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OUTBREAK INVESTIGATION, ISOLATION AND MOLECULAR CHARACTERIZATION OF SHEEP AND GOAT POX VIRUS IN CENTERAL ETHIOPIA

Msc Thesis

By

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Department of Veterinary Microbiology, Immunology and Public Health

Msc program in Veterinary Micro-biology

June, 2018

Bishoftu, Ethiopia

OUTBREAK INVESTIGATION, ISOLATION AND MOLECULAR CHARACTERIZATION OF SHEEP AND GOAT POX VIRUS IN CENTERAL ETHIOPIA

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

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Aberaham Damena Aweke

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Entitled: **Outbreak Investigation, Isolation and Molecular Characterization of Sheep and Goat Pox Virus in Central Ethiopia**

And recommend that it be accepted as fulfilling the thesis requirement for the degree of: Masters of ……………………………………………………………………………….

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DEDICATION

This piece of work is dedicated to my grand Mather Ms. Mulunesh Tesema Duressa.

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STATEMENT OF AUTHOR

First, I declare that this thesis is my bona fide 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 Msc 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 solemnly declare 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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LIST OF ABBREVIATIONS

AGID	Agar gel immune diffusion
CaPV	Capri pox Virus
CIE	Counter Immune electro phorosis
CPE	Cytophatic effect
DNA	Deoxy nuceic acid
EDTA	Ethyline diamine tetra acetic acid
ELISA	Enzyme linked immune sorbent assay
ESGPIP	Ethiopian sheep and Goat production improvement program
EU	European Union
GDP	Gross domestic product
GMEM	Glasgow's modified minimum essential medium
GTPV	Goat pox Virus
ICTV	International committee taxonomy of viruses
IEF	Immune electro focusing
LSDV	Lumpy Skin Disease Virus
NVI	National Veterinary Institute
OIE	Office international des epizootic
PBS	Phosphate buffer solution
PCR	Polymerase chain reaction
PPR	Pest des petits Ruminants
REA	Restriction enzyme analysis
RNA	Ribo nucleic acid
SGP	Sheep and Goat pox
SPV	Sheep pox virus
$TCID_{50}$	Tissue culture infective dose $_{50}$

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ABSTRACT

A cross sectional observational study was conducted from September, 2017 to March, 2018 to outbreak investigation, isolation and molecular characterization of *sheep and goat pox virus* in different districts of central Ethiopia. Study was engaged different approaches especially questionnaire survey, virus isolation using vero cell line, classical PCR for amplification of the DNA, gel-electrophoresis for identification of the specific band, real time PCR for further genotyping, sequencing the RP030 gene for identification of genetic relationship of *sheep and goat pox virus* isolate from field with other *Capri pox virus* (CaPV).The questionnaire survey results indicated that sheep and goat pox was the most common disease in all the study areas and the disease was frequently seen. The disease outbreak more (52.94%) observed in rainy season. Additionally, non vaccinated animals, female and young age shoats were more affected ($P< 0.05$) by pox virus. A total of 712 sheep and goats (603 sheep and 109 goats) were clinically examined for the presence of pox lesions on their skin and 35.82% sheep and 28.44% goats had pox lesions. Generally, high mortality (9.95%) rate was observed in sheep than goats respectively. The virus was isolated from 16 skin samples (13 sheep and 3 goats) and the cell culture showed a typical characteristic of pox virus induced-cytopathic effect with destruction of monolayer and rounding of the cell. Similarly, the conventional PCR revealed that 16 out of 16 tested samples were positive by developing band size of 172bp (Goat pox virus). Further more real time PCR 16 tested samples were positive for goat pox virus with the melting temperature of (56^0C/72.5^0C). Phylogenetic tree analysis revealed that the RP030 gene (606 nucleotides) sequences of the present isolates originated from both sheep and goats were clustered with the goat pox virus group. Even though the existing information suggested that *capri pox virus* is strictly host specific, but in the current study the PCR and RP030 gene sequencing result confirmed that sheep were infected by goat pox virus similarly to goat pox virus and hence classification of pox virus based on infected host in small ruminant has been found to be inconclusive.

Keywords: Central Ethiopia**,** outbreak investigation, *sheep and goat pox viruses*, PCR, Sequencing.

1. INTRODUCTION

Ethiopia have the largest livestock population in Africa with sheep and goat populations exceeding 49 million, which is one of the largest populations of small ruminants in Africa (CSA, 2013). Small ruminants (sheep and goats) have a unique role in small holder 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 (Notter, 2012). In Ethiopia, sheep are the second most important livestock species next to cattle (Gizaw *et al*., 2007).

Capri poxviruses are the most important poxviruses of animals, causing diseases in sheep, goats or cattle. *Capri pox viruses* are responsible for some of the most economically signific ant diseases of domestic ruminants in Africa and Asia (Bhanuprakash *et al*., 2006).Various strains of *Capri pox virus* are responsible for the disease and these are antigenically and serologically indistinguishable from strains causing sheep pox and goat pox but distinct at the genetic level (Babiuk *et al*., 2008).The genus *Capripoxvirus*, member of the *poxviride* consists of lumpy skin disease virus, *goat pox virus* and *sheep pox virus* (Buller *et al*., 2005).

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 is endemic as compared with exotic breeds. Indigenous animals are more likely infected by the disease in areas where it has been not found or dormant for a period of time, when intensive husbandry methods are introduced (ESGPIP, 2009). The virus that causes sheep and goat pox is sheep and goat pox virus of family *poxviridae,* genus *capripoxvirus*, one of the largest (170-260 nm by 300-450 nm), enveloped double stranded DNA viruses (Tulman *et al*., 2002).

Sheep and goat pox is one of the most important diseases of sheep and goats in Ethiopia following *pest des petites ruminants* (PPR) and *Caprine pleuropneumonia* (CCPP). Sheep and *goat pox* is a highly contagious viral disease of sheep and goats (ESGPIP, 2009). In Ethiopia, sheep pox is a serious problem and has been reported from different regions of the country. Generally, sheep pox is a disease of considerable economic importance for Ethiopian sheep farming (MORAD, 2010).

Sheep and goat pox diseases are a weighty problem in sheep and goats production. In fact they are most serious of all pox diseases of domestic animals.(Bhanuprakash *et al*., 2006).Sheep and goat pox are important Office International des Epizooties (OIE) notifiable and trans boundary diseases of sheep and goats, respectively (OIE, 2008). Clinically, both the diseases are characterized by fever and generalized pock lesions. Occurrence of these diseases is associated with high morbidity, mortality and export-import restriction of sheep and goats and their by-products and hence is economically important (Babiuk *et al*., 2008). Mortality in young animals can exceed 50%. Indigenous sheep and goats exhibit some natural immunity, while the European breeds of sheep and goats are more susceptible to infection with these viruses with occasional mortality up to 100% in native animals (Bhanuprakash *et al*., 2006).

CaPVs are generally considered to be host specific, because disease outbreaks or virus isolates may preferentially occur or cause disease in one host species (Munz and Dumbell, 1994). This has been shown specifically for Nigerian, Middle Eastern, and Indian strains of SPPV and GTPV and for LSDV. However, the ability of SPPV and GTPV strains to naturally or experimentally cross-infected and cause disease in both host species (Kitching and Taylor, 1986). This apparent variability in SPPV and GTPV host range, the clinical similarity between sheep pox and goat pox, and the inability to differentiate the two diseases by serological assays have led to the suggestion that sheep pox and goat pox are part of a disease complex caused by a single viral species and that observable host range specificities are the result of regional virus adaptations to sheep or goat hosts (Ewing and Green, 1998). CaPV genomes are very similar to each other, averaging no less than 96% nucleotide identity over their entire length. SPPV, GTPV, and LSDV contain the same repertoire of orthologous genes, with the exception that SPPV and GTPV lack nine LSDV genes with likely CaPV virulence and host range functions. SPPV and GTPV genomes sequenced are phylogenetically distinct from each other and from LSDV, and they contain species-specific nucleotide differences that may be associated with aspects of host range. Relatively few genomic changes in SPPV and GTPV vaccine viruses account for viral attenuation (Tulman, 2002).

In general, capripoxviruses (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). However, no reports exist on CaPV infecting all three species: sheep, goats and cattle (Davies, 1976).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).

In Ethiopia 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 sheep and goat pox virus in selected areas of Amahara region (North Gonder,West Gojjam, South Gonder, Awi, East Gojjam) and Afar region (Chifira, Adaar, Amibra, Awash and Fen ta) Administrative zone 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 imp ortance of sheep and Goat pox: A review on past and current aspects indicated that the disease distributed in all regions of Ethiopia and economically important due to production loss and direct death (Yune and Abdela, 2017). But circulating sheep and goat pox virus strain isolation and characterization are still under estimate in Ethiopia. Therefore, the study was initiated with the following objectives;

- General objective

To investigate the outbreak of sheep and goat pox, isolation and molecular characterization of *Capri pox virus* (*Sheep and goat pox viruses*) in central Ethiopia.

- Specific objective

The study was conducted with the following specific objectives;

- ✓ To investigate outbreaks of sheep and goat pox disease in the study areas.
- ✓ To determine season and frequency of the outbreak occurrence of sheep and goat pox in study area.
- ✓ To determine the associated risk factors of sheep and goat pox in the study area.
- ✓ To isolate and molecular characterization of Capri pox virus (*Sheep and goat pox viruses*) from outbreak samples collected in the study area.
- ✓ To sequence and see the genetic relation of *sheep and goat pox virus* with other *Capri pox viruses*.

2. LITERATURE REVIEW

2.1. Etiology

2.2.1. Sheep and goat pox virus

Virions are brick shaped, enveloped with complex symmetry and about 300×270×200 nm in size (Deshprakash *et al*., 2015 and Davies, 1976). The sheep pox virus (SPV) and goat pox virus (GPV), of family *poxviridae*, subfamily *Chrodopoxvirinae*, genus *capripoxvirus*.The pox viruses of sheep and goats (*capripoxviruses)* are closely related, both antigenically and physicochemical have unable to distinguish poxvirus from each with serological techniques. 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, but recomb ination can occur between them, however some *capripoxivirus* are not host specific. Kenyan sheep and goat pox virus and Yeman and Oman infect both sheep and goat (CFSPH, 2008).

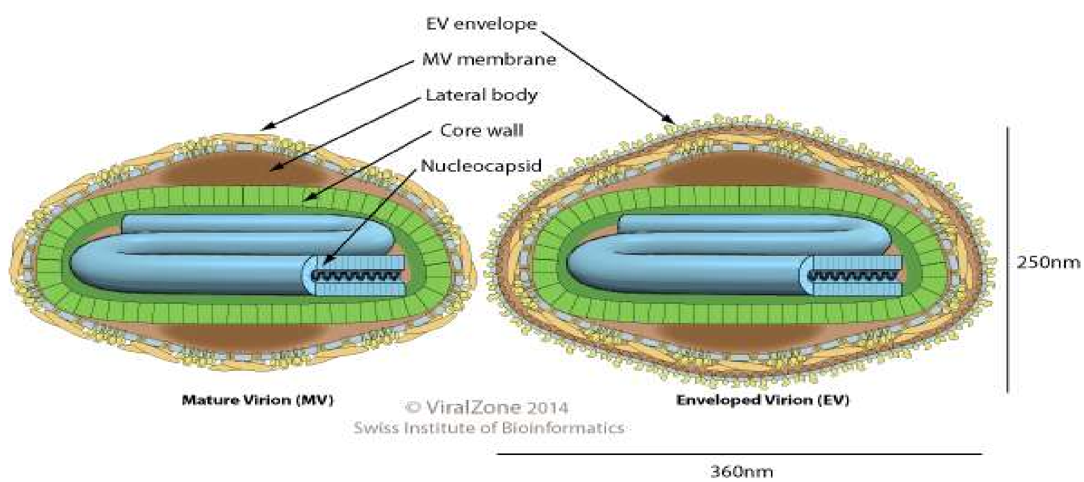


Figure 1: Morphology of pox virus
Source: Viral zone (2009).

2.2. Classification of *Capri pox virus*

According to the international committee on Taxonomy of viruses, the virus has the followi ng classification: Family *poxoviride* contain, *Entomopoxvirinae*, the poxviruses of insects and *Chordopoxvirinae,* the poxviruses of vertebrates.The sub family *Chordopoxvirinae* is comprised of eight genera, namely *orthopoxivirus, parapoxvirus capripoxvirus, avipoxvirus*, *Leporipoxvirus, Suipoxvirus, Molluscipoxvirus and Yatapoxvirus*.

Genetic recombination within genera results in extensive serological cross-reactions and cross-protection (Quinn *et al*., 2002).Genus: *Capri pox virus*, containing three species: *Goat pox virus* (GTPV) *Sheep pox virus* (SPPV), Lumpy skin disease virus (Buller *et al*., 2005).

2.3. Epidemiology

2.3.1. Geographic distribution

Sheep and goat pox are prevalent in parts of, central Asia, Africa except in South Africa, and the Middle Eastern countries. Goat pox is first reported in 879 in Norway and was later observed in Macedonia during the First World War. *Capri pox virus* is found in the Middle East, in Africa north of equator, India, Pakistan, Turkey and Iran. In Vietnam goat pox has been introduced in 2005. The outbreak of goat pox was occurred in Chinese Taipei in 2008 and in 2010 the disease reoccurred and was declared endemic (OIE, 2012).

The geographic range of sheep pox and goat pox has been restricted in the last 50 years mainly to Asia and Africa, extending from Africa north of the Equator (Kitching *et al*., 1989; Mariner *et al*., 1991; Achour and Bouguedour, 1999), into the Middle East (Daoud, 1997),Turkey (Oguzoglu *et al*., 2006), and Asia including regions of the former Soviet Union (Orlova *et al*., 2006), India (Mondal *et al*., 2004; Bhanuprakash *et al*., 2005) and China (Zheng *et al.,* 2007). Sheep pox or goat pox extended their range into Bangladesh in

1984 (Kitching *et al*., 1987) and Vietnam (2005 and 2008) and Mongolia (2006 and 2007) in the east, and repeated incursions have been reported in Greece in southern Europe (2007) (World Animal Health Information Database, 2009).

In Ethiopia, the disease is found in all regions (Yune and Abdela, 2017). In 2007/2008, the Animal and Plant Health Regulatory Directorate received 893 SGP outbreak reports from all regions except Gambella, Harari and Dire-dawa (ESGPEP, 2009).

2.3.2. Hosts of capri pox virus

The classification of pox viruses was made on the basis of the host from which these viruses were isolated. Serologically, all these three viruses (*Sheep pox virus*, *Goat pox virus* and *lumpy skin disease virus*) are identical and usually cross react, but can be differentiated by using molecular techniques (Christian *et al.,* 2009). It has been noted that the pox disease can infect all breeds of domestic and wild sheep and goats; 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). The genus *Capri pox virus*, member of the *poxviridae*, consists of *lumpy skin disease virus*, *goat pox virus* and *sheep pox virus*. Sheep pox 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).

An attenuated vaccine of Romanian sheep pox strain was incorporated to enhance the immunity of sheep but recently a severe outbreak of *Capri pox virus* 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 strains of *Capri pox virus* causing disease in sheep and goats are not host-specific and may either affect both or one species (Kitching, 2003). The local strain of goat pox virus 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).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).

2.3.3. Transmission of capri pox virus

The virus of sheep and got pox is highly contagious. Virus enters via respiratory tract and transmission is mostly by aerosol through contact with infected animal or fomite. Sheep and goats can be infected experimentally via intradermal inoculation and oral or intranasal administration of SPPV and GTPV (Bowden *et al*., 2008). Infected sheep and goats shed virus in oral, nasal and ocular secretions as well as in scabs that have fallen off the animal and transmission occurs through aerosols and direct contact (Kitching and Mellor, 1986; Bowden *et al*., 2008). Due to the stability of the virus, SPPV and GTPV may persist in the environment for prolonged periods of time, leading to infection of naïve animals. Unlike LSDV, insect vectors are not required for the transmission of SPPV and GTPV, although due to high viral loads in the skin, mechanical transmission may occur by insect vectors. *Stomoxys calcitrans* (stable fly) has been shown experimentally to transmit SPPV and GTPV; whereas *Mallophaga* species, *Damalinia* species, *Hydrotaea irritans* and *Culicoides nubeculosus* were not able to transmit SPPV despite the virus being isolated from *Hydrotaea irritans* after feeding on infected sheep (Kitching and Mellor, 1986).

Additionally the occurrence and spread of this skin disease are associated with poor management, climatic factors, feed scarcity and inadequate veterinary services. The increasing threat of skin diseases to the development of sheep production warranting an urgent control intervention is indicated (Woldemeskel and Ashenafi, 2003).It was noted that sheep pox, goat pox and lumpy skin diseases differ from each other but their viruses may transmit through a similar way like mechanically by biting insects (Carn, 2002)

2.3.4. Replication cycle of pox virus

The poxviruses replicate entirely in the cytoplasm. The assembly of progeny virions is located in specific cytoplasmic places called viroplasm or viral factories, which can be separated from other cellular structures in the form of virosomes. The cytoplasmic mode of replication implies that the machinery necessary for RNA synthesis and modification has to be carried by the incoming particles in order for the early viral genes to be expressed. Consequently among the active enzymes found in proteins included in the capsid, there are an RNA polymerase, capping and methylating enzymes, and a ploy A polymerase. These enzymes upon un coating of the virus, start the transcription of a series of early genes, among which are those responsible for DNA synthesis, such as DNA polymerase, ligase, thymedine kinase and thymedine sythatase. DNA synthesis has taken place the mode of transcription changes to a discontinuous one to transcribe the so-called late genes (structural genes).The life cycle is divided into several steps. Penetration and un coating (30 min), early transcription (1-2 hr), DNA synthesis (2-4hr), late transcription and assembly (4-6 hr), and release (budding) of the virions. During the late transcription period many early genes cease to be transcripted. It is during this phase of the life cycle that the structural viral proteins are expressed. An early effect of poxvirus infection on the host cell is the inhibition of cellular protein and DNA synthesis (Talavera and Javier, 1991).

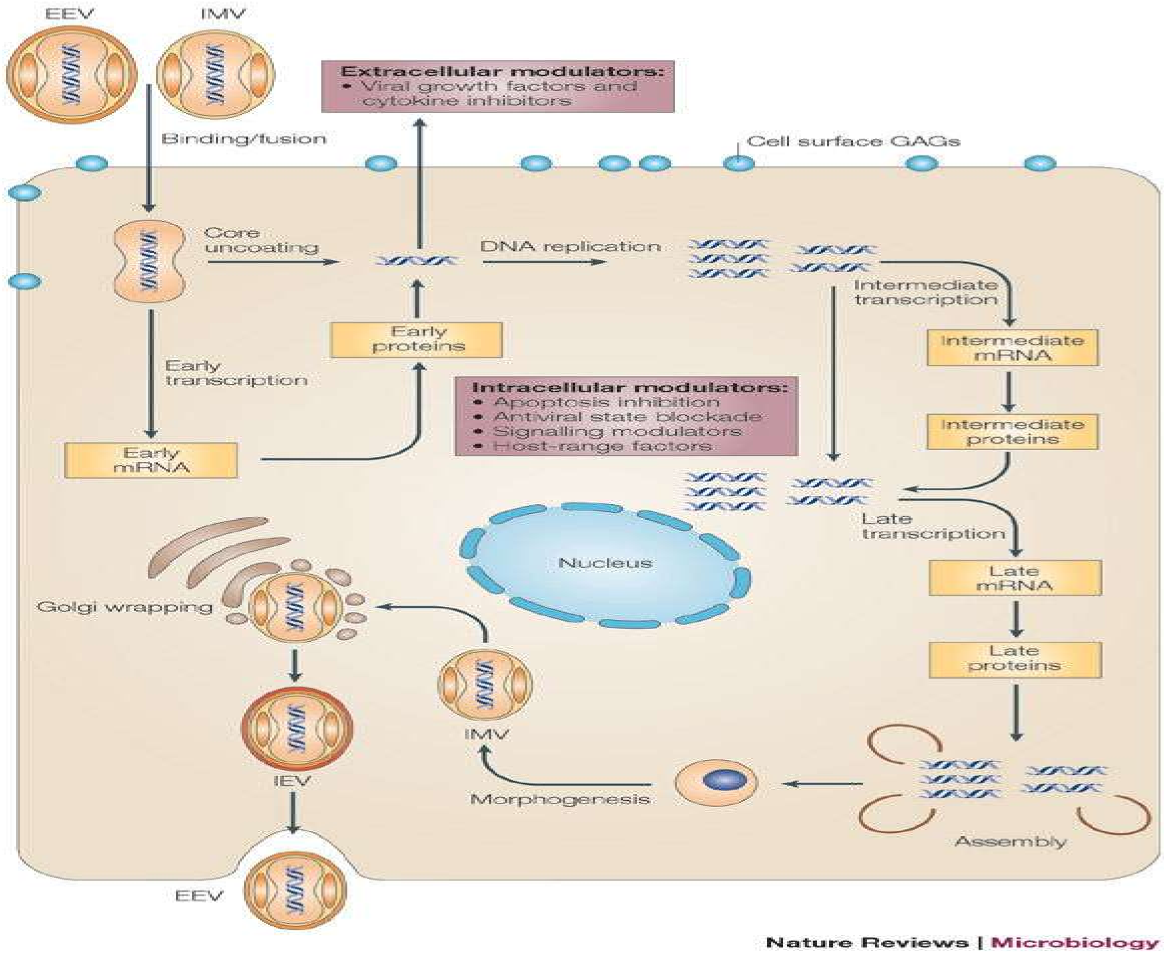


Figure 2: Replication cycle of pox virus

Source: Viral zone, 2017

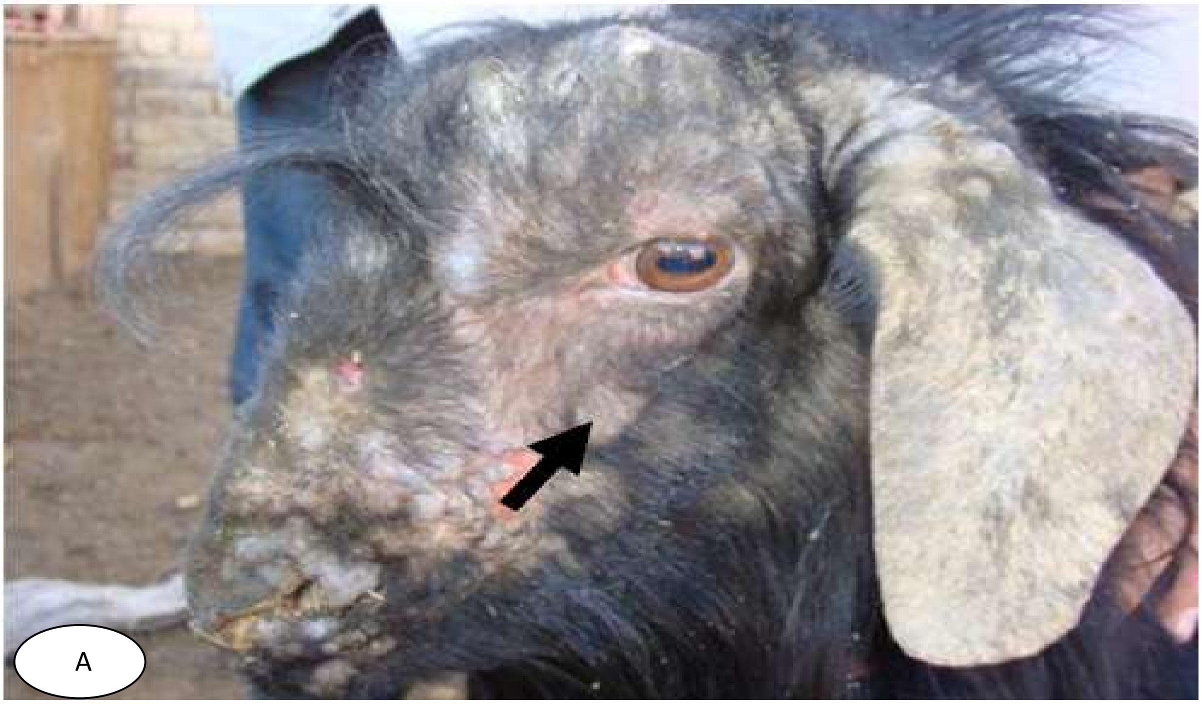
2.4. Pathogenesis

Incubation period of sheep pox is 4-8 of that of goat pox is 4-15 days. After it enters, sheep and goat pox virus replicates locally in the tissues. Since the virus is epitheliotropic, it will infest the epithelium tissues of the organism. On the 7^{th} day post-inoculation, the virus titer reached to its peak. The virus spread to the regional lymph nodes, after 3-4 days of 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 pre scapular lymph nodes. Excessive salivation can also occur after infection (OIE, 2012).

There are five stages in the development of pox infection. Roseola stage is stage in which Skin lesions typically begin with small red spots within three 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 roseola stage. Nodular skin lesions that are developed from roseola 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. Quantitative analysis using real-time PCR and isolation of the pathogenesis of sheep pox virus and goat pox virus in their respective hosts revealed high viral loads in skin (Bowden *et al*., 2008).

2.5. Clinical Signs

Both sheep and goat pox have similar clinical sign (Kitching *et al*., 1985). The incubation of SGP is between 4-15 Days in field condition (House, 1992). 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 occulonasal discharge and pox lesion on unwooled skin are manifested in malignant form of sheep pox. The diseases are more severe in young animals than adults. In benign form of sheep pox only skin lesions occur particularly under the tail. This form of sheep pox is common in adult. There is no systemic reaction and the animal recovers in 3-4 weeks. Abortion and secondary pneumonia are complications. In young the mortality rate may reach 100% while the overall mortality may be 50% of the flock. Lesions may be seen on the vulva, under tail, premium, nostril and mucous membranes of the mouth. If lesion is present in the lung acute respiratory distress occurs (AUSVETPLAN, 1996).


A


B

Figure 3: A, B and C Typical sign of Sheep and Goat pox
Source: Marzaie *et al.*, (2015).

2.6. Postmortem Lesions

Upon death, the skin contains congested bloody, swollen and necrotic lesions. The mucous membranes of the eyes, nose, mouth, vulva and prepuce (fore skin of male genitals) may be necrotic or ulcerated and all the body lymph nodes are enlarged and swollen. The lungs often contain severe and extensive pox lesions. Pox lesions are common on the abomasal mucosa, the rumen, large intestine, tongue, pharynx, trachea and esophagus. Pale areas of approximately 2 cm in diameter may occasionally be seen on the surface of the kidney and liver. Lymph nodes draining infected areas are enlarged up to eight times normal size, swollen with body fluids and may be congested and hemorrhagic (ESGPIP, 2009).

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The nodular lesions in intestine and lungs were recorded on necropsy (Parimal *et al.,* 2008). 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).

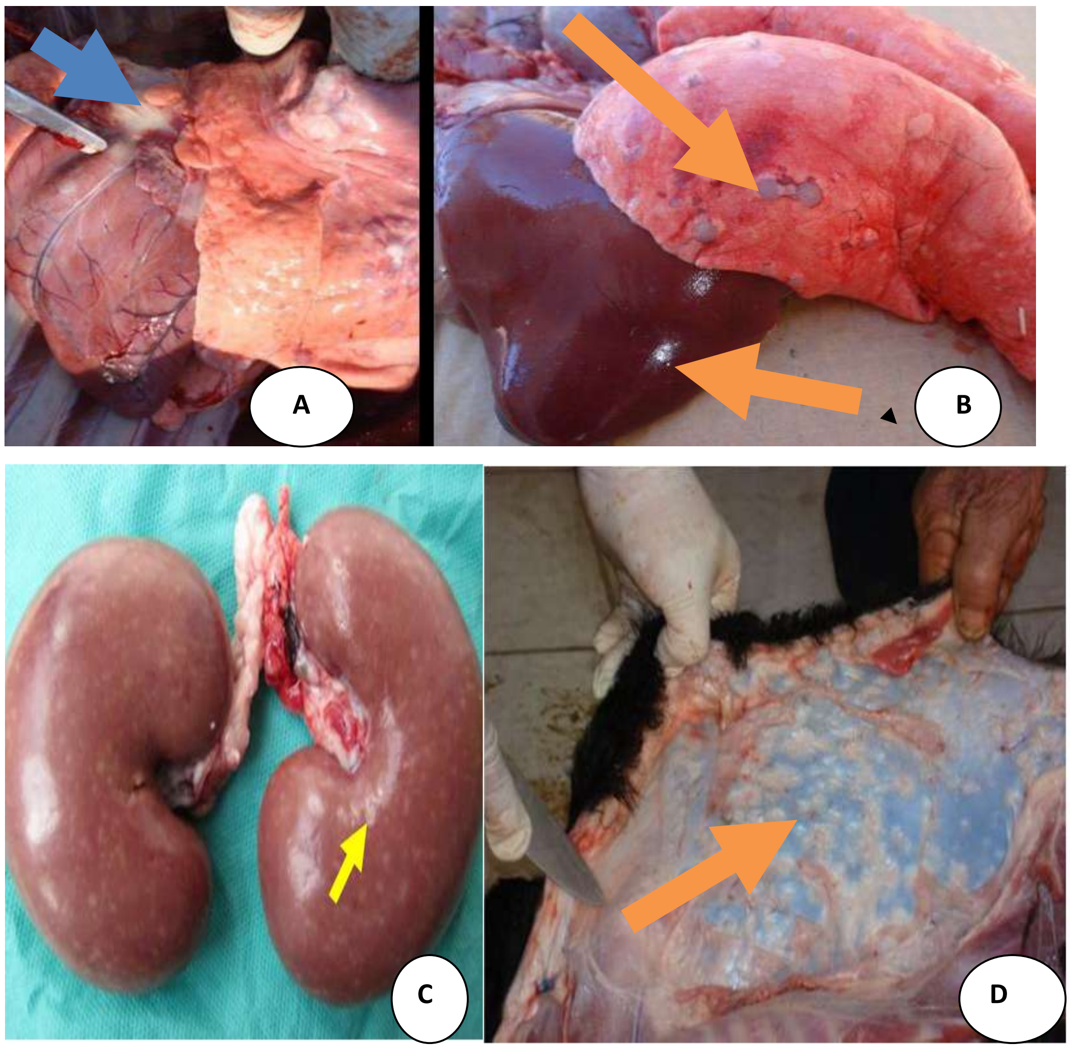

Figure 4.Postmortem lung (A), liver (B), kidney (C) and skin lesion (D)occurs in the case of sheep and goat pox.
Source: Marzaie *et al.*, (2015).

2.7. Histopathology

Microscopically, epidermal thickening, hyperplasia, acanthosis, hydropic degeneration of prickle cell layer, microvesiculation and necrotizing vasculitis were observed. Characteristic large intracytoplasmic eosinophilic inclusion bodies were conspicuously noticed in dermal cells (Pawaiya *et al.,* 2008). The microscopic lesions corroborated the macroscopic lesions and were characterized by the entry of sheep pox cells and lymphocytes in lung, skin, heart and spleen while skin scabs revealed hydropic changes, acanthosis and necrosis in epithelial layers. Sometime intracytoplasmic inclusion bodies were found in the epithelial and sheep pox cells (Singh *et al.,* 2007). Histopathological examination revealed marked proliferative change especially in the ectodermal layer with presence of intracytoplasmic acidophillic inclusion bodies (Joshi *et al.,* 1996).

2.8. Genomic Organization of Sheep and Goat Pox Virus

Virions are brick shaped, enveloped with complex symmetry and about 300×270×200 nm in size. Double stranded genomic DNA is about 154 kbp size (Anonymus, 1996) with less variable central region bounded by two identical inverted terminal repeats (ITR) at the ends (Deshprakash *et al*., 2015 and Davies, 1976). Within subfamily Chordopoxvirinae, CaPVs have highest A-T content that means73-75%. An extensive DNA cross hybridization between species of the genus is reported (Declekq and Goris, 2004). Viral genome shares 147 putative genes which encode proteins of 53-2,027 amino acids in size likely involved in replication, structure, virulence and host range functions (Davies, 1976 and Diallo, 2002).

The coding region of CaPV genome has 1-156 ORFs in which central ORFs (024-123) are conserved genes involved in replication and transcription mechanisms (Domench *et al*., 2006) whereas, the terminal ORFs (001-023 and 124-156) are variable in nature involving in host immune evasion and host-range functions (Deshprakash *et al*., 2015). Recent hypothesis suggests that GTPV and LSDV are more closely related to each other than to

SPPV and they are emerged from a common ancestor close to SPPV based on the phylo-genetic studies on different genomic segments (Babiuk *et al.,* 2008; Fenner, 1988).

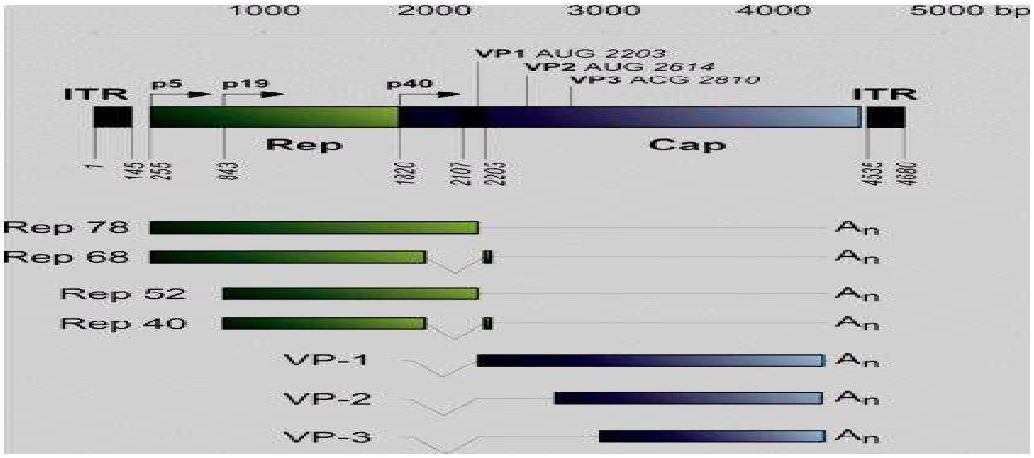

Figure 5: Genomic structure of pox virus
Source: Viral zone

2.9. Physiochemical Properties

CaPVs are generally resistant to drying and freezing-thawing. Sensitivity of heat differs between isolates (Balamurugan *et al*., 2009). Infectivity of virus is sensitive to ether trypsin, chloroform and formalin (Fulzele *et al*., 2006; Garner and lack, 1995).Virus can be destroyed at 56°C for 2 h or 65°C for 30 min but remains viable in wool for 3 months. High alkaline and acidic pH is detrimental to SPPV. Infectivity of virus gets affected by repeated freezing and thawing (Garnar *et al*., 2000).

2.10. Morbidity and Mortality

Morbidity and mortality rates vary according to breed, previous exposure to the virus, and the strain of the virus. Mild infections are common among indigenous breeds in endemic areas, but more severe disease can occur in young or stressed animals, animals suffering from other infections, or animals introduced from places where SGP is not present. Morbidity rates in indigenous breeds can be 70-90% with mortality ranging from 5-10%. Mortality and morbidity rates in newly imported animals can reach100% (ESGPIP, 2009).

Figure 6: Mass mortality and morbidity of sheep and goat pox.
Source: Marzaie *et al.*, (2015).

2.11. Diagnostic Methods of Sheep and Goat Pox Virus

2.11.1. Cell culture

Cultivation of GPV in cell culture of lamb kidney and tests (Kitching *et al.,* 1986) embryoni c caprine lung (ELZein *et al.,* 1982), Sheep thyroid (Nitzschke, 1967), chicken embryo fibro blast (Rao and Malik, 1982), Calf kidney (Tantawi *et al.,* 1980), Vero cell line (Assefa, 2017). Inoculated onto a confluent monolayer of Verocells in a $25cm^2$ tissue culture flask co ntaining10 mL of Glasgow minimum essential medium (SigmaAldrich) supplemented with 10% fetal calf serum (Gibco).Cell cultures were incubated at37^oC at 5% CO_2 and observed daily for the development of CaPV specific cytopathic effects (Gelaye *et al.,* 2013).

2.11.2. Virus Neutralization

A test serum can either be titrated against a constant titre of Capri poxvirus 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 and Carn, 1996). Because of the variable sensitivity of cell culture to *Capri pox virus*, and to consequent difficulty of ensuring the use of 100TCID50, the neutralization index is the preferred method. The test is described using 96-well flat-bottomed tissue-culture grade micro titre 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 used of Vero cells in the virus neutralization test has been reported to give more consistent results (OIE, 1996).

2.11.3. Agar gel precipitation Test (AGID)

A gel diffusion technique for the diagnosis of sheep and goat pox was introduced as early as the 1960s, using either homologous or heterologous (Uppal and Nilakantan, 1967) antiserum. Currently, a more efficient agar gel precipitation test (AGPT) using better diagnostic reagents, such as the soluble antigens, is available (Rao and Negi, 1997). In addition, the use of *methioninelabeled* antigen preparations considerably improves the sensitivity of the AGPT in the detection *Capri pox virus* of antibody (Kitching *et al*., 1986).

2.11.4. Indirect fluorescent antibody test

Capri pox virus-infected cell culture grown on flying cover-slips or cell culture microscope slides can be used for the indirect fluorescent antibody test. Uninfected cell culture control, and positive and negative control sera, should be included in the test. The infected and control cultures are fixed in acetone at -20ºC for 10 minutes and stored at 4°C. Dilutions of test sera are made in PBS, staring at 1/5, and positives are identified using an anti-sheep gamma-globulin conjugated with *fluoroscein thiocyanate*. Cross reaction can occur with Orf, Bovine popular stomatitis virus and perhaps other poxviruses (Davies and Atema, 1978).

2.11.5. Western blot analysis

Western blotting of test sera against *Capri pox virus*-infected cell lysate provides a sensitive and specific system for the detection of antibody to *Capripox* structural proteins, although the test is expensive and difficult to carry out (Chand *et al*., 1994).

2.11.6. Enzyme-linked immune sorbent assay

The enzyme-linked immunosorbent assay (ELISA) is now used widely to detect antibodies and antigens in a variety of test systems, and is more sensitive than virus -neutralization tests. Various methods of ELISA are available to diagnose goat and sheep pox, but 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. Recently, an avidin–biotin ELISA was introduced for the detection of antibodies to GPV in goat sera; it uses an isolated fraction of the soluble antigens that substantially reduces the background reaction in the assay. This assay is a reliable test for *Capri pox virus* antibody that may be used to assess immunity to goat and possibly sheep pox, and can also be used in epidemiological studies (Rao *et al*., 1999).

2.11.7. Polymerase chain reaction

Multiplex PCR is a fast and simple method for *Capri pox virus* species identification. The method was based on multiplex polymerase chain reaction (MPCR) with specific primers for each species. (Orlova *et al.,* 2006). The field isolated samples were declared positive by using PCR. Envelope protein gene PCR amplicons (192 bp) of Indian sheep pox viruses isolated in 1997 and 2003 were sequenced to analyze nucleotide divergence. Analysis revealed that 2003 isolates possessed 100% nucleotide identity (Parthiban *et al.,* 2005).

PCR were used for the detection of virus epidemic in Central Anatolia (Oguzoglu *et al.,* 2006). Multiplex PCR which is a single-step procedure was used for the amplification of *Capri pox virus* in skin biopsies. They used one specific primer for alpha-tubulin and two specific primers for *Capri pox virus*. 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 the assay of alpha-tubulin primers (Markoulatos *et al.,* 2000).

For many years, CaPV genotyping has been based on the electrophoretic patterns of viral ge nome isolates following digestion with restriction enzymes (Kitching *et al*., 1989). This met hod is time consuming and requires large amount of viral material, therefore cannot be appli ed in a routine basis. More recently, gene sequencing has been proposed for CaPV genotypi ng (LeGoff *et al*., 2009). However, this can only be applied for selected samples owing to its cost. 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 (Lamen*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 re solution mealting of small sized PCR products in the presence of saturating DNA dye such a s 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 count ries endemic for LSD, SPP and GTP virus (Wittwer *et al*., 2003; Liewel *et al*., 2004).

2.12. Economic Importance of Sheep and Goat pox virus

Sheep poxvirus and *goat pox virus* in endemic areas are associated with significant production losses because of reduced milk yield, decreased weight gain, 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).

Presence of sheep pox in a country limits the trade of new breeds and development of intensive sheep production. The disease has major impact on the economy with average morbidity and mortality rates of 50 and 100%, respectively. The effect is such that it would take 6 years for a flock or herd to recover from an outbreak with average income losses up to 30–43% of total annual revenue depending on flock type and owners actions. The level of impact varies from country-to-country both qualitatively and quantitatively. SGPV is one of the animal bioterrorist agents as it causes high morbidity and mortality, has potential for rapid spread, potential to cause serious socio-economic or public health consequences and is of major importance in the international trade of animals and animal products. Sheep pox virus is one of the 15 animal pathogens listed by Animal World Health Organization (OIE) and 23 by Animal and Plant Health Inspection Agency (USDA, 2002) which can be used as an animal biological warfare agent.

2.13. Prevention and Control

Uncontrolled movement of infected animals in SGP-endemic areas poses serious difficulties in efficient control of the disease. Therefore, it is essential to vaccinate sheep flocks regularly, on an annual basis, with a safe and efficient vaccine, for the control of this serious and economically important disease in endemic regions (Yeruham *et al*., 2007).

Sheep and goats introduced to endemic areas should be quarantined for twenty-one days. Movement of sheep and goats from infected to non-infected areas should be restricted. Movement of products; meat, wool, hair and skin from infected areas should also be controlled. The virus may persist for up to six months in shaded, unclean shelters and for a few months in dry scabs on the skin, tools and equipment that have been in contact with infected animals must be cleaned and disinfected with disinfectants such as ether, formalin, sodium hypochlorite, 2% hydrochloric acid or phenol. It will help to remove part of the top soil and burn it. In a sheep and goat pox outbreak, affected animals should be isolated immediately. Shelters should be cleaned and disinfected. Sheep and goats around the outbreak area should be vaccinated as soon as possible. In areas of frequent SGP occurrence, the most effective means of controlling is annual vaccination. Carcasses and contaminated materials should be buried or burned. Massive vaccination followed by cessation of vaccination and control of animal movements can be an effective strategy to control SGP if the disease has spread extensively in an area. Quarantine of areas and premises containing infected or exposed animals is required to prevent disease spread. Infected herds and sick animals should be isolated for at least 45 days after recovery (ESGPIP, 2009).

National programs for control and finally eradication of SGP, need fortifying of the veterinary infrastructure, reporting system, technology and financial resources, whereas developing nations lack some of these elements and thus suffer economic losses from endemic diseases (Breeze, 2006).

2.14. Status of Sheep and Goat pox virus in Ethiopia

In Ethiopia a total of 57,638 sheep and goats contracted the disease and 4,853,347 sheep and goats were at risk in areas where outbreaks occurred. Out of the 57,638 sick sheep and goats, 6,401 animals died. In the outbreak areas, The disease reporting rate in Ethiopia is only about 35-40%. The actual figures in terms of affected, vaccinated and dead animals is, therefore expected to be higher than the reported figures (ESGPEP, 2009).

According to the AU-IBAR the number of African countries affected by sheep pox and goat pox (SGPV) had, before 2011, shown an increasing trend for three consecutive years. The number of countries reportedly affected by SGPV in 2011 reduced remarkably from the previous year. In 2011, twelve countries reported occurrence of SGPV 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 three 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:Countries reporting sheep pox and goat pox to AU-IBAR in 2011 Country Outbreaks Cases Deaths Slaughtered.

Country	Out break	Cases	Deaths	Slaughter
Algeria	44	306	14	0
Cameron	2	15	0	0
Ethiopia	223	4827	815	90
Ghana	1	2	0	0
Kenya	2	9	0	0
Leseto	1	5	0	0
Niger	41	945	235	NS
Nigeria	2	33	7	9
Senegal	7	410	28	0
Somalia	170	2393	324	49
Sudan	29	859	185	5
Tunisia	19	128	11	5
Total	541	9932	1619	158

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

3. MATERIALS AND METHODS

3.1. Study Area

The study was conducted to investigate the outbreak, isolate and characterize sheep and goat pox virus in selected central parts of Ethiopia from September, 2017 to March, 2018. Active outbreaks of sheep and goat pox virus were investigated in five districts Tiyo, Yaya-Gulale, Sululta, Mulo and Adea-berga (Fig. 7). Tiyo district is located at about 167 km south east of Addis Ababa and at the foot slopes of Mount Chilalo in the eastern side. Tiyo has a total area of 65,000 hectares of land and from these 25,060 hectares is used for crop cultivation, 9,697 hectares for grazing, 3,959 hectares for forest, 9,479 hectares is covered by bush and shrub, 10,828 hectares is barren and 5,977 hectares used for other purposes. Tiyo has diverse climatic conditions; weynadega (52%), dega (37%), and kola (11%) agro- ecologies with altitude ranging from below 2300 to over 3200 meters above sea level. Tiyo has got 1300mm to 1350mm annual rainfall and an average temperature of 18 ^{o}C to 25 ^{o}C during dry season and 5 ^{o}C to 10 ^{o}C during wet season. The area experiences bimodal rainfall, that is long rainy season occurring from June to August and short rainy season from February to April. It has very productive environmental conditions due to its climate and soil. The dominant cereals cultivated in the area are wheat and barley (FAO, 2006).

Yaya-gulale wereda's capital city is Fital, 48 km from zonal capital, Fitche and 116 kms from Adiss Abeba. The wereda is bordered in the North by Girar Jarso, Degem in west, Sululta-Mulo and Wuchale-Jida in south, Wuchale-Jida in south-east, Adea Berga wereda of West Shewa Zone in the south-west and Amahara Region in north-east. Yaya Gulale- Debra Libanos wereda has an astronomical location of $9^{0}30$'- $9^{0}46$'N latitude and $38^{0}37$'- $38^{0}50$'E longitude.Yaya Gulale wereda has a total area of about 633.19 square kms, accounting for about 5.3% of the total area of the zone. The agro-climatic zones of the wereda are Beda (temperate) 2300-3300m, covers 50%, Bada-Dare (sub-tropical) 1500-2300m, covers 25% and Gamoji (tropical) 500-1500m, covers 25% of the total area of the wereda. The average annual rainfall and temperature were about 1000 mm and 15^0c respectively. There is no weather station in the wereda (Oromia finace and economic bureau, 2011)

Sululta district is one of the six districts of Oromia Special Zone Surrounding Finfinne of Oromia National Regional State. The districts' capital town, Chancho, is 40 kms away from Addis Ababa towards the North-west. It lies on the geographical coordinates of 9° 11' 0" N latitude, 38° 45' 0" E longitude. The area is characterized by shallow valley with an elevation of 2500 meters above sea level, almost completely surrounded by mountains with numerous small rivers which drain into the Muger. The average annual temperature in Sululta is 14.7 ^{0}C with an average rainfall of 1119 mm (SDAO, 2012).

Mulo district is one of the districts of Finifine Zuria special zone of Oromia region. It is situated about 73 kms to the northwest of Addis Ababa. Adea-berga and Welmera districts border in west and south directions. Sululta district also surrounds in the north and east. The agro-climatic zones of the district are temperate, sup-tropical and tropical (CSA, 2008).

Adea-berga district is located in West Shewa Zone, Oromia region, Ethiopia at 9^0 16'N latitude and 38^0 23'E longitude. In the district, the rainfall pattern is bimodal, with a short rainy period from March to May and a long rainy season from June to September and the rest of the months are dry. The annual temperature and rainfall ranges from 18°C to 24°C and 1000 to 1225 mm, respectively. The farming system is semi intensive where the animals pass their time mostly by grazing and practice also an indoor feeding (Tamirat *et al.,* 2016).

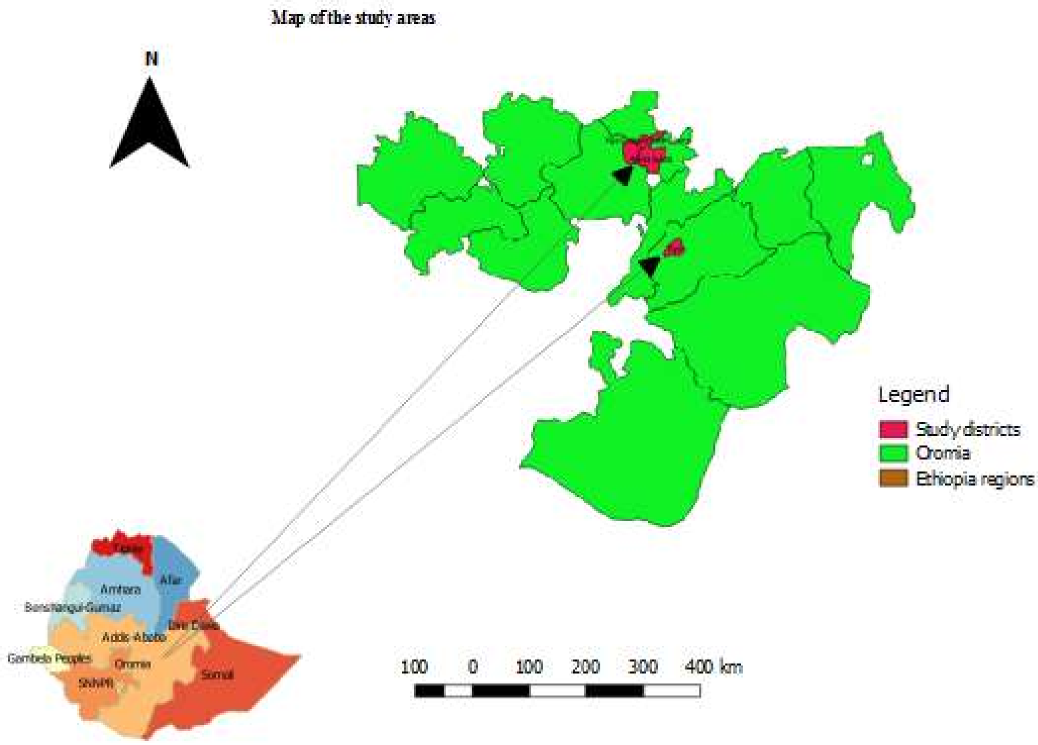

Figure 7. Map showing study area

Source: GIS (Geographical information system)

3.2. Study Animals

Local sheep and goat breed of all age and both sex groups under extensive management system by small holder farmers with 2 to 40 sheep and goat were included in the study. Sheep and goats with typical signs of the pox like skin lesion were purposively sampled for virus isolation , molecular characterization and sequencing.

3.3. Study Design

Cross sectional observational study design (Dohoo *et al*., 2003; Thrusfield, 2005) was condu cted from September 2017 up to March 2018. Active out break were assessed by frequent observation and communicating zonal, regional laboratories and district animal health professionals who are working in the district of veterinary clinic. Field investigation was conducted during the study period to identify the clinical sign of pox virus, estimate mortality, Morbidity and case fatality. Clinical and epidemiological data were gathered by observing clinically sick animals and interviewing animal health experts working in the respective areas for determination of associated risk factor for the occurrence of sheep and goat pox. Data on Age, Sex, herd size, animal management, body condition affected, before or after vaccination practices disease occurred, animal spices more affected, number of shoat at risk, number of case, death and hypothesized risk factors were recorded. All relevant information was carefully recorded on a designed sheet and appropriate viral samples were collected for virus isolation and characterization at National Veterinary Institute (NVI) of Ethiopia.

3.4. Sample Size

During the study period the total animal examined was 712. A field investigation was conducted purposively at the specific site of the outbreak at central Ethiopia.13 Sheep and 3 goat with the clear signs, symptoms and suspected to be Pox diseased in both sheep and goat were purposively selected and sampled for virus isolation and molecular characterizati on.

3.5. Questionnaire Survey

A structured questionnaire format was prepared to interview individual sheep and goat owners. Total of 102 respondents 22 from Mulo and 20 from Adea-berga, Sululta, Tiyo and Yaya-Gulales were randomly selected and interviewed for determine the frequency of disease and season of occurrence in study area.

3.6. Sample Collection Methodology

3.6.1. Clinical examination

After arriving at specific outbreak site during disease outbreak investigation, the shoat were examined for presence of nodules on the head and neck, under tail, perineum, udder and testicles. Temperature of shoat was measured by thermometer to check whether it had fever or not.

3.6.2. Sample collection

According to the procedure of OIE (2017) samples for virus isolation and molecular characterization were collected from clinically sick sheep and goat skin nodules. Tissue samples of skin biopsies were collected from the outbreak area from clinically sick animals (13 from sheep and 3 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. Collected samples were placed in a 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. Once the samples arrived at NVI, it was placed at -20 ^{o}C until analysis.

3.7. Laboratory Technique

3.7.1. Preparation of glassware for cultivation and maintenance of Vero cell line

Glassware, reagents and media were prepared and sterilized according to the standard operating procedures of NVI. The used glass wares was dipped in surf detergent, brushed thoroughly and washed in running tap water for 20 minutes and washed ten times with de-ionized water. The washed glass wares were kept inverted on a clean surface table top to drain out the water content and to dry. The glass wares were wrapped in wrapping papers and aluminum foils. All the glass wares including fresh were placed in hot air oven at 180 ^{0}C for 30 minutes.

3.7.2. Sample processing

The skin biopsy samples were thawed at room temperature and washed three times using sterile PBS at a pH of 7.2 under B.S safety cabinet. 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 10 ml of sterile phosphate buffer saline (PBS) containing pencillin and streptomysin. The tissue suspension was centrifuged at 1,500 rpm for 15 min. The supernatant was collected, filtered through 0.45mµ membrane filter and preserved at -80 ^{o}C until use.

3.7.3. Preparation of Vero cell monolayer

Vero cell line provided by NVI was grown in 25cm^2 tissue culture flask 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 rinse, washed twice with 10 ml PBS and covered with 5 ml of sterile trypsin for about 3 minutes in an incubator at 37^{o}C. 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.

3.7.4. Inoculation of virus

The field sample suspension 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 exhausted medium from the tissue culture flask was discarded and filtrate 1 ml of sample suspension then 8ml of sterile maintenance medium added per flask ($25m^2$). Three flasks were used. The two flasks was inoculated with 1 ml field virus suspension and the medium of the third was replace and kept as control flask. Each flask were observed for any cytopathic effect (CPE) daily. At 24 hours post-incubation, the tissue culture flask was passed through two regular freeze and thaw processes, and 2 ml inoculums were harvested, filtered through a syringe filter and then again inoculated into next flasks. All the flasks, including control flasks, were incubated. The medium (GMEM) was changed every 48hours.

3.7.5. DNA extraction

Virus DNA was extracted from the tissue suspension and homogenate using DNeasy® Blood and Tissue Kit (QIAGEN, Germany) following the manufacturer's instruction in the facilities of the molecular biology of the National Veterinary Institute (NVI). The eluted virus DNA was kept at -20ºC in a labeled eppendorf tube. All steps were carried out within in a Microbiological Safety Cabinet IIA. Details of the steps to be followed for DNA extraction are described in Annex (6).

3.7.6. Classical (conventional) PCR.

Conventional PCR was carried out according to the protocol described by Mangana-Vougiouka *et al*.,(2000) to amplify a small fragment of the 30KDa RNA Polymerase sub unit RP030 gene Lamien *et al*., (2011) gene. By using Capri pox virus specific primers of SPGP RNA Pol forward 5pm/µl and primer RNAPol reverse 5pm/µl synthesized by VBC bi otech (Vienna, Austria).

Table 2: Specific primer used for conventional PCR

Primer	Primer sequence	Length	Reference
For ward	5'TCTATGTTCTTGATATGTGGTGGTAG3'	26	Lamien *et al*., (2011)
Reverse	5'AGTGATTAGGTGGTGTATTATTTTCC3'	26	

PCR was done in a reaction volume of 20 µl contain RNase free water 3 µl, forward primer 2 µl , Reverse primer 2 µl, IQ super mix 10 µl, Template DNA sample 3 µl. The PCR tube was transferred in to a thermal cycler and amplification was conducted. The PCR tubes with all the components were transferred to a thermal cycler (Applied Bio Systems). The PCR protocol was performed with an initial denaturation at 95°C for 5min, followed by 40 cycles of denaturation at 95°C for 30, annealing 50°C for 30s and extension 72°C for 30sec, and final extension at 72°C for 7 min by 1cycle. PCR products were loaded and separated using electrophoresis apparatus (BIORAD) at 120 Volts for 1:20hrs in 3% agarose gel stained with gel red (NVI, SOP). The presence of a 21 nucleotide deletion in the RPO30 gene of SPPV strains and absent in GTPV strains is the base of differentiation (Fig. 8)

```
>KF495233.1 Goatpox virus isolate Maharashtra/Goat/19 RNA polymerase 30 kD
subunit (RPO30) gene, complete cds
ATGGATGATGATAATATTAATTCATATAGTGATAATACTACCCCCACATATCAAGACATAGAAGATATAA
TTTATAAATATGTAAAAGAAAAATCAAAGGTAAAAGAAATATTAAAATGGGCAACAGACAAAGCTTCCAA
GTTTTATATAAGAAATATTATTAATACAAAGTCAAATATAGAAGAAACTAAATTTGAACCAAGAAACAAC
ATAGGTATTGAATACTCAAAAGATTCAAAAAACAAATTATCGTGTAGAAACAAGCCTTTAATAGAGACAA
ATAAAGATTATTCTGACATATGTAATCTTATACGTACTACAAATGGAACAGAGAAAGAAATTTTAAGATA
TATACTTTTTGGAATAAAATGTGTTCAAAAAAATGTAGAATTCAATATAGACGATATTAGAGATATAAAT
TACGAAGAATATTTTAATGTTTTAGATAAAAAGTATAACCTCCCATGCCCTGAGTGCAAAAGTAAAAACA
CTATTCCCGTCATGATACAAACAAGAGCAGCAGATGAACCACCATTAGTTATGCATTCTTGTAGAGACTG
CAAGAAAAATTTTAAACCTCCGAAGTTTAGAGCTGTAGAAAAATAA
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>NC_004002.1:c28087-27503 Sheep pox virus 17077-99, complete genome
ATGGATGATGATAATACTACCCCCACATATCAAGACATAGAAGATATAATTTATAAATATGTAAAAGAAA
AATCAAAGGTAAAAGAAATATTAAAATGGGCAACAGACAAAGCTTCCAAGTTTTATATAAGAAATATTAT
TAATACTAAGTCAAATATAGAAGAAACTAAATTTGAACCAAGAAACAACATAGGTATTGAGTACTCAAAA
GATTCAAAGAACAAATTATCGTATAGAAACAAGCCTTTAATAGATACAAATAAAGAGTATTCTGACATAT
GTAATCTTATACGTACAACAAATGGAACAGAGAAAGAAATTTTAAGATATATACTTTTTGGAATAAAATG
TGTTCAAAAAAATGTAGAATTTAATATAGACAATATTAGAGATATAAATTACGAAGAATATTTTAATGTT
TTAGATAAAAAGTATAACCTCCCATGCCCTGAGTGCAAAAGTAAAAACACTATTCCCGTCATGATACAAA
CAAGAGCAGCAGATGAACCACCATTAGTTATGCACTCTTGTAGAGACTGCAAAAAAAAATTTAAACCTCC
AAAGTTTAGAGCTGTAGAAAAATAA
```

Figure 8: Nucleotide sequence of 30KDa RNA polymerase sub unit RP030 Sheep and Goat pox virus (NCBI).

3.7.7. Agarose gel electrophoresis of PCR product.

Agarose gel electrophoresis provides a means of analyzing DNA by separating molecules based on size amplified products were analyzed by agarose gel electrophoresis as described Manganavougiouka *et al*., (1999). The presence of DNA was checked agarose gels of 3% prepared in Tris/Acctate/EDTA (TAE). Amplified products were analyzed with a component 4 µl gel red loading dye and 10 µl PCR product and 10 µl marker (ladder) loaded to walls in prepared gels and run at 120V/1:20 hrs. Parallel with DNA molecular weight marker in electrophoresis apparatus until the DNA samples have migrated a sufficient distance trough the gel. DNA bands were visualized using UV transilluminater at a wave length of 590 nm and positive results were confirmed according to the size of the bands formed on agarose gel (NVI, SOP). The PCR results were considered as positive for sheep pox virus 151bp and goat pox virus 172bp Lamien *et al.,* (2011).

3.7.8. Real-time PCR

The recently developed species specific real time PCR method using unlabeled snap back primer and ds DNA intercalating dye assay targeting the CaPV RP030 gene was used to confirm the CaPV identify of the field isolates and determine the genotype Gelaye *et al*. (2013). Real time PCR was performed at the molecular biology laboratory of NVI using the amplification primers described by Gelaye *et al.,* (2013). Briefly the PCR was set up in react ion volume of 20 µl where 4.84 µl of RNase free water, 2µl of forward Primer CP HRM sb For5pm/µl (5'GGTGTAGTACGTATAAGATTATCGTATAGAAACAAGCCTTTA3'), 0.16µl reverse primer CP HRM/1REV5Pm/µl (5'AATTTCTTTCTCTGTTCCATTTG 3'), 10µl SsO fast Eva Green super mix (Biored) and 3µl sample template (DNA). PCR was performed with an initial denaturation step at 95^{o}C for 3mintes/1-Cycle,followed by 49 Cycles at 95^{o}C for 15sec and 58^{o}C for 1.20mint. Then product was then denatured at 95^{o}C for 1mint (held for 1minut), cooled to 40^{o}C (held for 1 minute) and heated continuously at 0.5^{o}C for 10sec with fluorescence acquisition from 45^{o}C to 85^{o}C. Finally pair of melting temperature each for snap back tail and full amplicon was recorded as GTPV (56^{o}C/ 72.5^{o}C) and SPPV (51^{o}C/72.50^{o}C), LSD (50^{o}C/73.5^{o}C) for genotyping of the tested isolates.

3.7.9. RP030 gene amplification for sequencing

Two set of primer for the RP030 gene were used for the amplification of the RPO30 gene as briefly described by Gelaye *et al*. (2015). The aim of using this over lapping primers was to generate the full length RP030 gene. PCR was conducted in reaction volume of 20 µl containg 2µl for ward primer (gene sequence RNA Pol OP1Fow 5' CAGCTGTTTGTTTAC ATTTGATTTTT3' RNA Pol OP1 Rev 5'TCGTATAGAAACAAGCCTTTAATAGA 3') 2 µl reverse primer (RNA Pol-Fow-5'TTTGAACACATTTTATTCCAAAAAG3' RNA Pol-OP2 Rev AACCTACATGCATAAACAGAAGC) 2.5µl dNTPS, 2.5µL10×PCR buffer (Qu agen), 0.5 µl Taq polymerase (Quiagen) 5.5 µl RNase free water and 5 µl template DNA. The initial denaturation at 95^{0}C for 4mint was followed by 40 cycles at 95^{O}C for 30 sec, 55^{o}C for 30sec and 72^{o}C for 7mint. Aliquots of PCR products were checked using electrophoresis on 1.5% agarose gel stained with gel red.

3.7.10. Sequencing and sequence analysis

The positive PCR product of the amplified RPO30 gene were purified using the wizard SV Gel and PCR clean up system kit (Promega Germany). The concentration of the purified PCR product was quantified using the Nano drop 200°C spectrometer (Thermo scientific USA). The concentration of each purified product was adjusted and prepared according to the instruction recommended by sequencing providing company. The purified PCR product were mixed with the sequencing primers and submitted for sequencing to the commercially sequencing LGC Genomics (Berlin Germany). The row sequence data were edited and fragments were assembled using vector NTI Advance ™ 11.5 Soft ware (invetrogen carls bad CA, USA). For each isolate the fragments produced with both set over lapping primers of the RPO30 gene were edited and assembled together and the clean gene sequence was extracted. Multiple sequence aliments were performed using the Clustal Walgorithm implemented in Bio edit software package to compare the RPO30 gene of outbreak isolates and the reference strain. For comparative studies Blast in was used to collect additional Capri pox virus RP030 Sequence from gene bank for inclusion in data set .For construction of phylogenetic tree multiple sequence alignments were performed to align the sequences as codons using the muscle algorism in MEGA6 (Tamura *et al*., 2013). The neighbuor joining algorthim was used with the maximum composite likely hood nucleotide substitution model with the pair wise deletion option was used. For construction of phylogenic tree 1000 boots trap replicate was used.

3.8. Ethics Statement

Samples were collected from diseased sheep and goats during outbreak for disease confirmation. No animal experiment was conducted. All efforts were made to minimize animal suffering during the course of sample collection in pox suspected outbreak areas. Consent was obtained from the animal owners for the collection of tissue samples. Sample collection and their use were according to National Veterinary Institute laboratory animal use ethics (OSP, NVI).

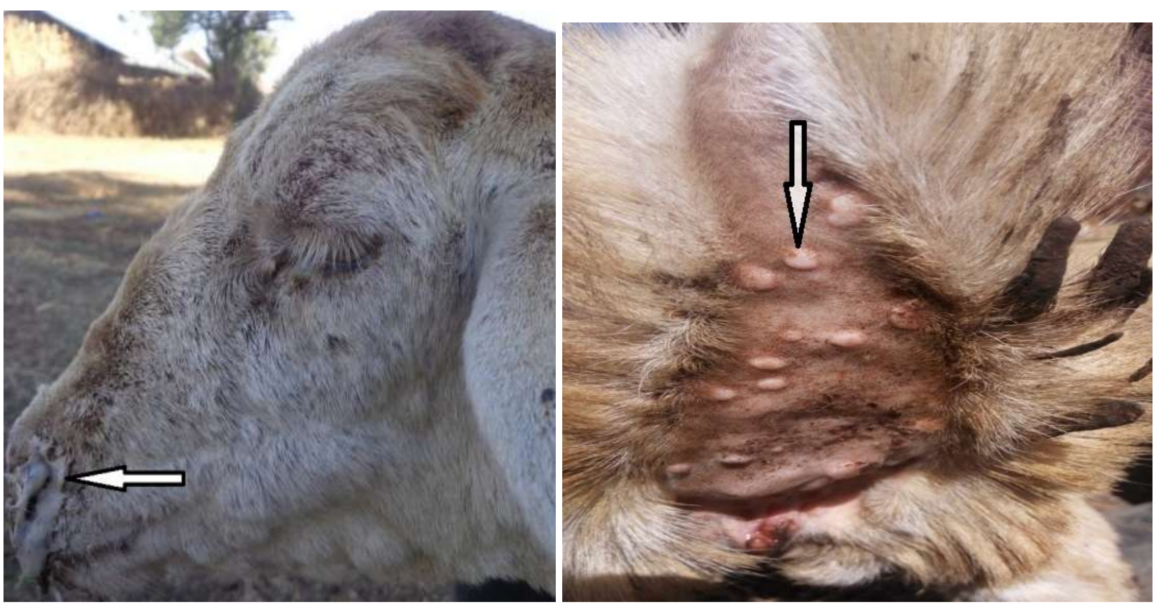
3.9. Data Management and Analysis

Data obtained from all field and laboratory investigation was coded and stored in micro soft office Excel 2007 spread sheets. Data collected during observation of clinical signs while investigating the outbreak, sample collection, virus isolation using cell culture, gene amplification using classical PCR, Further genotyped by real time PCR were record and stored. Statistical analysis was done using statistical procedures for social science (SPSS) version 20. The association of potential risk factor with sheep and goat pox were computed by chi square test. P value < 0.05 was set of detecting statistically significant finding and percentage were used to calculate morbidity, mortality, case fatality, season and frequency of outbreak occurrence .The nucleotide aliment and phylogenetic tree constriction, was compute using MEGA6 soft ware (Tamura *et al*., 2013).

4. RESULTS

4.1. Out Break Investigation

The common clinical sign observed in sheep and goat pox virus was fever, depression, loss of appetite different size skin nodules, necrotic nodule under the tail, perineum, udder and testicles, lacrimation, nasal discharge, cutaneous papules and nodules in areas of skin with less hair were prominent signs of the disease. During outbreak investigation from the total of 603 local sheep and 109 goats examined, 216 sheep were showed pox lesions where as 31 goats were positive for pox lesion. From the affected groups, 60 sheep and 4 goats were died. Overall 35.82%, 9.95% and 27.7% morbidity, mortality and case fatality where observed in sheep respectively. Whereas 28.44%, 3.66% and 12.9% morbidity, mortality and case fatality rate respectively, were observed in goats.



[A] **[B]**

Figure 9.Characteristic sign of sheep and goat pox observed during the study period circum scribed skin nodule under tail [B] and Nasal discharge [A]

Table 3. Morbidity rate, Mortality rate and case fatality rate of sheep pox in study area

District	Number of susceptible Sheep	Number of affected sheep	Number of death	Morbidity rate	Mortality rate	Case fatality
Mulo	78	46	14	58.97%	17.94%	30.43%
Sululta	165	54	18	32.72%	10.9%	33.33%
Adea- berga	82	26	6	31.70%	7.31%	23.07%
Yaya- gulale	196	70	18	35.71%	9.18%	25.71%
Tiyo	82	20	4	24.39%	4.87%	20%
Total	**603**	**216**	**60**	**35.82%**	**9.95**	**27.7%**

Table 4 Morbidity rate, Mortality rate and case fatality rate of goat pox in study areas

District	Number of susceptible Goat	Number of affected Goat	Number of death	Morbidity rate	Mortality rate	Case fatality
Mulo	27	3	1	11.11%	3.7%	33.3%
Sululta	42	9	2	21.42%	4.76%	22.22%
Adea- berga	15	2	0	13.33%	0.00	0.00
Yaya- gulale	16	5	1	31.25%	6.25%	20%
Tiyo	9	2	0	22.22%	0.00	0.00
Total	**109**	**31**	**4**	**28.44%**	**3.66%**	**12.9%**

4.2. Quationary Survey Results

According to the owner of the study area the local name for sheep and goat pox is Fino about of 97 respondents said that the sheep and goat pox outbreak re-occurs frequently in one year. While 5 individuals said the disease occurred in travel of two years.

54/102 (52.94%), 28/102 (27.45%) and 20/102 (19.6%) of the respondents also reported that the occurrence of the disease increased in rainy season 54/102 (52.94%), dry season 28/102 (27.45) and in both seasons (Rainy and dry) 20/102 (19.6%) respectively.

4.3. Result of Risk Factor Associated With Disease Occurrence.

102 sheep and goat affected by sheep and goat pox were assessed for their risk factors. pertaining to the possible risk factors, the occurrence of sheep and goat pox virus increase in non vaccinated shoat (sheep and goat) (85.29%), sheep species (80.39%), poor body condition (76.47%), in Female (71.56%), in young animal less than one year (64.7%), poor management (64.7%), in presence of sheep and goats living together (52.94%) and greater than 30 heard size (50.98%), respectively. The sheep and goat pox occurrence was significantly ($P<0.05$) associated with risk factors (Table 5).

Table 5. Chi square test at ($P<0.05$) for risk factor associated with disease occurrence (n=102)

No	Risk factors	Observed number	%	X^2	P-value
1	**Sex shoat**				
	Male	29	28.44	18.980	.0001
	Female	73	71.56		
2	**Age**				
	< 1 year	66	64.7	8.824	.003
	>1 year	36	35.3		
3	**Body condition**				
	Good body condition	2	1.96	91.294	.0001
	Medium body condition	22	21.56		
	Poor body Condition	78	76.47		
4	**Vaccine states**				
	Vaccinated	15	14.71	50.824	.0001
	Non vaccinated	87	85.29		
5	**Herd states**				
	Presence of cattle, sheep and goat	30	29.41		
	Presence cattle and sheep	16	15.69	57.843	.0001
	Presence cattle and goat	2	1.96		
	Presence of sheep and goat	54	52.94		
6	**Herd size**				
	>30	52	50.98		
	20-30	29	28.43	37.255	.0001
	10-19	15	14.7		
	<10	6	5.88		
7	**Animal management**				
	Good management	8	7.84	51.059	.0001
	Medium management	28	27.45		
	Poor management	66	64.7		
8	**Species affected**				
	Sheep	80	78.43	32.980	.0001
	Goat	22	21.56		

4.4. Virus Isolation

Characteristic pox virus cytopathic effect (CPE) was observed in infected Vero cell line within 7 days of post inoculation after one blind passage in all of the 13 representative skin nodules sampled from sheep and 3 typical skin nodule biopsies collected from goats. On the second passage CPE was observed within three days of post inoculation without any blind passage. The CPE were characterized by rounding of single cells, aggregation of dead cells and destruction of the monolayer (figure 10).

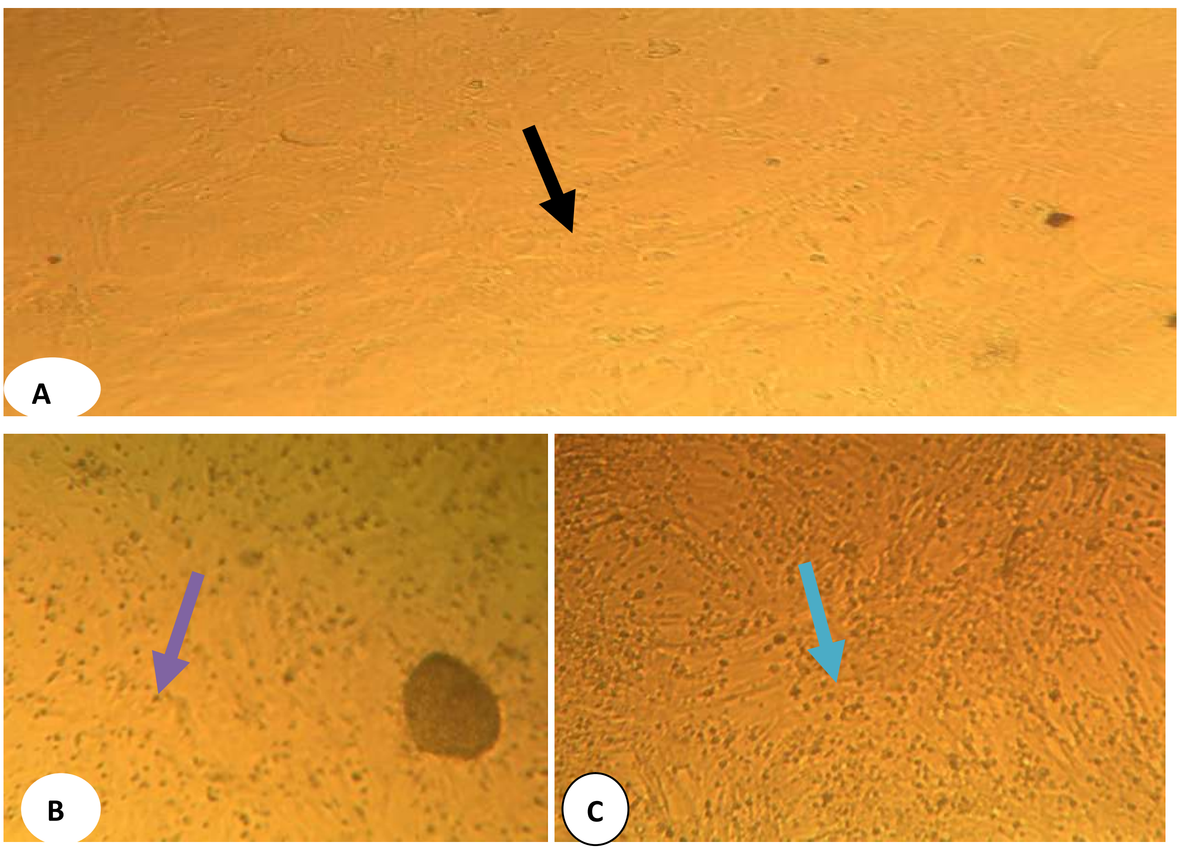


Figure 10: Pictures showing the isolation of infections Capri pox virus using vero cell line there [A] confluent non infected mono layer vero cell. [B] and [C] cytophatic effect induced by infectious sheep and goat pox virus

4.5. Classical PCR gene identification

The extracted DNA of 16 specimen that means 13 specimens for sheep pox and 3 specimens from goat pox was amplified using gene specific primers. The PCR product had molecular weight of 151 bp for sheep pox and 172 bp for goat pox which is the expected band size for sheep and goat pox genomic region targeted. PCR products on a 3% high resolution agarose gel DNA fragment of 172 bp size for sheep pox and 172 bp for goat pox was observed in all 13 sheep pox and 3 goat pox tested samples and non amplification seen on the non template control.

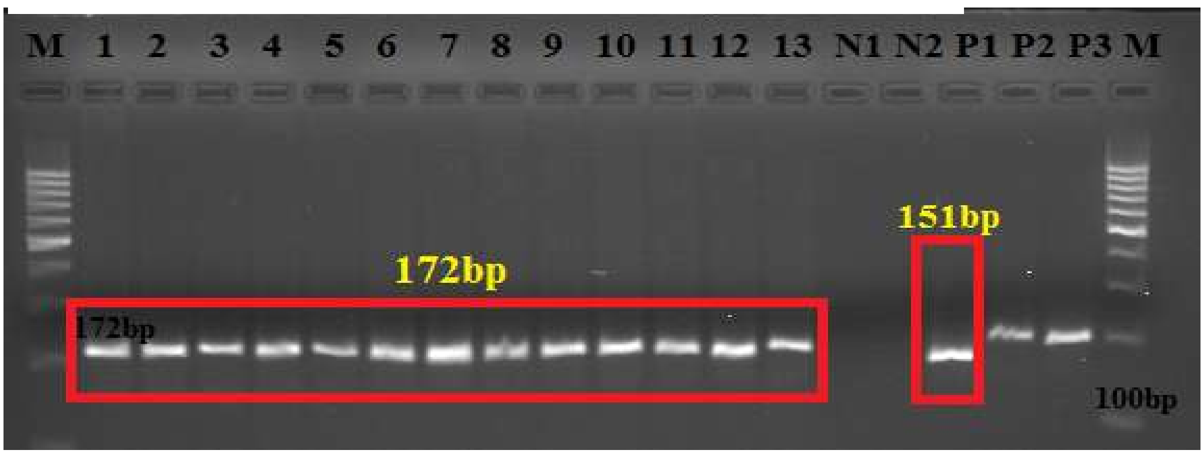


Figure 11: PCR-based detection of Goat pox virus originated from sheep on gel electrophoresis.

Lane **M**:DNA ladder; lane **1-13** represent positive sample of goat pox virus; lane **N1-** negative template control for sheep pox; **N2-** negative template control for goat pox; lane **P1-**posetivecontrol for sheep pox; **P2-**posetive control for goat pox virus; **P3-**Posetive control for LSDV.

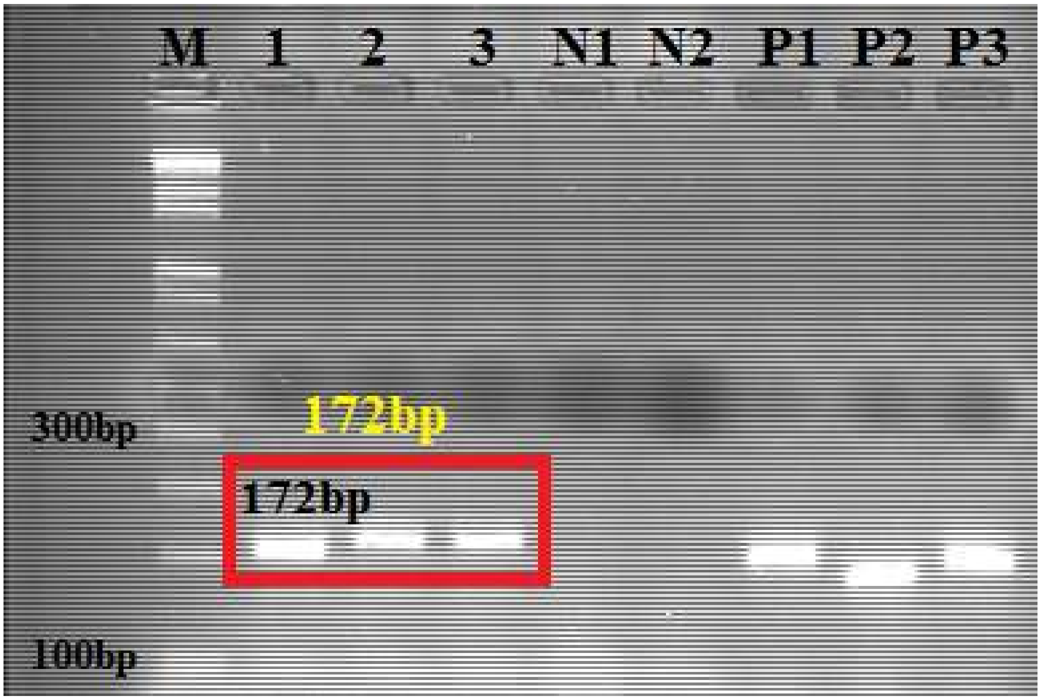
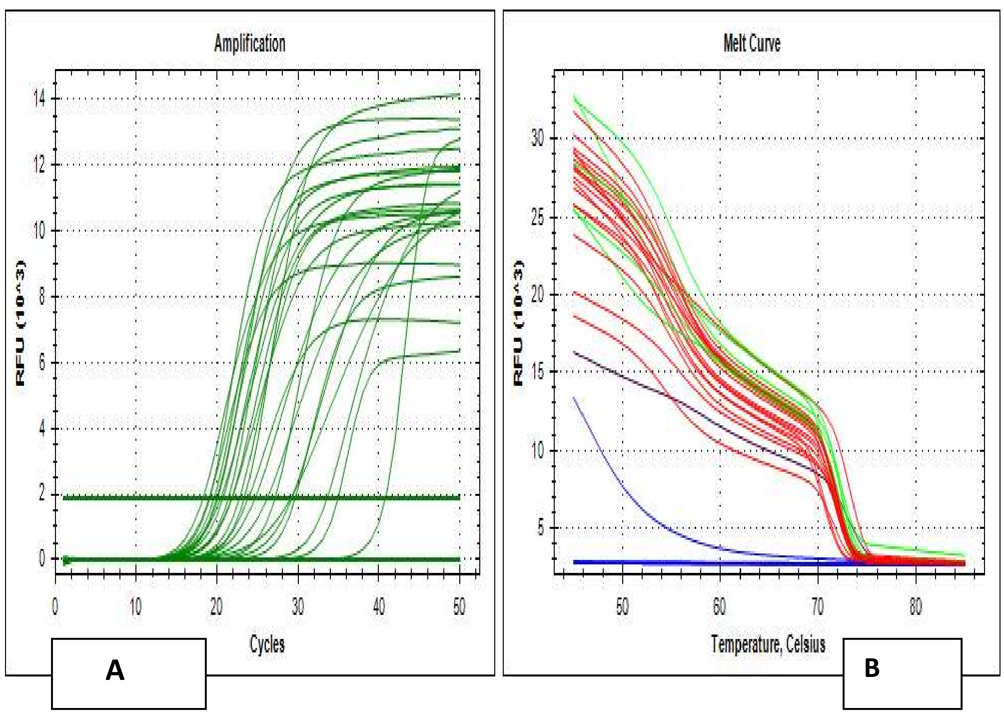


Figure 12: PCR based detection of goat pox virus collected from goats on gel electrophoresis.

Lane **M**:DNA ladder;lane**1-3** represent positive sample of goat pox; lane **N1-** negative template control for sheep pox;**N2-** negative template control for goat pox; lane **P1-** posietive control for goat pox; **P2-**posietive control for sheep pox virus; **P3-**Posietive control for LSD.

4.6. Real-Time PCR

The presence of CaPV on samples from sheep and goat was confirmed and farther genotyped using gene specific real time PCR method .The snap back assay targeting the RP030 gene. The results in all new 13 field isolate recovered from sheep were genotyped as goat pox with melting temperature 56.0 ^{o}C/72.50 ^{o}C for snap back and full length amplicons respectively (Fig.14).The goat pox field isolate was also tested and confirmed to be as goat pox virus. There was a 100% agreement between the result of the classical PCR and the real time PCR method.



A: Amplification plot **B**: Melting curve plot

Figure 13: Amplification plot and melting curve plot

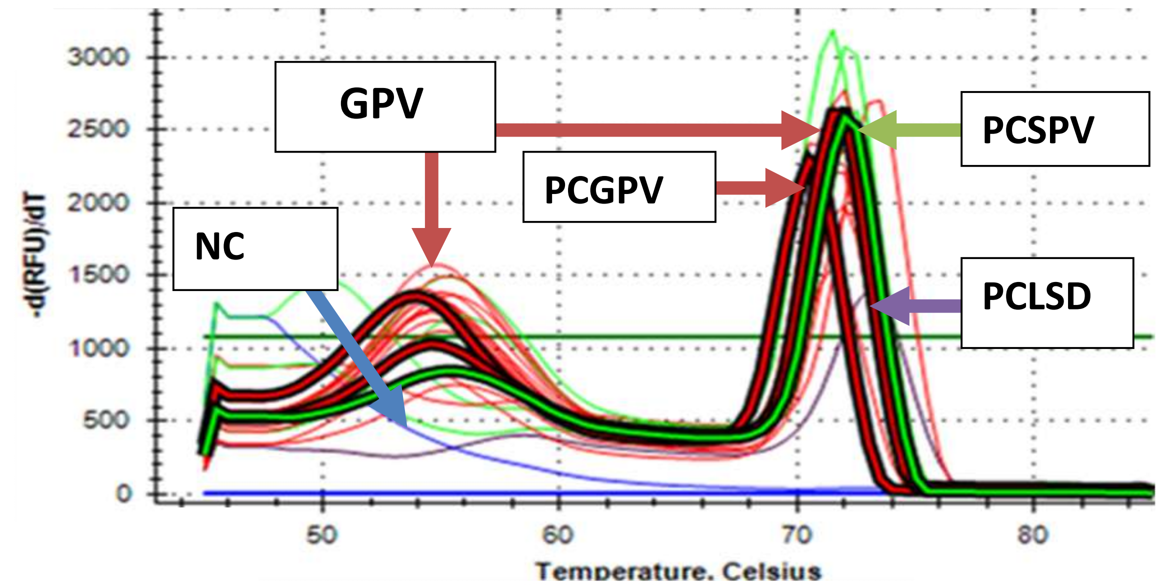
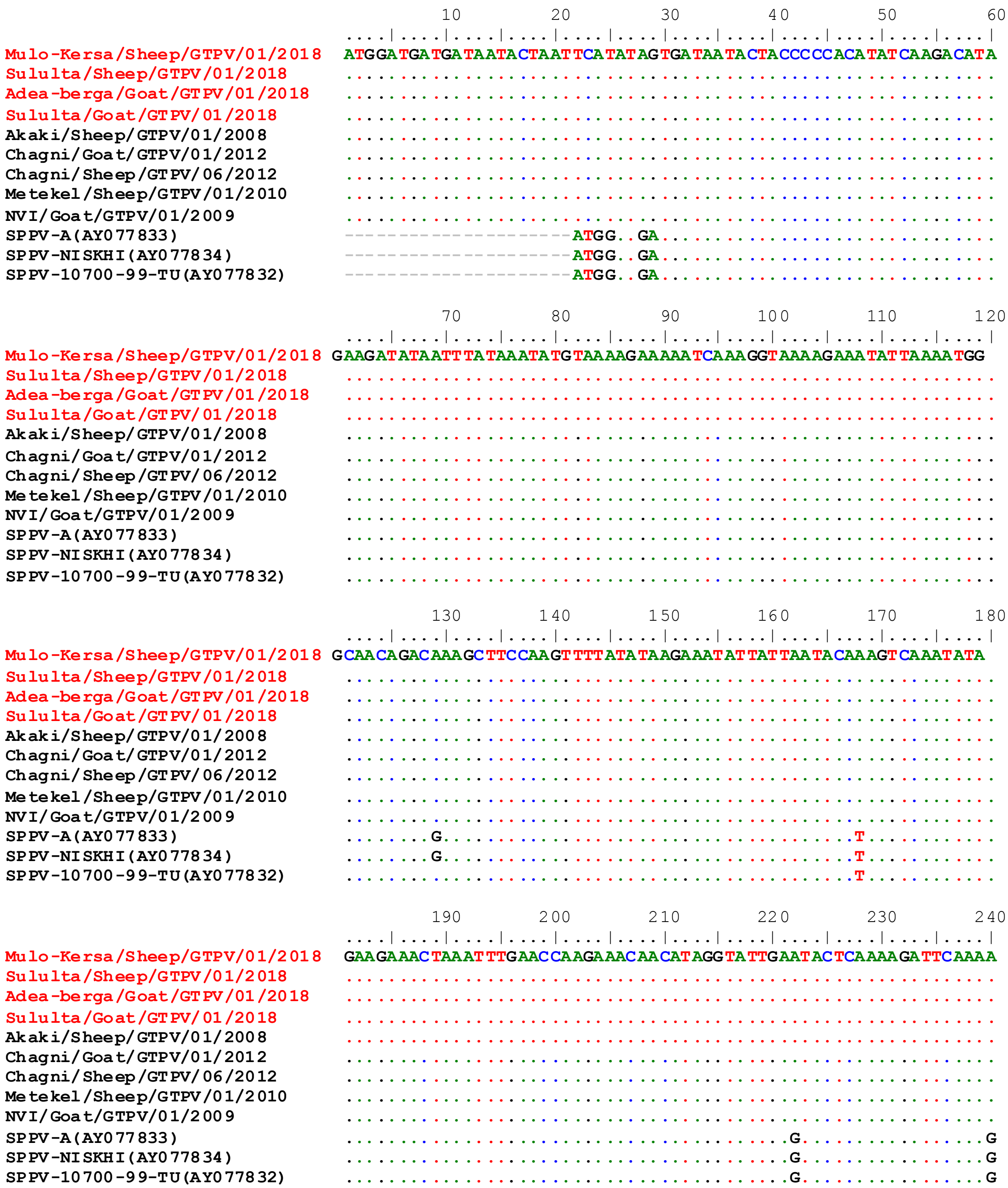


Figure 14: Melting curve analysis of the field sheep and goat pox virus isolates collecte from sheep and goat.

GPV: Positive sample for goat pox virus, **PCGPV**: positive control for goat pox virus, **PCSPV**: Positive control for sheep pox virus, **PCLSD**: positive control for LSD virus, **NC**: Negative control.

4.7. Result of RP030 gene sequencing analysis

Non synonymous mutation were observed in the RP030 gene of goat pox virus isolate from Mulo- Kersa, Sululta, Adea- berga, sheep and goats as compared to other Ethiopian goat isolate from sheep and goat (Fig 15).



N

```
                                      250       260       270       280       290       300
                                  ....|....|....|....|....|....|....|....|....|....|....|....|
Mulo-Kersa/Sheep/GTPV/01/2018     AACAAATTATCGTATAGAAACAAGCCTTTAATAGAGACAAATAAAGATTATTCTGACATA
Sululta/Sheep/GTPV/01/2018        ............................................................
Adea-berga/Goat/GTPV/01/2018      ............................................................
Sululta/Goat/GTPV/01/2018         ............................................................
Akaki/Sheep/GTPV/01/2008          ............................................................
Chagni/Goat/GTPV/01/2012          ............................................................
Chagni/Sheep/GTPV/06/2012         ............................................................
Metekel/Sheep/GTPV/01/2010        ............................................................
NVI/Goat/GTPV/01/2009             ............................................................
SPPV-A(AY077833)                  ..T................................T...........G............
SPPV-NISKHI(AY077834)             ..T................................T...........G............
SPPV-10700-99-TU(AY077832)        ...................................T...........G............

                                      310       320       330       340       350       360
                                  ....|....|....|....|....|....|....|....|....|....|....|....|
Mulo-Kersa/Sheep/GTPV/01/2018     TGTAATCTTATACGTACTACAAATGGAACAGAGAAAGAAATTTTAAGATATATACTTTTT
Sululta/Sheep/GTPV/01/2018        ............................................................
Adea-berga/Goat/GTPV/01/2018      ............................................................
Sululta/Goat/GTPV/01/2018         ............................................................
Akaki/Sheep/GTPV/01/2008          ............................................................
Chagni/Goat/GTPV/01/2012          ............................................................
Chagni/Sheep/GTPV/06/2012         ............................................................
Metekel/Sheep/GTPV/01/2010        ............................................................
NVI/Goat/GTPV/01/2009             ............................................................
SPPV-A(AY077833)                  .................A..........................................
SPPV-NISKHI(AY077834)             .................A..........................................
SPPV-10700-99-TU(AY077832)        .................A..........................................

                                      370       380       390       400       410       420
                                  ....|....|....|....|....|....|....|....|....|....|....|....|
Mulo-Kersa/Sheep/GTPV/01/2018     GGAATAAAATGTGTTCAAAAAAATGTAGAATTTAATATAGACGATATTAGAGATATAAAT
Sululta/Sheep/GTPV/01/2018        ............................................................
Adea-berga/Goat/GTPV/01/2018      ............................................................
Sululta/Goat/GTPV/01/2018         ............................................................
Akaki/Sheep/GTPV/01/2008          ............................................................
Chagni/Goat/GTPV/01/2012          ............................................................
Chagni/Sheep/GTPV/06/2012         ............................................................
Metekel/Sheep/GTPV/01/2010        ............................................................
NVI/Goat/GTPV/01/2009             ............................................................
SPPV-A(AY077833)                  ..........................................A.................
SPPV-NISKHI(AY077834)             ..........................................A.................
SPPV-10700-99-TU(AY077832)        ..........................................A.................

                                      430       440       450       460       470       480
                                  ....|....|....|....|....|....|....|....|....|....|....|....|
Mulo-Kersa/Sheep/GTPV/01/2018     TACGAAGAATATTTTAATGTTTTAGATAAAAAGTATAACCTCCAATGCCCTGAGTGCAAA
Sululta/Sheep/GTPV/01/2018        ............................................................
Adea-berga/Goat/GTPV/01/2018      ............................................................
Sululta/Goat/GTPV/01/2018         ............................................................
Akaki/Sheep/GTPV/01/2008          ............................................................
Chagni/Goat/GTPV/01/2012          ............................................................
Chagni/Sheep/GTPV/06/2012         ............................................................
Metekel/Sheep/GTPV/01/2010        ............................................................
NVI/Goat/GTPV/01/2009             ............................................................
SPPV-A(AY077833)                  ...........................................C................
SPPV-NISKHI(AY077834)             ...........................................C................
SPPV-10700-99-TU(AY077832)        ...........................................C................
```

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                                       490       500       510       520       530       540
                                  ....|....|....|....|....|....|....|....|....|....|....|....|
Mulo-Kersa/Sheep/GTPV/01/2018     AGTAAAAACACTATTCCCGTCATGATACAAACAAGAGCAGCAGATGAACCACCATTAGTT
Sululta/Sheep/GTPV/01/2018        ............................................................
Adea-berga/Goat/GTPV/01/2018      ............................................................
Sululta/Goat/GTPV/01/2018         ............................................................
Akaki/Sheep/GTPV/01/2008          ............................................................
Chagni/Goat/GTPV/01/2012          ............................................................
Chagni/Sheep/GTPV/06/2012         ............................................................
Metekel/Sheep/GTPV/01/2010        ............................................................
NVI/Goat/GTPV/01/2009             ............................................................
SPPV-A(AY077833)                  ............................................................
SPPV-NISKHI(AY077834)             ............................................................
SPPV-10700-99-TU(AY077832)        ............................................................

                                       550       560       570       580       590       600
                                  ....|....|....|....|....|....|....|....|....|....|....|....|
Mulo-Kersa/Sheep/GTPV/01/2018     ATGCATTCTTGTAGAGACTGCAAGAAAAATTTTAAACCTCCGAAGTTTAGAGCTGTAGAA
Sululta/Sheep/GTPV/01/2018        ............................................................
Adea-berga/Goat/GTPV/01/2018      ............................................................
Sululta/Goat/GTPV/01/2018         ............................................................
Akaki/Sheep/GTPV/01/2008          ............................................................
Chagni/Goat/GTPV/01/2012          ............................................................
Chagni/Sheep/GTPV/06/2012         ............................................................
Metekel/Sheep/GTPV/01/2010        ............................................................
NVI/Goat/GTPV/01/2009             ............................................................
SPPV-A(AY077833)                  .....C.................A.................A..................
SPPV-NISKHI(AY077834)             .....C.................A.................A..................
SPPV-10700-99-TU(AY077832)        .....C.................A.....A...........A..................

                                  ....|.
Mulo-Kersa/Sheep/GTPV/01/2018     AAATAA
Sululta/Sheep/GTPV/01/2018        ......
Adea-berga/Goat/GTPV/01/2018      ......
Sululta/Goat/GTPV/01/2018         ......
Akaki/Sheep/GTPV/01/2008          ......
Chagni/Goat/GTPV/01/2012          ......
Chagni/Sheep/GTPV/06/2012         ......
Metekel/Sheep/GTPV/01/2010        ......
NVI/Goat/GTPV/01/2009             ......
SPPV-A(AY077833)                  ......
SPPV-NISKHI(AY077834)             ......
SPPV-10700-99-TU(AY077832)        ......
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Figure 15: Nucleotide sequence alignment of RP030 gene of filed isolate.

The name indicated by red color is field isolate and the normal one are isolate of Ethiopian GTPV from gene bank.

4.8. Result of Phylogenetic Tree analysis

Phylogenetic tree analysis was performed to determine the genetic relationship among Ethiopian Capri pox virus isolate as well as other Capri pox virus isolate in other country. The Phylogenetic analysis of RP030 field isolate from sheep and goat were classified as GTPV (Fig16). All field isolate from sheep and goat were closely related to GTPVETH/Aka ki/2008/ (KP663669), ETH/Assosa/2010/Goat/KP663670, ETH/Chagni/01/2012/Goat (KP6 63674), Yemen/83/GTPV/ (GU119972) and differ from other GTPV isolate from other country (Fig 16).

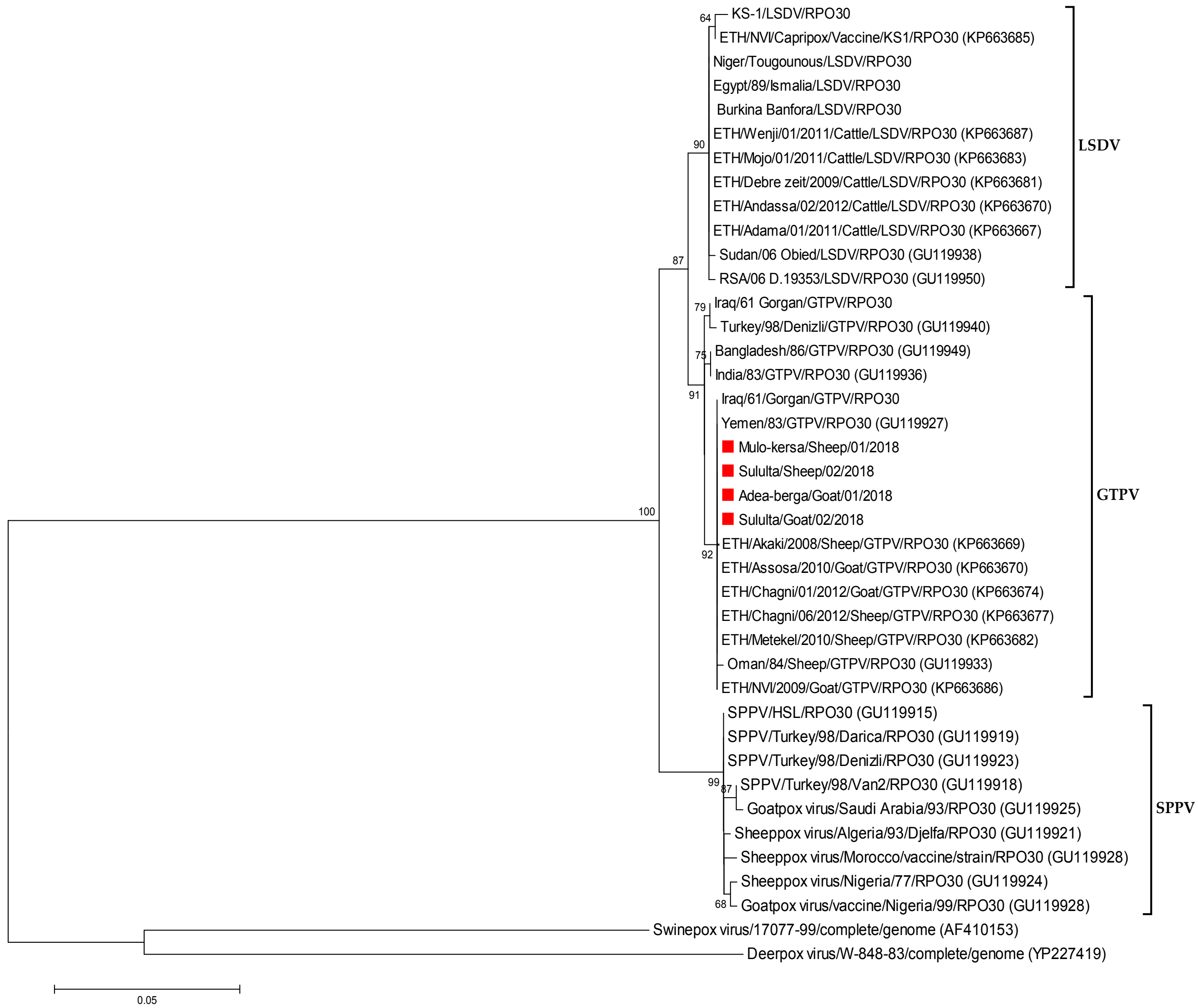

Figure 16: Phylogenetic tree analysis of Capri pox viruses

Phylogenetic analysis of 40 capripoxviruses based on nucleotide sequences of the RPO30 gene. Four Ethiopian current outbreak isolates and the sequences retrieved from the GenBank were used. The Neighbor-Joining method with the maximum composite likelihood nucleotide substitution model and the pair wise deletion option was compute using MEGA6. The percentage bootstrap scores above 50% (out of 1000 replicates) are shown next to the branches. The homologue gene sequence from one Deer poxvirus and one Swine poxvirus isolates retrieved from the GenBank were used as out-group. The isolates sequenced in this study are indicated red color rectangle.

5. DISCUSSION

The survey results revealed that majority of the respondents (95%) had previously experienced sheep and goat pox disease in their herds and familiar with the clinical sign of the disease, which they locally called *''Finno hoolaa* in Afan Oromo". Similarly, Mersha (2011); Teferi (2014) and Assefa (2017) also reported that the disease was named with similar local name in other parts of Ethiopia.

According to observation (78.43 %) sheep and goat pox disease is more important in sheep than goats. The difference could be attributed to the genetic difference of sheep and goat to resist the disease. This study are disagree with the study of Bidjeh *et al.* (1991) which reported that difference of sensitivity between sheep and goats was statistically insignificant. Additionally, 64.7% of the total observation young age groups are more susceptible than adult age group. The result was consistent with the findings of Schwabe *et al*. (1977) and Assefa (2017) who described that the morbidity and mortality by sheep and goat pox was high in young than adult age groups and young age group are more susceptible to sheep and goat pox disease, respectively.

Overall, 71.6% female sheep and goats are more susceptible than male. This might be related to female shoats physiology is correlated with lactating and pregnancy which could result poor immunity system than male shoats. According to observation poor body condition score and poor management are more pre disposing factors of sheep and goat pox disease in study areas. Furthermore, they revealed that large herd size and sheep and goat hearded together are pre disposing factors for the occurrence of the disease ($P<0.05$). This finding was correlated with the findings of Murray *et al. (*2003) who mentioned that significant association of sheep and goat pox disease occurrence with environment related factors. Similarly, the influence of various bio-meteriological factors on SPGV occurrence has also been reported in Algeria (Achour and Bouguedour, 1999).

The highest number (58.97%) of sheep affected by sheep pox virus was observed in Mulo district and the lowest number (24.4%) of sheep affected in Tiyo district. The highest number (24.39%) of goat affected by pox virus was in Yaya gulale district and the lowest number (11.11%) of goat affected by pox virus in Mulo district. This may be due to the geographical location of the districts, number of animal, movement of animal from district to district, trade of live animal of the districts, climatic factors, the grazing and migration pattern of sheep and goat in the districts and extensive grazing system of sheep and goat vary the number of affected sheep and goat between districts. During outbreak investigation the common clinical sign observed in sheep and goat pox virus was fever, depression ,loss of appetite different size skin nodules, necrotic nodule under the tail ,perineum ,udder and testicles ,lacrimation, nasal discharge, cutaneous papules and nodules in areas of skin with less hair were prominent signs of the disease. These findings were related with previous studies (Kitching *et al*., 1986, 1987, 1989; Kitching and Taylor, 1985) and Chaudhary *et al.,* 2009. The lesions found under the surface of tail, udder, perineum, head and neck. observations were also similar to that of (Singh *et al*., 1979; Davies, 1981; Sharma *et al*., 1986; Mersha, 2011; Radostits *et al*., 1994) who observed nodular form of pox as round firm flat surface nodules on the lateral aspect of abdomen and thoracic and face in some cases.

Overall, the morbidity of sheep and goat pox within species was 35.82% in sheep and 28.44% in goats, respectively. The mortality rate within species was 3.66% in goats and 9.95% in sheep, respectively. These results were not far from the results of Assefa (2017) who reported the morbidity of sheep and goat pox within species in the outbreak area of Adea berga were 32.1% as well as 29.4% in sheep and goats, respectively. The author also reported that the mortality rate within species was 6.5% in goat and 4.7% in sheep in the same area. But the slight differences could be related to management differences, large number of sheep population found in the study areas and CaPVs have traditionally been causing outbreaks in a preferred host.

Out of 13 tissue sample taken from sheep and 3 from goat, 100% sheep and 100% goats samples showed typical pox lesions after inoculation on Vero cells detect typical CPE to sheep and goat pox virus. In present study, the goat pox virus induced CPE such as small syncitia, cell ballooning, rounding, aggregation and detachment was observed within 7-10 days of incubation. Out of 16 skin biopsy samples, goat pox was isolated from all samples using Vero cell line. These findings were in agreement with (Sajid *et al*., 2013; Teferi, 2014) and (Assefa, 2017) who reported CPE development within 7-10 days.

In the present study, 16 samples yielded a product size of 172bp on agarose gel electrophoresis and the same result yielded in the snap back assay targeting the Rp030.The result of all new 13 field isolate recovered from sheep were genotyped as goat pox. The goat pox field isolate was also tested and confirmed to be as goat pox virus. There was a 100% agreement between the result of the classical PCR and the real time PCR method. Therefore, the virus isolated from both sheep and goats were not SPPV since the gel electrophoresis was greater than 151bp. This finding was in agreement with the pervious finding Lamien *et al*. (2011) who reported, the genotyping result of SPPV was 151bp and of goat was 172bp. Based on the findings of the PCR result, the present samples collected from sheep and goat population of different districts showed that the pox virus circulating in sheep and goat were characterized as goat poxvirus; whereas sheep pox virus were not identified from a single sample. This result clearly explain that both sheep and goats were equally susceptible to goat pox virus and it was only goat poxvirus circulating and causing pox disease in both sheep and goat population. The present molecular finding was in agreement with the previous report of Le Goff *et al*. (2009); Lamien *et al.* (2011); Gelaye *et al*. (2013) and Assefa (2017) who reported that goat pox virus was identified from pox lesion collected from clinically diseased sheep from different countries of the world. Additionally, the results agreed with Heine *et al.* (1999) who mentioned sheep and goat pox virus are not considered as host specific and although 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. Additionally, Gelaye *et al*., (2013) also reported that GTPV/SPPV(Tm=72.5^{o}C) and the snap back melting temperature to differentiate SPPV (51 ^{o}C) from GTPV (56 ^{o}C).

In present study RP030 gene sequences of GTPV isolates recovered from both sheep and goat are infect and provoke disease in both sheep and goat. The present RP030 sequence analysis were concord with the preceding report of Gelaye *et al*. (2015) who reported complete similarity of the GPCR and RP030 gene sequences of GTPV isolates recovered from both sheep and goats it is likely that the Ethiopian GTPV isolates are equally able to infect and induce disease in both sheep and goat. Similarly infection of sheep by GTPV have been reported previously in Oman and China respectively (Lamien *et al*.,2011;Le Goff *et al*., 2009 and Yan *et al.,* 2012).Additionally the present RP030 gene sequence were suggested that all field isolates classified as GTPV are genetically highly closely related to the previous field isolate in different geographical area of Ethiopia and other country (fig13).The current study is similar with Gelaye *et al*.,(2015) reported that only a few genetically distinct LSDV and GTPV isolates are circulating in Ethiopia. Furthermore RP030 gene of GTPV isolate from both sheep and goat is non synonymous mutation were observed as compared to other Ethiopian goat isolate. This report were similar to Gelaye *et al.,*(2015) who report RP030 gene of the Ethiopian GTPV isolate were identical within each of the genotype.

6. CONCLUSION AND RECOMMENDATIONS

Pox infection is a very common disease of sheep and goats in the study areas causing huge economic losses to the farming community, leather industry and even to the national GDP of Ethiopia.

The field virulent goat pox was easily isolated on African Green Monkey kidney (Vero cell) line within 7-10days of inoculation. Goat pox virus produces similar CPE with the recommended susceptible lines for isolation. Conventional PCR using RPO30 gene based genotyping, Real-time PCR and Sequencing confirmed that goat pox virus could cause pox outbreaks in both sheep and goat herds. This study approved that host specificity classification of CaPV is inaccurate at least for GTPV. The current finding may provide new insight on the micro-biology of sheep pox and goat pox in Ethiopia. It has also important implication in the control of the disease.

According to the current results, pox disease is more prevalent in rainy season. Furthermore, the disease was reported mainly to affect young sheep and goat. Generally, the disease associated morbidity; mortality and case fatality were high in sheep species when compared to goats. The disease was also commonly seen in unvaccinated shoats, young age groups less than one year, female sheep and goats, large heard size, poor body condition, poor management and mixed heard size of sheep and goats.

Therefore, based on the present results forwarded the following recommendations.

- There should be strict quarantine measures (control of illegal animal movement) especially the disease outbreak areas and a ring vaccination should be given regularly to the animals.
- Awareness should be created to animal health professionals and concerned bodies on inaccurateness of previous assumption of CaPVs host specificity.

- ❖ Further studies should be conducted on isolation of Circulating sheep and goat Virus strain and their vaccine matching.
- ❖ Comparative studies of Primary and cell lines for isolation of sheep and goat pox virus should be conducted.
- ❖ Further studies should be conducted to discover the effect of nucleotide and amino acid change on pathogen city of virus and immunogenicity of vaccine.

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8. ANNEXES

Annexs 1: Glasgow,s modified Minimum essential media Composition

- (GMEM) powder 12.5g
- De ionized water 1000ml

Preparation Glasgow's modified Minimum essential media.

- GMEM is dispensed into 100-200 ml amounts in screw cap bottles
- Store the bottles at 40C after sampling sterility testing
- If antibiotics are to be used add the antibiotic stock solution (Penicillin 5X 106IU vial + streptomycin 5 X 1g vial in 100ml of PBSA) to have it at a final concentration of 0.2%

Annexs 2: PBSA (Dulbecco‟s Phosphate buffered Saline) Composition

- NaCl 8.0g
- KCl 0.2g
- KH2 PO4 0.2g
- Na2HPO4. 2H2O 1.44g

Preparation of dulbecco's phosphate buffered saline

- Make up with distilled water to 1000ml
- Dissolve, than make up volumetrically to desired volume
- Mark liquid level before autoclaving
- Adjust the PH to 7.2 at 20 ^{0}C or 7.4 at 36.5 ^{0}C with sterile 1M Na OH
- Dispense into 200ml bottles
- Sterilize by autoclaving at 121 ^{0}C at 15min.
- Store the solution bottles at +4 ^{0}C

Annexs 3: Trypsin 2.5 % (W/V) solution (10 X stock solution preparation) Composition

- NaCl 8.0g
- KCl 0.4g
- Na2HPO4 0.0475g
- KH2 PO4 0.06g
- NaHCO3 0.35g
- Trypsin (1:25) 25g

- Deionized and distilled water to 1000ml

Preparation Trypsin 2.5% solution

- Dissolve by string over night at +4 ^{0}C Sterilize by filtration though a Seitz EK pad or a µm membrane filter
- Distribute aseptically into 100ml volumes
- Take sample for sterility tests
- Store at -20 ^{0}C
- For use add 100ml to 900ml sterile PBSA and adjust PH to 7.8 by the addition of sterile 1M NaOH

Trypsin 0.05% EDTA 0.02% solution for sub culturing Composition

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

EDTA (Versene) 2% stock solution Composition

- NaCl 8.0g
- KCl 0.20g
- Na2HPO4 1.15g
- KH2 PO4 0.2g
- EDTA di-sodium salt.2H2O 22.14g
- Phenol red 1.0 g

Preparation EDTA 2% Working solution

- Dissolve and make up 1000ml with de ionized distilled water
- Distribute in 100ml volume in screw cap bottles
- Autoclave at 121 0C for 15 min.
- Take sample for sterility tests
- Store at -20 0C

Annexs 4: Penicillin and Streptomycin stock solution Composition

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

Preparation of penicillin and streptomycin working solution

- Add aseptically 5 ml PBSA to each vial
- Leave for a few minutes to dissolve
- Remove the PBSA from the vials and back to the PBSA 100ml bottle
- Mix well
- Dispense 10ml into sterile containers and stor at -20oC

Annexs 5: Extraction kit master mix, gel, TAE buffer, Gel Red, Loading dye, Molecular ladder.

DNA Extraction kit Composition

- QIAamp spin columns
- Collection tubes
- Buffer AL
- Buffer AW1
- Buffer AW1
- Buffer AE 1
- Proteinase K

Master mix for pox virus DNA convectional for one reaction

- RNA free water 3µl
- Primer SPGP RNA pol forward 2 µl
- Primer SPGP RNA pol reverse 2µl
- IQ supper mix SPGP l0 µl
- Template DNA 3 µl

Master mix for pox virus DNA real time for one reaction

- RNA free water 4.84µl
- Primer CP-HRMSb –Fow-5pm/ µl 2 µl
- Primer CP-HRM/1REV-5pm/ µl 0.16 µl
- IQ supper mix SPGP l0 µl
- Template DNA 3 µl
- Eva green super mix 10 µl

Then mix and agitate divided into PCR tube Gel electrophoreses Composition

- Agarose gel 3g
- Distilled water 100m

Preparation of Gel electrophoreses

- Mix very well and boiled with micro- oven at 180 ^{o}C temperature for 3 min. until melting
- Cool at 54 ^{o}C and pour into gel tank
- Insert the gel comb into melting agarose gel
- Dry for 20 minute

Gel red Composition

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

Preparation of gel red

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

- The general rule of thumb is to use 1µL of gel loading dye per 5µL of sample PCR ladder The PCR 100 bp molecular ladder (fermentas) has been used for size determination of PCR generated DNA fragments. The recommended agarose gel concentration was 3.0%. The ladder contains 10 bands, 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.

Annexs 6: **DNA Extraction**

The sample suspensions were used for DNA extraction. DNA was extracted by using the DNeasy Tissue Kit (Qiagen, Germany) according to the manufacturer's instructions. The tissue suspensions are centrifuged at maximum of $5x10^6$ cells for 5 minutes at 3000×g (190rpm). The resuspend in PBS in 200µl, added 20µl proteinase K procceded to and then added 200µl buffer AL following that mix thoroughly by vortexing , incubated samples in water bath at 56 ^oC for 30minutes. Added 200µl ethanol (99%), mix thoroughly by vortexing and pipetted the mixture into a dneasy mini spin column placed in a 2ml collection tube. Centrifuged at $\geq$ 6000xg (8000rpm) for 1 minute. Discarded the flow-through and collection tube .Placed the spin column in a new 2 ml collection tube .Add 500 µl buffer AW1.Centrifuge for 1minute at $\geq$ 6000xg(8000rpm)for 1minutes , discard the flow- through and collection tube. Place the spin column in a new 2 ml collection tube. Add 500 µl buffer AW2. Centrifuged for 3minute at $\geq$ 20,000 x g (14,000rpm) and discarded the flow- through and collection tube. Transfer the spin column to a new 1.5ml micro centrifuge tube. Elute the DNA by adding 200µl Buffer AE to the center of the spin column membrane. Incubate for 3 minute at room temperature (24 ^oC).Centrifuge for 1minutes at $\geq$ 6000xg then put the DNA extracted at -20 ^oC temperature.

Annexs 7: Quationarry survey format for season and frequency of outbreak occurred in sheep and goat pox virus.

The purpose of this questionnaire is to gather information regarding sheep and goat pox virus. The information will be kept confidential and be only applied for the study. Your right information helps to reach goals of the study. Thank you for investing your time and honesty completing this questionnaire. Please remember that there are neither rights nor wrong answers. Put “X” mark on the box.

Back Ground information Owner ID--------------District----------------Keble--------------

1. Disease Detail

1.1. Which Season outbreak is Occur?

- ☐ Rainy season
- ☐ Dry season
- ☐ Both of them

1.2. How frequent sheep and goat pox out break occur in the area

- ☐ Every year
- ☐ Every two year
- ☐ Greater than 3 year

2.3. Local name of sheep and goat pox in your area ……………………………

Annexs 8: Outbreak investigation recording format

1. Total number of sheep affected in your heard-----------------
2. Total number of goat affected in your heard------------------
3. Total number of sheep dead by sheep and goat pox -------------
4. Total number of goat dead by sheep and goat pox------------------
5. Major clinical sign observed..
6. Total sheep and goat herd you have……………………………….

Annexs 9: Table of Sample collection format

Serial number	Sample identification code	Animal speces	Type of sample collected	Collection site	Collection date

Annexs 10: Risk factor associated to disease occurrence recording format.

Sex of sheep and goat affected by goat pox	**Number of animal**
Male	
Female	
Age of sheep and goat affected	
< 5 year	
>5 year	
Body condition affected to disease	
Good body condition	
Medium body condition	
Poor body Condition	
Vaccine states of sheep and goat affected to disease	
Vaccinated	
Non vaccinated	
Herd states of sheep and goat affected	
Presence of cattle, sheep and goat	
Presence cattle and sheep	
Presence cattle and goat	
Presence of sheep and goat	
Herd size sheep and goat	
>30	
20-30	
10-19	
<10	
Animal management system	
Good management	
Medium management	
Poor management	
Species more affected	

Sheep	
Goat	

Body condition score of the animal

During the study period, body condition of animals was classified in to poor, medium and good based on the following body condition scoring method.

Body condition score 1	The individual Spinous process is sharp when to touch and easily distinguished.
Body condition score 2	The Spinous process can be identified individually when touched but feel round rather than sharp.
Body condition score 3	The Spinous process can only felt with very firm pressure and area of either side of the tail and head have some fat cover.
Body condition score 4	Fat cover around tail and head is easily seen as slight mouds, soft to touch, the Spinous process cannot be felt.
Body condition score 5	The bone structure of the animal is no longer noticeable and the tail and head are almost completely buried in fatty tissue .

Body condition score 1 and 2= Poor

Body condition score 3 =Medium

Body condition score 4 and 5=Good

Source: (Heinonen, M., 1989)

Age determination (young and adult)

Young <5 years old

Adult > = 5 years (Melaku *et al*., 2012)