20%), and some detergents, e.g. sodium dodecyl sulphate in addition, the viruses are susceptible to sodium hypochlorite (2–3%), phenol 2% for15 minutes, quaternary ammonium compounds (0.5%). The Viruses are susceptible to sunlight, but survive well at cold temperatures for up to 6 months (EFSA, 2015).The virus inactivated by heating for 1 hour at 55°C. The virus is present in nasal, lachrymal and pharyngeal secretions, semen, milk and blood and it may remain in saliva for up to 11 days and in semen for 22 days (Awad *et al.,* 2010).

2.4. Epidemiology

LSDV infections, mortality usually remains below 10%, although mortality rates over 75% had recorded. Morbidity rates may vary from 5%–45% and sometimes be up to 100%. Highly contagious GTPV and SPPV may cause very high morbidity rates (70%–90%) in domestic animals. The mortality may be up to 50% and as high as 100% in young or naive animals. The virulence of different CaPVs may vary, but the severity of clinical disease often depends on immune status, age and the infected species. Mortality and morbidity numbers in wild animals are poorly recorded (Lamien *et al.,* 2011).

Lumpy skin disease is an important, economically devastating, notifiable disease which brought production loss in cattle due to generalized malaises and chronic debility (Molla *et al.*, 2017). Good understanding of epidemiological aspects LSDV related to pathogen, host and environment might aid for prevention mechanisms. Particular emphasis was given to exposure of hosts and pathogen in suitable environment that was facilitating transmission and distribution of the disease. The frequency of morbidity and mortality of the disease, its geographic distribution and mode of transmission in large herds of cattle were observed to cause severe economic losses (Salib and Osman, 2011).

Whereas Sheep pox and Goat pox affect sheep and goats of all breeds, both sexes, and all ages but more severe and common in exotic and young animals than adult and indigenous breeds (Heine *et al.,* 1999). Virus transmission can occur through infected aerosols due to saliva, conjunctiva secretions, nasal discharge, urine and fecal from infected animals and indirectly through mechanical transmission by vectors or direct abraded skin contact (Kitching and Taylor., 1985). Under experimental conditions, the extensive involvement of skin and associated viral load can result in mechanical transmission by insect such as *Stomoxys calcitrans* is capable of transmitting sheep pox and goat pox mechanically. The virus can persist on the hair or wool as long as three months after infection and maybe longer in scabs (Domenech *et al.,* 2006).

2.5. Geographical Distribution

CaPVs considered as highly host specific, but exceptions recorded sporadically. There are distinct differences between the host species and the geographic distribution of SPPV, GTPV, and LSDV. Historically CaPVs have no zoonotic potential and they infect only domestic ruminants. Globally, the geographical range of the three species of *Capripox viruses* are expanding their range and have the potential of becoming emerging disease threats because of increasing trade in animals and animal products and global climate change were reported in different places at different times (Givens, 2018). The geographic

distribution of GTPV and SPPV extends from Africa north of the Equator, across the Middle East and Turkey, to the Indian subcontinent and Asia (Figure3).

Skin disease for the first time in 1929 with new clinical sign occurred in Zambia (Mweene *et al*., 1996). That time LSDV considered as either plant poisoning or an allergic response of insect bite caused it and after fourteen years, in October 1943, another outbreak of the disease occurred in Botswana and named it provisionally as "Ngamiland cattle disease" as the case occurred for the first time in Ngamiland (Gabalebatse *et al*., 2013). After two years, 1945 the disease spreads to Zimbabwe and South Africa where the disease named as the lumpy skin disease and demonstration of transmission of the infectious agent by inoculation of cattle with suspension of the skin nodules was determined. The disease was diagnosed in Kenya in 1957; Sudan in 1971; Chad and Niger in 1973; Nigeria in 1974 and Somalia in 1983 (Swiswa *et al.,* 2017). In 1988, the first outbreak occurred in Egypt in Ismailia and although they try to control and eradicate, but the disease remains endemic in these areas. In 1989, the disease clinically observed in Israel dairy farms, which was suggested as it was spread from Egyptian outbreaks by insect vectors carried by wind. The disease primarily considered as an endemic disease to Africa and Middle East and other areas (Abutarbush *et al.,* 2015).

LSD has become endemic in Ethiopia since it was reported for the first time in 1981 in northwestern part of the country (Mebratu *et al*., 1984).In Ethiopia LSDV is distributed almost in all regions and is regarded as one of the most important livestock diseases and can cause economic loss in the country (Ayelet *et al*.,2014).

Goat pox and sheep pox was reported for the first time by Hansen in 1879 from Norway while sheep pox was first confirmed in Central Asia and spread to many Western countries (Rao *etal*., 2000). Sheep pox and goat pox recognized as exotic to the EU and classified in the notifiable diseases list of the OIE. (Tasioudi *et al.,* 2016).

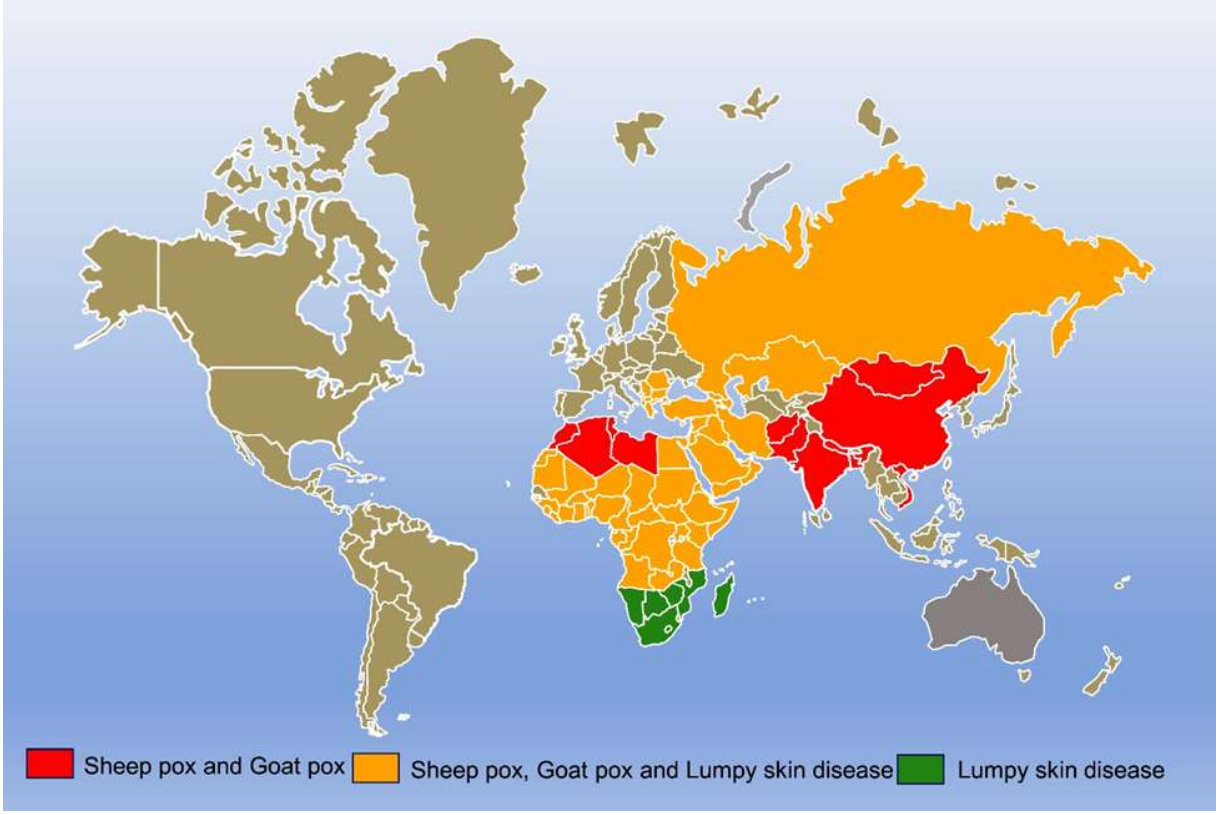


Figure 3: Worldwide distribution of Sheep pox, Goat pox and Lumpy skin disease.

Source: www.capri poxviruses distribution map, OIE-WAHID database records by March 2019.

The red color indicates the presence of SPPV and GTPV, the orange color shows of SPPV, GTPV and LSDV, whereas green indicate areas affected by LSDV only (Figure 3).

2.6. Host specificity

CaPVs are considered to be host-specific, causing clinical disease in either sheep, goats, or cattle (Tuppurainen *et al.,* 2017). CaPVs are generally considered to be host specific and some strains have equal virulence in both goats and sheep while, most SPPV and GTPV isolates show divergent host preferences with more severe disease evident in the homologous host (Babiuk *et al*., 2008). Usually, nomenclature of LSDV, GTPV and SPPV

based on animal species from which the virus was first identified but cross-species transmission may make difficult the situation. Sheep pox and goat pox are impossible to be distinguished in serological and physical assays due to their antigenic and structural relatedness but possible at molecular level targeting host range genes, immune dominant genes and virulence genes. Some Middle-Eastern and African strains have equal pathogenicity for goats and sheep. The GTPV associated with disease in sheep was confirmed in China by P32 and RP030 gene based species-specific PCR methods and presence of SPPV signature residue of P32 gene in goat poxvirus infecting goats identified in India (Santhamani *et al.,* 2013).

The previously published P32 gene sequences of SPPV, including strains from India were matching to SPPV field strains from Mongolia. The GTPV strains from China were identical to GTPV field strains from Mongolia and a recent outbreak in Vietnam (Babiuk *et al.*, 2009). In a study, CaPVs outbreaks in goats and sheep in Ethiopia were solely associated with GTPV (Gelaye *et al.*, 2015). LSDV natural infections have been reported in Asian water buffalo (Bubalusbubalis) (Gari *et al.,* 2011). Clinical signs of LSDV reported in impala and giraffe after experimental inoculation with LSDV. Natural infection by LSDV was reported in an Arabian oryx (*Oryx leucoryx*) in Saudi Arabia, springbok antelope in Namibia, Oryx gazelle in South Africa, and Asian water buffalo in Egypt (Tuppurainen & Oura, 2012). In the skin samples collected from springbok antelope of South Africa was detected as LSDV nucleic acid (Lamien *et al.,* 2011). CaPVs antibodies were detected in serum samples collected from African buffaloes, greater kudu, waterbuck, reedbuck, impala, and giraffe (Tuppurainen *et al.,* 2017). Though, the role of wildlife in the epidemiology of lumpy skin disease virus is not significant in the preservation or spread of LSDV (Hosamani *et al.*, 2004)**.** Neither goat pox nor sheep pox were reported in goats or sheep in South Africa however LSDV is endemic. The CaPVs diseases are not considered as zoonotic agents as there is no apparent evidence of these malignant diseases causing infection in human (Bhanuprakash *et al.*, 2011).

3. MATERIALS AND METHODS

3.1. Study Area

This present study conducted in different regional state of Ethiopia including the central part of the country, Addis Ababa (Yeka) located at 38.8^0E longitude and 9.05^0N latitude. Oromia regional state, Sebeta and Awash Melka areas and 250 Km far from Addis Ababa to the west, starting from Ginchi, Ambo, Guder and to Bako areas those located between 37.2 to 38.6^0E longitude and 8.7 to 9.05^0N latitude. Amhara regional state Motta, Maksegnet and Zigam located between 36.4 to 37.8^0E longitude and 10.7 to 12.4^0N latitude. Tigray regional state, Mekele and Raya areas located between at 39.4 to 39.5^0E longitude and 12.4 to 13.5^0N latitude, SNNPR regional state Humbo and Loma areas located between 37.2 to 37.8^0E longitude and 6.7 to 6.8^0N latitude. All the above areas are the active outbreak cases reported in the country (Figure 4).

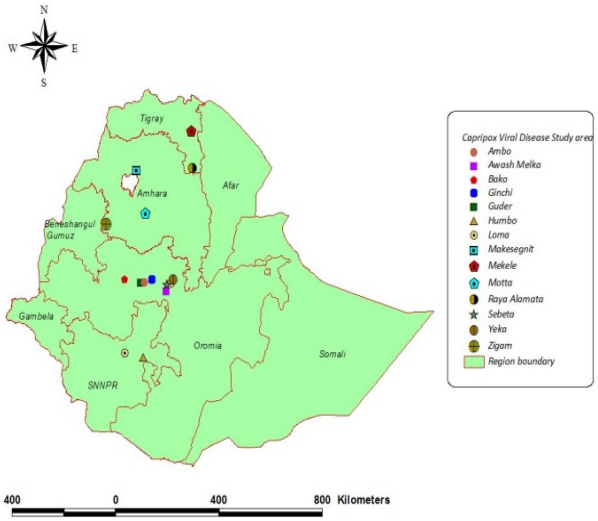


Figure 4: Map of Ethiopia showing the sampling sites in different color

3.2. Study Animal

Local and crossbred of cattle, goats and sheep of all age and both sex groups under extensive and intensives management systems having typical signs of the pox lesions are sampled for virus detection and genotyping of *Capripoxvirus*. Samples like skin scrap, buffy coat, and nasal swabs are collected.

3.3. Study Design

An active disease investigation was carried out based on the reports of the outbreak case information gathered through veterinary professionals who were working in the regional laboratories, zonal veterinary offices and district veterinary clinics from December 2019 until April 2020. The samples are collected from four regional states of the country and one city administration was the active outbreak cases occurred. Diagnosis of *Capri poxviruses* was depend on investigation of active outbreak cases on basis of the signs and lesions of affected domestic ruminants, as well as the animal owners interviewed using structured questionnaire format. The differentiating from other skin diseases like streptothricosis, Orf and mange were carried out in the laboratory using molecular virus detection and genotyping of Capri poxvirus.

3.4. Sample size and sample collection

Cattle, sheep, and goats that developed clinical symptoms with high fever between 39 to 41°C observed and those animals with suspected cases selected and sampled purposefully. The information gathered through questionnaires. About 2gm of skin scrap, blood samples for buffy coat, and nasal swabs collected aseptically from four regional states of the country and one city administration. During the active outbreak cases a total of 86 samples were collected from 59 animals of Yeka (Addis Ababa) Ambo, Awash Melka, Bako, Ginchi, Guder and Sebeta (Oromia) Maksegnet, Motta and Zigam, (Amhara) Mekele and Raya (Tigray) Humbo and Loma (SNNPR) areas, and these samples were aseptically collected and put in virus transport media (Table1 & Table 2) .

Table 1: Active outbreak cases collected from cattle.

Region	District	Host	Sample type	Number of samples
Oromia	Sebeta	Cattle	Skin scrap/nasal swab/buffy coat	28
	Bako	Cattle	Skin scrap/nasal swab/buffy coat	26
	Awash Melka	Cattle	Skin scrap	3
	Ambo	cattle	Skin scrap	2
	Ginchi	Cattle	Skin scrap	2
	Guder	Cattle	Skin scrap	1
Amhara	Motta	Cattle	Skin scrap	4
	Maksegnet	Cattle	Skin scrap	3
A.A	Yeka	Cattle	Skin scrap	3
SNNPR	Humbo	Cattle	Skin scrap	2
	Loma	Cattle	Skin scrap	2
Tigray	Raya	Cattle	Skin scrap	3
	Mekele	Cattle	Skin scrap	2
Total				81

Table 2: Active outbreak case collected from small ruminant.

Region	District	Host	Sample type	Number of samples
Oromia	Sebeta	Sheep	Skin scrap	2
Tigray	Mekele	Goat	Skin scrap	2
Amhara	Zigam	Goat	Nasal swab	1
Total				5

All collected clinical samples submitted to National Animal Health Diagnostic and Investigation Center (NAHDIC) laboratory under cold chain for confirmation of Capri poxviruses using molecular detection and genotyping techniques (OIE, 2017).

3.5. Processing of blood and tissue samples

The skin scrap samples minced using sterile scissors and forceps and were grinded with sterile sand in a mortar using a pistil. Suspension of suspect tissue samples (skin scrap) homogenized in phosphate buffer saline (PBS) containing [penicillin (100 U/ml), streptomycin (100µg/ml), neomycin (2.5mg/ml) and nystatin (50 U/ml)]. The homogenized suspension of clinical sample is clarified using a bench top centrifuge by centrifugation at 3000 rpm for 10 minutes to remove tissue debris and the suspension of samples were used for extraction of viral DNA in molecular laboratory (OIE, 2017).

Buffy coats were prepared from 7.5 ml heparinized whole blood by mixing with 25ml of PBS and with 15 ml of Ficoll solution by centrifugation at 3500 rpm for 35 minutes at -20^0C. The mononuclear layer carefully removed using a sterile Pasteur pipette into 50 ml falcon tube. Buffy coat is separated to test for the presence of *Capripoxviruses* (Murray, and Rajeevan., 2013).

3.6. Laboratory Diagnostic Methods

3.6.1. DNA extraction

DNA of the pox viruses were extracted using QIAmp viral DNA mini kit (Qiagen), according to the manufacturer's protocol. First 20 µl of proteinase K is added to all tubes according to the sample size, and then 200 µl-collected supernatant was added and 200µl AL buffer was added and mixed together by vortex mixer and incubated at 56°C for 10 minutes in water bath and it was briefly centrifuged. 200µl ethanol (96-100%) is added to bind the nucleic acid on mini spin column, and mixed using vortex mixer for 15 sec and briefly centrifuged. This above mixture transferred to the QIAamp mini spin column and centrifuged at 6000xg for 1 minute. The spin column having the DNA was transferred in to new 2 ml collection tube and the first washing buffer 500µl AW1 added and centrifuged at 6000xg for 1 min. 500µl of second washing buffer, which is AW2, was added and

centrifuged at 14000 rpm for 3 minutes and the filtrate was discarded. This step was repeated for 1 minute without adding any buffer. Then the mini spin column transferred to micro centrifuge tube and 200 µl of AE elution buffer added and incubated at room temperature for 1-5 minutes to increase the yield of DNA and eluted by centrifugation at 6000xg for 1 minute using fifth edition QiAamp DNA extraction protocol from Qiagen, 2016.

3.6.2. Conventional PCR

For molecular detection of *Capripoxviruses*, conventional PCR were performed with the following set of primers (Chibssa *et al*.,2018):-
SPPVDIVFowared:5'-ATCTGCTACAAGTTTTAACGAACTTA-3′ and
SPPVDIVReverse:5'-TGAATGTGATCTCATATCCTTATTG-3′ for amplification.
Using the above primers the conventional PCR method is that can differentiate SPPV Vaccine from other *Capripoxvirus* while detecting any circulating Capripoxvirus. Then PCR was conducted containing 2µl of each reverse and forward primer, and 2µl of 0.2mM dNTPs, 0.25µl Taq DNA polymerase (QIAGEN), 2µl of 10 x PCR buffer, and 2µl template DNA in a reaction volume of 20µl. The thermal cycler program is as follow: 95°C for 4 min followed by 35 cycles of 95°C for 30 sec, 58°C for 30 sec, and 72°C for 30 sec and a final extension at 72°C for 2 min to complete the amplification process (Chibssa *et al*., 2018). The PCR products separated by electrophoresis, using 2% agarose gel containing DNA staining dye (gel red) for 1hr at 100 V and subsequently visualized under UV light.

3.6.3. Genotyping by High Resolution melting (HRM) RT PCR

The HRM assay provides a highly specific and sensitive fluorescent detection based on real time PCR. The method differentiate SPPV vaccine from other three CaPV strains(Chibssa *et al*., 2019) and at the same time all Capri poxvirus from each other. By using the following primers: Cap-B22RDIV-Forward: 5- TATGGATTTAGGAGTAGA-3 Cap- B22RDIV-Reverse: 5- GCTTTACTTTAATATCATTG -3. The PCR was set up in a

10µl reaction mixture consisting of 2.5X Light scanner master mix, 250 nM of each reverse and forward primers and 2 µl of the sample DNA. The PCR reaction first performed with an initial denaturation at 95 °C for 4min, continued by 42 cycles of 95 °C for 5sec, 58 °C for 5sec and 72 °C for 5sec. For melting curve analysis the PCR products denatured at 95 °C for 30sec, cooled to 65 °C for 1min, and melted from 65 °C to 90 °C for 10 sec. In each set of reactions, negative controls consisting of nuclease-free water and positives control plasmids were included.

The HRM test conducted in a thermal cycler of Applied Biosystems by thermo fisher scientific 7500 Real time PCR system covered by US patent, and made in Singapore. The positive samples were noted with amplification fluorescence curves, while the negative were not amplified. The Ct values lower than 40 indicated as positive suggesting presence of the virus from the tissue specimens. Melting curve analysis was performed using the HRM to differentiate PCR product into one of the following for CaPV groups such as SPPV vaccines, SPPV field isolates, GTPVs and LSDVs due to the noticeable difference in the melting temperatures (Tm).

3.7. Data Management

The collected data during the active outbreak investigation entered into Microsoft Excel spreadsheet based on region, site of collection, number of animal at risk and number of death. The percentage of mortality calculated as number of death x 100 over number of animals at risk and the fatality rate calculated as number of death x 100 over number of sick animals.

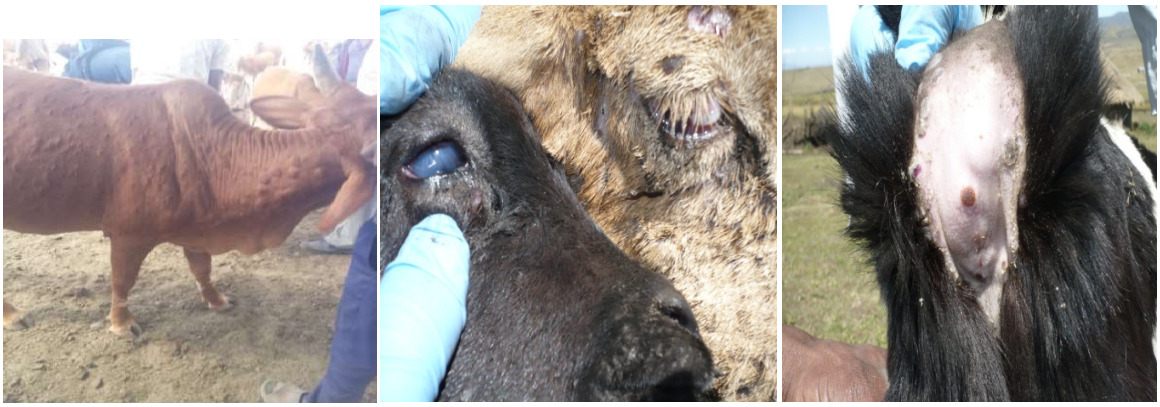
3.8. Ethical Clearance

Ethical clearance obtained from ethical committee of College of Veterinary Medicine, Addis Ababa University before starting the research work.

4. RESULT

4.1. Outbreak Investigation

In this study, active outbreaks were investigated between December 2019 and April 2020 from four Regional states; Oromia (four district), Amhara (three districts), SNNP (two districts), Tigray (two districts) and A.A city one administration (Yeka kifleketema) and a total of 86 samples from 59 animals were collected. The most commonly observed clinical signs of CaPVs were fever, depression, in appetence, salivation, and nasal discharge which may be mucoid or mucopurulent, skin nodules on different body parts, enlarged peripheral lymphnodes, lameness, disinclination to move, where as affected sheep and goats shows skin lesion under tail, lacrimation, conjunctivitis and sometimes followed by corneal opacity, and blindness (Figure 5).



A B C

Figure 5: Clinical signs of goat pox disease in goat and lumpy skin disease in cattle in Ethiopia.

Cattle with lumpy skin disease (A), blindness due to goat pox (B), pox lesion on hairless part of goat tail (C).

Capri poxvirus outbreaks on cattle and small ruminants had experienced with morbidity and mortality during the study period (Table 3&4). The data showed that the cattle had morbidity of 6.34 % and mortality of 1.02%, where as in small ruminants has the morbidity and mortality rates were 15.93 % and 10.85% respectively (Table 3). The data also revealed that small ruminants are more affected than cattle by *Capri poxvirus* during these outbreaks (Table 4).

Table 3: The morbidity and mortality rate of cattle affected with *Capri poxvirus* in Ethiopia.

Region	**Collected site**	**Host**	**Number of animals at risk**	**Morbidity rate in %**	**Number of death**	**Mortality rate in %**	**Case fatality rate in %**
Oromia	Bako	Cattle	241	15(6.22)	4	4(1.65)	26.66
	Sebeta		87	28(32.18)	6	6(6.89)	21.43
	Ginchi		52	2(3.84)	0	0	0
	Ambo		80	2(2.5)	0	0	0
	Guder		43	3(2.32)	1	0	0
	Awash Melka		72	3(4.16)	1	1.38	33.33
A.A	Yeka		20	3(15)	0	0	0
Amhara	Motta		74	4(5.40)	0	0	0
	Maksegnet		200	3 (1.5)	0	0	0
SNNPR	Humbo		62	3(4.83)	0	0	0
	Loma		55	1(1.81)	0	0	0
Tigray	Mekele		66	4(6.06)	0	0	0
	Raya		115	3(2.60)	0	0	0
Total			1167	74(6.34)	12	1.02	16.21

Table 4: The morbidity and mortality rate of small ruminant affected with *Capri poxvirus* in Ethiopia.

Region	Collected site	Host	Number of animals at risk	Morbidity rate in %	Number of death	Mortality rate in %	Case fatality rate in %
Amhara	Zigam	Goat	260	40(15.38)	30	11.53	75
Tigray	Mekele	Goat	20	4(20)	1	5	25
Oromia	Sebeta	sheep	15	3(20)	1	6.66	33.33
Total			295	47(15.93)	32	10.85	68.08

4.2. Detection of Capri poxvirus by Conventional PCR

Conventional PCR run targeting the region between DNA ligase and B22R homologous genes of the collected samples. Analysis of PCR products from the isolate of the Capri poxvirus from different samples by 2% agar gel electrophoresis showed that, the size of the fragment from 302bp-338bp for *Capri poxvirus* (Figure 6).

The results obtained using the conventional PCR for skin scrap, nasal swab and buffy coat in cattle were 85.36% (35/41), 69.23% (9/13) and (0/27) respectively. The conventional PCR results in cattle also indicated that detection of LSDV is high in the skin scrap than in nasal swab. In conventional PCR, there is no positive sample for buffy coat. In small ruminants, all samples were 100% positive by conventional PCR in both skin scrap and nasal swab samples (Table 7&8).

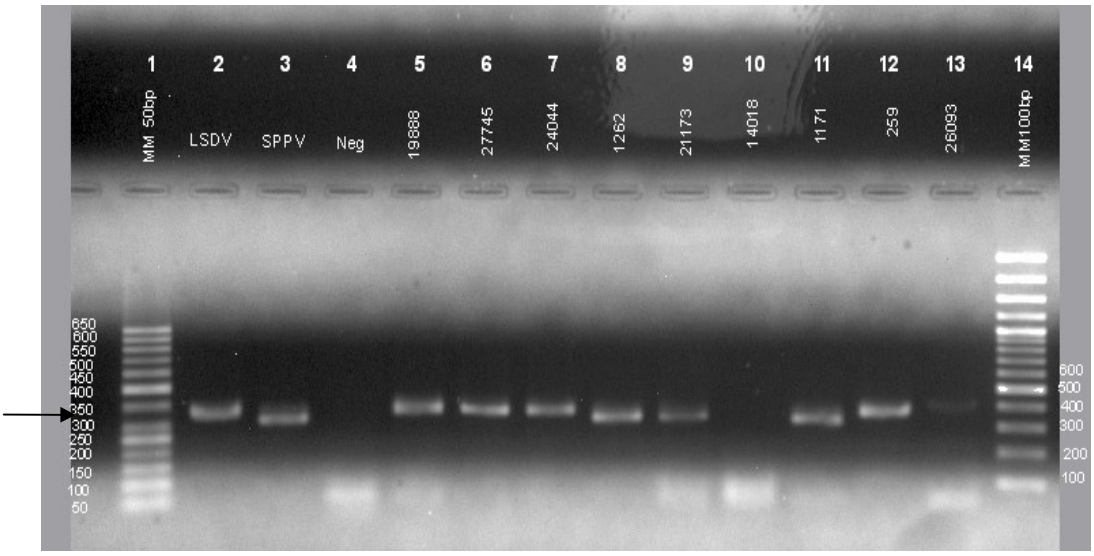


Figure 6: Conventional PCR method to detect *Capripoxvirus* (CaPV) field strain.

The arrow showed of 302bp-338bp, the PCR product of *Capri poxviruses*. Lane 1: 50bp DNA ladder; Lane 2: positive control plasmid of the LSDV strain (338bp); Lane 3: positive control plasmid of the SPPV field strain (302bp); Lane 4: Negative control (no DNA template); Lanes 5-7: LSDV strains; Lanes 8-9: GTPV; Lanes 11: GTPV field strains, Lanes 12: LSDV field strain.

4.3. Real time Polymerase chain reaction for detection and genotyping

The HRM result for RT-PCR detection for skin scrap, nasal swab and buffy coat were 92.68% (38/41), 69.23% (9/13), and 70.37% (19/27) positive for LSDV respectively (Table 7). In addition, in small ruminants, both skin scrap and nasal swab samples were 100% positive for goat pox (Table 8).

Table 5: Cattle samples were genotyped.

Region	District	Host	Number Positive	Tm	Genotype
Oromia	Bako	Cattle	13	80.9-81.3	LSDV
	Sebeta	Cattle	11	80.9-81.3	LSDV
	Awash Melka	Cattle	3	80.9-81.3	LSDV
	Ambo	cattle	2	80.3-81.4	LSDV
	Ginchi	Cattle	2	80.9-81.3	LSDV
	Guder	Cattle	1	81.6	LSDV
Amhara	Motta	Cattle	4	80.9-81.3	LSDV
	Maksegnet	Cattle	3	80.9-81.3	LSDV
A.A	Yeka	Cattle	1	80.9	LSDV
SNNPR	Humbo	Cattle	2	80.9-81.3	LSDV
	Loma	Cattle	2	80.9-81.3	LSDV
Tigray	Raya	Cattle	3	80.9-81.7	LSDV
	Mekele	Cattle	2	80.9-81.7	LSDV
Total			49		

Table 6: Small ruminant samples were genotyped.

Region	District	Host	Number positive	Tm	Genotype
Oromia	Sebeta	Sheep	2	80.6 -80.9	GTPV
Tigray	Mekele	Goat	2	80.9-81.3	GTPV
Amhara	Zigam	Goat	1	81.6	GTPV
Total			5		

Table 7: Result of cattle samples, Conventional and HRM RT-PCR Capri poxvirus analysis in skin scrap, nasal swab and buffy coat.

Region	District	Host	Conventional			Real time		
			Skin Scrap	Nasal swab	Buffy coat	Skin Scrap	Nasal swab	Buffy coat
Amhara	Motta	Cattle	4/4			4/4		
	Maksegnet	Cattle	1/3			3/3		
A.A	Yeka	Cattle	1/3			1/3		
SNNPR	Humbo	Cattle	2/2			2/2		
	Loma	Cattle	2/2			2/2		
Tigray	Raya	Cattle	3/3			3/3		
	Mekele	Cattle	1/2			2/2		
	Awash M	Cattle	3/3			3/3		
Oromia	Bako	Cattle	8/9	1/2	0/15	8/9	1/2	11/15
	Sebeta	Cattle	5/5	8/11	0/12	5/5	8/11	8/12
	Ambo	Cattle	2/2			2/2		
	Ginchi	Cattle	2/2			2/2		
	Guder	Cattle	1/1			1/1		
Total			35/41 (85.36%)	9/13 (69.23%)	0/27	38/41 (92.68%)	9/13 (69.23%)	19/27 (70.37%)

Table 8: Result of small ruminant samples, Conventional and HRM RT-PCR Capri poxvirus analysis in skin scrap and nasal swab.

Region	District	Host	Conventional PCR		Real time PCR	
			Skin Scrap	Nasal swab	Skin Scrap	Nasal swab
Amhara	Zigam	Goat		1/1		1/1
Tigray	Mekele	Goat	2/2		2/2	
Oromia	Sebeta	Sheep	2/2		2/2	
Total			4/4 (100%)	1/1 (100%)	4/4 (100%)	1/1 (100%)

In this study high resolution melting (HRM) assay differentiated *Capri poxvirus* into LSDV in cattle and GTPV from both sheep and goats (Table 5&6). As shown in the plots, the amplification and the melting profile of each DNA sample was generated for GTPV 80.6-81.3 ºC and LSDV 80.9-82.0 ºC with reference plasmid. Melting curves and melting picks shown in figures 7, 8 & 9.

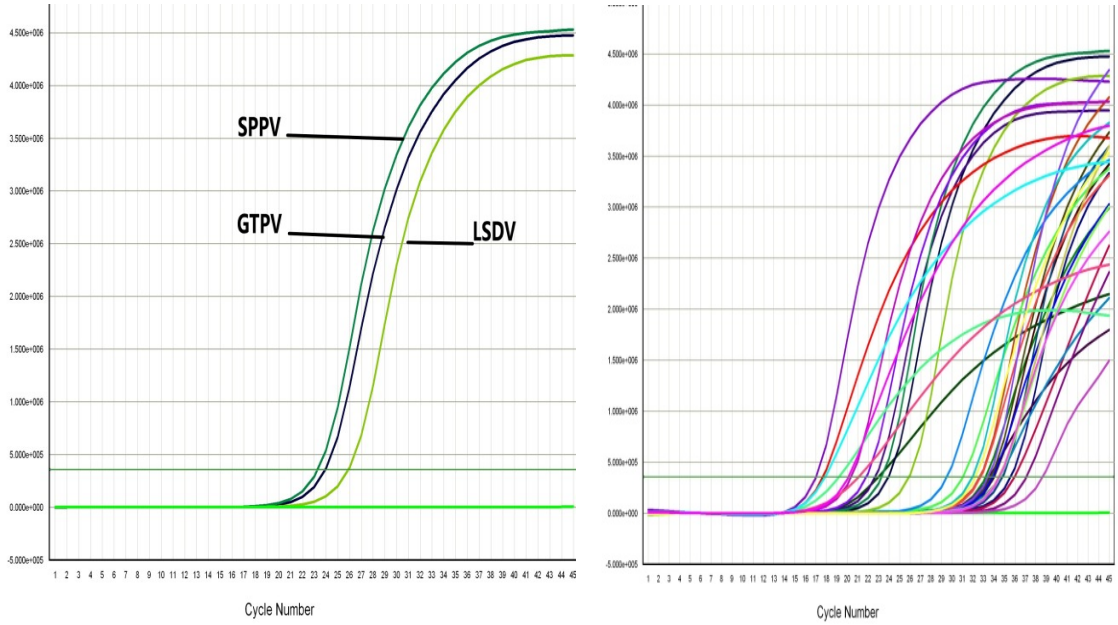


Figure 7: Real time PCR amplified samples (right) and controls (left*).

*Controls: - SPPV, GTPV, and LSDV

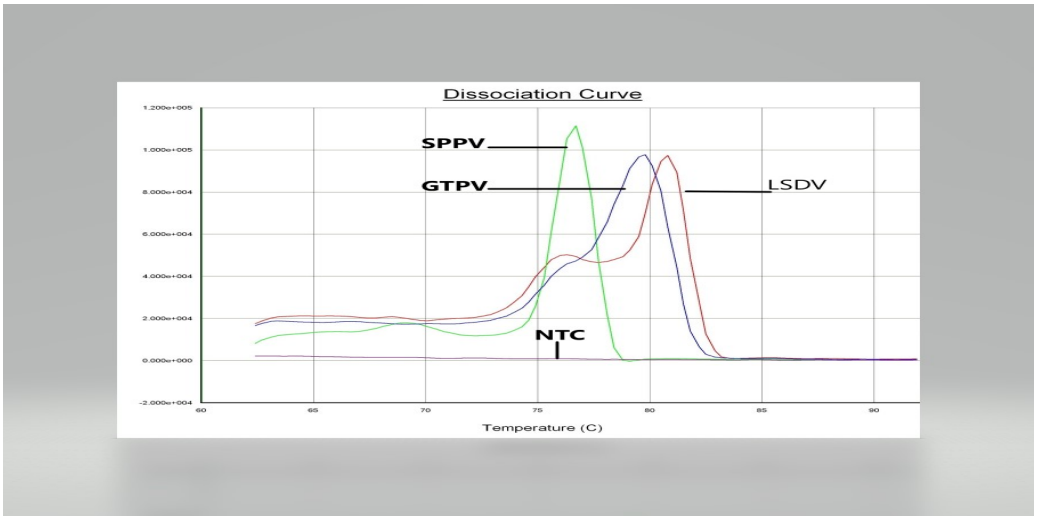


Figure 8: Real time PCR amplification curves for controls.

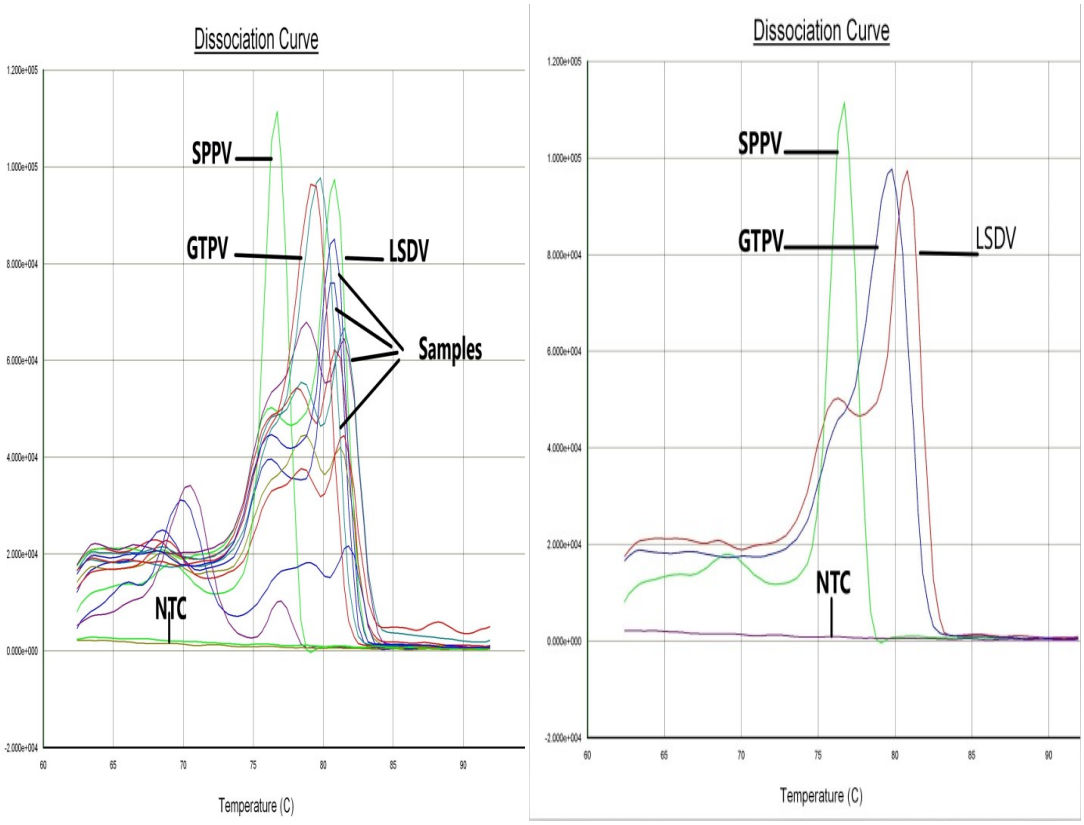


Figure 9: HRM assay for differentiation of SPPV and other Capri poxvirus disease (controls with samples (left), and controls (right).

The genotyping of the suspected Capri poxvirus samples were successfully run on applied bio-system platform with an average of melting temperature of 77.1^0c for SPPV vaccine, 79.3^0C for SPPV field, 80.9^0C for GTPV and 81.4^0C for LSDV (Table 9).

Table 9: The platform compatibility test

Virus Genotype	Current RT-PCR machines with Tm values		Previous study RT-PCR machines with Tm values (Chibssa *et al*, 2019)			
	AB System	Average	CFX96(Bio-Rad)	LC480 II(Roche)	QS6 (life tech)	RG-Q Qiagen
SPPV vaccine	77.0-77.3	77.1	75.8–76.3	76.59–76.88	76.45–76.74	77.2–77.54
SPPV field	79.1-79.6	79.3	78.9–79.4	79.87–80.26	79.74–80.22	80.46–80.9
GTPV	80.6-81.3	80.9	80.4–80.4	81.39–81.52	81.29–81.38	82–82.2
LSDV	80.9-82.0	81.4	80.9–80.9	82.07–82.16	81.96–82.06	82.6–82.66

5. DISCUSSION

In this study 1167 cattle were at risk, 74 were sick, and the overall morbidity, mortality and case fatality rates of LSD were (6.34%), (1.02%) and (16.21%) respectively, indicating that the mortality and case fatality rate was higher in Oromia regional state with zero mortality and case fatality rate in other regional states of the country. In addition, 295 small ruminants were at risk, 47 were sick and the mortality and case fatality rates were 10.85% and 68.08% respectively.

In the present study, we observed morbidity rate of 6.34 % of LSDV in cattle, and Alemayehu *et al*., (2015) reported 6.1%, which slightly lower than the current finding.
Other authors reported wide ranges of morbidity rates of cattle ranged from 3% up to 85%; Tuppurainen *et al*., (2012). The present finding indicates the mortality rate 1.02% and case fatality rate 16.21% of LSDV in cattle which is lower than Ayelet *et al*., (2014) report, who reported the mortality rate 4.97% and case fatality rate 36.49% and Alemayehu *et al*., (2015) who reported 1.8% and 30%, respectively.

These variations could be from the differences in geographic location and climate, the management conditions, immune status and condition of the animals, virulence of the virus, and the number and types of insect vectors (Tuppurainen and Oura, 2011).

In this study, the morbidity, mortality, and case fatality rate of goat pox disease in affected sheep and goat were 15.93%, 10.85% and 68.08% respectively. The data also revealed that small ruminants more affected by goat pox disease than cattle by lumpy skin disease.

The invention of conventional PCR and highly resolution melting real-time PCR has led to many major scientific advances. Though both methods are still regularly used in laboratories, real-time PCR is gaining popularity and quickly becoming the most cost and time effective method for analyzing DNA products.

The conventional PCR assay used in this study was identified a suitable target in CaPV genome to discriminate LSDV strains from SPPV field isolates. It showed high specificity as a unique band of the expected size 302bp (SPPV), and 338bp (LSDV) obtained for DNA samples derived from skin lesion, nasal swab and buffycoat. Out of 59 animal samples, conventional PCR detects 90.7% (49/54) for lumpy skin disease in cattle and 100% (5/5) were positive for goat pox diseases in sheep and goats. The method can detect any *Capripoxvirus* genome that can help for the screening of lumpy skin disease, sheep poxvirus and goat poxvirus diseases from cattle and shoats. Although goat pox band shows a little variation with LSDV, reported by Chibssa *et al*, (2018).

Real time PCR assays based on HRM to differentiate GTPV and LSDV was used for both detection and genotyping of CaPV diagnosis (Chibssa *et al*., 2019). The results of HRM real time amplify CaPV for the current work is indicated in Figure 7& 8. All samples were positive for a small ruminant and buffy coat samples from cattle were 70.37% was positive by the quantitative real time PCR. Therefore, real time PCR was more sensitive to detect buffy coat samples than conventional PCR.
The positive samples were genotyped as the same time and the result shows that LSDV was isolated from cattle and GTPV from sheep and goat (Table 5&6), and this result is supported by Galaye *et al*., (2015) and which indicates that only GTPV was isolated from sheep and goat in Ethiopia.

In this study HRM assay results performed on Applied Biosystems agreed with Gelaye *et al*., (2013), and Chibssa *et al.*, (2019) by using four other different quantitative PCR instruments for the discrimination of CaPV in to different species.

Therefore, both conventional and HRM assay used for screening and conformation of samples to undertake investigations when a Capri poxvirus outbreak occurs in a small ruminant or cattle herds in the country.

6. CONCLUSSION AND RECOMMENDATION

This is the first multi samples collection evidence of *Capripoxvirus* presence in Ethiopia and study indicates a wide spread distribution of CaPV in different regional state of the country. The widespread occurrence of CaPV observed in this study indicates distribution of the disease in the country and the disease is endemic in the country. SGPV and LSDV categorized by the OIE and distinguished as transboundary animal diseases of developing world and there is always a regular threat to their potential and substantial economic impact. Cattle are mainly at risk to Lumpy skin disease virus (LSDV), which is a vector borne disease where as SGPV transmitted by direct contact and clinically the disease is characterized by distinctive nodular lesions principally on the skin and underlying tissues of affected animals with occasional involvement of different parts of the body. These results in overwhelming economic losses due to deep pox lesions in the skin and these may also impose dramatic effects on rural livelihoods, which are strongly dependent on livestock, with significant production losses.

In the context of Ethiopia where livestock movement is uncontrolled, direct and indirect animal contacts during grazing, watering, and trading are the most important transmission means in intensive and extensive production system. This study provided information to a better understanding of the disease, and give further insight to improve control strategies in Capri pox infection.

Therefore, in order to overcome across this economically significant disease, the following recommendations forwarded:

- Active pox disease search, detecting and characterization of the Capri poxvirus should be continued.
- Strategic plan combined with well-organized annual vaccination campaign using quality and protective vaccine can control the Capri-pox diseases.

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8. ANNEXES

Annex 1: Questionnaires

I) History

1. Owner name _____Address/Location_____

2. Animal description and disease dissemination

a) Breed: ----------b) Species: _____

c) Sex: M_____F_____

d) Age: 1-2 yrs._____ 2-4 yrs._____ >4 yrs._____

e) Animal tag/brand/color: _____

3. Body Condition: a) Excellent b) Good c) poor

4. Production type: a) Intensive b) Extensive

5. Is there a skin disease in your herd or flock? Yes/No

6. How long the disease has been present? _____

a) Season _____b) Month_____ c)Year_____

7. Have you seen such outbreak in the area before? Yes/No

8. What is the magnitude of the problem? _____

9. How frequent skin disease occurs in the area? _____ Dry /wet season: ----------

10. Herd /Flock size: _____

11. Herd/flock composition: Adult M/F_____ young M/F_____Lactating _____ Calf/lamb/kids_____

12. Number of cases: _____

13. Population at risk: Adult_____ Young_____ Calf/Lamb/Kid_____

14. Is there death in the herd or flock? Yes/No

15. If Yes how many animals were died? _____

16. Is the animals were Vaccinated? Yes/No

17. If yes frequency of vaccination? _____

II) Clinical examination

18. Fever_____
19. Enlarged lymph node yes/ No
20. Other clinical sign observed_____

III) Movements of animals and other possible sources of infection:

21. Is there a new animals recently introduced into a herd or flock? Yes/No
22. What is their origin: _____
23. Are there animals that have left the herd or flocks and what is their destination? ---------
24. Are there contacts with other herds or flocks and use of communal grazing and contacts with wild ruminants? Yes/NO
25. Is there treatments and ruminants health records? Yes/NO
26. If yes what type of treatment? _____
27. Is there artificial inseminator visits and use of a breeding bull? Yes/No
28. How frequent milk collectors vehicle introduced to site? _____
29. How frequent animal trader/slaughter house transport vehicle visits the site? _____
30. Is there potential vector activity and presence of vector breeding sites such as lakes, rivers: Yes/NO
31. Is there other fomite that may have spread the disease? Yes/No

IV) Major economic impact due to loss of production

32. Is there Abortion in the herd/flock? Yes/No
33. Is there loss of fertility/ sterility? Yes/No
34. Is there extensive damage to hide and skin? Yes/No
35. What is the type of skin nodules? _____
36. Does the lowered milk production after infection Yes/No
37. Is there mastitis problem after the outbreak? Yes/No
38. Is there the depreciation of hide and skin quality because of the outbreak? Yes/No

39. Is there loss of draft from lameness? Yes No

40. What is the estimated cost due to the outbreak losses? _____

V) Post mortem Cases

41. Is there a lesion in the mucous membranes throughout the GI tract? Yes /No

42. Have you seen nodules in lungs/ Hemorrhages in spleen, liver, rumen? Yes/No

VI) Type of sample taken

a) Skin scrap b) Whole blood c) Milk d) nasal swab

43. What do you think about the skin disease transmission? _____

VII) Data collected by _____Signature _____Date_____

Annex 2: Outbreak cases of Capri poxviruses follow up format

S/N	Date	Owners name	Address	Spp	Sex	Age	Animal ID	Sample type	T^0c	Remark

Annex 3: DNA extraction Procedure

1. Pipet 20 µl QIAGEN Protease (or proteinase K) into the bottom of a 1.5 ml microcentrifuge tube.
2. Add 200 µl sample to the microcentrifuge tube.
3. Add 200 µl Buffer AL to the sample. Mix by vortex mixer for 15 s.
4. Incubate at 56°C for 10 min.
5. Briefly centrifuge the above mixture in 1.5 microcentrifuge tube to remove drops from the lid.
6. Add 200 µl ethanol (96–100%) to the mixture of sample, and mix well by vortex mixer for 15 s. After mixing, briefly centrifuge of the mixture to remove drops from the lid.
7. Carefully apply the above mixture to the QIAamp Mini spin column of 2 ml collection tube without wetting the rim. Close the cap, and centrifuge at 6000xg for 1 min. Place the QIAamp Mini spin column in a clean 2 ml collection tube (provided), and discard the tube containing the filtrate.
8. Carefully open the QIAamp Mini spin column and add 500 µl Buffer AW1 without wetting the rim. Close the cap and centrifuge at 8000 rpm for 1 min. Place the QIAamp Mini spin column in a clean 2 ml collection tube (provided), and discard the collection tube containing the filtrate.
9. Carefully open the QIAamp Mini spin column and add 500 µl Buffer AW2 without wetting the rim. Close the cap and centrifuge at full speed (14,000 rpm) for 3 min.
10. Recommended: Place the QIAamp Mini spin column in a new 2 ml collection tube (not provided) and discard the old collection tube with the filtrate. Centrifuge at full speed for 1 min.
11. Place the QIAamp Mini spin column in a clean 1.5 ml microcentrifuge tube (not provided), and discard the collection tube containing the filtrate. Carefully open the QIAamp Mini spin column and add 200 µl Buffer AE or distilled water. Incubate at room temperature (15–25°C) for 1 min, and then centrifuge at 6000 xg for 1 min.

Incubating the QIAamp Mini spin column loaded with Buffer AE or water for 5 min at room temperature before centrifugation generally increases DNA yield. A second elution step with a further 200 µl Buffer AE will increase yields by up to 15%.

Annex 4**:** Animal sampled by region

Region	**Host**	**Number of animal sampled**
Oromia	Cattle	35
	Sheep	2
Amhara	Cattle	7
	Goat	1
Tigray	Cattle	5
	Goats	2
SNNPR	Cattle	4
Addis Ababa	Cattle	3
Total		**59**

Annex 5: Active outbreak cases from domestic ruminants

Host	Skin srap	Nasal swab	Buffy coat	Total
Cattle	41	13	27	81
Sheep	2	0	0	2
Goats	2	1	0	3
Total	45	14	27	86

Annex 6: Detection of Capri poxviruses by conventional PCR

Host	Skin Scrap	Nasal swab	Buffy coat
Cattle	35/41	9/13	0/27
Sheep	2/2		
Goats	2/2	1/1	
Total	39/45	10/14	0/27

Annex 7: Detection of Capri poxviruses by real time PCR

Host	Skin Scrap	Nasal swab	Buffy coat
Cattle	38/41	9/13	19/27
Sheep	2/2		
Goats	2/2	1/1	
Total	42/45	10/14	19/27

Annex 8: Study area