Thesis Ref. No._____

ISOLATION AND MOLECULAR CHARACTERIZATION OF POX VIRUSES CIRCULATING IN SHEEP AND GOAT FROM OUTBREAK CASES IN SELECTED DISTRICT OF ZONE ONE OF AFAR, ETHIOPIA

Addis Ababa University, College of Veterinary Medicine and Agriculture, Department of Veterinary Microbiology, Immunology and Public Health, MVSc Program in Veterinary Micro-biology

MVSc. Thesis

By

AGERITU MOHAMMED SEID

August, 2020

Bishoftu, Ethiopia

ISOLATION AND MOLECULAR CHARACTERIZATION OF POX VIRUSES CIRCULATING IN SHEEP AND GOAT FROM OUTBREAK CASES IN SELECTED DISTRICT OF ZONE ONE OF AFAR, ETHIOPIA

A Thesis Submitted to College of Veterinary Medicine and Agriculture of Addis Ababa University in Partial fulfillment of the requirements for the degree of Master of Veterinary Science in Veterinary Micro-biology

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Department of Veterinary Microbiology, Immunology and Public Health

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As MVSc research advisor, we here by certify that we have read and evaluated this thesis prepared under our guidance by entitled: "***Isolation And Molecular Characterization Of Pox Viruses Circulating In Sheep And Goat From Outbreak Cases in selected district of Zone one of Afar , Ethiopia"*** we recommended that it can be submitted as fulfilling the MVSc Thesis requirement.

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Declaration

This piece of work is dedicated to my grandfather Mr. Seid Ahmed Ali.

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As members of the Examining Board of the final MVSc open defense, we certify that we have read and evaluated the thesis prepared by Ageritu Mohammed entitled ***"ISOLATION AND MOLECULAR CHARACTERIZATION OF POX VIRUSES CIRCULATING IN SHEEP AND GOAT FROM OUTBREAK CASES IN SELECTED DISTRICT OF ZONE ONE OF AFAR, ETHIOPIA"*** And recommended that it be accepted as fulfilling the thesis requirement for the Degree of Master of Veterinary Science in Veterinary Microbiology.

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SIGNED DECLARATION SHEET

First, I declare that this thesis is my original work and that all sources of materials used for this thesis have been duly acknowledged. This thesis has been submitted in partial fulfillments of the requirement for MVSc degree at College of Veterinary Medicine and Agriculture and is deposited at the college library to be made available to readers under rules of the library. I am solemnly declared that this thesis is not submitted to any other institution anywhere for the award of any academic degree, diploma, or certificate.

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Date of submission: _____

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LIST OF ABBREVIATIONS

µl	Micro litter
AGID	Agar gel immune diffusion
ANRS	Assessment of Afar National Regional State
AU-IBAR	African Union Inter African Bureau for Animal Resources
bp	base pair
CaPV	Capri pox Virus
CIE	Counter Immune electrophoresis
CFSPH	Center for Food Security Public Health
CPE	Cytopathic Effect
CSA	Central Statistic Authority
DNA	Deoxyribonucleic
dsDNA	double stranded Deoxyribonucleic acid
EDTA	Ethylene demine tetra acetic acid
ELISA	Enzyme linked immune sorbent assay
ESGPIP	Ethiopian sheep and goat productivity improvement program
EU	European Union
GMEM	Glasgow Minimum Essential Medium
GTPV	Goat pox Virus
ICTV	International committee taxonomy of viruses
IEF	Immune electro focusing
Kbp	Kilo base pair
KSGP	Kenyan sheep and goat pox
LSDV	Lumpy Skin Disease Virus
OIE	Office international des epizooties
NVI	National veterinary institute
PBS	Phosphate buffer solution
PCR	Polymerase chain reaction

PPR	Pest des petites Ruminants
REA	Restriction enzyme analysis
RNA	Ribonucleic acid
Rpm	Revolution per minute
RPO30	RNA polymerase subunit gene
SGP	Sheep and goat pox
SPPV	Sheep pox virus
TCID50	Tissue culture infective dose 50
UV	Ultra Violate

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ABSTRACT

Sheep and Goat poxvirus disease outbreak investigation was conducted to isolation and molecular characterization of pox viruses circulating in sheep and goat in outbreak cases in Mille district from December 2019 to April 2020 on a total 738 local breeds of sheep and goat in selected district of zone one of Afar region. A total of 738 sheep and goats (353 sheep and 385 goats) from Weranso and Geraro Kebele (in which the outbreaks occurred) were clinically examined for the presence of pox lesion on their skin. Out of these, 63 (8.5%) of sheep where as 123 (16.7%) of goats were found to be positive for pox lesion on their skin. The overall proportion of morbidity and mortality of SGPV was 25.2% and 2.3% respectively. Based on the observation the average morbidity and mortality of SGPV was high in goat than sheep, in young than adult and in male than female. The sampling methods was purposive and a skin nodular tissue samples were taken from 14 species from both (species, breed and sex) in order to isolate the field strain of SGPV circulating in and around Mille district of zoon one Afar. From that 14 tissue samples the virus was isolated on Vero cells line. Additionally, the isolated virus was identified by Conventional Polymerase Chain Reaction (CPCR) and Real time Polymerase Chain Reaction technique which is more sensitive molecular advanced technique for diagnosis of *Capri pox virus*. The tissue culture showed a typical characteristic of pox virus, cytopathic effect of cell ballooning, aggregation, rounding of cells and detaching of cells on Vero cell culture. Similarly, the conventional PCR revealed that out of 14 tested samples 11 samples were positive by developing band size of 172bp, where as three of them could not produce any band size on gel electrophoresis. In conclusion as complained by the owners and during field examination, SGPV disease was found to be a serious disease in the study area. Accordingly, further investigation is needed on identification of the causative agents and molecular characterization of SGPV and risk factors of the disease in selected district of zone one of Afar region.

Key words: *Afar region***,** *Mille district***,** *Out-break; Polymerase chain reaction*, *Sheep and goat poxvirus, virus isolation.*

1. INTRODUCTION

1.1. Background

Capri pox viruses (CaPVs) are large double-stranded DNA viruses belonging to the family Poxviridae. The genus includes sheep pox virus (SPPV), goat pox virus (GTPV) and lumpy skin disease virus (LSDV) (Fauquet *et al*., 2005; Murphy *et al*., 2012). SPPV is the type species among them (Bouden *et al*., 2009). CaPVs are responsible for some of the most economically significant diseases of domestic ruminants in Africa and Asia (Tulman *et al*., 2002; Stram *et al*., 2008). Their occurrence is associated with high morbidity like reduced milk yield and weight gain, increased abortion rates, damage to wool and hide, increased susceptibility to pneumonia and fly strike, high mortality and export–import restriction of their by-products (Bhanuprakash *et al*., 2011; Babiuk *et al*., 2008; Venkatesan *et al*., 2014).

In general, Capri pox viruses (CaPV) are considered to be very host-specific (Babiuk *et al.*, 2009). In addition to the isolate KSGP O-240, only a few other SPPV and GTPV strains have been known to affect both sheep and goats (Yan *et al*., 2012). In Ethiopia, where sheep, goats and cattle are all affected, a live attenuated vaccine strain (KS1-O180) is used for immunization of both small ruminants and cattle. Although occurrences of the disease in vaccinated cattle are frequently reported, information on the circulating isolates and their relation to the vaccine strain in use are still missing (Gelaye *et al*., 2015).

Sheep pox and goat pox (SGP) are a group of viral disease that causes highly infectious disease in sheep and goats. Generally the disease is less commonly seen in indigenous breeds in area where it's endemic as compared with exotic breeds. Indigenous animals are more likely infected from the disease in areas where it has been not found or dormant for a period of time, when intensive husbandry methods

are introduced (Hamito, 2009; ESGPIP, 2009). SGP virus diseases are caused by infection by genus Capri pox virus, family poxviaride (Buller & Palumbo, 1991) one of the largest (170-260 nm by 300-450 nm), enveloped double stranded DNA viruses (Tulman *et al*., 2002).

SGP geographical distribution has been relatively stable. SGP have seen in North and Central Africa, Middle Eastern countries, Asia and the former Soviet Union (Rao & Bandyopadhyay, 2000; Radostits *et al*., 2006; OIE, 2010). Ethiopian sheep and goat have been facing poxvirus infection for so many years. Updated information about SGP has a paramount importance to minimize the occurrence of this disease so as to encourage intervention options. However, there were a few works conducted so far on the isolation and characterization of CaPV affecting the livestock population. Additionally, there is a little information currently exists about the cross reactivity of the CaPV infections. In Ethiopian situation sheep and goat pox is found all region of the country (ESGPIP, 2009). In recent study around Gondar a total of 1296 ruminants studied for skin disease, the prevalence of sheep and goat pox is 77 (48.12%) or 64 (40%) or 13 (8.12%) respectively (Daniel & Samuel, 2015; Teshome, 2016).

1.2. Statement of the problem

In our country very limited works has been done on sheep and goat pox virus some reporters have been made on disease surveillance, Sero prevalence, risk factor and distribution of SGPV in selected areas of Amahara region (North Gonder,West Gojjam, South Gonder, Awi, East Gojjam) and Afar region (Chifira, Adaar, Amibra, Awash and Fenta) Administrative zone three in northwestern part of Ethiopia and North eastern part of Ethiopia(Getachew *et al.*,2015 and Tsegaw *et al.*,2017). A report on epidemiology and economic importance of SGPV: A review on past and current aspects indicated that the disease distributed in all regions of country and economically important due to production loss and direct death (Yune and Abdela, 2017). But circulating SPGV strain isolation and characterization are still under

estimate in our country. Additionally, in Afar region, so far there was no work done on isolation and identification of Sheep and Goat pox in the region in spite of a number of clinical signs based outbreak reports from the Region. Therefore, the present study was initiated with the following objectives:

GENERAL OBJECTIVE

- To investigate and isolate Sheep and goat pox viruses from outbreak cases and to characterize the virus from in selected district of zone one of Afar, Ethiopia.

SPECIFIC OBJECTIVES

- To investigate sheep and goat pox outbreak in selected district of zone one of Afar, Ethiopia
- To isolate the virus from outbreak samples by Vero cell culture investigate its CPE
- To identify and characterize the virus based on molecular technique (PCR) and genomic sequencing

2. LITERATURE REVIEW

2.1. Definition of Sheep and goat pox disease viruses

The disease of Sheep and goat pox (SGP) is an acute to chronic disease of sheep and goats characterized by generalized pox lesions all the skin and mucous membranes, a persistent fever, lymphadenitis, and often a focal viral pneumonia with lesions distributed uniformly throughout the lungs (Davies, 1976).

2.2. Etiology of sheep and goat pox viruses

Sheep and goat pox viruses are the causative agent of sheep and goat pox which are members of CaPVs and which are enveloped, brick shaped with complex symmetry, measuring 300x270x200 nm in size (Shakya, 2001). Mature *Capripoxvirions* have a more oval profile and larger lateral bodies than *Orthopoxvirions* (Abdulqa *et al*., 2016). These viruses are generally resistant to drying, survive freezing and thawing, and remain viable for months in the lyophilized state. Sensitivity to heat differs among strains (Rao & Bandyopadhyay, 2000).

The poxviruses of sheep and goats (SGP) are closely related, both physico chemically and antigenically. We have unable to distinguish poxvirus from each with serological techniques including serum neutralization, and were once thought to be strains of a single virus. SGP viruses are usually species specific; however, strains do exist that can infect both sheep and goats. Genetic sequencing has now confirmed that these viruses are distinct, however, recombination can occur between them, but some CaPV are not host specific. Kenya SGPV and Yemen and Oman infect both sheep and goat (CFSPH, 2008).

2.2.1. Taxonomy

SGP are grouped under the family of *poxviridae*. The family *Poxviridae* is subdivided into two subfamilies: *Chordopoxvirinae* (poxviruses of vertebrates) and *Entomopoxvirinae* (poxviruses of Insects). The subfamily *Chordopoxvirinae* is subdivided into eight genera i.e. *orthopoxvirus, parapoxvirus, capripoxvirus, suipoxvirus, leporipoxvirus, yatapoxvirus, avipoxvirus* and *molluscipoxvirus* and the genus *Capripox virus* contains 3 important viruses; i.e. LSDV, SPPV and GTPV (Lefkowitz *et al*., 2006).

2.2.2. Physico-chemical property of SGP

Sheep and goat pox are very resistant to physical and chemical agents. The electron microscopy of vesicle or scab material is an effective means of rapid diagnosis; poxviruses and herpes viruses are readily distinguished, and the characteristic morphology of par poxviruses can be identify (Bhanuprakash *et al*., 2011). Electron microscopic studies revealed that goat pox and sheep pox viruses are morphologically similar to members of orthopox genus. They are enveloped, brick shaped with complex symmetry, measuring 300x270x200 nm in size (Diallo & Viljoen, 2007). Mature *Capri poxvirions* have a more oval profile and larger lateral bodies than *Orthopoxvirions* (Diallo & Viljoen, 2007).The morphology and morphogenesis of GTPV showed by layer envelope at an early developmental stage with maximum protein and minimum lipid composition. The lipid content increased with development of virion (Madhavan *et al*., 2016).

2.2.3. Genome structure of SGP Viruses

CaPVs are double-stranded DNA viruses with genomes approximately 150 kbp in size. LSD virus has an additional nine genes that are non-functional in SPP and GTP viruses, some of which are likely responsible for their ability to infect cattle (Tulman *et al*., 2002). CaPV isolates are extremely conserved with genome identities of at least 96% between SPPV, GTPV and LSDV (Babiuk *et al*., 2008).

Even if CaPVs share high nucleotide sequence identity, they are phylogenetically distinct. The phylogenetic analysis showed that members of genus could be delineated into 3 distinct clusters of GPV, SPV and LSDV based on the P32 genomic sequence. There is an additional aspartic acid at 55th position of P32 present in SPPV which is absent in GTP and LSD virus (Hosamani *et al*., 2004)

The genome is flanked by inverted terminal repeat (ITR) sequences which are covalently-closed at their extremities. And the virion is enveloped, brick-shaped, 300×270x200nm. The surface membrane displays surface tubules or surface filaments. Two distinct infectious virus particles exist, the intracellular mature virus (IMV) and the extracellular enveloped virus (EEV) (GPBR, 2008).

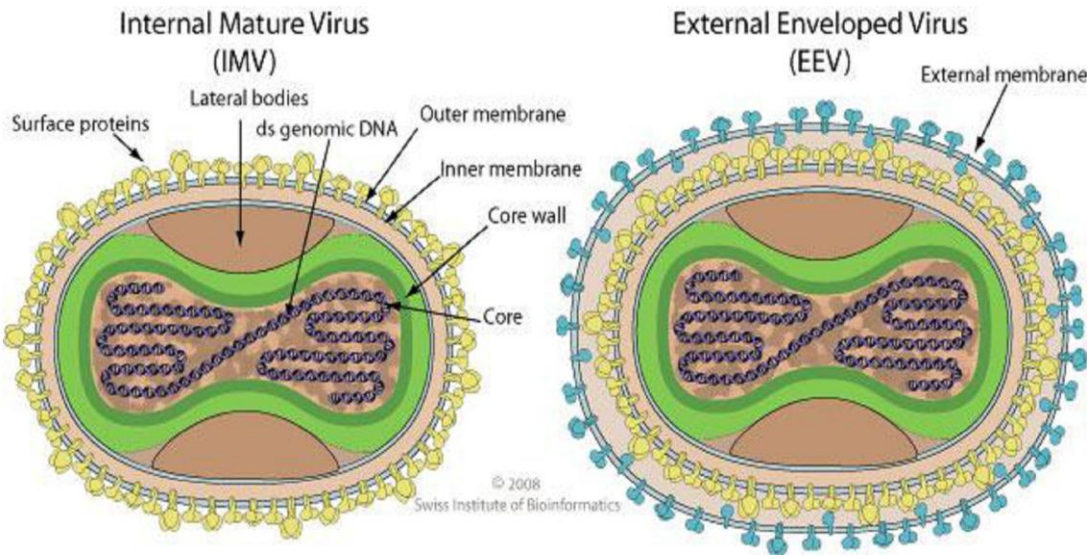


Figure 1, Morphological structures of SGP

Source: (Hulo *et al*., 2010).

2.2.4. Viral replication of SGP

All viruses grouped under family of poxviridae have the same way replication of the poxvirus involves several stages. The first thing the virus does is to bind to a receptor on the host cell surface; the receptors for the poxvirus are thought to be

Glycosaminoglycans (gags). After binding to the receptor, the virus enters the cell where it uncoats. Uncoating of the virus is a two step process. Firstly the outer membrane is removed as the particle enters the cell; secondly the virus particle (without the outer membrane) is uncoated further to release the core into the cytoplasm. The pox viral genes are expressed in two phases. The early genes are expressed first. These genes encode the non-structural protein, including proteins necessary for replication of the viral genome, and are expressed before the genome is replicated. The late genes are expressed after the genome has been replicated and encode the structural proteins to make the virus particle (Kara *et al*., 2003).

The replication of poxvirus is unusual for a virus with double-stranded genomic DNA because it occurs in the cytoplasm. Poxvirus encodes its own machinery for genome transcription, a DNA dependent RNA polymerase, which makes replication in the cytoplasm possible. Most dsDNA viruses require the host cell's proteins to perform transcription. These host proteins are found in the nucleus, and therefore most dsDNA viruses carry out a part of their infection cycle within the host cell's nucleus (Kara *et al*., 2003).

2.3. Epidemiology of SGP

2.3.1. Geographic and seasonal distribution

Outbreaks are recorded during all months of the year, but mostly occur between November and May, and the peak outbreaks occurred during March. During cold seasons, sheep are exposed to low temperature exerting stress which could suppress the immune system and ultimately the sheep become vulnerable to infection. The seasonality of SGP observed could be explained either by the capability of the viruses to survive for many months in wet and cold weather, by association with the lambing season, or by the poor physiological condition of flocks in the autumn (Bhanuprakash *et al*., 2005; (Yeruham *et al*., 2007; Zangana & Abdullah, 2013).

Its epidemiology is characterized by periodic outbreaks and the Outbreaks of the disease and the geographic range has been restricted in the last 50 years mainly to Asia and Africa, extending from Africa north of the Equator (Kitching *et al*., 1989; Achour & Bouguedour, 1999) into the Middle East (Daoud, 1997), Turkey (Oğuzoğlu *et al*., 2006) and Asia including regions of the former Soviet Union (Orlova *et al*., 2006), India (Mondal *et al*., 2004), China (Zheng *et al*., 2007).

The spread of SGP into new areas is predominantly associated with the increase of illegal animal movement via trade 7 (Domenech *et al*., 2006) as well as inadequate or breakdown of veterinary services (Rweyemamu *et al*., 2000) Countries free of SGPV usually have in place legislation based on OIE recommendations that attempt to prevent the trans-boundary spread of production-limiting diseases, but increasingly these are becoming more difficult to enforce, including on the border of the European Union. Biting flies have also been implicated in the spread of CaPV (Yeruham *et al*., 2007).

CaPV are not present in north, central or south America, South East Asia (excluding Vietnam) or Australasia. The impacts of global climate change on insect vectors, established as a route of transmission for SGPV (because of very high viral loads in the skin), suggest that there are real risks of further spread of these diseases into other geographic regions (Domenech *et al*., 2006).

In Ethiopia, the CaPV disease is endemic in all regions. In 2007/2008, the Animal and Plant Health Regulatory Directorate received 893 SGP outbreak reports from all regions except Gambela, Harari and Dire Dawa. Out of the 57,638 sick sheep and goats, 6,401 (11.1%) died. The disease reporting rate in Ethiopia is only about 35-40%. The figures in terms of affected, vaccinated and dead animal are expected to be higher than the reported figures (Sileshi, 2009).

According to the AU-IBAR the number of African countries affected by SGP had, before 2011; indicate an increasing trend for 3 consecutive years. The number of countries reportedly affected by SGP in 2011 reduced remarkably from the previous year. In 2011, 12 countries reported occurrence of SGP in their territories, which is a 46% reduction from the 26 countries affected by the disease in 2010 (AU-IBAR, 2011). There is no plausible explanation for this decrease in reporting as there is no ongoing continental program against SGP although there might be national interventions against the disease. The top 3 countries that recorded the highest number of outbreaks in 2011 include Ethiopia (223), Somalia (170) and Algeria (44). Overall, a total of 541 epidemiological units were affected on the continent involving 9932 cases and 1619 deaths, with a case fatality rate of 16.3% (AU-IBAR, 2011).

Table 1: The countries reporting about SGP to AU-IBAR in 2011

Country	Outbreaks	Cases	Deaths	Slaughtered	Destroyed
Algeria	44	306	14	0	0
Cameroon	2	15	0	0	
Ethiopia	223	4827	815	90	
Ghana	1	2	0	0	0
Kenya	2	9	0	0	0
Lesotho	1	5	0	0	0
Niger	41	945	235	NS	NS
Nigeria	2	33	7	9	5
Senegal	7	410	28	0	0
Somalia	170	2393	324	49	47
Sudan	29	859	185	5	0
Tunisia	19	128	11	5	15
Total (12)	141	9932	1619	158	67

NS: Not specified (**Source**: AU-IBAR, 2011)

2.3.2. Host Range

The classification of pox viruses was made on the basis of the host from which these viruses were isolated. Serologically, all these 3 viruses of CaPV are identical and usually cross react, but can be differentiated by using molecular techniques (Christian *et al.,* 2009). However, some strains are restricted to one species only. Native breeds in endemic areas are far less susceptible than introduced breeds of European or Australian origin (OIE, 2008). SPP is a highly contagious, host specific, viral infection, and causes a high rate of mortality and morbidity in sheep, irrespective of age, sex and breed (Singh *et al*., 2007).

CaPV only infect some ruminant species and have a tropism for certain cell types (McFadden, 2005). They are not infectious to humans (Regnery, 2007). SPPV and GTPV cause clinical disease in sheep and goats, respectively; however, there is a wide range of clinical disease seen with different field isolates (Davies , 1976). The nomenclature is largely made up of the location (country) and the species from which it has been isolated (sheep or goat or sheep and goat). This issue of naming strains SPP, GTP or SGP remains problematic because this has been depend on field observation of the species affected whether sheep or goats (McFadden, 2005).

Mostly, SPPV and GTPV infect and cause clinical disease in either sheep or goats, respectively, and most isolates induce more severe disease in either sheep or goats and only mild or sub-clinical infection in the other species (Babiuk *et al*., 2008). However, some isolates are pathogenic for sheep and goats, particularly some strains from central Africa (Davies , 1976) and the Middle East (Kitching & Mellor, 1986).

To date, there is, , no evidence of SPPV and GTPV viruses in wildlife, and it is assumed that wildlife do not play a relevant role in the epidemiology of SPP and GTP (Babiuk *et al*., 2008), although it cannot be excluded that wild sheep and wild goats can be infected 10 with SPPV. In support of this fact, the LSDV, closely related to SPPV/GTPV, has been isolated from wild ruminants (Tuppurainen & Oura, 2012).

The strains of CaPV causing disease in sheep and goats are not host-specific and may either affect both or one species (Kitching, 2003). The local strain of GTPV was studied in sheep and goats. Only goats died a few days after the inoculation (55%) and no mortality was recorded in the sheep. The difference of sensitivity between sheep and goats was statistically insignificant (Bidjeh *et al*., 1991). An attenuated vaccine of Romanian SPP strain was incorporated to enhance the immunity of sheep but recently a severe outbreak of CaPV has been seen only in the goats in a mixed flock of sheep and goats. The high affinity for goats was confirmed by experimental infection of sheep and goats with that virus strain (Elzein *et al.*, 2004).

The virus strain circulating in Chad region seemed to be host-specific for goats since sheep kept in contact with goats did not suffer from the disease (Bidjeh *et al.*, 1990). The British breeds of sheep and goats with the isolate of SPP and GTP from Kenya, India, Nigeria, turkey, Sudan, Yemen, Arab republic and Pakistan and were inoculated (Bidjeh *et al.*, 1990).

2.3.3. Routes of Transmission

2.3.3.1. Direct transmission

The virus has been isolated from nasal, ocular, and pharyngeal secretions, semen, milk and blood, which might be the source for transmission (Irons *et al*., 2005). The main mode of transmission of SPPV is direct contact between an infectious and a susceptible animal. Upon infection, animals develop pox lesions in the mucous membranes of the mouth, nasal cavities and conjunctiva, excreting the virus in saliva and ocular and nasal discharge. The virus is spread in droplets/aerosols via coughing, sneezing, head shaking, vocalizations and breathing. Naive animals acquire the virus via inhalation, orally or via skin abrasions (Babiuk *et al*., 2008). The high concentrations of the virus in the skin may also contribute to the spread of SPP and GTP via insect vectors (Babiuk *et al*., 2008). There is evidence that stable flies (*Stomoxys calcitrans*) can act as an efficient mechanical vector of SPPV and GTPV (Mellor *et al*., 1987).

Transmission is usually by aerosol after close contact with severely affected animals containing ulcerated papules on the mucous membranes. There is reduced transmission once papules have become necrotic and neutralizing antibody is produced. Animals with mild localized infections also rarely transmit disease. Infection may also occur through other mucous membranes or abraded skin. Indirect transmission by contaminated implements, vehicles or products occurs. Indirect transmission by insects (mechanical vectors) has been established (OIE, 2009).

The virus transmits to susceptible sheep and goats by flies, and the virus can remain viable for four days in some flies. High virus titers and intrinsic resistance of the virus, vectors with large mouthparts and their frequent feeding habits are the basic factors favoring 12 mechanical transmissions. No transmission was detected with biting (*Mallophaga* spp.) and sucking lice (*Damalinia* spp.), sheep head flies (*Hydrotaea irritans*) and midges (*Culicoides nubeculosus*), even though virus could be isolated from previously infected sheep, which was the host for feeding of the aforementioned flies. There is no evidence that SPPV can replicate in arthropod vectors (Mellor *et al*., 1987).

2.3.3.2.Indirect transmission

The viruses in saliva, ocular and nasal discharge, skin lesions and scabs, urine and faces may contaminate feed, water, wool and the environment, leading to an indirect transmission of the virus either orally or via skin abrasions. Infectious virus is well protected inside scabs, which are shed by infected animals; when scabs dissolve, the virus may be released into the environment and this may continue for several months after the outbreak (Rao & Bandyopadhyay, 2000). Unfortunately, no reports have been published on survival of SPPV in litter, fodder and feed. Untreated skins, hides and wool collected from infected animals may contain skin lesions and scabs with viable virus and, therefore, they may be a source of infection for naive sheep and goats (Rao & Bandyopadhyay, 2000).

The CaPV prevalence is expanding, which is evident from outbreaks in Vietnam, Ethiopia, Mongolia, Egypt, Greece and Israel. The poor quarantine measures and trade across the border of live animals may lead to further spread of the disease (Babiuk *et al*., 2008).The grazing and migration pattern indicates that the disease was probably introduced to the farm in Jammu, India by local sheep (Mondal *et al*., 2004).

2.4. Postmortem lesions of SGP

The nodular lesion in intestine and lungs were recorded on necropsy. Typical pock lesions disperse over the body of the affected animals with nodular lesions observed in the lung tissue of the dead animals (Mondal *et al*., 2004). Postmortem of dead animal's shows vesicles and pock lesions on affected skins. The lungs are diffusely inflamed (bronchopneumonia) with dispersed Pock lesion (Dubaib, 2002).

2.5. Risk factors of SGP

2.5.1. Host risk factor

CaPV can affect sheep, goat and cattle. Virus of goat pox is highly host-specific, infecting only goats; however, from isolate to isolate host specificity varies. Group of Sheep and goat of all age, breed and sex are susceptible to SGP. In areas where SPP is enzootic, imported breeds such as Merinos or some European 14 breeds may show greater susceptibility than the native stock. SGP infect only sheep and goat and have no zoonosis. Wild ungulate is not reservoir for this disease (ESGPIP, 2009).

2.5.2. Pathogen risk factor

Poxviruses are via to have prolonged survival in inactivated and environment by drying, freezing, thawing, and remain viable for months in the lyophilized state. However, it is sensitive to 1% of formalin and extreme PH. It can remain infectious for up to six months in sheep pens, and may also be found on the wool or hair for as

long as three months after infection (Sharma *et al*., 1988).The CaPV are highly stable in normal environment condition and can survive for prolonged time, with or without susceptible animal. They are inactivated by sun light and heat, but can survive in cool dark environment for up to 6 month (Davies & Otema, 1981).

2.5.3. Environment risk factor

It had impact on the agent, host and vectors as well as interaction between them. The environmental determinants play a great role in the occurrence of SGP. These (agent, host and vectors) predisposing factors have a great role in maintenance of *Stomoxys calcitrans* and the tsetse fly to susceptible animals which are the vectors for transmission of disease (Webbs *et al*., 1980).

2.6. Pathogenesis of SGP

The poxviruses are generally epitheliotrophic and can cause localized or systemic disease. Initial multiplication of the virus occurs at the entry site of the virus into the body of the host. In systemic infections, further viral replication takes place in the draining lymph nodes, followed by viraemia and further viral multiplication in many different organs including the liver, spleen and lungs (Fenner *et al*., 1987). Viral replication takes place in the cytoplasm of cells. Viral particles are enveloped when mature virus particles move to the Golgi complex; most particles are however non-enveloped and are release by cell disruption. Both enveloped and non-enveloped particles are infectious (Fenner *et al*., 1987).

The incubation period of SPP is 4-8 days of that of GTP is 4-15 days. After it inters, goat pox virus replicates locally in the tissues. Since the virus is epitheliotropic, it will infest the epithelium tissues of the organism. On the 7th day post-inoculation, the virus titer reached to its peak. The virus spread to the regional lymph nodes, after 3-4 days of 15 primary viremia. The viremia spread in the body, and affected spleen, lungs and liver. The virus inhaled may also cause lungs lesions. In skin nodules from

7 to 14 days after inoculation, the virus titers persisted and decreased with the development of serum antibodies. Within 24 hours of the appearance of generalized papules, affected animals develop conjunctivitis, rhinitis and enlargement of all the superficial lymph nodes, in particular the prescapular lymph nodes. Excessive salivation can also occur after infection (OIE, 2012).

Rosella stage is stage in which Skin lesions typically begin with small red spots within 3 days of infection which is followed by papules. The affected animals are febrile at this stage. The second stage of pox lesion is Papules which develops after 3 days of reseola stage. Nodular skin lesions that are developed from reseola stage (red spots) those are hard during palpation. Papules within 5-6 days are changed to vesicles and known as vesicular stage. Pustular stage develops after 3 days of vesicular stage. The last stage of pox lesion is scab (Bowden *et al*., 2008).

2.7. Clinical sign of SGP

Pox lesions have seen in the heart muscles in this form of the disease, most rarely. In the benign form, more common in adults, only skin lesions occur, particularly under the tail, and there is no systemic reaction and animals recover in 3-4 weeks. Abortion and secondary pneumonia are complications (Iran Veterinary Organization, 2014).

The incubation period of the disease varies from 4 to 21 days with an initial rise in temperature, pulse and respiratory rates, edema of the eyelids, nasal discharge, in appetence, arched back, lacrimation, coughing, salivation and nasal discharge leading to crust formation, pneumonia, hypersensitivity, constipation and scanty urine (Singari *et al*., 1990). Usually, the first manifestation of the disease is swelling of nostrils, followed thick discharges from the nose and watery discharges from the eyes (Figure 1). High body temperature (41 to 42°C) is found in infected animals, and keratitis may develop (Daoud, 1997).

A) Face and neck region B) perennial region C) Face D) face and perennial region

Figure 2, Pox lesion showed on clinical diseased sheep.

Source: (Assefa , 2017).

The clinical sign of sheep pox can be either malignant or benign. The malignant form of sheep pox is mostly common in lamb. Affected lambs may die without observable pox lesion. Fevers which peak at 40-42°C, dyspnea, and ocular nasal discharge and pox lesion on unwooled skin are manifested in malignant form of sheep pox (AUSVETPLAN, 1996).

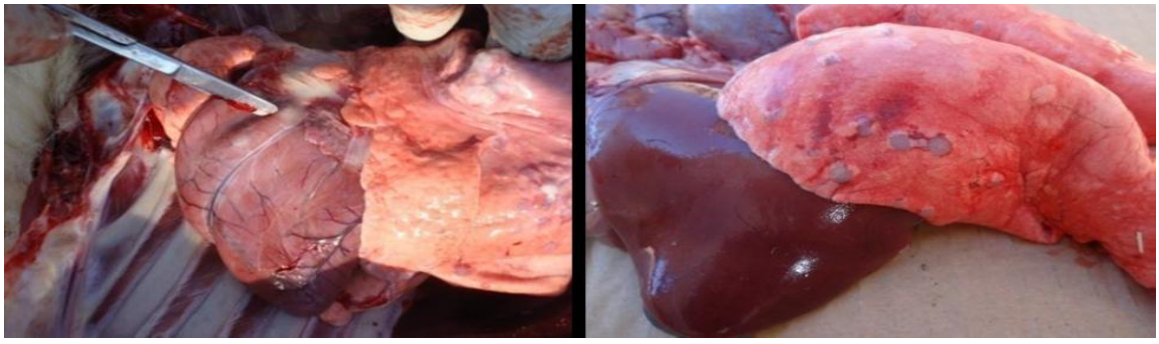


Figure 3, Malignant form of SGP: Pox lesions on heart muscles (left), & lungs (right),

Source: (Marzaie *et al.*, 2015).

2.8. Morbidity and mortality of SGP

Morbidity and mortality vary with the breed of the animal, its immunity to Capri pox viruses, and the strain of the virus. Mild infections are common among indigenous breeds in endemic areas, but more severe disease can be seen in young or stressed animals, animals with concurrent infections, or animals from areas where pox has not occurred for some time. Reported morbidity rates in indigenous breeds range from 1% up to 75% or higher. Although the mortality rate is often less than 10%, case fatality rates of nearly 100% have been reported in some young animals. Imported breeds of sheep and goats usually develop severe disease when they are moved into an endemic area. The morbidity and mortality rates can approach 100% in newly imported, highly susceptible flocks (ESGPIP, 2009).

Figure 4, Mass mortality and morbidity of sheep and goat pox.

Source: (Marzaie *et al.*, 2015).

2.9. Diagnosis of SGP

SGP can be diagnosed depends on the observable clinical sign like, dyspnea, fever and pox lesion in different parts of the unwoolen skin (Gitao *et al*., 2017). Epidemiology of the disease is also important in diagnoses of SGP. Clinical pathology and species of affected host are also important in the diagnosis of this disease. As the viruses of SGP are very closely related it's indistinguishable by serologically. It appears that the host preference shown by these viruses with respect to either sheep or goats, accompanied by the case history, may be regarded as partially affirmative for either SPP or GTP, but confirmatory diagnosis requires laboratory studies (Yune & Abdela, 2017).

The differential diagnoses include contagious ecthyma (contagious pustular dermatitis), bluetongue, dermatophilosis/ streptothricosis, mange (e.g., psoroptic mange/sheep scab), photosensitization or urticaria, peste des petits ruminants, parasitic pneumonia, multiple insect bites and caseous lymphadenitis (Rao & Bandyopadhyay, 2000). SGP can be tentatively diagnosed by electron microscopy; because the morphology of the virus particle is characteristic, capripoxviruses can be differentiated from most poxviruses that cause lesions in small ruminants. Histopathology can also be helpful (Rao & Bandyopadhyay, 2000).

The clinical diagnosis is confirmed by various diagnostic techniques including; virus isolation in cell cultures, transmission electron microscopy, immunohistochemistry, direct and indirect fluorescent antibody tests, agar-gel immunoduffusion, and enzyme linked immunosorbent assay (ELISA), western blot and serum neutralization test (SNT). Molecular diagnostic methods being used include conventional PCR (Tuppurainen *et al*., 2005; Orlova *et al*., 2006; Zheng *et al*., 2007); real-time PCR (Babiuk *et al*., 2008) and dot blot hybridization.

2.9.1. Virus isolation

During clinical examination samples for virus isolation and antigen detection were collected from clinically sick animals according to the procedures of (OIE, 2010), Confirmation of SGP in a new area requires virus isolation and identification. SGP will grow in tissue culture of ovine or caprine origin, although maximum yield is obtained using lamb testis. CaPV antigens can be demonstrated in tissue culture using immunoperoxidase or immunofluorescent staining and the virus can be neutralized using specific anti sera (Abdulqa *et al*., 2016; OIE, 2017).

Before collecting or sending any samples, the proper authority's samples should only be sent under secure conditions and to authorized laboratories to prevent the spread of the disease. For virus isolation samples must be sent to the laboratory as soon as possible. They should be kept cold and shipped on gel packs If these samples must be shipped long distances without refrigeration, glycerol (10%) can be added; tissue samples must be large enough that glycerol does not penetrate into the centre of the tissue and destroy the virus (Davies, 1981).

Cultivation of GPV in cell culture of lamb kidney and tests (Kitching *et al*., 1986). embryonic Caprine lung (Elzein & Crowther, 1982), Sheep thyroid (Nitzschke *et al*., 1967), chicken embryo fibroblast (Rao & Malik, 1982), Calf kidney (Tantawi *et al*., 1980), Vero cell line (Assefa, 2017). Cell cultures were incubated at 37^{O}C at 5% CO2 and observed daily for the development of CaPV specific cytopathic effects (Gelaye *et al.,* 2013).

2.9.2. Virus neutralization test (VNT)

VNT is the only validated serological test available. A test serum can either be titrated against a constant titer of CaPV 100 TCID50 (50% tissue culture infective dose). A standard virus strain can be titrated against a constant dilution of test serum in order to calculate a neutralization index (Kitching & Carn, 1996). The test is

described using 96-well flat-bottomed tissue-culture grade micro titer plates, but it can be performed equally well in tissue culture tubes with the appropriate changes to the volumes used, although it is more difficult to read an end-point in tubes. The use Vero cells in the virus neutralization test have been reported to give more consistent results (OIE, 1996).

2.9.3. Agar gel immune diffusion test (AGID)

It is less specific than the VNT due to cross-reactions with antibody to other poxviruses. A gel diffusion technique for the diagnosis of SGP was introduced as early as the 1960s, using either homologous or heterologous (Uppal & Nilakantan, 1967) antiserum. The use of *methioninelabeled* antigen preparations considerably improves the sensitivity of the AGPT in the detection CaPV of antibody (Kitching *et al*., 1986). Unfortunately, AGPT does not distinguish between CaPV and CPD due to antigen cross-reactivity (OIE 2000) and is also less sensitive (Mangana-Vougiouka *et al*., 2000).

2.9.4. Indirect fluorescent antibody test (IFAT)

It is also less specific than the VNT due to cross-reactions with antibody to other poxviruses. An indirect FAT is more sensitive than direct FAT because of the availability of additional binding sites. CaPV infected cell culture grown on flying cover-slips or cell culture microscope slides can be used for the indirect fluorescent antibody test. This technique has been successfully applied: (1) to detect and locate the SPV antigen, (2) epidemiological 19 investigations (Davies, 1976), (3) pathogenesis (Gurel, 1979), (4) sequential growth of SPV in vitro (Sarkar *et al*., 1980), (5) cross-reactivity of SPV, GPV and cowpox and antigenic relationship between CaPV and orthopox viruses (Ramyar & Hessami, 1970), (6) for providing rapid diagnosis (Mangana-Vougiouka *et al*., 2000), (7) to study the replication site of the virus and timing of appearance of antigens in cell cultures (Soman, 1986), (8) to assess the extent of cell-to-cell virus spread and synthesis in cell cultures and (9) immune status of animal (Debnath *et al*., 1992).

2.9.5. Immune blotting (Western blotting)

It is using the reaction between the P32 antigens of SGP with test sera is both sensitive and specific, but is difficult and expensive to carry out (Abera *et al*., 2015). Its test sera against CaPV infected cell lysate provide a sensitive and specific system for the detection of antibody to CaPV structural proteins (Chand *et al.*, 1994; OIE, 2017).

2.9.6. Enzyme-linked immune sorbent assay (ELISA)

It is now used widely to detect antibodies and antigens in a variety of test systems, and is more sensitive than virus -neutralization tests. Some antibody-detecting ELISAs have been described but none is sufficiently validated to be recommended for use (OIE, 2017; (Bowden *et al*., 2008). An indirect ELISA depend on SPV P32 recombinant fusion protein expressed in Escherichia coli was found rapid, reliable, non-infectious and detected antibodies to CaPV post-infection earlier than VNT (Carn *et al.*, 1994) as the latter test is besotted with disadvantages as discussed elsewhere.

SGP are diagnose by various methods of ELISA; However, problems such as a considerable background reaction and the requirement for special reagents, such as recombinant proteins often limit their use as routine screening tests. Hence, an immune capture ELISA has been developed as a relatively simple assay (Carn, 1995).The detection of GPV and SPV antigens in scab suspensions. However, this assay also has a limitation in that it is best used only in combination with the CIE test for accurate and confirmative diagnosis. A dot ELISA, carried out on nitrocellulose strips or paper, is a valuable addition to the battery of diagnostic methods for goat pox and is about three times more sensitive than the single radial hemolysis test (Carn, 1995).

2.9.7. Single radial hemolysis test (SRH)

The test is the lysis of erythrocytes when the antigen–antibody complexes using complement are formed. The test is very simple and used successfully for quantifying the antigen, antibody and post-vaccinal immune response (Kataria & Sharma, 1992) of SPP. The test gives a simple and quantitative estimate of antigen and antibody and is used successfully to diagnose sheep and goat pox (Tiwari & Negi, 1996).

2.9.8. Single radial immune diffusion (SRID)

This test has been successfully used for the quantification of immune response against SPV. The Immune response in terms of IgG in lambs vaccinated with SPV was estimated depending upon the diameter of the precipitation ring (Sharma, 1990).

2.9.9. Polymerase chain reaction (PCR)

It is rapid molecular laboratory confirmation of SGPV based on clinical signs, electron microscopy and previously mentioned serological tests are not always reliable. To overcome these limitations, simple, rapid and specific PCR techniques have been developed to detect CPV DNA in infected cell culture supernatants, biopsy samples (Heine *et al*., 1999) and also to differentiate between SGPV and LSDV on the basis of unique restriction sites in the corresponding PCR fragments.

Multiplex PCR is a fast and simple method for CaPV species identification. The method was based on multiplex polymerase chain reaction (MPCR) with specific primers for each species (Orlova *et al*., 2006). It is a single-step procedure was used for the amplification of CaPV in skin biopsies. They used one specific primer for alpha-tubulin and two 21 specific primers for CaPV. The technique was optimized with the standardization of different concentrations like primer, magnesium and dNTPs. Sometimes due to DNA amplification inhibitors, false negative results occur that may be corrected by the addition in assay of alpha-tubulin primers (Markoulatos *et al*., 2000).

The only rapid method available so far for a routine genotyping of the three CaPVs is a real time PCR assay based on dual hybridization probe technology (Lamien *et al*., 2011). However the use of this method for virus detection and genotyping is costly since it require the use of two fluorescently labeled probes and specialized real time PCR machines. High resolution melting of small sized PCR products in the presence of saturating DNA dye such as LC green and Eva green offers means of CaPV genotyping. This is a cost effective, rapid, highly sensitive and specific, and easy to perform method in diagnostic laboratories in countries endemic for LSD, SPP and GTP virus (Wittwer *et al*., 2003; Liew *et al*., 2004).

PCR technique becomes more effective for the diagnosis of SPV and GPV from field samples when combined with restriction enzyme analysis (REA) of PCR-amplicons (Rao and Bandyopadhyay, 2000). Recently, GPV and SPV from infected cell culture supernatants and skin biopsy were clearly differentiated by REA of PCR amplified P32 gene products (Hosamani *et al.*, 2004).

2.10. Economic importance of Sheep and Goat and Pox disease in Ethiopia

The World Organization for Animal Health (OIE) categorizes SGP as a notifiable disease because of the substantial economic impact of an outbreak. Small ruminants (Sheep and Goats) have a unique role in smallholder agriculture as they require small investments; faster growth rates, have shorter production cycles, and greater environmental adaptability as compared to large ruminants. They are important protein sources in the diets of the poor and help to provide extra income and support survival for many farmers in the tropics and sub-tropics (Tibbo *et al*., 2006; Nottor, 2012). SGP in endemic areas are associated with significant production losses since decreased weight gain, reduced milk yield, increased abortion rates, damage to wool and hides, and increased susceptibility to pneumonia and fly strike, while also being a direct cause of mortality (Yeruham *et al*., 2007).

2.11. Prevention and control of SGP

SGP immunization has been practiced since the early attempts by Borrel in 1903. The control measures with the exception of vaccination are usually not effective. Vaccination will greatly reduce the morbidity and epizootic but may not completely limit the extension. In endemic countries, vaccination is considered the only economically feasible way to control the spread of SGP and improve cattle productivity (Abera *et al*., 2015; OIE, 2017).

The numerous live attenuated vaccines have been developed and used worldwide, while inactivated vaccines are considered less effective (Boumart *et al*., 2016). Only live attenuated vaccines are currently available and are cheap and provide good protection if sufficient herd immunity (over 80%) is maintained by carrying out annual vaccinations (Tuppurainen *et al*., 2017).

Therefore, outbreaks can be eradicated by strict quarantines to avoid introduction of infected animals into safe herds, isolation and prohibition of animal movements, slaughtering of all sick and infected animals (Depopulation of infected and exposed animals), proper disposal of carcasses (Incineration), cleaning and disinfection of the premises and insect control (Abera *et al*., 2015).

The local controls in the semi-arid zone of Eastern Europe, Asia and Africa, where SGP is endemic, the problem of disease control are great. The range is not enclosed, there is animal's movement in search of water and grazing and mixing of different population groups is inevitable in these extensive husbandry systems. Trade is also continuous, extensive and largely beyond in any institutional control, with little regards for national boundaries. In this situation SGP control is demanding and difficult. The individual animal owners practice vaccination and national schemes may achieve extensive vaccination cover over very large areas. Drought, civil unrest or other similar events can totally destroy all the progress, which has been made, however, and large regionally coordinated schemes may be required to have any lasting impact (Prasad and Datt, 1993).

The countries free from Capri pox viruses maintain their disease - free status by the restriction of imports of animal products and livestock from affected areas. The rapid implementation of a radical slaughter policy and severe movement restriction, coupled with a ring vaccination of radius 25-50 km should result in elimination of disease in the case of countries from enzootic areas (Carn, 1993).

In endemic areas, for winter housing a regular cleaning program is essential to eliminate any residual virus that may remain dormant. Poxviruses are capable of long intervals between animal to animal transmissions. The owners often report the appurtenance of cases when they house the animals for the winter period. The virus maybe persists for several months in organic matter and this is even more essential, if there have been case of disease. Via the removal of the dung and subsequent treatment with phenol, alkali or other suitable disinfectant is advisable to eliminate any residual viruses. The detergents will kill the virus by dissolving the outer membrane of the virus (Nandi *et al*; 1999).

3. MATERIAL AND METHODS

3.1. Description of Study Area

The study was conducted starting from December 2019 to April 2020 in mille district of Zone one of Afar Region, Ethiopia. The Afar National Regional State is one of the ten administrative regions of Ethiopia located in the north-eastern part of the country. The region is divided into five zones, 32 districts and 102 Kebeles. Geographically, the region is located between 8° 49' - 14° 30'north latitude and 39° 34' - 42° 28' east longitude. Administrative Zone 1 is one of five Zones of the Afar Region of Ethiopia. This zone is bordered on the south by Administrative Zone 3, on the southwest by Administrative Zone 5, on the west by the Amhara Region, on the northwest by Administrative Zone 4, on the north by Administrative Zone 2, on the northeast by Eritrea, and on the east by Djibouti. Active outbreaks of sheep and goat pox virus were investigated in five selected pastoral association of Mille district and in two Kebele (Geraro and Weraniso) of Mille district, which are included based on the occurrence of outbreak for the isolation and characterization of the virus from SGP poxvirus. Mille district is named for the Mille River, a tributary of the Awash River. It is bordered on the south by the Administrative Zone 3, on the southwest by Administrative Zone 5, on the west by the Amhara Region, on the northwest by Chifra, on the northeast by Dubti, and on the southeast by the Somali Region. Towns in Mille district include Mille and Eli-Wuha. Geographically Mille district is located at 51 km south of samara capital city of Afar and 594 km from Addis Ababa (ANRS, 2010).

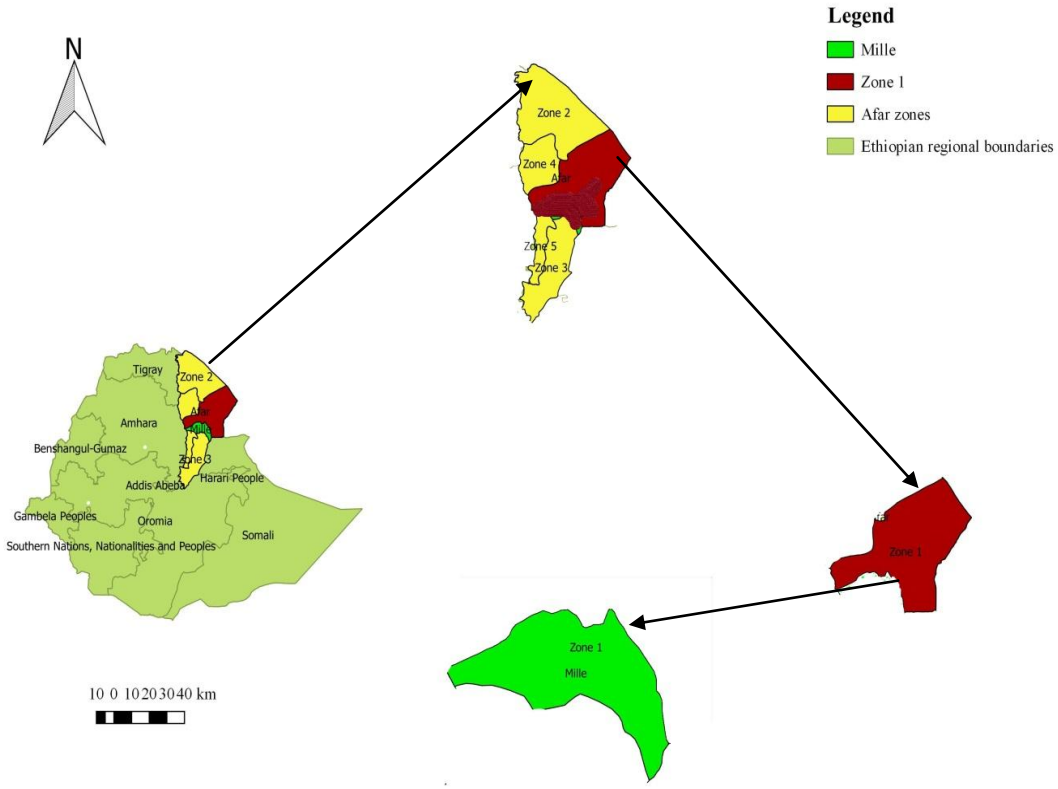


Figure 5; Map of the Mille District

Source: Ethiopia GIS map, 2007

3.2. Study population

The study population was local sheep and goat breed of all age and both sex which were found in flock that reported outbreak of sheep and goat pox from December 2019 to April 2020. Based on this, outbreaks were reported from two kebeles of Mille district in zoon one of Afar region, Ethiopia. Animals were managed under pastoral production system. All animals which experienced clinical presentation and sign of sheep and goat pox were included in the study area.

3.3. Study design and sampling strategy

3.3.1. Study design

The study was conducted from December 2019 to April 2020 for the isolation and molecular characterization of pox virus circulating in sheep and goat pox virus from purposively selected sheep and goat in flocks who reported outbreak of the disease and the animals showed the clinical sign of the disease. The herds were purposively selected based on the occurrence of suspected cases of sheep and goat pox and based on the lists of outbreak reports obtained from the livestock and fishery office of Mille district. When an active outbreak of sheep and goat pox was encountered or reported, field investigations was conducted and information was gathered by interviewing sheep and goat owners, district animal health workers and clinical examination of the cases at field level.

3.3.2. Sampling strategy

Purposive sampling strategy was employed based on follow up of an active outbreak of SGP reports, then based on the report, field investigations was conducted and information was gathered by interviewing sheep & goat owners and district animal health workers. Samples for virus isolation were collected within the first and second week of the occurrence of clinical signs, Tissue samples of skin biopsies were collected from Sheep and goat showing suspected CaPV lesions during the study period. About 3 g of tissue samples were collected and placed in a bottle with a 50% phosphate buffer saline (PBS) at a pH of 7.2 – 7.6 with antibiotics (Gentamycin).

3.3.3. Sample Size

During the study period in outbreak area, the total animal examined was 738. Out of 186 cases the field investigation was conducted purposively at the specific site of the outbreak cases from five Sheep and nine goats (7.5 %) with the clear signs, symptoms and suspected to be Pox disease were sampled purposively and that fresh (unfixed) samples were submitted to the virology and molecular biology laboratory at NVI, Bishoftu, Ethiopia within 24 to 48 hrs of collection.

3.4. Methodology

3.4.1. Observation

A total of two kebeles (Weranso and Geraro) from Mille district of the outbreak occurred were purposively selected and interviewed the animal owners about the outbreak occurred to collected data on the number of sick and dead animals and vaccination history of the affected animals as indicated in (Annex 1) to supplement the work. As soon as particular outbreak areas were reported, after arriving at that specific outbreak site during that disease outbreak investigation, the individual animals was conducted on a total of 186 shoat. These exposed animals were inspected for developing typical sheep and goat pox clinical sign such as, fever, ocular nodule on the skin with different size and lameness, lacrimation, salivation and discharges from the nose and eyes.

3.4.2. Sample collection

The samples for isolation and molecular characterization were collected from clinically sick sheep and goat skin nodule. A total of 63 sheep and 123 goats suspected for pox virus infection were carefully examined for the presence of clinical lesion on their skin. The diagnosis of sheep and goat pox disease was done on the basis of clinical observation of pox lesions. That tissue Samples collected from the outbreak area of clinically sick animals (five tissue from Sheep and nine tissue from goat) showing suspected Capri pox lesion during the study period. About 3 g of tissue samples were

collected aseptically by washing and cleaning the area and removing the hairs with the help of sterile scalpel blade. The collected samples were placed in a universal bottle with a 50% phosphate buffer saline (PBS) at a pH of 7.2–7.6 with antibiotics (Gentamycin) and anti fungal. Species, identification number, sex, age and village was labeled, and immediately placed in a cold box and transported to National Veterinary Institute (NVI), Bishoftu, Ethiopia. Once the samples arrived at NVI, it was placed at -20 ºC until processed.

3.5. Laboratory Diagnosis Techniques

3.5.1. Sample processing

During clinical examination samples for virus isolation and antigen detection were collected from clinically sick animals according to the procedures of OIE (2010), The skin nodule samples were thawed at room temperature and washed three times using sterile PBS containing antibiotics (penicillin) and antifungal at a pH of 7.2 under Bio-safety cabinet class II. About 1 g of the samples was minced using sterile scissors and forceps. Additionally, the minced samples were crashed by using sterile mortar and pestle by adding 9 ml of sterile phosphate buffer saline (PBS) containing penicillin and streptomycin (OIE; 2010). The tissue suspension was centrifuged at 3500 rpm for 10 mints. The supernatant was collected, filtered through 0.45mμ membrane filter and preserved at -80 ºC until use.

3.5.2. Preparation of Vero cell monolayer

Isolation of virus was done by using African Green Monkey kidney Cell line (Vero) (AU-PANVAC, Ethiopia). Glasgow Modified Eagle‟s Medium (GMEM) was prepared (Annex 2) according to the manufacture instruction for cell line propagation and virus isolation (HiMedia, India). The GMEM solution was supplemented with 10% inactivated calf serum, 10% tryptose phosphate Broth (TPB) (Oxoid, England), 1% gentamycin solution prior to used. Vero cell line

provided by NVI, Bishofitu, Ethiopia, was grown in 25cm^2 plastic tissue culture flask (Roux flask) in the facilities of Virology Laboratory of Research and Development Section, having confluent monolayer (90%) observed under inverted microscope. These cell lines were processed for harvesting and transferring to new vessels. The growth medium overlaying the cell monolayer was pour off in a sterile beaker under sterile conditions. The monolayer was rinsed, washed twice with 10 ml sterile PBSA and covered with 5 ml of sterile 0.25% trypsin for about 3 - 5 minutes in an incubator at 37ºC. The monolayer was periodically observed under an inverted microscope for rounding and detachment of cells. The trypsin was removed quickly to avoid wastage of detached cells. The cells detached from the flasks was collected and mixed to form homogenous cell suspension. Equal volume of the cell suspension added to each of the three tissue culture flasks already containing growth medium with 10% fetal calf serum. The whole process was carried out under aseptic and sterile conditions (Bio-safety cabinet Class II). Those flasks were placed horizontally in the incubator at 37ºC. After three days, all the flasks had developed a confluent monolayer with typical cell sheet with light frosted glass appearance, having clearly visible fibroblastic whirls. The cells in that phase was considered fit for sub-culturing and virus infection.

3.5.3. Virus Isolation

Isolation of pox virus was carried out according to a previously described protocol (OIE, 2012). The filed sample suspensions were inoculated on Vero cell lines according to the method of (Balinsky *et al*., 2008). When a complete monolayer of the Vero cell line was formed and almost 80 % confluence was obtained, the processed supernatants kept at -80ºC were thawed in advance of inoculation. The exhausted medium from the tissue culture flask was discarded and filtrate 1 ml of sample suspension then 9ml of sterile maintenance medium added per flask (25cm^2). Five flasks were used.

The following incubation at 37°C for 2 hours for virus adsorption the inoculate were discarded, the flasks were washed three times in the medium, followed by the addition of maintenance medium containing 2% calf serum, penicillin 10,000 UI/ml, streptomycin 100 µg/ml, kanamycin 50 µg/ml and amphotericin B 2.5 µg/ml. The third flask containing confluent monolayer cells was filled with media only and kept as control flask. Those three flasks per sample were incubated at 37ºC and each flask was observed daily for 7 – 10 days under the inverted microscope for any cytopathic effect (CPE) development. The medium having 2% calf serum was changed every 48 hours. When 80% CPE was observed, the flasks were frozen at -20ºC (after pH adjustment). The virus was harvested after two freeze-thaw cycles. When no CPE is visible until day 14, the culture was freeze–thawed three times, and clarified supernatant inoculated on to fresh Vero cell culture. In general, two more blind passages were carried out for samples that were initially negative for CPE.

3.6. Molecular identification

3.6.1. DNA Extraction

The virus DNA extraction was conducted in molecular biology laboratory of the national veterinary institute (NVI), Ethiopia. The virus DNA was extracted from the tissue suspension and homogenate by using DNeasy® blood Tissue Kit (QIAGEN, Germany) according to the manufacturer's instructions. The nucleic acid bound to the silica membrane was eluted and the eppendorf tube was labeled properly and kept at -20ºC until analysis. The whole steps were carried out within microbiological safety cabinet IIA and the details of the steps to be followed for DNA extraction indicated in (Annex 3).

3.6.2. Conventional Polymerase chain reaction (PCR)

The viral DNA was extracted from that cell culture that to do Polymerase chain reaction (PCR) assay was used to detect the virus with CaPV specific primers. PCR protocol described by (Mangana-Vougiouka *et al.*, 2000) was followed. The technique was performed aiming to amplify a small fragment of the 30KDa RNA polymerase subunit (RPO30) gene of CaPV. The technique is able to differentiate SPPV from GTPV, since, the harbor gene a well conserved sequence signature for the genotyping and differentiation of the two poxviruses. Accordingly, technique was conducted to amplify a small fragment of the RPO30 genes using forward and reveres primers and the protocol described by (Lamien *et al.*, 2011). The specific forward and reverse primers had the sequences (SpGpRNApol-F and SpGpRNApol R with sequences 5'TCTATGTTCTTGATATGTGGTGGTAG-3'and 5'AGTGATTAGGTGGTGTATTATTTTCC-3', respectively) that encodes for the text gene and synthesized by VBC Biotech (Vienna, Austria).

The presence of 21 nucleotide deletion in the RPO30 gene of SPPV strains and absent in GTPV strains is the bases of differentiation. The strategy was that the primers flanking region containing in SPPV sequence so that the PCR amplification products from SPPV isolates would be shorter in comparison to those from GTPV isolates (151bp for SPPV and 172bp for GTPV). A total of 14 tissue samples representing from different animal species and geographical area were analyzed by conventional PCR.

The PCR technique was carried out in reaction volume of 20µl containing 3µl RNase free water, 2µl forward, 2µl reverse primers , 10µl IQ super mix and 3µl viral DNA as template. The PCR tube containing 20µl final volumes were transferred into thermal cycler (Applied Biosystems). The thermal cycler (PCR) protocol was performed with an initial denaturation 95ºC for 5minute, followed by 40 cycle of denaturation at 95ºC for 30 second, Annealing 50ºC for 30 second and extension 72ºC for 30 second, final extension at 72ºC for 7 minute by 1 cycle.

3.6.3. Agarose gel electrophoresis of PCR product

The PCR product were analyzed by 3% agarose gel electrophoresis as described by (Lamien *et al*., 2011). The presence of DNA was checked by agarose gel of 3gm and 100ml of Tries/Acetate/EDTA (1X - TAE) buffer. Briefly, the mixture was boiled by micro oven to dissolve and cooled at 55ºC. 4µl GelRed nucleic acid stain (loading dye) was added. The gel was poured on gel caster that placed horizontally and the comb was placed on the caster. After the gel poured when the gel was completely solidified after 15 minutes, then the gel was placed in the electrophoresis tank containing the 1X-TAE running buffers and the comb was removed carefully. The amplified products were analyzed within the first lane 10µl 50 bp DNA markers (Fermentas, Lithuania) was added, while in the remaining lanes 10µl PCR product, non template and positive control of SPPV and mixed with 4µl loading were loaded in each wells in prepared gel by using micropipettes but the micropipette tips changed for each sample, then the gel running tank was connected to power supply and run at 120 voltage for 1:20 hours.

The DNA molecular weight marker parallel with in electrophoresis apparatus until DNA sample have migrated a sufficient distance via the gel. The gel was observed under the UV trans-illuminator gel documentation system and DNA bands picture was captured by using Polaroid photographed camera. The results confirmed according to the size of the bands formed on the agarose gel. Finally, the band size were considered as positive for GTPV 172bp and SPPV 151bp (Lamien *et al*., 2011).

3.6.4. Real-time PCR

The real-time PCR was performed at the molecular biology laboratory NVI, Ethiopia and described by Gelaye *et al*., (2013). The recently developed for species specific methods using unlabeled snapback primer and dsDNA intercalating dye. The assay targeting and used to confirm the CaPV RPO30 gene identify the field isolated and determine the genotype Gelaye *et al*., (2013). The real - time PCR was set up in 20µl

total reaction volume were 4.84µL of RNA free water, 2µl of SpGp RNA pol- F primer, 0.16µl SpGpRNApol-R primer and 10µl of 1xSso fast eva green super mix. Additionally, then 3 µL of sample extracted DNA; no-template (RNAase free water); and positive LSDV, GTPV and SPPV controls was added in duplicate fashion. The PCR protocol was done an initial denaturation at 95ºC for 30 minute/1-cycle, followed by 45 cycle at 95ºC for 15 second and 58ºC for 1: 20 minute. PCR products was denatured at 95ºC for 1-minute, cooled to 40º for 1-minute and heated continuously at 0.5ºC for 10 second with fluorescence acquisition from 45ºC to 85ºC. Lastly the pairs of melting temperature for each snapback tail and the full amplicon was recorded as SPPV 51ºC / 72.50ºC, GTPV 56ºC / 72.5ºC and LSDV 50ºC/73.5ºC for genotyping of tested isolates.

3.7. Ethical clearance

The study was approved by Addis Ababa University, College of Veterinary Medicine Animal research ethical review committee ref. no. VM/ERC /31/03/12/2020 (Annex 6).

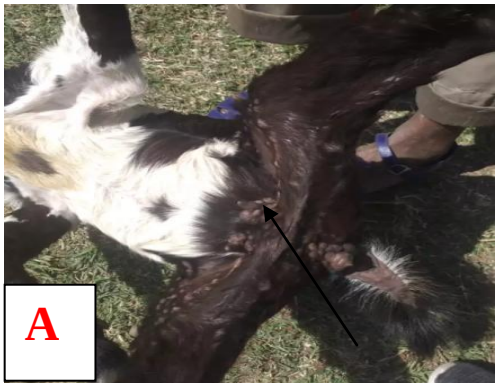
3.8. Data Analysis and Management

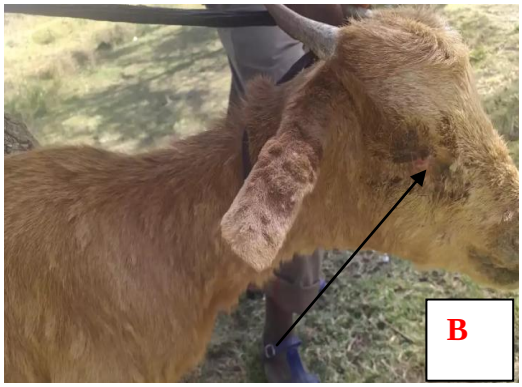
All the data obtained from field during sampling and laboratory analysis was entered and stored into Microsoft office excel 2007 spread sheet. Briefly, the data collected during observation of clinical signs while investigating the outbreak, sample collection, virus isolation, gene amplification, further genotyping by real-time PCR were recorded and stored. The data were thoroughly screened before subjecting to statistical analysis. The data were imported to SPSS (Version 23). Descriptive data analysis was used to summarize the data of lesion and laboratory findings. Morbidity and mortality were determined at district level.

4. RESULTS

4.1. Observation of field clinical examination

During the study period, once outbreak was encountered and investigated. The outbreak occurred on December, 2019 in Mille district at Weranso and Geraro Kebele. The owner of sheep and goat indicated that SGPV was a common disease reported the frequent occurrence of pox disease in their sheep and goat herds. All the owners informed that the disease equally affected both sheep and goat. The owner also added that the protective level of vaccine has not given and the source of outbreaks was new purchased goat from the market of Mille district. As the owners said the local name of the disease is "korboda". The common clinical sign observed in sheep and Goat affected by SGPV were fever, depression, circumscribed nodules on the skin with different size, necrotic nodules under the tail, loss of appetite, nasal discharge, lacrimation, deep scap formation and lameness (Fig .6). Accordingly, they were papules and nodules varying the size on the vulvas, skin, udder, testicles and in the whole free part of the skin. These nodules could be confluence and form lesions that look like skin tumors (fig .6, A, B, C and D), the nodular lesions were increased in number and when palpated felt hard. Moreover, the surviving animals showed skin lesion and scabs toward the end of disease evaluation. During outbreak investigation from the total of 353 local sheep and 385 goats examined.





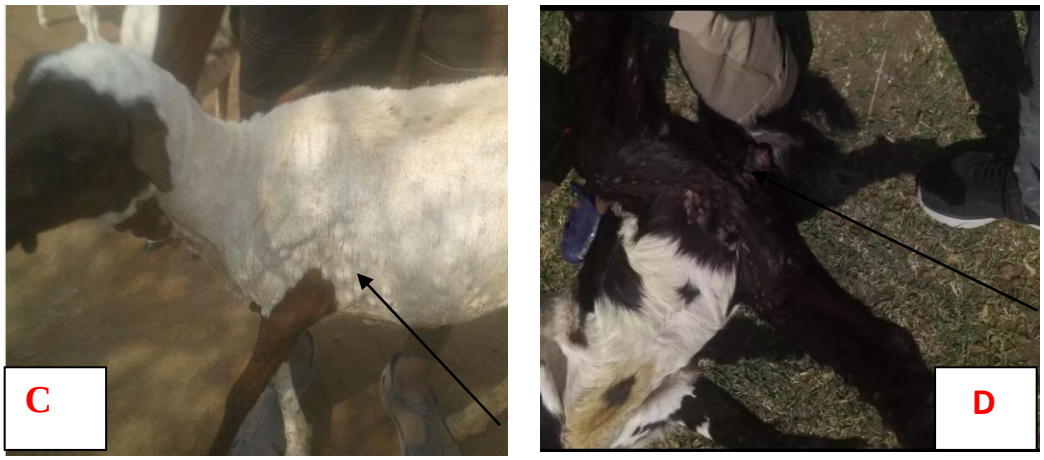


Figure 6: pox lesions showed on clinically diseased sheep and goat

A. Perepennial region, **B**. lacrimation and face region **C.** circumscribed nodules over the entire body and lameness. **D.** circumscribed nodules under the tail

Out of 738 sheep and goat examined, 63 (8.5%) of sheep where as 123 (16.7%) of goats were found to be positive for pox lesion on their skin. The highest number of pox lesion on sheep and goat was observed in Geraro (33.02%) the lowest pox lesion was observed in Weranso (14.6%) in sheep and goat respectively. Accordingly, 14 tissue samples (five sheep and nine goat) with sever pox lesion on their body were sampled (table.2).

Table 2: Number of sheep and goats clinically showed pox lesion and sampled

PAs	**Animals examined**		**Animals with pox lesion**		**Number of sampled**	
	Sheep	Goat	Sheep	Goat	Sheep	Goat
Geraro	187	237	43	97	4	7
Weranso	165	149	20	26	1	2
Total	353	385	63	123	5	9
Over all Total	**738**		**186**		**14**	

Out of 738 examined and 186 sick animals, the morbidity of SGPV within species during outbreak in two kebeles was 17.8 % and 31.9 % respectively. Within different age groups, the morbidity was 33.3 % in young and 21.5 % in adult age groups. Based on the clinical signs the overall morbidity of sheep and goat pox virus was 25.2 % (table 3) which was largely observed on young age groups (77 case/ 231 observations). Statistically there was significance difference among Age group and species ($P < 0.05$) but there was no statistical significance among sex groups ($P > 0.05$).

Table 3: Proportion of morbidity by SGPV within species, age group and sex groups

Risk factors	**Category**	**Animals Examined**	**Animals Sick**	**Morbidity Rate (%)**	**Chi-square**	**P-Value**
Species	Sheep	353	63	17.8 %		
	Goat	385	123	31.9 %	0.463	.0001
Age	Adult	507	109	21.5%		
	Young	231	77	33.3 %	1.363 %	.0001
Sex	Female	301	69	22.9 %		
	Male	437	117	26.8 %	1.157 %	.0001
The Overall Proportion		**738**	**186**	**25.2 %**		

Based on clinical sign the mortality rate with in species was 2.6 % in goat, 1.98 % in sheep and within different age groups, 1.2 % in adult and 4.8 % in young age groups and within different sex groups, 2.97 % in male and 1.3 % in Female. This reveals that the average mortality of sheep and goat pox virus was 2.3 % % (table 4). From this observation we can conclude that there is the higher mortality in Goat than in sheep, in young age groups than adult age group and in male than in female. Generally there was significant difference between age group ($p < 0.05$) but there was no significance difference b/n species and sex group ($p > 0.05$) and 95% CI.

Table 4: Proportion of mortality by SGPV within species, age group and sex groups

Risk factors	Category	N.o of animals examined	N.o of animals died	Mortality rate (%)	P - value
species	Sheep	353	7	1.98 %	
	Goat	385	10	2.6 %	.0001
age	Adult	507	6	1.2 %	
	Young	231	11	4.8 %	.002
sex	Female	301	4	1.3 %	
	Male	437	13	2.97 %	.0001
The overall proportion		**738**	**17**	**2.3 %**	

4.2. Virus Isolation

The suspensions obtained from processed skin tissue were inoculated in to African Green monkey kidney cells (Vero cells) after filtration through 0.22 µm filter. Out of the 8 inoculated samples 3 of them produced cytopathic effect at the 3rd passage (fig. 9) characterized by small rounding of single cells aggregation of dead cells and distraction of the monolayer on Vero cell line culture starting from the 7th day up to 14th day post-inoculation. But none of negative control produced any CPE. The virus was harvested within 7-14 day of inoculation for further diagnosis.

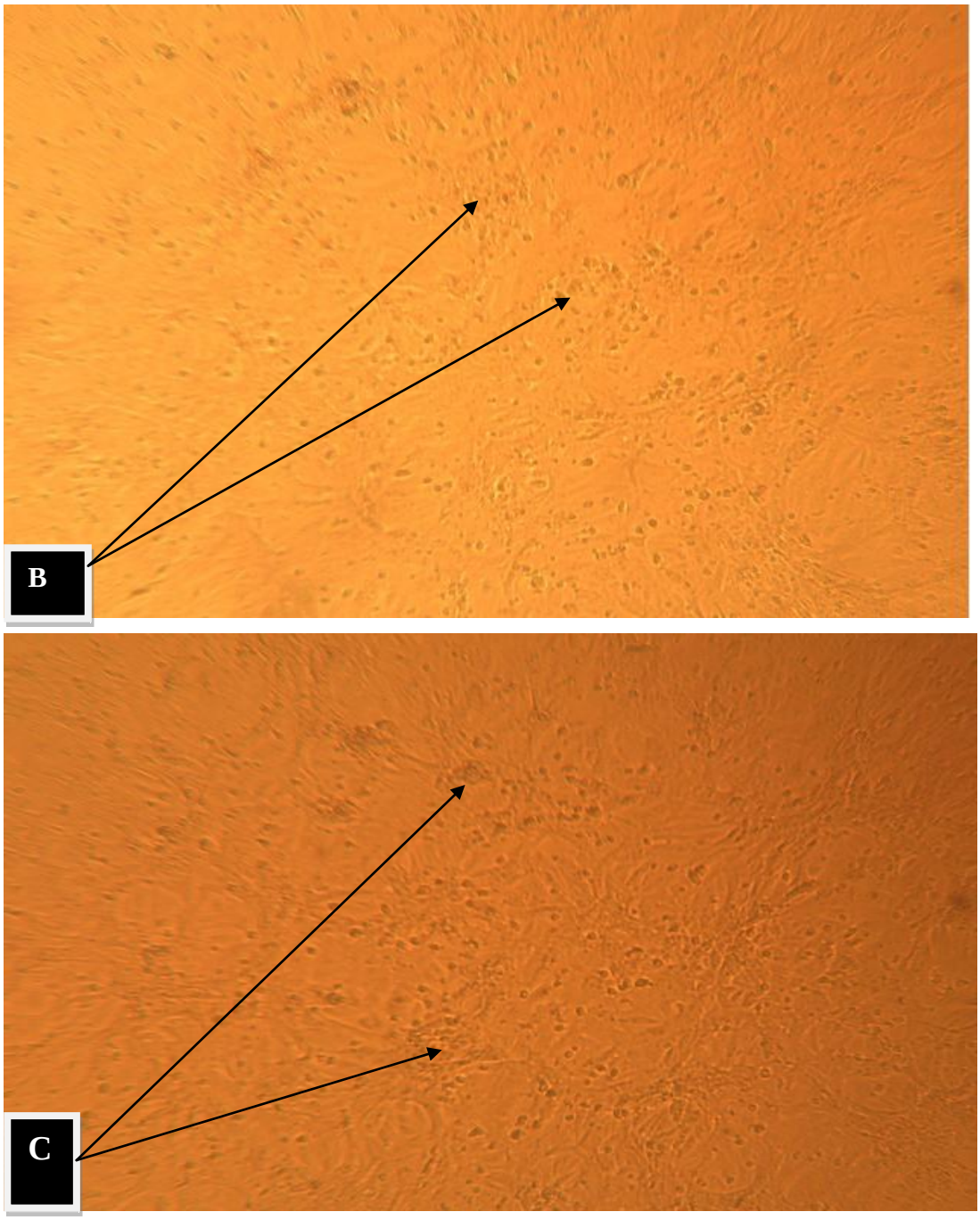


Figure 7: The CPE picture taken by using camera fitted inverted microscope

A). none infected Vero cell (Negative control)

B) & **C).** Cells developed characteristics CPE of SGPV at day 13 & passage 3

4.3. Conventional polymerase chain reaction (PCR)

The extracted viral DNA of all 14 specimens (nine from goats and five from sheep) were analyzed by standard PCR. Similarly, out of these, 11 (78.6 %) specimens were positive; with respective to two kebeles, 11specimens produce band size of 172 bp on agarose gel electrophoresis (fig 10). In spite of the fact that, three specimens from Geraro and Weranso, i.e., Geraro-2 on lane 3, Weranso- 6 on lane 9 and Weraso- 9 on lane 13 were again negative since, they could not produce any band on agarose gel electrophoresis. Positive bands of 172 bp size fragments of GPV on 3% agarose gel electrophoresis as indicated in the following picture below.

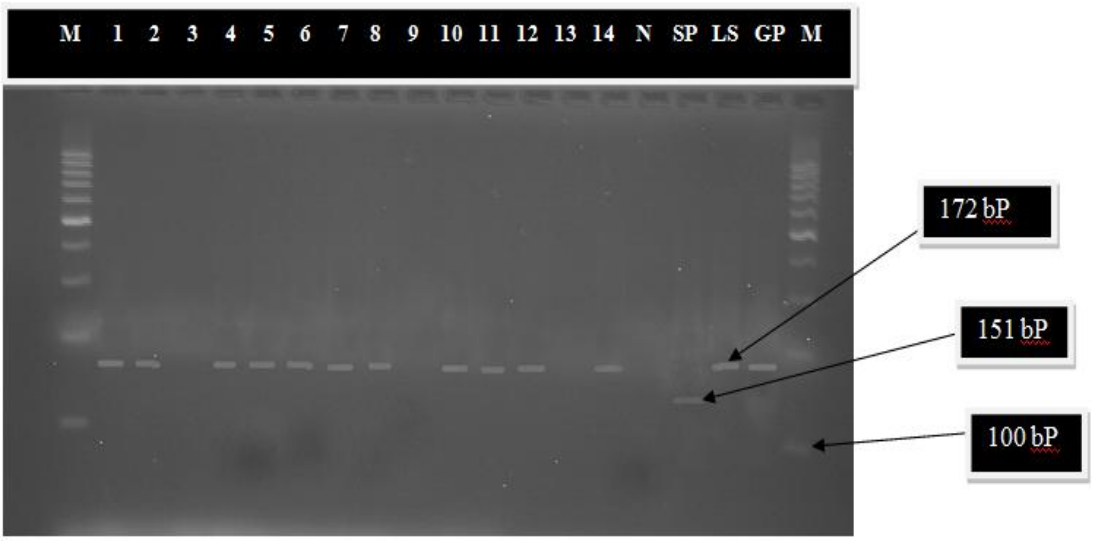


Figure 8: Agarose gel electrophoresis on fragment of the RPO gene of *SPV, GPV and LSDV* by the use of specific primer.

Where; **Lane M**: Molecular marker started at 100bp (Ladder, Fermentas), **1, 2, 4, 5, 6, 7, 8, 10, 11, 14** Positive field samples and **3, 9, 13** negative field samples, **N**: Negative controls without template- no amplification, **SP:** Sheep pox virus positive control, **LS:** Lumpy skin disease virus positive control, **GP:** Goat pox virus positive control.

4.4. Real time PCR

The classical PCR could not differentiate GTPV from LSDV since both have 172bp PCR product, in spite of the fact, it could be differentiate SPPV (151bp) from LSDV (172bp) as shown in the gel picture. So, the presence of CaPV from sheep and goat samples was confirmed and farther genotyped using gene specific Real-time PCR method. Such because of the snap beak assays targeting the RPO30 gene. The extracted viral DNA was again subjected to Real-time PCR. Additionally, the peak melting curve of real-time PCR revealed that all 5 field virus isolates recovered from sheep were characterized as GTPV since their snapback of melting peak were at 56ºC while the 2nd melting peaks were at 72.50ºC (Fig. 10). Similarly, the GTPV field isolates was tested and confirmed to be as GTPV. The real - time PCR assay detected differences in the melting point temperatures for GTPV, SPPV and LSDV after fluorescence melting curve analysis from each other (Fig. 10). Generally, the result between conventional PCR and Real- time PCR techniques was 100% agreement.

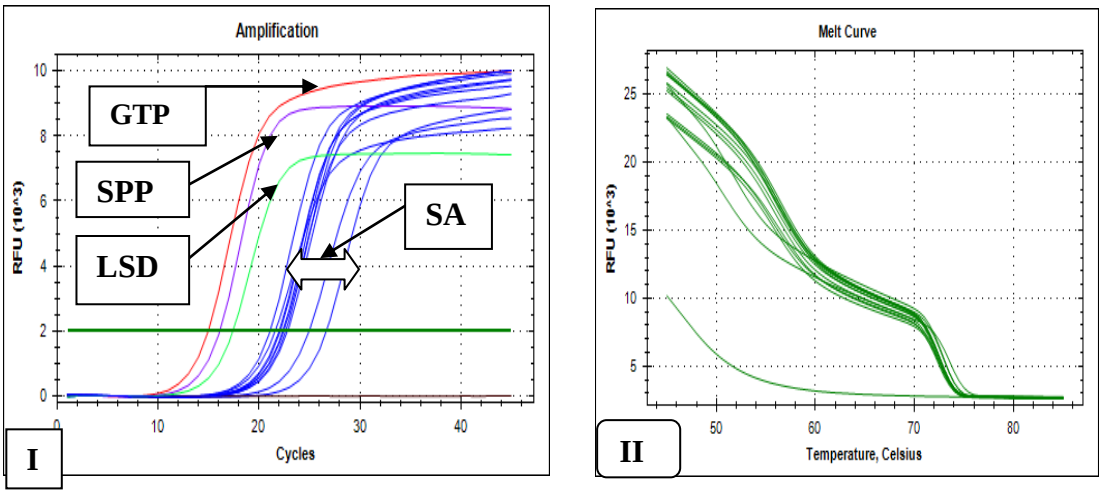


Figure 9: **I,** Amplification plot and **II,** Melting curve plot

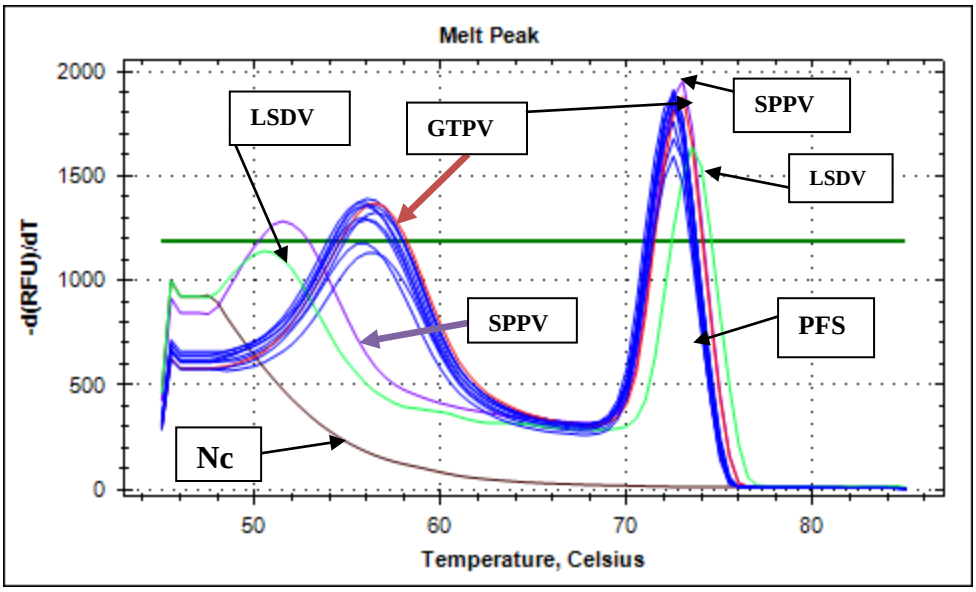


Figure 10: Differences in the melting curve of Known positive CaPV and field isolates of SGPVs

Where: **GTPV**: Positive sample for goat pox virus, **SPPV**: Positive control for sheep Pox virus, **LSDV**: positive control for LSD virus, **FS:** Positive field Samples and **Nc:** Negative control.

5. DISCCUSSION

The present study was conducted to provide the adequate information regarding the occurrence of SGPV outbreak causes in selected district of zone one of Afar region. The diseases are common in Ethiopia and cause a very huge economic loss to the leather industry and farming community. SGPV disease is endemic and one of listed disease in the country (OIE, 2009). In spite of the fact that, there are no detailed studies on prevalence of SGPV in Ethiopia especially in Afar region, although, some reports indicate that SGPV is common small ruminant production problem and widely distributed in the country (Mersha, 2011).

In the present study the present data collected have provided a reliable indication of extent and that severity of SGPV in selected district of zone one of Afar region specifically in Mille district. The outbreak was confirmed by clinical finding, virus isolation and PCR diagnosis that are caused by SGPV disease. According to observation the clinical signs were fever, in appetence, depression, circumscribed skin nodules, necrotic nodules under the tail, perineum, testicle and udder, nasal discharge, lacrimation, salivation and death. These clinical signs have been documented as the characteristic clinical features of SGPV (Carn and kitaching, 1995; Radostits *et al.,* 2007). The lesions found under the skin surface of perineum, udder, tail, head and neck. These observations were also similar to that of (Singh *et al.,* 1979; Davies, 1981; Sharma *et al.,* 1986; Mersha, 2011; Radostits *et al*., 1994; Abraham; 2019) who observed the nodular form of pox lesion as round firm flat surface nodules on the thoracic and the lateral aspect of the abdomen.

The local name given to SGPV by the farmers is " *Koriboda*" but that the disease was named with different in other part of Ethiopia, which they called "*Finno hoolaa"* in Afan Oromo and "*Fentata*" in Amharic, Similarly, Mersha (2011), Teferi (2014), Assefa (2017) and Damena (2019) was also reported. According to the owners response the occurrence of SGPV high at rainy season and decrease in dry season. This result is supported by the work of other researchers who showed the incidence of SGPV to be high during wet seasons when the biting –fly populations are abundant and it decrease during dry seasons (Ali *et al*., 2006; Radostits *et al*., 2006; Gari *et al*., 2010) and the disease is often observed throughout summer and the beginning of the spring season (Assefa, 2017).

In the present study all owners informed that the disease are equally affected both goat and sheep with more morbidity and mortality in goat population. This study is disagreement with the study of Assefa (2017) which reported that more morbidity in sheep population but the study is in agreement with that young age groups are more susceptible than adult age groups of both species and high mortality in young age groups. This study was in consistence with the finding of Schwab *et al*., (1977), Assefa (2017) and Damena (2019) who reported the morbidity and mortality of SGPV was high in young age groups are more susceptible than adult age groups of both species and high mortality in young age groups than adult age group. The overall, male sheep and goats are more susceptible than female. This study is disagreement with Damena (2019) who reported female sheep and goat are more susceptible than male. The significant association of SGPV occurrence with various type of physiographic, ecosystem, soil type, rainfall, relative humidity and temperature has been studied and all these factors have strong influence on disease occurrence (Murray *et al*., 2003).

The present study according to the observation the overall, 25.2 % morbidity and 2.3 % mortality of SGPV with in species 17.8 %, 1.98 % in sheep and 31.9 %, 2.6 % in goat respectively. The present study result were not far from the result of Assefa (2017) who reported morbidity and mortality of SGPV with in species in outbreak area of Adea berga district were 32.1 %, 4.7% in sheep as well as 29.4 %, 6.5% in goats respectively, and Damena (2019) who reported in outbreak area of central Ethiopia were 35.82 %, 9.95% in sheep and 28.44 %, 3.66% in goat respectively.

The laboratory conformation was made by virus isolation on cell culture, out of 9 tissue samples taken from goats and 5 from sheep, 11 (78.6b%) samples showed typical pox lesion after cultivation on Vero cell lines that are to detect typical cythopatic effect (CPE) to SGPV. The result of CPE in present study, the goat pox virus induced CPE like cell ballooning, very rounding, aggregation and detachment of the cell was observed within 7-10 days of incubation. Out of 14 tissue samples, goat pox virus was isolated from 11 samples using Vero cell line, while two samples could not develop any CPE in three passages. These studies were in agreement with Sajid *et al*., (2013) and Teferi (2014) reports who reported development of CPE within 7-10 days.

The PCR reaction is sensitive, quick and reliable methods as antigenic resemblance of SGPV with LSDV that makes the diagnosis through routine serological tests difficult (Tuppurainen *et al.,* 2005; Anonymous, 2010). In the present study, all isolates were identified as GTPV after sequential diagnosis using classical PCR and real-time PCR. Out of 14 samples 11 samples yielded a PCR product size of 172bp on agarose gel electrophoresis. Accordingly, the virus isolated from both goats and sheep were not SPPV because the PCR product of gel electrophoresis is greater than 151bp. The results of all new 11 field isolates recovered from sheep were genotyped as GTPV. The present study is in agreement with the previous conducted study of Le Goff *et al*., (2009), Lamien *et* al., (2011), Gelaye *et al.,* (2013), Assefa (2017) and

Damena (2019) who reported the genotyping result of SPPV was 151bp and GTPV was 172bp and the pox lesions collected from clinically diseased sheep from different countries of the world. This result clearly explained that both goat and sheep were equally susceptible to GTPV. There was a 100% in agreement between the result of conventional and real-time PCR techniques.

SGPV are not considered as host specific and in spite of the fact that, the majority of strain shows a host preference, a single strain may cause disease in both sheep and goat. Similarly, the authors described that sheep may become infected with virulent goat strain. This result agreed with Heine *et al*., (1999). Moreover, that also described by Gelaye *et al.*, (2013, the GTPV/SPPV (Tm=72.5ºC) and the snapback melting temperature to differentiate SPPV (51ºC) from GTPV (56ºC) during the real- time PCR method.

6. CONCLUSION AND RECOMMENDATIONS

SGPV disease was known to be the major sheep and goat health problem causing huge economic losses due to reduced weight gain, permanent damage to hides, temporary or permanent infertility and abortion. The present study approved that host specificity classification of CaPV is inaccurate at least for GTPV. GTPV is well known by farmers in selected zone of Afar region especially in Mille district and the virus circulating in the study area. According to this study result GTPV is more prevalent in rainy season. GTPV caused moderately high morbidity and mortality in study area which may be associated with direct and indirect economic losses. Furthermore, the disease mainly affected male and young age goats. The Illegal animal movement was the major risk factor for transmission of GTPV in study area. The current study may provide a new insight in micro-biology of SGPV in Ethiopia for a big implication of disease control. Vaccination is the only effective method to control GTPV disease in the country like Ethiopia. In spite of the fact that, the current study result showed the accessibility of vaccine and the presence of vaccine failure is in the study area. Therefore, based on the above conclusion the following recommendations are forwarded:

- As the SGPV is widely occurring in Afar Region, coordinated and sustained control program using vaccination should be practice in the affected areas.
- At a national level, the government should design strategic policies for effective control and eradication of the SGPV disease.
- Further studies are needed to investigate the epidemiology and risk factors for SGPV infection in Ethiopia especially in Afar region.
- Further isolation and molecular characterization of SGPV should be conducted so as to identify strain of the virus in Ethiopia in order to produce effective vaccine for circulating viruses.

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8. ANNEXS

Annex 1: Questionnaire format

1. Background information:

   Region: _____

   Zone: _____

   District : _____

   Keble: _____

   Date: _____

2. Number of Sheep and Goat per PA : _____
3. Disease Detail
   a. Local Name of the disease: _____, meaning:_____
   b. Season of Occurrence

      ☐ Dry Season
      ☐ Rainy Season
      ☐ Any Season

   c. Frequency of out break

      ☐ Every year
      ☐ Every two year
      ☐ Every three year
      ☐ Greater than three year
4. Animal at risk

Species		Age		Sex		Animal at risk
Ovine	Caprine	Adult	Young	Male	Female	

5. Animals Affected

Species		Age		Sex		Animals affected
Ovine	Caprine	Adult	Young	Male	Female	

6. Clinical Sign Observed

Fever		Lesion on skin		Presence of nodular Lesion		Lameness	
Yes	No	Yes	No	Yes	No	Yes	No

7. Source of Outbreaks

☐ Introduction of infected Animals

☐ Movement of infected Animals

☐ Contact at Communal points

8. Total number of Animals Dead

Species		Age		Sex		Total Animals Died
Ovine	Caprine	Adult	Young	Male	Female	

9. Vaccination History

d. Have you vaccinated your animals against SGP Yes ☐ No ☐

e. If the answer 9.1, Yes, Who gave the Vaccine? _____

f. Number of get Sick (N.o of Sick/total N.o Vaccinated) _____

g. Number of dead (N.o of dead/total N.o of Sheep and Goat? _____

h. Opinion of the owners about the vaccine? _____

i. What should be done in the future? _____

10. Impact of SGPV disease

S/N	**Impact of SGP type**		Yes	No
1	Animal body weight loss			
2	Loss of production	Milk yield		
		Meat yield		
		skin and hide production		
3	Abortion			
4	Traction Power			
5	Mortality			

Annex 2: Laboratory Procedures for virus isolation

I. Propagation of Vero Cell

Vero cell for virus isolation, Vero mono layer cell sheets were prepared from previous available cell lines according to the following procedures:

1. Prepare the required materials such as , sterile flasks ($75m^2$) and pipettes
2. The monolayer cell line was washed by using PBS solution that containing antibiotics to avoid bacterial contamination.
3. 2 ml of Trypsin was discarded and that flask was incubated at 37ºC for 3 – 5 minutes to digest and detachment of the cell to joining /linking the single cell.
4. 10 ml of GMEM mediums was added to the flask to stop the trypsin action and mixed very well by pipe ting to break the remaining cell bonds that are not separated by Trypsin
5. Then the additional 10ml of GMEM was added up to the 30ml mark of the flask
6. Then 20 ml of the solution was transferred in to five other sterile flasks (10ml for each flask) by using pipette
7. All the five flasks were incubated at 37ºC for 24 hours
8. After that 24 hours we were obtained five clean Vero monolayer cell lines

A. Glasgow's modified minimum Essential Media (GMEM) composition

- ✓ GMEM Powder 12.5 g
- ✓ De ionized Water 1000ml

✶ Preparation of GMEM (10%)

- ✓ Dissolve the GMEM powder and De ionizes water then dispense into 100-200 ml screw cap bottle
- ✓ Incubate the bottle at 4ºC after sampling for sterility testing
- ✓ Added the Antibiotic stock solution (penicillin, 5X 106 IU vial + streptomycin 5X Ig vial in 100ml of PBSA) to have it at a final concentration of 0.2%.

B. Dulbecco's Phosphate Buffered Saline (PBSA) Composition

- NaCl 8.0g
- KCl 0.2g
- KH2 PO4 0.2g
- Na2H PO4 $2H_2O$ 1.44g
- Distilled Water 1000ml

✶ Preparation of PBSA

- ✓ Make up with 1000ml distilled water
- ✓ Dissolve, then make up to that desired volume
- ✓ Mark the liquid level before autoclaving
- ✓ Adjust the PH to 7.2 at 20ºC or PH to 7.4 at 36.5ºC with IM Na OH
- ✓ Then dispense into that 200ml bottles
- ✓ Sterilize by autoclaving at 121ºC for 15minute
- ✓ Finally store the solution bottles at +4ºC

C. Trypsin 2.5 % solution (10 X stock solution) Composition

- Deionized and distilled water 1000ml
- Trypsin (1:25) 25g
- NaCl 8.0g
- KCl 0.4g
- $Na_2 HPO_4$ 0.0475g
- KH_2PO_4 0.06g
- $NaHCO_3$ 0.35g

✶ Preparation of Trypsin 2.5%

- ✓ Dissolve by string over night at +4ºC
- ✓ Sterilize by filtration via a µm membrane filter
- ✓ Distribute aseptically into 100ml tube
- ✓ Take sample for sterility test and store at -20ºC for use add 100ml to 900ml sterile PBSA and adjust PH to 7.8 by addition of sterile IM NaOH .

★ Trypsin 0.05% EDTA 0.02% solution for sub culturing composition

- PBSA 970ml
- Trypsin 2.5% 20ml
- EDTA* 2% stock solution 10ml

D. EDTA (Versene) 2% stock solution composition

- ✓ EDTA di-sodium salt $2H_2O$ 22.14g
- ✓ KCl 0.20g
- ✓ NaCl 8.0g
- ✓ Phenol red 1.0g
- ✓ $Na2HPO_4$ 1.15g
- ✓ KH2PO4 0.2g

★ Preparation of EDTA 2% working solution

- Dissolve and make up 1000ml with de ionized distilled water
- Distribute that in 100ml volume into screw cap bottles
- Autoclave that at 121ºC for 15min
- Take the sample for sterility test
- Finally store at - 20ºC

E. Streptomycin and penicillin stock solution composition

- ✓ Streptomycin sulphate 5 x 1g vial
- ✓ Sodium penicillin 5 x 106 i.u vial
- ✓ PBSA 100ml

★ Preparation of penicillin and streptomycin working solution

- Add 5ml PBSA to each vial aseptically
- Leave that for few minutes to dissolve
- Remove the PBSA from vials and back to the PBSA 100ml bottle then mix well
- Finally dispense 10ml into sterile containers and store at -20ºC

II. Propagation of tissue sample in Vero cells

- The deep freezed skin tissue samples were taken from -20 refrigerators and incubated at 37ºC for some minutes to warm the sample suspension and to melt the virus transportation medium (PBS + Antibiotics).
- About 1 gm of skin tissue Sample was taken and placed on a mortar, washed for three times with PBS solution containing antibiotics.
- The tissue was cut into smaller pieces using scissors and forceps then grinded with pestles
- 9 ml of PBS solution containing antibiotic was added in to the mortar by using pipette, very well mixed then transferred in to test tubes
- Then that test tubes were centrifuged at 3500 rpm for 10 minute, the supernatant was taken using a disposable syringe and transferred in to a bottle that have a filter paper with a diameter of 0.22 µm which do not pass large particles
- The Vero monolayer cell lines were washed using PBS and about 0.5 ml of the filtered supernatant was inoculated and flasks were incubated for one hour at 37ºC prior to addition of GMEM medium.
- Then 2% GMEM medium was added to the flasks and incubated at 37ºC for daily follow up for any CPE on the cell lines
- Five samples show the CPE at the 10^{th} day post inoculation and place at deep freeze until processed to prevent cell again.

Annex 3: Laboratory procedures for DNA Extraction and Amplification

I. DNA Extraction composition

✓ QIAamp spin columns	14 samples
✓ Collection tubes	750µl
✓ Buffer AL	
✓ Beffer AW1	
✓ Buffer AW1	
✓ Buffer AE	
✓ Protinase K	

II. DNA Extraction procedures

- The deep freezed tissue samples were freeze and thawed many times for homogenizing / melting and then were transferred in to cry vial tube and transferred to molecular biology laboratory.
- About 200 µl cultured virus was taken and transferred to 1.5ml micro centrifuge tube and equal amount lyses buffer (Buffer ATL) then mixed by vortexing.
- Added 20µl proteinase K enzyme, mixed by vortexing and incubate at 56ºC for 1 hour in water bath and then again mixed by vortexing.
- Then added 200µl of buffer AL and mixed by vortexing
- 200 µl 96 – 100% ethanol was added and again mixed by vortexing
- The mixture was pipe ting in to DNease mini spin column that placed in 2ml collection tube and that centrifuged at 8000rpm for 1 minute but that the collection tube and flow through was discarded.
- The DNease mini spin was placed in new 2ml collection tube and 500µl buffer AW1 was added and centrifuged at 8000rpm for 1 minute and that the collection tube and flow through was discarded.
- The DNease mini spin was placed in new 2ml collection tube and 500µl buffer AW2 was added and centrifuged at 14000rpm for 3 minute to dry the DNease membrane. the flow through and the collection tube was discarded.
- The DNease mini spin was placed in new 1.5 ml micro centrifuge tube and elute the DNA by adding about 200µl buffer AE to the center of DNease spin column membrane. Then incubate that at room temperature for 1 minute and centrifuged at 8000rpm for 1 minute.
- Finally the solution which is extracted that contains viral DNA was obtained which needs conformation through polymerase chain reaction (PCR) assay.
- The extracted viral DNA was stored at -20ºC until processed through PCR assay.

Annex 4: polymerase chain reaction (PCR) assay

I. Conventional polymerase chain reaction (PCR)

1. Selection of primers appropriately and preparation of working solution

- ✓ SpGpRNAPol-F and SpGpRNAPol-R with sequences from 5' to 3' 5'TCTATGTCTTGATATGTGGTGGATAG-3' and 5'AGTGATTAGGTGGTGTATTATTTTCC-3' respectively were selected
- ✓ According to manufacturer instruction the two coated primers were diluted with RNA free water of appropriate volume to prepare a stock solution
- ✓ About 20µl of the stock solution was mixed with 200µl of RNA free water to prepare a working solution and then putted in ice.

2. master mix preparation was prepared by mixing

- RNA free water 3µl
- Primer SPGP RNA pol - forward 2µl
- Primer SPGP RNA pol - Reveres 2µl
- 1µl of 25 mm MgCl
- 0.5 µl of Taq DNA polymerase. → IQ supper mix SPGP
- 2 µ l of each dNTP 10µl
- 5 µl of dream taq buffer.
- Template (extracted viral DNA) 3Ml

3. The PCR Protocol

- ✶ About 17µl of master mix solution was transferred in to a micro centrifuge tube
- ✶ About 2oµl of master mix solution was added in to one tube as a control
- ✶ Than 3µl of extracted viral DNA was added to the 20µl tubes but not for the control tube and centrifuged to that mix the Extract DNA with the master mix solution

- The mixture was inserted in to thermo cycler for amplification and adjusted to 4 steps, such like; denaturation, Annealing, Elongation and final elongation. 1st cycle: at 95ºC for 5 minute (initial denaturation), 2nd cycle: 94ºC for 1 minute, 55ºC for 1 minute, 72ºC for 2 minute and last cycle 72ºC for 7 minute.
- Finally hold that at 4ºC until analysis.

4. preparation of TAE (Tris Acetate EDTA) Buffer

- Reagents (per litter 50 x solution)
  - ✓ Tris base 242g
  - ✓ 0.5 M EDTA 100ml
  - ✓ Glacial acetic acid 57.1ml
- Measure 600ml bi-distilled water by using graduated cylinder and pour into Pyrex beaker
- Stir bar was added and place on stir plate to begin stirring.
- Then 242g tris, 0.5M EDTA and 57.1ml acetic acid was added
- That was allow stir to until tris goes to solution and if needed adjest the PH 8.0
- Then brining 1µl volume, transfer to a clean glass bottle and store at room temperature
- Finally, dilute 1x before use (2ml 50x TAE per 98ml waer)

5. Composition of Gel Red dye (10ml)

- Glycerol 7.5ml
- Bromophenol blue 2mg
- 0.5M EDTA 20µl
- 1M tris 100µl
- Xylene cyanol 2mg
- 10 SDS 200µL
- H_2O 2,2ml

6. Protocol and Preparation of Agarose gel electrophoresis

- ✓ The recommended agarose gel concentration was 3.0%.
- ✓ 3% agarose gel prepared in Trise/Acetate/EDTA (TAE) buffer and mixed very well
- ✓ Then until melting boiled with micro- oven at 180ºC for 3 minute
- ✓ Then a comp was putted in to a gel forming dish and the melted gel solution was dispensed over the dish and allowed to settle for 30 minutes at room temperature.
- ✓ A bout 2µl of loading buffer was added to a new tube and mixed with 10µl of above solution.
- ✓ All 12µl mixture was taken from the tube in to the wells of the comb on the gel dish
- ✓ Then added the negative control after one gap following the last test mixture
- ✓ The gel electrophoresis was connected to power and switched on.
- ✓ It was allowed to stand for 1 hour and 42 minutes to allow the DNA to move from the negative pole to the positive.
- ✓ Then the gel was taken and observed under UV light for flouresencing of base pairs.
    - ✶ The thumb rule is to use 1µl of gel loading dye pr 5µl of sample PCR leader.
    - ✶ PCR 100 bp molecular ladders (fermentas) have been used for size determination of PCR generated DNA fragments.
    - ✶ The ladder ranking from 100-1000bp.
    - ✶ The ladder is supplied as a solution in 10 mM tris HCL, PH 7.5-8.0, with 1.0 mM EDTA.

7. Preparation and protocol of Gel red dye (loading buffer)

- To make 10mg/ml stock weight 1.0 g and add it to 100ml distilled water
- Then add a stir bar and let it dissolve for several hours to overnight
- Finally store it in brown bottle (good for just all applications)
- Gel Red is a non mutagen but must be handled with care.

Annex 5: Real Time PCR

1. Appropriate selection of primers and preparation of working solutions

- SpGpRNA pol forward and reverse with sequence from 5' to 3' 5'GGTGTAGTACGTATAAGATTATCGTATAGAAACAAGCCTTT 3') and 5'AATTTCTTTCTCTGTTCCATTTG-3 respectively.

2. Preparation of master mix solution

- PCR was set up in reaction volume of 20µl
- RNA free water 4.84µl
- SpGp RNA pol forward 2µl
- SpGp RNA pol reverse 0.16µl
- 1x S so fast Eva Green super mix (Biored) 10µl
- Then 3µl of sample extracted viral DNA (Template), no template (RNA free water), positive controls LSDV ,GTPV and SPPV was added in duplicate fashion

3. **The PCR was performed in a CFX96TM real time PCR detection system with an;**

- ♥ Initial denaturation step at 95ºC for 3 minutes
- ♥ That followed by 45 cycle of 95ºC for 15 sec, and 58ºC for 1:20minute by using a Low Profile Hard-Shell H 96-well PCR plate (Bio-Rad).
- ♥ The PCR product was denatured at 95ºC (hold for 1 minute) and cooled at 4ºC (hold for 1 minute).
- ♥ Then heated continuously at 0.5º C for 10 second with fluorescence acquisition from 40ºC to 85ºC.
- ♥ The melting temperatures were analyzed by using the CFXTM manager software version 2.0(Bio-Rad).
- ♥ 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 NA sequences, was also used to plot the melting profile of the three genotypes using the Precision Melt Analysis TM Software (Bio-Rad).
- ♥ Normalized melt curves and difference in curves were acquired by selecting pre-and post-melt regions for amplicons separately.

Annex 6: Ethical clearance certificate

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ADDIS ABABA UNIVERSITY
College of Veterinary Medicine
and Agriculture
Bishoftu/Debre Zeit

Animal Research Ethics Review Committee

Ethical clearance certificate

Certificate Ref. No: VM/ERC/31/03/12/2020

Name of Applicant: Ageritu Mohammed (DVM, MVSc fellow)

Address: College of Veterinary Medicine and Agriculture (Addis Ababa University)

Title of the project: *Isolation and molecular characterization of poxviruses circulating in sheep and goats from outbreak cases in selected districts of Zone one of Afar Region, Ethiopia*

Date of application:	**28/02/2020**
Nature of the project:	**mildly invasive**
Target animal species:	**Sheep and Goats**
Number of animals involved:	**600**
Study area:	**Afar Region, Ethiopia**

Minutes No. and date of review: **VM/ERC/03/12/020, 11/03/2020**

The above indicated research project is acceptable from ethical perspective, relevance, originality and technical competence points of view. Hence the project is ethically sound to be executed provided that:

1. All procedures and conditions stipulated in the proposal are respected, minor comments are corrected and any deviation or changes be reported to the committee
2. The project activities be open for occasional supervision by the committee when this is deemed necessary

Dr Getachew Terefe
Chairman

Signature

The Federal Democratic Republic of Ethiopia
Addis Ababa University
College of Veterinary Medicine and Agriculture

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