1. INTRODUCTION

Small ruminant have higher reproductive capacity and growth rates and are ideally suited to production by resource-poor small holders (Tibbo, 2006). Indigenous goat and sheep are resistant to some infectious diseases and parasites, good flocking instinct, ability to walk long distances in search of feed, high tolerance to adverse climatic conditions, endurance to droughts and low and fluctuating nutrient availability (Kosgey *et al*., 2008). They require smaller investments, have shorter production cycles and greater environmental adaptability, and hence have a unique niche in smallholder agriculture. Ethiopia has about 25 million sheep and 23 million goats (CSA, 2008).

Capripox (Goat pox, Sheep pox and Lumpy skin disease) is the most serious of the pox diseases of livestock and can cause heavy production losses in small ruminants (goat and sheep). The economic losses occur in the form of mortality, reduced productivity and lower quality of wool and leather (Parthiban *et al.,* 2005). Moreover, the slow recovery execrates production losses (Fenner *et al*., 1987). Above all, Capripox has a significant impact on hard currency earning of Ethiopia since it is associated with trade restrictions (APHRD, 2010).

Capri pox is a highly contagious viral disease affecting goat and sheep (Carn, 1993). The disease is characterized by fever, ocular and nasal discharges and pox lesions on the skin and on the respiratory and gastro-intestinal mucosae (OIE, 2012). It is caused by Goat Pox virus (GTPV) belongs to *Capripox* genus of the *Poxviridae* family (Mangana-Vougiouka *et al.*, 1999). The transmission of virus mainly occurs through direct contact via the aerosol, but indirect contact and mechanical transmission by insects can also occur. In endemic areas, spread of Goat pox mainly occurs in summer (Bhanuprakash *et al.*, 2006).

Severity of the disease depends on breed, age, nutritional and immune status, virus strain, virulence, the nature of the secondary infection and organs involved. Generally, the disease and associated mortality are less commonly seen in indigenous breeds in endemic areas as compared with exotic breeds (Sileshi, 2009). These animals may exhibit mild form of the disease characterized by mild and few skin lesions on certain areas such as the ears and around the tail.

However, indigenous animals are more likely to suffer from the disease in areas where it has been absent or dormant for a period of time.

Sheep and goat pox occur throughout Africa, the Middle East, and the Indian sub-continent, central and eastern Asia (Kitching *et al*., 1985; Geering *et al*., 1995). In endemic areas, most cases are seen in young animals and outbreaks are sporadic. The disease is also endemic in all regions of Ethiopia (APHRD, 2010) for instance; the Animal and Plant Health Regulatory Directorate received 389 Sheeppox and Goatpox outbreak reports from all regions in 2009/2010. This year report indicated that a total of 2,177,741 sheep and goats contracted the disease and 6,522 sheep and goats were at risk in areas where outbreaks occurred. However, the fatality rate was as low as 4.63% of the sick goats and sheep (APHRD, 2010).

The control strategy of goat pox varies according to the disease status of the country. Pox free countries rely upon quarantine barriers to prevent entry of infected animals to maintain their disease free status of a country. Usually these quarantine barriers are coupled with a policy of "stamping out" whenever the disease occur, to rapidly re-establish disease-freed status. On the other hand, endemic countries such as Ethiopia focus on vaccination strategy to control or prevent pox disease. Ring vaccination and restriction animal movement are used to control outbreaks cases. For effective control of pox disease through vaccination, proper diagnosis of disease outbreak and identification of the effective vaccine is essential. Though various kinds of vaccines are available; the live attenuated vaccine is the best choice (Bhanuprakash *et al*., 2006). Live attenuated vaccines are considered more effective, providing immunity for 12 months or longer (Munz and Dumbell, 1994).

Prompt and accurate diagnosis tool is paramount for successful prevention or control of goat pox. Its diagnosis is based on gross lesions, clinical signs, virus isolation, and serology. But all these diagnostic procedures have limited value in accurate diagnosis of the disease in one way or another. For example, gross clinical findings can easily be confused with disease like contagious ecthyma and contagious pustular dermatitis. Similarly, the isolation and serological techniques have limited specificity and accuracy due to antigenic and biochemical similarities of causative

agents with other poxviruses such as orthopox virus. These methods are also time consuming, laborious, and require sterile conditions (Kitching and Carn, 1996).

Ethiopian sheep and goat has been facing poxvirus infection for so many years .11.88% morbidity and 0.13% mortality was recorded based on one-year outbreak report (APHRD, 2008) and vaccination has been implemented for the control of the disease in the country. However, there are few works conducted so far on the isolation and identification of *Capri poxvirus* affecting the livestock population.

Therefore, the objectives of the present study were:

- To investigate sheep and goat pox outbreaks in Ethiopia
- To isolate and identify *Pox virus* circulating in sheep and goats in Ethiopia using cell culture method
- Molecular characterization of *Capripoxvirus* isolated from pox cases of small ruminants using conventional and real-time PCR methods

2. LITERATURE REVIEW

2.1. **History and Occurrence**

Goat pox is contagious diseases which were first reported in 1763. The causative agent and pathogenesis were first studied in 1868. Borrel in 1903 documented a detailed study on sheep and Goat pox disease. Goat pox are presently endemic in the Middle East, including Egypt, Iran, Afghanistan, Africa north of the equator, Turkey, Iraq and the Indian subcontinent. In South-Eastern Europe, sporadic outbreaks occur (OIE, 2012).

2.2. **Epidemiology**

Epidemiologically, the disease is very important and causes a huge morbidity and mortality in the endemic areas, ranging from 70-90% and 5-10%, respectively. The mortality may reach 100% in imported animals. Capripoxvirus is endemic in Pakistan, India, Iran, Afghanistan, Nepal, and parts of China, and, since 1984, Bangladesh. Recently, southern Europe is also invaded by the disease (OIE, 2008). In Bangladesh, it has been observed that Capripoxvirus causes huge mortality. Initially, the disease was restricted to the western region but later on it spread to the northern and central regions, according to Kitching *et al.*, (1987).

In Ethiopia, the disease is endemic in all regions. In 2007/2008, the Animal and Plant Health Regulatory Directorate received 893 SGP outbreak reports from all regions except Gambella, Harari and Dire Dawa. 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 (Sileshi, 2009).

In Ethiopia, little information is available on the morbidity and mortality of Sheep Pox and GTP in the country. However, Mersh (2011) was found about 49.5% morbidity and 10.42% mortality from central highland of the country based on the clinical signs and histopathological lesion. In addition, 11.88% morbidity and 0.13% mortality was recorded based on one year outbreak report (APHRD, 2008). This figure might not be exact picture of what is happening in the country since

the former study was done in one district of the country and latter figure is taken from poor reporting system data which is 35-40% rate of reporting.

2.3. **Economic impact**

Among livestock, sheep and goat are a major part and play a very pivotal role by contributing to the world economy (Abdul, 2010). Similarly, surveys conducted in the Ethiopia revealed that the disease has major impact on the economy. The direct impact of the disease is substantial losses in the production and productivity of sheep and goat. The disease also results in damage to the quality of skins and wool; as the result, it reduce price of the animals and their products (Sileshi, 2009). Therefore, the disease has a significant impact on livelihood resource-poor small holders, which mainly rely on small ruminants for living (Tibbo, 2006). Beyond these, existence of the disease in any country severely affects trade of animals and their products (Sileshi, 2009; Abdul, 2010). This in turn results in loss of export earnings. Moreover, the disease is one of the challenges for introduction of exotic breeds to improve indigenous breeds, thereby further developing of intensive farming system in the developing countries (Sileshi, 2009**).**

2.4. **Hosts**

The classification of pox viruses was made on the basis of the host from which these viruses were isolated. Serologically, all these three viruses (sheeppox virus, goatpox 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, 2012). Goat pox is a highly contagious, host specific, viral infection, and causes a high rate of mortality and morbidity in sheep and goat, 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 *Capripox 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).

During documentation of an outbreak in Saudi Arabia and in an experimental study, it was observed that both the naturally and experimentally induced disease showed its host inclination to goats (Elzein *et al.,* 2003). The strains of Capripox 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 poxvirus 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). The British breeds of sheep and goats with the isolate of sheep pox and goat pox from Kenya, India, Nigeria, turkey, Sudan, Yemen, Arab republic and Pakistan and were inoculated. It was not possible to differentiate the disease based on clinical manifestation only because the disease is caused by different isolates (Kitching and Taylor, 1985).

2.5. **Transmission**

Sheep and goat pox disease is usually transmitted by the respiratory route during close contact while inhalation of virus containing air droplets. Infectious virus is found in all secretions, excretions, and the scabs from skin lesions. The disease may also enter the body through other mucous membranes or abraded skin when broken surfaces contact contaminated objects, feed and wool. Wading of sheep and goat through bushes and thorny plants, like Acacia to nibble leaves damages the skin facilitating the disease transmission from the infected to the susceptible sheep. These viruses can remain infectious for up to six months in shaded sheep pens. They may also be found on the wool or hair for as long as three months after infection, and possibly longer in scabs. Moreover, biting flies, especially *Stomoxys calcitrans* are an efficient mechanical vector of capripox (Mellor *et al*., 1987; OIE, 2012). Pre-infected flies transmit the virus to susceptible goats and the virus remains alive for 4 days in some flies. High virus titres and

intrinsic resistance of the virus, vector with large mouth with coarse parts and their frequent feeding habits are the basic factors favouring mechanical transmission (Bhanuprakash *et al*., 2006).

2.6. **Etiology**

Goat pox disease is caused by a virus in the family Poxviridae and genus Capripox virus and is highly contagious. Among the viruses it is the largest one with brick shaped morphology of 170 to 260 by 300 to 450-nm-diameter capsid. Its genome is double-stranded DNA, non-segmented, and linear of approximately 150 kilo bases. A false lipid envelope surrounds the genome. The virus is endemic in Africa, the Middle East, India, and Asia (Kitching, 2003; ICTV, 2006) reported that Capripoxvirus caused goat pox, sheep pox and lumpy skin disease, in goats, sheep and cattle respectively.

2.7. **Pathogenesis**

The pathogenicity of the field isolates was documented by injecting them intradermally in lambs, showing different cutaneous lesions with increasing body temperature and death. By using serum neutralization test, the isolate gave high titers of antibody to the experimental infection (Baroodi, 2009). Clinically, it was noticed that virus isolated from Yemen was more pathogenic in goats, causing 100% morbidity and mortality as compared to sheep with 0% mortality and 100% morbidity. While the virus isolated from Vietnam in goats gave 100% morbidity with 33% mortality, while in sheep, it gave mild morbidity and 0% mortality (Babiuk *et al.*, 2009). After inoculation, goat pox virus replicates locally in the tissues. On the 7^{th} day post-inoculation, the virus titer reached to its peak. After 3-4 days of primary viremia, the virus spread to the regional lymph nodes. 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 - 14 days after inoculation, the virus titers persisted and decreased with the development of serum antibodies. Usually, the virus spread through skin scabs but the nasal and ocular secretions may also be infectious .When sheep were inoculated by the intranasal and intradermal routes all KLP infected lambs survived. Lambs infected with KLP showed extensive reduction in fever response, viremia, gross lesions and virus-shedding (Balinsky *et al.*, 2007).

For documenting the pathogenesis of these diseases, an experimental study has been conducted by inoculating Nigerian sheep pox virus or Indian goat pox virus intradermally in their respective hosts. After 2-3 weeks after inoculation, viremia developed which was confirmed by real-time PCR and virus isolation. Within 10 and 14 days after inoculation, shedding of virus and viral DNA in conjunctival, nasal, and oral secretions occurred, and this shedding remained for 3 to 6 weeks more with low quantity of virus in these discharges. Macroscopic changes developed in many organs but only the skin gave highest viral titers and in gastrointestinal tract and oronasal tissues (Timothy *et al.*, 2007). Poxviruses can enter into the host body through different routes like skin, respiratory tract and the oral route. Poxviruses usually cause acute infections rather than chronic, with less chances of recurrence. The infection may be local or systemic (Buller and Palumbo, 1991).

2.8. **Diagnosis of Goat poxvirus**

The diagnosis of the disease is based on gross lesions, clinical signs, isolation, and serological techniques. However, all these diagnostic procedures have limited value in accurate diagnosis of the disease in one way or another (Kitching and Carn, 1996). Because of the importance and permanent threat to goat population, there is a dire need to rapidly and specifically diagnose the causative agent in order to find out new ways and means for an efficient control of this menace

2.9. **Clinical Signs**

Goat pox is highly contagious diseases characterized by an initial rise in temperature 40–42ºC, increased pulse and respiratory rate, salivation, edema of eyelids, hypersensitivity, arched back, in-appetence, lacrimation, coughing, nasal discharge leading to crust formation, scanty urine, pneumonia and constipation (Afshar *et al.*, 1986; Bhanuprakash *et al.*, 2006).

The characteristic pox lesions usually become visible on the skin and on the mucosa of GIT and respiratory tract. After infection, the skin lesions usually erupt within 24 to 48hrs. The skin lesions usually spread mostly on areas of little or no hair, like on the groin, face, under the tail ears and maxillae. These lesions also occur on mucous membranes of the vulva, nostrils and mouth. The development of these lesions occurs by passing through the typical pox lesion developmental stages including, skin erythema, papule and vesicle with clear fluid that convert

to pustule containing pus, which, on rupture, form crusts on the lesion, and finally scabs formation occur. The skin lesions usually heal within 5-6 weeks, which is very slow. Mostly the mortality occurs within two weeks after infection but the animal may die at any stage of the disease (Gulbahar *et al.,* 2006). It is noted that different symptoms developed by one virus strain in multiple breeds of different age group animals usually mixed with those developed by other viruses within the same group, or even different groups of poxviruses (Kitching, 1983).

2.10. **Postmortem Lesions**

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). Post-mortems of dead animals showed vesicles and pock lesions on affected skin. The lungs are diffusely inflamed (bronchopneumonia) with dispersed pock lesions (Dubaib, 2002).

2.11. **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 goat 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 cells (Singh *et al.,* 2007). The histopathological examination of infected skin showed hydropic degeneration and eosinophilic intracytoplasmic inclusion bodies in epidermal cells. Microscopic examination of skin sections showed epithelial hyperplasia, cellulitis and intracytoplasmic viral inclusions. Lung sections showed the alveoli full of inflammatory cells and presence of viral inclusions (Dubaib, 2002).

Histopathological examination revealed marked proliferative change especially in the ectodermal layer with presence of intracytoplasmic acidophillic inclusion bodies (Joshi *et al.*, 1996). In early stages of lesions, an inflammatory response in lymph nodes and vasiculitis with associated thrombosis and infraction manifests in necrosis and edema (Prozesky and Barnard, 1982).

2.12. **Virus Isolation and Propagation**

2.12.1. Sample collection and preparation

Sample for virus isolation is usually biopsy or at post-mortem from skin papules, lung lesions or lymph nodes. Samples are taken from sick animal within the first week of the occurrence of clinical signs, before the development of neutralizing antibodies. Buffy coat from blood collected into ethylene diamine tetracetic acid (EDTA) during the viraemic stage, before generalization of lesions or within 4 days of generalization, can also be used for virus isolation (OIE, 2012).

The tissue is minced using sterile scissors and forceps, and then ground with a sterile pestle in a mortar with sterile sand and an equal volume of sterile phosphate buffered saline (PBSA) containing antibiotic The homogenized suspension is freeze–thawed three times and then partially clarified by centrifugation using a bench centrifuge at 3000 rpm for 10 minutes. In cases where bacterial contamination of the sample is expected, the supernatant can be filtered through a 0.45μm pore size filter after the centrifugation step.

2.12.2. In vivo virus isolation

Sheep is the highly susceptible and natural host for sheep and goat poxvirus. The virus yield is high in Kivrcik and Merino sheep. Even today, sheep are the host of choice for propagation of different virulent SPPV and GTPV. Propagation of SPPV and GTPV in embryonated chicken eggs is inconclusive. Cytoplasmic inclusions, as well as inflammatory cells and necrosis in the infected chorioallantoic membrane (CAM) have been observed (Tantawi *et al.*, 1980; Fehri *et al.*, 1984; Joshi *et al.*, 1996; Rao and Bandyopadhyay, 2000).

2.12.3. In vitro virus isolation

Recent advances in vitro methods for propagation of viruses coupled with stringent laws about in-humane animal treatment prompted scientists to use in vitro methods. Until mid-seventies, SPPV had been propagated in sheep skin for vaccine production. This technique was practically tedious, uneconomical and unsafe. Therefore, most of the researchers shifted to different cell culture systems for propagation of SPPV and GTPV. Tissue cultures of skin from sheep, its

embryo and fetuses have been used for propagation of SPPV and GTPV. Sheep fetal skin cultures after infection with GTPV produced CPE and enhanced titer (Lang and Leftheriotis, 1961) and the virus was safe and immunogenic after 15 passages.

Currently, primary lamb kidney or primary lamb testis cells are the most commonly used cells for isolation (Kalra and Sharma, 1981; Zhou *et al*., 2004). They induce the formation of distinct plaques (Soman and Singh, 1980) with a cytopathic effect characterized by elongated cells (Jassim and Keshavamurthy, 1981). However, primary cells have several disadvantages including the need to constantly establish new cultures, cell lot variation, and contamination with extraneous agents. A lamb testis secondary cell line (OA3.Ts) has been evaluated as a replacement for primary cells (Babiuk *et al*., 2007).

Capripoxvirus isolation can be confirmed by immunostaining using anti-capripoxvirus serum (Gulbahar *et al*., 2006; Babiuk *et al*., 2007) but it is not yet possible to differentiate between SPPV, GTPV and LSDV, as there is only a single capripoxvirus serotype (Kitching, 1986). Immunostaining also allows easier visualization of capripoxvirus plaques (Babiuk *et al*., 2007).

2.13. **Detection viral antibody**

Serological test can identify GTPV and SPPV as capripox viruses, but cannot distinguish these two viruses from each other. Antibodies to capripox viruses can be found approximately one week after the skin lesions appear

2.13.1. Latex agglutination test (LAT)

Antigens can be coated very effectively on to latex beads (carriers), which can be applied in agglutination test. This test is economical, rapid, reliable, easy to perform and successful in the detection of antigen–antibody systems. LAT is more efficient than CIEP in the diagnosis of sheep pox (Rao *et al*., 1997). LAT has also been employed for diagnosis of goat pox .The gene Q13L coding for the Capripoxvirus group specific structural protein P32 was expressed in Escherichia coli using plasmid pGEX-2T as a fusion protein with glutathione-s-transferase and

purified on glutathione sepharose affinity chromatography column. The protein was then employed for diagnosis of sheep and goat pox by latex agglutination test (LAT) (Muinamia *et al.*, 2007)

2.13.2. Neutralization test

A test serum can either be titrated against a constant titre of capripoxvirus (100 TCID50 [50% Tissue Culture Infective Dose]) or a standard virus strain can be titrated against a constant dilution of test serum in order to calculate a neutralization index. Because of the variable sensitivity of tissue culture to capripoxvirus, and the consequent difficulty of ensuring the use of 100 TCID50, the neutralization index is the preferred method, although it does require a larger volume of test sera. The test is described using 96-well flat-bottomed tissue culture grade microtitre plates. The use of Vero cells in the virus neutralization test has been reported to give more consistent results (Kitching and Taylor, 1985). Neutralization is most specific but not sensitive enough to identify animals that have had contact with the virus and developed only low level neutralizing antibody. This is because immunity to capripox virus (GTPV) infection is predominantly cell mediated. Even though, neutralization had been satisfactory for diagnosis (Arik, 1967).

2.14. **Detection of Antigen**

2.14.1. Immunohistochemistry

This technique is used to detect antigen in tissue section together with the specific lesion (Rodriguez *et al.*, 1996). Over the last two decades immunohistochemistry has had an immense impact on the practices of diagnostic pathology. The identification of highly specific cellular epitopes in routinely processed paraffin wax-embedded tissue sections with specific antibodies are used, and a suitable labeling system is a usual practice in many pathological laboratories. Immunohistochemistry is used to identify cellular or tissue antigens by means of antigen-antibody reaction. The antigen and antibody complex is being identified either by use of a secondary labeling or direct labeling of the antibody method. Different methods were developed for detection of antigens in tissue sections. Both direct and indirect techniques are widely used in

routine practices. The most common methods used are the streptavidin-biotin complex horse radish peroxides method (StreptABC/HRP) and the labeled streptavidin-biotin (LSAB) method. The antigen was found in the degenerated epithelial cells of the lungs, skin, GIT with pox lesions in the cytoplasm of pox cells (Gulbahar *et al.*, 1999).

2.14.2. Agar gel precipitation test (AGPT)

The agar gel immuno-difussion test is based on the property of proteins to randomly diffuse in an agar of certain concentration. Antibodies and antigen are placed in wells at a defined distance. After incubation, at the site in the agar gel where antibodies and the antigen meet and bind, precipitation lines are formed, visible under a beam of intense oblique light against a black background. It was application in diagnosis of sheep and goat pox was reported much later using either homologous or heterologous serum (Soad *et al*., 1996). AGPT does not distinguish between capripox and CPD due to antigen cross-reactivity and is less sensitive (Mangana-Vougiouka *et al.*, 2000). An efficient AGPT using soluble antigens has been reported (Rao *et al*., 1997). It had improved the sensitivity of AGPT using (35S) methionine labelled antigen.

2.14.3. Counter immunoelectrophoresis (CIEP)

Counter immunoelectrophoresis (CIEP) is the most rapid test for viral antigen detection. It is carried out on a horizontal surface using a suitable electrophoresis bath, which consists of two compartments connected through a bridge. The apparatus is connected to a high-voltage source. Agar or agarose (1–2%, [w/v]) dissolved in 0.025 M barbitone actate buffer is dispensed on to microscope slides in 3-ml volumes. From six to nine pairs of wells are punched in the solidified agar. The electrophoresis bath is filled with 0.1 M barbitone acetate buffer. The pairs of wells in the agar are filled with the reactants: sera in the anodal wells and antigen in the cathodal wells.

Counter immunoelectrophoresis has been found more sensitive and rapid than AGPT for the detection of GTPV. Additionally, CIEP has been more effecting in detecting GTPV specific antigen in infected tissues (skin, lymph nodes, lungs, liver) as well as cell cultures than AGPT

because of its relatively more sensitivity (Uppal and Nilakantan, 1967). Similarly, CIEP has also been reported to be more sensitive than AGPT for detection of vaccinia antigen in calf, rabbit, and human skin lesions as well as infected CAM (Mangana-Vougiouka *et al.,* 2000**;** Amal et *al*., 2008).

2.14.4. Fluorescent antibody technique (FAT)

Fluorescent antibody test (FAT) is used for detection of pathogens in animal tissues or fluids, using specific antibodies against the targeted antigen. The antibodies are labeled with fluorescent dye, most commonly, fluorescein isothiocyanate (FITC). Sample antigen and labeled antibody complex results in visible fluorenscence light under microscopic examination. Test is reliable and simple for the detection of GTPV (Davies and Otema, 1978). Using this technique, it is possible to detect GTPV antigen in infected edema fluid, skin, LK and LT cells, chicken embryo and bovine kidney fibroblast monolayer (OIE, 2012).

2.14.5. Enzyme linked immunosorbent assay (ELISA)

This ELISA test is an indirect ELISA one based on SPPV P32 recombinant fusion protein expressed in *Escherichia coli* was found rapid, reliable, non-infectious and detected antibodies to capripox virus post-infection. The anti-recombinant P32 antibody based ELISA has been developed. Unlike in immunofluorescence and AGID, no cross-reactions have been reported with orthopoxviruses and parapoxviruses in P32 ELISA (Heine *et al.,* 1999). An antigen-trapping ELISA has also been developed which was compared well with virus isolation from skin biopsy samples of sheep, goats and cattle and reduced the reliance of diagnostic laboratories on tissue culture facilities. The test is most suitable to detect Capripox virus in tissue culture. Recently, a relatively simple immunocapture ELISA for the detection of SPPV and GTPV antigens in scab suspensions has been developed. Post-vaccinal immune response has been efficiently monitored with different kinds of ELISAs. Development of MAbs against SPPV and GTPV will definitely pave the way for differentiation of SPPV and GTPV thereby providing specific diagnosis (Carn, 1995).

2.14.6. Western blotting

Immunoblotting combines the high resolution of gel electrophoresis with the specificity of immunochemical detection and offers a means of identifying immunodominant proteins recognised by antibodies from infected animals or monoclonal antibodies (MAbs) directed against the target agent. The procedure can have six steps: Preparation of the sample, Resolution of the antigen by gel electrophoresis, Transfer of the separated polypeptides to a membrane support (nitro-cellulose membrane), Blocking nonspecific binding sites on the membrane, Incubation with detecting antibody, and Detection of bound antibody. Western blotting of test sera against capripox virus infected cell lysate provides a sensitive and specific system for the detection of antibody to capripox virus structural proteins. However, the test is expensive and difficult to carry out (Chand *et al*., 1994). Positive test samples and controls produce a consistent reaction with the major structural proteins of capripox virus of molecular weights 67, 32, 26, 19 and 17 kDa, whereas negative serum samples will not react in this pattern.

2.15. **Molecular Diagnosis Techniques**

2.15.1. Conventional Polymerase chain reaction (PCR)

Sheep and goat pox virus is belongs to *Capripoxvirus* (CaPV) genus of the *Poxviridae* family of viruses also comprises GTPV, and LSDV viruses, Members of the CaPV genus are closely related, with genomic identities ranging from 96% between viral species to 99% between isolates of the same species (Tulman *et al*., 2002). PCR can detect capripox viruses in blood, nasal or oral swabs, scabs, skin lesions and tissue samples and can be taken after neutralizing antibodies have developed. PCR techniques have been developed to detect CPV DNA in infected cell culture supernatants, biopsy samples and to differentiate between SPPV, GTPV and LSDV on the basis of unique restriction sites in the corresponding PCR fragments. The PCR technique becomes more effective for the diagnosis of GTPV from field samples when combined with restriction enzyme analysis (REA) of PCR-amplicons. Recently, SPPV and GTPV from infected cell culture supernatants and skin biopsy were clearly differentiated by REA of PCR amplified P32 gene products (Hosamani *et al*., 2004). To identify a diagnostic target for strain genotyping,

the CaPV homologue of the Vaccinia virus gene which encodes the 30 kDa DNA-dependant RNA polymerase subunit, RPO30 was used. Most CaPVs fit into one of the three different groups according to their host origins: the SPPV, the GTPV and the LSDV group. A unique 21-nucleotide deletion was found in all SPPV isolates which was exploited to develop a RPO30-based classical PCR test to differentiate SPPV from GTPV that was allow rapid differential diagnosis of disease during CaPV outbreaks in small ruminants .Primers used study bind and amplify the partial RPO30 gene including the 21 nucleotide deletion. As a result SPPV yields the product size (152 bp) which is lesser than the GTPV product size (172 bp). This genotyping PCR was successfully used for identifying SPPV and GTPV. The same PCR was also used by for CaPV species identification (Senthilkumar *et al.,* 2006; Lamien *et al.,* 2011; Yan *et al.,* 2012)

2.15.2. Real-time Polymerase Chain Reaction (RTPCR)

The dynamics of Real-time PCR are typically observed through DNA binding dyes like SYBR green or DNA hybridization probes such as molecular beacons (Strategene) or Taqman probes (Applied Biosystems). The basis of real-time PCR is a direct positive association between a dye with the number of amplicons. The plot of logarithm 2- based transformed fluorescence signal versus cycle number will yield a linear range at which logarithm of fluorescence signal correlates with the original template amount. A baseline and a threshold can then be set for further analysis. The cycle number at the threshold level of log-based fluorescence is defined as Ct number, which is the observed value in most real-time PCR experiments, and therefore the primary statistical metric of interest

The CaPV real-time PCR assay provides a rapid, sensitive test for CaPV that is able to detect SPPV and GTPV prior to the onset of clinical disease. For preclinical detection of GTPV, the sample of choice was nasal swabs, which provided better sensitivity than buffy coat samples. For clinical identification of GTPV, scabs or skin lesion biopsies provided high sensitivity and specificity and were easy to collect without the need for euthanizing suspect animals (Balinsky *et al*., 2007)

To use of the real-time PCR for the detection of viral DNA in the clinical samples SYBR Green-based real-time PCR assay for the detection of goat pox virus using a plasmid construct carrying one of the highly conserved genes encoding the virion envelope protein (P32) as a template.

After 40 amplification cycles, a melting analysis was carried out to verify the correct product by its specific melting temperature (Tm). This method does not require post-PCR manipulation because the melt curve data allow to verifying amplification products, thus diminishing the potential contamination risk. No primer–dimers were observed in the amplification products when analysed by melting curve (Tian *et al.*, 2012).

A cost-effective, cross-platform compatible and easy-to-perform real time PCR assay was developed for CaPV genotyping using a snapback primer and the dsDNA intercalating EvaGreen dye. A snapback probe element added to the 5'end of the forward primer allowed the formation of a second melting peak during the melting of the PCR products, corresponding to the melting of the snapback stems. Using a combination of the melting of the snapback stems and those of the ampicons, we were able to develop a new approach for CaPV genotyping. The genotyping was achieved by using information from both snapback and amplicons melting. The melting of the amplicons was used to differentiate LSDV (Tm = 73.5ºC) from GTPV/SPPV (Tm =72.5ºC). Because the melting peaks separation can be more accurately determined in this region due to height of the peaks as compared to, the snapback melting peaks of SPPV (52.0ºC) and LSDV (51.0ºC) which are more flat (Zhou *et al.,* 2008; Tian *et al.*, 2012; Gelaye *et al.,* 2013).

2.16. Differential diagnosis

A number of diseases may show symptoms akin to sheep and goat pox and some of them warrant differential diagnosis. Bluetongue (BT). Animals are depressed and have a nonpurulent conjunctivitis. The muzzle is swollen, congested, edematous, and there may be coronitis. Deformed aborted foetuses and newborn lamb and kids may be encountered. Peste des petits ruminants (PPR). Conjunctivitis, rhinitis, and oral lesions (white, raised and necrotic) are common. Pneumonia, diarrhea, and mortality approaching 90% in lambs and kids under 1 month of age are characteristic. Contagious ecthyma (contagious pustular dermatitis, Orf). This is most severe disease in lambs and kids and the proliferative pox lesions are common on the muzzle and eyes of affected neonates; mortality may approach 50%. Nursing females may have proliferative pox lesions on the teats and muzzle. It is a zoonotic disease and the lesions in attendants are not uncommon. Photosensitized, dry, flaky and inflamed areas are confined to the non-pigmented parts of the skin. Severe signs of respiratory distress with extensive parasitic lesions but no pox

lesion on the skin. Focal, raised lesions on the skin represent caseous abscesses, which are absent in sheep pox infections. Streptothricosis (*Dermatophilus congolensis* infection). Lesions are superficial and often moist and are common in the skin of neck, axillary region, inguinal region and perineum. Giemsa staining (Bhanuprakash *et al*, 2006) may demonstrate the organism.

2.17. Prevention and Control

The most likely manner for Sheep and goat poxvirus to enter a new area is by introduction of infected animals. Restrictions on the movement of animals and animal products (meat, hair, wool, and hides) are essential to prevent introduction of SPV. Wool, hair, and hides must be subjected to suitable decontamination procedures before entry into non-endemic areas. If a new case is confirmed in a new area before extensive spread occurs, the area should be quarantined, infected and exposed animals should be slaughtered, and the premises cleaned and disinfected. Vaccination of susceptible animals on premises surrounding the infected flock(s) should be considered.

If the disease has spread over a large area, the most effective means of controlling losses from GTPV is vaccination; however, consideration should be given to eliminating infected and exposed flocks by slaughter; properly disposing of animals and contaminated material; and cleaning and disinfecting contaminated premises, equipment, and facilities. A carrier state has not been shown for GTPV. However, the virus may persist for many months on contaminated premises. The imposition of quarantines on areas and premises containing infected or exposed animals is required to prevent disease spread. If the disease has spread extensively, massive vaccination followed by cessation of vaccination and control of animal movements from the area represent a strong strategy to control and then eradicate Sheep and goat pox disease (Bhanuprakash *et al.*, 2006).

2.18. Vaccines

As there are no broad-spectrum antiviral pharmaceuticals available, hygienic measures to limit exposure and vaccination are the only means to prevent or control viral infections. Because of

viruses are highly variable, and many viral infections are due to viruses with multiple serotypes. Sheep and goat pox is a major health hazard of sheep and goat, which can be controlled mainly by vaccination. Various kinds of vaccines are available but the live attenuated vaccine is the best choice (Bhanuprakash *et al.*, 2006).

The current vaccine produce in Ethiopia is in national veterinary institute at Debre Ziet. Vaccine is live freeze dried *Capripoxvirus* KSGP-0180 strain cultured on VERO-cells freeze dried (lyophilized) with a minimum titer of 2.5 $TCID_{50}$ per field dose the vaccine is available. Vaccine should be stored at a temperature of -20ºc reconstitution and dilution of vaccine is in 100 ml of cool and sterile saline water. 5ml or 20ml vial of 100 doses 1 ml of diluted vaccine injected subcutaneously on the inner face of the thigh and both for sheep and goat. Immunity is develops eight days after vaccination and may last for two years (NVI, 2012).

3. MATERIALS AND METHOD

3.1. **Study Area**

The study was conducted from November 2013 to May 2014 in districts located in three National Regional States of Ethiopia (Benshangul Gumuz, Amhara and Oromia Regional States) which were purposively included in the present study since pox outbreaks were occurred during the study period. Hence, Assosa town and its suburb areas from Benshangula Gumuz Regional States; Debre Berhan town and its suburb areas from Amhara Regional States; and Adama, Bishoftu, Fiche and Mojo towns and their suburb areas from Oromia Regional States were the specific study districts/towns included in the study based on pox outbreak reports in small ruminants during the study period (Figure 1).

Assosa area altitude ranges from 550 to 2,500 meters above sea level. Although the average temperature in the area is 2.16ºC; average temperature exceeds 27ºC during hottest six months. Its average annual temperature is 21.6ºC. However, average high temperatures become 27.8ºC while 15.6ºC of average low temperatures. The annual rainfall varies from 500-1800 mm with 1056 mm of average precipitation. The area mainly gets rain within May to October period (NMSA, 2003). The rainy season spreads through May to October (NMSA, 2003). The total small ruminant population of the area is estimated to be sheep 4778, and goats 24139 head. The livestock management system is mixed farming system.

Adama, Bishoftu and Mojo areas are situated on the top of Great Rift Valley. Thus, these areas have similar agro-climate condition with minimum variation. Bishoftu town is situated 47 km while Mojo 88 km and Adama 99 km distant south east of the capital city. Their altitude ranges 1700 to 1900 meter above sea level. The total population of small ruminants in the area is 134 thousand.

Debre-Berhan and Fiche Woredas are found in the centre of highland plateau of the country and both districts almost have similar agro-climate conditions. Fiche town is located 85 km while Debre Berhan town 120 km north of the capital city. Most areas of the districts have altitude of

more than 2500 meter above sea level. The rainfall patterns is bimodal type and mean annual rainfall varies from 900 to 1360 mm. the average relative humidity in a year is 62%. 21ºC is the recorded average annual temperature in the areas. However, cold harsh climate is very common between November and January. In study area, Debre Berhan small ruminant population is estimated at Sheep 200676 and 635422 head. In Fiche the district's small ruminant population is estimated at, 80 900 sheep and 16 491 goats head.

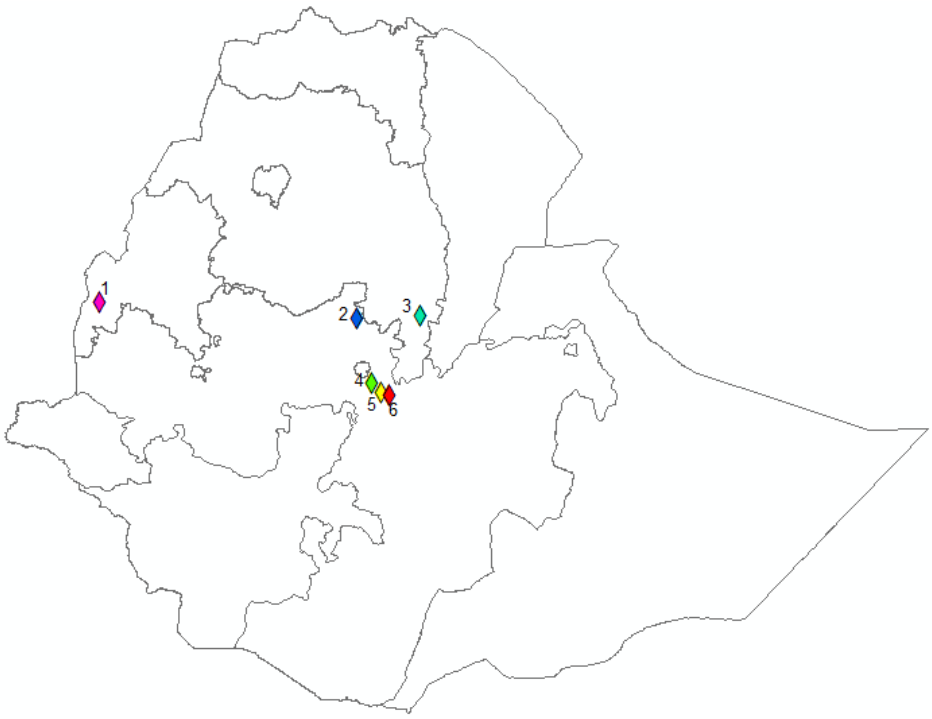

Figure 1. Map of Ethiopia showing the study areas where outbreak pox samples were collected from sheep and goats. Where 1 = Assosa, 2 = Fiche, 3= Debre Berhan, 4 = Debre zeit, 5 = Mojo, and 6 = Adama.

3.2. **Study Animals**

The study had covered three regions of Ethiopia rearing sheep and goats. Almost all small ruminants are indigenous breeds, but there are very few numbers of sheep and goats having exotic blood. The indigenous breeds included in this study were Arsi-Bale and Menz breeds of sheep and Western lowland breed of goats. The study sheep were managed under small holder farming system, but study goats were reared under semi-intensive farming system in Assosa

town. Female were predominant in small ruminant herds, though some households managed only male for fattening. The study included adult above 5 months of both sexes.

3.3. **Sampling Strategy and Size**

Purposive sampling was employed to get appropriate study animals. The National Regional States and their respective woredas were purposively selected based on the occurrence of suspected sheep and goat pox outbreaks during the study period. From the selected zones, villages were also purposively chosen based on their proximity to the towns and access of transportation service. Five herds from Adama, two herds from Assosa, Bishoftu, Debre-Berhan and Fiche; and three herds from Modjo were selected and included for taking samples based on presence of sheep and goats pox disease with typical pox lesions in the herd and owner's willingness to cooperate with study was also considered. Form each pox infected herd one sample was collected.

3.4. **Field Clinical Examination**

A total of 352 sheep and 376 goats of 16 herds from three agro-ecological regions were visually inspected for pox lesion development. However, 102 sheep and 50 goats were purposively selected and thoroughly examined by palpation. Physical examination focused on all parts of the body including the mucous membranes, mouth, the ears, perineum, less wool covered body parts and scrotal areas. Rectal temperature was also taken.

3.5. **Questionnaire Survey**

The questionnaire survey was carried out in woredas where outbreak occurs. The respondents from each village were purposively selected from households rearing small ruminants. A total of 100 respondents were interviewed using semi-structured questions to assess the disease status and seasonality. Majority (98%) of the respondents were male while 2% were female. Ten from Assosa; fifteen each from Adama and Modjo, twenty each from Bishoftu, Fiche, and Debre

Berhan respondents were interviewed. The semi-structure question was prepared to gather information on age; sex, herd size, animal management, and outbreak seasonality (Annex 8).

3.6. **Sample Collection**

A total of sixteen sheep and goat suspected for poxvirus infection were examined carefully for the presence of clinical lesion on their skin. The diagnosis of sheep and goat pox disease was done on the basis of clinical observation of pox lesions. Tissue samples of skin biopsies were collected from the outbreak areas. About 3 gm of tissue samples was taken from goats and sheep showing typical pox lesions. The samples were placed in sterile universal bottle containing 50% phosphate buffer saline A (PBSA) at a pH of 7.2 with 1% Gentamycin. Species, sample code, sex, age and village was labeled on the bottles. The samples were transported using cold box to the National Veterinary Institute (NVI), Bishoftu and after arrival they were kept at -20ºC until processed.

3.7. **Samples Processing**

The biopsy samples were thawed at room temperature and washed three times using sterile PBSA containing antibiotics and antifungal at a pH of 7.2 in Bio-safety cabinet Class II. About 1 gm of the samples was ground using sterile mortar and pestle by adding 9 ml of sterile PBSA. The tissue suspension was centrifuged at 3,500 rpm for 10 min at 4ºC. The supernatant was collected, filtered through 0.45 µm membrane filter and preserved at -80ºC until use.

3.8. **Preparation of Vero Cell Monolayer**

African Green Monkey kidney Cell line (Vero) (AU-PANVAC, Ethiopia) was used for isolation of virus. Dulbecco's Modified Eagle's Medium (DMEM) was prepared (Annex1) according to the Manufacture instruction for cell line propagation and virus isolation (HiMedia, India). The DMEM solution was supplemented with 10% inactivated calf serum, 10% tryptose phosphate Broth (TPB) (Oxoid, England), 1% gentamycin solution prior to use. Vero cell line was grown in

Roux flask in the facilities of Virology Laboratory of Research and Development Section, NVI. Cells were observed daily for their confluent monolayer under inverted microscope.

This cell line was processed for harvesting and transferring to new 25 cm^2 plastic tissue culture flasks. The growth medium overlaying the cell monolayer was pour off in a sterile beaker under sterile conditions. The monolayer was rinsed, washed twice with 10 ml sterile PBSA and covered with 5 ml of sterile 0.25% trypsin for about 5 minutes in an incubator at 37ºC. The trypsin was removed quickly to avoid wastage of detached cells. 10 ml of the complete media was added and rolled with jerking and hitting avoiding damage to 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 flasks already containing 10 ml growth medium with 10% fetal calf serum. The whole process was carried out under aseptic and sterile conditions under Bio-safety cabinet Class II. These flasks were placed horizontally in the incubator at 37ºC. After three days, all the flasks had developed a confluent monolayer with typical cell sheet with light frosted glass appearance, having clearly visible fibroblastic whirls. The cells in that phase was considered fit for sub-culturing and virus infection.

3.9. **Isolation of Virus**

Isolation pox virus was carried according to previously described protocol (OIE, 2012). Briefly, inoculation of the field virus was made on two days after sub-culturing when monolayer reached 80% confluence. The processed supernatants kept at -80ºC were thawed in advance of inoculation. The exhausted medium from the flask having confluent monolayer was discarded and the monolayer was washed with sterile PBSA. Three flasks were used. Monolayer of Vero cells grown in 25 cm^2 tissue culture flasks were inoculated with 1 ml of sample supernatant using two flasks per sample. Following incubation at 37°C for 2 hours for virus adsorption the inocula were discarded, the flasks were washed three times in the medium, followed by the addition of maintenance medium containing 2% calf serum, penicillin 10,000 UI/ml, streptomycin 100 µg/ml, kanamycin 50 µg/ml and amphotericin B 2.5 µg/ml. The third flask containing confluent monolayer cells was filled with media only and kept as control flask.

Three flasks per sample were incubated at 37ºC and each flask was observed daily for 7 – 10 days under the inverted microscope for any cytopathic effect (CPE) development. The medium having 2% calf serum was changed every 48 hours. When 80% CPE was observed, the flasks were frozen at -20ºC (after pH adjustment). The virus was harvested after two freeze-thaw cycles. When no CPE is visible until day 14, the culture was freeze–thawed three times, and clarified supernatant inoculated on to fresh Vero cell culture. In general, two more blind passages were carried out for samples that were initially negative for CPE.

3.10. **Polymerase Chain Reaction (PCR)**

The confirmatory diagnosis of the cell culture positive sample was made by conventional and real-time PCR using primers that amplify RNA polymerase subunit 30 kDa (RPO30) gene which could enable to differentiate goat pox virus from other Capri poxviruses.

3.10.1. DNA Extraction

DNA extraction was conducted in the Molecular Biology laboratory of the National Veterinary Institute. Extraction of DNA from 10% (w/v) tissue sample suspension and/or cell culture homogenate was carried out using DNeasy Blood and Tissue Kit (Qiagen, Germany) following the manufacturer's instruction. Accordingly, 200µl cell culture suspension was transferred into a labelled 1.5 ml eppendorf tube. 20 µl proteinase K and 200 µl Buffer AL was added for each tube and mixed by vortexing and incubated at 56ºC for 30 minutes (until completely lysed). 200µl 96% ethanol was added per tube and mixed thoroughly gently by vortexing. The mixture was transferred to a labelled DNeasy mini spin column placed in a 2ml collection tube and centrifuged for 1 minute at 12000rpm. The collection tube was changed by new one and 500µl Buffer AW1 was added into the spin column and centrifuged for 1 minute at 12000rpm. The collection tube was again changed by new tube and 500µl Buffer AW2 was added and centrifuged for 3minutes at 20000rpm.

Finally, the spin column was transferred into a labelled 1.5ml eppendorf tube and 40 µL Buffer AE (elution buffer) was added to the center bottom of the column and the content was incubated for 3 minutes at room temperature and centrifuged for 1 minute at 10,000 rpm to elute the DNA

into the eppendorf tube. The nucleic acid bound to the silica membrane was eluted and the tube was labeled properly and kept at -20ºC until analysis.

3.10.2. Conventional PCR

Polymerase Chain Reaction (PCR) protocol described by Mangana-Vougiouka *et al.,* (1999) was followed. Conventional PCR was performed aiming to amplify a small fragment of the 30KDa RNA polymerase subunit (RPO30) gene of capripoxviruses. The method is able to differentiate goat poxvirus from sheep poxvirus since the gene harbor a well conserved sequence signature for the differentiation and genotyping of the two poxviruses. Accordingly, PCR was conducted to amplify small fragment of the RPO30 gene using the primers and protocol described by Lamien *et al*. (2011). The primers used were SpGpRNApolF (5'-TCTATGTCTTGATATGTGGTGGTAG-3') and SpGpRNApolR (5'-AGTGATTAGGTGGTGTATTATTTTCC-3') and synthesized by VBC Biotech (Vienna, Austria) and purified by reverse phase high-performance liquid chromatography.

The strategy was the primers flanking the region containing a 21-nucleotide deletion in SPPV sequences so that the PCR amplification products from SPPV isolates would be shorter in comparison to those from GTPV isolates (151bp for sheep poxvirus and 172bp for goat poxvirus). A total of 16 samples representing from different geographical area and animal species were analyzed by PCR.

PCR was carried out in a volume of 20µL containing 2µL forward primer, 2µL reverse primer, 10µL iQ supermix (BioRad, Germany), 4µL DNase free water and 2µL viral DNA. No-template, positive and negative controls for each genotype were included.

The PCR tubes containing 20 µl final volume were transferred into the thermal cycler (2720, Applied BioSystems). The thermal cycling protocol was first initial denaturation for 5 min at 95ºC followed by 40 cycles of 95ºC for 30 sec, 55ºC for 30 sec and 72ºC for 30 sec; and final extension at 72ºC for 2 min.

3.10.3. Agarose Gel Electrophoresis of PCR Product

PCR products were analyzed by 3% agarose gel electrophoresis as described by Lamien *et al*. (2011). Briefly, 3 gm Agarose was added into a flask containing 100 ml of 1X TAE (Tris-acetate-EDTA) buffer. The mixture was boiled to dissolve and cooled to 55ºC. 5 µl GelRed nucleic acid stain (Biotium, Germany) was added. The gel was poured on gel caster placed horizontally and the comb was placed in the caster. When the gel was completely solidified after 20 minutes, the gel was placed in the electrophoresis tank containing 1X TAE running buffer and the comb was removed carefully.

In the first lane 10 µl 50 bp DNA ladder (Fermentas, Lithuania) was loaded, while in remaining lanes 10 µl sample PCR products, Positive control of sheep pox and non template and mixed with 2 µl DNA 6x loading dye were loaded in each wells by using micropipettes. Micropipette tips were changed for each sample. The gel-running tank was connected to the power supply. The voltage was adjusted to 100 volt and run for 1 hour. The gel was then observed under the UV trans-illuminator gel documentation system and gel picture was captured using a Polaroid photograph camera. Virus genotyping was determined and recorded based on the band size of the PCR product.

3.10.4. Real time PCR

In this study, a real-time PCR assay was applied for the simultaneous detection and genotyping of the 16 poxvirus infection suspected samples using the method developed by Gelaye *et al*., (2013). The method is cost-effective, fast and easy to perform and designed based on the principle of using snapback primer and dsDNA intercalating dye. The real time PCR was executed at the Molecular Biology Laboratory, NVI. Each sample was tested in duplicate and every PCR run included no-template and positive GTPV, LSDV and SPPV controls. Primers used were snapback forward primer 5''-ggTGT**A**GTACGTATAAGATTATCGTATAGAAACAAGCCTTTA-3' and reverse primer 5'-AATTTCTTTCTCTGTTCCATTTG-3'. The PCR was set in a 20 µL reaction volume containing 2µL forward primer, 0.16µL reverse primer, 10µL 1x SsoFast EvaGreen Supermix (BioRad, German), 5.48µL DNase free water and 2µL sample DNA. The PCR assay was completed the result within 2:30 hours and was used.

No-template (RNAase free water); positive GTPV (Goat Pox Virus), LSDV (Lumpy Skin Disease Virus) and SPPV (Sheep Pox Virus) controls were included for each run. All samples and controls were added in duplicate fashion.

The PCR mix was transferred into Low Profile Hard-Shell H 96-well PCR plate (Bio-Rad, German) sealed with Microseal B adhesive seals, and the detection and genotyping assay was completed within 2:30 hours. PCR was performed in a CFX96^{TM} real-time PCR detection system (BioRad, Germany) with an initial denaturation step at 95ºC for 3 minutes, followed by 45 cycles of 95ºC for 15 seconds and 58ºC for 80 second. The PCR product was then denatured at 95ºC (held for 1 minute), cooled to 40ºC (held for 1 minute), and heated continuously at 0.5ºC/10 seconds with fluorescence acquisition from 40ºC to 85ºC. The melting temperatures were analyzed using the CFX^{TM} Manager Software Version 2.0 (Bio-Rad, Germany) and the correspondent curves were displayed as negative first-derivative plots of fluorescence with respect to temperature. Genotyping of the virus was achieved by observing the melting temperature of snapback stems of the hairpins and those of the full-length amplicons (SPPV: 51.5ºC/72.5ºC, GTPV: 56.0ºC/72.5ºC and LSDV: 51.0ºC/73.5ºC), respectively for each tested samples as stated in Gelaye *et al.* (2013).

3.10.5. PCR for Sequencing and PCR Product Purification

Sixteen samples were again subjected for conventional PCR aiming to amplify the full RPO30 gene sequence using the over-lapping primers and thermal cycling protocol recommended by Gelaye *et al*. (unpublished). 5µL from each PCR product was checked by gel electrophoresis. PCR positive product was purified individually using Wizard® SV Gel and PCR product purification kit (Promega, Germany) following the manufacturer's instruction. Briefly, and the remaining 45µL PCR product was mixed with equal volume of membrane binding solution and let it for 1minute. The mixture was transferred to a labeled SV mini-column, and incubated for 1minute at room temperature and centrifuged at 13,000 rpm for 1 minute. The flow-through was discarded into bowel and mini-column was reinserted into new collection tube. Then 700µl membrane washing solution was added into mini-column and centrifuged at 13,000 rpm for 1 minute to remove residuals. The flow-through was discarded and mini-column was reinserted to a new collection tube. 500 µl membrane washing solution was added into mini-column and centrifuged at 13,000 rpm but for 5 minutes and the flow-through with collection tube was

discarded. Finally, the mini-column was transferred into empty collection tube and centrifuged the column for 1 minute while the micro-centrifuge lid open to evaporate any residual washing solution.

The mini-column was transferred into a labeled sterile 1.5ml eppendorf tube and 50 µl of nuclease free water (elution buffer) was added to the center bottom of the column. The tubes were incubated for three minutes and centrifuge at 13,000 rpm for three minutes to elute the DNA. The purified virus DNA concentration and purity was quantified using micro-volume spectrophotometer (Nanodrop 2000c, USA) with Software version V1.0. The concentration of the quantified purified PCR product was adjusted following the requirements set by the sequencing service company (LGC Genomics, German). Either forward or reverse sequencing primers were added into the labelled eppendorf tube containing the quantified virus DNA. The labeled eppendorf tubes containing mixture of DNA and primer are ready to be shipped for sequencing Service Company and kept at +4ºC.

3.11. **Data Analysis**

The collected data during sampling and laboratory analysis was entered and stored into Microsoft office Excel spread sheet 2007. The data were thoroughly screened before subjecting to statistical analysis. Descriptive statistics was used to summarize data of lesion, questionnaire and laboratory findings. PCR product of 151 bp for SPPV, 172 bp for GTPP and LSDV band size on agarose gel electrophoresis was used for genotyping CaPV using convention PCR. The melting temperatures in real-time PCR assay were analyzed using the CFX^{TM} Manager Software Version 2.0 (Bio-Rad, Germany).

4. RESULT

4.1. **Questionnaire Survey**

In this study, the result of the questionnaire survey indicated that sheep and goat pox was common disease in the study areas in which 96% (n=100) of the respondents reported the frequent occurrence of pox disease in their sheep and goat herds. Similarly, with regard to seasonal occurrence, 84 of the respondents informed that the disease was more prevalent during summer followed by spring season of the year and the remaining respondant did not associate the disease occurrence with the season. According to their explanation, the disease is often observed throughout summer and the beginning of the spring season. All respondents informed that the disease equally affected both sheep and goat; with more sever in sheep population. They also explained similar susceptibility of all age groups of both species.

4.2. **Field Clinical Examination**

Out of 102 sheep examined, 34.3% sheep developed pox lesions on their skins where as eight goats from the total of 50 were found to be positive for pox lesion (Table 1). The pox lesion was observed on 28 (n=82) sheep from Oromia, 7 (n=20) sheep for Amhara, and 8 (n=50) goats from Benishagul Gumuz Regional States.

Table 1. Sheep and goats clinically examined for pox lesion and sampled

Town	No. of examined herd	Species of animal examined	No. of examined animal	No. of animal with pox lesion	No. sampled animal
Assosa	2	Caprine	50	8	2
Debre Berhan	2	Ovine	20	7	2
Adama	5	Ovine	25	8	5
Bishoftu	2	Ovine	15	6	2
Fiche	2	Ovine	18	5	2
Modjo	3	Ovine	24	9	3
Total	**16**	**----**	**152**	**43**	**16**

Most of animals having the pox lesion were adult and more prevalent in sheep. During clinical examination few sheep and goats with typical pox lesions showed fever, 104-108ºF, lacrimation, nasal discharge, loss of appetite and reluctance to move (Fig. 2). However, most sick sheep and goats manifested only skin pox lesions without any systemic signs. The pox lesions were found all over the skin but were mostly confined to the areas with little or no hair, such as the face, ears, groin, and perennial region, and under the tail. The pox lesions like macules, pustules and scabs involving most of the body with more lesions around the eyes, lips, nares, the thighs and the upper neck region were encountered.

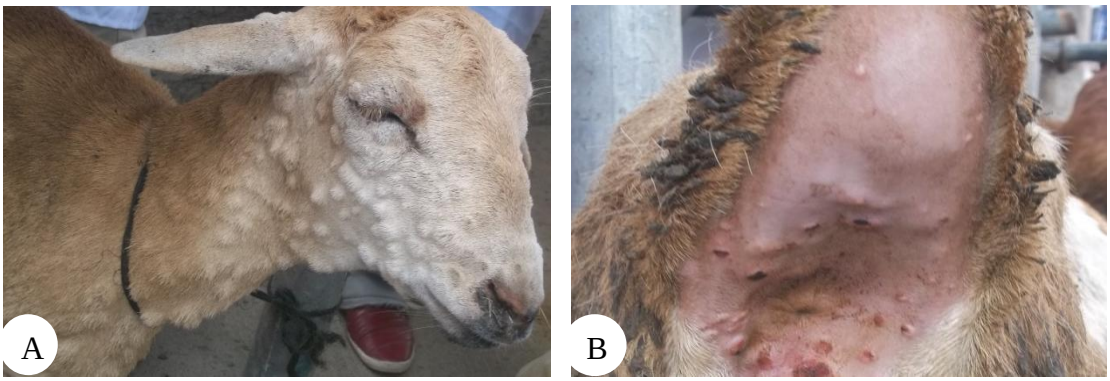

Figure 2. Pox lesions in clinically diseased sheep: A) face and neck region and B) perennial region.

4.3. **Virus Isolation**

Out of the 16 tissue samples processed, 14(87.5%) of them were produced cyptopathic effect (CPE) on Vero cell line at the first passage (Table 2). However, Adama-5 and Modjo-C samples could not develop any CPE on Vero cell line until second blind passage incubated for 10 days.

Table 2. Number of samples developed characteristic pox virus CPE with area of collection

Town	No. sample processed	Result	
		Positive	Negative
Adama	5	4	1
Assosa	2	2	-
Bishoftu	2	2	-
Debre Berhan	2	2	-
Fiche	2	2	--
Modjo	3	2	1
Total	**16**	**14**	**2**

The observed CPE were characterized by small syncytia, cell ballooning, rounding, aggregation and detachment (sloughing) of the cell sheet (Fig. 3). The infected cell lines were showed the early CPE at an average of 6 days post inoculation. The virus was harvested within 7–10 day of inoculation for further diagnosis.

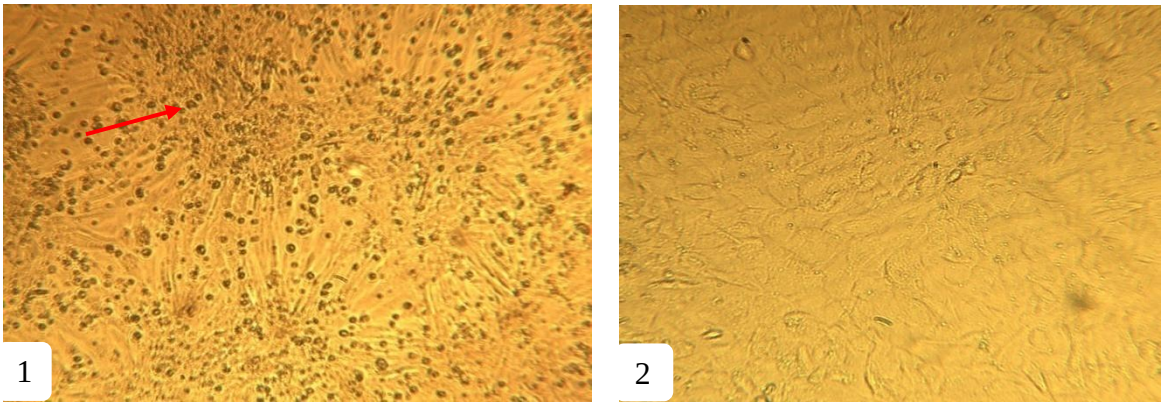

Figure 3. Picture taken using camera fitted inverted microscope: 1) cells developed characteristics CPE of pox virus as shown by arrow, and 2) Normal Vero cell monolayer.

4.4. **Conventional Gel-Based PCR Result**

All 16 skin samples were analyzed by conventional genotyping PCR. Similarly, out of those, 14(87.5%) samples were positive for *goat pox virus*. With respective to National Regional State,

12 twelve of 10 the samples collected from Oromia and two samples from each Assosa and Amhara produced band size of 172 bp on agarose gel electrophoresis (Fig 4). However, two samples from Oromia, i.e., Adama-5 on lane 5 and Modjo-C on lane 8 were again negative by conventional PCR since they could not produce any band on gel electrophoresis (Fig 4).

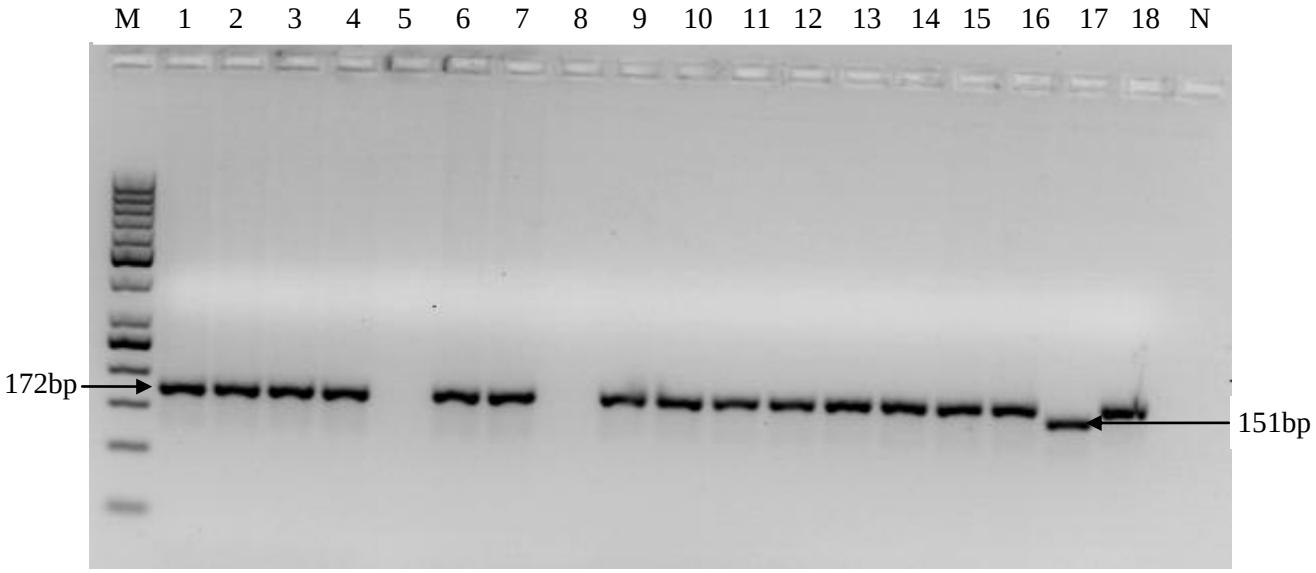

Figure 4 . Classical PCR for differentiating GTPV from SPPV based on a 21-nucleotide deletion in the SPPV RPO30 gene. The PCR products were separated by electrophoresis on a 3% high resolution agarose gel. This gel picture shows the PCR results of different pox samples. Where lane 1-5 = Adama; 6-8 = Mojo; 9-10 = Bishoftu; 11-12 = Debre Berhan; 13-14 = Assosa; and 15-16 = Fiche; 17 = Sheep poxvirus (positive control, 151bp); 18 = Goat poxvirus (positive control, 172bp); N = No-template control; and M = Molecular marker 50bp (Fermentas).

4.5. **Real Time PCR Results**

From a total of 16 samples tested by real-time PCR 14 samples were positive. However, 12 samples were early amplified at 19-25 cycle beyond cycle threshold and two samples showed by 1 and 2 line on Fig. 5 were lately amplified at 38-39 cycle. But, the two samples (Adama-5 and Modjo-C) did not amplify even at 50 cycles.

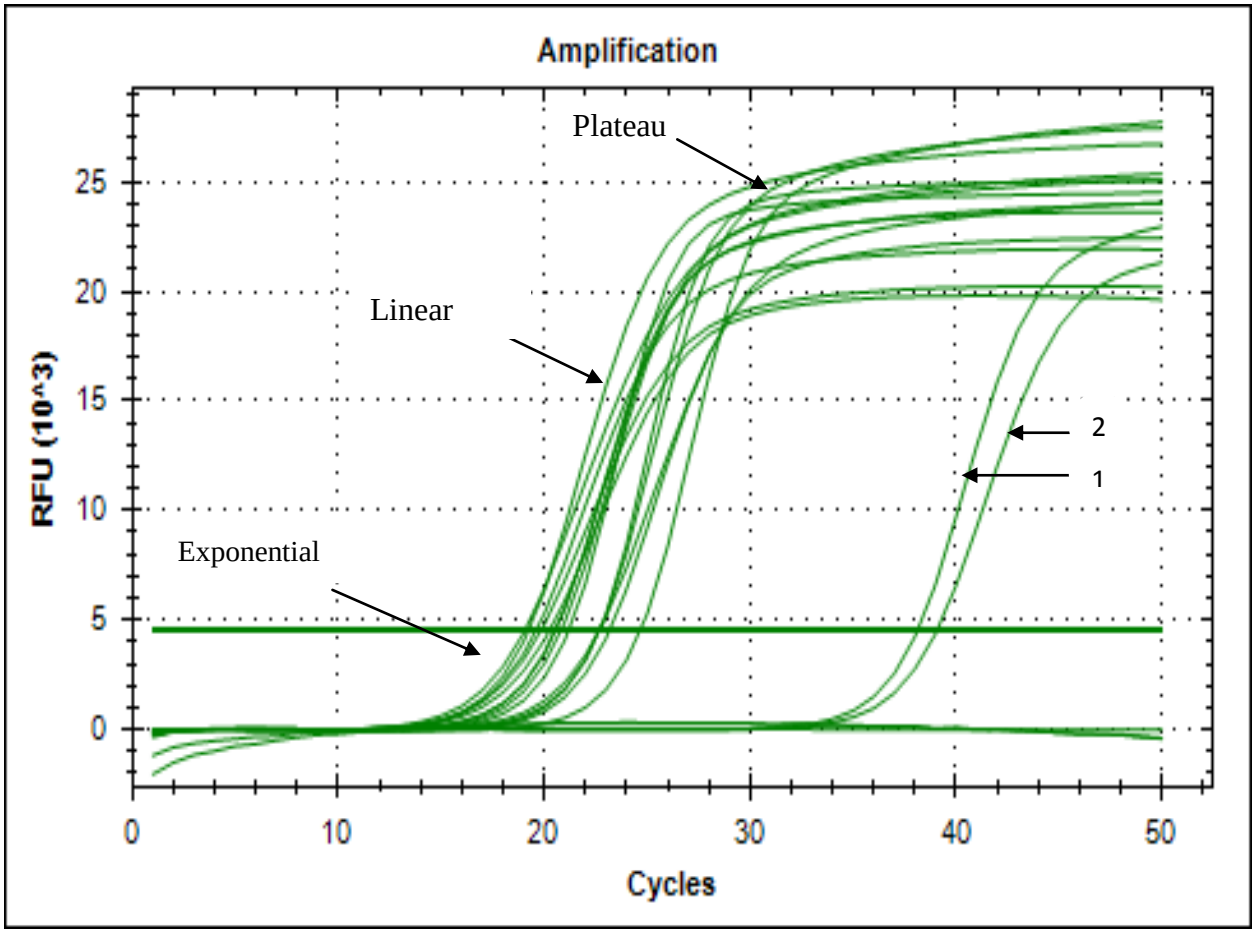

Figure 5. PCR amplification: Plot of a PCR reaction performed on a Bio-Rad CFX96 real-time PCR instrument with duplicates of each sample. X-axis: cycle number; Y-axis: amount of DNA fluorescence. Notice that the three PCR phases-exponential, linear and plateau vary for the sixteen samples.

The melting peak of three positive control *capripox viruses* was separately analyzed. The snapback melting peak of LSDV was lower temperature at 50.5ºC for followed by SPPV at 51.0ºC while GTPV snapback melting peak was relatively high temperature at 56ºC (Fig. 6). On the other hand, the full-length amplicons of GTPV and SPPV produced melting peak at 72.5ºC; whereas LSDV full-length amplicon melting peak was slightly high temperature of 73.5ºC (Fig. 6).

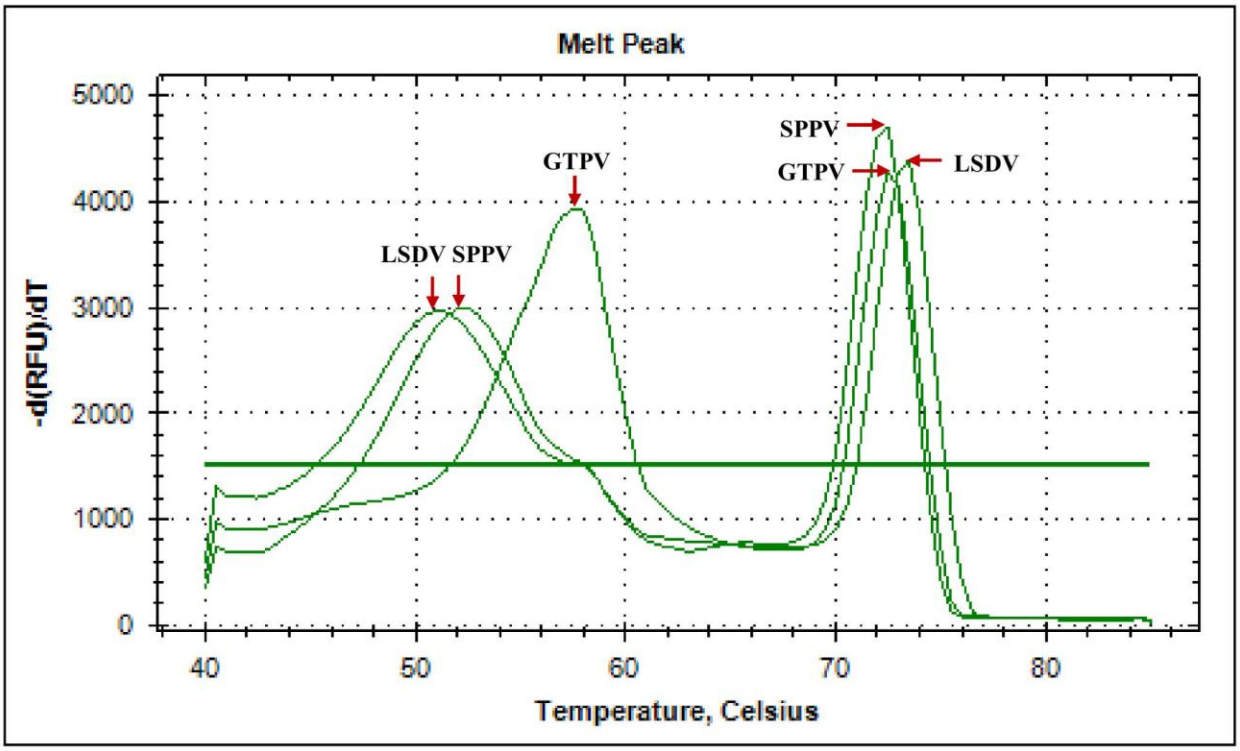

Figure 6. The fluorescence melting curve analysis of the PCR products shows two melting peaks for each of the CaPV three genotypes (GTPV, SPPV and LSDV).

The snapback and full-length amplicon melting of 14 samples became peak at 56 ºC and 72.5ºC, respectively. The melting peak graph of 14 samples was perfectly matched with GTPV as shown in Fig. 7. Non-template control (negative control) and LSDV melting peak graph lines completely differed from the sample viruses melting peak graph. However, SPPV amplicon melting peak graph was coincided with sample viruses and GTPV amplicon melting peak. In contrary, SPPV snapback melting peak was differ from both sample viruses and GTPV graphs. Therefore, all 14 samples were found to be categorized as GTPV genotype.

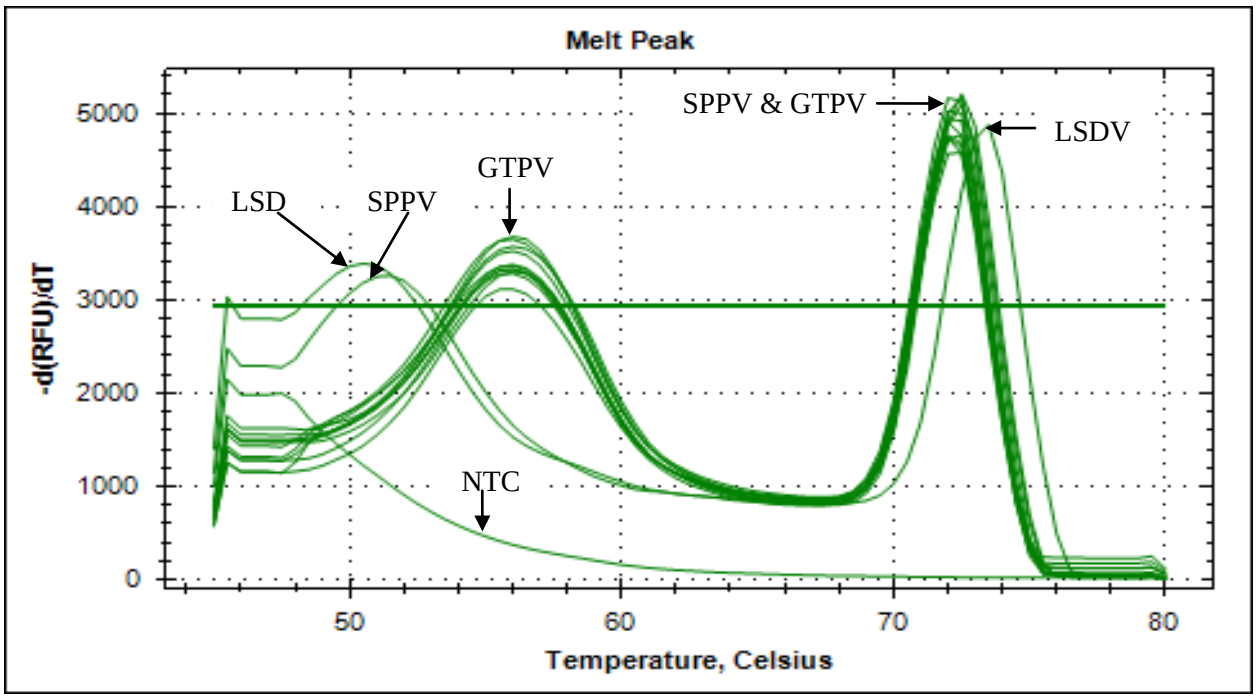

Figure 7. Melting peaks for each tested samples with *Capripoxvirus* three genotype positive controls. NTC: No-template control.

The melting curve graph of 14 samples was perfectly matched with melting curve of positive control GTPV revealed melting peaks at 56ºC and 72.5ºC for snapback and full-length amplicons, respectively. The melting regions of the PCR products duplexes are located between the two colored thick vertical lines; the purple lines are flanking the melting region of snapback hairpins (50–60ºC) while the red lines are flanking the full-length amplicons (70–74ºC) as shown in figure 8.

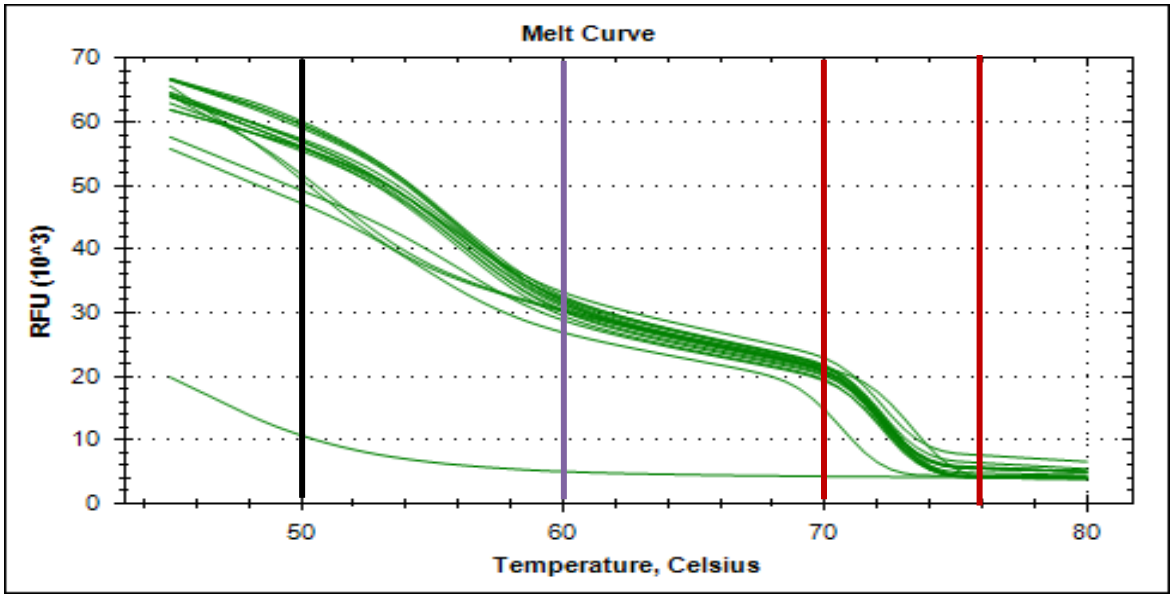

Figure 8. Melting curve of capripoxviruses showing two melting regions for a single tested samples.

4.6. **PCR Product Purification Result**

The concentration of purified PCR product of 12 samples varied from 1.80 to 1.86 ng/µl. The purity of all PCR products were found to be above the recommended values for sequencing (≥1.70 ng/µl); which meant all PCR products were suitable for further diagnosis, particularly for genome sequencing.

Table 3. Purified PCR product concentration and purity

Sample code	**Host**	**PCR concentration ng/µl (1)**	**Purity 260/280 (1)**	**PCR concentration ng/µl (2)**	**Purity 260/280 (2)**
Mojo -A	sheep	110.7	1.81	107.4	1.81
Mojo -B	sheep	122.00	1.83	110.5	1.83
Adama-1	sheep	106.4	1.82	104.8	1.82
Adama-2	sheep	99.5	1.80	96.0	1.85
Adama-4	sheep	93.0	1.83	111.2	1.85

Fiche -1	sheep	104.1	1.83	116.7	1.84
Fiche -2	sheep	107.4	1.84	107.2	1.86
Debre Berhan -1	Sheep	104.4	1.81	113.6	1.84
Debre Berhan -2	Sheep	102.5	1.81	106.2	1.86
Assosa -1	Goat	119.4	1.82	110.9	1.84
Assosa -2	Goat	89.7	1.82	112.9	1.82
Debre Zeit	Sheep	115.5	1.83	111.5	1.80

5. DISCUSSION

In the present study *poxvirus* was isolated using cell culture technique and confirmed to be *goat poxvirus* by using virus specific genotyping methods of conventional PCR (Lamien *et al*., 2011) and real-time PCR (Gelaye *et al*., 2013) targeting the RPO30 gene of poxvirus from clinical samples collected from diseased sheep and goat reared in different geographical areas of the country.

The result of the questionnaire survey indicated that sheep and goat poxes were common disease in the study areas. The survey revealed that majority of the respondents had previously experienced disease in their herds and familiar with the clinical signs of the disease, which they locally called "*fentata*". Mersha (2010) reported that this disease is named with similar local name in another parts of the country. As the respondents informed that, the disease is mainly observed summer followed by spring and autumn seasons. This might be due to favorability these seasons for insects, which possibly have a contribution for the disease spreading.

The pox lesions were observed all over the body but mostly predominant on the face, ears, groin, perennial region, and under the tail. 34.3% sheep (n=102) and eight goats (n=50) developed pox lesions on their skins during clinical examination. Few infected sheep and goats were exhibited systemic signs: febrile (104- 108ºF), lacrimation, nasal discharge, loss of appetite and reluctance to move. Clinical findings were in conformity with Mersh (2011); Senthilkumar *et al*. (2006); Woldemeskel and Ashenafi (2003).

Thought lamb testis and lamb kidney cell lines are considered to susceptive for *Capripox virus* (OIE, 2012); Sajid *et al*. (2013) and Abdula (2010) revealed that *goatpox virus* grow well on Vero cell line. Out of 16 skin biopsy samples, goat pox virus was isolated from 14 samples using Vero cell line while two samples could not develop any CPE in two passages. In present study, the *goatpox virus* - induced CPE such as small syncitia, cell ballooning, rounding, aggregation and detachment was observed within 7–10 days of incubation. These findings were agreement with Sajid *et al*. (2013) and Abdula (2010) reports of similar CPE development with 7 -10 days after inoculation.

The PCR is generally a sensitive and reliable technique for confirmation of virus in cell culture and skin biopsy samples. In this study, the primers amplify the partial RPO30 gene sequence flanking the 21 nucleotide deletion in case of SPPV but in not in GTPV sequence. This genotyping conventional PCR was successfully used for identifying SPPV and GTPV based on product sizes: 151 bp for SPPV and 172 bp for GTPV. In the present study, 14 samples out of 16 yielded a product size of 172 bp on agarose gel electrophoresis (Fig 4). Therefore, the virus isolated from both sheep and goats were not SPPV since the gel electrophoresis was greater than 151 bp. The finding of GTPV from pox lesions collected from clinically diseased sheep is in agreement with the genotyping findings previously reported by Lamien *et al.* (2011). And this finding explains that goat pox virus can infect and cause pox lesion in sheep and goat population.

In this study, a snapback real-time PCR genotyping method was applied and evaluated for the detection and genotyping of GTPV genotype from all the tested field samples. From the tested 16 samples originated from different sites, 14 samples were positive while two samples were negative for capripoxviruses. Thus, the genotyping findings generated by conventional PCR aligned with the results of real-time PCR that all 14 samples collected from clinically diseased sheep and goat are goat poxvirus.

In case Real time PCR the melting temperature was used to differentiate GTPV from SPPV/LSDV due to the noticeable difference in the melting temperatures of the GTPV (Tm = 56ºC) which matched perfectly, and those of SPPV and LSDV (Tm = 52.0ºC and 51.0ºC respectively). Each sample of tested Tm was 56ºC and 72.5ºC. The shape and position of this DNA melting curve functions are to differential of desired products can distinguished from undesired products. A snapback tail of 16 bases length was designed to match 100% with GTPV, and presented a T: A mismatch with SPPV and a T: G mismatch with LSDV, and attached to the 5’ end of the forward primer to provide a more targeted genotyping.

The genotyping was achieved by using information from both snapback stem and full-length amplicons melting temperature respectively for LSDV (Tm = 51.0ºC/73.5ºC), SPPV (Tm = 52.0ºC/72.5ºC) and GTPV (Tm = 56.0ºC/72.5ºC). GTPV is clearly differentiated from

SPPV/LSDV by observing the Tm of snapback. Likewise, LSDV is differentiated from SPPV/GTPV by observing the Tm of full-length amplicons.

Considering the high cost and complexity of gene sequencing, the method is not likely to be applied for routine screening and it is not affordable in most laboratories in the affected regions. It is well established that most of CaPV strains especially those affecting small ruminants are not strictly host specific and can cross-infect both sheep and goats. It was confirmed that sheep and goat was infected by caused by GTPVs. The availability of cost-effective diagnostic tools for routine determination of CaPV genotype was assist to clarify the epidemiological picture in the affected regions Diallo (2007); Le Goff *et al.* (2009); Lamien *et al.* (2011); Gelaye *et al.* (2013).

Based on the findings of the present study sheep and goat population were affected by goat poxvirus from samples collected from different parts of the country (Figure 1); whereas sheep poxvirus was not identified from a single sample. This result clearly explains that both sheep and goats are equally susceptible to goat poxvirus and it's only goat poxvirus circulating and causing pox disease in sheep and goat population. The present molecular findings are in agreement with the previous report of Le Goff *et al*. (2009), Lamien *et al*. (2011), and Gelaye *et al*. (2013) that all reported goat poxvirus was identified from pox lesions collected from clinically diseased sheep from different countries of the world. The country is not able to export animal and animal products to the developed world because of the presence of international trade restriction. The tanneries located in different corners of the country are getting poor quality and down-graded hides, and this directly affects the national economy.

The capripox disease in sheep is caused by a GTPV or a goat is affected by SPPV without genetic characterization of the virus isolate. Considering the high cost of gene sequencing, the method is not likely to be applied for routine screening and it is not affordable in most laboratories in the affected regions. It is well established that most of CaPV strains especially those affecting small ruminants are not strictly host specific and can cross-infect both sheep and goats. It was confirms this finding out breaks in sheep caused by GTPVs. The availability of a cost-effective diagnostic tool for routine determination of CaPV genotype was assist to clarify

the epidemiological picture in the affected regions Diallo, (2007); Le Goff *et al.* (2009); Lamien *et al.* (2011).

The concentration of purified PCR product of 12 samples varied from 1.80 to 1.86 ng/µl. The purity of all PCR products were found to be above the recommended values for sequencing (≥1.70 ng/µl); which meant all PCR products were suitable for further diagnosis, particularly for genome sequencing similarly to Smith and Ballantyne (2007).

In the present study, 12 samples from sheep and two samples from goats was found to be GTPV using both convention PCR and Real-time PCR. It is often believed that the majority of CaPVs strains are host-specific. The criterion used for distinguishing CaPVs strains within the genus is based on the animal species from which the viruses are isolated (Babiuk *et al*., 2009). However, in advancement of diagnostic technology, there are increasing indications of such classifying method inaccurateness (Babiuk *et al*., 2009; Lamien *et al*., 2011; Gelaye *et al*., 2013) since some CaPVs strains were found outside the group corresponding to their host of origin. One GTPV suspected sample from Saudi Arab and GTPV vaccine Nigeria/99 strains were relocated to the SPPV group (Lamien *et al*., 2011; Gelaye *et al*., 2013). Similarly, the causative agent of one outbreak of pox in goats in India was confirmed as SPPV (Bhanuprakash *et al*., 2010). Conversely, one from Oman, three from Kenya and one from Ethiopia SPPV suspected samples were confirmed to be GTPV (Lamien *et al*., 2011; Gelaye *et al*., 2013). There was also a report of the association of goat pox virus to sheep pox outbreaks in china (Yan *et al*., 2012). The present study in agreement with later authors, 12 SPPV suspected isolates from sheep pox were group as GTPV. This was the second to isolate goat pox viruses from Ethiopian sheep. However, this classification method seems to be working for LSDV except one sample from sheep (SPPV KS1) grouped as LSDV (Lamien *et al*., 2011; Gelaye *et al*., 2013).

6. CONCLUSION AND RECOMMENDATION

Pox disease is one of well-known diseases of sheep and goats in the study areas. The present study proved that pox disease is more prevent in rainy seasons followed by spring and autumn season, respectively. The field virulent goatpox virus can easily be isolated on African Green Monkey Kidney (Vero) cell line within 7-10 days of inoculation. Goatpox virus produces similar CPE with the recommended susceptible cell lines for isolation. Convention PCR using RPO30 gene based genotyping and Snapback SYBER green Real-time PCR confirmed that goatpox virus could cause pox outbreaks in both sheep and goat herds. This study approved that host-specificity classification of CaPVs is inaccurate at least for GTPV. This finding may provide new information on the epidemiology of sheep pox and goat pox in Ethiopia. It has also important implications in the control of the diseases of sheep and goats caused by the viruses of genus of CaPVs.

Therefore, based on these study findings the following recommendations are suggested:

- Further studies should be conducted on the investigation of outbreaks and identification of the circulating *capripoxviruses*
- Detailed studies on the evaluation of available vaccines and the development of a vaccine containing goat poxvirus antigen for small ruminants
- Study should be under taken on the susceptibility of sheep and goats for SPPV
- Awareness should be given to animal health professionals and concerned bodies on inaccurateness of previous assumption of CaPVs host-specificity.

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8. ANNEX

Annex 1: Dulbecco's modification of Eagle's Minimum essential

Composition

- (DMEM) powder 12.5 g
- Deionised water 1000 milliliter (ml)

Preparation

- Dissolve, then make up volumetrically to desired volume
- Check if pH = 7.2
- Dispense into 200 ml aliquots.
- sterilised by filtration by EKS2.
- Check sterility by broth culture.
- Store at room temperature away from direct sunlight.

Dulbecco's Minimum essential Medium10%

Composition

- DMEM 80 ml
- TPB 10 ml
- Foetal calf serum 10 ml

Preparation

- DMEM is and dispensed into 100-200 ml amounts in screw cap bottles.
- Store the bottles at 4^{o}C after sampling for sterility testing
- If antibiotics are to be used add the Antibiotic stock solution (Penicillin 5X 106 IU vial + streptomycin 5 X 1g vial in 100ml of PBSA) to have it at a final concentration of 0.2 %.

Annex 2: PBSA (Phosphate buffered saline

Composition

NaCl	8.0 g
KCl	0.2 g
KH_2PO_4	0.2 g
$Na_2HPO_4.2H_2O$	1.44g
Make up with distilled water to	1000 ml

Preparation

- Dissolve, then make up volumetrically to desired volume
- Mark liquid level before autoclaving
- Adjust the pH to 7.2 at 20ºC or 7.4 at 36.5ºC with sterile 1M NaOH
- Dispense into 200ml bottles.
- Sterilize by autoclaving at 121ºC at 15 min.
- Store the solution bottles at +4ºC.

Annex 3: Trypsin 2.5 % (W/V) solution (l0X stock solution preparation

Composition

NaCl	8 g
KCl	0.4 g
Na_2HPO_4	0.0475 g
KH_2PO_4	0.06 g
$NaHCO_3$	0.35 g
Trypsin (l: 250)	25 g
Deionized and distilled water to	1000 ml

Preparation

- Dissolve by stirring overnight at 4ºC.
- Sterilize by filtration through a Seitz EK pad or a 0.2 µm membrane filter.
- Distribute aseptically into l00 ml volumes.
- Take sample for sterility tests.
- Store at -20ºC.

- For use add l00 ml to 900 ml 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 20 ml
- PBSA 970 ml
- EDTA* 2% stock solution 10 ml

EDTA (Versene) 2% stock solution

Composition

- NaCl 8 .0 g
- KCl 0.20 g
- Na_2HPO_4 1.15 g
- KH_2PO_4 0.2 g
- EDTA di-sodium salt.$2H_2O$ 22.14 g
- Phenol red 1.0 g

Preparation

- Dissolve and make up to l000 ml with deionized distilled water.
- Distribute in 100 ml volumes in screw cap bottles.
- Autoclave at 121^oC for 15 min.
- Take sample for sterility tests.
- Store at 4ºC.

Annex 4: Penicillin and streptomycin stock solution

Penicillin and streptomycin stock solution

Sodium penicillin	5x 10^6 i.u. vials
Streptomycin sulphate	5x 1g vials
PBSA	100 ml

Preparation

- 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 store at -20ºC

Annex 5: DNA extraction Kits master mix, gel, TAE buffer, GelRed, loading dye, molecular ladder

DNA extraction Kits

- QIAamp spin columns 250samples
- Collection tubes (2 ml) 750ml
- Buffer AL 54 ml
- Buffer AW1 95 ml
- Buffer AW2 66 ml
- Buffer AE 110 ml
- Proteinase K 6 ml

Master mix for pox virus DNA conventional reaction

- RNA free water 4µl
- primer SpGPRNApol forward 1µl
- primer SpGPRNApol reverse 1µl
- IQ supper mix SPGP 10µl

Then mix and agitate divided into PCR tube

- **Gel Electrophoreses**

Composition

- Agarose gel 3g
- Distilled water 100ml

Preparation gel electrophoreses

- Mix very and boiled with Micro- oven at 180ºC temperature for 3minute until melting

- Cool 54^{O}c and pour in to gel tank
- Insert the gel comb in to melting agarose gel
- Dry for 20 minute

Preparation of TAE Buffer

Reagents: (per Liter of 50X solution)

- Tris-base, 242 g
- Glacial acetic acid, 57.1 ml
- 0.5M EDTA, 100 ml

Preparation

- Measure 600 ml of bi distiller water using the graduated cylinder and pour into pyrex beaker.
- Add stir bar, place on stirplate, and begin stirring.
- Add 242 g tris, 57.1 ml acetic acid, and 100 ml 0.5M EDTA.
- Allow to stir until the tris goes into solution.
- Adjust the pH to 8.0 if needed.
- Bring the volume to 1L.
- Transfer to a clean glass bottle.
- Store at room temperature.
- Dilute to 1X before use (2ml 50X TAE per 98ml water).

GelRed is a non mutagen but must be handled with care. To make a 10mg/ml stock weigh 1.0g and add it to 100ml distiller 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 about all applications)

- Glycerol 7.5ml
- Bromophenol Blue 2mg
- Xylene Cyanol 2mg
- 1M Tris 100µL
- 0.5M EDTA 20µL
- 10% SDS 200µL

- H2O 2.2ml
- Total 10ml

The general rule of thumb is to use 1µL of gel loading buffer dye per 5µL of sample

PCR ladder

The PCR 50 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 13 bands, ranging from 50–1000 bp.

DNA Sizes: base pairs (bp)

1000	300
900	250
800	200
700	150
600	100
500	50
400	

The ladder is supplied as a solution in 10 mM Tris-HCl, pH 7.5-8.0, with 1.0 mM EDTA.

ANNEX 6: Materials Real time PCR master mix and Reaction

Materials

- 2×reaction mix (10 mM MgCl2,1 mM each dNTP, DNA polymerase, SsFastEvaGreen Dye)
- 50 µM forward primer
- 50 µM reverse primer
- RNase-free water
- DNATemplate
- 1.5-ml microcentrifuge tubes for preparing master mix
- Thin-walled PCR tubes (BioRad)
- Real-time thermal cycler CFX96 (BioRad)

Table 1. Components of the Real-Time PCR Reaction Mix

Component	Volume/reaction	16× master mix
iQ™ SsFastEvaGreen Dye supermix (2x)	10 µl	160 µl
Forward primer	2 µl	32 µl
Reverse primer	0.6 µl	9.6 µl
RNase-free water	4.84 µl	77.4
Template DNA (added to each reaction tube)	3 µl	-
Total	**20 µl**	**189 µl**

Table 2. Real-Time PCR protocol

Thermal Cycling protocol	Time	Temperature
Step Initial PCR activation step	3 min	95ºC
Cycle Denaturation	30 sec	95ºC
Annealing	30 sec	58ºC
Extension	30 sec	72ºC
Repeat cycles	45 times	
Final extension	2min	72ºC
Melting curve analysis	0.5ºC/sec	56,72.5

Annex 7: Melting peaks temperature of samples and positive capripox virus

Table 3. Melting peaks of samples and positive capripox virus (SPPV, GTPV and LSDV)

Strain Name Area	SPP	Two Melting Peak (ºC)		Genotyping
		Snapback	Full-length Amplicon	
SPPV	Positive Control	52.0	72.5	SPPV
GTPV	Positive Control	56.0	72.5	GTPV
LSDV	Positive Control	51.0	73.5	LSDV

Mojo -A	Sheep	56.0	72.5	GTPV
Mojo -B	Sheep	56.0	72.5	GTPV
Mojo -C	Sheep	-	--	Negative
Adama-1	Sheep	56.0	72.5	GTPV
Adama-2	Sheep	56.0	72.5	GTPV
Adama-3	Sheep	56.0	72.5	GTPV
Adama-4	Sheep	56.0	72.5	GTPV
Adama-5	Sheep	--	--	Negative
Debre Ziet 1	Sheep	56.0	72.5	GTPV
Debre Ziet 2	Sheep	56.0	72.5	GTPV
Fiche -1	Sheep	56.0	72.5	GTPV
Fiche -2	Sheep	56.0	72.5	GTPV
Debre Berhan -1	Sheep	56.0	72.5	GTPV
Debre Berhan -2	Sheep	56.0	72.5	GTPV
Assosa-1	Goat	56.0	72.5	GTPV
Assosa-2	Goat	56.0	72.5	GTPV

Annex 9: Goat and sheep pox diseases survey assessment questionnaires form

Region ----------

Zone ----------

Type disease ----------

Date ----------

Table 4. Goat and sheep pox diseases survey assessment questionnaires form

Wereda	Species	Herd	Number	Age	Sex	Breed	Management practices	Disease Seasonal